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Somatic Embryogenesis from Immature Cotyledons of Some European Chestnut (*Castanea sativa* Mill.) Cultivars

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Abstract: In 4 European chestnut (*Castanea sativa* Mill.) cultivars ('Hacıibiş', 'Karamehmet', 'Osmanoğlu' and 'Sarıaşlama'), somatic embryogenesis from immature cotyledons of open-pollinated seeds collected 5, 6, 7, and 8 weeks after anthesis and cultured on Driver and Kuniyuki walnut (DKW) medium was determined in this study. The percentage of embryogenic cotyledons and the number of embryos per embryogenic cotyledon were recorded at the end of the second subculture. The results showed that the highest percentage of embryogenesis ranged from 40% to 70% and the number of embryos per embryogenic cotyledon varied from 4.04 to 7.70 among the cultivars. Cotyledons collected 5, 6, and 7 weeks after anthesis were the most embryogenic.

Key Words: Chestnut, *Castanea sativa* Mill., somatic embryogenesis

Bazı Avrupa Kestane (*Castanea sativa* Mill.) Çeşitlerinde Olgunlaşmamış Kotiledonlardan Somatik Embriyogenesis

Özet: Bu çalışmada, dört Avrupa kestane (*Castanea sativa* Mill.) çeşidinde (Hacıibiş, Karamehmet, Osmanoğlu ve Sarıaşlama) tam çiçeklenmeden 5, 6, 7 ve 8 hafta sonra toplanan ve Driver ve Kuniyuki ceviz (DKW) ortamında kültüre alınan serbest tozlanmış tohumların olgunlaşmamış kotiledonlarında somatik embriyogenesis belirlenmiştir. Embriyogenik kotiledon yüzdesi ve embriyogenik kotiledon başına embriyo sayısı ikinci alt kültürün sonunda kaydedilmiştir. Sonuçlar, çeşitlere göre embriyogenesis oranının % 40-70 ve embriyogenik kotiledon başına embriyo sayısının 4.04-7.70 arasında değiştiğini göstermiştir. Tam çiçeklenmeden 5, 6 ve 7 hafta sonra toplanmış kotiledonlar çok embriyogenik olmuştur.

Anahtar Sözcükler: Kestane, *Castanea sativa* Mill., somatic embriyogenesis

Introduction

Chestnut trees are well suited for low input agroforestry systems and offer advantages as a profitable multiple use crop for producing nuts and timber. However, loss of trees, particularly due to fungal diseases such as ink disease caused by *Phytophthora* sp., and chestnut blight canker caused by *Cryphonectria parasitica* (Murr.) Barr., has created a worldwide shortage of chestnuts. Biotechnology offers a set of very valuable tools that can help meet the increasing demand for chestnuts and guarantee the sustainability of chestnut-based industries (Vieitez and Merkle, 2005). Somatic embryogenesis, which is a field of biotechnology, offers the advantages both for gene transfer and mass clonal propagation of elite chestnut genotypes (Xing et al., 1999; Robichaud et al., 2004) and hybrids. Induction of somatic embryogenesis on various media such as Murashige and Skoog (MS) (1962) medium (Gonzales et al., 1985; Vieitez et al., 1990; Piagnani and Eccher, 1990; Corredoira et al., 2003), woody plant medium (Lloyd and McCown, 1980) (WPM) (Merkle et al., 1991), Schenk and Hildebrandt (1972) medium (SH) (Leva et al., 1993) or Teasdale's (1992) P24 medium (Sauer, 1999) has been attempted with many different explant types

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such as embryonic axes, cotyledon pieces (Gonzales et al., 1985; Vieitez et al., 1990), ovules (Merkle et al., 1991; Xing et al., 1999), and leaf (Corredoira et al., 2003) from various species and hybrids of chestnut in previous studies (Vieitez and Merkle, 2005). However, results on percentage of embryogenesis were not high. Merkle et al. (1991) reported that only 7 cultures (1.75%) produced either direct somatic embryos or proembryogenic masses on variations of Lloyd and McCrown's (1980) WPM from over 400 ovules and embryos explanted from 60 nuts collected from American chestnut tree-1 at approximately 3, 6, and 9 weeks after anthesis. In the present study, we report somatic embryogenesis from immature cotyledons of open-pollinated seeds collected 5, 6, 7, and 8 weeks after anthesis and cultured on DKW medium in 4 European chestnut (*Castanea sativa* Mill.) cultivars.

Materials and Methods

Burs that had open-pollinated seeds were collected from 4 European chestnut (*C. sativa* Mill.) cultivars ('Hacıibiş', 'Karamehmet', 'Osmanoğlu', and 'Sarıaşlama') at weekly intervals beginning 5 weeks after anthesis and continuing until 8 weeks (July 16, July 23, July 30, and August 6, 2004, respectively). Burs were opened with a knife and seeds were taken out. They were sterilized by immersion in 3.75% (v/v) sodium hypochlorite for 25 min, followed by rinsing 3 times for 5 min in sterile distilled water. The seeds were opened and immature cotyledons as a whole and/or approximately pieces of 5 mm in diameter based on the developmental stage of cotyledons were removed under sterile conditions. These explants were placed in 100 x 10 mm petri dishes on the initial medium consisting of a DKW basal medium (Driver and Kuniyuki, 1984) containing 4.4 µM (1 mg l^{-1}) BAP, 9.3 µM (2 mg l^{-1}) kinetin, 0.05 µM (0.01 mg l^{-1}) IBA, and 1.7 mM (250 mg l^{-1}) L-glutamine (Tulecke and McGranahan, 1985). In our preliminary studies, we found that this medium greatly induced somatic embryo formation in chestnut cotyledon explants. The media were supplemented with 3% (w/v) sucrose and 0.21% Gelrite (w/v) (Merck Co.), and the pH was adjusted to 5.7 before autoclaving at 121 °C for 20 min. Cotyledon explants were cultured on the initial medium for 4 weeks and then subcultured twice for 4-week periods on a DKW basal medium without growth regulators and L-glutamine. All cultures were incubated at 25 °C in the dark. The number of cotyledon explants that formed somatic embryos and somatic embryos per embryogenic cotyledon was determined at the end of the second subculture. Each parameter for somatic embryogenesis consisted of 10 dishes with 5 cotyledon pieces per dish.

The data were analyzed using analysis of variance (ANOVA) at $P \leq 0.05$, 0.01, or 0.001 in Minitab software (MINITAB Inc.). The means were separated by Duncan's new multiple range test ($P \leq 0.05$). The percentage data were transformed into angle values prior to analysis.

Results and Discussion

As shown in Table 1, statistical analyses showed that the interaction between sampling dates and cultivars was not significant for the percentage of embryogenic immature cotyledons. In addition, there were no significant differences among the cultivars. However, differences among sampling dates were significant and the optimum cotyledon collection date for induction of somatic embryos was 5, 6, and 7 weeks after anthesis ($P = 0.000$) (Table 1). The percentages of cotyledons that formed embryos on these dates were 50.0%, 31.3%, and 47.9%, respectively. The interaction between sampling dates and cultivars was significant for the number of somatic embryos per cotyledon explant. The highest somatic embryo formation was observed in 'Osmanoğlu' and 'Sarıaşlama'. In 'Sarıaşlama', the percentage of embryogenesis was 70.0% and the number of embryos per embryogenic cotyledon collected 7 weeks after anthesis was 7.41. The same values in 'Osmanoğlu' were 60.0% and 7.70, respectively, which were obtained 5 weeks after anthesis (Table 1 and Figure 1b). Although the cotyledon explants were examined at the end of the second subculture (after 12 weeks cultured on the initial medium), the first embryos on cotyledon pieces were observed in the sixth week of cultures. Vietiez et al. (1990) reported that embryogenic cultures from immature seeds collected from *C. sativa* x *C. crenata* clones approximately 8-10 weeks postanthesis were induced on MS medium. Leva et al. (1993) cultured cotyledon explants of *C. sativa* on semi-solid SH medium and found that the best stage for sampling explants was 50 days (approximately 7 weeks) after full blooming.

Table 1. The percentages of embryogenic immature cotyledons and number of somatic embryos per cotyledon explant in 4 European chestnut genotypes, *C. sativa* Mill.

Cultivar	Sampling date	Cotyledon explants with somatic embryos (%)	No. of somatic embryos / embryogenic cotyledon explant		
Hacıbiş	5	50.0	3.29 ± 0.82	ab[b]	BC[b]
	6	40.0	2.79 ± 0.35	ab	A
	7	48.9	4.06 ± 1.34	a	B
	8	18.0	1.67 ± 0.33	b	A
Karamehmet	5	40.0	3.74 ± 0.51	a	B
	6	37.1	4.04 ± 1.30	a	A
	7	48.9	2.49 ± 0.36	ab	B
	8	17.8	1.40 ± 0.19	b	A
Osmanoğlu	5	60.0	7.70 ± 1.23	a	A
	6	40.0	5.03 ± 1.78	b	A
	7	24.0	4.43 ± 1.04	b	B
	8	6.0	1.00 ± 0.00	c	A
Sarıaşlama	5	50.0	1.30 ± 0.24	c	C
	6	48.0	4.98 ± 0.53	b	A
	7	70.0	7.41 ± 1.13	a	A
	8	40.0	3.26 ± 0.76	bc	A
Mean	5	50.0 a[a]			
	6	31.3 ab			
	7	47.9 ab			
	8	20.4 b			
Cultivar		ns[c] (P = 0.076)	ns (P = 0.081)		
Sampling date		*** (P = 0.000)	** (P = 0.006)		
Cultivar x sampling date		ns (P = 0.420)	** (P = 0.006)		

[a] Mean separations within a column followed by the same letter are not significantly different at P ≤ 0.05 by Duncan's multiple range test.

[b] Different small letters within the same column indicate significant differences among sampling dates in each cultivar, different capital letters within the same column show significant differences among cultivars in each sampling date by Duncan's multiple range test, P ≤ 0.05.

[c] ns, *, **, ***Nonsignificant or significant at P ≤ 0.05, 0.01, or 0.001, respectively.

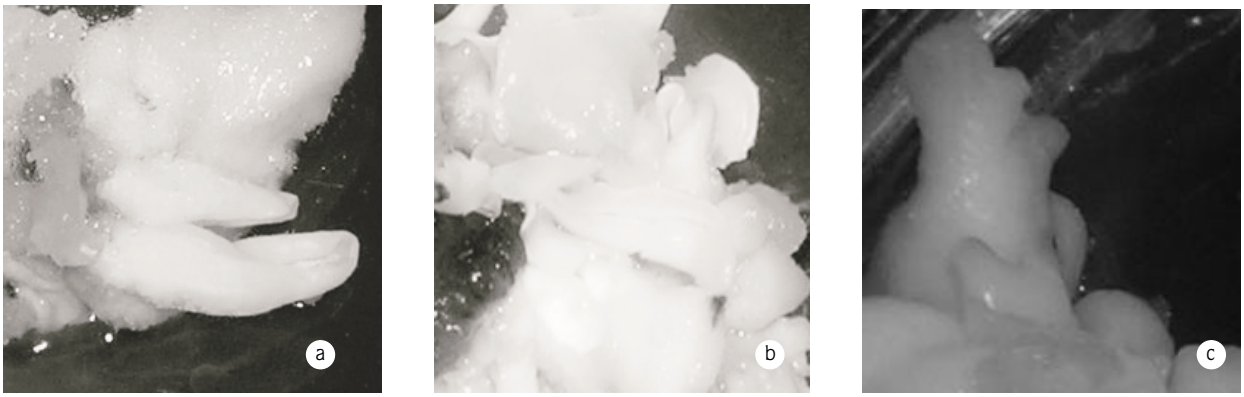

Figure 1. (a) Somatic embryogenesis from an immature cotyledon explant collected 5 weeks after anthesis in European chestnut, *C. sativa* Mill., (b) Somatic embryos of 'Osmanoğlu' cultivar, (c), Somatic embryos of 'Karamehmet' cultivar.

This finding for the developmental stage of explants is in agreement with our results.

In addition to differences in species, there is the possibility that medium x growth regulator interactions could account for the differences in response on somatic embryogenesis (Merkle et al., 1991). We used DKW basal medium, which was not used previously in somatic embryogenesis of chestnut genotypes. The results showed that DKW medium was highly inductive on somatic embryogenesis in chestnut cotyledons (Table 1 and Figure 1). The effects of basal media and various supplements on somatic embryogenesis are currently being investigated in our laboratory.

Conclusions

The highest induction frequencies of somatic embryogenesis from immature cotyledons were obtained 5-7 weeks after anthesis in chestnut cultivars. Driver and Kuniyuki (1984) medium (DKW) could be used for studies on somatic embryogenesis from immature cotyledons in chestnuts.

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