INDUCTION OF EMBRYOGENIC TISSUE FROM IMMATURE ZYGOTIC EMBRYOS IN *Pinus nigra* SUBSPECIES *pallasiana* LAMB.

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Prof. Dr. Canan Özgen
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Semra Kocabıyık
Head of the Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. Sertaç ÖNDE	Prof. Dr. Zeki KAYA
Co-Supervisor	Supervisor

Examining Committee Members

Asist. Prof. Dr. Ayşegül ÇETİN GÖZEN	(METU,BIO)	_____
Prof. Dr. Zeki KAYA	(METU,BIO)	_____
Prof. Dr. Hüseyin Avni ÖKTEM	(METU,BIO)	_____
Dr. Songül GÜREL	(Şeker Enst.)	_____
Dr. Tolga YILDIRIM	(Akdeniz Uni.BIO)	_____

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name : Zeynep ÖZKURT

Signature :

ABSTRACT

INDUCTION OF EMBRYOGENIC TISSUE FROM IMMATURE ZYGOTIC EMBRYOS IN *Pinus nigra* SUBSPECIES *pallasiana* LAMB.

Özkurt, Zeynep

Ms., Department of Biology
Supervisor : Prof.Dr. Zeki KAYA
Co-Supervisor : Assoc.Prof.Dr. Sertaç Önde

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Cloning of trees using somatic embryogenesis could have a major impact on tree breeding and commercial plantation forestry. To initiate somatic embryogenesis in Anatolian black pine (*Pinus nigra* Arnold. subspecies *pallasiana*), one-year old cones containing immature seeds were collected from eight trees located in METU campus, Ankara. Embryogenic tissues were derived from immature zygotic embryos excised from the seeds. The zygotic embryos at the time of collection were at the precotyledonary stage of development. For this study, Douglas-fir cotyledon revised medium (DCR) supplemented with 13.6μM 2,4-D, 2.2μM BAP, 0.5 g/L casein hydrolysate, 0.25 g/L L-glutamine and 3% sucrose was used. The media was solidified with 0.2% gelrite. Embryogenic tissue initiation was calculated for each genotype and collection date. Overall initiation frequencies were recorded as 0.92% for 2004 and 1.96% for 2005. Highest initiation frequency was calculated for 5-July 2005 sampling time (4.06). ANOVA revealed significant differences between trees and collection date for initiation frequencies. Also,

ECL (Established cell lines) recorded after five subcultures. Overall, 0.38% and 0.62% of the initial explants were converted into ECLs for 2004 and 2005 respectively.

Keywords: *Pinus nigra* subsp. *pallasiana,* somatic embryogenesis, initiation, Douglas-fir cotyledon revised medium, Established cell lines.

ÖZ

Pinus nigra SUBSPECIES *pallasiana* LAMB.'da OLGUNLAŞMAMIŞ ZİGOTİK EMBRİYODAN EMBRİYOGENİK DOKU İNDÜKLEMESİ

Özkurt, Zeynep

Yüksek Lisans, Biyoloji Bölümü
Tez yöneticisi : Prof. Dr. Zeki KAYA
Ortak tez yöneticisi : Doç. Dr. Sertaç Önde

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Ağaçların somatik embriyogenez kullanılarak klonlanması ağaç ıslahında ve ticari ormancılıkta önemli bir etkiye sahip olabilir. Anadolu karaçamı'nda (*Pinus nigra* Arnold. subspecies *pallasiana*), somatik embriyogenezi indüklemek için ODTÜ kampusü Ankara'da bulunan 8 ağaçtan, olgunlaşmamış tohum içeren bir yaşındaki kozalaklar toplandı. Embriyogenik dokular tohumlardan kesip çıkarılan olgunlaşmamış zigotik embriyolardan elde edildi. Zigotik embriyolar toplanma zamanında kotiledon gelişimini tamamlamışlardı. Bu çalışma için Revize Edilmiş Adi-Duglas Kotiladon (DCR) besiyeri, 13.6µM 2,4-D, 2.2µM BAP, 0.5 g/L hidrolize kazein, 0.25 g/L L-glutamin ve %3 sukroz ilave edilerek kullanıldı. DCR besiyeri %0.2 gellan gum agarı ile katılaştırıldı. Her bir ağaç ve örnekleme zamanı için embriyogenik doku indüklemesi hesaplandı. Ortalama indükleme frekansı 2004 için %0.92 ve 2005 için %1.96. En yüksek indükleme frekansı 5-Temmuz 2005 örnekleme zamanında %4.06 olarak hesaplandı. Varyans analizi sonucuna göre, ağaçlar ve örnekleme zamanları indükleme frekansları bakımından önemli farklılıklar gösterdi.

Ayrıca ECL (Süreklilik gösteren hücre hattı) beş alt kültür sonra hesaplandı. Başlangıç materyalinin ortalama %0.38 (2004) ve %0.62'si (2005) süreklilik gösteren hücre hattına dönüştü.

Anahtar kelimeler: *Pinus nigra* subsp. *pallasiana*, somatik embriyogenez, indükleme, Revize edilmiş Adi-Duglas Kotiladon besiyeri, Süreklilik Gösteren Hücre Hattı.

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To my husband and parents and sisters

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2,4-D	2,4-dichlorophenoxyacetic acid
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
HCl	Hydrochloric acid
INFREQ	Initiation frequency
$INFREQ_N$	Number of explants showing initiation
LP	Liquid Proliferation
MS	Mean square
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
PGR	Plant growth regulators
SE	Somatic embryogenesis
VC	Variance component

CHAPTER 1

INTRODUCTION

The propagation of plants is a fundamental occupation of humankind. Human society could not continue to live on exist without the availability of the special kinds of food, fiber and amenities provided by cultivated plants, most of which must be maintained under man's surveillance or they would not continue to exist. Plant selection and breeding provide the plants which are most useful to promote man's well-being. Plant propagation multiplies these plants and preserves their essential genetic characteristics (Hartmann *et al.* 1997).

The history of selection and improvement of many agricultural crops contrasts with that for forest tree species, where the shift from exploitation to domestication is much more recent and progressing. Forests were considered to be as renewable things. But the experience of the last twenty years has demonstrated that forests once considered inexhaustible can rapidly be exhausted. Because forest production is inefficient and its wastage is high; forests suffer from the wood consumption demands. Over the last five years, the world suffered a net loss of over 37 million hectares of forest, according to data from the United Nations Food and Agriculture Organization. Forest plantations can alleviate logging pressure on natural forest areas.

1.1. Propagation of coniferous species

Forest productivity can be increased by planting tree farms with large numbers of elite, high-value trees (Pullman *et al*. 2005). Thus, cloning of

trees is important for commercial plantation forestry. Clonal forestry may be broadly defined as any use of clonally propagated trees in forestry, including the use of bulk-propagated families. However, more restrictively, it refers to the use of only tested clones in plantation forestry. Currently, clonal forestry has been successfully practiced with some hardwood species. Recently, however, the opportunities for clonal forestry with conifer species have generated considerably interests among forestry organizations (Park, 2002; Högberg *et al*, 1998; Sutton, 2002).

There are several advantages associated with practice clonal forestry. These include:

(1) Consistent production of the same genotypes over time,

(2) Capture of larger genetic gains including the capture of specific combining ability of crosses, than possible with any conventional tree breeding technique,

(3) Flexibility to deploy suitable clones with respect to changing breeding goals and /or environmental conditions,

(4) Ability to manage genetic diversity and genetic gain in plantation forestry (Park *et al*. 1998a).

Whatever the goals for forest management and conservation, the methods of clonal propagation provide powerful options for breeding and management (von Arnold *et al*. 2005). Cloning can be very effective if used in conjunction with breeding programs. Based on their applicability to forest trees, there are two ways of clonal propagation: (1) traditional macropropagation and, (2) micropropagation (Dunstan, 1988; Bonga & von Aderkas, 1992).

1.1.1. Macropropagation of conifers

There are several ways of macropropagation. The three main types in forest tree propagation could be stated as grafting, air-layering and the use of cuttings.
Macropropagation by grafting on juvenile rootstock can generally be performed with trees of any age as longs as there is no problem of compatibility. It is effective when only a small number of propagules are required and when a tree form that may deviate from the one of the ortet is not a problem. Grafting is generally limited to the creation of seed and breeding orchards and propagation of fruit trees (Hartmann *et al*, 1997; Bonga & von Aderkas, 1992).

Air layering seldom is used on plants which root easily by other less complicated methods. Nevertheless, it is useful for rooting ornamental plants such as ornamental figs, dieffenbachia, croton and others with herbaceous nature. Woody plants frequently propagated in this manner include magnolia, holly, camelia, azalea and many of the fruit and nut bearing plants such as citrus, apple, pears and pecans.

Today the common way to propagate plants vegetatively is through cuttings. However, large scale cutting propagation may be limited to some species owing to problems with rooting, aging of mother trees and survival of cuttings, as well as high costs (von Arnold *et al*. 2005). Furthermore, coniferous species, large-scale commercial use of cutting propagation methods is hindered by rooting problems. Rooting frequency varies considerably between genotypes, and decreases with increasing age of the donor tree. Rooted cuttings may also show the undesirable plagiotrophic growth pattern (Ahuja & Libby, 1993; Browne *et al*, 1997; Niemi *et al*, 2000).

These three types are collectively referred to as vegetative propagation, as an alternative to micropropagation which covers tissue culture or *in vitro* culture methods.

1.1.2. Micropropagation of conifers

Due to the commercial importance of some conifer species, advances in conifer propagation especially micropropagation is important for forestry. The three types of micropropagation are considered as: (1) axillary shoot elongation (2) organogenesis (3) somatic embryogenesis.

The first method of propagation is more common with hardwood species than with conifers. It is preferred for the commercial propagation of hardwoods since it generally is the easiest method available (Bonga & von Aderkas, 1992).

The second method, organogenesis is another tissue culture pathway that allows for *in vitro* regeneration of conifers. This technique has been tested with several coniferous species as a method of *in vitro* propagation. Plantlets from a large number of conifer species have been successfully propagated via organogenesis using various explants (Abdullah & Grace, 1987; Garcia-Ferriz *et al*. 1994; Saborio *et al*. 1997). Radiata pine is a good example of commercial propagation success using organogenesis in conifers (Menzies *et al*. 2001). With most other coniferous species, the method has only been applicable on a limited research scale, which is primarily because of the rooting problems and plagiotrophic growth nature of *in vivo* cuttings (Supriyanto & Rohr, 1994; Höggman *et al*, 1996; Drake *et al*, 1997; Gonzales *et al*, 1998; Tang & Guo, 2001).

The last of the micropropagation methods, somatic embryogenesis is potentially powerful tool for improvement of forest trees and is considered to be the *in vitro* plant regeneration system of choice in woody plants (Gupta *et al*. 1993a). In the area of tree biotechnology, propagation via somatic embryogenesis is regarded as a system of choice for mass propagation of superior tree genotypes (Cervelli *et al*. 1995).

In vitro culture has the potential to play a considerable role in tree improvement programs. Its chief advantage is the ability to multiply plants in very large quantities and to make improved or selected specimens available for use in a shorter time than by conventional means.

1.2. Somatic Embryogenesis as a Micropropagation Technique

Somatic embryogenesis (SE), the process by which asexual or somatic cells are induced to form embryos in culture, occupies a prominent role in clonal propagation. When integrated with conventional breeding programs and molecular and cell biological techniques, SE provides a valuable tool for increasing genetic improvement of trees (Stasolla & Yeung, 2003). It is accomplished by taking cultures through a series of developmental stages that are similar to zygotic embryogenesis.

Somatic embryo development in liquid medium with automation offers tremendous potential as a method for mass propagation of superior conifer genotypes (Gupta & Timmis, 2005). SE in conifers has been regarded as a model for large-scale propagation of tree species throughout the world. To date cloning of embryos by SE is considered by many as the lowest-cost method for producing uniform plants, especially for those species which are difficult to propagate by conventional cuttings.

This is why in the last few years there has been a tremendous effort towards the development and optimization of protocols for the induction and maturation of somatic embryos of both flowering plants and coniferous species (Thorpe & Stasolla, 2001; Stasolla et al. 2002).

1.2.1. Advantages of somatic embryogenesis

Conifer SE is a good example of a potential biotechnology application to conventional tree improvement. When compared to other *in vitro* propagation methods, SE offers several advantages:

(1) Immature embryos selected from seeds of superior trees and put under appropriate culture conditions can produce a mass of embryogenic tissues from which several thousands of somatic embryos can be obtained. Thus, SE allows the production of a large number of trees from a single seed and can accelerate tree breeding cycles (Gupta & Timmis, 2005).

(2) Both root and shoot meristem developments occur in the same step of the process. That is, bipolar embryos with organized shoot and root portion are obtained (Jain, 1999).

(3) Under suitable conditions, unlimited quantities of genetically identical embryos can be produced from small amounts of starting tissue (Gupta *et al*. 1993b).

(4) Embryogenic cultures of several conifer species can be grown as liquid suspension cultures (Tautorus *et al*. 1991). Suspension cultures allow a faster increase in culture fresh matter than culture on semi-solid medium, reducing the manipulation and the risk of contamination (Silveira *et al*. 2004).

(5) The most important advantage of conifer propagation by SE is that the embryogenic clonal lines can be cryopreserved in liquid nitrogen. By using

SE in conjunction with cryopreservation, it is possible to develop high-value clonal varieties by retrieving from liquid nitrogen those clones that in genetic tests had been the test performers and by subsequently propagating these (Park *et al*. 1998b). Elite clones can then be used for advanced breeding programmes and commercial forestry (Aitken-Christie, 2001).

(6) SE, which could be a useful and efficient method for genetic transformation. It provides a regeneration system for gene transfer studies (Toribio *et al*. 2004).

(7) SE has another advantage, including the use of somatic synthetic seed technologies. Synthetic seed technology requires the inexpensive production of large numbers of high-quality somatic embryos. Production of artificial seeds has been reported in many species in conifers as well as in angiosperms (Gupta *et al*. 1993c).

1.3. Review of Literature

Somatic embryogenesis is the most important *in vitro* culture system for conifer propagation. SE has been reported for many commercially important gymnosperms and especially conifers including many genera and species (Fowke *et al*, 1993; Park, 2002; Sutton 2002; Tautorus *et al,* 1991; Attree & Fowke, 1993).

1.3.1. Literature review on somatic embryogenesis in conifers

SE of coniferous species was first reported more than 20 years ago. The first description of embryo-like structures was independently reported in *Pinus banksiana* (Chalupa *et al*, 1976; Durzan & Chalupa, 1976), *Picea glauca* (Durzan & Steward, 1968), *Picea abies* (Chalupa & Durzan, 1973),

Pseudotsuga menziesii (Durzan, 1980). After several years, morphologically and physiologically mature somatic embryos were obtained from embryogenic tissue induced from immature embryos of *Picea abies* (Chalupa, 1985; Hakman *et al*, 1985) and *Larix decidua* (Nagmani & Bonga, 1985). Over the past 20 years, tremendous progress has been made towards the development of systems for the induction and development of somatic embryos of coniferous species. (Becwar *et al*, 1990; Lelu *et al*, 1994; Li *et al*, 1998; Park *et al*, 1998b; Schuller *et al*, 2000; Stasolla *et al*, 2002).

In the genus *Abies*, SE was initiated from immature (Schuller *et al*, 1989; Norgaard & Krogstrup, 1991; Krajnakova & Höggman, 1997; Vookova *et al*, 1997/98) as well as from mature zygotic embryos (Hristoforoglu *et al*, 1995; Norgaard & Krogstrup, 1995). The embryogenic tissue appeared on the suspensor of immature zygotic embryos (Salajova *et al*, 1996) or on the hypocotyl of mature zygotic embryos (Guevin *et al*, 1994). After these studies, SE in hybrid firs from immature zygotic embryos (Salajova *et al*, 1996), preliminary results of SE on hybrid *Abies alba* x *Abies cephalonica* mature zygotic embryos (Salajova & Salaj, 1998), and SE from mature embryos excised from stored hybrid *Abies alba* x *Abies cephalonica* seeds (Salaj & Salaj, 2003) were reported. Also SE has been induced in *Abies fraseri* (Guevin *et al*, 1992).

High initiation frequencies were reported for *Picea* from immature (Hakman & von Arnold, 1985, Lu & Thorpe 1987) as well as mature zygotic embryos (Tremblay, 1990). Also, embryogenic cell lines were established from zygotic embryos of Norway spruce (von Arnold *et al*, 2005).

1.3.1.1. Literature review on somatic embryogenesis in pines

Several economically important tree species belong to the genus *Pinus* and many of them form the ecological base of forest ecosystems. Pine wood is an important raw material for the forest industry and many of the pine species have been involved in conventional tree improvement programmes (Höggman *et al*, 2006).

In general, the genus *Pinus* is considered to be recalcitrant to somatic embryogenesis. In comparison with other conifers, *Pinus* species have shown lower initiation frequencies and survival of initiated cell lines (Salajova *et al,* 1999; Salajova & Salaj, 2005).

In *Pinus radiata* only early stage somatic embryos were developed (Chandler & Young, 1995)**.**

Partial or complete embryogenesis has been reported in *Pinus elliotii* (Liao & Amerson, 1995), *Pinus lambertiana* (Gupta & Durzan, 1986), *Pinus patula* (Jones *et al*, 1993), *Pinus sylvestris* (Lelu *et al*, 1999), *Pinus strobus* (Finer *et al*, 1989). Embryogenic callus was developed from cotyledonary somatic embryos but only one plant was obtained (Kaul, 1995). Experiment focused on the maturation of *Pinus strobus* somatic embryos led to the improvement of somatic embryo maturation and plantlet regeneration in high frequency was described (Klimaszewska & Smith, 1997).

Plantlet regeneration occurred in *Pinus caribaea* from embryogenic callus and also protoplast-derived somatic embryos were obtained (Laine & David, 1990).

In *Pinus pinaster* (maritime pine), SE initiation has been achieved (Bercetche & Paques, 1995; Lelu *et al*, 1999). In these studies, only four (Bercetche & Paques, 1995) and three (Lelu *et al*, 1999) open-pollinated trees have been used as zygotic embryo donors. After these studies, 20 open-pollinated families have been examined for their capacity to undergo SE (Miguel *et al*, 2004).

Embryogenic cultures have been initiated and established in *Pinus roxburghii* (chir pine) using immature megagametophytes (Mathur *et al*, 2000).

The first report of SE in *Pinus taeda* (loblolly pine) occurred in 1987 (Gupta & Durzan, 1987).Since then several reports have focused on loblolly pine maturation (Uddin *et al,* 1990; Becwar & Pullman, 1995; Li *et al*, 1997, 1998; Tang *et al*, 1998) along with abundant patent activity (Rutter *et al*, 1998a, 1998b; Pullman & Gupta, 1991; Uddin, 1993).

In Turkey, SE was studied in Turkish red pine (*Pinus brutia* TEN.) from immature zygotic embryos (Yıldırım, 2005).

In *Pinus nigra*, SE was published over ten years ago (Salajova & Salaj, 1992). The early events in somatic embryo development were reported (Jasik *et al*, 1995) and physiological, biochemical and structural aspects have also been summarized (Salajova *et al*, 1995). SE has been initiated from immature zygotic embryos. The initiation frequencies are lower in comparison with other conifer species and reduced by the maturation stage of zygotic embryos used as explants (Salajova *et al*, 1995).

In *Pinus nigra* embryogenic cultures, the less organized somatic embryos were obtained with abnormal development. However, they were resulted no vigorous plantlet production (Salajova & Salaj, 2005).

1.3.2. Factors affecting somatic embryogenesis in conifers

The process of somatic embryogenesis is defined as a sequence of three steps including: (1) induction and proliferation of embryogenic tissue, (2) embryo maturation, (3) embryo germination and conversion to plants (Stasolla & Yeung, 2003).

The complete process of conifer plant regeneration through SE encompasses a series of defined developmental steps, which require the proper execution of each step for successful completion of the next (Stasolla & Yeung, 2003).

As reviewed by Stasolla *et al*. (2002a,b), several factors, including choice of explant, culture environment, and growth regulators must be judiciously tested at each step to allow proper development of the embryos in culture.

The induction of embryogenic tissue in coniferous species is strictly dependent upon the choice of explant. The choice and response of initial explants for the induction of SE in conifers have been very limited. Mature zygotic embryos, seedling cotyledons, cotyledonary stage immature zygotic embryos and haploid megagametophytes are some of the sources used for raising embryogenic tissue in a number of species (Attree & Fowke, 1993; Tautorus *et al*, 1991). As reported by Roberts *et al*. (1989). In fact, different tissue types within the same plant or the same tissue at various stages of development produce different responses to *in vitro* culture conditions.

The immature zygotic embryo is usually the most 'responsive' tissue when used as the explant source. In pines, SE has been initiated in *P. caribaea* (Laine & David, 1990), *P. sylvestris* (Keinonen-Mettala *et al*, 1996), *P. elliottii* (Newton *et al*, 1995), *P. lambertiana* (Gupta & Durzan, 1986), *P. nigra* (Salajova *et al*, 1995), *P. palustris* (Nagmani *et al*, 1993a), *P. patula* (Jones & van Staden, 1995), *P. taeda* (Becwar *et al*, 1990) using immature zygotic embryos and in *P. korainensis* using mature zygotic embryos. However, mature seed explants generally yield a substantially lower frequency of initiation (Bozhkov *et al*, 1997; Garin *et al*, 1998).

Culture of whole female gametophytes or segments of cotyledons have been utilized in several conifer species, including *Picea abies* (Krogstrup, 1986), *Picea mariana* (Lelu & Bornman, 1990), *Pinus taeda* (Becwar *et al*, 1990).

Several factors have been reported to affect initiation from immature zygotic embryos in *Pinus* species; these are:

(1) Genotype,
(2) Collection time
(3) Media
(4) Plant growth regulators
(5) Gelling agent concentration

(1) The quality of embryogenic tissue and somatic embryos obtained in culture and their conversion rate, defined as the ability of the embryos to generate a functional root and shoot system is strictly dependent upon the genotype of the original plant (Stasolla & Yeung, 2003). Evidence from several pine species has indicated that the capacity to initiate SE varies greatly among families (Becwar *et al*, 1990; Keinonen-Mettala *et al*, 1996; Garin *et al*, 1998; Percy *et al*, 2000).

(2) It has been reported that the initiation of embryogenic cell lines is influenced by the collection time or development stage of zygotic embryos (Becwar *et al*, 1990; Nagmani *et al*, 1993b; Liao & Amerson, 1995). The maturation stage of zygotic embryos is critically important, especially pine species.

In *Pinus strobus,* the most responsive stage of zygotic embryo development is immediately following fertilization. This is evidenced by a sharp increase in induction rate during the anticipated fertilization period and subsequent gradual decline as zygotic embryo development continues (Klimaszewska *et al*, 2001). Careful monitoring of the zygotic embryo development stage may result in high initiation rates.

(3) Depending on the species, several basal media have been utilized during the induction process (Gupta & Grob, 1995). Two basal media may use for initiation process; DCR, Douglas-fir cotyledon revised medium, (Gupta & Durzan, 1985), and ½ Litvay's medium (Litvay *et al*, 1985), MSG (Becwar *et al*. 1990). SE in pine was influenced by medium composition (Becwar *et al*. 1990).

(4) All the significant results have always been obtained in the presence of plant growth regulators (PGR), an auxin (2,4-dichlorophenoxyacetic acid, 2,4-D associated with a cytokinin (benzyladenine, BA). A few attempts have been made to induce embryonal masses on growth regulator-free medium but with little success (Becwar *et al*. 1990; Nagmani *et al*, 1993a; Jones & van Staden, 1995).

(5) Gelling agent concentration (Becwar *et al*, 1995), myo-inositol (Li & Huang, 1996) and silver nitrate (Li & Huang, 1996) are important for SE.

Also, as reviewed by Tautorus *et al*. (1991), other factors of the culture conditions, such as pH, agar type, and nitrogen level can affect the generation of embryogenic tissue.

Depending on the species and genotype, embryogenic tissue becomes visible after several weeks of culture in dark. In contrast to non-embryogenic tissue, which is often dark in color, embryogenic tissue can be characterized

as a translucent mass of immature somatic embryos, often referred to as filamentous embryos or embryonal suspensor masses. Within the growing embryogenic mass, two different cell types can be distinguished based on their morphology. Small, densely cytoplasmic cells compose the embryonic head of the filamentous embryos, whereas elongated cells with large vacuoles form the suspensor apparatus (Stasolla & Yeung, 2003).

1.4. Taxonomy and distribution of the European black pine (*Pinus nigra* Arnold)

Gymnosperms are generally divided into two main groups: the coniferophytes and cycadophytes (Page, 1990). Conifers are the most important prominent components of the extant flora, with a very long history and a particularly rich fossil record, beginning in pre-Permian times (Rothwell, 1982). The Pinaceae family consists of 11 genera. *Pinus,* with over 100 extant species, is the largest genus of conifers and the most widespread genus of trees in the Northern Hemisphere (Price *et al*, 1998; Wolff *et al*, 2001).

Taxonomy of the black pine dates back to 1768 (Vidakovic, 1991). The European black pine (*Pinus nigra* Arnold) is indigenous to central and southern Europe as well as to the Crimea and Turkey (Figure 1.1). It is found at elevations ranging from sea level to 2,000 m, most commonly from 250-1,600 m.

European black pine, also called Austrian pine is medium to fast-growing, reaching 40-60 feet in the landscape. It is native to Europe. Its range extends from longitude 5^0 W. in Spain and Morocco to about 40^0 E. in eastern Turkey; and from latitude 35^0 N. in Morocco and Cyprus to 48^0 N. in northeastern Austria and to 45^0 N. in the Crimea (Critchfield & Elbert, 1966). Black pine grows widely throughout southern Europe from the eastern half of Spain, southern France and Italy to Austria; south throughout Yugoslavia

(Albania, Republic of Macedonia), western Romania, Bulgaria, and Greece on the Balkan Peninsula; east to southern Russia in the Crimea and southern, northern and western Turkey; and on the islands of Cyprus, Sicily, and Corsica, with outliers in Algeria and Morocco (Mirov, 1967).

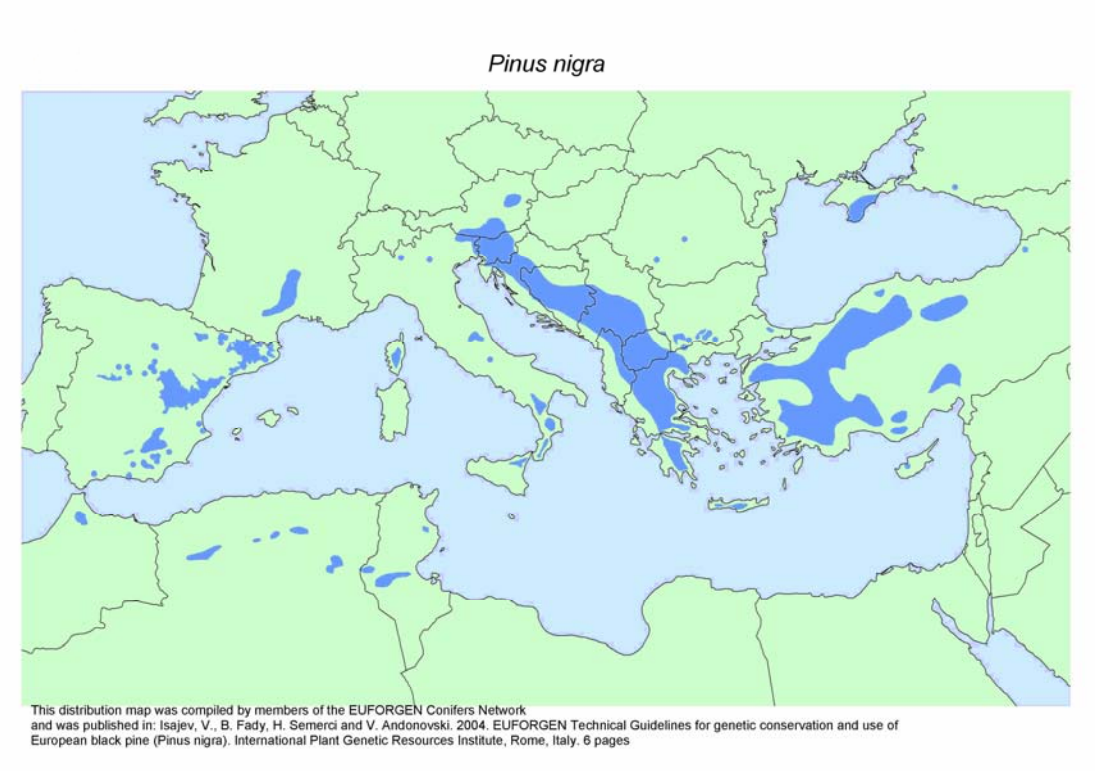


Figure 1.1. The distribution of black pine (*Pinus nigra* Arnold)

Black pine is one of the major species for afforestation of arid and rocky terrain in the sub-Mediterranean region. It most often occurs on limestone and dolomite mother rock, but also on silicates. It usually forms pure stands. It is resistant to wind, drought and quite tolerant to urban conditions (Çengel, 1998).

There are five subspecies of European black pine (Blecic, 1967) as follows: *Pinus nigra* subsp. *nigra* is found in the east of the range, from Austria, north-east and central Italy.

Pinus nigra subsp. *salzmannii* occupies the mountains of the western Mediterranean region, at altitudes of (250-) 500-1600 m.
Pinus nigra subsp. *laricio* grows in Corsica, Sardinia and Calabria.
Pinus nigra subsp. *dalmatica* grows in the north-west of the former Yugoslavia.
Pinus nigra subsp. *pallasiana*

1.4.1. Taxonomy and distribution of the Anatolian black pine (*Pinus nigra* subsp. *pallasiana* Lamb.)

European black pine includes sufficient morphological variation that taxonomists have recognized several nomenclaturally distinct species, subspecies, or varieties to describe this variation.

The subspecies of European black pine found in Turkey is known as Anatolian black pine (*Pinus nigra* subsp. *pallasiana* (Lamb.) Holmboe) and is a widely distributed timber species. Pure and mixed stands occupy more than 2,204 million hectares of forest land (Figure 1.2).

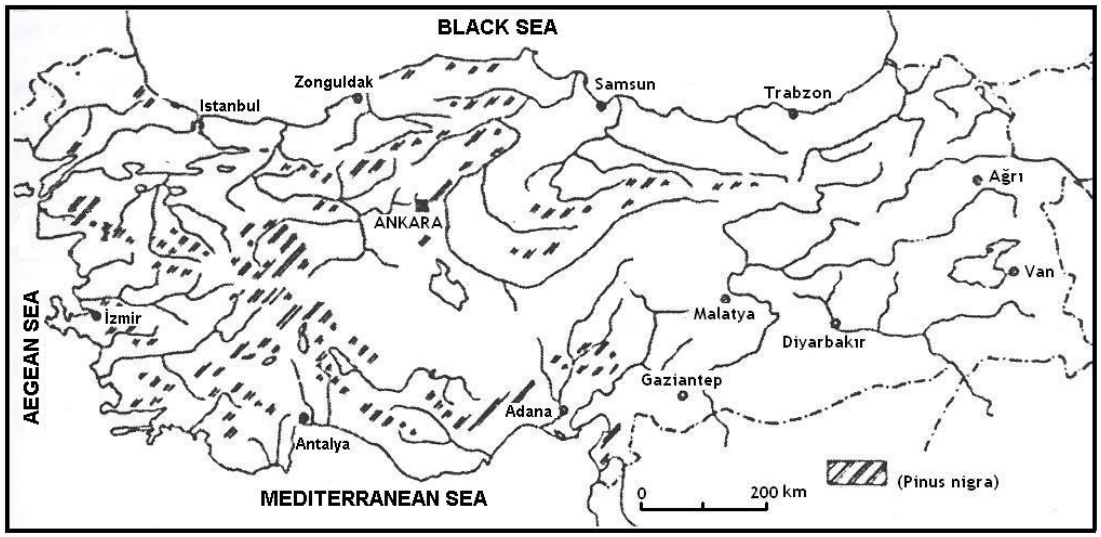


Figure 1.2. (Kaya & Gökçe, 1997) The distribution of Anatolian black pine (*Pinus nigra* subsp. *pallasiana* Lamb.)

The altitudinal distribution of the species ranges from 1000 to 2000 meters in the Taurus Mountains in southern Turkey and from 400 to 2000 meters in western and northern Turkey (Kaya & Gökçe, 1997).

There are four varieties of Anatolian black pine (Anşin, 1993) occurring in Turkey:
var. *pallasiana*;
var. *pyramidata* (Acatay) Yalt.;
var. *şeneriana* (Saatçioğlu) Yalt.;
var. *yaltırıkiana* Alptekin.

1.5. Significance of the study

Forest trees play a vital role in the lives of humans and functioning ecosystems. They provide renewable sources of wood, fibers and chemicals for human societies.

They provide habitats for numerous organisms and essential ecological functions such as water purification and carbon storage (von Arnold *et al*. 2005). As wood is one of the most important natural products, if consumption continues at the current rate, the world will soon be faced with a shortage of wood and natural forests (Lane, 2004).

The FAO estimates that at their peak, forests covered 30% of the earth surface or about 3.9 billion ha. Of this area, approximately 80% has been affected in some way by human activities such as logging, agriculture, and settlement. With current projections of wood usage, it appears that there may soon not be enough forestland to support human expansion. Since drastic conservation efforts appear slow and ineffective, there is an obvious need for a more efficient approach to producing wood and meeting consumption demands (Strauss, 1999).

In Turkey, according to data of “Ormancılık Ana Planı”, current stock of over 20 million hectares of forestland is not enough to meet the consumption demands for wood and wood products. Wood shortage is estimated to be 6.6 million cubic meters by 2010 and minimum 40 million cubic meters by 2020 (DPT: 2531-ÖİK: 547). The pressure to increase productivity of the forest will increase dramatically in the future. At the same time, pressure will be brought to bear to increase forest conservation and sustainability (von Arnold *et al*. 2005).

Anatolian black pine is an important timber species and the first choice for afforesting the high Anatolian steppes (Kaya & Temerit, 1994). Large areas have been afforested in Turkey with species in recent years. By reforestation volume, Anatolian black pine is the second most important tree species in Turkey, preceded only by *Pinus brutia*.

During the last 30 years, 24% of the artificial and 19% of the natural regeneration area have been planted with Anatolian black pine. However, because its natural distribution is greater than that of any other species, it has a great importance in Turkish forestry (Koski, 1993).

Somatic embryogenesis occupies a prominent role in clonal propagation. When integrated with conventional breeding programs and biotechnological studies, somatic embryogenesis provides a valuable tool for increasing the pace of genetic improvement of trees (Stasolla & Yeung, 2003). Also somatic embryogenesis provides a great potential to propagate genetically superior clones of *Pinus nigra* subspecies *pallasiana.* Altough there are reports on induction and development of somatic embryogenesis in European black pine (Salajova *et al,* 1999; Salajova & Salaj, 2005), there is no published study on the induction of embryogenic tissues and somatic embryogenesis about Anatolian black pine. There is an *in vitro* study on adventitious buds and shoots from excised embryos of *Pinus nigra* subspecies *pallasiana* (Kaya & Gökçe, 1997). The results of this research will be very useful for propagation of superior Anatolian black pine clones. In addition, this development system can be used for genetic transformation studies.

1.6. Objectives of the study

The main objective of this study was to obtain embryogenic tissue which could be used to develop an efficient micropropagation system for Anatolian black pine by using somatic embryos. The specific objectives of this study could be outlined as:

(1) induction of embryogenic tissue from immature zygotic embryos;

(2) testing the influences of the collection date on induction responses of the cones as an embryogenic tissue

(3) testing the effects of genotypes on induction responses as an embryogenic tissues

CHAPTER 2

MATERIALS AND METHODS

2.1. Plant Material

The experiments were performed in 2004 and 2005. Green, female cones containing immature seeds were collected randomly from open-pollinated (OP) cones of *Pinus nigra* subsp *pallasiana* (Lamb.) in the campus of METU.

In 2004, five or six immature cones per selected tree (genotype) were collected weekly starting from June 22 to July 27th (June 22nd, 29th, July 6th, 13th, 20th, 27th) from each of 8 randomly chosen trees. In 2005, the cones were collected on June 21–August 2nd (June 21st, 28th, July 5th, 12th, 19th, 26th and August 2nd) from 8 genotypes represent 8 different Anatolian black pine trees which were unrelated to the trees used in 2004 since trees sampled in 2004 did not yield sufficient cones to be sampled in 2005. These trees were chosen with the following restrictions:

(1) Parent trees were chosen randomly in the METU campus.
(2) Parent trees had to be approximately at the same age.

Whole cones were disinfected for 2 minutes with 70% alcohol and then sterilized with 30% (v/v) commercial bleach (5% active chloride) with a few drops of detergent for 15 minutes and rinsed several times in sterile distilled water and then the cones were stored in jars at 4^0 C for several days until utilized (Figure 2.1).

Figure: 2.1. Stored cones in jars at refrigerator.

Afterwards, the immature seeds were removed from the cones in the laminar flow bench (Figure 2.2).

Figure: 2.2. Seeds removed from the cones.

They were surface sterilized with 5-10% H_2O_2 for 5 minutes then rinsed three times with sterile distilled water for 2 minutes each (Figure 2.3).

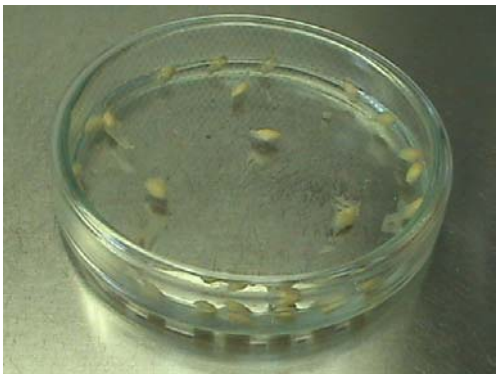


Figure: 2.3. Surface sterilization of seeds.

2.2. Initiation of Embryogenic Tissue (ET)

Developmental stage of the zygotic embryo is important. To check the development stage of the zygotic embryo, seeds were excised and examined using a dissecting microscope. In year 2004, cones were collected weekly and then seeds were examined starting from June 22 to August 3. Megagametophytes contained immature zygotic embryos at the pre-cotyledonary stage were used as explants. Because the embryos were at the cotyledonary stage, the last week's (August 3rd in 2004) cones were not useful for this experiment. In the year 2005, the procedure of the collection of the cones was same, but the last week's cones were used as explants. Since the embryos at the precotyledonary stage could be useful for initiation of embryogenic tissue.

In experiments carried out in 2004 and 2005, megagametophytes with the enclosed immature zygotic embryos were aseptically and carefully removed from the seeds and used as explants (Figure 2.4). This type of explant provides two advantages; first, it is easier to culture, and second, it has high initiation frequency. Also, it was important not to wound the megagametophytes during excision.

Figure: 2.4. Immature zygotic embryos used as explants.

2.2.1. Medium preparation

Only the standard initiation medium, Douglas-fir cotyledon revised medium, DCR (Gupta & Durzan, 1985) consists of DCR basal salts, nicotinic acid, pyridoxine.HCl, thiamine.HCl, glycine, 2,4-dichlorophenoxyacetic acid (2,4-D), and 6-benzylaminopurine (BAP), myo-inositol, casein-hydrolysate, L-glutamine, sucrose and gellan gum (Gelrite®) was used for initiation experiment (Table 2.1). The DCR medium was seleted because DCR medium is widely used for initiation of embryogenic tissue in *Pinus nigra* (Salajova *et al,* 1999; Salajova & Salaj, 2005).

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

Inorganics, mg/L		**Plant growth regulators, mg/L**	
NH_4NO_3	400.0	2,4-D	3.0
KNO_3	340.0	BAP	0.5
$Ca(NO_3)_2.4H_2O$	556.0		
$MgSO_4 7H_2O$	370.0		
KH_2PO_4	170.0	**Carbohydrate and gelling agent, g/L**	
$CaCl_2.2H_2O$	85.0	Sucrose	30.0
KI	0.83	Gellan gum	1.5
H_3BO_3	6.20		
$MnSO_4.H_2O$	22.30		
$ZnSO_4.7H_2O$	8.60	**Vitamins and amino acid, mg/L**	
$Na_2MoO_4.2H_2O$	0.25	Nicotinic acid	0.5
$CuSO_4.5H_2O$	0.25	Pyrodoxine.HCl	0.5
$CoCl_2.6H_2O$	0.025	Thiamine.HCl	1.0
$NiCl_2.6H_2O$	0.025	Glycine	2.0
$FeSO_4.7H_2O$	27.8		
Na_2EDTA	37.3		

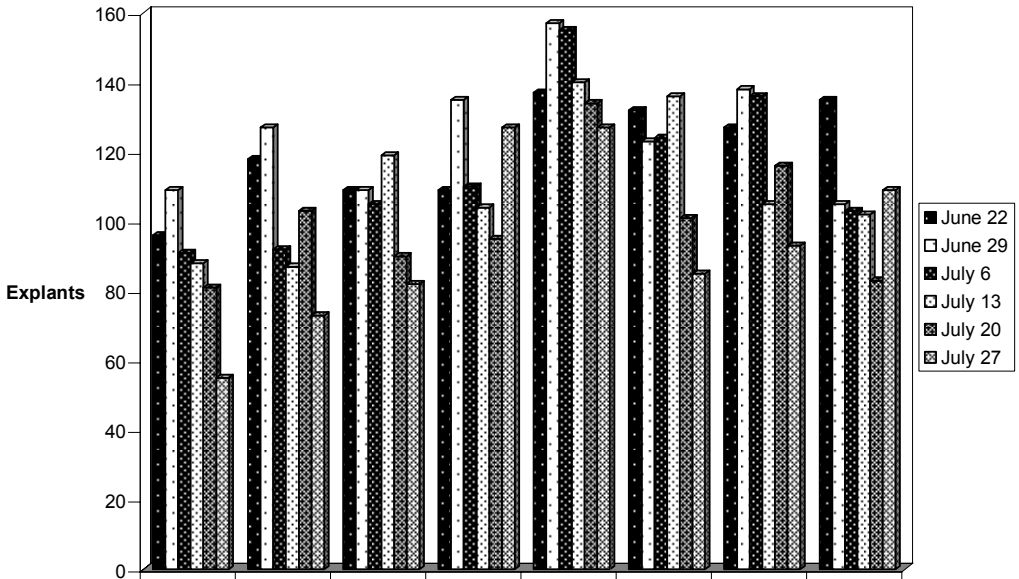
All of the chemicals and plant growth regulators used in medium preparation were obtained from Duchefa Biochemi B.V. (The Netherlands) and Sigma Chemical Company (USA).

Before sterilization, the pH of all media was adjusted to 5.8 with NaOH and HCl. After that, 1.5 g/L gelrite was added. The media was then sterilized by autoclaving at 15 psi and 121^0C for 20 minutes. After autoclaving, filter-sterilized (0.22m CA membrane, 25 mm diameter syringe filter) stock solutions of L-glutamine and casein hydrolysate were added to the cooling medium. Approximately 25 ml of initiation medium were poured into a sterile petri plate (90 X 10 mm).

2.2.2. Culture condition

About ten explants were cultured in each petri plate. The perimeter of each plate was wrapped twice with Parafilm “M” (American National Can^{TM}). The cultures were kept in the dark at 22^{0}C for 8-10 weeks. After plating, visual observations were made.

Each collection-date/genotype combination contained the same number of explants (40-60) if there was no explant contamination. There are two replications in each year. Eighty to one-hundred twenty explants were cultured per selected tree. In total, 10 463 explants were cultured on initiation medium in 2004(Figure 2.5) and 2005(Figure 2.6); 5317 explants for 2004 and 5146 explants for 2005.

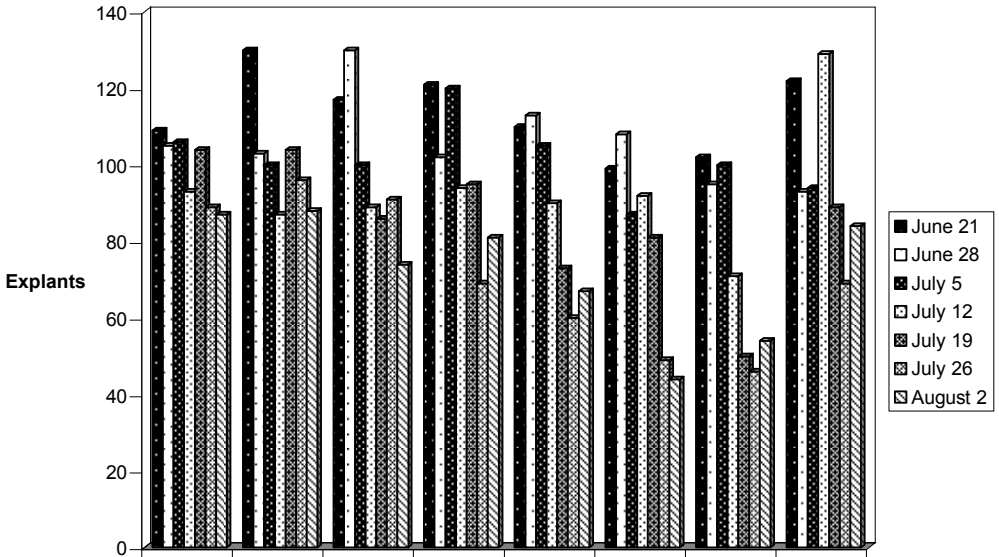


	1	2	3	4	5	6	7	8
June 22	96	118	109	109	137	132	127	135
June 29	109	127	109	135	157	123	138	105
July 6	91	92	105	110	155	124	136	103
July 13	88	87	119	104	140	136	105	102
July 20	81	103	90	95	134	101	116	83
July 27	55	73	82	127	127	85	93	109

Clones

Figure 2.5. Overall explants cultured on initiation medium in 2004.

Figure 2.5. displays overall explants cultured on initiation medium in 2004. The overall mean of cultured explants was 886 seeds weekly and Figure 2.6. shows overall explants in 2005 and overall mean was 735.



	1	2	3	4	5	6	7	8
June 21	109	130	117	121	110	99	102	122
June 28	105	103	130	102	113	108	95	93
July 5	106	100	100	120	105	87	100	94
July 12	93	87	89	94	90	92	71	129
July 19	104	104	86	95	73	81	50	89
July 26	89	96	91	69	60	49	46	69
August 2	87	88	74	81	67	44	54	84

Clones

Figure 2.6. Overall explants cultured on initiation medium in 2005.

2.3. Proliferation of ET

Initiation (Figure 2.7.) and extrusion (Figure 2.8.) were recorded and depending on culture development, either 8 or 10 weeks after culture initiation, proliferating embryogenic tissues were isolated as new explants and transferred to fresh medium. These embryogenic tissues were subcultured every 3-4 weeks.

The same initiation medium was used for subculturing. In each subculture, four or five embryogenic tissue clumps depending on availability of tissue, were cultured per petri dish and maintained under the same conditions as used for initiation.

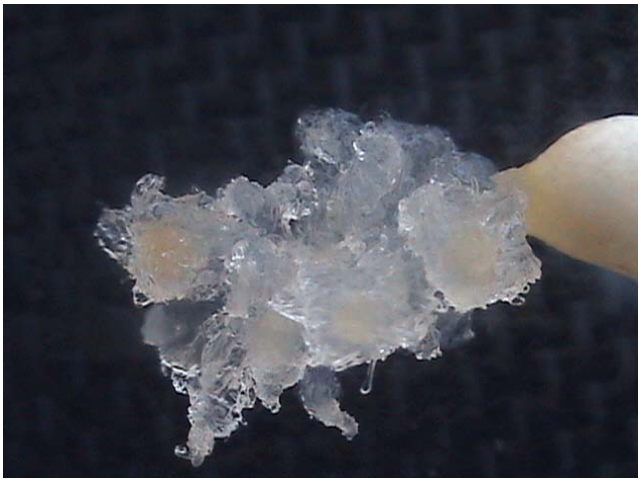


Figure 2.7. Picture of the initiation.

Figure 2.8. Picture of the extrusion.

2.4. Data collection and statistical analyses

After 8 weeks culture, explant initiation frequencies (INFREQ) and extrusion frequencies (EXFREQ) were calculated for each replications. These frequencies calculated for each genotype values were used in ANOVA and genotype mean comparisons within each collection date by use of Tukey's multiple comparison test. For proliferation frequency, the established cell lines (ECL) were recorded after nearly 15 weeks or 5 subculturing period. It is calculated by dividing the number of cell lines growing after 5 subculturing period to the initial number of explants. Also conversion frequencies (CF) were determined. This was calculated by dividing the number of ECL (ECL_N) to the number of explants showing initiation ($INFREQ_N$).

For the statistical analyses, one-way ANOVA (Analyses of variance) used to detect the differences between the collection dates and genotypic variation within each collection date. The GLM procedure of SAS (SAS Institude) was used for analysis of variance.

CHAPTER 3

RESULTS

3.1. Initiation of Embryogenic Tissue (ET)

This study consisted of two independent experiments; the first one was carried out in 2004 while the second one was performed in 2005. The procedures for these experiments were the same for each year, but genotypes and collection dates were differed. The sampling period was 6 weeks in 2004, but it was 7 weeks in 2005. Because of this, we reported the results for 2004 and 2005 separately.

3.1.1. The results of the year 2004

In 2004, 5317 explants were cultured on initiation medium. Initiation and extrusion frequencies were calculated for collection dates and genotypes. One-way ANOVA (Analysis of variance) was used to determine the significance of the differences between the collection dates and the genotypic variation for each collection date.

Figure 3.1 shows the overall distribution of the initiation (INFREQ) and extrusion frequencies (EXFREQ) by collection dates 2004. No ET initiation was recorded on 27-July. The lowest value was detected on 13-July as 0.30%. Then, the values were 0.71% for 20-July, 0.87% for 29-June, and 1,53% for 6-July. The highest value was recorded on 22-June as 1,82%.

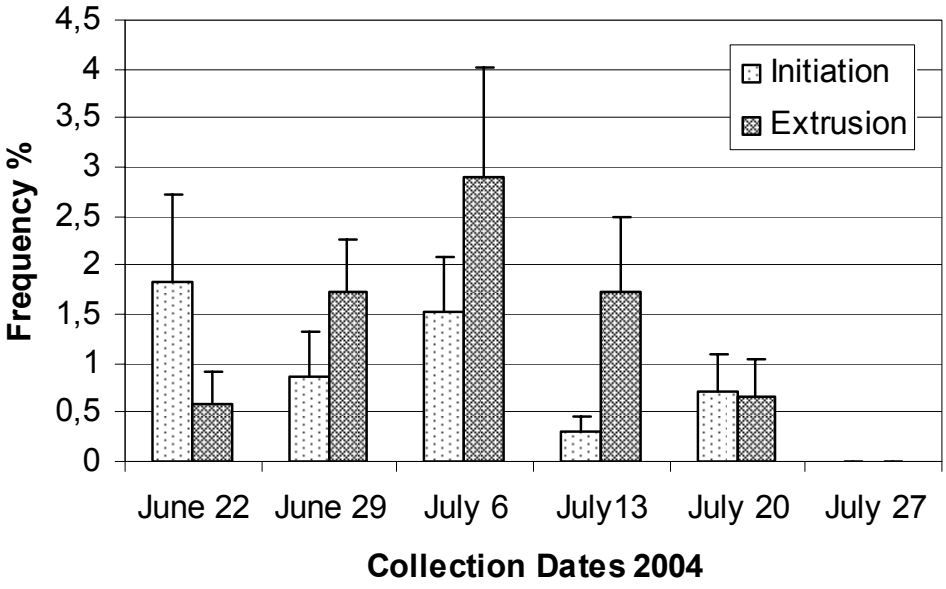


Figure 3.1. Overall distribution of initiation and extrusion frequencies along collection dates (2004).

In addition to the INFREQ, the collection date means for EXFREQ were displayed in Figure 3.1. EXFREQ were higher than INFREQ and estimated values were 1,72%, 2.90%, 1,72% for the 29-June, 6-July, 13-July, respectively.

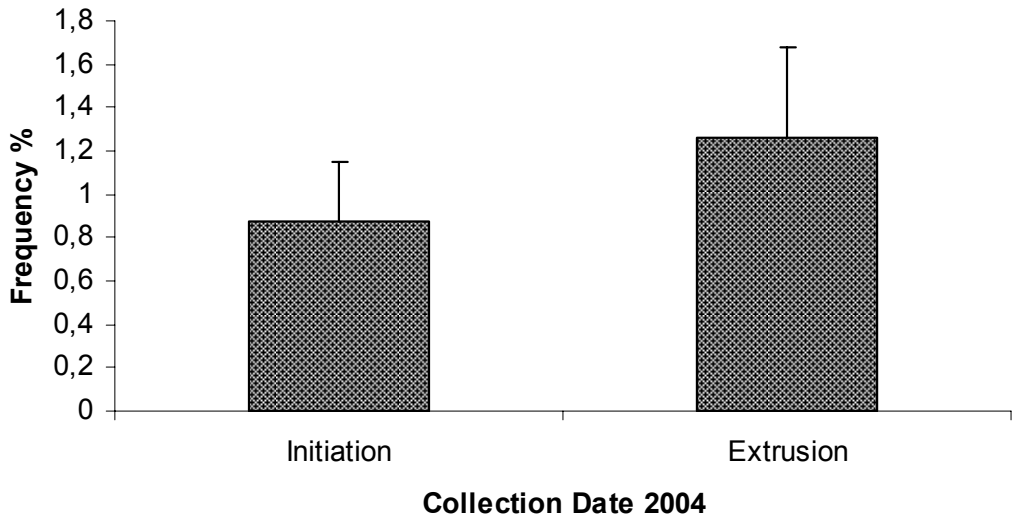


Figure 3.2. Overall initiation and extrusion frequencies for collection dates (2004).

Figure 3.2 shows the overall INFREQ and EXFREQ values for overall collection dates in 2004. Overall EXFREQ value was higher than INFREQ value. Overall EXFREQ and INFREQ values were 1.26% and 0.87%, respectively.

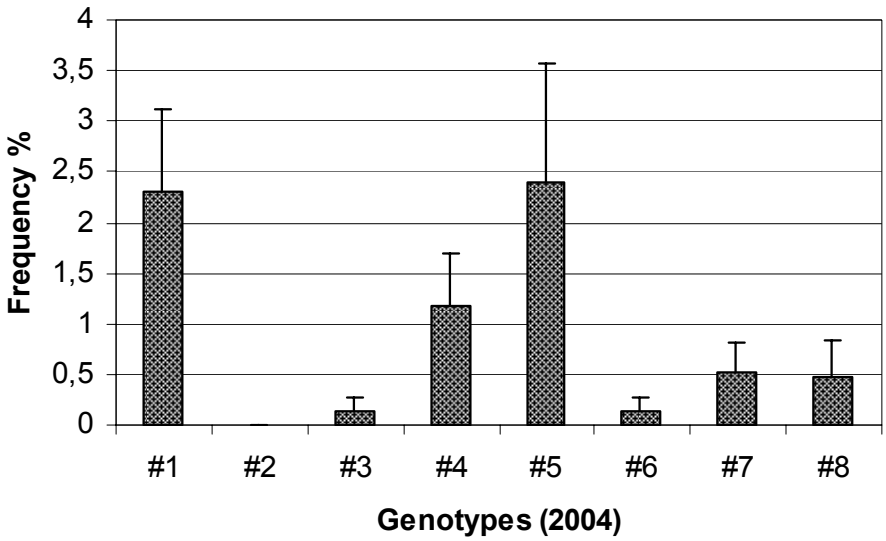


Figure 3.3. Overall distribution of the INFREQ of the genotypes 2004.

Figure 3.3 shows overall distribution of the mean ET initiation of the genotypes studied in 2004. The highest initiation frequency was observed for Genotype 5 (2.40%). No response was determined for Genotype 2. The lowest INFREQ was recorded for Genotype 6 and 3 (0.14). Thereafter, the values were 2.31% (Genotype 1), 1,17% (Genotype 4), 0.53% (Genotype 7), 0.48% (Genotype 8).

3.1.2. The results of the year 2005

The second part of the study was conducted in 2005. In this year, 5146 explants were cultured on initiation medium. Initiation and extrusion frequencies were calculated for collection dates and genotypes as in year 2004. One-way ANOVA was used to determine the significance of the differences between the collection dates and the genotypic variation for each collection date like, treatment of the results of 2004.

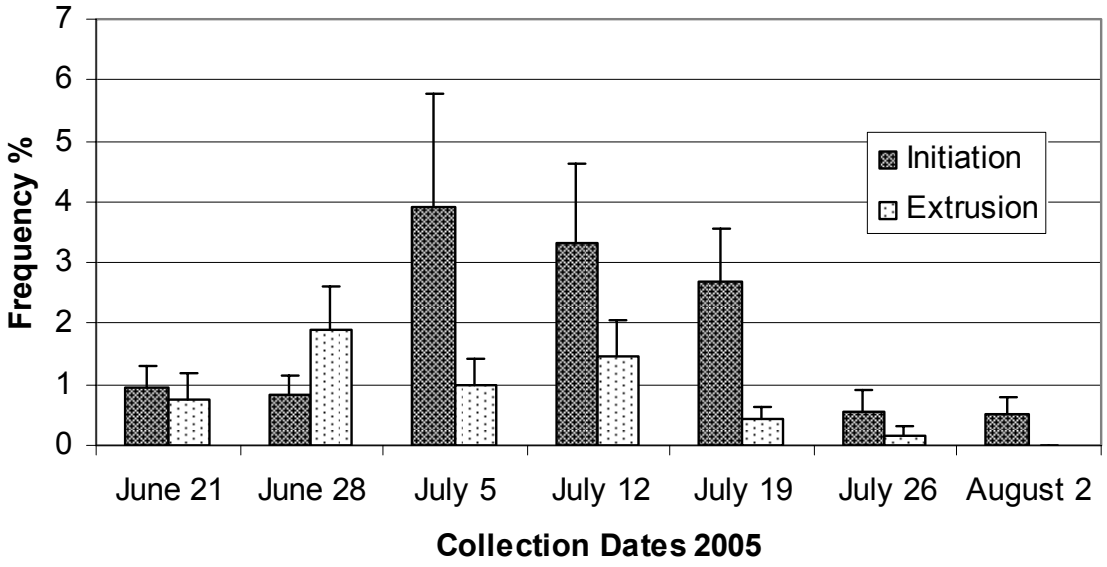


Figure 3.4. Overall distribution of initiation and extrusion frequencies along collection dates (2005).

Figure 3.4 shows the overall distribution of the initiation (INFREQ) and extrusion frequencies (EXFREQ) by collection date in 2005. The lowest value was recorded on 2-August as 0.50%. Then, under 1%, there were three collection dates; 26-July (0.57%), 28-June (0.82%) and 21-June (0.94%). There were two collection dates between 1 and 3.5%; 19-July (2.70%) and 12-July (3.31%). The highest value was recorded on 5-July as 3.91%.

The means of collection dates for EXFREQ are shown in Figure 3.4. INFREQ were higher than EXFREQ for the six collection dates; 21-June (INFREQ:0.94 ;EXFREQ:0.74), 5-July(INFREQ:3.91; EXFREQ:0.99), 12-July (INFREQ:3.31; EXFREQ:1.45), 19-July(INFREQ:2.70;EXFREQ:0.42), 26-July (INFREQ:0.57;EXFREQ:0.16) 2-August (INF:0.50;EXF:0.0).

Figure 3.5. Overall initiation and extrusion frequencies for collection dates (2005).

Figure 3.5 shows the overall INFREQ and EXFREQ values for collection dates in 2005. Overall INFREQ value was higher than EXFREQ value. INFREQ value was 1.82% and EXFREQ value was 0.81%.

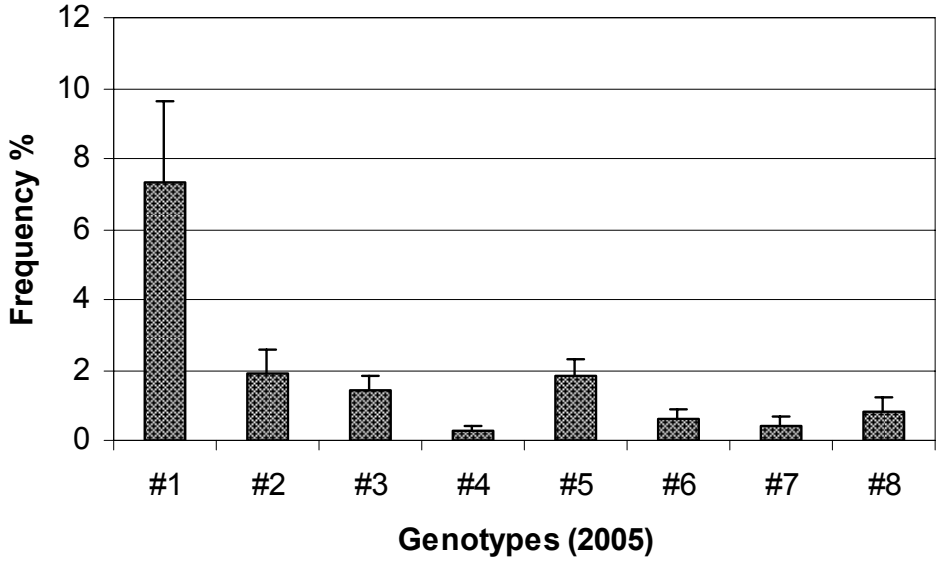


Figure 3.6. Overall distribution of the INFREQ of the genotypes 2005.

Figure 3.6 displays overall distribution of the mean ET initiation of the genotypes studied in 2005. The highest initiation frequency was observed for Genotype 1 (7.32%). The lowest INFREQ was recorded for Genotype 4(0.24%).

3.1.3. The results of ANOVA (2004)

Table 3.1. The results of ANOVA for initiation and extrusion frequencies (2004).

		Initiation frequency			Extrusion frequency		
Source	**df**	**MS[1]**		**VC[2] (%)**	**MS**		**VC (%)**
Replication	1	0.0044	NS		0.0160	NS	
Collection Date	5	0.0132	*	5.41	0.0276	**	12.67
Genotype	7	0.0200	**	15.13	0.0198	*	10.45
Genotype*Coll. Date	35	0.0070	NS	16.50	0.0079	NS	2.00
Error	45	0.0046		62.96	0.0075		74.89

****significant at p<0.01 *significant at p<0.05**

df: degrees of freedom NS: Not Significant

[1] MS: Mean squares [2] VC: Variance component

The results of Analysis of variance (ANOVA) revealed that there was no significant difference between replications for initiation and extrusion frequencies. However there were significant effects of genotypes and collection dates on initiation and extrusion frequencies (Table 3.1.)

Variance component (VC) analysis showed that 5.41% and 15.13% of the variation were based on the collection dates and genotypes, respectively for initiation (Table 3.1.).

3.1.4. The results of ANOVA (2005)

Table 3.2. The results of ANOVA for initiation and extrusion frequencies (2005).

		Initiation frequency		Extrusion frequency	
Source	**df**	**MS** [1]	**VC**[2] **(%)**	**MS**	**VC (%)**
Replication	1	0.0000 NS		0.0016 NS	
Collection Date	6	0.0364 **	11.93	0.0360 **	20.85
Genotype	7	0.0605 **	26.53	0.0143 **	5.25
Genotypes*Coll. Date	42	0.0110 **	20.73	0.0081 *	23.61
Error	55	0.0054	40.81	0.0042	50.29

****significant at p<0.01 *significant at p<0.05**

df: degrees of freedom NS: Not Significant

[1] **MS: Mean squares** [2] **VC: Variance component**

The results of analysis of variance (ANOVA) showed that there was no significant difference between replications for initiation and extrusion frequencies in 2005. Initiation and extrusion frequencies were significantly affected by the cone collection date and genotypes (Table 3.2.).

Variance component (VC) analysis showed that 11.93% and 26.53% of the variation were based on the collection dates and genotypes, respectively for initiation (Table 3.2.).

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3.2. Proliferation of Embryogenic Tissue (ET)

3.2.1. Values of INFREQ, ECL and CF (2004)

Table 3.3. The results of INFREQ, ECL and CF among collection dates (2004).

Collection Date	Initial # of explants	INFREQ(%)	ECL(%)	CF(%)
June 22	963	1.82 a (19)[1]	0.62 (6)[2]	31.58
June 29	1003	0.87 b (8)	0.20 (2)	25.00
July 6	916	1.53 a (13)	0.76 (7)	53.85
July 13	881	0.30 c (3)	0.00 (0)	0.00
July 20	803	0.71 b (6)	0.37 (1)	16.67
July 27	751	0.00 d (0)	0.00 (0)	0.00
Overall	**5317**	**0.92 (49)**	**0.38 (20)**	**40.82**

[1] the # of initiated explants [2] the # of ET after 5 subculture
Values followed by different letters are significantly different at *P< 0.05* using Tukey's HSD post-hoc test.

Established cell lines (ECL) were recorded after five subcultures. Table 3.3. showed that ECL percentages were lower than INFREQ for all collection dates. ECL values varied between 0.2% (29-June) and 0.76% (6-July). For 13-July and 27-July collection date, none of the initiated embryogenic tissues generated ECL.

The highest conversion frequency (CF) was recorded for the 6-July collection date (53.85%). Highest conversion frequency seems to be associated with high INFREQ.

Tukey's HSD post-hoc test showed that collection date June-22 and July-6 are different from the other collection times (Table 3.3.)

Table 3.4. The results of INFREQ, ECL and CF among genotypes (2004).

Genotype	# of explants	INFREQ (%)	ECL (%)	CF (%)
1	520	2.31 a (12)[1]	0.96 (5)[2]	41.67
2	600	0.00 b (0)	0.00 (0)	0.00
3	614	0.14 b (1)	0.00 (0)	0.00
4	680	1.17 a (8)	0.44 (3)	37.50
5	850	2.40 a (19)	0.82 (7)	36.84
6	701	0.14 b (1)	0.00 (0)	0.00
7	715	0.53 b (5)	0.42 (3)	60.00
8	637	0.48 b (3)	0.31 (2)	66.67
Overall	**5317**	**0.92 (49)**	**0.38 (20)**	**40.82**

Values followed by different letters are significantly different at *P< 0.05* using Tukey's HSD post-hoc test.
[1] the # of initiated explants [2] the # of ET after 5 subculture

Overall comparison of INFREQ, ECL and CF values among genotypes for 2004 was presented in Table 3.4. Genotype 1 had the highest ECL percentages (0.96%), but it was still lower than the initiation frequencies. Among the remaining genotypes, Genotype 5 had the highest ECL value (0.82%). The lowest ECL percentage was found for Genotype 8 (0.31%). Genotype 2, Genotype 3 and Genotype 6 had no ECL.

Ranking of the INFREQ and ECL values were similar, but CF did not follow the same pattern. Although Genotype 7 and Genotype 8 had lower initiation frequencies, they had higher conversion frequencies 60.00% and 66.67%, respectively.

Genotype 1 had highest INFREQ, but it had 41.67% CF. Other CFs were 37.50% for Genotype 4 and 36.84% for Genotype 5. Overall CF was found to be 40.82%.

Differences among genotypes tested by Tukey's HSD post-hoc test at a significance level of α=0.05 was provided in Table 3.4. This test showed that Genotype 1, 4 and 5 are significantly different from Genotype 3, 6, 7 and 8.

3.2.2. Values of INFREQ, ECL and CF (2005)

Table 3.5. The results of INFREQ, ECL and CF among collection dates (2005).

Collection Date	Initial # of explants	INFREQ(%)	ECL(%)	CF(%)
June 21	910	0.94 abc (9) [1]	0.44 (2)[2]	22.22
June 28	849	0.82 bc (7)	0.00 (0)	0.00
July 5	812	3.91 a (33)	1.48 (16)	48.49
July 12	745	3.31 a (25)	1.21 (8)	32.00
July 19	682	2.70 ab (20)	0.73 (5)	25.00
July 26	569	0.57 c (4)	0.18 (1)	25.00
August 2	579	0.50 c (3)	0.00 (0)	0.00
Overall	**5146**	**1.96 (101)**	**0.62 (32)**	**31.69**

[1] the # of initiated explants [2] the # of ET after 5 subculture
Values followed by different letters are significantly different at *P< 0.05* using Tukey's HSD post-hoc test.

Established cell lines (ECL) were recorded after five subcultures as 2004. Table 3.5. revealed that ECL percentages were much lower than INFREQ for all collection dates. There were no initiated embryogenic tissues generated ECL at 28-June and 2-August. The higher ECL was 1.48% at the collection date 5-July. 0.18% (26-July) was the lower value. The other values were 0.44% (21-June), 0.73% (19-July) and 1.21% (12-July). Overall ECL value was 0.60% in 2005.

At 5-July, CF was highest value that is, 48.49% of the ETs was converted into ECL. The lowest value was 22.22% at 21-June. The other CF values were 25.00% (19-July and 26-July) and 32.00% (12-July).

Tukey's HSD post-hoc test showed that collection date July-5 and July-12 are different from the other collection times (Table 3.5.)

Table 3.6. Genotype means for INFREQ, ECL and CF in year 2005.

Genotype	# of explants	INFREQ (%)		ECL (%)		CF (%)
1	693	7.32 a	(52) [1]	2.17	(15) [2]	28.85
2	708	1.93 b	(13)	0.57	(4)	30.77
3	687	1.39 b	(10)	0.73	(5)	50.00
4	682	0.24 b	(2)	0.15	(1)	50.00
5	618	1.80 b	(11)	0.00	(0)	0.00
6	560	0.63 b	(4)	0.55	(3)	75.00
7	518	0.42 b	(2)	0.19	(1)	50.00
8	680	0.84 b	(7)	0.44	(3)	42.86
Overall	**5146**	**1.96**	**(101)**	**0.62**	**(32)**	**31.68**

Values followed by different letters are significantly different at *P< 0.05* using Tukey's HSD post-hoc test.
[1] the # of initiated explants [2] the # of ET after 5 subculture

Overall comparison of INFREQ, ECL and CF values among genotypes for 2005 were presented in Table 3.6. Genotype 1 had the highest ECL percentages 2.17%, lower than the initiation frequencies.

Among the remaining genotypes, Genotype 3 had 0.73% ECL values. The lowest ECL percentage was observed in Genotype 4 (0.15%). Genotype 5 had no ECL. The other values were 0.57% (Genotype 2), 0.55% (Genotype 6), 0.44% (Genotype 8) and 0.19% (Genotype 7). Overall ECL percentage was 0.62%.

Grading of the INFREQ, ECL and CF values were not similar. Although Genotype 5 had higher initiation frequencies, they had no ECL and no conversion frequencies. Overall CF value was 31.68%.

Comparisons of genotypes were revealed in Table3.6. by using Tukey's HSD post-hoc test at a significance level of α=0.05. Genotype 1 was significantly different from other genotypes.

CHAPTER 4

DISCUSSION

This study is the first report on the initiation of embryogenic tissue for *Pinus nigra* subsp. *pallasiana.* Although, embryogenic tissue initiation has been reported for *Pinus nigra* earlier (Salajova *et al,* 1999; Salajova & Salaj, 2005) genotypes and genotypic influences have not been dealt in these studies.

The initiation of somatic embryogenesis (SE) is affected by the developmental stage of zygotic embryos, the genotype, the formulation of tissue culture medium, plant growth regulators and gelling agent concentration (Park *et al*, 1993; Höggman *et al*, 1999; Klimaszewska *et al*, 2001; Pullman *et al*, 2004). In conifers, SE has initiation, maturation and germination steps, and each step has its own challenges, but; in this study our focuses was only the initiation step. Other steps will be studied in the future.

In *Pinus* species, developmental stage of zygotic embryos affected the initiation frequencies (Klimaszewska & Smith, 1997; Höggman *et al*, 1999; Miguel *et al*, 2000). In our experiments, embryogenic tissue was obtained from immature zygotic embryos that were at the precotyledonary stage of development. Commonly immature zygotic embryos in precotyledonary stages of development have been shown to be excellent explants for embryogenic tissue initiation in *Pinus* (Bercetche & Paques, 1995; Laine & David, 1990; Finer *et al*, 1989); and choosing explants in the precotyledonary stage of development is known to be a very important factor for the embryogenic tissue initiation process.

There are numerous examples when ET have been obtained from the precotyledonary stage of embryos. Experiments in *Pinus nigra* showed that explants collected in the second half of June were superior for embryogenic callus initiation (Salajova & Salaj, 1992; Salajova *et al*, 1995). In that study, histological analysis showed that zygotic embryos were at the precotyledonary stage of development (Salajova *et al,* 1999).

In *Pinus brutia*, precotyledonary stages of zygotic embryos were used as explant. The "window" for ET initiation was about four weeks, throughout July for year 2003 (Yıldırım, 2005). In *Pinus elliottii* (Jain *et al*, 1989), *Pinus taeda* (Becwar *et al*, 1990), *Pinus caribaea* (Laine & David, 1990), *Pinus patula* (Jones *et al*, 1993), and *Pinus palustris* (Nagmani *et al*, 1993a), the precotyledonary embryos were the optimal response for ET initiation. In *Pinus pinaster*, the best results were obtained at Stage 4 (zygotic embryo from precotyledonary to late cotyledonary stage (Lelu *et al*, 1999). Also, Becwar *et al*. (1989) were reported that only precotyledonary embryos formed embryogenic callus and the origin of the callus was the suspensor region.

Finer *et al*. (1989), in reporting on *Pinus strobus* somatic embryogenesis, displayed that high induction rates were attained when precotyledonary embryos enclosed within the megagametophytes were used as explants.

The highest initiation frequency was obtained with cotyledons excised from seeds at an early stage of germination in *Pinus radiata* (Schestibratov *et al*, 2003). In *Pinus sylvestris*, the highest response was obtained with Stage 1 and Stage 2 corresponding to four-celled embryo and to cleavage polyembryogeny respectively (Lelu *et al*, 1999).

In this study, “window” for ET initiation was extended over approximately 3 weeks, from the first week of July to the third week of July, according to the results of 2005 (Figure 4.1). The results of the 2004 were somewhat problematic. Because the cones were stored in refrigerator long time and some contamination occurred. This contamination affected the initiation frequencies negatively in this year.

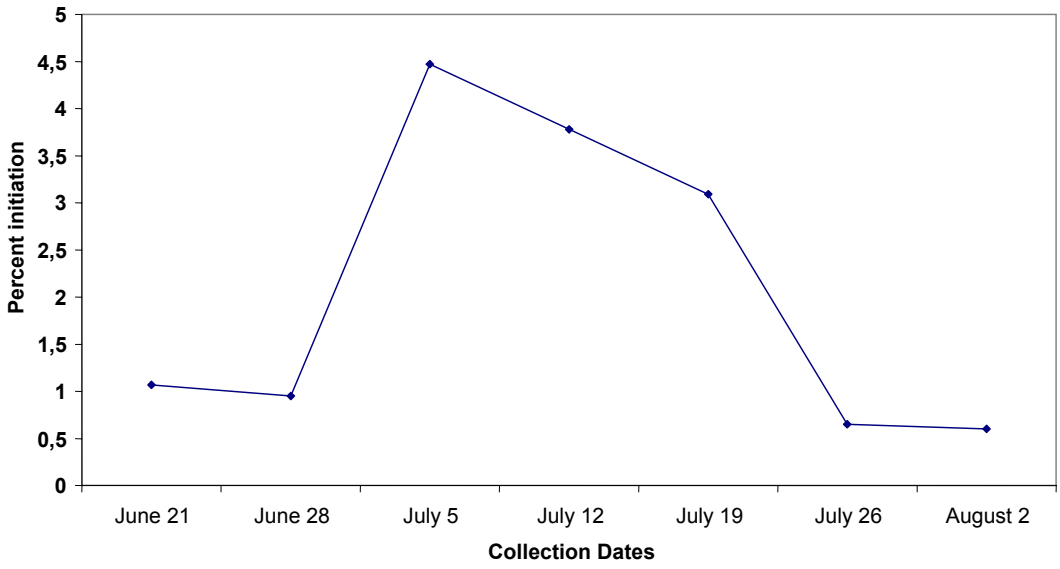

Figure 4.1. Initiation of SE in Anatolian black pine by collection dates (2005).

In our experiment, we did not do histological study in detail. Although this is important to observe the stages of embryo development for ET initiation process, formulation of medium, plant growth regulators and gelling agent concentration is also important.

In pines, DCR, LM, MSG media could be used for initiation. DCR basal medium supplemented with sucrose as a carbon source and gelrite and phytagel as a gelling agent was used respectively in *Pinus nigra* & *Pinus strobus* and *Pinus brutia* to initiate embryogenic callus (Salajova & Salaj, 2005; Yıldırım, 2005). Also DCR basal medium supplemented with sucrose and gellan gum was used in *Pinus roxburghii* (Mathur *et al*, 2000).

Half strength LP (liquid proliferation) basal medium containing sucrose and agar was utilized in *Pinus radiata* (Schestibratov *et al*, 2003). BM_1 (Gupta & Pullman, 1990) basal medium was superior so higher frequency was obtained in *Pinus taeda* and phytagel was used as gelling agent (Li, *et* al, 1998). Plant growth regulators, auxins and cytokinins play an important role in the initiation and proliferation of embryogenic tissues. The most commonly used auxin is 2,4-D and cytokinin is BA.

Genetic influences for SE in conifers is well-known (Park *et al*, 2006).In this study, there is high variability among genotypes for embryogenic tissue initiation. The source of this variability is the genes which is responsible for somatic embryogenesis. For example, experiments for somatic embryogenesis in cucumber showed that, obtaining more embryos related to the determination of the third dominant genes (Parrott *et al*. 1993).

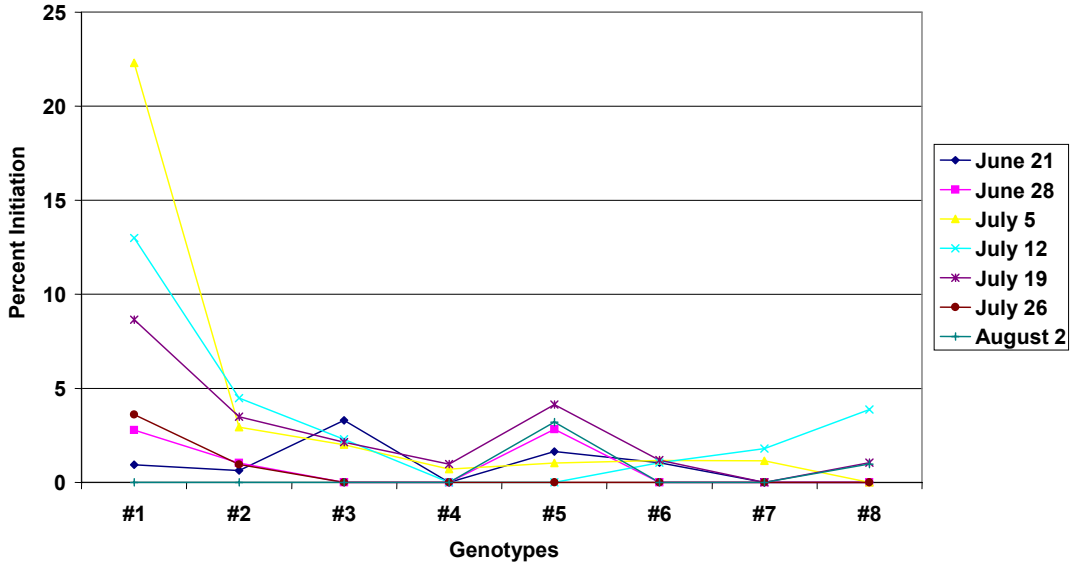

Figure 4.2. Comparisons of genotypes by collection dates (2005).

Comparison of genotypes between all collection dates analyzed separately revealed consistency in rankings of somatic embryo initiation. There were significant differences among genotypes, collection dates and genotypes X collection dates interactions (Figure 4.2).

In 2004, EXFREQ normally distributed around July-6, but INFREQ shows no particular pattern. This may be consequences of the contaminations of some cones that reduced the sample size. In 2005, it is obtained quite a different overall distribution pattern was observed than that was for 2004. In 2005, because immature zygotic embryos were quickly removed from seeds due to the experience gained in the previous year, cones were utilized without contamination.

The overall ET initiation frequency was 1.96% for 2005. The highest value in 2005 was 4.06% in third week of sampling time. In the experiments with *Pinus nigra* the initiation frequencies were relatively low 3.06% (Salajova & Salaj, 2005). The highest values were reported in *Pinus strobus*, 35% (Finer *et al*, 1989), *Pinus pinaster*, 20.1% (Miguel *et al*, 2004) and *Pinus brutia*, 11.6% (Yıldırım, 2005).

In *Pinus*, the total number of explants showing initiation ($INFREQ_N$) may not form the ECL, that is; not all initiated tissues may be converted into ECLs. In these experiments, overall 0.38% for 2004 and 0.62% for 2005 of the initial explants were converted into established cell lines. These are quite low when compared to the previous studies.

For example, in *Pinus brutia*, it was 3.4% (Yıldırım, 2005), in *Pinus pinaster*, 12% (Höggman *et* al, 1999; Miguel *et al*, 2004) and in *Pinus strobus*, 2.1% (Garin *et al*, 1998), of the initial explants were converted into ECLs.

In the future experiments:

Cones can be collected in three weeks (in July) instead of seven weeks for initiation step. Reducing of collection date would save time for optimizing culture medium formulation and plant growth regulators.

Microscopic observations could be done for characterization of embryogenic tissue to determine exact developmental stage of zygotic embryos.

The other steps of somatic embryogenesis; maturation and germination steps should be also optimized after this initiation and proliferation steps.

CHAPTER 5

CONCLUSION

The main objective of this study was the induction of embryogenic tissue from immature zygotic embryos in *Pinus nigra* subsp. *pallasiana*. For this goal, independent experiments were done conducted in 2004 and 2005.

The other specific objectives were determined influences between

(1) collection date and initiation frequencies of embryogenic tissue
(2) genotype and initiation frequencies of embryogenic tissue

Specifically the following findings could be reported based on the results of experiments carried out in years 2004 and 2005:

- 0.92% in 2004 and 1.96% in 2005 initiation frequencies were obtained which are low compared to other studies, but with collection time selection these figures could be improved further.
- The developmental stage of zygotic embryo is significantly important for obtaining SE initiation. For central Turkey conditions, it appears that collection date is the first, second and third weeks of July.
- Also, results indicate that genotype effects had a large influence on SE initiation and significant genotype X cone collection time interaction was observed. Coincide with proper developmental stage of embryo to be used ET initiation.

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