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High Efficiency Somatic Embryogenesis from Immature Zygotic Embryos of Grapevine: The Effect of Genotype, Media, 2,4-D, and Incubation Conditions

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Abstract: Immature zygotic embryos of 41 B grape rootstock (*V. vinifera* L. cv. 'Chasselas' × *V. berlandieri*) and Yalova İncisi (*V. vinifera* L.) were cultured on MS, NN, and B5 media supplemented with 0.5, 1, 2, and 4 mg l^{-1} 2,4-D at 16/8 h light/dark photoperiod and complete darkness. For 41 B rootstock, the highest somatic embryo formation was obtained from dark condition on B5 media containing 0.5 and 1 mg l^{-1} 2,4-D with the rates of 30% and 28.9%, respectively. Yalova İncisi zygotic embryos produced 5% somatic embryos only at 16/8 h light/dark photoperiod of MS supplemented with 1 mg l^{-1} 2,4-D, and also in 16/8 h light/dark photoperiod and complete darkness somatic embryos were produced at the rates of 6.3% and 2.3%, respectively, in cultures of NN containing 0.5 mg l^{-1} 2,4-D. After 8 months of culture, 559 embryos at torpedo stage were identified on B5 + 1 mg l^{-1} 2,4-D + dark cultures of 41 B and 912 embryos at torpedo stage were identified on NN + 0.5 mg l^{-1} 2,4-D + light cultures of Yalova İncisi. The highest germination and plantlet conversion rates were obtained from 41 B on free NN medium (58% and 75%, respectively) and from Yalova İncisi on free MS medium (77.4% and 45%, respectively). Of the regenerated somatic plantlets, 91.9% were successfully transferred to soil.

Key Words: Grapevine, zygotic embryo, somatic embryo, regeneration

Asmada Olgunlaşmamış Zigotik Embriyolardan Yüksek Oranda Somatik Embriyo Oluşumu: Genotip, Besi Ortamı, 2,4-D ve İnkübasyon Koşullarının Etkisi

Özet: 41 B Amerikan asma anacı (*V. vinifera* L. cv. 'Chasselas' × *V. berlandieri*) ve Yalova İncisi üzüm çeşidinin (*V. vinifera* L.) olgunlaşmamış zigotik embriyo eksplantları, içerisinde 0.5, 1, 2 ve 4 mg l^{-1} 2,4-D bulunan MS, NN ve B5 besi ortamlarında, fotoperyodu 16/8 saat ışık/karanlık ve tamamen karanlık koşullarda kültüre alınmıştır. 41 B anacında en yüksek oranda somatik embriyo oluşumu karanlık koşullardaki 0.5 ve 1 mg l^{-1} 2,4-D içeren B5 ortamlarında sırasıyla % 30 ve % 28.9 oranında saptanmıştır. Yalova İncisi zigotik embriyoları yalnız 1 mg l^{-1} 2,4-D içeren MS ortamının fotoperyodu 16/8 saat ışık/karanlık kültürlerinde % 5; 0.5 mg l^{-1} 2,4-D içeren NN ortamının fotoperyodu 16/8 saat ışık/karanlık ve tamamen karanlık kültürlerinde ise sırasıyla % 6.3 ve % 2.3 oranlarında somatik embriyo oluşturmuştur. Sekiz aylık kültür süresi sonunda 41 B'nin B5+1 mg l^{-1} 2,4-D+karanlık kültürlerinde 559 adet, Yalova İncisi'nin NN+0.5 mg l^{-1} 2,4-D+aydınlık kültürlerinde ise 912 adet embriyonun torpedo evresinde olduğu belirlenmiştir. En yüksek çimlenme ve tam bitkiye dönüşüm oranı hormonsuz NN ortamında kültüre alınan 41 B' den (sırasıyla % 58 ve % 75); Yalova İncisi'nde ise hormonsuz MS ortamında (sırasıyla % 77.4 ve % 45) elde edilmiştir. Rejenerasyon gösteren somatik bitkiciklerin % 91.9' u başarıyla toprağa aktarılmıştır.

Anahtar Sözcükler: Asma, zigotik embriyo, somatik embriyo, rejenerasyon

Abbreviations: MS, Murashige and Skoog medium; NN, Nitsch and Nitsch medium; B5, Gamborg medium; 2,4-D, 2,4-Dichlorophenoxy acetic acid

Introduction

Grapevine (*Vitis* spp.) is one of the most important fruit crops worldwide. Many table, wine and raisin grape varieties are grown commercially. However, there is always a need to new breed cultivars. Genetic improvement of grape through conventional breeding is

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severely limited by several factors such as long pre-bearing period, polyploidy, and the highly heterozygous nature of existing cultivars (Jayasankar et al., 1999). Plant biotechnology could be an attractive means for improving grape (Kuksova et al., 1997); however, this would be possible only if there is an effective regeneration system from somatic tissues of mature plants. In vitro techniques have been known as a convenient tool for vine breeding to overcome the difficulties in conventional breeding studies and obtaining new cultivars (Maitz et al., 2000).

One of these techniques is somatic embryogenesis. Plant regeneration via somatic embryogenesis using nucellar tissue of *Vitis vinifera* L. cv 'Cabernet Sauvignon' on liquid media is the first reported in grape (Mullins and Srinivasan, 1976). Later on somatic embryogenesis technique was significantly improved in several genotypes and from various explants. Thereafter stem segments of Seyval, a French hybrid grape (Krul and Worley, 1977), leaves and petiole of *V. rupestris* and *V. rotundifolia* (Martinelli et al., 1993; Robacker, 1993), zygotic embryos of *V. rupestris* and *V. vinifera* (Emershad and Ramming, 1994), tendrils of *Vitis vinifera* L. cv Thompson, Sonaka and Tas-e ganesh (Salunkhe et al., 1997), stigma-style culture of different grapevine cultivars (*Vitis vinifera* L.: cvs. 'Bombino Nero', 'Greco di Tufo', 'Merlot', and 'Sangiovese') (Carimi et al., 2005), low frequency somatic embryos were obtained.

In this paper we describe procedures for somatic embryo initiation, obtaining high frequency torpedo shape somatic embryo formation and their germination from the immature zygotic embryo explants of 2 economically important grapevine genotypes.

Materials and Methods

In this study, immature zygotic embryo explants of 41 B American rootstock (*V. vinifera* L. cv. 'Chasselas' × *V. berlandieri)* and Yalova İncisi (*V. vinifera* L.) grape cultivar were used.

The immature zygotic embryo explants were isolated under aseptic conditions from the ovules of unripe berries of 2 genotypes that were collected from the experimental vineyard of Faculty of Agriculture, Çukurova University, Adana, Turkey. Unripe berries were collected 5 to 7 weeks after flowering (Emershad and Ramming, 1994; Tangolar et al., 1998) and used after surface sterilization with 1.5% sodium hypochlorite for 1 h. Then the immature embryos were cultured on MS (Murashige and Skoog, 1962), NN (Nitsch and Nitsch, 1969), and B5 medium (Gamborg et al., 1968) containing 0.5, 1, 2, and 4 mg l^{-1} 2,4-D and incubated in 16/8 h light/dark photoperiod and complete darkness. All media containing 3% sucrose were solidified with 0.8% agar and pH was adjusted to the 5.8 prior to autoclaving at 105 kPa and 121 °C for 15 min.

Calli obtained from immature zygotic embryos on all media were sub-cultured at 3-month intervals. At the end of the second three-month culture period, formation of the embryos at different stages on calli obtained from zygotic embryos were observed.

Somatic embryos with torpedo shape were cultured on the same media with no hormone for germination in light conditions. Globular and heart shaped somatic embryos were cultured on $^1/_2$ MS medium for development of torpedo shape embryos in 16/8 h light/dark photoperiod and complete dark conditions. Then, 1 to 3 mm long white somatic embryos isolated from embryogenic cultures of genotypes were transferred to hormone free solid MS, NN, and B5, individually. Somatic embryos were incubated in 16/8 h light/dark photoperiod at 25 ± 1 °C, and after shoot and root development (in 3 to 4 weeks), the plantlets were transferred to individual test tubes containing hormone free media. Within 6 weeks, plantlets were transplanted to the pots containing sterile soil. Plantlets were initially covered with plastic during the hardening off period. After 2 weeks, plants were transferred to the greenhouse at 24 to 28 °C, and under natural light.

The observations were carried out until the end of 8th month. The rate (%) of callus and embryo formation and the numbers of torpedo shaped embryos were recorded. Germination and plantlet rates (%) were also determined.

Somatic embryo formation and germination trials were performed with 10 replications comprising 5 explants each. Mean values were given with standard errors.

Results

41 B American Rootstock

In the explants cultured on 3 medium with all 2,4-D concentrations, calli formation rates varied between 90%

and 100% both at 16/8 h light/dark photoperiod and dark conditions. All callus obtained (100%) were embryogenic. In dark conditions, the highest embryo formation rates were 30% and 28.9% for B5 media containing 0.5 and 1 mg l^{-1} 2,4-D, respectively. The highest number of torpedo shaped embryos was found in dark conditions with 1 mg l^{-1} 2,4-D (559 n explants^{-1}) on the same medium (Table 1 and Figure 1a).

After somatic embryos had been obtained from different media, they were cultured on the same media with no hormone and different germination rates were recorded. Somatic embryos with torpedo shape germinated on NN, MS, and B5 media at the rate of 58.0%, 53.6%, and 51.8%, respectively. The whole plant formation rates were 75.0% on MS and NN media and 60.7% on B5 media (Table 2). Nearly all of the regenerated somatic plantlets (91.9%) were successfully transferred to soil (Table 3 and Figure 1 b).

Yalova İncisi Grapevine Cultivar

The results presented in Table 4 indicate that the callus formation rates obtained on MS, NN, and B5 media supplemented with 4 mg l^{-1} 2,4-D at 16/8 h light/dark photoperiod and dark conditions were lower compared to the other 2,4-D concentrations. Rates of calli formation were found to be 90% on MS, 98% on NN, and more than 71.5% on B5 medium. All calli obtained (100%) were found to be embryogenic.

Embryo formation was observed only on MS medium containing 1 mg l^{-1} 2,4-D at 16/8 h light/dark photoperiod conditions, and on NN medium containing 0.5 mg l^{-1} 2,4-D at 16/8 h light/dark photoperiod and dark conditions. Embryo formation was determined to be 5% on MS medium supplemented with 1 mg l^{-1} 2,4-D, and 6.3% on NN medium supplemented with 0.5 mg l^{-1} 2,4-D at 16/8 h light/dark photoperiod and 2.3% in dark conditions (Table 4).

Table 1. Callus, embryo formation rates, and number of torpedo shaped embryos obtained from immature zygotic embryos of 41 B rootstock*

Medium	2,4 D mg l^{-1}	Light regime	Callus formation (%)	Embryo formation (%)	Torpedo shaped embryo (n explant^{-1})
MS	0.5	Light	96.5 ± 1.9	9.4 ± 4.1	8 ± 0
		Dark	90.0 ± 3.1	22.7 ± 4.7	186 ± 64
	1	Light	94.7 ± 2.5	3.5 ± 1.9	24 ± 2
		Dark	92.0 ± 3.8	21.5 ± 6.2	90 ± 42
	2	Light	96.7 ± 1.7	3.8 ± 2.7	8 ± 0
		Dark	90.8 ± 4.8	12.0 ± 3.8	157 ± 71
	4	Light	90.5 ± 3.6	2.4 ± 1.6	8 ± 2
		Dark	96.0 ± 2.9	6.7 ± 3.2	169 ± 75
NN	0.5	Light	94.7 ± 2.4	16.7 ± 4.9	46 ± 30
		Dark	91.2 ± 3.6	20.0 ± 5.0	370 ± 110
	1	Light	91.9 ± 4.0	7.7 ± 3.8	86 ± 43
		Dark	100	24.3 ± 6.4	166 ± 50
	2	Light	98.7 ± 1.3	8.9 ± 3.7	153 ± 59
		Dark	100	25.5 ± 6.1	314 ± 173
	4	Light	95.4 ± 2.4	6.7 ± 2.5	162 ± 82
		Dark	90.9 ± 3.1	8.0 ± 3.3	135 ± 26
B5	0.5	Light	97.6 ± 1.6	1.1 ± 1.1	0
		Dark	100	30.0 ± 5.4	98 ± 51
	1	Light	100	5.0 ± 2.3	203 ± 16
		Dark	95.7 ± 2.3	28.9 ± 5.4	559 ± 230
	2	Light	100	2.1 ± 1.5	50 ± 4
		Dark	100	23.2 ± 4.1	253 ± 66
	4	Light	96.3 ± 2.7	0	0
		Dark	98.8 ± 1.2	5.0 ± 2.9	385 ± 172

* Callus and embryo formation were calculated from 50 explants for each variant (10 replications with 5 explants for each). The number of different stages of embryos was counted from the explants which produced embryos. Light means: 16/8 h light/dark photoperiod, dark means complete darkness.

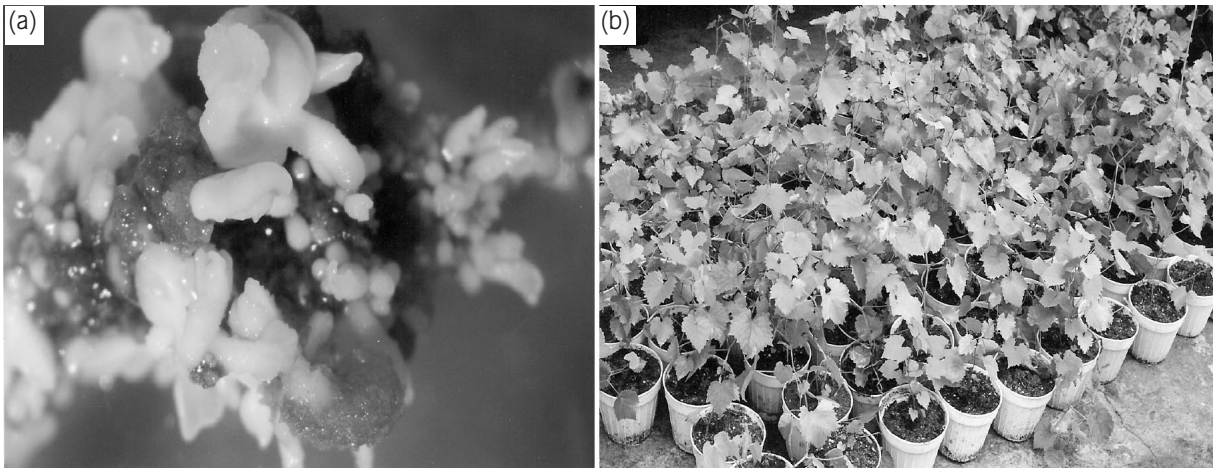

Figure 1. Various developmental stages during plant regeneration from immature zygotic embryos (a) different stages of somatic embryos on callus (b) transplanted plants to the pots.

Table 2. Germination and plantlet formation rates of 41 B rootstock somatic embryos cultured on MS, NN, and B5 hormone free media.

Media	Germination rate (%)	Plantlet formation rate (%)
MS	53.6 ± 3.4	75.0 ± 24.9
NN	58.0 ± 7.6	75.0 ± 24.9
B5	51.8 ± 18.5	60.7 ± 10.7

Table 3. Acclimation of plantlets formed through germination of somatic embryo obtained from 41B rootstocks (after 30 days).

Plantlet numbers transferred to soil (n)	Survived plants numbers (n)	Survival rate (%)
99	91	91.9

A total of 981 and 912 torpedo shaped embryos were determined on MS, NN medium at 16/8 h light/dark photoperiod, respectively, but only 108 torpedo shaped embryos were detected on NN medium in dark conditions (Table 4).

When torpedo shaped somatic embryos obtained from Yalova İncisi grape cultivar were transferred to the same media with no hormone, germination rates and plantlet rates were 77.4% and 45.0% on MS, 74.3% and 38.5% on NN medium, respectively (Table 5).

Discussion

In this study a protocol was developed for the initiation and development of somatic embryos from immature zygotic embryo explants of 2 grapevine genotypes. Yalova İncisi is of great importance as a table grape variety and 41 B, as a rootstock, is resistant to calcareous soil. Although plant regeneration has been reported previously in *Vitis* sp., the rate of regeneration has been very low. In this study, high frequency somatic embryogenesis was obtained for 2 important grape cultivars for which somatic embryogenesis have not been previously reported. When the studies on obtaining somatic embryos from vine are evaluated, it is seen that embryogenic callus formation is usually induced by the use of distinct concentrations of NOA (2-naphthoxyacetic acid) and BA (6-benzyladenine). The calli obtained are then transplanted to a hormone free medium to induce embryo formation (Carimi et al., 2005). In our research, somatic embryo formation was achieved by the effects of diminishing 2,4-D concentration during 3-month culture periods.

Dark brown and unorganized friable callus were obtained from the immature zygotic embryos of 2 grapevine genotypes after the second sub-culture. It was thought that these calli were similar to those obtained from leaves and leaf petioles of *Muscadine* grapes 'Regale' and 'Fry' by Robacker (1993) in terms of callus and somatic embryo structure. Similar calli were also obtained from leaf petiole of *V. rupestris* by Martinelli et al. (1993), tendrils of *V. vinifera* cvs. 'Thompson', 'Sonaka' and 'Tas-e-Ganesh' by Salunkhe et al. (1997) and anthers of *V. latifolia* by Salunkhe et al. (1999). The brownish

Table 4. Callus, embryo formation rates, and number of torpedo shaped embryos obtained from immature zygotic embryos of Yalova İncisi*.

Medium	2,4 D mg l^{-1}	Light regime	Callus formation (%)	Embryo formation (%)	Torpedo shaped embryo (n $explant^{-1}$)
MS	0.5	Light	100	0	0
		Dark	96.4 ± 2.3	0	0
	1.0	Light	90.0 ± 10.0	5.0 ± 2.9	981 ± 113
		Dark	100	0	0
	2.0	Light	100	0	0
		Dark	91.4 ± 4.2	0	0
	4.0	Light	80 ± 0	0	0
		Dark	50.5 ± 6.1	0	0
NN	0.5	Light	100	6.3 ± 6.3	912 ± 393
		Dark	98.2 ± 1.8	2.3 ± 2.3	108 ± 19
	1.0	Light	100	0	0
		Dark	100	0	0
	2.0	Light	100	0	0
		Dark	98.0 ± 1.9	0	0
	4.0	Light	80.0 ± 8.0	0	0
		Dark	64.0 ± 10.3	0	0
B5	0.5	Light	94.3 ± 3.6	0	0
		Dark	78.6 ± 7.6	0	0
	1.0	Light	97.5 ± 2.4	0	0
		Dark	76.9 ± 7.9	0	0
	2.0	Light	77.1 ± 9.3	0	0
		Dark	71.5 ± 9.0	0	0
	4.0	Light	38.3 ± 15.2	0	0
		Dark	23.8 ± 5.8	0	0

* Callus and embryo formation were calculated from 50 explants for each variant (10 replications with 5 explants for each). The number of different stages of embryos was counted from the explants which produced embryos. Light means: 16/8 h light/dark photoperiod, dark means complete darkness.

Table 5. Germination and plantlet rates of somatic embryo obtained from immature zygotic embryo of Yalova İncisi cultured on MS and NN hormone free media.

Media	Germination rate (%)	Plantlet formation rate (%)
MS	77.4 ± 6.0	45.0 ± 5.1
NN	74.3 ± 12.3	38.5 ± 6.5

calli formations were considered as a result of rich phenolic compounds in grape explants. Jayasankar et al. (1999) also observed phenolic compounds.

In this study, mature embryos were successfully obtained by consecutive transplanting calli to fresh media, practiced with the intervals of 1-3 months. However, Coutos-Thevenot et al. (1992) stated in their study on suspension culture of 41 B rootstock that the development of somatic embryos was ceased at heart shape stage, they struggled to complete embryogenesis stages, and mature embryo formation was only achieved by means of sub-culturing every day. The procedure was not useful since it requires heavy labor work, high costs, and is also susceptible to infection. Lopez-Perez et al. (2006), who obtained somatic embryos and plant regeneration from the *Vitis vinifera* cvs 'Sugraone', 'Crimson Seedless' and 'Don Mariano', reported 5 different morphologies in their germinated embryos, however neither abnormalities nor different morphologies were observed in this study.

Plant growth regulators were used for callus initiation but not for germination in our study. Some researchers have been successful in their studies related to somatic embryo formation but with slightly different approaches. For example, Stamp and Meredith (1988) cultured excised zygotic embryos from mature seeded grapes on a hormone-containing medium and were able to induce direct somatic embryogenesis. Plant development was on either a growth regulator supplemented or hormone free media. Rajasekaran et al. (1982) reported that in maturing grape somatic embryos ABA accumulation may be involved with dormancy, and chilling of these embryos decreased ABA levels and induced germination. Researchers have observed that chilling grape somatic embryos increases germination and their development into plants (Rajasekaran and Mullins, 1979). In our study, hormone free media were found to be enough for germination.

The results obtained in this study indicated that genotype might influence somatic embryogenesis. During induction of somatic embryogenesis, differences were observed between cultivars not only in the efficiency of embryogenesis but also in the behavior of embryogenic calli, e.g., calli of Yalova İncisi produced less embryos than that of 41 B.

Stamp and Meredith (1988), Matsuta and Hirabayashi (1989), Gray (1992), Mozsar and Viczian (1996), and Gribaudo et al. (2000) were also reported differences between the genotypes used.

In conclusion, our results show that immature zygotic embryo culture of 2 important grapevine genotypes on different 2,4-D concentration and media composition leads to formation of somatic embryos. Utilizing our regeneration system and existing transformation technologies in grape breeding program could provide an additional strategy for the enhancement of grape germplasm.

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