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Research Article

Induction of Embryogenic Tissue from Immature Zygotic Embryos in *Pinus nigra* J.F.Arnold subsp. *nigra* var. *caramanica* (Loudon) Businsky

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Abstract: Embryogenic cultures (EC) of Anatolian black pine (*Pinus nigra* J.F.Arnold subsp. *nigra* var. *caramanica* (Loudon) Businsky) were initiated from immature pre-cotyledonary zygotic embryos sampled weekly from 16 different trees in 2 consecutive years. Douglas-fir cotyledon revised (DCR) medium supplemented with 13.6 µM of 2,4-D and 2.2 µM of BAP was used for initiation and maintenance of EC. Overall initiation frequencies of EC in the study were 0.92% in 2004 and 1.96% in 2005; tree values ranged from 0.0% to 7.32%. Overall, 0.38% and 0.62% of the initial explants were converted into established cell lines in 2004 and 2005, respectively.

Key Words: *Pinus nigra* subsp. *nigra* var. *caramanica*, somatic embryogenesis, initiation, embryogenic cultures

Pinus nigra subsp. *nigra* var. *caramanica* (Loudon) Businsky Olgunlaşmamış Zigotik Embriyolarından Embriyogenik Dokuların İndüklemesi

Özet: Karaçam embriyogenik dokuları (ED), birbirini takip eden iki yıl boyunca haftalık olarak 16 farklı ağaçtan toplanan olgunlaşmamış henüz kotiledon gelişimi göstermeyen zigotik embriyolardan elde edildi. ED'lerin indüklemesi ve sonrasında çoğaltılması için, 13,6 µM 2,4-D ve 2,2 µM BAP ilave edilmiş Douglas-fir cotyledon revised (DCR) besiyeri kullanıldı. ED genel indükleme frekansları 2004 yılında %0,92, 2005 yılında ise %1,96 olarak gerçekleşti; farklı ağaçlar için değerler sıfır ile %7,32 arasında değişti. ED indüklemesinde kullanılan explantlar, 2004 ve 2005 yıllarında yapılan örneklemelerde sırasıyla %0,38'i ve %0,62'si çoğalmaya devam eden hücre hatlarına dönüştüler.

Anahtar Sözcükler: *Pinus nigra* subsp. *nigra* var. *caramanica*, somatik embriyogenez, indükleme, embriyogenik doku

Introduction

When integrated into conventional tree breeding programmes, somatic embryogenesis (SE) provides a valuable tool for clonal propagation of superior trees. Of those advantages, SE offers the use of cryopreservation, enabling researchers to preserve embryogenic cell lines in liquid nitrogen until their field performance is evaluated. It also provides a regeneration system to incorporate genes that are not available in the breeding species to obtain transgenic trees (Attree & Fowke, 1991; Park, 2002; Stasolla & Yeung, 2003).

SE has been reported for many commercially important conifers, mainly in the genera of *Abies* Mill., *Picea* A.Dietr., and *Pinus* L. (Tautorus et al., 1991). Although *Pinus* spp. are considered to be recalcitrant to SE, proper explant selection and manipulation of culture conditions have both increased the number of species studied and resulted in higher induction rates of embryogenic tissue (ET) in various pine species, including *Pinus brutia* Ten. (Yildirim et al., 2006), *Pinus caribaea* Morelet (Laine & David, 1990), *Pinus nigra* J.F.Arnold (Salajová et al., 1999), *Pinus palustris* Mill. (Nagmani et

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al., 1993), *Pinus patula* Schltdl. & Cham. (Jones & van Staden, 1995), *Pinus pinaster* Aiton (Lelu et al., 1999), *Pinus roxburghii* Sarg. (Mathur et al., 2000), *Pinus sylvestris* L. (Keinonen-Mettälä et al., 1996), *Pinus strobus* L. (Finer et al., 1989), and *Pinus taeda* L. (Gupta & Durzan, 1987; Becwar et al., 1990).

Pinus nigra J.F.Arnold. (European black pine) is indigenous to central and southern Europe, as well as to the Crimea and Turkey. It is one of the major species used for afforestation of arid and rocky terrain in the sub-Mediterranean region (Cengel et al., 2000; Tolun et al., 2000; Isajev et al., 2004). The subspecies of European black pine found in Turkey is known as Anatolian black pine (*Pinus nigra* subsp. *nigra* var. *caramanica* (Loudon) Businsky. It is an important timber species and the first choice for high altitude Anatolian steppes (Kaya & Temerit, 1994). Based on reforestation volume, Anatolian black pine is the second most important tree species in Turkey, preceded only by *Pinus brutia*. In the last 30 years 24% of the total artificial and 19% of the total natural regeneration area have been planted with Anatolian black pine. Since its natural distribution is wider than that of any other species, it is of great importance to Turkish forestry (Koski & Antola, 1993).

Although there are reports on the induction of ET and the development of somatic embryos of European black pine (Salajova & Salaj, 1992; Salajova et al., 1999; Salajova & Salaj, 2005), there is no published study on the induction of ET and SE in Anatolian black pine. The main objectives of the present study were: (1) to initiate embryogenic cultures (EC) that could be used to develop an efficient micropropagation system for Anatolian black pine using SE; (2) to determine the developmental time period; and (3) to test the effect of genotype on the initiation of ET. The results of this research are expected to be very useful for mass propagation of selected genotypes of Anatolian black pine, clonal forestry, and seed orchard establishments.

Materials and Methods

We collected 1-year-old green female *Pinus nigra* subsp. *nigra* var. *caramanica* cones enclosing pre-cotyledonary immature zygotic embryos from open-pollinated (OP) trees located on the campus of Middle East Technical University in Ankara, Turkey (lat 40°02'N, long 32°54'E, and altitude: 850 m). In 2004 and 2005 the cones were collected weekly between June 22 and July 27 (6 sampling times), and between June 21 and August 2 (7 collection times). In each year 8 trees (genotypes) were sampled; in 2005, due to insufficient cone production by the trees sampled in 2004, 8 different trees were sampled. These trees were numbered from 1 to 8 in 2004, and from 9 to 16 in 2005. From each tree (planted in 1975; based on the approximate age of seedlings, the trees are probably around 35 years old), 5-6 cones were collected from the upper one-third of the crown, to avoid self pollination products, and stored for a maximum of 4 weeks in paper bags at 4 °C until dissection. Before sterilisation the green cones were washed in water with a few drops of dishwashing detergent, disinfected with 30% (v/v) commercial bleach (ACE, 5.25% NaClO) for 15 min, and then rinsed 3 times in sterile H_2O. Immature seeds were removed from cones in a laminar flow bench and sterilised with 5% commercial bleach for 5 min, followed by 3 rinses with sterile H_2O. Megagametophytes containing immature embryos were dissected out aseptically and used as explants.

Douglas-fir cotyledon revised medium (DCR) (Gupta & Durzan, 1985) with a combination of 13.6 µM of 2,4-D and 2.2 µM of BAP was used for initiation. All of the chemicals and plant growth regulators 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 and then 2 g/l of Gelrite® gellan gum was added. The medium was autoclaved at 121 °C (1.05 kg cm^{-2}) for 20 min. After autoclaving, filter-sterilised solutions of casein hydrolysate (500 mg/l) and L-glutamine (250 mg/l) were added to the cooling medium. Ten explants were cultured in a 90 × 10-mm petri dish. Cultures were maintained in the dark at 24 °C for 8-10 weeks. For each collection date, 40-80 seeds of each of the 16 genotypes were used as explants. In total, 5317 and 5146 explants were cultured in 2004 and 2005, respectively, with 2 replications each year.

After an initiation period of 8-10 weeks, proliferating EC were separated from the megagametophyte and subcultured to freshly prepared DCR initiation medium every 3-4 weeks. Following 4-5 subculture periods, actively growing ECs were recorded as established cell lines (ECLs).

ET initiation frequencies (INFREQ) were determined after 8 weeks of culture (Figure). After 5 subculturing periods (15 weeks) the embryogenic cell lines with ≥ 200

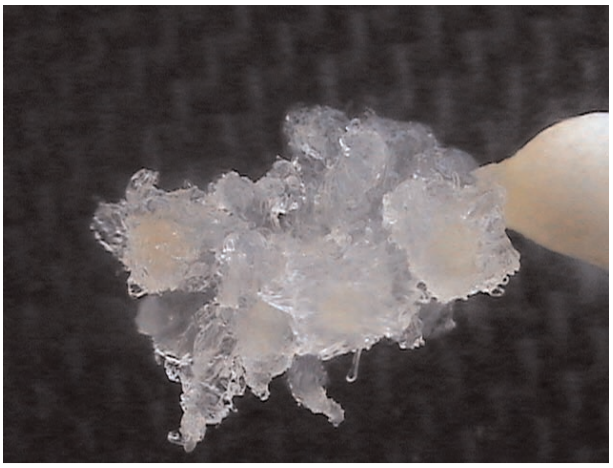

Figure. Photo of EC initiation from an immature zygotic embryo.

mg of fresh tissue weight were accepted as ECLs (Miguel et al., 2004), and their frequencies were recorded for each tree and sampling time.

To determine the differences in the occurrence of initiation between collection dates and trees, one-way analysis of variance (ANOVA) was performed separately for each year because a different set of trees was sampled each year. Data were normalised by ArcSin$\sqrt{x}$, where x represents observed frequencies prior to analysis, in order to meet the assumptions of ANOVA. The GLM procedure of SAS (SAS Institute Inc., 2001) was used for ANOVA. All main effects were considered fixed effects. Multiple comparisons of the collection dates and clones within each year were made using Tukey's HSD post-hoc test.

Results

The results of ANOVA showed that the initiation of EC conducted in 2004 and 2005 was significantly influenced by both cone collection date and tree genotype (Table 1). Overall distribution of INFREQ values across collection dates clearly shows that the initiation process was most successful during the first half of July 2005 (on July 5, 12, and 19) (Table 2). There was no clear trend in the results from 2004. Differences among the trees tested by Tukey's HSD post-hoc test (at $P< 0.05$) are shown in Table 3. Among the 16 studied trees, trees 2 and 9 had the lowest and highest INFREQ values, respectively (Table 3).

Table 1. ANOVA table for INFREQ.

Source	204		2005	
	df	MS	df	MS
Replication	1	0.004 NS	1	0.000 NS
Collection date	5	0.013 *	6	0.036 **
Trees	7	0.020 **	7	0.061 **
Collection date x Trees	35	0.007 NS	42	0.011 **
Error	45	0.005	55	0.005

df: degrees of freedom; MS: mean square;
* $P < 0.05$; ** $P < 0.01$; NS: Not significant

Table 2. Overall comparison of collection times for INFREQ and ECL values.

Collection date	$N_{Explant}$ / INFREQ[1] (%) / ECL (%)					
	2004			2005		
June 21	963	1.82a	0.62	910	0.94abc	0.44
June 28	1003	0.87b	0.20	849	0.82bc	0.00
July 5	916	1.53a	0.76	812	3.91a	1.48
July 12	881	0.30c	0.00	745	3.31a	1.21
July 19	803	0.71b	0.37	682	2.70ab	0.73
July 26	751	0.00d	0.00	569	0.57c	0.18
Aug 2	–	–	–	579	0.50c	0.00
Overall	5317	0.92	0.38	5146	1.96	0.62

[1] Within this column, values followed by different letters are significantly different at $P < 0.05$ using Tukey's HSD post-hoc test.

Table 3. Overall comparison of trees according to INFREQ and ECL.

	$N_{Explant}$ / INFREQ[1] (%) / ECL (%)						
	2004				2005		
#2	600	0.00a	0.00	#12	682	0.24a	0.15
#3	614	0.14a	0.00	#15	518	0.42a	0.19
#6	701	0.14a	0.00	#14	560	0.63a	0.55
#8	637	0.48a	0.31	#16	680	0.84a	0.44
#7	715	0.53a	0.42	#11	687	1.39a	0.73
#4	680	1.17b	0.44	#13	618	1.80a	0.00
#1	520	2.31b	0.96	#10	708	1.93a	0.57
#5	850	2.40b	0.82	#9	693	7.32b	2.17

The frequency of ECLs was much lower than the frequency of ET initiation for all collection dates (Table 2). No ECLs were obtained from the sampling dates of 12 July 2004 and 28 June 2005. ECL values for the other collection dates for both years were low; varying from 0.18% to 1.48%. Overall, 0.38% of the 5317 explants in 2004, and 0.62% of the 5146 explants in 2005 formed ECLs (Table 2). The ECL frequency for all 16 trees ranged from 0.00% to 2.17%. Ranking of the trees according to ECL did not follow the same order as for the frequency of initiation (Table 3).

Discussion

ET initiation depends mainly on the collection of immature zygotic embryos at certain stages. Pre-cotyledonary immature zygotic embryos were reported in previous studies to be the most responsive explant type in *Pinus* (Finer et al., 1989; Becwar et al., 1990). The results of the present study showed that the "window" for ET initiation was about 2-3 weeks, which corresponded with the first half of July 2005; however, a similar "window" was observed in 2004.

The overall ET INFREQ in 2004 (0.92%) was about 50% less than in 2005 (1.96%). Among the other published SE studies of the genus *Pinus*, the ET frequency was quite low: 35% for *Pinus strobus* (Finer et al., 1989) and 20.1% for *Pinus pinaster* (Miguel et al., 2004). However, these frequencies are not as low as those reported for European black pine. Salajová and Salaj (1992) first reported a 2% overall INFREQ value. Later, Salajová et al. (1999) obtained an ET frequency as high as 24.1% from one collection date. In the latest study on European black pine, 3.06% and 5.54% of the megagametophytes formed ET in 2 consecutive years of sampling (Salajová & Salaj, 2005). They reported that the studied material was collected from open-pollinated *Pinus nigra*; however, there was no information concerning the effect of genotype differences on ET initiation. Compared to Salajova's study, the results of the present study (in which 16 trees in 2 consecutive years were tested for the initiation of ET) demonstrated that the effect of genotype is clearly important, ranging from 0.0% (tree 2) to 7.32% (tree 9).

The low frequency of initiation observed in the present study could be further improved by testing different basal media, plant growth regulator combinations, and carbohydrates. For example, Salajová and Salaj (2005) reported a higher INFREQ in European black pine by using maltose instead of sucrose. Additionally, the results of the present study for 2005 indicated a significant genotype × collection time interaction, implying a variation in the developmental stage of immature embryos sampled from a given tree, which may require further attention. For instance, Salajova and Salaj (2005) examined a sample of explants and found high variability in the developmental stage of the zygotic embryos, which could have been responsible for low INFREQ.

In vitro conditions, developmental stage of sampled zygotic embryos, and differences among genotypes are deterministic factors involved in the development of successful SE protocols for any pine species. Aside from these factors, handling of the explants at the beginning could be critical for the remaining SE stages. For example, Yildirim et al. (2006) stressed the importance of dissecting megagametophytes without causing

damage. Even the harshness of seed sterilisation and the storage of cones before dissection would make a difference, since *P. nigra* has relatively small seeds with thin coats. The effects of handling needs to be elaborated in future studies of Anatolian black pine.

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