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Production of Artificial Seeds from Somatic Embryos Obtained from Immature Zygotic Embryos of Vines

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Summary

In this study, immature zygotic embryo explants of 41 B American rootstock (*V. vinifera* L. cvs. 'Chasselas' x V. *berlandieri*) were cultured on B5 medium (Gamborg et al. 1968) supplemented with 0.5, 1.0, 2.0 and 4.0 mg l^{-1} 2,4-D (2,4 dichlorophenoxy acetic acid) in light and dark conditions. The highest somatic embryo formation was obtained from dark condition in the media containing 0.5, 1.0 and 2.0 mg l^{-1} 2,4-D with the rates of 27.5, 22.0 and 20.0 %, respectively. The highest number of torpedo shaped somatic embryos was also obtained from dark condition with B5 medium supplemented with 1.0 mg l^{-1} 2,4-D (565 embryos per explant). These torpedo shaped somatic embryos were used to produce artificial seeds.

The somatic embryos were encapsulated in calcium alginate gel using various concentrations of sodium alginate (3 and 4 %) and calcium chloride (75, 100 and 125 mM) in order to determine the effect of rigidity of bead on conversion frequency of somatic embryos. The mixture of 3 % sodium alginate and 125 mM calcium chloride was found to be the optimal combination for encapsulation. The encapsulated somatic embryos were transferred to the hormone free MS medium (Murashige and Skoog 1962) for germination and the germination rates were between 54 and 74 %.

Key words. grapevine – vine rootstock – artificial seed – *in vitro* – somatic embryos – zygotic embryos

Introduction

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. The term of somatic embryogenesis is described as embryo formation from somatic cell or tissue. The embryos obtained with somatic embryogenesis are similar to the zygotic embryos formed by fertilization (Hatipoglu 1997).

Somatic embryogenesis being one of the *in vitro* techniques that produce high regeneration rates, serves for high frequency propagation, gene transfer and germplasm storage.

An important application of somatic embryogenesis is the production of artificial seeds (Merkle et al. 1990). Although it exhibits a great potential for artificial seed production, there are still many limitations to its practical implementation (Gray et al. 1995; Standardi and Piccioni 1998).

Artificial seeds can be obtained by encapsulation of somatic embryos with sodium alginate. Alginate gel beads provide easy handling, nutrition and protection for naked somatic embryos (Redenbaugh and Walker 1990; Redenbaugh et al. 1993). The technology can also be used in germplasm storage and transportation of elite genotypes (Gray and Compton 1993). The potential application of artificial seed technology has been demonstrated for many plant species such as carrot (Kitto and Janick 1985), sandalwood (Bapat and Rao 1988), eggplant (Lakshmana Rao and Singh 1991), white spruce (Attree et al. 1994), pepper (Büyükalaca and Mavituna 1995), pistachio (Onay et al. 1996), sweet potato (Bhatti et al. 1997), M26 apple rootstock (Piccioni 1997), camellia (Janeiro et al. 1997), lilac (Refouvelet et al. 1998), mango (Ara et al. 1999), citrus (Antonietta et al. 1999) and asparagus (Mamiya and Sakamoto 2001). So far there are some studies to induce somatic embryos (Jayasankar et al. 2001, 2002; Martinelli et al. 2003; Miaja et al. 2004; Kikkert et al. 2005; Lopez-Perez et al. 2006), no attempt to produce grapevine artificial seeds has hitherto been reported.

This paper describes production of somatic embryos from immature zygotic embryos and, germination and plantlet regeneration from encapsulated somatic embryos of grapevine. 1–3 mm-long torpedo shaped somatic embryos were encapsulated to demonstrate the applicability of encapsulation technology in grapevine and to determine the optimum concentration for sodium alginate beads.

Materials and Methods

Production of somatic embryos

The immature zygotic embryo explants were isolated under aseptic conditions from the ovules of unripe berry of

41 B MG American rootstock (collected from the experimental vineyard of Agriculture Faculty, Çukurova University, Adana, Turkey). Unripe berries were collected five to seven weeks after flowering (EMERSHAD and RAMMING 1994; TANGOLAR et al. 1998) and they were used after surface sterilization with 1.5 % sodium hypochlorite for 1 h. The immature embryos were then cultured on the B5 medium (GAMBORG et al. 1968) containing 0.5, 1.0, 2.0 and 4.0 mg l^{-1} 2,4-D (2,4 dichlorophenoxy acetic acid) and incubated in 16/8 h light/dark photoperiod and complete darkness. All media, which contain 3 % sucrose, were solidified with 0.8 % agar and pH was adjusted to 5.8 prior to autoclaving at 105 kPa and 121 °C for 15 min.

Callus was sub-cultured three months intervals. At the end of the second three-month culture period, formation of the embryos at different stages on the callus obtained from zygotic embryos were observed.

The observations were carried out until end of 8th month. The rate (%) of callus, embryogenic callus and embryo formation and, the numbers of globular, heart and torpedo shaped embryos were recorded.

Encapsulation of somatic embryos

To determine the optimum concentration for calcium alginate capsules, two different concentrations of sodium alginate (3 and 4 %) and three different concentrations of calcium chloride (75, 100 and 125 mM) prepared in MS basal medium (MURASHIGE and SKOOG 1962) containing 3 % sucrose were tested. All stages of the procedure were carried out under aseptic conditions. Torpedo-shaped somatic embryos obtained from dark conditions of the B5 medium supplemented with 1.0 mg l^{-1} 2,4-D were removed from embryo cluster under binocular microscope after eight month culture and used for encapsulation. The isolated embryos (1–3 mm long) were dipped into sodium alginate solution. Each embryo was picked up using a Pasteur pipette and dropped into calcium chloride solution, stirred slowly by a magnetic stirrer, and allowed to remain for 15 min for the beads to harden.

Somatic embryo formation experiments were performed with 10 replications each comprising 5 explants. Mean values were given with standard errors. Germination experiments were carried out with 5 replications each of which contains five artificial seeds. One way variance analyses was conducted on germination data using ANOVA program and LSD test was applied to evaluate differences between means for the germination of artificial seeds.

Results and Discussion

In the explants cultured on the B5 medium with four 2,4-D concentrations in combination with light and dark conditions, the callus formation rates were in eight of the ten combinations 100.0 %. Only the combinations 0.5 mg l^{-1} 2,4-D/light and 4.0 mg l^{-1} 2,4-D/dark reacted less with 95.0±3.3, 98.0±2.0 respectively. All the callus obtained (100 %) were found to be embryogenic. The highest embryo formation rates were 27.5, 22.0, and 20.0 % for 0.5, 1.0 and 2.0 mg l^{-1} 2,4-D concentrations of dark conditions, respectively (Table 1).

Dark-brown friable callus were obtained from immature zygotic embryos of 41 B after the second sub-culture. The callus was similar to those obtained from leaves and leaf petioles of *Muscadine* grapes Regale and Fry by ROBACKER (1993) as in callus and somatic embryo structure. In the same manner, similar callus was 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). These brownish callus formations were considered as a result of rich phenol compounds in grape explants. This interpretation was also supported by JAYASANKAR et al. (1999).

The highest numbers of globular shaped embryos were obtained from dark conditions of media supplemented with 2.0, 1.0 and 4.0 mg l^{-1} 2,4-D with the values of 721, 491 and 347 per explants respectively. Heart shaped embryo formation were found to be higher in the

Table 1. Embryo formation as well as number of different stages of embryo obtained from immature zygotic embryo of 41B (*V. vinifera* L. cvs. 'Chasselas' x V. *berlandieri*) rootstock cultured in B5 medium containing different concentrations of 2,4-D under dark and light conditions*.

2,4-D (mg l^{-1})	Light regime	Embryo formation (%)	Stages of embryo (number per explant)		
			globular shaped	heart shaped	torpedo shaped
0.5	light	2.5 ± 2.5	121 ± 41	1 ± 0	4 ± 2
	dark	27.5 ± 6.5	117 ± 102	128 ± 90	64 ± 18
1.0	light	4.5 ± 3.0	180 ± 58	365 ± 57	156 ± 62
	dark	22.0 ± 7.0	491 ± 163	502 ± 184	565 ± 248
2.0	light	4.0 ± 2.7	30 ± 21	82 ± 3	33 ± 10
	dark	20.0 ± 7.3	721 ± 452	290 ± 140	261 ± 83
4.0	light	2.0 ± 2.0	26 ± 11	13 ± 4	2 ± 1
	dark	8.0 ± 4.4	347 ± 40	228 ± 137	246 ± 34

*Embryo formation was calculated from 50 explants for each variant (10 replication with 5 explants for each).
The number of different stages of embryos was counted from the explants which produce embryos.

Table 2. Germination rate of the 41B (*V. vinifera* L. cvs. 'Chasselas' x *V. berlandieri*) American rootstock somatic embryos encapsulated with different concentrations of sodium alginate and calcium chloride.

Sodium alginate (%)	Calcium chloride (mM)	Average (%) [x]
3	75	64 b
	100	68 ab
	125	74 ab
4	75	65 b
	100	58 b
	125	54 b
Naked embryos		91 a
LSD (0.05): 24.1[y]		

[x]Averages followed with same letters are not significant different at LSD 0.05 level

[y]This value was calculated using angle values (arcsin $\sqrt{x}$) of data

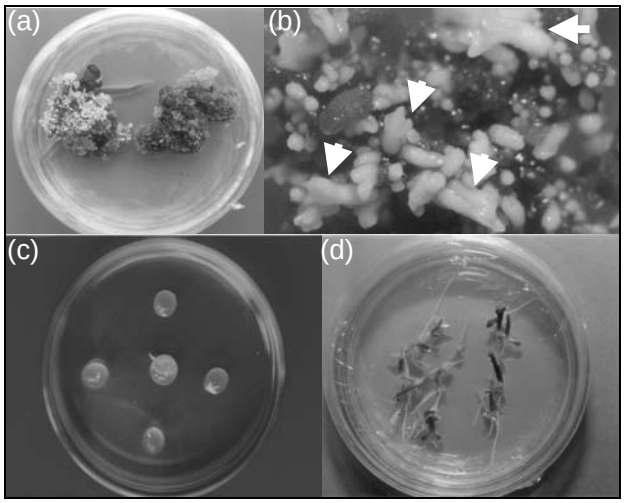


Fig. 1. Various developmental stages during plant regeneration via artificial seeds in 41B (*V. vinifera* L. cvs. 'Chasselas' x V. *berlandieri*) American rootstock (a) different stages of somatic embryos on explant (b) torpedo-shaped embryos used encapsulation (c) encapsulated somatic embryos (d) germination of encapsulated embryos.

media of both light (502 per explant) and dark conditions (365 per explant) supplemented with 1.0 mg l^{-1} 2,4-D than the other 2,4-D concentrations. The highest number of torpedo shaped embryos were obtained from dark conditions with 1.0 mg l^{-1} 2,4-D (565 per explant) (Table 1 and Fig. 1a, b).

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 stages and they struggled to complete embryogenesis stages and mature embryo formation was occurred by the way of subculturing everyday. These procedures were not useful as they require longer time, higher costs and having more infection risks. In our study, mature embryos were succeeded to be obtained by consecutive transplantations to fresh media carried out in 1–3 months intervals.

When the studies on obtaining somatic embryos of vine were evaluated, usually embryogenic callus formation is induced by the use of distinct concentrations of 2,4-D and BA. The obtained callus is then transplanted to a medium without hormone to induce embryo formation. In our research, it was considered that the somatic embryo formations were achieved by the effects of diminishing 2,4-D during three months culture period.

Since concentration of sodium alginate and calcium chloride affected the rigidity and uniformity of the beads, different concentrations were tested in our study. In preliminary experiments, it was found that beads formed with the low concentration (1–2 %) of sodium alginate were too soft to handle while beads formed with high concentration (5 %) were too hard for emerging. The beads with uniform shape and optimum rigidity were formed with 3 and 4 % sodium alginate (Fig. 1c). The encapsulated and the naked somatic embryos were germinated on hormone-free MS medium in order to investigate the effect of encapsulation on germination of embryos. The results of the naked embryos and the embryos encapsulated with different concentrations of sodium alginate and calcium chloride are given in Table 2. As can be seen from the table, germination rate increases slightly parallel to the increase in calcium chloride concentration when 3 % sodium alginate concentration was used. However, in the case of 4 % sodium alginate, germination rate decreases with increasing calcium chloride concentration. Germination rate of naked embryos was found to be higher (91 %) than those of all the encapsulation treatments (54–74 %). Similar results were observed on the other studies carried out with asparagus (Ghosh and Sen 1994), pepper (Büyükalaca and Mavituna 1995) and papaya (Castillo et al. 1998). Among the encapsulated embryos, the best germination was obtained from 3 % sodium alginate and 125 mM calcium chloride combination. The results reveal that influence of sodium alginate concentration on germination was more pronounced than calcium chloride concentration (Table 2).

Roots of the embryos emerged from the beads 6 weeks later from artificial seeds cultured on hormone-free MS medium (Fig. 1d). The rigidity of the bead played an important role for the germination of embryos.

The period of time for germination is different in the encapsulated and the naked embryos. Germination started in 10 d in the naked embryos, while it took 6 weeks in the encapsulated ones. The reason for this is that the naked embryos can contact the medium directly and they use the moisture easily, whereas a certain period of time is needed for the encapsulated embryos to utilize the moisture and the medium.

The germination rates of the encapsulated embryos were varied between 54–74 %. These rates were affected from the concentrations of calcium chloride and sodium alginate.

The production and encapsulation of somatic embryos offer mass propagation of elite genotypes. In this study, we present a suitable protocol to obtain uniform and firm beads from grapevine somatic embryos.

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