INDUCTION OF SOMATIC EMBRYOGENESIS IN *PINUS KESIYA* ROYLE EX. GORD

By

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CERTIFICATE

I, Chitta Ranjan Deb, hereby, declare that the subject matter of the thesis entitled "Induction of somatic embryogenesis in *Pinus kesiya* Royle ex. Gord." is the record of work done by me, that the contents of this 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 submitted by me for any research degree in any other University/Institute.

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

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Chapter - 1

Somatic embryogenesis in conifers – a review of current status

Both the biodiversity and the extent of world's forest resources are depleting at an alarming rate due to indiscriminate felling of forest trees for timber and ruthless commercial exploitation of other forest plants. In addition, the rapid and disastrous effects of diseases, pests and fires today may jeopardize the very existence of certain species. During the 20th century the human population has increased 3-fold and consumption rate of fossil fuel energy 12-fold and it seems that the carrying capacity of earth would saturate by the middle of the 21st century (Myers, 1990). The ever-increasing demand for wood for pulp, paper, timber and furniture industries has resulted in unimaginable shortfalls. To accommodate this demand, the forest productivity of our remaining lands will have to be increased, while other areas are set aside untouched (Gupta *et al.*, 1995a). Currently there are about 100 million hectares of industrial tree plantation world

wide, of which 70-80% comprises conifers, made up about equally of long-rotation and medium-rotation species. To meet the predicted requirements for industrial timber, the acreage might need to be doubled. It is likely that, a major proportion of this additional area will be located in the tropics, comprising tropical conifers, *Eucalyptus* and other tropical hard wood trees (Haines, 1993).

Traditionally, forests have been regenerated from seedlings derived from bulked seeds collected in nature, or more recently from seeds collected from randomly pollinated 'plus' ('+') trees. In most of these forests, there is a large variation in growth, form and vigour. Besides, there are forest tree species that are characterized by poor and irregular seed set, while in others the seed may be prone to genetic damage or rapid loss of viability. Clonal forestry has been practiced in many countries like Japan to establish 'Sugi' (*Cryptomeria japonica*) forests, in Germany and Sweden to establish Norway spruce (*Picea abies*) forests, in Brazil *Eucalyptus* and in New Zealand radiata pine (*Pinus radiata*) for centuries for commercial forestry (Ahuja, 1993).

By employing conventional methods of vegetative propagation, the number of plants that can be propagated from a tree species in a growing season often are relatively small. The limiting factors for a

mass scale utilization of selected materials are restricted availability of improved genotype as planting material and the available space. Furthermore, in many tree species, such as *Quercus, Fagus, Eucalyptus* and in most conifers, woody cuttings from mature trees are generally difficult to root or the rooting frequency may be rather low. In still others, such as European aspen (*Populous tremuloides*), root initials may be lacking and difficult to induce in the woody cuttings (Girouard, 1974; Libby, 1974; Hackett, 1988).

Today, trees of poor or heterogeneous quality and slow or unreliable propagation methods are no longer acceptable. There is an urgent need to make a broad and concerted effort in domesticating and propagating trees with superior wood quality, optimal stem form and uniformity, rapid growth rates, short-rotation and high production index (stem: total tree biomass), resistance to diseases and pests, the ability to adopt to new climates and extreme environmental variables (including pollution) and the ability to respond well to silviculture practices (Thorpe and Biondi, 1984). The biotechnological approaches offer opportunities not only for mass cloning of the selected genotypes round the year but also for the genetic modification of tree species. Serious consideration has been given to genetic improvement of forest

tree species in the past few decades. This involves hybridization between superior trees and establishment of seed orchards. In order to achieve genetic gains in forest tree species, it would be necessary to breed selected trees for at least a few generations, each requiring anywhere from 15 to 50 years. Regeneration through 'somatic embryogenesis' of trees with selected genotypes seem to offer a great potential in this regard. Somatic embryogenesis is the process of embryo formation from somatic cells to give rise to whole plant without fusion of gametes. This is different from zygotic embryogenesis where gametic fusion during sexual reproduction is an absolute requirement. The phenomenon of somatic embryogenesis was first reported in *Daucus carota* (Steward *et al.*, 1958; Reinert, 1958, 1959). Since then micropropagation by somatic embryogenesis is a promising avenue for clonal propagation in plants and numerous angiosperm species have been regenerated *in vitro* by this method, such as *Prunus persica* (Bhansali *et al.*, 1991), *Cyclamen persicum* (Kreuger *et al.*, 1995), *Mangifera indica* (Alfaro *et al.*, 1996), *Cymbopogon martinii* (Patnaik *et al.*, 1997), *Heracleum candicans* (Wakhlu and Sharma, 1998), *Panax ginseng* (Choi *et al.*, 1999), *Quercus robur* (Cuenca *et al.*, 1999), *Medicago truncatula* (das Neves

Table 1. The current status of somatic embryogenesis in conifers

Species	Explants	Response	Established in soil	References
Abies alba	Female gametophyte	SE	-	Schuller *et al.*, 1989
	Mature zygotic embryos	SE, PL	-	Hristoforoglu *et al.*, 1992
A. fraseri	Mature zygotic embryos	SE, PL	-	Guevin and Kirby, 1997
A. balsamea	Mature embryos	SE	-	Guevin *et al.*, 1992
A. fraseri	Mature zygotic embryos	SE	-	Gueven *et al.*, 1992
A. nordmanniana	Immature embryos	SE, PL	-	Norgaard and Krogstrup, 199
	Immature embryos	SE, PL	Yes	Norgaard, 1997
Agathis australis	Mature zygotic embryos	SE, PL	-	Aitken-Christie *et al.*, 1992
Larix decidua	Female gametophyte	SE	-	Von Aderkas *et al.*, 1987
	Female gametophyte	SE, PL	-	Von Aderkas and Bonga, 1988; Nagmani and Bonga, 1985
	Female gametophyte	SE	-	Von Aderkas *et al.*, 1990
	Immature embryos	SE	-	Von Aderkas *et al.*, 1990
L. occidentalis	Immature embryos	SE, PL	Yes	Thompson and Von Aderkas, 1992
L. decidua x L. leptolepis	Immature embryos	SE	-	Von Aderkas *et al.*, 1990
	Immature embryos	SE, PL	Yes	Klimaszewska, 1989
	Female gametophyte	SE	-	Von Aderkas *et al.*, 1990
L. leptolepis x L. decidua	Immature embryos	SE, PL	Yes	Klimaszewska, 1989
	Female gametophyte	SE	-	Simola and Santanen, 1990
L. leptolepis	Immature embryos	SE	-	Von Aderkas *et al.*, 1990
	Female gametophyte	SE	-	Von Aderkas *et al.*, 1990
	Immature embryos	SE, PL	Yes	Kim *et al.*, 1999
Picea abies	Immature embryos	SE, PL	Yes	Chalupa, 1985
	Immature embryos	SE	-	Hakman *et al.*, 1985
	Immature embryos	SE, PL	-	Hakman and Von Arnold, 1985
	Immature embryos	SE, PL	-	Becwar *et al.*, 1987
	Immature embryos	SE, PL	Yes	Von Arnold and Hakman, 1988
	Mature embryos	SE, PL	Yes	Chalupa, 1985
	Mature embryos	SE, PL	-	Gupta and Durzan, 1986a
	Mature embryos	SE, PL	Yes	Von Arnold and Hakman, 1988
	Mature embryos	SE, PL	-	Verhagen and Wann, 1989

Contd...

Species	Explants	Response	Established in soil	References
	Mature embryos	SE, PL	Yes	Gupta *et al.*, 1991
P. abies	Cotyledons	SE, PL	-	Krogstrup, 1986; Lelu *et al.*, 1987, 1990
	Needles (one year old embling)	SE	-	Ruaud *et al.*, 1992
P. jezoensis	Mature embryos	SE, PL	Yes	Ishii, 1991
Picea glauca	Immature embryos	SE, PL	Yes	Attree *et al.*, 1990a; Dunstan *et al.*, 1993
		SE, PL	-	Lu and Thorpe, 1987
	Mature embryos	SE, Pl	Yes	Tremblay, 1990
	Cotyledons	SE	-	Attree *et al.*, 1990b; Lelu and Borman, 1990
P. omorika	Mature embryos	SE, PL	Yes	Budimir and Vujicic, 1992
P. pungens	Mature embryos	SE, PL	Yes	Afele *et al.*, 1992
P. glauca x	Immature embryos	SE	-	Roberts *et al.*, 1989
P. engelmannii	Immature embryos	SE, PL	-	Webb *et al.*, 1989
complex	Immature embryos	SE, PL	Yes	Roberts *et al.*, 1990; Webster *et al.*, 1990
	Cotyledons	SE	-	Eastman *et al.*, 1991
P. mariana	Immature embryos	SE, PL	-	Hakman and Fowke, 1987a
	Mature embryos	SE	-	Tautorus *et al.*, 1990
	Immature embryos	SE	-	Tautorus *et al.*, 1990
	Mature embryos	SE, PL	Yes	Attree *et al.*, 1990b
P. ruben	Mature embryos	SE, PL	Yes	Tremblay and Tremblay, 1991; Harry and Thorpe, 1991
P. sitchensis	Immature embryos	SE, PL	Yes	Krogstrup *et al.*, 1988
	Immature embryos	SE, Pl	Yes	Roberts, 1991
	Mature embryos	SE, PL	Yes	Krogstrup, 1990
P. wilsonii	Immature embryos	SE, PL	-	Ying-Hong and Zhong-Shen, 1990
Pinus caribaea	Female gametophyte	SE, PL	-	Laine and David, 1990
Pinus elliottii	Immature embryos	SE	-	Jain *et al.*, 1989
P. lambertiana	Immature embryos	SE, PL	-	Gupta and Durzan, 1986a
P. nigra	Immature and mature embryos	SE	-	Salajova and Salaj, 1992

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Species	Explants	Response	Established in soil	References
P. taeda	Female gametophyte	SE, PL	Yes	Gupta and Durzan,
P. taeda	Female gametophyte	SE	-	Becwar *et al.*, 1990
	Immature embryos	SE, PL	Yes	Gupta and Pullman, 1990
P. radiata	Immature embryos	SE, PL	Yes	Aitken-Christie *et al.*, 1996
P. serotina	Female gametophyte	SE	-	Becwar *et al.*, 1988
P. strobus	Female gametophyte	SE	-	Finer *et al.*, 1989
	Immature embryos	SE	-	Finer *et al.*, 1989
P. sylvestris	Immature embryos	SE, PL	Yes	Keinonen *et al.*, 1996
P. palustris	Immature embryos	SE	-	Nagmani *et al.*, 1993
	Female gametophyte	SE	-	Nagmani *et al.*, 1993
Pseudotsuga menziesii	Immature embryos	SE, PL	Yes	Durzan and Gupta, 1987
	Mature embryos	SE, PL	-	Durzan and Gupta, 1987
	Mature embryos	SE, PL	Yes	Gupta and Pullman, 1991
	Immature embryos	SE, PL	Yes	Gupta and Pullman, 1991
Sequoia sempervirens	Mature embryos	SE, PL	-	Bourgkard and Favre, 1988
	Cotyledons	SE	-	Bourgkard and Favre, 1988

SE – Somatic embryos; PL – Plantlets

et al., 1999), *Vitis vinifera* (Jayasankar *et al.*, 1999), *Chamomilla recutia* (Kintzios and Michaclakis, 1999).

Somatic embryogenesis in conifers was first documented from immature zygotic embryos of *P. abies* (Hakman *et al.*, 1985; Chalupa, 1985). In the same year somatic embryogenesis was established from the female gametophyte of *Larix decidua* (Nagmani and Bonga, 1985) which resulted in the production of haploid embryos. Since then several papers have been published on successful regeneration of conifer plantlets via somatic embryogenesis (Attree and Fowke, 1993; Dunstan *et al.*, 1993; Gupta and Grob, 1995; Gupta *et al.*, 1993, 1995a, 1995b; Jain *et al.*, 1995; Keinonen *et al.*, 1996; Li and Huang, 1996; Guevin and Kirby, 1997; Barrett *et al.*, 1997; Li *et al.*, 1998; Kim *et al.*, 1999). The current status of conifer somatic embryogenesis is shown in table 1.

Somatic embryogenesis may be direct, when the embryos develop directly from the somatic or vegetative cells of the explant without an intervening callus phase or it may be indirect, when the embryos develop from an undifferentiated mass of cells. Embryogenesis can be induced very easily in some cells like the cells of zygotic embryos and these 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 as embryos only when they are subjected to major manipulations. These cells are the induced embryonic determined cells or potentially competent cells for example, the cells of the explants in culture. There are again, cells, which are so well differentiated that any efforts to induce embryogenesis in them meet with failure. These are the non-competent cells. Just as zygotic embryos, the somatic embryos also develop in a bipolar fashion. Hence, a somatic embryo should ultimately possess both a root and a shoot meristem and be capable of forming a complete plant. Somatic embryos develop from embryogenically competent somatic cell *in vitro*. In contrast to organogenesis, where shoots and roots differentiate sequentially on different media, somatic embryogenesis is a one step process. That is not to say that, one does not need different media for the induction, developmental and maturation phases of somatic embryos *in vitro*. Rather, the developmental genetic programme in the embryogenically competent somatic cells, under the influence of specific phytohormones, proceeds in the same or closely similar fashion as it would in a zygote for the development of a zygotic embryo. In *P.*

glauca and most of the conifer somatic embryos closely resemble developing zygotic embryos (Attree and Fowke, 1991). But, somatic embryos are not very precisely organized like zygotic embryos. The suspensor region may consist of loosely associated cells, and many embryos may share a common suspensor system (Hakman and Fowke, 1987b). Somatic embryogenesis proceeds directly or indirectly after exposure of responsive explants to critical concentrations of exogenously supplied plant growth regulators during the initial culture phase (Gupta and Grob, 1995).

Somatic embryogenesis offers a tremendous potential for an elegant system for large-scale propagation of superior genotypes. It potentially provides many production advantages:

i) a large number of plantlets can be produced inexpensively,

ii) both root and shoot meristem development occur in one step process,

iii) quick and easy scale-up can be achieved via suspension culture,

iv) long- term germplasm storage via cryopreservation can be achived,

v) manufactured seeds or a direct delivery system can be used for emblings (plantlets regenerated from somatic embryos), and

vi) genetic gains of forest trees can be captured through somatic embryogenesis.

The commercial importance of conifers for their wood and wood products has drawn considerable attention of plant biotechnologists to evolve means for rapid clonal propagation of selected genotypes. In contrast to the angiosperm trees, conifers have received lesser attention as far as their *in vitro* propagation is concerned (Kumar and Tandon, 1994, 1995). The potential of somatic embryogenesis as a method for rapid *in vitro* multiplication of conifers has been emphasized by many workers (Thorpe and Biondi, 1984; Dunstan, 1988; Wann, 1988; Gupta *et al.*, 1991, 1995a, 1995b; Bonga *et al.*, 1995; Gupta and Grob, 1995; Attree *et al.*, 1995; Barrett *et al.*, 1997; Guevin and Kirby, 1997; Rajbhandari and Stimp, 1997; Find *et al.*, 1998; Kim *et al.*, 1999). Considerable efforts have been directed towards somatic embryogenesis of conifers for many years. Studies have been conducted on the growth, metabolism and developmental patterns that characterize callus and cell suspensions of conifers (Durzan and Steward, 1968; Durzan *et al.*, 1976; Durzan, 1980). Plant regeneration by somatic embryogenesis in coniferous species also provides an ideal *in vitro* system for basic studies of plant cell biology

Figure 1. Schematic diagram of zygotic embryogenesis from free nuclear to mature seed embryo in pinaceae (modified after Buchholz and Steimert, 1945 and Dogra, 1967)

A-C. Free nuclear proembryos

D. Cellular proembryos with primary upper tier (pU) and primary embryonal tier (pE).

E. Cellular proembryos with upper tier of 4 cells (U4), suspensor tier of 4 cells (S4), substitute suspensor segments of 4 cells ($E_1$4) and embryonal cells (E4)

F-I. Different successive developmental stages of pre-cotyledonary embryo formation in cleavage and non-cleavage types, represented by *Pinus* and *Picea*, respectively. (Note the proximal cells (E_t), in between embryonal cells (e′ for *Pinus* and e for *Picea*) and suspensor (S) and its additional tiers of cells (E'_1, E''_2, E'_3 for *Pinus* and E_1, E_2, E_3, E_4 for *Picea*)

J. Pre-cotyledonary embryo with an apical dome

K-L. Cotyledonary embryos

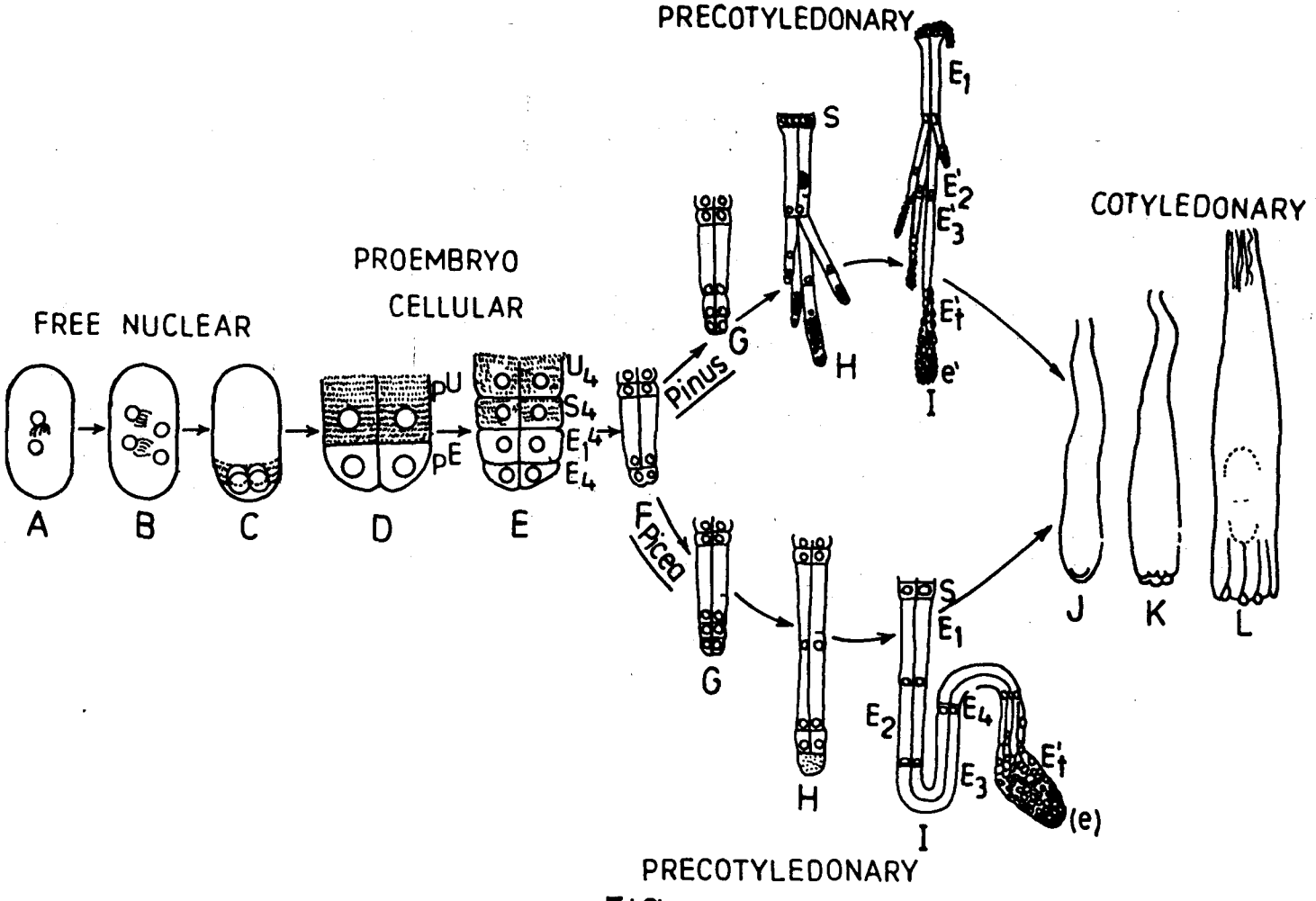

PRECOTYLEDONARY
COTYLEDONARY
PROEMBRYO
CELLULAR
FREE NUCLEAR
Pinus
Picea
PRECOTYLEDONARY
A
B
C
D
E
F
G
H
I
J
K
L
pU
pE
(e)

Figure 1

and embryo development. Somatic embryogenesis is important in conifer biotechnology for two major reasons. Firstly, this system offers the capability to produce unlimited numbers of propagules by bioreactor, ultimately in the form of artificial seeds (Lulsdorf *et al.*, 1993; Attree *et al.*, 1994). In forestry, the production of manufactured/synthetic seeds round the year provides a complementary technology which will reduce risks related to seed orchards where seed production is limited and uncertain (Redenbaugh and Ruzin, 1989; Onishi *et al.*, 1994). Secondly, genetic transformation could be achieved by using embryogenic cultures (Kim *et al.*, 1999).

Polyembryogenesis is a common and natural phenomenon in conifers. The process of natural polyembryony in conifers is shown in figure 1. Most conifers undergo one of the two types of polyembryony, either simple or cleavage. Simple polyembryony is the fertilization of more than one egg per ovule and occurs in both *Picea* and *Pinus* (Singh, 1978). In simple polyembryony, each pro-embryo is a result of fertilization by gametes from separate pollen grains, and therefore every proembryo is genetically different. One pro-embryo usually dominates and continues development while the others abort.

In *Pinus* species it is cleavage polyembryogenesis. Cleavage embryos result from a separation of the apical-tier cells of an individual proembryo into four files of cells, each of which may develop into a separate embryo. The resulting embryos are therefore, genetically identical. One of these embryos becomes dominant and the other embryos usually cease development. Polyembryony is believed to be a primitive gymnosperm character and may be an effective means for elimination of unfit embryos (Buchholz, 1918). The mechanism by which the successful embryo inhibits the growth of other embryos is unknown, although factors such as mechanical, nutritional and growth inhibiting influences of the dominating embryo probably have some role to play (Dogra, 1967; Owens and Blake, 1985 ; Tautorus *et al.*, 1991; Von Aderkas *et al.*, 1991).

Somatic polyembryogenesis has been described in *P. abies* (Gupta and Durzan, 1986b; Dunstan *et al.*, 1995), *P. lambertiana* (Gupta and Durzan, 1986b), *P. taeda* (Gupta and Durzan, 1987), and *P. menziesii* (Durzan and Gupta, 1987). They termed the embryogenic tissues as 'embryonal suspensor masses' (ESMs) due to their high degree of organization. Most embryogenic cultures of conifers have been induced from the members of family pinaceae and are similar in

appearance. The exception is redwood (*Sequoia sempervirens*), which is a member of the family taxodiaceae. They have a distinctly different phenotype compared to callus of pinaceae (Attree and Fowke, 1991).

The ESMs are usually described as white, translucent appearances which when cultured on semi-solidified medium appear glisten due to the production of mucilage. They anatomically consist of early stage embryos, which have embryonal head and a suspensor system (Gupta and Durzan, 1987). On the other hand, non-embryogenic callus appears opaque, friable and green when exposed to light. This type of callus may or may not have an anatomical organization.

There are three different methods for initiation of embryogenic cultures in conifers: a) through the continuation of natural cleavage polyembryony of embryonal heads of explanted immature embryos (Durzan and Gupta, 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, 1987).

The female gametophyte may play an important role in the induction of ESMs. Initiation of embryogenic cultures from *Pinus* species has been most successful when intact female gametophytes were placed on initiation medium without excision of immature embryos. Embryogenic cultures were later extruded from the micropylar ends of the female gametophyte (Becwar *et al.*, 1990).

Mostly zygotic explants have so far been used to initiate the embryogenic cultures in conifers. These tissues include immature embryos dissected from seeds collected during the growing seasons, mature zygotic embryos dissected from stored seed and tissues from seedlings. In addition, megagametophytes of several species have been cultured with varying success. It is interesting that the same general principles and strategies that have been used successfully during the early 1980s for the initiation of somatic embryogenesis in recalcitrant cereals and grasses can also be applied to conifers. A key factor to many of the successes with the cereals and conifers has been the choice of explants. 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 1). *Pinus* is the largest and most important genus of conifers,

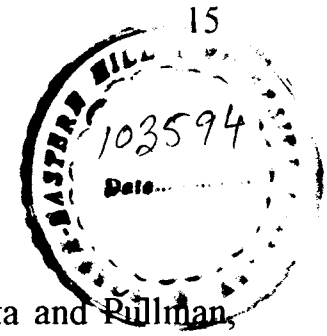
comprising approximately 95 species widely scattered over the Northern Hemisphere (Preston, 1989).

Use of zygotic explants offers several disadvantages like, heterogeneity due to open cross-pollination, which is the main limiting factor for multiplication of forest trees as it leads to the generation having inferior characters. The disadvantages associated with using zygotic explants could be overcome, if multiplication of forest trees is achieved by using vegetative tissues like, apical dome from mature *'elite'* trees, as the regenerants are uniform with *'elite'* characters as parents. All reports to date on initiation of embryogenic cultures of various conifers are restricted to immature zygotic embryos, mature zygotic embryos, cotyledons of germinated seedlings, buds, needles from 6-7 years old trees (Westcott, 1992) but there are no reports of induction of somatic embryogenesis from vegetative explants of mature conifer trees.

Despite growing practical experience, a lack of biochemical and physiological background knowledge prevents us from more deep understanding the events of somatic embryogenesis. These is almost unambiguous consent on the crucial role of plant growth regulators in the regulation of somatic embryogenesis. Plant growth regulators

control all the main developmental events in somatic embryogenesis from embryogenic culture induction to somatic embryo germination. Changes of plant growth regulators in the environment surrounding embryogenic culture are considered to be the most important trigger for accomplishment of the desired developmental steps. The experience has been gained with regard to the effect of growth regulators exogenously added to the media (Vagner *et al.*, 1998), while, little information is available so far about the levels of endogenous growth regulators during somatic embryogenesis in general in conifer species (Dunstan *et al.*, 1995).

For initiation of embryogenesis in conifers from different explant sources, several media have been used by the workers in various modified forms of basal media. Media requirements thus, for the initiation of embryogenic cultures do not appear to be very specific. Embryonal suspensor masses have been initiated on several media such as LV (Litvay *et al.*, 1981), DCR (Gupta and Durzan, 1985), DCR_1 (Becwar *et. al.*, 1995), modified Murashige and Skoog (mMS) (Gupta and Durzan, 1986b), BLG (Verhagen and Wann, 1989), P_6 (Gupta and Pullman, 1990), BM_1 (Gupta and Pullman,



1991), LP (Quoirin and Lepoivre, 1977), WTC (Gupta and Pullman, 1991) etc. In all these cases it has been found that the main modification appeared in nitrate salt concentration, especially of NH_4NO_3 and KNO_3. According to Barrett *et al.* (1997) removal of organic nitrogen sources is beneficial for *P. glauca.* It has been observed that low percentages of sucrose (1-2%) produced more ESMs (Von Arnold, 1987; Becwar *et al.*, 1988). Gelrite was found to be a better gelling agent when compared with agar for the initiation of ESMs for *P. strobus* (Finer *et al.*, 1989), while phytagel (gellan gum) was effective for *P. taeda* (Li *et al.*, 1998).

Amongst the different auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) has been the preferred auxin for the initiation of ESMs of most conifer species (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). However, no significant difference has yet been reported in ESMs proliferation or embryo development in conifers with NAA versus 2,4-D as the sole auxin source (Gupta and Grob, 1995). Li *et al.* (1998) induced somatic embryo in *P. taeda* and Kim *et al.* (1999) in *L. leptolepis* using 2,4-D

as sole auxin source. In most of the cases addition of either N^6- benzyl amino purine (BAP) or kinetin has proved to be beneficial. Initiation of ESMs from *Abies* species were best initiated with cytokinin (BAP or kinetin) alone. The incorporation of auxin was found to be inhibitory for the initiation of embryogenic cultures in *A. nordmanniana* (Norgaard and Krogstrup, 1991; Norgaard *et al.*, 1992).

Initiation of ESMs in culture has been achieved with different concentrations of hormones, such as 2,4-D (2-10 mg/l) and BA (0.5-5 mg/l) in *Larix* (Cornu and Geoffrion, 1990; Bonga *et al.*, 1995), NAA and BAP (2 mg/l each) and BA and thidiazuron (2.0 mg/l) in *A. fraseri* (Gueiven and Kirby, 1997), 2,4-D (10-110 mg/l) in *P. abies* (Gupta *et al.*, 1991), and NAA (5.0 mg/l) with BA and kinetin (55 mg/l each) (Becwar *et al.*, 1988), 2,4-D (2-10 mg/l) along with BA (0.5-2.5 mg/l) in *P. palustris* (Nagmani *et al.*, 1993). The pH of the medium is also crucial and in general pH value 5.5 to 6.0 proved to be effective. In most of the conifers the induction of somatic embryogenesis was accomplished in the dark. However, in Norway spruce somatic embryogenesis could be induced in the light (Verhagen and Wann, 1989).

Genetic markers are important tools for forest tree improvement. Isozyme markers have been applied extensively during the last 20 years (Neale *et al.*, 1992). Induction of somatic embryogenesis is influenced by various factors, like light, temperature, RH, pH of the medium, media composition, organic carbon source. Some enzymes like peroxidase, IAA oxidase, polyphenol oxidase and soluble proteins have been reported to play a significant role in initiation of somatic embryogenesis (Pitel *et al.*, 1992).

Since the first report of somatic embryogenesis with carrot, till date, the carrot system is the most comprehensively studied with respect to culture conditions and developmental physiology and biochemistry of somatic embryogenesis. Hence, it has been a useful model for the investigation of the mechanisms controlling somatic embryogenesis, embryo specific genes and proteins have extensively searched for and studied (Mc. William *et al.*, 1974; Nomura and Komamine, 1985; Roberts, 1991; Komamine *et al.*, 1992; Zimmerman, 1993).

Peroxidase activity in embryogenic cells and its role on induction of somatic embryogenesis has been studied by many

workers (Cordewener *et al.*, 1991; Zhou *et al.*, 1992; Rawal and Mehta, 1982; Jain *et al.*, 1990). Zhou *et al.* (1992) studied the correlation between peroxidase activity and somatic embryogenesis in *Lactuca sativa* and found that, the embryogenic calli were associated with the synthesis of specific iso-peroxidases. They also reported an increase in peroxidase activity prior to initiation of embryogenesis, which was not observed in case of non-embryogenic callus.

In zygotic embryo development, secretion of extracellular proteins has been considered a physiological event regulated by embryo specific genes. These extracellular proteins have various functions during embryo differentiation and development, such as metabolism, nutrient storage, phytohormone synthesis and transportation. There is increasing evidence to indicate that, genes, reflective of normal requirements of embryo development also developmentally regulate the secretion of extracellular proteins in vitro in embryogenic suspension cultures (Van Engelen *et al.*, 1991). Van Engelen *et al.* (1991) identified an extracellular protein which is only secreted by non-embryogenic cells, while Sterk *et al.* (1991) have

Figure 2 a. Distribution of *Pinus kesiya* in North-East India
b. A view of a pine forest.

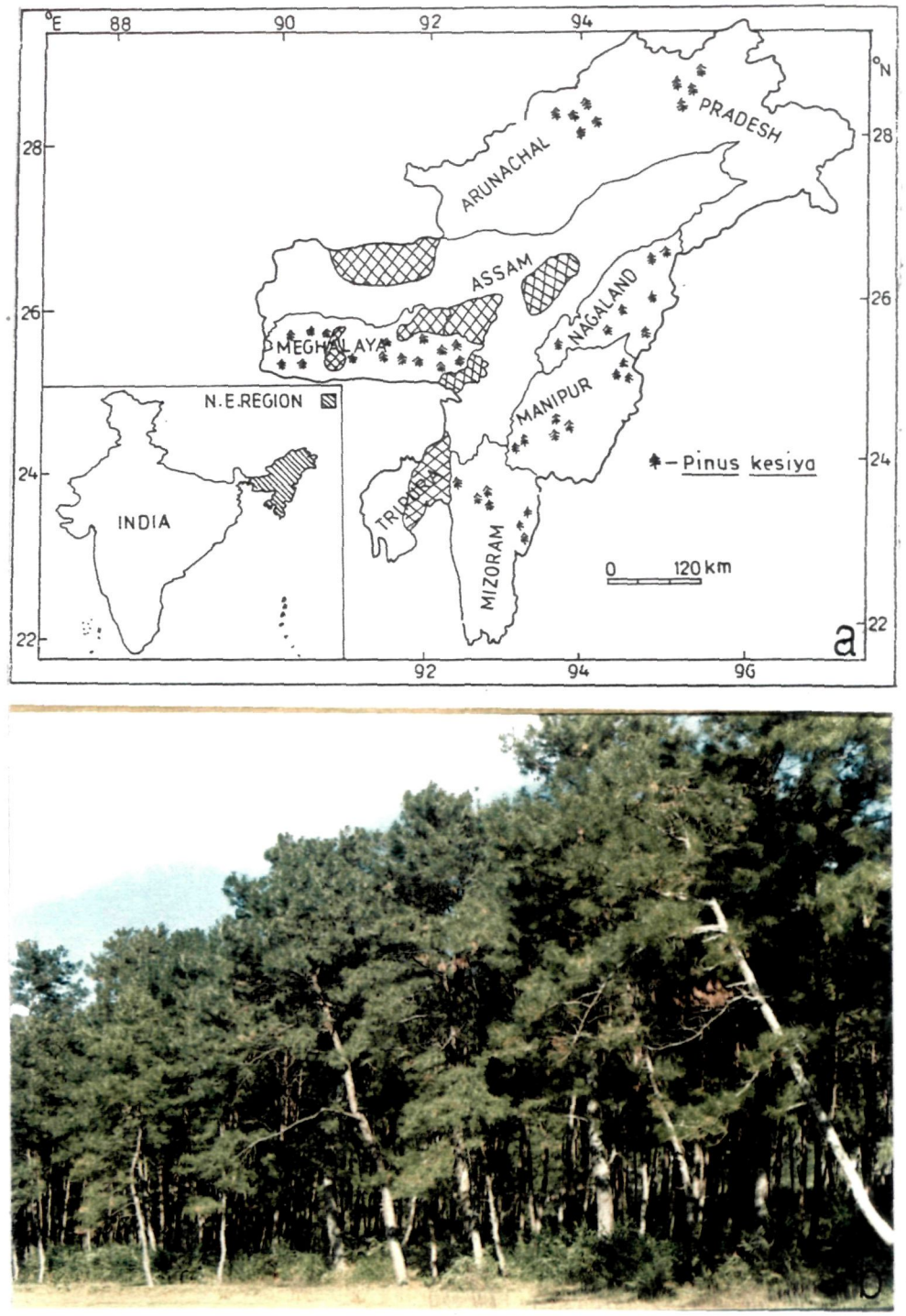

°E
88
90
92
94
°N
28
26
24
22
ARUNACHAL
PRADESH
ASSAM
NAGALAND
MEGHALAYA
MANIPUR
TRIPURA
MIZORAM
N.E.REGION
INDIA
Pinus kesiya
0 120 km
a
b

Figure 2

identified the second protein which acts as lipid transfer protein and is synthesized by only embryogenic cells and somatic embryos.

Egertsdotter *et al.* (1993) reported that the extracellular protein profiles of Norway spruce (*Picea abies*) embryogenic suspension cultures were different in those cultures which resulted in maturation of cotyledonary somatic embryos and those which were not capable of maturation. Donga and Dunstan (1994) observed that intracellular protein content is maximum during the early stage (at day 9) of culture and decreased at later stage of culture.

The North-East region of India accounts roughly 8.0% of the total geographical area of the country. The region lies between 21°51′N to 29°28′N latitudes and from 89°40′E to 97°25′E longitude and about half of the total geographical area of North-East India (about 22.5 million hectares) is under forests with valuable timber-yielding trees. The economy of the tribal people of the region is based mainly on agriculture and forests.

Pinus kesiya Royle ex. Gord. is an economically important early successional tree species which is predominant in the subtropics (800 - 2000 m) of North-East India (especially East Khasi Hills of Meghalaya, India) (Figure 2a), Myanmar (Burma) and Philippines. *P.*

kesiya is the major source of timber in Khasi hills of Meghalaya where it covers about 30% of the total forest area. Some genotypes have tremendous biomass potential and oleo-resin prospects. The wood of this tree is moderately hard, pale brown to red, very resinous and is used in the Khasi hills for building of houses. The resinous wood is also used for torch-wood, the production of which causes considerable injury to the trees. A hole is cut in the bole in order to induce the flow of resin, forming in time a large wound from which pieces of resinous wood are constantly removed until the tree is nearly cut through ultimately leading to its fall. Moreover, the other reason for the dwindling population of pine trees is deforestation as a result of slash and burn cultivation practiced in North-East India. A view of *P. kesiya* forest is shown in figure 2b.

The propagation of *P. kesiya* by conventional methods faces constraints mainly because the seed orchards show great variations and at times seed germination is very poor. A large number of seedlings are destroyed by fire, low winter temperature, heavy rainfall, cattle grazing etc. Weed infestation is another cause of seedling mortality. The vegetative propagation method used for its multiplication from economically favoured genotypes is rather

difficult as this pine reaches sexual maturity at an early stage after which rooting ability of cutting decreases resulting in poor regeneration. These problems indicate that, there is an urgent need for large-scale afforestation programme using cloning material. Although some efforts have been made to micropropagate *P. kesiya* (Nadgauda *et al.*, 1993; Kumar and Tandon, 1994, 1995), somatic embryogenesis in this conifer has not been attempted so far.

Keeping this in mind, it was decided to work on the induction of somatic embryogenesis in *Pinus kesiya* with the following objectives.

1. Initiation of somatic embryogenesis on semisolid medium from various explant sources like mature zygotic embryos, secondary needles and apical dome sections from mature '+' trees.
2. Optimization of physico-chemical factors controlling initiation, development and maturation of somatic embryos.
3. Establishment of an embryogenic suspension culture system using different explants.
4. Assay of peroxidase activity and total protein contents during induction of somatic embryos.

Chapter - 2

Materials and Methods

Conventional breeding of conifers is a long-term endeavour. Vegetative propagation would alleviate time constraints, but has a low success rate and is especially difficult with mature trees. However, it is the mature trees that are of most interest for establishing clonal orchards as desirable traits can be readily identified. *In vitro* culture techniques, particularly somatic embryogenesis, provide an alternative means to rapidly clone a large number of genetically identical plants with '*elite*' characters for direct use by forest nurseries in tree improvement programmes. Somatic embryogenesis is the development of embryos from somatic cells. Somatic embryogenesis in angiosperms has been studied for more than 40 years, whereas in conifers it was started 15-years back. Since the first report of conifer somatic embryogenesis, our knowledge and understanding pertaining to the factors controlling induction and development of somatic embryos has made remarkable progress (Attree *et al.*, 1995; Carrier *et*

al., 1997; Vagner *et al.*, 1998). Till date, the induction of somatic embryogenesis in conifers is restricted to explants like mature and immature zygotic embryos, cotyledons from germinating seeds, secondary needles, female gametophytes etc. Success in the field of somatic embryogenesis in conifers to a greater extent depends on the selection of right explant, its collection period, storage and priming, media composition, exogenous growth regulators and culture conditions.

2.1 Plant materials

Seeds: Mature cones of *Pinus kesiya* Royle ex. Gord. were collected from '+' trees identified by the Forest Department, Government of Meghalaya, during late January to March. Seeds were extracted by air-drying the cones at 25-30°C. The extracted seeds were stored at 4°C in sealed polythene bags till used.

Secondary needles: Young seedlings from field (after release of the first batch of secondary needles) were obtained round the year. The seedlings were also raised in the glasshouse [~20 seeds were placed equidistant from each other in an open plastic petridish (90 mm diameter) containing moist cotton pad] in normal day light and were harvested after release of the first batch of secondary needles (~5-6

weeks old). The seedlings collected from both field and glasshouse were placed in water containing 100 mg/l polyvinyl pyrollidone (PVP) and secondary needles were dissected out in this solution.

Apical domes: Shoot tips (2~3 cm) were collected prior to emergence of needles round the year from mature '+' trees. Immediately after harvesting they were transferred to anti-oxidant (200 mg/l PVP) solution to prevent exudation of phenolic compounds.

2.2 Sterilization of plant materials

Seeds: Stored seeds were surface cleansed with 5% (v/v) commercially available sodium hypochlorite (0.2% available chlorine) for 5 min and thereafter washed under running tap water for 15 min. They were further treated with 6% (v/v) hydrogen peroxide (H_2O_2) for 10 min and washed with sterilized pure water (from MiliQ water purification system) for 4-5 times. The treated seeds were stratified at 4°C for 24 h. Prior to dissection of embryos, the seeds were sterilized with mercuric chloride ($HgCl_2$) (0.5% w/v) with few drops of 'Tween-20' (as wetting agent) 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) for 5 min and thereafter washed under

* [equivalent to Cetrimide IP 20% (w/v) and Isopropyl alcohol IP (w/v), make-ICI]

running tap water for 15 min. These were sterilized with $HgCl_2$ (0.25% w/v) with few drops of 'Tween-20' for 3-5 min and washed 4-5 times with sterilized pure water. Finally the needles were treated with 70% ethanol for about 30 sec and washed with sterilized pure water before inoculation.

Apical dome: Young needles, scales etc. were removed from shoot tips and apical dome sections measuring about 1.0 cm were cut. These were surface cleansed with 'Labklin' (1.0% v/v) (commercial laboratory detergent) for 10 min and washed under running tap water for 20-30 min. They were sterilized with $HgCl_2$ (0.25% w/v) for 5 min and washed several times with sterilized pure water and finally treated with 70% ethanol for 1 min followed by washing with sterilized pure water.

2.3. Priming of plant materials

Zygotic embryos: To initiate the cultures from mature zygotic embryos, one set of sterilized seeds were stratified on moist filter paper (~60 seeds per 90 mm size petridish) and incubated at 4°C for 0-96 h. In another sets 30 seeds each were cultured on ½MS basal medium containing sucrose (2.0%) in 250 ml culture bottles at 25±2°C for 1 week.

Table 2. Composition of different media used for induction of somatic embryogenesis

Components	MS	mMS	DCR	LP
Inorganic salts (mg/l)				
NH_4NO_3	1650.00	550.00	400.00	400.00
KNO_3	1900.00	4460.00	340.00	1800.00
$Ca(NO_3)_2.4H_2O$	-	-	556.00	1200.00
H_3BO_3	6.20	6.20	6.20	6.20
KH_2PO_4	170.00	170.00	170.00	270.00
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
KI	0.90	0.90	0.83	0.08
$CoCl_2.6H_2O$	0.10	0.10	0.025	0.025
$CaCl_2.6H_2O$	440.00	440.00	85.00	-
$MgSO_4.7H_2O$	370.00	370.00	370.00	360.00
$MnSO_4.4H_2O$	22.30	22.30	22.30	1.00
$ZnSO_4.7H_2O$	9.00	9.00	8.60	8.60
$CuSO_4.5H_2O$	0.10	0.10	0.25	0.025
Na_2EDTA	37.00	37.00	37.30	40.00
$FeSO_4.7H_2O$	28.00	28.00	27.80	30.00
$NiCl_2$	-	-	0.025	-
Organic nutrients (mg/l)				
Thiamine HCl	1.00	1.00	1.00	1.00
Pyridoxine HCl	0.50	0.50	0.50	0.50
Nicotinic acid	0.50	0.50	0.50	0.50
Glycine	2.00	2.00	2.00	2.00
Meso-inositol	*	*	*	*
L-glutamine	*	*	*	*
Casein-hydrolysate	*	*	*	*
PVP	*	*	*	*
Citric acid	*	*	*	*
Sucrose	*	*	*	*
Gelling agents (g/l)				
Agar-Agar	7.00	7.00	7.00	7.00
PGRs	*	*	*	*
pH	5.5	5.5	5.5	5.5

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

Apical dome sections: Sterilized apical domes were cut into thin transverse sections (0.2–1.0 mm thick). 30 sections were cultured per 250 ml culture bottles on ½DCR medium containing sucrose (2.0%) and activated charcoal (0–1.0%) at 4°C and 25°C for 0–168 h in the dark.

2.4 Tissue culture

Media: For initiation of embryogenic cultures, various media like MS, ½MS, modified MS (mMS), ½mMS, DCR, ½DCR, LP and ½LP were used (Table 2). All the half strength media contained half of inorganic salts and full strength of organic nutrients. The mature zygotic embryos, secondary needles and apical dome sections were inoculated on all the above mentioned media, which were supplemented with casein-hydrolysate (CH) (0-1500 mg/l); myo-inositol (0-1500 mg/l); L-glutamine (0-1000 mg/l) and sucrose (0 to 3.0%, as organic carbon source) along with growth regulators as mentioned below. Polyvinyl pyrollidone and citric acid (at a range of 0-400 mg/l singly or in combination) were used to prevent browning.

For the culture of zygotic embryo, the media were supplemented with 2,4-D, NAA (each at 0–10.0 mg/l singly or in combination) and BAP (0–5.0 mg/l). In case of secondary needles, the

media were supplemented with 2,4-D, NAA (each at 0–5.0 mg/l singly or in combination) and BAP (0–5.0 mg/l). Apical dome sections were cultured in the media containing 2,4-D, NAA (each at 0-7.0 mg/l singly or in combination) and BAP (0–5.0 mg/l). For all the above mentioned explants another experiment was also carried out with BAP and kinetin (each at 0-5.0 mg/l singly or in combination) in the media devoid of any auxin.

Difco-bacto agar (0.7%) was added as gelling agent for preparation of semisolid medium. A range of pH (between 4.25 to 7.5 with an increment of 0.25 – the pH range at which medium solidified) was used to study its effect on initiation of embryogenic cultures. About 15 ml medium was dispensed in each test tube (size 150 mm x 25 mm) and 30 ml in a 250 ml culture bottle. 15 ml of liquid medium was dispensed in 50 ml conical flask. The media were autoclaved at 1.06 kg/cm^2 pressure for 15 min. About 20 ml of autoclaved medium was poured in each disposable petridish (size- 90-mm diameter, Tarson make). L-glutamine and abscisic acid (ABA) were filter sterilized and incorporated into medium after autoclaving using a laminar flow table.

2.4.1 Induction of somatic embryogenesis on semisolid medium

2.4.1.A Initiation of cultures

Zygotic embryos: The embryos were dissected out from stratified seeds using a laminar flow table and cultured on different media. Two embryos were cultured in each test tube. For each treatment 50 embryos were cultured and experiments were repeated thrice.

Secondary needles: Five numbers of sterilized needles were cultured in different media in each petridishes ensuring that whole needle surface is in full contact with the medium. The petridishes were sealed with parafilm (American National Can). For each treatment 50 needles were cultured and experiments were repeated thrice.

Apical dome sections: The primed sections were cultured on various media as mentioned above. Two sections were inoculated per test tube in up side up orientation. 50 explants were cultured for each treatment and the experiments were repeated thrice.

All the three types of explants above were grown in dark and in the light (20 and 1900 lux) at 12 h photoperiod at 25 ± 2°C. The zygotic embryos and needles (from optimum growth conditions) were subcultured after 2 weeks for 2-3 passages at 2 weeks interval. The

apical dome sections were subcultured for 2 passages and then cold-treated at 4°C for 0-72 h.

2.4.1.B Maintenance of cultures

The embryogenic cultures obtained from zygotic embryos, secondary needles and apical dome sections were transferred to respective initiation medium (mMS, MS and ½DCR) containing sucrose (2.0%) and reduced growth regulators (0, $1/5^{th}$ and $1/10^{th}$ of initiation medium). They were subcultured for 2-3 passages at 2 weeks interval for the formation of proembryonal masses (PEMs). Thereafter the cultures were transferred to respective initiation medium devoid of any growth regulators and subcultured twice at 2 weeks interval.

2.4.1.C Embryo development and maturation

Zygotic embryo and secondary needles: The cultures from zygotic embryos and secondary needles (as stated above) showing proembryos were transferred to respective media (mMS and MS) containing varying concentrations of sucrose (0-5.0%) and ABA (0-8.0 mg/l) singly or in combination and maintained for 3-4 passages at 2 weeks interval. The resulting cotyledonary embryos were transferred on mMS and MS media devoid of growth regulators, CH and L-glutamine but contained activated charcoal (0.0-0.6%), reduced myo-

inositol (100 mg/l) and sucrose (0-4.0%). The cultures were maintained at 12 h photoperiod of 1900 lux light intensity provided by cool white fluorescent tubes.

Apical dome sections: The cultures with globular embryos were cultured on ½DCR medium containing sucrose (0-5.0%) and ABA (0-8.0 mg/l) singly and or in combination for 3-4 passages at 2 weeks interval.

2.4.1.D Germination of somatic embryos

The embryos were singulated from the clumps. The cotyledonary embryos (developed from zygotic embryos and secondary needles) were transferred on respective media containing sucrose (3.0%), kinetin (0-7.0 mg/l) and NAA (0-3.0 mg/l) singly and or in combination. The cultures were maintained in 1900 lux light at 12 h photoperiod.

2.4.2 Induction of somatic embryogenesis in suspension cultures

2.4.2.A Initiation of cultures

The embryogenic cultures (white, soft, translucent and gelatinous) were pressed gently with a scalpel blade to form small pieces. About 50-300 mg inoculum was transferred to 50 ml conical flask containing 15 ml of respective media and shaken at 120 rpm on

an 'orbital shaker' (B. Braun, model-Certomat U) in the dark at 25±2°C unless mentioned otherwise.

Zygotic embryo: The embryogenic cultures raised on semisolid medium were transferred in liquid mMS medium containing sucrose (2.0%), 2,4-D, NAA (each at 0-10.0 mg/l singly or in combination) and BAP (0-5.0 mg/l) with other adjuvants as used in the initiation medium.

Secondary needles: The embryogenic cultures were transferred in MS liquid medium containing sucrose (2.0%), 2,4-D, NAA (each at 0-5.0 mg/l singly or in combination) and BAP (0-5.0 mg/l) with other adjuvants as included in the initiation medium.

Apical dome sections: The white and soft calli obtained from pre-treated apical dome sections at 4°C for 0-72 h and were chopped into very small pieces and cultured in liquid ½DCR medium containing sucrose (2.0%), 2,4-D and NAA (each at 0-7.0 mg/l singly or in combination) and BAP (0-5.0 mg/l) along with other adjuvants as used earlier.

The liquid media were replaced with fresh media at 6-7 day interval for 3-4 passages in case of cultures initiated from zygotic embryos and secondary needles. For the cultures initiated from apical

dome sections the media were replaced initially at 3-4 days interval for 2-3 passages and later at an interval of 6-7 days. The single cells thus obtained in culture were separated from mother tissues. The cultures obtained from zygotic embryo and secondary needle were diluted at 1:4 ratio at every subculture whereas, from apical dome sections at 1:3 initially for 2 passages followed by 1:5 ratio at later stages. For each treatment, five numbers of culture vessels were used and experiments were repeated thrice.

2.4.2.B Maintenance of cultures

The suspension cultures with elongated single cells obtained from various explants were transferred to respective basal media containing sucrose (2.0%) and reduced growth regulators (0, $1/5^{th}$ and $1/10^{th}$ of initiation medium) and maintained for 4-5 passages. Thereafter the cultures were grown in growth regulator-free liquid media for 3-4 passages at 100 rpm.

2.5 Assay of peroxidase activity and estimation of protein

Analytical procedures

Tissues: The tissues for analyses comprised of explants before culture and during the initiation of somatic embryogenesis. The cultured explants were transferred to fresh medium after 20 days of inoculation

and thereafter at every 14 days interval. The tissues were harvested at 5 days intervals.

Tissue extracts: About 150 mg tissue was homogenized in 5 ml chilled 0.2 M sodium phosphate buffer (pH 6.0) using a chilled tissue grinder. The homogenates were squeezed through cheese cloth and centrifuged at 5,000 g in a Sorval Refrigerated Centrifuge (Model-RC5C) for 30 min at 2-4°C. The supernatant was used for the assay.

Quantitative estimation

Peroxidase: Peroxidase (EC 1.11.1.7) activity was measured following Tandon and Arya (1982). The rate of decomposition of H_2O_2 by the enzyme with O-dianisidine as hydrogen donor was determined spectrophotometrically by measuring the rate of colour development at 460 nm.

To a 10 mm cuvette, 2.5 ml of 0.1 M phosphate buffer (pH 6.0), 0.1 ml of 1 mM H_2O_2 and 0.2 ml of enzyme solution were added. The absorbance was calibrated to zero at 460 nm and 0.2 ml of 2 mM O-dianisidine (dissolved in methanol) was added to the reaction mixture and mixed quickly. The absorbance was recorded at 20 sec interval for 3 min. The peroxidase activity is expressed as change in absorbance at 460 nm per min per mg protein.

Protein: Protein was estimated by the method of Lowry *et al.* (1951) from the same supernatants prepared for the assay of peroxidase. The protein was precipitated by adding 10.0% trichloroacetic acid to the tissue extract in a ratio of 1:1 (V/V), and centrifuged at 5,000 g for 10 min. The supernatant was discarded and the precipitate was washed twice with pure water and dissolved in 1.0 ml of 0.5 N NaOH. The volume of 0.2 ml of the extract was made to 1.5 ml with pure water. To this, 5.0 ml freshly prepared alkaline solution [1.0 ml of 1% copper sulphate and 1.0 ml of 2.7% sodium-potassium tartarate mixed with 100 ml of 2.0% sodium carbonate solution] was added by mixing and allowed to stand for 10 min at room temperature. After incubation, 0.5 ml Folin-Phenol reagent (equally diluted with water) was added with immediate shaking and held at room temperature for 30 min and the colour was read at 750 nm. Protein is expressed as mg/g fresh weight of tissue using a standard curve prepared with bovine serum albumin.

Chapter - 3

Results

3.1 Induction of somatic embryogenesis on semisolid medium

3.1.A Effect of priming of explants

a) Effect of seed stratification period

A 24 h stratification on moist filter papers at 4°C resulted in optimum induction of embryogenic cultures (Figure 3). With increase in this period a gradual decline in initiation of embryogenic cultures was observed. The control seeds showed a poor response of ~18.0%. The pre-germinated zygotic embryos were found to be suitable for initiation of embryogenic cultures, whereas, germinating zygotic embryos (1 week culture in the medium at 25°C) mostly resulted in poor embryogenic cultures.

b) Effect of pre-treatment of apical dome sections with activated charcoal and temperature

The pre-culture of apical dome sections at 4°C on ½DCR medium containing lower concentrations of activated charcoal helped

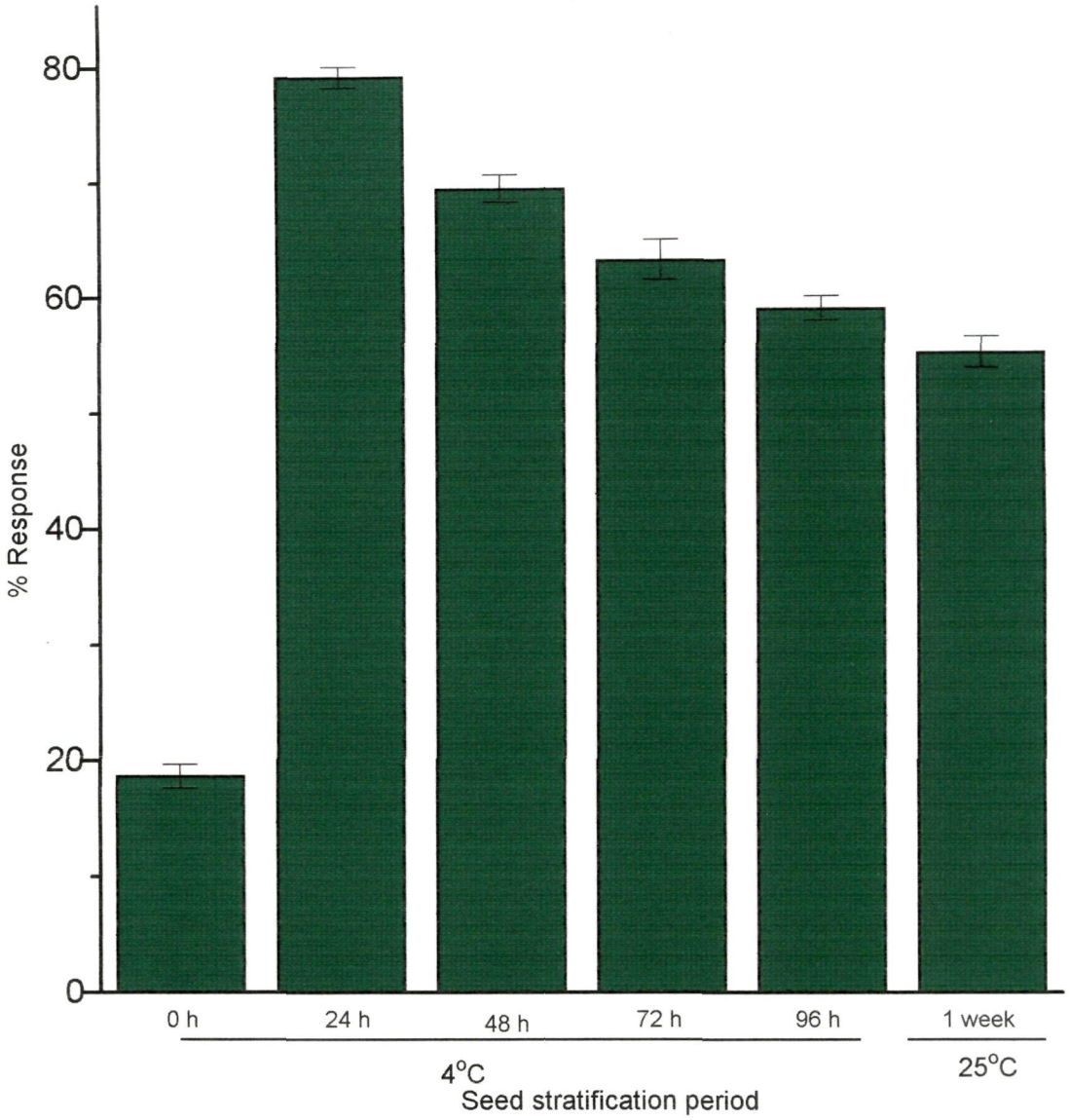


Figure 3. *Effect of seed stratification period on induction* of embryogenic cultures from mature zygotic embryos (details are provided in the materials and methods)

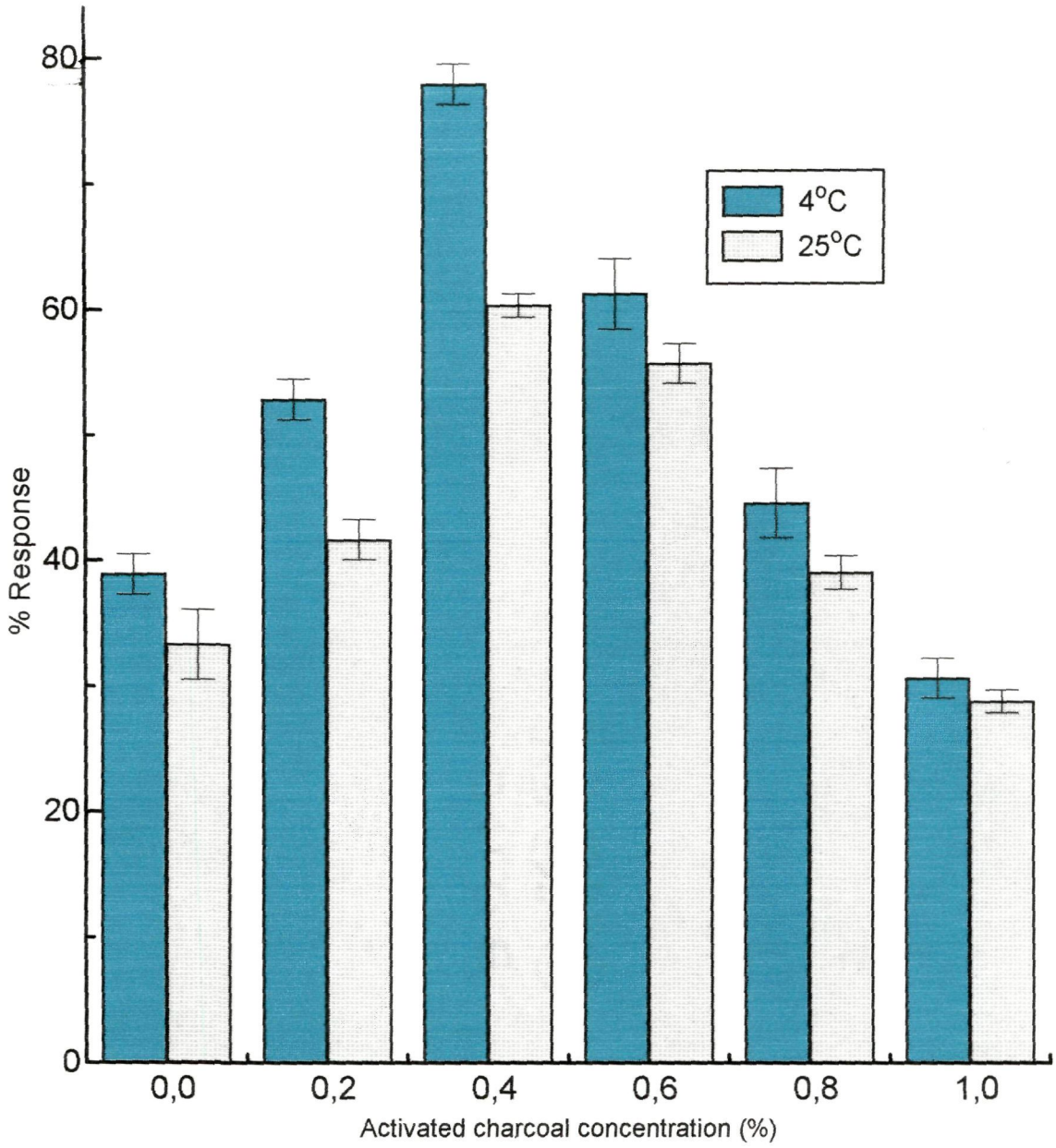


Figure 4. Effect of activated charcoal and temperature as priming factors on induction of embryogenic cultures from apical dome sections

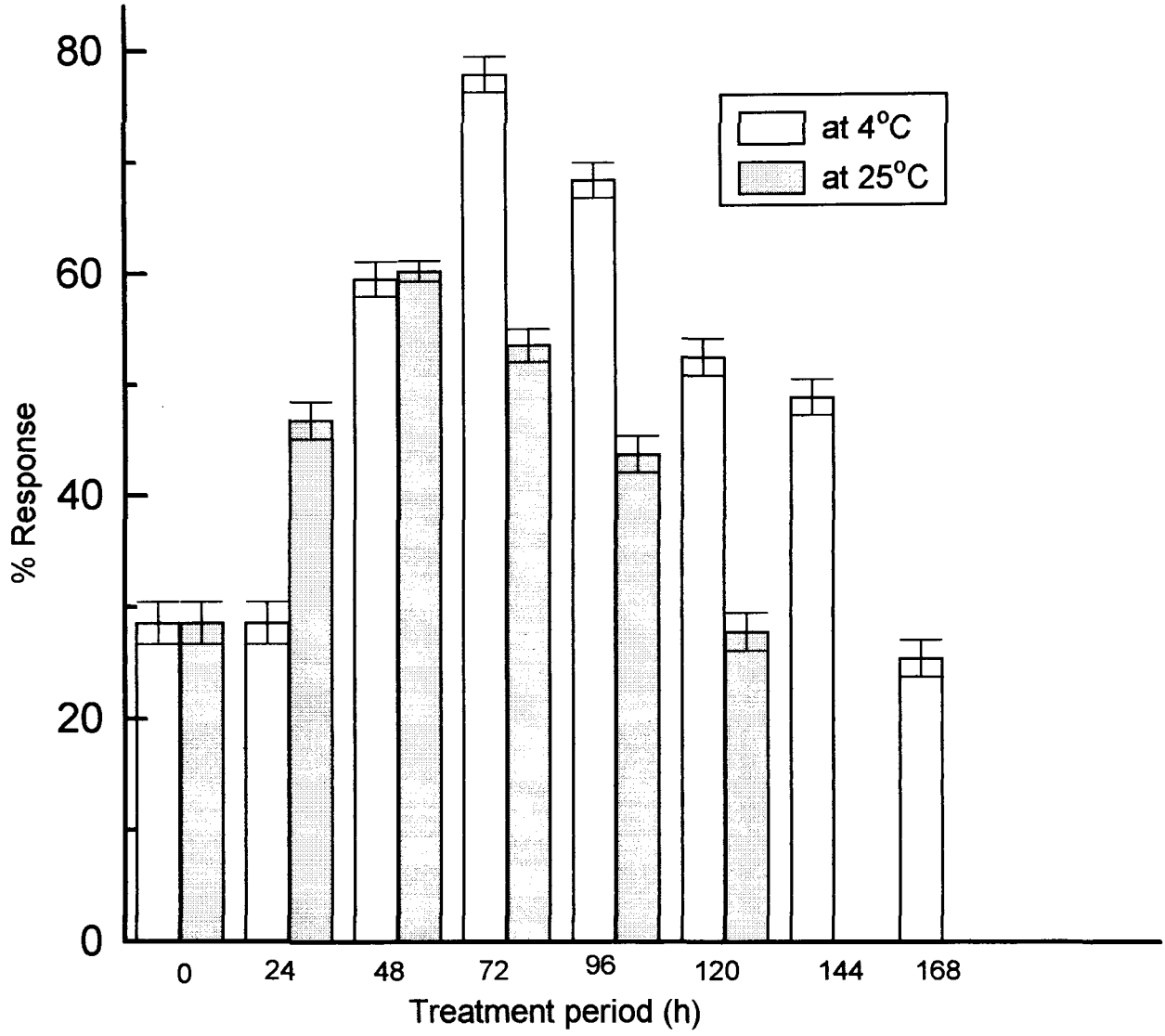


Figure 5. Effect of duration and temperature of pre-treatment with activated charcoal (0.4%) on induction of embryogenic cultures from apical dome sections

in induction of embryogenic cultures (Figure 4). As compared to control, a ~2 fold increase in the induction of embryogenic cultures grown at 4°C was recorded in the medium containing 0.4% activated charcoal. However, the response of explants cultured at 25°C was much lower. Hard calli resulted in the medium containing 0.2% activated charcoal. Increase in the concentration of activated charcoal beyond 0.4% resulted in a sharp decline in formation of embryogenic cultures and the cultures degenerated upon subculture. Even the lower temperature treatment could not make any difference in the embryogenic response in these cases. The cultures grown at 25°C exhibited browning and ultimately degenerated. It was noted that, use of thick sections (>0.5 mm) resulted in poor response and formation of hard calli.

With increase in duration of activated charcoal treatment upto 72 h, a marked increase in embryogenic culture formation was observed which subsequently declined (Figure 5). The promotive effect of low temperature culture at 4°C was exhibited from 72-144 h treatment. However, a temperature of 25°C was found to be better at 24 h of culture. Beyond 120 h at 25°C, the growth of the cultures was completely inhibited and the explants degenerated.

Table 3. Relationship between seed collection season and induction of embryogenic cultures

Month of collection	Type of response[@]	Performance*
January	Seeds collected during first half of the month formed very poor callus, but white and soft calli formed throughout the embryo from seeds collected during the end of the month	++
February	Very soft, white, translucent calli formed throughout the embryo from seeds collected during the entire month	+++
March	Seeds collected during this period formed very poor embryogenic calli and even the growth was very poor	+

@: on mMS medium containing sucrose (2.0%), 2,4-D and NAA (each at 5.0 mg/l), BAP (2.5 mg/l), CH and myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). *: +: poor response, ++: moderate response and +++: optimum response.

Table 4. Relationship between harvesting season of secondary needles from *in vivo* grown seedlings and induction of embryogenic cultures

Month of collection	Type of response@	Performance*
January	No response	-
February	Only swelling of explant	-
March	About half of the needles callused from the base, (calli were white and soft), other parts showed swelling only	++
April	White, soft translucent callus developed throughout the needle (callusing started from the base)	+++
May	-do-	+++
June	Basal part of the needle callused and the rest swelled	++
July	-do-	+
August	Callus induction was very poor and formed hard calli	+
September	No response	-
October	-do-	-
November	-do-	-
December	-do-	-

@ on MS medium containing sucrose (2.0%), 2,4-D and NAA (each at 3.0 mg/l), BAP (1.0 mg/l), CH and myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). *-: no response; +: poor response; ++: moderate response and +++: optimum response.

Table 5. Relationship between harvesting season of apical domes and induction of embryogenic cultures

Month of collection	Type of response[a]	Performance*
January	Hard and green calli were produced	+
February	Explants swelled and some of them produced white and hard calli	+
March	Calli were white but very few of them were soft and translucent	+
April	Calli were white, soft and moderately translucent	++
May	Calli were white, soft and translucent in about 90 % cultures	+++
June	-do-	+++
July	-do-	+++
August	Calli were white, most of them were hard and very few were soft	+
September	Hard and green calli were produced	+
October	-do-	+
November	-do-	+
December	-do-	+

a: on ½DCR medium containing sucrose (2.0%), 2,4-D and NAA (each at 5.0 mg/l), BAP (2.5 mg/l), CH and myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). * +: poor response; ++: moderate response and +++: optimum response.

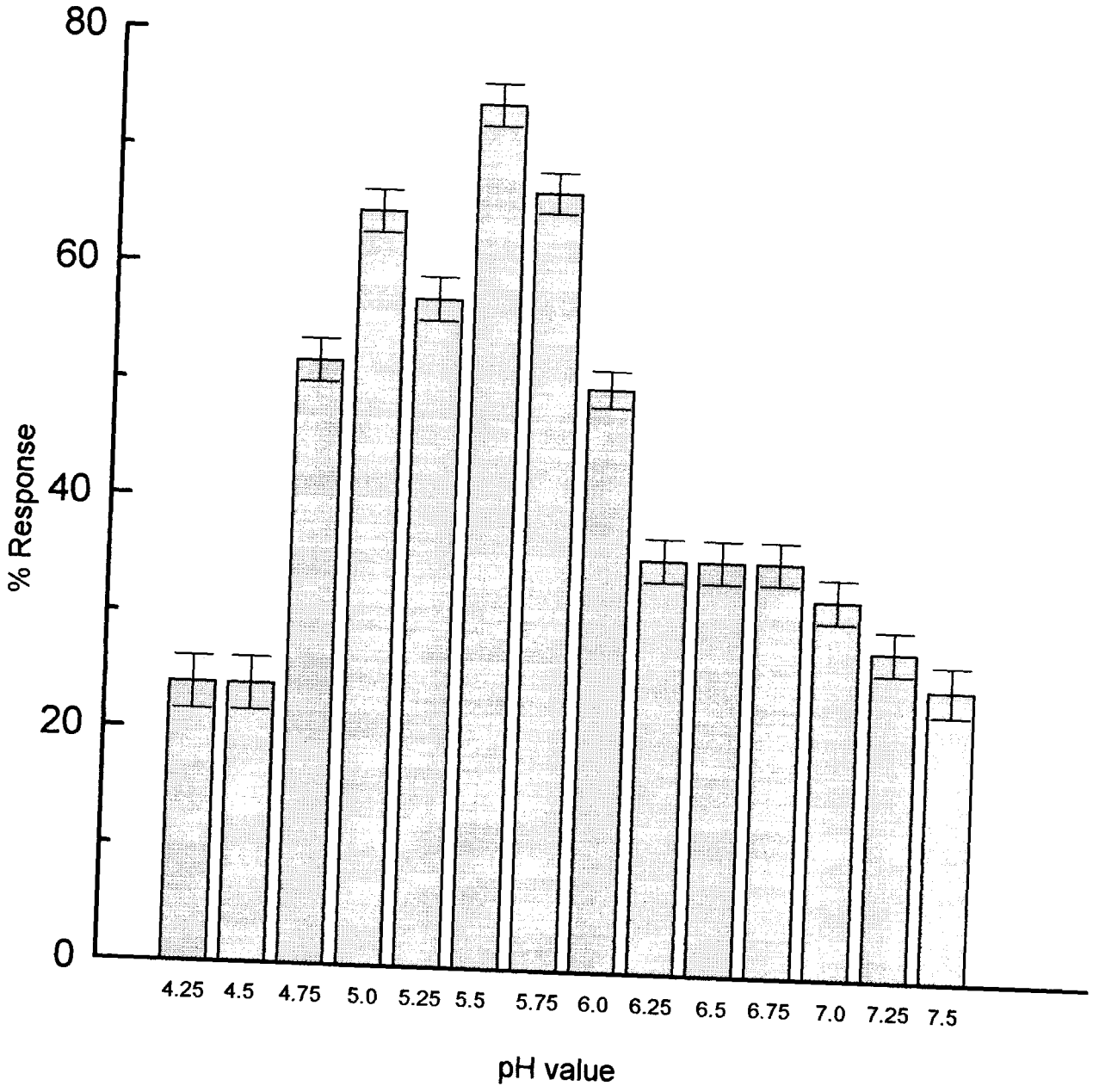


Figure 6. Effect of pH of mMS medium on initiation of embryogenic cultures from zygotic embryos

3.1.B Effect of collection period of explants

Seeds: Seeds collected during late January to late February were ideal, as this is the period, when mature seeds dehisced. The seeds collected during March exhibited poor embryogenic response (Table 3).

Secondary needles: The secondary needles collected during March to June produced more embryogenic cultures (Table 4). The optimum response was recorded in the explants collected during April and May. The whole needle surface exhibited white, soft and translucent callus.

Apical dome: Apical domes from '+' trees collected during May to July before emergence of the needles produced better embryogenic cultures (Table 5). About 90.0% of the cultures were white, soft and translucent. If the apical domes were collected after emergence of the needles, the response declined considerably.

3.1.C Culture media conditions for initiation of cultures

a) Effect of pH of the medium

The pH of mMS medium exhibited a pronounced effect on the induction of embryogenic cultures from zygotic embryo (Figure 6). The pH 4.25 and 4.5 in the lower range and 6.25 to 7.5 of the medium were not conducive for the formation of embryogenic cultures. With increase in pH upto 5.5 an increase in embryogenic culture formation

Table 6. Effect of anti-oxidants on prevention of browning of zygotic embryos and secondary needles and culture media

PVP conc. (mg/l)	Citric acid conc. (mg/l)	Responses@	Performance*
0	0	Prominent browning, poor callus development	-
100	0	Very little browning existed and moderately healthy calli were formed	++
200	0	No browning and very healthy calli were formed	+++
300	0	No browning existed but callus growth was slightly retarded	++
400	0	Callus growth inhibited	-
0	100	Very little browning existed but callus turned slightly brown	+
100	100	Browning of medium eliminated but callus turned slight brown	++
200	100	-do-	++
300	100	No browning of medium but cultures turned brown	-
400	100	No response	-
0	200	Very little browning of medium existed but callus turned slight brown	+
100	200	No browning of the medium but callus growth retarded	+
200	200	-do-	+
300	200	Cultures turned brown	-
400	200	-do-	-
0	300	No browning of medium but callus growth retarded	+
100	300	-do-	+
200	300	No response	-
300	300	-do-	-
400	300	-do-	-
0	400	Cultures turned brown	-
100	400	No response	-
200	400	-do-	-
300	400	-do-	-
400	400	-do-	-

@: on mMS (for zygotic embryos) and MS (for secondary needles) media containing sucrose (2.0%), 2,4-D and NAA (each at 5.0 mg/l for zygotic embryo and 3.0 mg/l each for secondary needles), BAP (2.5 mg/l for zygotic embryo and 1.0 mg/l for secondary needles), CH and myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). * -: very poor response; +: poor response; ++: moderate response and +++: optimum response

Table 7. Effect of anti-oxidants on prevention of browning of apical dome sections and culture medium

PVP conc. (mg/l)	Citric acid conc. (mg/l)	Response@	Performance*
0	0	Medium turned brown due to leaching and growth retarded	-
100	0	Slight reduction in browning	+
200	0	Very little browning existed, the cultures were healthy	++
300	0	-do-	++
400	0	Browning eliminated but growth inhibited	+
0	100	Medium turned brown	+
100	100	Browning moderately controlled	++
200	100	Browning completely eliminated, healthy growth of cultures	+++
300	100	Browning eliminated but growth retarded	++
400	100	-do-	+
0	200	Browning exists in the medium	+
100	200	-do-	+
200	200	Browning controlled completely but growth retarded	++
300	200	-do-	+
400	200	No browning but very poor growth	-
0	300	Little browning existed and growth was retarded	+
100	300	-do-	+
200	300	-do-	+
300	300	Browning controlled but growth inhibited	-
400	300	No response	-
0	400	Very poor response	-
100	400	No response	-
200	400	-do-	-
300	400	-do-	-
400	400	-do-	-

@: on ½DCR medium containing sucrose (2.0%), 2,4-D and NAA (each at 5.0 mg/l), BAP (2.5 mg/l), CH and myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). * -: very poor response; +: poor response; ++: moderate response and +++: optimum response.

Table 8. Effect of different media and sucrose concentrations on initiation of embryogenic cultures from mature zygotic embryos

Media*	Sucrose conc. (%)	Type of response	% response (±SE[1])	Performance*
½ MS	0.0	No response	0.0 (±0.0)	-
	1.0	Most part of the explant produced hard callus and very little white and soft callus was formed	18.5 (±1.85)	+
	2.0	-do-	29.6 (±1.85)	+
	3.0	As above but in subsequent sub-culture callus turned slightly brown	25.9 (±1.85)	+
MS	0.0	No response	0.0 (±0.0)	-
	1.0	Very little soft embryogenic callus and rest hard and green callus	20.4 (±1.85)	+
	2.0	-do-	25.0 (±1.85)	+
	3.0	-do-	24.1 (±1.85)	+
½ mMS	0.0	No response	0.0 (±0.0)	-
	1.0	White, soft, translucent and gelatinous callus formed but not very healthy	42.6 (±1.83)	++
	2.0	As above but comparatively healthy cultures	57.4 (±1.85)	+++
	3.0	White callus which in subsequent subculture turned slightly brown	48.1 (±0.70)	++
mMS	0.0	No response	0.0 (±0.0)	-
	1.0	White and soft embryogenic healthy callus was produced	71.3 (±2.44)	+++
	2.0	As above but growth very satisfactory	79.6 (±1.85)	++++
	3.0	Though initial calli were healthy but in subsequent subcultures turned brown	64.8 (±1.86)	+++
½ DCR	0.0	No response	0.0 (±0.0)	-
	1.0	Partially soft pinkish callus and rest hard and green	36.1 (±1.23)	++
	2.0	As above but comparatively better healthy callus produced	55.5 (±1.23)	+++
	3.0	As above but in subsequent subcultures callus turned brown	40.7 (±1.84)	++
DCR	0.0	No response	0.0 (±0.0)	-
	1.0	Soft & healthy callus but reddish white	41.0 (±1.36)	++
	2.0	As above but comparatively more white callus	57.4 (±1.85)	+++
	3.0	As above but in subsequent subcultures callus turned brown	38.1 (±1.85)	++
½ LP	0.0	No response	0.0 (±0.0)	-
	1.0	Very little soft callus & rest of the explant only swelled	17.8 (±2.07)	+
	2.0	-do-	24.2 (±1.85)	+
	3.0	Very poor callusing	18.5 (±1.84)	+
LP	0.0	No response	0.0 (±0.0)	-
	1.0	Moderately soft and white callusing	40.3 (±2.65)	++
	2.0	As above but healthy	51.8 (±1.83)	+++
	3.0	As above but in subsequent subcultures the calli tend to degenerate	42.6 (±1.84)	++

* Additional adjuvants: CH (1000 mg/l); myo-inositol (1000 mg/l); L-glutamine (500 mg/l); PVP (200 mg/l); 2,4-D and NAA (5.0 mg/l each) and BAP (2.5 mg/l). 1: standard error. *a* -: no response; +: poor response; ++: moderate response; +++: good response and ++++: optimum response.

Figure 7. Stages of induction of somatic embryos using zygotic embryo

a. Excised zygotic embryos before culture
b. Initiation of callusing at the base of the embryo (after 1 week)
c. Embryogenic culture (after 3 weeks)
d. Culture showing proembryonal masses and proembryos
e. Culture showing cotyledonary embryos
f. Culture showing 7-8 embryos clumped together with fused cotyledons

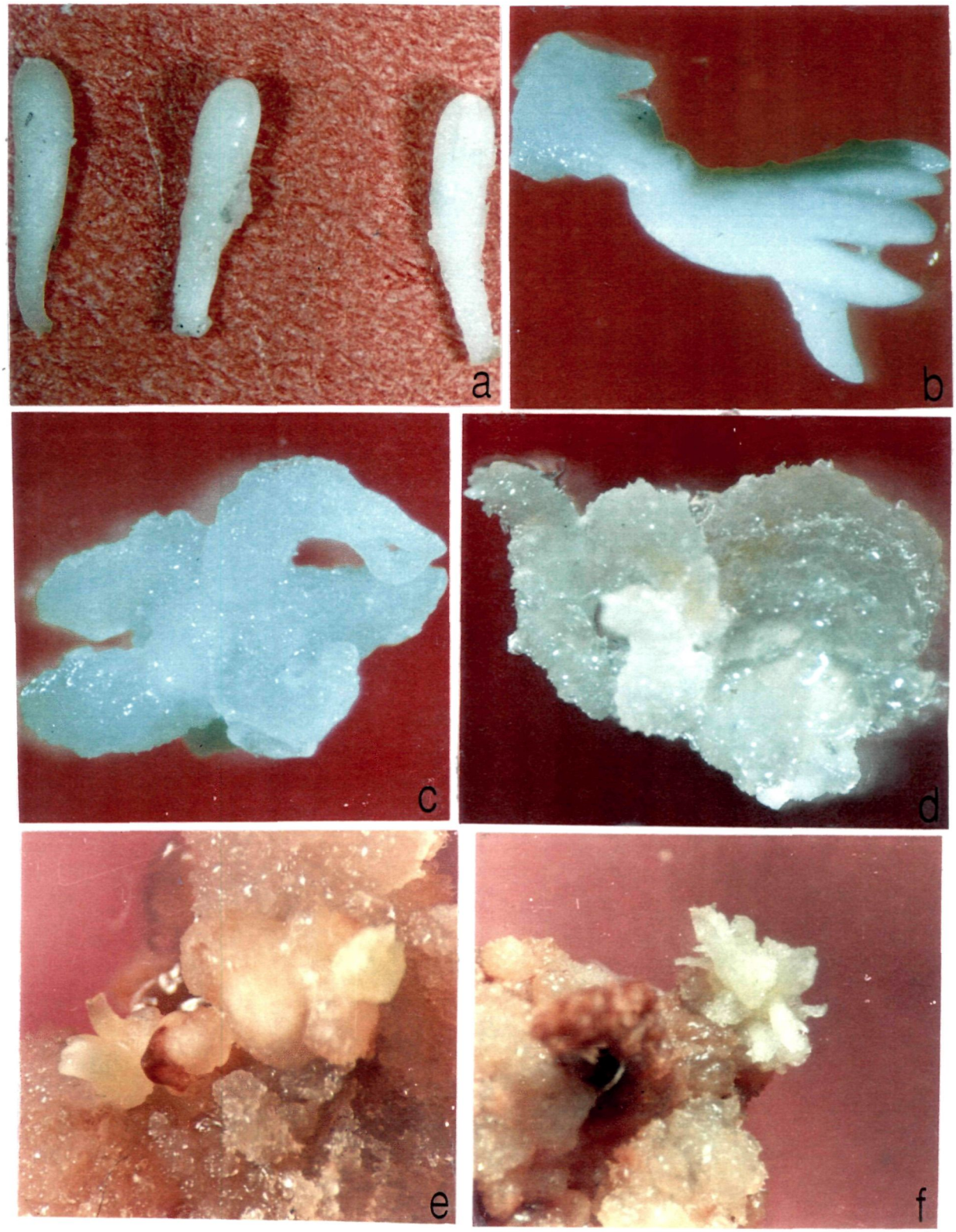


Figure 7

was recorded. The response exhibited by secondary needles and apical dome sections were similar to zygotic embryos.

b) Effect of anti-oxidants

The incorporation of PVP and citric acid helped in preventing the browning of both explants and media. In case of zygotic embryos and secondary needles PVP alone was found to be better than citric acid singly or in combination (Table 6). A concentration of 200 mg/l of PVP was found to be very effective in preventing browning and resulted in growth of healthy cultures. In case of apical dome sections the combined effect of PVP and citric acid was more pronounced than either of them used singly (Table 7). The combination of PVP (200 mg/l) and citric acid (100 mg/l) completely eliminated the browning of the medium and the healthy growth of the cultures resulted.

c) Effect of media

Zygotic embryos: Of the different media tested, the best embryogenic cultures resulted in mMS medium followed by ½mMS, DCR and ½DCR media (Table 8). The excised embryos (Figure 7a) started callusing at the base (Figure 7b) after 1 week of culture in mMS medium. Within 3 weeks, the zygotic embryos exhibited a) white, soft, translucent and gelatinous embryogenic (79.6%) (Figure 7c) and b)

Figure 8. Stages of induction of somatic embryos using secondary needles

a. Freshly harvested secondary needles
b. Needle showing embryogenic callus
c. Globular and cotyledonary embryos all over the callused part of needle

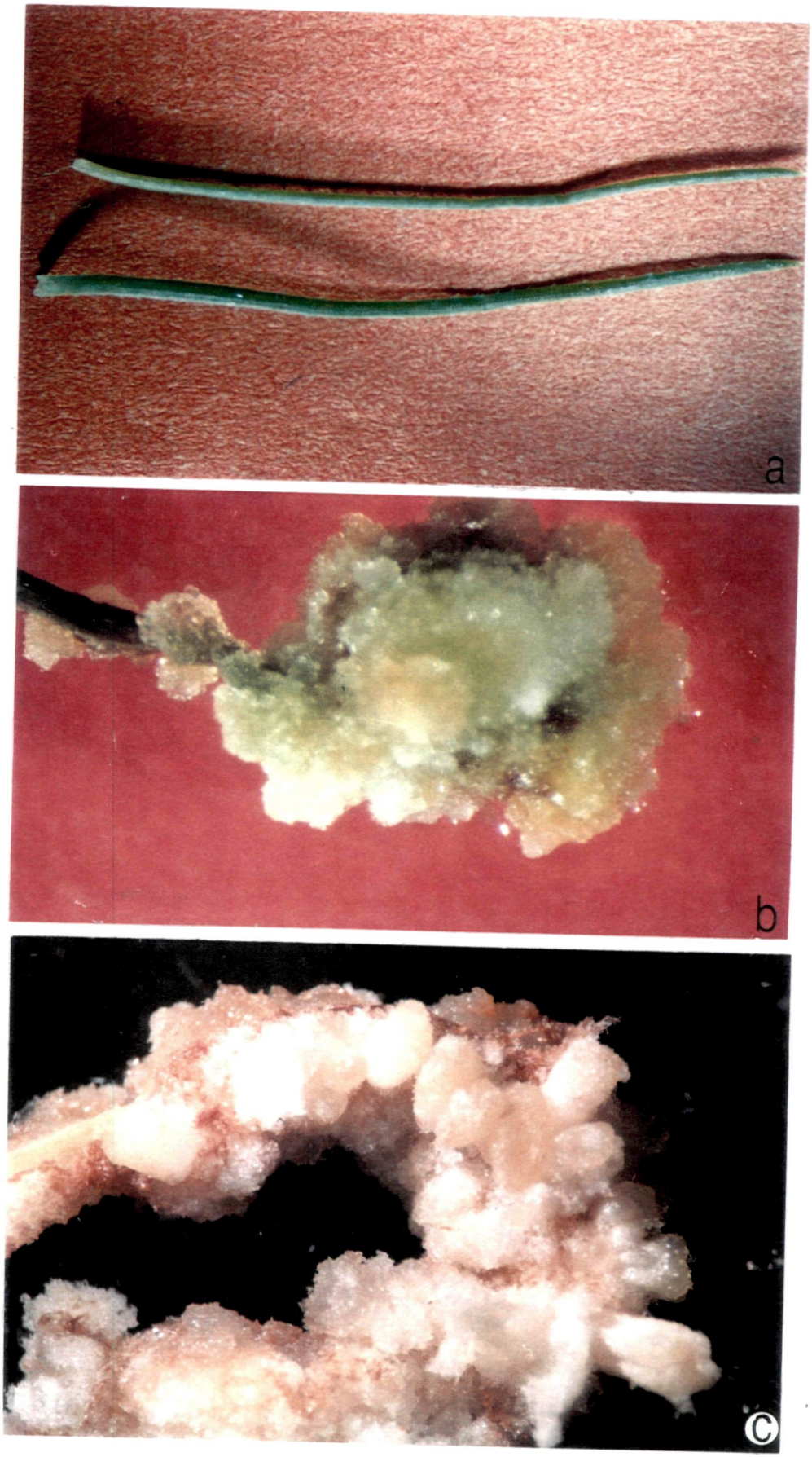


Figure 8

Table 9. Effect of different media and sucrose concentrations on initiation of embryogenic cultures from secondary needles

Media*	Sucrose conc. (%)	Type of response	% response (±SE[1])	Performance[a]
½ MS	0.0	No response	0.0 (±0.0)	-
	1.0	Soft callusing restricted only at the basal part of the needle	42.1 (±2.85)	++
	2.0	Few needles callused throughout and the rest only at the base	59.3 (±2.54)	+++
	3.0	As above	55.5 (±2.67)	+++
MS	0.0	No response	0.0 (±0.0)	-
	1.0	Moderately soft callus formed all along the needle	73.6 (±2.36)	+++
	2.0	Calli were soft, white and translucent and very healthy	88.6 (±2.82)	++++
	3.0	As above but slight browning of callus	80.0 (±2.88)	+++
½ mMS	0.0	No response	0.0 (±0.0)	-
	1.0	Soft callusing restricted only at the basal part of the needle	42.1 (±1.84)	++
	2.0	As above	43.6 (±2.60)	++
	3.0	-do-	41.4 (±2.10)	++
mMS	0.0	No response	0.0 (±0.0)	-
	1.0	Moderately healthy callus and few needles callused throughout the needles	55.0 (±2.67)	+++
	2.0	-do-	76.4 (±1.79)	+++
	3.0	Callus slightly brown	67.8 (±2.64)	+++
½ DCR	0.0	No response	0.0 (±0.0)	-
	1.0	Basal portion callused, very few needles formed callus all along	34.3 (±2.54)	++
	2.0	-do-	37.8 (±2.40)	++
	3.0	As above but slightly brown	32.1 (±2.64)	++
DCR	0.0	No response	0.0 (±0.0)	-
	1.0	Moderately soft and white callus formed. In some needles only basal portion callused	44.3 (±2.02)	++
	2.0	-do-	50.7 (±2.64)	++
	3.0	slightly browning of callus	50.6 (±2.02)	++
½ LP	0.0	No response	0.0 (±0.0)	-
	1.0	No response	0.0 (±0.0)	-
	2.0	Almost no response, only basal parts callused	10.0 (±2.18)	+
	3.0	-do-	10.7 (±1.30)	+
LP	0.0	No response	0.0 (±0.0)	-
	1.0	Only swelling observed	15.0 (±1.67)	+
	2.0	Very poor response and most of them swelled	20.0 (±1.88)	+
	3.0	-do-	20.7 (±2.02)	+

*Additional adjuvants: CH , myo-inositol (each at 1000 mg/l); L-glutamine (500 mg/l); 2,4-D and NAA (3.0 mg/l each) and BAP (1.0 mg/l).1: standard error; *a*: -: no response; +: poor response; ++: moderate response; +++: good response and ++++: optimum response.

Figure 9. Stages of induction of somatic embryos using apical dome sections

a. Primed section before culture
b. Soft and translucent embryogenic callus
c. White but hard non-embryogenic callus
d. Proliferated embryogenic callus with PEMs and proembryos after giving cold treatment to stage b
e. Callus became hard and turned green when culture exposed to light
f. Cultures with globular embryos

Figure 9

Table 10. Effect of different media and sucrose concentrations on initiation of embryogenic cultures from apical dome sections

Media*	Sucrose conc. (%)	Type of response	% response (±SE[1])	Performance[@]
½ MS	0.0	No response	0.0 (±0.0)	-
	1.0	Very poor callusing	11.1 (±1.0)	+
	2.0	-do-	29.5 (± 1.86)	+
	3.0	-do-	22.2 (±1.20)	+
MS	0.0	No response	0.0 (±0.0)	-
	1.0	Very poor callus and callus slightly brown	11.1 (±0.54)	+
	2.0	-do-	27.8 (±0.73)	+
	3.0	-do-	18.5 (±1.85)	+
½ mMS	0.0	No response	0.0 (±0.0)	-
	1.0	White callus but very poor growth	29.6 (±1.85)	+
	2.0	White and soft callus with moderate callus growth	59.3 (±1.85)	+++
	3.0	As above but in subsequent sub-cultures callus turned brown	57.4 (±1.86)	+++
mMS	0.0	No response	0.0 (±0.0)	-
	1.0	Poor callus growth	20.4 (±1.85)	+
	2.0	-do-	38.9 (±2.63)	++
	3.0	As above and turned brown in subsequent sub-cultures	22.2 (±2.72)	+
½ DCR	0.0	No response	0.0 (±0.0)	-
	1.0	Moderate soft callus but growth is not satisfactory	59.2 (±1.85)	+++
	2.0	Very soft and healthy callus	92.6 (±1.84)	++++
	3.0	As above but in subsequent sub-cultures callus turned slight brown	90.7 (±1.85)	++++
DCR	0.0	No response	0.0 (±0.0)	-
	1.0	Poor callus growth	33.3 (±2..02)	++
	2.0	Moderate callus growth and growth is triggered when medium is exhausted	64.8 (±1.85)	+++
	3.0	As above but after sub-cultures turned brown	51.8 (±1.85)	++
½ LP	0.0	No response	0.0 (±0.0)	-
	1.0	No response	0.0 (±0.0)	-
	2.0	Very poor callus formation	11.1 (±1.10)	+
	3.0	-do-	11.1 (±1.230)	+
LP	0.0	No response	0.0 (±0.0)	-
	1.0	-do-	11.1 (±1.0)	+
	2.0	Callus turned brown after one sub-culture	37.0 (±1.85)	++
	3.0	-do-	35.2 (±1.85)	++

*Additional adjuvants : CH, myo-inositol (each at 1000 mg/l), L-glutamine (500 mg/l), 2,4-D and NAA (each at 5.0 mg/l) and BAP (2.5 mg/l). 1: standard error; @: -: no response, +: poor response, ++: moderate response, +++: good response and ++++: optimum response.

hard, green non-embryogenic cultures. In ½LP medium, a poor response was observed.

Secondary needles: The needles (Figure 8a) exhibited the best embryogenic cultures on MS medium followed by mMS and ½MS media (Table 9). The poor induction of embryogenic cultures resulted on LP and ½LP media. Within 3 weeks in culture, the needles exhibited the formation of 2 types of cultures a) white, soft and translucent embryogenic (Figure 8b) and b) hard non-embryogenic cultures.

Apical dome sections: The optimum induction of embryogenic cultures was recorded in ½DCR medium followed by DCR, ½mMS media (Table 10). The pre-treated apical dome sections (Figure 9a) exhibited white and soft embryogenic cultures (Figure 9b) in 92.6% of the explants within 2 weeks of culture. In rest of the explants, white and hard non-embryogenic cultures (Figure 9c) developed. Both MS and LP media in full and half strength did not result in good embryogenic cultures.

The relative response of zygotic embryos, secondary needles and apical dome sections cultured on different media is shown in

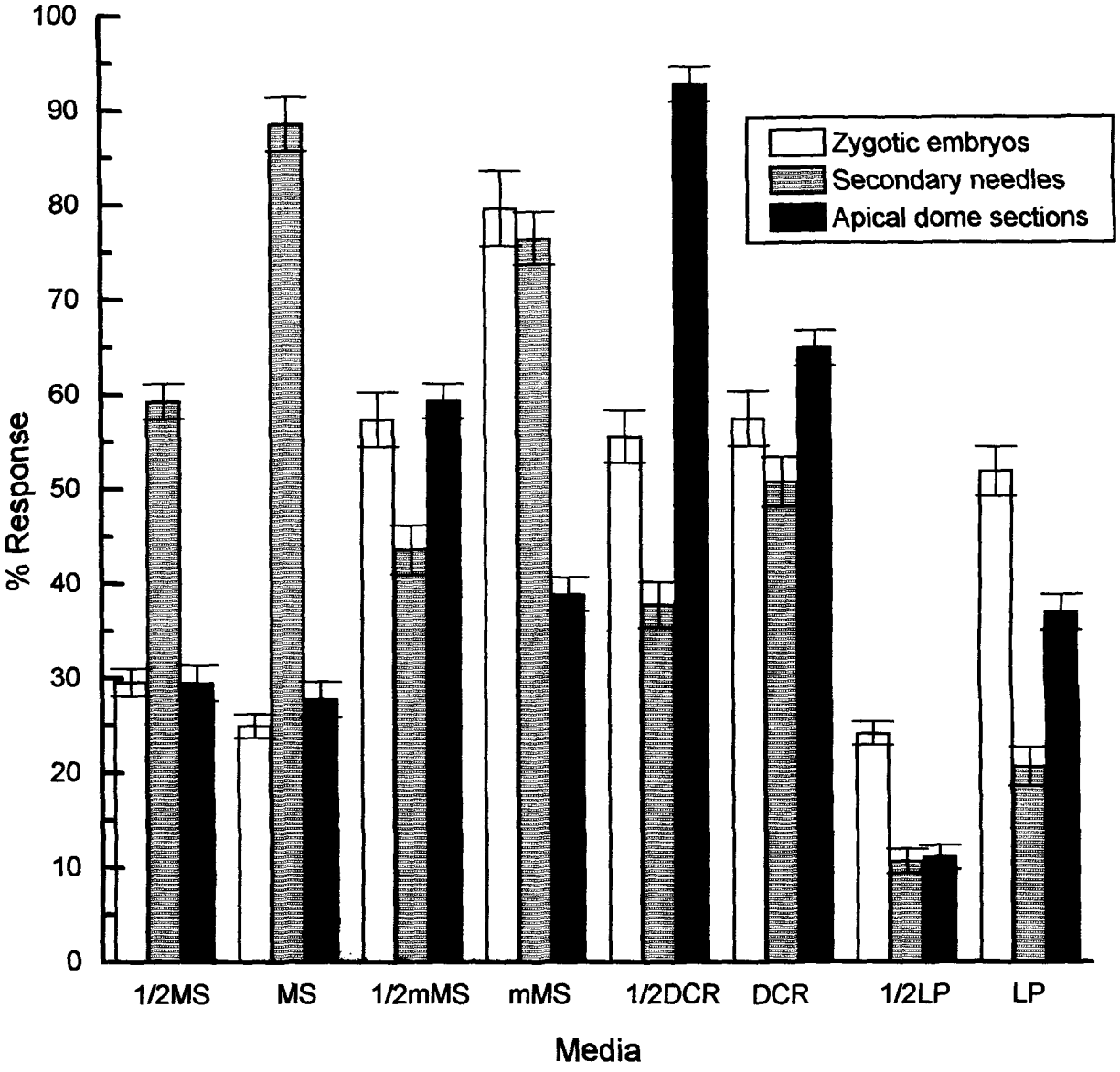


Figure 10. Effect of media composition on initiation of embryogenic cultures from various explants

figure 10. A perusal of this shows that marked variation existed between the response of different explants with regard to media used.

d) Effect of sucrose

Zygotic embryo: Sucrose was essential as a carbon source for culture initiation. In its absence no growth was recorded. In general, the increase of sucrose concentration from 1.0-2.0% in the different media resulted in higher production of embryogenic cultures. The response declined with increase in concentration of sucrose to 3.0%. The optimum embryogenic cultures were observed in mMS medium containing 2.0% sucrose (Table 8).

Secondary needles: In general, the incorporation of 2.0% sucrose in different media exhibited better embryogenic culture formation (Table 9). While the optimum response was recorded in MS medium, it was poor in ½LP and LP media. Media devoid of sucrose could not support any growth. Increase in sucrose concentration to 3.0% resulted in a decline in response.

Apical dome sections: There was no growth in media without sucrose. In general, the incorporation of 2.0% sucrose in different media exhibited better response compared to other concentrations (Table 10). However, the difference in response between 2.0% and

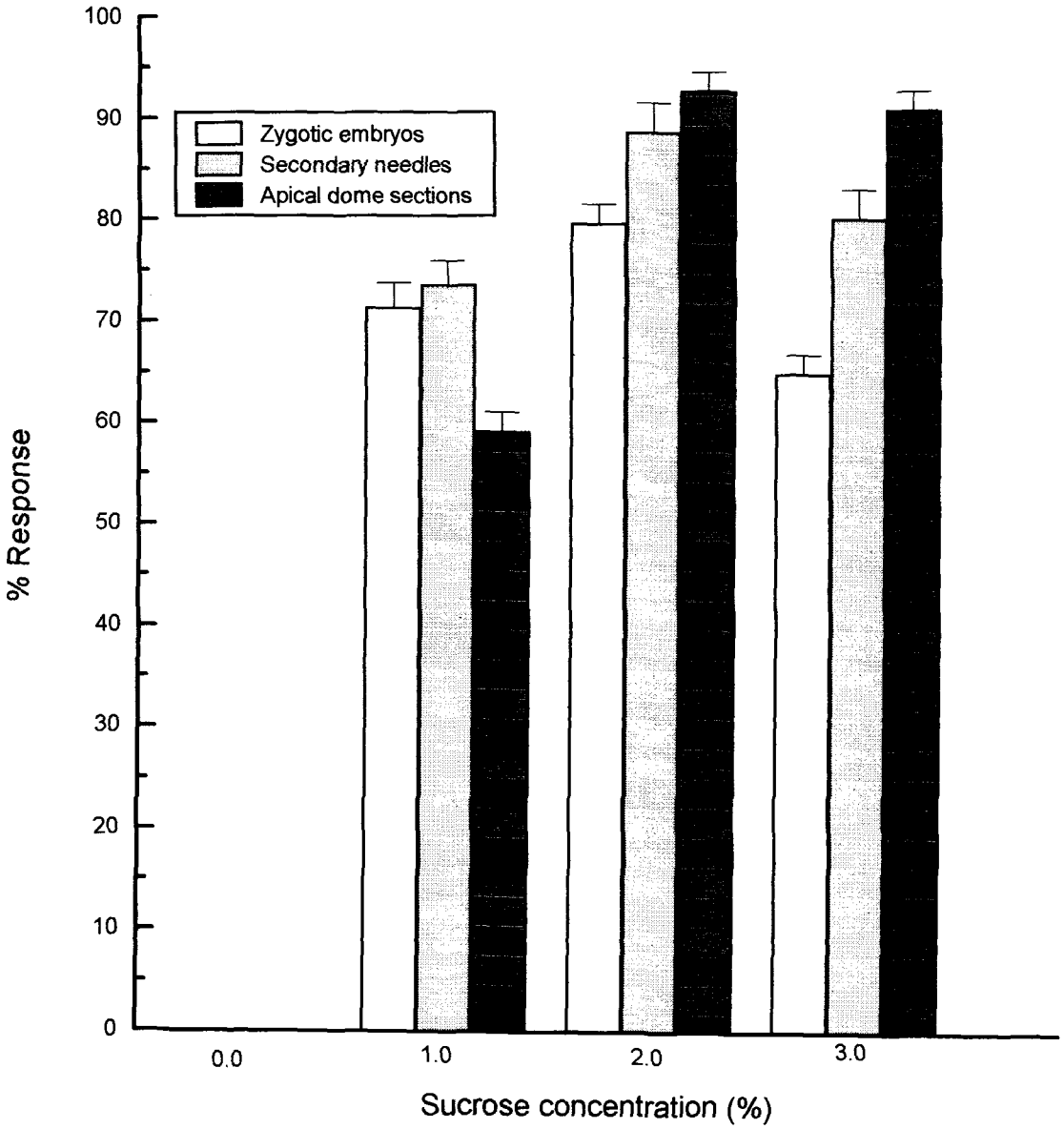


Figure 11. Effect of sucrose concentration on induction of embryogenic cultures from different explants

Table 11. Effect of CH and myo-inositol on initiation of embryogenic cultures from mature zygotic embryos, secondary needles and apical dome sections

CH conc. (mg/l)	Myo-inositol conc. (mg/l)	Type of response	Performance*
0	0	Very poor callus developed	+
250	0	-do-	+
500	0	Hard callus developed	+
750	0	Moderately healthy and translucent callus	+
1000	0	-do-	++
1250	0	Soft but slightly brown callus	+
1500	0	-do-	+
0	250	Poor and hard callus	+
250	250	-do-	+
500	250	-do-	+
750	250	-do-	+
1000	250	Moderately soft and healthy callus	++
1250	250	Slightly brown callus developed	+
1500	250	-do-	+
0	500	Hard callus developed	+
250	500	-do-	+
500	500	As above but comparatively healthy	+
750	500	-do-	++
1000	500	Moderately soft and translucent callus resulted	++
1250	500	As above but slightly brown callus developed	+
1500	500	-do-	+
0	750	White and hard callus formed	+
250	750	-do-	+
500	750	-do-	+
750	750	White soft and moderately translucent callus formed	++
1000	750	-do-	++
1250	750	As above but callus turned brown	++
1500	750	-do-	+
0	1000	White but semisoft callus formed	++
250	1000	-do-	++
500	1000	-do-	++
750	1000	-do-	++

Contd...

1000	1000	White, soft, translucent and gelatinous healthy callus formed	+++
1250	1000	As above but slightly brown callus formed	++
1500	1000	Brown callus formed	+
0	1250	White and semisoft callus formed	+
250	1250	-do-	+
500	1250	-do-	+
750	1250	Semisoft callus formed but slightly brown	++
1000	1250	-do-	++
1250	1250	Poor callus formation and callus turned brown	++
1500	1250	-do-	+
0	1500	Semi-hard and brown callus developed	+
250	1500	-do-	+
500	1500	-do-	+
750	1500	-do-	+
1000	1500	Soft but brown callus formed	++
1250	1500	-do-	+
1500	1500	-do-	+

@: on mMS and ½DCR media for zygotic embryo and apical dome sections respectively containing 2,4-D and NAA (each at 5.0 mg/l), BAP (2.5 mg/l), and on MS medium for secondary needles containing 2,4-D and NAA (each at 3.0 mg/l), BAP (1.0 mg/l). All the media contained with sucrose (2.0) and L-glutamine (500 mg/l). * +: poor response; ++: moderate response and +++: optimum response.

3.0% sucrose treatment was not pronounced. Increase in sucrose concentration from 1.0% to 2.0% in ½DCR medium resulted in marked increase in the formation of embryogenic cultures. This treatment was optimum (92.6% response) for the formation of embryogenic cultures.

The effect of sucrose on induction of embryogenic cultures from different explants on respective media (optimum) is shown in figure 11. In the absence of sucrose there was no growth and cultures were not established.

e) Effect of casein-hydrolysate and myo-inositol

In the absence of both CH and myo-inositol, very poor callus formation was recorded (Table 11). Incorporation of CH singly in the medium did not help in formation of the embryogenic cultures except for 750 and 1000 mg/l where moderately healthy and translucent cultures were formed in all the different explants. Higher concentrations (1250 and 1500 mg/l) resulted in browning of the cultures and they exhibited poor growth. The optimum response was recorded at 1000 mg/l each of CH and myo-inositol. In this case cultures were white, soft, translucent and gelatinous.

Table 12. Effect of L-glutamine on initiation of embryogenic cultures from mature zygotic embryos, secondary needles and apical dome sections

L-glutamine conc. (mg/l)	Type of response@	Performance*
0	Very poor callus developed. Explants callused partially	+
100	-do-	+
200	-do-	+
300	Moderate callusing but not completely translucent	++
400	-do-	++
500	Explants callused completely and formed soft, translucent and gelatinous callus	+++
600	As above but callus slightly turned brown	++
700	Callus turned brown	+
800	-do-	+
900	-do-	+
1000	-do-	+

@: on mMS and ½DCR media for zygotic embryo and apical dome sections respectively containing 2,4-D, NAA (each at 5.0 mg/l), BAP (2.5 mg/l), and on MS medium for secondary needles containing 2,4-D, NAA (each at 3.0 mg/l) and BAP (1.0 mg/l). All the media contained with sucrose (2.0%) and CH, myo-inositol (each at 1000 mg/l). * +: poor response; ++: moderate response and +++: optimum response.

Table 13. Effect of 2,4-D and NAA in mMS medium[@] on initiation of embryogenic culture from mature zygotic embryos

2,4-D conc. (mg/l)	NAA conc. (mg/l)	Type of response	% response (±SE[1])	Performance*
0.0	0.0	No response	-	-
2.5	0.0	Very little portion callused and callus not healthy	20.40 (±1.85)	+
5.0	0.0	Partial soft callus and the rest hard	29.60 (±1.84)	+
7.5	0.0	Comparatively more soft but calli turned lightly brown	20.40 (±1.85)	+
10.0	0.0	-do-	18.50 (±1.84)	+
0.0	2.5	Almost no response	9.25 (±1.85)	-
2.5	2.5	Moderate callusing with some hard portions	50.00 (±1.85)	++
5.0	2.5	-do-	44.40 (±2.54)	++
7.5	2.5	As above but cultures turned slightly brown, not very healthy	31.50 (±1.84)	++
10.0	2.5	-do-	33.30 (±1.30)	++
0.0	5.0	Partially soft and partially hard callus	51.85 (±1.85)	++
2.5	5.0	-do-	50.00 (±1.76)	++
5.0	5.0	White, soft, translucent and gelatinous callus and very little hard callus formation	79.6 (±1.84)	++++
7.5	5.0	As above but callus growth drastically retarded	42.60 (±1.84)	++
10.0	5.0	-do-	29.60 (±1.84)	+
0.0	7.5	Partially soft callus, but turned brown	40.70 (±1.84)	++
2.5	7.5	-do-	38.90 (±1.84)	++
5.0	7.5	White and soft callus with retarded growth	46.30 (±1.86)	++
7.5	7.5	Comparatively better callus growth but turned brown	55.50 (±1.76)	+++
10.0	7.5	Callus growth retarded and turned brown	31.50 (±1.84)	++
0.0	10.0	Very little callusing	20.35 (±1.84)	+
2.5	10.0	Very poor callusing with retarded growth	35.20 (±1.85)	++
5.0	10.0	-do-	27.80 (±1.50)	+
7.5	10.0	-do-	33.30 (±2.10)	++
10.0	10.0	Comparatively better callus growth but turned brown	42.60 (±1.84)	++

@: containing sucrose (2.0%), BAP (2.5 mg/l), CH and myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). 1: standard error * -: no response; +: poor response; ++: moderate response; +++: good response and ++++: optimum response.

f) Effect of L-glutamine

Table 12 shows that incorporation of L-glutamine into the medium was highly beneficial for the formation of embryogenic cultures. In case of the control, very poor and partial callus formation on the explants resulted. The optimum response was recorded in the medium containing 500 mg/l L-glutamine where cultures were soft, translucent and gelatinous. With increase in its concentration, the cultures turned brown and exhibited poor growth.

g) Effect of plant growth regulators

Zygotic embryo

Auxin: There was no embryogenic response in the medium-free of 2,4-D and NAA but containing 2.5 mg/l BAP (Table 13). NAA as an auxin source was found superior than 2,4-D. By increasing the concentration of NAA from 2.5 to 5.0 mg/l in media, about 5 fold increase in embryogenic culture formation was recorded. However, further increase in NAA concentration resulted in decrease in the response. The incorporation of both 2,4-D and NAA each at 5.0 mg/l in conjunction with BAP (2.5 mg/l) resulted in optimum (79.6%)

Table 14. Effect of BAP in mMS medium[@] on initiation of embryogenic cultures from mature zygotic embryos

BAP conc. (mg/l)	Type of response	% response (±SE[1])	Performance*
0.0	White and semi-soft callus was formed	38.0 (±2.38)	+
0.5	-do-	33.3 (±2.37)	+
1.0	-do-	45.2 (±2.38)	+
1.5	White, soft callus formed	54.8 (±2.38)	++
2.0	-do-	59.5 (±2.37)	++
2.5	White, soft, translucent and gelatinous healthy callus produced	71.4 (±2.38)	+++
3.0	As above but slight greenish callus	66.7 (±2.38)	++
3.5	Slightly hard and green callus along with soft embryogenic callus	47.6 (±2.38)	+
4.0	-do-	47.6 (±2.38)	+
4.5	-do-	42.8 (±2.38)	+
5.0	As above but harder callus	40.5 (±2.38)	+

@: medium containing sucrose (2.0%), 2,4-D, NAA (5.0 mg/l each), CH and myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). 1: standard error * +: poor response; ++: moderate response and +++: optimum response

Table 15. Effect of BAP and kinetin in mMS medium* on initiation of embryogenic cultures from mature zygotic embryos

BAP conc. (mg/l)	Kinetin conc. (mg/l)	Type of response	% response
0.0	0.0	No response	0.0
1.0	0.0	-do-	0.0
3.0	0.0	Only swelling- green in colour	0.0
5.0	0.0	-do-	0.0
0.0	1.0	No response	0.0
1.0	1.0	-do-	0.0
3.0	1.0	All the explants turned green and swelled	0.0
5.0	1.0	-do-	0.0
0.0	3.0	-do-	0.0
1.0	3.0	-do-	0.0
3.0	3.0	-do-	0.0
5.0	3.0	Only swelling- green in colour	0.0
0.0	5.0	-do-	0.0
1.0	5.0	-do-	0.0
3.0	5.0	-do-	0.0
5.0	5.0	Swelling- green in colour very little hard callus	0.0

* containing sucrose (2.0%), CH and myo-inositol (each at 1000 mg/l), L-glutamine (500 mg/l) and without any auxin.

Table 16. Effect of 2,4-D and NAA in MS medium[a] on initiation of embryogenic cultures from secondary needles

2,4-D conc. (mg/l)	NAA conc. (mg/l)	Type of response	% response (±SE[1])	Performance*
0.0	0.0	No response	0.0	-
1.0	0.0	Very few needles swelled and little callusing observed	26.7 (±2.10)	+
3.0	0.0	Needles callused at the base and remaining showed swelling	30.7 (±1.63)	+
5.0	0.0	-do-	30.7 (±2.66)	+
0.0	1.0	Whitish callus formed but not very soft, callusing restricted only to the basal part	37.0 (±2.67)	++
1.0	1.0	Moderately soft and white callus	60.0 (±2.10)	+++
3.0	1.0	-do-	68.0 (±2.49)	+++
5.0	1.0	Callus slight brown and showed poor growth	46.7 (±2.10)	++
0.0	3.0	White and soft callus, some needles callused completely	61.3 (±1.33)	+++
1.0	3.0	As above but more healthy callus	65.3 (±2.49)	+++
3.0	3.0	Soft and white and healthy callus formed all along the needles	88.6 (±1.82)	++++
5.0	3.0	As above but cultures turned slightly brown	73.3 (±2.11)	+++
0.0	5.0	Very few healthy callus and rest turned brown	28.0 (±2.48)	+
1.0	5.0	Moderate callus growth with slight browning	60.0 (±2.10)	+++
3.0	5.0	As above	64.0 (±1.63)	+++
5.0	5.0	Most of the calli turned brown in next sub-culture and ultimately degenerated	44.0 (±1.64)	++

a: containing sucrose (2.0%) and BAP (1.0 mg/l). CH. myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). 1: standard error. * -: no response; +: poor response; ++: moderate response; +++: good response and ++++: optimum response.

embryogenic culture formation. The cultures were white, soft, translucent and gelatinous in texture.

N^6-benzyl amino purine: In the absence of BAP in mMS medium (containing 5.0 mg/l each of 2,4-D and NAA), white and semisoft cultures were formed in 38.0% of the explants (Table 14). With increase in BAP concentration to 2.5 mg/l in the medium, increase in the formation of embryogenic cultures was observed. This concentration was found to be optimum (71.4% response) for healthy embryogenic culture formation. At higher concentrations of BAP in the medium the response declined.

Kinetin and BAP: In the absence of cytokinins and auxins in the mMS medium no callus formation could be observed. Both kinetin and BAP singly or in combination in the medium devoid of any auxin, failed to initiate embryogenic cultures (Table 15). In general, slight swelling of the explants resulted which was green in colour.

Secondary needles

Auxin: The initiation of embryogenic cultures did not occur in auxin-free MS medium containing BAP (1.0 mg/l) (Table 16). Using NAA singly as an auxin source resulted in better embryogenic culture initiation as compared to 2,4-D. However, combined influence of both

Table 17. Effect of BAP in MS medium@ on initiation of embryogenic cultures from secondary needles

BAP conc. (mg/l)	Type of response	% response (±SE[1])	Performance*
0.0	Moderate callus growth but semi-soft in appearance	70.0 (±2.23)	+++
0.5	As above but comparatively healthy cultures	72.0 (±2.54)	+++
1.0	Callus soft and translucent. Needles callused all along the length and satisfactory proliferation of cultures in subsequent subcultures	88.0 (±2.0)	++++
1.5	As above	83.0 (±2.54)	++++
2.0	Slightly hard callus resulted	77.0 (±2.55)	+++
2.5	-do-	65.0 (±1.58)	+++
3.0	-do-	65.0 (±1.58)	+++
3.5	More hard callus resulted	56.0 (±1.87)	+++
4.0	-do-	52.0 (±1.22)	+++
4.5	-do-	49.0 (±1.87)	++
5.0	-do-	46.0 (±1.00)	++

@ containing sucrose (2.0%), 2,4-D and NAA (each at 3.0 mg/l), CH, myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). 1: standard error.* ++: moderate response, +++: good response and ++++: optimum response.

Table 18. Effect of BAP and kinetin in MS medium@ on induction of embryogenic cultures from secondary needles

BAP conc. (mg/l)	Kinetin conc. (mg/l)	Type of response	% response
0.0	0.0	No response	0.0
1.0	0.0	-do-	0.0
3.0	0.0	Very few needles swelled	0.0
5.0	0.0	-do-	0.0
0.0	1.0	No response	0.0
1.0	1.0	No response	0.0
3.0	1.0	At the base of the needles swelling observed	0.0
5.0	1.0	-do-	0.0
0.0	3.0	-do-	0.0
1.0	3.0	-do-	0.0
3.0	3.0	-do-	0.0
5.0	3.0	Whole needle swelled	0.0
0.0	5.0	Swelling at the base	0.0
1.0	5.0	-do-	0.0
3.0	5.0	Whole needle swelled	0.0
5.0	5.0	-do-	0.0

@: containing sucrose (2.0%), CH, myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l) and without any auxin.

Table 19. Effect of 2,4-D and NAA in ½ DCR medium@ on initiation of embryogenic cultures from apical dome sections

2,4-D conc. (mg/l)	NAA conc. (mg/l)	Type of response	% response (±SE[1])	Performance*
0.0	0.0	No response	0.0	-
1.0	0.0	Very little portion callused and rest swelled	34.2 (±2.65)	++
3.0	0.0	-do-	37.0 (±2.74)	++
5.0	0.0	Calli were white but not very soft and healthy	48.1 (±2.34)	++
7.0	0.0	As above but slight turned brown	37.9 (±2.65)	++
0.0	1.0	Most of the sections swelled, very little hard callus resulted	26.8 (±0.92)	+
1.0	1.0	Moderate callusing but hard	52.8 (±2.37)	+++
3.0	1.0	-do-	51.8 (±2.74)	++
5.0	1.0	As above but more soft	59.2 (±2.74)	+++
7.0	1.0	As above but slightly brown	58.3 (±2.37)	+++
0.0	3.0	Hard callus and little soft callus	44.4 (±2.27)	++
1.0	3.0	Healthy callus but not soft	74.0 (±1.83)	++
3.0	3.0	Comparatively more white and soft callus	75.9 (±2.27)	+++
5.0	3.0	White and soft callus but growth was not satisfactory	64.0 (±2.74)	+++
7.0	3.0	As above but slightly brown	59.2 (±2.74)	+++
0.0	5.0	White callus	49.0 (±2.23)	++
1.0	5.0	-do-	71.3 (±2.65)	+++
3.0	5.0	More white and soft callus and very few proembryos developed	81.4 (±2.34)	+++
5.0	5.0	White, soft and translucent callus formed. Growth was smooth and good number of pro-embryos developed	92.6 (±1.84)	++++
7.0	5.0	As above but less number of pro-embryos observed and cultures turned brown	76.8 (±2.65)	+++
0.0	7.0	Soft but callus turned brown in subsequent subcultures	34.2 (±2.23)	++
1.0	7.0	-do-	76.8 (±2.22)	+++
3.0	7.0	-do-	77.8 (±2.02)	+++
5.0	7.0	-do-	72.2 (±2.02)	+++
7.0	7.0	Brown callus degenerated in subsequent subcultures	55.5 (±2.48)	+++

@: containing sucrose (2.0%), CH_2 myo-inositol (each at 1000 mg/l), L-glutamine (500 mg/l) and BAP (2.5 mg/l). 1 :. standard error. * -: no response, +: poor response, ++: moderate response, +++: good response and ++++: optimum response.

these auxins and BAP exhibited pronounced embryogenic culture formation. At 3.0 mg/l each of 2,4-D and NAA in the medium, soft, white and healthy embryogenic cultures resulted on the entire surface of the needle (88.6% response). With the increase in auxin concentration in the medium a decline in response was recorded.

N^6-benzyl amino purine: In MS medium devoid of BAP, but containing 3.0 mg/l each of 2,4-D and NAA, 70.0% of the explants exhibited moderate growth of cultures that were semisoft in texture (Table 17). A concentration of 1.0 mg/l BAP was found to be optimum for embryogenic culture formation. At this concentration the entire surface of the needle exhibited callus formation. By increasing the concentration of BAP, the response declined and beyond 3.0 mg/l BAP in the medium harder calli resulted.

Kinetin and BAP: In the absence of both cytokinins and auxins in MS medium, callus formation did not take place (Table 18). BAP and kinetin singly or in combination could not stimulate the initiation of embryogenic process. However, at higher concentrations, swelling of the needle was noticed.

Table 20. Effect of BAP in ½DCR medium@ on initiation of embryogenic cultures from apical dome sections

BAP conc. (mg/l)	Type of response	% response (±SE[1])	Performance*
0.0	White but hard callus and very little soft callus produced	47.8 (±2.22)	++
0.5	-do-	50.0 (±1.75)	++
1.0	Comparatively more soft callus produced	51.1 (±2.07)	++
1.5	-do-	63.3 (±2.21)	+++
2.0	White and soft callus growth moderately satisfactory and very few pro-embryos were observed	84.4 (±2.08)	+++
2.5	Callus white and soft with more pro-embryos and growth very satisfactory in subsequent subcultures	92.6 (±1.84)	++++
3.0	-do-	87.8 (±2.07)	+++
3.5	Callus appeared comparatively hard	72.2 (±1.76)	+++
4.0	-do-	53.3 (±2.22)	+++
4.5	-do-	53.3 (±2.83)	+++
5.0	More hard and green callus	46.7 (±2.83)	++

@ containing sucrose (2.0%), 2,4-D and NAA (each at 5.0 mg/l), CH, myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). 1: standard error. * +: poor response, ++: moderate response, +++: good response and ++++: optimum response.

Table 21. Effect of BAP and kinetin in ½DCR medium@ on initiation of embryogenic cultures from apical dome sections

BAP conc. (mg/l)	Kinetin conc. (mg/l)	Type of response	% response (±SE [1])	Performance*
0.0	0.0	No response	0.0	-
1.0	0.0	-do-	0.0	-
3.0	0.0	Greenish swelling	0.0	-
5.0	0.0	-do-	0.0	-
0.0	1.0	No response	0.0	-
1.0	1.0	-do-	0.0	-
3.0	1.0	Slightly hard brown callusing	0.0	-
5.0	1.0	-do-	0.0	-
0.0	3.0	No response	0.0	-
1.0	3.0	Very poor and hard callusing	0.0	-
3.0	3.0	Reddish translucent callus but no proembryos were formed	47.2 (±2.78)	++
5.0	3.0	-do-	38.9 (±2.77)	++
0.0	5.0	No distinct callusing	0.0	-
1.0	5.0	-do-	0.0	-
3.0	5.0	Only swelling,, no distinct callusing	30.5 (±2.77)	+
5.0	5.0	-do-	0.0	-

@ containing sucrose (2.0%), no auxin, CH and myo-inositol (each at 1000 mg/l) and L-glutamine (500 mg/l). 1: standard error. * -: no response, +: Poor response and ++: moderate response.

Apical dome sections

Auxin: The callus formation did not occur in ½DCR medium free of 2,4-D and NAA, but containing BAP (2.5 mg/l) (Table 19). There was an increase in embryogenic culture formation with incorporation of both 2,4-D and NAA either singly or in combination. Enriching the medium with 2,4-D as an auxin resulted in poor embryogenic cultures compared to NAA. A ~2 fold increase in formation of embryogenic cultures was observed by increasing NAA from 1.0 mg/l to 5.0 mg/l singly in the medium. An optimum response of 92.6%, was recorded with incorporation of both 2,4-D and NAA each at 5.0 mg/l in the medium. In this treatment, the cultures were white, soft and translucent. Both 2,4-D and NAA singly or in combination at higher concentrations were inhibitory and the cultures turned brown and degenerated.

N^6-benzyl amino purine: About 48.0% explants formed white but hard callus in ½CDR medium containing 2,4-D and NAA (5.0 ml/l each) but without BAP (Table 20). By increasing BAP concentration upto 3.0 mg/l, softer and embryogenic cultures could be produced. An optimum response was recorded in the medium with 2.5 mg/l BAP.

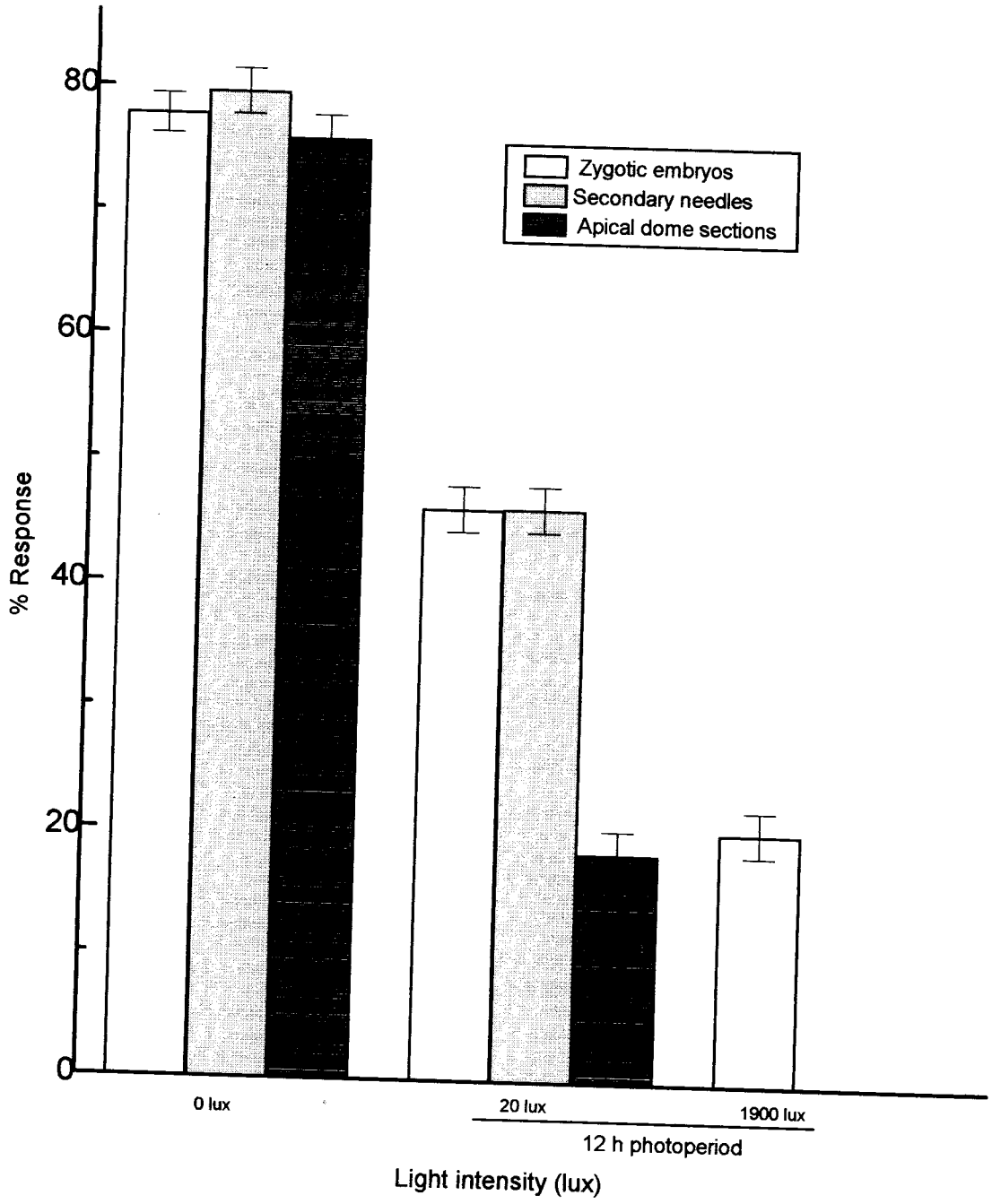


Figure 12. Effect of light on initiation of embryogenic cultures from various explants

Table 22. Effect of growth regulators in media@ on maintenance of embryogenic cultures obtained from zygotic embryos, secondary needles and apical dome sections

Growth regulators' level*	Type of response	Performance@
$1/5^{th}$	Cultures remained undifferentiated. In some cases continued proliferation and in some cases turned brown. No proembryo formation observed	++
$1/10^{th}$	Cultures were healthy and proliferation slowed. Distinct PEMs and proembryo formation observed in all types of explants	+++
0	Cultures ceased proliferation. No PEMs and proembryo formation noticed. Cultures turned brown and subsequently degenerated	+

@: mMS, MS and ½DCR media for zygotic embryos, secondary needles and ½DCR for apical dome sections respectively. The media were supplemented with sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). *: compared to respective initiation medium. @: +: poor response, ++: moderate response and +++: optimum response.

The formation of hard calli and decline in response were recorded in media containing more than 3.0 mg/l BAP.

Kinetin and BAP: In the absence of both cytokinins and auxins in ½DCR medium, no callus formation could be observed (Table 21). Both BAP and kinetin singly did not promote any callus formation. However, reddish translucent cultures were obtained in the medium containing 3.0 mg/l each of BAP and kinetin, which subsequently degenerated. At higher concentrations no callus formation was observed.

h) Effect of light

The optimum initiation of embryogenic cultures from various explant sources was accomplished in the dark. As shown in figure 12, light inhibited the initiation of embryogenic cultures. At 20 lux light intensity, embryogenic culture formation in the case of apical dome sections was lower compared to other two explants. The initiation of embryogenic cultures was completely inhibited in apical dome sections and secondary needles at 1900 lux light intensity. Also a marked decline in response of zygotic embryo was noticed. The cultures turned hard and green on subsequent subcultures in the light (Figure 9d).

3.1.D Maintenance of cultures

After 2-3 subcultures, the embryogenic cultures were transferred on respective maintenance medium (Table 22). If left on the initiation medium they cease to proliferate and turned brown.

Zygotic embryos: The PEMs and proembryos were formed after 2-3 passages on mMS medium containing reduced growth regulators. The optimum response was observed on medium containing $1/10^{th}$ growth regulators of initiation medium i.e., 2,4-D, NAA (0.5 mg/l each) in conjunction with BAP (0.25 mg/l) (Figure 7d).

Secondary needles: The embryogenic cultures proliferated on MS medium containing $1/10^{th}$ growth regulators of initiation medium i.e., 2,4-D, NAA (each 0.3 mg/l) and BAP (0.1 mg/l). The PEMs and proembryos developed within 3 weeks in culture on MS medium without growth regulators. The proembryos subsequently converted into globular embryos in the next 2-3 weeks in culture.

Apical dome sections: The embryogenic cultures were not very soft and translucent after 2 subcultures on initiation medium. At this stage, a cold treatment of cultures at 4°C for 24 h resulted in softer and translucent cultures. The cold treated cultures formed PEMs and proembryos in 3-4 weeks time on ½DCR medium containing $1/10^{th}$

Table 23. Effect of ABA and sucrose on development and maturation of somatic embryos on semisolid media obtained from zygotic embryos and secondary needles

Abscisic acid conc.(mg/l)	Sucrose conc.(%)	Type of response	Performance*
0	0	No response	-
	1	-do-	-
	2	-do-	-
	3	-do-	-
	4	Cultures turned brown	-
	5	-do-	-
2.0	0	No response	-
	1	Proembryos slightly increased in size but no distinct embryos formed	-
	2	-do-	-
	3	-do-	+
	4	-do-	+
	5	Cultured turned slightly brown	-
4.0	0	No response	-
	1	Proembryos slightly increased in size but no distinct embryos formed	-
	2	-do-	+
	3	Distinct embryos with cotyledons formed	++
	4	Distinct embryonal head and cotyledon formation observed, cultures were healthy	+++
	5	As above but cultures turned slightly brown	+
6.0	0	No response	-
	1	Distinct embryos with cotyledons formed	+
	2	As above but cultures turned slightly brown	++
	3	-do-	+
	4	-do-	++
	5	Cultures turned brown	-
8.0	0	Culture degenerated	-
	1	No embryonal head formation was observed	-
	2	-do-	-
	3	Cultures turned brown	-
	4	-do-	-
	5	-do-	-

* -: no response; +: poor response; ++: moderate response and +++: optimum response.

growth regulators of initiation medium i.e., 2,4-D and NAA (each 0.5 mg/l) and BAP (0.25 mg/l) (Figure 9c). The proembryos converted into globular embryos within 3 weeks in culture on ½DCR medium without any growth regulators but containing 3.0% sucrose (Figure 9f).

In general, if the embryogenic cultures were grown along with the non-embryogenic cultures, they tend to convert into non-embryogenic type and ultimately degenerated.

3.1.E Embryo development and maturation

Zygotic embryo and secondary needles: The pro- and globular embryos produced on maintenance media from both zygotic embryos and secondary needles could not proceed to next developmental stage until they were cultured on respective media containing ABA and sucrose. An optimum response of cotyledonary embryo formation in cultures was recorded with combined treatment of both sucrose and ABA (4.0% and 4.0 mg/l, respectively) (Table 23). Figure 7e and 8c show distinct cotyledonary embryos in cultures obtained from zygotic embryos and secondary needles, respectively. In case of cultures from zygotic embryos, about 7-8 embryos were found clumped together at

Table 24. Effect of activated charcoal on somatic embryo (obtained *from zygotic embryos)* elongation

Activated charcoal conc. (%)	Sucrose conc. (%)	Type of response@	Performance*
0	0	No response	-
	1.0	No response	-
	2.0	Very poor embryo elongation	+
	3.0	-do-	+
	4.0	-do-	+
0.2	0	No response	-
	1.0	Elongated embryos but unhealthy	+
	2.0	Moderately healthy elongated embryos	++
	3.0	Healthy elongated embryos	+++
	4.0	Elongated embryos turned brown	++
0.4	0	No response	-
	1.0	Poor embryo health and turned slightly brown	+
	2.0	Embryos turned slight brown	+
	3.0	As above	++
	4.0	Embryos turned brown	+
0.6	0	Culture turned brown	-
	1.0	-do-	+
	2.0	-do-	+
	3.0	-do-	+
	4.0	-do-	+

@: on mMS medium containing myo-inositol (100 mg/l). * -: no response; +: poor response; ++: moderate response and +++: optimum response.

Table 25. Effect of activated charcoal on somatic embryo (obtained from secondary needles) elongation

Activated charcoal conc. (%)	Sucrose conc. (%)	Type of response@	Performance*
0	0	No response	-
	1.0	No response	-
	2.0	Very poor embryo elongation	+
	3.0	-do-	+
	4.0	-do-	+
0.2	0	No response	-
	1.0	Elongated embryos but unhealthy	+
	2.0	Moderately elongated embryos	+
	3.0	Moderately healthy elongated embryos	++
	4.0	Embryos turned slightly brown	+
0.4	0	No response	-
	1.0	Moderate embryo health and elongation	++
	2.0	Healthy elongated embryos	+++
	3.0	As above but slightly brown	++
	4.0	Embryos turned slightly brown	+
0.6	0	Culture turned brown	-
	1.0	-do-	+
	2.0	-do-	+
	3.0	-do-	+
	4.0	-do-	+

@: on mMS medium containing myo-inositol (100 mg/l). * -: no response; +: poor response; ++: moderate response and +++: optimum response.

Table 26. Effect of kinetin and NAA on somatic embryo germination

Kinetin conc. (mg/l)	NAA conc. (mg/l)	Type of response@	% response (±SE[1])	Performance*
0	0	No response	-	-
1	0	No response	-	-
3	0	Poor rooting	10.0 (±1.23)	+
5	0	-do-	11.50 (±0.50)	+
7	0	-do-	7.0 (±1.0)	+
0	1	Poor shoot formation observed	9.0 (±1.60)	+
1	1	-do-	10.0 (±0.50)	+
3	1	Rooted plantlets formed	32.50 (±1.95)	++
5	1	Well rooted plantlets formed	40.70 (±1.23)	+++
7	1	Rooted plantlets formed	30.50 (±0.70)	++
0	3	Poor shoot formation observed	15.5 (±1.23)	+
1	3	Embryos trend to form callus	17.0 (±1.73)	+
3	3	-do-	17.0 (±0.70)	+
5	3	Rooted plantlets formed but showed callusing at the base of the shoot	15.30 (±0.50)	+
7	3	As above	12.0 (±0.70)	+
0	5	Embryo callused	-	-
1	5	As above	-	-
3	5	As above	-	-
5	5	Rooted plantlets formed but showed callusing at the base of the shoot	13.0 (±1.70)	+
7	5	As above	12.5 (±0.70)	+
0	7	Embryo callused	-	-
1	7	As above	-	-
3	7	As above	-	-
5	7	As above	-	-
7	7	As above		+

@: on mMS medium containing sucrose (3.0%), myo-inositol (100 mg/l).1: standard error.* -: no response, +: poor response, ++: moderate response and +++: optimum response.

the base with fused cotyledons (Figure 7f). Increase in concentrations of both ABA and sucrose resulted in browning of cultures.

The cotyledonary somatic embryos (obtained from zygotic embryos) elongated in 2-3 weeks in culture on mMS medium free of ABA, CH and L-glutamine, but containing activated charcoal (0.2%), myo-inositol (100 mg/l) and sucrose (3.0%), under 12 h photoperiod (Table 24). It was noticed that elongation of embryos could not occur in the medium free of activated charcoal and its concentration higher than 0.2% resulted in browning of cultures.

The cotyledonary somatic embryos (obtained from secondary needles) elongated within 2 passages on MS medium devoid of any growth regulators, CH, L-glutamine but containing myo-inositol (100 mg/l), sucrose (2.0%) and activated charcoal (0.4%) under 12 h photoperiod (Table 25).

Apical dome sections: The cultures with globular embryos obtained from apical dome sections were very healthy. Despite of growing these cultures on basal medium with various levels of sucrose and ABA singly or in combination for several passages, the globular embryos could not convert into cotyledonary embryos.

3.1.F Germination of somatic embryos

Figure 13. Embling formation from germinating somatic embryos

Figure 13

Table 27. Complete media formulation for induction of somatic embryogenesis from mature zygotic embryos, secondary needles and apical dome section in semisolid cultures

Components	Initiation and proliferation of embryogenic cultures			Maintenance of cultures			Somatic embryo development			Somatic embryo maturation			Germination of somatic embryo		
	a	b	c	a	b*	c**	a	b	c	a	b	c	a	b	c
Basal medium	mMS	MS	½DCR	mMS	MS	½DCR	mMS	MS	½DCR	mMS	MS	½DCR	mMS	-	-
Supplements (g/l)															
Sucrose	20.0	20.0	20.0	20.0	20.0	20.0	40.0	40.0	40.0	30.0	20.0	-	30.0	-	-
Casein-hydrolysate	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	-	-	-	-	-	-
Myo-inositol	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.1	0.1	0.1	-	-	-
L-glutamine	0.5	0.5	0.5	0 5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-	-
PVP	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-	-
Citric acid	-	-	0.1	-	-	0.1	-	-	0.1	-	-	-	-	-	-
Activated charcoal	-	-	-	-	-	-	-	-	-	2.0	4.0	-	-	-	-
Growth regulators (mg/l)															
2.4-D	5.0	3.0	5.0	0.5	0.3	0.5	-	-	-	-	-	-	-	-	-
NAA	5.0	3.0	5.0	0.5	0.3	0.5	-	-	-	-	-	-	1.0	-	-
BAP	2.5	1.0	2.5	0.25	0.1	0.25	-	-	-	-	-	-	-	-	-
Kinetin	-	-	-	-	-	-	-	-	-	-	-	-	5.0	-	-
ABA	-	-	-	-	-	-	4.0	4.0	-	-					

a : zygotic embryos, b : secondary needles and c : apical dome sections.* from this stage cultures were transferred to growth regulators free medium. ** Embryogenic cultures were cold treated at 4°C for 24 h.

Table 28. Effect of 2,4-D and NAA in mMS medium@ on initiation of embryogenic suspension culture from zygotic embryos

2,4-D conc. (mg/l)	NAA conc. (mg/l)	Type of response	Performance*
0	0	Cultures degenerated	-
2.5	0	Poor culture proliferation and few single cells formed	+
5.0	0	As above	+
7.5	0	Moderate culture proliferation and singulation of cells. Cultures turned slightly brown	+
10.0	0	Cultures turned brown	+
0	2.5	Poor culture proliferation and separation of cells was not satisfactory	+
2.5	2.5	As above	+
5.0	2.5	Moderate culture proliferation and singulation of cells	+
7.5	2.5	As above	+
10.0	2.5	Moderate culture proliferation and separation of cells but cultures turned brown	+
0	5.0	Moderate singulation of cells and culture proliferation	+
2.5	5.0	As above but slightly better in appearance	+
5.0	5.0	Very good singulation of cells. Cultures were healthy and overall performance was satisfactory	+++
7.5	5.0	Culture proliferation and separation of cells was good and moderately healthy cultures	++
10.0	5.0	As above but cultures turned slightly brown	+
0	7.5	Moderate singulation of cells and culture proliferation	+
2.5	7.5	As above	+
5.0	7.5	Moderate singulation of cells and culture proliferation	++
7.5	7.5	Moderate culture proliferation but cultures turned brown	+
10.0	7.5	As above	+
0	10.0	Moderate separation of cells but cultures turned slightly brown	+
2.5	10.0	As above	+
5.0	10.0	Cultures turned brown	+
7.5	10.0	Good singulation of cells but cultures turned browned	+
10.0	10.0	Cultures turned browned and degenerated	+

@: containing BAP (2.5 mg/l), sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). * -: no response, +: poor response, ++: moderate response and +++: optimum response

Zygotic embryos: In the absence of kinetin and NAA, embryos could not germinate (Table 26). With kinetin in the medium poor rooted plantlets were formed. Using NAA alone at lower concentrations, poor shoot formation occurred while at higher concentrations callusing of embryos resulted. An optimum response of 40% embling formation was recorded in the medium with combination of kinetin (5.0 mg/l) and NAA (1.0 mg/l) at 1900 lux light of 12 h photoperiod (Figure 13). Higher concentrations of both kinetin and NAA in the medium resulted in callusing of somatic embryos.

Secondary needles: The cotyledonary embryos cultured on MS medium containing various levels of sucrose, kinetin and NAA singly or in combination and exposed to dark and 12 h photoperiod did not germinate and subsequently degenerated.

The complete media formulation for induction of somatic embryogenesis on semisolid culture is shown in table 27.

3.2 Induction of somatic embryogenesis in suspension culture

3.2.A Initiation of cultures

a) Effect of plant growth regulators

Zygotic embryos

Auxins: In the control, the cultures degenerated in the medium free of 2,4-D and NAA but containing BAP (2.5 mg/l) (Table 28). In general,

Table 29. Effect of BAP in mMS medium@ on initiation of embryogenic suspension cultures from zygotic embryos

BAP conc. (mg/l)	Type of response	Performance*
0	Very poor culture growth and formation of a few single cells	+
0.5	As above	+
1.0	Moderate culture growth and singulation of cells	+
1.5	As above	+
2.0	Moderately healthy cultures with single cells. Culture proliferation was moderate	++
2.5	Healthy culture growth and good singulation of cells	+++
3.0	As above but slightly poorer culture growth	++
3.5	Very few single cells and more cell clumps	+
4.0	As above	+
4.5	Growth of cultures was poor and only cell clumps observed. Cells were attached to the parent tissues	+
5.0	As above	+

@: mMS medium supplemented with 2,4-D and NAA (5.0 mg/l each), sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). * +: poor response, ++: moderate response and +++: optimum response.

Table 30. Effect of 2,4-D and NAA in MS medium[@] on initiation of embryogenic suspension cultures from secondary needles

2,4-D conc. (mg/l)	NAA conc. (mg/l)	Type of response	Performance*
0	0	Cultures degenerated	-
1.0	0	Poor culture proliferation and few single cells formed	+
3.0	0	Moderate proliferation and singulation of cultures	+
5.0	0	Moderate culture proliferation and singulation of cells. Cultures turned slightly brown	+
0	1.0	Poor culture proliferation and separation of cells was not satisfactory	+
1.0	1.0	As above	+
3.0	1.0	Moderate culture proliferation and singulation of cells and healthy culture growth	++
5.0	1.0	As above but cultures turned slightly brown	+
0	3.0	Moderate singulation of cells and culture proliferation	+
1.0	3.0	As above but slightly better in appearance	++
3.0	3.0	Good cultures growth and single cell formation	+++
5.0	3.0	Good culture proliferation and separation but cultures slightly turned brown	++
0	5.0	Good singulation of cells and culture proliferation but cultures turned brown	+
1.0	5.0	As above	+
3.0	5.0	Good singulation of cells and culture proliferation but browning of cultures observed	+
5.0	5.0	Cultures turned brown and degenerated	+

@: containing BAP (1.0 mg/l), sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). * -: no response, +: poor response, ++: moderate response and +++: optimum response

with the increase in concentrations of auxins, better culture proliferation and singulation of cells was observed. NAA singly was found to be more effective than 2,4-D. In the medium containing 2,4-D and NAA (5 mg/l each), good singulation of cells with healthy growth of the cultures was recorded. With further increase in concentration of auxins resulted in browning and degeneration of cultures.

N^6-benzyl amino purine: The growth of embryogenic suspension cultures and singulation of cells varied with different concentrations of BAP in the medium supplemented with 2,4-D and NAA (5.0 mg/l each) (Table 29). In the absence of BAP, very poor culture growth resulted with formation of a few single cells. Healthy culture growth and good singulation of cells was recorded in the medium containing BAP (2.5 mg/l). Higher concentrations of BAP were found to be inhibitory.

Secondary needles

Auxins: In the control, cultures degenerated in the absence of 2,4-D and NAA in the medium (Table 30). Both 2,4-D and NAA in the medium at 3.0 mg/l each resulted in good culture growth and single cell formation. At higher concentrations of both 2,4-D and NAA

Table 31. Effect of BAP concentration in MS medium@ on initiation of embryogenic suspension cultures from secondary needles

BAP conc. (mg/l)	Type of response	Performance*
0	Very poor culture growth and formation of a few single cells	+
0.5	Moderately healthy cultures with single cells. Cultures proliferation was moderate	++
1.0	Healthy culture growth and very good singulation of cells	+++
1.5	As above but slightly poor culture growth	++
2.0	Very few single cells and more cell clumps	+
2.5	Very few single cells and more cell clumps	+
3.0	Growth of cultures was poor and only cell clumps observed. Cells were attached to the parent tissues	+
3.5	As above	+
4.0	As above	+
4.5	As above	+
5.0	As above	+

@: containing 2,4-D and NAA (3.0 mg/l each), sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). * +: poor response, ++: moderate response and +++: optimum response.

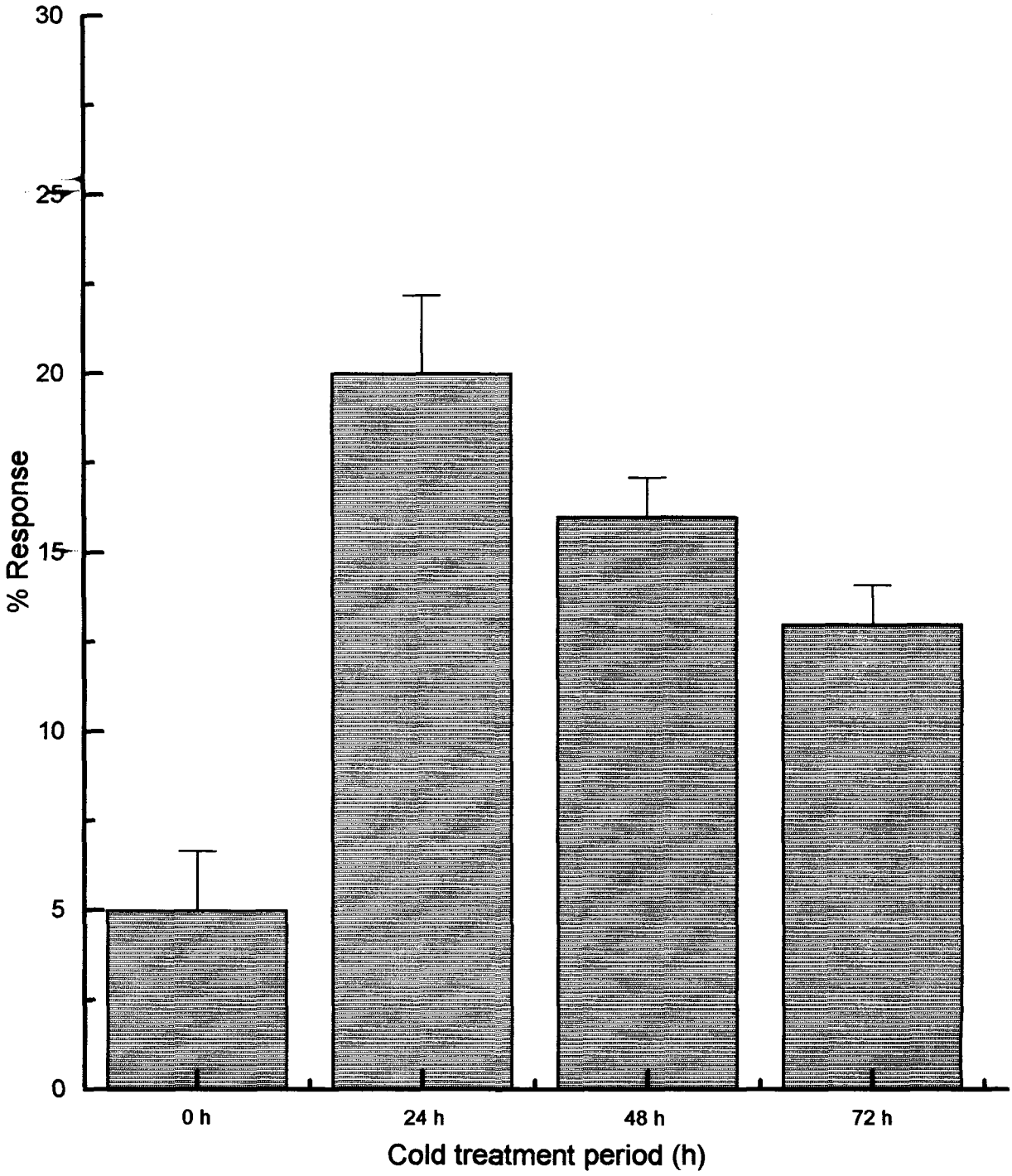


Figure 14. Effect of duration of cold pre-treatment (4°C) on initiation of embryogenic suspension cultures from apical dome sections

Table 32. Effect of 2,4-D and NAA in ½DCR medium[@] on initiation of embryogenic suspension cultures from apical dome sections

2,4-D conc. (mg/l)	NAA conc. (mg/l)	Type of response	Performance*
0	0	Cultures degenerated	-
1.0	0	Poor culture proliferation and few single cells formed	+
3.0	0	As above	+
5.0	0	Moderate culture proliferation and singulation of cells	+
7.0	0	Cultures turned brown	+
0	1.0	Poor culture proliferation and separation of cells was not satisfactory	+
1.0	1.0	As above	+
3.0	1.0	Moderate culture proliferation and singulation of cells	+
5.0	1.0	As above	+
7.0	1.0	Moderate culture proliferation and separation of cells but cultures turned brown	+
0	3.0	Moderate singulation of cells and culture proliferation	+
1.0	3.0	As above but slightly better in appearance	+
3.0	3.0	As above	++
5.0	3.0	Culture proliferation and separation of cells was good and moderately healthy cultures	++
7.0	3.0	As above but cultures turned slightly brown	+
0	5.0	Moderate singulation of cells and culture proliferation	+
1.0	5.0	As above	+
3.0	5.0	Moderate singulation of cells and culture proliferation	++
5.0	5.0	Very good singulation of cells. Cultures were healthy and overall performance was satisfactory	+++
7.0	5.0	As above but cultures turned slightly brown	++
0	7.0	Moderate separation of cells but cultures turned slightly brown	+
1.0	7.0	As above	+
3.0	7.0	Cultures turned brown	+
5.0	7.0	Good singulation of cells but cultures browned	+
7.0	7.0	Cultures browned and degenerated	+

@: containing BAP (2.5 mg/l), sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). * -: no response, +: poor response, ++: moderate response and +++: optimum response

singly or in combination poor growth and browning of the cultures resulted.

N^6-benzyl amino purine: In the absence of BAP in the medium very poor culture growth and formation of few single cells was observed (Table 31). At 1.0 mg/l BAP both healthy culture growth and very good singulation of cells resulted. However, with increase in BAP concentrations in the medium, poor growth of cultures and formation of cell clumps was recorded.

Apical dome sections

The embryogenic suspension cultures from apical dome sections required a cold treatment at 4°C before transfer to liquid medium. As a result of this treatment, the cultures became softer and suspension cultures could be raised easily. About 4-fold increase in initiation of suspension culture was recorded in 24 h cold treatment which declined with increase in duration of treatment (Figure 14). Though cold treatment for 48 and 72 h produced softer cultures, they turned brown when shifted to room temperature.

Auxins: Initiation of embryogenic suspension cultures could not be accomplished from apical dome sections in the medium devoid of 2,4-D and NAA but containing BAP (2.5 mg/l) (Table 32). Use of NAA

Table 33. Effect of BAP in ½DCR medium@ on initiation of embryogenic suspension cultures from apical dome sections

BAP conc. (mg/l)	Type of response	Performance*
0	Poor culture growth and formation of a few single cells observed	+
0.5	As above	+
1.0	Moderate culture growth and single cell formation noticed	+
1.5	As above	+
2.0	Moderately healthy cultures with single cells. Cultures proliferation was moderate	++
2.5	Healthy culture growth and good singulation of cells	+++
3.0	As above but slightly poor culture growth	++
3.5	Very few single cells and more cell clumps	+
4.0	As above	+
4.5	Growth of cultures was poor and only cell clumps observed. Cells were attached to the parent tissues	+
5.0	As above	+

@: ½DCR medium containing 2,4-D and NAA (5.0 mg/l each), sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). * +: poor response, ++: moderate response and +++: optimum response.

Table 34. Effect of inoculum size on initiation of embryogenic suspension cultures[@] from zygotic embryos, secondary needles and apical dome sections

Amount of inoculum (mg)	Type of response	Performance*
50	Good singulation of cells but, culture growth was very poor	+
100	As above but comparatively better growth	++
150	Very good singulation of cells. Cells were elongated except apical dome sections where these were spherical.	+++
200	As above	+++
250	Singulation of cells was not satisfactory. Poor cell growth due to over crowding of tissues and cells	++
300	As above but cell growth was very poor	+

@: mMS, MS and ½DCR media for zygotic embryos, secondary needles and apical dome sections respectively. The mMS and ½DCR media were supplemented with 2,4-D and NAA (5.0 mg/l each), BAP (2.5 mg/l) whereas, MS medium was supplemented with 2,4-D and NAA (3.0 mg/l each) and BAP (1.0 mg/l). All the media contained sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). *: +: poor response, ++: moderate response and +++: optimum response.

Figure 15. Stages of development of somatic embryos in suspension cultures

a. Initial stage in suspension culture (1 week old) started releasing single cells
b. Characteristic embryogenic elongated cells along with initiation of cleaving
c. Cleavage polyembryony (initial stage)
d. Embryonal suspensor masses
e. Initiation of embryonal suspensor masses accumulation

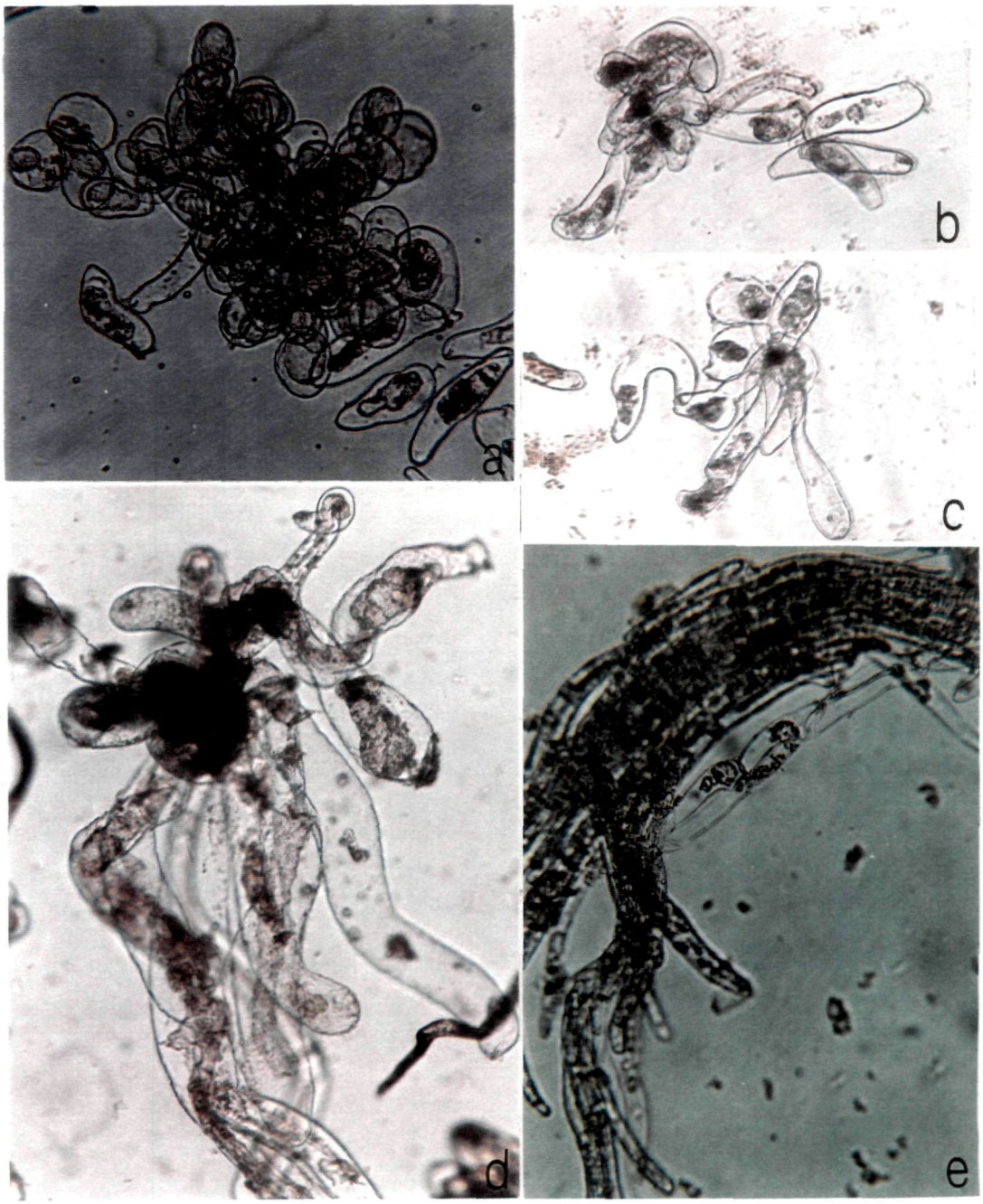


Figure 15

singly was better compared to 2,4-D. At 5.0 mg/l each of 2,4-D and NAA in the medium, healthy growth of the cultures and very good singulation of cells was observed. However, higher concentrations were found to be inhibitory.

N^6-benzyl amino purine: The growth of embryogenic suspension cultures and singulation of cells varied with different concentrations of BAP in the medium supplemented with 2,4-D and NAA (5.0 mg/l each) (Table 33). In the absence of BAP, very poor culture growth resulted with formation of a few single cells. Healthy culture growth and good singulation of cells was recorded in the medium containing 2.5 mg/l BAP. Higher concentrations of BAP were found to be inhibitory.

b) Effect of inoculum size

The inoculum size exhibited a pronounced effect on initiation of suspension cultures from all the explants (Table 34). Using 50 mg inoculation per 15 ml of medium resulted in very poor culture growth. However, singulation of cells was good. About 150-200 mg inoculum size was found to be suitable for initiation of suspension cultures and singulation of cells. Inoculum size higher than this resulted in poor response.

Table 35. Effect of growth regulators in media# on maintenance of embryogenic suspension cultures obtained from zygotic embryos, secondary needles and apical dome sections

Growth regulators' level*	Type of response	Performance@
$1/5^{th}$	Very few cells showing cleaving and it was not distinct. Very few ESMs formation noticed	++
$1/10^{th}$	Cultures were healthy and cleavage polyembryony observed in almost all the cells. ESMs were distinct in cultures from all the different explants	+++
0	Single cells did not cleave. Cultures turned brown and ultimately degenerated	+

#: mMS medium for zygotic embryos, MS medium for secondary needles and ½DCR medium for apical dome sections. All the media were supplemented with sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). *: compared to respective initiation medium. @: +: poor response, ++: moderate response and +++: optimum response.

The culture dilutions at the ratio of 1:4 for both zygotic embryos and secondary needles and 1:5 for apical dome sections were found to be optimum. The formation and release of single cell from mother tissues was observed after 1 week of culture (Figure 15a). It was necessary to free the single cells from parent tissues at the early stage of culture failing which they exhibited degeneration.

The characteristic elongated single cells obtained from cultures of zygotic embryos and secondary needles, upon subculture for 3-4 passages, started cleaving (Figure 15b). The single cells in suspension cultures, obtained from apical dome sections, did not show any cleavage during 2-3 passages of subcultures. They elongated in subsequent 2-3 passages and started cleaving (Figure 15c).

3.2.B Maintenance of cultures

The elongated single cells showing initiation of cleavage were maintained on the same respective basal medium with sucrose (2.0%) and various levels of reduced growth regulators (Table 35) for 4-5 passages. No further cleavage of single cell was recorded in the media without any growth regulators and the cultures turned brown and degenerated. Very few cells exhibited cleavage and ESMs formation in the medium containing $1/5^{th}$ of growth regulators. With further

Table 36. Complete media formulation for induction of somatic embryogenesis from zygotic embryos, secondary needles and apical dome sections in liquid cultures

Components	Initiation and proliferation of embryogenic cultures			Maintenance of cultures			Somatic embryo development		
	a	b	c	a	b	c	a	b	c
Basal medium	mMS[l*]	MS[l]	½DCR[l]	mMS[l]	MS[l]	½DCR[l]	mMS[l]	MS[l]	½DCR[l]
Supplements (g/l)									
Sucrose	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	30.0
Casein-hydrolysate	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Myo-inositol	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
L-glutamine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
PVP	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Citric acid	-	-	0.1	-	-	0.1	-	-	0.1
Growth regulators (mg/l)									
2,4-D	5.0	3.0	5.0	0.5	0.3	0.5	-	-	-
NAA	5.0	3.0	5.0	0.5	0.3	0.5	-	-	-
BAP	2.5	1.0	2.5	0.25	0.1	0.25	-	-	-

a: zygotic embryo, b: secondary needles, c: apical dome sections. l: Liquid medium

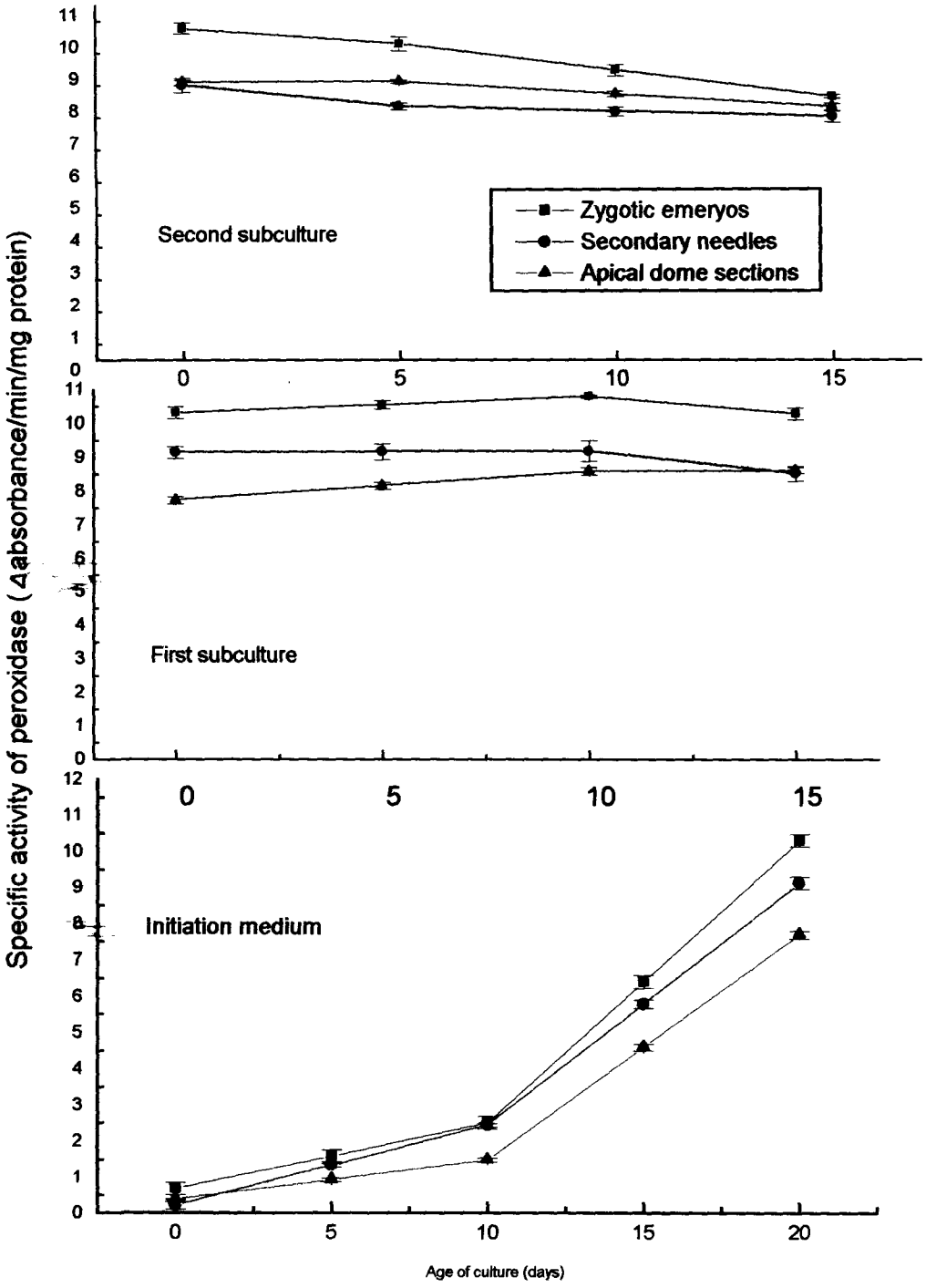


Figure 16. Specific activity of peroxidase in embryogenic cultures obtained from zygotic embryos, secondary needles and apical dome sections

reduction of growth regulators to $1/10^{th}$ in the medium the cultures were healthy and showed cleavage polyembryony in all most all the cases with distinct ESMs formation (Figure 15d). The ESMs elongated further and started to come in close proximity to each other within 3-4 passages when transferred on respective basal medium free of growth regulators but containing various levels of sucrose (Figure 15e). In case of cultures obtained from zygotic embryos and secondary needles, a sucrose concentration 2.0% was found to be very effective whereas it was 3.0% for cultures from apical dome sections.

The complete media formulation for induction of somatic embryogenesis in suspension cultures is shown in table 36.

3.3 Assay of peroxidase activity and estimation of protein

Peroxidase

Before culture, zygotic embryos, secondary needles and apical dome sections showed a very low specific activity of peroxidase. During culture on initiation medium it increased gradually upto day 10 and subsequently a marked increase was recorded till day 20 of culture (Figure 16). On day 15 and 20 the embryogenic callus was separated from explants and used for assay. The activity of peroxidase was maximum in zygotic embryos followed by secondary needles and

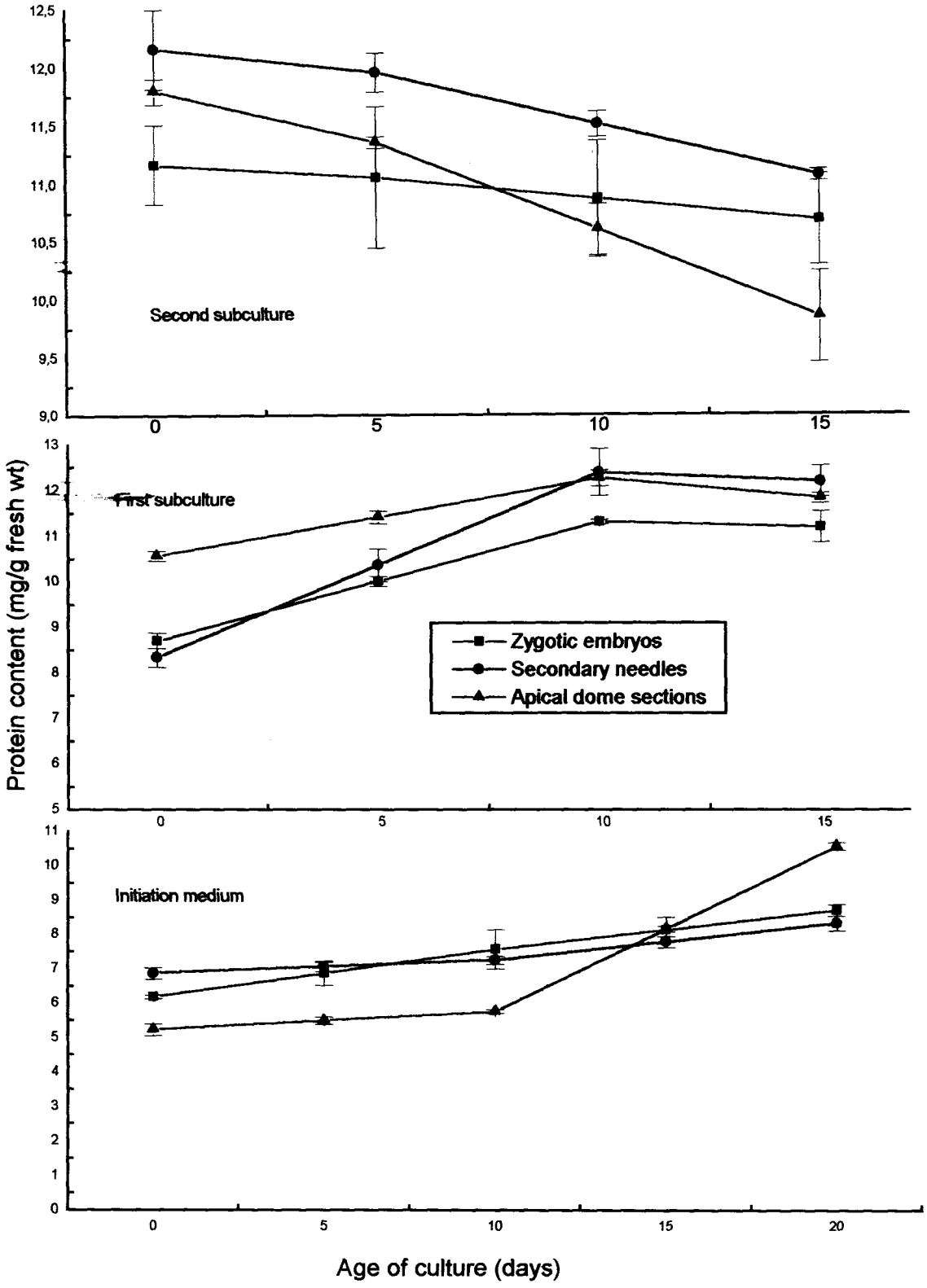


Figure 17. Total protein content in embryogenic cultures obtained from zygotic embryos, secondary needles and apical dome sections

apical dome sections. During the first subculture the activity of peroxidase in embryogenic cultures from all the explants showed a gradual increase in activity upto day 10 and thereafter showed a slight decline. During second subculture the peroxidase activity showed a marked decline during 15^{th} day of cultures.

Protein

The protein content was maximum in secondary needles followed by zygotic embryos and apical dome sections before their culture (Figure 17). In the former two cases the protein content increased steadily upto day 20 of growth. In case of apical dome sections, there was a slight increase in protein content upto day 10 and thereafter a marked increase was recorded. The protein content in all the cultures increased considerably upto day 10 of first subculture and then showed a gradual decline on day 15. During the entire period of second subculture the protein content decreased in all the cultures.

Chapter - 4

Discussion

The somatic embryogenesis in conifers is restricted to explants like mature and immature zygotic embryos, cotyledons from germinating seeds, secondary needles, female gametophytes etc. But till date there is no report of induction of somatic embryos in any conifer using explants from mature trees. In *Pinus kesiya* it was possible to induce somatic embryogenesis from mature zygotic embryos, secondary needles from seedlings grown in a glasshouse and apical dome sections from mature trees.

Dissecting immature zygotic embryos from seeds throughout the growing season to provide sufficient embryogenic material for experimentation is very labour-intensive and the material is available for only a short duration annually. The induction of somatic embryogenesis from mature embryos dissected from stored seed can extend the period during which embryogenic material can be

generated from just a few months to more than a decade (Gupta and Durzan, 1986a; Tautorus *et al.*, 1990). Since mature seeds contain embryos of similar developmental stage which are available throughout the year, the variability of response observed with immature embryos of differing maturity is eliminated, thus permitting detailed studies of factors influencing induction. Mature seeds of *P. kesiya* collected during late January to late February produced better embryogenic cultures (Table 3). This is the period when seeds dehisced. The seeds collected during March gave poor response, as the embryos in these seeds were not healthy.

In the present study, a poor embryogenic response was recorded in case of zygotic embryos dissected from dry seeds. The stratification of seeds for 24 h at 4°C prior to dissection of embryos exhibited a pronounced promotive effect on the formation of embryogenic cultures. Increase in the stratification period and use of 1 week old germinating embryos (cultured at 25°C) resulted in poor embryogenic response. The effect of seed imbibition period on induction of somatic embryogenesis was investigated by various workers. Tremblay (1990) reported better frequency of induction of embryogenic cultures following a 4 h imbibition of *P. glauca* seeds. Other workers like

Hakman *et al.* (1985) in *P. abies*; Hakman and Fowke (1987a) in black and white spruce; Finer *et al.* (1989) in *P. strobus*; Attree *et al.* (1990b) in black and white spruce; Nagmani *et al.* (1993) in *P. palustris*; Lelu *et al.* (1994) and Bonga *et al.* (1995) in *L. decidua* reported that, pre-cotyledonary to pre-germinating embryos were suitable for initiation of embryogenic culture. In *P. strobus* (Finer *et al.*, 1989) reported that the best stage of embryo development 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 germinating embryos of *P. kesiya* could be due to the fact that, biochemical and molecular events may have set in which lead to germination.

Secondary needles of *P. kesiya* from 5-6 weeks old seedlings collected during April to May produced better embryogenic cultures than collected during the other parts of the year. A heavy rainfall and moderate temperature are experienced which are ideal during April to May, which are ideal for seed germination and formation of healthy needles in the natural conditions. From June onwards the secondary needles become harder and show poor initiation of callus, which is

mostly non-embryogenic, and hard in texture. There are several reports available in literature on the formation of embryogenic cultures using secondary needles. In *P. abies* embryogenic callus was established from explants excised from 7 day 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 and 10 year stored seeds (Attree *et al.*, 1990b). Ruaud *et al.* (1992) described induction of somatic embryogenesis of *P. abies* using needles of 14 months old somatic seedlings growing in a greenhouse. Wescott (1992) could induce embryogenic callus from buds and needles of seven year old trees of *P. abies*.

The sections of apical dome of *P. kesiya* collected during May to July (before flushing of needles) formed better embryogenic cultures. During this period pine forests rejuvenate and cambial activity is maximum. The apical domes are also highly meristematic. The thinner the apical dome sections (0.2-0.5 mm thick) better was the embryogenic response. Priming of apical dome sections at 4°C for 72 h on ½DCR medium containing 0.4% activated charcoal resulted in optimum embryogenic cultures (Figure 4 and 5). A higher temperature of 25°C resulted in browning of cultures. The low temperature

treatment of explants in the medium containing activated charcoal may have helped in adsorption of phenolic compounds and other inhibitors. The promotary effect of activated charcoal on the initiation of polyembryogenesis has been documented. Webb and Flinn (1991) added 1.0% activated charcoal in the initiation medium which triggered embryogenic callus formation in *P. strobus*. To induce polyembryogenesis in *P. menziesii*, Gupta *et al.* (1995b) used activated charcoal in the medium for absorbing endogenous ABA along with other plant growth regulators and other inhibitory metabolic by-products.

In the present study, the browning of the medium was maximum in case of apical dome sections followed by secondary needles and zygotic embryos. The browning of the medium could be prevented by incorporating different anti-oxidants (200 mg/l PVP for zygotic embryos and secondary needles and 200 mg/l PVP + 100 mg/l citric acid for apical dome sections). Gupta (1980) reported that in case of shoot tip cultures of apple and teak agitating the plant materials for an hour or so in an liquid medium containing PVP, β-mercaptoethanol, dithiothreitol, glutathione (0.5-2%) helped in preventing the oxidation of phenolic compounds. Narayanaswamy (1994) discussed that culture

medium fortified with anti-oxidants like ascorbic acid and citric acid (500-2000 mg/l) can curtail the effects of phenolic oxidation.

The effect of different media and inorganic and organic nitrogen sources on somatic embryogenesis in conifers has been extensively studied (Chirstie and Butler, 1994; Bonga *et al.*, 1995; Barrett *et al.*, 1997; Norgaard, 1997). Amongst the different media tried, mMS medium for zygotic embryos, MS medium for secondary needles and ½DCR medium for apical dome sections were found suitable for initiation of embryogenic cultures in *P. kesiya*. In the present study, pH levels of 5.5-5.75 were found to be suitable for culture of all the explants. For initiation of embryogenic cultures of *P. palustris*, the pH of the medium was adjusted to 6.0 (Nagmani *et al.*, 1993). The zygotic embryos showed better embryogenic response in MS medium when KNO_3 level was increased to 4460 mg/l and NH_4NO_3 was decreased to 550 mg/l whereas secondary needles responded well on MS full strength inorganic salts and organic nutrients (Table 8 and 9 respectively). The apical dome sections produced better embryogenic cultures on half strengths of all inorganic salts and full organic nutrients of DCR medium. All the three explants of *P. kesiya* also required higher concentrations of CH (1000 mg/l) and L-glutamine

(500 mg/l) besides inorganic nitrogen sources for initiation of embryogenic cultures. Gupta and Durzan (1986b) used MS medium with modified levels of NH_4NO_3 (550 mg/l), KNO_3 (4676 mg/l). A half strength of modified MS medium supplemented with CH (500 mg/l), myo-inositol (1000 mg/l), L-glutamine (450 mg/l) and sucrose (3.0%) was used for somatic polyembryogenesis of *P. abies*. For induction of embryogenic callus formation in *P. strobus*, in MSG medium (Wann *et al.*, 1987a; Becwar *et al.*, 1988) NH_4NO_3 was completely replaced by 1450 mg/l glutamine, KNO_3 was reduced from 1900 to 100 mg/l and KCl was added at 745 mg/l. Furthermore 1.0% activated charcoal was used. Finer *et al.* (1989) used DCR medium containing 50 mg/l glutamine to induce embryogenesis in *P. strobus*. For mature zygotic embryos comparisons of full strength LP and half strength Litvay media gave similar induction frequencies when tested for both *P. glauca* (Tremblay, 1990) and *P. mariana* (Tautorus *et al.*, 1990). In these cases CH was included in most of the media tested. In a comparative study Hristoforoglu *et al.* (1995) reported, an increase of number of *P. abies* embryogenic lines by three times in a medium containing glutamine and asparagine than the one containing NH_4NO_3 and KNO_3. Embryogenic lines proliferated faster on medium

containing glutamine and CH than medium lacking them. Norgaard (1997) reported better embryogenic culture formation of *A. nordmanniana* on ½BLG medium (Verhagen and Wann, 1989) when NH_4NO_3 was removed completely, reduced KNO_3 (50 mg/l) and KCl (372.5 mg/l) added along with L-glutamine (750 mg/l) and L-asparagine (50 mg/l). Barrett *et al.* (1997) investigated the effects of glutamine-based dipeptides, glutamine and CH as well as deletion of organic nitrogen on induction of somatic embryogenesis in *P. glauca*. They reported that the removal of organic nitrogen sources was promotive for embryogenesis. 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*. It was further reported that BM_1 medium enhanced extrusion and proliferation frequency compared to DCR_1 basal medium, while LP basal salts were ineffective for initiation of culture. Kim *et al.* (1999) used LM (Litvay *et al.*, 1985), LP and MS media for induction of somatic embryogenesis from immature zygotic embryos of *L. leptolepis* and recorded 60.0%, 67.0% and 59.0% embryogenic tissue formation respectively. Though LP medium was slightly better at the initial stage, with time LM medium proved to be

more effective in that species since embryogenic tissue on LP medium failed to proliferate.

In general, glutamine is beneficial for the induction of conifer somatic embryogenesis. Negative effects have only rarely been reported (Barrett *et al.*, 1997). Von Arnold (1987) found that the rate of initiation of *P. abies* embryogenic cultures was higher in NH_4NO_3-containing medium without glutamine. Incorporation of myo-inositol and CH both at 1000 mg/l in the medium resulted in better embryogenic cultures of all the explants of *P. kesiya* compared to use of either of these compounds singly.

Generally for initiation of somatic embryogenesis in conifers, lower concentration of organic carbon sources were more effective (Von Arnold and Hakman, 1986; Von Arnold, 1987; Becwar *et al.*, 1988; Nagmani *et al.*, 1993; Bonga *et al.*, 1995; Li *et al.*, 1998). In the present study, for initiation of embryogenic cultures, lower concentration of sucrose (2.0%) was found to be very effective for zygotic embryos and secondary needles, except apical dome sections where no significant difference in embryogenic response was recorded between media containing 2.0% and 3.0% sucrose. Becwar *et al.* (1988) reported that low sucrose concentration (1.0%) produced more

ESMs. Using maltose, glucose and sucrose as organic carbon source for initiation of embryogenic cultures of *P. palustris* from zygotic embryos and female gametophytes the better embryogenic cultures could be initiated on medium containing 3.0% sucrose. Kim *et al.* (1999) reported that 2.0% sucrose in the medium resulted in better initiation of embryogenic cultures of *L. leptolepis*.

Usually both an auxin and a cytokinin are necessary for induction of embryogenic cultures in conifers (Attree and Fowke, 1991). 2,4-D generally has been the preferred auxin for the initiation of ESMs of most conifer species (Gupta *et al.*, 1991; Tautorus *et al.*, 1991). NAA has also been successfully used in some cases. Verhagen and Wann (1989) found that 2,4-D and NAA were equally effective in promoting induction of somatic embryogenesis from mature embryos of Norway spruce. Von Arnold (1987) obtained slightly higher frequencies with 2,4-D (at 20 μM) compared to NAA at the same concentration, but NAA was more effective at lower concentrations (5 μM). Jain *et al.* (1988) found that NAA was more effective than either IAA or 2,4,5-T, but did not compare 2,4-D in the same experiment. For *P. kesiya* though 2,4-D and NAA were the preferred auxins like other conifers (Verhagen and Wann, 1989; Gupta *et al.*, 1991;

Tautorus *et al*, 1991; Gupta and Grob, 1995; Gupta *et al*., 1995a, 1995b; Norgaard, 1997; Li *et al*., 1998; Kim *et al*., 1999), NAA singly was found to be more effective than 2,4-D for all the explants. 2,4-D and NAA at 5.0 mg/l each were highly effective in initiation of embryogenic cultures for zygotic embryos and apical dome sections while for secondary needles 3.0 mg/l each of 2,4-D and NAA were required for optimum response. Combined effect of 2,4-D and NAA was beneficial compared to use of either of them alone. It was observed that incorporation of BAP in auxin rich medium was stimulatory for initiation of embryogenic cultures. The present investigation is in agreement with the observations of other reports on conifers (Cornu and Geoffrion, 1990; Gupta *et al*., 1991; Nagmani *et al*., 1993; Bonga *et al*., 1995; Guevin and Kirby, 1997). Li *et al*. (1998) reported that a combination of 3.0 mg/l 2,4-D and 0.5 mg/l BA was found better than higher concentrations for initiation of embryogenic cultures in *P. taeda*. In *P. kesiya*, the media containing cytokinins (BAP and kinetin) were was found to be inhibitory for embryogenic culture formation except apical dome sections where BAP and kinetin (each at 3.0 mg/l in combination) could produce little reddish brown callus which subsequently degenerated. However, BAP

in the medium along with auxins accelerated embryogenic culture formation but at higher concentrations resulted in hard non-embryogenic cultures. ESM culture from *A. nordmanniana* were best initiated with cytokinins (BA and kinetin) alone, with auxin found to be inhibitory (Norgaard and Krogstrup, 1991).

In the present study, light was found to be inhibitory for initiation of embryogenic cultures, which produced more green and non-embryogenic cultures. Dark was preferred but light at 20 lux formed moderate embryogenic cultures except apical dome sections, where completely hard and green callus resulted. In general, induction of embryogenic cultures in conifers has mostly been done in the dark (Gupta and Durzan, 1986a; Von Arnold, 1987; Gupta and Grob, 1995) except in case of Norway spruce where initiation of embryogenic culture was equally well under light and dark (Verhagen and Wann, 1989). Von Arnold (1987) reported that in Norway spruce, culture under light of 20 h photoperiod was inhibitory for induction of embryogenic cultures. Further, lower induction frequencies were reported in the light compared to the dark, but no significant differences existed.

Cultures on semisolid medium from all the explants after few subcultures ceased proliferation and in some cases started browning on auxin rich medium (Table 22). It was necessary to transfer the cultures on basal media containing reduced levels of growth regulators before culture proliferation ceased. The gradual removal of growth regulators resulted in the formation of PEMs and proembryos. Higher auxin concentration interfered with the development of polarity. When auxins were withdrawn gradually, the polarity developed followed by the formation of proembryos. It was observed that complete removal of growth regulators resulted in degeneration of cultures. Durzan and Gupta (1988) maintained the ESMs in the medium containing lower concentrations of plant growth regulators (1-2 mg/l 2,4-D and 0.1 mg/l kinetin and BA). Nagmani *et al.* (1993) reduced the 2,4-D levels gradually to 0.5 mg/l for maintenance of long leaf pine cultures and transferred them from dark to diffuse light conditions. 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*.

In the present study, the culture raised from apical dome sections on semisolid medium were not very soft and translucent until

they were cold treated at 4°C for 24 h before transferring in liquid medium. After cold treatment softer embryogenic cultures resulted.

For initiation of suspension cultures of *P. kesiya*, lower inoculum size (150-200 mg/15 ml medium) was found to be suitable. By decreasing the inoculum (<150 mg/15 ml medium) a poor culture growth was recorded which may be due to inadequate culture density. Higher inoculum at the initial stage slowed the proliferation of cultures which may be due to over crowding of cells. Finer *et al.* (1989) reported that suspension cultures proliferated well with a low culture density in *P. strobus* while, Krogstrup (1990) reported that culture density was crucial and determined the quality of early stage embryos in suspension cultures in *P. sitchensis*.

In the present study, it was necessary to separate embryogenic cultures from non-embryogenic parts grown on semisolid medium. It was necessary to free single cells from mother tissues in suspension cultures failing which degeneration of cultures resulted. The ESMs formation resulted from elongated single cells through cleavage in the suspension medium with reduced growth regulators. They started accumulation in the medium free of growth regulators but containing higher sucrose concentrations. The cultures required low agitation for

proembryonal head formation because at higher speed the embryonal suspensor cells maintained a distance which was not enough for accumulation of cells.

Abscisic acid has been used for cotyledonary embryo development in many plant species (Skriver and Mundy, 1990) including conifers (Gupta and Durzan, 1987). In the present study, the proembryos converted into cotyledonary embryos on respective semisolid basal media containing higher concentrations of sucrose in conjunction with ABA (Table 23). It was observed that neither sucrose nor ABA singly could promote maturation of somatic embryos. A concentration of 4.0% sucrose and 4.0 mg/l ABA was found to be effective for somatic embryo maturation. It is believed that the higher osmoticum helped in the development of embryos while ABA caused desiccation stress resulting in maturation of somatic embryos. 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 can serve as an osmotic agent and as a carbon and energy source. Black spruce somatic embryo maturation. Further, the maturation medium containing mannitol could not promote somatic

embryo maturation. Norgaard (1997) reported that maltose (3.4-4.3%) used singly was better organic carbon source than sucrose (3.0%) for maturation of somatic embryos and germination in *A. nordmanniana.* The combination of both maltose and sucrose was more effective than either of them used singly. Carrier *et al.* (1997) studied the effect of exogenous sucrose on maturation and germination of somatic embryo of interior spruce and 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 the available sucrose in the medium. This exogenously supplied sucrose promoted the formation of linolenic acid, which participated in the maturation of embryos. Durzan and Gupta (1987) discussed that ABA inhibits cleavage polyembryony and allows embryo singulation and further development in Douglas fir. Roberts (1991) reported that a higher concentration of mannitol (6.0%) as a organic carbon source promoted the formation of globular embryos in callus culture of spruce, but few of them could convert into cotyledonary embryos. Like mannitol, ABA also promotes formation of globular embryos. But combination of mannitol and ABA enhanced the effect of ABA on production of globular embryos. Higher concentration of mannitol was inhibitory.

Although mannitol promoted formation of globular embryos, it inhibited the maturation of the embryos. Webster *et al.* (1990) reported that 40 µM ABA was optimum for the production of mature embryos. In *P. abies*, the embryo development occurred following the removal of auxins and cytokinins and addition of ABA (Gupta *et al.*, 1995a).

Vagner *et al.* (1998) reported that with increase in exogenous ABA levels (5-40 µM), an increase in somatic embryo maturation of *P. abies* was recorded both in semisolid and liquid medium. In the absence of the exogenous ABA, embryo yield was negligible. Embryogenic cultures on auxin rich medium produce ethylene which act as inhibitor for somatic embryo development (Biddington *et al.*, 1993). They 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* cultures. It was observed that more ethylene was produced in non-embryogenic than in embryogenic cultures. Ethylene interfered with the development of polarity. The media supplemented with ethylene inhibitors like ABA inhibited ethylene formation and promoted somatic embryo maturation. Kong

and Yeung (1994) studied the influence of ethylene on somatic embryo maturation of white spruce by incorporating various ethylene inhibitors in the medium. They reported that the addition of ABA in the development and maturation medium caused decrease in ethylene production. In the absence of any ethylene inhibitor, the cotyledonary embryo formation was very poor. ABA decreased ethylene formation in the initial stage of cultures but the production of ethylene increased during later. It was further reported that polarity developed with decrease in ethylene production.

In the present study, the somatic embryos (developed from zygotic embryos and secondary needles) became distinct and elongated on basal media without any growth regulators, CH and L-glutamine but containing activated charcoal (0.2% and 0.4% respectively). Activated charcoal adsorbed all the endogenous growth regulators and growth inhibitors and stimulated better singulation and elongation of embryos. Becwar *et al.* (1989) transferred the Norway spruce cultures with ESMs on hormone-free medium containing activated charcoal for 1 week. It was thought that the charcoal adsorbed cytokinins and auxins, which caused the cultures to cease cleavage polyembryony. Pullman and Gupta (1991) could produce

cotyledonary embryos of *P. abies* on medium containing ABA (50 mg/l) and activated charcoal (1.25 g/l). Further, the combined ABA and charcoal treatment improved embryo quality. Gupta *et al.* (1993) reported that a combination of increased osmolarity with ABA and activated charcoal produced good quality cotyledonary embryos. In *P. menziesii* the embryo development and maturation was promoted by amending the medium with activated charcoal (1.25 g/l) and ABA (30 mg/l) (Gupta *et al.*, 1995a).

In the present study, somatic embryos germinated in the light (1900 lux) at 12 h photoperiod and 40% germination was recorded. The embryos of *P. abies* germinated best after 7 days in the dark followed by continuous light and 80-90% germination was recorded (Gupta *et al.*, 1995b).

A very low specific activity of peroxidase and protein content were recorded in all the explants of P. kesiya before culture. With growth on respective initiation media, the peroxidase activity and protein content increased gradually and were maximum at day 10 of the first subculture. This was the period when embryogenic cultures started forming PEMs. The peroxidase activity and protein content declined subsequently during second subculture. Increase in

peroxidase activity and protein content was correlated with the initiation of somatic embryogenesis. Once the process initiated, the peroxidase activity and protein content decreased. The peroxidase activity and protein content could be used as useful markers to identify the onset of embryogenesis (Jain *et al.*, 1990). Zhou *et al.* (1992) studied the role of peroxidase on induction of somatic embryogenesis in *L. sativa*. They reported the peroxidase activity increased prior to visual manifestation of embryoids and also synthesized specific iso-peroxidases. Once this process started, peroxidase activity decreased. This phenomenon was not observed in the non-embryogenic cultures.

Pitel *et al.* (1992) studied the activities of 10 different enzymes and protein contents during maturation of somatic embryos of *L. eurolepis* hybrid. Further, there was no significant difference in protein contents and patterns in non-embryogenic and embryogenic cell lines on maintenance medium. On maturation medium, all the 10 enzyme activities increased upto 15 days of culture after which gradually decreased. In cultures grown on maturation medium, the protein content started increasing after day 9 and reached the peak on day 15 followed by gradual decrease in the quantity and number of bands. Donga and Dunstan (1994) reported that proteins rapidly

accumulated to the highest value (1109 mg/l) at day 9 at the rate of 94.7 mg/l/d during early stages of initiation of embryogenesis of *P. glauca*. The intracellular protein content decreased after day 9 of culture.

In the present study, the initiation, development and maturation of *P. kesiya* somatic embryos have been achieved. This opens up a great potential for large-scale propagation of this species which is facing rapid denudation. Further work on somatic embryo development in liquid medium and increase in conversion frequency of somatic embryos developed on semisolid medium need to be worked out in future.

Chapter - 5

Summary

Pinus kesiya seeds collected during late January to late February were ideal for initiation of embryogenic cultures, whereas the seeds collected during March exhibited poor embryogenic response. The optimum embryogenic response was recorded in the secondary needles collected during April and May where whole needle surface was full of white, soft and translucent callus. About ~90.0% embryogenic response was recorded in apical domes (collected during May to July) before emergence of the needles. This response declined considerably in apical domes showing emergence of needles.

Incorporating PVP and citric acid in the culture media prevented the browning of both explants and media. 200 mg/l of PVP was found to be very effective for zygotic embryos and secondary needles whereas, a combination of 200 mg/l PVP and 100 mg/l citric acid for apical dome sections.

The optimum embryogenic cultures (79.6%) from zygotic embryos (from seeds stratified at 4°C for 24 h) resulted within 3 weeks in mMS medium containing 2,4-D, NAA (5 mg/l each), BAP (2.5 mg/l), sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l). The embryogenic cultures were white, soft, translucent and gelatinous whereas, the non-embryogenic cultures were hard and green. The embryogenic response in ½mMS (57.4%), DCR (57.4%), ½DCR (55.5%), LP (51.8%) and ½LP (24.2%) media were recorded in decreasing order. In case of secondary needles, the use of MS medium containing 2,4-D, NAA (3.0 mg/l each), BAP (1.0 mg/l), sucrose (2.0%), CH and myo-inositol (1000 mg/l each) and L-glutamine (500 mg/l) was optimum for initiation of embryogenic cultures (88.6% response) followed by mMS (76.4%), ½MS (59.3%), DCR (50.7%) and ½ mMS (43.6%) media. Pre-culture of the apical dome sections on ½DCR medium containing activated charcoal (0.4%) at 4°C for 72 h was found suitable for initiation of embryogenic cultures. The culture of these sections in ½DCR medium containing 2,4-D, NAA (5.0 mg/l each), BAP (2.5 mg/l), sucrose (2.0%), CH and myo-inositol (1000 mg/l each), L-glutamine (500 mg/l) resulted in optimum initiation of embryogenic cultures (92.6%)

followed by DCR (64.8%), ½mMS (59.3%), mMS (38.9%) and LP (37.0%) media. For all the explants, cytokinins alone were found to be inhibitory for initiation of embryogenic cultures. The embryogenic cultures from all the explants were obtained in the dark at 25 ±2°C. Light was inhibitory for initiation of embryogenic cultures particularly for apical dome sections. The cultures turned hard and green in the light.

The embryogenic cultures obtained from different explants proliferated well on respective initiation medium when cultured at 2 weeks interval for 2-3 passages. Softer embryogenic cultures were obtained when embryogenic cultures from apical dome sections were cold-treated at 4°C for 24 h after two subcultures on initiation medium. PEMs and proembryos were formed on maintenance medium (respective basal medium) containing $1/10^{th}$ growth regulators of initiation medium along with other adjuvants. The proembryos, developed from zygotic embryos and secondary needles, converted into cotyledonary embryos within 2 passages on respective basal medium devoid of auxin and cytokinin but containing ABA (4.0 mg/l) and sucrose (4.0%). It was not possible to convert pro- and globular embryos into cotyledonary embryos in case of apical dome sections.

The cotyledonary embryos, obtained from zygotic embryos, elongated on mMS medium without any growth regulators, CH, L-glutamine but containing myo-inositol (100 mg/l), sucrose (3.0%) and activated charcoal (0.2%) at 12 h photoperiod (1900 lux light). These somatic embryos germinated in 3-4 weeks time on mMS medium containing sucrose (3.0%), kinetin (5.0 mg/l) and NAA (1.0 mg/l) at 12 h photoperiod. A 40% conversion frequency of somatic embryos to emblings was recorded.

The cotyledonary somatic embryos, obtained from culture of secondary needles, elongated on MS medium free of all growth regulators, CH, L-glutamine and contained myo-inositol (100 mg/l), sucrose (2.0%) and activated charcoal (0.4%) under 12 h photoperiod (1900 lux light). However, it was not possible to convert cotyledonary embryos into emblings.

About 150-200 mg inoculum per 15 ml of medium was found suitable for initiation of suspension cultures. The elongated single cells formed in the culture were proliferated by subculturing at 6-7 days interval for 3-4 passages. The cultures were diluted at 1:4 ratio at every subculture for zygotic embryos and secondary needles and for apical dome sections at 1:5.

In 3-4 passages, the ESMs formed from single cells through cleavage in respective basal medium containing $1/10^{th}$ growth regulators of initiation medium. The suspensor cell masses started accumulating and formed proembryonal head in growth regulators free medium containing sucrose (2.0% for zygotic embryos, secondary needles and 3.0% for apical dome sections) at 100 rpm.

It was observed that the specific activity of peroxidase increased after 10 days of culture on initiation medium. It was maximum at 10^{th} day of first subculture and followed a declining trend during the second subculture. Increase in total protein content was recorded upto 10^{th} day of first subculture after which it declined considerably which followed a similar pattern as that of peroxidase activity.

Chapter - 6

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Sincerely Yours'

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