5.1. INTRODUCTION

The development of embryos from somatic cells of any explant through the embryological states stimulating zygotic embryos from the embryonic potential of the somatic plant cell of the callus tissue or cells of suspension culture is called somatic embryos or embryoids and this process is called somatic embryogenesis. This was first observed in carrot (Daucus carota) root tissues by Steward (1958). Differentiation and development of somatic embryos required lowering the concentration or complete elimination of auxin used in the induction medium. Somatic embryogenesis has acquired much importance in agriculture, silviculture, horticulture and industries involved in continued supply of basic plant materials of elite varieties.

The term somatic means that ameristematic (i.e., not containing shoot apex) plant tissues such as leaf, stem, or other tissues such as wound or callus tissues. The term embryogenesis consists of two words: ‘embryo’ which describes a structure that resembles a seed embryo and the word ‘genesis’ means to give forth. The process of somatic embryogenesis begins with the chemical signal that result in the formation of embryo-like structures from somatic (i.e., non-meristematic) tissues without a sexual phase. Over 6000 somatic embryos can be formed from one gram of callus tissue, so the process is remarkably efficient at producing very large number of plants (Fay, 1994).

Potentiality of plant cell to regenerate the entire organism (plant) is termed is ‘totipotency’. This potentiality has been exploited through the culture of protoplasts, cells, tissues and organs in vitro. In cultured materials it has been possible to induce such processes as differentiation of parenchyma cell into tracheid (cyto differentiation), organ formation (organogenesis) and somatic embryo formation (somatic embryogenesis). In vitro culture studies such as direct organogenesis, indirect organogenesis via., callus pathway, somatic embryogenesis, somaclonal variation, regeneration through protoplast culture, somatic hybridization, haploid culture etc., have great impact on crop improvement and medicinal plant conservation.
Somatic embryogenesis is an ideal method of mass propagation of plants possessing the same phenotypic and genotypic characters and for the production of transgenic plants and mutagenesis (Ammirato, 1983). *In vitro* regeneration of plants via, somatic embryogenesis has much potential for use in plant propagation and gene transfer (Durham and Parrott, 1992). However, an efficient conversion of somatic embryos into plants remains a problem (Baker *et al.*, 1995). Advances in plant cell and tissue culture may provide novel ways to recognize, select, modify and transfer the genes involved in disease resistance. However, a pre-requisite for applying these methods to any species is the availability of reproducible plant regeneration protocol from explants or protoplasts (Eapen and George, 1993).

Multiplication of plants through plant tissue culture can be achieved by any one of the methods. The basic concept is to achieve rapid multiplication without creating unwanted somaclonal variation. Therefore, axillary and adventitious budding and somatic embryogenesis are most frequently used methods of micropropagation. Micropropagation is defined as production of miniature plantlings [seeds (somatic embryo) or plantlets] in large number by vegetative multiplication through regeneration.

Conservation of the endemic and threatened flora is carried out using different strategies. Micropropagation constitutes a powerful tool for *ex situ* conservation programs of rich flora, especially for species with vary reduced populations or low seed production (Fay, 1994; Krogstrup *et al.*, 1992). This technique facilitates the rapid establishment of large number of stock plants, from minimum of the original plant material and they impose minimum impact on the endangered wild population (Debnath, 2004). With this technology various endangered and endemic species from the Iberian Peninsula have been successfully conserved (Marco and Ibanez, 1998 and Lledo *et al.*, 1995).

Tissue culture techniques are used for clonal multiplication and *in vitro* conservation of valuable indigenous germplasms threatened with extinction. Greater demand for *Plectranthus barbatus* especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats. Micropropagation offers a great potential for large scale multiplication of such useful species and subsequent exploitation (Anand and Jeyachandran, 2005).
The supply of excess nutrients to cell cultures may result in a loss of totipotency and may encourage the continued proliferation of non embryonic callus (Lee et al., 2001). The enhancement of somatic embryogenesis following long term culture and subsequent reduction of nutrients has been reported in culture of Angelica gigas (Choi and Soh, 1993). Somatic embryogenesis too might not only allow the clonal propagation of valuable genotypes, but also facilitate genetic engineering (Corredoira et al., 2002). Recently, in vitro regeneration of complete plantlets through somatic embryogenesis was reported in many medicinal plants.

Somatic embryos have been obtained from cultured internode explants of Paulownia elongata (Ipekci and Gozukirmizi, 2003) and Solanum tuberosum (Sharma and Millam, 2004), leaf explants of Simmondsia chinesis (Hamama et al., 2001), Oncidium spp. (Chen and Chang, 2002), Coffea arabica (Molina et al., 2002), Helianthus eggertii (Yordanov et al., 2002), Echinops spinosissimus (Murch et al., 2003), Ostericum koreanum (Cho et al., 2003) and Naregamia alata (Sebastian et al., 2006b), petiole explants of Hylomecon vernalis (Kim et al., 2003), Eipremnum aureum (Zhang et al., 2005) and Syngonium podophyllum (Zhang et al., 2006), root explants of Spinacia oleracea (Leguillon et al., 2003) and Medicago truncatula (Iantheva et al., 2005), hypocotyl explants of sunflower (Petitprez et al., 2005), hypocotyl and cotyledon explants of Gymnema sylvestre (Ashokkumar et al., 2002) and Cucumis melo (Rhimi et al., 2006), zygotic embryos explants of immature Catharanthus roseus (Kim et al., 2004) and Azadirachta indica (Chaturvedi et al., 2004) and mature Liriope platyphylla (Kim et al., 2000), Ulmus minor and Ulmus glabra (Corredoira et al., 2002), Cocos nucifera (Fernando et al., 2003) and Picea abies (Ramarosandratana and Staden, 2003) and inflorescence stem segments of Cynodon dactylon and Cynodon transvaalensis (Chaudhury and Qu, 2000).

Recently, there has been a great deal of interest in vitro regeneration through somatic embryogenesis. Somatic embryogenesis and plant regeneration have been reported in many species such as Ranunculus kazusensis (Min et al., 2007), Dianthus caryophyllus (Seo et al., 2007), Jatropha curcas (Jha et al., 2007), Bactris gasipaes (Steinmacher et al., 2007), Catharanthus roseus (Junaid et al., 2007), Hippophae
rhamnoïdes (Liu et al., 2007), Cucurbita moschata (Zhang et al., 2008), Brasenia schreberi (Oh et al., 2008), Picea koraiensis (Li et al., 2008), Arachis hypogaea (Joshi et al., 2008), Gossypium hirsutum (Wang et al., 2008), Ricinus communis (Ganeshkumari et al., 2008), Saposnikovia divaricata (Qiao et al., 2009), Physalis ixocarpa (Guzman et al., 2009), Brasenia schreberi (Oh et al., 2008), Picea koraiensis (Li et al., 2008), Arachis hypogaea (Joshi et al., 2008), Gossypium hirsutum (Wang et al., 2008), Ricinus communis (Ganeshkumari et al., 2008), Saposnikovia divaricata (Qiao et al., 2009), Physalis ixocarpa (Guzman et al., 2009), Phoenix dactylifera (Othmani et al., 2009), Quercus rubra (Vengadesan and Pijut, 2009), Trifolium nigrescens Konieczny et al., 2010) and Vigna mungo (Muruganantham et al., 2010).

Sharma and Millam (2004) defined the process of somatic embryogenesis as the development of a bipolar structure with both root and shoot poles from any sporophytic part of the plant occurring through the same key stages of embryo development. Zygotic embryogenesis (i.e., globular heart and torpedo stages) was yet another way of exhibiting totipotency by plant cells, whereby they first dedifferentiate and then redetermine towards the embryogenic pathway as has been reported in many cases.

Application of *in vitro* techniques have been routinely practiced for the multiplication of many medicinal plants to meet the demand of the pharmaceutical firms and to protect the natural population of rare and endangered plant species (Nayak, 2002; Nagaraja et al., 2003; Loc et al., 2005 and Mehash and Jeyachandran, 2005). *In vitro* propagation is considered as a viable technology to conserve the plant species (Anupama et al., 2005).

Hence, this present study describes the successful experiments of somatic embryogenesis which includes initiation, proliferation and development of somatic embryos and encapsulation and their germination pattern *in vitro* and *in vivo* conditions using leaf, petiole, internode and root explants of *Plectranthus barbatus* (selected on the basis of response) on solid medium. Furthermore, this report describes an efficient method for initiation, proliferation and development of somatic embryos in suspension culture. In the first step, efficient somatic embryogenic system from leaf, petiole, internode and root explants of *Plectranthus barbatus* was developed. Secondly, experiments were conducted for encapsulation of somatic embryos obtained from both solid and liquid culture through the phase of direct and indirect somatic embryogenesis.
5.2. REVIEW OF LITERATURE

Somatic embryogenesis, to date attributed only to plants, was first observed in *Oenanthe aquatica* (Waris, 1957) and *Daucus carota* (Steward *et al.*, 1958) of the Apiaceae. Somatic embryogenesis offers a potentially novel method for producing potato nuclear seed material in an efficient and economical manner. Additionally somatic embryogenesis system in potato may also be useful in transformation-related studies as the unicellular mode of origin would enable the regeneration of completely transformed plants without the creation of Chimeras as has been reported (Sharma and Millam, 2004).

Somatic embryogenesis was studied in *Vigna radiata* from leaf callus explant as has been reported (Patel *et al.*, 1991). Organogenic callus and somatic embryos are found to be from cotyledon explants on MS medium supplemented with cytokinin and auxin. Plantlets were recovered from the callus on modified MS medium containing BAP 3.0mg/l with NAA 0.5mg/l. Mostly plant regeneration was achieved via., somatic embryogenesis (Sharma *et al.*, 2003). Direct somatic embryogenesis or formation of embryoids in callus culture usually gave rise to complete plantlets (Patel *et al.*, 1991). Direct somatic embryogenesis with singular shoot initiation was seldom observed. In the light of above, genetic instability of the callus cells is expected and possibilities of somaclonal variations cannot be ruled out as was observed by Chintapalli *et al.* (1997). Moghaieb *et al.* (2006) studied the somatic embryos which directly emerged from the body of the explants or indirectly germinated from the embryonic callus.

Sahrawat and Chand (2001) developed a protocol for rapid and continuous production of somatic embryogenesis from hypocotyl segments of *Psoralea corylifolia*. Embryogenic callus was induced on MS medium supplemented with 2.7-10.8µM NAA and 2.2µM BAP. Somatic embryogenesis was achieved after transfer of embryogenic callus clumps to MS medium supplemented with 1.4µM NAA and 2.2µM BAP alone or in combination with 0.9µM ABA. The addition of 1.2mM L-glutamine to the MS medium containing 2.7µM NAA, 2.2µM BAP and 0.9µM ABA significantly enhanced maturation of somatic embryos to cotyledonary stage. Well developed embryos germinated on MS medium containing 6.6µM BAP.
Corredoira et al. (2002) achieved a protocol for somatic embryogenesis via a straightforward procedure of culturing immature zygotic embryos in MS medium containing 2,4-D (0.2mg/l). Somatic embryos at different stages of development, from globular shaped to cotyledonary shaped, had arisen by this time. The frequency of embryogenic cultures was the greatest (12.5%) for both explants collections of *Ulmus minor* and *Ulmus glabra*. The formation of callus was an essential step not only for the induction of embryogenic masses, but also for the maintenance of embryogenic competence through successive subculture of callus on induction media supplemented with 0.1mg/l BA.

Somatic embryogenesis and plant regeneration were achieved by Ashokkumar et al. (2002) using callus culture derived from hypocotyl, cotyledon and leaf explants of *Gymnema sylvestre*. Embryogenic callus was induced on MS medium containing 2,4-D (2.0µM) + BA (1.0µM) and 2% (w/v) sucrose. These embryogenic callus produced torpedo, cotyledon and plantlets upon frequent subculturing on embryo maturation medium containing MS salt, B5 vitamins, 0.5µM BA and 2% sucrose.

Lee and Lee (2003) induced plant regeneration via somatic embryogenesis from *in vitro* derived callus of *Dicentra spectabilis*. Embryogenic callus formation was observed on media containing 1.0mg/l 2,4-D under dark conditions. Somatic embryogenesis on MS basal media with 1.0mg/l of KN was excellent under light conditions. Somatic embryos were rooted by transferring them to half strength MS medium containing 2.0mg/l phytogel.

High frequency somatic embryogenesis and plant regeneration were described by Kim et al. (2003) using petiole and leaf derived embryogenic cell suspension cultures of *Hylomecon vernalis*. Petiole explants formed embryogenic calluses when cultured on B5 medium supplemented with 13.6µM 2,4-D alone and leaf responses at a combination 4.52µM 2,4-D and 2.22µM BA. Cell suspension cultures were established with petiole derived embryogenic calluses using liquid B5 medium with 4.52µM 2,4-D. Upon planting into B5 basal medium, cell suspension cultures produced numerous somatic embryos which then developed into plantlets.
In vitro plant regeneration was achieved by Saradamani et al. (2003) using immature inflorescence explants of *Sorghum bicolor* through the embryogenic callus. Callus induction was obtained from the explant on MS medium supplemented with 2% sucrose and 1.5mg/l 2,4-D. Maximum shoot induction was noticed on MS medium at 1.0mg/l of BAP and root induction on MS medium at 1.0mg/l IAA. Hu et al. (2005) cultured petiole derived callus of *Amorphophalus rivieri* on MS medium supplemented with high concentration of auxin with dose of 13.57µM 2,4-D and 8.88µM BA or 21.48µM NAA and 6.66µM BA to promote the somatic embryogenesis.

Somatic embryo induction studies by Jeyaseelan and Rao (2005) showed that 2,4-D (9.04µM) promoted the highest (60.8) percentage of somatic embryo formation from leaf callus followed by 2,4-D + BA (9.04 + 0.44µM) and induced 61.2% and 69.32% embryos recorded from leaf and internode derived callus. Among the auxins, 2,4-D has been particularly found to be an effective inducer of somatic embryogenesis in more than 50% of plant taxa reported for successful production of somatic embryos (Wakhlu and Sharma, 1998). Embryogenic callus was induced from cotyledon and leaf explants of *Solanum surattense* on MS medium fortified with 6.0mg/l NAA + 0.5mg/l BAP and 4.0mg/l NAA+0.5mg/l BAP, respectively for high frequency rate. Well developed cotyledonary stage embryos were germinated on MS medium supplemented with 0.5mg/l IAA + 2.0mg/l BAP, with plantlets formation, but they did not germinate on half strength MS basal medium, which were studied by Ramaswamy et al. (2005).

Aoshima (2005) developed a technique for plantlet regeneration through somatic embryogenesis and adventitious bud formation. An efficient somatic embryogenesis protocol has been obtained for callus derived from shoot apex of tea plant. The addition of mannitol at 0.31M 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 (5-10mg/l), the differentiation rate increased up to 43%.

George et al. (2005) evolved a protocol for direct shoot organogenesis and somatic embryogenesis of *Centella asiatica* using leaf and internode derived calluses on MS medium having BA (0.5mg/l) in combination with NAA (0.5mg/l), respectively for direct regeneration. Subsequently transferred callus were shoot differentiated on MS Medium
containing BA (3.0mg/l). Efficient roots were obtained on half MS medium enriched with IBA (0.5mg/l). Somatic embryos were obtained from MS medium containing 2,4-D (0.5mg/l) + KN (0.25mg/l). A protocol has been developed for plant regeneration from encapsulated somatic embryos of *Hyoscyamus muticus* L. (Pandey and Chand, 2005). Somatic embryogenesis were achieved in callus cultures derived from cotyledonary leaf pieces on MS medium enriched with NAA 2.24μM and BAP 2.22μM. Well developed plantlets were obtained from somatic embryos encapsulated using 3% sodium alginate and 75mM CaCl₂ as gel matrix, cultured on MS basal medium. Plantlets with well developed shoots and roots were transferred to pots containing autoclaved mixture of peat moss, compost and soil (1:1:1).

*In vitro* plant regeneration was obtained from leaf, petiole, internode and somatic embryo explants of *Solanum violaceum* (Raghu et al., 2006b). Embryogenic callus was initiated from *in vitro* derived leaf discs on MS medium containing 2,4-D (2.26-6.78μM) and KN (2.32-6.97μM). Reduced concentration of 2,4-D and KN facilitated the development and maturation of embryos within 3-4 weeks on suspension cultures. The somatic embryos were bipolar that developed into complete plantlets.

Direct somatic embryogenesis has been established from petiole explant of *Syngonium podophyllum* var. ‘variegatum’ (Zhang et al., 2006) Somatic embryos were directly formed at one or two sides of petiole explants on MS medium supplemented N-phenyl-N’-1,2,3-thiadiazol-5-ylurea (TDZ 2.5mg/l) with NAA (0.5mg/l) or TDZ (2.0mg/l) with NAA (0.5mg/l) with 0.2 and 0.5mg/l 2,4-D respectively. The frequency of somatic embryos produced from petiole explants was as high as 86% when cultured on MS containing TDZ (2.5mg/l) with NAA (0.5mg/l). Upto 85% of somatic embryos were able to germinate after transferring onto medium containing BA (2.0mg/l) and NAA (0.2mg/l). Approximately 50-150 plantlets were regenerated from a single petiole explant.

Sebastian *et al.* (2006b) have achieved plant regeneration from leaf explants of *Naregamia alata* through direct and indirect somatic embryogenesis. Direct somatic embryogenesis was obtained on MS medium supplemented with 2,4-D (0.1mg/l). Indirect somatic embryogenesis was induced by subculturing embryogenic calli obtained on MS medium with 2,4-D (1.0mg/l) to basal liquid medium. Plantlets were hardened in the greenhouse and transferred to the field.
Hypocotyl, cotyledon and zygotic embryo explants from two Tunisian *Cucumis melo* L. cultivars *Beji* and *Maazoan*, cultured on MS medium added with 2,4-D (0.25mg/l) and BA (0.50mg/l) produced calluses with somatic embryos after 3 weeks of culture. For *Beji* cv. the highest percentage (62.50%) of embryogenesis was observed for cotyledons. Embryogenesis induction for zygotic embryos reached 33.50%. The embryogenic ability of hypocotyle did not exceed (12.50%). Somatic embryogenesis for *Maazoan* cv. explant was less efficient. Embryo formation was observed only for cotyledons (29%) and zygotic embryos (25%). Cotyledonary stage embryo, when transferred to hormone free MS medium, germinated. The maximum germination rates were 51.50 and 44.50% respectively for *Maazoan* and *Beji* cv. The highest percentage (36.50%) of survival at *in vitro* plants was noted for *Beji* cv. (Rhimi *et al*., 2006).

Tejavathi and Anitha (2006) have offered an efficient protocol for somatic embryos on MS medium supplemented with NAA from hypocotyl derived callus of *Linum usitatissimum* for three elite varieties of flax –R552, Jeevan and Gaurav. MS medium supplemented with 2,4-D (0.90µM), GA₃ (0.23µM) and Zeatin (4.56µM) was found to be the best for the development of embryo into plantlets from the cultures of R552 and Jeevan. Conversion of somatic embryos into plantlets was not observed in the cultures of Gaurav.

Ranjithakumari *et al*., (2006) established an efficient protocol for the induction of somatic embryogenesis and plant regeneration from embryogenic axes of explants of *Glycin max*. Somatic embryos were established on MS medium supplemented with 180.8µM 2,4-D and 2.22µM BA, respectively. Embryo development and maturation was achieved on MS medium supplemented with 2.26µM 2,4-D and 8.88µM BA. The well formed embryos germinated into complete plantlets on MS medium containing 8.88µM BA and 1.34µM NAA. Regenerated plantlets were transferred to plastic cups and later into pots for maturation.

High frequency plant regeneration *via*., somatic embryogenesis from cell suspension cultures of *Ranunculus kazusensis* were described by Min *et al*., (2007). Zygotic embryos formed white nodular structures and pale-yellow calluses at a frequency of 84.9% when cultured on half strength Schenk and Hildebrandt (SH) medium.
supplemented with 0.1mg/l 2,4-D. However, the frequency of white nodular structure and off-white callus formation decreased with an increasing concentration of 2,4-D up to 10mg/l, when the frequency reached 25%. Cell suspension cultures were established from zygotic embryo derived calluses using half strength SH medium supplemented with 0.1mg/l of 2,4-D. Upon plating onto half strength SH basal medium, over 90% of cell aggregates gave rise to numerous somatic embryos and developed into plantlets. Regenerated plantlets were successfully transplanted to potting soil and grown to maturity at a survival rate of over 90% in a growth chamber.

Swaroop et al. (2007) reported encapsulation of in vitro derived nodal segment or shoot tips of Coleus forskohlii (Willd.) Briq. in 3.5% Sodium alginate for germplasm storage and exchange purposes. Encapsulated buds regenerated complete plantlets on MS optimized medium with varied concentrations of growth regulators. High percentage frequency of regeneration from alginate coated micropropagules is an encouraging system and this is an easy method of germplasm storage and exchange. Encapsulated buds could be stored for six months at 4°C for acclimatization with 61% success rate. Forskolin content is also stable in plants regenerated from encapsulated buds.

In vitro storage and regeneration of encapsulated micropropagules in sugarcane were carried out by Neelamathi et al. (2007). Tiny groups of shoots (0.2 - 0.5 cm) excised from the tuft of multiple shoots of sugarcane clones CO 8021 and CO 86032 were encapsulated with Sodium alginate 3% in distilled water and MS medium and incubated with Calcium chloride (2.5%). Encapsulated and non encapsulated micropropagules were stored at 4°C, 10°C and 25°C for 30 and 60 days. Storage at 10°C showed 91% and 90% survival in 30 days and 60 days and 80% and 85% survival in 30 and 60 days in distilled water and medium encapsulated micropropagules, respectively. Non encapsulated micropropagules stored at 10°C and 25°C showed poor response of 42% and 33% survival in 30 days and 8% and 11% in 60 days.

You et al. (2007) established the protocol for plant regeneration of Panax japonicus via., direct somatic embryogenesis. Somatic embryos were directly obtained from the segments of zygotic embryos on MS medium with 4.4µM 2,4-D. Thereafter, somatic embryos were produced by repetitive secondary somatic embryogenesis.
Frequency of secondary somatic embryo formation from cotyledon segments was lowered by plasmolyzing pretreatment, but the number of somatic embryos per explants was greatly increased. Plasmolyzing pretreatment resulted in retardation of embryo growth and required subculture to fresh medium for further growth of embryos into cotyledonary stage. Without plasmolyzing pretreatment, cotyledonary embryos were obtained after 8 weeks of culture. All the cotyledonary somatic embryos germinated by 5.0µM GA$_3$ treatment, but only 15.3% were germinated on hormone free medium. Choi and Soh (1997) reported that 1.0 M sucrose pretreatment of cotyledon segments of *Panax ginseng* for 24 hrs induced a high frequency of single cell derived somatic embryos. In zygotic embryos of *Eleutherococcus senticosus*, plasmolyzing pretreatment strongly enhanced the frequency of direct somatic embryos (You *et al*., 2006). You *et al*.* (2006) reported that enhanced single cell derived somatic embryogenesis directly from the surface of explants coincided with the rapid accumulation of callose in the cell wall due to plasmolyzing pretreatment. Stimulation of germination of somatic embryos by GA$_3$ treatment has been reported in *Panax ginseng* and *Eleutherococcus senticosus* by Choi *et al*.* (1999)*. Choi *et al*.* (1999)* interpreted the requirement of GA$_3$ for the germination of somatic embryos as due to the dormancy of somatic embryos.

Root explants excised from carnation plants maintained *in vitro* formed white, friable calluses after three weeks of culture on MS medium supplemented with 1.0mg/l TDZ and 1.0mg/l NAA. These calluses were subsequently transferred to MS basal medium where, after an additional four weeks of culture, approximately 50% of the calluses formed somatic embryos. However, calluses formed on root explants that had been cultured on MS medium supplemented with 2,4-D did not produce somatic embryos upon transfer to MS basal medium. Somatic embryos developed into plantlets and subsequently were grown to maturity (Seo *et al*.*., 2007)*. Somatic embryos have been formed directly from root explants in a few species, including *Lotus corniculatus* (Akashi *et al*.*., 1998)* and plant regeneration from root derived callus via*, somatic embryogenesis was reported in hybrid tea rose (Kamo *et al*.*., 2004)* and *Tylophora indica* (Chaudhuri *et al*.*., 2004)*.*

Steinmacher *et al*.* (2007)* described a protocol for somatic embryogenesis in peach palm (*Bactris gasipaes* Kunth). Younger inflorescences were more competent to respond to SE induction than more mature inflorescence and the use of a pretreatment with 2,4-D
(200µM) in liquid MS culture medium also increased the embryogenic capacity, and diminished the development of flower buds. Higher oxidation rates were observed in explants maintained on 2,4-D supplemented culture medium, while on 300µM or 600µM Picloram and Dicamba lower oxidation rates were observed. Picloram also enhanced the embryogenic induction rate more than 2,4-D and Dicamba, and among the concentrations evaluated 300µM Picloram enhanced induction for both genotypes, with significant differences between genotypes.

Somatic embryogenesis from callus derived from leaf and nodal segments of strawberry were reported by Biswas et al. (2007). The highest percent of cultures with somatic embryos was achieved on MS medium supplemented with 1.0mg/l 2,4-D, 0.5mg/l BAP and 50% proline. Darkness was the best condition for incubation with daily light periods over 6 hrs reducing the frequency of embryogenesis. Regenerated plants (90–95%) were successfully transferred to soil and showed normal morphology.

Embryogenic callus in *Catharanthus roseus* was initiated from hypocotyl on MS medium supplemented with 1.0 - 2.0mg/l of 2,4-D or chlorophenoxyacetic acid (CPA). Calli from other sources were non-embryogenic. Numerous somatic embryos were induced from primary callus on MS medium supplemented with NAA within two weeks of culture. Embryo proliferation was much faster on medium supplemented with BAP. After transfer to medium with GA₃, 1.0mg/l mature green embryos were developed and germinated well into plantlets on MS liquid medium supplemented with 0.5mg/l BAP (Junaid et al., 2007).

Plant regeneration via., direct somatic embryogenesis from cotyledons, hypocotyls and leaves in seabuckthorn (*Hippophae rhamnoides* L.) was achieved (Liu et al., 2007). The highest frequency of somatic embryos production and germination was obtained on Schenk and Hildebrandt (SH) medium supplemented with 1.0mg/l KN and 0.2, 0.5mg/l IAA. Granulated sugar was the optimal carbon source. The embryo-derived plantlets with well developed roots and shoots were transferred successfully to the greenhouse with a maximum survival rate of 55%. The effectiveness of a carbon source in plant tissue culture depended on the plant species, the genotype, and the explants used. Abdoulaye (2000) reported that glucose, sucrose and fructose resulted in higher somatic embryogenesis than maltose and sorbitol in cacao, while maltose was more efficient than sucrose, fructose, glucose in apple and cassava somatic embryogenesis (Daigny et al., 1996; Li et al., 1999).
Chapter – V Somatic Embryogenesis

Somatic embryogenesis, a powerful tool of plant biotechnology for faster and quality plant production has been successfully applied to regenerate plants in *Jatropha curcas* (Jha et al., 2007). Embryogenic calli were obtained from leaf explants on MS basal medium supplemented with only 9.3µM KN. Induction of globular somatic embryos from 58% of the cultures was achieved on MS medium with different concentrations of 2.3 - 4.6µM KN and 0.5 - 4.9µM IBA; 2.3µM KN and 1.0µM IBA proved to be the most effective combination for somatic embryo induction. Addition of 13.6µM AdS stimulated the process of development of somatic embryos. Mature somatic embryos were converted to plantlets on half strength MS basal medium with 90% survival rate in the field conditions. The formation of distinct bipolar somatic embryos with root and shoot poles may be attributed to the presence of cytokinins, as cytokinins stimulate shoot and tap root formation (Chang, 1991). Somatic embryogenic cells can act independently from neighboring cells and undergo somatic embryogenesis, or they can continue to differentiate into secondary embryogenesis (Raemakers et al., 1995).

An improved protocol for high frequency plant regeneration via*, somatic embryogenesis from zygotic embryo derived cell suspension cultures of *Brasenia schreberi* has been developed by Oh et al. (2008). Zygotic embryos formed pale-yellow globular structures and white friable callus at a frequency of 80% when cultured on half strength MS medium supplemented with 0.3mg/l 2,4-D. However, the frequency of formation of pale-yellow globular structures and white friable callus decreased slightly with increasing concentration of 2,4-D upto 3.0mg/l. Cell suspension cultures from zygotic embryo derived white friable callus were established using half strength MS medium supplemented with 0.3mg/l 2,4-D. Upon plating of cell aggregates on half strength MS basal medium, approximately 8.3% gave rise to somatic embryos and developed into plantlets. However, the frequency of plantlet development from cell aggregates sharply increased, when activated charcoal and zeatin were applied.

Plant regeneration *via*, somatic embryogenesis from open pollinated immature zygotic embryos of nine genotypes of elite trees (*Picea koraiensis*) were established (Li et al., 2008). Immature zygotic embryos were cultured onto WP modified medium with 21.48µM NAA, 2.22µM BA, and 2.32µM KN. The average frequency for all nine genotypes was 74.2%. Embryogenic calluses of the nine genotypes of elite trees were
subcultured on RJW basal medium containing 8.06µM NAA, 1.11µM BA, and 1.16µM KN. Embryos was greatly reduced and the optimal medium for plant conversion was ½ WPM. In *Picea*, the modulation of the water content of somatic embryos is required for the stimulation of their germination by dehydration or by the addition of osmotically active solutes or by increasing the medium’s gel strength (Stasolla and Yeung, 2003; Jones and Staden, 2007).

*Zhang et al.* (2008) developed multiple shoots from pumpkin (*Cucurbita moschata*), cotyledon explants excised from various ages of seedlings after *in vitro* germination were cultured on MS augmented with different concentrations of BA. The highest frequency of shoot regeneration (63.7%) was observed from seven days old cotyledon explants cultured on MS containing 0.5mg/l BA. Multiple shoots elongated on MS supplemented with 0.1mg/l BA and 5 - 7 shoots per regenerated explant were recovered. Elongated shoots were rooted on MS, which was easier than that on 2/3 MS, ½ MS, or MS supplemented with 0.1mg/l NAA.

*Joshi et al.* (2008) established normal embryo differentiation by culturing the embryogenic masses in embryo development medium containing 2,4-D and various concentrations of TDZ. Although this was not achieved due to restricted somatic embryo development in the presence of TDZ, bud-like projections appeared in the embryogenic masses when these were cultured in media containing combinations of 2,4-D and TDZ.

A highly reproducible system for efficient plant regeneration from protoplast via somatic embryogenesis was developed by *Wang et al.* (2008) in cotton (*Gossypium hirsutum* L.).Embryogenic callus, somatic embryos and suspension culture cells were used as explants. Callus-forming frequency (82.86 %) was obtained in protoplast cultures from suspension culture cells in medium with 0.45µM 2,4-D, 0.93µM KN, 1.5 % glucose and 1.5 % maltose. The calli from protoplast culture were transferred to somatic embryo induction medium and 12.7% of normal plantlets were obtained on medium contained 3% maltose or 1% of each sucrose + maltose + glucose, 2.46µM IBA and 0.93µM KN.

*An in vitro* propagation system was developed *Ganeshkumari et al.* (2008) using castor-bean (*Ricinus communis* L. cv. TMV 6) through cotyledon derived callus cultures. Green compact nodular organogenic callus was obtained on the medium fortified with MS
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salts, B5 vitamins, 2.0mg/l BA and 0.8mg/l NAA. Multiple shoot proliferation from the
callus cultures was achieved on the medium with MS salts, B5 vitamins, 2.5mg/l TDZ,
0.4mg NAA and 15mg/l glutamine. The proliferated shoots were elongated on the medium
comprising MS salts, B5 vitamins, 1.5mg/l TDZ and 0.3mg/l GA3. The elongated shoots
were rooted on the medium containing MS salts, B5 vitamins, 0.3mg/l IBA and 0.6mg/l
silver nitrate.

Guzman et al. (2009) carried out eight cultivars and two accessions of Physalis
ixocarpa Brot. for their capacity to regenerate embryos and plants from anther cultures.
Anthers were pretreated at 4˚C for 2 days and then at 35˚C for 8 days in the dark while
cultured on MS medium supplemented with 0.045µM 2,4-D + 0.03mg/l vitamin B12 or
with 2.26µM 2,4-D + 0.1mg/l vitamin B12. Androgenetic responses were cultivar and
culture medium dependent, with the greatest embryo yields recorded for cv. Chapingo
(36.3%) and with wild type 2 (21.8%). Further development of regenerated embryos was
promoted on MS medium supplemented with 0.54µM NAA, 8.88µM BA and 50mg/l
casein hydrolysate. The regenerated plants were cultured on half strength mineral salts MS
medium with 2.85µM IAA to enhance root formation.

Plant regeneration through somatic embryogenesis from young leaf explants
adjacent to the apex of 5 - 6 year old offshoots of Tunisian date palm (Phoenix dactylifera
L.), cultivar Boufeggous was successfully achieved (Othmani et al., 2009). The highest
induction frequencies of embryogenic calli occurred after 6 - 7 months on MS medium
supplemented with 10mg/l 2,4-D and 0.3mg/l activated charcoal. Fine chopping and
partial desiccation of embryogenic calli with proembryos prior to transfer to MS medium
supplemented with 1.0mg/l ABA stimulated the rapid maturation of somatic embryos.
Subsequent substitution of ABA in MS medium with 1.0mg/l NAA resulted in the
germination and conversion of 81% of the somatic embryos into plantlets with normal
roots and shoots.

Efficient somatic embryogenesis and in vitro flowering and fruiting were achieved
by Qiao et al. (2009) in Saposnikovia divaricata. Friable embryogenic callus developed
from the root, internode, and leaf explants on MS with 2.26µM 2,4-D and subsequently
developed into somatic embryos on MS medium containing 4 - 5% sucrose, 1.74µM
NAA, 4.44µM BA, and 1.90µM abscisic acid (ABA). Then the mature embryos were separated and transferred onto MS with 3% sucrose and 0.6% agar for further development and conversion to plantlets.

A somatic embryogenesis protocol for plant regeneration of northern red oak (*Quercus rubra*) was established from immature cotyledon explants (Vengadesan and Pijut, 2009). A higher response (66%) of embryogenic callus was induced on 0.45µM 2,4-D. Higher number of globular - (31), heart - (17), torpedo - (12), and cotyledon stage - (8) embryos per explant were obtained by culturing embryogenic callus on MS with 3% sucrose and devoid of growth regulators after 8 weeks cultures in darkness. Cotyledon-stage embryos subjected to desiccation and chilling treatment cultured on MS with 3% sucrose, 0.44µM BA and 0.29µM GA₃ germinated at a higher frequency (61%) than with 0.44µM BA alone and control cultures.

Direct somatic embryogenesis of *Ricinus communis* L. was achieved by Ganeshkumari and Jayabalan (2009) using hypocotyl explants on MS medium with 2,4-D (5.0mg/l) and KN (0.5mg/l). After two passages with one week interval, different stages of somatic embryos were obtained with higher frequency from the embryogenic callus and showed approximately 64% of response with 40-60 embryos/hypocotyl. The embryo inoculated on Pluronic F68 (0.3mg/l) produced root and shoot within one week of inoculation.

Konieczny *et al.* (2010) developed a plant regeneration protocol for *Trifolium nigrescens* via somatic embryogenesis. Immature zygotic embryos at torpedo (TsE) and cotyledonary stage (CsE) were cultured on media with different auxins and cytokinins at different concentrations. The cultural requirements for SE differed between the explants used. The addition of KN or 2iP along with 2,4-D or NAA was needed to elicit the embryogenic response of CsE, but an exogenous cytokinin totally inhibited 2,4-D-induced SE from TsE. When applied alone, neither the cytokinin nor NAA induced SE in TsE or CsE. At comparable concentrations 2,4-D was a more potent SE inducer than NAA. Plant regeneration were achieved after transfer of somatic embryos or embryo-derived first shoots to medium without plant growth regulators.
Direct somatic embryogenesis from pericycle cells of broccoli were established by Yang et al. (2010). The frequency of direct somatic embryo formation was 100% when root explants were cultured in liquid medium. All broccoli genotypes examined had 100% somatic embryo induction efficiency, and the number of somatic embryos per 0.8 mm root segment ranged from 22.9 in ‘Luhui’ to 26.0 in ‘Haizi’. The number of normally developed somatic embryos in culture increased with increasing 2,4-D concentration. Plantlet regeneration frequency was the highest (73.3%) when germinated plantlets were transferred to ½ strength MS medium containing 1.0mg/l BA.

Somatic embryogenesis was achieved for Chamelaucium uncinatum and C. repens. Somatic embryos from young leaves of C. uncinatum and C. repens were induced in vitro on MS medium containing 20g/l sucrose and 2,4-D. For C. uncinatum, up to 4% of explants developed somatic embryos at 20μM 2,4-D and for C. repens, up to 3% developed somatic embryos at 5.0μM 2,4-D. Somatic embryos of C. uncinatum were also induced from immature seeds—a maximum of 6% of seed explants producing somatic embryos on MS medium containing 0.05μM BA and 0.5μM NAA. Somatic embryo cultures maintained on MS medium supplemented with 0.1μM 2,4-D were induced to develop into plantlets after transfer to a hormone-free medium under light (Ratanasanobon and Seaton, 2010).

A protocol for plant regeneration via somatic embryogenesis were developed by Ghanti et al. (2010) using two chickpea (Cicer arietinum L.) cultivars ICCV-10 and Annigeri. Somatic embryos were induced from immature cotyledons on MS medium supplemented with different concentrations of 2,4-D, 2,4,5-T, NAA and picloram alone or in combination with 0.5 - 2.0mg/l BA or KIN. NAA was better for somatic embryo induction compared to other auxins. The well formed, cotyledonary shaped embryos germinated into plantlets with 36.6% frequency on MS medium supplemented with 2.0mg/l BA + 0.5mg/l ABA.

The regeneration of plants via somatic embryogenesis liquid shake culture of embryogenic calluses were achieved by Muruganantham et al. (2010) using Vigna mungo (L.) Hepper (blackgram). The production of embryogenic callus was induced by seeding primary leaf explants of V. mungo onto MS medium supplemented with 1.5mg/l 2,4-D.
The embryogenic callus was then transferred to liquid MS medium supplemented with 0.25mg/l 2,4-D. Globular, heart-shaped, and torpedo-shaped embryos developed in liquid culture. The optimal carbohydrate source for production of somatic embryos was 3% sucrose. L-Glutamine (20mg/l) stimulated the production of all somatic embryo stages significantly. Torpedo-shaped embryos were transferred to containing 0.5mg/l ABA to induce the maturation of cotyledonary-stage embryos. Cotyledonary-stage embryos were transferred to ½ MS semi-solid basal medium for embryo conversion.

5.3. MATERIALS AND METHODS

5.3.1. Plant Material

The root explant was obtained from 15 days old in vitro shoot raised roots and other explants such as leaf, internode and petiole from in vivo field grown plants of Plectranthus barbatus from our garden as described in Chapter -1.

5.3.2. Methodology for Somatic Embryogenesis

5.3.2.1. Selection of Explants

Based on the response, only four kinds of explants (leaf, petiole, internode and root) were used as the source of explants.

5.3.2.2. Inoculation of Explants

The leaf explants were cut into 1.0 cm² bits followed by petiole, internode and root explants cut into 6 - 8 mm length and placed horizontally on MS medium containing 0.7% (w/v) agar, 30 g/l sucrose with suitable hormone concentration described below. The leaf explant was placed with either abaxial or adaxial surface down in 25 x 150 mm culture tubes or in 250 ml conical flask containing 15 ml and 50 ml, respectively in semisolid medium. The cultures were frequently subcultured an intervals of 3 - 4 weeks on embryo proliferation medium. The cultures were subcultured to a photoperiod of 16 hrs light and incubated at 25 ± 2° C.

5.3.2.3. Effect of Media and carbohydrates for Somatic Embryogenesis

Different (MS, MS + B5, half MS and B5) media with plant growth regulators were used. The half strength MS medium was selected for further studies of somatic embryo induction. The effect of different sugars on somatic embryogenesis was
investigated by supplementing the basal medium containing growth regulators with sucrose, fructose, dextrose and maltose separately. To study the effect of different concentrations of sucrose on somatic embryogenesis, sucrose at 1, 2, 3, 4 and 5% (w/v) was incorporated into the medium. Based on the preliminary experiment, 3% (w/v) sucrose was selected for further studies.

5.3.3. Direct Somatic Embryogenesis

5.3.3.1. Embryo Formation

The explants (leaf, petiole, internode and root) were placed in culture tubes and conical flask. The induction medium was half strength MS Basal medium augmented with 30 g/l sucrose and different concentrations of 2,4-D (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0mg/l) in combination with KN/ TDZ (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2mg/l). The pH of the medium was adjusted to 5.8, 0.7% (w/v) agar was added and autoclaved at 121°C for 15 min. The cultures were incubated for 3 - 4 weeks at 25 ± 2°C under TL 40w/54w cool white fluorescent light at 80μE m⁻² s⁻¹ with 16 hrs photoperiod. At the end of 5th week percentage of explants with somatic embryogenesis (explants with globular and later stages of embryos visible to naked eye and microscopically) was recorded.

5.3.3.2. Development and Maturation of Somatic Embryos

Globular embryos formed after 3 - 4 weeks of culture were separated and transferred to MS basal medium augmented with 30g/l sucrose, 7g/l agar and different growth regulators and incubated for two weeks under the conditions described above. Half strength MS basal medium supplemented with 2,4-D (0.5mg/l) and different concentrations of BAP/KN (0.2 - 1.2mg/l) were tested for further development and maturation of somatic embryos, while half strength MS basal medium lacking growth regulators served as the control. The cultures were subjected to a photoperiod of 16 hrs light and incubated at 25 ± 2°C.

5.3.3.3. Germination of Mature Somatic Embryos

Fully developed torpedo or cotyledonary stage embryos were transferred to half strength MS basal medium (3% w/v sucrose) with different concentrations of BAP/KN/GA₃ (0.0 - 2.0mg/l) and NAA (0.0 – 0.5mg/l) for germination and conversion
into plantlets. The embryos were incubated for 15 days at 25 ± 2°C under TL 40w/54w cool white fluorescent light at 80 µE m⁻² s⁻¹ with 16 hrs photoperiod.

5.3.3.4. Development of Plants

Fully germinated embryos with well developed root and shoot systems were transferred for further growth on half MS basal medium with 0.7% (w/v) agar. The sucrose content was kept as 3% (w/v). Total number of plantlets with well developed root and shoot systems were counted. The ratio of plantlets formed from the total number of germinated embryos were calculated as the conversion percentage.

5.3.3.5. Hardening and Acclimatization

Complete plantlets with well developed roots in the tubes were subjected to the following hardening procedure. After loosening the cotton plugs, plants were washed in the running tap water. Thereafter, the plantlets were transferred to the paper cups containing 1:1:1 mixture of river sand, garden soil and saw dust and later established in pots.

5.3.4. Indirect Somatic Embryogenesis

5.3.4.1. Callus Initiation and Maintenance

The leaf, petiole and internode explants were dissected from in vivo field grown plants and roots from in vitro shoot derived roots and inoculated in culture tubes containing 15 ml of the MS medium + 7g/l agar + 30g/l sucrose + 2,4-D/NAA (1.0 – 3.0mg/l) and BAP/KN (0.2 - 1.2mg/l) for callus induction. Callus cultures were maintained at 25 ± 2°C under 16 hrs photoperiod at 80 µE m⁻² s⁻¹ light intensity (TL 40w/54w cool white fluorescent lamps). Calli obtained were subcultured after 15 - 21 days either in the same medium for callus via., somatic embryogenesis or transferred to the liquid medium to establish cell suspensions for further experiments.

5.3.4.2. Cell Suspension Culture Induction and Proliferation

After callus cultures were maintained on the initiation medium, 500 mg of tissue was placed in 50 ml of suspension culture induction medium. This induction medium contained half strength MS basal medium, 3% (w/v) sucrose, 2,4-D (0.0 to 3.0mg/l) and KN (0.4mg/l) or TDZ (0.6mg/l) without agar. The culture shaker was agitated at 100 rpm at 25 ± 2° C. Large clumps of embryogenic tissue, which were seen first in 15 - 28 days
following initiation of suspension culture, were transferred to the embryo proliferation medium. The embryo proliferation medium was the same as that used for induction include KN (0.4mg/l) and 2,4-D (1.0mg/l) was used as growth regulators. Proliferated cell suspensions were subcultured weekly to monthly depending on subculture inoculum density. Weekly subculture was necessary if 25 ml of an old culture was removed and replenished with 25 ml of fresh medium. A monthly subculture or longer was warranted if a single clump (500mg) of early stage embryonic tissue was used to inoculate 50 ml of fresh medium. Samples of suspension cultures were taken at random at the end of 21 days of incubation in liquid medium and the number of somatic embryos was counted under a microscope during the culture period. Counts were made from 35 different independent cultures and the percentage of embryos was calculated based on the total number of embryos present in each culture.

5.3.4.3. Embryo Development

For embryo development, embryogenic tissues were subcultured on half strength MS medium containing 2,4-D (0.5mg/l) + BAP/KN (0.8mg/l). Different stages such as globular, heart, torpedo and dicotyledonous shaped embryos were observed. Finally matured embryos were washed with hormone free proliferation medium and transferred to semi solid medium. The tissue was allowed to settle and the liquid medium was decanted and discarded. Embryos at different stages of development were separated manually. Mature embryos, which were obtained in 28 days on the semisolid medium then they were placed in germination medium. Samples were photographed at different stages during growth period.

5.3.4.4. Germination of Mature Embryos and Plant Development

Mature embryos obtained from plated cultures were placed in culture tubes containing half strength MS basal medium, 3% (w/v) sucrose, KN (1.0mg/l), BAP (1.0mg/l) and GA$_3$ (0.5mg/l) individually or in combination with NAA (0.0 – 0.5mg/l). Embryos were incubated at 25 ± 2°C and 80µE m$^{-2}$S$^{-1}$ light intensity. After root and shoot elongation, the plantlets were transferred initially to the plastic cups containing 1:1:1 mixture of river sand, garden soil and saw dust and later established in pots.
5.3.5. Synthetic seed production

5.3.5.1. Encapsulation of somatic embryos

Somatic embryos obtained from both direct and indirect in solid and liquid (suspension) cultures were blot dried on filter paper and dipped in sodium alginate gel (0.5 - 5% w/v) few seconds. They were dropped into CaCl₂ (2.5% w/v) solution in a 500 ml of flask for encapsulation.

5.3.5.2. Storage and germination of encapsulated embryos

The encapsulated embryos were kept for 30 minutes on a shaker (100 rpm) in light conditions. After incubation period, CaCl₂·2H₂O solution was removed by decanting and capsules were washed with sterile distilled water at 10°C in a refrigerator for various (1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50 and 60) days storage was done in dark by covering the sterile petriplates with aluminium foil in refrigerator.

The synthetic seed germination was determined by incubating the capsules on half strength MS medium under both light and dark condition at 25 ± 2°C. Encapsulated embryos were sown on half strength MS medium containing BAP/KN/GA₃ alone (0.0 – 2.5mg/l) or in combination with NAA (0.0 – 0.5mg/l) for germination of synthetic seeds. Healthy “syn seeds” were placed on various substrates such as half strength MS solid medium and sterilized moist cotton containing BAP/KN/GA₃ + NAA combinations.

5.3.6. Hardening and Acclimatization

Acclimatization and hardening as described in the above section 5.3.3.5.

5.3.7. Statistical Analysis

5.3.7.1. Observation of Cultures and Presentation of Results

The cultures were observed periodically and the somatic embryo Induction, maturation and germination percentages were recorded on the basis of visual and microscopic observation. Whenever possible, the effects of different treatments were quantified on the basis of percent of cultures showing the response per culture. The experimental design was completely randomized design (CRD) and factorial with auxin and cytokinin as independent variables. Each treatment consists of at least 7 explants and the experiment was repeated five times. Mean separation was calculated using Duncan's new Multiple Range Test (DMRT).
5.3.7.2. Calculation

The experimental results were calculated as follows:

\[
\text{Percentage of embryogenesis} = \frac{\text{No. of explants showed embryogenic cell}}{\text{Total No. of explants cultured}} \times 100
\]

\[
\text{Maturation response} = \frac{\text{No. of mature embryos formed}}{\text{No. of embryogenic calli cultured}} \times 100
\]

\[
\text{Conversion frequency} = \frac{\text{No. of plants produced}}{\text{No. of mature embryos cultured}} \times 100
\]

5.4. RESULTS

All the steps comprising somatic embryo induction, maturation, germination, acclimatization and field transfer have been affected by important factors include explant type, media formulation, growth regulators and culture conditions. All these factors were investigated in the somatic embryogenesis of *Plectranthus barbatus*. Both direct and indirect somatic embryogenesis was observed using auxin (2,4-D) singly. Addition of cytokinins like KN or TDZ had significantly influenced to produce somatic embryogenesis, but this combination was comparatively less somatic embryos produced than 2,4-D alone. The somatic embryos were produced both solid and liquid medium. Among the different types of (leaf, petiole, internode and root) explants, the frequency of somatic embryos induction and number of somatic embryos per culture was optimum in leaf explant followed by root, internode and petiole explants. The highest percentage of culture response and optimal number of somatic embryos from different explants and different ways are discussed in Fig. – 5.1, 5.2 and 5.3. Following main steps in regeneration of plants through somatic embryogenesis is given in Fig. – 5.6, 5.7 and 5.8.

5.4.1. Direct Somatic Embryogenesis on solid medium

Direct somatic embryos were formed within 25 – 30 days of inoculation on basal media containing different concentrations of either 2,4-D or in combination with cytokinins (KN and TDZ). The somatic embryos developed from the explants (leaf, petiole, internode and root) portions depending upon the strength of induction medium.
Significant variations in the percentage of responding cultures and number of somatic embryos per explant were also observed. All the explants directly formed embryos without intermediate callus phase.

**5.4.1.1. Induction of Somatic Embryogenesis**

The four explants with three sets of growth regulators (2,4-D, 2,4-D + KN and 2,4-D + TDZ) were tested for induction of direct somatic embryogenesis. The results indicate that 2,4-D singly yielded the highest percentage of response and higher number of embryos while other combinations induced low. After 4 weeks of culture, white granular calli were formed on leaf and root but not on the other explants. The granular callus gave rise numerous somatic embryos as the culture proceeded, but only nodules appeared on the other explants (petiole and internode).

In many instances the epidermal cell layer of the explants split up and organized structures enlarged from the subepidermal layers. The number of organized structures were higher on leaf compared to petiole explants. These organized structures closely resembled globular and heart shaped somatic embryos. Nodular structures were seen in most of the combinations with varied response. Hereafter, these nodular structures are referred as embryogenic masses, since the development of embryos was restricted to these protruberances. The frequency of embryogenic mass formation varied with the type and concentration of growth regulators used.

**5.4.1.2. Effect of Growth Regulators on embryo induction**

a. **Effect of 2,4-D**

Half MS basal medium fortified with various concentrations of 2,4-D (0.0 to 3.0mg/l) were examined for their embryo induction efficiency. Among various concentrations, 2,4-D at 1.5mg/l was found to be the best for maximum percentage of somatic embryos developed directly on the explants. The frequency of direct somatic embryo induction was highest in the leaf explants (71.4%) as compared to petiole (68.6%) followed by internode (68.6%) and root (54.3%) explants listed in Table - 5.1 and Table - 5.2.

The highest number of somatic embryos was noticed as in 23.2 embryos on leaf explant (Plate - 5.1 a, b and c), 17.9 embryos on petiole explant (Plate - 5.1 g, h and i), 18.2 embryos on internode explant (Plate - 5.1 d, e and f) and 19.0 embryos on root
explant (Plate- 5.1 j, k and l) within 4 weeks of culture (Table - 5.1 and Table - 5.2). In media supplemented with higher concentration or low concentration from the optimal level of 1.5mg/l 2,4-D had reduced in the induction frequency and number of embryos formation in all the explants.

b. Effect of 2,4-D in combination with KN

Among different concentrations of KN (0.2 - 1.2mg/l) optimal concentration of 2,4-D (1.5mg/l) produced somatic embryos without callus phase. Somatic embryos formed directly from leaf explants within 25 days were cultured half strength MS medium supplemented with 1.5mg/l 2,4-D + 0.4mg/l KN (Table - 5.1). Somatic embryos were observed after 4 weeks on petiole, internode and root explants cultured half MS medium with these hormone supplementation. Embryos were white or green, small and globular, appearing individually or in cluster (Plate - 5.1). The maximum percentage of response was observed on medium supplemented with 2,4-D (1.5mg/l) + KN (0.4mg/l) combination in leaf (62.9%), petiole (57.1%), internode (65.7%) and root (48.6%). The maximum of 18.2 embryos from leaf, 15.7 embryos from petiole, 17.1 embryos from internode and 16.8 embryos from root explant within a month of culture. The number of embryos varied with hormone concentration and types of explant used.

c. Effect of 2,4-D in combination with TDZ

Fixed concentration of 2,4-D (1.5mg/l) combined with different concentrations of TDZ (0.2 – 1.2mg/l) were tested for induction of somatic embryos listed in Table- 5.1 and Table- 5.2. Somatic embryos formed directly at cut edges, base near the midrib or on the leaf surface. Petiole and internode explants slightly expanded and became a little longer and wider 3 weeks after culturing on the medium. Somatic embryos generally appeared from cut ends of roots that touched on half MS medium supplemented with 1.5mg/l 2,4-D + 0.6mg/l TDZ. Small, round somatic embryos directly produced from one or two sides of petiole, internode and root explants surfaces. The protruding somatic embryos developed into globular form, appearing either in clusters or individually.

The 2,4-D at 1.5mg/l + TDZ at 0.6mg/l produced the maximum frequency of embryogenic response in leaf (57.1%), petiole (54.3%), internode (54.3% and root (48.6%).
The maximum number of somatic embryos in leaf was 16.1 embryos per explant whereas in petiole it was 13.9 embryos per explant followed by internode produced 15.8 embryos per explant and root produced 15.2 embryos per explant within 35 days culture (Table- 5.1 and Table- 5.2). Statistical analysis showed significant correlation between hormone concentration 2,4-D (1.5mg/l) + TDZ (0.6mg/l) and embryo induction response per responding culture (P < 0.05).

5.4.1.3. Development and Maturation of embryos

For further development and maturation the leaf, petiole, internode and root explants directly derived globular and heart shaped somatic embryos with mother explants were transferred to half strength MS basal medium supplemented with 3% sucrose and different concentrations of BAP or KN (0.2 – 1.2mg/l) and 2,4-D (0.5mg/l). The maturation efficiency of somatic embryos varied with different concentrations of 2.4-D and BAP or KN. The globular and heart shaped embryos developed into torpedo and cotyledonary stage in this medium. Medium without PGR less promote the growth of the embryos and embryo development was observed. Embryos were very loosely arranged on the surface of the explants. Maturation increased with increasing in the BAP/KN concentration up to optimum level in the medium. The combination of BAP (0.8mg/l) and 2,4-D (0.5mg/l) was found to be the best for highest frequency of somatic embryos maturation (Table- 5.8 and Plate- 5.3).

5.4.1.4. Germination of Somatic embryos

Isolation of matured embryos from cultures were transferred to germination medium containing 3% (w/v) sucrose and different concentrations of either BAP/KN or GA₃ (0.0 – 2.5mg/l) singly or optimum level of these hormone in combination with NAA (0.0 – 0.5mg/l) for further growth. Germination of embryos was characterized by simultaneous production of root and shoot systems. The embryos turned green and developed by elongation, reveals the folded cotyledon in the germination medium. Later, the cotyledons unfolded and the shoot region developed further with the emergence of first leaves (Plate - 5.4). When the half strength MS basal medium supplemented with different concentration and combination of PGR used, both shoot and root poles developed in all the combinations with varying frequencies.
**Chapter – V Somatic Embryogenesis**

**a. Effect of BAP**

For germination of somatic embryos, various concentrations of BAP (0.0 to 2.5mg/l) were used individually and among these, 1.0mg/l BAP induced the highest percentage of embryo germination (68.6 %) and 20 embryos germinated within 21 days (Table- 5.9).

**b. Effect of BAP in combination with NAA**

The optimum concentration of BAP (1.0mg/l) optimized in the previous experiment and various concentrations of NAA (0.0 - 0.5mg/l) were tested for somatic embryos germination. Of these, BAP (1.0mg/l) and NAA (0.3mg/l) combination stimulated the maximum percentage of embryo germination (88.6%) and maximum number of embryos germinated (26) within 21 days of culture (Table- 5.9).

**c. Effect of KN in combination with NAA**

The synergistic effect of different concentrations of NAA (0.0 – 0.5mg/l) combined with optimum concentration of KN (1.0mg/l) were tested for somatic embryo germination. Among these, NAA (0.3mg/l) and KN (1.0mg/l) combination showed the maximum percentage of germination response (80.0) and optimum of 24 embryos were germinated within 21 days of culture (Table - 5.9).

**d. Effect of GA\textsubscript{3} in combination with NAA**

Different concentrations of NAA (0.0 – 0.5mg/l) and combined with optimized GA\textsubscript{3} (0.5mg/l) were used for embryos germination. Among these combinations, the GA\textsubscript{3} (0.5mg/l) and NAA (0.3mg/l) combination showed maximum percentage (85.7%) of response with 25 germinated embryos were obtained within 21 days of culture (Table - 5.9). Statistical analysis showed significant variations between hormone concentration and germination response per responding of culture (P < 0.05).

**5.4.1.5. Acclimatization and Hardening**

The process of the embryos growth round oval shape delayed somatic embryogenesis at early stages. Cotyledon initiation occurred at one end of the oval shaped somatic embryos and latter globular stage was clearly evident when elongation started radicle formation was followed by cotyledon elongation, as occurs in zygotic
developmental stages. Both radicle and cotyledons were conspicuous by torpedo stage, after which morphology of somatic embryos was clearly established. After germination, cotyledons turned dark green and shoots elongated. Finally, complete plantlets developed from bipolar (shoot & root) somatic embryogenesis were obtained (Plate - 5.4).

The somatic embryos derived plantlets were shifted to plastic cups (Plate - 5.5) containing garden soil, river sand and vermiculite (1:1:1) and then finally transferred to field conditions.

5.4.2. Indirect Somatic Embryogenesis

The most important factors affecting the induction of embryogenic callus and plant regeneration through somatic embryogenesis include the explant type, media formulation and growth regulators. The significant feature of all these factors were investigated with reference to the indirect somatic embryogenesis of *P. barbatus*.

5.4.2.1. Somatic Embryogenesis on solid medium

5.4.2.1.1. Callus induction

A model where somatic embryogenesis is divided into two different steps can be considered. The first step an induction phase of the explant can be approached by the number of embryogenic callus mass. The second step, expression of embryogenic potential can be evaluated by the number of embryos formed and developed. Callusing efficiency of *in vitro* raised explant like root and *in vivo* explant like leaf, petiole and internode were tested on MS medium containing 1.0 – 3.0mg/l 2,4-D/NAA singly or in combination with BAP/KN (0.2 – 1.2mg/l). Swelling of explants occurred within a week and explants began to produce callus after two weeks of inoculation. Among the four explants, internode explants produced maximum frequency of callus followed by petiole, leaf and root explants. Leaf explants produced greenish brown and friable callus on 2,4-D (2.0mg/l) and light brown and less compact callus on 2,4-D (2.0mg/l) + KN (0.6mg/l). Petiole explants produced greenish white and friable callus on 2,4-D (2.0mg/l) and light brown and friable callus on 2,4-D (2.0mg/l) + KN (0.6mg/l). Internode explants produced light brown and friable callus on 2,4-D (2.0mg/l) singly or in combination with BAP (0.6mg/l) and root explants produced light brown and friable callus on 2,4-D/NAA (2.0mg/l) singly or in combination with BAP or KN (0.6 mg/l).
Among the two auxins (2,4-D and NAA) used, 2,4-D induced maximum amount of friable and embryonic callus while NAA had no similar response. In addition of BAP/KN enhanced optimum amount of friable callus. The callus formation occurred at cut ends and then began to spread to parts of the explant especially leaf explant midrib, venal or intravenal region and from both adaxial and abaxial surfaces. For further proliferation the callus was subcultured on the same medium (Table - 5.3). Prolonged culture period resulted in browning and death of callus and also callus failed to initiate on growth regulator free MS medium. The callusing response from both explants varied depending on the concentration of PGR used. The variation seen in callus derived from both explant were similar. The percentage of response of leaf, petiole, internode and root explants for callus induction on various concentrations and combinations of PGR is given in the Table - 5.3. Calli with greenish brown or light brown colour and friable were visually selected and subcultured for further embryogenic response.

5.4.2.1.2. Induction of Somatic Embryogenesis

Highly organized, rounded and brown colour embryonic mass development was observed from all over the surface of the callus within 14 days of culture. Somatic embryos were produced half strength MS basal medium supplemented with different concentrations of 2,4-D (0.0 - 3.0mg/l) or in combination with KN or TDZ (0.2 to 1.2mg/l). The frequency of embryogenic mass formation varied with the type and concentration of auxin used. Repeated subcultures on the same medium composition increased the frequency of somatic embryos at 15 days of each culture. Leaf explant derived callus was found to be the best explant source for embryogenesis followed by root, internode and petiole explants derived callus. The data showed that the embryogenic mass induction was higher in 2,4-D (1.0mg/l), which was highly significant at the level of P < 0.05 (Table- 5.4 and Table- 5.5).

At lower concentrations of 2,4-D (0.5mg/l) or absence of 2,4-D on the medium, the initial hump formation was noted at low frequency. At higher concentration (above optimal level) of 2,4-D embryogenic mass induction was low by the end of the sixth week and later tissues became brown. Of the 2,4-D alone or in combination with KN or TDZ tested, 2,4-D was found to be superior for embryogenic mass induction (Plate- 5.2) and various stages of embryos were observed in the same induction medium.
a. Effect of 2,4-D

To find out the role of 2,4-D in somatic embryo induction, different concentrations of 2,4-D (0.0 - 3.0mg/l) were used individually and 2,4-D (1.0mg/l) produced the highest frequency of somatic embryogenesis (91.4% and 88.6%) on leaf and internode callus and maximum number of 41.5 and 34.9 embryos per callus, respectively followed by petiole and root derived callus which produced maximum response (85.7% and 77.1%) with 32.5 and 37.0 embryos per callus, respectively (Table - 5.4; Table - 5.5) within 28 days with two subculture (Plate - 5.2).

b. Effect of 2,4-D in combination with KN

Somatic embryos were induced from various concentrations of KN (0.2 - 1.2mg/l) with optimum concentration of 2,4-D (1.0mg/l). The combination of 2,4-D (1.0mg/l) + KN (0.4mg/l) induced the optimum percentage of somatic embryos in leaf (82.9%) and petiole (74.3%) explants derived callus (Table - 5.4). The highest number of somatic embryos was noticed in leaf (38.8 embryos/callus) and petiole (29.9 embryos/callus) explant derived callus within 28 days of culture followed by internode derived callus which showed 77.1% embryogenic response with 32.2 embryos per callus and root callus showed optimum of 65.7% embryogenic response with 33.7 embryos per callus (Table - 5.5 and Plate - 5.2).

c. Effect of 2,4-D in combination with TDZ

The effect of 2,4-D (1.0mg/l) combined with different concentrations of TDZ (0.2 – 1.2mg/l) were tested and 2,4-D (1.0mg/l) and TDZ (0.6mg/l) combination produced the highest frequency of somatic embryos (77.1, 65.7, 74.3 and 60.0%) and maximum number of somatic embryos (36.4, 26.3, 29.1 and 27.5 embryos/callus) in leaf, petiole, internode and root explants derived callus, respectively within 35 days of culture (Table - 5.4 and Table - 5.5).

5.4.2.1.3. Development and Maturation of Somatic Embryos

Somatic embryos were developed on leaf, petiole, internode and root explants derived callus. Somatic embryos obtained in each treatment were transferred to embryo development and maturation medium. Somatic embryos developed within 15 - 21 days. Each group of embryonic masses were transferred to half strength MS medium containing
0.5mg/l 2,4-D and different concentrations of BAP/KN (0.2 - 1.2mg/l) for further development and maturation. On maturation media embryos turned green and developed further into mature embryos like torpedo and cotyledon stages (Plate - 5.3). Half strength basal medium supplemented with 0.5mg/l 2,4-D with 0.8mg/l BAP was found to be best combination for maturation of embryos. Maturation response increased with increasing concentration of BAP/KN from 0.2 - 0.8mg/l and a further increasing concentration of BAP/KN, the embryogenic maturation frequency declined.

5.4.2.1.4. Germination of Somatic Embryos

After maturation of somatic embryos, they were transferred to half strength MS solid medium containing three combinations of PGR for germination. Further details are as described in the previous section 5.4.1.4.

5.4.2.1.5. Acclimatization and Hardening

As described in the previous section 5.4.1.5.

5.4.2.2. Somatic embryogenesis in liquid medium (Suspension cultures)

5.4.2.2.1. Callus induction

As described in the above section 5.4.2.1.1.

5.4.2.2.2. Cell Suspension Culture

All the explants derived greenish brown or light brown, friable and fast growing differentiated callus individually were subcultured on half strength MS, MS, MS + B5 and B5 liquid medium containing 2,4-D (0.0 -3.0mg/l) alone or in combination with KN or TDZ (0.2 - 1.2mg/l) for embryo initiation. The dispersion mainly composed of free cells in small clusters of 15 - 20 cells and irregular aggregates. Most of the cells aggregated randomly on the callus, however some globular organization of dividing cells may occur in three weeks and in older callus when they were transferred to liquid medium, the callus broke up into single cells and small cell groups.

The results of embryogenesis from both explants derived callus with various growth regulators concentrations and combinations summarized in Table - 5.6 and Table - 5.7. The results indicate that half strength MS basal liquid medium yields the highest amount of active division and growth of embryogenic cells.
5.4.2.2.3. Induction of somatic embryogenesis

Suspension cultures were agitated with 100 rpm in complete darkness for 3 days. 500mg of callus were subcultured into half strength MS liquid medium with suitable growth regulators. This single cell stopped dividing and differentiated into large, curved and vacuolated cells, some cells within the cell groups are small, relatively round, dense and often green or greenish brown. These cells keep dividing to form a tight, globular proembryo masses. The central core of the proembryo from the suspension culture continue to grow on solid medium and outer enlarged, vacuolated cells become crescent shaped and were lost from the surface. Whole embryo became round and light brown or white brown. This was early globular stage of development of somatic embryogenesis and it was equivalent to the zygotic embryos but without the attached suspensor.

**a. Effect of 2,4-D**

The effect of 2,4-D was studied for embryo induction from callus on half strength MS liquid medium containing different concentrations of 2,4-D (0.0 – 3.0mg/l). The highest frequency of somatic embryogenesis (100% and 88.6%) and maximum number of somatic embryos (75.8 and 59.1 embryos/culture) occurred in leaf and petiole derived callus suspension culture, respectively on 2,4-D (1.0mg/l) within 28 days of culture (Table - 5.6). Followed by internode, root derived callus suspension culture showed maximum percentage of culture response (94.3 and 97.1%) with optimum number of 65.4 and 67.2 embryos per culture, respectively (Table - 5.7). The frequency of somatic embryogenesis decreased with increasing concentration of 2,4-D (above optimal level) in the medium and the higher frequency was observed at 1.0mg/l.

**b. Effect of 2,4-D in combination with KN**

Somatic embryos were induced from various concentrations of KN (0.2 - 1.2mg/l) with 2,4-D (1.0mg/l) combinations. These combinations produced the maximum percentage of somatic embryos in leaf (94.3%), petiole (80.0%), internode (91.4%) and root (91.4%) with an average of 66.4, 54.2, 58.7 and 61.0 embryos per culture was noticed on 2,4-D (1.0mg/l) + KN (0.4mg/l) within 28-35 days of culture (Table- 5.6 and Table- 5.7).
c. Effect of 2,4-D in combination with TDZ

Optimum concentration of 2,4-D (1.0 mg/l) with various concentrations of TDZ (0.2 – 1.2mg/l) combinations were used for embryos induction. Among these, 2,4-D (1.0mg/l) + TDZ (0.6mg/l) combination induced the maximum percentage of somatic embryos in leaf (88.6%), petiole (74.3%), internode (85.7%) and root (88.6%) derived callus suspension culture while in the other, the optimum number was 59.6 embryos per leaf callus culture, 49.7 embryos per petiole callus culture, 54.5 embryos per internode callus culture and 55.8 embryos per root callus culture within 28 - 35 days of inoculation (Table - 5.6 and Table - 5.7). The effect of different hormones 2,4-D (1.0mg/l) and TDZ (0.6mg/l) combination on percentage of response and number of somatic embryos were statistically significant at P < 0.05 level.

5.4.2.2.4. Development and Maturation of Somatic embryos

Proembryos underwent further internal cell divisions to give rise to the globular embryos on a medium containing 2,4-D with BAP/KN. The changes from globular to torpedo shaped embryo was brought about by increasing mitotic cell divisions in the shoot apical region and mid central region of embryos and further divisions in the cotyledon. The frequency of somatic embryogenesis was studied in the cell suspension, after 28 days of culture. More than 85% of the cells were converted into different stages of somatic embryos (Plate - 5.3).

Somatic embryos at late torpedo stage were transferred to 250 ml flask containing 25 ml of half strength MS medium fortified with various growth regulator concentrations of 2,4-D, BAP and KN and placed on a rotary shaker.

a. Effect of 2,4-D

Different concentrations of 2,4-D (0.0 – 2.0mg/l) were tested for efficiency of development and maturation of somatic embryos. At 0.5mg/l of 2,4-D there was maximum frequency of maturation response (85.7%) with maximum number of matured embryos (23.0 globular stage, 18.4 heart-shaped stage and 11.2 torpedo-shaped stage) within 28 days of culture (Table - 5.8).
b. Effect of 2,4-D in combination with BAP

Optimum concentration of 2,4-D (0.5mg/l) combined with various concentrations of BAP (0.2 - 1.2mg/l) was investigated for their maturation efficiency. Among these, 2,4-D (0.5mg/l) + BAP (0.8mg/l) combination showed the maximum percentage of maturation response (97.1%) with optimum of 34.3 globular stage, 26.6 heart-shaped stage and 15.8 torpedo-shaped somatic embryos per culture within 21 days of culture (Table - 5.8). Globular and heart-shaped embryos were developed into torpedo and cotyledonary stage in this medium (Plate - 5.3).

c. Effect of 2,4-D in combination with KN

At 0.5mg/l 2,4-D with additions of various concentrations of KN (0.2 - 1.2mg/l) for maturation of somatic embryos. 2,4-D (0.5mg/l + KN (0.8mg/l) combination showed the highest percentage of embryos maturation (91.4%). The highest number of globular stage was in (28.4), heart-shaped stage (22.2) and torpedo-shaped stage (13.0), somatic embryos from the culture within 21 - 28 days of inoculation (Table - 5.8). The effect of hormone 2,4-D (0.5mg/l) and KN (0.8mg/l) concentration on maturation of somatic embryos was statistically significant at P < 0.05 level.

5.4.2.2.5. Germination of Somatic embryos

After maturation of somatic embryos, they were transferred to half strength MS solid medium containing three combinations of growth regulators for germinations as described in the previous section 5.4.1.4.

5.4.2.2.6. Acclimatization and Hardening

As described in the previous sections 5.4.1.5.

5.4.3. Synthetic seed Production

The encapsulation of somatic embryos, conversion of encapsulated embryos into plants and factors affecting the conversion frequency are described below.

Abundant somatic embryos were directly produced from the explants on half MS medium containing 2,4-D (1.5mg/l) alone or in combination with KN (0.4mg/l) or TDZ (0.6mg/l) in solid medium. Indirect somatic embryogenesis was observed through the callus phase on half strength MS medium containing 2,4-D (1.0mg/l) alone or in combination with KN (0.4mg/l) or TDZ (0.6mg/l) in both solid and liquid medium.
5.4.3.1. Encapsulation of somatic embryos and germination

Sodium alginate (0.5 – 5.0%) with CaCl₂ (2.5%) was tested for preparation of synthetic seeds. Among these, 3.0% of sodium alginate was found to be suitable for encapsulation. For maximum frequency of “syn seed” germination sodium alginate 3.0% was more suitable than other concentration. The maximum frequency of response was 91.4 % and the optimum number of 27 “syn seeds” germinated in 3.0% of alginate concentration with suitable hormone combination, thereafter there was the reduced germination response in low and high concentrations of sodium alginate with 25 days of culture (Table - 5.10). Hence 3.0% sodium alginate and 2.5% CaCl₂ solution was selected for synthetic seeds preparation (Plate - 5.4).

Various substrates were tested for “syn seeds” germination on half strength MS solid medium and sterilized moist cotton containing half strength MS salts with growth regulators such as BAP (1.0 mg/l) and NAA (0.3mg/l) and of these, half strength MS solid medium showed 91.4% of syn seeds germination in 25 days of culture (Plate - 5.4). The encapsulated seeds germinated at very low percentage on sterilized moist cotton with MS nutrients and hormones. The synthetic seeds retained their viability upto 15 days and gradually there was a declined.

5.4.3.2. Storage effect of Synthetic seeds on Germination

The effect of storage conditions on encapsulated embryos was studied for various days upto two months (1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50 and 60 days). The maximum percentage of response was 91.4% and totally 27 syn seeds germinated out of 35 syn seeds that were cultured in 15 days of stored conditions. After 15 days stored syn seeds germination was gradually reduced. The effect of synthetic seed storage on germination was statistically significant at P < 0.05 level (Table - 5.11).

Various concentrations of BAP (0.5 - 2.5mg/l) were tested for syn seeds germination and BAP at 1.0mg/l showed maximum percentage (74.3%) of germination response with 22 syn seeds germination on half strength MS medium. The combination of BAP (1.0mg/l) and NAA (0.3mg/l) showed optimum of 91.4% response with 27 syn seeds germination. KN (1.0mg/l) + NAA (0.3mg/l) combination showed 82.9% embryos response with 25 syn seeds germination and the combined effect of GA₃ (0.5mg/l) + NAA
(0.3mg/l) showed 88.6% of response with 26 syn seeds germination (Table - 5.10). The effect of BAP (1.0mg/l) and NAA (0.3mg/l) combination was significant on “syn seeds” germination and survival was statistically significant at P < 0.05 level.

The encapsulated embryos germinated within three weeks of culture. The alginate matrix ruptured, green leaves emerged and roots developed from encapsulated embryos. The plantlets from encapsulated embryos were normal when compared to non-encapsulated embryos derived plants (Plate - 5.4).

5.4.3.3. Acclimatization and Hardening

As described in the previous section 5.4.1.5.

5.4.4. Effect of media

Leaf derived embryonic callus produced on MS solid medium was transferred to half strength MS, MS, MS + B5 and B5 liquid medium containing 2,4-D (0.0 - 2.5mg/l) for the induction and development of somatic embryos (Table - 5.12). The frequency of (97.1%) embryos formation and development of (34.3 globular stage, 26.6 heart shaped stage and 15.8 torpedo shaped stage) somatic embryos per culture was higher in the half MS liquid medium (Fig. – 5.4). Increasing the nutrient strength of half MS medium reduced the embryogenic frequency. Somatic embryo frequency was low in MS followed by in MS + B5 and B5 medium.

5.4.5. Effect of Carbohydrates

Four different carbohydrates (glucose, fructose sucrose and maltose) were tested at different levels to study their effect on somatic embryogenesis, producing the highest frequency and average number of somatic embryos (Table - 5.13). In the presence of glucose, fructose and maltose, the frequency was reduced than in sucrose.

Among the four carbohydrates, sucrose was the most promising carbohydrate for the induction and development of somatic embryogenesis. Of the different concentrations of sucrose tested, 3% concentration was the most effective and it increased the embryogenic potential. The highest frequency (97.1%) of embryogenic response and optimum number of (34.3 globular stage 26.6 heart-shaped stage and 15.8 torpedo-shaped embryos per culture) somatic embryos in leaf were the highest at sucrose 3% (w/v)
However, higher concentration of sucrose (4, 5% w/v) inhibited or decreased the embryogenesis. For sucrose, the frequency of somatic embryos and maturation increased as concentration increased to 3% (w/v) and then decreased with further increase (Table - 5.13).

5.5. DISCUSSION

The results demonstrate distinctly the induction of somatic embryogenesis from leaf, petiole, internode and root explants of *Plectranthus barbatus* through direct and indirect embryogenesis. This study has shown half strength MS medium supplemented with 2,4-D to be the suitable medium for induction of somatic embryogenesis. Induction of somatic embryogenesis in several species requires a high concentration of auxin in the culture medium especially 2,4-D or NAA. The usual procedure for induction of somatic embryos in both monocotyledonous and dicotyledonous species requires the explant transfer from an auxin supplemented induction medium to auxin free medium.

The ability to induce somatic embryos in tissue culture opens new vistas which was not available in plantlets regeneration via., organogenesis. The advantages of somatic embryogenesis was the simultaneous development of root and shoot systems. Plant regeneration with direct and indirect somatic embryogenesis from various explant sources of *Paulownia* species has been reported by Ipekci and Gozukirmizi (2003).

Reliable plant regeneration systems that culminate in the formation of plants are necessary for the successful utilization of biotechnological methods in conservation of medicinally important herbaceous plants. Somatic embryogenesis offers several advantages such as protoplast culture, gene multiplication in higher plants, production and synthetic seeds and possible use of bioreactors for producing large quantities of somatic embryos (Liu et al., 2007).

*In vitro* regeneration of complete plantlets through somatic embryogenesis was reported in many medicinal plants such as *Panax* (Tang, 2000), *Liriope platyphylla* (Kim et al., 2000), *Cynodon dactylon* (Chaudhury and Qu, 2000), *Ulmus minor* and *Ulmus glabra* (Corredoira et al., 2002), *Coffea arabica* (Molina et al., 2002), *Picea abies* (Ramarosandratanana and Staden, 2003), *Pinus taeda* (Pullman et al., 2003), *Catharanthus*
In the present study, we have attempted somatic embryogenesis and plantlets formation from various explants of medicinally important herb *Plectranthus barbatus*. The observations showed that different concentrations of 2,4-D and combinations of 2,4-D + KN and 2,4-D + TDZ were found to be essential for induction of both direct and indirect somatic embryogenesis in *Plectranthus barbatus*.

5.5.1. Direct somatic embryogenesis in solid medium

In the present study half strength MS medium having 2,4-D, 2,4-D + KN and 2,4-D + TDZ combination has produced embryos developed from leaf, petiole, internode and root explants.

Regeneration of plants through somatic embryogenesis, particularly direct somatic embryogenesis has been reported in *Paulownia elongata* (Ipekci and Gozukirmizi, 2003), *Azadirachta indica* (Chaturvedi et al., 2004), *Epipremnum aureum* (Zhang et al., 2005), *Syngonium podophyllum* (Zhang et al., 2006), *Bactris gasipaes* (Steinmacher et al., 2007), and *Hippophae rhamnoides* (Liu et al., 2007). Additionally, direct somatic embryogenesis has a lower probability of genetic variation than other propagation methods (Merkle, 1997). Propagation through the somatic embryogenesis, however, may have advantages over organogenesis particularly direct somatic embryogenesis, because it can potentially scale-up propagation using bioreactors and produce synthetic seeds (Rani and Raina, 2000). Regeneration of *Oncidium* through direct somatic embryogenesis has been achieved using leaf explant (Chen et al., 1999). Young leaves have a high regeneration capacity and may provide large number of embryos and plantlets in a short period of time. Direct somatic embryos formation on leaf explants was retarded by exogenous auxins, but promoted by exogenous cytokinins (Chen and Chang, 2000).
In the present experiment, there was much possibility of induction of direct somatic embryogenesis and plant regeneration from leaf, petiole, internode and root explants of *P. barbatus* using exogenous auxin (2,4-D at 1.5mg/l) alone or in combination with cytokinin (KN at 0.4mg/l or TDZ at 0.6mg/l). Induction of somatic embryogenesis was achieved on MS medium supplemented with 2,4-D (4.0mg/l) with KN (5.0mg/l) from the shoot primordial explants of *Gloriosa superba* (Jadhav and Hegde, 2001). Zhang *et al.* (2005) have reported direct somatic embryogenesis in golden pothos from the leaf explants in MS medium supplemented with 2.0mg/l KN and 0.5mg/l 2.4-D and induced high frequency of somatic embryos. The present investigations have some distinct advantages over previous reports where 2.4-D (1.5mg/l) only showed the highest frequency of (71.4%) response with an average of 23.2 embryos directly from the leaf explant followed by 2.4-D (1.5mg/l) with KN (0.4mg/l) which showed 62.9% response with 18.2 embryos from the explant.

Somatic embryogenesis can be considered as the ultimate expression of the totipotentiality of plant cells and it is well established that the expression of this feature is under genetic control (Ezhova, 2003). The influence of the genotype on somatic embryos induction in peach palm was observed using leaf sheaths as explants (Stein and Stephens, 1991) and inflorescence as explants (Steinmacher *et al.*, 2007). Some reports (Paterson and Everett, 1985; Chraibi *et al.*, 1991; Arditti and Ernst, 1993 and Yordanov *et al.*, 2002) show an increase in regeneration potential by supplementing the regeneration medium with cesein hydrolysate, amino acids, KNO₃ and AgNO₃. Chraibi *et al.* (1992) reported that the silver nitrate in the regeneration media increased the regeneration efficiency from cotyledons of *Helianthus annuus* by decreasing sensitivity of explants to endogenic ethylene. Our preliminary results showed that the regeneration efficiency was positively affected by the higher auxin content in the embryo induction medium. For further development of embryos, a transfer to a medium with lower auxin but with higher cytokinins content was made.

Somatic embryos were directly at cut edges or on the surface of leaf sections, around cut ends or along the side of petiole, internode and root explants. Somatic embryos were able to germinate directly on the initial induction medium. This is consistent with reports of Zhang *et al.* (2005), Zhang *et al.* (2006) and Ipekci and Gozukirmizi (2003).
5.5.1.1. Development and Maturation of Somatic embryos

The developmental stages of embryos at the time of culture is an important determinant of the morphogenic response (Custers and Bergervoet, 1990 and Chaturvedi et al., 2004). In present study different stages of development (globular, heart-shape stage, torpedo-shape stage and early dicotyledonous stage) were observed in the maturation medium containing 2,4-D (0.5mg/l) + BAP (0.8mg/l). TDZ treatment induced embryogenesis and enhanced the number of somatic embryos from explants initially cultured on medium containing 10mg/l TDZ alone and could be detected visually after transfer of the original explants into hormone-free MS basal medium after 4 weeks (Ipekci and Gozukirmizi, 2003). Their study showed that there were all stages of the somatic embryos from globular through cotyledonary embryos developed on the surface of the leaf and internode explants. The present study also has shown all the stages of (globular to early dicotyledonous shape) somatic embryos after transfer of original explants into maturation medium containing 2,4-D (0.5mg/l) + BAP (0.8mg/l) only. Maturation medium containing 2,4-D and BAP as well as those containing KN and NAA were not very suitable for development or were unable to stimulate an maturation of embryogenic response (Table - 5.8). In general leaf explants were more responsive than other such as petiole internode and root explants. Similar results were also reported by Kim et al. (2003) in Hylomecon vernalis, Jeyaseelan and Rao (2005) in Cardiospermum halicacabum, George et al. (2005) in Centella asiatica and Qiao et al. (2009) in Saposhnikovia divaricata.

Among concentrations and combinations of the growth regulators tested, 2,4-D (0.5mg/l) + BAP (0.8 mg/l) showed an increase in the embryogenic response of the explant. Normal growth and development of induced globular embryos then torpedo shaped embryos was obtained in cultured explants on half strength MS medium. This phenomenon has already been observed in many other species (Chaturvedi et al., 2004; Sellars et al., 1990; Fambrini et al., 1996 and Mandal and Datta, 2005).

5.5.1.2. Germination of somatic embryos

Regeneration through the direct somatic embryogenesis could be a potential solution to minimize the variation. Somatic embryos that passed through normal development stage underwent germination into complete plantlets with distinct shoot and
root systems, when taken out from suspension culture and cultured on MS basal medium (Sebastian et al., 2006b; Kim et al., 2000 and Kim et al., 2004). Somatic embryos that were germinated on half strength MS medium supplemented with 0.01mg/l BAP and 0.25mg/l ABA promoted maturation and germination of somatic embryos of *Acacia arabica* (Nanda and Rout, 2003). Similar observations have been made in some crop plants (Das et al., 1997 and Ortiz et al., 2000). This is in contrast to the present studies which have shown that conversion of somatic embryos into plants was dependent on the type and concentration of auxin and cytokinin used in the somatic embryo germination medium. The best plant conversion frequency was obtained when cultured embryos on half strength MS solid medium containing BAP (1.0mg/l) + NAA (0.3mg/l) and tiny plantlets were transferred to green house for further developments. There results corroborate the previous findings of Zhang et al. (2006) where 85% somatic embryos germinated 5 - 10 weeks after transferring onto medium containing 2.0mg/l BAP and 0.2mg/l NAA. Somatic embryos were able to germinate directly on the initial induction medium and complete embryos germinations and shoot development occurred in MS medium containing 2.0mg/l BAP and 0.2mg/l has been reported (Zhang et al., 2005).

In present investigation, a successful protocol was developed for somatic embryogenesis. Advanced stages of somatic embryos (globular to early dicotyledonous shaped) and tiny plantlets were obtained. However, fully developed plantlets were obtained on half strength MS basal medium with plant growth regulator supplementation. This is the first report of direct somatic embryogenesis and subsequent plant regeneration of *Plectranthus barbatus* through the non-meristematic tissues.

### 5.5.2. Indirect Somatic Embryogenesis

The process of somatic embryogenesis defined as the development of a bipolar structure with both root and shoot poles from any sporophytic part of plant occurring through the same key states of embryos development as zygotic embryogenesis is yet another way of exhibiting totipotency by plant cells, whereby they first dedifferentiate and them redetermine towards the embryogenic pathway.

Successful application of a somatic embryogenesis protocol largely depends on the ability of the somatic embryos to convert into plantlets. There are many reports on the successful induction of somatic embryogenesis and developments of plants through the
callus phase from leaf segments (Hamama et al., 2001; Cho et al., 2003; Murch et al., 2003; Lee and Lee, 2003; Jones et al., 2007 and Joshi et al., 2008), leaf and petiole segments (Kim et al., 2003), leaf and internode segments (Jeyaseelan and Rao, 2005; George et al., 2005 and Biswas et al., 2007), internode explants (Sharma and Millam, 2004) and root explants (Leguillon et al., 2003; Iantcheva et al., 2005 and Qiao et al., 2009).

The induction of somatic embryogenesis for in vitro plant regeneration provides several advantages over the traditional organogenesis (Wang and Bhalla, 2004). Somatic embryogenesis provides an excellent morphogenetic system for investigating the cellular and molecular process underlying differentiation (Benelli et al., 2001). In addition, somatic embryogenesis also provides the possibility to produce artificial seeds and valuable tools for genetic engineering and germplasm conservation via. cryopreservation (Litz and Gray, 1995 and Merkle, 1997).

Somatic embryogenesis has been currently used in several transgenic research programs (Rommens et al., 2004 and Walter, 2004). However, the process of embryo formation and conversion is complex and affected by several external factors (Vookova and Kormuak, 2006). Among them the role of plant growth regulators, especially of 2,4-D in determining the early stages of embryogenesis has been regularly emphasized (Davletova et al., 2001; Pasternak et al., 2002 and Wang et al., 2006). Cytokinins are usually less effective but not uncommon (Mujib et al., 1998 and Iantcheva et al., 1999). In the present study, an optimized regeneration system for long-term subculture and continuous high frequency of somatic embryogenesis and plantlets production in Plectranthus barbatus was developed. On the induction medium containing 2,4-D and KN, embryogenic callus was successfully induced from the leaf, petiole, internode and root explants (Table - 5.3).

5.5.2.1. Callus induction

Induction of calli is the first phase in the somatic embryogenesis. Soft, friable, greenish white or yellowish white callus is usually recommended for induction of somatic embryogenesis in herbaceous plants (Levanic et al., 2004; Eisa et al., 2005; Rhimi et al., 2006; Jones et al., 2007 and Wang et al., 2008). In the present investigation, response of
calli from different explants varied. Among the four explants, internode explants produced maximum frequency of callus followed by petiole, leaf and root explants. Similar results were also reported by Biswas et al. (2007) in strawberry, George et al. (2005) in *Centella asiatica* and Jeyaseelan and Rao (2005) in *Cardiospermum halicacabum*. This was at variance with the results obtained by Prakash et al. (2001) where hypocotyl explants proliferated faster than other aerial explants as in *Pimpinella tirupatiensis*. Followed by Ashokkumar et al. (2002) cultured hypocotyl explants also great effect on callus induction than leaf and cotyledon.

In present study, the type and morphological nature of callus induced from different explants also varied. Leaf explants produced greenish brown and friable callus on 2,4-D (2.0mg/l) and light brown and friable callus on 2,4-D (2.0mg/l) + KN (0.6mg/l). Petiole explants produced greenish white and friable callus on 2,4-D (2.0mg/l) and light brown and less compact callus on 2,4-D (2.0mg/l) + KN (0.6mg/l). Internode explants produced light brown and friable callus on 2,4-D (2.0mg/l) singly and in combination with BAP (0.6mg/l) and root explants produced light brown and friable callus on 2,4-D/NAA (2.0mg/l) singly or in combinations with BAP or KN (0.6mg/l). This results was in agreement with reports on *Hylomecon vernalis* by Kim et al. (2003) who reported that brownish friable calluses began to form on the cut ends of petiole and leaf explants, after culture on medium with 2,4-D and on medium with combination of 2,4-D and BAP. Followed by the pattern of embryogenic callus formation on internode, leaf and root explants of *P. barbatus* was in agreement with the results of Saposhnikovia divaricata (Qiao et al., 2009) in pedicel explants from *Chelidonium majus* (Woo et al., 1996), ovule explants from *C. majus* (Kim et al., 1999), leaf explants from *Ostericum korencnum* (Cho et al., 2003) and root from *Medicago truncatula* (Iantcheva et al., 2005). Long time exposure of these embryogenic calli on the induction medium tissue became highly browned or darkened and whitish nature might be due to depletion of 2,4-D in the medium declining the growth. This is in agreement with the reports of Lee and Lee (2003), Aoshima (2005) and Jones et al. (2007).

5.5.2.2. Induction and Development of Embryos in solid medium

The present studies have shown that it is possible to induce somatic embryogenesis and plant regeneration from leaf, petiole, internode and root explants of *Plectranthus barbatus*. Considering the efficiency of embryogenesis, media containing 2,4-D produced
the largest number of somatic embryos. When individual 2,4-D and combination with KN or TDZ were compared, the former was markedly more active for embryogenesis. Similar results were also reported by Kim et al. (2000), Corredoira et al. (2002), Kim et al. (2004) and Junaid et al. (2007). Somatic embryos are formed from embryogenic callus after transferring the calluses from MS medium containing 2,4-D to hormone free medium in many plants (Soh et al., 1996; Cho et al., 2003 and Sharma and Millam, 2004). But the present study did not show any somatic embryogenesis without growth regulator medium. This is in consistence with reports of Corredoira et al. (2002) and Fernando et al. (2003). George et al. (2005) achieved somatic embryogenesis when they cultured callus on MS medium supplemented with lower concentration of 2,4-D (0.5mg/l) and KN (0.25mg/l), followed by transfer to half strength MS medium containing 2,4-D (2.0mg/l) and embryogenic callus gave rise to numerous somatic embryos. Thus, these results are analogous to the present observations where the presence of auxin in the medium is generally essential for embryo induction and high frequency of somatic embryogenesis was recorded on half strength MS medium supplemented with 1.0mg/l 2,4-D and singly produced 41.5 embryos/leaf derived callus followed by addition of KN (0.4mg/l) with 2,4-D (1.0mg/l) that produced 38.8 embryos. The frequency of embryos formation increased with increasing concentration of 2,4-D upto 1.0mg/l. However, at higher concentration (3.0mg/l) of 2,4-D, the callus did not form embryogenic mass, but the callus became to brown. This result was in agreement with reports of Kim et al. (2000). The auxin in the culture medium prevents the development of somatic embryos from embryogenic cells (Cho and Soh, 1995). Addition of cytokinin (BAP/KN) to the medium promoted the growth, development, maturation and enhanced shoot morphogenesis (Hamama et al., 2001; Ashokkumar et al., 2002; Rhimi et al., 2006 and Wang et al., 2008).

In present study, both the frequency of somatic embryos maturation and germination were very high in the presence of cytokinin. Somatic embryo development stopped at the heart stage on the media containing 2,4-D with globular/heart stage embryos on induction medium. These embryos produced torpedo and cotyledon stage embryo upon subculturing on embryo maturation medium (Medium containing half MS + 2,4-D 0.5mg/l + BAP 0.8mg/l). Similar beneficial effect of 2,4-D + BAP combination on
somatic embryos maturation has been demonstrated (Rhimi et al., 2006 and Biswas et al., 2007). Later, for better germination (plant conversion) these matured embryos were individually placed on media supplemented with BAP (1.0mg/l) + NAA (0.3mg/l). Hamama et al. (2001) also reported same kinds of germination.

5.5.2.3. Somatic embryogenesis in liquid medium

Callus induction from both leaf, petiole, internode and root explants was observed on medium containing 2,4-D/NAA alone or in combination with BAP/KN. Callus was highly variable colour and texture. Typical colour of callus was green, yellow, white and brown. Some cultures were rough surfaced and friable white, others were smooth surfaced and hard calli were yellow to green colour and appeared friable, were visually selected and subcultured for cell suspension.

5.5.2.3.1. Induction of Embryos in cell Suspension

The use of suspension culture is an important component in the scale-up and synchronization of embryogenic culture (Silveria et al., 2004 and Jones et al., 2007). The regeneration of plants via., somatic embryogenesis through cell suspension culture is an useful and promising technique (Gamborg et al., 1983; Yang et al., 1990; Petitprez et al., 2005 and Oh et al., 2008) and has higher application for in vitro selection and for raising somaclonal variants (Adkins et al., 1992). Embryogenic cell suspension culture induced the process of somatic embryos development and maturation (Kim et al., 2004 and Min et al., 2007). In present the investigation, active cell division was noticed from 10 - 15 days of subculture and later the cell turned brown and the culture became thick and mucilaginous. The embryonic tissue formed large clumps of globular and later staged embryos had to be transferred individually with forceps or a spatula to the half strength MS medium darkened between subcultures and the embryos underwent proliferation and development in the same medium. Smooth round structures were present in the callus which had the appearance of globular embryos, but these structures developed into further stages. Somatic embryos were mostly induced from callus placed on induction medium supplemented with 2,4-D (1.0mg/l) singly or in combination with KN (0.4mg/l). Earlier reports by many authors (Min et al., 2007; You et al., 2007 and Othmani et al., 2009) also support the role of 2,4-D in somatic embryo induction.
5.5.2.4. Development and Maturation of embryos

Following transfer of embryogenic masses for the maturation medium containing 2,4-D with BAP/KN, the growth characters of the culture changed. The response of embryogenic masses following transfer to a medium depended on the use of hormones. Globular somatic embryos on subculturing in to the maturation medium containing 2,4-D (0.5mg/l) + BAP (0.8mg/l) were found to gradually convert into heart shaped, torpedo and cotyledonary stage embryos with bipolarity with globular stage embryos dominating in the culture (Table - 5.8). Our study required a maximum 4 to 6 weeks for conversion of globular embryos to germinated plantlets. The 2,4 D and BAP are known to enhance the efficiency of maturation of somatic embryos, which is a critical step in somatic embryogenesis. Earlier reports support the role of auxin and cytokinin in somatic embryo maturation (Cho et al., 2003; Jha et al., 2007 and Ganasan et al., 2007).

The frequency of embryo development and maturation increased slightly with increasing concentration of cytokinin (BAP) up to 0.8mg/l and further increase in concentration of BAP decreased their maturation potentiality. Similar results were also reported by Oh et al. (2008) in Brasenia schreberi and Mandal and Datta (2005) in Chrysanthemum.

5.5.2.5. Germination of Somatic Embryos

Somatic embryos were subsequently rooted and developed into plantlets at a higher frequency (George et al., 2005 and Min et al., 2007). In present study, for shoot and root induction when the somatic embryos at the cotyledonary stage were inoculated on half strength MS medium supplemented with BAP (1.0mg/l) and NAA (0.3mg/l) for up to 5 weeks they produced maximum plantlets with germination. Du and Bao (2005) also reported that BAP with NAA were effective with optimum germination. You et al. (2007) characterized that somatic embryos were rapidly germinated when they were transferred onto MS medium containing GA3 within 5 weeks of culture.

In the present study, a critical step of conversion of mature somatic embryos into somatic plantlets was obtained on transfer of 6 weeks old mature somatic embryos on maturation medium to conversion medium for 2 - 3 weeks. The formation of distinct bipolar somatic embryos with root and shoot poles may be attributed to the presence of
cytokinins, as cytokinins stimulate shoot and tap root formation (Chang, 1991 and Jha et al., 2007). After a certain developmental stage, transfer of embryos to growth regulator containing (BAP + NAA) half strength MS medium resulted in germination forming complete plantlets. Stimulation of germination of somatic embryos by cytokinin alone or containing auxin has been reported (Hamama et al., 2001; Ashokkumar et al., 2002; Junaid et al., 2007 and Wang et al., 2008).

5.5.2.6. Hardening and Acclimatization

Germinated somatic embryos subsequently developed into plantlets (shoot and root) at higher frequency of 90%. The plantlets were subsequently transferred to plastic cups containing garden soil, river sand and vermiculite (1:1:1) (Biswas et al., 2007) and maintained at 80 - 90% humidity by covering with transparent polythene bags in a growth chamber for two weeks.

The plants were irrigated once in 3 days and transferred to green house with nutrient solution. After one month regenerated plantlets were transferred to pots and they survived at a frequency of approximately 80 - 90% and grew to morphologically normal appearance.

5.5.3. Synthetic seed technology

Main objective of this investigation was to develop a procedure for the encapsulation of somatic embryos of *P. barbatus* to the production of synthetic seeds as a new population method. Synthetic seed technology is a potential method for delivering clonal plant material produced from tissue culture to the field. Syn seeds are composed of somatic embryos encased in a protective coating that mimics the shape and function of zygotic seeds. Synthetic seeds have multiple advantages, long term storage, higher scale-up capacity uniformity in production, potential for automation of the whole production process, seeding of clonal varieties and may provide a means for maintenance of elite germplasm (Singh et al., 2007). Somatic embryos of *Quercus serrata* were encapsulated in 4% sodium alginate with half strength MS medium (Ishii et al., 1999). To cope with these difficulties, the embryogenic masses of plants have been encapsulated in sodium alginate gel using the method of Redenbaugh *et al.* (1987). Somatic embryos subjected to encapsulation process induce the maturation of uniform growth than non encapsulated
embryos. Various syn seed systems have been proposed in the present study, but the focus was on using a hydrated gel capsule composed of alginate to encapsulate single hydrated somatic embryos. The potentiality of the axillary buds for alginate encapsulation and use of the alginate encapsulated buds for propagation were evaluated in *Ocimum* (Mandal *et al.*, 2000).

The reports on synthetic seed production in *Plectranthus barbatus* are scanty. Nethertheless, the success achieved in *P. barbatus* on encapsulation of somatic embryos and subsequent germination of embryo to produce plants indicated the possibility of applying regenerative potency and it should not be difficult to encapsulate single embryos raised plants.

The effect of different days stored artificial seeds was investigated for embryo germination. In present study, the encapsulated embryos showed 91.4% germination when stored for one day to fifteen days at 10˚C and thereafter a decline in the frequency of germination was noticed. The viability percentage decreased with increase in storage time and temperature. Likewise, the regeneration percentage of MS medium encapsulated propagules stored at 10˚C showed similar response (81%) in 30 days and when stored at 25˚C the survival rate was 45% in 30 days as reported by Neelamathi *et al.* (2007). The encapsulated micropropagules in sugarcane were stored up to 20 days under culture conditions (Jalaja, 2000). According to Datta *et al.* (1999) the artificial seeds stored at 4˚C for 120 days showed no reduction in viability. In contrast, encapsulates somatic embryos stored at 4˚C in sealed petriplates remained viable for up to 75 days with 6.9% germination, whereas non-encapsulated embryos remained viable for up to 45 days with 24.9% germination (Singh *et al.*, 2007). Synthetic seeds were stored at 4˚C for two months without significant loss of conversion capacity as reported by Pintos *et al.* (2008).

Among the two substrates (half MS solid and sterilized moist cotton) used, half MS solid medium only showed higher germination rate than moist cotton. BAP and NAA combination showed the highest frequency of (91.4%) germination response in half strength MS solid medium. Plant derived from syn seeds was normal compared to non-encapsulated somatic embryos derived plants as reported by Singh *et al.* (2007).
However a detailed study on the nutrient requirements of encapsulated buds towards developing a more suitable nutrient reservoir for the encased plant tissue is now necessary to improve the frequency of plant retrieval. The storage of encapsulated micropropagules, somatic embryos, buds and shoot tips at low temperature also offer the possibility of using this method for ex situ germplasm conservation of medicinally and commercially important plants (Neelamathi et al., 2007).

5.5.4. Effect of Media on Somatic Embryogenesis

Nutrient concentrations and their ratios in the medium significantly affected the induction and development of somatic embryos. The effects of growth regulators and nutrient factors were intensively studied on somatic embryogenesis in many important crops (Zimmerman, 1993; Pedroso and Pais, 1995 and Chen and Chang, 2002). In our experiment, modified half strength MS salts as basal medium was found to be highly suitable for the induction, maturation and germination of somatic embryos than full strength MS, MS + B5 and B5 media. Vookova and Kormuak (2001) reported that there were no significant differences between the MS and SH Medium on induction and germination of somatic embryos. In this study, however, the results were not in accordance with their report. Half MS basal medium was proven to be the best among the four kinds of basal media tested. Kulothungan (1997) reported that higher percentage of nitrogen in MS and MS + B5 liquid medium supported the induction of higher number of globular embryos than B5 media. This is in contrast to the present study for half strength MS liquid medium supported the initiation of higher number of globular embryos than MS, MS + B5 and B5 media.

5.5.5. Effect of Carbohydrates on Somatic Embryogenesis

Among the four different carbohydrates investigated for their influence on somatic embryogenesis, optimum response was observed in sucrose followed by glucose, fructose and maltose. The most commonly used carbohydrate in plant tissue culture is sucrose. The influence of carbon source on somatic embryogenesis has been reported for other plant species (Parrot and Bailey, 1993; Daigny et al., 1996; Abdoulaye, 2000; Jha et al., 2007 and Oh et al., 2008). Abdoulaye (2000) reported that sucrose, glucose and fructose resulted in higher somatic embryogenesis than maltose. However, maltose was unsuitable
for somatic embryogenesis (Ladyman and Girard, 1992). In nature, carbohydrate is transported within plant tissue as sucrose and tissue may have an inherent capacity for uptake transport and utilization of sucrose. Sucrose at 3% produced the best results in *P. barbatus* followed by glucose 4%, fructose 4% and maltose 2%. In this study which corroborates with the reports of Kulothungan (1997) in cowpea and Liu *et al.* (2007) in *Hippophae rhamnoides*. 