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Somatic Embryogenesis from Immature Cotyledons of Apomictic and Non-Apomictic Seeds in Walnut (*Juglans regia* L.)

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Abstract: Somatic embryogenesis from immature cotyledons of apomictic and open-pollinated seeds in some walnut (*Juglans regia* L.) genotypes was investigated. To obtain apomictic seeds, female flowers were bagged and/or pollinated with pollen of the apple cv. 'Golden Delicious' (*Malus* x *domestica* Borkh.). The best cotyledon stage for somatic embryogenesis was determined in open-pollinated seeds of 10 walnut genotypes. Immature cotyledons were cultured 8, 9, 10, 11 and 12 weeks after anthesis. As a result of this experiment, cotyledons of seeds thought to be of apomictic origin were cultured 8 weeks after anthesis. Driver and Kuniyuki walnut (DKW) medium supplemented with 1 mg l^{-1} 6-benzylaminopurine (BAP), 2 mg l^{-1} kinetin, 0.01 mg l^{-1} indole-3-butyric acid (IBA) and 250 mg l^{-1} L-glutamine was used in initial cultures. Explants were transferred to DKW medium without growth regulators and L-glutamine in subcultures. The percentage of embryogenic cotyledons that originated from apomictic and non-apomictic seeds ranged from 3.6% to 25% and the number of embryos per cotyledon ranged from 1 to 9.7 at the end of the fourth subculture. A repetitively embryogenic embryo line originating from immature cotyledons of apomictic seeds of the Tokat-1 walnut genotype was maintained by secondary embryogenesis.

Key Words: Walnut, *Juglans regia* L., somatic embryogenesis, immature cotyledon, apomictic seed

Cevizde (*Juglans regia* L.) Apomiktik ve Apomiktik Olmayan Tohumların Olgunlaşmamış Kotiledonlarından Somatik Embriyogenesis

Özet: Bazı ceviz (*Juglans regia* L.) genotiplerinde apomiktik ve açıkta tozlanmış tohumların olgunlaşmamış kotiledonlarından somatik embriyogenesis araştırılmıştır. Apomiktik tohumları elde etmek için dişi çiçekler keselenmiş ve/veya 'Golden Delicious' (*Malus* x *domestica* Borkh.) elma çiçek tozları ile tozlanmıştır. Somatik embriyogenesis için en iyi kotiledon aşaması, on ceviz genotipinin açıkta tozlanmış tohumlarında belirlenmiştir. Olgunlaşmamış kotiledonlar tam çiçeklenmeden 8, 9, 10, 11 ve 12 hafta sonra kültüre alınmıştır. Bu denemenin sonucuna göre apomiktik orijinli olduğu düşünülen tohumların kotiledonları tam çiçeklenmeden 8 hafta sonra kültüre alınmıştır. Başlangıç kültürlerinde 1 mg l^{-1} 6-benzilaminopürin (BAP), 2 mg l^{-1} kinetin, 0.01 mg l^{-1} indol-3-bütirik asit (IBA) ve 250 mg l^{-1} L-glutamin ile desteklenmiş Driver ve Kuniyuki ceviz (DKW) ortamı kullanılmıştır. Eksplantlar alt kültürlerde büyümeyi düzenleyici maddeler ve L-glutamin bulunmayan DKW ortamına transfer edilmiştir. Dördüncü alt kültürün sonunda apomiktik ve apomiktik olmayan tohumlardan orijinini almış embriyogenik kotiledonların oranı %3.6'dan %25'e kadar değişmiştir ve kotiledon başına embriyo sayısı 1'den 9.7'ye değişmiştir. Somatik embriyogenesis ile 'Tokat-1' ceviz genotipinin apomiktik tohumlarının olgunlaşmamış kotiledonlarından orijinini almış tekrar eden bir embriyogenik embriyo hattı sağlanmıştır.

Anahtar Sözcükler: Ceviz, *Juglans regia* L., somatik embriyogenesis, olgunlaşmamış kotiledon, apomiktik tohum

Introduction

Although somatic embryogenesis from zygotic tissues of open-pollinated seeds has been successful in members of the Juglandaceae (Tulecke and McGranahan, 1985; Cornu, 1988; Long et al., 1992, 1995; Neuman et al., 1993; Pijut, 1993; Tang et al., 2000; Dandekar et al., 2005), the use of nonzygotic tissues as a source of somatic embryos is advantageous to avoid meiosis. Apomixis, embryo sacs and embryos formed without meiosis or fertilization in ovules (Koltunow, 1993), is a vegetative propagation method by seed. The percentage of apomictic nut was generally below 17.3% in the Juglandaceae (Badalov, 1989; Valdiviesso, 1990; Terziiski and Stefanova, 1990; Gao et al., 1999; Zhang

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et al., 2000). This amount was very poor for direct seed production in walnut. Although the percentage of apomixis was reportedly very high and ranged from 23.5% to 81.2% in some genotypes (Loiko, 1990; Solar et al., 1995), apomictic nuts could always not be obtained. However, somatic embryogenesis from immature cotyledons of a few apomictic seeds could provide the possibility of mass clonal propagation of cultivars in walnut species that are difficult to use in in vitro techniques such as shoot tip culture.

The objective of the present study was to obtain repetitively embryogenic embryo lines originating from immature cotyledons of seeds thought to be of apomictic origin in Turkish walnut genotypes (*J. regia* L.).

Materials and Methods

Apomixis experiments

The female flowers were bagged approximately a week before pistillate blooming in 10 Turkish walnut (*J. regia* L.) genotypes (Yalova-1, Şebin, Bilecik, KR-1, KR-2, Şen-2, 07-KOR-1, Tokat-1, Kaman-1 and Kaman-5) in 2001 and 2002. In addition, pistils of 4 genotypes (Bilecik, 07-KOR-1, Tokat-1 and Kaman-1) that had apomictic seeds in 2001 and/or 2002, were pollinated with pollen of the apple cv. 'Golden Delicious' (*Malus* x *domestica* Borkh.) to stimulate an apomictic fruit set in 2003. In the apomixis experiments, female flowers were up to about 200 on 3 trees for each genotype and each year. The pollination bags were removed 2 weeks later and the formation of apomictic seeds and fruit set in open-pollinated flowers was determined 8 weeks after anthesis.

Somatic embryogenesis experiments

To determine the best cotyledon stage for somatic embryogenesis, immature fruits of 10 open-pollinated genotypes were collected at weekly intervals beginning 8 weeks after anthesis and continuing until 12 weeks. As the result of this experiment, fruits thought to be apomictic origin were only collected 8 weeks after anthesis. Fruits were sterilized by immersion for 25 min in 3.75% (v/v) sodium hypochlorite, followed by three 5-min rinses in sterile distilled water. The fruits were opened and approximately 5 mm pieces of cotyledon 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 1 mg l^{-1} BAP, 2 mg l^{-1} kinetin, 0.01 mg l^{-1} IBA and 250 mg l^{-1} L-glutamine (Tulecke and McGranahan, 1985). All 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 3 weeks and then subcultured at 4-week intervals 4 times on a DKW basal medium without growth regulators and L-glutamine. The numbers of cotyledon explants that formed somatic embryos and somatic embryos from an explant were determined at the end of each subculture. Proliferated embryos were removed from cotyledons and subcultured every 8-10 days on a DKW basal medium without growth regulators and L-glutamine for secondary (repetitive) embryogenesis. All cultures were incubated at 25 °C in the dark. Each parameter for somatic embryogenesis and secondary embryogenesis consisted of 5 dishes with 10 embryos per dish. All experiments were repeated twice.

Statistical analysis

Data were analyzed using ANOVA by Minitab software (MINITAB Inc.) in accordance with an F-test ($P = 0.05$) and means were compared by Duncan's new multiple range test ($P \leq 0.05$). Transformed angle values were used for percentage data.

Results and Discussion

As shown in Table 1, the results of somatic embryogenesis from the immature cotyledons of open-pollinated seeds of 10 walnut cultivars during 2001 and 2002 indicated that generally the optimum cotyledon collection time for induction of somatic embryos was 8 weeks after anthesis. At this stage, the percent of embryogenic cotyledons ranged from 6.7% to 17.0% (Table 1) and the number of embryo per cotyledon explant varied from 1.7 to 9.7 (Table 2) among the genotypes. In addition, somatic embryos from cotyledon explants formed 9-12 weeks after anthesis in some genotypes. Our results on the optimum cotyledon development stage for somatic embryogenesis are consistent with other studies on *Juglans* spp. Tulecke and McGranahan (1985) reported that the optimum stage of cotyledon development for the induction of somatic embryos was 6-11 weeks after pollination, the cotyledon pieces that were embryogenic ranged from 10% to 44%,

Table 1. The percentages of embryogenic immature cotyledons from open-pollinated seeds in 10 walnut genotypes, *J. regia* L. Cotyledon explants were cultured on the initial medium consisting of DKW basal medium containing 1 mg l^{-1} BAP, 2 mg l^{-1} kinetin, 0.01 mg l^{-1} IBA and 250 mg l^{-1} L-glutamine (Tulecke and McGranahan, 1985) for 3 weeks and then subcultured at 4-week intervals 4 times on a DKW basal medium without growth regulators and L-glutamine.

Weeks after anthesis	No. of subculture							
	1	2	3	4	1	2	3	4
	Cotyledon explants with somatic embryos (%)							
	YALOVA-1				SEN-2			
8	0	1.4 a	5.7 a	8.6 a	3.5 a	7.1 a	12.9 a	15.3 a
9	0	0 b	0 b	0 b	0 b	0 b	3.2 b	3.2 b
10	0	0 b	0 b	0 b	0 b	0 b	0 b	0 b
11	0	0 b	0 b	0 b	0 b	0 b	3.5 b	2.4 b
12	0	0 b	0 b	0 b	0 b	0 b	0 b	0 b
	SEBIN				07-KOR-1			
8	0	0	5.3 a	9.5 a	4.0 a	8.0 a	4.0 a	8.0 a
9	0	0	1.0 b	1.0 b	0 b	0 b	1.1 b	0 b
10	0	0	0 b	0 b	0 b	0 b	0 b	0 b
11	0	0	0 b	0 b	0 b	0 b	0 b	0 b
12	0	0	0 b	0 b	0 b	0 b	0 b	0 b
	BILECIK				TOKAT-1			
8	6.7 a	4.4 a	10.0 a	8.9 a	2.2 a	3.3 a	6.7 a	6.7 a
9	0 b	0 b	0 b	0 b	1.1 a	0 b	1.1 b	4.2 ab
10	0 b	0 b	0 b	0 b	0 a	0 b	0 b	0 b
11	1.1 b	1.1 ab	0 b	0 b	0 a	0 b	0 b	0 b
12	0 b	0 b	0 b	0 b	0 a	0 b	0 b	0 b
	KR-1				KAMAN-1			
8	3.2 a	0 b	15.8 a	15.8 a	4.3 a	6.4 a	11.4 a	10.0 a
9	0 b	1.3 a	0 b	0 b	2.4 ab	2.4 b	8.2 a	5.9 a
10	0 b	0 b	0 b	0 b	0 b	0 b	0 b	0 b
11	0 b	0 b	0 b	0 b	0 b	0 b	0 b	0 b
12	0 b	0 b	0 b	0 b	0 b	0 b	0 b	0 b
	KR-2				KAMAN-5			
8	4.0 a	1.3 a	9.3 a	6.7 a	6.0 a	13.0 a	17.0 a	12.0 a
9	4.3 a	4.3 a	4.3 ab	4.3 ab	1.0 b	0 b	0 b	1.0 b
10	0 a	0 a	0 b	0 b	0 b	0 b	0 b	3.2 b
11	2.2 a	2.2 a	0 b	2.2 ab	0 b	0 b	0 b	0 b
12	0 a	0 a	1.1 b	1.1 b	0 b	0 b	0 b	0 b

Within each column of genotypes, values followed by the same letter are not significantly different at P = 0.05 level according to Duncan's new multiple range test.

Table 2. Number of somatic embryo per cotyledons explant from open-pollinated seeds in ten walnut genotypes, *J. regia* L. See the legend of Table 1 for the culture conditions.

Weeks after anthesis	No. of subculture							
	1	2	3	4	1	2	3	4
	No. of somatic embryos / embryogenic cotyledon explant							
	YALOVA-1				SEN-2			
8	-	2.0	1.2	1.5	1.7	1.0	1.4	1.6
9	-	-	-	-	-	-	1.5	2.0
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	1.2	1.0
12	-	-	-	-	-	-	-	-
	SEBIN				07-KOR-1			
8	-	-	2.0	1.7	2.3	1.3	1.7	1.3
9	-	-	1.0	2.0	-	-	2.0	-
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
	BILECIK				TOKAT-1			
8	2.7	1.3	2.2	2.0	2.5	2.3	4.5	9.7
9	-	-	-	-	2.0	-	2.0	4.2
10	-	-	-	-	-	-	-	-
11	2.0	1.0	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
	KR-1				KAMAN-1			
8	4.3	-	3.5	4.5	1.7	1.0	3.0	2.7
9	-	2.0	-	-	1.0	2.0	2.8	2.8
10	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
	KR-2				KAMAN-5			
8	3.5	1.0	1.4	2.0	3.2	2.8	3.0	1.6
9	2.5	1.3	3.7	2.0	1.0	-	-	1.0
10	-	-	-	-	-	-	-	1.2
11	1.0	1.5	-	1.5	-	-	-	-
12	-	-	4.0	1.0	-	-	-	-

and the number produced per cotyledon piece varied from 0 to 26. Pijut (1993) suggested that the highest frequency (42%-56%) of cotyledon stage somatic embryo development of butternut (*J. cinerea* L.) was found in explants collected 9 or 8 weeks postanthesis. According to Neuman et al. (1993), the greatest percentage of cultures with somatic embryos (78%-80%) and the highest number of individual somatic embryos (12-13) of Eastern black walnut (*J. nigra* L.) were observed 10 weeks after anthesis on media containing the lowest concentration of 2,4-D (0.02 mg l^{-1} or 0.2 mg l^{-1}) and the highest concentrations of TDZ (0.1 mg l^{-1} or 1.1 mg l^{-1}). Long et al. (1995) reported that the maximum embryogenesis of *J. nigra* L. occurred 12 weeks after anthesis.

In our somatic embryogenesis studies, explants from immature cotyledons of seeds thought to be of apomictic origin of 4 genotypes were cultured 8 weeks after anthesis according to the results of the previous experiment. The percentage of cotyledons that formed embryos ranged from 3.6% to 25% and the number of somatic embryos per embryogenic cotyledon explant from apomictic seeds was 1 or 2 (Table 3) (Figure 1A). The frequency of somatic embryos producing somatic embryos from immature cotyledons of apomictic and non-apomictic seeds ranged from 76% to 93%, with the mean number of embryos per explant ranging from 4.4 to 7.7, both parameters being significantly influenced by the genotypes (Table 4). A repetitively embryogenic embryo line originating from immature cotyledons of seeds thought to be of apomictic origin of the Tokat-1 walnut genotype was maintained by secondary embryogenesis (Figure 1B). The repetitively embryogenic embryo lines arose from immature cotyledons of open-pollinated seeds of other Turkish walnut genotypes.

Table 3. Somatic embryogenesis from the immature cotyledons of seeds thought to be apomictic in 4 walnut genotypes, *J. regia* L. Cotyledon explants were cultured 8 weeks after anthesis on the initial medium consisting of DKW basal medium containing 1 mg l^{-1} BAP, 2 mg l^{-1} kinetin, 0.01 mg l^{-1} IBA and 250 mg l^{-1} L-glutamine (Tulecke and McGranahan, 1985) for 3 weeks and then subcultured at 4-week intervals 4 times on a DKW basal medium without growth regulators and L-glutamine.

Weeks after anthesis	Cotyledon explants with somatic embryos (%)				No. of subculture	No. of somatic embryos / embryogenic cotyledon explant			
	1	2	3	4		1	2	3	4
					2001				
BILECIK	-	-	-	-		-	-	-	-
07-KOR-1	0	0	20	0		-	-	1	-
TOKAT-1	-	-	-	-		-	-	-	-
KAMAN-1	-	-	-	-		-	-	-	-
					2002				
BILECIK	0	0	0	0		-	-	-	-
07-KOR-1	-	-	-	-		-	-	-	-
TOKAT-1	0	0	20	0		-	-	1	-
KAMAN-1	0	0	0	25		-	-	-	1
					2003				
BILECIK	0	0	0	0		-	-	-	-
07-KOR-1	0	0	0	0		-	-	-	-
TOKAT-1	0	0	0	3.6		-	-	-	2
KAMAN-1	0	0	0	0		-	-	-	-

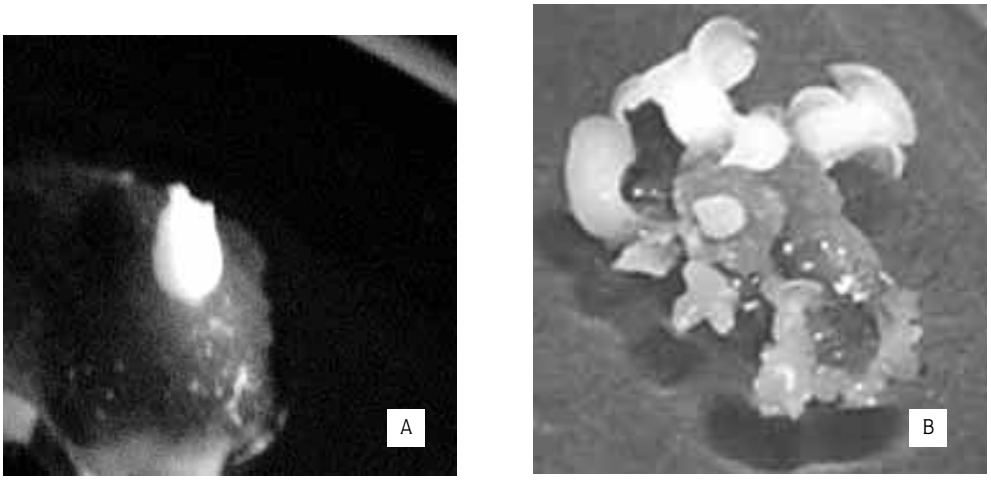

Figure 1. Somatic embryos originating from immature cotyledons of seed thought to be apomictic in walnut, *J. regia* L. (Tokat-1) (A) Somatic embryogenesis from an immature cotyledon explant. The explant was firstly cultured on the initial medium consisting of DKW containing 1 mg l^{-1} BAP, 2 mg l^{-1} kinetin, 0.01 mg l^{-1} IBA and 250 mg l^{-1} L-glutamine (Tulecke and McGranahan, 1985) for 3 weeks and then subcultured on a DKW basal medium without growth regulators and L-glutamine for 4 weeks at 25 °C in the dark. (B) Secondary embryogenesis from a somatic embryo explant. Proliferated embryos were removed from the cotyledon and subcultured every 8-10 days on a DKW basal medium without growth regulators and L-glutamine for secondary (repetitive) embryogenesis at 25 °C in the dark.

Table 4. Secondary embryogenesis from somatic embryos originating from seeds thought to be apomictic and non-apomictic in walnut genotypes, *J. regia* L. Somatic embryo explants were cultured on DKW basal medium without growth regulators and L-glutamine for 3 weeks.

Genotype	Somatic embryo explants with secondary embryos (%)	No. of secondary embryos / embryogenic somatic embryo explant
YALOVA-1	77 b	4.4 d
SEBIN	86 b	7.1 ab
BILECIK	76 b	6.2 bc
KR-1	85 b	4.7 d
KR-2	83 b	4.7 d
07-KOR-1	93 a	7.7 a
TOKAT-1	80 b	4.7 d
TOKAT-1 (apomictic)	77 b	4.6 d
KAMAN-1	86 b	5.2 cd
KAMAN-5	83 b	5.4 cd

Within each column, values followed by the same letter are not significantly different at P = 0.05 level according to Duncan's new multiple range test.

Conclusions

Somatic embryogenesis from immature cotyledons of a few seeds thought to be of apomictic origin could provide the possibility of mass clonal propagation of walnut (*J. regia*). The embryogenic embryo lines obtained from immature cotyledons of seeds produced by open-pollinated and non-pollinated pistils with walnut pollen in this study could be useful in further biotechnological research on walnut breeding.

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