INDUCTION OF EMBRYOGENIC TISSUE AND DEVELOPMENT OF SOMATIC EMBRYOS IN *Pinus brutia* TEN.

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ABSTRACT

INDUCTION OF EMBRYOGENIC TISSUE AND DEVELOPMENT OF SOMATIC EMBRYOS IN *Pinus brutia* TEN.

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Conifer species are subjected to major time constraints in tree improvement because of their long regeneration cycle and large sizes. However, integration of developing biotechnologies could significantly reduce this time limitation in tree breeding programs. In this regard, somatic embryogenesis (SE) offers a great potential in commercially important Turkish red pine (*Pinus brutia* TEN.) for rapid production of larger number of clones as well as capture of greater genetic gains. In this study, seven collections were done to sample precotyledonar zygotic embryos for induction of embryogenic tissue (ET) from 15 clones located in Antalya. Afterwards, abscisic acid, carbohydrates, polyethylene glycol (PEG), and gellan gum were tested to obtain mature somatic embryos in maturation experiments. Analyses of variance showed a significant variation among

collection dates (43.1% of total variance) and clones studied (18.8% of total variance) for induction of ETs. Overall initiation frequency of ET in this study was 11.6% with clonal range of 4.7 – 24.1%. Of those tested maturation treatments, 80µM ABA, sucrose and maltose at 3 and 6%, 3.75% PEG combined with 1% gellan gum were found to be suitable for maturation of somatic embryos in Turkish red pine. Sixty nine somatic embryos were obtained from Clone 22, which was one of tested clones. Induction frequencies could be further improved by using different basal media and/or manipulating media components, such as plant growth regulators. For proper maturation of somatic embryos, embryogenic lines need to be screened to find suitable lines, which are developmentally responsive to ABA treatment.

Keywords: Somatic embryogenesis, somatic embryos, embryogenic tissue, maturation, abscisic acid.

ÖZ

Pinus brutia TEN.'de EMBRİYOGENİK DOKU İNDÜKLEMESİ VE SOMATİK EMBRİYO GELİŞİMİ

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Konifer türleri uzun yaşam döngüleri ve büyük boyutlarda olmaları nedeniyle ıslah çalışmalarında zaman kısıtlamasına maruz kalmaktadırlar. Gelişmekte olan biyoteknoloji tekniklerinin ıslah çalışmalarına entegrasyonu zaman problemini önemli ölçüde azaltabilir. Bu bağlamda, somatik embriyogenez tekniği ticari öneme sahip Kızılçam (*Pinus brutia* TEN.) klonlarının daha kısa bir sürede çoğaltılması ve daha fazla genetik kazanç elde edilmesi bakımından büyük bir potansiyel taşımaktadır. Bu çalışmada Antalya'da kurulu olan tohum bahçesinde bulunan 15 klondan embriyogenik doku indüklemesi sağlamak için kotiledon gelişimini tamamlamamış zigotik embriyoları örneklemek amacıyla yedi defa tohum toplandı. Bu aşamadan sonra olgunlaşma deneylerinde olgun somatik embriyo elde etmek için absisik asit (ABA), karbonhidrat, polietilen glikol (PEG) ve gellan gum agarı

içeren besiyerleri test edildi. Varyans analizi örnekleme tarihleri (toplam varyasyonun %43.1'i oranında) ve çalışılan klonlar arasında (toplam varyasyonun %18.8'i oranında) istatistiksel önemde farklılıklar olduğunu gösterdi. Bu çalışmada ortalama embriyogenik doku indükleme frekansı %11.6 ve klonlar için de %4.7 – 24.1 aralığında elde edildi. Test edilen olgunlaşma uygulamaları arasından, %1 gellan gum agarı ilave edilen 80μM ABA, %3 sükroz veya %3 ve 6 oranında maltoz ve %3.75 PEG içeren besiyerlerinin Kızılçam somatik embriyo oluşumu için uygun olduğu belirlendi. Test edilen klonlardan biri olan Klon 22'den altmış dokuz adet olgun somatik embriyo elde edildi. İndükleme oranları, farklı besiyeri kullanarak veya besiyeri bileşenlerini değiştirerek, örneğin bitki büyüme hormonları gibi, artırılabilir. Somatik embriyo oluşumunu sağlamak için, ABA uygulamasına cevap verebilecek gelişim aşamasında bulunan uygun hatların bulunabilmesi gereklidir. Bu nedenle olgunlaşma deneylerine başlamadan önce embriyogenik hatların bir ön taramadan geçirilmesi elde edilecek embriyo sayısının artması bakımından faydalı olacaktır.

Anahtar kelimeler: Somatik embriyogenez, somatik embriyo, embriyogenik doku, olgunlaşma, absisik asit.

To my wife and grandparents

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2,4-D	2,4-dichlorophenoxyacetic acid
ABA	Abscisic acid
AC	Activated carbon
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
CF	Conversion frequency
DCR	Douglas-fir cotyledon revised medium
ECL	Established cell line
ECL_N	Number of established cell lines
ET	Embryogenic tissue
EXFREQ	Extrusion frequency
GLM	General linear model
HCl	Hydrochloric acid
INFREQ	Initiation frequency
$INFREQ_N$	Number of explants showing initiation
MS	Mean square
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
PEG	Polyethylene glycol
PEM	Proembryogenic mass
PGR	Plant growth regulators

SCV	Settled cell volume
SE	Somatic embryogenesis
VC	Variance component

CHAPTER 1

INTRODUCTION

An ever-increasing demand for forest products has led to development of biotechnologies assisting traditional tree breeding programs to produce phenotypically superior and genetically improved trees. Clonal propagation can be very effective together with breeding. Based on their applicability to forest trees, there are two ways of clonal propagation: (1) traditional macropropagation from seed and (2) micropropagation (Dunstan, 1988; Bonga & von Anderkas, 1992).

Macropropagation includes grafting on juvenile rootstock and rooting of cuttings that are mostly used for fruit trees and conifers with juvenile material. Micropropagation is divided into three types: (1) axillary shoot elongation; (2) organogenesis; and (3) somatic embryogenesis. The first one has a very limited use in conifers; the latter two are based on plant tissue culture techniques. In organogenesis, plantlets are regenerated either from preexisting meristems (e.g. axillary buds) or from adventitious buds. Although utility of organogenesis has been proven in the clonal propagation of several commercially important forest trees, such as *Eucalyptus* spp. and radiata pine (*Pinus radiata*) (Aitken-Christie *et al*., 1988; Becwar *et al*., 1988), its major limitation for mass production on a commercial scale is the high production cost of individual plantlets. The last one, somatic embryogenesis (SE), is the development of embryos from embryogenic tissue (ET), which

can be cultured to form an organized bipolar structure having shoot and root connected through functional vascular tissue (Cheliak & Rogers, 1990). Somatic embryogenesis offers tremendous potential over micropropagation via organogenesis for large-scale plant production in forestry. Its advantages can be summarized as follows (Attree & Fowke, 1991; Gupta, 1988): (1) large number of plantlets can be produced rapidly; (2) it is amenable to suspension culture which can reduce labor time and cost; (3) complex root induction treatments are not necessary; (4) it provides a regeneration system for gene transfer studies; (5) cryopreservation of embryogenic cultures permits long-term storage of genotypes during performance trials in the field; (6) potential of being encapsulated to produce synthetic seeds.

1.1. Literature review on somatic embryogenesis in conifers

Successful SE in woody perennials has been reported for about 30 years, including more than 25 families, 44 genera, 60 species and numerous cultivars of trees (Tulecke, 1987). In conifers, D.J. Durzan and colleagues conducted many studies about the growth, metabolism, and developmental patterns to characterize callus and suspension cultures from about 1968 to 1980. Although they reported polarized embryo-like structures (embryoids) and long suspensor-like cells in cultures of *Pinus banksiana* (Durzan & Steward, 1968; Chalupa *et al*. 1976; Durzan & Chalupa, 1976), *Picea glauca* (Durzan & Steward, 1968), *Picea abies* (Chalupa & Durzan, 1973), and *Pseudotsuga menziesii* (Durzan, 1980), no further development into embryos

or plantlets were obtained.

The first successful reports on SE and plantlet regeneration were in *Picea abies* (Chalupa, 1985) and *Larix decidua* (Nagmani & Bonga, 1985), initiated from zygotic embryos and megagametophytes, respectively. After these studies, many conifer explants have been induced to form somatic embryos including examples from the generas of *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, and *Sequoia* (Tautorus *et al*. 1991).

Conifer zygotic embryogeny is divided into two phases: (1) a brief phase during 16-celled proembryo consisting of four tiers of four cells each is formed within the archegonium; and (2) an embryo phase starting with the elongation of third suspensor tier pushing the embryonal cells into the gametophyte corrosion cavity, followed by tissue differentiation and storage product deposition (Figure A.1 and A.2 A-E). In conifers, polyembryony is a common phenomena in which several embryos begin to develop at the same time. There are two types of polyembryony, simple and cleavage. Simple polyembryony occurs due to fertilization of more than one egg per ovule by gametes from different pollen grains resulting in genetically different proembryos. This is observed both in *Picea* and *Pinus* species. The latter species also undergo cleavage polyembryony that takes place when the tiers of cells in the very early stage of embryo development, destined to become suspensors, are functionally separated. Each has a potential to develop into genetically identical embryos. In both types of cleavage, one proembryo later dominates and others degenerate (Figure A.1 and A.2 F-H, both figures are given in the Appendix with their captions; Owens and Blake, 1985).

Current knowledge about the origin and development of conifer somatic embryos is quite limited. Even the type of explant used can be effective (Nagmani *et al*. 1987; Finer *et al*. 1989). Hakman *et al*. (1987) have suggested three different processes for the origin of somatic embryos: (1) arise from single cells or small cell aggregates by an initial asymmetric division that delimits the embryonal apex and suspensor region; (2) develop from small meristematic cells within suspensor; (3) arise by a mechanism similar to cleavage polyembryony, with the initial separation occurring in the embryogenic region.

Generally, there are two important factors affecting the success of SE: (1) explant selection and culture conditions; and (2) embryo maturation and development. The first one, explant selection is very critical in the induction of SE in conifers and is depended on the species studied. So far, embryogenic cultures of conifers have been initiated from immature zygotic embryos, mature zygotic embryos, and excised tissues from young seedlings (mainly cotyledons) (Attree & Fowke, 1991). In both *Picea* and *Pinus* species, often one cone collection date during maturation period corresponding to particular developmental stage of zygotic embryo are proven to be the most responsive to induction of embryogenic culture, such as *Picea glauca* (Lu & Thorpe, 1987), *Larix decidua* (von Aderkas *et al*. 1987), *Pinus caribaea* (Laine & David, 1990), *Pinus taeda* (Becwar *et al*. 1988, 1990), *Pinus strobus* (Finer *et al*. 1989). Usually, precotyledonary and cotyledonary immature zygotic embryos are best explants for embryogenic tissue induction in *Pinus* and *Picea* species, respectively. The induction of

embryogenic tissue from mature explants is mainly pertinent to *Picea* species with the exception of sugar pine (*Pinus lambertiana*) in which mature zygotic embryos were used. For example, Attree *et al*. (1990) obtained embryogenic tissue from 12 to 30 days old seedlings of *Picea mariana* and *Picea glauca*. In *Pinus* species, more studies need to be done to obtain embryogenic tissue from mature explants. The principal advantage of using mature zygotic embryos is the availability of mature seeds year-round.

Different types of media are used to induce ET in conifers, such as in *Pinus* species, half-strength (1/2) LP with *Pinus caribaea* (Laine & David, 1990), ½ MS, and full strength DCR (Douglas-fir cotyledon revised medium) with loblolly pine (*Pinus taeda*) (Gupta & Durzan, 1987; Becwar *et al*. 1990). A full list of media used for induction of SE is given by Tautorus *et al*. (1991). In *Pinus* species, both megagametophytes containing immature embryos and excised immature zygotic embryos (at precotyledonary stage) are used for SE. Former explants have two main advantages over excised ones. First, it is much easy to culture the megagametophyte, and second is the higher initiation frequency. Nevertheless, it is important not to wound the megagametophytes during dissection.

Once the megagametophytes are placed on initiation medium, they display a number of responses within the first few weeks. Some explants exhibit no response at all or form non-embryogenic callus originating from gametophytic cells. Extrusions from the micropylar end of the gametophytes are preliminary indicators for a positive response. However, proliferation from extrusions could be arrested at some point for unknown reasons.

Therefore, the initiation frequency of embryogenic tissue (ET) is generally determined after 7 – 9 weeks of culture (Lelu *et al*. 1999; Percy *et al*. 2000). After the initiation period, ET is separated from mother explant. Afterwards they are subcultured mostly onto same medium used for induction. Depending on the species studied, the plant growth regulator (PGR) regime is kept either same or lowered.

The second important factor in SE is the embryo maturation and plantlet development. This is a major limitation for the commercial utilization of this technology (Dunstan, 1988; Tautorus *et al*. 1991). Once the induction of ET is completed, the induction medium is generally repressive for further development because of the plant growth regulators used; 2,4-D (2,4-dichlorophenoxyacetic acid) and BAP (6-benzylaminopurine). Therefore, they are usually transferred to different medium for maturation. The reduction or elimination of phytohormones is not sufficient for formation of somatic embryos and conversion into plantlets, sporadic plantlet formation and infrequent growth are quite common in such embryos (Hakman & Fowke, 1987; Gupta & Durzan, 1987; Bourgkard & Favre, 1988). Application of abscisic acid (ABA) and increased osmolarity are required for production of cotyledonary stage embryos in many coniferous species. Von Arnold & Hakman (1988) first made the classification of somatic embryo maturation in their study with *Picea abies*. They defined four distinct stages, which are useful to follow the developmental progress: Stage 1, proembryos with translucent suspensors; Stage 2, embryos with smooth outline, opaque in appearance; Stage 3, embryos with cotyledons; Stage 4, plantlet with

rudimentary radicle development. This classification has been used as a guideline in conifers somatic embryogenesis studies. The ABA in the maturation medium promotes the accumulation of storage lipids and proteins and prevents precocious germination (Bonga & von Aderkas, 1992). Its working concentration mainly depends on the species studied. For example, in *Picea* species successful maturation is possible by applying 16 - 50μM ABA for a period of 4 to 6 weeks (Egertsdotter & von Arnold, 1998; Stasolla & Yeung, 2001). On the other hand, the studies in *Pinus* species demonstrated that ABA the requirements are quite different, such as 60 - 120μM ABA and a period of 8 to 12 weeks for maturation are reported (Lelu et al. 1999; Miguel *et al*. 2004).

High osmolarity is provided by either with plasmolysing agents (sucrose, maltose, mannitol, and amino acids) or with non-plasmolysing agents (polyethylene glycol (PEG), sorbitol, and dextran) (Stasolla *et al*. 2002). Former agents can cross the plasma membrane and be absorbed by the symplast of the cells but, after certain time, an osmotic recovery would take place and the state of low tissue water content would be lost. Also, such agents at high concentrations are toxic for the tissue and could interfere with maturation and cause necrosis (Attree & Fowke, 1993). For conifers, PEG is the most commonly used osmotic agent combined with ABA for promoting maturation of somatic embryos. In Norway spruce (*Picea abies*) and white spruce (*Picea glauca*), 3.75 and 5% PEG were the optimum concentrations for Stage 3 embryo formation, respectively (Svobodova *et al*. 1999; Attree *et al*. 1995). Compared to *Picea* genus, maturation media containing 7.5 and

10% PEG resulted in higher number mature embryos in *Pinus taeda* (Li *et al*. 1997), and *Pinus patula* (Jones & van Staden, 2001). Klimaszewska and Smith (1997) first demonstrated that high gellan gum concentration in maturation media could be used to restrict the availability of water physically instead of using non-plasmolysing osmotica in *Pinus strobus*. They found optimum maturation response at 1.0% gellan gum (with 80μM ABA) among the tested range, 0.4 – 1.2%. Following this report, beneficial effect of using high levels of gellan gum was also confirmed in other pine species, such as *Pinus sylvestris*, *P. pinaster*, *P. monticola*, and *P. maritime*. This suggests that decreasing the water availability of the medium is more important than increasing its osmotic potential for development of Stage 3 embryos (Lelu *et al*. 1999; Garin *et al*. 2000; Percy *et al*. 2000; Ramarosandratana *et al*. 2001; Miguel *et al*. 2004). Although inclusion of PEG has contributed to maturation positively, several researchers reported problematic germination and incomplete root development, which affect their subsequent establishment in field (Bozhkov & von Arnold, 1998; Ramarosandratana *et al*. 2001b). Therefore, the applicability of both PEG-mediated and high level of gellan gum maturation needs to be empirically determined for species under study.

Of the carbohydrates tested for maturation, sucrose and maltose are the most common types both in pine and in spruce species. They are generally added to media at 3, 6, or 9% level. Majority of the published somatic embryogenesis papers support the use of 3% sucrose in maturation medium (Attree *et al*. 1995; Garin *et al*. 1998; Lelu *et al*. 1999; Miguel *et al*. 2004). On the other hand, some researchers harvested more Stage 3

embryos by adding 6% sucrose in contrast to using 3% (Haggman *et al*. 1999; Percy *et al*. 2000; Ramarosandratana et al. 2001). Laine & David (1990) tested sucrose concentration lower than 2% at 0.5% and reported a loss embryogenic capacity. Jones & van Staden (1995) also reported poor maturation on a medium containing 1% sucrose. There are only a limited number of studies in favor of maltose use. For example, use of 6 and 9% maltose yielded the highest number of somatic embryos (Salajova *et al*. 1999). They suggested that maltose is broken down more slowly than sucrose by which it metabolizes over a longer period to provide high osmolarity required for maturation.

Some of the SE protocols apply a pre-maturation step to avoid possible carryover effect of 2,4-D during maturation. Since, synthetic auxins like 2,4-D is less metabolized by the cells than other auxins (von Arnold *et al*. 2002). For this purpose, ET is transferred onto PGR-free medium that includes 0.5 – 1.0% activated carbon (AC) for 3 to 14 days on a semi-solid medium (Haggman *et al*. 1999; Mathur e*t al*. 2000). The other widely used strategy is to collect tissue from embryogenic calli after 7 days of subculturing, and then to wash this tissue with PGR-free liquid media before placing them on ABA containing maturation medium (Miguel *et al*. 2004). Several studies have pointed out that the selection or targeting the most responsive embryo developmental stage is an important parameter for successful maturation in pine species (Lelu *et al*. 1999; Klimaszewska *et al*. 2001). This responsive stage corresponds to Stage 1, small embryos consisting of an embryogenic region of small, densely cytoplasmic cells

subtended by a suspensor comprised of long and highly vacuolated cells. Mathur *et al*. (2000) sampled embryogenic tissue (ET) only when they formed a bullet-shaped embryo with suspensors on maintenance medium and used such ET for maturation in *Pinus roxburghii*. In *Pinus pinaster*, authors grouped embryogenic calli as spiky versus smooth type while on maintenance medium, and evaluated their maturation performances. Spiky type with more than 20 Stage 1 embryos per cm^2 visible at the periphery of colony was found more productive than the smooth type with less than five protruding Stage 1 per cm^2 (Ramarosandratana *et al*. 2001a).

Filonova *et al*. (2000) studied the developmental pathway of somatic embryogenesis in *Picea abies* in detail by time-lapse tracking technique. They showed that Stage 1 somatic embryos develop from proembryogenic (PEM) mass. Such mass passes through a series of three characteristic stages that each is distinguished by cellular organization and cell number; PEM I, PEM II, and PEM III. PEM I is a cell aggregate composed of a small compact clump of cytoplasmic cells adjacent to a single vacuolated cell. PEM II aggregates hold more than one vacuolated cell. The last stage, PEM III has an enlarged clump of densely cytoplasmic cells that appears loose rather than compact. Somatic embryos specifically develop from PEM III in absence of plant growth regulators. Once these Stage 1 embryos formed, ABA is required for further somatic embryo development and maturation. Bozhkov *et al*. (2002) further studied this mechanism and demonstrated that PEM-to-somatic embryo transition is a key developmental switch, which is induced by the withdrawal of PGRs in cell suspension. Along with this

transition a concomitant cell death was also found that is mediated by extracellular acidification. Both studies show the importance of close monitoring of the embryogenic cell lines for proper sampling of the responsive cell population to ABA treatment for embryo maturation.

Once the maturation completed, fully matured Stage 3 embryos are transferred to germination medium under sterile conditions. No PGR is used in this medium and the carbon source is generally reduced. Depending on the species studied, a partial drying treatment (PDT) is applied to reduce the water content of somatic embryos for better subsequent germination, which is a common approach in *Picea* and *Abies* species (Find, 1997; Svobodova *et al*. 1999; Norgaard, 1997). It has been demonstrated that no PDT treatment is required if the maturation has taken place on high gellan gum concentration (0.8 – 1.0%). After collecting somatic embryos from maturation treatment, they are transferred onto germination medium containing lower concentration of gellan gum, such as 0.4 or 0.6%. This second approach has been widely employed in *Pinus* species (Klimaszewska *et al*. 2000; Ramarosandratana *et al*. 2001b; Klimaszewska *et al*. 2001). After the germination phase is completed, seedlings pass through acclimatization process after which planted under field conditions.

Practicing of clonal forestry has started to become a reality through development of SE technology at least for some conifer species. The most important element of this technology is the ability to cryopreserve the embryogenic cultures (EC) of clonal lines in liquid nitrogen until the corresponding trees are tested in the field (Park, 2002). Cryopreservation of

ECs has been successful for at least 26 conifer species of which 18 of them were in *Picea* (8) and *Pinus* (10) genus. During early 90s several clone banks were established for long-term clonal programs. The material used for SE is obtained from controlled pollinations of elite trees. *Picea abies*, *P. glauca*, *P. glauca* x *engelmannii*, *Pinus radiata*, *P. strobus*, *P. taeda*, and *P. pinaster* are the species of interest for which SE clonal programs have been started (Cyr & Klimaszewska, 2002). Regarding commercial production, most of the clonal selection efforts are conducted primarily in the private sector. For example, CellFor (Canada) is producing about 2 million somatic seedlings from elite control-pollinated families of loblolly pine (*Pinus taeda*) and Douglas fir (*Pseudotsuga menziesii*) yearly (Sutton, 2002). Other companies known to be active are Bioforest (Chile), Carter-Holt Harvey (New Zealand), International Paper (USA), J.D. Irving (Canada), Rayonier (New Zealand & USA), Westvaco (USA), and Weyerhaeuser (USA).

Current tree improvement programs have been using the existing genetic base of conifer species. On the other hand, there are traits that are not readily available in the breeding population or even in the gene sources of trees. Such traits could be incorporated into target species through genetic transformation. This requires a tissue culture system, which enables transformation of individual cells and subsequent regeneration through a reliable regeneration system; SE meets these criteria suitably. Currently, transformation studies in conifers have focused on certain traits. These are herbicide and insect resistance, lignin content of wood, sexual sterility, and phytoremediation (Poupin and Arce-Johnson, 2005). For herbicide

resistance, *aro A* and *bar* gene are being used to confer resistance against the herbicides used for eradication of weeds in nurseries, and in plantation areas where weed pressure is heavy or mechanical weed control is uneconomical (Walter, 2004). Expression of endotoxins encoded by *Bt* genes in transformed trees provide resistance against defoliating insects (Campbell *et al*. 2003). Manipulation of lignin content of wood has been the primary target of forest biotechnology. Since lignin constitutes 15 – 35% of the dry weight of trees that requires use of toxic chemicals during pulping process. Reduced lignin content is highly desirable from both an economic and environmental point of view. Manipulating the expression of genes involved in lignin synthesis has produced promising results (Merkle & Dean, 2000). Production of sterile trees is very attractive from two standpoints. First, it will ease the pressure on release of transgenic trees and help the acceptance of gene technologies in forestry. Second, the energy invested in reproductive process can be redirected into vegetative growth thereby enhancing more wood (Poupin & Arce-Johnson, 2005). Phytoremediation is an another target for engineered trees that makes trees ideal tool for inexpensive clean up of polluted areas because of their large biomass and longevity (Rugh *et al*. 1998). In the studies of transgenic conifers, microprojectile bombardment and *Agrobacterium*-mediated transformation protocols have been widely employed. However, most of the recent protocols are favoring the use of *Agrobacterium* because observed advantages that are low copy number, less fragmentation of the transgenes, and the precision of gene integration (Kumar & Fladung, 2001).

So far, tremendous progress has been accomplished in the development of SE protocols in conifer species. However, there are technical issues that need to refined and developed for successful implementation of SE in clonal forestry. The first is the obtainment of high SE initiation and subsequent plant conversion rates. This is important to maintain genetic diversity of clonal plantations. Although initiation rates as high as 65% is possible in *Picea* genus (using immature zygotic embryos), such rates are lower than 35% in *Pinus* genus that require detailed studies to pinpoint the factors involved in initiation for optimization (Park, 2002). The second is the genetic stability of cryopreserved clones. The study in *Picea glauca* demonstrated that the genetic integrity is maintained during cryogenic storage (Park *et al*. 1998). De Verno *et al*. (1999) studied the genetic stability of randomly selected clones using RAPD technique in *Picea glauca*. Their results emphasized the importance of avoiding prolonged subculture and selecting somatic embryos of normal morphology. The third is the development efficient techniques or procedures for handling somatic embryos, such as artificial seeds. Since, germination and transplantation of somatic embryos are very labor intensive and thus expensive process. Automating any of these steps would certainly reduce the production cost, but an efficient artificial seed technology has not been developed, yet. In summary, elucidation of the mechanisms involved in initiation and conversion, and development of technologies for automation of somatic embryos will enable us to realize the benefits of clonal forestry effectively in near future.

1.2. Rationale and significance of the study

The demand for wood products and ecological benefits of forests has been increasing in Turkey. Besides these, regulation of water regimes, protection of natural balance, and meeting recreational demand are also drawing attention. Current stock of over 20 million hectares of forestland is not enough to meet this ever-increasing demand. It is projected that 40 million cubic meter of wood will be needed in Turkey by 2020 (DPT, 2001). Increasing forest productivity on our lands will be the major way to keep pace with consumption. This can be accomplished by deploying fast-growing tree species with desired characteristics.

Turkish red pine (*Pinus brutia* TEN.) is a fast growing native tree species and therefore it has been selected as one of the target species for reforestation efforts and tree breeding projects in Turkey (DPT, 2001). To use this potential, Turkish Ministry of Environment and Forestry has established seed orchards by selecting phenotypically superior trees of *P. brutia*. Seeds from these orchards have been used for new plantations. However, incomplete ongoing progeny trials, pollen contamination, and risk of insufficient seed production depending on yearly climatic changes in seed orchards lead us to find new alternatives.

The deployment of genetic variation (both additive and non-additive) captured in seed orchards can be enhanced by integrating micropropagation methods into existing tree improvement programs. Especially, somatic embryogenesis (SE) offers a great potential for propagation of genetically

superior individuals of *P. brutia*. So far, there is no published study on somatic embryogenesis about Turkish red pine. There is only a single *in vitro* study on adventitious shoot formation from mature embryos and cotyledonary tissues of *P. brutia* (Abdullah *et al*. 1985). The results and experience gained from this research will be used for clonal propagation of superior *P. brutia* clones as well as other Turkish conifers, regarding desired features. In addition, the development such system will also provide an efficient tool for genetic transformation studies involving this species in the future.

1.3. Goal and objectives of the study

The major goal of this study was to develop a reliable and efficient *in vitro* culture system for the production of somatic embryos from Turkish red pine (*Pinus brutia* TEN.) The specific objectives were as follows: (1) induction of embryogenic tissue from immature zygotic embryos; (2) testing various ABA concentrations, osmotic agents, and carbon sources for optimum embryo development and maturation; (3) germination of somatic embryos.

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant material

One-year-old green, female cones enclosing immature zygotic embryos were collected from open-pollinated (OP) trees in clonal seed orchard established in Antalya/Turkey by Ministry of Environment and Forestry. The origin of trees used in this orchard is Çameli-Göldağ, Denizli (800m). In 2003, the cones were sampled weekly starting from June 10 to July 22, representing 15 different plus trees cloned into the seed orchard (Clones 3, 5, 6, 8, 9, 10, 12, 13, 14, 17, 19, 22, 24, 25, and 28). From each tree, five to six cones were taken from the upper one-third of Turkish red pine trees (17 years old), and stored in paper bags at 4°C until dissection for a maximum of 4 weeks. Before sterilization, intact cones first washed with a few drops of dish soap afterwards disinfected with 20% (v/v) commercial bleach (ACE, 5.25% NaClO) for 15 minutes and finally rinsed three times in sterile H_2O. Immature seeds removed from the cones in the laminar flow bench and sterilized with 5% commercial bleach for 10 minutes followed by three rinses with sterile H_2O. Megagametophytes containing immature embryos were dissected out aseptically using sterile hemostat type forceps.

2.2. Initiation of embryogenic tissue (ET)

Only single basal media, Douglas-fir cotyledon revised medium, DCR (Gupta & Durzan, 1985) was used for initiation experiments. The complete formulation of DCR medium is listed in Table 1. All of the chemicals and plant growth hormones used in medium preparation were obtained from Duchefa Biochemie B.V. (The Netherlands). Before autoclaving, the pH of the medium was adjusted to 5.8 with 0.5 N NaOH and 0.5 N HCl and 2 g/L gellan gum (Gelrite®) was added. Medium was autoclaved at 15 psi and 121°C for 20 minutes. After autoclaving, filter-sterilized (0.22µ CA membrane, 25mm diameter syringe filter) solutions of casein hydrolysate (500 mg/L) and L-glutamine (250 mg/L) were added to the cooling medium. Ten explants were cultured in each of 90 x 10 mm Petri dish. The perimeter of each plate was wrapped twice with Parafilm "M" (American National Can™). Cultures were maintained in dark at 23°C for 8-10 weeks. For each collection-date/clone combination, 30-90 explants were used depending availability of seeds extracted from the cones. Overall, 13044 explants were cultured on initiation medium in two replications; 6822 and 6222 explants in first and second replication, respectively.

2.3. Characterization of embryogenic tissue (ET)

A piece of ET was placed on a glass slide and stained with 0.5% acetocarmine diluted to 1/10 with dH_2O, and pressed gently with cover slide.

Slide preparations were observed and photographed with light microscope to determine the embryogenic features (Becwar *et al*. 1990).

Table 2.1. Formulation of DCR medium (Gupta & Durzan, 1985).

Inorganics, mg/L		**Vitamins and amino acid, mg/L**	
NH_4NO_3	400.0	Nicotinic acid	0.5
KNO_3	340.0	Pyridoxine·HCl	0.5
$Ca(NO_3)_2.4H_2O$	556.0	Thiamine·HCl	1.0
$MgSO_47H_2O$	370.0	Glycine	2.0
KH_2PO_4	170.0		
$CaCl_2.2H_2O$	85.0	**Carbohydrate and gelling agent, g/L**	
KI	0.83		
H_3BO_3	6.20	Sucrose	30.0
$MnSO_4.H_2O$	22.30	Gellan gum	2.0
$ZnSO_4.7H_2O$	8.60		
$Na_2MoO_4.2H_2O$	0.25	**Plant growth regulators, mg/L**	
$CuSO_4.5H_2O$	0.25		
$CoCl_2.6H_2O$	0.025	2,4-D	3.0
$NiCl_2.6H_2O$	0.025	BAP	0.5
$FeSO_4.7H_2O$	27.8		
Na_2EDTA	37.3		

2.4. Proliferation of ET

After 8-10 weeks of initiation period, proliferating ETs were separated from megagametophyte and transferred to fresh medium. The same initiation medium was used for subculturing, which
was repeated every 3-4 weeks. Following 4-5 subculture period, actively growing ETs were recorded as established cell lines (ECL). Conversion frequencies (CF) were also calculated for each collection date and clone by dividing the number of ECL to the initial number explants used.

2.5. Maturation of ET

For maturation, a single line from different clones was selected. Suspension culture technique was used to obtain a sufficient amount of ET. Such cultures were initiated by transferring 200-500 mg ET to 50 ml of liquid initiation medium in 300 ml erlenmayer flask on orbital shaker at 80-100 rpm. Once suspension cultures started, 50 ml fresh liquid medium was added to scale up the growth. After they formed an actively growing suspension culture, they were subcultured weekly by dividing 100 ml culture into half, and completing the culture volume to 100 ml again with fresh medium. ET growth in each flask was measured by pouring the culture into sterile graduated cylinder. Then, the amount of settled cell volume (SCV) was recorded after one hour. One line from clone 25 was destructively sampled to convert SCV

into weight in milligrams. Embryogenic tissue was transferred onto 70 mm in diameter filter paper (Schleicher & Schuell, Germany; Grade 597) by using a Büchner funnel on suction flask applying suction for three seconds to discard liquid medium by a vacuum pump (KNF-Germany, Diaphragm type). By this method, 17 SCV corresponded to about 2200 mg of ET, which means that one SCV is equal to about 130 mg. This number was accepted as a reference for the calculations made to figure out the amount of ET to be transferred onto maturation treatments. No other line was calibrated individually to avoid any contamination problem among the suspension cultures. For all of the maturation experiments, 70mm in diameter filter paper circles were used as a carrier of ET, which facilitates the handling (Schleicher & Schuell, Germany; Grade 597). The abscisic acid (ABA, Duchefa) was prepared as 4mg/ml stock using 1N NaOH as a solvent diluted with dH_2O, and filter sterilized and used fresh. Polyethylene glycol (PEG 4000, Duchefa) was dissolved in dH_2O and autoclaved separately and then added to the cooling medium, since they form a solid precipitation at the bottom of media flask if they are coautoclaved with other medium components.

Overall, two different maturation experiments were conducted to obtain mature somatic embryos. Experimental layouts for these experiments are given in Table 2. The following codes were established to identify the DCR media: supplemented with maltose ‘M’, and sucrose ‘S’ at 3% ‘S3/M3’ or 6% ‘S6/M6’ or 9% ‘S9/M9’, polyethylene glycol at 0% ‘P0’ or 3.75% ‘P3’ or 7.5% ‘P7’. Thus, S6P3 in Experiment - 1 is a medium containing sucrose at

6%, PEG at 3.75% and solidified with corresponding gellan gum concentration depending on the experiment set.

Experiment -1: In the Experiment-1, four clones were used; #6, #8, #25, and #28. Selection of the clones was based on their success in forming stable suspension cultures. Four levels of ABA (0, 10, 40, and 80μM), two types of carbohydrate at two levels (sucrose and maltose at 6 and 9%) combined with three levels of PEG (0, 3.75, and 7.5%) were tested. Together with controls, there were 48 treatments (4 x 2 x 2 x 3 = 48), each with six replicates (petri dishes), totally 288 petri dishes. About 300 mg of ET was transferred to each petri dish. The gellan gum (Gelrite®) at 0.4% was used for all 48 treatments as a solidifying agent.

Experiment -2: In the Experiment-2, three clones were used; #22, #25, and #82. Unlike Experiment-1, a pretreatment was applied to the whole set, in which all ET carrying membranes were cultured hormone PGR-free DCR medium containing 1% activated charcoal, 3% sucrose, and 0.4% gellan gum. After this pretreatment, four levels of ABA (0, 40, 80, and 120μM), two types of carbohydrate at two levels (sucrose and maltose at 3 and 6%) combined with two levels of PEG (0, and 3.75) were tested. There were 24 treatments including controls. In this set, PEG was incorporated into the medium only for control 0μM ABA and 80μM ABA, not tested in 40 and 120μM ABA. There was no PEG in 40 and 120μM ABA containing treatments. In this set, the amount of ET on each petri dish was reduced to 200mg, and gellan gum concentration was fixed for all treatments as 1.0%.

2.6. Germination of somatic embryos

After 16 weeks of maturation period, somatic embryos obtained from Experiment-2 maturation experiments were transferred to germination medium. This medium was a hormone free DCR medium solidified with 0.6% gellan gum. Somatic embryos laid horizontally on germination medium and and kept for 8 weeks until germination completed. Afterwards, germinated embryos were transferred onto same medium vertically for further growth and development.

Table 2.2. Layout for Experiment-1 and Experiment-2 maturation treatments.

ABA concentrations (µM)				
0	10	40	80	120
Experiment - 1*				
S6P0	S6P0	S6P0	S6P0	—
S6P3	S6P3	S6P3	S6P3	—
S6P7	S6P7	S6P7	S6P7	—
S9P0	S9P0	S9P0	S9P0	—
S9P3	S9P3	S9P3	S9P3	—
S9P7	S9P7	S9P7	S9P7	—
M6P0	M6P0	M6P0	M6P0	—
M6P3	M6P3	M6P3	M6P3	—
M6P7	M6P7	M6P7	M6P7	—
M9P0	M9P0	M9P0	M9P0	—
M9P3	M9P3	M9P3	M9P3	—
M9P7	M9P7	M9P7	M9P7	—
Experiment - 2**				
S3P0	—	S3P0	S3P0	S3P0
S3P3	—	—	S3P3	—
S6P0	—	S6P0	S6P0	S6P0
S6P3	—	—	S6P3	—
M3P0	—	M3P0	M3P0	M3P0
M3P3	—	—	M3P3	—
M6P0	—	M6P0	M6P0	M6P0
M6P3	—	—	M6P3	—

***** 0.4% and ******1.0% gellan gum held constant in Set-1 and Set-2, respectively. For codes of treatments, please consult to the section 2.5 of the Materials & Methods of the thesis.

2.7. Data collection and statistical analysis

For initiation experiments, explant (megagametophyte containing immature zygotic embryo) initiation and extrusion frequencies were determined after 8 weeks culture for each replications (Figure 1). After five subculturing period (15 weeks), the embryogenic lines with at least 200 mg fresh weight or greater were accepted as established cell line (ECL) and their frequencies were also recorded for each clone.

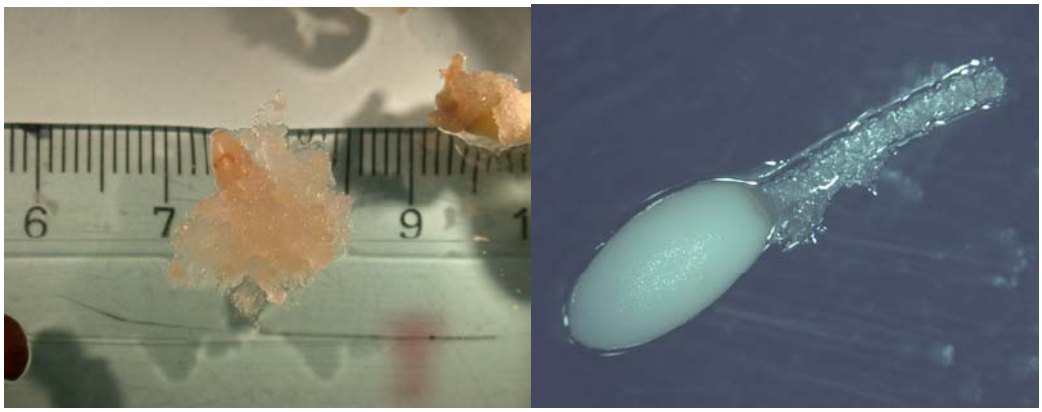


Figure 2.1. Pictures of initiation (left) and extrusion (right).

For maturation experiments, developmental stages of the somatic embryos were determined and each petri was assigned as 0 or 1 for absence or presence of Stage 1, 2, and 3. These numbers were used to calculate the percentages for evaluation of the treatments. For example, if there are three responsive petries having Stage 3 embryo out of six, it was calculated as 50% (3/6) for this treatment. These percentage values were used as a

reference to draw conclusions about the effects of each or group of treatment(s). Staging of somatic embryo development is done based on the classification proposed by Hakman and von Arnold (1988) for *Picea glauca*. The “Stage 1” embryos are small embryos consisting of an embryogenic region of small, densely cytoplasmic cells subtended by a suspensor comprised of long and highly vacuolated cells. The “Stage 2” embryos have a prominent embryogenic region (bullet shaped) that is more opaque and more smooth and glossy surface than “Stage 1” embryos. The “Stage 3” embryos have an elongated embryogenic region with visible cotyledons (Figure 2.2). Full list of traits recorded and analyzed are given Table 2.3.

Table 2.3. List of traits recorded and analyzed/evaluated

Traits	Symbol	Explanation
Initiation & Extrusion	INFREQ & EXFREQ	Frequencies calculated for each plate values were used in ANOVA and clone mean comparisons within each collection date Tukey`s multiple comparison test
Established cell lines	ECL	Frequencies calculated by dividing the number of cell lines growing after five subcultures to number of cell lines initiated
Conversion frequency	CF	CF (ECL_N/$INFREQ_N$), where ECL and INFREQ are the of explants
Stage 1	Stage 1	Presence/absence of Stage 1 embryos calculated for all plates of each treatment and used for drawing general conclusions on maturation, no statistics performed
Stage 2	Stage 2	Presence/absence of Stage 2 embryos calculated for all plates of each treatment and used for drawing general conclusions on maturation, no statictics performed
Stage 3	Stage 3	Presence/absence of Stage 3 embryos calculated for all plates of each treatment and used for drawing general conclusions on maturation, no statistics performed

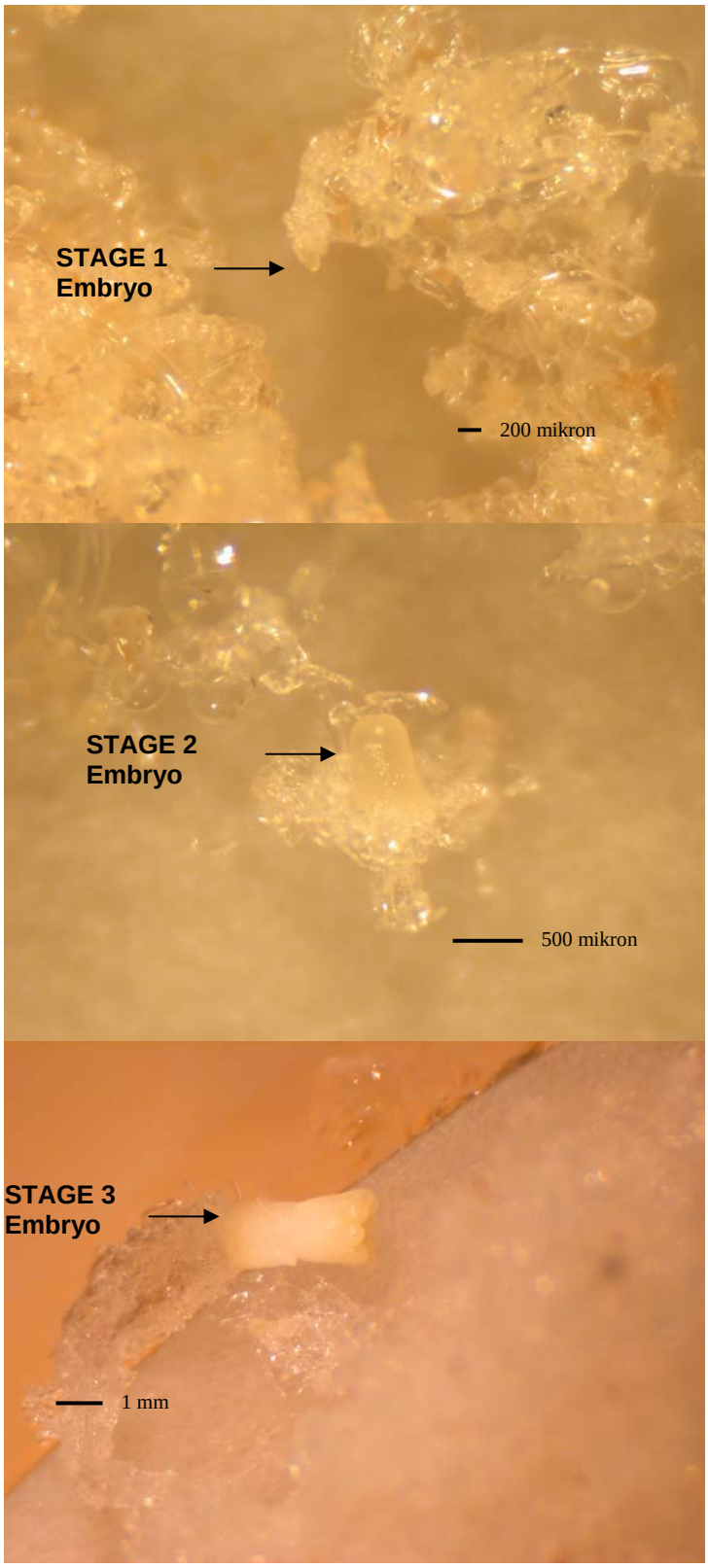


Figure 2.2. The classification scheme for somatic embryo developmental stages.

One-way ANOVA (Analysis of variance) was carried out to determine differences between the collection dates and the clonal variation within each collection date. Data were normalized by ArcSin √P transformation prior to analysis. The following model was used in the data analysis:

$$Y_{ijk} = \mu + R_i + D_j + C_k + e_{ijkl}$$

Where Y_{ijk} is the percentage of lth plate of the ith replication of jth collection date for kth clone; μ is the experimental mean; R_i is the effect of ith replication; D_j is the effect of jth collection date; C_k is the effect of kth clone; and e_{ijkl} is the random error component. All main effects were considered as fixed effects. Multiple comparisons of clones within each collection date using Tukey's HSD post-hoc test. The GLM procedure of SAS (SAS Institute) was used for analysis of variance by considering the statistical model given above.

CHAPTER 3

RESULTS

3.1. Initiation of embryogenic tissue (ET)

The results of analysis of variance (ANOVA) showed that both initiation and extrusion of ET were significantly influenced by the cone collection date (Table 3.1). Variance component (VC) analysis showed that 43.1% and 78.1% of the variation were originated from the collection dates for initiation and extrusion, respectively.

Table 3.1. ANOVA results for ET initiation and extrusion frequencies

		Initiation frequency			Extrusion frequency		
Source	df	MS*		VC** (%)	MS		VC** (%)
Replication	1	0.047	NS		0.609	NS	
Collection date	6	7.062	***	43.1	14.840	***	78.2
Clones (dates)	98	0.236	***	18.8	0.063	***	3.4
Error	1188	0.033		38.1	0.019		18.3

df: degrees of freedom *** $p < 0.001$, NS: Not significant

* MS: Mean square ** VC: Variance component

The means of ET initiation for each collection date are presented in Figure 1. No response was observed on 10-June. The lowest value was recorded on 17-June as 0.2%. Thereafter, the values were 2.6%, 18.3%,

17.1%, 22.3%, and 21.0% for the rest collections made in June 24 through July 22. Additional to the initiation frequencies (INFREQ), mean extrusion frequencies (EXFREQ) were also presented in the same graph next to INFREQ values. Such values were even higher than INFREQ for the last three collections (8, 15, 22-July). Overall INFREQ and EXFREQ values were 11.6% and 13.6%, respectively.

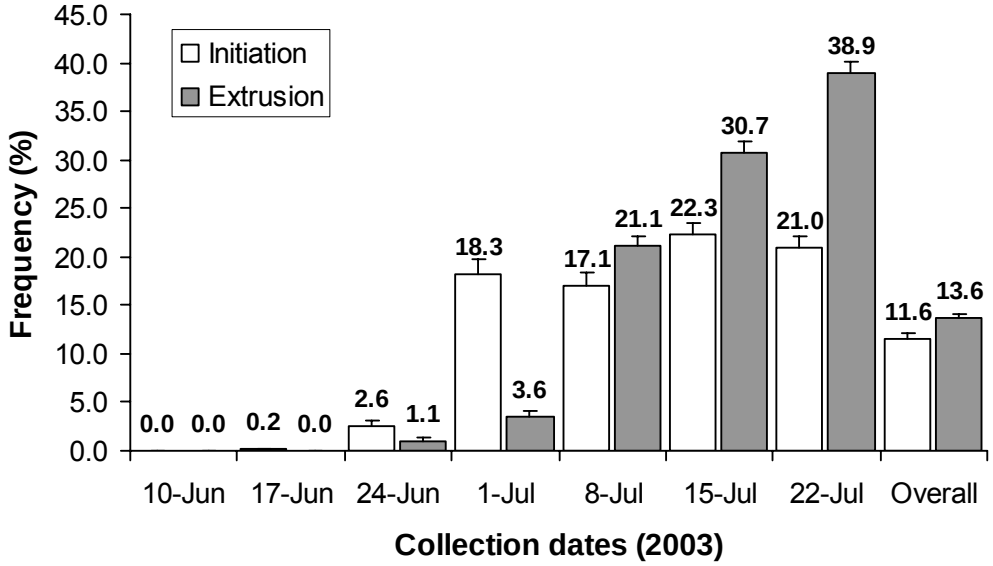


Figure 3.1. Overall distribution of initiation and extrusion frequencies along collection dates.

Figure 3.2 shows the overall mean INFREQ for the clones. Under 10%, there were seven clones: Clone 8 (4.7%), Clone 12 (5.2%), Clone 3 (6.6%), Clone 5 (7.1%), Clone 9 (7.6%), Clone 22 (8.2%), and Clone 17 (8.9%). There were seven clones between 10 and 20%; Clone 19 (10.4%), Clone 10 (11.1%), Clone 25 (12.5), Clone 6 (14.1), Clone 13 (14.8), Clone 24

(17.6%), and Clone 14 (19.3%). Over 20%, there was only single clone that is Clone 28 with 24.1% of INFREQ (Figure 3.2).

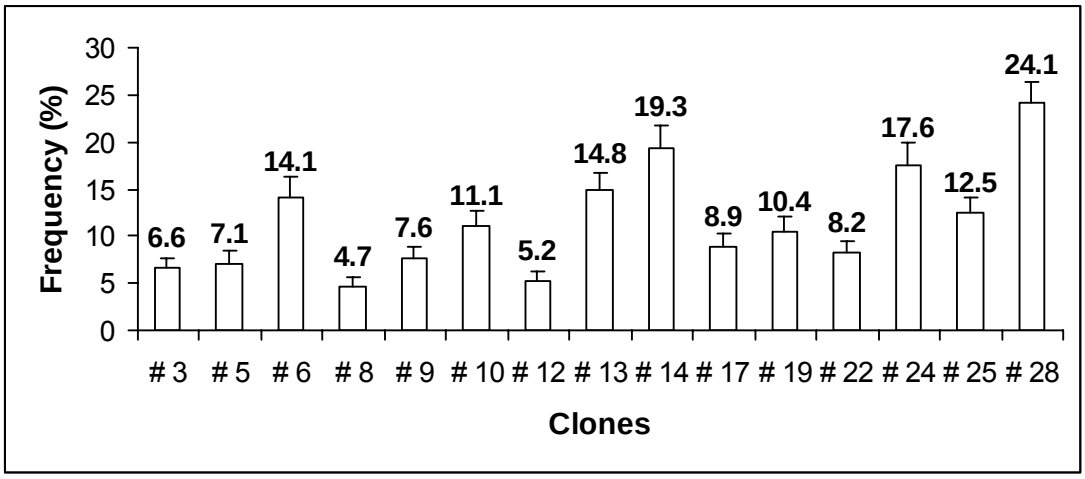


Figure 3.2. Distribution of overall mean INFREQ of the clones studied.

Based on ANOVA results presented in Table 1, variation of INFREQ values of the clones within each collection date were significant and considerable high ($p < 0.001$). Clonal differences for the last five responsive collections tested by Tukey's HSD post-hoc test at a significance level of $\alpha = 0.05$ are documented in Table 3.2.

Clones 8 and 12 showed the lowest INFREQ for all of collection dates (Table 3.2). Clone 28 was the most responsive one ranked in the first in all three-collection dates. Rest of the clones had different profiles within each sampling time; no general pattern was noticeable.

Table 3.2. Multiple comparisons of clones within each collection date using Tukey's HSD post-hoc test.

Clone	24-Jun	Clone	1-Jul	Clone	8-Jul	Clone	15-Jul	Clone	22-Jul
5	0.0	5	4.2 a	12	3.6 a	3	7.1 a	8	6.4 a
8	0.0	12	4.2 a	19	7.4 a	12	11.4 ab	12	14.2 a
10	0.0	22	5.0 a	9	8.1 ab	8	13.1 abc	13	15.0 ab
12	0.0	3	6.3 a	8	8.3 ab	13	13.3 abc	22	15.8 abc
19	0.0	8	7.1 a	10	9.3 ab	17	14.5 abc	5	16.2 abc
9	0.8 a	6	7.7 a	17	10.0 ab	5	16.7 abc	9	16.9 abcd
14	0.8 a	9	8.3 a	22	10.5 ab	22	18.4 abcd	24	18.4 abcd
17	0.8 a	10	10.2 a	3	11.3 ab	19	18.8 abcd	3	20.0 abcd
6	0.9 a	19	13.8 ab	5	12.5 ab	9	22.4 abcd	14	20.3 abcd
25	1.5 a	17	15.4 abc	25	13.6 ab	10	24.5 abcde	17	21.5 abcd
3	3.1 ab	25	21.5 abc	24	18.3 abcd	14	28.4 bcde	25	21.5 abcd
13	4.6 ab	13	29.2 bc	6	26.9 bcde	25	33.0 cde	6	24.6 abcd
22	7.5 ab	24	31.5 cd	28	34.7 cde	28	36.9 de	10	33.9 bcd
28	7.5 ab	14	47.9 de	14	36.4 de	6	37.1 de	28	34.2 cd
24	10.8 b	28	51.5 e	13	39.3 e	24	43.1 e	19	35.3 d
Mean	2.6	Mean	18.3	Mean	17.1	Mean	22.3	Mean	21.0

Values followed by different letters are significantly different for α = 0.05 as evaluated by Tukey's HSD multiple comparisons as described in Materials and methods.

As explained in Materials and Method section after initiation of ETs, they were subcultured onto fresh medium (used for initiation) and their establishment abilities were recorded after 15 weeks. The established cell line (ECL) percentages were much lower than the initiation percentages for all collections (Table 3.3). At the sampling date of 17 and 24-June, none of initiated ETs formed ECL. ECL values for the other collection dates were 1.8% (1-July), 4.8% (8-July), 8.5% (15-July), and 8.6% (22-July). Overall, 3.4% of the explants (N = 13044) used for ET initiation formed ECL. However, cultures of last two collection dates were more successful in terms of conversion into ECL, e.g., 37.9 and 40.9% of the initiation converted into stable lines for sampling time of Jul-15 and July-22, respectively.

Table 3.3. Comparison of INFREQ, ECL, and CF values among collection dates.

Collection	$N_{Explant}$	INFREQ (%)	ECL (%)	CF ($ECL_N/INFREQ_N$)
10-Jun	1845	0.0	0.0	0.0
17-Jun	1830	0.2 (3)*	0.0	0.0
24-Jun	1887	2.6 (47)	0.0	0.0
1-Jul	1854	18.3 (331)	1.8 (34)*	10.0
8-Jul	2006	17.1 (338)	4.8 (97)	28.4
15-Jul	1903	22.3 (425)	8.5 (162)	37.9
22-Jul	1719	21.0 (359)	8.6 (141)	40.9
	13044	11.6 (1503)	3.4 (441)	29.3

* Number in paranthesis gives the sample size used in calculation.

Table 3.4 presents the clonal ECL frequencies for the last five responsive collections. Clone 8 and 12 had the lowest ECL percentages, 0.9% and 1.7%, similar to their initiation values. On the other hand, Clone 28 had the highest value of 12.9% of ECL. For the rest of the clones, the ECL values ranged between 2.2% (Clone 9) to 9.6% (Clone 6). ECL ranking of the clones did not follow the same order as observed in initiation values. Although Clones 3, 6, and 19 had lower initiation percentages, they were more efficient in forming stable lines compared to Clone 28, e.g., CF values were 45.7%, 50.4%, and 41.6% versus 38.0%. Overall, 4.7% of the 9369 explants established stable embryogenic lines and about a quarter of the ET initiations converted into stable lines in total.

Table 3.4. Overall comparison of INFREQ, ECL, and CF values among clones for the last five responsive collections.

Clone No	$N_{Explant}$	INFREQ (%)	ECL (%)	CF ($ECL_N/INFREQ_N$)
3	633	9.5 (59)*	4.3 (27)*	45.7
5	589	10.2 (57)	3.4 (20)	35.1
6	617	19.6 (117)	9.6 (59)	50.4
8	572	6.7 (37)	0.9 (5)	13.5
9	584	10.4 (60)	2.2 (13)	21.6
10	653	15.2 (100)	2.6 (17)	17.0
12	642	6.9 (44)	1.7 (11)	25.0
13	643	20.3 (133)	5.0 (32)	24.0
14	636	27.1 (174)	7.1 (45)	25.8
17	645	12.2 (78)	3.4 (22)	28.2
19	620	14.5 (89)	6.0 (37)	41.6
22	610	11.4 (67)	4.1 (25)	37.3
24	638	24.4 (152)	3.1 (20)	13.2
25	650	17.9 (117)	4.0 (26)	22.2
28	637	33.4 (216)	12.9 (82)	38.0
	9369	16.00 (1500)	4.7 (441)	29.4

* Number in paranthesis gives the sample size used in calculation.

3.2. Characterization of embryogenic tissue (ET)

The embryogenic callus was mucilaginous, transparent, and whitish in color Non-embryogenic callus had a compact structure and yellowish in color (Figure 3.3). Observation of the slides prepared both from embryogenic and non-embryogenic callus shows the differences between them at the cellular level. Embryogenic callus is composed of an embryogenic region of small, densely cytoplasmic cells (stained bright red with acetocarmine) subtended

by a suspensor comprised of long and highly vacuolated cells. On the contrary, non-embryogenic callus is made of unorganized isodiametric cells with no suspensor cells. This type of callus formed mostly on megagametophyte and they did not grow after they were separated and subcultured Figure 3.4).

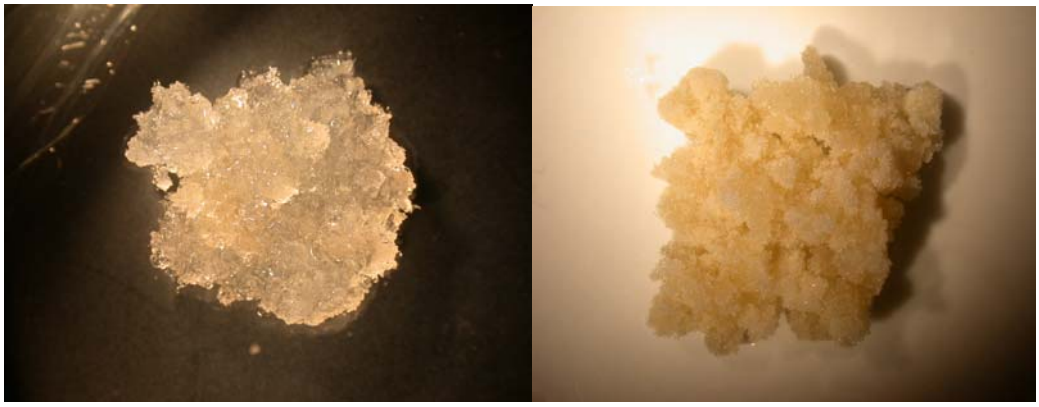


Figure 3.3. The morphology of embryogenic (left) versus non-embryogenic (right) callus.

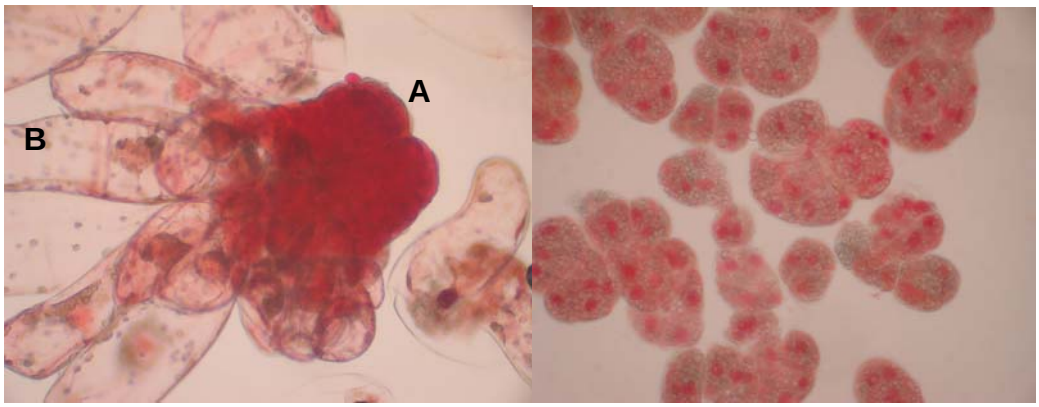


Figure 3.4. Slides of embryogenic (left) versus non-embryogenic (right) cells under light microscope; (A) embryogenic region, (B) suspensor cell.

3.3. Maturation of somatic embryos (SE)

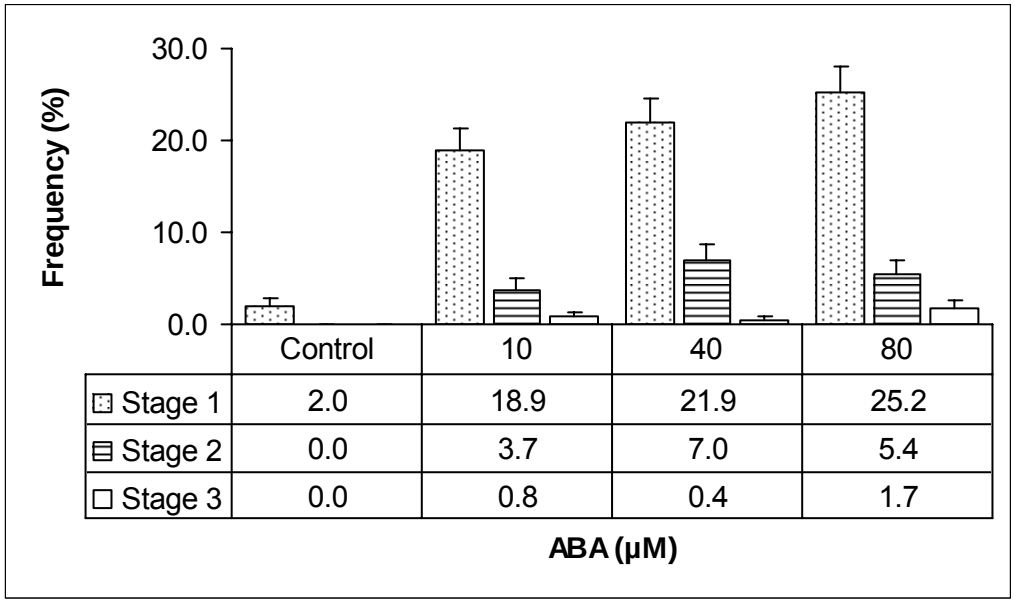
In Experiment-1, the gellan gum concentration and the amount of tissue transferred from suspension onto membrane kept fixed for all 48 treatments as 0.4% and 300 mg/plate. No pretreatments were applied to ET after growing them in suspension.

For Experiment-1, the overall ABA effect is presented in Figure 3.5. The percentage of petri dishes with Stage 1 embryos for 10 to 80μM ABA was increased steadily from 18.9% to 25.2%, but only 2% of the control group formed Stage 1 embryos. No Stage 2 and Stage 3 embryos were observed in control. The same values dropped sharply in all ABA treatments. The ABA with 40μM formed more Stage 2 embryos compared 10 and 80μM of ABAs. On the other hand, more Stage 3 was developed on 80μM ABA, i.e., 1.7% versus 0.8 and 0.4% for 10 and 40μM.

The carbohydrate type and concentration acted on maturation differently (Figure 3.6). All of them supported Stage 1 embryo formation, ranging from 10.8% (S9) to 22.7% (M6). Stage 2 formation was the highest on M6 (7.2%) followed by S6 (4.2%), M9 (3.6%), and S9 (1.2%) in decreasing order. The Stage 3 embryos were observed only on maltose containing media; M6 (2.0%) was the highest one. All sucrose concentrations did not lead to any Stage 3 embryo formation.

Compared to P0 and P3, use of P7 (7.5%) had a positive effect on maturation (Figure 3.7). Stage 1, Stage 2, and Stage 3 values for P7 treatments were 21.7%, 9.0%, and 1.2%, respectively. PEG at 3.75%

resulted in slight increase in Stage 2 and Stage 3 formation compared to control, but it had a lower Stage 1 formation compared to the control plates.



	Control	10	40	80
Stage 1	2.0	18.9	21.9	25.2
Stage 2	0.0	3.7	7.0	5.4
Stage 3	0.0	0.8	0.4	1.7

Figure 3.5. Effect of ABA concentrations on formation of Stage 1-3 embryos in maturation Experiment-1.

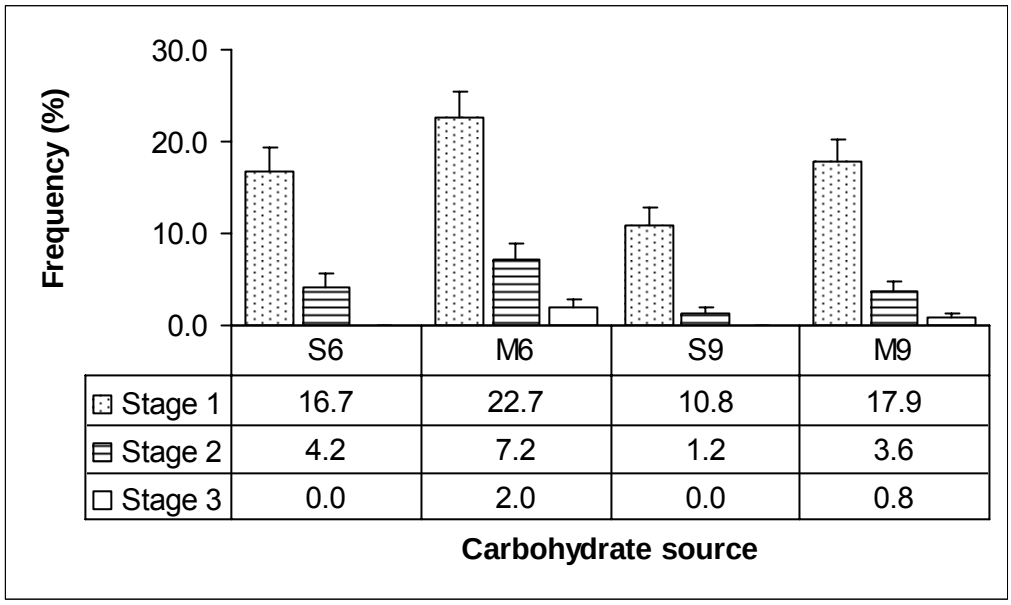


Figure 3.6. Effect of carbohydrate source and levels on formation of Stage1-3 embryos in maturation Experiment-1.

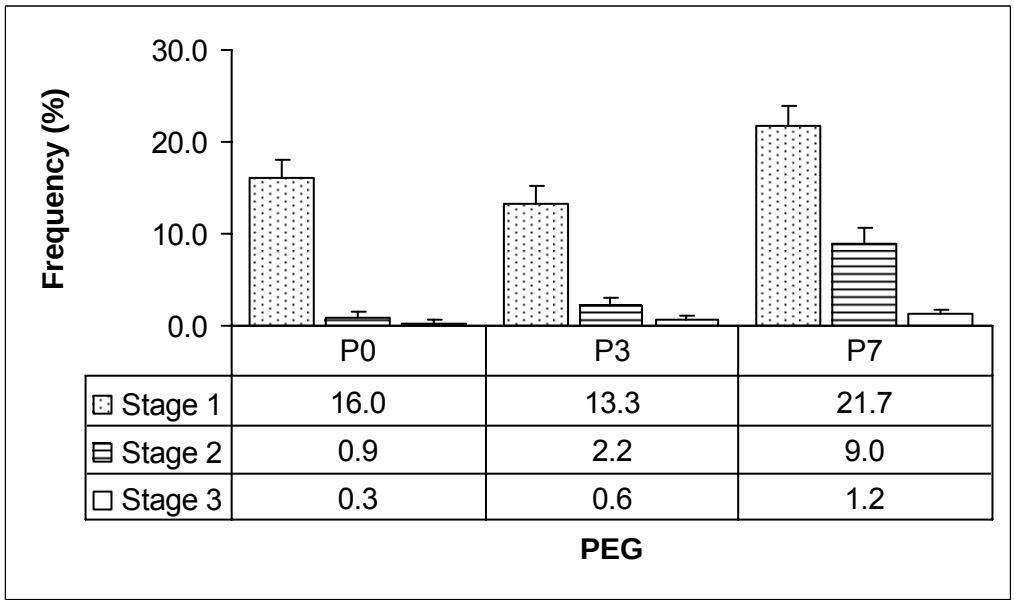
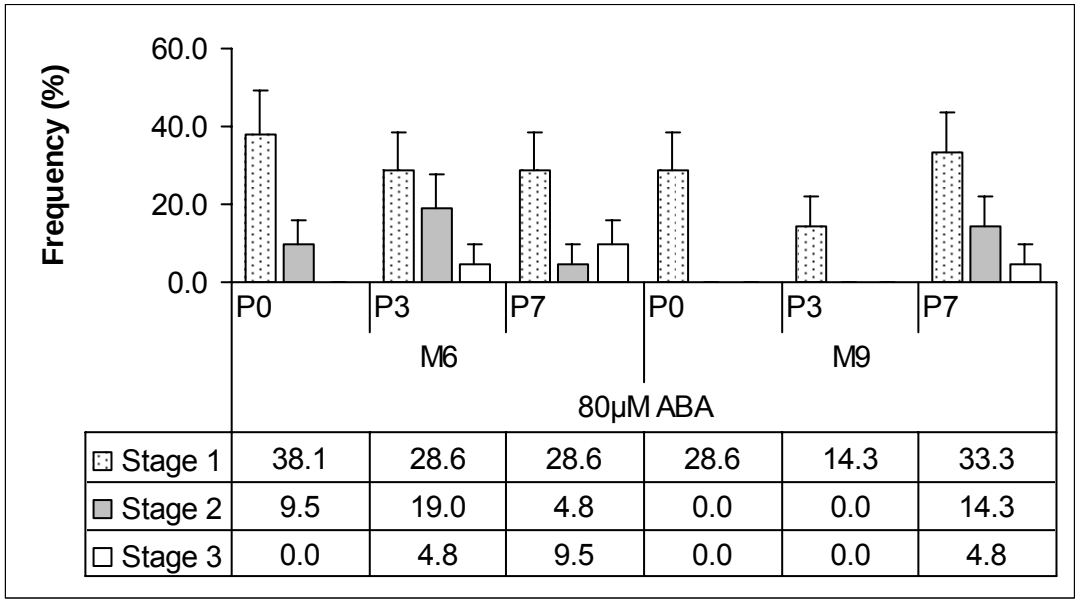


Figure 3.7. Effect of PEG concentrations on formation of Stage 1-3 embryos in maturation Experiment-1.

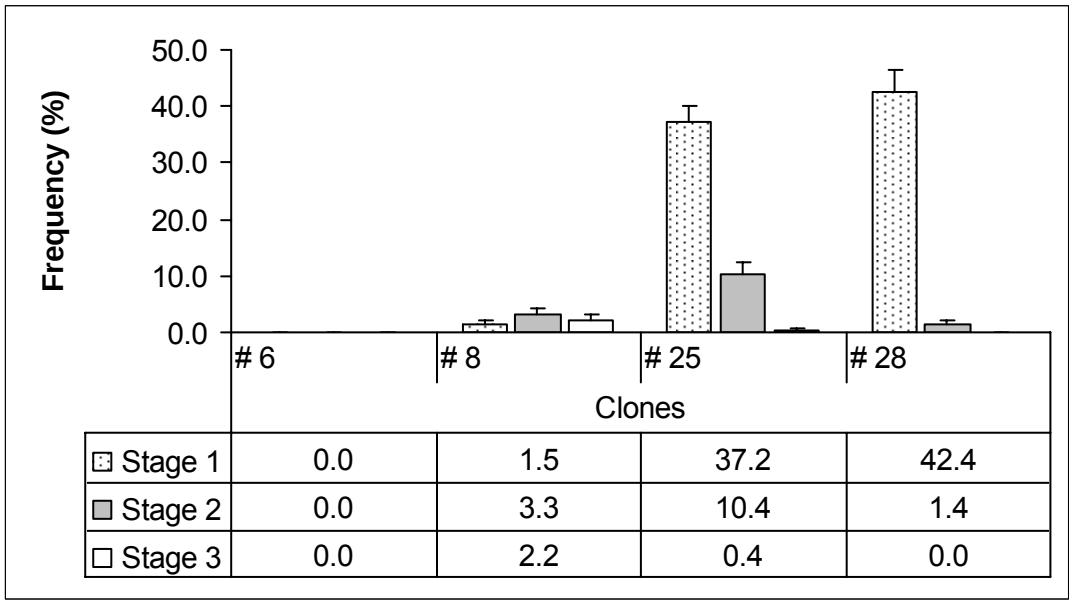
The results of optimum treatments at 80µM ABA were given in Figure 3.8. High maltose (M9) application did not support Stage 2 and Stage 3 formation, unless used with PEG at 7.5% (P7). On the contrary, addition of PEG to M6 treatments led to Stage 2 and Stage 3 embryo formation at P3 and P7. The treatments of M9P7 and M6P3 plates produced similar values for Stage 1, Stage 2, and Stage 3 embryo formations.

Clonal analysis showed that only Clone 8 and 25 formed Stage 3 embryos of 2.2% and 0.4%, respectively (Figure 3.9). Although Clone 25 had a certain number of Stage 1 and Stage 2 development, they did not turn into cotyledonous embryos.



	P0	P3	P7	P0	P3	P7
Stage 1	38.1	28.6	28.6	28.6	14.3	33.3
Stage 2	9.5	19.0	4.8	0.0	0.0	14.3
Stage 3	0.0	4.8	9.5	0.0	0.0	4.8

Figure 3.8. Graphical presentation of optimum treatments at 80µM ABA concentration in maturation Experiment-1.



	# 6	# 8	# 25	# 28
Stage 1	0.0	1.5	37.2	42.4
Stage 2	0.0	3.3	10.4	1.4
Stage 3	0.0	2.2	0.4	0.0

Figure 3.9. Overall maturation performances of the clones (single ECL) in maturation Experiment-1.

In Experiment-1, there were three major problems adversely affecting the embryo maturation. The first one was the overgrowth of ET on petri dishes (Figure 3.10A). Recallusing of the Stage 2 embryos was the second matter that interfered the transition to final Stage 3 (Figure 3.10B). The last one was the formation of abnormal Stage 3 embryos, and none of these embryos attained to fully mature state. Either such Stage 3 embryos turned into green without forming fully developed cotyledons (Figure 3.11A) or they had underdeveloped hypocotyls (Figure 3.11B). The most common problem in this set was the overgrowth of ET on petri dishes.

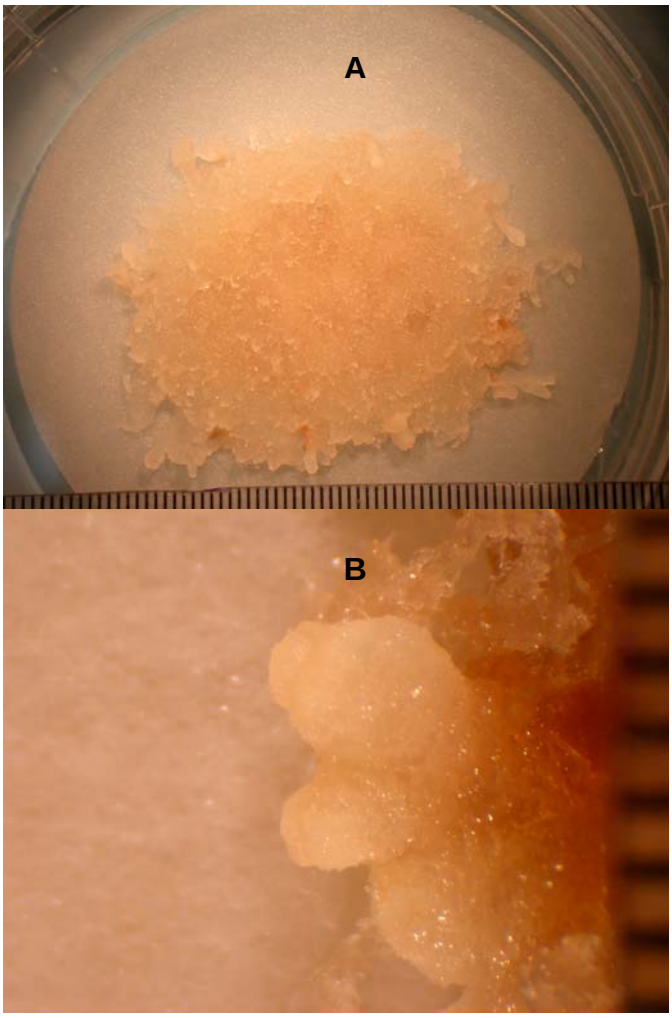


Figure 3.10. Pictures showing overgrowth of ET (A), and recallusing of Stage 2 embryos (B).

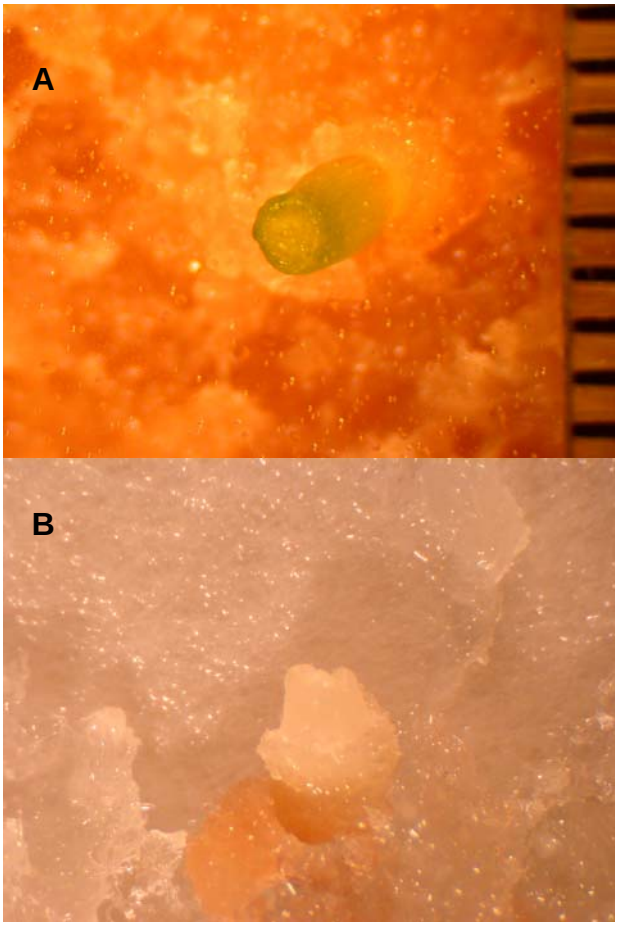


Figure 3.11. Pictures showing abnormal Stage 3 somatic embryos, green without cotyledon formation (A) and underdeveloped hypocotyl (B).

In the Experiment-2, 1.0% gellan gum was used for all of the treatments and the amount of tissue reduced to about 200 mg per plate. One percent (1.0%) active charcoal pretreatment was also applied to ET before transferring onto maturation treatments. The purpose was to reduce or eliminate any hormone carryover effect that was possibly found in suspension cultures, and caused possible ET growth during maturation (Figure 3.12).

Figure 3.12. Picture showing the embryogenic tissue activated charcoal medium.

The overall ABA effect for this set is presented in Figure 3.13. Including the control, all ABA concentrations supported Stage 1 embryo formation. The percentages of Stage 1 were 40.0%, 52.8%, 73.3%, and 70.8% for the concentrations of 0μM, 40μM, 80μM, and 120μM ABA, respectively. Stage 2 values also followed a similar trend, although the values less than S1; steady increase until 80μM ABA (20.0 for 0μM, 38.9 for 40μM, and 55.8% for 80μM) then a slight decline (47.2% for 120μM of ABA) was observable. ABA at 80μM was the optimum concentration that supported the S3 embryo formation compared to 0.8% at control and 4.2% at 40μM ABA.

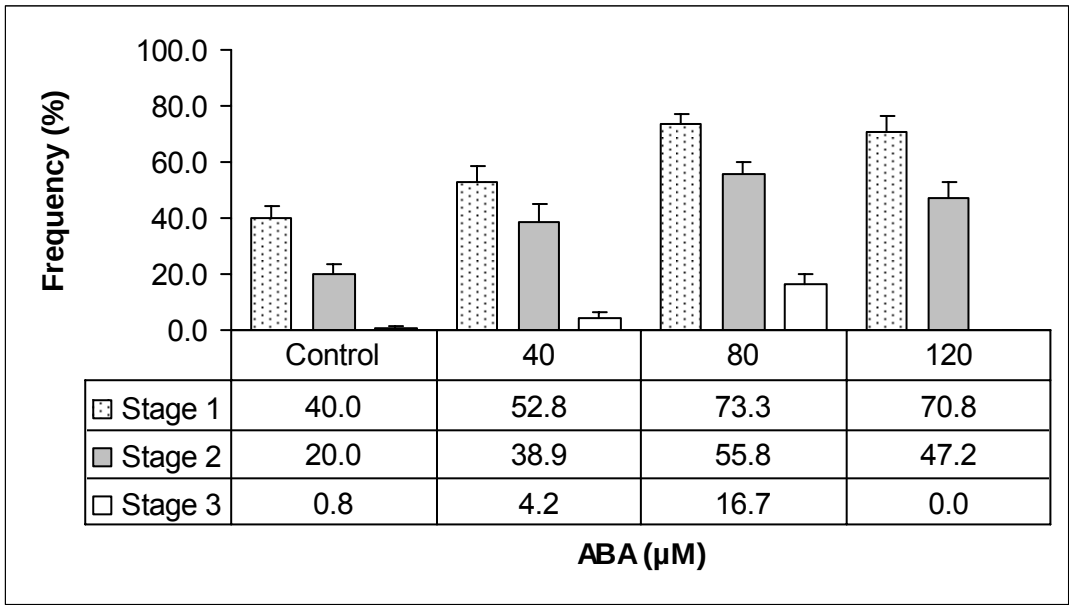


Figure 3.13. The effect of ABA concentrations on formation of Stage 1-3 embryos in maturation Experiment-2.

Based on the results of carbohydrate test in the first set, 6% concentration was kept same, but 9% was replaced by 3% (Figure 3.14). At 3%, both sucrose and maltose produced similar results. Stage 3 formation was same, 9.4%. Three percent maltose was slightly better than 3% sucrose, 46.9% versus 37.5%. Stage 1 embryo formation rates were 63.5% and 60.4% for S3 and M3, respectively. Use of 6% maltose resulted in higher values for all developmental stages, Stage 1 (67.7%), Stage 2 (45.8%) and Stage 3 (6.3%). In contrast to 6% maltose, 6% sucrose produced only Stage 1 and Stage 2 embryos with 42.7% and 29.2% percent of the plates, respectively. S6 was not successful in conversion of Stage 1 and 2 embryos to Stage 3. However, Stage 3 embryo formation on M6 was lower than M3 application (Figure 3.14).

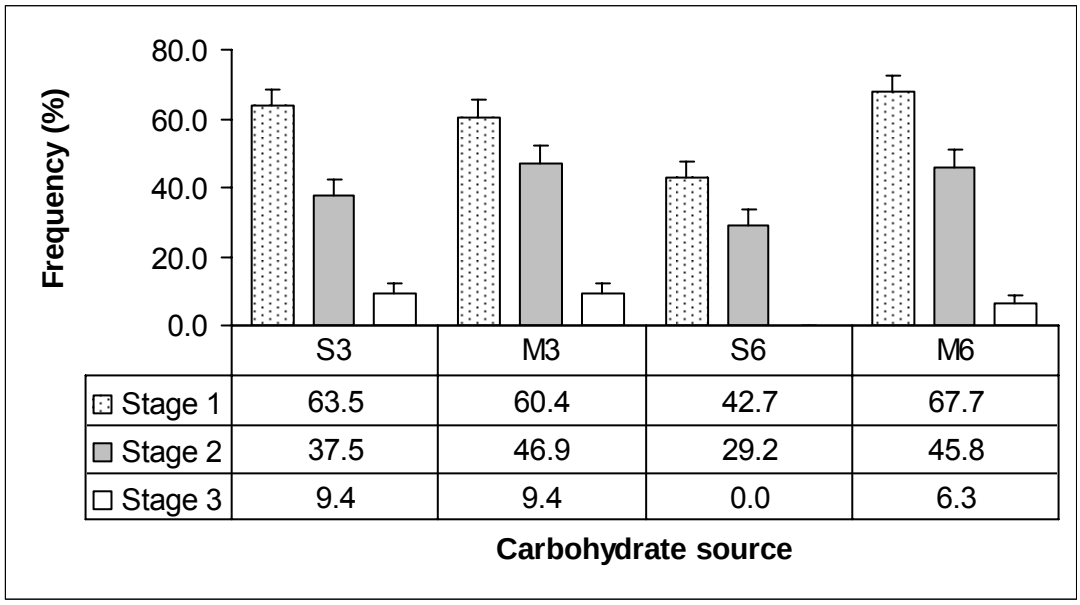


Figure 3.14. The effect of carbohydrate concentrations and levels on formation of Stage 1-3 embryos in maturation Experiment-2.

In Experiment-2, only 3.75% PEG was tested with 80µM ABA treatments against the control group. The forty and 120µM ABA treatments did not include PEG. Positive effect of the PEG application is presented in Figure 3.15. Addition of 3.75% PEG augmented the number of plates that had all stages. Of embryos on PEG containing plates, Stage 1, Stage 2, and Stage 3 frequencies were 84.4%, 51.0%, and 14.6% comparing to 50%, 36.1%, and 3.5% in control plates.

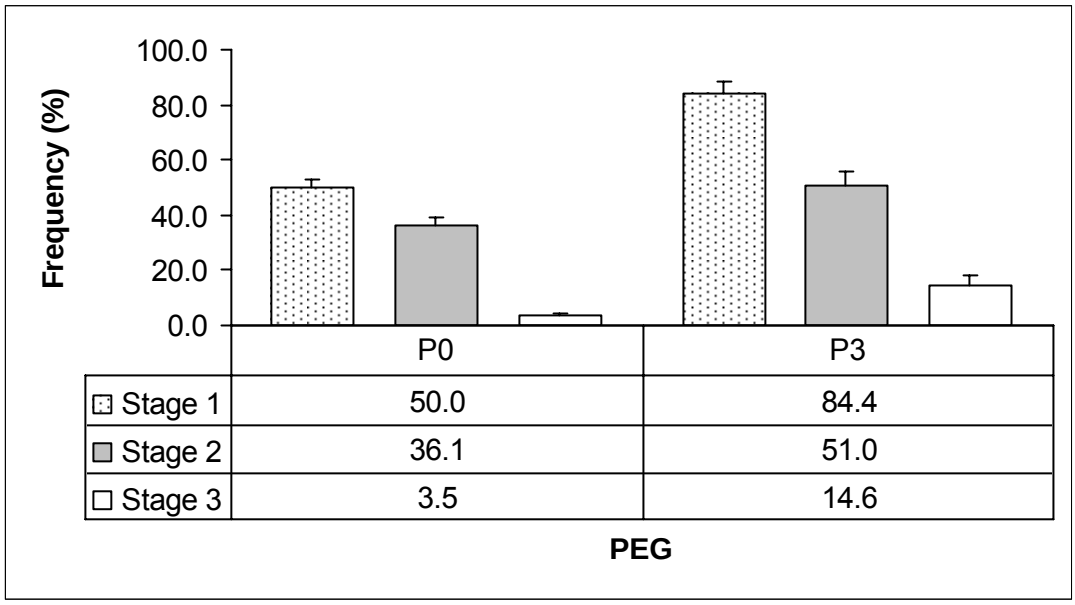
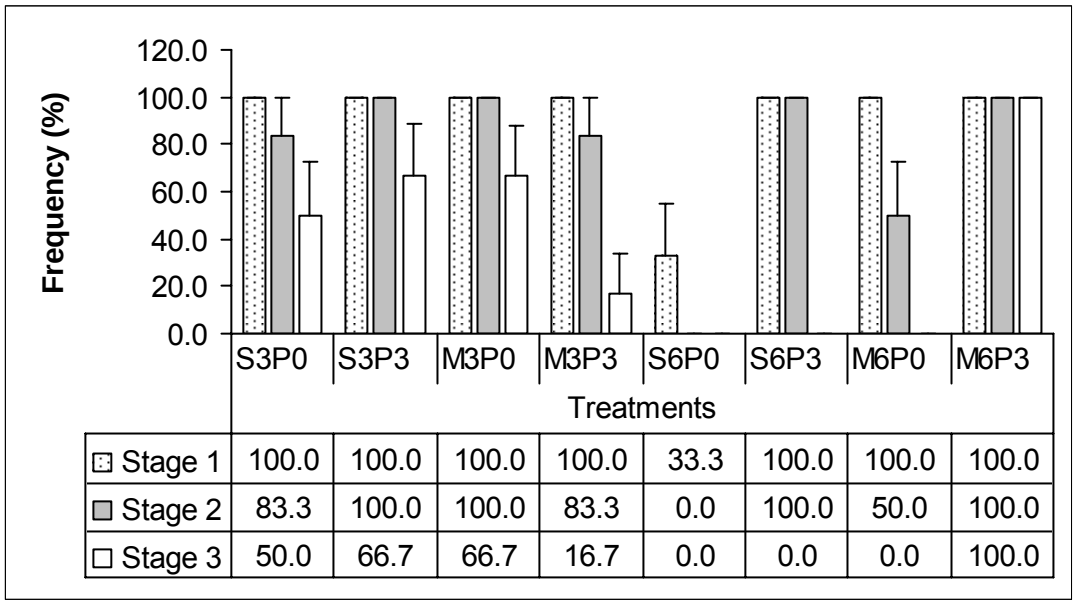


Figure 3.15. The effect of PEG concentrations on formation of Stage 1-3 embryos in maturation Experiment-2.

Although the gellan gum concentration was higher and the clones used were different in Experiment-2 (except Clone 25), 80μM ABA again was the optimum concentration, which favored all embryo stages as in Experiment-1. The effects of PEG, and carbohydrate sources applied at 80μM ABA are presented in Figure 3.16.

Sucrose at 6% concentration did not support any Stage 2 and Stage 3 embryo development, whereas 3% sucrose without PEG yielded Stage 1, Stage 2, Stage 3 embryos as 100.0%, 83.3%, and 50.0%, respectively. Addition of PEG in S6 medium resulted in Stage 1 and Stage 2 embryo formation in all plates, but it did not support any Stage 3 embryo formation. In S3 treatments, presence of PEG further improved the formation of somatic embryos on which Stage 2 and Stage 3 formation rose to 100% and 66.7%,

respectively. At higher carbohydrate levels (S6 and M6), Stage 3 formation did not take place at all without PEG. As opposed to M6P0, PEG free low maltose medium was permissive for Stage 3 development. The Stage 1-3 embryo values were next to M6P3 and as high as S3P3. A combination of PEG with M6 was the best treatment of Experiment-2 in which Stage 3 formation observed in all replicates.

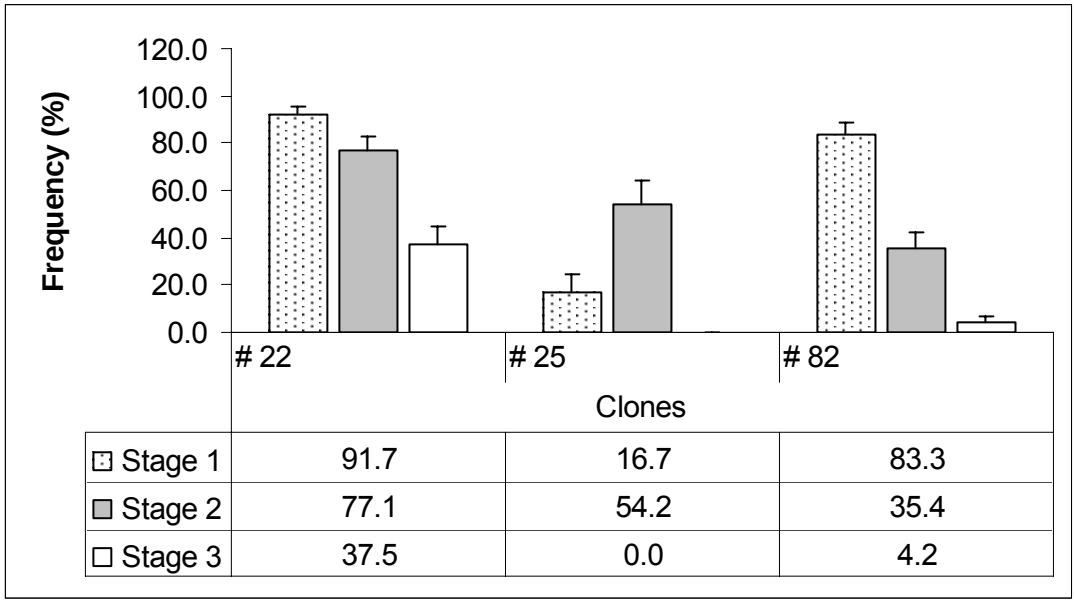


	S3P0	S3P3	M3P0	M3P3	S6P0	S6P3	M6P0	M6P3
Stage 1	100.0	100.0	100.0	100.0	33.3	100.0	100.0	100.0
Stage 2	83.3	100.0	100.0	83.3	0.0	100.0	50.0	100.0
Stage 3	50.0	66.7	66.7	16.7	0.0	0.0	0.0	100.0

Figure 3.16. A graphical presentation of optimum treatments at 80µM ABA concentration in maturation Experiment-2.

A clonal variation was also observed for embryonal stages in Experiment-2 (Figure 3.17). Clone 25 did not form Stage 3 embryos at all, only Stage 1 and Stage 2 with frequencies of 16.7% and 54.2%, respectively. Clone 82 performed better than Clone 25, with 83.3% Stage 1, 35.4% Stage

2, and 4.2% Stage 3 embryos. Clone 22 was the best performer in this experiment producing 91.7% Stage 1, 77.1% Stage 2, and 37.5% Stage 3 embryos. So far, well-developed Stage 3 embryos were collected for germination only from this clone.



	# 22	# 25	# 82
Stage 1	91.7	16.7	83.3
Stage 2	77.1	54.2	35.4
Stage 3	37.5	0.0	4.2

Figure 3.17. Overall maturation performances of the clones (one ECL for each clone) in maturation Experiment-2.

3.4. Germination of somatic embryos (SE)

After 16 weeks of maturation period (Figure 3.18), 69 somatic embryos from Clone 22 were collected from the second maturation experiments, which consist 1% gellan gum supplemented with 80µM ABA. Afterwards, they were transferred to germination medium. The treatments and the numbers of mature somatic embryos in parenthesis are as follows:

S3P0 (5), S3P3 (8), M3P0 (6), M3P3 (6), and M6P3 (44). Most of the germinating embryos did not have well formed roots (Figure 3.19) or hypocotyl (Figure 3.20). Figure 3.21 and Figure 3.22 show well developed somatic embryos with elongating root. Due to insufficient number of conversion (root elongation and epicotyl development), germinated somatic embryos were not transferred into soil for further development and acclimatization procedures.

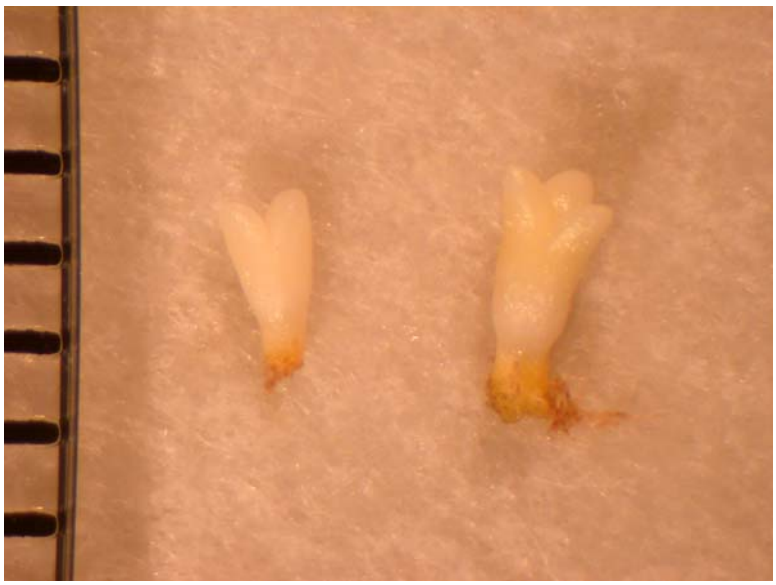


Figure 3.18. View of somatic embryos after 16 weeks of maturation.

Figure 3.19. An example of germinating somatic embryo without a root.

Figure 3.20. An example of germinating somatic embryos without hypocotyl.

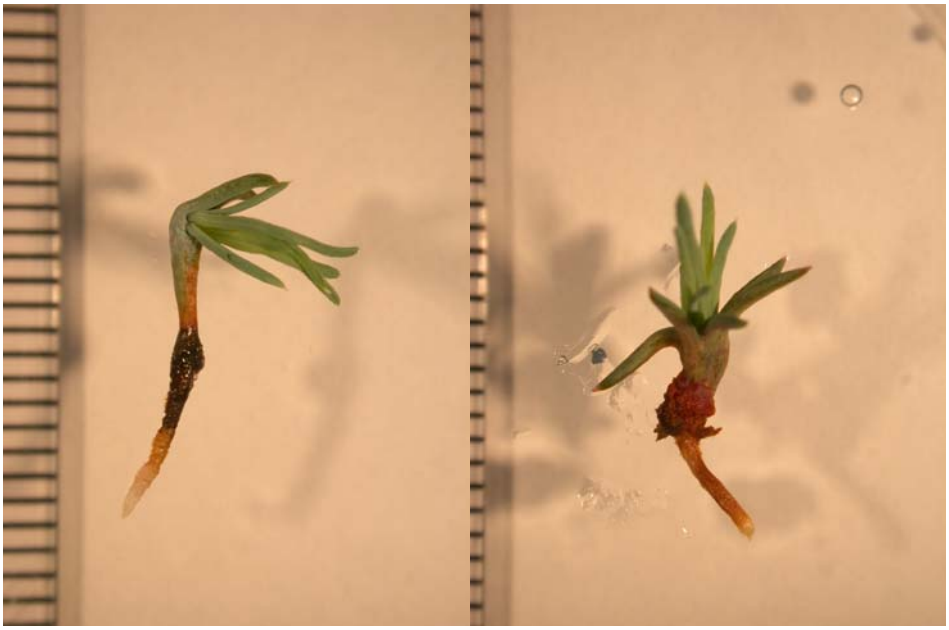


Figure 3.21. An example of germinating somatic embryos with well-developed root.

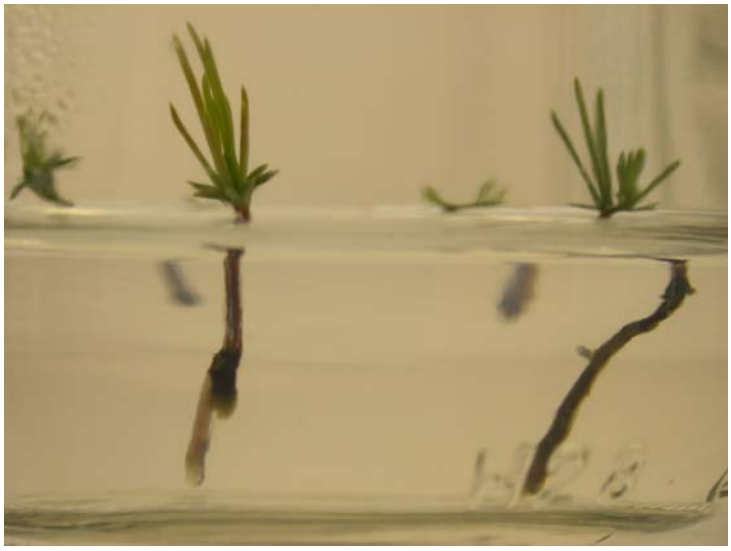


Figure 3.22. Appearance of germinated somatic embryos in baby jar.

CHAPTER 4

DISCUSSION

This study is the first report on the initiation of embryogenic tissue (ET) in Turkish red pine. Of ET, the initiation is mainly dependent on the timing of collections to sample the immature zygotic embryos in certain stage of development. The previous studies reported precotyledonary stage as the most responsive explant type in *Pinus* genus (Finer *et al*. 1989; Becwar *et al*. 1990). The results of this study showed that the "window" for ET initiation was about four weeks, throughout July for year 2003. A time-based determination of appropriate stage of immature embryo may not be as effective as embryo size and morphology. Since, changes in the environmental parameters and the high variability observed in developmental stages even among seeds from a single tree could limit the efficiency of time-based sampling. For example, Becwar *et al*. (1990) reported that precotyledonary embryos less than 0.5 mm in length (excluding suspensor) were suitable explants for initiation of EC in *Pinus taeda*. Even the stage of the embryo would make a difference in outcome. The studies with *Pinus sylvestris* and *Pinus pinaster* showed that Stages 1 and 2, which correspond to four-celled embryo to cleavage polyembryony and Stage 4 immature zygotic embryos from precotyledonary to late cotyledonary stage were more responsive explant types, respectively (Lelu *et al*. 1999). Due to workload, no detailed histological observations were made. Only limited number of

megagametophytes dissected from various clones to confirm their precoytledonary nature. In future studies, histological work should be included to pinpoint the exact stage of embryo development.

The overall ET initiation frequency of this study covering seven collections was 11.6%. Among the other published SE studies with *Pinus* genus, this frequency is one of the highest value reported so far, except for *Pinus strobus* (Finer *et al*. 1989, 35%) and *Pinus pinaster* (Miguel *et al*. 2004, 20.1%). High initiation frequency obtained at the last collection date (22-July) suggests that the possibility of more EC induction could be obtained by extending the sampling time. All of the clones studied in this study formed ETs varying from 4.7% (Clone 8) to 24.1% (Clone 28). Observed clonal variation was more pronounced within sampling times of which initiation frequency can be over 50% for a given clone (Clone 28; July 1, 2003). Among Eurasian and Mediterranean pines, the initiation frequencies of the studied clones for *Pinus sylvestris* and *Pinus pinaster* were in the range of 1-22.5% (of 10 clones) and 5-19% (of 4 clones), respectively (Lelu *et al*. 1999; Bercetche & Pagues, 1995). High clonal variation within each sampling time would explain the close average initiation values obtained across the last four collections.

Although time-dependent sampling of immature zygotic embryos is a prerequisite for induction, proper *in vitro* conditions should be provided to get healthy embryogenic cultures. Different types of basal media, PGR regimes, carbohydrates, and gelling agents have been in use. Regarding pines DCR, MSG, LM, LP, and BM media are widely used. In this study DCR medium

gave satisfactory results. Besides DCR, MSG (Becwar *et al*.1990), BM (Li *et al*.1998), and Westvaco WV5 medium (Coke J.E. United States Patent No. 5,534,433) were also tested for a limited number of clones to find out their potential use for EC initiation. These trials demonstrated that BM and WV5 basal media could be also used for initiation of embryogenic cultures as an alternative to DCR medium (personal observation), but this observation needs further testing before reaching solid conclusion. In this study, only a single hormone regime was used in the initiation medium (13.6µM 2,4-D and 2.2µM BAP). In *Pinus sylvestris* and *Pinus strobus*, use of low PGR concentrations resulted in higher initiation frequencies (Lelu *et al*. 1999; Klimaszewska *et al*. 2001). Although inclusion of 3% sucrose is widespread in initiation medium (as in this study), some researchers obtained better response by using either lower sucrose concentration or maltose (Garin *et al*. 1998; Keinonen-Mettala *et al*. 1996). Observance of high extrusion rates in this study (specifically for the last three collection dates) suggests that initiation of embryogenic cultures in Turkish red pine could be improved by using different basal media, lower PGR regime, and different carbohydrate source. Such factors should be tested individually or in combinations for this purpose.

Following the removal from the megagametophyte after initiation, a portion of cell lines may not continue to their proliferation, which is quite common in both *Pinus* and *Picea* species (Finer *et al*. 1989; Webb *et al*. 1989). In this study, overall 3.4% of the initial explants (441/13044) or 29.3% (441/1503) of ET initiations were converted into established cell lines (ECLs).

ECL values for clones ranged from 0.9% to 12.9% for the last five collection dates. In *Pinus strobus*, Garin *et al*. (1998) reported overall 2.1% ECL and a clonal range of 0.5 to 4.0% ECL for one year of study. Similar values were also reported for *Pinus sylvestris* (1.1% overall with 0.2 – 4.0% clonal range) and *Pinus pinaster* (12.0% overall with 2.0 – 35.8% clonal range) (Haggman *et al*. 1999; Miguel *et al*. 2004). For Turkish red pine, it seems that only a third of the ET initiations had the capacity for long-term proliferation. This sharp decrease may be related to media formulation, i.e. use of single PGR regime. Klimaszewska and Smith (1997) developed a simple technique that involves suspending and dispersing the ET in liquid medium of the same composition, and transferring the ET onto filter paper disc after which placed on the same initiation/maintenance medium. It appears that media optimization and handling of ET would raise the proportion of established cell lines further in Turkish red pine.

The maturation medium composition (the levels of ABA, gellan gum, and levels and source of carbohydrate source), the tested line, and the morphology of embryogenic tissue are the deterministic factors involved in the maturation of SE into cotyledonary somatic embryos and their subsequent conversion into plantlets. Use of ABA is indispensable for maturation. Among the ABA concentrations tested in the Experiment-1 maturation treatments for Turkish red pine (0, 10, 40, 80, 120µM), Stage 3 embryos were observed only in treatments containing 80µM ABA, but they were abnormal SE regarding morphology. No Stage 3 embryos were harvested on treatments containing either 40 or 120µM ABA. When gellan

gum concentration was raised to 1% in the second set, the number of replicates with normal Stage 3 embryos increased sharply, from 1.7% to 16.7%. In *Pinus strobus* and *Pinus pinaster*, the same gellan gum concentration was found to be optimal for somatic embryo formation as well (Garin *et al*. 1999; Ramarosandratana *et al*. 2001). In other pines, normal embryo development was reported using ABA as low as 15μM in *Pinus sylvestris* and as high as 120μM in *Pinus maritime* and *Pinus strobus* (Keinonen-Mettala *et al*. 1996; Klimaszewska *et al*. 2001; Miguel *et al*. 2004). However, studies showed that use of ABA lower than 15μM could not support somatic embryo development beyond Stage 2 (Jain *et al*. 1989; Becwar *et al*. 1990). It seems that appropriate ABA level is more of species dependent and needs to be empirically determined for the species in concern.

In addition to ABA, osmotic stress inducers are widely used for maturation that triggers the accumulation of storage lipids and proteins as well as prevents precocious germination. Osmotic stress could be provided by using either permeating or non-permeating osmotic agents. In this study, two types of carbon source at three levels (sucrose and maltose – 3, 6, and 9%) and PEG at three levels (0, 3.75, and 7.5%) were tested as permeating and non-permeating type, respectively. Although Experiment-1 treatments resulted in incomplete somatic embryos, it nevertheless provided useful clues for the second set treatments. Neither 6% nor 9% sucrose supported Stage 3 embryo formation with or without PEG. However, maltose treatment

showed positive response and more Stage 3 embryos were obtained at 6% concentration.

Among PEG applications, 7.5% favored more Stage 3 embryos compared to other concentrations (0% and 3.75%). A closer look at 80µM ABA combined with maltose and PEG demonstrated a possible synergistic PEG effect. Without PEG, no maturation was observed except for PEG 3% at 9% maltose. In Experiment-2 maturation treatments, same carbon sources were used, but 9% was replaced by 3% and PEG (3.75%) was solely added to 80µM ABA treatments. Promoting PEG effect was more pronounced in this set even at a lower concentration (3.75%). This outcome was probably mediated by high gellan gum concentration employed in this set. This implies that high levels of osmotic stress is necessary for maturation of Turkish red pine somatic embryos. In *Pinus sylvestris*, *Pinus patula*, and *Pinus taeda,* Stage 3 embryos were obtained by using 5% and 7.5% PEG in maturation medium at 0.25% and 0.3% gellan gum and 0.6% agar, respectively (Haggman *et al*. 1999; Jones & van Staden, 2001; Li *et al*. 1997). However, in *Pinus patula*, Jones & van Staden (2001) also reported inhibitory post-effects of PEG on germination. In *Pinus strobus*, no cotyledonary embryos observed on PEG containing treatments (Klimaszewska & Smith, 1997). Ramarosandratana *et al*. (2001) noted that Stage 3 embryos developed on M6P6G4 (maltose 6%, PEG 6%, and gellan gum 0.45%) medium were short and they had no root cap development compared to non-PEG treatments. Similar type of embryos was also noted for Turkish red pine on M6P3 (containing 1% gellan gum) treatment in the

second set of maturation experiments; only 3 out of 44 cotyledonary embryos had a root cap. Further maturation experiments are necessary with additional genotypes to reach any conclusion about PEG use maturation media.

In addition to ABA and PEG, the type and level of carbon source used for maturation appear to have an effect on the number and quality of somatic embryos formed. The results of the present study showed that 6 and 9% sucrose concentrations are not suitable for embryo development in Turkish red pine whether combined with PEG or not. However, 6% sucrose was found to be superior in *Pinus monticola* (Percy *et al*. 2000) and *Pinus pinaster* (Ramarosandratana *et al*. 2001) over 3% sucrose and maltose, and 6% maltose. In this study, both maltose at 3 and 6% as well as sucrose at 3%, supported Stage 3 embryo formation, specifically in Experiment-2 treatments. Meanwhile, Salajova *et al*. (1999) and Jones & van Staden (2001) reported best results with 6% maltose. However, the use of 3% sucrose is quite common in *Pinus strobus*, *Pinus pinaster*, *Pinus sylvestris*, *Pinus taeda*, *Pinus maritime*, and *Pinus roxburghii* (Garin *et al*. 1998; Lelu et al. 1999; Li *et al*. 1997; Miguel *et al*. 2004; Mathur *et al*. 2000).

Both maturation experiments conducted in this study demonstrated that inclusion of 1% gellan gum was clearly superior over 0.4%. In total, all 69 somatic embryos obtained were from the treatments containing higher gellan gum concentration. Klimaszewska and Smith (1997) first reported the stimulatory effect of high concentration of gellan gum with *Pinus strobus*. Later, Klimaszewska *et al*. (2000) tested different gelling agents extensively,

which again resulted in favor of gellan gum concentrations of 0.8 and 1.0%. They found a positive correlation between the number of mature somatic embryos and the relative gel strength. In other words, the physical restriction of water availability imposed by 1% gellan gum alone was more effective compared to use of osmotic agents, such as PEG (Garin *et al*. 2000). At 0.4% concentration, abundant tissue proliferation was common on maturation treatments in Experiment-1. Microscopic observation showed that suspensor-type cells were mostly responsible for this proliferation. Recallusing of the Stage 2 embryos mostly prevented the development of normal Stage 3 embryos. Occasionally abnormal S3 embryos were formed, but they were either with incomplete cotyledon primordia or had hypocotyl malformation. Similar results also reported in *Pinus strobus* (Klimaszewska and Smith, 1997; Ramarosandratana *et al*. 2001).

Once embryogenic cell lines (ECLs) are established, there is no guarantee that each line will form a normal Stage 3 embryos. In *Pinus sylvestris*, 107 out of 194 ECLs produced Stage 3 embryos (Keinonen-Mettala *et al*. 1996). Klimaszewska *et al*. (2001) obtained normal somatic embryos almost from all of the ECLs tested (145/152). However, among ECLs of *Pinus pinaster* only 11% of 896 lines had the capacity to form Stage 3 embryos (Miguel *et al*. 2004). Percy *et al*. (2000) commented that the maturation response is mostly dependent on the ECL tested; not on the clone or family used. All of these studies emphasized the importance of the determination of cellular stage of ECL that is suitable for development of somatic embryo. This is the stage where ECL has well-developed somatic

embryos during the proliferation/maintenance. Such embryos correspond to Stage 1, bullet-shaped head with suspensors (Mathur *et al*. 2000). Ramarosandratana *et al*. (2001) classified the ECLs in terms morphology as "spiky" or "smooth" appearance of which former type had more than 20 Stage 1 embryos per cm^2 compared to latter type with less than five Stage 1 embryos per cm^2. Furthermore, they tested three types of sampling from embryogenic calli - outer, inner, and whole. They reported that spiky lines sampled from the periphery of calli produced more Stage 3 embryos.

Turkish red pine maturation experiments produced cotyledonary Stage 3 embryos only from a single line of Clone 22 from the second set of experiments, out of seven clones tested; no line differences within clones were studied. ECLs used in Turkish red pine maturation were obtained via suspension culture to produce sufficient amount of tissue earlier than regular subculturing of each ECL on a semi-solid proliferation medium. Most of the referred studies above harvested their material by collecting the tissue from several clumps of the same ECL after 7 days of subculturing. Some researchers also applied an activated charcoal (AC) pretreatment before applying maturation treatment(s) to alleviate possible carry over effects of auxin (2,4-D) used in proliferation medium (Keinonen-Mettala *et al*. 1996; Percy *et al*. 2000; Haggman *et al*. 1999). This approach was used in the Set-2 maturation experiments of Turkish red pine. Tissue of each ECL from suspension culture was transferred onto filter disc after which transferred onto PGR-free medium containing 1% activated charcoal for a week. Although high gellan gum concentration combined with PEG could be

responsible for somatic embryos obtained from Clone 22, the pretreatment would have contributed this outcome positively. Nevertheless, this point needs further verification by running controls to reach any firm conclusion.

Finer *et al*. (1989) also used embryogenic suspension cultures to obtain embryogenic tissue for maturation of *Pinus strobus*, but they stressed on the importance of preserving low-density suspension culture to avoid embryogenic quality degrades. In addition, the suspension environment would place a selection pressure favoring fast-growing culture type, which could have altered responses upon exposure to exogenous ABA to promote maturation (Dunstan *et al*. 1993). These comments imply that the dynamics of suspension culture for each ECL needs a careful monitoring for proper sampling of Stage 1 embryos for future maturation experiments of Turkish red pine ECLs. Another option would be that first to scale up ECL through suspension culture then to plate the tissue back onto proliferation medium and to harvest them after a week or until Stage 1 embryos observed on embryogenic calli.

Besides the morphology of the ECL before maturation, microscopic observations of EC slides could provide a better criterion for selecting the potential lines. For this purpose, Jalonen and von Arnold (1991) characterized the ECL of Norway spruce (*Picea abies*) as Type A and B. Their microscopic observations showed that Type A line is mainly composed of somatic embryos polarized with distinct embryo-heads and well developed suspensors. On the contrary, Type B embryos had embryo heads comprised of only a few loosely aggregated clusters of cells inseparable from the

vacuolated suspensor cells. They reported mature somatic embryos only from Type A ECLs. Such findings suggest that before starting maturation experiments with Turkish red pine ECLs should be screened carefully to find out potential lines.

Filonova *et al*. (2000) further studied the developmental pathway of Type A lines for somatic embryogenesis via time-lapse tracking of *Picea abies*. They described three types of proembryogenic masses (PEM) as PEM I, II, and III based on their developmental state. In the presence of PGR used in proliferation medium, such cell masses cycle through PEM I to PEM III. Removal of PGR is prerequisite for transdifferentiation of somatic embryos from PEM III at which they become responsive to ABA for development into Stage 3 embryos. Somatic embryos at this stage correspond to Stage 1 embryos. Bozhkov *et al*. (2002) further studied this model, and proposed a developmental switch triggered by withdrawal of growth regulators. They used suspension culture system to study this mechanism and found that highest number of somatic embryos from PEM III population formed after 7 days of subculture into PGR-free medium, as high as 75% of the entire population. Thereafter, somatic embryos started to degenerate. This period was closely associated with concomitant cell death and extra cellular acidification. The studies conducted in *Picea* genus clearly explain the critical factors pertaining to proper maturation protocols. All these factors have to be assessed carefully for developing efficient and repeatable somatic embryo maturation protocol for Turkish red pine.

CHAPTER 5

CONCLUSION

As stated in the introduction part, the major goal of this study was to develop a reliable and efficient protocol for the production of somatic embryos from Turkish red pine (*Pinus brutia* TEN.). To reach this goal different set of experiments were conducted for each specific objective. The outcomes of this research could be listed under two headings: (1) initiation of embryogenic tissue, and (2) maturation of somatic embryos.

Initiation of embryogenic tissue

- Compared to literature, high ET initiation frequency (11.6%, overall) was obtained. In addition, the clonal variation suggested that initiation frequency is affected significantly by the genotype.

- Obtainment of high extrusion frequencies along with initiation suggests that optimization of initiation phase could be possible through further testing of different basal media, and manipulation of PGR regimes.

- Conversion of the initiations into established cell lines (ECLs) would be improved by changing culture conditions, such as lowering plant growth regulators or applying a membrane plating technique.

Maturation of somatic embryos

- Of those 7 ECLs tested for maturation, only the one of them belonging to Clone 22 was competent for maturation into Stage 3 embryos.

- The suspension used for proliferation ET for maturation needs to revised or used in combination with semi-solid media to sample the competent embryogenic cells for proper maturation.

- In the selection of ECL for maturation, the morphology of the ECL could be a useful criterion along with microscopic observation to target Type A cell lines as described in *Picea abies*.

- Development cryopreservation technique ET could be necessary for long-term preservation of the ECL and protecting them against possible adverse effects of continuous subculturing.

- A combination of 80µM ABA, 1% gellan gum, and 3.75% PEG was found optimum for maturation of Turkish red pine somatic embryos. However, this observation needs further experimentation by using ECLs responsive to ABA treatment. Carbohydrate sources would also require repeating.

- Only a limited number of mature somatic embryos were obtained in this study. Therefore, no germination experiments were not conducted. Optimization of maturation process in future studies will provide enough material for germination studies in Turkish red pine somatic embryos.

In conclusion, somatic embryogenesis technology offers a great potential for clonal propagation elite Turkish red pine trees. Implementation of this technology will be possible through optimization of both initiation and maturation steps. Aside from clonal propagation, embryogenic tissue itself is one of the most suitable materials for genetic transformation studies in the future.

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APPENDIX A

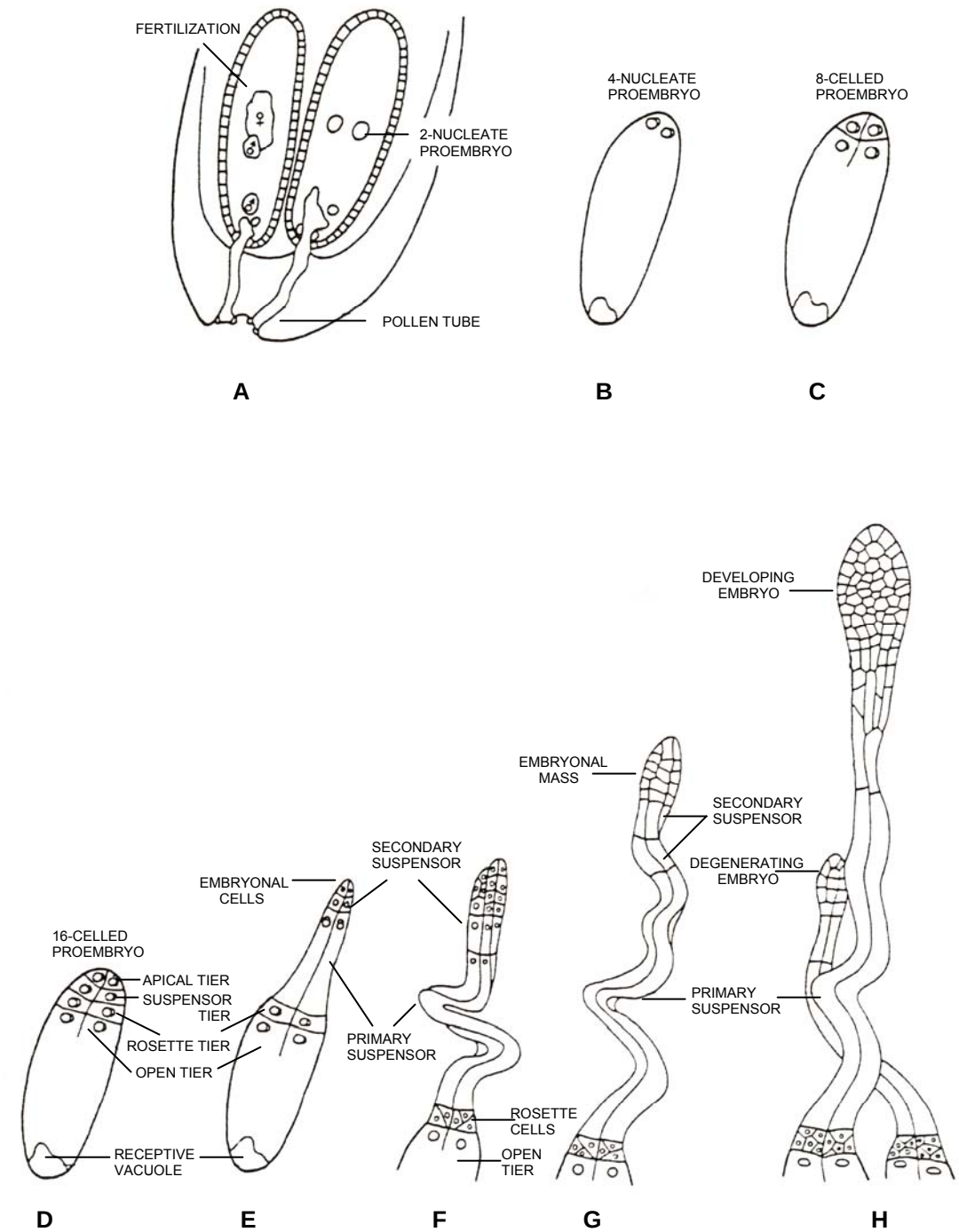

Figure A.1 Fertilization and simple polyembryony in *Picea*.

Proembryo development begins when the fertilized egg nucleus divides into two (A), then four. The nuclei become oriented into a single tier (B), and each nucleus divides. Cell wall forms between the eight nuclei to create two tiers of four cells each (C). Each divides again, forming a 16-celled proembryo consisting of four tiers of four cells each (D). The third tier of cells (suspensor tier) elongates and thrusts the distal apical tier out of the archegonial jacket into the female gametophytic tissue (E). This ends proembryo stage. The early embryo stage then begins (E). Cells of the apical tier divide forming terminal embryonal cells and subterminal secondary suspensors. This forces the terminal embryonal cells still deeper into the female gametophyte. The embryonal cells divide, creating an embryonal mass (F, G). The basal cells of the embryonal mass continue to divide and elongate, contributing to the thick secondary suspensor. Distal cells of the embryonal mass divide to form a club-shaped embryo (H). Simple polyembryony occurs when more than one egg is fertilized within an ovule from separate grains. The resulting embryos have different genotypes. One embryo is usually more vigorous and continues development while the others degenerate (H) (Owens & Blake, 1985).

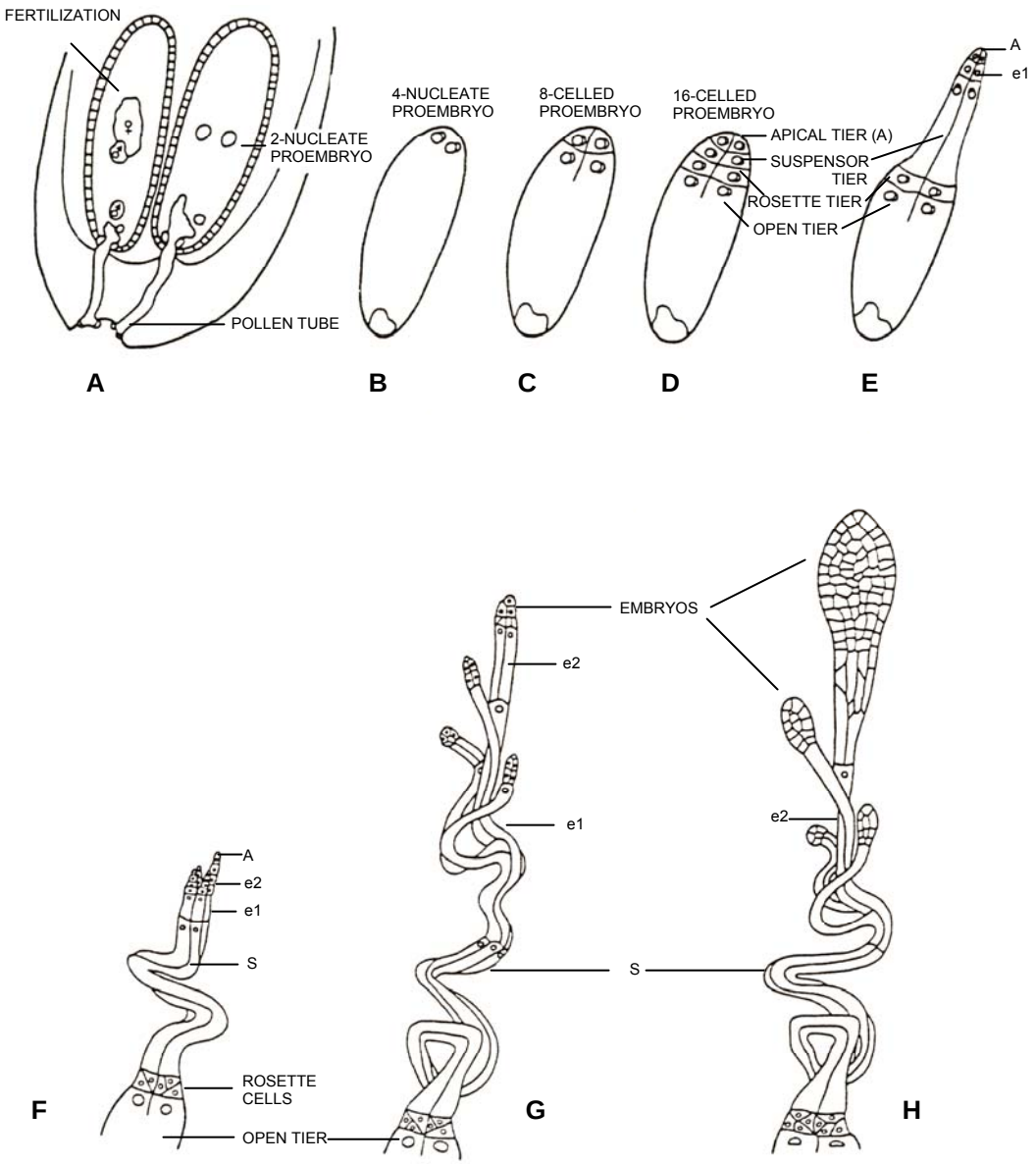

Figure A.2 Fertilization and cleavage polyembryony in *Pinus*.

See Figure 1.1 for description of stages A-E. After the suspensor tier elongates and embryonal tubes form (e1, e2), the apical tier separates into four files of cells (cleavage polyembryony) (F). Despite their genetic identity, one of these embryos soon becomes the most vigorous and other embryos

degenerate (G, H). The club-shaped embryo that survives the polyembryonic selection process is pushed by the suspensors to the middle of the female gametophytic tissue. Here, the embryo enlarges rapidly (H) (Owens and Blake, 1985).

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