CHAPTER IV

SOMATIC EMBRYOGENESIS
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4.1 INTRODUCTION

Somatic embryogenesis is a process where groups of somatic cells/tissues lead to the formation of somatic embryos which resemble the zygotic embryos of intact seeds and can grow into seedlings on suitable medium. In 1952, Steward initiated work on cultured carrot explants and used coconut milk as a nutrient that ultimately led to the discovery of embryogenesis (Steward, 1958). Plant regeneration via somatic embryogenesis from single cells, that can be induced to produce an embryo and then a complete plant, has been demonstrated in many medicinal plant species. Arumugam and Bhojwani (1990) noted the development of somatic embryos from zygotic embryos of Podophyllum hexandrum on MS medium containing 2 μM BA and 0.5 μM IAA. Ghosh and Sen (1991) reported regeneration and somatic embryogenesis in Asparagus coo peri on MS medium having 1.0 mg/L NAA and 1.0 mg/L KN. Embryogenic calluses and germination of somatic embryos in nine varieties of Medicago sativa has been achieved (Fuentes et al., 1993). Using a medium containing 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and TDZ, Zhou et al., (1994) have achieved the induction of somatic embryogenesis in cells from Cayratia japonica.

Somatic embryogenesis and subsequent plant regeneration from callus derived from immature cotyledons of Acacia catechu has also been achieved on medium supplemented with 13.9 μM KN and 2.7μM NAA (Rout et al., 1995). Gastaldo and Caviglia (1996) induced somatic embryogenesis from bark callus of Aesculus hippocastanum on MS medium supplemented with 2.0 mg/L KN, 2.0 mg/L 2,4-D and 2.0 mg/L NAA. High-frequency somatic embryogenesis and plant regeneration from suspension cultures of Acanthopanax koreanum have been reported on a medium containing 4.5 μm 2, 4-D (Choi, 1997).

Das et al., (1999) Reported high frequency somatic embryogenesis in Typhonium trilobatum on medium containing 1.0 mg/L KN and 0.25 mg/L NAA. The suspension culture of Catharanthus roseus from stem and leaf explants on
medium containing NAA and KN has been established by Zhao et al., (2001) Chand et al., (2002) have reported the somatic embryogenesis of *Psoralea corylifolia* L. from root explants on medium supplemented with NAA and BA.

Efficient development and germination of somatic embryos are prerequisites for commercial plantlet production. Scientists have proved that germination of the somatic embryos is achievable on MS medium without the growth regulator. However, Arumugam and Bhojwani (1990) noted that inclusion of BA (2 μM) and gibberellic acid (GA₃, 2.8 μM) in the medium stimulated embryo development of *Podophyllum hexandrum*, although 75% of the embryos germinated on basal MS medium devoid of growth regulator. Similar results were reported on the germination of embryos of *Psoralea corylifolia* (Chand et al., 2002). Wakhlu et al., (1990) have reported that the somatic embryos of *Bunium persicum* matured and germinated on the basal MS medium supplemented with 1.0 mg/L KN.

Somatic embryogenesis can be used for scale up in liquid culture (i.e., Bioreactors) and for direct delivery to the greenhouse or field as artificial seeds. Such features make it likely that cloned propagules produced via somatic embryogenesis will have significantly lower cost per unit than those produced using other micropropagation systems (Merkle, 1995).

Rangasamy (1986) pointed out that somatic embryogenesis has acquired much importance in agriculture, silviculture, horticulture and industries involved in continuous supply of basic plant materials of elite varieties. In *Arachis hypogaea* and *Cyamopsis tetragonoloba* same auxin (2, 4-D) was used for both induction and development of embryo. On the other hand 2, 4-D in the induction medium was substituted by NAA for embryo development in alfalfa, *Medicago sativa* (Kao and Michyluk, 1981). Somatic embryos observed in suspension culture of *Vigna aconitifolia* on MS medium supplemented with KN (0.5 μm) and 2, 4-D (0.4 and 0.9 μm) (Bhargava and Chandra, 1983).

Gamborg et al., (1984) reported production of somatic embryos from cultured cells of Glycine species in liquid medium. Somatic embryos can be encapsulated and used as artificial seeds. In addition to that, there have been notable
achievements in somatic embryogenesis in few grain legumes like *Glycine max* (Lippmann and Lippmann, 1984; Li, *et al.*, 1985). According to Grant (1984), *Glycine canescens* required 10 times more minor salts, whereas in *Phaseolus coccineus* the concentration of MS major and minor nutrients had to be reduced to half for the callus induction from immature cotyledon (Genga and Allavena, 1991).

Li *et al.*, (1985) reported that 2, 4-D (1.0–40 mg/l) was individually needed for the callus induction and subsequent embryo development in *Glycine max*. Grant (1984) reported that it was essential to supplement low concentration of NAA (0.1-0.5 μm) with any one of the cytokinins like Zeatin and KN for *Glycine canescens* and KN for *Pisum sativum* (Jacibsib and Jysektm, 1984). Suspension cultures of Soybean were initiated from hypocotyl or cotyledon callus tissue for several Soybean genotypes. When these were grown in L2 medium with 0.4 mg/l 2, 4-D, several genotypes produced numerous embryoids while others produced only few such structures (Kerns *et al.*, 1986).

### 4.2 REVIEW OF LITERATURE

Stejskal and Griga (1992) reported somatic embryogenesis and plant regeneration in *Pisum sativum*. Zhou *et al.*, (1992) induced somatic embryos in lettuce cotyledons cultured on MS medium containing either 2.0 mg/l BA and 0.2 mg/l NAA or 0.2 mg/l IBA and 2.0 mg/l NAA.

Sagare *et al.*, (1993) reported plant regeneration via, somatic embryogenesis in chick pea (*Cicer arietinum*) and maximum frequency of globular embryo formation was noticed in chick pea cotyledonal segments on MS medium with 2, 4, 5-7 (30 mg/l). At the cotyledonal stage, the embryos germinated on half strength MS medium with ZN (1.0 mg/l). The regenerated plants were transferred to soil and grown to maturity. Eapen *et al.* (1990) reported the use of GA₃ in the embryo development medium and it was proved to fasten the growth and maturation of somatic embryos.
Using chick pea leaf explants, on MS medium supplemented with 2, 4-D (1.25 mg/l) and KN (0.25 mg/l), Kumar et al., (1994) developed an efficient method for plant regeneration via., somatic embryogenesis from cotyledonary explants of soybean cultivars (Glycine max). Lou and Kako (1995) reported the role of high sugar concentrations including somatic embryogenesis from Cucumber cotyledons.

Venkatachalam et al. (1997) reported the formation of embryogenic calli from hypocotyl explants of groundnut (Arachis hypogaea) cultured on a medium containing different concentrations of 2, 4-D or NAA in combination with 0.5 mg/l BAP. The type of auxin, concentration and genotypes influenced somatic embryogenesis and 2, 4-D was found to be the best somatic embryo inducer. Germination of embryo into plantlet occurred on MS medium supplemented with BAP (0.5-2.0 mg/l) and NAA (0.25 mg/l). According to Burns and Wetzstem (1997), the cultures were repetitively embryogenic and proliferated in growth regulator free medium.

Pollegrineschi et al. (1997) studied the factors responsible for successful rescue of immature embryos and in vitro embryo development in cowpea (Vigna unguiculata) and V. vexillata. A new basal medium for embryo development in vitro was formulated on the basis of the mineral composition of embryos and sucrose, fructose and glucose were compared as carbohydrate sources. According to Holme et al., (1997) the proline addition affected the formation of embryogenic callus and the growth and improvement of suspension cultures depends on the proline concentrations and the basal salts of the medium.

Sreenivasu et al., (1998) reported the plant regeneration via somatic embryogenesis in Pigeon pea (Cajanus cajan). Ramesh Anbazhagan and Ganapathi (1999) reported the somatic embryogenesis in cell suspension cultures of Pigeon pea (Cajanus cajan). Zhang et al., (2005) reported that somatic embryos were directly formed at cut edges or on the surface of leaf explants, around cut ends or along side surfaces of petiole and stem explants of ‘Golden pathos’ [Epipremnum aureum (Lindl. & Andre) Bunt.]. Somatic embryos were produced on MS medium containing 2.0 mg/l KN and 0.5 mg/l 2, 4-D from leaf and petiole explants, MS
medium supplemented with 2.0 mg/l CPPU and 0.5 mg/l 2, 4-D from petiole and stem explants, and 2.0 mg/l TDZ and 0.2 mg/l or 0.5 mg/l 2, 4-D from stem explants.

Somatic embryos matured and grew into multiple buds, shoots or even plantlets after 2-3 months on the initial culture medium. Germination was optimal on MS medium containing either 2 mg/l 6-bezylaminopurine BA and 0.2 mg/l NAA or 2 mg/l Zeatin and 0.2 mg/l NAA. Shoots elongated better and roots developed well on MS medium with no growth regulators. Approximately 30-100 plantlets were regenerated from each explant. The regenerated plants grew vigorously after transplanting to a soil-less container substrate in a shaded green house.

Magali et al. (2002) described an efficient method for embryogenic callus induction and plant regeneration from bahi grass (cv. Tifton 9) seed explants. MS medium containing 30 μm dicamba and 5 μm 6-benzyladeneine (BA) was optimal for callus induction and growth. Out of 9734 seeds cultured, 65.7% germinated and 21.4% produced embryogenic callus on this medium. Shoot formation was optimal when embryogenic calluses induced in this medium were transferred to MS medium supplemented with 5 μm BA and 1 μm gibberellic acid with 1640 plantlets formed per gram fresh weight of callus tissue. When transferred to hormone-free SH medium, shoot system produced well-developed root systems. The resulting plantlets grew normally and produced viable seeds when transferred to soil in the green house.

Development of somatic embryogenesis has been grossly divided into two process induction and expression. In the former, differentiated somatic cells acquire embryogenic competence and proliferate as embryogenic cells and cell aggregates (Komamine et al., 1992), i.e., cells with dense cytoplasm that divide very rapidly (Kiyosue et al., 1993; Nuti-Ronchi and Giorgetti, 1995). During expression of somatic embryogenesis, embryogenic cells aggregate and continue their growth by passing through a series of developmental stages, namely (in dicots) globular, oblong, heart-shaped and finally torpedo-shaped (Schiavone and Cooke, 1985).
Carrot embryogenic cultures have been used as a model system to study somatic embryogenesis in plants (Komamine et al., 1992).

Calluses with somatic embryos were produced after 3 weeks of culture, when Rhimi et al., (2006) cultured hypocotyl, cotyledon and zygotic embryo explants from two Tunisian (Cucumis melo L.) cultivars Beji and Maazoun, on the MS medium added with 0.25-1 mg/l 2, 4-D and 0.10-0.5 mg/l BA. For Beji c.v. the highest percentage (62.50%) of embryogenesis was observed for cotyledons. Cotyledonary staged embryos, when transferred to hormone free MS medium germinated. The maximum germination rates were 51.50 and 44.50% respectively for Maazoun and Beji c.v. The highest percentage (36.50%) of survival plants was noted for Beji c.v. Regenerants were diploids (2n = 2x = 24) and morphologically similar to their parents issued from seeds.

Rashmi and Rout, (2003) achieved in vitro somatic embryogenesis and subsequent plant regeneration in callus cultures derived from immature zygotic embryos of Acacia arabica on semi-solid MS basal salts and vitamins supplemented with 8.8 μm of BA, 6.78 μm 2, 4-D and 30 g/l (W/v) sucrose. The maximum number of somatic embryos per callus was 72.6 after 8 weeks of culture on medium containing 6.66 μm of BA and 6.78 μm of 2, 4-D. The isolated somatic embryos germinated on half strength basal MS salts and vitamins supplemented with 0.04 μm of BA, 0.94 μm of ABA and 2% (W/V) sucrose. The embryo derived plantlets were acclimatized in the greenhouse and subsequently showed normal growth.

Ipekci and Gozukirmizi (2003) developed a reproducible system for efficient direct somatic embryogenesis from leaf and internodal explants of Paulownia elongata. The somatic embryos obtained were subsequently encapsulated as single embryos to produce synthetic seeds. The highest induction frequencies of somatic embryos were obtained from leaf and internode explants on MS medium supplemented with 3% sucrose, 0.6% phytgel, 500 mg/l case in hydrolysate and 10 mg/l TDZ (medium MS 10). Subsequent withdrawal of TDZ from the induction medium resulted in the maturation and growth of the embryos.
into plantlets on MS basal medium. Somatic embryos obtained directly on leaf explants were used for encapsulation in liquid MS medium containing different concentrations of sodium alginate with a 30-min exposure to 50 mm CaCl₂. A 3% sodium alginate concentration provided a uniform encapsulation of the embryos with survival and germination frequencies of 73.7% and 53.3% respectively.

A simple and effective method of regenerating *Syngonium podophyllum* 'variegatum' via direct somatic embryogenesis has been established on MS medium supplemented with N-(2-Chloro - 4-pyridyl) – N-Pheny urea (CPPU) or N Phenyl – N’- 1, 2, 3- thia diazol – 5-Ylurea (TOZ) with either NAA or 2, 4-D. The frequency of petiole explants with somatic embryos was produced as high as 8.6% when cultured on medium containing 2.5 mg/l TDZ with 0.5 mg/l NAA. Upto 85% of somatic embryos were able to germinate after transferring on to medium containing 2.0 mg/l BA and 0.2 mg/l NAA. Approximately 50-150 plantlets were regenerated from a single petiole explant (Qian Zhang et al., 2006).

Somatic embryogenesis and whole plant regeneration were achieved in callus cultures derived from hypocotyl, cotyledon and leaf explants excised from seedlings of *Gymnema sylvestre*. Embryogenic callus was induced on MS medium containing 2, 4-D (0.5-5.0 μm) + BA (0.5-2.0 μm) and 2% (W/v) sucrose in 6-8 weeks of culture. Globular / heart stage embryos developed on induction medium. These embryos produced torpedo and cotyledon stages embryos upon sub-culturing on embryo maturation medium EM8 (Medium containing MS salts, B5 vitamins, 0.5 μm BA and 2% sucrose). Embryo germination and plantlet formation was achieved by sub-culturing mature embryos on fresh EM8 medium. The plantlets were acclimatized in the greenhouse.

Mandal and Datta, (2005) obtained direct somatic embryogenesis from ray florets explants of five *Chrysanthemum* cultivars within 12-15 days on MS medium supplemented with 2,4-D and BA. Somatic embryos developed asynchronously on the adaxial surface of explants. Among the five cultivars tested, Birbal sahani was best responding (40% explants responded on 4 mg/l 2, 4-D and 2 mg/l BA supplemented medium). Precocious germination of somatic embryos was noticed
on the same medium. The best sucrose concentration in the medium was found to be 60 g/l where 70% explants responded while 55% embryogenic response was obtained on medium supplemented with 400 mg/l inositol. Plants developed from Somatic embryos were transferred to soil and produced true to type flowers.

Immature zygotic embryos of *Catharanthus roseus* (Madagascar periwinkle) ‘Little Bright Eye’ formed off-white, friable calluses at a frequency of 20% on MS medium supplemented with 4.52 μm of 2, 4-D after 8 weeks of culture. After a second subculture using MS basal medium at 4 week intervals, off-white friable calluses formed a small quantity of yellowish, compact embryogenic calluses. Upon transfer to MS basal medium embryogenic calluses gave rise to numerous somatic embryos. Cell suspension cultures were established with embryogenic cultures using liquid MS medium supplemented with 4.52 μm of 2, 4-D. Embryogenic cell clumps from cell suspension cultures developed into plantlets at a frequency of 56.7% when placed on to MS basal medium. Plantlets were transplanted to potting soil and grown to maturity in a growth chamber (Suk *et al.*, 2004). Yoich Aoshima (2005) developed an efficient method for Somatic embryogenesis from callus derived from shoot apex of tea *Camellia chinensis* (L.) O.Kuntze. The addition of mannitol at 0.31 m improved somatic embryogenesis relative to that on medium without mannitol (25% and 7% respectively). When the callus was cultured on medium with small amounts of hygromycin (5-10 mg/l), the differentiation rate increased up to 43% that of control. Results suggested that culturing with osmotica or antibiotics was quite effective to induce Somatic embryogenesis from begitative-derived callus tissue of tea plants.

Kim *et al.*, (2003) described culture condition for high frequency Somatic embryogenesis and plant regeneration in petiole and leaf explant cultures and petiole derived embryogenic cell suspension cultures of *Hylomecon vernalis* Max. Petiole explants fromed embryogenic calluses at a frequency of 53% when cultured on B5 medium supplemented with 13.6 μm 2, 4-D alone. Leaf explants formed embryogenic calluses at a frequency of 21% when cultured at a combination of 4.52 μm 2, 4-D and 2.22 μm 6-benzyladenine. Cell suspension cultures were established
with petiole-derived embryogenic calluses using liquid B5 medium with 4.52 μm 2, 4-D. Upon planting onto B5 basal medium, cell suspension cultures produced numerous somatic embryos, which then developed into plantlets. Regenerated plantlets were transplanted to potting soil and grown to maturity in a greenhouse.

Duck et al., (2003) cultured the leaf explants of *Ostericum koreanum* on MS medium supplemented with 5.37 μm NAA and 0.44 μm BA and did not transfer to growth regulator-free medium for somatic embryogenesis. The explants were exposed to low pH for only several days at the early period of culture. On medium of pH 4.3 the production of somatic embryos was enhanced to six times in comparison with that on medium of pH 5.8. The average regeneration rate of total somatic embryos produced on medium of low pH was over 10% higher than that at pH 5-8. The regeneration of cup-shaped embryos was improved from 33% on medium of pH 5-8 to 67% on medium of pH 4.3. Therefore, the production and regeneration of somatic embryos were enhanced by the temporary exposure of leaf explant in the medium of low pH, even though somatic embryogenesis substantially occurred on medium of nearly routine pH.

Modified ½ MS medium (containing 85 mg/l KH₂PO₄) supplemented with 10-20 g/l sucrose, 170 mg/l NaH₂PO₄ and 0.5 g/l peptone, significantly promoted direct somatic embryogenesis from the leaf tip explants of Oncidium “Gower Ramsay” (Jen-Tsung and Weichin Chang, 2002). Embryo induction and regeneration from suspension culture of two *Medicago truncatula* CV. S (CV R 1081 and CV. Jemalong) have been studied. The influence of osmotic pre-treatment (1M solution of sucrose for 48 h and 72 h) of roots as an initial explant, on embryogenic efficiency of the suspension culture was assessed. The shortest regeneration period and the highest percent of conversion to plants were found in CV. R 1081 after 72 h pre-treatment of roots (Anelia et al., 2005).

Raina and Irfan (1998) reported high frequency embryogenesis and plantlet development from microspores isolated from anthers of two *indica* (IR-43, IR-54) and a *japonica* (T-309) rice cultivars, without prior nutrient preculture of anthers. Among the indica cultivars, the maximum response was obtained in the
basal medium M-019. Plantlet regeneration occurred in about 9% (T-309), 7% (IR-43) and 2% (IR-54) of the transferred embryo like structures.

Sections from mature zygotic embryos of Norway spruce exhibited different capacities of somatic embryo initiation. The upper hypocotyl part (Zone 2) was the most embryogenic, followed by the lower hypocotyl (Zone 3) and the apical zone (Zone 1); the root part (Zone 4) never initiated embryonal suspensor masses (ESM) (Ramarosandratana and van Staden, 2003).

Embryogenic callus was induced from hypocotyl segments of *Psoralea corylifolia* Linn, an endangered medicinal plant belonging to family Fabaceae, on MS medium supplemented with 2.7-10.8 μm NAA and 2.2 μm BAP. High frequency somatic embryogenesis was achieved after 16 weeks of culture on MS medium containing 2.7 μm NAA, 2.2 μm BA, 0.94 μm ABA and 1.2 mm L-glutamine. Well-developed embryos germinated on 1/2 MSO, MSO (MS medium without any growth regulator) and also on MS medium supplemented with BAP (2.2 – 8.8 μm). Somatic embryo-derived plants were transferred to pots, where they grew well and attained maturity (Ashok Kumar *et al.*, 2001).

Hamama *et al.* (2001) developed a protocol for the induction, maturation and germination of somatic embryo from leaf tissue of Jojoba (*Simmondsia chinensis* (Link) Schneider). Explants were placed on their adaxial sides in petridishes and maintained in darkness on half strength MS basal medium MS/2 combinations of 2, 4-D (1.35-4.52 μm) with BA (1.33-4.43 μm) and synthetic cytokinins, N- (2-chloro – 4pyridyl) – N-phenylurea (1.21- 4.03 μm) or L-6- (3-trifluorom ethyl – but 2-enylamino) purine (1.11-3.71 μm) resulted in formation of embryogenic cultures and somatic embryos. Somatic embryo maturation, germination and plantlet formation were achieved using NAA (3.75 μm) or IBA (3.44 μm) in combination with BA (0.44 or 1.33 μm) or Fzip (0.37 or 1.11 μm).

Mature zygotic embryos of *Liriope platyphylla* Wang et Tang formed embryogenic calluses at a frequency of 33% when cultured on MS medium supplemented with 4.52 μm 2, 4-D. Upon transfer to half strength MS basal medium, embryogenic calluses gave rise to numerous somatic embryos, which then
developed into plantlets. Regenerated plantlets were successfully transferred to potting soil where they were subsequently grown to maturity (Suk et al., 2000). Embryogenic cell suspension cultures of Garlic (Allium sativum L.) were initiated in liquid medium from friable embryogenic tissue. The optimal parameters for culture maintenance were: (1) an initial cell density of 1-4% V/V; (2) medium renewal every 14 days and subculturing every 28 days; and (3) a low 2, 4-D concentration (0.1-0.3 mg/l). Cultures regenerated during 14 month period. The cell suspension cultures differentiated embryos following transfer to a semi-solid embryo induction medium (Fereol et al., 2005).

Mikhail et al., (2006) studied the effects of different factors on the embryogenesis and plant regeneration from mature embryos of Russian Spring and Winter genotypes of Wheat. Embryogenic callus induction was achieved on MS medium supplemented with different concentrations of 2, 4-D, 2, 4, 5-7 (2, 4- 5-trichlorophenoxy acetic acid) or Dicamba (3, 6-dichloro-O-anisic acid) when contact of explants with callus induction medium was increased from 7 to 21 days the rate of somatic embryogenesis and number of regenerated plants per embryogenic callus gradually increased from 13.0 to 38.4% and 3.6 to 8.0% respectively. Supplement of additional auxins (IAA, IBA and NAA) to callus induction medium with Dicamba had a positive effect on the rate of embryogenic callus formation, while the average number of regenerated shoots was not affected. The optimal rate of somatic embryogenesis was observed at the addition of 0.5 mg/l IAA with Dicamba (61.0%).

Sharry et al., (2006) developed a highly efficient somatic embryogenesis system and subsequent plant regeneration of Chinaberry (Melia azedarach L). Plants were regenerated from indirect somatic embryogenesis induction. Novel features of this improved protocol, include embryogenic callus induction with no addition of 2, 4-D in the culture media,

a) Somatic embryos differentiation was achieved by using high concentration of cytokinin (BAP 10 mg/l.) and adenine.
b) 100% conversion of somatic embryos to plant was practically obtained and 100% of plants survived under greenhouse conditions.

c) Addition of putrescine improved somatic embryos germination.

The amount of somatic embryos produced by the pathway of indirect embryogenesis was 413 per gram of fresh weight callus. Regenerated plants were phenotypically normal. The developed protocol established the potential to produce plantlets from cotyledon explants through somatic embryogenesis.

Somatic embryogenesis has been reported for a growing list of woody angiosperm and conifer tree species including Betula (Chalupa 1992, 1995), Citrus (Gosal et al., 1995), Eucalyptus (Muralidharan and Mascarenhan, 1987), Fraxinus (Precece et al., 1989), Hevea (Montoro et al., 1993), Juglans (Pijut 1993), Liriodendron (Merkle and Sommer, 1986), Pinus (Newton et al., 1995), Picea (Ellis et al., 1993), Populus (Michler 1995), Prunus (Camara Machado et al., 1995) and Salia (Gronroos et al., 1989).

Somatic embryogenesis has been demonstrated with several woody legumes reviewed in Trigiano et al., (1992) including Acacia (Skolmen 1986), Albizia (Gargal and Maheshwari, 1981; Tomar and Gupta, 1988), Cercis (Geneve and Kester, 1990; Trigiano et al., 1988), Dalbergia (Muralidhar Rao and Lakshmi Sita, 1996) and Robinia (Arrillaga et al., 1994; Merkle 1992; Merkle and Wiecko, 1989; Woo et al., 1995b). All these somatic embryos were derived from either seeds or seedlings.

4.3 Synthetic Seeds

Synthetic seeds refer to somatic embryos or shoot apex or any component having regenerative potential, encapsulated in a synthetic polymer like sodium or calcium alginate, which mimics the functions of natural endosperm and seed coat and thereby allows a normal germination after exposure to favourable humidity and temperature. The technique offers an early storage and transportation of otherwise difficult plant materials.
The higher degree viability and percent germination had added significance to the technique of synthetic seeds. The term synthetic seeds have also been applied to shoot meristem, apical and axillary shoot buds and callus derived adventitious buds (Bapat et al., 1987; Ahuja et al., 1989). This is in fact a new dimension to the concept of synthetic/artificial seeds which is applicable even to crops which don't reproduce through seeds in nature. Recently the encapsulation of somatic embryos of woody trees like *Lantalam album* and *Morus indica* has provided enough evidence of the advantages of synthetic seeds (Bapat et al., 1987 and 1988).

**MATERIALS AND METHODS**

4.4 SOMATIC EMBRYOGENESIS

4.4.1 Explant Selection

From the 10 days-old in vitro raised seedlings/shoot tip, node, axillary bud and cotyledonary node were selected as explants.

4.4.2 Callus Induction

Semi-solid MS basal medium containing MS vitamins, 0.8% Agar, 30 g/l Sucrose and 2, 4-D (0.0-3 mg/l) was used as inoculum and explants placed horizontally and also vertically on the medium. The shoot tip explants were cut into 2-3 cm. These leaf bits were placed in 25 x 150 mm tubes containing 15 ml semi-solid MS medium. The induced callus was subcultured at an interval of 10 days on the MS medium with different concentrations of BAP (0.5-5 mg/l).

4.4.3 Suspension Culture

Initiation of cell suspension culture was done by transferring 10-days-old greenish white friable calli (1 g fresh mass) from semisolid MS medium. They were aseptically transferred to 150 ml Erlenmeyer flask containing 50 ml of MS liquid medium per flask, with BAP (0.0-5 mg/l) individually or along with BAP or KN (0.1-0.5 mg/l), 3% sucrose, and pH was adjusted to 5.8 before autoclaving at 121°C for 15 minutes. The suspension was agitated on a gyratory Shaker (1000-110 rpm). Cultures were maintained at light intensity of 1.5 μm m⁻² s⁻¹, 16 h photoperiod and
25 ± 2°C. Further subcultures involved the replacement of 10 ml of the cell suspension with fresh medium of the same formulation at 6 day intervals. Cultures were filtered through 125 μm stainless steel sieves to separate individual cells and small cell clumps. Cells from the suspension were observed with a microscope during the culture period and the growth rate of the cells was monitored from the 12th day by determining the Packed Cell Volume (PCV) of samples from 10 replicates. PCV was measured after centrifuging the suspension at 200 rpm for 10 minutes in a graduated centrifuge tube (Kumar et al., 1988). MS basal medium lacking BAP served as control. Embryos at different stages of development were separated manually.

4.4.4 Development of Somatic Embryos

Globular stage embryos which formed after 20 days in MS liquid medium were subcultured in the same medium for further development. After 6 days of subculture, heart shaped embryos were formed from globular stage embryos. After a period of further growth, heart shaped embryos were separated and transferred to MS liquid medium with different concentrations of BAP for the development of torpedo shaped embryos.

4.4.5 Ontogenic Study of Somatic Embryos

The cultures in liquid medium were examined every day during the experimental period to trace the ontogeny of somatic embryos. Samples from 20 replicate flasks per BAP treatment were taken at random in order to determine the frequency of embryogenic initials and different stages of somatic embryos during somatic embryogenesis. The percentage of somatic embryogenesis was determined by the total number of cells cultured (Eapen and George, 1990).

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\text{Percentage of response} = \frac{\text{No. of embryos}}{\text{Total no. of embryo formed}} \times 100
\]

4.4.6 Germination of Somatic Embryos

The torpedo shaped embryos were transferred to MS liquid medium having 3% sucrose, BAP (0.0-5.0 mg/l) alone or together with GA₃ (0.1-0.5 mg/l), 2ip (0.1-0.5 mg/l) and several other techniques such as hormone free MS basal, half
strength MS, desiccation and cold treatment were studied for germination. The embryos were incubated under different physical conditions as explained earlier and kept in an orbital Shaker (50 rpm) to obtain tiny plantlets. The tiny plantlets were transferred to MS solid medium for germination. Photographs were taken with computerized Grand Tec. VCD stereomicroscope (IOX).

4.4.7 Statistical Analysis

Each treatment consisted of at least 50 explants and the experiment was repeated 5 times. A complete randomized design was used in all experiments and analysis of variance and mean separations were carried out using Duncan’s Multiple Range Test (DMRT). Significance was determined at the 5% level (Gomez and Gomez, 1976).

4.5 OBSERVATIONS

4.5.1 Indirect Somatic Embryogenesis

A protocol for highly efficient somatic embryogenesis system and subsequent plant regeneration of *Pueraria phaseoloides* has been achieved. Plants were regenerated successfully from indirect somatic embryogenesis induction. Novel features of this protocol include:

a) Embryogenic callus induction with no addition of 2, 4-D in the culture media.

b) Differentiation of somatic embryos was evolved by using high concentration of cytokinin (BAP 5 mg/l).

The amount of somatic embryogenesis was 413 per gram of fresh weight callus. The developed protocol established the potential to produce plantlets from shoot tip explants through somatic embryogenesis. It also presents itself a highly efficient method for mass clonal propagation and conservation of *Pueraria phaseoloides*.

Callusing efficiency of *in vitro* raised plant explants like shoot tip, node, axillary bud and cotyledonary node were tested on MS medium containing 0.05-5mg/l, BAP, .05-4mg/l KN, 0.05-2mg/l NAA, 0.05-2mg/l IAA & 0.05-2mg/l 2,4-D.
Explants initiated to produce callus after ten days of inoculation. Among the four explants, shoot tip explants produced maximum induction of callus followed by cotyledonary node, node and axillary bud explants. Shoot tip explants produced yellowish green and friable callus on BAP (5mg/l). Cotyledonary node explants produced pale white and friable callus on (4mg/l) KN. Node and axillary bud explants produced yellow coloured friable callus on 4mg/l KN. BAP and KN induced maximum amount of friable and embryogenic callus, while auxins had no similar responses. All the 3 auxins used (2, 4 −D, NAA and IAA) induced compact calli but cytokinin induced friable and high mass weight of callus (Table - 8), (Plate - 18).

4.5.2 Differentiation of embryogenic cells in callus

Explants collected from in vitro plants placed on MS medium fortified with BAP, 5mg/l and KN 4 mg/l showed maximum response. Among all the explants tested, only shoot tip explants produced yellowish green and friable calli from the cut ends on medium supplemented with 5 mg/l BAP after 10 days of inoculation. Efficiency of callus induction and embryogenetic response was higher from 20th day onwards. The calli were subcultured in 10 days of interval. After the third subculture the differentiated calli were transferred into suspension culture for development and separation.

4.5.3 Cell Suspension Culture

Shoot tip derived, yellowish, green, friable and differentiated calli were sub cultured on, ½ MS, medium containing BAP 5 mg/l and KN 4 mg/l. Active division and growth of cells were observed in ½ MS medium supplemented with BAP 5 mg/l. KN 4 mg/l also produced the same result from 8-10th days of culture and slowly reached the stationary phase. The declining phase started from 12th day onwards, after which the culture became thick, mucilaginous and brown in colour.

4.5.4 Induction of Somatic Embryogenesis

The shoot tip callus maintained up to 2 weeks on MS solid medium when transferred to MS, liquid media containing BAP (0-5 mg/l) globular, heart shaped
and torpedo shaped embryos were formed (Table 16). Frequency of somatic embryogenesis induction was higher in MS liquid medium containing 5 mg/l BAP.

4.5.5 Ontogeny of Somatic Embryos

Microscopic observation on the initiation and development of somatic embryos (plate-17) showed the following details. Initially the callus showed highly vacuolated cells. After the completion of 10\textsuperscript{th} day the callus showed morphologically distinct cells, namely spherical cells with visible cytoplasm and nucleus. This spherical embryogenic cells, proliferated in the form of tubular out growth, followed by transverse division, resulting in two and four cell stages upper globular and linear elongated lower cell. Then the later by transverse division formed a long linear suspensor while the former by divisions in different angles produced globular proembryos. At the end of 20\textsuperscript{th} day, the callus cultures maintained maximum number of pro embryos. These embryos by further divisions at different angles formed globular embryos within two days in the MS liquid medium containing 5 mg/l BAP. After the development of globular embryo, the suspensor disappeared. The globular embryos differentiated into heart shaped embryos followed by torpedo shaped embryos in the same media composition (Plate - 18, 19).

4.5.6 Maturation of Somatic Embryos and Regenerations

Torpedo shaped embryos matured and germinated into tiny plantlets (Plate - 20) on MS semi solid medium free of hormones. The tiny plantlets were obtained at very low frequency. The germinated plantlets were hardened and transplanted into soil.

4.5.7 Encapsulation of Somatic Embryos

For successful germination of somatic embryos, the torpedo shaped embryos were further subjected to encapsulation study. The encapsulated somatic embryos started to develop on the 10\textsuperscript{th} day of planting on the basal medium. Tiny plantlets were emerged from encapsulated beads (Plate - 21, 22).
4.6 Discussion

In the present study 5 mg/l BAP was found to be suitable for organogenetic callus with shoot buds in the case of shoot tip explants and 4 mg/l KN for node, axillary bud and cotyledonary node explants. In *Cajanus cajan* 1.0 mg/l BAP and 0.1 mg/l NAA induced regeneration in different explants such as cotyledon, hypocotyls, epicotyl and cotyledonary node (Geetha *et al.*, 1998). This is in contrast to the present study where 5 mg/l BAP was found to be effective in inducing regeneration from shoot tip explants. And 4 mg/l KN was found to be effective in inducing regeneration from cotyledonary node explants. Callusing of seedling explants like shoot tip, node, axillary bud and cotyledonary node was tested on MS medium containing 0-5 mg/l BAP, 2,4-D, NAA and IAA.

The path way of somatic embryogenesis obtained was indirect because it needed to pass through a callus phase. Seedling tissues started de-differentiation after 10 days of culture. After 20 days of culture, embryogenic calli were formed from shoot tip, node, axillary bud and cotyledonary node explants in half MS medium. They were then sub cultured to liquid medium for somatic embryo development. Embryogenic callus were heterogenous, light brown to green in colour in shoot tip, yellowish to green colour in axillary bud, and pale yellow to green colour in cotyledonary node. The embryogenic calli were friable and with high number of somatic embryos in different states, easy to separate from callus surface. As can be observed, the efficiency of embryogenic callus formation per initial explants was very high. The reproducibility was also high. All the different stages of somatic embryogenesis were easily observed after 15 to 20 days on liquid medium (from globular to mature somatic embryos) as can be seen in Plate -18.

The standard process for somatic embryos consist in callus formation in media containing auxins and afterwards subculture to hormone free medium. (Reinert, 1967). The presence of an auxin is usually required in the medium in order to maintain the growth of sub cultures (Halperin, 1995). Moreover the type and concentration of auxin employed are critical for the induction and formation of somatic embryogenesis. Auxin 2, 4-D has been the most efficient plant growth
regulator for the induction of somatic embryogenesis (Merkle, 1995). However in some cases auxin was not necessary in the development of somatic embryogenesis (Smith and Krikorian, 1990).

In the present study it is clear that though 2, 4-D has positive effect in callus formation, it had no effect on induction of morphogenic callus. On the other hand we found that cytokinin concentration was very important for somatic embryogenesis development in this species. In the present study callus occurred with BAP and KN and it seemed that it is important to have a high concentration of BAP (5mg/l) alone for somatic embryo differentiation. Effect of induction of cytokinin over the somatic embryogenesis process was reported also by (Kavithakar et al., 1978), Jha et al., (1981) and Desai et al., (1986). BAP was the most suitable cytokinin for Pueraria phaseoloides though KN also induced organogenic calli.

4.6.1 Somatic Embryo Germination and Conversion

Somatic embryos were green in colour and they had to be separated from callus and subcultured to fresh medium, or necrosis occurred. The right stage of somatic embryogenesis to be subcultured was torpedo and cotyledon stage (plate.20). For embryo germination, the transfer to a free hormone medium or with low concentration of BAP or BAP+KN (0.01+0.01mg/l) without auxin was effective. The germination occurred in each of the three types of embryo formed from shoot tip, node, axillary bud and cotyledonary node explants in half MS medium.
**TABLE – 16**

Influence of different concentrations of BAP on induction of somatic embryogenesis from shoot tip derived callus on MS liquid medium.

<table>
<thead>
<tr>
<th>BAP mg/l</th>
<th>PCV (Packed Cell Volume) on 10th day</th>
<th>Embryogenic callus on 20th day</th>
<th>Different stages of somatic embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Globular</td>
</tr>
<tr>
<td>0.0</td>
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<td>0.0</td>
<td>0.0</td>
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<tr>
<td>0.5</td>
<td>1.46±0.04&lt;sup&gt;lm&lt;/sup&gt;</td>
<td>7.2±0.24&lt;sup&gt;lm&lt;/sup&gt;</td>
<td>3.6±0.48&lt;sup&gt;m&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>2.46±0.04&lt;sup&gt;ik&lt;/sup&gt;</td>
<td>16.1±0.21&lt;sup&gt;kl&lt;/sup&gt;</td>
<td>8.4±0.48&lt;sup&gt;ik&lt;/sup&gt;</td>
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<td>26.5±0.49&lt;sup&gt;ij&lt;/sup&gt;</td>
<td>14.6±0.48&lt;sup&gt;ih&lt;/sup&gt;</td>
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<tr>
<td>2.0</td>
<td>3.16±0.04&lt;sup&gt;ih&lt;/sup&gt;</td>
<td>40.4±0.49&lt;sup&gt;ib&lt;/sup&gt;</td>
<td>15.8±0.48&lt;sup&gt;ib&lt;/sup&gt;</td>
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<td>2.5</td>
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<td>54.7±0.45&lt;sup&gt;fg&lt;/sup&gt;</td>
<td>18.4±0.48&lt;sup&gt;fg&lt;/sup&gt;</td>
</tr>
<tr>
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<td>71.5±0.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.6±0.48&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.5</td>
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<td>91.4±0.49&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31.4±0.48&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>112.4±0.49&lt;sup&gt;e&lt;/sup&gt;</td>
<td>39.4±0.48&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>4.5</td>
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<td>134.5±0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>45.4±0.48&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>5.0</strong></td>
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<td><strong>48.6±0.48&lt;sup&gt;a&lt;/sup&gt;</strong></td>
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<tr>
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<td>19.5±0.49&lt;sup&gt;k&lt;/sup&gt;</td>
<td>7.4±0.48&lt;sup&gt;kl&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean values within a column having the same alphabet are not statistically significant (p=0.05) according to New Duncan’s multiple Range test.
Influence of different concentrations of BAP on
induction of somatic embryogenesis from shoot tip

Different stages of somatic embryos

No. of embryos
PLATE 17. Various developmental stages of callus.

a. Elongated and vacuolated cells.

b. Elongated and vacuolated cells.

c. 1-6, callus cells develop globular, heart-shaped and torpedo-shaped embryos.

d. 1-4, callus cells develop globular, heart-shaped and torpedo-shaped embryos.

e. Plantlet derived from somatic embryos.

PLATE: 18. Somatic embryogenesis

- Different developmental stages

a, b, c, d, e, f - globular embryo formation

h, i, j, k, l - heart shaped embryos

m, n - torpedo shaped embryos

o - cotyledonary stage of embryos

a. 2 celled stage
b. 4 celled stage
c. 4 celled stage
d. Embryonal developmental stages.
e. Embryonal developmental stages.
f. Embryonal developmental stages.
g. Embryonal developmental stages.
h. Embryonal developmental stages.
PLATE: 20. Developing somatic embryos

a. Embryonal development leading to tiny plantlets
b. Embryonal development leading to tiny plantlets
c. Embryonal development leading to tiny plantlets
d. Embryonal development leading to tiny plantlets
e. Embryonal development leading to tiny plantlets
f. Embryonal development leading to tiny plantlets
g. Tiny plantlets

a. Somatic embryos in suspension culture.
b. Synthetic seeds
c. Synthetic seeds
d. Synthetic seeds
e. Synthetic seeds
f. Synthetic seeds
g. Synthetic seeds cultured on 1/2 MS medium
h. Germination of synthetic seeds.
PLATE: 22. Synthetic seeds containing the somatic embryos at various stages.

\begin{itemize}
\item [a.] Synthetic seeds with globular somatic embryo
\item [b.] Synthetic seeds with globular somatic embryo
\item [c.] Synthetic seeds with heart shaped somatic embryos
\item [d.] Synthetic seeds with heart shaped somatic embryos
\item [e.] Synthetic seeds with heart shaped somatic embryos
\item [f.] Synthetic seeds with heart shaped somatic embryos
\item [g.] Synthetic seeds with torpedo stage of somatic embryos
\item [h.] Synthetic seeds with torpedo stage of somatic embryos
\item [i.] Synthetic seeds with torpedo stage of somatic embryos
\item [j.] Synthetic seeds with cotyledonary stage of somatic embryos
\item [k.] Synthetic seeds with cotyledonary stage of somatic embryos
\item [l.] Synthetic seeds cultured on 1/2 MS medium
\item [m.] Tiny plantlets formation from synthetic seeds
\item [n.] Shoot elongation
\item [o.] Hardening
\end{itemize}