DEVELOPMENT OF AN EMBRYOGENIC SYSTEM FOR MASS PROPAGATION OF *PINUS KESIYA* ROYLE EX. GORD. AND FIELD PERFORMANCE OF THE REGENERANTS

BY

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CERTIFICATE

I, Hiranjit Choudhury, hereby, declare that the subject matter of the thesis entitled " **Development of an embryogenic system for mass propagation of *Pinus kesiya* Royle ex. Gord. and field performance of the regenerants**" 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 in any other University/Institution.

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

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Chapter 1

Introduction

The world's forest resources have been subjected to tremendous pressure due to ever increasing requirement of forest and forest products. The demand for wood for pulp, paper, timber and furniture industries, urbanization and industrialization have resulted in inconceivable deficit of wood and a gradual decline of forests world wide. The forests are being cleared in South-east Asia, Africa and South America at an estimated rate of 5 million ha, 2 million ha, and 8 million ha yr^{-1} respectively (Kumar, 1997). Therefore, to meet the demand of timber, the acreage of forests might need to be doubled. At present, there are about 100 million ha of industrial plantations worldwide. Of these, 70-80% is comprised of conifers, made up about equally of long-rotation and medium-rotation species. It is likely that, a major part of this additional area of plantation will be located in the tropics, comprising tropical conifers, *Eucalyptus*, and other tropical hard wood trees (Haines, 1993).

India is facing the problem of large-scale exploitation of forests and deforestation. The forest cover of India as per the present

assessment is 63.73 million ha. The seven North-Eastern states together comprise 25.7% of the total forest cover. But most of the states show a decline in forest cover. North-Eastern region lies between $21^{\circ}\ 51'$ N to $29^{\circ}\ 28'$ N latitudes and from $89^{\circ}\ 40'$ E to $97^{\circ}\ 25'$E longitude. This region accounts for only about 8% of the total geographical area of the country. Almost half of the total geographical area of North-East India (about 22.5 million hectares) is under forests with valuable timber yielding trees including pines. The economy of the indigenous people of this region is solely based on agriculture and forest. The forests of the region are getting denuded due to shifting cultivation, indiscriminate felling of forest trees for timber, population explosion, industrialisation, and unplanned construction of dams and roads. The area affected by shifting cultivation in different states of North-East during 1987-97 was- 0.18 million ha in Meghalaya, 0.36 million ha in Manipur, 0.38 million ha in Mizoram, 0.39 million ha in Nagaland, 0.23 million ha in Arunachal Pradesh, 0.13 million ha in Assam and 0.06 million ha in Tripura (SFR, 1999).

More than 94 species of *Pinus* (family Pinaceae; Order Coniferales) are distributed in the Northern Hemisphere, from the polar region to Guatemala, North Africa and Indonesia. Of these, six species are indigenous to India and are found scattered in the Himalayan region

from Jammu to North-East. These species are *P. roxburghii* Srag. (syn. *P. longifolia* Roxb., *P. serenagensis* Madden, Chirpine); *P. wallichiana* A.B Jacks (syn *P. excelsa*, Blue pine, Kail); *P. gerardiana* Wall ex. Lamb (Chilgoza pine, Neoza pine); *P. kesiya* Royle ex. Gord (syn *P. insulris*, Khasi pine); *P. merkusii* Jungh. (Merkus pine) and *P. armandii* Franchlet (Armandi's pine). Chirpine is the most widespread and important species of Indian subcontinent and Khasi pine is the most important in North-East India that provides valuable natural resources and influence the ecology and economy of the region to a great extent.

Pinus kesiya, commonly known as Khasi pine is an early successional tree species and is an active colonizer of degraded sites. This species is predominant in subtropics (800m-2000m) of North-East India (Meghalaya, Manipur, Mizoram, Nagaland and Arunachal Pradesh) and Myanmar and Philippines (Figure 1a-b). Khasi pine is a major source of timber and resin in Meghalaya and it covers about 30% of the total forest area. The wood of this species is moderately hard, pale brown to red in colour and resinous. Various parts of the tree are valuable and are used in one form or the other. The timber is used for building houses and other construction purposes, in making poles and posts, packing boxes, match sticks, match-boxes and plywood. Some

Figure 1. a. Distribution of *Pinus kesiya* in North-East India

b. A natural stand of *P. kesiya* forest

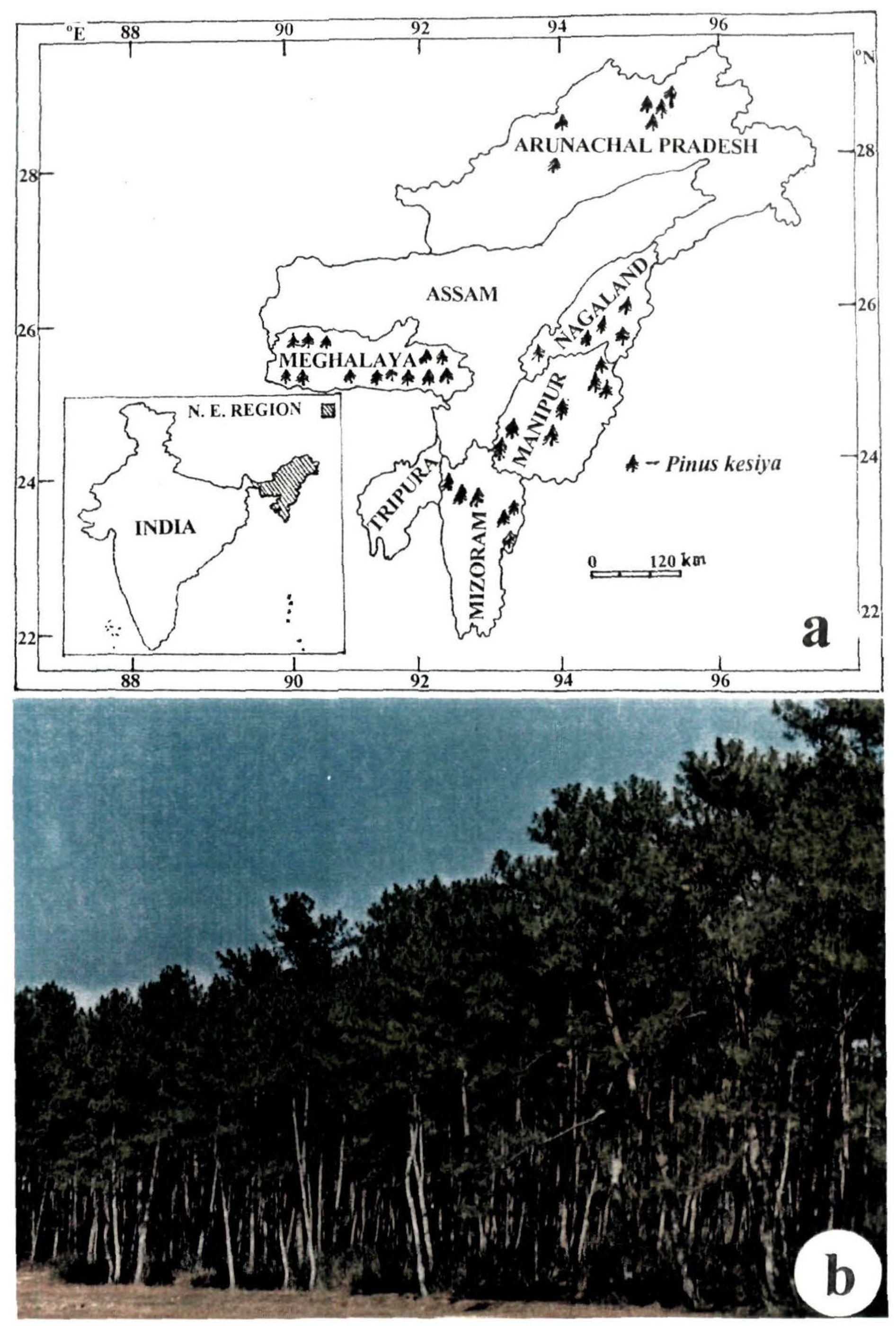

Figure 1

genotypes have tremendous biomass potential and oleo-resin prospects. The resinous wood, besides being used as torch-wood, yields a good quality ole-resin. The average yield of resin per tree (dia. 30-45 cm) has been estimated at 2.3-2.7 kg per tapping season (Chadha, 1977). The average composition (by weight) of turpentine oil from *P. kesiya* is as follows: α-pinene 30-40%, β-pinene 60-65%, other terpenes 5-10%; some workers have also reported the presence of Δ-3-carene and longifolene (Singh and Mehra, 1977). The oil of turpentine viz., α-pinene, β-pinene, Δ-3-carene and longifolene are materials of considerable commercial importance finding use in the manufacture of essential commodities like soaps and cosmetics; scents and incense; detergents; paints and varnishes; rubber and adhesives; disinfectants; insecticides and pesticides etc. But so far the oil of turpentine from khasi pine has not been utilized to its fullest extent that would otherwise prove to be a great revenue earner for NE region. Needles are used in making mattresses and cushions and local people use mature ripe female cones as fuel. Charcoal obtained from khasi pine is used as a source of domestic fuel.

Taxonomically, *P. kesiya* is an evergreen and monoecious, tree up to 25m high and girth 2m, bearing branches in whorls, crown on mature trees are broad. Bark is 2-4 cm thick, grayish, fissured and scale

like in younger trees while on mature trees it is dark gray to brownish, deeply fissured and scaly. Shoots are light brownish, and are of two kinds viz., dwarf or spur shoot and long shoot of unlimited growth. Leaves are dimorphic: (i) brown, small, thin scale leaves which occur on long as well as on dwarf shoots and (ii) needle like green foliage leaves developing in cluster of three at the apex of dwarf shoot. Needles are 18-20 cm long. Male cones are borne in catkin-like fashion (Figure 2a). They are yellow, orange or light red in colour and composed of many spirally arranged miscrosporophylls with two pollen sacs. Mature male cones are about 20-30 mm long. Pollen grains are winged and are shed and dispersed by wind during February to April. The female cones are purplish to deep green in colour (Figure 2b) and borne on the apices of new shoots either solitary or in a whorl of 2-5 (Figure 2c-f). The young cones are erect and ovoid with fertile scales of inflorescence spirally arranged, with a small bract scale above each of them. The scales on the cone close immediately after pollination and the cone turns inverted or horizontal on the branch to hibernate the cold winter. The cones again show active growth, turn green in the beginning of second season. Fertilization occurs during that time i.e. one year after the event of pollination. The cones are pushed to the lateral position on the branch due to the growth of new shoots. With the advent of winter, the cones

Figure 2. Different vegetative and reproductive parts of *Pinus kesiya*

a. A twig with secondary needles and male cones

b. A green immature second year female cone

c. A mature solitary female cone

d. Mature female cones in a cluster of two

e. Mature female cones in a cluster of three

f. Mature female cones in a cluster of five

g. A megasporophyll with two winged seeds at the base

h. An enlarged view of winged seeds (0.63 x 6.5)

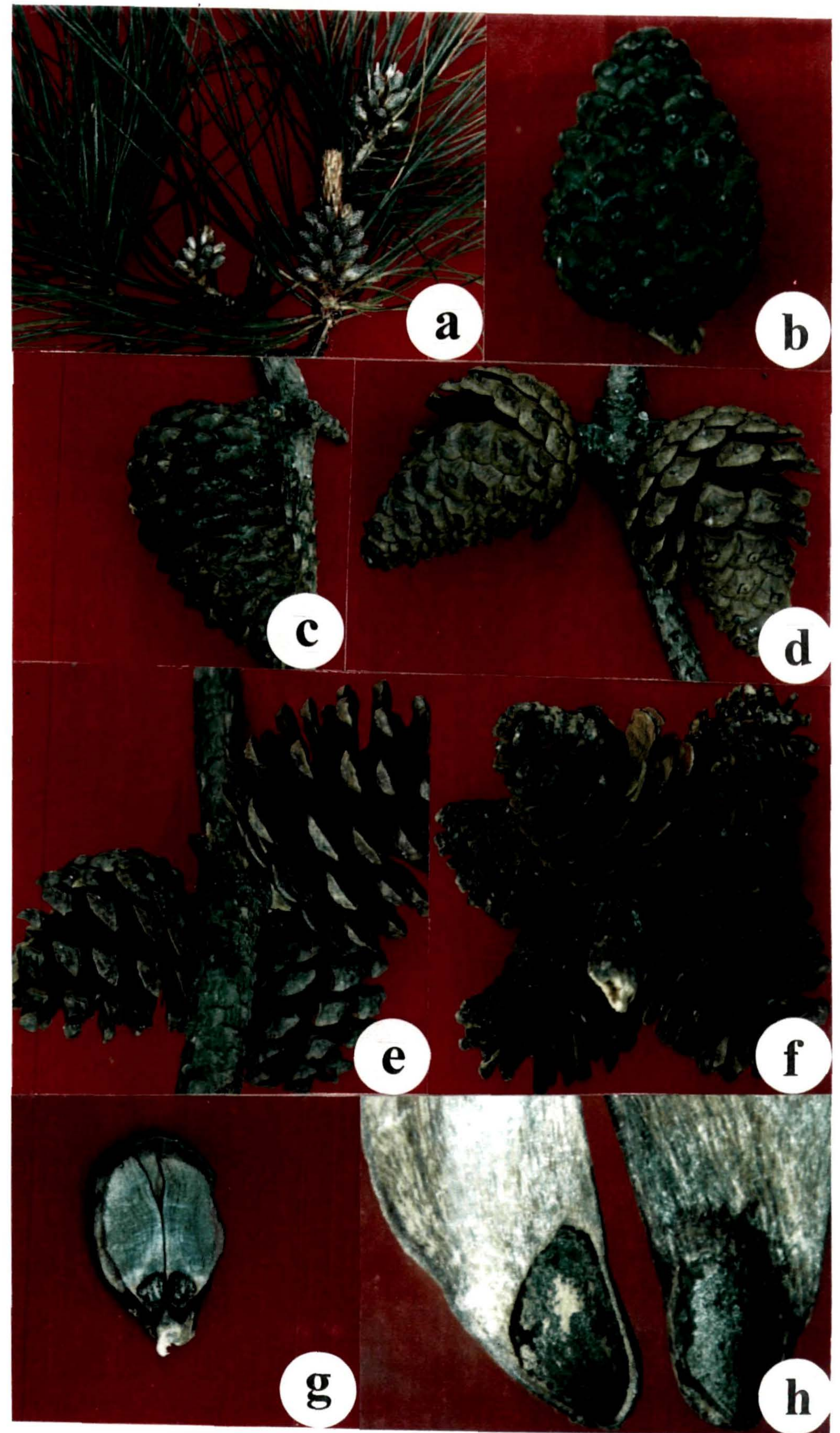

Figure 2

gradually turn brown and hard, attaining their full development. In the third season i.e. 24-26 months after appearance of the female cones, they begin to open from last week of December and dispersal of seeds continue up to March in dry weather dispersing the winged seeds. Fully ripened cones of third year are on an average 60 mm long and 40 mm broad and are composed of many spirally arranged, thick, woody scales (Figure 2f-g). Two seeds are attached at the base of the woody scale (Figure 2g-h). On an average seeds are 5.7 mm long and 3.9 mm wide, winged, wing about 18-20 mm long and cotyledons are 6-7 in number.

Forest department of North-Eastern states have been involved in forest plantations over the years particularly in areas affected by shifting cultivation. Among different plant species, Khasi pine has been used extensively for afforestation programmes. *P. kesiya* plantations in different states of North-East by State Forest Departments are shown in table 1. It is evident from this table that khasi pine plays a very crucial role in afforestation as well as in industrial plantation to meet the various needs of this tree. 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 uniformity of planting material with desired characteristics.

Table 1. ***Pinus kesiya*** **plantation by State Forest Departments of North-East India***

State	Year	Area (ha)	% of total plantation
Meghalaya	1978 up to 1998	2,000.37	8.90
Manipur	- up to 1997	82, 000.42	63.00
Mizoram	1951 up to 1999	32,000.63	17.20
Nagaland	- up to 1997	10,000.15	6.30

* Data obtained from State of Forest Report 1999, Forest Survey of India (Ministry of Environment and Forests, Dehra Dun)

Natural regeneration of Khasi pine occurs through seeds. Seeds are dispersed by air from last week of December to April and germinate after receiving a couple of showers. Factors influencing natural regeneration of pines include- seed viability, light, moisture, soil condition, undergrowth, fire, grazing etc. Fire as such has a beneficial effect on natural regeneration but high and yearly incidence of fire results in death of new seedlings.

Conventionally, forests have been regenerated from seedlings obtained from bulked seeds collected in nature and more recently from seeds collected from randomly pollinated '+' trees. But this method in itself has many varied problems like the seeds show loss of viability during extraction and storage, poor seed set and consumption by rodents and birds. 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 hence slashing down the seed reserves considerably. Besides, cone crops are often characterized by poor and irregular seed set, and most pine species require at least 10-15 years to yield a substantial amount of seed. Again, a large number of seedlings are destroyed every year by fire, low winter temperature, heavy down pour, grazing, poor storage etc. Weed infestation is another factor adding to the cause of seedling mortality.

In traditional methods of vegetative propagation, the numbers of plants that can be propagated from a tree species in a growing season are relatively small. The limiting factors for a large-scale utilization of selected materials are restricted availability of improved genotype as planting material and the available space. Moreover, in many tree species, for instance, *Quercus*, *Fagus*, *Eucalyptus* and in most conifers, cuttings from mature trees are generally difficult to root or rooting frequency may be rather low. It has already been reported that with increase in the age of mother tree, the rooting ability of cuttings is reduced considerably. Furthermore, the vegetative propagation method used for multiplication of Khasi pine is rather difficult as it reaches sexual maturity at an early stage after which rooting ability of cuttings decrease leading to poor regeneration. In addition, collection of cuttings, transportation, and rooting of cuttings are labour and cost intensive and this process cannot be automated due to the complexity associated with the tasks.

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 stands. Many fungi are responsible for causing diseases like stem rot, needle rust and seed borne diseases.

These results in tremendous loss to annual yield of timber and other products in plantation as well as in natural pine stands.

Today, trees having poor or heterogeneous character, slow and unreliable propagation methods are no longer economically feasible and acceptable. The need of the hours, is to make a broad and concerted effort in domestication and propagation of trees with superior wood quality, straight bole, optimal stem form and uniformity, rapid growth rates, short-rotation and high production index (stem: total tree biomass), resistance against diseases and pests, adaptability to new climates and extreme environmental variables (including pollution) and the ability to respond easily to silviculture practices (Thorpe and Biondi, 1984). Non-conventional methods of propagation hold potential of mass multiplication in a short period of time to achieve the afforestation targets. Regeneration of Khasi pine through 'somatic embryogenesis' with selected genotypes seems to offer a great potential in plantation programmes. This method can produce plantlets on mass scale in a short period of time.

The biotechnological approaches including plant tissue culture offer tremendous opportunities in basic as well as applied studies including commercial application. In the past few decades, serious effort has been made for genetic improvement of forest trees, involving

hybridization between superior trees and establishment of seed orchards. Besides, newly emerged recombinant DNA technology provides a powerful tool for the study of the molecular basis of plant development, genetic manipulation and their improvement. Different experimental techniques have been developed for the delivery, stable integration and expression of foreign genes into the plants. The biotechnological approaches have resulted in remarkable advancement in our understanding of plant improvement together with the production of transgenic plants with desirable agronomic characters. Till date many transgenic plants have been introduced to field for trial and would be commercially available very soon (Bishop-Hurley *et al.*, 2001; Donahue *et al.*, 1994; Levee *et al.*, 1997, 1999; Walter *et al.*, 1998, 1999).

Tissue culture and biotechnological methods can increase the productivity both quantitatively and qualitatively. It provides many advantages over conventional system of propagation. The potential benefits of forest biotechnology include:

☞ Rapid multiplication of selected superior genotypes

☞ Quick and easy scale up via suspension culture

☞ Cloning of phenotypically superior trees

☞ Seasonal independence of the process

☞Little space requirement to carry out the operation

☞Long-term germplasm storage via cryopreservation

☞Manipulation of specific traits like disease, pest and herbicide resistance

☞Artificial seed production or a direct delivery system can be used for emblings

☞Genetic gains can be captured through somatic embryogenesis

☞Increased genetic pool through somaclonal variation

Keeping this in mind, the present study on somatic embryogenesis of *P. kesiya* was taken up with the following objectives :

1. Studies on induction of somatic embryogenesis in semi-solid and suspension cultures using various explant sources and factors controlling somatic embryogenesis.
2. Conversion of somatic embryos to plantlets and establishment of regenerants in soil.
3. Comparative studies of the growth characteristics of somatic embryo derived emblings and seedlings under field condition.

Chapter 2

Somatic embryogenesis in conifers-A review of current status

Plant tissue culture is a general term applied to aseptic culture of plant parts like cells, tissues, organs or even protoplasts in *in vitro* system under controlled environment. The technology owes its origin to the idea of the German plant physiologist Haberlandt (1902), who introduced the 'concept of totipotency' i.e. 'all living plant cells containing a normal complement of chromosomes is capable of regenerating into a complete plant'.

An important breakthrough in tissue culture came from the studies of White (1934, 1937) where he maintained continuously growing aseptically excised root tip cultures of tomato. Gautheret (1934) for the first time reported the formation of callus from woody tree species. The tree species of *Acer pseudoplatanus, Ulmus campestris, Robinia pseudoaccacia* and *Salix caprea*. Further studies led to the independent demonstration by Gautheret (1939), Nobecourt (1939) and White (1939) that cells in culture can be made to proliferate continuously and undergo differentiation as well. A new era began in tissue culture during 1960s

where eradication of viruses was achieved through meristem culture (Morel and Martin, 1952), cultivation of single cells and suspension cultures (Muir *et al.*, 1954), auxin-cytokinin basis of organogenesis (Skoog and Miller, 1957), somatic embryogenesis (Reinert, 1958; Steward *et al.*, 1958), production of protoplasts (Cocking, 1960), anther culture (Guha and Maheswari, 1964), regeneration of plants from single cells (Vasil and Hildebrandt, 1965) uptake of DNA by plant cells (Bishop-Hurley *et al.*, 2001) were made.

Somatic embryogenesis

Somatic embryogenesis is the development of embryos from somatic cells. A somatic embryo ultimately possesses both a root and a shoot meristem and is capable of forming a complete plantlet. This is different from zygotic embryogenesis where fusion of gametes during sexual reproduction is an absolute requirement. The somatic embryos can be produced directly on the explant or indirectly from callus or cell suspension cultures. This morphogenic process is divided into four stages viz., induction, proliferation, maturation and germination.

The phenomenon of somatic embryogenesis was reported for the first time in *Dacus carrota* (Steward *et al.*, 1958; Reinert, 1958, 1959). Since then micropropagation by somatic embryogenesis has opened up a promising avenue for clonal propagation in plants. Numerous

angiospermic tree species have been regenerated *in vitro* by somatic embryogenesis such as *Cyclaman persicum* (Kreuger *et al.*, 1995), *Mangifera indica* (Alfaro *et al.*, 1996), *Cymbopogon martini* (Patnaik *et al.*, 1997), *Panex ginseng* (Choi *et al.*, 1999), *Quercus robur* (Cuenca *et al.*, 1999), *Medicago truncatula* (das Neves *et al.*, 1999), *Vitis vinifera* (Jayasankar *et al.*, 1999), *Chamomilla reticulata* (Kintzois and Michaclakis, 1999).

Somatic embryogenesis in conifers was first reported from immature and mature zygotic embryos of *Picea abies* (Hakman *et al.*, 1985; Chalupa, 1985). In the same year somatic embryogenesis was documented from the female gametophyte of *Larix decedua* (Nagmani and Bonga, 1985) which led to the production of haploid embryos. Thereafter, several reports have been published on successful regeneration of conifer via somatic embryogenesis (Attree and Fowke, 1993; Dunstan *et al.*, 1993; Gupta and Grob, 1995; Gupta *et al.*, 1993, 1995a,b; Jain *et al.*, 1995; Keinonen *et al.*, 1996; Guevin and Kirby, 1997; Barrett *et al.*, 1997; Li *et al.*, 1998; Kim *et al.*, 1999; Salajova *et al.*, 1999; Ford *et al.*, 2000). The current status of conifer somatic embryogenesis is shown in table 2.

Induction of embryogenesis is the redirection of the morphogenic fate of cells in explant to form embryogenic masses or embryoids. The

Table 2. Somatic emryogenesis in conifers

Species	Explant	Response	Established in soil	Reference
Abies alba	FG	SE	-	Schuller *et al.*, 1989
	MZE	SE, PL	-	Hristoforoglu *et al.*, 1992
A. fraseri	MZE	SE, PL	-	Guevin and Kirby, 1997
	MZE	SE	-	Guevin *et al.*, 1992
A. balsamea	MZE	SE	-	Guevin *et al.*, 1992
A. nordmanniana	IZE	SE, PL	-	Norgaard and Krogstrup, 1991
	IZE	SE, PL	+	Norgaard, 1997
Agathis australis	MZE	SE, PL	-	Aitken-Christie *et al.*, 1992
Larix decidua	FG	SE	-	von Aderkas *et al.*, 1987
	FG	SE, PL	-	von Aderkas and Bonga, 1988; Nagmani and Bonga, 1985
	FG	SE	-	von Aderkas *et al.*, 1990
	IZE	SE	-	von Aderkas *et al.*, 1990
L. occidentalis	IZE	SE, PL	+	Thompson and von Aderkas, 1992
L. decidua x L. leptolepis	IZE	SE	-	von Aderkas *et al.*, 1990
	IZE	SE, PL	+	Klimaszewaska, 1989
	FG	SE	-	von Aderkas *et al.*, 1990
	FG	SE	-	Simola and Santanen, 1990
L. leptolepis	IZE	SE	-	von Aderkas *et al.*, 1990
	FG	SE	-	von Aderkas *et al.*, 1990
	IZE	SE, PL	+	Kim *et al.*, 1999
Picea abies	IZE	SE, PL	+	Chalupa, 1985
	IZE	SE	-	Hakman *et al.*, 1985
	IZE	SE, PL	-	Hakman and von Arnold, 1985
	IZE	SE, PL	-	Becwar *et al.*, 1987
	IZE	SE, PL	+	von Arnold and Hakman, 1988
	MZE	SE, PL	+	Chalupa, 1985
	MZE	SE, PL	-	Gupta and Durzan, 1986a
	MZE	SE, PL	+	von Arnold and Hakman, 1988
	MZE	SE, PL	-	Verhagen and Wann, 1989
	MZE	SE, PL	+	Gupta *et al.*, 1991
	Cot.	SE, PL	-	Krogstrup, 1986; Lelu *et al.*, 1987, 1990
	Needles (1 yr old embling)	SE	-	Ruaud *et al.*, 1992
P. jezoensis	MZE	SE, PL	+	Ishii, 1991
P. glauca	IZE	SE, PL	+	Attree *et al.*, 1990a; Dunstan *et al.*, 1993
	IZE	SE, PL	-	Lu and Thorpe, 1987
	MZE	SE, PL	+	Tremblay, 1990
	Cot.	SE	-	Attree *et al.*, 1990b, Lelu and Borman, 1990
P. omorika	MZE	SE, PL	+	Budimir and Vujicic, 1992
P. pungenes	MZE	SE, PL	+	Afele *et al.*, 1992

contd...

Species	Explant	Response	Established in soil	Reference
P. glauca x P. engelmani complex	IZE	SE	-	Roberts *et al.*, 1989
	IZE	SE, PL	-	Webb *et al.*, 1989
	IZE	SE, PL	+	Roberts *et al.*, 1990; Webster *et al.*, 1990
	Cot.	SE	-	Eastman *et al.*, 1991
P. mariana	IZE	SE, PL	-	Hakman and Fowke, 1987a
	IZE	SE	-	Tautorus *et al.*, 1990
	MZE	SE	-	Tautorus *et al.*, 1990
	MZE	SE, PL	+	Attree *et al.*, 1990b
P. ruben	MZE	SE, PL	+	Tremblay and Tremblay, 1991; Harry and Thorpe, 1991
P. sitchnensis	IZE	SE, PL	+	Krogstrup *et al.*, 1988
	IZE	SE, PL	+	Roberts, 1991
	MZE	SE, PL	+	Krogstrup, 1990
P. wilsonii	IZE	SE, PL	-	Ying-Hong and Zhong-Shen, 1990
Pseudotsuga menziesii	IZE	SE, PL	+	Durzan and Gupta, 1987
	MZE	SE, PL	-	Durzan and Gupta, 1987
	MZE	SE, PL	+	Gupta and Pullman, 1991
	IZE	SE, PL	+	Gupta and Pullman, 1991
Sequoia sempervirens	MZE	SE, PL	-	Bourgkard and Faver, 1988
	Cot.	SE	-	Bourgkard and Faver, 1988
Pinus caribaea	FG	SE, PL	-	Laine and david, 1990
	IZE	SE, CP, PL	-	Laine *et al.*, 1992
P. contorta	Needle	Proembryo	-	Mac Dougall *et al.*, 1988
P. elliottii	IZE	SE	-	Jain *et al.*, 1989
	IZE	SE	-	Newton *et al.*, 1994
	IZE	SE	-	Liao and Amerson, 1995a,b
	IZE	SE, PL	-	Tang *et al.*, 1997
P. koraiensis	MZE	SE	-	Bozkov *et al.*, 1997
P. lambertiana	IZE, MZE	SE, PL	-	Gupta and Durzan, 1986a,b
P. massoniana	MZE	SE	-	Huang *et al.*, 1995
P. nigra	IZE, MZE	SE	-	Salajova and Salaj, 1992
	IZE	SE	-	Jasik *et al.*, 1995
	IZE	SE, PL	-	Salajova *et al.*, 1999
P. palustris	IZE, FG	SE	-	Nagmani *et al.*, 1993
P. patula	IZE	SE, PL	-	Jones *et al.*, 1993
	IZE	SE, PL	-	Jones and van Staden, 1995
	IZE	SE, CP, PL	-	Ford *et al.*, 2000
P. pinaster	IZE	SE, PL	-	Lelu *et al.*, 1999
P. roxbughii	IZE	SE	-	Arya *et al.*, 2000
	IZE	SE	-	Mathur *et al.*, 2000
P. serotina	FG	SE	-	Becwar *et al.*, 1988
P. strobus	IZE, FG	SE	-	Finer *et al.*, 1989
	IZE	SE, PL	-	Klimaszewaska and Smith, 1997
	IZE, MZE	SE, PL	-	Garin *et al.*, 1998
	IZE	SE, PL	-	Klimaszewaska *et al.*, 2000

contd...

Species	Explant	Response	Established in soil	Reference
P. sylvestris	IZE	SE	-	Percy *et al.*, 2000
	IZE	SE, PL	+	Keinonen *et al.*, 1996
	IZE	SE, CP	-	Haggman *et al.*, 1998
	IZE	SE	-	Haggman *et al.*, 1999
	IZE	SE, PL	-	Lelu *et al.*, 1999
P. taeda	IZE	SE, PL	+	Gupta and Durzan, 1987a, b
	IZE	SE, PL	+	Gupta and Pullman, 1990
	FG	SE	-	Becwar *et al.*, 1990
	IZE	SE	-	Becwar *et al.*, 1991
	IZE	SE	-	Li *et al.*, 1997, 1998
	IZE	SE, PL	-	Tang *et al.*, 1998a, b
	IZE	SE	-	Tang 2000.

IZE: Immature zygotic embryo; FG: Female gametophyte; MZE: Mature zygotic embryo; Cot: Cotyledon; SE: Somatic embryo; CP: Cryopreservation; PL: Plantlet; +: yes; -: No

explants used for somatic embryogenesis include female gametophyte containing developing zygotic embryos (immature embryos), mature zygotic embryos, hypocotyls, cotyledons, secondary needles and recycled cotyledons of somatic embryos. Majority of investigations so far revealed that mature and immature zygotic embryos are the most preferred explants for induction of embryogenesis in pines.

Somatic embryogenesis may follow two different paths of development viz., direct and indirect. In direct somatic embryogenesis, the embryos develop directly from the somatic or vegetative cells of the explant without any intermediate callus phase. In indirect embryogenesis, the somatic embryos develop from an undifferentiated mass of cells. Embryogenesis can be induced very easily in some cells like the cells of zygotic embryos. These easily inducible cells are called competent or pre-embryonic determined cells. However, there are a wide variety of cells, which are well differentiated and can be made to behave like embryos only through major manipulations of different factors. These cells are called as the induced embryogenic determined cells or potentially competent cells e.g. the cells of the explant in culture. Besides, there are some cells, which are so highly differentiated that it is almost impossible to induce embryogenesis in them. These cells are designated as the non-competent cells. Somatic embryos

develop from embryogenically competent somatic cells *in vitro* and are a one step process. In contrast, organogenesis shows sequential shoot and root differentiation on different media. Developmental phases in embryogenically competent somatic cells under the influence of specific phytohormones generally proceed in the same or closely similar fashion like that of the development of a zygotic embryo. In *P. glauca* and most of the conifers, somatic embryos closely resemble developing zygotic embryos (Attree and Fowke, 1991). However, somatic embryos are not very precisely organized as zygotic embryos. The suspensor like cells may be formed of loosely arranged elongated cells and many embryos may share a common suspensor system (Hakman and Fowke, 1987b). Somatic embryogenesis proceeds directly or indirectly when responsive explants are exposed to critical doses of exogenous growth regulators at the initial phase of culture (Gupta and Grob, 1995a,b).

Occurrence of polyembryogenesis is a very common and natural phenomenon in conifers. Most of the conifers exhibit either simple or cleavage polyembryony. In conifers simple polyembryony results from fertilization of more than one egg cell within a single ovule and leads to initial development of multiple zygotic embryos with different genotypes (Singh, 1978). One pro-embryo usually dominates and continues to develop while the others abort. In *in vitro* condition,

through manipulation of culture environment, these subordinate embryos may lead to initiation of embryonal tissues (ETs) and subsequently to multiple somatic embryos as reported in *P. lambertiana* (Gupta and Durzan, 1986b), *P. taeda* (Gupta and Durzan, 1987a,b) and *P. mengiesii* (Durzan and Gupta, 1987a,b). These workers termed the 'embryogenic tissues' as 'embryonal suspensor masses' (ESMs) due to their high degree of organization. The ESMs are generally white, translucent cellular mass, which glisten due to the production of mucilage. They show variable mixture of elongated cells, embryo initials, early stage embryos, which have embryonal head and suspensor system and sometimes, later stage embryos (Gupta and Durzan, 1987a,b; Finer *et al.*, 1989; Laine and David, 1990). Non-embryogenic callus, on the other hand, appears opaque, friable and turn green when exposed to light.

In conifers, three different methods could be used for initiation of embryogenic cultures. These are: (a) through the continuation of natural cleavage polyembryony of embryonal heads of cultured immature embryos (Durzan and Gupta, 1988), (b) through cell division in the epidermal and sub-epidermal layers of hypocotyl, cotyledons or needles resulting in calli which then rapidly organize to form embryonal suspensor masses (Nagmani *et al.*, 1987) and (c) through cell division of

small cells within the suspensor system of the explanted immature embryo (Gupta and Durzan, 1987a,b).

Proper explant selection has been critical in order to achieve successful induction of somatic embryogenesis in conifers. Various tissues of the same plant or tissues at various stages of development can differ in their responses when cultured *in vitro* (Attree and Fowke, 1991). Pines are responsive towards embryogenesis at early stages of zygotic embryo development i.e. at the early stage of polyembryony (Gupta and Durzan, 1987b; Laine and David, 1990). Finer *et at.* (1989) found immature zygotic embryo just prior to development of cotyledon-primordia as the optimal stage for initiation of embryogenesis in *P. strobus* and induction frequency of embryogenic callus declined with the emergence of cotyledon primordia. Jones and Van Staden (1995) observed embryogenesis at all stages of embryo development except at very young and cotyledonary stages in *P. patula.*

In most studies with *Pinus*, female gametophytes were found superior over excised immature zygotic embryos. Embryogenic cultures were later extruded from the micropylar ends of the female gametophyte (Becwar *et al.*, 1990; Haggman *et at.*, 1998; Li *et al.*,1998; Salajova *et al.*, 1999; Arya *et al.*, 2000; Ford *et al.*, 2000; Mathur *et al.*, 2000).

Mostly zygotic explants have so far been used for the induction of embryogenesis in conifers. These tissues include immature embryos dissected from seeds collected during the growing seasons, mature zygotic embryos from stored seeds and tissues from seedlings. Female gametophytes of several species have also been cultured with varying success. Explants from various conifers that have been induced to form somatic embryos include examples from following genera: *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga* and *Sequoia* (Table 2). *Pinus* is the largest and most important genus of conifers consisting approximately 95 species widely distributed over the Northern Hemisphere (Preston, 1989).

Several physico-chemical factors have been reported to influence the induction of embryogenesis from immature zygotic embryos in *Pinus* species. These factors include –basal medium (Liao and Amerson, 1995a; Li and Huang, 1996), concentrations of plant growth regulators (Becwar *et al.*, 1988; Nagmani *et al.*, 1993), concentration of gelling agent (Li *et al.*, 1997a,b; 98), myo-inositol and silver nitrate content in the medium (Li and Huang, 1996), organic nitrogen (Barett *et al.*, 1997), concentration of organic carbon (von Arnold, 1987; Becwar *et al.*, 1988a,b), pH of the medium in induction of somatic embryogenesis. Generally pH value from 5.5 to 6.0 was found to be effective. In most of

the conifers the induction of somatic embryogenesis was accomplished in the dark however, it could be induced in the light in case of *P. abies* (Verhagen and Wann, 1989).

For induction of embryogenesis in conifers from different explant sources, several media have been used in original as well as in modified form. Media requirement for the initiation of embryogenic cultures do not seem to be very specific. ESMs have been initiated on several media such as LP (Quoirin and Lepoivre, 1977), Litvay's (Litvay *et al.*, 1985), DCR (Gupta and Durzan, 1985), DCR_1 (Bewar *et al.*, 1995), modified Murashige and Skoog [mMS (Gupta and Durzan, 1986b], BLG (Verhagen and Wann, 1989), P_6 (Gupta and Pullman, 1990), BM_1 (Gupta and Pullman, 1991), WTC (Gupta and Pullman, 1991), modified HLM medium (Tremblay, 1990) etc. In all these media it has been found that the main modification was made in nitrate salt concentration, especially- NH_4NO_3 and KNO_3. Barrett *et al.* (1997) reported that removal of organic nitrogen sources like casein hydrolysate (CH) and L-glutamine is beneficial for *P. glauca*. It has been observed that low percentage of sucrose (1-2%) resulted more ESMs formation (von Arnold, 1987; Becwar *et al.*, 1988a,b). Gelrite was found to be a superior gelling agent when compared with agar for the initiation of

ESMs from *P. strobus* (Finer *et al.*, 1989), while phytagel (gellan gum) was effective for *P. taeda* (Li *et al.*, 1998).

In spite of constantly growing knowledge, there is a lack of proper understanding of biochemical and physiological events involved in somatic embryogenesis. Majority of the workers unambiguously consented to the crucial role of plant growth regulators in the regulation of somatic embryogenesis. 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. The effect of exogenously added growth regulators in media was studied by Vagner *et al.* (1998) but so far there is very little information available about the state of endogenous growth regulators in conifer somatic embryogenesis (Dunstan *et al.*, 1995).

Usually, both auxins and cytokinins are necessary for somatic embryogenesis. Amongst the various auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) is the most preferred auxin for the initiation of ESMs in most conifers (Gupta *et al.*, 1991; Tautorus *et al.*, 1991). Naphthaleneacetic acid (NAA) has also been used for ESMs induction in *P. abies* (Verhagen and Wann, 1989). But no significant difference has been reported in the proliferation of ESMs or development of embryos in conifers with NAA versus 2,4-D as the sole auxin source

(Gupta and Grob, 1995). Induction of somatic embryos using 2,4-D as sole auxin source was reported in *P. taeda* (Li *et al.*, 1998), *L. leptolepis* (Kim *et al.*, 1999). Incorporation of either N^6-benzylaminopurine (BAP) or kinetin in the medium was found to be beneficial in most of the cases. Initiation of ESMs from *Abies* species was best obtained with cytokinin (BAP or kinetin) alone. Auxin proved to be inhibitory for the initiation of embryogenic cultures in *A. nordmanniana* (Norgaard and Krogstrup, 1991; Norgaard *et al.*, 1992). Initiation of ESMs have been achieved with different concentrations of hormones, like 2,4-D (2-10 mgl^{-1}) and BA (0.5-5 mgl^{-1}) in *Larix* (Cornu and Geoffrion, 1990; Bonga *et al.*, 1995), NAA and BAP (2 mgl^{-1} each) and BA and thidiazuron (2 mgl^{-1} each) in *A. fraseri* (Gueiven and Kirby , 1997), 2,4-D (10-110 mgl^{-1}) in *P. abies* (Gupta *et al.*, 1991), 2,4-D (2-10 mgl^{-1}) along with BA (0.5-2.5 mgl^{-1}) in *P. palustris* (Nagmani *et al.*, 1993). In *P. serotina* (Becwar *et al.*, 1988) and *P. taeda* (Li *et al.*, 1998) higher induction frequency of embryogenic callus was reported at lower concentrations of phytohormones. Nagmani *et al.* (1993) on the contrary, reported an increased induction frequency at higher level of plant growth regulators in *P. palustris.*

Maintenance and proliferation of embryogenic culture is important so as to increase its availability for regeneration and genetic

manipulations. Proliferation is usually done on medium containing lower concentrations of growth regulators. Different species and genotypes show different frequency of proliferation of embryogenic cultures in the medium. In some species and genotypes, embryogenic cultures proliferate readily on induction medium while in some others, reformulation of medium may be required (Gupta and Grob, 1995).

Laine and David (1990) reported genotypic variability in pro-embryo formation from cones collected from different trees of *P. caribaea*. They found some of the embryogenic callus lines produced embryos spontaneously even on the maintenance and proliferation medium while others required supplementation of abscisic acid (ABA) in the differentiation medium prior to proembryo formation. Role of ABA has been studied in relation to somatic embryogenesis in conifers. ABA plays several important roles in conifer embryo development and has been used in different concentrations. 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 (Durzan and Gupta, 1987; Roberts *et at.*, 1990; Attree *et al.*, 1991; Dunstan *et al.*, 1993). Salajova and Salaj (1992) demonstrated the role of ABA in somatic embryo maturation in

P. nigra where no somatic embryo maturation was found without exogenous ABA. Finer *et at.* (1989) observed that the increase in the concentration of ABA from 0-38 μM enhanced the recovery of stage-II somatic embryos in *P. strobus*. Promotive effect of ABA on maturation of somatic embryos was reported in *P. sylvestris* and *P. pinaster* (Lelu *et al.*, 1999). A similar effect of ABA along with gellan gum was reported in *P. roxbrughii* (Mathur *et al.*, 2000).

Role of organic carbon in conifer somatic embryogenesis has been studied extensively by various workers. In *P. strobus*, 6% sucrose along with 10 mgl^{-1} ABA promoted the development of stage II somatic embryos. With the decrease of sucrose concentration from 20 gl^{-1} to 5 gl^{-1} a decrease in proembryo formation and loss of embryogenic potential was reported in *P. caribaea* (Laine and David, 1990). The physiological and osmotic roles of sucrose during black spruce (*P. mariana*) embryo maturation have been investigated by Tremblay and Tremblay (1995). A comparative study of role of maltose and sucrose on total number of mature somatic embryo formation was reported by Garin *et al.* (2000) in *P. strobus*. Glutamine has been used successfully at lower concentrations for the development of sugar pine (Gupta and Durzan, 1986a,b) and loblolly pine somatic embryos (Gupta and Durzan, 1987). Role of polyethylene glycol (PEG) in maturation of

P. taeda somatic embryos was reported by Li *et al.* (1997, 1998). The final step in conifer somatic embryogenesis is the successful germination of embryoids into emblings. This step is generally achieved on media free of any growth regulators. So far there is limited report of success in establishment of regenerants in conifers. Gupta and Durzan (1987) first reported germination and continued growth of a somatic seedling of loblolly pine (*P. taeda*) under non-axenic conditions. From this study one somatic seedling from a single culture genotype was transferred to soil (Pullman and Gupta, 1991) which is growing at the Weyerhaeuser Company in Washington (Gupta and Durzan, 1991).

Research at the Westvaco Corporation Forest Science Laboratory in Summerville, South Carolina has established three separate field plantings of somatic seedlings of loblolly pine. A total of 583 somatic emblings derived from 77 different genotypes of loblolly pine were successfully established in the field during 1991, 1992 and 1993. The somatic seedlings were derived from embryogenic cultures initiated from immature zygotic embryos of 14 different open pollinated families and 5 different controlled crosses of loblolly pine. So far, successful germination and subsequent field 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* (Keinonen *et al.*, 1996; Lelu *et al.*, 1999), *P. elliottii* (Tang *et al.*, 1997), *P. strobus* (Klimaszewska and Smith, 1997; Garin *et al.*, 1998; Klimaszewska *et at.*, 2000), *P. nigra* (Salajova *et al.*, 1999) and *P. pinaster* (Lelu *et al.*, 1999).

Unlike angiosperms, somatic embryogenesis and subsequent regeneration of emblings in conifers is difficult especially in pines including *P. kesiya.* But recent knowledge of conifer somatic embryogenesis has opened up the route for mass propagation of conifers from superior planting stocks using suspension culture and bioreactors. Large-scale plant regeneration of conifers via somatic embryogenesis and their use in plantation programmes has found its application in different countries like New Zealand, Canada, USA etc. However, no concerted efforts have been made in India- a country that is expected to benefit immensely by industrial plantations of conifers.

Chapter 3

Materials and Methods

Conventional methods of breeding conifers include the use of seeds collected from seed orchards, grafting and rooting of cuttings. But conventional breeding is a long-term endeavour. Vegetative propagation would alleviate time constraints, but has a low success rate and is especially difficult with mature trees. *In vitro* culture techniques, especially somatic embryogenesis, provides an alternative routes to clone rapidly a large number of genetically alike plants with 'elite' characters for direct use by forest nurseries involved in tree improvement programmes. Somatic embryogenesis refers to the development of embryos from somatic cells. In angiosperms, somatic embryogenesis has been studied for more than four decades but in contrast, conifer somatic embryogenesis is recent and started only one and a half decade back. Since the first report of conifer somatic embryogenesis, our knowledge and understanding pertaining to factors controlling induction and development of somatic embryos has made a remarkable progress (Attree *et al.*, 1995; Carrier *et al.*, 1997; Vagner *et al.*, 1998; Arya *et al.*, 2000; Mathur *et al.*, 2000). Different explants

have been used for induction of somatic embryogenesis in conifers. Mature and immature zygotic embryos, cotyledons from germinating seeds, secondary needles, female gametophytes etc. are used as explants for somatic embryogenesis. The success of somatic embryogenesis in conifers largely depends on the selection of explants at the right developmental stage, their collection period, storage, media composition, growth regulators and culture conditions.

Plant materials

Attempts were made to induce somatic embryogenesis using the following explants in the present study:

i) Female gametophytes

ii) Mature zygotic embryos

iii) Secondary needles

iv) Apical dome sections

Female gametophytes: Immature second year green cones from open pollinated trees of *P. kesiya* Royle ex. Gord. were collected from Barapani, during the months of May-October from '+' trees identified by Forest Department, Government of Meghalaya, India, at 15-days interval. Five trees were selected for collection of cones. The cones were stored for a maximum of 15 days at 4°C in cardboard boxes.

Mature zygotic embryos: Mature cones were collected from above mentioned '+' trees during late January to March. Seeds were extracted by air-drying the cones at 20-25°C. The seeds were stored at 4°C in sealed polythene bags till used.

Secondary needles: Secondary needles were harvested from the same '+' trees round the year. After collection needles were placed in water containing 100 mgl^{-1} polyvinyl pyrollidone (PVP) as an anti-oxidant.

Apical dome sections: Shoot tips (2-3 cm) were harvested before the emergence of needles round the year from mature '+' trees. Immediately after collection, these were transferred to PVP (100 mgl^{-1}) solution to prevent exudation of phenolics.

Sterilization of explants

Female gametophytes: The green cones were cut into two halves and immature seeds were removed carefully and surface cleansed with Labklin (1% v/v) (commercial laboratory detergent) for 10 min and washed thoroughly under tap water for 10-15 min. The immature seeds were then sterilized with 0.1% mercuric chloride ($HgCl_2$) for 10 min followed by 5-6 rinses with sterile ultra pure water (from Milli Q water purification system).

Mature zygotic embryos: Stored seeds were surface cleansed with 5% (v/v) commercially available (Qaligens) sodium hypochlorite

(5% v/v; 0.2% available chlorine) in a beaker using a magnetic stirrer for 5 min and thereafter washed under running tap water for 15 min. These were further treated with 6% (v/v) hydrogen peroxide (H_2O_2) for 10 min and washed with sterilized pure water for 4-5 times. The treated seeds were stratified at 4^0C for 24 h. Prior to dissection of embryos, the seeds were again sterilized with $HgCl_2$ (0.5% w/v) for 3-5 min and washed with sterilized pure water for 4-5 times.

Secondary needles: The healthy needles were surface cleansed with 'Cetrimide*'{(1:100 v/v), * equivalent to cetrimide IP 20% w/v} for 5 min and thereafter washed under running tap water for 10-15 min. These were then sterilized with $HgCl_2$ (0.25% w/v) for 3-5 min and washed 4-5 times with sterile ultra pure water. Finally the needles were dipped in 70% ethanol followed by immediate rinse with sterilized ultra pure water before inoculation.

Apical dome sections: Young needles, scales etc. were removed from shoot tips and apical dome sections measuring about 1 cm were cut. These were surface cleansed with 'Labklin' (1% v/v) for 10 min and washed under running tap water for 10-15 min. These were then sterilized with $HgCl_2$ (0.20% w/v) for 3 min and washed several times with sterile ultra pure water and finally treated with 70% ethanol for 30 sec, followed by a couple of rinses with sterile ultra pure water.

Tissue Culture

Media

The nutrient media used in the experiments for initiation of embryogenic cultures included MS (Murashige and Skoog, 1962), ½MS, modified MS [mMS (Gupta and Durzan, 1986b)], ½mMS, DCR (Gupta and Durzan, 1985), Litvay's (Litvay *et al.*, 1985), ½Litvay's, BM_1 (Gupta and Pullman, 1991), ½BM_1 (Table 3). All the half strength media contained half inorganic and full strength of organic nutrients. The female gametophytes, mature zygotic embryos, secondary needles and apical dome sections were explanted on all the above-mentioned media. Sucrose, maltose, lactose and fructose were incorporated in the media singly as organic carbon source (0-4%). A range of concentrations of different plant growth regulators (PGRs) was used in the media singly or in various combinations. 2,4-D, NAA (each at 0-10 mgl^{-1} singly or in combination) and BAP (0-5 mgl^{-1}) were used in different media for the culture of female gametophytes. Zygotic embryos were cultured in the media containing 2,4-D, NAA (each at 0-10 mgl^{-1} singly or in combination) and BAP (0-5 mgl^{-1}). In case of secondary needles 2,4-D, NAA (each at 0-5 mgl^{-1} singly or in combination) and BAP (0-5 mgl^{-1}) were incorporated in the media mentioned above. Apical dome sections were cultured on media

Table 3. Composition of different media used for initiation of somatic embryogenesis

Components	MS	mMS	DCR	BM_1	Litvay's
Inorganic salts (mgl^{-1})					
NH_4NO_3	1650.00	550.00	400.00	603.80	1650.00
KNO_3	1900.00	4460.00	340.00	909.00	1900.00
$Ca(NO_3)_2.4H_2O$	-	-	556.00	236.20	-
H_3BO_3	6.20	6.20	6.20	15.50	31.00
KH_2PO_4	170.00	170.00	170.00	136.10	340.00
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.125	1.25
KI	0.90	0.90	0.83	4.15	4.15
$CoCl_2.6H_2O$	0.10	0.10	0.025	0.125	0.125
$CaCl_2.6H_2O$	440.00	440.00	85.00	-	22.00
$MgSO_4.7H_2O$	370.00	370.00	370.00	246.50	1850.00
$MnSO_4.4H_2O$	22.30	22.30	22.30	10.50	21.00
$ZnSO_4.7H_2O$	9.00	9.00	8.60	14.40	43.00
$CuSO_4.5H_2O$	0.10	0.10	0.25	0.125	0.50
Na_2EDTA	37.00	37.00	37.30	9.33	37.20
$FeSO_4.7H_2O$	28.00	28.00	27.80	6.95	27.80
$NiCl_2$	-	-	0.025	-	-
$Mg(NO_3)_2.6H_2O$	-	-	-	256.50	-
$MgCl_2.6H_2O$	-	-	-	50.00	-
Organic nutrients (mgl^{-1})					
Thiamine HCl	1.00	1.00	1.00	1.00	1.00
Pyridoxine HCl	0.50	0.50	0.50	0.50	0.50
Nicotinic acid	0.50	0.50	0.50	0.50	0.50
Glycine	2.00	2.00	2.00	2.00	2.00
Carbohydrate	*	*	*	*	*
Gelling agents gl^{-1}					
Difco bacto agar	7.50	7.50	7.50	7.50	7.50
PGRs	*	*	*	*	*
PH	5.80	5.80	5.80	5.80	5.80

* Concentrations are mentioned in the materials and methods for different explants

PGRs: Plant growth regulators

consisting of 2,4-D, NAA (each at 0-7 mgl^{-1} singly or in combination) and BAP (0-5 mgl^{-1}). With all the above-mentioned explants, a set of experiment was carried out where the media were supplemented only with cytokinins (BAP and kinetin each at 0-5 mgl^{-1} singly or in combination) but lacking auxins.

For gelling the media, Difco-bacto agar (0.75-0.8%) was used. The pH of the medium was adjusted to 5.8 using 0.1N NaOH and 0.1 N HCl. About 15 ml medium was dispensed in each test tube (150x25 mm). 10 and 50 ml of liquid medium were dispensed in 100 and 250 ml Erlenmeyer flasks, respectively. The media were autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 15 min. About 12 ml and 20 ml autoclaved medium containing agar was poured in sterile disposable petri plates (Tarson) of 60 mm and 90 mm diameter respectively. The petri plates were sealed with parafilm (American National Can). 150-200 ml of sterilized liquid medium was dispensed in 500 ml capacity bubble column bioreactors (BCBs). The plant growth regulators (Hi-Media) were added to the media before adjustment of pH and autoclaving. The thermo labile chemicals like ABA was filter sterilized and added to the luke warm autoclaved media in a laminar flow table.

Induction of somatic embryogenesis on semi-solid media

Initiation of cultures

Female gametophytes: The female gametophytes were dissected out from sterilized immature seeds in a laminar flow table and cultured on different media. Five to six mega-gametophytes were cultured in each 60 mm sterile disposable petri plates.

Effect of developmental stage of female gametophytes on somatic embryogenesis: To identify the best developmental stage of the female gametophyte for embryogenesis, green cones were collected at regular time intervals as mentioned earlier. The mega-gametophytes containing the immature zygotic embryos (stage a, b, c, d and e defined after examination; Table 4) were cultured on different initiation media over a 5 month period (May-October).

Mature zygotic embryos: The embryos were dissected out from the seeds in laminar flow table and cultured on different media. Two embryos were cultured in each test tube.

Secondary needles: Sterilized needles were cultured in different media in petri-dishes (90mm dia). Five needles were cultured in each petri plate. Needles were inoculated in such a way that the whole surface of the needle remained in contact with the medium.

Table 4. Developmental stages of immature zygotic embryos 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

Apical dome sections: The sections were cultured on various media as stated earlier. Two sections were inoculated per test tube with up side up orientation.

Incubation of cultures

All the four types of explants were incubated in the dark for the induction of embryogenic cultures and their multiplication at 25±2°C. The first sub-culturing of female gametophytes was done just after the extrusion from micropylar ends (i.e. after 4-6 weeks). Thereafter, these were sub-cultured after 2 weeks for 1-2 passages till the extrusion showed considerable increase in size. The zygotic embryos, needles and apical dome sections were subcultured after 2 weeks for 1-2 passages depending on the proliferation of cultures. In case of zygotic embryo, needle and apical dome section, callusing started within 10-15 days time.

Maintenance and multiplication of cultures

The embryogenic calli (soft, white and translucent) obtained from female gametophytes, zygotic embryos and soft-white calli from secondary needles and apical dome sections were transferred to respective initiation media (½Litvay's, mMS, MS and ½DCR, respectively) containing sucrose and maltose (3% each singly) and a range of growth regulators (0, 1/5th, 1/10th and normal concentrations of

the initiation medium) for further multiplication and development. Thereafter, these media were referred to as maintenance media.

Somatic embryogenesis in suspension culture

Initiation of suspension cultures in shake flask and bubble column bioreactor

Suspension cultures were established using rapidly growing embryogenic calli in both shake flask (SF) and bubble column bioreactor (BCB). Embryogenic suspensions were initiated by the addition of approximately 2 g fresh weight of callus into 50 ml of medium (i. e. 40 gl^{-1} medium) in 250 ml Erlenmeyer flasks. Besides above mentioned inoculum size, a range of inoculum density (5-100 gl^{-1} medium) was used in the initiation experiment. The cultures were maintained in the dark at 25±2°C on an orbital shaker (B. Braun, Certomat U) at 100rpm.

Female gametophytes: The embryogenic cultures raised on semisolid medium were transferred in ½Litvay's liquid medium containing sucrose, maltose, lactose and fructose (1-4% each singly), 2,4-D (0-10 mgl^{-1}), NAA (0-10 mgl^{-1}), BAP (0-5 mgl^{-1}) and kinetin (0-5 mgl^{-1}) with other adjuvants as used in semisolid initiation medium.

Mature zygotic embryos: The embryogenic cultures were transferred in mMS liquid medium containing sucrose, maltose, lactose and fructose (1-4% each singly), 2,4-D, NAA (0-10 mgl^{-1} each), BAP (0-5 mgl^{-1}) and kinetin (0-5 mgl^{-1}) and other adjuvants as incorporated in the semi-solid initiation medium.

Secondary needles: The soft-white calli were cultured in MS liquid medium containing sucrose, maltose, lactose and fructose (1-4% each singly), 2,4-D, NAA (0-7 mgl^{-1} each), BAP (0-5 mgl^{-1}) and kinetin (0-5 mgl^{-1}) and other adjuvants as used in the semi-solid initiation medium.

Apical dome sections: The soft-white calli obtained from apical dome sections were cultured in ½DCR liquid medium containing sucrose, maltose, lactose and fructose (1-4% each), 2,4-D and NAA (0-7 mgl^{-1} each), BAP (0-5 mgl^{-1}) and kinetin (0-5 mgl^{-1}). Other adjutants were the same as used in the semi-solid initiation medium.

Suspension cultures in BCBs

The embryogenic calli obtained from female gametophytes, zygotic embryos and soft white calli from secondary needles and apical dome sections were transferred to different media as used in SFs. The initial inoculum density was maintained like that of SF (2g fresh wt/50 ml medium). The cultures were maintained in self-designed BCBs

(Figure 3) in the dark at 25±2°C. BCB vessels were fabricated of autoclaveable glass. The shape and structure of the BCB vessels were based on the necessary requirement and resource availability. Capacity of each BCB vessel was 500ml. The upper side of the vessel consisted of a detachable cap, with two channels for air inlet and air over pressure outlet. The glass air inlet tube was prolonged to the bottom of the vessel and consisted of an opening of 0.5mm size, which acted as air sparger. The glass tubings were connected with autoclavable silicon tubing and connectors that were ultimately connected to the air pump. Multiple use autoclaveable glass jar humidifiers with half full of sterile pure water were connected to the airflow pump. The bioreactors were supplied with air from an ordinary aqua-pump (used for aeration in aquarium). Both air inlet and air outlet channels were fitted with 0.45μ membrane filters (Millipore). The dry air from the pump was moistened by allowing it to flow through humidifying jar mentioned above. The upper end of the BCB vessel was broad while the bottom of the vessel was narrow and cone shaped to avoid accumulation of cells. Agitation in the bioreactor was achieved entirely by the air bubbles rising through the medium. The flow rate of air was maintained between 240 to 320 bubbles/min.

The detachable cap with air inlet-cum-sparger and air outlet, filter valves, tubings and connectors were assembled under aseptic conditions

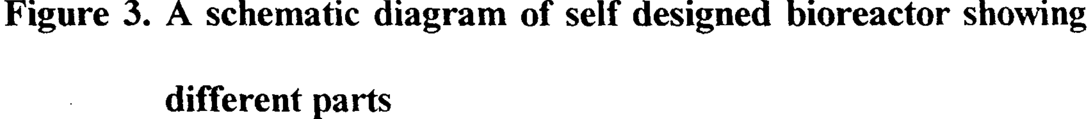

Figure 3. A schematic diagram of self designed bioreactor showing different parts

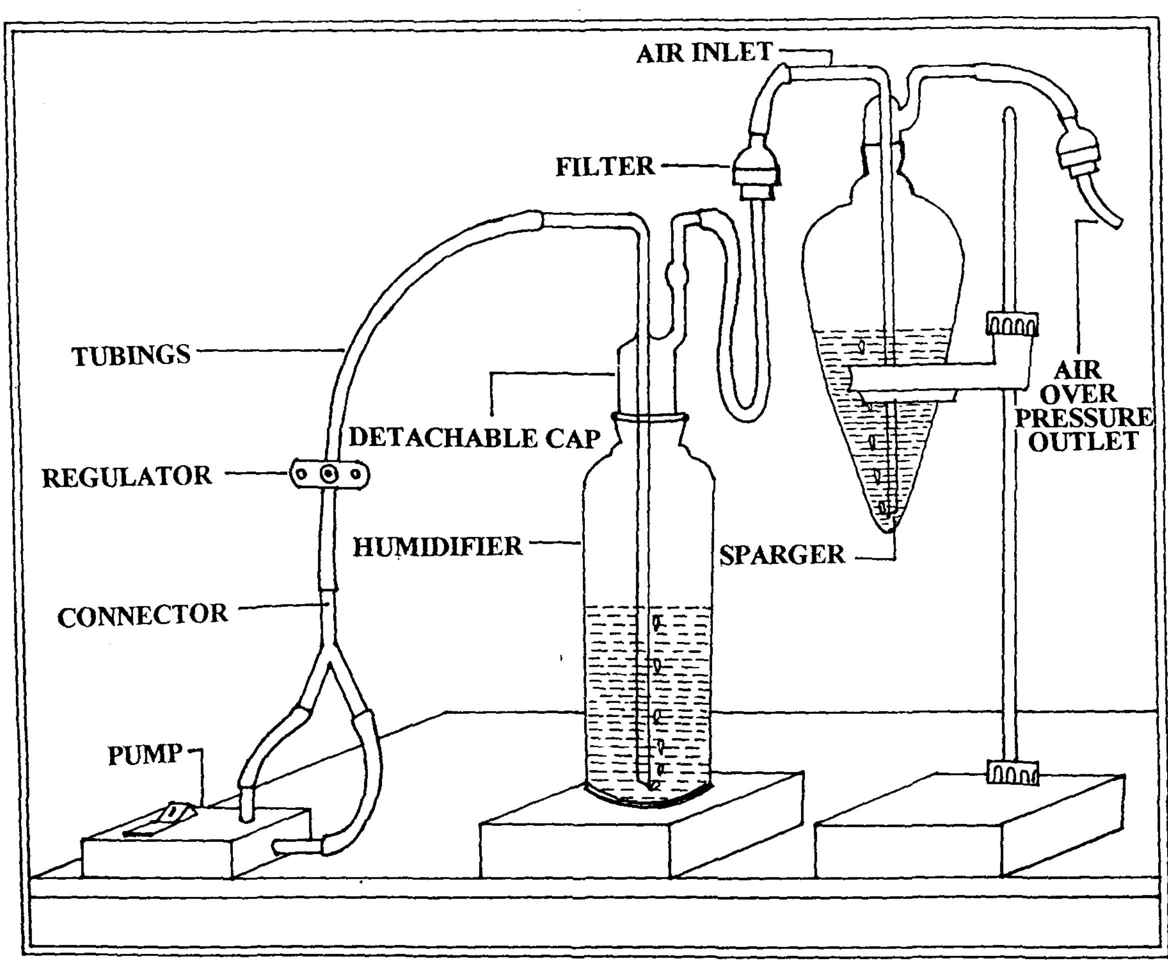

Figure 3

and connected to the vessels, which were charged with media mixed with the cultures. The air, which was made to flow through the spargers supplied by a pump, was used for both mixing and aeration of the cultures, thus relieving the need for mechanical stirring.

The sub-culturing was done with fresh liquid medium at 7 days interval. Before sub-culturing the cells were allowed to settle down for 30 mins. The spent medium was removed with the help of an autoclaved pipette connected to a sterile rubber bulb replenished with fresh medium. The cultures were maintained in the dark at 25±2°C.

Establishment of suspension cultures in SF and BCBs

Once suspension culture was initiated, the embryogenic suspensions were established by sub-culturing at 1 week interval by adjusting the sedimented culture volume (SCV) to 10±1% (v/v) with fresh medium. For adjustment of SCV, the cultures were aseptically poured into 100 ml sterilised measuring cylinders in a laminar flow table. After 30 min of sedimentation, the supernatant was discarded leaving 10 ml of sedimented cells. New medium was added to a total volume of 100 ml and the suspensions were poured into 250 ml Erlenmeyer flasks or BCB vessels and incubated aseptically as mentioned above. Also a range of SCV (5-100%) was tried to see the effect of SCV on establishment of suspension cultures.

Maintenance and multiplication of suspension cultures in SF and BCB vessels

After 3-4 passages in the initiation medium, the embryogenic cultures from various explants were transferred to respective basal media containing sucrose or maltose (3%) and a range of growth regulators (0, $1/5^{th}$, $1/10^{th}$ and normal concentration to that of the initiation medium). The cultures were maintained for 4-5 passages in the above mentioned media (thereafter referred to as maintenance media) for further multiplication and development. During multiplication and maintenance, the inoculum concentration was maintained at 100 ml SCV l^{-1} medium. All cultures were grown in the dark at 25±2°C.

Embryo development and maturation

The embryogenic cultures were transferred to respective media (½Litvay's, mMS, MS and ½DCR) containing varying concentrations of organic carbon (sucrose 0-12% singly and sucrose and mannitol each at 0-6% in combination) with or without ABA (0-10 mgl^{-1}). The media mentioned above were referred to as maturation media.

Embryogenic tissues were collected 1 week following subculture and allowed to undergo maturation. Three methods were employed for maturation experiments: (1) 1 g embryogenic cultures containing

pro-embryos and stage-I embryos were transferred directly to agar solidified medium (*Direct culture method*) (2) embryogenic cultures from different culture vessels were collected, combined and suspended in respective liquid growth regulator-free media, and shaken vigorously by hand for a few seconds. 3ml of the embryogenic tissue suspension were than distributed on to sterile filter paper (Whatman # 1) (*Filter paper base method*). The liquid medium was drained for 10 sec and the filter papers with the attached layer of tissues were placed on the maturation medium. Petri dishes were then sealed with parafilm. Four weeks after the beginning of the maturation period, filter papers and their embryogenic tissues were transferred onto fresh maturation media (3) embryogenic liquid suspension cultures from SFs and BCBs were transferred to petri dishes (3ml/plate) containing respective maturation media. Each medium consisted of a thin layer of agar-solidified medium covered with 7ml liquid medium (*submerge culture method*). The submerged cultures were agitated on an orbital shaker at 60 rpm. All the cultures were incubated at 25±2°C in the dark.

Growth parameters of embryogenic suspensions

A 6-month-old embryogenic suspension was used for growth studies. Fresh weight (FW), dry weight (DW), sedimented culture volume (SCV) and packed cell volume (PCV) were measured at 2-day

interval from day 0 (day of subculture) to day 14 (day of subculture). SCV was determined by pouring the content from each flask into a 100 ml graduated cylinder and allowing the cultures to settle for 30 min. Fresh weight was obtained by removing the medium from 10 ml of suspension by passing it through a pre-weighed filter paper followed by re-weighing. The dry weight was recorded after drying the sample for 24 h at 80°C to a constant weight. PCV was determined by centrifuging 10 ml of suspension at 2000 rpm for 5 min. Another 10 ml of suspension was used to determine the number of stage-III embryos (described later) per ml.

For semi-solid culture, the increase in fresh weight of callus and number of stage III embryos formed g^{-1} fresh weight of callus was determined using a 6-month old culture.

For growth parameter studies, embryogenic cultures from different cell lines were pooled together both for suspension and semi-solid cultures.

Microscopic observation

Cultures were routinely observed under stereo-zoom (Leica Wild M3Z) and inverted microscope (Leitz Fluovert FU) to study the nature of embryogenic and non-embryogenic cultures.

Germination of somatic embryos

The cotyledonary somatic embryos (after 10-12 weeks of maturation) obtained from embryo development and maturation medium from SFs and semi-solid medium were singulated and cultured in respective germination media without growth regulators (½ and ¼Litvay's for female gametophyte and mMS and ½mMS for zygotic embryo cultures) containing sucrose (1-3%) devoid of growth regulators but containing 0.8% Difco-bacto agar. These were referred to as germination media. No somatic embryo (SE) formation was observed form the cultures obtained from secondary needles and apical dome sections and therefore, no germination treatment was given. For germination, 10 SEs were cultured in each culture vial using both *direct culture* and *submerge culture- methods*. The cultures were maintained at 25±2°C and under 0-2200 lux and 0-12h photoperiods. The SEs were subcultured at 2-week interval. SEs were considered germinated as soon as radicle and hypocotyl elongation occurred.

After 3-4 weeks, SE showing elongated radicle and hypocotyl were counted and the germination percentage was evaluated [(Number of germinated SE x 100) ÷ Total number of mature SE]. Conversion of germinated SE to embling was scored based on the presence of an epicotyl (ca 0.5cm). The number of SEs germinated and converted to

emblings were determined after 7-10 weeks of culture on germination medium and the emblings were then hardened.

Hardening and establishment of emblings in soil

When epicotyls were about 1cm long (ca 8-10 weeks on germination medium), emblings were transferred to a substrate of mixture of peat: vermiculite: pumice (1: 1: 1) and soil obtained from Khasi pine forests. The emblings were then placed in a growth room under a 12h photoperiod (1900 lux) at 24±2^{0}C. For the first 2 weeks, each embling was covered by transparent poly bag and watered once a day to keep the humidity near the saturation point. After 1-2 weeks, the poly bags were gradually removed to reduce humidity, this was achieved by removing the polythene bags on the 3rd week. On the first day of 3rd week the plantlets were kept without bags for 1h, 2nd day for 2h, 3rd day for 4h, 4th day for 6h, 5th day for 8h, 6th day for 10h & 7th day for 12h. After this gradual treatment emblings were maintained for another 2-3 weeks in 12h photoperiod of 1900 lux at 24±2°C). Thereafter, the acclimatized emblings were kept for 4 weeks in a green house fitted with misting pipes.

Field performance of regenerants

The pots containing emblings were taken out from the green house after 4 weeks and were kept outside in the shade and watered after every 2 days. After two weeks of growth in the shade the emblings were brought to sun. The growth performance of the emblings was evaluated at 45d, 90d, and 180 days after transfer from green house to the open area. Survival rate of emblings was evaluated at 45, 90 and 180 days. The growth performance of seed derived plantlets was evaluated side by side. The growth pattern of emblings and the seedlings were compared to find the performance of emblings in natural conditions.

For evaluation of growth following parameters were taken: at each evaluation date, shoot length, root length and biomass per plant was determined. For determination of biomass the entire embling or seedling was oven dried at 80°C for 48h and their final weight was taken. Before determination of biomass, roots were thoroughly washed to remove adhering soil particles. Five emblings and seedlings each were used on every evaluation date.

Survivability of the regenerants

The survivability of the emblings was evaluated on 45, 90 and 180 day and the percentage survivability was calculated as- (Number of

emblings survived x 100) ÷ Total number of emblings transferred to pots). Survivability of emblings and seedlings of different age groups were carried out on each evaluation date.

Chapter 4

Results

Induction of somatic embryogenesis on semi-solid medium

Effect of developmental stage and collection period of explants

Female gametophytes: The development stages of zygotic embryo in the female gametophytes (Figure 4a) and their response to induction of somatic embryogenesis were examined. The stage 'c' embryos (Figure 4b) with head size 0.2-1.1mm was highly responsive and most appropriate stage showing 35% somatic embryogenesis. The stage 'a' embryos resulted in poor embryogenic response (1.6%), which could not be maintained for 3½ months. The embryogenic callus from stages - 'b' 'c' and 'd' were 3.4, 15.2 and 3.5 % respectively (Table 5) after 3½ months of culture.

Mature zygotic embryos: While the seeds collected during December to late February were suitable for initiation of embryogenic cultures, the seeds collected during March exhibited poor response. An embryogenic response of 12.5% was recorded in mMS medium after 1½ month that declined to 2% after 3½ months of culture (Table 5).

Figure 4. Different stages of somatic embryogenesis from female gametophyte explants in semi-solid cultures

a. Female gametophyte explants (0.63 x 10)

b. Isolated stage-c zygotic embryo (40x)

c. Female gametophyte with embryogenic extrusion (0.63 x 25)

d. Embryogenic callus from extrusion (0.63 x 16)

e. Two cotyledonary SEs attached to the callus by suspensor like cells (0.63 X 40)

f. SEs turned green when kept in the light (0.63 x 10)

g. Germinated SEs

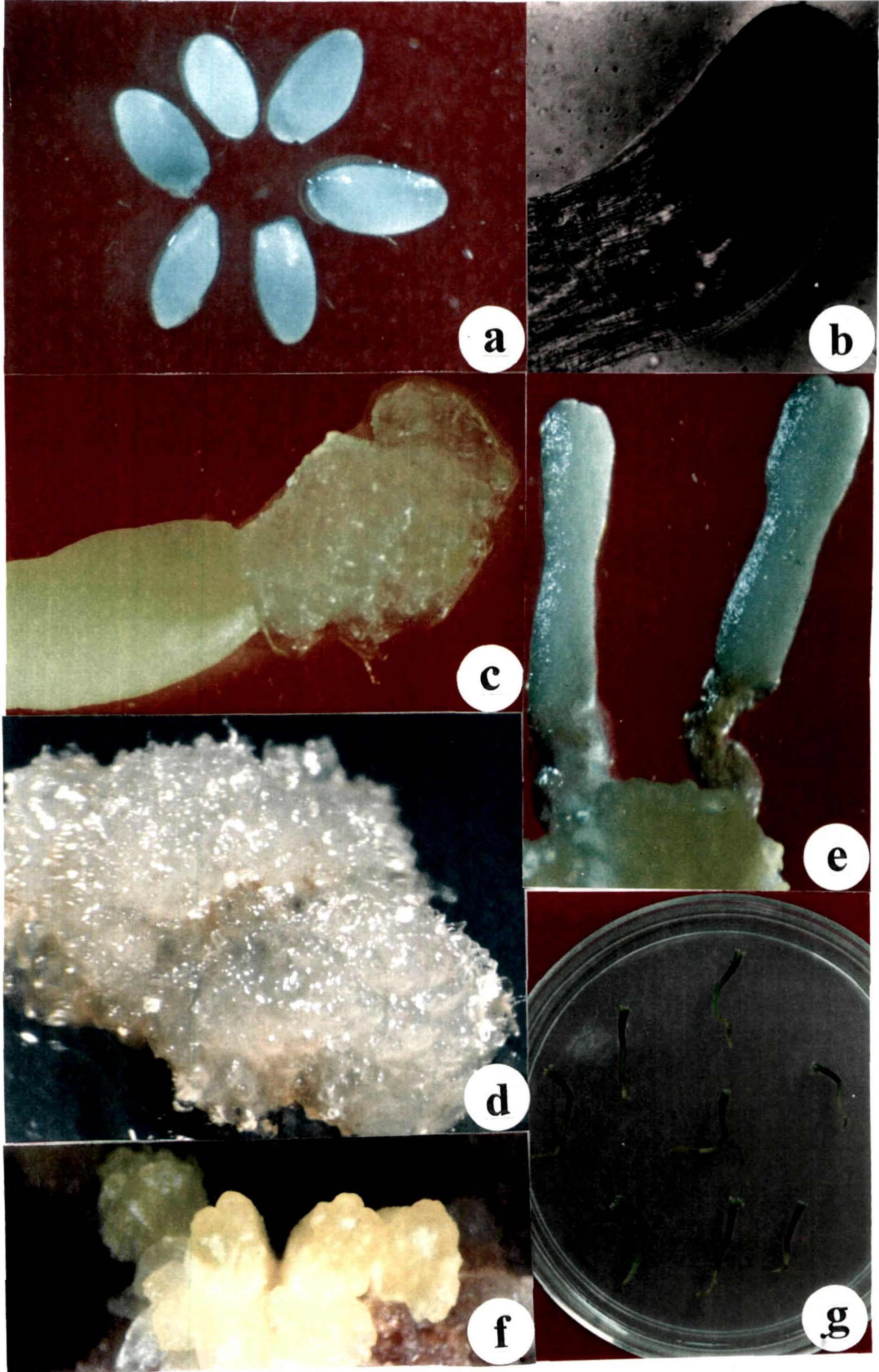

Figure 4

Table 5. Effect of developmental stages of female gametophytes containing immature zygotic embryos on frequency of somatic embryogenesis

Defined stage of zygotic embryo	Number of explants cultured	Embryogenic calli induced after 1½ months (%)	Embryogenic calli after 3½ months (%)* (±SE)[1]
Stage 'a'	500	1.60 (±1.22)	0.00 (±1.51)
Stage 'b'	500	7.00 (±1.45)	3.40 (± 1.18)
Stage 'c'	500	35.00 (±1.40)	15.20 (± 1.27)
Stage 'd'	500	16.10 (±1.37)	3.50 (± 1.29)
Stage 'e'	500	14.50 (±1.59)	2.00 (± 1.42)
Mature zygotic embryo	500	12.50 (± 1.38)	2.00 (± 1.30)

Female gametophytes (with immature zygotic embryos) were cultured on ½ Litvay's medium containing 2,4-D (5 mgl^{-1}), NAA and BAP (2.5 mgl^{-1} each), sucrose (3.0%) and Difco-bacto agar (0.75%), whereas, mature zygotic embryos were cultured on mMS medium containing 2,4-D and NAA (5 mgl^{-1} each) and BAP (2.5 mgl^{-1}) and adjuvants used were same as stated above.

* The embryogenic cell lines get established and continue to proliferate up on subculture

[1]: Standard error

Secondary needles: Secondary needles collected during March to June produced healthy, white calli.

Apical domes: Apical domes collected during May to July produced healthy, white calli.

Culture media (semi-solid) for initiation of embryogenesis

Effect of media and carbohydrates

Female gametophytes: The female gametophytes containing stage 'c' embryo (Figure 4a-b) exhibited the best embryogenic cultures on ½Litvay's medium followed by BM_1 and DCR (Table 6). In 4-6 weeks in culture, the female gametophytes exhibited the formation of two types of extrusions: (a) white, soft and translucent embryogenic extrusion (Figure 4c) and (b) hard brownish and small extrusion (Figure 5a). The hard brownish extrusion turned totally brown in 1-2 weeks in culture and died or formed non-embryogenic calli (Figure 5b-c)

Sucrose was essential as a carbon source for initiation of cultures. No growth was recorded in its absence. In general, the increase of sucrose concentration from 1-3% in various media resulted in higher embryogenic cultures (Table 6). However, 4% sucrose in the medium resulted in rapid browning of cultures.

Maltose was also found to be effective in initiation of cultures. An increase in maltose concentration from 1-3% showed increased

Figure 5. Female gametophyte explant showing non-embryogenic cultures in semi-solid medium

a. Hard non-embryogenic extrusion (0.63 x 25)

b. Hard non-embryogenic callus from extrusion (0.63 x 16)

c. Non-embryogenic callus turned brownish (0.63 x 16)

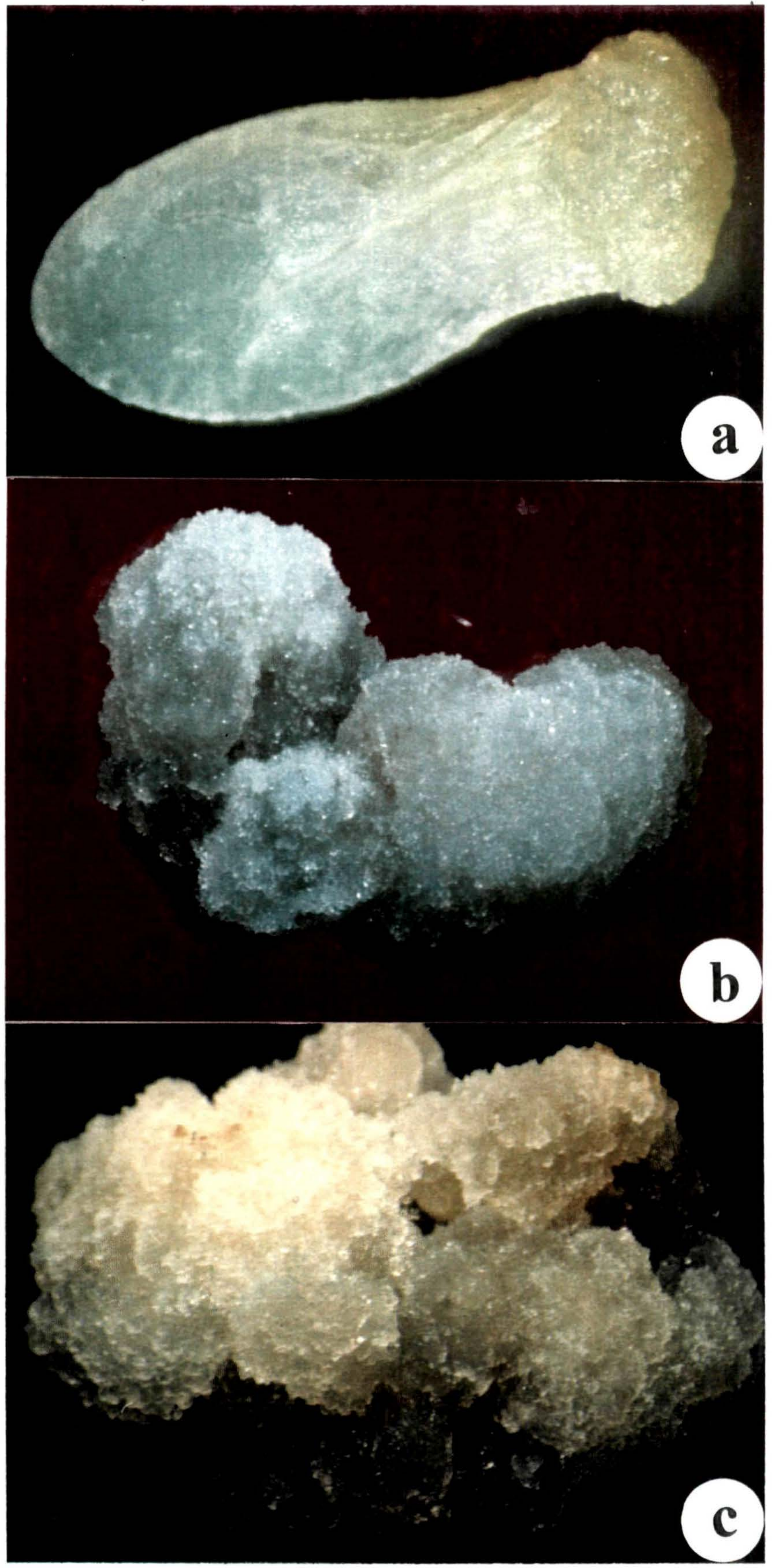

Figure 5

Table 6. Effect of different media and sucrose concentrations on initiation of embryogenic cultures from female gametophytes containing stage 'c' embryos

Media*	Sucrose (%)	Type of response**	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Explants produced soft extrusions and in subsequent subcultures extrusions turned slightly brown	1.70 (±1.03)	20.53 (±1.45)
	2.0	Explants produced soft-translucent extrusions but most of these turned non-embryogenic and brownish	3.50 (±1.01)	55.10 (±1.70)
	3.0	-do-	4.65 (±1.80)	60.00 (±1.85)
	4.0	-do-	2.00 (±1.20)	28.21 (±1.32)
MS	0.0	No response	0.00	0.00
	1.0	Very little soft-translucent extrusions which turned brownish in subsequent subcultures	1.23 (±1.33)	32.43 (±1.31)
	2.0	-do-	2.30 (±1.81)	52.00 (±1.10)
	3.0	-do-	4.90 (±1.22)	60.54 (±1.55)
	4.0	-do-	1.50 (±1.20)	25.35 (±1.32)
½mMS	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed but cultures were not very healthy and failed to proliferate	4.50 (±1.48)	38.00 (±1.88)
	2.0	As above but comparatively healthy cultures most of these turned brownish in subsequent subcultures	4.92 (±1.33)	44.10 (±1.78)
	3.0	-do-	7.51(±1.43)	48.10 (±1.81)
	4.0	-do-	4.00 (±1.31)	38.50 (±1.63)
mMS	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed and very few cultures proliferated	5.20 (±1.21)	50.19 (±1.76)
	2.0	As above but exhibited comparatively healthy culture proliferation	8.15 (±1.09)	55.25 (±1.78)
	3.0	Extrusions were fairly healthy with better proliferation	12.38 (±1.55)	61.50 (±1.98)
	4.0	Extrusions turned slight brownish	6.00 (±1.35)	23.00 (±1.54)
½DCR	0.0	No response	0.00	0.00
	1.0	Explants produced soft-translucent extrusions but were not very healthy and most of which failed to proliferate	4.10 (±1.23)	26.20 (±1.67)
	2.0	As above but comparatively healthy and most of the cultures turned light brown in subsequent subcultures	4.50 (±1.34)	28.10 (±1.41)
	3.0	-do-	5.25 (±1.34)	35.00 (±1.76)
	4.0	Extrusions turned light brownish	4.00 (±1.25)	30.40 (±1.95)

contd...

DCR	0.0	No response	0.00	0.00
	1.0	Explants produced soft-translucent extrusions, cultures were moderately healthy and exhibited some proliferation and did not turn brownish	10.00 (±1.30)	28.20 (±1.90)
	2.0	Extrusions were healthy and exhibited good proliferation, did not turn brownish on subsequent subcultures	17.50 (±1.40)	22.32 (±1.44)
	3.0	Explants showed very healthy extrusions and proliferation was very much satisfactory and did not turn brownish in subsequent subculture	29.00 (±1.38)	20.13 (±1.01)
	4.0	Extrusions turned light brownish	9.00 (±1.86)	29.00 (±1.91)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions, cultures moderately healthy and exhibited some proliferation, cultures did not turn brownish in subsequent subcultures	10.00 (±1.35)	39.50 (±1.56)
	2.0	As above but cultures were comparatively healthier	15.20 (±1.39)	26.80 (±1.88)
	3.0	Explants produced very healthy extrusions and proliferation was very satisfactory	35.00 (±1.40)	22.50 (±1.76)
	4.0	Extrusions turned light brownish	11.30 (±1.28)	42.00 (±1.79)
Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed but very few cultures proliferated	4.00 (±1.50)	29.00 (±1.74)
	2.0	As above and some of the cultures turned slightly brownish in subsequent subcultures	4.73 (±1.66)	35.00 (±1.62)
	3.0	-do-	11.00 (±1.56)	30.34 (±1.36)
	4.0	Extrusions turned light brownish	4.00 (±1.31)	28.79 (±1.89)
½BM_1	0.0	No response	0.00	0.00
	1.0	Explants produced soft extrusions and in subsequent subculture turned slightly brownish	4.00 (±1.22)	35.00 (±1.54)
	2.0	Explants produced soft-translucent extrusions but most of these turned no-embryogenic and brownish	4.80 (±1.10)	33.45 (±1.67)
	3.0	-do-	7.21 (±1.23)	31.22 (±1.55)
	4.0	Extrusions turned light brownish	4.30 (±1.20)	35.00 (±1.66)
BM_1	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed and very few cultures proliferated	15.50 (±1.38)	32.15 (±1.46)
	2.0	As above but exhibited comparatively healthy culture proliferation	19.24 (±1.35)	30.22 (±1.72)
	3.0	-do-	30.68 (±1.77)	27.33 (±1.98)
	4.0	Extrusions turned light brownish	11.50 (±1.46)	37.20 (±2.12)

* Additional adjuvants: 2,4-D (5 mgl^{-1}); NAA and BAP (2.5 mgl^{-1} each) and Difco-bacto agar (0.75%); [1]: Standard error.

** Extrusions started in 4-6 weeks time

embryogenic culture formation (Table 7). But initiation and proliferation of cultures was quite slow with maltose as compared to sucrose. In case of sucrose containing medium, the extrusion started in 4-6 weeks while with maltose the extrusion started after 6-8 weeks. Proliferation of cultures was rapid in sucrose containing medium but was slow in medium containing maltose. However, browning of cultures was absent or very less in maltose containing medium as compared to sucrose. Maltose higher than 3% was harmful resulting in browning of cultures.

Both lactose and fructose did not promote either embryogenic or non-embryogenic calli formation (Table 8 and 9). A maximum of 5.5% embryogenic cultures were produced in ½Litvay's medium containing 3% lactose and 5.1% in the same medium but containing 3% fructose. These cultures could not be maintained due to rapid browning.

Mature zygotic embryos: Of the different media tested, the optimum embryogenic cultures resulted in mMS medium followed by DCR and BM_1 (Table 10). The excised embryos (Figure 6a and 7a) started callusing within 12-14 days in the culture medium (Figure 6b and 7b). In 3 weeks the zygotic embryos exhibited: (a) white, soft, translucent embryogenic callus (Figure 6c) and (b) hard, non-embryogenic callus (Figure 7b-c). The cultures in ½MS and MS resulted in poor embryogenic response.

Table 7. Effect of different media and maltose concentrations on initiation of embryogenic cultures from female gametophytes containing stage 'c' embryos

Media*	Maltose (%)	Type of response**	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Explants produced soft extrusions and in subsequent subcultures extrusions did not proliferate much	1.56 (±1.13)	19.50 (±1.31)
	2.0	Explants produced soft-translucent extrusions but most of these turned non-embryogenic	4.00 (±1.20)	45.50 (±1.56)
	3.0	-do-	4.65 (±1.77)	52.30 (±1.85)
	4.0	Extrusions turned slight brownish	2.20 (±1.11)	25.00 (±1.51)
MS	0.0	No response	0.0	0.00
	1.0	Very few soft-translucent extrusions with slow proliferation which turned non-embryogenic in subsequent subcultures	1.40 (±1.23)	30.00 (±1.65)
	2.0	-do-	2.0 (±1.31)	50.00 (±1.20)
	3.0	-do-	4.90 (±1.42)	57.54 (±1.75)
	4.0	Extrusions turned slight brownish	1.68 (±1.23)	24.00 (±1.63)
½mMS	0.0	No response	0.0	0.00
	1.0	Soft-translucent extrusions were formed but cultures were not very healthy and failed to proliferate	4.00 (±1.18)	31.00 (±1.81)
	2.0	As above but comparatively healthy cultures most of these turned non-embryogenic in subsequent subcultures	4.52 (±1.39)	40.70 (±1.68)
	3.0	-do-	7.00(±1.63)	45.10 (±1.91)
	4.0	Extrusions turned slight brownish	4.20 (±1.17)	35.80 (±1.93)
mMS	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed and very few cultures proliferated. Initial culture proliferation was slow	4.75 (±1.50)	47.19 (±1.60)
	2.0	As above but exhibited comparatively healthy culture proliferation. Initial culture proliferation was slow	6.00 (±1.20)	52.00 (±1.38)
	3.0	Extrusions were fairly healthy. Initial proliferation was slow but subsequently showed better proliferation	11.00 (±1.49)	58.50 (±1.78)
	4.0	Extrusions turned slight brownish	4.80 (±1.27)	20.00 (±1.90)
½DCR	0.0	No response	0.00	0.00
	1.0	Explants produced soft-translucent extrusions but cultures were not very healthy and most of these failed to proliferate	3.75 (±1.50)	22.00 (±1.71)
	2.0	As above but comparatively healthy with slow proliferation and most of the cultures turned non-embryogenic in subsequent subcultures	4.00 (±1.41)	25.20 (±1.39)
	3.0	-do-	4.80 (±1.44)	30.00 (±1.51)
	4.0	Extrusions turned slight brownish	3.80 (±1.16)	25.00 (±1.51)

contd...

DCR	0.0	No response	0.00	0.00
	1.0	Explants produced soft-translucent extrusions, cultures were moderately healthy and though slow in growth, exhibited some proliferation and did not turn brownish	8.00 (±1.30)	24.20 (±1.85)
	2.0	Extrusions healthy and exhibited slow but good proliferation, did not turn brownish on in subsequent subcultures	15.20 (±1.40)	23.00 (±1.73)
	3.0	Explants showed very healthy extrusions and initial proliferation was slow but later on proliferation was very much satisfactory and did not turn brownish in subsequent subcultures	26.00 (±1.38)	22.50 (±1.01)
	4.0	Extrusions turned slight brownish	9.55 (±1.33)	26.20 (±1.83)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions, cultures moderately healthy and though slow in growth, exhibited some proliferation, cultures did not turn brownish in subsequent subcultures	10.00 (±1.15)	36.00 (±1.96)
	2.0	As above but cultures were comparatively healthier	14.50 (±1.22)	23.50 (±1.75)
	3.0	Explants produced very healthy extrusions, initial proliferation was slow but subsequently proliferation was satisfactory	30.00 (±1.40)	20.00 (±1.49)
	4.0	Extrusions turned slight brownish	9.00 (±1.23)	38.50 (±2.34)
Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed but very little cultures proliferated	3.90 (±1.25)	27.00 (±1.57)
	2.0	As above and some of the cultures turned slightly brownish in subsequent subcultures	4.00 (±1.38)	30.20 (±1.82)
	3.0	-do-	10.00 (±1.76)	32.13 (±1.29)
	4.0	Extrusions turned slight brownish	3.55 (±1.15)	25.00 (±1.93)
½BM_1	0.0	No response	0.00	0.00
	1.0	Explants produced soft extrusions and in subsequent subculture turned slightly brownish	3.00 (±1.42)	30.00 (±1.38)
	2.0	Explants produced soft-translucent extrusions but most of these turned non-embryogenic and brownish	4.00 (±1.25)	31.45 (±1.90)
	3.0	-do-	6.50 (±1.50)	31.00 (±1.70)
	4.0	Extrusions turned slight brownish	2.35 (±1.14)	29.00 (±1.60)
BM_1	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed and very few cultures proliferated	9.00 (±1.38)	30.15 (±1.51)
	2.0	As above but exhibited comparatively healthy culture proliferation	16.50 (±1.35)	28.00 (±1.90)
	3.0	-do-	28.00 (±1.77)	25.40 (±1.80)
	4.0	Extrusions turned slight brownish	10.00 (±1.05)	32.50 (±1.53)

* Additional adjuvants: 2,4-D (5 mgl^{-1}); NAA and BAP (2.5 mgl^{-1} each) and Difco-bacto agar (0.75%);
[1]: Standard error.
** Extrusions started after 6-8 weeks showing slow growth and proliferation of cultures

Table 8. Effect of different media and lactose concentrations on initiation of embryogenic cultures from female gametophytes containing stage 'c' embryos

Media*	Lactose (%)	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Explants produced soft-translucent extrusions and in subsequent subcultures they turned brownish	1.50 (±0.91)	13.81 (±1.21)
	2.0	-do-	1.93 (±1.34)	14.55 (±1.35)
	3.0	-do-	2.50 (±1.02)	15.10 (±1.30)
	4.0	Extrusions turned brownish	1.55 (±1.17)	13.50 (±1.71)
MS	0.0	No response	0.00	0.00
	1.0	Explants produce soft, yellow extrusions which turned brownish	0.00	14.90 (±1.31)
	2.0	-do-	0.00	17.50 (±1.42)
	3.0	-do-	0.00	20.11 (±1.22)
	4.0	Hard-brownish extrusions		
½mMS	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were produced by the explants most of which turned brownish in subsequent subcultures	1.56 (±0.97)	14.50 (±1.17)
	2.0	-do-	2.32 (±1.27)	16.20 (±1.21)
	3.0	-do-	3.10 (±1.31)	19.00 (±1.19)
	4.0	Extrusions turned brownish	1.90 (±1.10)	15.00 (±1.93)
mMS	0.0	No response	0.00	0.00
	1.0	Explants produce soft-translucent extrusions most of which failed to proliferate in subsequent subcultures and turned brownish	1.62 (±1.00)	18.00 (±2.10)
	2.0	-do-	2.11 (±0.98)	20.10 (±1.98)
	3.0	-do-	2.85 (±1.11)	21.20 (±1.78)
	4.0	Extrusions turned brownish	1.50 (±1.19)	19.10 (±1.80)
½DCR	0.0	No response	0.00	0.00
	1.0	Explants produce soft extrusions most of which turned brownish	0.98 (±0.50)	11.00 (±1.32)
	2.0	-do-	1.58 (±0.89)	13.20 (±1.09)
	3.0	-do-	2.30 (±0.99)	15.70 (±1.24)
	4.0	Extrusions turned brownish	1.12 (±1.09)	11.00 (±1.90)
DCR	0.0	No response	0.00	0.00
	1.0	Explants formed soft, white, translucent extrusions most of which turned brownish in subsequent subcultures	1.50 (±1.00)	15.20 (±1.11)
	2.0	-do-	2.90 (±1.45)	18.32 (±1.90)
	3.0	-do-	3.65 (±1.34)	20.25 (±2.10)
	4.0	Extrusions turned brownish	1.79 (±1.13)	16.00 (±1.85)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions formed but not very healthy and turned brownish in subsequent subcultures	2.80 (±0.97)	13.10 (±1.32)
	2.0	-do-	4.00 (±1.20)	16.35 (±1.39)
	3.0	-do-	5.50 (±1.20)	19.35 (±1.55)
	4.0	Extrusions turned brownish	2.00 (±1.16)	14.50 (±1.61)

Contd...

Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed but cultures failed to proliferate and turned brownish	2.00 (±1.05)	12.50 (±1.70)
	2.0	-do-	3.10 (±1.08)	15.60 (±1.43)
	3.0	-do-	4.20 (±1.10)	18.54 (±1.27)
	4.0	Extrusions turned brownish	2.43 (±1.14)	14.00 (±1.99)
½BM_1	0.0	No response	0.00	0.00
	1.0	Explants produced hard-yellowish extrusions which turned brownish in subsequent subcultures	0.00	13.20 (±1.30)
	2.0	-do-	0.00	15.45 (±1.50)
	3.0	-do-	0.00	15.91 (±1.13)
	4.0	Hard-brownish extrusions	0.00	11.80 (±1.94)
BM_1	0.0	No response	0.00	0.00
	1.0	Soft, white, translucent extrusions most of which turned brownish in subsequent subcultures	3.20 (±0.83)	15.65 (±1.11)
	2.0	-do-	4.00 (±0.98)	18.20 (±1.35)
	3.0	-do-	5.00 (±1.09)	21.78 (±1.41)
	4.0	Extrusions turned brownish	1.80 (±1.11)	16.50 (±1.78)

*Additional adjuvants: 2,4-D (5 mgl^{-1}); NAA and BAP (2.5 mgl^{-1} each) and Difco-bacto agar (0.75%); [1]: Standard error.

Table 9. Effect of different media and fructose concentrations on initiation of embryogenic cultures from female gametophytes containing stage 'c' embryos

Media*	Fructose (%)	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Explants produced hard extrusions	0.00	18.80 (±1.38)
	2.0	Explants produced hard extrusions which turned brownish in subsequent subcultures	0.00	20.30 (±1.43)
	3.0	-do-	0.00	21.40 (±1.58)
	4.0	Hard-brownish extrusions	0.00	20.00 (±1.97)
MS	0.0	No response	0.00	0.00
	1.0	Explants produced hard and non-embryogenic extrusions	0.00	19.50 (±1.98)
	2.0	As above and turned brownish in subsequent subcultures	0.00	20.30 (±1.74)
	3.0	-do-	0.00	21.42 (±1.86)
	4.0	Hard-brownish extrusions	0.00	19.00 (±1.91)
½mMS	0.0	No response	0.00	0.00
	1.0	Explants produced soft, white, translucent extrusions but turned brownish in subsequent subcultures	1.60 (±1.33)	9.30 (±1.71)
	2.0	-do-	2.00 (±1.43)	12.13 (±1.64)
	3.0	-do-	2.54 (±1.48)	13.11(±1.71)
	4.0	Extrusions turned slight brownish	1.50 (±1.20)	10.00 (±1.53)
mMS	0.0	No response	0.00	0.00
	1.0	Explants produced soft-translucent extrusions but turned brownish very rapidly	1.68 (±1.21)	12.50 (±1.75)
	2.0	-do-	2.30 (±1.67)	12.50 (±1.66)
	3.0	-do-	2.91 (±1.41)	13.20 (±1.34)
	4.0	Extrusions turned light brownish	1.75 (±1.03)	12.00 (±1.99)
½DCR	0.0	No response	0.00	0.00
	1.0	Explants produced hard extrusions which turned brownish	0.00	12.00 (±1.51)
	2.0	-do-	0.00	12.50 (±1.79)
	3.0	-do-	0.00	14.10 (±1.47)
	4.0	Hard-brownish extrusions	0.00	11.00 (±1.96)
DCR	0.0	No response	0.00	0.00
	1.0	Explants produced soft, white, translucent extrusions which turned brownish rapidly in subsequent subcultures	2.10 (±1.40)	13.30 (±1.67)
	2.0	-do-	2.72 (±1.48)	14.00 (±1.52)
	3.0	-do-	3.80 (±1.38)	15.20 (±1.76)
	4.0	Extrusions turned slight brownish	1.50 (±1.10)	13.00 (±1.69)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions which turned brownish rapidly in subsequent subcultures	1.31 (±1.01)	10.10 (±1.70)
	2.0	As above, but cultures were healthier	2.60 (±1.11)	13.00 (±1.50)
	3.0	-do-	5.10 (±1.32)	15.20 (±1.71)
	4.0	Extrusions turned slight brownish	3.00 (±1.21)	11.00 (±1.58)

contd...

Litvay's	0.0	No response	0.00	0.00
	1.0	Explants produced soft, slight yellowish extrusions which did not proliferate much	0.00	10.40 (±1.21)
	2.0	-do-	0.00	12.10 (±1.34)
	3.0	-do-	0.00	13.55 (±1.41)
	4.0	Hard-brownish extrusions	0.00	11.30 (±2.18)
½BM_1	0.0	No response	0.00	0.00
	1.0	Explants showed soft-yellowish extrusions and failed to proliferate	0.00	10.32 (±1.23)
	2.0	-do-	0.00	12.60 (±1.39)
	3.0	-do-	0.00	14.45 (±1.33)
	4.0	Hard-brownish extrusions	0.00	10.00 (±1.96)
BM_1	0.0	No response	0.00	0.00
	1.0	Soft-translucent extrusions were formed but cultures failed to proliferate and turned brownish very rapidly	1.70 (±0.90)	13.80 (±1.10)
	2.0	-do-	2.27 (±1.00)	14.10 (±1.44)
	3.0	-do-	4.00 (±1.32)	15.30 (±1.71)
	4.0	Extrusions turned slight brownish	2.00 (±1.15)	11.50 (±1.48)

* Additional adjuvants: 2,4-D (5 mgl^{-1}); NAA and BAP (2.5 mgl^{-1} each) and Difco-bacto agar (0.75%); [1]: Standard error.

Table 10. Effect of different media and sucrose concentrations on initiation of embryogenic cultures from mature-zygotic embryos

Media*	Sucrose (%)	Type of response**	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Most part of the explants produced hard calli. Very little soft, white, translucent calli were formed which turned brownish in subsequent subcultures	1.20 (±0.23)	20.00 (±1.12)
	2.0	-do-	2.70 (±0.76)	30.50 (±1.70)
	3.0	-do-	3.40 (±0.98)	35.90 (±1.81)
	4.0	Calli turned brownish	1.50 (±1.10)	20.21 (±1.69)
MS	0.0	No response	0.00	0.00
	1.0	Explants produced hard calli with small patches of soft, translucent callus which turned brownish in subsequent subcultures	3.80 (±0.95)	20.50 (±1.56)
	2.0	-do-	4.20 (±0.89)	31.60 (±1.63)
	3.0	-do-	6.30 (±1.10)	40.00 (±1.78)
	4.0	Calli turned brownish	3.50 (±1.21)	22.50 (±1.92)
½mMS	0.0	No response	0.00	0.00
	1.0	Soft, whitish, translucent calli formed but not very healthy and turned brownish in subsequent subcultures	0.85 (±0.67)	37.20 (±1.71)
	2.0	As above but the cultures did not turn brownish in subsequent subcultures and showed very poor proliferation	2.32 (±1.05)	43.60 (±1.53)
	3.0	-do-	2.88 (±1.10)	44.70 (±1.32)
	4.0	Calli turned brownish	1.50 (±1.08)	38.00 (±1.59)
mMS	0.0	No response	0.00	0.00
	1.0	White, soft, translucent calli were formed	6.80 (±1.10)	70.20 (±1.43)
	2.0	White, soft, translucent calli formed and cultures were moderately healthy	9.10 (±1.33)	78.20 (±2.20)
	3.0	White, soft, translucent calli formed and cultures were healthy and exhibited satisfactory proliferation	12.50 (±1.35)	85.00 (±2.45)
	4.0	Calli turned brownish	5.00 (±1.13)	57.50 (±1.86)
½DCR	0.0	No response	0.00	0.00
	1.0	Partially soft translucent calli formed and rest hard green	0.75 (±0.87)	38.00 (±1.23)
	2.0	As above but in subsequent subcultures calli turned brownish	1.12 (±0.95)	45.00 (±1.23)
	3.0	As above but comparatively healthy cultures	2.20 (±1.00)	53.22 (±1.39)
	4.0	Calli turned brownish	1.50 (±1.12)	40.00 (±1.75)
DCR	0.0	No response	0.00	0.00
	1.0	Soft-translucent calli but not very healthy and did not proliferate much	4.50 (±0.99)	41.00 (±1.31)
	2.0	As above but calli were moderately healthy and showed some proliferation	7.45 (±1.12)	50.20 (±1.58)
	3.0	As above but exhibited much better proliferation	10.00 (±1.22)	57.30 (±1.75)
	4.0	Calli turned brownish	6.45 (±1.07)	46.10 (±1.81)

contd...

½ Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent calli which were moderately healthy and exhibited some proliferation, cultures did not turn brownish in subsequent subcultures	4.80 (±0.88)	47.50 (±1.90)
	2.0	As above but cultures were comparatively healthier	7.90 (±0.98)	54.30 (±1.98)
	3.0	-do-	9.00 (±1.33)	61.20 (±2.45)
	4.0	Calli turned brownish	6.00 (±1.25)	50.00 (±1.59)
Litvay's	0.0	No response	0.00	0.00
	1.0	Very little soft translucent calli and rest formed hard callus	1.90 (±0.76)	25.60 (±1.65)
	2.0	As above but calli slightly healthier	3.20 (±0.90)	38.10 (±1.81)
	3.0	As above but calli quite healthy	5.70 (±1.10)	45.25 (±1.55)
	4.0	Calli turned brownish	2.40 (±1.13)	31.00 (±1.93)
½BM_1	0.0	No response	0.00	0.00
	1.0	Soft-translucent calli which did not proliferate much	3.30 (±0.87)	65.11 (±1.78)
	2.0	-do-	4.70 (±0.96)	70.20 (±1.66)
	3.0	As above but cultures exhibited some proliferation	5.75 (±0.89)	71.25 (±2.21)
	4.0	Calli turned brownish	2.32 (±1.20)	68.00 (±1.87)
BM_1	0.0	No response	0.00	0.00
	1.0	Soft-translucent calli with moderate proliferation	7.30 (±0.93)	69.00 (±2.20)
	2.0	As above but cultures were healthy and exhibited better proliferation	8.00 (±1.09)	73.00 (±2.67)
	3.0	-do-	10.00 (±1.13)	75.10 (±2.45)
	4.0	Calli turned brownish	7.00 (±1.53)	50.00 (±1.76)

* Additional adjuvants: 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

** Callusing started in 10-14 days time

Figure 6. Different stages of somatic embryogenesis from mature zygotic embryo explant cultured in semi-solid medium

a. Mature zygotic embryo explant (0.63 x 25)
b. Initiation of embryogenic callus (0.63 x 25)
c. Embryogenic callus (0.63 x 10)
d. Two cotyledonary SEs attached to the callus by suspensor like cells (0.63 x 16)
e. A mature cotyledonary SE attached to the callus (0.63 x 25)

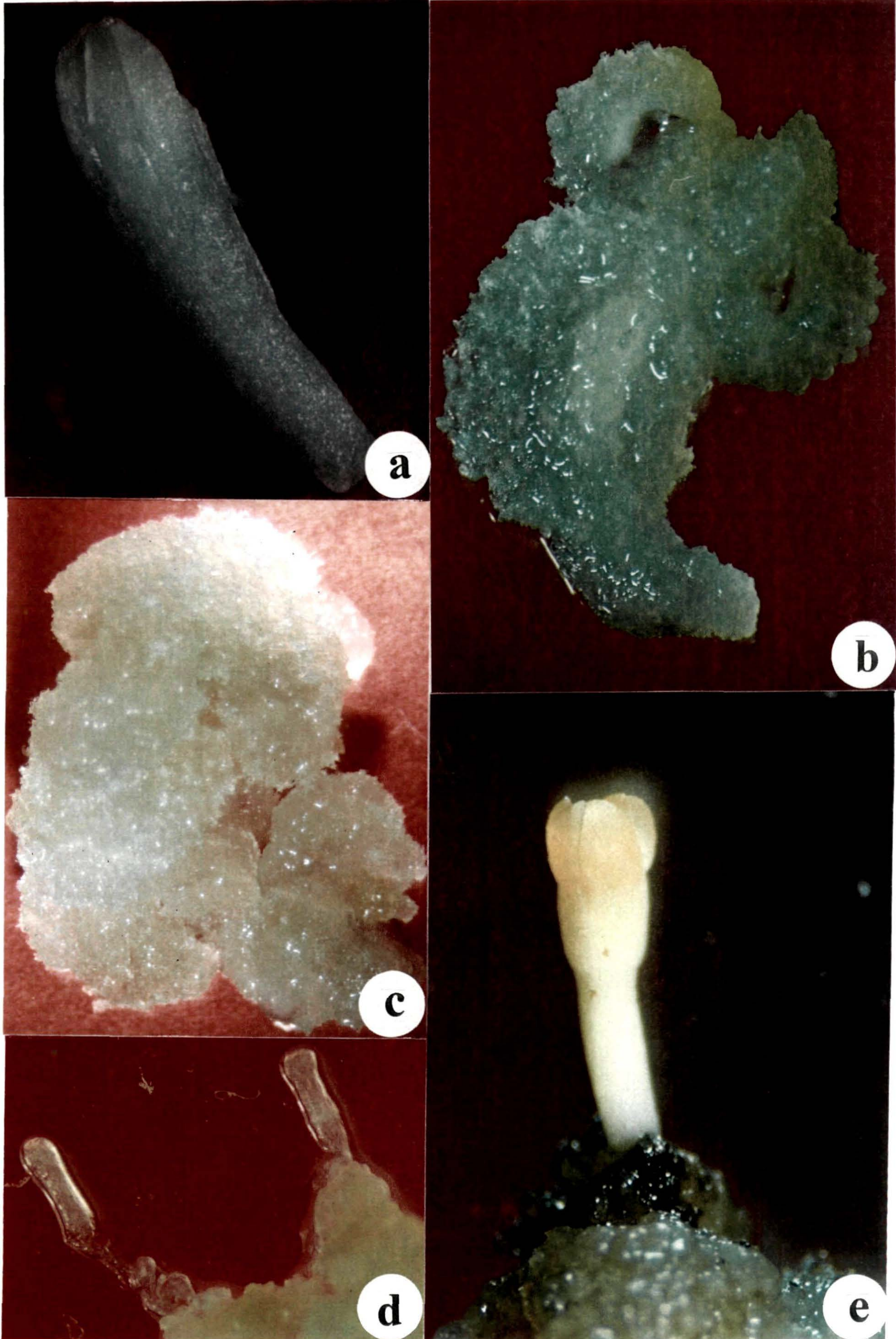

Figure 6

Figure 7. Zygotic embryo explant showing non-embryogenic cultures in semi-solid medium

a. Mature zygotic embryo explant (0.63 x 25)

b. Callusing of the explant (0.63 x 16)

c. Non-embryogenic callus (0.63 x 16)

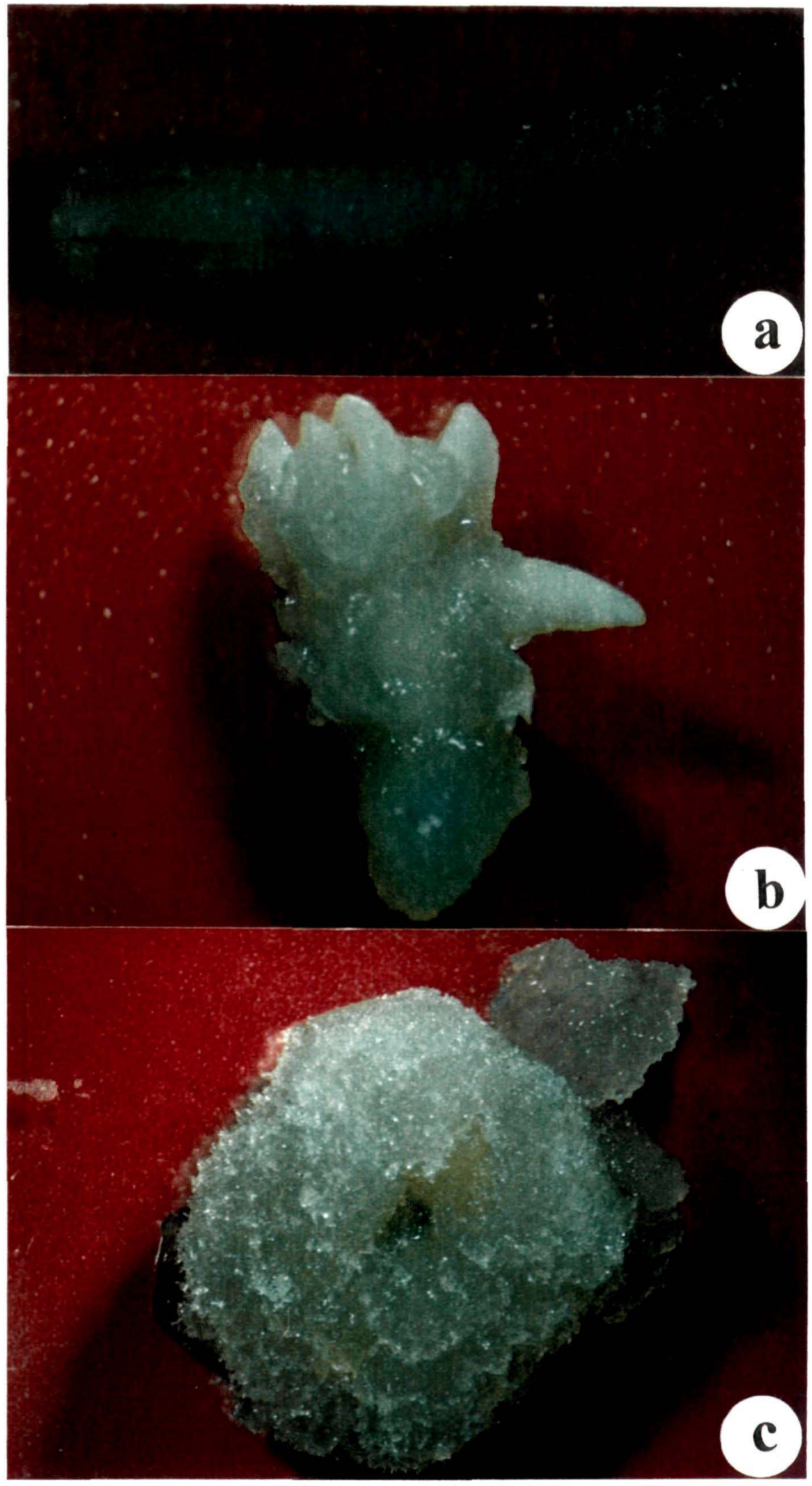

Figure 7

Sucrose was found slightly better in initiation of embryogenic cultures (12.5%) (Table-10) as compared to maltose (11.5%) (Table-11). A delay in callusing and slow proliferation was observed in maltose containing medium compared to responses obtained in the medium containing sucrose. In sucrose containing medium, callusing of explants started within 10-14 days but in maltose supplemented medium the initiation process was delayed by 7-10 days. Culture proliferation was rapid with sucrose but associated with varying degrees of culture browning. With maltose in the medium there was no or little browning of cultures. Lactose and fructose were ineffective in embryogenic culture initiation (Table 12 and 13). In the media containing these carbohydrates only non-embryogenic cultures were produced.

Secondary needles: The needles (Figure 8a) exhibited only non-embryogenic cultures. The best callusing was recorded on MS followed by mMS and ½MS media (Table 14). Poor callusing was observed in ½BM and ½DCR media. The soft white callus (Figure 8b-c) did not show any pro-embryos.

Very high frequency of soft, white, non-embryogenic callus formation was recorded in media containing sucrose (75.5%) (Table 14), maltose (71.35%) (Table 15) and lactose (65%) (Table 16). Fructose (Table 17) proved to be the least effective in inducing callus formation

Table 11. Effect of different media and maltose concentrations on initiation of embryogenic cultures from mature zygotic embryos

Media*	Maltose (%)	Type of response**	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Most part of the explants produced hard calli. Little soft translucent calli were formed which turned hard in subsequent subcultures	1.35 (±0.89)	23.00 (±1.21)
	2.0	-do-	2.50 (±0.93)	29.30 (±1.34)
	3.0	-do-	3.10 (±1.05)	32.20 (±1.93)
	4.0	Calli turned slight brownish	1.50 (±1.23)	24.50 (±1.65)
MS	0.0	No response	0.00	0.00
	1.0	Explants produced hard calli mostly mixed with patches of soft-translucent calli. Most of these translucent patches turned hard in subsequent subcultures	2.50 (±0.78)	20.00 (±1.30)
	2.0	-do-	4.00 (±0.89)	28.50 (±1.32)
	3.0	As above but cultures were healthy	6.50 (±0.93)	37.30 (±1.44)
	4.0	Calli turned slight brownish	3.20 (±1.08)	22.50 (±1.53)
½mMS	0.0	No response	0.00	0.00
	1.0	Soft, whitish, translucent calli formed but not healthy and most of the cultures turned hard and did not proliferate much	1.00 (±0.57)	30.00 (±1.41)
	2.0	-do-	2.10 (±1.00)	38.45 (±1.52)
	3.0	As above but cultures moderately healthy	3.00 (±1.12)	46.00 (±1.31)
	4.0	Calli turned slight brownish	1.50 (±1.14)	33.00 (±1.63)
mMS	0.0	No response	0.00	0.00
	1.0	White, soft, translucent calli formed throughout the explants with patches of hard calli	5.00 (±1.12)	70.00 (±2.34)
	2.0	As above and cultures were moderately healthy	8.30 (±1.23)	70.20 (±1.96)
	3.0	White, soft, translucent healthy calli which exhibited satisfactory proliferation	11.50 (±1.34)	75.11 (±2.60)
	4.0	Calli turned slight brownish	3.50 (±1.17)	54.50 (±1.73)
½DCR	0.0	No response	0.00	0.00
	1.0	Hard calli with patches of soft translucent portions, cultures not very healthy	1.00 (±0.78)	30.10 (±1.34)
	2.0	-do-	1.78 (±1.09)	39.00 (±1.54)
	3.0	As above but cultures were moderately healthy	2.00 (±1.10)	40.50 (±1.41)
	4.0	Calli turned slight brownish	1.25 (±1.07)	32.00 (±1.69)
DCR	0.0	No response	0.00	0.00
	1.0	Soft-translucent calli but not very healthy and exhibited very slow proliferation	3.80 (±0.90)	35.00 (±1.39)
	2.0	As above but calli were moderately healthy	6.00 (±1.11)	43.00 (±1.42)
	3.0	As above but with better proliferation	8.50 (±1.20)	50.10 (±1.51)
	4.0	Calli turned slight brownish	3.00 (±1.14)	36.00 (±1.90)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent calli formed in patches, cultures were not healthy and exhibited very slow proliferation	3.10 (±0.88)	36.20 (±1.40)
	2.0	As above but cultures were moderately healthy and exhibited some proliferation	7.00 (±1.05)	45.00 (±1.52)
	3.0	-do-	8.50 (±0.94)	57.10 (±1.80)
	4.0	Calli turned slight brownish	2.50 (±1.03)	38.00 (±1.83)

contd...

Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-translucent calli formed but there was very little culture proliferation in subsequent subcultures	2.30 (±0.65)	23.20 (±1.22)
	2.0	-do-	3.50 (±0.85)	28.00 (±1.40)
	3.0	As above with better proliferation	5.00 (±1.15)	35.00 (±1.56)
	4.0	Calli turned slight brownish	1.70 (±1.19)	25.00 (±1.85)
½BM_1	0.0	No response	0.00	0.00
	1.0	Explants formed hard calli with patches of soft translucent parts which did not proliferate much in subsequent subcultures	2.80 (±1.10)	21.30 (±1.21)
	2.0	-do-	3.50 (±1.16)	26.00 (±1.53)
	3.0	-do-	5.50 (±1.25)	39.10 (±1.67)
	4.0	Calli turned slight brownish	2.00 (±1.18)	22.50 (±1.85)
BM_1	0.0	No response	0.00	0.00
	1.0	Patches of soft-translucent calli formed along with hard calli which failed to proliferate	3.10 (±1.21)	30.00 (±1.28)
	2.0	As above but cultures moderately healthy	7.00 (±1.30)	35.00 (±1.41)
	3.0	-do-	9.50 (±1.37)	32.00 (±1.39)
	4.0	Calli turned slight brownish	2.75 (±1.26)	30.00 (±1.78)

*Additional adjuvants: 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

** Callusing started in 17-24 days time showing slow growth and proliferation of cultures

Table 12. Effect of different media and lactose concentrations on initiation of embryogenic cultures from mature zygotic embryos

Media*	Lactose (%)	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Explants produced hard-white calli	0.00	17.50 (±1.41)
	2.0	As above but the cultures turned brownish in subsequent subcultures	0.00	20.00 (±1.34)
	3.0	-do-	0.00	23.10 (±1.89)
	4.0	Calli turned brownish	0.00	18.80 (±1.88)
MS	0.0	No response	0.00	0.00
	1.0	Explants produced hard, pinkish-white calli which turned brownish in subsequent subcultures	0.00	18.00 (±1.13)
	2.0	-do-	0.00	20.45 (±1.47)
	3.0	-do-	0.00	21.58 (±1.77)
	4.0	Calli turned brownish	0.00	18.00 (±1.63)
½mMS	0.0	No response	0.00	0.00
	1.0	Explants produced hard, reddish-white calli which turned brownish in subsequent subcultures	0.00	16.20 (±1.45)
	2.0	As above but cultures turned brown very rapidly in subsequent subcultures	0.00	18.50 (±1.35)
	3.0	-do-	0.00	22.90 (±1.64)
	4.0	Calli turned brownish	0.00	17.00 (±1.73)
mMS	0.0	No response	0.00	0.00
	1.0	Partially soft-pinkish calli which turned brownish in subsequent subcultures	0.00	15.00 (±1.25)
	2.0	-do-	0.00	20.10 (±1.39)
	3.0	-do-	0.00	25.24 (±1.53)
	4.0	Calli turned brownish	0.00	18.50 (±1.71)
½DCR	0.0	No response	0.00	0.00
	1.0	Partially soft-white calli which turned brownish	0.00	18.00 (±1.87)
	2.0	-do-	0.00	21.00 (±1.65)
	3.0	-do-	0.00	24.75 (±2.10)
	4.0	Calli turned brownish	0.00	18.60 (±2.53)
DCR	0.0	No response	0.00	0.00
	1.0	Partially soft-whitish calli which turned hard and brownish in subsequent subcultures	0.00	14.20 (±1.70)
	2.0	-do-	0.00	20.00 (±1.49)
	3.0	-do-	0.00	27.90 (±1.55)
	4.0	Calli turned brownish	0.00	17.00 (±2.23)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Partially soft-whitish calli which turned hard and brown in subsequent subcultures	0.00	16.50 (±1.76)
	2.0	-do-	0.00	22.45 (±1.88)
	3.0	-do-	0.00	29.00 (±1.81)
	4.0	Calli turned brownish	0.00	18.10 (±1.59)
Litvay's	0.0	No response	0.00	0.00
	1.0	Partially soft calli which turned brownish in subsequent subcultures	0.00	14.00 (±1.79)
	2.0	-do-	0.00	17.86 (±1.88)
	3.0	-do-	0.00	24.38 (±2.05)
	4.0	Calli turned brownish	0.00	15.00 (±1.93)

contd...

½BM_1	0.0	No response	0.00	0.00
	1.0	Partially soft calli which turned brownish in subsequent subcultures	0.00	14.32 (±1.98)
	2.0	-do-	0.00	20.00 (±2.01)
	3.0	-do-	0.00	24.50 (±2.27)
	4.0	Calli turned brownish	0.00	19.00 (±1.96)
BM_1	0.0	No response	0.00	0.00
	1.0	Calli moderately soft but turned brownish in subsequent subcultures	0.00	15.50 (±1.67)
	2.0	-do-	0.00	20.14 (±2.06)
	3.0	-do-	0.00	22.15 (±1.98)
	4.0	Calli turned brownish	0.00	16.50 (±1.76)

*Additional adjuvants: 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

Table 13. Effect of different media and fructose concentrations on initiation of embryogenic cultures from mature zygotic embryos

Media*	Fructose (%)	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Explants produce hard calli which turned brownish very fast	0.00	12.20 (±1.33)
	2.0	As above and cultures turned brownish in subsequent subcultures	0.00	15.50 (±1.45)
	3.0	-do-	0.00	25.50 (±1.51)
	4.0	Calli turned brownish	0.00	14.00 (±1.83)
MS	0.0	No response	0.00	0.00
	1.0	Explants produced hard-white calli which turned brownish very quickly	0.00	15.30 (±1.35)
	2.0	-do-	0.00	28.00 (±1.42)
	3.0	-do-	0.00	36.00 (±1.42)
	4.0	Calli turned brownish	0.00	20.00 (±1.75)
½mMS	0.0	No response	0.00	0.00
	1.0	Explants produced hard-white calli which turned brownish very quickly in subsequent subcultures	0.00	15.75 (±1.38)
	2.0	-do-	0.00	22.60 (±1.41)
	3.0	-do-	0.00	40.00 (±2.31)
	4.0	Calli turned brownish	0.00	18.00 (±1.73)
mMS	0.0	No response	0.00	0.00
	1.0	Explants produced hard-white calli throughout, which turned brownish very quickly	0.00	17.00 (±1.410
	2.0	-do-	0.00	28.00 (±1.52)
	3.0	-do-	0.00	34.35 (±1.65)
	4.0	Calli turned brownish	0.00	22.00 (±2.13)
½DCR	0.0	No response	0.00	0.00
	1.0	Explants produced hard-pinkish calli which turned brownish very quickly in subsequent subcultures	0.00	13.00 (±1.29)
	2.0	-do-	0.00	15.25 (±1.34)
	3.0	-do-	0.00	23.80 (±1.49)
	4.0	Calli turned brownish	0.00	13.50 (±1.93)
DCR	0.0	No response	0.00	0.00
	1.0	Explants produce hard-whitish calli which turned brownish in subsequent subcultures	0.00	16.00 (±1.23)
	2.0	-do-	0.00	25.00 (±1.39)
	3.0	-do-	0.00	35.50 (±1.93)
	4.0	Calli turned brownish	0.00	18.65 (±1.87)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Explants produced hard calli which turned brownish in subsequent subcultures	0.00	11.75 (±1.07)
	2.0	-do-	0.00	23.45 (±1.11)
	3.0	-do-	0.00	29.70 (±1.31)
	4.0	Calli turned brownish	0.00	15.00 (±2.21)

contd...

Litvay's	0.0	No response	0.00	0.00
	1.0	Explants produced hard-white calli with greenish patches, cultures turned brownish in subsequent subcultures	0.00	13.00 (±1.41)
	2.0	-do-	0.00	20.00 (±1.39)
	3.0	As above but cultures turned brownish very quickly	0.00	39.00 (±1.58)
	4.0	Calli turned brownish	0.00	18.00 (±1.95)
½BM₁	0.0	No response	0.00	0.00
	1.0	Explants produced hard-whitish calli which turned brown in subsequent subcultures	0.00	12.00 (±1.10)
	2.0	-do-	0.00	18.50 (±1.23)
	3.0	-do-	0.00	26.00 (±1.41)
	4.0	Calli turned brownish	0.00	14.00 (±1.92)
BM_1	0.0	No response	0.00	0.00
	1.0	Explants produced hard-white calli which turned brownish in subsequent subcultures	0.00	14.00 (±1.50)
	2.0	As above but cultures turned brownish very rapidly	0.00	20.00 (±1.67)
	3.0	-do-	0.00	31.50 (±1.61)
	4.0	Calli turned brownish	0.00	17.00 (±1.82)

*Additional adjuvants: 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

Figure 8. Secondary needle explants showing non-embryogenic cultures in semi-solid medium

a. Secondary needle explants (0.63 x 6.5)

b. Callusing of secondary needle (0.63 x 25)

c. Hard non-embryogenic callus from secondary needles (0.63 x 16)

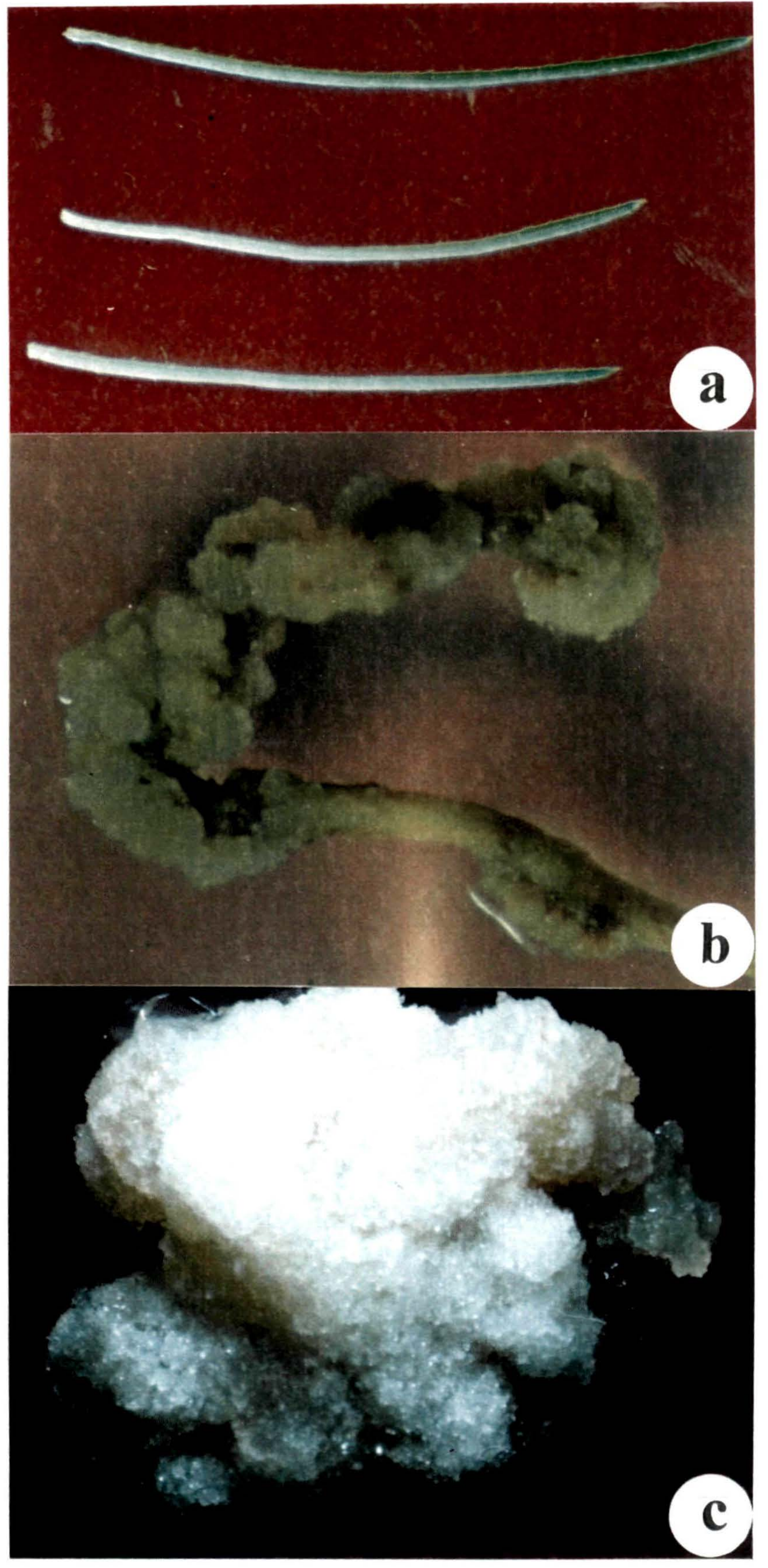

Figure 8

Table 14. Effect of different media and sucrose concentrations on initiation of embryogenic cultures from secondary needles

Media*	Sucrose (%)	Type of response**	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½ MS	0.0	No response	0.00	0.00
	1.0	Soft-white calli formed at the basal part of the needles and turned hard in subsequent subcultures	0.00	40.10 (±2.58)
	2.0	Some needles formed soft-whitish calli throughout the length and the rest only at the basal part. The cultures turned hard in subsequent subcultures	0.00	55.00 (±2.67)
	3.0	-do-	0.00	70.50 (±2.45)
	4.0	Calli turned brownish	0.00	35.59 (±1.98)
MS	0.0	No response	0.00	0.00
	1.0	Soft-white callusing all along the needles, cultures very healthy but turned hard in subsequent subcultures	0.00	70.10 (±1.84)
	2.0	-do-	0.00	72.00 (±2.10)
	3.0	-do-	0.00	75.50 (±2.20)
	4.0	Calli turned brownish	0.00	60.00 (±1.92)
½ mMS	0.0	No response	0.00	0.00
	1.0	Soft-white calli formed only at the basal part of the explants. Cultures turned hard in subsequent subcultures	0.00	40.00 (±1.83)
	2.0	-do-	0.00	41.50 (±2.10)
	3.0	-do-	0.00	44.00 (±2.30)
	4.0	Calli turned brownish	0.00	34.00 (±2.15)
mMS	0.0	No response	0.00	0.00
	1.0	Soft-white moderately healthy calli formed. Few explants showed callusing throughout. All the cultures turned hard in subsequent subcultures	0.00	52.10 (±2.56)
	2.0	As above but cultures were moderately healthy	0.00	66.50 (±2.46)
	3.0	As above but the cultures were very healthy	0.00	71.00 (±2.33)
	4.0	Calli turned brownish	0.00	50.00 (±1.91)
½ DCR	0.0	No response	0.00	0.00
	1.0	Basal portion callused, very few needles formed calli all along the length. Cultures turned hard slowly	0.00	32.50 (±2.16)
	2.0	-do-	0.00	35.00 (±2.38)
	3.0	-do-	0.00	39.15 (±2.10)
	4.0	Calli turned brownish	0.00	30.00 (±2.10)
DCR	0.0	No response	0.00	0.00
	1.0	Moderately soft-white calli formed but turned hard in subsequent subcultures	0.00	40.50 (±1.98)
	2.0	-do-	0.00	43.15 (±2.15)
	3.0	-do-	0.00	51.00 (±2.56)
	4.0	Calli turned brownish	0.00	36.45 (±2.74)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Moderately soft-white calli formed. In some needles only basal part showed callusing	0.00	37.19 (±1.87)
	2.0	As above but the cultures turned hard in subsequent subcultures	0.00	40.55 (±2.07)
	3.0	-do-	0.00	46.11 (±2.25)
	4.0	Calli turned brownish	0.00	30.00 (±1.85)

contd...

Litvay's	0.0	No response	0.00	0.00
	1.0	Some needles formed soft-whitish calli throughout the length and the rest only at the base	0.00	30.00 (±1.98)
	2.0	As above but the cultures turned hard in subsequent subcultures	0.00	33.15 (±1.48)
	3.0	-do-	0.00	39.50 (±2.33)
	4.0	Calli turned brownish	0.00	23.80 (±2.28)
½ BM_1	0.0	No response	0.00	0.00
	1.0	Basal part of the needles formed soft calli. In most of the needles hard calli developed throughout the length	0.00	21.70 (±1.68)
	2.0	-do-	0.00	28.50 (±2.17)
	3.0	-do-	0.00	32.35 (±2.10)
	4.0	Calli turned brownish	0.00	18.00 (±1.71)
BM_1	0.0	No response	0.00	0.00
	1.0	Moderately soft-white calli formed. Few needles showed callusing throughout but the cultures turned hard in subsequent subcultures	0.00	36.50 (±1.78)
	2.0	-do-	0.00	39.00 (±2.11)
	3.0	-do-	0.00	41.55 (±2.43)
	4.0	Calli turned brownish	0.00	29.00 (±2.10)

*Additional adjuvants: 2,4-D and NAA (3 mgl^{-1} each); BAP (1mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

** Callusing started in 10-14 days time

Table 15. Effect of different media and maltose concentrations on initiation of embryogenic cultures from secondary needles

Media*	Maltose (%)	Type of response**	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½ MS	0.0	No response	0.00	0.00
	1.0	Soft-white calli formed at the basal part of the needles. Proliferation of cultures was very slow and no browning was observed	0.00	40.00 (±2.08)
	2.0	Some needles formed soft-whitish calli throughout the length and the rest at the base. Though culture proliferation was very slow but no browning was observed	0.00	54.23 (±2.43)
	3.0	-do-	0.00	57.55 (±2.35)
	4.0	Calli turned slight brownish	0.00	37.00 (±2.26)
MS	0.0	No response	0.00	0.00
	1.0	Soft-white calli all along the explants, proliferation was very slow but cultures very healthy	0.00	68.50 (±2.03)
	2.0	As above but cultures exhibited better proliferation	0.00	70.00 (±2.20)
	3.0	-do-	0.00	71.35 (±2.34)
	4.0	Calli turned slight brownish	0.00	53.80 (±2.19)
½ mMS	0.0	No response	0.00	0.00
	1.0	Soft-white calli formed only at the base of the explants. No browning of cultures but proliferation was very slow	0.00	37.58 (±1.87)
	2.0	-do-	0.00	39.65 (±2.13)
	3.0	-do-	0.00	41.25 (±2.40)
	4.0	Calli turned slight brownish	0.00	32.00 (±1.56)
mMS	0.0	No response	0.00	0.00
	1.0	Soft-white moderately healthy calli, few needles showed callusing. Culture proliferation was very slow but no browning was observed	0.00	50.10 (±2.35)
	2.0	As above but proliferation of culture was much better	0.00	62.25 (±2.76)
	3.0	-do-	0.00	67.50 (±2.31)
	4.0	Calli turned slight brownish	0.00	43.90 (±2.11)
½ DCR	0.0	No response	0.00	0.00
	1.0	Basal part of the explants callused, very few needles callused all through, no browning was found	0.00	30.00 (±2.07)
	2.0	-do-	0.00	33.00 (±2.37)
	3.0	-do-	0.00	36.00 (±2.15)
	4.0	Calli turned slight brownish	0.00	25.13 (±1.83)
DCR	0.0	No response	0.00	0.00
	1.0	Moderately soft-white calli formed. Culture proliferation was very slow but no browning was observed	0.00	38.66 (±1.85)
	2.0	As above but with better proliferation	0.00	41.00 (±2.09)
	3.0	-do-	0.00	58.90 (±2.54)
	4.0	Calli turned slight brownish	0.00	31.00 (±1.69)

contd...

½ Litvay's	0.0	No response	0.00	0.00
	1.0	Moderately soft-white calli formed. Culture proliferation was slow but there was no browning of culture	0.00	31.35 (±1.85)
	2.0	As above but exhibited better proliferation	0.00	38.45 (±2.33)
	3.0	-do-	0.00	41.55 ((±2.47)
	4.0	Calli turned slight brownish	0.00	24.00 (±1.70)
Litvay's	0.0	No response	0.00	0.00
	1.0	Soft-white calli, proliferation was slow but no culture browning observed	0.00	30.14 (±1.76)
	2.0	As above but with better culture proliferation	0.00	32.53 (±1.77)
	3.0	-do-	0.00	36.37 (±1.98)
	4.0	Calli turned slight brownish	0.00	22.00 (±1.84)
½ BM_1	0.0	No response	0.00	0.00
	1.0	In most of the explants hard-white calli developed. Browning of cultures was not observed	0.00	20.00 (±1.78)
	2.0	As above but with better culture proliferation	0.00	25.33 (±2.32)
	3.0	-do-	0.00	30.00 (±2.09)
	4.0	Calli turned slight brownish	0.00	17.50 (±2.13)
BM_1	0.0	No response	0.00	0.00
	1.0	Moderately soft-white calli with slow proliferation	0.00	32.15 (±1.86)
	2.0	As above but exhibited better culture proliferation	0.00	34.75 (±2.21)
	3.0	-do-	0.00	39.00 (±2.35)
	4.0	Calli turned slight brownish	0.00	23.85 (±1.96)

*Additional adjuvants: 2,4-D and NAA (3 mgl^{-1} each); BAP (1mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

** Callusing started in 17-24 days time showing slow growth and proliferation of cultures

Table 16. Effect of different media and lactose concentrations on initiation of embryogenic cultures from secondary needles

Media*	Lactose (%)	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½ MS	0.0	No response	0.00	0.00
	1.0	Soft-white calli at the base which turned brownish in subsequent subcultures	0.00	30.50 (±1.79)
	2.0	Most of the needles callused at the base and few needles showed callusing throughout. Cultures turned brownish in subsequent subcultures	0.00	34.55 (±1.82)
	3.0	-do-	0.00	41.37 (±2.10)
	4.0	Hard-brownish calli	0.00	26.45 (±2.17)
MS	0.0	No response	0.00	0.00
	1.0	Soft-white calli which turned hard	0.00	50.00 (±1.86)
	2.0	Soft-white calli which turned hard brownish in subsequent subcultures	0.00	56.50 (±2.13)
	3.0	-do-	0.00	65.00 (±2.36)
	4.0	Hard-brownish calli	0.00	43.57 (±2.29)
½ mMS	0.0	No response	0.00	0.00
	1.0	Basal part of the explants produced hard calli	0.00	27.35 (±2.11)
	2.0	-do-	0.00	32.00 (±1.97)
	3.0	As above and the cultures browned in subsequent subcultures	0.00	37.25 (±1.76)
	4.0	Hard-brownish calli	0.00	21.00 (±1.91)
mMS	0.0	No response	0.00	0.00
	1.0	Soft-white calli but turned brown in subsequent subcultures	0.00	51.00 (±1.97)
	2.0	-do-	0.00	59.55 (±2.32)
	3.0	-do-	0.00	60.45 (±1.85)
	4.0	Hard-brownish calli	0.00	46.15 (±2.27)
½ DCR	0.0	No response	0.00	0.00
	1.0	Hard callusing at the basal portion and very few needles formed calli all along the length. Cultures turned brownish in subsequent subcultures	0.00	28.45 (±1.78)
	2.0	-do-	0.00	33.75 (±1.83)
	3.0	-do-	0.00	36.00 (±2.12)
	4.0	Hard-brownish calli	0.00	22.44 (±1.71)
DCR	0.0	No response	0.00	0.00
	1.0	Moderately soft calli formed but turned brownish in subsequent subcultures	0.00	35.15 (±1.68)
	2.0	-do-	0.00	39.50 (±2.15)
	3.0	-do-	0.00	41.23 (±2.40)
	4.0	Hard-brownish calli	0.00	29.00 (±1.87)

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½ Litvay's	0.0	No response	0.00	0.00
	1.0	Very poor callusing which turned brownish in subsequent subcultures	0.00	23.30 (±1.38)
	2.0	Moderate callusing but turned brownish in subsequent subcultures	0.00	27.15 (±2.11)
	3.0	-do-	0.00	34.23 (±2.31)
	4.0	Hard-brownish calli	0.00	19.50 (±1.46)
Litvay's	0.0	No response	0.00	0.00
	1.0	Some needles formed soft-whitish calli throughout the length and the rest only at the base. The cultures turned brownish in subsequent subcultures	0.00	29.00 (±1.76)
	2.0	-do-	0.00	31.50 (±2.21)
	3.0	-do-	0.00	35.75 (±2.37)
	4.0	Hard-brownish calli	0.00	23.50 (±1.66)
½ BM_1	0.0	No response	0.00	0.00
	1.0	In most of the needles hard calli developed which turned brownish in subsequent subcultures	0.00	17.50 (±1.81)
	2.0	-do-	0.00	22.47 (±2.10)
	3.0	-do-	0.00	26.54 (±2.41)
	4.0	Hard-brownish calli	0.00	15.00 (±2.76)
BM_1	0.0	No response	0.00	0.00
	1.0	Moderately soft calli formed in the beginning but turned hard and brownish in subsequent subcultures	0.00	30.00 (±1.89)
	2.0	-do-	0.00	34.40 (±2.18)
	3.0	-do-	0.00	37.15 (±2.33)
	4.0	Hard-brownish calli	0.00	23.00 (±1.97)

*Additional adjuvants: 2,4-D and NAA (3 mgl^{-1} each); BAP (1mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

Table 17. Effect of different media and fructose concentrations on initiation of embryogenic cultures from secondary needles

Media*	Fructose (%)	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½ MS	0.0	No response	0.00	0.00
	1.0	No response	0.00	0.00
	2.0	Almost no response, only basal part callused and turned brown very rapidly	0.00	10.00 (±1.97)
	3.0	-do-	0.00	11.20 (±2.10)
	4.0	Hard-brownish calli	0.00	8.50 (±1.75)
MS	0.0	No response	0.00	0.00
	1.0	Hard callusing at the basal part which turned brownish very rapidly in subsequent subcultures	0.00	15.23 (±1.92)
	2.0	-do-	0.00	19.10 (±1.88)
	3.0	-do-	0.00	21.50 (±1.84)
	4.0	Hard-brownish calli	0.00	10.00 (±1.95)
½mMS	0.0	No response	0.00	0.00
	1.0	Only swelling observed	0.00	11.30 (±1.71)
	2.0	Very poor response, only hard calli formed which turned brown very rapidly in subsequent subcultures	0.00	13.70 (±2.02)
	3.0	-do-	0.00	15.10 (±2.11)
	4.0	Hard-brownish calli	0.00	9.00 (±1.84)
mMS	0.0	No response	0.00	0.00
	1.0	Only hard calli formed which turned brown very rapidly in subsequent subcultures	0.00	13.00 (±1.67)
	2.0	-do-	0.00	15.50 (±1.87)
	3.0	-do-	0.00	19.00 (±2.15)
	4.0	Hard-brownish calli	0.00	8.50 (±1.73)
½DCR	0.0	No response	0.00	0.00
	1.0	Almost no response and only swelling of explants which turned brown and eventually died	0.00	5.62 (±1.68)
	2.0	Very poor callusing at the basal part. Calli turned brown in subsequent subcultures	0.00	8.45 (±1.97)
	3.0	-do-	0.00	10.71 (±2.05)
	4.0	Hard-brownish calli	0.00	7.00 (±1.90)
DCR	0.0	No response	0.00	0.00
	1.0	Hard, faint-greenish calli formed which turned brownish very rapidly in subsequent subcultures	0.00	11.00 (±1.71)
	2.0	-do-	0.00	12.50 (±2.13)
	3.0	-do-	0.00	15.15 (±2.11)
	4.0	Hard-brownish calli	0.00	11.50 (±1.16)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Almost no response and only swelling of the explants which turned brown and eventually died	0.00	3.30 (±1.70)
	2.0	Very poor callusing at the basal part and cultures turned brownish very rapidly	0.00	8.00 (±1.69)
	3.0	-do-	0.00	9.50 (±1.88)
	4.0	Hard-brownish calli	0.00	5.95 (±1.69)

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Litvay's	0.0	No response	0.00	0.00
	1.0	Hard, light-greenish calli which turned brown very rapidly in subsequent subcultures	0.00	9.10 (±1.61)
	2.0	-do-	0.00	14.36 (±2.05)
	3.0	-do-	0.00	19.51 (±2.30)
	4.0	Hard-brownish calli	0.00	11.00 (±1.98)
½BM₁	0.0	No response	0.00	0.00
	1.0	Almost no response and only swelling of explants observed	0.00	3.50 (±1.78)
	2.0	Very poor callusing at the base which turned hard and brownish in subsequent subcultures	0.00	7.11 (±1.88)
	3.0	-do-	0.00	8.00 (±1.91)
	4.0	Hard-brownish calli	0.00	5.00 (±1.65)
BM_1	0.0	No response	0.00	0.00
	1.0	Hard-white calli which turned brown very rapidly in subsequent subcultures	0.00	7.32 (±1.80)
	2.0	-do-	0.00	11.50 (±2.14)
	3.0	-do-	0.00	16.11 (±2.17)
	4.0	Hard-brownish calli	0.00	9.00 (±1.88)

*Additional adjuvants: 2,4-D and NAA (3 mgl^{-1} each); BAP (1 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

(21.5%). Calli developed in media containing lactose and fructose turned brownish very rapidly.

Apical dome sections: Apical domes collected during May to July (second flushing) produces healthy, white and soft calli. The apical dome sections (Figure 9a) showed best callusing on ½DCR medium followed by DCR and ½mMS medium (Table 18). They produced white and soft callus (Figure 9b-c) in 81.5% of the explants within 2-3 weeks of culture. A low frequency of callusing resulted in MS, Litvay's and BM_1 media (full and half strength). Amongst different media used, the lowest response of callusing of 20% was obtained in ½BM medium (Table 18). All these cultures were found to be without pro-embryos revealing the non-embryogenic nature of the callus.

There was no growth of explants in media lacking carbohydrates. In general, incorporation of 3% each of sucrose or maltose resulted in very high callusing of 81.5% and 75%, respectively (Table 18 and 19). Lactose and fructose at 3% level resulted in low callusing (Table 20 and 21) as compared to sucrose and maltose. Culture browning was very rapid in the media with fructose and lactose. Maximum culture browning was found in the media containing fructose followed by lactose and sucrose and it was least with maltose. However, culture initiation and proliferation were best in the medium containing sucrose.

Figure 9. Apical dome section explants showing non-embryogenic cultures in semi-solid medium

a. Explants from apical dome sections (0.63 x 10)

b. Hard-slight greenish non-embryogenic callus (0.63 x 16)

c. Hard-white non-embryogenic callus (0.63 x 16)

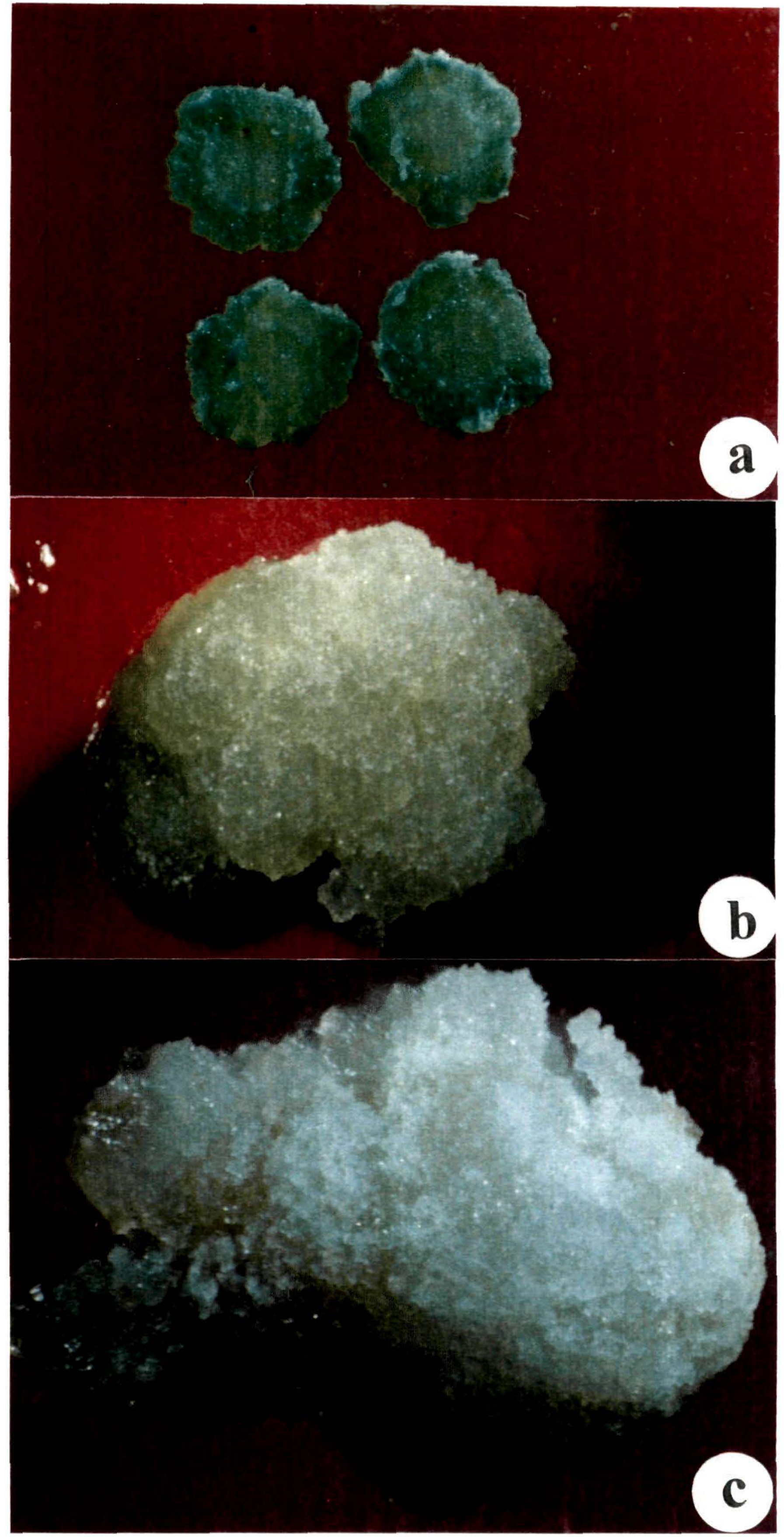

Figure 9

Table 18. Effect of different media and sucrose concentrations on initiation of embryogenic cultures from apical dome sections

Media*	Sucrose (%)	Type of response**	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Very poor callusing	0.00	10.00 (±1.34)
	2.0	-do-	0.00	20.30 (±1.89)
	3.0	-do-	0.00	27.50 (±1.85)
	4.0	Very poor callusing. Calli brownish	0.00	15.75 (±1.94)
MS	0.0	No response	0.00	0.00
	1.0	Very poor growth of calli and slight brownish in colour	0.00	10.50 (±1.68)
	2.0	-do-	0.00	16.75 (±1.97)
	3.0	-do-	0.00	25.68 (±2.03)
	4.0	Very poor callusing. Calli brownish	0.00	15.00 (±1.81)
½mMS	0.0	No response	0.00	0.00
	1.0	White-soft calli but poor growth	0.00	30.50 (±1.88)
	2.0	White-soft calli and moderate growth	0.00	56.85 (±1.76)
	3.0	As above and turned brown in subsequent subcultures	0.00	60.00 (±2.30)
	4.0	Brownish calli	0.00	43.00 (±2.34)
mMS	0.0	No response	0.00	0.00
	1.0	Poor growth of calli	0.00	20.00 (±1.95)
	2.0	-do-	0.00	23.10 (±2.04)
	3.0	As above and turned brown in subsequent subcultures	0.00	40.00 (±2.21)
	4.0	Brownish calli	0.00	20.15 (±1.79)
½DCR	0.0	No response	0.00	0.00
	1.0	Moderately soft calli but culture growth was not satisfactory	0.00	63.80 (±2.13)
	2.0	Soft-white healthy calli but turned hard in subsequent subcultures	0.00	78.00 (±2.34)
	3.0	-do-	0.00	81.50 (±2.19)
	4.0	Brownish calli	0.00	52.00 (±1.88)
DCR	0.0	No response	0.00	0.00
	1.0	Poor growth of calli	0.00	31.50 (±1.99)
	2.0	Soft-white calli with moderate growth but turned hard and brown in subsequent subcultures	0.00	49.70 (±1.61)
	3.0	-do-	0.00	62.80 (±1.87)
	4.0	Brownish calli	0.00	35.13 (±2.21)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Moderately soft calli but growth was not satisfactory	0.00	33.00 (±1.43)
	2.0	As above but with better culture growth	0.00	39.40 (±2.05)
	3.0	-do-	0.00	42.00 (±1.76)
	4.0	Brownish calli	0.00	35.50 (±1.83)

contd...

Litvay's	0.0	No response	0.00	0.00
	1.0	Poor calli growth	0.00	25.00 (±1.71)
	2.0	Moderate calli growth but turned brown in subsequent subcultures	0.00	31.55 (±1.46)
	3.0	-do-	0.00	37.00 (±2.11)
	4.0	Brownish calli	0.00	27.00 (±1.94)
½BM$_1$	0.0	No response	0.00	0.00
	1.0	Poor calli growth	0.00	16.32 (±1.71)
	2.0	-do-	0.00	16.50 (±1.45)
	3.0	-do-	0.00	20.00 (±2.17)
	4.0	Brownish calli	0.00	15.85 (±1.64)
BM$_1$	0.0	No response	0.00	0.00
	1.0	White-soft calli with moderate growth but turned brown in subsequent subcultures	0.00	37.00 (±1.86)
	2.0	-do-	0.00	40.23 (±2.11)
	3.0	-do-	0.00	44.10 (±1.91)
	4.0	Brownish calli	0.00	38.50 (±1.80)

*Additional adjuvants: 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%);
[1]: Standard error.
** Callusing started in 10-14 days time

Table 19. Effect of different media and maltose concentrations on initiation of embryogenic cultures from apical dome sections

Media*	Maltose (%)	Type of response**	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Callusing was very poor	0.00	9.70 (±1.45)
	2.0	-do-	0.00	21.00 (±1.76)
	3.0	-do-	0.00	26.35 (±1.70)
	4.0	-do-	0.00	14.00 (±1.64)
MS	0.0	No response	0.00	0.00
	1.0	Poor callusing	0.00	10.50 (±1.54)
	2.0	-do-	0.00	14.75 (±1.77)
	3.0	-do-	0.00	23.50 (±1.80)
	4.0	Poor callusing. Calli slight brownish	0.00	12.25 (±1.99)
½mMS	0.0	No response	0.00	0.00
	1.0	Soft-white calli but proliferation was very slow	0.00	26.78 (±1.76)
	2.0	Soft-white calli with moderate growth but turned hard in subsequent subcultures	0.00	38.45 (±1.72)
	3.0	-do-	0.00	47.66 (±2.45)
	4.0	Calli slight brownish	0.00	35.00 (±1.96)
mMS	0.0	No response	0.00	0.00
	1.0	Poor growth of calli	0.00	18.25 (±1.87)
	2.0	As above and turned hard in subsequent subcultures	0.00	21.10 (±1.45)
	3.0	-do-	0.00	37.55 (±1.59)
	4.0	Calli slight brownish		20.00 (±1.65)
½DCR	0.0	No response	0.00	0.00
	1.0	Moderately soft calli but proliferation was very slow	0.00	55.60 (±1.98)
	2.0	As above with moderate proliferation	0.00	76.10 (±1.91)
	3.0	-do-	0.00	86.45 (±2.36)
	4.0	Calli slight brownish	0.00	58.00 (±2.34)
DCR	0.0	No response	0.00	0.00
	1.0	Poor growth of calli	0.00	28.00 (±1.63)
	2.0	Moderate growth of calli but turned hard in subsequent subcultures	0.00	50.00 (±1.78)
	3.0	-do-	0.00	52.00 (±1.52)
	4.0	Calli slight brownish	0.00	30.00 (±1.82)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Moderately soft calli but culture growth was not satisfactory	0.00	30.45 (±1.59)
	2.0	As above but cultures were comparatively healthier	0.00	37.55 (±1.96)
	3.0	-do-	0.00	41.00 (±2.57)
	4.0	Calli slight brownish	0.00	32.00 (±1.39)

Contd...

Litvay's	0.0	No response	0.00	0.00
	1.0	Poor calli growth	0.00	23.00 (±1.35)
	2.0	Moderate calli growth but turned hard in subsequent subcultures	0.00	27.15 (±1.55)
	3.0	-do-	0.00	34.75 (±1.86)
	4.0	Calli slight brownish	0.00	24.10 (±1.80)
½BM$_1$	0.0	No response	0.00	0.00
	1.0	Poor calli growth	0.00	15.00 (±1.78)
	2.0	-do-	0.00	15.50 (±1.38)
	3.0	-do-	0.00	19.39 (±1.23)
	4.0	Calli slight brownish	0.00	15.20 (±1.77)
BM$_1$	0.0	No response	0.00	0.00
	1.0	White-soft calli but culture proliferation was very slow	0.00	32.78 (±2.17)
	2.0	White-soft calli with moderate proliferation but turned hard in subsequent subcultures	0.00	38.41 (±1.68)
	3.0	-do-	0.00	40.95 (±2.07)
	4.0	Calli slight brownish	0.00	34.50 (±1.91)

*Additional adjuvants: 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%);
[1]: Standard error.
** Callusing started in 17-24 days time showing slow growth and proliferation of cultures

Table 20. Effect of different media and lactose concentrations on initiation of embryogenic cultures from apical dome sections

Media*	Lactose (%)	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Very poor callusing observed	0.00	8.25 (±1.45)
	2.0	As above and cultures turned brown in subsequent subcultures	0.00	12.00 (±1.67)
	3.0	-do-	0.00	20.00 (±1.23)
	4.0	Brownish calli	0.00	10.00 (±1.74)
MS	0.0	No response	0.00	0.00
	1.0	Very poor calli growth and browning of cultures	0.00	7.51 (±1.72)
	2.0	-do-	0.00	14.00 (±1.44)
	3.0	-do-	0.00	23.10 (±2.31)
	4.0	Brownish calli	0.00	9.50 (±1.90)
½mMS	0.0	No response	0.00	0.00
	1.0	Hard-white calli and exhibited very poor growth	0.00	16.30 (±1.46)
	2.0	Hard-white calli with moderate growth but cultures turned brown in subsequent subcultures	0.00	25.00 (±1.62)
	3.0	-do-	0.00	35.45 (±2.01)
	4.0	Brownish calli	0.00	17.90 (±1.75)
mMS	0.0	No response	0.00	0.00
	1.0	Hard-white calli with very poor growth	0.00	14.00 (±1.31)
	2.0	As above and turned brown in subsequent subcultures	0.00	20.55 (±1.76)
	3.0	-do-	0.00	29.38 (±1.93)
	4.0	Brownish calli	0.00	16.20 (±1.85)
½DCR	0.0	No response	0.00	0.00
	1.0	Moderately soft-white calli but growth was not very satisfactory	0.00	26.54 (±1.81)
	2.0	Soft-white moderately healthy calli but turned brown in subsequent subcultures	0.00	39.00 (±2.11)
	3.0	-do-	0.00	40.50 (±2.63)
	4.0	Brownish calli	0.00	32.10 (±1.80)
DCR	0.0	No response	0.00	0.00
	1.0	Poor growth of calli and subsequent browning of cultures	0.00	17.11 (±1.54)
	2.0	Moderate calli growth but turned brown in subsequent subcultures	0.00	26.00 (±1.67)
	3.0	-do-	0.00	38.70 (±2.39)
	4.0	Brownish calli	0.00	21.00 (±1.64)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Hard-white calli and exhibited poor growth	0.00	14.00 (±1.71)
	2.0	As above and cultures turned brown in subsequent subcultures	0.00	21.50 (±2.40)
	3.0	-do-	0.00	30.00 (±1.97)
	4.0	Brownish calli	0.00	15.50(± 1.63)
Litvay's	0.0	No response	0.00	0.00
	1.0	Hard-white calli and very poor growth	0.00	14.50 (± 1.57)
	2.0	As above and cultures turned brown in subsequent subcultures	0.00	21.00 (±1.78)
	3.0	-do-	0.00	32.00 (±2.41)
	4.0	Brownish calli	0.00	18.60 (±1.82)

Contd...

½BM_1	0.0	No response	0.00	0.00
	1.0	Very poor calli growth	0.00	10.00 (±1.75)
	2.0	As above and turned brown in subsequent subcultures	0.00	12.50 (±1.81)
	3.0	-do-	0.00	16.75 (±1.63)
	4.0	Brownish calli	0.00	11.25 (±1.84)
BM_1	0.0	No response	0.00	0.00
	1.0	Moderately soft calli but turned hard and brown in subsequent subcultures	0.00	16.00 (±1.59)
	2.0	-do-	0.00	24.50 (±1.73)
	3.0	-do-	0.00	28.00 (±2.32)
	4.0	Brownish calli	0.00	21.00 (±1.70)

*Additional adjuvants: 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

Table 21. Effect of different media and fructose concentrations on initiation of embryogenic cultures from apical dome sections

Media*	Fructose (%)	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
½MS	0.0	No response	0.00	0.00
	1.0	Very poor callusing and cultures turned brown very rapidly	0.00	8.00 (±1.70)
	2.0	-do-	0.00	11.40 (±1.84)
	3.0	-do-	0.00	16.00 (±1.87)
	4.0	Brownish calli	0.00	9.50 (±1.74)
MS	0.0	No response	0.00	0.00
	1.0	Very poor calli growth and rapid browning of culture	0.00	9.00 (±1.61)
	2.0	-do-	0.00	11.00 (±1.72)
	3.0	-do-	0.00	20.15 (±2.31)
	4.0	Brownish calli	0.00	10.20 (±1.88)
½mMS	0.0	No response	0.00	0.00
	1.0	Hard-white calli which turned brown very rapidly in subsequent subcultures	0.00	13.50 (±1.77)
	2.0	-do-	0.00	20.00 (±1.91)
	3.0	-do-	0.00	27.00 (±2.11)
	4.0	Brownish calli	0.00	17.15 (±1.98)
mMS	0.0	No response	0.00	0.00
	1.0	Hard-white calli exhibited very poor growth	0.00	11.50 (±1.68)
	2.0	As above and turned brown in very rapidly in subsequent cultures	0.00	18.00 (±1.79)
	3.0	-do-	0.00	23.40 (±2.15)
	4.0	Brownish calli	0.00	15.00 (±2.24)
½DCR	0.0	No response	0.00	0.00
	1.0	Moderately soft-white calli but with poor growth	0.00	15.00 (±1.47)
	2.0	Moderate calli growth but turned brown rapidly in subsequent subcultures	0.00	24.00 (±2.03)
	3.0	-do-	0.00	31.50 (±2.41)
	4.0	Brownish calli	0.00	19.00 (±1.98)
DCR	0.0	No response	0.00	0.00
	1.0	Poor growth of calli and turned brown very rapidly	0.00	11.51 (±1.63)
	2.0	Moderate calli growth but turned brown in subsequent subcultures	0.00	21.00 (±2.07)
	3.0	-do-	0.00	27.41 (±2.18)
	4.0	Brownish calli	0.00	17.68 (±1.94)
½ Litvay's	0.0	No response	0.00	0.00
	1.0	Poor growth of calli	0.00	10.00 (±1.44)
	2.0	Hard-white calli, turned brown rapidly in subsequent subcultures	0.00	14.00 (±1.79)
	3.0	-do-	0.00	23.40 (±2.01)
	4.0	Brownish calli	0.00	11.30 (±1.79)
Litvay's	0.0	No response	0.00	0.00
	1.0	Hard-white calli and very poor growth	0.00	10.00 (±1.34)
	2.0	As above and turned brown rapidly in subsequent subcultures	0.00	20.00 (±1.75)
	3.0	-do-	0.00	22.00 (±1.36)
	4.0	Brownish calli	0.00	16.80 (±2.11)

Contd...

½BM$_1$	0.0	No response	0.00	0.00
	1.0	Very poor growth of calli	0.00	10.10 (±1.49)
	2.0	As above and turned brown very rapidly in subsequent subcultures	0.00	13.20 (±1.69)
	3.0	-do-	0.00	15.00 (±1.84)
	4.0	Brownish calli	0.00	11.20 (±1.69)
BM$_1$	0.0	No response	0.00	0.00
	1.0	Hard-white calli and very poor growth	0.00	10.40 (±1.59)
	2.0	Moderate culture growth but turned brown rapidly in subsequent subcultures		21.17 (±1.74)
	3.0	-do-	0.00	23.00 (±2.43)
	4.0	Brownish calli	0.00	17.95 (±1.80)

*Additional adjuvants: 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: Standard error.

The effect of different media on initiation of embryogenic cultures using the above mentioned explants is summarised in figure 10.

The effect of different carbohydrates on initiation of embryogenic cultures from different explants on respective media is shown in figure 11. Secondary needles and apical dome sections failed to produce embryogenic cultures with sucrose, maltose, lactose and fructose in the respective initiation media. Zygotic embryos did not form embryogenic cultures on lactose and fructose containing medium but produced embryogenic callus on medium containing sucrose and maltose. Female gametophytes produced embryogenic cultures on all the carbohydrates used. But the cultures obtained from lactose or fructose containing medium turned brown very rapidly and could not be maintained.

Effect of plant growth regulators

Female gametophytes

i. Auxin: There was no callusing in ½Litvay's medium lacking 2,4-D and NAA but containing 2.5 mgl^{-1} BAP (Table 22). Incorporation of either 2,4-D or NAA singly resulted in low frequency of embryogenic culture formation. Inclusion of 2,4-D (5 mgl^{-1}) and NAA (2.5 mgl^{-1}) in conjunction with BAP (2.5 mgl^{-1}) resulted in highest embryogenic response (35%). However, further increase in 2,4-D and NAA were

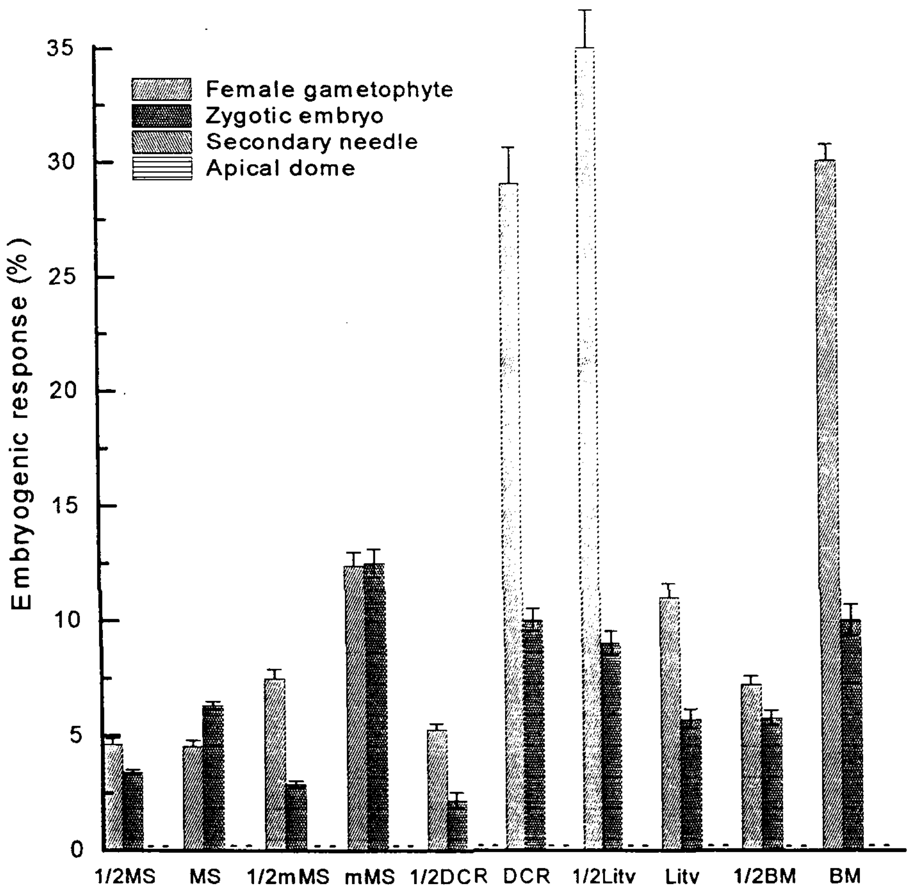

Figure 10. Effect of media (containing 3% sucrose) on initiation of embryogenic cultures from various explants

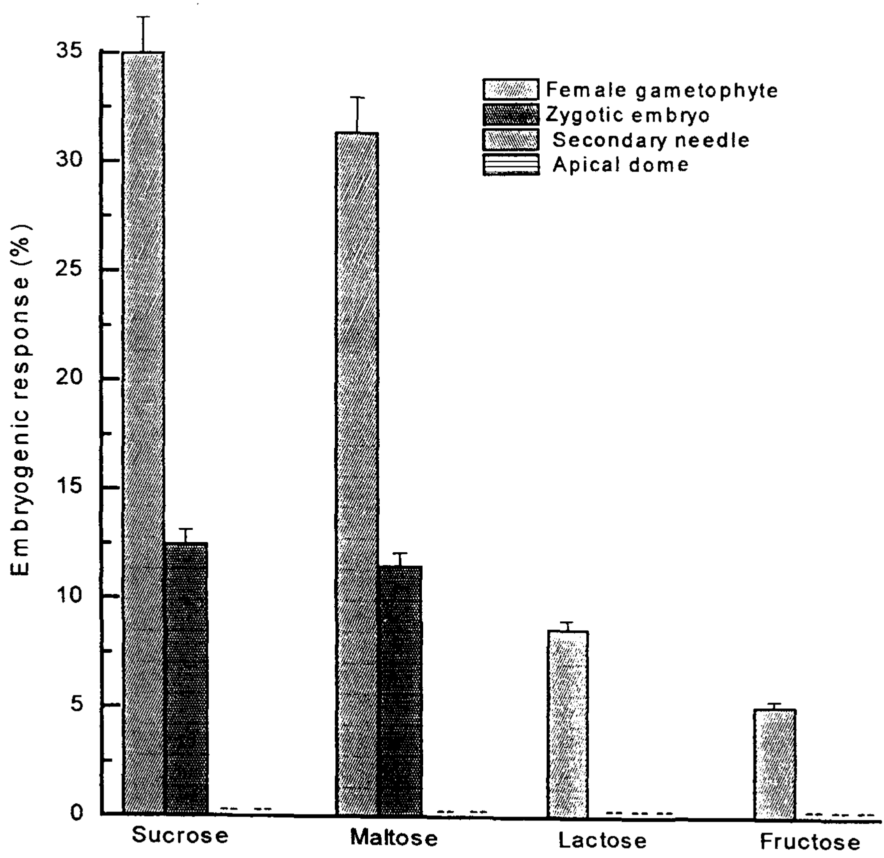

Figure 11. Effect of different carbohydrates (3%) on initiation of embryogenic cultures from different explants cultured on respective initiation medium

Table 22. Effect of 2,4-D and NAA* on initiation of embryogenic cultures from female gametophytes

2,4-D (mgl^{-1})	NAA (mgl^{-1})	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
0.0	0.0	No response	0.00	0.00
2.5	0.0	Very little extrusions and not very healthy	4.51 (±1.20)	20.45 (±1.49)
5.0	0.0	Moderately healthy extrusions and proliferation	10.90 (±1.45)	39.00 (±1.97)
7.5	0.0	-do-	12.00 (±1.69)	41.00 (±2.17)
10.0	0.0	Extrusions turned brown	6.00 (±1.51)	35.00 (±2.32)
0.0	2.5	Poor extrusions and proliferation	2.75 (±1.39)	15.50 (±1.98)
2.5	2.5	Moderate extrusions and proliferation	6.00 (±1.58)	38.50 (±2.45)
5.0	2.5	Healthy extrusions and proliferation	35.00 (±1.40)	22.00 (±1.70)
7.5	2.5	As above but cultures turned brownish	18.50 (±1.71)	26.40 (±2.12)
10.0	2.5	Extrusions turned brownish	9.00 (±1.84)	35.60 (±1.89)
0.0	5.0	Moderately healthy extrusions but with poor proliferation	10.00 (±1.72)	28.90 (±1.91)
2.5	5.0	-do-	16.19 (±1.68)	45.00 (±2.58)
5.0	5.0	As above but cultures turned slight brownish	25.50 (±1.94)	15.00 (±1.42)
7.5	5.0	Extrusions turned brownish	18.00 (±1.32)	29.80 (±1.95)
10.0	5.0	-do-	16.87 (±1.47)	47.10 (±2.60)
0.0	7.5	Moderately healthy extrusions but turned hard	15.45 (±1.37)	26.00 (±1.81)
2.5	7.5	Extrusions turned hard and brownish	12.15 (±1.48)	25.00 (±1.72)
5.0	7.5	-do-	13.00 (±1.91)	31.70 (±1.88)
7.5	7.5	-do-	10.50 (±1.84)	45.10 (±2.10)
10.0	7.5	-do-	10.50 (±1.93)	40.00 (±1.94)
0.0	10.0	Extrusions with retarded growth and proliferation	14.25 (±1.76)	25.00 (±1.47)
2.5	10.0	As above and cultures turned brown	12.50 (±1.68)	37.90 (±1.51)
5.0	10.0	-do-	11.56 (±1.97)	30.40 (±1.90)
7.5	10.0	Culture browning was very rapid	9.00 (±1.46)	28.50 (±1.85)
10.0	10.0	Extrusions turned brownish very rapidly	9.00 (±1.78)	25.10 (±1.90)

* ½Litvay's medium containing sucrose (3.0%), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%);
[1]: standard error

found to be inhibitory. The cultures exhibited white, soft and translucent nature.

ii. N^6-benzylaminopurine: In ½ Litvay's medium devoid of BAP, but containing 5 mgl^{-1} 2,4-D and 2.5 mgl^{-1} NAA a low embryogenic response of 16.51% was recorded (Table 23). Increase in BAP concentration to 2.5 mgl^{-1} in the medium resulted in an increase in the embryogenic cultures (35%). However, further increase in BAP concentrations was found to be inhibitory.

iii. BAP and kinetin: No callusing was observed in ½Litvay's medium in the absence of cytokinins and auxins. Both BAP and kinetin singly or in combination in the medium devoid of auxin failed to produce embryogenic extrusions.

Mature zygotic embryos

i. Auxins: No embryogenic cultures were produced in mMS medium devoid of 2,4-D and NAA but containing 2.5 mgl^{-1} BAP (Table 24). Incorporation of both 2,4-D and NAA at equimolar concentrations of 5 mgl^{-1} each together with 2.5 mgl^{-1} BAP resulted in the best (12.5%) embryogenic culture formation. Other combinations of 2,4-D and NAA in conjunction with 2.5 mgl^{-1} BAP however, showed a decline in embryogenic response.

Table 23. Effect of BAP* on initiation of embryogenic cultures from female gametophytes

BAP (mgl^{-1})	Type of response	% Response (±SE)[1]	
		Embryogenic	Non-embryogenic
0.0	Moderately soft, white, translucent extrusions formed	16.51 (±1.94)	45.00 (±2.34)
0.5	-do-	18.00 (±2.12)	40.00 (±2.14)
1.0	-do-	21.41 (±2.03)	38.15 (±1.84)
1.5	Soft, white, translucent, extrusions formed	24.70 (±1.78)	32.00 (±1.97)
2.0	-do-	28.50 (±1.93)	30.00 (±1.59)
2.5	Soft, white, translucent and very healthy extrusions formed	35.00 (±1.78)	22.25 (±1.97)
3.0	-do-	27.11 (±1.75)	31.60 (±1.91)
3.5	As above but turned slightly hard and brownish	18.23 (±1.91)	39.10 (±2.02)
4.0	-do-	16.79 (±1.76)	47.00 (±2.18)
4.5	-do-	15.67 (±1.54)	47.50 (±1.98)
5.0	-do-	15.10 (±1.31)	49.00 (±2.31)

* ½Litvay's medium containing sucrose (3.0%), 2,4-D (5 mgl^{-1}), NAA (2.5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]:standard error

Table 24. Effect of 2,4-D and NAA* on initiation of embryogenic cultures from mature zygotic embryos

2,4-D (mgl^{-1})	NAA (mgl^{-1})	Type of response	% Response (±SE)[1]	
			Embryogenic	Non-embryogenic
0.0	0.0	No response	0.00	0.00
2.5	0.0	Very small part of explants callused and calli not healthy	2.48 (±1.13)	21.50 (±1.85)
5.0	0.0	Patches of soft and hard calli formed	6.60 (±1.43)	30.60 (±1.78)
7.5	0.0	Comparatively soft calli formed but turned slightly brown	7.75 (±1.71)	20.50 (±2.12)
10.0	0.0	-do-	6.15 (±1.25)	18.00 (±1.90)
0.0	2.5	Almost no callusing	0.00	10.00 (±1.46)
2.5	2.5	Moderately soft calli with small patches of hard portions	10.00 (±1.84)	48.00 (±2.46)
5.0	2.5	-do-	8.50 (±1.73)	42.40 (±1.98)
7.5	2.5	As above but cultures turned slightly brown	7.50 (±1.65)	30.00 (±1.55)
10.0	2.5	-do-	8.00 (±1.48)	34.00 (±2.08)
0.0	5.0	Partially soft calli with patches of hard parts, not very healthy	5.15 (±1.24)	50.13 (±2.46)
2.5	5.0	Mostly soft calli, very little hard calli formed	10.50 (±1.76)	48.50 (±1.79)
5.0	5.0	White, soft, translucent calli and very little hard calli formed	12.50 (±1.35)	85.00 (±2.45)
7.5	5.0	As above but cultures slightly brownish and growth retarded	8.68 (±1.39)	40.00 (±1.98)
10.0	5.0	-do-	6.45 (±1.47)	28.00 (±1.84)
0.0	7.5	Partially soft calli, turned brownish	8.50 (±1.61)	39.00 (±1.69)
2.5	7.5	-do-	7.00 (±1.90)	36.50 (±1.99)
5.0	7.5	White-soft calli but with slow proliferation	8.90 (±1.74)	43.00 (±1.58)
7.5	7.5	White-soft calli with better proliferation but turned brownish	9.21 (±1.33)	51.50 (±1.85)
10.0	7.5	Retarded growth and browning of calli	6.50 (±1.22)	30.00 (±2.09)
0.0	10.0	Very poor callusing	2.10 (±1.70)	19.00 (±1.81)
2.5	10.0	Very poor callusing with retarded growth	2.60 (±1.36)	33.00 (±1.94)
5.0	10.0	As above and cultures turned brown	3.65 (±1.42)	25.00 (±1.50)
7.5	10.0	-do-	4.55 (±1.60)	30.00 (±2.23)
10.0	10.0	Comparatively better calli growth but turned brown	8.41 (±1.93)	34.00 (±2.04)

* mMS medium containing sucrose (3.0%), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%);
[1]: standard error

ii. N^6-benzylaminopurine: In the absence of BAP, but containing 5 mgl^{-1} each of 2,4-D and NAA, 8% explants exhibited embryogenic callusing (Table 25). Increase in BAP concentration to 2.5 mgl^{-1} in the medium enhanced the embryogenic culture (12.5%) formation. Further increase of BAP concentration brought about a gradual fall in embryogenesis.

iii. BAP and kinetin: In the absence of both cytokinins and auxins in mMS medium, callus formation did not occur. BAP and kinetin singly or in combination could not initiate embryogenesis. At higher concentrations, only swelling of explants was observed.

Secondary needles

i. Auxin: There was no callusing of the explant in MS medium devoid of auxin but containing BAP (1 mgl^{-1}) (Table 26). Embryogenesis was not recorded with all the combinations of 2,4-D and NAA in the medium (Table 26). Incorporation of 2,4-D and NAA singly or in combination in the medium in presence of 1 mgl^{-1} BAP resulted in initiation of white, partially soft or hard non-embryogenic calli. A high percentage of 75.5% non-embryogenic cultures resulted from secondary needles when cultured in the medium containing 3 mgl^{-1} each of 2,4-D and NAA in conjunction with 1 mgl^{-1} BAP.

Table 25. Effect of BAP* on initiation of embryogenic cultures from mature zygotic embryos

BAP (mgl^{-1})	Type of response	% Response (±SE)[1]	
		Embryogenic	Non-embryogenic
0.0	Moderately soft calli with small patches of hard part	8.00 (±1.78)	37.50 (±1.98)
0.5	-do-	8.65 (±1.49)	32.00 (±1.77)
1.0	-do-	9.00 (±1.61)	40.00 (±2.12)
1.5	White-soft calli formed	9.46 (±1.76)	51.50 (±2.19)
2.0	-do-	10.65 (±1.41)	57.50 (±2.23)
2.5	White, soft, translucent and healthy calli formed	12.50 (±1.40)	85.00 (±2.45)
3.0	As above but slightly brownish tinge observed	10.86 (±1.31)	60.00 (±1.78)
3.5	Patches of calli turned slightly hard and brownish	9.18 (±1.46)	45.00 (±2.25)
4.0	-do-	8.20 (±1.66)	45.50 (±2.31)
4.5	-do-	8.20 (±1.79)	38.00 (±2.14)
5.0	-do-	8.00 (±1.57)	35.00 (±2.33)

* mMS medium containing sucrose (3.0%), 2,4-D and NAA (5 mgl^{-1} each) and Difco-bacto agar (0.75%); [1]: standard error

Table 26. Effect of 2,4-D and NAA* on initiation of embryogenic cultures from secondary needles

2,4-D (mgl^{-1})	NAA (mgl^{-1})	Type of response	% Response (±SE)[1]	
			Embryogenic[#]	Non-embryogenic
0.0	0.0	No response	0.00	0.00
1.0	0.0	Only few needles swelled and little callusing observed	0.00	25.50 (±2.19)
3.0	0.0	Needles callused at the base but turned hard	0.00	29.00 (±1.74)
5.0	0.0	-do-	0.00	30.50 (±1.85)
0.0	1.0	White calli formed but not very soft and callusing confined only to the basal part	0.00	35.40 (±2.54)
1.0	1.0	Moderately soft-white calli	0.00	57.80 (±2.10)
3.0	1.0	-do-	0.00	65.00 (±2.39)
5.0	1.0	Calli slight brownish	0.00	40.00 (±2.12)
0.0	3.0	Moderately soft-white calli but turned hard	0.00	59.00 (±1.35)
1.0	3.0	-do-	0.00	61.57 (±2.31)
3.0	3.0	Soft-white calli but turned hard	0.00	75.50 (±2.20)
5.0	3.0	As above but cultures turned slightly brown	0.00	69.00 (±1.77)
0.0	5.0	Calli turned hard and brownish	0.00	27.88 (±1.79)
1.0	5.0	Moderately soft-white calli but turned brownish	0.00	58.00 (±1.95)
3.0	5.0	-do-	0.00	60.50 (±2.17)
5.0	5.0	-do-	0.00	38.55 (±1.95)

* MS medium containing sucrose (3.0%) and BAP ($1mgl^{-1}$) and Difco-bacto agar (0.75%); [1]: standard error

[#] **embryogenesis not recorded**

ii. N^6-benzylaminopurine: MS medium devoid of BAP but containing 3 mgl^{-1} each of 2,4-D and NAA resulted in white, moderately soft callus (67.5%) (Table 27). Supplementation of medium with 1 mgl^{-1} BAP exhibited 75.5% callusing. However, all the combinations of BAP in conjunction with 2,4-D and NAA failed to induce embryogenesis in this explant.

iii. BAP and kinetin: In the absence of both auxins and cytokinins in MS medium, no callusing was observed. Both BAP and kinetin singly or in combination but lacking any auxin did not promote callusing.

Apical dome sections

i. Auxin: All the combinations of 2,4-D and NAA along with BAP resulted in only non-embryogenic callus formation (Table 28). The explants did not show callusing in ½DCR medium devoid of 2,4-D and NAA but containing 2.5 mgl^{-1} BAP. Increased callus formation was achieved with the addition of both 2,4-D and NAA either singly or in combination. Maximum non-embryogenic callus formation (81.5%) was obtained with 5 mgl^{-1} each of 2,4-D and NAA and 2.5 mgl^{-1} BAP.

ii. N^6-benzylaminopurine: Explants formed white and hard callus (46.5%) in the medium containing 2,4-D and NAA (5 mgl^{-1} each) but without BAP (Table 29). However, with 2.5 mgl^{-1} BAP in conjunction with 2,4-D and NAA (each at 5 mgl^{-1}) resulted in highest

Table 27. Effect of BAP* on initiation of embryogenic cultures from secondary needles

BAP (mgl^{-1})	Type of response	% Response (±SE)[1]	
		Embryogenic[#]	Non-embryogenic
0.0	Moderate calli growth but not very soft	0.00	67.50 (±2.21)
0.5	-do-	0.00	70.00 (±2.45)
1.0	Soft-white calli but turned hard and brownish	0.00	75.50 (±2.20)
1.5	-do-	0.00	68.50 (±1.95)
2.0	White but hard calli	0.00	60.00 (±2.14)
2.5	-do-	0.00	57.00 (±1.89)
3.0	-do-	0.00	50.00 (±1.85)
3.5	White-hard calli	0.00	46.00 (±1.87)
4.0	-do-	0.00	42.00 (±1.79)
4.5	-do-	0.00	39.00 (±1.77)
5.0	-do-	0.00	35.00 (±1.45)

* MS medium containing sucrose (3.0%), 2,4-D and NAA (each at 3 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: standard error
[#] **embryogenesis not recorded**

Table 28. Effect of 2,4-D and NAA* on initiation of embryogenic cultures from apical dome sections

2,4-D (mgl^{-1})	NAA (mgl^{-1})	Type of response	% Response (±SE)[1]	
			Embryogenic[#]	Non-embryogenic
0.0	0.0	No response	0.00	0.00
1.0	0.0	Very little callusing	0.00	26.00 (±2.41)
3.0	0.0	-do-	0.00	30.00 (±1.98)
5.0	0.0	White calli but not very soft and healthy	0.00	35.00 (±1.75)
7.0	0.0	As above but cultures turned brownish	0.00	23.24 (±2.02)
0.0	1.0	Only swelling of explants and little hard calli formed	0.00	20.00 (±1.85)
1.0	1.0	Moderately hard calli	0.00	41.00 (±1.97)
3.0	1.0	-do-	0.00	39.00 (±2.41)
5.0	1.0	Moderately soft calli	0.00	47.50 (±1.34)
7.0	1.0	As above but slightly brownish	0.00	45.60 (±2.32)
0.0	3.0	Hard white calli	0.00	33.55 (±2.25)
1.0	3.0	Healthy but hard calli	0.00	63.00 (±1.85)
3.0	3.0	Comparatively more white and soft calli	0.00	66.00 (±2.27)
5.0	3.0	As above but slightly brownish	0.00	53.50 (±2.47)
7.0	3.0	-do-	0.00	48.50 (±2.74)
0.0	5.0	White calli formed	0.00	40.00 (±1.75)
1.0	5.0	-do-	0.00	61.40 (±2.61)
3.0	5.0	White soft calli with very healthy growth	0.00	72.00 (±2.33)
5.0	5.0	-do-	0.00	81.50 (±2.19)
7.0	5.0	As above but cultures turned brown	0.00	67.00 (±1.89)
0.0	7.0	Soft white calli but turned brownish	0.00	38.20 (±1.94)
1.0	7.0	-do-	0.00	65.90 (±2.31)
3.0	7.0	-do-	0.00	68.00 (±2.11)
5.0	7.0	-do-	0.00	61.70 (±2.01)
7.0	7.0	-do-	0.00	45.00 (±1.78)

* ½DCR medium containing sucrose (3.0%), BAP (2.5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: standard error

[#] **embryogenesis not recorded**

Table 29. Effect of BAP* on initiation of embryogenic cultures from apical dome sections

BAP (mgl^{-1})	Type of response	% Response (±SE)[1]	
		Embryogenic[#]	Non-embryogenic
0.0	White-hard calli	0.00	46.50 (±2.18)
0.5	Moderately soft calli	0.00	49.75 (±1.75)
1.0	-do-	0.00	50.00 (±2.07)
1.5	-do-	0.00	62.50 (±2.20)
2.0	Soft calli, turned brownish	0.00	70.51 (±2.15)
2.5	-do-	0.00	81.50 (±2.19)
3.0	-do-	0.00	73.50 (±2.09)
3.5	Comparatively hard calli	0.00	70.00 (±1.84)
4.0	-do-	0.00	51.50 (±2.40)
4.5	-do-	0.00	50.00 (±1.78)
5.0	Hard-greenish calli	0.00	45.50 (±1.98)

* ½DCR medium containing sucrose (3.0%), 2,4-D and NAA (each at 5 mgl^{-1}) and Difco-bacto agar (0.75%); [1]: standard error

[#] **embryogenesis not recorded**

non-embryogenic culture formation (81.5%). No embryogenic response was observed with apical dome sections.

iii. BAP and kinetin: The callus formation was not noticed in the medium without cytokinins and auxins. Both BAP and kinetin singly did not promote callusing.

Maintenance of cultures

After 2-3 subcultures, the embryogenic calli were transferred to respective maintenance media as they turned brownish if maintained on the initiation medium.

Female gametophytes: The cultures with pro-embryos were transferred to maintenance medium for further proliferation. The best response was observed on medium containing 1/10th growth regulators to that of initiation medium i.e. 2,4-D (0.5 mgl^{-1}), NAA and BAP (0.25 mgl^{-1} each) and 3% sucrose. When cultured on growth regulator free medium, culture proliferation was retarded considerably. Use of maltose at 3% level was found to be less effective in embryogenic culture proliferation as compared to sucrose at the same level of concentration (Table 30 and 31).

Mature zygotic embryos: The embryogenic cultures proliferated on mMS medium containing 1/10th growth regulators to that of initiation medium i.e. 2,4-D and NAA (each at 0.5 mgl^{-1}) in conjunction with

Table 30. Effect of growth regulators* on maintenance and multiplication of embryogenic calli from female gametophytes

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response	Final wt. of callus (g)** (±SE)[1]	Fold increase
5.00	2.50	2.50	Culture proliferation was satisfactory but rapid browning was observed	1.89 (±1.11)	3.78
1.00	0.50	0.50	Culture growth was satisfactory and browning of cultures was reduced considerably	1.90 (±1.05)	3.80
0.50	0.25	0.25	Calli growth was satisfactory, cultures were healthy and no browning was observed	2.28 (±1.16)	4.55
0.00	0.00	0.00	Calli growth retarded considerably	2.050 (±1.13)	4.10

* ½Litvay's medium containing sucrose (3.0%) and Difco-bacto agar (0.75%); [1]: standard error.

** Data recorded after 28 days (ca. 0.5 g callus inoculum was used)

Table 31. Effect of growth regulators* on maintenance and multiplication of embryogenic calli from female gametophytes

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response	Final wt. of callus (g)** (±SE)[1]	Fold increase
5.00	2.50	2.50	Culture proliferation was slower than sucrose containing medium but browning was comparatively less	1.43 (±1.31)	2.85
1.00	0.50	0.50	Culture proliferation was slow but culture browning was negligible	1.60 (±1.12)	3.20
0.50	0.25	0.25	Calli proliferation were slow but cultures healthy and no browning was observed	1.88 (±1.42)	3.75
0.00	0.00	0.00	Calli growth retarded considerably no browning of cultures	1.75 (±1.16)	3.50

* ½Litvay's medium containing maltose (3.0%) and Difco-bacto agar (0.75%); [1]: standard error.

** Data recorded after 28 days (ca. 0.5 g callus inoculum was used)

BAP (0.25 mgl^{-1}) and 3% sucrose (Table 32). Maltose at 3% proved less effective than sucrose at the same concentration in proliferation of embryogenic cultures (Table 33).

Secondary needles: The cultures from secondary needles proliferated best on MS medium containing $1/10^{th}$ growth regulators of initiation medium i.e., 2,4-D and NAA (0.3 mgl^{-1} each) and BAP (0.1 mgl^{-1}). 3% sucrose (Table 34) was found to be better for proliferation of cultures than 3% maltose (Table 35) in the maintenance medium. However, somatic embryogenesis was not recorded in cultures obtained from secondary needle explants.

Apical dome sections: The cultures proliferated well when maintained on ½DCR medium with $1/10^{th}$ and $1/5^{th}$ growth regulators to that of initiation medium (Table 36). However in the latter treatment, the cultures showed browning. Culture proliferation declined considerably when maintained in growth regulator-free medium. In $1/10^{th}$ growth regulators and 3% sucrose containing medium (Table 36) better proliferation resulted compared to the cultures grown in medium containing 3% maltose (Table 37). The cultures, however, did not show embryogenesis.

Table 32. Effect of growth regulators* on maintenance and multiplication of embryogenic calli from zygotic embryos

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response	Final wt. of callus (g)** (±SE)[1]	Fold increase
5.00	5.00	2.50	Proliferation of calli was satisfactory but cultures turned brownish	1.70 (±1.07)	3.46
1.00	1.00	0.50	Proliferation of calli was satisfactory and browning of cultures was reduced considerably	1.83 (±1.12)	3.66
0.50	0.50	0.25	Proliferation of calli was satisfactory, cultures were healthy and no browning of cultures	2.10 (±1.25)	4.20
0.00	0.00	0.00	Cultures showed slow proliferation	1.90 (±1.19)	3.80

* mMS medium containing sucrose (3.0%) and Difco-bacto agar (0.75%); [1]: standard error
** Data recorded after 28 days (ca. 0.5 g callus inoculum was used)

Table 33. Effect of growth regulators* on maintenance and multiplication of embryogenic calli from zygotic embryos

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response	Final wt. of callus (g)** (±SE)[1]	Fold increase
5.00	5.00	2.50	Proliferation of calli was comparatively slow but culture browning was less	1.33 (±1.25)	2.65
1.00	1.00	0.50	Proliferation of calli was slow but culture browning was very negligible	1.63 (±1.16)	3.26
0.50	0.50	0.25	Proliferation of calli was slow but no browning of cultures	1.98 (±1.22)	3.96
0.00	0.00	0.00	Cultures showed very slow proliferation	1.78 (±1.34)	3.55

* mMS medium containing maltose (3.0%) and Difco-bacto agar (0.75%); [1]: standard error
** Data recorded after 28 days (ca. 0.5 g callus inoculum was used)

Table 34. Effect of growth regulators* on maintenance and multiplication of calli from secondary needles

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response#	Final wt. of callus (g)** (±SE)[1]	Fold increase
3.00	3.00	1.00	Proliferation of calli was good but cultures turned brownish very rapidly	2.00 (±1.31)	4.00
0.60	0.60	0.20	Proliferation of calli was satisfactory and culture browning was reduced considerably	2.13 (±1.11)	4.26
0.30	0.30	0.10	As above but without any browning of calli	2.30 (±1.14)	4.60
0.00	0.00	0.00	Cultures showed slow proliferation	9.50 (±1.14)	1.90

* MS medium containing sucrose (3.0%) and Difco-bacto agar (0.75%); [1]: standard error
** Data recorded after 28 days (ca. 0.5 g callus inoculum was used)
# **Embryogenesis not recorded; data shown above relate to non-embryogenic callus**

Table 35. Effect of growth regulators* on maintenance and multiplication of calli from secondary needles

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response[#]	Final wt. of callus (g)** (±SE)[1]	Fold increase
3.00	3.00	1.00	Proliferation of calli was slow but culture browning was less	1.88 (±1.24)	3.75
0.60	0.60	0.20	Proliferation of calli was slow but almost no browning of cultures	2.00 (±1.59)	4.00
0.30	0.30	0.10	As above but without any browning of calli	2.08 (±1.14)	4.15
0.00	0.00	0.00	Cultures showed very slow proliferation and no browning observed	0.75 (±1.14)	1.50

* MS medium containing maltose (3.0%) and Difco-bacto agar (0.75%); [1]: standard error
** Data recorded after 28 days (ca. 0.5 g callus inoculum was used)
[#] **Embryogenesis not recorded; data shown above relate to non-embryogenic callus**

Table 36. Effect of growth regulators* on maintenance and multiplication of calli from apical dome sections

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response#	Final wt. of callus (g)** (±SE)[1]	Fold increase
5.00	5.00	2.50	Proliferation of calli was satisfactory but culture turned brownish very rapidly	1.99 (±1.42)	3.98
1.00	1.00	0.50	Proliferation of calli was satisfactory and culture browning was reduced considerably	2.10 (±1.11)	4.20
0.50	0.50	0.25	As above and cultures were healthy and no browning observed	2.21 (±1.34)	4.42
0.00	0.00	0.00	Cultures exhibited slow proliferation	9.00 (±1.37)	1.80

* ½DCR medium containing sucrose (3.0%) and Difco-bacto agar (0.75%); [1]: standard error
** Data recorded after 28 days (ca. 0.5 g callus inoculum was used)
# **Embryogenesis not recorded; data shown above relate to non-embryogenic callus**

Table 37. Effect of growth regulators* on maintenance and multiplication of calli from apical dome sections

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response	Final wt. of callus (g) (±SE)[1]	Fold increase
5.00	5.00	2.50	Proliferation of calli was slow but culture browning was very less	1.25 (±1.33)	2.50
1.00	1.00	0.50	Proliferation of calli was slow but almost no browning of cultures	1.80 (±1.41)	3.60
0.50	0.50	0.25	As above and no browning observed	1.95 (±1.65)	3.90
0.00	0.00	0.00	Cultures exhibited slow growth but no browning	0.85 (±1.29)	1.70

* ½DCR medium containing maltose (3.0%) and Difco-bacto agar (0.75%); [1]: standard error
** Data recorded after 28 days (ca. 0.5 g callus inoculum was used)
[#] **Embryogenesis not recorded; data shown above relate to non-embryogenic callus**

Initiation and establishment of embryogenic suspension cultures in SF and BCB

Initiation of cultures

Effect of carbohydrates

Female gametophytes: Figure 12a shows the embryogenic extrusion from female gametophyte. No growth and proliferation of cultures was observed in the control (Table 38). The best cultures were obtained in ½Litvay's medium supplemented with 3% sucrose and exhibited satisfactory cleavage and healthy pro-embryo formation (Figure 12b-d). Increase in sucrose concentration beyond 3% resulted in browning of cultures (Table 38).

Culture initiation and establishment was quite effective in medium containing 3% maltose. However, ½Litavay's medium with maltose as carbohydrate source exhibited slow proliferation of ESMs and moderately healthy pro-embryos (Table 38) when compared with sucrose. When maltose concentration was increased beyond 3%, culture browning was observed. The browning was conspicuously less in the medium containing maltose compared to sucrose even at higher concentrations (Table 38).

Lactose and fructose incorporated separately in ½Litvay's medium were found ineffective in initiation and establishment of

Figure 12. Different stages of somatic embryogenesis from female gametophyte explants in suspension culture

a. Female gametophyte with embryogenic extrusions on semi-solid medium (0.63 x 40)
b. Two early stage pro-embryos with suspensor like cells (40x)
c. An enlarged view of pro-embryo (80x)
d. Early stage of SE (stage-I) formation with dense embryonal head and four long, vacuolated suspensor like cells (40x)
e. Two stage-II SEs sharing a common suspensor like cells (40x)
f. An advanced stage-II (bullet shaped) SE and long suspensor like cells (40x)

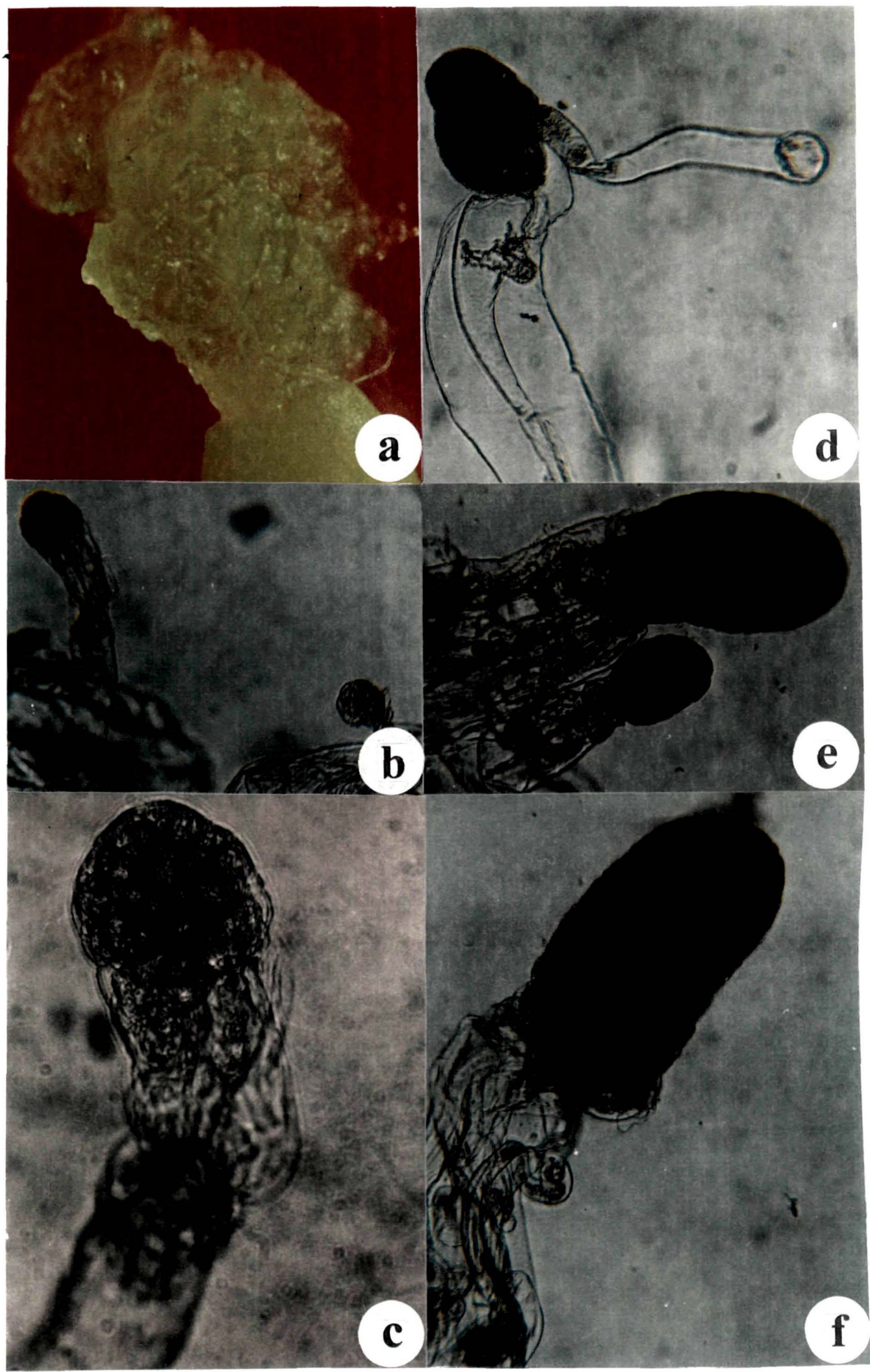

Figure 12

Figure 12. Different stages of somatic embryogenesis from female gametophyte explants in suspension culture

g. Stage-III (cotyledonary) SE showing initiation of cotyledonary bumps (40x)

h. Two cotyledonary stage SEs sharing a common suspensor like cells (0.63 x 40)

i. Many mature cotyledonary stage SEs (0.63 x 16)

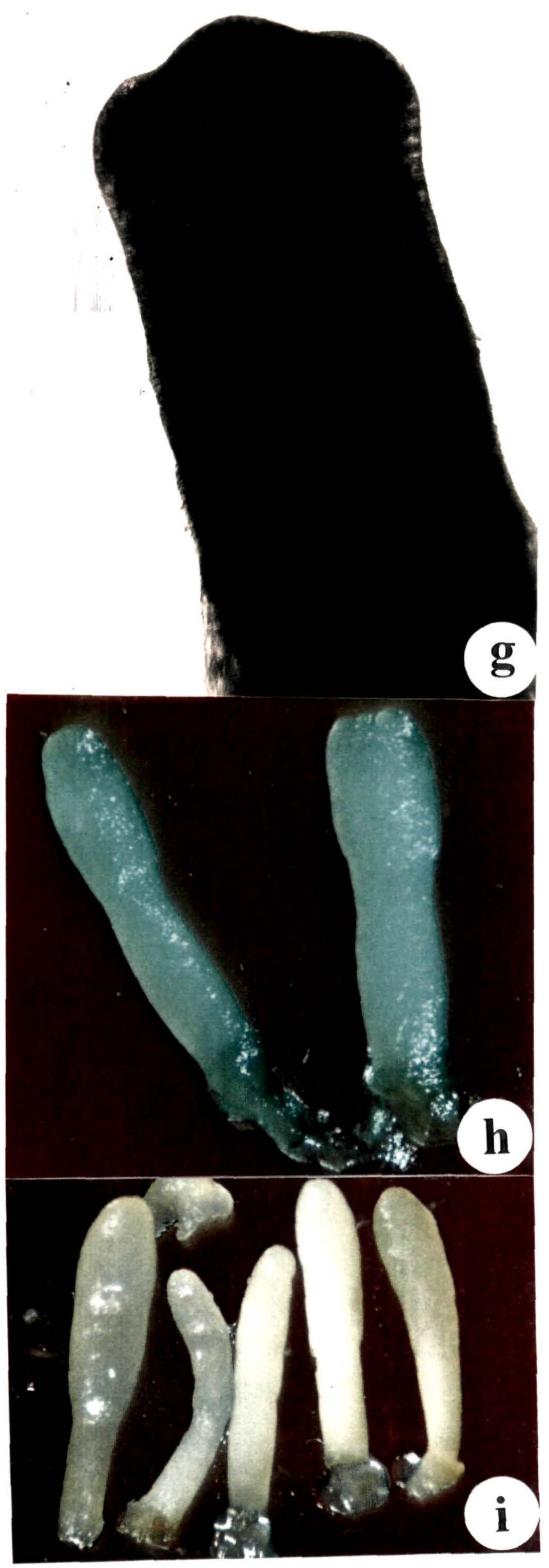

Figure 12

Table 38. Effect of different carbohydrates at varying concentrations on initiation of embryogenic suspension cultures from embryogenic extrusions of female gametophytes and embryogenic calli of zygotic embryos**

Carbohydrate	Carbohydrate (%)	Type of response	Performance*
Sucrose	0.0	Cultures degenerated	-
	1.0	Poor growth and proliferation of ESMs	+
	2.0	Moderate growth and proliferation of ESMs but cleaving was not satisfactory	++
	3.0	Culture proliferation was satisfactory, showed cleavage and healthy pro-embryos	++++
	4.0	Moderate culture proliferation, showed pro-embryos but cultures turned slight brownish	++
Maltose	0.0	Cultures degenerated	-
	1.0	Poor and slow proliferation of ESMs	+
	2.0	Slow growth and proliferation of ESMs, pro-embryos were not very healthy	++
	3.0	Slow proliferation of ESMs, cultures showed moderate cleavage and moderately healthy pro-embryos	+++
	4.0	Slow proliferation of ESMs, pro-embryos were fewer and slight browning observed	++
Lactose	0.0	Cultures degenerated	-
	1.0	Very poor growth and proliferation of ESMs	+
	2.0	Moderate proliferation and cleaving of ESMs but cultures turned slight brownish in subsequent subcultures	++
	3.0	-do-	++
	4.0	As above and cultures turned brownish very rapidly	+
Fructose	0.0	Cultures degenerated	-
	1.0	Very poor growth and proliferation of ESMs	+
	2.0	Moderate proliferation and cleaving of ESMs but cultures turned slight brownish in subsequent subcultures	++
	3.0	-do-	++
	4.0	As above and cultures turned brownish very rapidly	+

** Calli from female gametophytes were cultured in liquid ½Litvay's medium supplemented with 2,4-D (5 mgl^{-1}), NAA and BAP (2.5 mgl^{-1} each); Calli from zygotic embryos were cultured in liquid mMS medium supplemented with 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}); * - no, + poor, ++ moderate, +++ good and ++++ optimum embryogenic response
ESMs: embryonal suspensor masses

embryogenic suspension cultures. Even at lower concentrations of lactose and fructose (2%), culture browning was prominent.

Mature zygotic embryos: In control, the cultures degenerated in the medium free of carbohydrates. Cultures showed healthy pro-embryos (Figure 13a-e) with satisfactory proliferation and cleaving in mMS medium containing 3% sucrose (Table 38). Increase in sucrose level beyond 3% led to culture browning.

Maltose proved to be less effective in initiation and establishment of suspension cultures. The cultures showed moderate growth and proliferation in medium containing 2 and 3% maltose. In general, over all growth and proliferation of cultures was slow in medium containing maltose than sucrose at 2 and 3% level. Increasing maltose concentration beyond 3% resulted in slight browning of cultures. Culture browning was found to be distinct and more in sucrose as compared to maltose containing medium (Table 38).

Secondary needles: The non-embryogenic cultures degenerated in the liquid medium devoid of any carbohydrates (Table 39). 3% sucrose in the medium showed optimum growth and proliferation of non-embryogenic cell lines (Figure 14a-d). Cultures turned brown when sucrose concentration was raised beyond 3%.

Figure 13. Different stages of somatic embryogenesis from mature zygotic embryo explants in suspension cultures

a. Mature zygotic embryo explants (0.63 x 10)

b. Embryogenic callus on semi-solid medium (0.63 x 10)

c. Three early stage pro-embryos sharing common suspensor like cells (40x)

d. A proembryo with 4-8 embryonal head cells and 2-3 long suspensor like cells (40x)

e. An early stage SE (stage-I) with dense embryonal head and 3 long, vacuolated suspensor like cells (40x)

f. Two SEs (stage-I) sharing common suspensor like cells (40x)

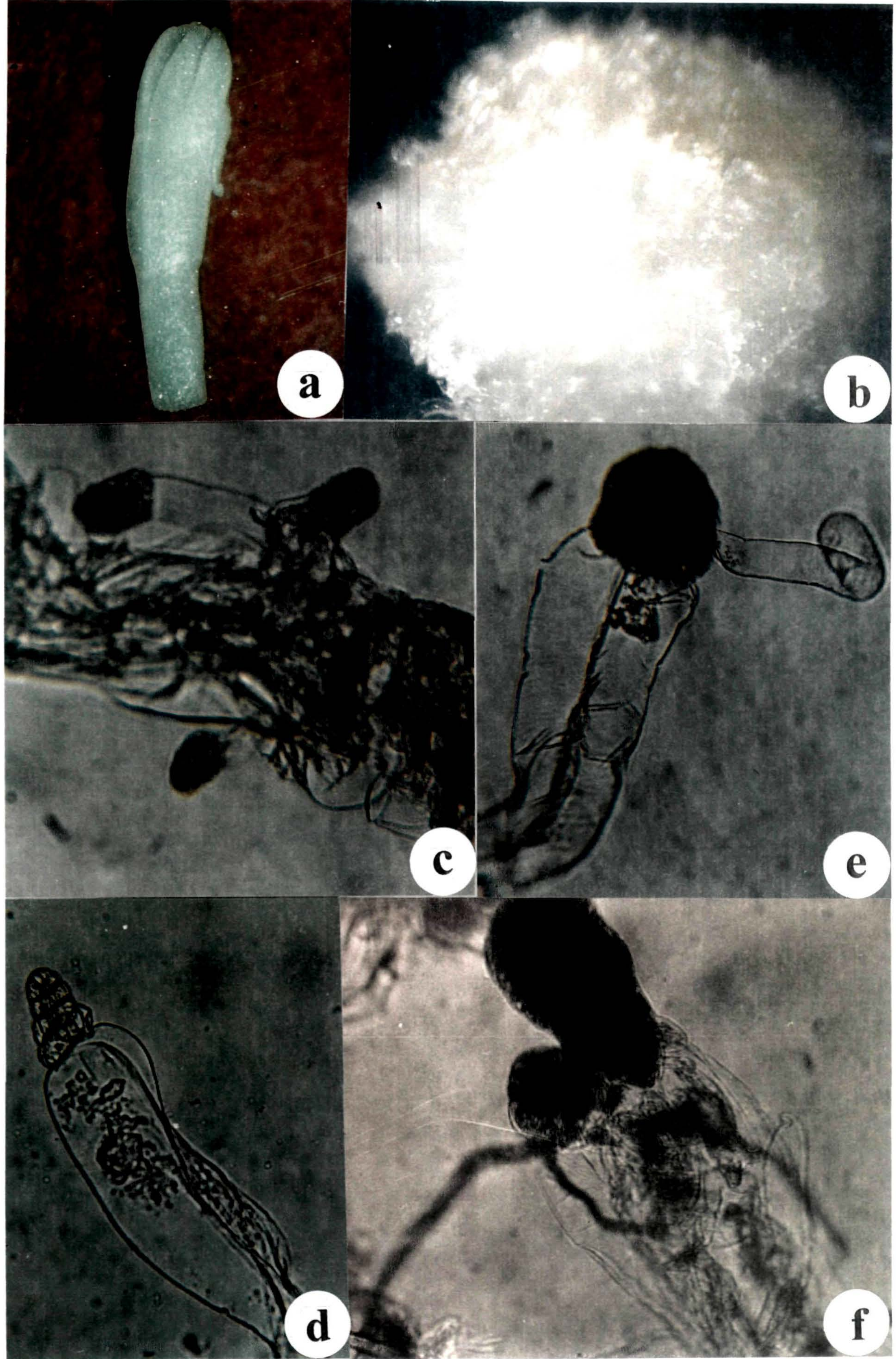

Figure 13

Figure 13. Different stages of somatic embryogenesis from mature zygotic embryo explants in suspension cultures

g. Two SEs (stage-I) sharing common suspensor like cells (40x)

h. Advanced stage SE (stage-II) showing two-three bullet shaped embryonal heads sharing common long suspensor like cells (40x)

i. Stage-III SE (cotyledonary stage) (0.63 x 25)

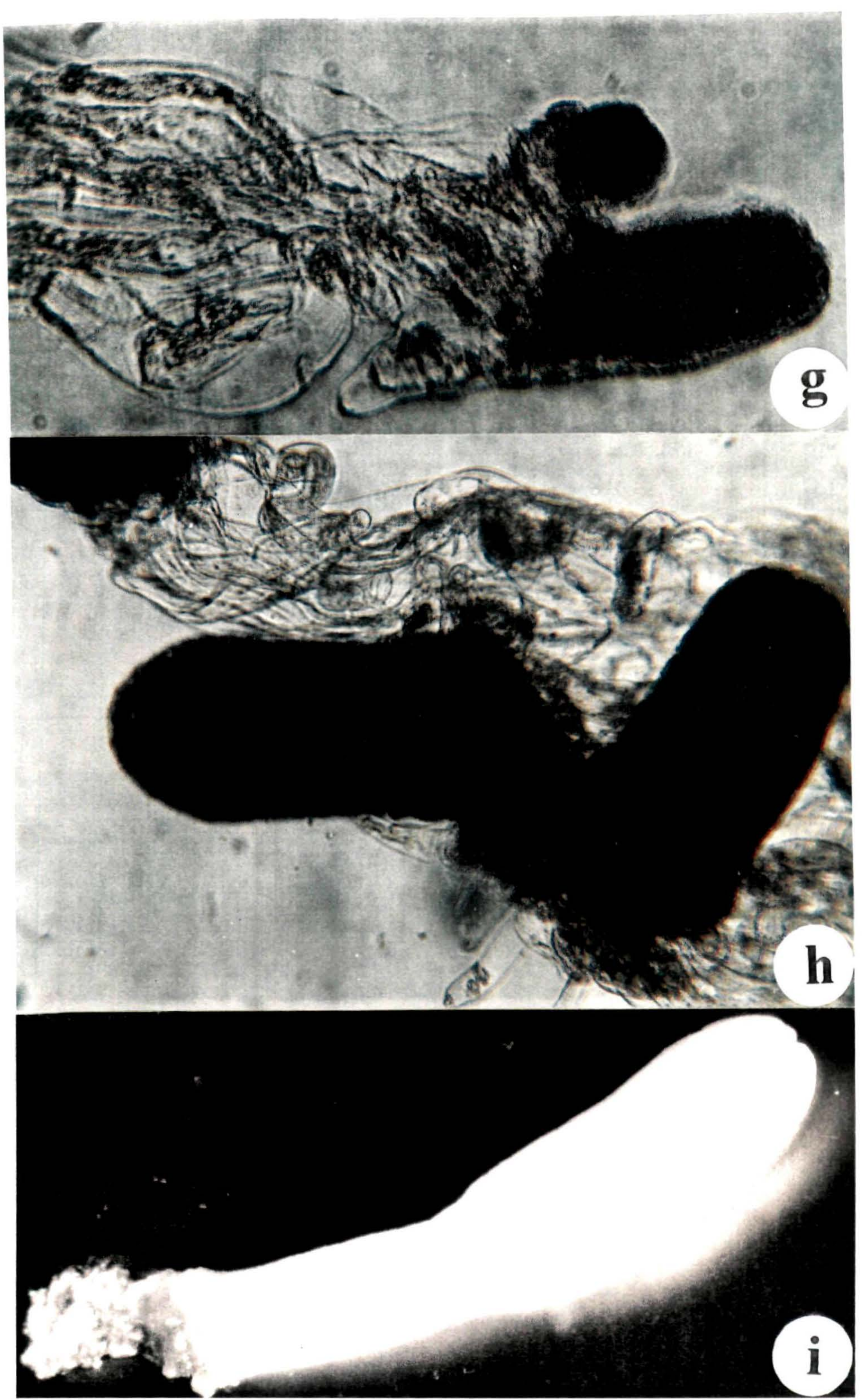

Figure 13

Table 39. Effect of different carbohydrates at varying concentrations on initiation of embryogenic suspension cultures from calli of secondary needles and apical dome sections**

Carbohydrates	Carbohydrate (%)	Type of response#	Performance*
Sucrose	0.0	Cultures degenerated	-
	1.0	Poor proliferation of cultures	+
	2.0	Moderate growth and proliferation of cultures. Cultures composed of mixture of spherical and elongated cells but no pro-embryos observed	++
	3.0	As above but culture proliferation was satisfactory	++
	4.0	Moderate culture proliferation, showed mixture of spherical and elongated cells but no pro-embryos. Cultures turned slight brownish	++
Maltose	0.0	Cultures degenerated	-
	1.0	Poor and slow proliferation of cultures	+
	2.0	Slow growth and proliferation of cultures. Cultures composed of spherical and elongated cells but no pro-embryos observed	++
	3.0	As above but cultures moderately healthy	++
	4.0	Slow proliferation of cultures, no pro-embryos observed	++
Lactose	0.0	Cultures degenerated	-
	1.0	Poor growth and proliferation of cultures	+
	2.0	Moderate proliferation of cultures but turned slight brownish. No pro-embryos observed	++
	3.0	-do-	++
	4.0	As above and cultures turned brownish very rapidly	+
Fructose	0.0	Cultures degenerated	-
	1.0	Poor growth and proliferation of cultures	+
	2.0	Moderate proliferation of cultures and turned slight brownish. No pro-embryos observed	++
	3.0	-do-	++
	4.0	As above and cultures turned brownish very rapidly	+

** Calli from secondary needles were cultured in liqiud MS medium supplemented with 2,4-D and NAA (3 mgl^{-1} each); BAP (1 mgl^{-1}); Calli from apical dome sections were cultured in liquid ½DCR medium supplemented with 2,4-D and NAA (5 mgl^{-1} each); BAP (2.5 mgl^{-1}); * - no, + poor, ++ moderate response

Embryogenesis not recorded

Figure 14. Suspension cultures from calli of secondary needle explants showing non-embryogenic cell lines

a. A small clump of rounded cells (40x)
b. A small non-embryogenic cell clump with rounded and elongated cells (40x)
c. Suspension with mixture of rounded, oblong and elongated suspensor like non-embryogenic cells (40x)
d. Non-embryogenic elongated suspensor like cells (40x)

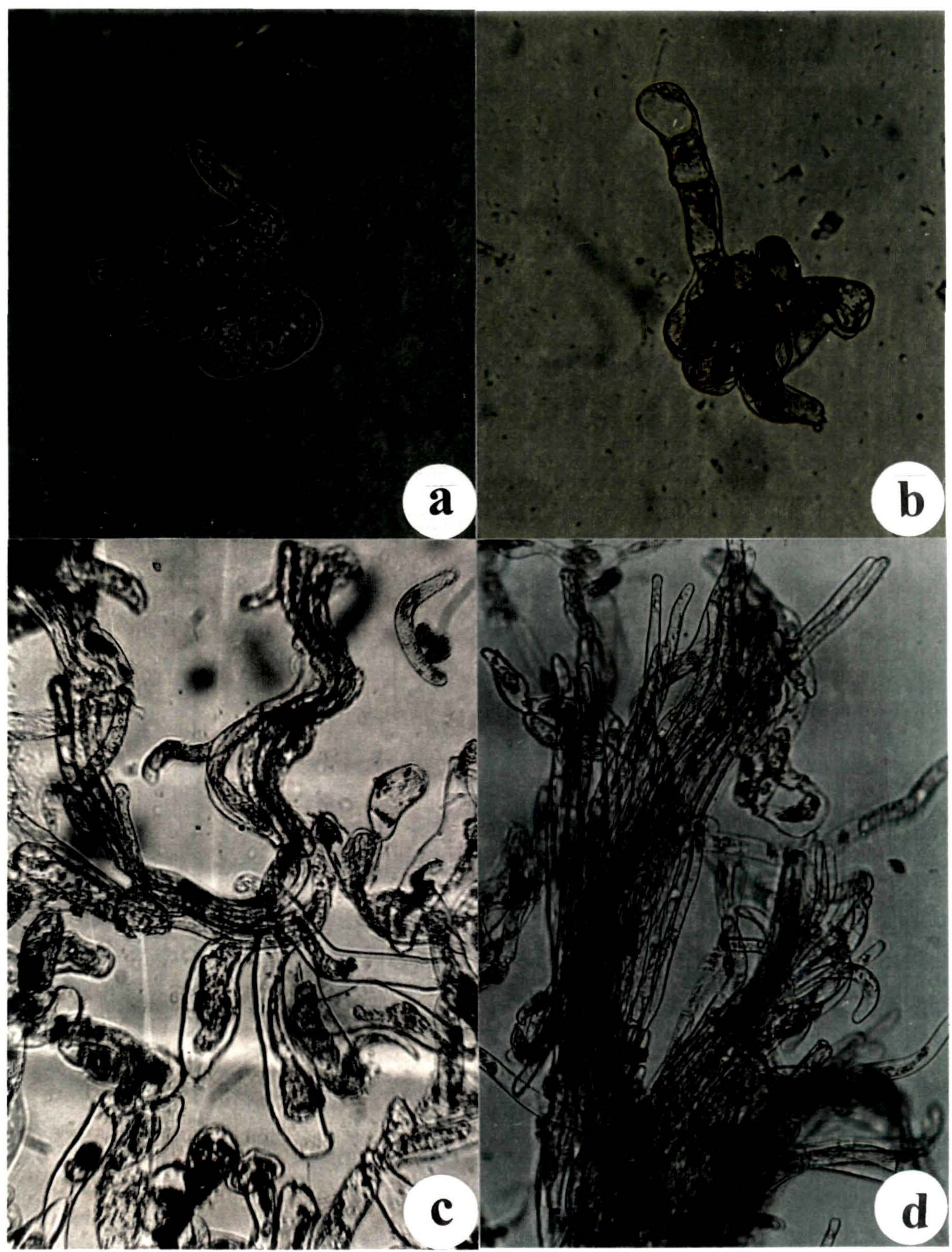

Figure 14

The optimum growth of non-embryogenic cell lines was found when MS medium was incorporated with 3% maltose. The cultures exhibited slow growth and proliferation in the medium with maltose as compared to sucrose. Media containing lactose and fructose were found least effective in initiation and establishment of culture. At low concentration of lactose and fructose (2%) there was culture browning (Table 39).

The cultures were found to consist of spherical and elongated suspensor like cells in all the above mentioned carbohydrate-containing medium. However, no pro-embryos or somatic embryos were observed in the cultures.

Apical dome sections: ½DCR medium free of carbohydrates resulted in degeneration of cultures (Table 39). Healthy culture growth and proliferation was obtained with 3% sucrose. Increase in sucrose concentration beyond 3% resulted in culture browning. The response was poor in the medium containing maltose at 2 and 3% level as compared to sucrose. Increase in the level of maltose beyond 3% brought about slight browning of cultures. However, intensity of browning was low in this treatment compared to sucrose. Unsatisfactory growth of cultures resulted in the media containing lactose and fructose. Culture browning set in even at 2% level of both lactose and fructose.

The cultures were composed of mixture of spherical and elongated suspensor like cells in the media with different carbohydrates tested. No pro-embryos or somatic embryos developed in the culture (Figure 15a-f).

Effect of plant growth regulators

Female gametophytes

i. Auxins: The embryogenic cultures failed to proliferate in the medium devoid of 2,4-D and NAA but containing 2.5 mgl^{-1} BAP. Increase in the concentration of auxins showed better growth and proliferation of cultures. The optimum response was observed with 5 mgl^{-1} 2,4-D and 2.5 mgl^{-1} NAA in conjunction with 2.5 mgl^{-1} BAP. Further increase in auxin concentration resulted in browning and degeneration of cultures (Table 40).

ii. N^6-benzylaminopurine: The growth and proliferation of embryogenic suspension cultures varied with different concentrations of BAP in the medium incorporated with 2,4-D (5 mgl^{-1}) and NAA (2.5 mgl^{-1}) (Table 41). Absence of BAP resulted in poor culture growth and cleavage. Healthy culture growth and proliferation with satisfactory cleavage of ESMs and somatic embryos were observed in the medium containing 2.5 mgl^{-1} BAP (Figure 12b-d). However, higher concentrations of BAP inhibited culture growth.

Figure 15. Suspension cultures from calli of apical dome sections showing non-embryogenic cell lines

a. Clumps of rounded cells emerging from inoculum (40x)

b. Rounded and elongated cell masses (40x)

c. A clump of rounded non-embryogenic cells (40x)

d. A non-embryogenic cell clump with rounded and elongated cells (40x)

e–f. Non-embryogenic elongated suspensor like cells (40x)

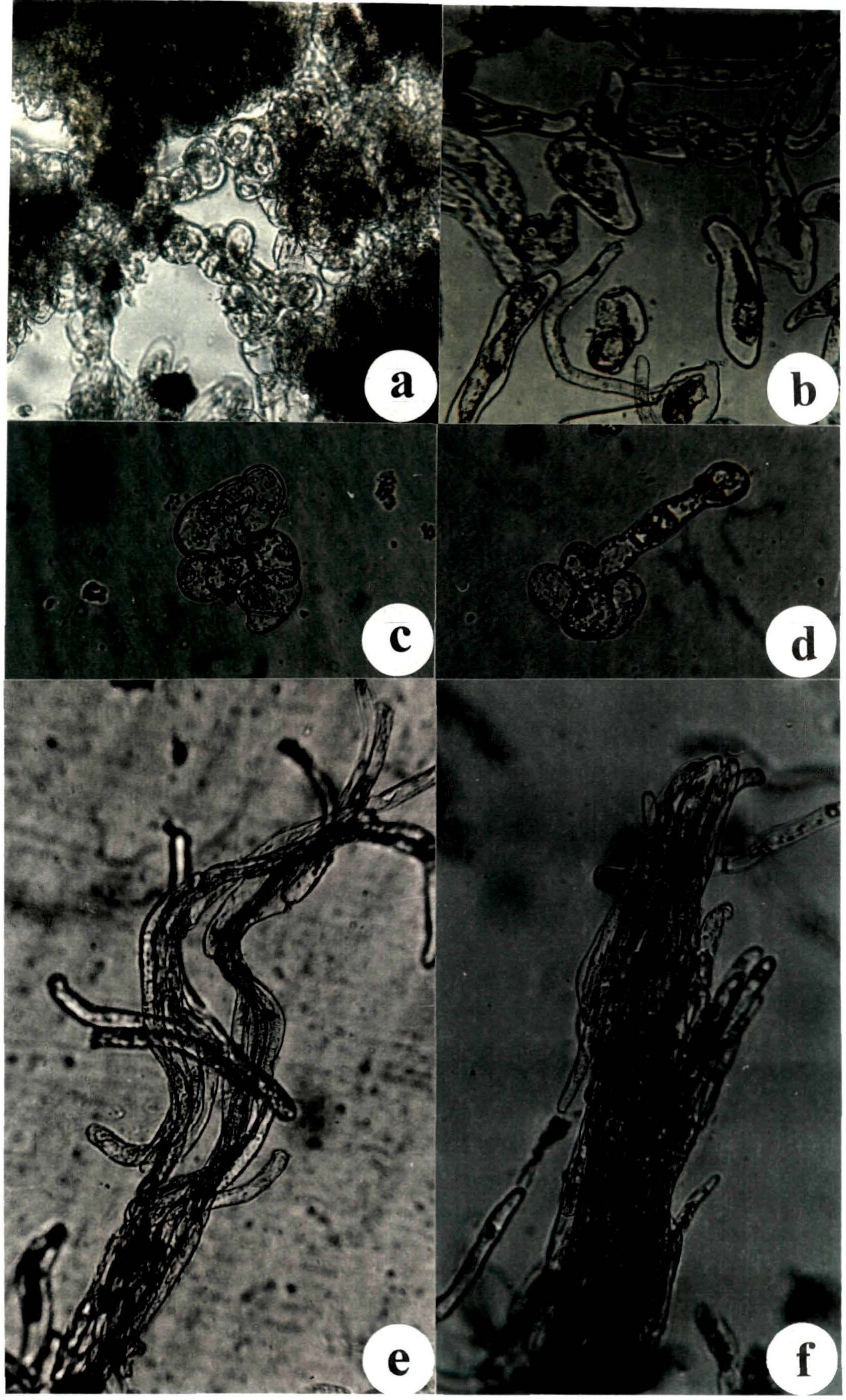

Figure 15

Table 40. Effect of 2,4-D and NAA in the media on initiation of embryogenic suspension cultures in SF and BCB from female gametophytes and zygotic embryos**

2,4-D (mgl^{-1})	NAA (mgl^{-1})	Type of response	Performance*
0.0	0.0	Cultures degenerated	-
2.5	0.0	Poor culture proliferation and cleaving of ESMs	+
5.0	0.0	Culture proliferation was moderate, showed cleavage and somatic embryos at different developmental stages	++
7.5	0.0	Moderate culture proliferation, cultures turned slightly brown	++
10.0	0.0	Cultures turned brown	+
0.0	2.5	Poor culture proliferation and cleaving	+
2.5	2.5	Poor culture proliferation and cleaving of ESMs was not satisfactory	+
5.0	2.5	Healthy cultures with optimum proliferation, showed cleavage and somatic embryos at different developmental stages	+++*[1]
7.5	2.5	Moderate culture proliferation, cultures turned brownish	++
10.0	2.5	Moderate culture proliferation but turned brownish	++
0.0	5.0	Poor culture proliferation and cleaving	+
2.5	5.0	As above but slightly better in appearance	+
5.0	5.0	Healthy cultures with optimum proliferation, showed cleavage and somatic embryos at different developmental stages	+++*[2]
7.5	5.0	Cultures turned brown	+
10.0	5.0	-do-	+
0.0	7.5	Poor culture proliferation and slightly brownish	+
2.5	7.5	-do-	+
5.0	7.5	Moderate culture proliferation but slight brownish	++
7.5	7.5	Moderate culture proliferation but cultures turned brown	++
10.0	7.5	Poor culture proliferation and turned brown	+
0.0	10.0	Poor proliferation and cultures turned brown	+
2.5	10.0	-do-	+
5.0	10.0	Cultures turned brown	+
7.5	10.0	Cultures turned brown and started degeneration	+
10.0	10.0	Cultures turned brown and degenerated	+

** Calli from female gametophytes were cultured in liquid ½Litvay's medium containing BAP (2.5 mgl^{-1}) and sucrose (3.0%); Calli from zygotic embryos were cultured in liquid mMS medium containing BAP (2.5 mgl^{-1}) and sucrose (3.0%); *- no, + poor, ++ moderate and +++ optimum embryogenic response; *[1] In cultures obtained from female gametophytes; *[2] In cultures obtained from zygotic embryos

ESMs: embryonal suspensor masses

Table 41. Effect of BAP in the media on initiation of embryogenic suspension cultures in SF and BCB from female gametophytes and zygotic embryos**

BAP (mgl^{-1})	Type of response	Performance*
0.0	Poor culture growth showing very little cleavage	+
0.5	-do-	+
1.0	Moderate culture growth and cleavage	++
1.5	-do-	++
2.0	Moderately healthy cultures. Culture proliferation and cleaving was good and showed somatic embryos at different developmental stages	+++
2.5	Healthy culture growth and proliferation. Optimum cleavage of ESMs observed and showed somatic embryos at different developmental stages	++++
3.0	Moderate culture growth and cleaving	++
4.0	-do-	++
4.5	Poor culture growth with little cleavage of ESMs	+
5.0	-do-	+

** Calli from female gametophytes were cultured in liquid ½Litvay's medium containing 2,4-D (5 mgl^{-1}), NAA and BAP (2.5 mgl^{-1} each) and sucrose (3.0%); Calli from zygotic embryos were cultured in liquid mMS medium containing 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and sucrose (3.0%), *- no, + poor, ++ moderate, +++ good and ++++ optimum embryogenic response
ESMs: embryonal suspensor masses

iii. BAP and kinetin: In the control, poor culture growth and proliferation as well as cleaving of embryogenic cultures were recorded in ½Litvay's medium lacking auxins and cytokinin. Both BAP and kinetin singly or in combination without auxins showed poor culture growth and cleavage of ESMs.

Mature zygotic embryos

i. Auxins: The embryogenic cultures degenerated in the medium devoid of 2,4-D and NAA but containing only 2.5 mgl^{-1} BAP. Increase in concentrations of auxins resulted in better culture growth and proliferation. NAA singly was found less effective than 2,4-D. 5 mgl^{-1} each of 2,4-D and NAA in conjunction with 2.5 mgl^{-1} BAP (Table 40) showed maximum culture growth and proliferation with cleavage of ESMs and somatic embryo formation at different developmental stages (Figure 13c-f). Further increase in concentration of auxins was found inhibitory and resulted in browning and degeneration of cultures.

ii. N^6-benzylaminopurine: In the absence of BAP in the medium very poor culture growth and cleavage of ESMs were recorded (Table 41). At 2.5 mgl^{-1} BAP healthy culture growth and cleavage of ESMs was noted. However, further increase in BAP concentration in the medium triggered retardation of culture growth.

iii. BAP and kinetin: The growth of embryogenic suspension cultures was very poor in mMS medium devoid of auxins and cytokinins. Both BAP and kinetin singly or in combination without auxins showed poor culture growth and no cleavage of ESMs.

Secondary needles

i. Auxins: In the control, cultures failed to proliferate and degenerated in absence of 2,4-D and NAA in the medium. Both 2,4-D and NAA (3 mgl^{-1} each) in the medium resulted in healthy culture growth and proliferation (Table 42). Higher concentrations of 2,4-D and NAA singly or in combination resulted in poor growth and browning of the cultures.

iii. N^6-benzylaminopurine: Poor culture growth was observed in the absence of BAP in the medium. 1 mgl^{-1} BAP was found to produce healthy culture growth (Table 43). With further increase in BAP concentrations, poor growth of culture was recorded.

The culture consisted of mixture of spherical and elongated suspensor like cells. Somatic embryogenesis was not recorded in the cultures (Figure 14a-d).

iii. BAP and kinetin: Culture growth was poor in MS medium devoid of auxins and cytokinins (control). BAP and kinetin singly or in combination without auxins resulted in poor culture growth.

Table 42. Effect of 2,4-D and NAA in the media on initiation of suspension cultures in SF and BCB from secondary needles and apical dome sections**

2,4-D (mgl^{-1})	NAA (mgl^{-1})	Type of response#	Performance*
0.0	0.0	Cultures degenerated	-
1.0	0.0	Poor culture proliferation	+
3.0	0.0	Moderate culture proliferation	++
5.0	0.0	Moderate proliferation of cultures. Only spherical and elongated suspensor like cell masses observed. Cultures turned slightly brown	++
7.0	0.0	Poor culture proliferation and cultures turned brownish	+
0.0	1.0	Poor culture proliferation	+
1.0	1.0	-do-	+
3.0	1.0	Moderate culture proliferation. Spherical and elongated suspensor like cell masses observed.	++
5.0	1.0	As above but cultures turned slightly brown	++
7.0	1.0	Poor culture proliferation and turned brownish	+
0.0	3.0	Poor culture growth	+
1.0	3.0	Moderate culture proliferation	++
3.0	3.0	Culture growth and proliferation was healthy and satisfactory. Only spherical and elongated suspensor like cell masses observed	+++*[1]
5.0	3.0	As above but cultures turned slightly brownish	++
7.0	3.0	Moderate culture growth and proliferation but cultures turned brownish	++
0.0	5.0	Poor culture growth and proliferation	+
1.0	5.0	As above and cultures turned slightly brown	+
3.0	5.0	Moderate culture growth and proliferation	++
5.0	5.0	Culture growth and proliferation was healthy and satisfactory. Only spherical and elongated suspensor like cell masses observed	+++*[2]
7.0	5.0	Culture turned brown	+
0.0	7.0	Poor culture growth and cultures turned brownish	+
1.0	7.0	-do-	+
3.0	7.0	Cultures turned brown	+
5.0	7.0	-do-	+
7.0	7.0	-do-	+
0.0	9.0	Very poor culture growth and proliferation. Cultures turned brown very rapidly	+
1.0	9.0	-do-	+
3.0	9.0	-do-	+
5.0	9.0	-do-	+
7.0	9.0	-do-	+

** Calli from secondary needles were cultured in liquid MS medium containing BAP (1 mgl^{-1}) and sucrose (3.0%); Calli from apical dome sections were cultured in liquid ½DCR medium containing BAP (2.5 mgl^{-1}) and sucrose (3.0%); * - no, + poor, ++ moderate and +++ optimum response; *[1] In cultures obtained from secondary needles; *[2] In cultures obtained from apical dome sections

 # Embryogenesis not recorded

Table 43. Effect of BAP in the media on initiation of suspension cultures in SF and BCB from secondary needles and apical dome sections**

BAP conc. (mgl^{-1})	Type of response[#]	Performance*
0.0	Poor culture growth	+
0.5	-do-	+
1.0	Healthy culture growth and proliferation but only spherical and elongated suspensor like cell masses were observed and no embryogenesis recorded	+++*[1]
1.5	Moderate culture growth, spherical and elongated suspensor like cell masses were present	++
2.0	-do-	++
2.5	Healthy culture growth and proliferation but only spherical and elongated suspensor like cell masses were observed and no embryogenesis recorded	+++*[2]
3.0	Moderate culture growth and only spherical and elongated cell masses observed	++
4.0	-do-	++
4.5	Poor culture growth	+
5.0	-do-	+

** Calli from secondary needles were cultured in liquid MS medium containing 2,4-D and NAA (3 mgl^{-1} each) and sucrose (3.0%); Calli from apical dome sections were cultured in liquid ½DCR medium containing 2,4-D and NAA (5 mgl^{-1} each) and sucrose (3.0%); * + poor, ++ moderate and +++ optimum response; *[1] In cultures from secondary needles; *[2] In cultures from apical dome section explants
[#] **Embryogenesis not recorded**

Using various plant growth regulators mentioned above embryogenic suspension cultures could not be obtained from calli of secondary needles.

Apical dome sections

i. Auxins: Cultures degenerated in the medium lacking 2,4-D and NAA but containing 2.5 mgl^{-1} BAP (Table 42). Both 2,4-D and NAA (5 mgl^{-1} each) brought about maximum culture growth and proliferation and resulted only non-embryogenic cultures. NAA singly was less effective as compared to 2,4-D. However, further increase in the concentrations of 2,4-D and NAA was associated with culture browning and their subsequent degeneration. The cultures revealed the formation of both spherical and elongated suspensor like cells but no pro-embryos or somatic embryos (Figure 15a-f).

ii. N^6-benzylaminopurine: Absence of BAP in ½DCR medium resulted in poor culture growth (Table 43). BAP at 2.5 mgl^{-1} was found to induce optimum culture growth without formation of embryogenic cell lines. Increase in concentration of BAP beyond 2.5 mgl^{-1} showed a negative effect resulting in retarded culture growth. However, the culture showed no pro-embryos or somatic embryos, and consisted only of spherical and elongated suspensor like cells (Figure 15a-f).

iii. BAP and kinetin: Culture growth was poor in ½DCR medium lacking auxins and cytokinin. Both BAP and kinetin singly or in combination in the medium but without auxins showed poor culture growth.

In all the plant growth regulator treatments mentioned above embryogenic cell lines could not be initiated.

Effect of inoculum density on initiation of suspension cultures

The inoculum density directly related to initiation and establishment of embryogenic suspension cultures from all the explants in SF and BCB. While inoculum density of 10 gl^{-1} resulted in poor culture growth, 40 gl^{-1} inoculum density was found to be most suitable for initiation of suspension cultures from calli from female gametophytes (Table 44), zygotic embryos (Table 44) and secondary needles (Table 45) & apical dome sections (Table 45). Inoculum density higher than this resulted in poor culture growth and browning. However, with secondary needles and apical dome sections, somatic embryogenesis was not recorded and culture consisted of only spherical and elongated suspensor like cells (Figure 14 and 15).

Effect of SCV on establishment of suspension cultures

At 10% SCV, cultures from female gametophyte and zygotic embryo in both SFs and BCBs were healthy, exhibited satisfactory

Table 44. Effect of inoculum density on initiation of embryogenic suspension cultures from embryogenic extrusions of female gametophytes and embryogenic calli of zygotic embryos**

Amount of inoculum (gl^{-1})	Type of response	Performance*
5	Culture growth was poor with very few pro-embryos	-
10	Culture growth was poor with a few pro-embryos	+
20	Moderate culture growth with some pro-embryos	++
30	Healthy culture growth with many pro-embryos	+++
40	Very healthy culture growth with numerous pro-embryos	++++
50	Moderate culture growth with some pro-embryos which were not very healthy	++
60	Slow and poor cell growth due to over crowding of cells and higher cell numbers. Cultures turned slight brownish	++
70	As above and browning was intense	+
80	As above with rapid and intense browning of cultures	+
90	Rapid and intense browning of cultures	-
100	-do-	-

** Calli from female gametophytes were cultured in liquid ½Litvay's medium supplemented with 2,4-D (5 mgl^{-1}), NAA and BAP (2.5 mgl^{-1} each) and sucrose (3.0%); Calli from zygotic embryos were cultured in liquid mMS medium containing 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and sucrose (3.0%)
*- very poor, + poor, ++ moderate, +++ good and ++++ optimum embryogenic response

Table 45. Effect of inoculum density on initiation of embryogenic suspension cultures from calli of secondary needles and apical dome sections**

Amount of inoculum (gl^{-1})	Type of response[#]	Performance[*]
5	Very poor culture growth. No pro-embryos observed	-
10	Culture growth was poor. No pro-embryos observed	+
20	Moderate culture growth but no pro-embryos observed	++
30	Healthy culture growth but no pro-embryos observed	+++
40	As above but cultures were very healthy	++++
50	Moderate culture growth but no pro-embryos	++
60	Slow and poor cell growth due to over crowding of cells and higher cell numbers. No pro-embryos observed. Cultures turned slight brownish	++
70	As above and browning was intense	+
80	As above with rapid and intense browning of cultures	+
90	Rapid and intense browning of cultures	-
100	-do-	-

** Calli from secondary needles were cultured in liquid MS medium supplemented with 2,4-D and NAA (3 mgl^{-1} each), BAP (1 mgl^{-1}) and sucrose (3.0%); Calli from apical dome sections were cultured in liquid ½DCR medium containing 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and sucrose (3.0%)
[*]- very poor, + poor, ++ moderate, +++ good and ++++ optimum response
[#] **Embryogenesis not recorded; data relate to non-embryogenic culture**

proliferation and composed of pro-embryos and somatic embryos at different developmental stages (Table 46). In establishing the cultures, an increase in SCV beyond 10% showed decline in growth and browning of cultures. A decrease below 10% SCV showed poor growth of cultures.

Culture growth was satisfactory in both SFs and BCBs with cultures from secondary needles and apical dome sections at 10% SCV (Table 47). Higher SCV resulted in inhibition of growth associated with culture browning during establishment of cultures. The cultures from secondary needles and apical dome sections comprised of only spherical and elongated suspensor like cells with no somatic embryogenesis.

Maintenance of suspension cultures

The cultures were maintained in the same respective basal media with sucrose (3%) or maltose (3%) and various levels of growth regulators for 4-6 passages.

Female gametophytes: The cultures showed better growth of pro-embryos in media without and 1/10th of growth regulators compared to 1/5th of initiation medium. The cultures turned brown in media containing full and 1/5th growth regulator. Sucrose (Table 48) was found to be more effective than maltose (Table 49) in culture growth. In

Table 46. Effect of culture volume in sucrose containing media on establishment of embryogenic suspension cultures in SF and BCB from cultures of female gametophytes and mature zygotic embryos**

SCV* (%)	Type of response	Performance[1]
5	Cultures moderately healthy and exhibited moderate proliferation	+++
10	Cultures very healthy and exhibited satisfactory proliferation and consisted of somatic embryos at different developmental stages	++++
20	Cultures moderately healthy and exhibited good proliferation but cultures showed tendency towards browning	+++
30	As above but with slight browning of cultures	++
40	Culture growth and proliferation retarded and browning of cultures was intense	+
50	Culture failed to proliferate and rapid browning was observed	+
60	Total inhibition of culture growth and started degenerating	+
70	No culture proliferation, rapid browning due to degeneration of cultures	+
80	-do-	+
90	-do-	+
100	-do-	+

** ½Litvay's and mMS media were used for raising cultures from female gametophytes and mature zygotic embryos respectively. ½Litvay's medium contained 2,4-D (5 mgl^{-1}), NAA and BAP (2.5 mgl^{-1} each) and mMS medium contained 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1})

*SCV: Sedimented culture volume

[1] + poor, ++ moderate, +++ good and ++++ optimum embryogenic response

Table 47. Effect of culture volume in sucrose containing media on establishment of suspension cultures in SF and BCB from cultures of secondary needles and apical dome sections**

SCV* (%)	Type of response#	Performance[1]
5	Cultures moderately healthy and exhibited moderate proliferation	+++
10	Cultures very healthy and exhibited satisfactory proliferation, no somatic embryos observed, only spherical and elongated cell masses were observed	++++
20	Cultures moderately healthy and exhibited good proliferation but cultures showed tendency towards browning	+++
30	As above but slight browning of cultures	++
40	Culture growth and proliferation retarded and browning of cultures was intense	+
50	Culture failed to proliferate and rapid browning was observed	+
60	Total inhibition of culture growth and started degenerating	+
70	No culture proliferation, rapid browning due to degeneration of cultures	+
80	-do-	+
90	-do-	+
100	-do-	+

** MS and ½DCR media were used for raising cultures from secondary needles and apical dome sections respectively. MS medium contained 2,4-D and NAA (3 mgl^{-1} each) and BAP (1 mgl^{-1}) and ½DCR medium contained 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1})

*SCV: Sedimentd culture volume

[1] + poor, ++ moderate, +++ good and ++++ optimum response

 # Somatic embryogenesis not recorded

Table 48. Effect of growth regulators* on maintenance and multiplication of embryogenic suspension cultures from female gametophytes

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type of response	Final SCV# (ml)	Fold increase
5.00	2.50	2.50	Culture proliferation was good but rapid browning of cultures observed	12.50	1.25
1.00	0.50	0.50	Culture proliferation was satisfactory and browning reduced considerably	13.00	1.30
0.50	0.25	0.25	Culture proliferation retarded to certain extent, cultures were very healthy with some increase in the size of pro-embryos	14.50	1.45
0.00	0.00	0.00	Cultures exhibited slow proliferation, no browning observed and cultures were healthy. Increase in the size of pro-embryos observed	15.00	1.50

* ½Litvay's medium containing sucrose (3.0%)

Cultures maintained at 10% SCV

Table 49. Effect of growth regulators* on maintenance and multiplication of embryogenic suspension cultures from female gametophytes

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type of response	Final SCV# (ml)	Fold increase
5.00	2.50	2.50	Culture proliferation was slow but browning was very less	11.00	1.10
1.00	0.50	0.50	As above and no browning of cultures	11.75	1.175
0.50	0.25	0.25	Culture proliferation was comparatively slower, cultures very healthy with increase in the size of pro-embryos	12.50	1.25
0.00	0.00	0.00	Cultures exhibited very slow proliferation, increase in the size of pro-embryos	12.80	1.28

* ½Litvay's medium containing maltose (3.0%)

Cultures maintained at 10% SCV

maintenance medium, cultures showed increase in the size of pro-embryos and formation of occasional stage-I SE (Figure 12b-d).

Mature zygotic embryos: Cultures from zygotic embryos exhibited better growth of ESMs and pro-embryos in media without and 1/10th of growth regulators (Table 50) compared to 1/5th of initiation medium. There was severe culture browning in the medium with the same concentration of growth regulators as that of initiation medium. A 1.27 fold increase in SCV was recorded in the medium containing 1/10th growth regulators and 3% sucrose where as, 1.3-fold increase in SCV was noted when cultured in growth regulator free maintenance medium. Sucrose was found superior over maltose (Table 51). A maximum of 1.28-fold increase in SCV was obtained in the medium containing maltose where as, it was 1.3-fold in sucrose containing medium. In maintenance medium, increase in the size of pro-embryos was observed along with formation of occasional stage-I embryos (Figure 13c-e).

Secondary needles: The medium containing 1/10th growth regulators and 3% sucrose resulted in optimum culture growth (Table 52). In growth regulator free medium there was pronounced decline in culture growth. The cultures turned brown rapidly when maintained in the same growth regulator concentrations as of initiation medium with 3% sucrose. However, culture browning was more in sucrose containing

Table 50. Effect of growth regulators* on maintenance and multiplication of embryogenic suspension cultures from mature zygotic embryos

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type of response	Final SCV# (ml)	Fold increase
5.00	5.00	2.50	Culture proliferation was satisfactory, but turned slightly brownish	11.00	1.10
1.0	1.0	0.50	Culture proliferation was satisfactory, cultures healthy and browning was reduced considerably	11.85	1.185
0.50	0.50	0.25	Culture proliferation declined but cultures very healthy with increase in the size of pro-embryos	12.70	1.27
0.00	0.00	0.00	Cultures showed very slow growth and proliferation but increase in the size pro-embryos observed	13.00	1.30

* mMS medium containing sucrose (3.0%)

 # Cultures maintained at 10% SCV

Table 51. Effect of growth regulators* on maintenance and multiplication of embryogenic suspension cultures from mature zygotic embryos

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type of response	Final SCV# (ml)	Fold increase
5.00	5.00	2.50	Culture proliferation was slow but browning was very less	10.90	1.09
1.0	1.0	0.50	Culture proliferation was slow. Browning not recorded	11.50	1.15
0.50	0.50	0.25	Culture proliferation was slow but cultures very healthy, increase in the size of pro-embryos	12.50	1.25
0.00	0.00	0.00	Cultures showed very slow growth but increase in the size of pro-embryos observed	12.80	1.28

* mMS medium containing maltose (3.0%)

 # Cultures maintained at 10% SCV

Table 52. Effect of growth regulators* on maintenance and multiplication of suspension cultures from secondary needles

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type of response**	Final SCV[#] (ml)	Fold increase
3.00	3.00	1.00	Culture proliferation was satisfactory but turned brownish rapidly	12.50	1.25
0.60	0.60	0.20	Culture proliferation was satisfactory and culture browning was reduced considerably	13.60	1.36
0.30	0.30	0.10	As above and no browning of cultures	14.50	1.45
0.00	0.00	0.00	Cultures showed very slow proliferation	11.50	1.15

* MS medium containing sucrose (3.0%)

[#]Cultures maintained at 10% SCV (sedimented culture volume)

**** Embryogenesis not recorded; data shown above relate to non-embryogenic cultures**

medium than maltose (Table 53). In both sucrose and maltose (3% each singly) containing medium, the cultures consisted only of spherical and elongated suspensor like cells and no somatic embryogenesis was recorded (Figure 14a-d).

Apical dome sections: A 1.48-fold increase in SCV was obtained when cultures were maintained in the medium with $1/10^{th}$ growth regulators and 3% sucrose (Table 54). The culture growth was retarded in growth regulator free maintenance medium. The cultures turned brown when maintained in the same growth regulator concentrations to that of initiation medium containing 3% sucrose. But browning was negligible at the same range of phytohormone concentrations as above but with 3% maltose (Table 55). However, sucrose proved to be better in promoting growth compared to maltose at the same range. In general, the cultures were composed of spherical and elongated suspensor like cell masses and somatic embryogenesis was not observed (Figure 15a-f).

Embryo development and maturation

The pro-embryos and stage-I embryos (Figure 12b-d and 13c-e) developed on maintenance medium in cultures of both female gametophytes and zygotic embryos. However, they failed to enter next developmental stage unless they were cultured on respective basal media containing ABA and a carbohydrate (maturation media). The

Table 53. Effect of growth regulators* on maintenance and multiplication of suspension cultures from secondary needles

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type of response**	Final SCV[#] (ml)	Fold increase
3.00	3.00	1.00	Culture proliferation was slow but browning was less	11.20	1.12
0.60	0.60	0.20	Culture proliferation was slow but no browning of cultures	13.30	1.33
0.30	0.30	0.10	Culture proliferation was slow but no browning of cultures	14.20	1.42
0.00	0.00	0.00	Cultures showed very slow proliferation, growth retarded but no browning was observed	11.50	1.15

* MS medium containing maltose (3.0%)

[#]Cultures maintained at 10% SCV

**** Embryogenesis not recorded; data shown above relate to non-embryogenic cultures**

Table 54. Effect of growth regulators* in ½DCR medium* on maintenance and multiplication of suspension cultures from apical dome sections

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response**	Final SCV# (ml)	Fold increase
5.00	5.00	2.50	Culture proliferation was satisfactory but culture turned brownish	11.50	1.15
1.00	1.00	0.50	Culture proliferation was satisfactory and culture browning was reduced considerably	13.40	1.34
0.50	0.50	0.25	As above and cultures were very healthy and no browning observed	14.80	1.48
0.00	0.00	0.00	Cultures exhibited very slow proliferation	11.20	1.12

* ½DCR medium containing sucrose (3.0%),

 # Cultures maintained at 10% SCV

**** Embryogenesis not recorded; data shown above relate to non-embryogenic cultures**

Table 55. Effect of growth regulators* in ½DCR medium* on maintenance and multiplication of suspension cultures from apical dome sections

2,4-D (mgl^{-1})	NAA (mgl^{-1})	BAP (mgl^{-1})	Type response**	Final SCV# (ml)	Fold increase
5.00	5.00	2.50	Culture proliferation was slow but culture browning was less	11.30	1.13
1.00	1.00	0.50	Culture proliferation was slow but almost no browning of cultures	13.10	1.31
0.50	0.50	0.25	As above and no browning observed	14.50	1.45
0.00	0.00	0.00	Culture exhibited very slow growth but no browning	11.0	1.10

* ½DCR medium containing maltose (3.0%)

#Cultures maintained at 10% SCV

**** Embryogenesis not recorded; data shown above relate to non-embryogenic cultures**

cultures obtained from secondary needles and apical dome sections did not show somatic embryogenesis therefore, cultures were not subjected to maturation treatment.

Effect of ABA, carbohydrates and culture technique

Female gametophytes: *Using filter paper base method of culturing,* the optimum response of stage-II (bullet shaped) and stage-III (cotyledonary) embryo formation (Figure 12e-i) was recorded with 8 mgl^{-1} ABA and 3% each of sucrose and mannitol (Table 56). Sucrose at 6% (Table 57) alone along with 8 mgl^{-1} ABA was not as effective as sucrose-mannitol combination.

Submerge culture method was also found effective in maturation of somatic embryos (Figure 12b-i). Optimum maturation was obtained with sucrose and mannitol (3% each) together with 8 mgl^{-1} ABA (Table 58). Sucrose at 6% alone with 8 mgl^{-1} ABA exhibited low maturation (Table 59).

Direct culture method was not very effective in embryo maturation and cotyledonary embryo formation. The optimum response of cotyledonary embryo formation in direct culture method was recorded with combined treatment of sucrose and mannitol (3% each) and 8 mgl^{-1} ABA (Table 60) followed by 6% sucrose singly (Table 61).

Table 56. Effect of ABA, sucrose and mannitol on development and maturation of somatic embryos on semisolid medium obtained from female gametophytes and zygotic embryos (*filter paper base method*)**

ABA (mgl^{-1})	Sucrose (%)	Mannitol (%)	Type of response	Performance*
0.0	0.0	0.0	No response	-
	1.0	1.0	Multiplication and proliferation of ESMs but no maturation of somatic embryos beyond stage-I	-
	2.0	2.0	-do-	-
	3.0	3.0	As above but with slight increase in the size of stage-I embryo	-
	4.0	4.0	Formation of few stage-II embryos but turned brownish	-
	5.0	5.0	As above and cultures turned brown	-
	6.0	6.0	-do-	-
2.0	0.0	0.0	No response	-
	1.0	1.0	Increase in the size of pro-embryo but no further growth, supported multiplication and proliferation of ESMs	-
	2.0	2.0	-do-	-
	3.0	3.0	-do-	-
	4.0	4.0	Development of few stage-II embryos but could not develop into stage-III embryo	-
	5.0	5.0	As above, cultures turned brown eventually	-
	6.0	6.0	Cultures turned brown and no maturation of somatic embryos observed	-
4.0	0.0	0.0	No response	-
	1.0	1.0	Slight increase in the size of pro-embryo, formation of few stage-II embryos	-
	2.0	2.0	Formation of stage-II and a few stage-III embryos	+
	3.0	3.0	Formation of stage-II and few stage-III embryos	++
	4.0	4.0	-do-	++
	5.0	5.0	Formation of stage-III embryos but turned brownish	++
	6.0	6.0	Cultures turned brown and no maturation of somatic embryos	-
6.0	0.0	0.0	No response	-
	1.0	1.0	Formation of very few stage-II embryos	-
	2.0	2.0	Formation of stage-II and few stage-III embryos	++
	3.0	3.0	-do-	++
	4.0	4.0	Formation of stage-III embryos but slight brownish	++
	5.0	5.0	As above and cultures turned brownish	+
	6.0	6.0	Cultures turned brown and no maturation observed	-
8.0	0.0	0.0	No response	-
	1.0	1.0	Formation of stage-II embryo, no stage-III embryos formed	-
	2.0	2.0	Formation of stage-II and stage-III embryos and embryos were healthy	+++
	3.0	3.0	Formation of many stage-III embryos and embryos were very healthy	++++
	4.0	4.0	As above and cultures turned slight brownish	+++
	5.0	5.0	Formation of stage-II and stage-III embryos but turned slight brownish	++
	6.0	6.0	Formation of few stage-III embryos but eventually turned brown	+
10.0	0.0	0.0	No response	-
	1.0	1.0	Formation of few stage-II embryos, no stage-III embryo developed	-
	2.0	2.0	Formation of stage-II and a few stage-III embryos	+
	3.0	3.0	Formation of few stage-III embryos	++
	4.0	4.0	As above but turned brownish	+
	5.0	5.0	Cultures turned brownish and no embryo maturation	-
	6.0	6.0	-do-	-

** Cultures obtained from female gametophytes were raised on ½Litvay's medium and cultures from zygotic embryos were raised on mMS medium. The media were solidified with Difco-bacto agar (0.8%); * - No, + poor, ++ moderate, +++ good and ++++ optimum response

ABA= Abscisic acid

Table 57. Effect of ABA and sucrose on development and maturation of somatic embryos on semisolid medium obtained from female gametophytes and zygotic embryos (*filter paper base method*)**

ABA (mgl^{-1})	Sucrose (%)	Type of response	Performance*
0.0	0.0	No response	-
	2.0	Only multiplication of ESMs with no signs of maturation of embryos beyond stage-I	-
	4.0	-do-	-
	6.0	-do-	-
	8.0	Appearance of few off-white stage-II embryos which turned brownish and died	-
	10.0	As above and cultures turned brownish	-
	12.0	Cultures turned brownish and no maturation observed	-
2.0	0.0	No response	-
	2.0	Slight increase in size of pro-embryos but no growth beyond that stage and supported multiplication of ESMs	-
	4.0	-do-	-
	6.0	-do-	-
	8.0	Formation of few stage-II embryos which eventually turned brownish and died	-
	10.0	As above and cultures turned brownish	-
	12.0	No maturation, cultures turned totally brownish	-
4.0	0.0	No response	-
	2.0	Slight increase in size of pro-embryos but no growth beyond that stage.	-
	4.0	Formation of few stage-II and stage-III embryos but no further development	+
	6.0	-do-	+
	8.0	-do-	+
	10.0	As above but cultures turned brownish and no maturation observed	-
	12.0	-do-	-
6.0	0.0	No response	-
	2.0	Formation of few stage-II embryos but no stage-III embryos formed	-
	4.0	Maturation of embryos to few stage-III embryos	++
	6.0	-do-	++
	8.0	Formation of few stage-III embryo, but turned brownish eventually	++
	10.0	-do-	+
	12.0	Cultures turned brown and no maturation observed	-
8.0	0.0	No response	-
	2.0	Formation of stage-II embryos but no stage-III embryos	-
	4.0	Maturation of embryos up to stage-III and embryos were healthy	+++
	6.0	Many stage-III embryos formed and embryos were healthy	++++
	8.0	As above but cultures slight brownish	++
	10.0	Formation of stage-II and stage-III embryos, but turned brownish	+
	12.0	Cultures turned brown and no maturation observed	-
10.0	0.0	No response	-
	2.0	Formation of few stage-II embryos but no stage-III embryos formed	-
	4.0	Formation of few stage-II and a few stage-III embryos but turned slight brownish	+
	6.0	-do-	+
	8.0	As above but cultures turned brown	+
	10.0	Cultures turned brownish and no maturation observed	-
	12.0	-do-	-

** Cultures obtained from female gametophytes were raised on ½Litvay's medium and cultures from zygotic embryos were raised on mMS medium. The media were solidified with Difco-bacto agar (0.8%); * - No, + poor, ++ moderate, +++ good and ++++ optimum response
ABA= Abscisic acid

Table 58. Effect of ABA, sucrose and mannitol on development and maturation of somatic embryos in liquid medium obtained from female gametophytes and zygotic embryos (*submerge culture method*)**

ABA (mgl^{-1})	Sucrose (%)	Mannitol (%)	Type of response	Performance*
0.0	0.0	0.0	No response	-
	1.0	1.0	Multiplication and proliferation of ESMs but no maturation of somatic embryos beyond stage-I	-
	2.0	2.0	-do-	-
	3.0	3.0	As above but slight increase in the size of stage-I embryo	-
	4.0	4.0	Growth and proliferation of ESMs retarded. Formation of few stage-II embryos but turned slight brownish	-
	5.0	5.0	As above and cultures turned brown	-
	6.0	6.0	-do-	-
2.0	0.0	0.0	No response	-
	1.0	1.0	Increase in the size of stage-I embryos, no further growth, supported multiplication and proliferation of ESMs	-
	2.0	2.0	-do-	-
	3.0	3.0	-do-	-
	4.0	4.0	Growth of ESMs retarded considerably. Development of few stage-II embryos but no stage-III embryo	-
	5.0	5.0	As above, cultures eventually turned brown	-
	6.0	6.0	ESMs turned brown and no maturation of somatic embryos observed	-
4.0	0.0	0.0	No response	-
	1.0	1.0	Growth and proliferation of ESMs retarded slightly. Slight increase in the size of stage-I embryos, formation of few stage-II embryos	-
	2.0	2.0	Growth and proliferation of ESMs retarded considerably. Formation of stage-II and few stage-III embryos	++
	3.0	3.0	-do-	++
	4.0	4.0	-do-	++
	5.0	5.0	Formation of stage-III embryos but turned brownish	+
	6.0	6.0	ESMs turned brown and no maturation of somatic embryos	-
6.0	0.0	0.0	No response	-
	1.0	1.0	Culture proliferation retarded to some extent. Formation of very few stage-II embryos	-
	2.0	2.0	Growth and proliferation of ESMs retarded considerably. Formation of stage-II and stage-III embryos	+++
	3.0	3.0	-do-	+++
	4.0	4.0	Formation of stage-III embryos but turned slight brownish	++
	5.0	5.0	As above cultures turned brownish	+
	6.0	6.0	Cultures turned brown and no maturation observed	-
8.0	0.0	0.0	No response	-
	1.0	1.0	Formation of stage-II embryo and few stage-III embryos	++
	2.0	2.0	Formation of stage-II and stage-III embryos, embryos healthy	+++
	3.0	3.0	Formation of many stage-III embryos, embryos were very healthy	++++
	4.0	4.0	As above and cultures turned slight brownish	++
	5.0	5.0	Formation of stage-II and stage-III embryos but turned brownish	+
	6.0	6.0	Formation of few stage-III embryos but eventually turned brown	+
10.0	0.0	0.0	No response	-
	1.0	1.0	Formation of few stage-II embryos, no stage-III embryo developed	-
	2.0	2.0	Formation of stage-II and few stage-III embryos	++
	3.0	3.0	-do-	++
	4.0	4.0	As above but turned slight brownish	+
	5.0	5.0	Cultures turned brownish and no embryo maturation	-
	6.0	6.0	-do-	-

** Cultures obtained from female gametophytes were raised in ½Litvay's medium and cultures from zygotic embryos were raised in mMS medium. The media were solidified with Difco-bacto agar (0.8%); * - No, + poor, ++ moderate, +++ good and ++++ optimum response

ABA= Abscisic acid

Table 59. Effect of ABA and sucrose on development and maturation of somatic embryos in liquid medium obtained from female gametophytes and zygotic embryos (*submerge culture method*)**

ABA (mgl^{-1})	Sucrose (%)	Type of response	Performance*
0.0	0.0	No response	-
	2.0	Multiplication of ESMs, presence of occasional stage-I embryo but no maturation beyond that stage	-
	4.0	-do-	-
	6.0	-do-	-
	8.0	Growth and multiplication of ESMs retarded. Appearance of few stage-II embryos but cultures turned brownish	-
	10.0	-do-	-
	12.0	Cultures turned brown	-
2.0	0.0	No response	-
	2.0	Multiplication of ESMs, occasional stage-I embryos formed but no maturation to cotyledonary stage embryo	-
	4.0	Growth of ESMs retarded slightly, increase in the size of stage-I embryos but no further maturation	-
	6.0	-do-	-
	8.0	Growt of ESMs declined sharply. Formation of few stage-II embryos but eventually turned brownish	-
	10.0	-do-	-
	12.0	Cultures turned brown	-
4.0	0.0	No response	-
	2.0	Slight increase in the size of pro-embryos and presence of occasionally formed stage-I embryos but no maturation thereafter	-
	4.0	ESMs proliferation retarded considerably. Formation of stage-II and a few stage-III embryos	+
	6.0	-do-	+
	8.0	As above but cultures turned brown	+
	10.0	Cultures turned brown	-
	12.0	-do-	-
6.0	0.0	No response	-
	2.0	ESMs proliferation reduced to some extent and stage-II embryos formed	-
	4.0	Proliferation of ESMs retarded considerably. Formation of stage-II and few stage-III embryos	++
	6.0	-do-	++
	8.0	Formation of stage-III embryos but turned brownish	++
	10.0	As above and cultures turned brown	-
	12.0	Cultures turned brown	-
8.0	0.0	No response	-
	2.0	Formation of stage-II and stage-III embryos	++
	4.0	Formation of stage-III embryos, embryos were healthy	+++
	6.0	Formation of stage-III embryos, embryos were very healthy	++++
	8.0	Formation of stage-II and stage-III embryos but turned brownish	++
	10.0	As above but cultures turned brown	-
	12.0	Cultures turned brown	-
10.0	0.0	No response	-
	2.0	Formation of a few stage-II and III embryos	+
	4.0	Formation of few stage-III embryos	++
	6.0	-do-	++
	8.0	As above but browning of cultures	+
	10.0	Cultures turned brown	-
	12.0	-do-	-

**: Cultures obtained from female gametophytes were raised in ½Litvay's medium and cultures from zygotic embryos were raised in mMS medium. The media were solidified with Difco-bacto agar (0.8%); * - No, + poor, ++ moderate, +++ good and ++++ optimum response

ABA= Abscisic acid

Table 60. Effect of ABA, sucrose and mannitol on development and maturation of somatic embryos on semisolid medium obtained from female gametophytes and zygotic embryos (*direct culture method*)**

ABA (mgl^{-1})	Sucrose (%)	Mannitol (%)	Type of response	Performance*
0.0	0.0	0.0	No response	-
	1.0	1.0	Multiplication and proliferation of ESMs but no maturation of somatic embryos beyond stage-I	-
	2.0	2.0	-do-	-
	3.0	3.0	As above but with slight increase in the size of stage-I embryo	-
	4.0	4.0	Culture growth retarded. Formation of few stage-II embryos but turned brownish	-
	5.0	5.0	As above and cultures turned brown	-
	6.0	6.0	-do-	-
2.0	0.0	0.0	No response	-
	1.0	1.0	Increase in the size of pro-embryos but no further growth, supported multiplication and proliferation of ESMs	-
	2.0	2.0	-do-	-
	3.0	3.0	-do-	-
	4.0	4.0	Culture growth growth reduced considerably. Development of few stage-II embryos but could not develop into stage-III embryo	-
	5.0	5.0	As above, cultures turned brown eventually	-
	6.0	6.0	Cultures turned brown and no maturation observed	-
4.0	0.0	0.0	No response	-
	1.0	1.0	Culture growth retarded slightly. Slight increase in the size of pro-embryos, formation of few stage-II embryos	-
	2.0	2.0	Culture growth retarded considerably. Formation of stage-II and a few stage-III embryos	+
	3.0	3.0	-do-	+
	4.0	4.0	-do-	+
	5.0	5.0	Sharp reduction in culture growth. Formation of stage-III embryos but turned brownish	++
	6.0	6.0	Cultures turned brown and no maturation of somatic embryos	-
6.0	0.0	0.0	No response	-
	1.0	1.0	Growth of ESMs retarded to some extent. Formation of very few stage-II embryos	-
	2.0	2.0	Growth of EMs retarded considerably. Formation of stage-II and few stage-III embryos	++
	3.0	3.0	-do-	++
	4.0	4.0	Formation of a few stage-III embryos	+
	5.0	5.0	As above cultures turned brownish	+
	6.0	6.0	Calli turned brown and no maturation observed	-
8.0	0.0	0.0	No response	-
	1.0	1.0	Formation of stage-II embryo and few stage-III embryos	++
	2.0	2.0	Formation of stage-II and stage-III embryos, embryos were healthy	+++
	3.0	3.0	Formation of many stage-III embryos, embryos were very healthy	++++
	4.0	4.0	As above and cultures turned slight brownish	++
	5.0	5.0	Formation of stage-II and stage-III embryos but turned slight brownish	+
	6.0	6.0	Formation of a few stage-III embryos but eventually turned brown	+
10.0	0.0	0.0	No response	-
	1.0	1.0	Formation of few stage-II embryos	-
	2.0	2.0	Formation of stage-II and a few stage-III embryos	+
	3.0	3.0	-do-	+
	4.0	4.0	As above but turned slight brownish	+
	5.0	5.0	Cultures turned brownish and no embryo maturation	-
	6.0	6.0	-do-	-

** Cultures obtained from female gametophyte were raised on ½Litvay's medium and cultures from zygotic embryos were raised on mMS medium. The media were solidified with Difco-bacto agar (0.8%); * - No, + poor, ++ moderate, +++ good and ++++ optimum response

ABA= Abscisic acid

Table 61. Effect of ABA and sucrose on development and maturation of somatic embryos on semisolid medium obtained from female gametophytes and zygotic embryos (*direct culture method*)**

ABA (mgl^{-1})	Sucrose (%)	Type of response	Performance*
0.0	0.0	No response	-
	2.0	Only multiplication of ESMs with no sign of maturation of embryos beyond stage-I	-
	4.0	-do-	-
	6.0	-do-	-
	8.0	Culture growth drastically reduced. Appearance of few off-white stage-II embryos which turned brownish	-
	10.0	As above and cultures turned brownish	-
	12.0	Cultures turned brownish and no maturation observed	-
2.0	0.0	No response	-
	2.0	Slight increase in size of pro-embryos but no growth beyond that stage and supported multiplication of ESMs	-
	4.0	-do-	-
	6.0	-do-	-
	8.0	Culture growth sharply reduced. Formation of few stage-II embryos which eventually turned brownish	-
	10.0	As above and cultures turned brownish	-
	12.0	No maturation, cultures turned totally brownish	-
4.0	0.0	No response	-
	2.0	Slight increase in size of pro-embryos but no growth beyond that stage. Culture growth was retarded slightly	-
	4.0	Formation of few stage-II and stage-III embryos but no further development	+
	6.0	-do-	+
	8.0	-do-	+
	10.0	As above but cultures turned brownish and no maturation observed	-
	12.0	-do-	-
6.0	0.0	No response	-
	2.0	Formation of few stage-II embryos but no stage-III embryos formed	-
	4.0	Maturation up to a few stage-III embryos	+
	6.0	Formation of few stage-III embryos	++
	8.0	Formation of few stage-III embryo, but turned brownish eventually	+
	10.0	-do-	+
	12.0	Cultures turned brown and no maturation observed	-
8.0	0.0	No response	-
	2.0	Formation of stage-II and a few stage-III embryos	+
	4.0	Maturation of embryos up to stage-III and embryos were healthy	+++
	6.0	Formation of many stage-III embryos, embryos were very healthy	++++
	8.0	As above but cultures slight brownish	++
	10.0	Formation of stage-II and stage-III embryos, but turned brownish	+
	12.0	Calli turned brown and no maturation observed	-
10.0	0.0	No response	-
	2.0	Formation of few stage-II embryos but no stage-III embryos formed	-
	4.0	Formation of few stage-II and stage-III embryos but turned slight brownish	++
	6.0	-do-	++
	8.0	As above but cultures turned brown	+
	10.0	Cultures turned brownish and no maturation observed	-
	12.0	-do-	-

** Cultures obtained from female gametophytes were raised on ½Litvay's medium and cultures from zygotic embryos were raised on mMS medium. The media were solidified with Difco-bacto agar (0.8%); * - No, + poor, ++ moderate, +++ good and ++++ optimum response

ABA= Abscisic acid

Out of three different culture methods, *submerge culture method* was found most effective in maturation of somatic embryos (Figure 16).

Mature zygotic embryos: A better maturation and cotyledonary embryo formation (Figure 13c-i) was achieved using *submerge culture method* (Table 58 and 59) followed by *filter paper base method* (Table 56 and 57) and *direct culture method* (Table 60 and 61). Effective embryo maturation and cotyledonary somatic embryo formation was obtained in the medium containing 8 mgl^{-1} ABA along with 3% each of sucrose and mannitol compared to using 6% sucrose alone.

Out of three different culture methods, *submerge culture method* was found most effective in maturation of somatic embryos (Figure 17).

Secondary needles and apical dome sections: The cultures from secondary needles and apical dome sections did not show somatic embryogenesis (Figure 14 and 15) and therefore, no maturation treatment was given.

<u>Embryo development and maturation in BCBs</u>

The pro-embryos and stage-I embryos developed in maintenance medium both from female gametophytes and zygotic embryos failed to enter next developmental stage unless they were cultured on respective basal media containing 8 mgl^{-1} ABA and 3% each of sucrose and mannitol or 6% sucrose (maturation medium).

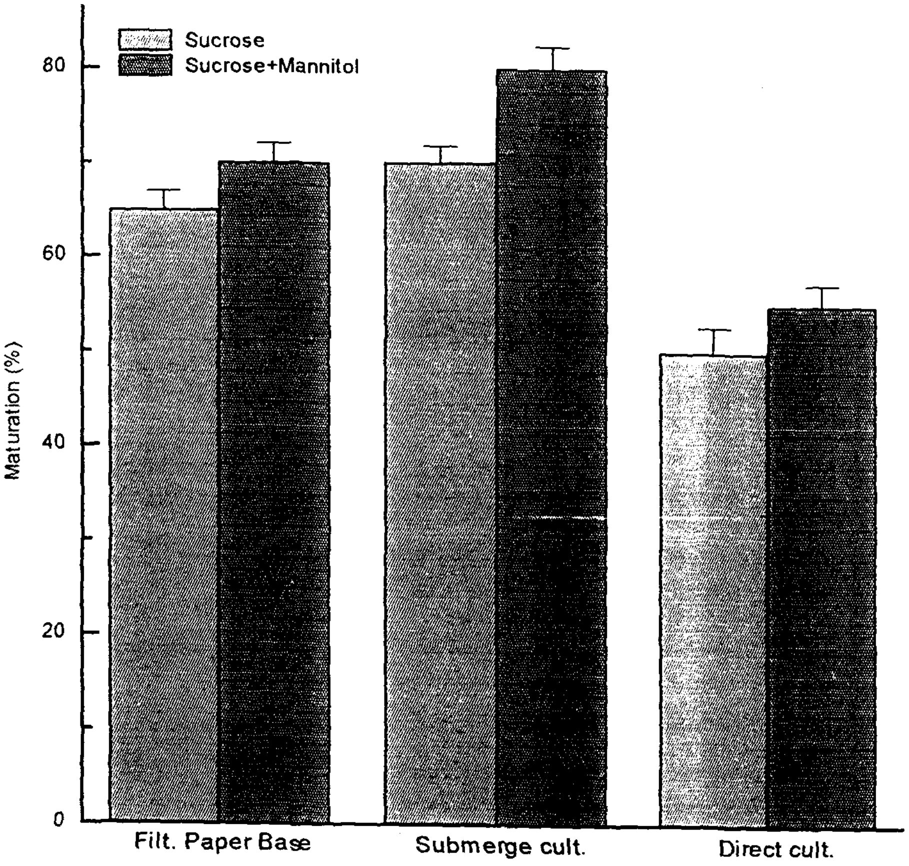

Figure 16. Maturation response of somatic embryos derived from cultures of female gametophytes using different culture methods

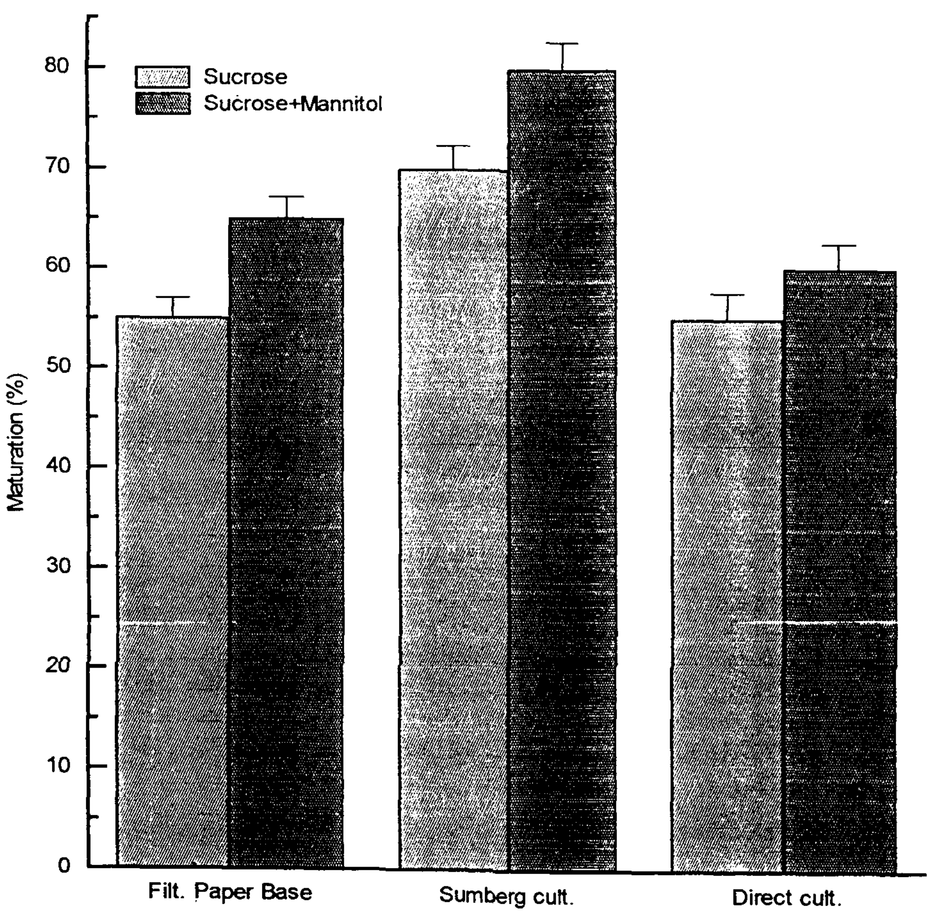

Figure 17. Maturation response of somatic embryos derived from cultures of zygotic embryos using different culture methods

Female gametophytes: In BCBs, stage-I and occasional stage-II embryos were formed when cultured in maturation medium mentioned above containing sucrose and mannitol in combination together with ABA (Table 62) or sucrose singly with ABA (Table 63). But maturation to stage-III embryos (cotyledonary embryo) was not obtained in all the combinations tested.

Mature zygotic embryos: Cotyledonary stage somatic embryos (stage-III) were not obtained in all the combinations tried in maturation medium (Table 62-63).

The conical shape of BCB (Figure 18) with a glass sparger at the base to act both as a support and air source was found to provide optimal mixing, aeration and minimal shearing damage compared to flat bottom vessels.

Growth parameters of SF suspension cultures

Female gametophytes: Growth kinetic studies of embryogenic cultures obtained from female gametophytes in ½Litvay's medium exhibited changes in total dry weight (DW) (Figure 19). The cultures did not show any lag phase, but exhibited a linear phase of 7 days followed by a stationary phase from day 8 to day 10. A maximum DW of 1.5 gl^{-1} was achieved in 3% each of sucrose and mannitol together with 8 mgl^{-1} ABA

Table 62. Effect of ABA and sucrose on development and maturation of somatic embryos obtained from cultures of female gametophytes and zygotic embryos in BCB**

ABA (mgl^{-1})	Sucrose (%)	Type of response#	Performance*
0.0	0.0	No response	-
	2.0	Multiplication of ESMs, presence of pro-embryos and occasional stage-I embryo but no maturation beyond that stage	-
	4.0	-do-	-
	6.0	-do-	-
	8.0	Growth and multiplication of ESMs retarded. Presence of only stage-I embryos no maturation beyond that stage	-
	10.0	-do-	-
	12.0	Cultures turned brown	-
2.0	0.0	No response	-
	2.0	Multiplication of ESMs, slight increase in the size of pro-embryos and occasional stage-I embryo formed but no maturation to cotyledonary stage	-
	4.0	Growth of ESMs retarded, increase in the size of pro-embryos and stage-I embryos but no further maturation	-
	6.0	-do-	-
	8.0	Growth of ESMs declined sharply. Formation of few stage-I embryos but eventually turned brownish	-
	10.0	-do-	-
	12.0	Cultures turned brown	-
4.0	0.0	No response	-
	2.0	Slight increase in the size of pro-embryos and presence of occasionally formed stage-I embryos but no maturation thereafter	-
	4.0	Proliferation of ESMs retarded considerably. Formation of only stage-I embryos but no maturation thereafter	-
	6.0	-do-	-
	8.0	As above but cultures turned brown	-
	10.0	Cultures turned brown	-
	12.0	-do-	-
6.0	0.0	No response	-
	2.0	Proliferation of ESMs reduced to some extent and only stage-I embryos formed but no further maturation	-
	4.0	Proliferation of ESMs retarded considerably. Formation of only stage-I embryos but no maturation thereafter	-
	6.0	-do-	-
	8.0	Formation of stage-I embryos and no maturation thereafter	-
	10.0	As above and cultures turned brown	-
	12.0	Cultures turned brown	-
8.0	0.0	No response	-
	2.0	Formation of stage-I embryos but no maturation thereafter	-
	4.0	-do-	-
	6.0	-do-	-
	8.0	Formation of stage-I embryos but turned brownish	-
	10.0	As above but cultures turned brown	-
	12.0	Cultures turned brown	-
10.0	0.0	No response	-
	2.0	Formation of few stage-I embryos but no maturation thereafter	-
	4.0	-do-	-
	6.0	-do-	-
	8.0	As above and browning of cultures	-
	10.0	Cultures turned brown	-
	12.0	-do-	-

** Cultures obtained from female gametophytes were raised in ½Litvay's medium and cultures from zygotic embryos were raised in mMS medium. The media were solidified with Difco-bacto agar (0.8%);
* - No response
ABA= Abscisic acid
 # No maturation observed

Table 63. Effect of ABA, sucrose and mannitol on development and maturation of somatic embryos obtained from cultures of female gametophytes and zygotic embryos in BCB**

ABA (mgl^{-1})	Sucrose (%)	Mannitol (%)	Type of response[#]	Performance*
0.0	0.0	0.0	No response	-
	1.0	1.0	Multiplication of ESMs, presence of pro-embryos and occasional stage-I embryo, no maturation beyond that stage	-
	2.0	2.0	-do-	-
	3.0	3.0	-do-	-
	4.0	4.0	Appearance of few stage-I embryos but no stage-II embryos and cultures turned slight brownish	-
	5.0	5.0	As above and browning of cultures observed	-
	6.0	6.0	Cultures turned brownish and no maturation observed	-
2.0	0.0	0.0	No response	-
	1.0	1.0	Multiplication of ESMs, slight increase in size of pro-embryos and occasional Stage-I embryos but no maturation thereafter	-
	2.0	2.0	Proliferation of ESMs retarded slightly, increase in the size of pro-embryos and stage-I embryos, no further maturation	-
	3.0	3.0	Slight increase in the size of proembryos but no further development	-
	4.0	4.0	Sharp decline in the growth of ESMs. Formation of few Stage-II embryos but eventually turned brownish	-
	5.0	5.0	As above and cultures turned slight brownish	-
	6.0	6.0	Cultures turned brown	-
4.0	0.0	0.0	No response	-
	1.0	1.0	Slight increase in the size of pro-embryos and formation of occasional stage-I embryos but no maturation after that stage	-
	2.0	2.0	Proliferation of ESMs retarded considerably. Formation of only stage-I embryos but no maturation thereafter	-
	3.0	3.0	-do-	-
	4.0	4.0	As above but cultures turned brown	-
	5.0	5.0	As above but cultures turned brown	-
	6.0	6.0	Cultures turned brown	-
6.0	0.0	0.0	No response	-
	1.0	1.0	Proliferation of ESMs reduced to some extent and only stage-I embryos formed but no further maturation	-
	2.0	2.0	Proliferation of ESMs retarded considerably. Formation of only stage-I embryos but no maturation thereafter	-
	3.0	3.0	-do-	-
	4.0	4.0	Formation of stage-I embryos and no maturation thereafter	-
	5.0	5.0	As above and cultures turned brown	-
	6.0	6.0	Cultures turned brown	-
8.0	0.0	0.0	No response	-
	1.0	1.0	Formation of few stage-I embryos but no maturation thereafter	-
	2.0	2.0	-do-	-
	3.0	3.0	-do-	-
	4.0	4.0	Formation of stage-I embryos but turned brownish	-
	5.0	5.0	As above but cultures turned brown	-
	6.0	6.0	Cultures turned brown	-
10.0	0.0	0.0	No response	-
	1.0	1.0	Formation of few stage-I embryos but no maturation thereafter	-
	2.0	2.0	-do-	-
	3.0	3.0	-do-	-
	4.0	4.0	As above and cultures turned brownish	-
	5.0	5.0	Cultures turned brown	-
	6.0	6.0	-do-	-

** Cultures obtained from female gametophytes were raised in ½Litvay's medium and cultures from zygotic embryos were raised in mMS medium. The media were solidified Difco-bacto agar (0.8%); * - No response
ABA= Abscisic acid

 # No maturation observed

Figure 18. Self designed bubble column bioreactor with suspension cultures from embryogenic calli of female gametophyte and zygotic embryo explants

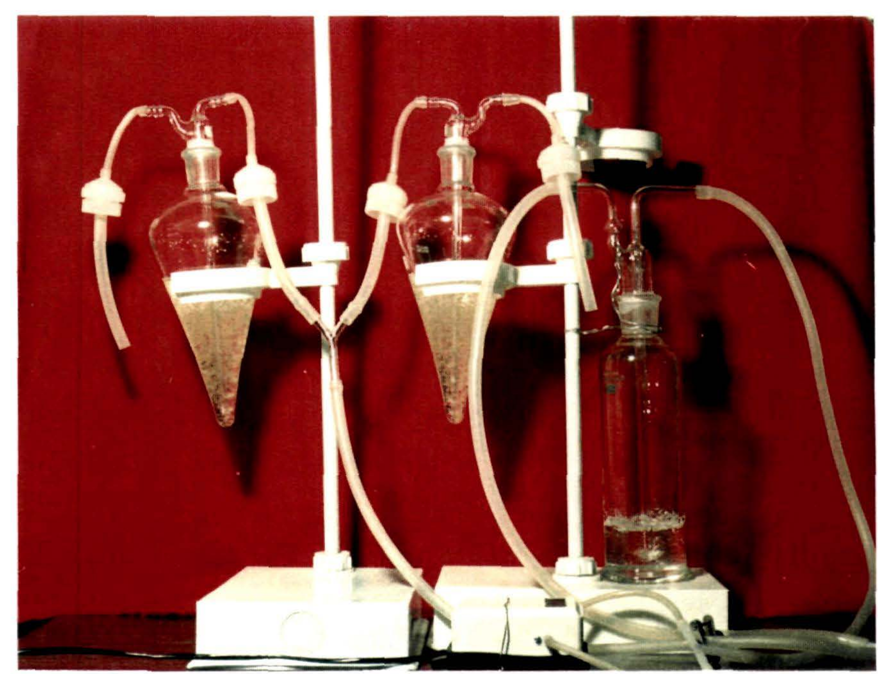

Figure 18

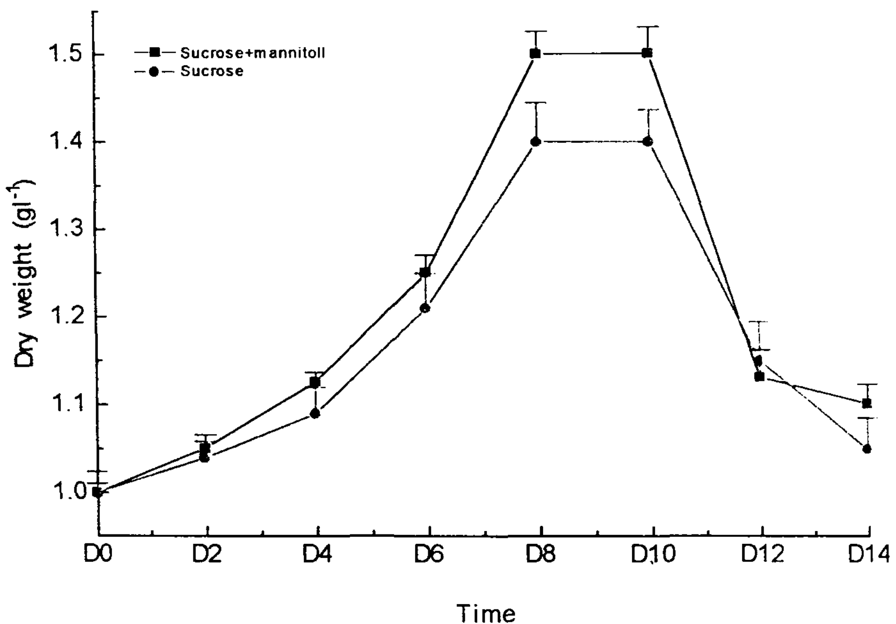

Figure 19. Growth of embryogenic suspensions in shake flasks obtained from cultures of female gametophytes in 1/2Litvay's medium containing sucrose (6%), sucrose and mannitol (3% each)

(Table 64). It was observed that number of somatic embryo formation was closely related to DW and FW (Table 64).

The maximum FW of embryogenic callus from female gametophytes was recorded to be 1.5 g in the medium containing 3% each of sucrose and mannitol together with 8 mgl^{-1} ABA. FW was $1.1g^{-1}$ when 6% sucrose was used singly along with 8 mgl^{-1} ABA in the medium.

Mature zygotic embryos: Growth kinetics of embryogenic cultures obtained from zygotic embryos in mMS medium exhibited changes in total DW (Figure 20). The culture showed a lag phase of 1 day, a linear phase of 7 days followed by a stationary phase from day 8. A maximum DW of 0.88 gl^{-1} was obtained in the medium containing 3% each of sucrose and mannitol and it was 0.85 gl^{-1} in 3% sucrose containing medium (Table 65).

In the callus culture, maximum FW of 1.3 g was recorded in the medium containing 3% each of sucrose and mannitol along with 8 mgl^{-1} ABA and it was 1 g in the medium containing 6% sucrose.

Effect of culture technique on number of somatic embryo formation

The optimum number of SE formation of 150 in cultures from female gametophytes and 35 in cultures from zygotic embryos was obtained using *submerge culture method* followed by *filter paper base*

Table 64. Growth characteristics of SF suspension cultures obtained from female gametophytes in ½Litvay's medium*

Growth characteristics	Treatment	
	Sucrose and mannitol (3.0% each)	**Sucrose (6.0%)**
Parameter		
Maximum FW (gl^{-1})	60.00	58.00
Time to maximum FW (day)	8-10	8-10
Maximum DW (gl^{-1})	1.50	1.40
Maximum number of embryos per ml	150.00	136.00
Maximum settled culture volume (ml)	4.00	3.80
Maximum packed culture volume (ml)	1.60	1.45
Lag phase length (day)	1.00	1.00
Linear phase length (day)	8.00	8.00

*: maturation medium containing ABA (8 mgl^{-1})

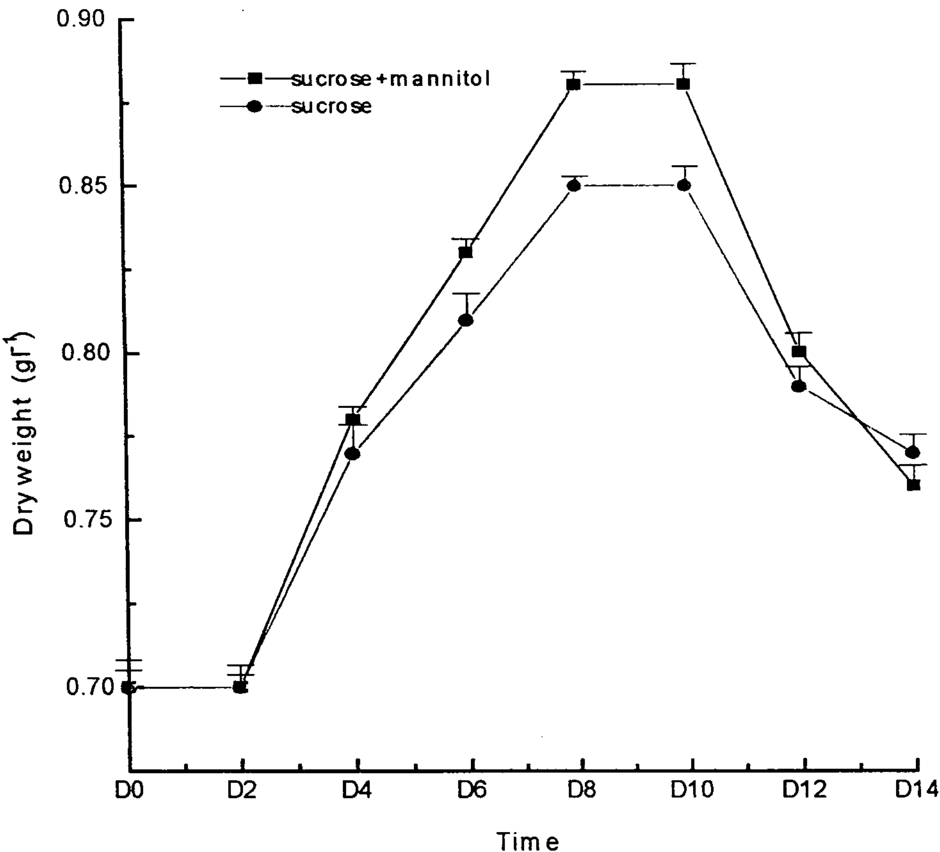

Figure 20. Growth of embryogenic suspensions obtained from cultures of zygotic embryos mMS medium containing sucrose (6%), sucrose and mannitol (3% each)

Table 65. Growth characteristics of shake-flask suspension cultures obtained from mature zygotic embryos in mMS medium*

Growth characteristics	Treatment	
	Sucrose and mannitol (3.0% each)	Sucrose (6.0%)
Parameter		
Maximum FW (gl^{-1})	48.50	46.50
Time to maximum FW (day)	8-10	8-10
Maximum DW(gl^{-1})	0.88	0.85
Maximum number of embryos per ml	35.00	31.00
Maximum settled culture volume (ml)	2.50	2.35
Maximum packed culture volume (ml)	0.89	0.87
Lag phase length (days)	1.00	1.00
Linear phase length (day)	8.00	8.00

*: maturation medium containing ABA (8 mgl^{-1})

method showing 20 and 10 SEs from cultures of female gametophytes and zygotic embryos respectively. The SE formation was found to be least effective in *direct culture method* as the number of SE was recorded to be 15 and 8 from cultures obtained from female gametophytes and zygotic embryos respectively (Figure 21 and 22).

Effect of semi-solid culture and suspension culture on number of somatic embryo formation

A comparative study of cultures grown in semi-solid and liquid suspensions showed a marked difference in number of embryo formation. A maximum of 15 SE g^{-1} callus were recorded in both the cultures from female gametophyte and zygotic grown in semi-solid medium. Under similar set of conditions, number of embryo formation per ml was found as high as 150 and 35 with suspensions derived from female gametophyte and zygotic embryos, respectively (Figure 21 and 22).

Microscopy of the embryogenic cultures

The microscopic observation of the cultures showed the presence of somatic embryos at different developmental stages (Figure 12b-e and 13c-g). Proembryos with dense embryonal head and suspensors of one to four elongated cells (Figure12b-d and 13c-e) or dense head with single suspensor cell (Figure 12b-c) was observed. In the initial and

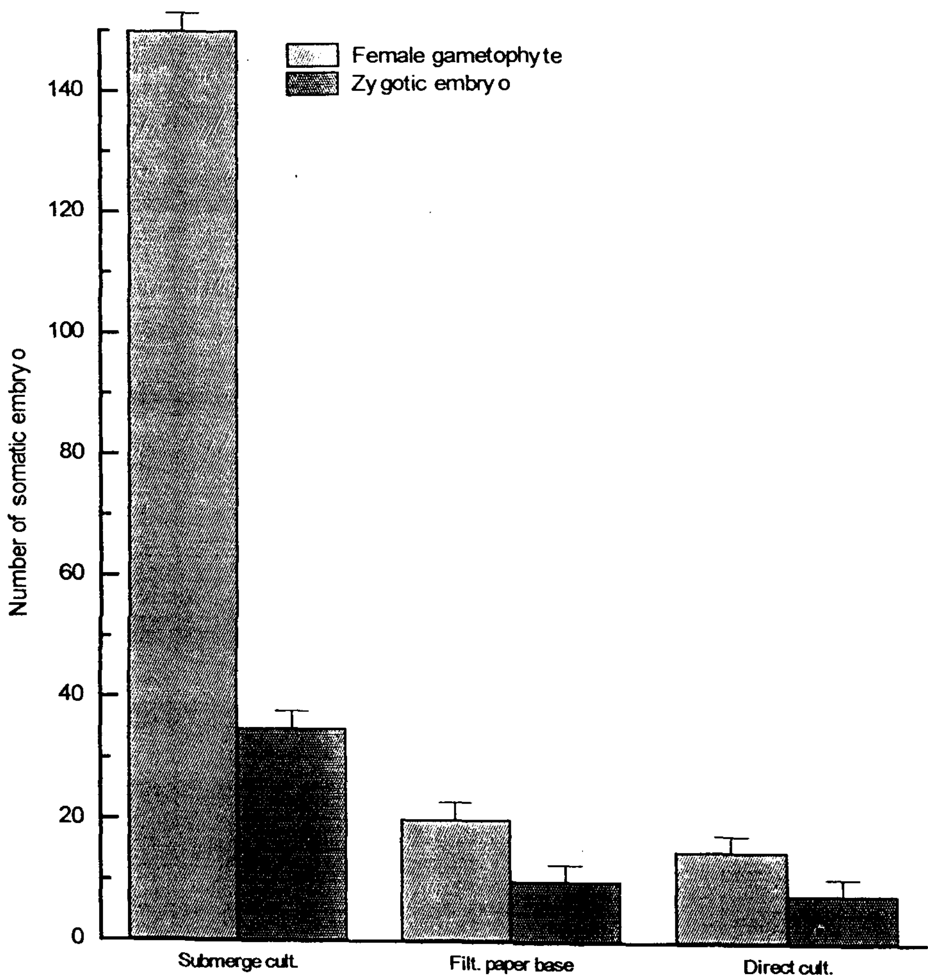

Figure 21. Effect of media type and culture methods on number of somatic embryo formation

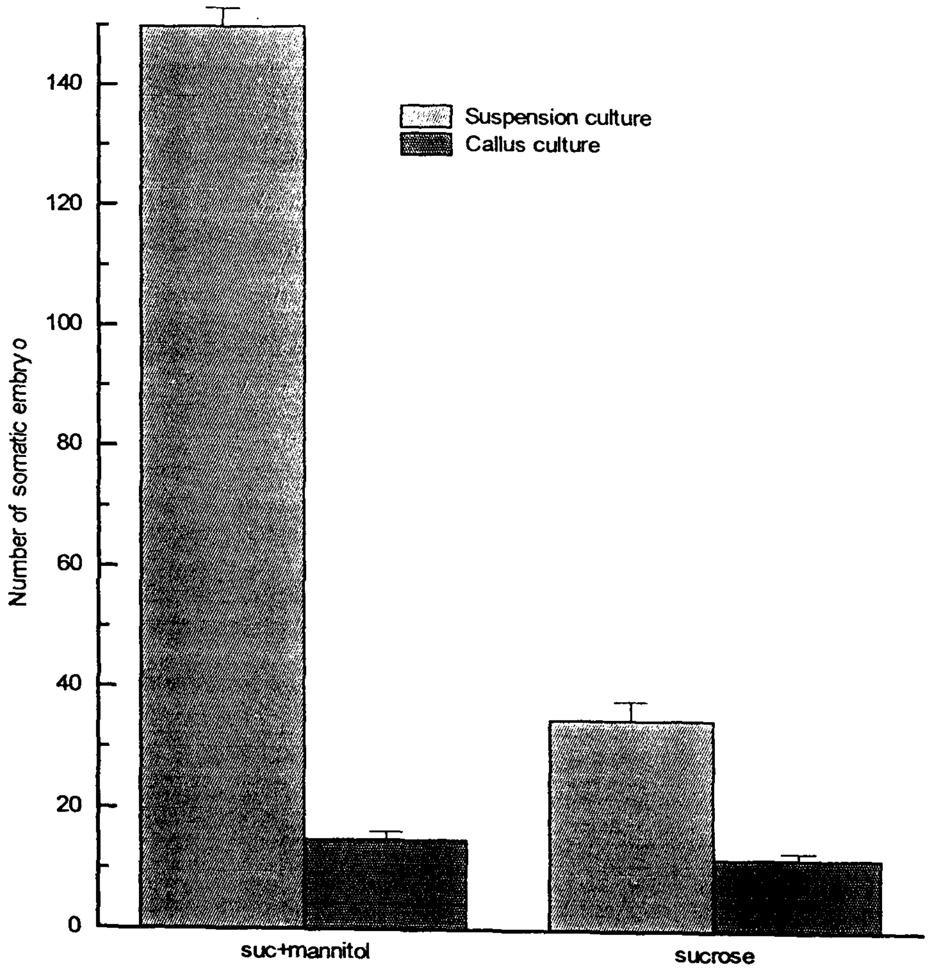

Figure 22. Effect of suspension culture and callus culture methods on number of somatic embryo formation

established cultures, somatic embryos with fused heads or fused suspensors (Figure 12e and 13f-g) were observed. With subsequent culturing these fused parts cleaved into many embryos connected to a common suspensor (Figure 12e and 13f-g). Presence of cleavage polyembryony provides an important method of multiplication of somatic embryos. In the established cultures and maturation media, cultures were seen to have SEs at advance stages of development. Stage-II (bullet shaped) SEs sharing a common suspensor like cells (Figure 12f and 13h) developed into stage-III (cotyledonary) SEs (Figure 12g-i and 13i) in the respective maturation media showing the complete process of somatic embryogenesis. In some of the cultures, mature cotyledonary SEs were observed to retain the common suspensor connections between two or more SEs (Figure 12h).

Germination of somatic embryo

Effect of strength of media

¼Litavy's medium and ½mMS medium were found to enhance germination of somatic embryos derived from cultures of both female gametophytes (Figure 23 and 24) and zygotic embryos (Figure 25 and 26) cultures respectively. ½Litvay's and mMS were comparatively less effective for germination.

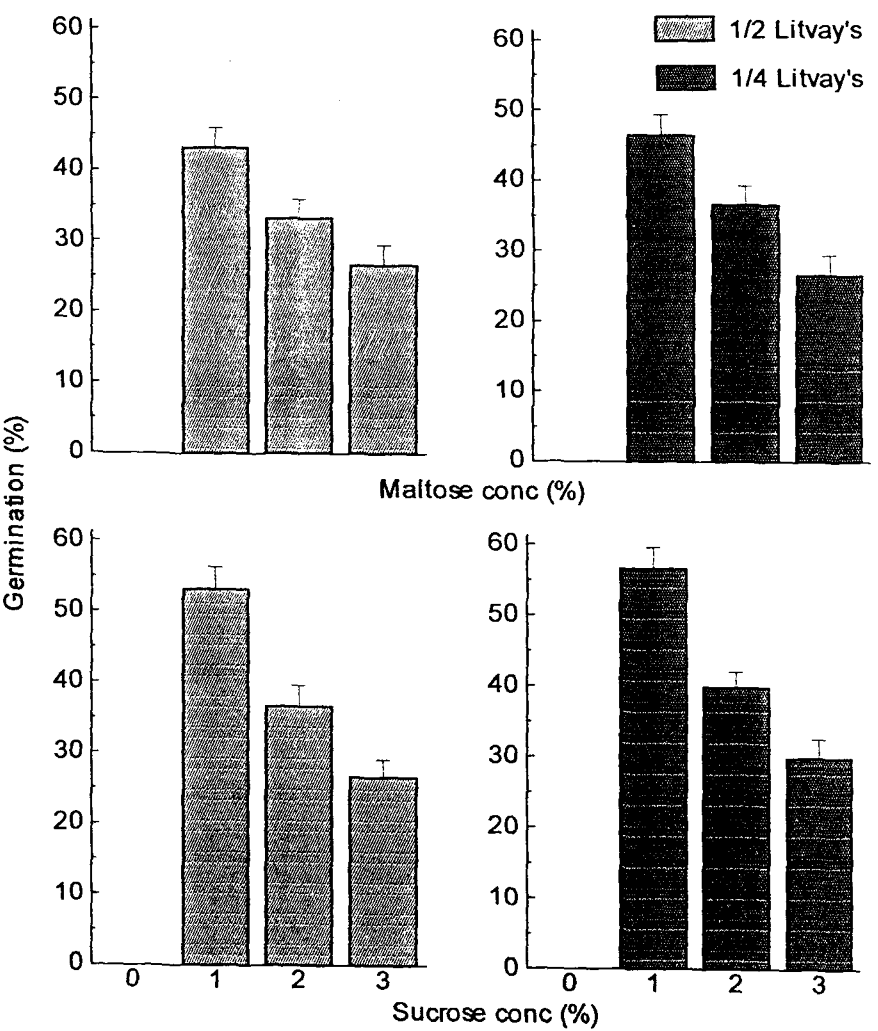

Figure 23. Effect of sucrose and maltose concentrations and strength of media on germination of somatic embryos derived from cultures of female gametophytes using direct culture method

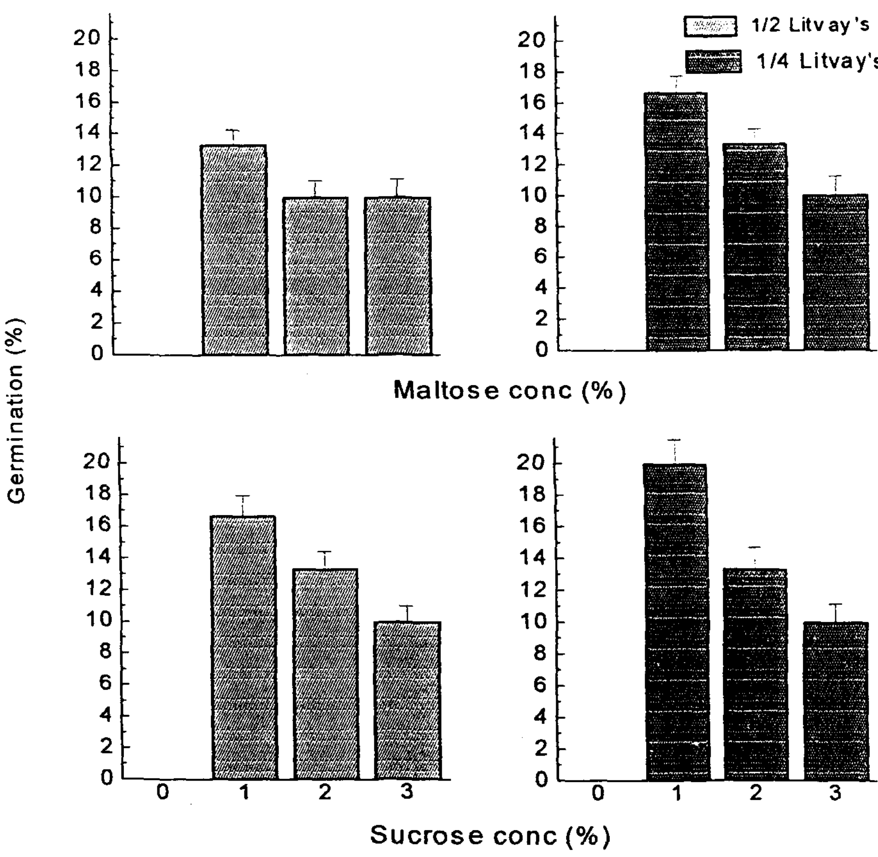

Figure 24. Effect of sucrose and maltose concentrations and strength of media on germination of somatic embryos derived from cultures of female gametophyte using submerge culture method

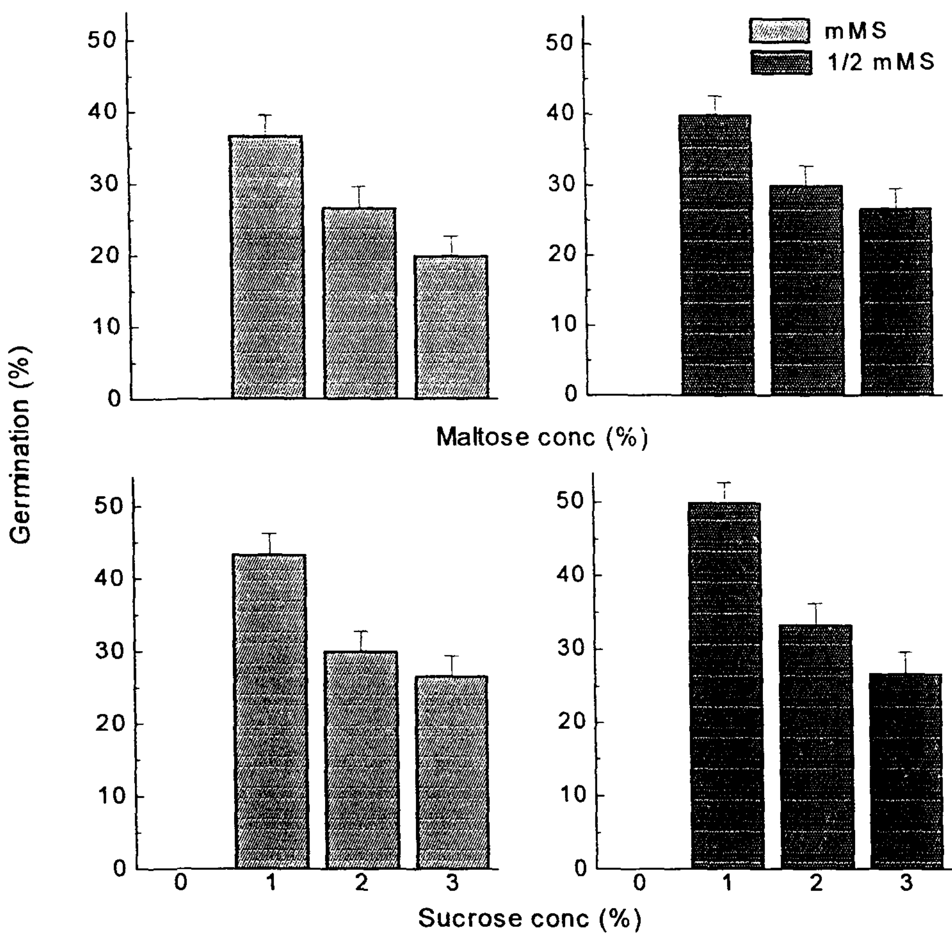

Figure 25. Effect of sucrose and maltose concentrations and strength of media on germination of somatic embryos derived from cultures of zygotic embryos using direct culture method

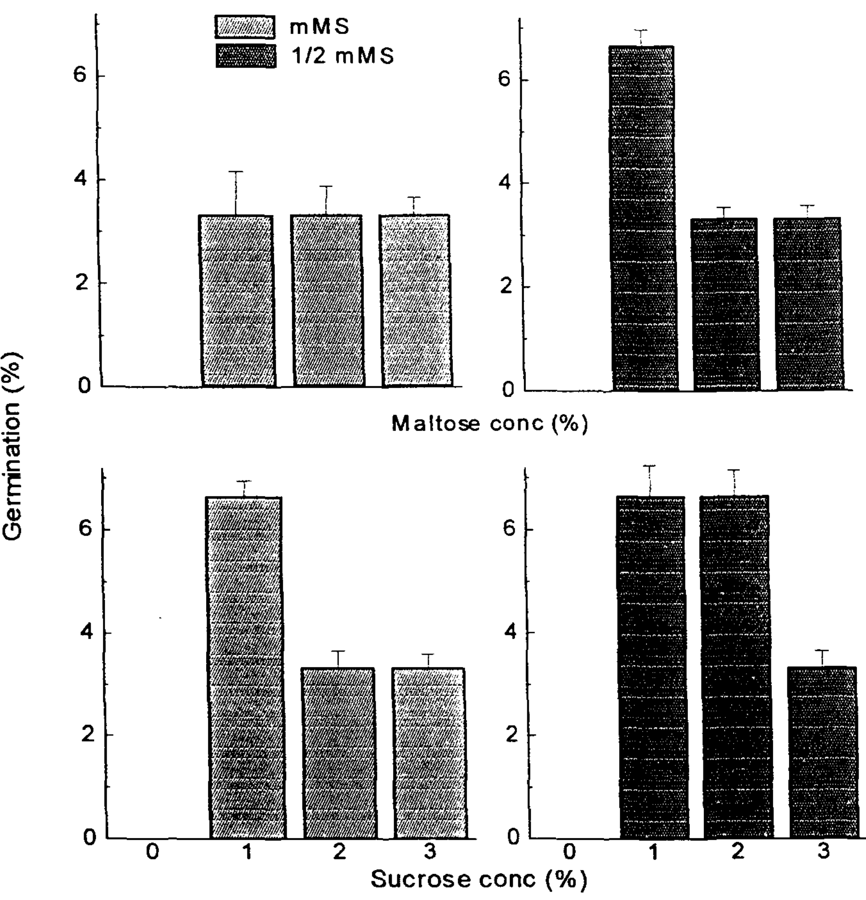

Figure 26. Effect of sucrose and maltose concentrations and strength of media on germination of somatic embryos derived from cultures of zygotic embryos using submerge culture method

Effect of carbohydrate concentration

For germination of somatic embryos, sucrose was found to be superior to maltose in the medium. Maximum germination of somatic embryos (56.66%) from female gametophyte cultures was recorded in ¼Litvay's medium containing 1% sucrose (Figure 23 and 24). Similar results were observed for germination of somatic embryos derived from cultures from zygotic embryo and maximum of 50% germination was noted when cultured directly in ½mMS medium containing 1% sucrose. A germination of 43.33% in ½ Litvay's medium supplemented with 1% maltose (Figure 23 and 24) and 40% in ½ mMS medium containing same concentration of maltose (Figure 25 and 26) were recorded for germination of SE obtained from female gametophyte and zygotic embryo cultures, respectively.

Effect of culture technique

Female gametophytes: Highest germination of 56.66% was observed in the SEs obtained from female gametophyte explants when cultured directly on ¼Litvay's medium containing 1% sucrose and solidified with 0.8% Difco-bacto agar (Figure 23). Only 20% germination was achieved in the SEs obtained from female gametophyte when cultured in ¼Litvay's medium containing 1% sucrose using *submerge culture*

method (Figure 24). However, these embryos were abnormal, with thick and fleshy hypocotyls and did not convert into emblings.

Mature zygotic embryos: With the SEs obtained from zygotic embryo cultures, a maximum of 50% germination (Figure 25) was recorded using direct culture method in ½mMS medium containing 1% sucrose and solidified with 0.8% Difco-bacto agar whereas it was found to be 6.66% using *submerge culture method* (Figure 26). The germinated embryos from *submerge culture method* were abnormal, with thick and fleshy hypocotyls and did not form emblings.

Effect of light intensity and photoperiod on germination of somatic embryos

Of different light intensities and photoperiods tried, 1900 lux light intensity and 12 h photoperiod resulted in optimum germination of SEs (Table 66; Figure 27a-c). 1900 lux light intensity and 6 h photoperiod was quite effective in radicle and hypocotyl elongation. 3 h photoperiod and 1900 lux light intensity was found to be least effective in germination.

Effect of humidity on hardening of emblings

Gradual decrease of humidity was found better in hardening of the emblings. It was found that when the emblings were hardened at higher RH value of 95±5%, there was about 95% survivability of

Table 66. Effect of light and photoperiod on germination of somatic embryos from female gametophyte and zygotic embryo**

Light intensity (lux)	Photoperiod (h)	Type of response	Performance*
0	0	Somatic embryos with thick and fleshy, slightly elongated radicle and hypocotyls, white to off white in colour i.e. non green in colour and failed to germinate	-
1000	0	As above and no germination observed	-
	3	Somatic embryos showed slight elongation of radicle and hypocotyls and faint green pigmentation, appeared etiolated. Failed to germinate	+
	6	As above but pigmentation was comparatively better	+
	12	As above but with much improved pigmentation	++
1300	0	Somatic embryos with thick and fleshy, slightly elongated radicle and hypocotyls, white to off white in colour i.e. non green in colour and failed to germinate	-
	3	Somatic embryos showed slight elongation of radicle and hypocotyls. Pigmentation was faint and appeared slightly etiolated. Failed to form emblings	+
	6	Comparatively better elongation of radicle and hypocotyls and pigmentation was also better but failed to form emblings	++
	12	As above but pigmentation was much better though failed to form emblings	++
1600	0	Somatic embryos with thick and fleshy, slightly elongated radicle and hypocotyls, white to off white in colour i.e. non green in colour and failed to germinate	-
	3	Somatic embryos showed slight elongation of radicle and hypocotyls. Pigmentation was faint and appeared slightly etiolated. Failed to form emblings	+
	6	Comparatively better elongation of radicle and hypocotyls and pigmentation was also better but failed to form emblings	++
	12	As above but pigmentation was much better and only very few formed emblings	+++
1900	0	Somatic embryos with thick and fleshy, slightly elongated radicle and hypocotyls, white to off white in colour i.e. non green in colour and failed to germinate	-
	3	Moderately elongated radicle and hypocotyls. Pigmentation not very satisfactory and failed to form emblings	++
	6	Comparatively better elongation of radicle and hypocotyls. Pigmentation was moderate and only few could form embling	+++
	12	Radicle, hypocotyls elongation and pigmentation was satisfactory. Somatic embryos showed higher rate of germination	++++
2200	0	Somatic embryos with thick and fleshy, slightly elongated radicle and hypocotyls, white to off white in colour i.e. non green in colour and failed to germinate	-
	3	Comparatively better elongation of radicle and hypocotyls. Pigmentation was moderate and only few could form embling	+++
	6	As above	+++
	12	Radicle and hypocotyls elongation was satisfactory. Pigmentation was very intense. Only few could develop into emblings	+++

**: ¼Litvay's medium and ½mMS medium for female gametophyte and zygotic embryo cultures respectively. Media were supplemented with only sucrose (1.0%) and solidified with Difco-bacto agar (0.8%); * - no, + poor, ++ moderate, +++ good and ++++ optimum response

Figure 27. Germinated SEs from embryogenic cultures of female gametophytes and zygotic embryos

a. Germinated SEs in a petri dish
b. Same as ‘a’ from female gametophyte cultures (0.63 x 6.5)
c. Same as ‘a’ from zygotic embryo cultures (0.63 x 10)

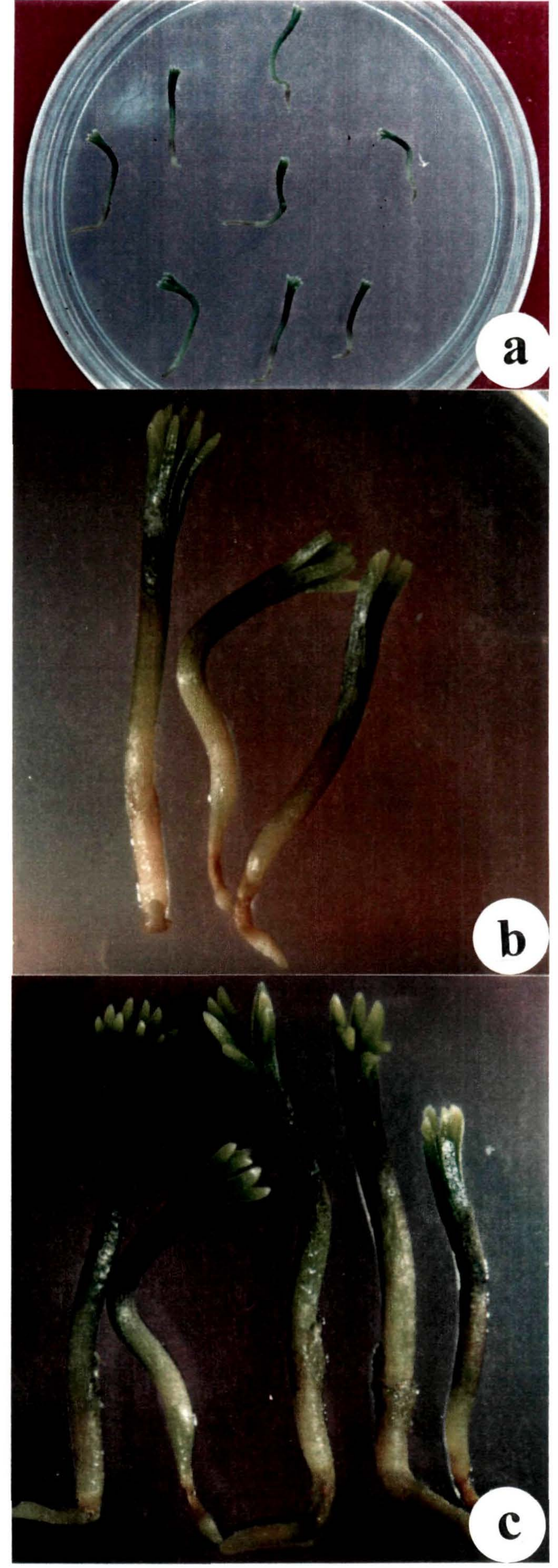

Figure 27

emblings. When the emblings were not hardened gradually and RH value was 50±5%, the survivability declined to 15% (Table 67) in the hardening of emblings obtained from both female gametophyte and zygotic embryo explants. The hardening of emblings is shown in figure 28.

The substrate of vermiculite: peat: pumice (1: 1: 1) as well as soil obtained from pine forests were found equally suitable for transplantation of emblings. Transfer of emblings needed around 1½ months hardening treatment before exposure to natural conditions.

Field performance of regenerants

Growth performance of regenerants

Shoot and root length: With 45d old emblings, 1.1-fold increase in shoot length was recorded over seedlings of the same age group. An increase of 1.1 and 1.4 fold in shoot length was found in with 90 and 180d old emblings respectively in comparison to the seedlings of same age. Similar trend of increased root length was noted with emblings. In 45d old emblings the increment of root length was recorded to be 1.14-fold and that of 90 and 180d emblings were 1.1 and 1.13-fold respectively as compared to the seedlings of the same age groups (Figure 29).

Table 67. Effect of humidity on hardening of emblings obtained from cultures of female gametophytes and zygotic embryos

	RH (%)	Type of response	Survivability* ($\pm$SE)[1] (%)
Poly covered pot	95±5	Emblings very healthy and green, no sign of wilting or drying. High survivability obtained in this method of hardening	95.00 (± 2.15)
Uncovered pot	50±5	Emblings moderately healthy. Many showed wilting and survivability declined drastically in this method of hardening	15.00 (± 1.57)

* Survivability of emblings on first day of complete removal of polybags

[1]:Standard error

Figure 28. Hardening of emblings

a. Emblings obtained from female gametophyte cultures

b. Emblings obtained from zygotic embryo cultures

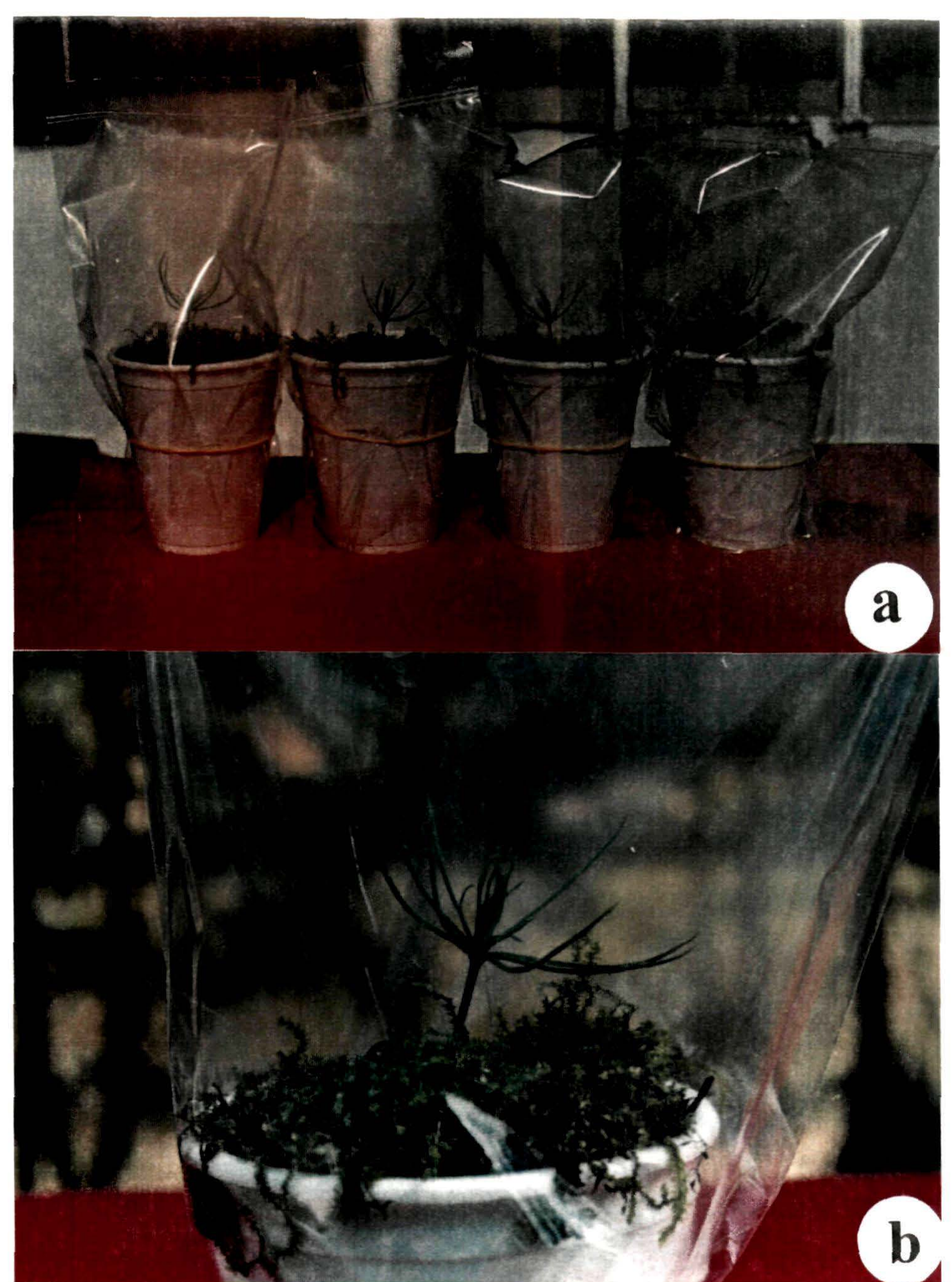

Figure 28

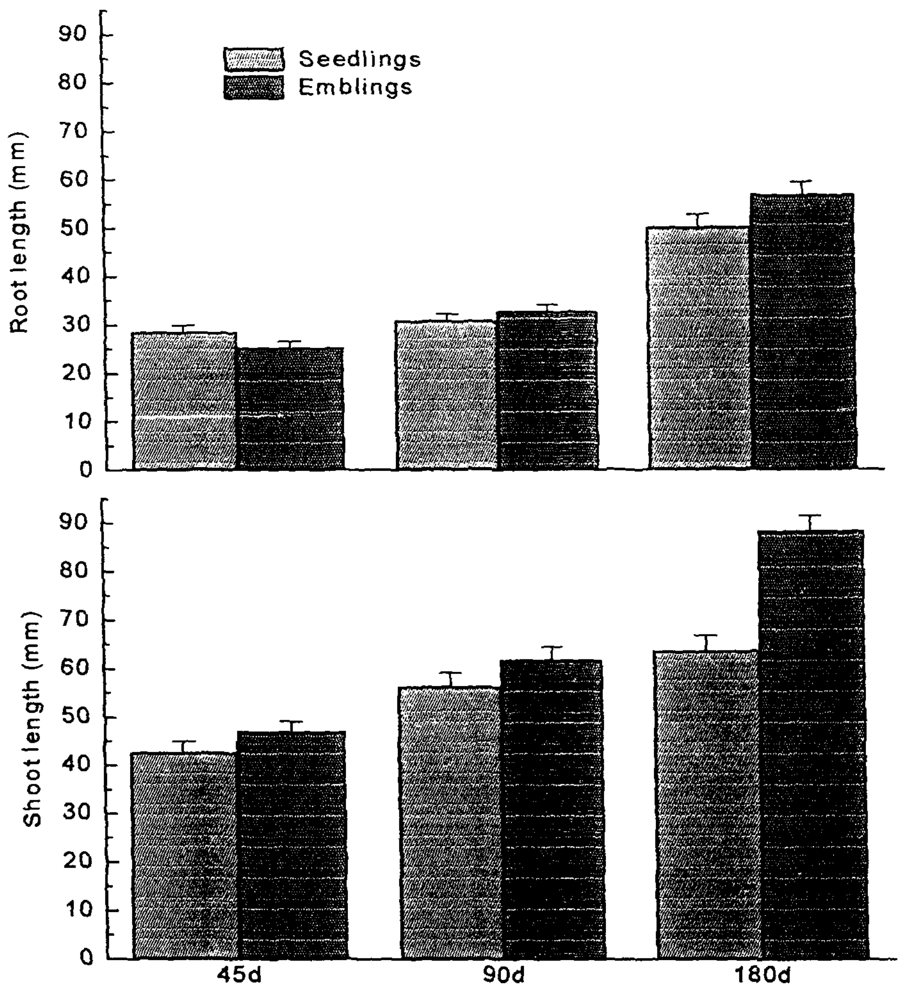

Figure 29. Shoot length and root length of emblings and seedlings of different age groups

Biomass evaluation: 45d, 90d and 180d old emblings showed 1.14-fold, 1.15-fold and 1.3-fold increase in biomass respectively as compared to the seedlings of the same age groups (Figure 30).

The study of biomass showed significant differences between the embling and seedling growth performance. Emblings showed better biomass accumulation than seedlings over a period of 180 days growth.

Survivability of regenerants in natural condition

The survivability of the emblings was 73.53% with 45d old emblings and it was 75% with the seedlings of the same age group. Survivability was recorded to be 50% and 51% with 180d old emblings and seedlings respectively (Figure 31) and the comparative study showed no significant difference in the survivability pattern of emblings and seedlings. The potted regenerants showed satisfactory growth and performance in the natural conditions (Figure 32).

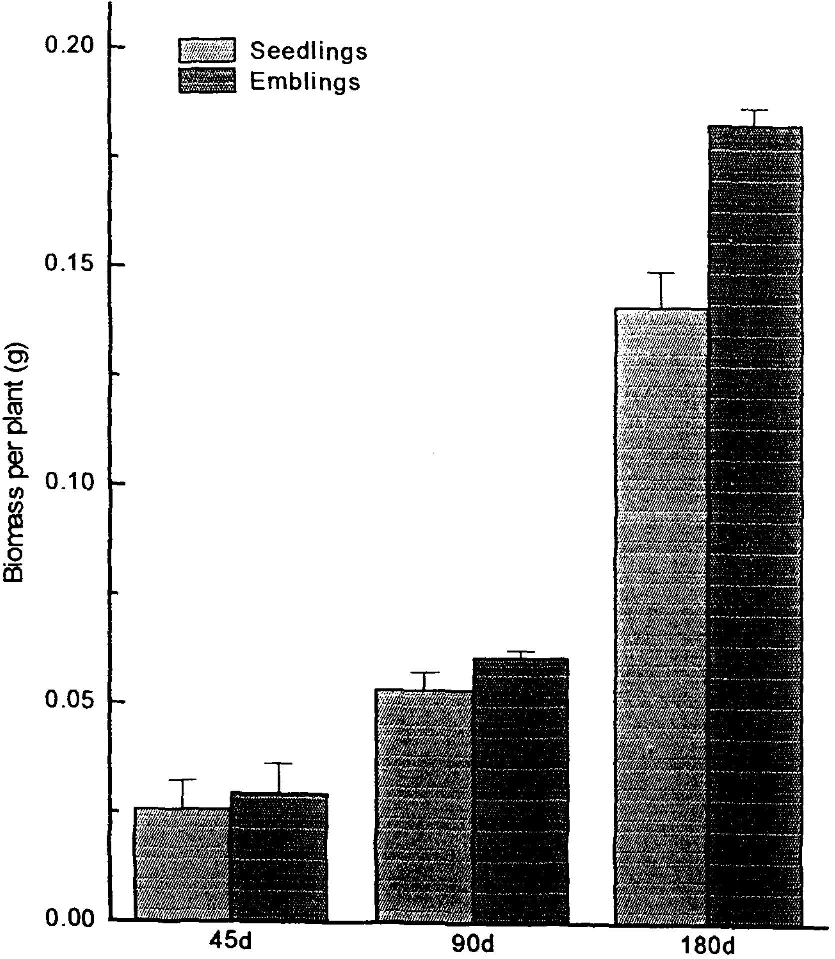

Figure 30. Biomass of seedlings and emblings of different age groups

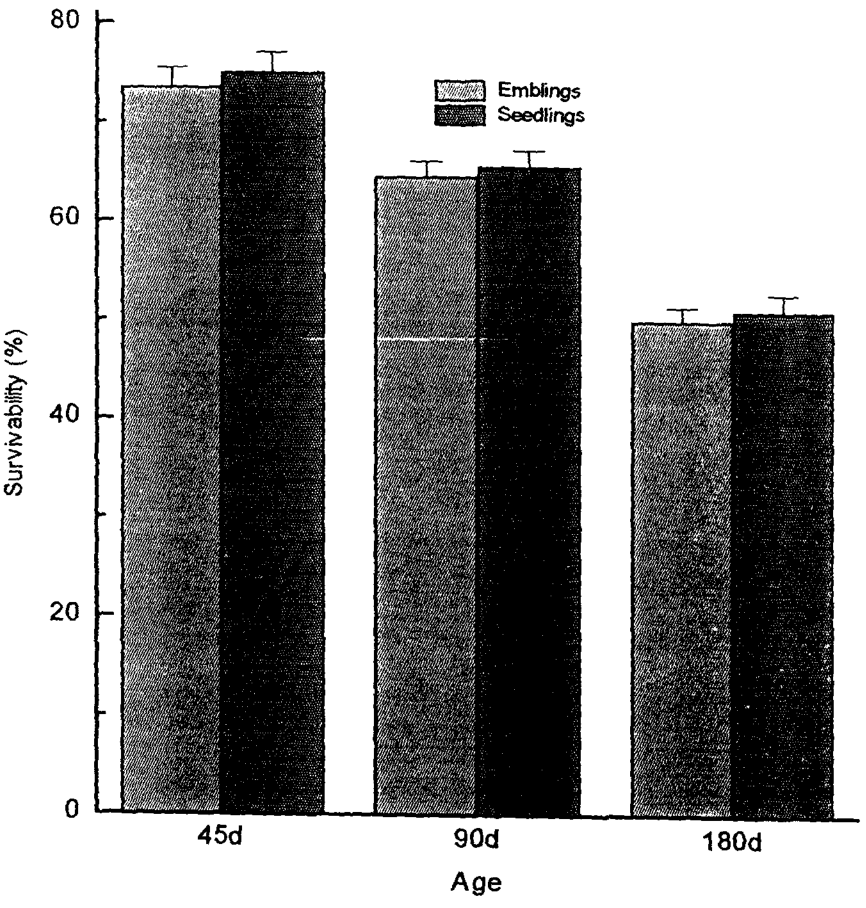

Figure 31. Survivability of emblings and seedlings of different age groups

Figure 32. Hardened potted emblings under natural environmental conditions

a. Hardened emblings of the somatic embryos from female gametophytes
b. Hardened emblings of the SE from zygotic embryos

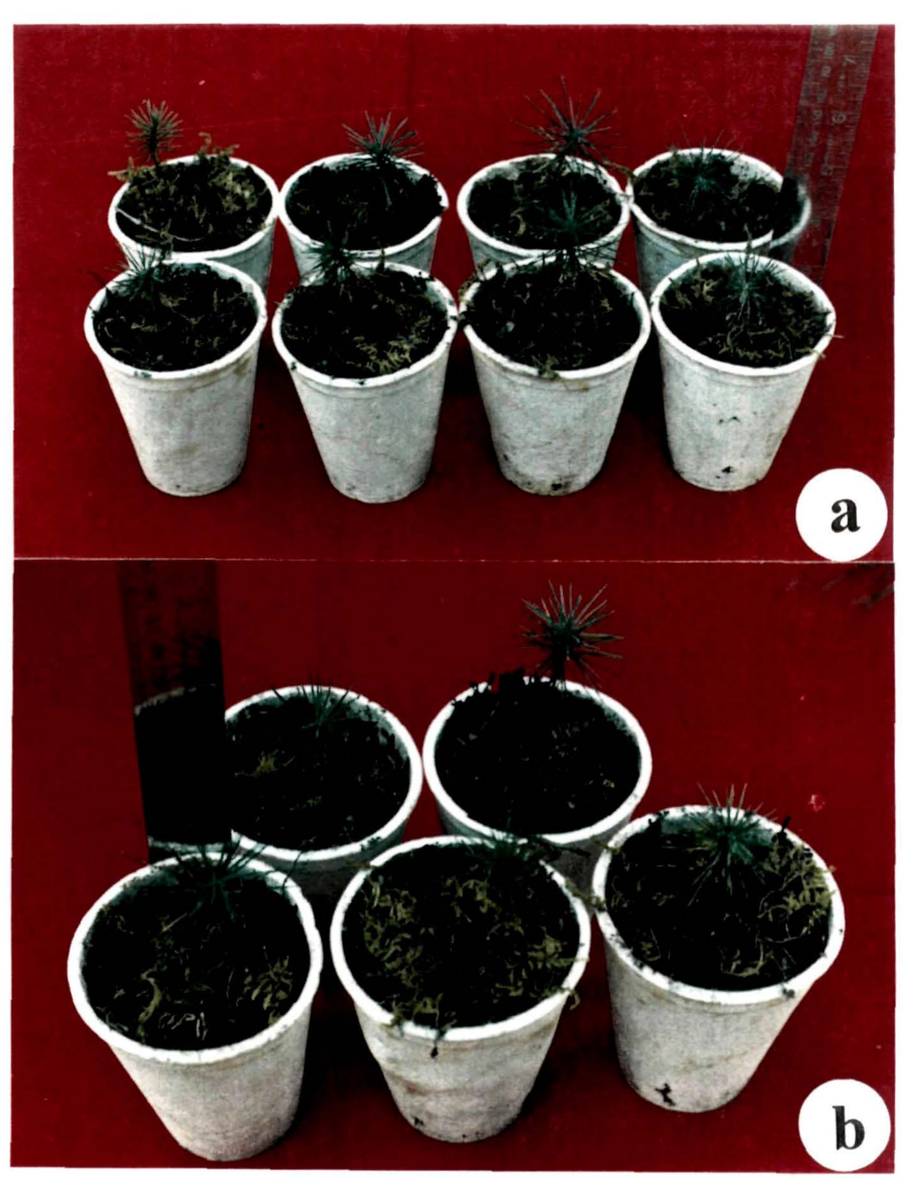

Figure 32

Chapter 5

Discussion

Somatic embryogenesis provides an ideal experimental system for investigation of the process of plant differentiation as well as the mechanism of expression of totipotency in plant cells. It is a process analogous to zygotic embryogenesis, but in somatic embryogenesis, a single cell or a small group of somatic cells are the precursors of somatic embryos. Unlike organogenesis, somatic embryogenesis recapitulates the events of zygotic embryogenesis with the production of a bipolar structure. Dunstan (1988), Wann (1988) and Gupta et al. (1991) have emphasized the potential of somatic embryogenesis as a method for rapid *in vitro* multiplication of conifers. Somatic embryos are also an important source of totipotent protoplasts useful for genetic transformation. They are useful for long-term germplasm storage using artificial seeds and cryopreservation (Chen and Kartha, 1987; Tautorus *et al.*, 1991).

Proper explant selection is critical to achieving successful induction of somatic embryogenesis in conifers. This is important

because various tissues of the same plant or tissues at various developmental stages can differ in their response *in vitro* (Roberts *et al.*, 1989; Arya *et al.*, 2000). Four explants viz., female gametophytes (with immature zygotic embryo), mature zygotic embryos, secondary needles and apical dome sections (shoot discs) from mature trees of *Pinus kesiya* were used for initiation of embryogenic cultures. It was possible to induce somatic embryogenesis using female gametophytes (with immature zygotic embryos) and mature zygotic embryos as explants whereas it was not possible when other explants were used. Frequency of induction of somatic embryogenesis with mature zygotic embryos of *P. kesiya* was found to be very low. The developmental stage i.e. age of the explant is a critical factor that decides its embryogenic potential. In the present study, the embryogenic potential of the explants declined with an increase in the age. This decrease is due to the progressive specialization of the tissues, reducing the plasticity and capacity of the cells to dedifferentiate. Abdullah *et al.* (1987b) had shown a lower response of explants with increasing age in *P. brutia*.

Majority of the reports regarding initiation of embryogenic cultures in *Pinus* species is restricted to immature zygotic embryo (Salajova and Salaj, 1992; Gupta and Grob 1995; Arya *et al.*, 2000, Tandon and Choudhury, 2002), while only few have reported induction

of embryogenesis from mature zygotic embryos (Bozhkov *et al.*, 1998). No reports are available in pines regarding development of mature somatic embryos from explants other than the zygotic embryos. However, in other conifers, somatic embryogenesis has been reported from immature and mature embryos (von Arnold and Hakman, 1986; Verhagen and Wann, 1989), cotyledons of germinated seedlings (Krogstrup, 1986; Lelu *et al.*, 1987), 20-30-day old seedlings (Attree *et al.*, 1990b), 14-month-old somatic emblings (Ruaud *et al.*, 1992) and buds and needles of 7-year-old trees (Westcott, 1992). Development of non-embryogenic callus from mature zygotic embryos in pines have been reported by Jain *et al.* (1989) and Salajova and Salaj (1992).

The importance of time of collection of explants in the present study suggested that there is a developmental period in which zygotic embryos are highly responsive towards somatic embryogenesis. Further, determination of optimum stage based on the time of season is ineffective because seed development may vary from year to year and influenced by latitude and altitude (Arya *et al.*, 2000). In the present investigation, immature zygotic embryos 10-12 weeks after fertilization were suitable for initiation of embryogenic cultures. Embryos 4-5 weeks after fertilization were most responsive in loblolly pine (Gupta and Durzan, 1987b). Although post fertilization period can serve as an

effective marker, the precise time of fertilization is very difficult to ascertain. This necessitated the need for identification of the optimum stage of explant for induction of somatic embryogenesis on the basis of size and morphology of the zygotic embryo. The advanced pre-cotyledonary stage i.e. stage-c (Figure 4b) with head size 0.2-1.1 mm was highly responsive and was found to be the most appropriate stage for induction of somatic embryogenesis in *P. kesiya*. This observation conforms to the report on suitability of pre-cotyledonary stage for initiation of somatic embryogenesis in *P. taeda* (Becwar *et al.*, 1990). The embryogenic potential has been found to decline with mature zygotic embryo in *P. kesiya* (Table 5). Jain *et al.* (1989) suggested that right stage of zygotic embryos for embryogenic callus induction is based on embryo length. They also suggested that embryos with attached suspensors were necessary for embryogenic culture production. In general, in *Pinus* species, pre-cotyledonary zygotic embryos are the best for inducing somatic embryogenesis (Gupta and Grob, 1995), where as in *Picea* species cotyledonary zygotic embryos are better (Becwar *et al.*, 1989; Tautorus *et al.*, 1991).

The pre-cotyledonary stage embryos were too small to be dissected out and were prone to desiccation during excision. Thus, intact

female gametophytes containing immature zygotic embryos were used as explants in *P. kesiya*. Female gametophytes containing immature zygotic embryos have been used as explants in many pine species (Becwar *et al*., 1988b, 1990; Salajova and Salaj, 1992; Nagmani *et al*., 1993). The female gametophytes with embryogenic extrusions (Figure 4c), when cut open longitudinally, showed that embryogenic extrusions were initiated from the suspensors of the developing zygotic embryo in most of the explants examined rather than the embryonal head. This examination revealed that the induction of embryogenic callus was in fact a continuation of the process of cleavage polyembryony that already exist in the suspensors of developing zygotic embryo. The stage 'a', 'b' and 'd' embryos with no or few cleavage embryos were responsive to a limited extent towards embryogenesis. A comparative study on the frequency of somatic embryogenesis with pre-cotyledonary zygotic embryos and those possessing cotyledonary primordia showed a decline in the embryogenic response with the cotyledonary stage embryo (Table 5). Tautorus *et al*. (1991) reported that somatic embryos could arise by the process similar to cleavage polyembryony occurring in nature.

The culture of immature zygotic embryos poses a very serious time constraint, because of the seasonal availability of the material

restricting its culture only for a short duration annually. The induction of somatic embryogenesis from mature embryos from stored seeds on the other hand extend the period from just a few months to more than a decade (Gupta and Durzan, 1986a; Tautorus *et al.*, 1990). But frequency of embryogenesis using mature zygotic embryos was very low after 3½ months in culture (Table 5). Mature seeds of *P. kesiya* during December to early February produced better embryogenic cultures (Table 5). The seeds collected during March resulted in poor response, as the embryos in these seeds were not healthy. The pre-cotyledonary to pre-germinating embryos were found suitable for initiation of embryogenic culture in *P. abies* (Hakman *et al.*, 1985), black and white spruce (Hakman and Fowke 1987a), *P. strobus* (Finer *et al.*, 1989), black and white spruce (Attree *et al.*, 1990b), *P. palustris* (Nagmani *et al.*, 1993), *L. decidua* (Lelu *et al.*, 1994; Bonga *et al.*, 1995). Finer *et al.* (1989) reported that the best stage of embryo of *P. strobus* for embryogenic culture initiation was prior to cotyledon development of the zygotic embryos. Cotyledonary stage embryos yielded embryogenic cultures with a frequency of ~0.1%. The decline in embryogenic response of the cotyledonary embryos of *P. kesiya* could be due to two reasons- (i) biochemical and molecular events may have set in which lead to germination and (ii) termination of cleavage embryony to a great

extent due to negligible amount or total absence of suspensor region in mature cotyledonary embryos.

Several reports are available on the formation of embryogenic cultures using secondary needles from *Picea* species. In *P. abies* embryogenic callus was established from explants excised from 7day old seedlings (Krogstrup, 1986; Lelu *et al.*, 1987). Embryogenic callus was also initiated from 12-30 day old seedlings of *P. glauca* and *P. mariana* germinated from 4 to 10 year old stored seeds (Attree *et al.*, 1990b). Ruaud *et al.* (1992) reported induction of somatic embryogenesis from *P. abies* using needles of 14 months old somatic seedlings grown in a green house. Westcott (1992) induced embryogenic callus from buds and needles of seven-year-old trees of *P. abies*. Deb and Tandon (2002) reported somatic embryogenesis from secondary needles of 5-6 week-old glasshouse-raised seedlings of *P. kesiya*. In the present study, mature secondary needles of *P. kesiya* collected during March to June produced healthy, white and soft callus and were much better in appearance than those collected during the other parts of the year. A heavy rainfall and moderate temperature are experienced which are ideal during this period. From July onwards the secondary needles become harder and show poor initiation of callus, which is mostly hard in texture. In this investigation, secondary needles from mature trees

though produced white, soft callus, but no somatic embryogenesis was recorded (Table 14).

The sections of apical dome of *P. kesiya* collected during May to July (during second flushing) formed very healthy, white and soft callus (Table 18). Both secondary needles and apical dome sections did not produce somatic embryos in all the media with different combinations and concentrations of plant growth regulators and organic carbons tried. Therefore, the callus obtained from these two explants could be regarded as non-embryogenic. These non-embryogenic calli so obtained, appeared opaque, friable and turned light green in the light as also reported by Tautorus *et al.* (1991). The non-embryogenic callus may or may not have anatomical organization and composed of isodiametric as well as elongated cells of variable size. The cells divided in disorganized fashion and no bipolar structures typical of embryogenic calli were present. A similar observation was made by Jasik *et al.* (1995) in *P. nigra.*

In the present study, five basal media viz., MS, mMS, DCR, BM_1 and Litvay's in full as well as half strength were tried (Figure 10). Amongst the different media tested, ½Litvay's medium for female gametophyte (Table 6) and mMS medium for zygotic embryos (Table 10) were found suitable for induction of embryogenic cultures in

P. kesiya. MS medium for secondary needles (Table 14) and ½DCR medium (Table 18) for apical dome sections were found better for initiation of soft, white non-embryogenic callus. Gupta and Durzan (1986b) used MS medium with modified levels of NH_4NO_3 (550 mgl^{-1}) and KNO_3 (4676 mgl^{-1}). A half strength modified MS medium supplemented with CH (500 mgl^{-1}) and sucrose (3%) was used for somatic embryogenesis of *P. abies*. For mature zygotic embryos, comparison of full strength LP and half strength LM (Litvay *et al*., 1985) media gave similar induction frequencies when tested for both *P. glauca* (Tremblay, 1990) and *P. mariana* (Tautorus *et al*., 1990). Li *et al*. (1998) reported that BM_1 medium (Gupta and Pullman, 1991) was superior over other media tried for initiation of embryogenic cultures from immature zygotic embryos of *P. taeda*. Kim *et al*. (1999) used LM, LP and MS media for induction of somatic embryogenesis from immature zygotic embryos of *L. leptolepis* and recorded 60%, 67% and 59% embryogenic tissue formation, respectively. Though LP medium was slightly superior at the initial stage, with time LM medium was found to be more effective in this species as embryogenic tissues failed to proliferate on LP medium.

For initiation of somatic embryogenesis in conifers, generally, lower concentrations of organic carbon sources were more effective

(von Arnold and Hakman, 1986; von Arnol, 1987, Becwar *et al.*, 1988; Nagmani *et al.*, 1993; Bonga *et al.*, 1995; Li *et al.*, 1998). In the present investigation, a comparative study on different media, carbohydrate types and their concentrations was studied on initiation of embryogenic cultures (Figure 10 and 11). Of various concentrations and types of carbohydrates, sucrose 3% was found to be most effective for embryogenic response using female gametophytes (35%) and zygotic embryos (12.5%) (Figure 11). 3% sucrose was also very effective in initiation of soft, white callus from secondary needles and apical dome sections. However, these two explants failed to produce embryogenesis. Maltose at 3% level was not very effective and could initiate embryogenesis only in female gametophytes and zygotic embryos. Lactose and fructose (3% each singly) were least effective, producing embryogenic cultures only in case of female gametophyte explants (Figure 11), which turned brownish in subsequent sub-culture and could not be maintained. Nagmani *et al.* (1993) also reported similar observations. They used maltose, glucose and sucrose as organic carbon source for initiation of embryogenic cultures in *P. palustris* from zygotic embryos and female gametophytes and found better initiation of embryogenic cultures on medium containing 3% sucrose. Kim *et al.* (1999) reported that 2% sucrose in the medium resulted in better

initiation of embryogenic cultures in *L. leptolepis*. To induce somatic embryogenesis in conifers, the most preferred and widely used organic carbon was sucrose at 3% level (Mathur *et al.*, 2000; Lelu *et al.*, 1999; Nagmani *et al.*, 1993; Becwar *et al.*, 1990)

Usually both an auxin and a cytokinin are used for initiation of embryogenic cultures in most of the conifer species (Attree and Fowke, 1991). 2,4-D generally has been the preferred auxin for the induction of ESMs in conifers (Gupta *et al.*, 1991; Tautorus *et al.*, 1991). NAA has also been successfully used in some cases. Verhagen and Wann (1989) found 2,4-D and NAA equally effective in initiation of somatic embryogenesis from mature embryos of Norway spruce. von Arnold (1987) found slightly higher frequency of embryogenesis with 2,4-D (at 20 μM) as compared to NAA at the same level, but NAA was more effective at lower concentrations (5 μM). Initiation of embryogenic culture has been achieved with different concentrations of plant growth regulators such as 10-110 mgl^{-1} 2,4-D (Gupta *et al.*, 1991). In *P. kesiya* 2,4-D and NAA were the preferred auxins like other conifers (Gupta *et al.*, 1991; Tautorus *et al.*, 1991; Gupta *et al.*, 1995a,b; Norgaard, 1997; Li *et al.*, 1998; Kim *et al.*, 1999). In the present study, female gametophyte explants exhibited optimum embryogenesis in the medium containing 5 mgl^{-1} 2,4-D and 2.5 mgl^{-1} NAA. 2,4-D and NAA at 5 mgl^{-1}

each were effective in initiation of embryogenic cultures from zygotic embryo explants and same concentration of auxins was promotive in soft, white callus induction from apical dome sections. For secondary needles, optimum callus induction was recorded using 2,4-D and NAA each at 3 mgl^{-1}. 2,4-D and NAA combination was highly beneficial in culture initiation compared to use of either alone. Incorporation of BAP in auxin rich medium was found to enhance embryogenic culture initiation. The present investigation is in conformity with the observation of other reports on conifer somatic embryogenesis (Gupta *et al.*, 1991; Nagmani *et al.*, 1993; Bonga *et al.*, 1995; Guevin and Kirby, 1997). The balance between auxin and cytokinin was highly beneficial than the absolute concentration of auxins. Li *et al.* (1998) in *P. taeda* found higher rate of extrusion and proliferation on lower auxin concentrations (3 mgl^{-1} 2,4-D) in combination with cytokinin (0.5 mgl^{-1} BA). In *P. kesiya*, the media containing only cytokinins (BAP and kinetin) were found to be ineffective in embryogenic culture initiation from female gametophyte and zygotic embryo. Secondary needles (Table 26 and 27) and apical dome sections (Table 28 and 29) as such did not produce any embryogenic culture in BAP and kinetin containing medium. However, BAP along with auxins in the medium promoted culture formation. BAP (2.5 mgl^{-1}) along with auxins (2,4-D and NAA;

Table 23 and 25) was found to accelerate embryogenic culture formation from female gametophyte and zygotic embryo explants. However, higher concentrations resulted in hard non-embryogenic cultures. In some conifers, cytokinins alone were found effective in initiation of embryogenesis. Norgaard and Krogstrup (1991) reported that the ESM cultures of *A. nordmanniana* were best initiated with cytokinins alone (BA and kinetin), while auxin was found to be inhibitory.

In the present study, induction of embryogenic cultures was done in the dark as also reported in most of the conifers (Tautorus *et al.*, 1991; Gupta and Grob, 1995). Embryogenesis is reported to occur in light in *P. abies* (Verhagen and Wann, 1989). Light was found to inhibit embryogenic culture initiation and resulted in hard and light-greenish cultures in *P. kesiya*.

The present investigation using female gametophytes indicated that higher rate of callus in the first one to one-and-half month of culturing could be due to the response of embryos towards injury or nourishment from female gametophytes. It was also recorded that although higher percentages of female gametophytes initiated the embryonal extrusion but only a limited number could survive sub-culturing over a longer period of time (Table 5). von Aderkas *et al.* (1990) and Becwar *et al.* (1988a) also suggested that megagametophytes

tissue supplied the nourishment necessary for embryogenic culture initiation. In *Picea* species also, only some embryogenic cultures that were initiated could be maintained for several months (Attree *et al.*, 1989; Webb *et al.*, 1989; Tautorus *et al.*, 1990).

In the present investigation, cultures on semi-solid medium from all the explants after few sub-cultures ceased to proliferate and started browning on growth regulator rich medium. Therefore, the cultures had to be sub-cultured on basal medium containing reduced level of growth regulators to sustain proliferation and multiplication. The cultures were transferred every 12-15 days on to fresh medium containing reduced level of growth regulators and maintained in the dark at 25±2°C. In the present investigation, optimal multiplication of cultures was achieved on $1/10^{th}$ growth regulator containing medium along with 3% sucrose (Table 30, 32, 34 and 36). Durzan and Gupta (1987a,b) also reported the use of lower concentrations of growth regulators for maintenance of the ESMs. Gupta *et al.* (1995a) reported that it was necessary to reduce the growth regulator levels in the maintenance medium compared to initiation medium for *P. abies*.

For initiation of suspension cultures of *P. kesiya* using calli from different explants, 3% sucrose was found to produce best results (Table- 38 and 39). Increase in sucrose concentration beyond 3% was

found inhibitory. Low concentration (30 mM) of sucrose has been reported beneficial in suspension cultures (Hakman and von Arnold, 1988; Tautorus *et al.*, 1992; Lulsdorf *et al.*, 1992; Dunstan *et al.*, 1993). Find *et al.* (1998) used 3% sucrose in suspension cultures of Norway spruce and Sitka spruce. Other organic carbon sources like maltose, lactose and fructose were found less effective in initiation of suspension cultures in *P. kesiya*.

In the present study, auxins and cytokinins at the same level of concentrations as used for initiation of cultures on semisolid medium were found equally effective in initiation of embryogenic suspension cultures from calli of female gametophytes and zygotic embryos in *P. kesiya* (Table 40 and 41). Healthy non-embryogenic suspension cultures from calli of secondary needles and apical dome sections were also initiated using the same growth regulator concentrations to that used in initiation of cultures in agar-solidified medium (Table 42 and 43).

Inoculum density exhibited a prominent bearing on initiation and establishment of suspension cultures. In the present study, 40 gl^{-1} (2 gml^{-50}) inoculum density was found to be optimum for initiation of suspensions (Table 44 and 45). An increase or decrease of inoculum density below 40 gl^{-1} resulted in poor culture growth (Table 44 and 45).

Dunstan *et al.* (1993) found 40 gl^{-1} as optimum inoculum density in *P. glauca* suspension culture. Lulsdorf *et al.* (1992) reported the same inoculum density for *P. glauca-engelmannii*-complex and *P. mariana* suspension cultures.

In the present investigation, 10% SCV was optimum for proliferation of embryogenic suspension cultures with all the explants. An increase in SCV resulted in culture browning (Table 46 and 47). By decreasing the SCV (below 10%) a poor culture growth was recorded which may be due to inadequate culture density. Higher SCV (> 10%) slowed down the growth of cultures associated with browning, which may be attributed to competition for nutrients due to over crowding of cells. Low SCV (< 10%) slowed down the culture growth, which could be due to low cell density in the culture environment. Krogstrup (1990) reported that culture density was crucial and determined the quality of early stage embryos in suspension cultures in *P. sitchensis*. Find *et al.* (1998) found 20% (v/v)±1% SCV suitable for maintenance of suspension cultures of Norway spruce and Sitka spruce. Ingram and Mavituna (2000) also reported 100 ml SCV l^{-1} medium (i.e. 10% SCV) was suitable for proliferation of embryogenic suspension cultures in *P. sitchensis*.

In *P. kesiya*, the suspension cultures from all the explants showed browning if maintained in the medium containing high concentrations of growth regulators. So the suspension cultures were required to be subcultured in basal medium containing reduced growth regulators. Optimum culture growth was found in 1/10th or 0 concentrations of growth regulators (Table 48, 50, 52 and 54) at 10% SCV and 3% sucrose. Durzan and Gupta (1987); Gupta *et al.* (1995a), reported the use of reduced growth regulator levels in the maintenance medium for *Picea* cultures.

A comparative study of sucrose and maltose (3% each) in maintenance medium showed that sucrose was superior over maltose in the increment of growth of suspension cultures. But browning of culture was found to set in rapidly in sucrose containing medium than maltose containing medium.

Microscopically, the embryogenic culture of *P. kesiya* showed the presence of somatic embryos at various developmental stages interspersed with elongated and spherical cell clusters. The present findings are similar to Gupta and Durzan (1987b) have discussed the similar constitution of embryogenic cultures in loblolly pine. The embryogenic cultures exhibited somatic embryos with fused suspensors and with subsequent culturing those fused parts cleaved into many

embryos connected to a common suspensor (Figure 12b-e and 13c-g). However, these embryos separated from each other on transfer to ABA containing medium (Figure 12f and 13h). These observations suggested that "cleavage polyembryony" occurred in embryogenic cultures that constitute a method of multiplication of somatic embryos. Similar results of polyembryony have also been reported by Gupta and Durzan (1986a,b), Durzan and Gupta (1987a,b), Arya *et al.* (2000).

Tautorus *et al.* (1991) reviewed the mode of origin of somatic embryos *in vitro* in conifers. Origin of somatic embryos may vary depending upon the type of explant material used (Nagmani *et al.*, 1987; Finer *et al.*, 1989). Three pathways have been suggested, which account for the origin of conifer somatic embryos (Hakman *et al.*, 1987)- (i) somatic embryos may arise from single cells or small cell aggregates by an initial asymmetric division that delimits the embryonal apex and suspensor region (Hakman *et al.*, 1987; Nagmani *et al.*, 1987; Jain *et al.*, 1989). In *P. abies* and *P. glauca*, the two-celled proembryos resulted from an unequal division of somatic cell, forming a dense cytoplasmic embryonal head-like cell and a vacuolated suspensor-like cell. (ii) somatic embryos may develop from small meristematic cells within the suspensor. Their initials could arise by asymmetric division of suspensor cells or from meristematic cells of the embryonal apex region

that have failed to elongate while being integrated into the suspensor (Hakman *et al.*, 1987). and (iii) somatic embryos could arise by a mechanism similar to cleavage polyembryogeny with the initial separation occurring in embryogenic region. A cleavage-like polyembryony has been described in somatic embryo cultures of *A. alba* (Schuller *et al.*, 1989), *L. deciduas* (Nagmani and Bonga, 1985; von Aderkas and Bonga, 1988), *Pinus* and *Picea* species (Becwar *et al.*, 1988a; von Arnold and Woodward, 1988) and *P. menziesii* (Durzan and Gupta, 1987).

In the present study, non-embryogenic callus appeared white, opaque, friable and turned light green in the light as also reported by Tautorus *et al.* (1991). The non-embryogenic callus may or may not have anatomical organization and composed of isodiametric cells of variable size. Non-embryogenic suspension cultures were composed of mixture of isodiametric and elongated suspensor like cells (Figure 14 and 15). The cells divided in disorganized fashion and no bipolar structures typical of embryogenic cultures were present. Similar observations were also made by Jasik *et al.* (1995) in *P. nigra*.

Abscisic acid has been used for cotyledonary embryo development in many plant species including conifers (Gupta and Durzan, 1987a,b). In the present study, the stage-I embryos (Figure 12d

and 13e) converted into stage-II (Figure 12f and 13h) and cotyledonary embryos (Figure 12g-i and 13i) in respective basal media containing higher concentrations of sucrose or combination of sucrose and mannitol (3% each) in conjunction with ABA (Table 56-63). *Submerge culture method* and *filter paper base method* were found to be superior over *direct culture method* of somatic embryo maturation (Figure 16 and 17). It was also found that neither sucrose nor ABA singly could promote maturation of somatic embryos. A concentration of 6% sucrose and 8 mgl^{-1} ABA was found to be quite effective in embryo development and maturation. A comparative study of sucrose and mannitol in maturation medium showed that use of sucrose and mannnitol at 3% level each in conjunction with 8 mgl^{-1} ABA was most effective and resulted optimum embryo development and maturation from cultures of female gametophytes and zygotic embryos (Table 58) using *submerge culture method*. It was believed that the higher osmoticum helped in the development of embryos while ABA brought about desiccation stress resulting in maturation of somatic embryos. Sucrose, mannitol and ABA at higher concentrations probably increased the medium osmoticum, thus limiting the water uptake by the tissue and causing water stress, which led to embryo maturation in *P. kesiya*. It has been suggested that the favourable effect of ABA could be due to increase in storage

reserves, such as storage proteins, triglycerides and lipids. Another effect of ABA is prevention of precocious germination (Lelu *et al.*, 1994). Finer *et al.* (1989) found that elevated sucrose levels (6-12%) were beneficial for somatic embryo maturation and differentiation in *P. strobus*. Tremblay and Tremblay (1995) reported that sucrose (4-6%) in the medium could serve as an osmoticum and as a carbon and energy source in black spruce somatic embryo maturation. Li *et al.* (1998) reported the promotory effect of maltose on embryo maturation in *P. taeda*. Norgaard (1997) reported that maltose (3.4-4.3%) used singly was better organic carbon source than sucrose (3%) in maturation and germination of somatic embryos in *A. nordmanniana*. The combined effect of both maltose and sucrose was more satisfactory than either of these used singly. Carrier *et al.* (1997) studied the effect of exogenous sucrose on maturation and germination of somatic embryo in interior spruce. They reported that embryos placed on the medium with added sucrose developed roots and epicotyls and increased their fresh mass by about 13 fold by consuming 25% of available sucrose in the medium. The exogenously supplied sucrose promoted the formation of linolenic acid, which participated in the maturation of embryos. Durzan and Gupta (1987a,b) reported that ABA inhibits cleavage polyembryony and allowed singulation of embryos and further development in Douglas fir.

Mannitol (6%) enhanced embryo maturation in interior spruce (Roberts, 1991). Webster *et al.* (1990) reported that 40 μM ABA was optimum for the production of mature embryos. Gupta *et al.* (1995a) reported that in *Picea abies*, embryo development occurred following the removal of auxins and cytokinins and addition of ABA.

In *P. abies*, an increase in exogenous ABA levels (5-40 μM) resulted in an increase in somatic embryo maturation both in semisolid and liquid medium (Vagner *et al.*, 1998). In the absence of exogenous ABA, yield of embryos was negligible. Embryogenic cultures on auxin rich medium has been found to produce ethylene which act as inhibitor in somatic embryo development (Biddington *et al.*, 1993). Further it was reported that ethylene inhibited induction of embryogenic cultures and development of somatic embryos. Accumulation of ethylene in embryogenic cultures in conifers was reported by Noland *et al.* (1986) in loblolly pine, Wann *et al.* (1987a) in *P. abies* and Kumar *et al.* (1989) in *P. glauca*. It was found that more ethylene was formed in non-embryogenic cultures. Ethylene interfered with the development of polarity. Ethylene inhibitors like ABA, when incorporated in the media, formation of ethylene was inhibited and that promoted somatic embryo maturation. Influence of ethylene and different ethylene inhibitors on somatic embryo maturation of white spruce was studied by Kong and

Yeung (1994). They reported that the presence of ABA in the development and maturation medium showed a decrease in ethylene production. Absence of any ethylene inhibitor resulted in very poor cotyledonary embryo formation. It was further reported that polarity development was better with decrease in ethylene production.

In the present study, the embryogenic cultures in BCBs from different explants produced stage-I somatic embryos. But these embryos could not develop further into cotyledonary embryo even in presence of ABA and sucrose or ABA and sucrose-mannitol in the medium. Failure in achieving cotyledonary somatic embryos could be attributed to problems linked to self-designed BCBs. In the present study, one of the most important reasons could be flow rate and dissolved oxygen in the medium, which might have affected and inhibited maturation of somatic embryos. Tautorus *et al.* (1992) aerated the embryogenic cultures of *Picea mariana* and *Picea glauca-engelmannii* at a rate of 0.2 to 0.6 vvm (volume air per volume culture per minute) and dissolved O_2 was maintained at above 80% saturation. Okamoto *et al.* (1996) reported the effect of oxygen concentration in the aeration gas on plantlet regeneration from rice cells in bioreactor cultures. They showed that the efficiency of regeneration in cultures aerated with gas mixture of over 40% oxygen was higher than that in a SF culture. In the present BCB

culture, though not severe, sedimentation occurred with the embryo cultures to some extent suggesting that the mixing in the BCB was insufficient to agitate the cultures thoroughly. Tanaka (1982) found accumulation of sedimented cells and dead zones in *C. tricuspidata (Capsicum)* culture at high cell densities and attributed that to the configurations of airlift bioreactors and bubble bioreactors. Ingram and Mavituna (2000) reported sedimentation of embryos of *P. sitchensis* within the bubble bioreactors. They reinforced that bioreactors could be used successfully for large-scale somatic embryogenesis so long as the bioreactor configuration, design and operating conditions are carefully chosen to suit the physiological, metabolic and morphological characteristics of the culture.

Measurement of cell growth and assessment of growth kinetics are important to optimize the methods for large-scale somatic embryo production (Ryu *et al.*, 1990). In the present investigation, growth kinetics were studied and analysed using an array of parameters like sedimented culture volume, packed culture volume. These were compared with fresh weight, dry weight and somatic embryo number in order to determine the parameters that were highly correlated with growth and embryo number. The embryogenic cultures of female gametophytes showed increased biomass in the media with sucrose and

mannitol (each at 3% level) compared to sucrose alone in the medium of female gametophytes (Table 64). In case of cultures from female gametophytes, a maximum dry weight of 1.5 gl^{-1} (in 10-days time) and 150 embryos ml^{-1} suspension (in ½Litvay's medium with 3% each of sucrose and mannitol and 8 mgl^{-1} ABA containing medium) was obtained (Table 64). Similarly, with cultures of zygotic embryo, a maximum DW of 0.88 gl^{-1} and 35 embryos ml^{-1} suspension was found in mMS medium with 3% each of sucrose and mannitol (Table 65). The effect of sucrose concentration on culture growth and number of somatic embryo formation was studied by Tautorus *et al.* (1992) in black spruce and interior spruce. They reported an increase of FW and DW with 60 mM and 90 mM sucrose in 10-12 days time in the culture medium. Fresh mass, dry mass and corresponding number of embryo formation was also correlated by Dong and Dunstan (1994) in *P. glauca.* Higher sucrose concentration has been found essential in somatic embryo formation and maturation. Lulsdorf *et al.* (1992) found that SF suspensions of interior spruce and black spruce became carbohydrate-limited in medium with low concentration of sucrose (30 mM). They also reported that sucrose depletion caused suspension cultures to enter a stationary growth phase, which was characterized by a decline in biomass and browning of embryos. In the present study of growth

kinetics, a lag phase was found with zygotic embryo suspension cultures but no lag phase was found in female gametophyte suspensions and exhibited a linear phase, stationary phase and decline phase (Figure 19 and 20). Similar growth patterns have been reported in embryogenic suspension cultures of *P. sitchensis* (Krogstrup, 1990), *P. glauca-engelmannii* (Lulsdorf *et al.*, 1992), *P. glauca-engelmannii* and *P. mariana* (Tautorus *et al.*, 1992), *P. glauca* (Dong and Dunstan, 1994).

Comparative study of growth parameters of callus culture and growth kinetics of suspension culture in the present study revealed the advantages and superiority of suspension culture over callus culture in somatic embryogenesis. This was evident from the fact that, from suspension culture, a maximum of 150 somatic embryos could be obtained from just 1 ml of suspension whereas the number of somatic embryo formed from 1 g callus was just 15 (Figure 22). Therefore, suspension culture method could be considered as the best potential method for mass-multiplication of somatic embryos and their use in clonal forestry.

Culture technique and media type (i.e. liquid culture and semi-solid culture) have been found to affect maturation of somatic embryos in conifers to some extent. In *P. kesiya* maturation of somatic

embryos were possible in all the culture methods tried i.e. *submerge culture method, filter paper base method* and *direct culture method.* There have been no reports of successful maturation of conifer somatic embryos and plantlet recovery following submerged liquid culture method (Attree *et al.*, 1994). Somatic embryo maturation has been restricted to solid medium (Tautorus *et al.*, 1994) or to solid supports soaked with liquid medium (Attree *et al.*, 1994). Ingram and Mavituna (2000) reported maturation of somatic embryos of SS03 *P. sitchensis* genotype using both solid medium and submerged culture method and that conforms to our result.

Development, maturation, germination and conversion of somatic embryos into emblings has been a persistent problem and remains as the limiting factor in regeneration of conifers through somatic embryogenesis (Gupta and Grob, 1995). In the present study, germination of somatic embryos was found better in a low strength medium with low concentration of carbohydrates. Somatic embryos obtained from cultures of female gametophytes showed optimum germination of 56.66% in ¼Litvay's medium containing 1% sucrose. 50% germination of somatic embryos was recorded in case of cultures derived from zygotic embryos and sucrose was found superior to maltose. Germination of somatic embryos with both the types of

cultures mentioned above were found to be better when cultured directly on semi-solid germination media (Figure 23 and 25). However, our study revealed that, though germination percentage was low (20% and 6.66% with female gametophyte and zygotic embryo cultures respectively) but it was possible to achieve germination using liquid-*submerge culture method* (Figure 24 and 26). But the germinated embryos from submerged cultures showed thick, fleshy hypocotyls and could not be converted into emblings. No successful reports are available on somatic embryo maturation and germination in liquid medium and have been restricted to solid medium (Tautorus *et al.*, 1994) or to solid supports soaked with liquid medium (Attree *et al.*, 1994). But in *P. sitchensis*, somatic embryo maturation has been reported in both solid medium and submerged culture (Ingram and Mavituna, 2000). Tremblay (1990) reported germination of *P. glauca* somatic embryos on ¼SH medium. Bomal and Tremblay (1999) studied the effect of desiccation on germination and plantlet regeneration from black spruce (*P. mariana*). They reported 93.3-100% germination rate and 33.3-40% germinated plantlets with epicotyls in black spruce. Kim *et al.* (1999) reported germination of *L. leptolepis* somatic embryos on ½LM medium with about 2% sucrose. 72% of *P. sylvestris* and 80% of *P. pinaster* somatic embryo germination was reported by Lelu *et al.*

(1999). In the present study, somatic embryos germinated well in light (1900 lux) at 12h photoperiod (Table 66) and a maximum of 56.66% and 50% germination was recorded from female gametophyte and zygotic embryo explants respectively. The embryos of *P. abies* germinated best at 7days in the dark followed by continuous light and 80-90% germination was recorded (Gupta *et al.*, 1995b). Cryopreserved somatic embryos of *P. patula* were allowed to germinate in the dark before being placed in the light (Ford *et al.*, 2000). Ramarosandratana *et al.* (2001) obtained about 47.8% germination of somatic embryos in maritime pine with 2% sucrose under 16h photoperiod (80 $\mu E\ m^{-2}\ s^{-1}$).

In the present study, gradual decrease of humidity was found better in hardening of the emblings. When the emblings were hardened at higher RH value of 95±5%, there was about 95% survivability of emblings (Table 67) in the hardening experiment. When the emblings were not hardened gradually and RH value was 50±5%, the survivability declined to 15% in both female gametophyte and zygotic embryo derived emblings.

The substrate of vermiculite: peat: pumice (1: 1: 1) as well as soil obtained from pine forest were found equally suitable for transplantation of emblings. Transfer of emblings needed around 1½ months hardening treatment before exposure to natural condition.

In the present study, growth parameters of emblings and seedlings of same age group were evaluated. Measurements of shoot length and root length showed variations in their growth and emblings showed better growth and an increment of 1.4-fold and 1.13-fold in shoot and root length respectively in 180d old emblings over seedlings of same age (Figure 29). Similarly biomass evaluation of 40d, 90d, 180d old (i.e. DW of shoot and root) emblings and seedlings revealed significant difference in the growth performances. The comparative study showed that morphology and growth rates of emblings were better than the seedlings. 45d and 180d old emblings showed 1.14-fold and 1.3-fold increase in biomass respectively as compared to the seedlings of the same age groups (Figure 30). One of the possible reasons for better growth performance of emblings over seedlings could be the capture of '+' characters from the parent. Gupta *et al.* (1995b) reported normal range of growth rates and morphology of emblings of *P. menziesii*. Normal emblings with normal growth rates to that of seedling was also reported by Webster *et al.* (1990) in interior spruce. Morphological similarity of somatic embryo-derived plantlets and the control plantlets obtained from seed germination was reported in *P. glauca* (Tremblay, 1990).

In the present study, no significant difference was noted in the survivability performance of emblings to that of seedlings. The survivability of the emblings was about 73.53% with 45d old emblings and it was 50% with 180d old emblings (Figure 31). The survivability of 75% and 51% was recorded in 45d and 180d old seedlings respectively. The potted regenerants showed satisfactory growth and performance in the natural conditions (Figure 32). Plantlets from somatic embryos have been successfully established in soil for *P. menziesii* (Gupta and Durzan 1987a), *P. taeda* (Gupta and Durzan, 1987b), *P. abies* (von Arnold and Hakman, 1988), *L. decidua* x *L. leptolepis* (Klimaszewska, 1989), *P. glauca* (Tremblay, 1990), *P. abies* (Gupta *et al.*, 1991), *P. jezoensis* (Ishii, 1991), *L. occidentalis* (Thompson and von Aderkas, 1992), *P. glauca* (Dunstan *et al.*, 1993), *A. nordmanniana* (Norgaard, 1997), *P. strobus* (Garin *et al.*, 1998), *L. leptolepis* (Kim *et al.*, 1999), *P. sylvestris* and *P. pinaster* (Lelu *et al.*, 1999).

In the present study, the initiation, development, maturation and subsequent conversion of somatic embryos to emblings have been achieved using both semisolid and suspension culture methods. Emblings were established in the pots under natural conditions. Enhancement in number of somatic embryo production through suspension cultures opens up a great potential for large-scale

propagation of this species. Further studies are essential for better understanding of the developmental processes in order to ascertain the best media supplement and other factors to enhance the number of somatic embryo formation in suspension culture and bioreactors. Germination and conversion frequency of SEs to emblings and their successful establishment in soil needs to be worked out in detail in future studies.

Chapter 6

Summary

Pines constitute one of the most divergent and economically important groups of plants that provide valuable natural resources and contribute significantly to the economy and ecology of the country. *Pinus kesiya* (Khasi pine) is an important pine species, which yields timber and resin of commercial value that has multifarious uses in the industries. In the recent past, pine resources have dwindled considerably due to over-exploitation, shifting cultivation etc. Harvesting from naturally occurring stands with minimum conservation and reforestation has led to decline in pine forest cover. Forest regeneration after harvest is often left to natural processes, although prompt artificial regeneration provides the most effective means to increase forest yield. The major conventional methods of pine propagation include seeds and rooting of cuttings. Propagation through seeds and cuttings are beset with varied problems. The major problem, which limits application of rooted cuttings in plantation programmes, is the reduced growth and rootability potential of cuttings of mature Khasi pine.

To get a sustainable supply of Khasi pine, it is necessary to raise them on mass scale in plantations. Vegetative propagation as such is associated with various problems and there is a need to switch over to non-conventional methods, which hold potential of mass production in a short period of time. Somatic embryogenesis is one of the non-conventional methods that can be utilized successfully for this purpose. With this perspective, the present investigation was carried out to develop protocols for mass multiplication using various explants. The salient findings that come up during the course of study are summarised below.

Attempts were made to induce somatic embryogenesis from various explants viz., female gametophytes (with immature zygotic embryo), isolated mature zygotic embryos, mature secondary needles and apical dome sections. Somatic embryogenesis was successfully induced from immature and mature zygotic embryos, while other explants failed to show somatic embryogenesis and produced non-embryogenic callus.

The immature seeds containing zygotic embryos were collected from '+'trees marked by Department of Forest, Barapani, Govt. of Meghalaya over a period of 6 months (May-October) at 15day intervals. The immature zygotic embryos ranged from pre-cotyledonary to

cotyledonary stages and were categorized into stages 'a', 'b', 'c', 'd' and 'e' according to developmental stages of the embryos. The pre-cotyledonary stage embryos were too small to dissect out and also prone to desiccation during excision, so they were cultured along with the megagametophytes. The mature cotyledonary zygotic embryos were not prone to desiccation and easy to dissect out and cultured. Pre-cotyledonary stage and cotyledonary stage zygotic embryos were responsive towards somatic embryogenesis. Stage 'c' zygotic embryos were most responsive (35%) towards somatic embryogenesis. Induction frequency declined with stage 'a', 'b', 'd', 'e' and mature zygotic embryo.

The optimum embryogenic cultures (extrusion) of 35% resulted from stage 'c' embryos with in 4-6 weeks in ½Litvay's medium containing 2,4-D (5 mgl^{-1}), NAA and BAP (2.5 mgl^{-1} each) and sucrose (3%). The embryogenic response in BM_1 (30.68%), DCR (29%), mMS (12.38%) and Litvay's (11%) media were recorded in decreasing order. MS, ½MS, ½mMS, ½DCR and ½BM_1 showed very poor embryogenic response. Out of different carbohydrates tested, sucrose at 3% level was very effective in embryogenic culture production followed by maltose. Lactose and fructose were poor in embryogenic culture induction.

In case of mature zygotic embryos, use of mMS medium containing 2,4-D and NAA (5 mgl^{-1} each) and BAP (2.5 mgl^{-1}) and sucrose (3%) was optimum for initiation of embryogenic cultures (12.5%). Frequency of embryogenic culture initiation in various media containing 3% sucrose was found to be 10% in both DCR and BM_1, 9% in ½Litvay's, 6.3% in MS, 5.75% in ½BM_1, 5.7% in Litvay's, 3.4% in ½MS, 2.88% in ½mMS and 2.2% in ½DCR media. Use of sucrose at 3% level was most effective in initiation of embryogenic cultures (12.5%) form zygotic embryos followed by maltose (11.5%). Lactose and fructose containing medium did not show somatic embryogenesis. Maltose was found to delay the process of callusing by 7-10 days with female gametophyte as well as zygotic embryo explants.

With secondary needles and apical dome sections, no embryogenesis was observed, but high frequency of callus formation was exhibited by both the explants. In case of secondary needles, the use of MS medium containing 2,4-D and NAA (3 mgl^{-1} each) and BAP (1 mgl^{-1}) and sucrose (3%) was optimum for initiation of callus (75.5%). Frequency of callusing was 71.35% in maltose (3%) and 65% in lactose (3%) containing media. Fructose (21.5%) was very poor in initiation of callus. The callus induction frequency in various media with 3% sucrose was 71% in mMS, followed by 70.5% in ½MS, 51% in DCR, 46.11% in

½Litvay's), 44% in ½mMS, 41.55% in BM_1, 39.5% in Litvay's and 32.35% in ½BM_1. With apical dome sections, ½DCR medium supplemented with 2,4-D and NAA (5 mgl^{-1} each), BAP (2.5 mgl^{-1}) and sucrose (3%) resulted in optimum initiation of callus (81.5%) followed by DCR (62.8%), ½mMS (60%), BM_1 (44.1%), ½Litvay's (42%), mMS (40%), Litvay's (37%), ½MS (27.5%), MS (25.68%) and ½BM_1 (20%). Sucrose in ½DCR medium was most effective (81.5%) than maltose (75%) at 3% level. Lactose and fructose showed 40.5% and 31.5% callusing respectively in ½DCR medium.

The embryogenic calli (from female gametophytes and zygotic embryos) and non-embryogenic calli (from secondary needles and apical dome sections) multiplied and proliferated well on respective initiation medium but containing 1/10th and 0 growth regulators to that of the initiation medium along with other adjutants. The calli from female gametophytes and zygotic embryos showed SEs at different developmental stages (pro-embryos and stage-I embryos).

About 2 g inoculum ml^{-50} medium was found to be optimum for initiation of suspension cultures using same basal medium and growth regulator concentrations as used in callus initiation medium. 3% sucrose showed healthy growth of embryogenic suspension cultures. Maltose at 3% level resulted in slightly retarded growth. However, browning of

cultures was comparatively lesser in maltose containing medium than sucrose. Lactose and fructose (3%) showed browning of cultures. Suspension cultures of secondary needles and apical dome sections showed presence of healthy spherical and elongated cells in sucrose and maltose (3% singly) containing medium but no pro-embryos were recorded. Lactose or fructose at 3% level showed rapid browning of cultures.

Concentration of growth regulators used in suspension cultures was same as that of semisolid initiation medium with different explants. In case of cultures obtained from female gametophytes, ½Litvay's medium containing 2,4-D (5 mgl^{-1}), NAA and BAP (each at 2.5 mgl^{-1}) and 3% sucrose was found optimum for initiation of embryogenic suspensions. Suspension cultures from the calli of zygotic embryos showed optimum growth in mMS medium with 2,4-D and NAA (5 mgl^{-1} each) and BAP (2.5 mgl^{-1}) and 3% sucrose. Cultures from secondary needles exhibited optimum growth in MS liquid medium containing 2,4-D and NAA (3 mgl^{-1} each) and BAP (1 mgl^{-1}). In case of apical dome sections, suspension cultures were best initiated in ½DCR medium containing 2,4-D and NAA (5 mgl^{-1}) and BAP (2.5 mgl^{-1}) with other supplements.

In the establishment of embryogenic suspensions, 10 gl^{-1} inoculum density resulted in poor culture growth but 40 gl^{-1} inoculum density was optimum with different explant sources. In the establishment of suspension cultures, the growth of cultures was directly affected by the SCV. Optimum growth and proliferation of embryogenic cultures in established suspensions was recorded with 10% SCV. Decrease in SCV level below 10% resulted in slow and poor growth and increase in SCV level beyond 10% resulted in slow growth and browning of cultures with all the explant sources.

The established embryogenic and non-embryogenic suspensions were best maintained in 3% sucrose containing media with out growth regulators. Embryogenic cultures from female gametophytes exhibited 1.5-fold increment in growth regulator free maintenance medium but containing 3% sucrose. Growth of embryogenic cultures was less in maltose containing medium as compared to sucrose at same concentration and it showed 1.28-fold increase in FW. Cultures from zygotic embryos showed 1.3-fold increase in the medium devoid of growth regulators (0 concentration) but containing 3% sucrose. Though maintenance was optimum in 0 and $1/10^{th}$ growth regulator containing medium, but no somatic embryogenesis was recorded in cultures obtained from secondary needles and apical dome sections.

The embryogenic cultures containing stage-I somatic embryos did not mature into stage-III (cotyledonary) somatic embryos unless cultured in ABA containing medium and higher level of carbohydrates. With female gametophyte and zygotic embryo cultures, optimum stage-III embryo formation was found in respective maturation media containing 8 mgl^{-1} ABA and 3% each of sucrose and mannitol. Sucrose at 6% level singly with ABA (8 mgl^{-1}) was not as effective as that of sucrose-mannitol combination. In the present investigation, out of three different culture techniques employed in maturation of somatic embryos, *submerge culture method* was found optimum in maturation of somatic embryos. However, no somatic embryo formation was observed with cultures obtained from secondary needles and apical dome sections. In BCBs, though stage-I somatic embryos were formed from the cultures of female gametophytes and zygotic embryos but further development and maturation into stage-III cotyledonary somatic embryos was not achieved in all the combinations of carbohydrate and ABA tested.

Growth parameter studies revealed better growth of cultures obtained from female gametophytes and number of somatic embryo formed ml^{-1} suspension was 150 in the medium containing 3% each of

sucrose and mannitol as compared to 35 SE from zygotic embryo cultures under same set of conditions.

Different culture techniques showed different degree of SE formation. 150 SE formation was obtained with female gametophyte cultures using *submerge culture method* while same method resulted in 35 SE formation form cultures of zygotic embryo. SE formation was lowest with cultures from both female gametophytes (15 SE) and zygotic embryos (8 SE) using *direct culture method. In filter paper base method*, embryo formation was slightly higher. Female gametophyte cultures produced 20 SEs and zygotic embryo cultures formed 10 SEs in the above culture method.

A comparative study of semisolid and suspension culture showed a marked difference in number of SE formation. In semisolid culture a maximum of 15 SE g^{-1} callus was obtained from both female gametophyte and zygotic embryo calli. Under same set of conditions, number of SE formation ml^{-1} suspension was 150 and 35 with female gametophyte and zygotic embryo suspension cultures, respectively.

The microscopic observation of the embryogenic cultures showed the presence of somatic embryos at different developmental stages. Proembryos with dense embryonal head and suspensors of one to four elongated cells or dense head with single suspensor cell was observed.

In the initial and established cultures, somatic embryos with fused heads or fused suspensors were observed. With subsequent culturing these fused parts cleaved into many embryos connected to a common suspensor. Presence of cleavage polyembryony provides an important method of multiplication of somatic embryos. In the established cultures and maturation media, cultures were seen to have SEs at advance stages of development. Stage-II (bullet shaped) SEs sharing a common suspensor like cells developed into stage-III (cotyledonary) SEs in the respective maturation media showing the complete process of somatic embryogenesis. In some of the cultures, mature cotyledonary SEs were observed to retain the common suspensor connections between two or more SEs. The non-embryogenic cultures showed the presence of mixture of spherical, oblong and elongated suspensor like cells but no somatic embryos were seen to develop.

In germination of SEs, low concentrations of inorganic nutrients and carbohydrates were found effective as compared to initiation medium. ¼Litvay's medium and ½mMS medium along with 1% sucrose resulted in 56.66% and 50% germination of SEs from female gametophyte and zygotic embryo cultures, respectively. Maltose at 1% level was found less effective and resulted in 46.66% and 40%

germination under similar set of conditions from female gametophyte and zygotic embryo cultures, respectively.

In germination of SEs, *direct culture method* was superior to *submerge culture method.* Optimum germination of 56.66% was found with female gametophytes when cultured on ¼Litvay's medium containing 1% sucrose and solidified with 0.8% Difco-bacto agar. A germination of only 20% was recorded when cultured in ¼Litvay's medium containing 1% sucrose using *submerge culture method.* With zygotic embryo cultures, an optimum germination response of 50% was recorded using *direct culture method* in ½mMS medium containing 1.0% sucrose and 0.8% Difco-bacto agar and it was recorded to be 6.66% using *submerge culture method.* Out of different light intensities and photoperiods tested, 1900 lux and 12h photoperiod showed optimum germination.

Hardening of emblings was optimum when humidity was decreased gradually from 95±5%, than low humidity treatment of 50±5%. A survivability rate of 95% was achieved in the hardening experiments when emblings are hardened gradually at 95±5% RH.

In growth performance study of regenerants, 45d and 180d old emblings showed 1.14-fold and 1.3-fold increase in biomass respectively as compared to the seedlings of the same age groups. The

study of biomass showed significant difference between the embling and seedling growth performance. Emblings showed better biomass accumulation than seedlings over a period of 180 days growth. The survivability of the emblings was 73.53% with 45d old emblings and it was 75% with the seedlings of the same age group. Survivability was recorded to be 50% and 51% with 180d old emblings and seedlings respectively and the comparative study showed no significant difference in the survivability pattern. The potted regenerants showed satisfactory growth and performance in the natural condition.

Chapter 7

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Educational qualification

Exam	Board/Univ.	Div./Class	Year	Rank
Ph.D.	NEHU (to submit shortly)	-	2002	-
M.Sc.	NEHU	I	1994	**1st (Gold medal)**
B.Sc. (Hons.)	NEHU	I	1992	**1st**
PU.Sc.	NEHU	II	1987	-
HSLC	MBOSE	II	1984	-
Ph. D.	NEHU Thesis submitted		2002 December	

Title of Ph. D. thesis

- "Development of an embryogenic system for mass propagation of *Pinus kesiya* Royle. ex. Gord. and field performance of the regenerants"

Specialization

- **Plant Biotechnology**

Research experience

- Six years research experience in Plant Biotechnology
- Four years experience (w.e.f. 1st March 1997- 31st Oct. 2001) as JRF in DBT sponsored project entitled " **Development of an embryogenic system for genetic improvement and mass propagation of *Pinus kesiya*"**

Teaching experience

- Lecturer, Department of Botany, St. Edmund's College, Shillong (w.e.f 1st September 2001- Till date)
- Ad-hoc Lecturer, Dept. of Botany, Sankardev College, Shillong (w.e.f 1st July 2000-31st August 2001)

Scholarship/Fellowship

- Junior Research Fellowship from DBT (1997- 2001)
- NEHU Merit Scholarship 1992-1994

Awards

- **NEHU Gold Medal (M. Sc. Botany) 1994**
- **NEHU Merit Prize** for securing **First Class First Rank in Botany (Hons)** in the **Degree Examination of B.Sc. 1992**
- **Talents Club Award** for securing **First Rank** in B.Sc. (Hons.) Botany in the Final Degree Examination of NEHU 1991-1992

- **T. C. Dutta Award for the highest marks in Botany** secured by a **First Class (Hons.)** student in the **Degree Examination of B.Sc. 1992**

- **St. Anthony's College Award** for the highest marks in the college secured by a Second Class (Pass) student in the **Degree Examination of Bachelor of Sciences 1991**

Extra and co-curricular activity

- Participated in the Three Days "Workshop on Ecology and Environment" conducted by All India Association for Christian Higher Education and the National Council of Y. M. C. As of India,North East Region held at Aizawl from 16-18 September 1991

- Participated in the "17th Annual Science Talent Competition (Meghalaya Science Society)" and bagged **Second Prize** in the Group-G Chart Category held from 2-6 September 1991

- Participated in M. Y. W. O. organized Flood Relief Run '91 of St. Anthony's College held on 9 August 1991 and secured runners position

- Participated in the "16th Annual Science Talent Competition (Meghalaya Science Society)" and bagged **Special Prize** in the Group-D Chart (Life Science) Category held at St. Edmund's College on 23-25 August 1990

- Participated in NSS (1984)

Conf/Workshop/Training Attended

- 72nd Annual Session of the National Academy of Sciences & National Symposium on 'Biodiversity: A Scientific Approach- *Agenda for the 21st Century*' organized by North Eastern Hill University. October 25-27, 2002, North Eastern Hill University, Shillong

- Training Course on "Basic Computer Concepts and Applications" conducted by the Bioinformatics Centre. June 12-14, 2000, North-Eastern Hill University, Shillong

- "Application of Radioisotopes and Radiation Technology" organized by National Association For Application of Radio Isotope & Radiation in Industry (NAARRI) Mumbai & Regional Sophisticated Instrumentation Centre (RSIC) Shillong. November 26-27, 1999, Shillong

- 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, Banaras Hindu University. October 23-25, 1998, North-Eastern Hill University, Shillong

- International Geosphere Biosphere Programme (IGBP) sponsored "Workshop on Agriculture, Biodiversity and Climate Change". Shillong. March 9-10, 1998, North-Eastern Hill University, Shillong

- "National Symposium on Role of Plant Biochemistry and Biotechnology in Improving Crop Productivity". March 18-20, 1997. Sponsored by Society for Plant Biochemistry and Biotechnology, New Delhi & ICAR Research Complex for NEH Region, Barapani, Meghalaya

Publications

- Choudhury H and Tandon P. 2002. Induction of somatic embryogenesis in *Pinus kesiya* Royle ex. Gord. from female gametophyte and mature zygotic embryo. Plant Cell Reports (Communicated)

- Choudhury H and Tandon P. 2002. Effect of developmental stage of zygotic embryo and media composition on initiation of somatic embryogenesis in Khasi Pine (*Pinus kesiya* Royle ex. Gord.). Oral Presentation (Full paper). *In*: 72nd Annual Session of the National Academy of Sciences & National Symposium on 'Biodiversity: A Scientific Approach- *Agenda for the 21st Century*' organized by North Eastern Hill University, October 25-27, 2002. (Abstract p. 8-9, Section of Biological Sciences)

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