

Somatic embryogenesis and regeneration of *Vitis* sp.

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Summary: *In vitro* regeneration from cell to plant, considered to be an important precondition of gene transfer, has been attempted in *Vitis vinifera*, *V. amurensis* as well as in some of their interspecific hybrids, starting with different organs of the plants (leaf blade, petiole, anther, pistil) as inocula. First an embryogenic callus has been induced in a solid *Murashige-Skoog* medium supplemented with 2,4 D and benzyladenin. Four *V. vinifera* cultivars (*Chardonnay*, *Chasselas blanc*, *Pinot noir*, *Riesling*) produced embryogenic callus lines. All of them conserved its embryogenic capacity after several transfers. Somatic embryos appeared on 1/2 strength MS medium, either solidified or liquid, without hormones added. Entire plants were succeeded with the variety *Chardonnay*. Germinating embryos have been put on hormonfree, half strength, solidified MS medium where regular shoot and root development ensued.

Introduction

Traditional methods of the breeding of grapes require much time and are a tedious procedure. In addition, new, cross bred grape genotypes have little chance to become popular because of the conservatism of consumers, consequently of the wine industry and trade. New names and qualities appearing on the market receive a dubious acceptance (Mullins et al., 1990). A reasonable solution of that inconvenience would be the genetic transformation of existing and successful varieties, which could be improved without endangering the identity of the make accustomed.

A basic precondition of attempting successful genetic transformation from somatic cells is an efficient protocol of cell-plant regeneration. First publications on successful somatic embryogenesis of grape are found already as early as by Mullins & Srinivasan (1976) and on adventitious organogenesis by Rajasekaran & Mullins (1981). Undoubtedly, the organogenesis is less suitable for our purpose as chimeric plants are of dubious value (Colby et al., 1991). Embryogenic callus was derived from diverse organs. Unfertilised ovules (Mullins & Srinivasan, 1976), pistils (Gray & Mortensen, 1987), zygotic embryos (Stamp & Meredith, 1988a), leaf tissues (Krul & Worly, 1977; Stamp & Meredith, 1988b; Nakano et al., 1994; Scorza et al., 1996) as well as segments of the petiole and stem (Krul & Worly, 1977) served equally as sources of embryogenic callus lines. Most frequently, however, anthers produced embryogenic structures, as Rajasekaran & Mullins (1979); Bouquet et al. (1982), Gray & Mortensen (1987), Pearl et al. (1995), Torregrosa (1995). Liquid or solidified media proved to be equally useful. The success depended highly from the genotype. A number of American *Vitis* species and their

interspecific hybrids have reliable regeneration technology protocols. In *Vitis vinifera* too, some 25 varieties proved to produce embryogenic cell suspensions suitable for transformation (Pearl & Eshdat, 1998). Our objective was to widen the circle of grape genotypes producing embryogenic callus for the purpose of breeding, and to study the process of embryogenic regeneration.

Material and methods

Somatic embryogenesis was attempted in grape varieties belonging to different *V. vinifera* varieties and *V. amurensis* genotypes, as well as American and *V. amurensis* interspecific hybrids. The large number of genotypes involved was justified with the fact of contradictory results found in the literature concerning the regenerating ability of individual varieties. For starting embryogenic cultures, anthers, pistils, leaf blade- and petioles were used (Table 1).

Induction of embryogenic callus

Plant parts for tissue culture were collected from cuttings raised in greenhouse or from buds outdoors closely before blooming. The flower clusters in bud stage were cut and dipped in sodium hypochlorite solution (Clorox 10 %) for 15 minutes, then rinsed three times in distilled water for 10 minutes. Excised anthers were placed on the medium together with the filaments. Pistils have been cut longitudinally before using it for inoculation. In one Petri dish 50 anthers or pistils are incubated, altogether 200 pieces per variety. Explants of leaves and petioles were taken from *in vitro* grown plants. The medium for inducing embryo-

Table 1 Origin of grape varieties and genotypes and the plant parts used as explants.

Cultivars (genotypes)	Genetic origin	Explant origin			
		anthers	pistils	leaves	petioles
Riesling	<i>V. vinifera</i> convar. occidentalis, subcon. gallica, provar. microcarpa	+		+	+
Pinot-noire	<i>V. vinifera</i> , convar. occidentalis subcon. gallica	+			
Chardonnay	<i>V. vinifera</i> , convar. occidentalis, subconvar. gallica, provar. microcarpa, subprov. 'Noirien'	+			
Chasselas blanc	<i>V. vinifera</i> , convar. orientalis, subconv. caspica, provar. aminea, subprovar. vulgaris	+			
Kismis chorniy	<i>V. vinifera</i>	+	+		
Katta kurgan	<i>V. vinifera</i>	+	+		
Moldova	Seyve-Villard 12 375 x Guzal kara	+	+		
Georgikon 28	<i>V. berlandieri</i> x <i>V. riparia</i> x <i>V. vinifera</i>			+	+
Teleki 5C	<i>V. berlandieri</i> x <i>V. riparia</i>	+			
<i>Vitis amurensis</i>		+	+		
Kunleány	(<i>V. amurensis</i> x <i>V. vinifera</i>) F ₂ x Afuz Ali	+			
A 109 (Amadeus)	(<i>V. amurensis</i> x <i>V. vinifera</i>) F ₂ x Chardonnay			+	+
V 9	(<i>V. amurensis</i> x <i>V. vinifera</i>) F ₂ x Chardonnay	+		+	+
A 214 (Odysseus)	[(<i>V. amurensis</i> x <i>V. vinifera</i>) F ₂ x Thallóczy L. musk.] x Pinot gris	+		+	+
N 82	(<i>V. amurensis</i> x <i>V. vinifera</i>) BC ₂ x Moldova			+	+

genic callus development was first described by *Mozsár & Süle* (1994) and used as MSE medium which contained in addition to components of *Murashige & Skoog* (1962) 20 g/l sacharose, 1.1 mg/l 2,4-D, 0.2 mg/l BA. The pH was

adjusted to 6.4 and Fe-EDTA (5 ml/l) was added after sterilisation only. Solidification was due to 7 g/l Oxoid agar.

After inoculation, the cultures were incubated in darkness at 26–28 °C. Callus development was checked after 30 days and the cultures were transferred to new media of the same composition.

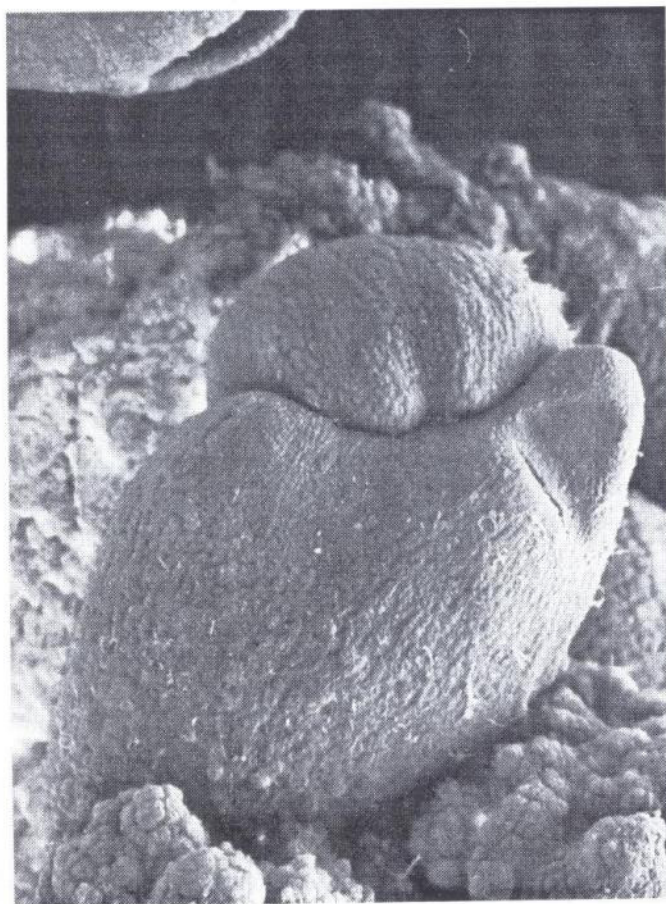


Figure 1 Somatic embryo with distinct cotyledons (Chardonnay). Scanning elektron microscope picture, magnification: 200 x

Regeneration of plants

The induction of somatic embryos was attempted in solid as well as in liquid media. The solid medium contains half strength of MS salts and vitamins plus sacharose (10 g/l) and agar (6 g/l), whereas the liquid variant with a full dose of MS components was shaken. Induction occurred also in darkness at 26–28 °C.

After one month, cultures on solid medium were exposed to light for a week. Then germinating embryos were separated and transferred to tubes containing 10 ml hormonfree, half strength MS solidified medium.

One month old cultures raised in liquid medium were also exposed to light for a week. The germinating embryos starting to get green were equally transferred in tubes containing 10 ml hormonfree, half strength MS, solidified medium, at a photoperiod of 16 hours at 24–26 °C. After two weeks, the plantlets with regular shoots and roots were individually passed into tubes with the same medium.

Plantlets with shoots and roots of normal appearance obtained from either solid or liquid media were planted into soil of the greenhouse.

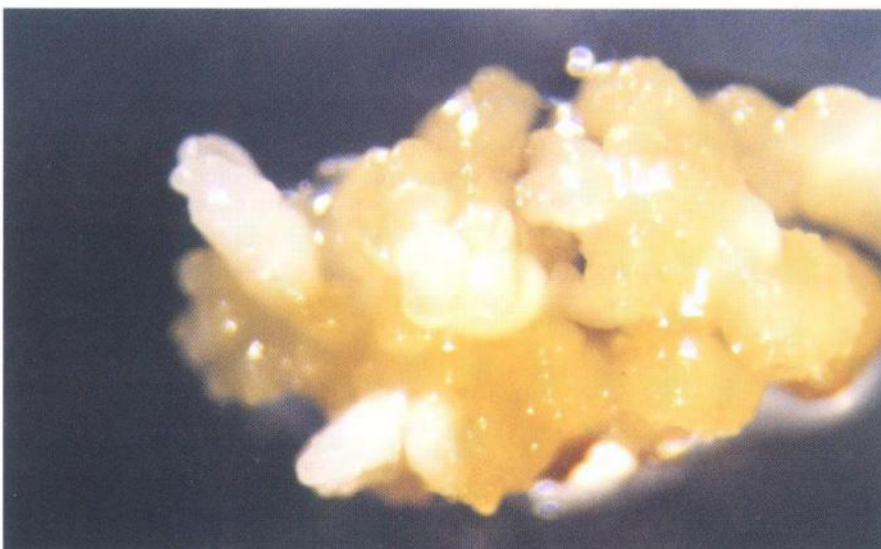
Results and discussion

Induction of embryogenic callus

The induction was attempted on explants derived from four different types of plant parts: leaves, petioles, pistils and

Table 2 Results of embryogenic callus induction on excised anthers

Varieties (genotypes)	Results
Riesling	embryogenic callus
Pinot noire	embryogenic callus
Chardonnay blanc	embryogenic callus
Chasselas blanc	embryogenic callus
Kismis chorniy	no callus developed
Katta kurgan	no callus developed
Moldova	callus developed
Teleki 5C	callus developed
Vitis amurensis	callus developed
Kunleány	callus developed
V 9	no callus developed
A 214 (Odysseus)	callus developed

**Figure 2a** Phases of somatic embryogenesis and plant regeneration on solid medium (Chardonnay) – embryogenic callus**Figure 2b** – torpedo and cotyledonary stage of embryos

anthers. Different types of callus appeared in the cultures. Embryogenesis was observed in those callus cultures which displayed yellow colour and their consistency was more solid showing a granular surface as if some signs of differentiation were in course (*Figure 2 a*). The rest of calli was more light, more soft, plastic and homogenous on the surface.

Calli raised from leaf and petiole explants did not produce any embryoid structures in the varieties studied (*V 9, A 109, A 214, N 82, G 28* and *Riesling*). Similarly, neither calli raised from pistils of the varieties *Kismis chorniy, Katta kurgan, Moldova* and *V. amurensis* were of embryogenic type.

Embryogenic callus was obtained from anthers only as shown in *Table 2*. It is evident that embryogenic callus could not be obtained but in varieties of *V. vinifera* (*Chasselas, Chardonnay, Pinot noir, Riesling*). According to Pearl & Eshdat (1998) in the variety *Riesling*, embryogenic calli suitable for genetic transformation are entirely lacking among the known laboratories.

Our results suggest that the other grape varieties of different origin owed their successful regeneration to the modification of the medium used for induction. The sole exception was the species *Vitis amurensis* - which received double dose of 2,4-D in the medium. Higher concentration of 2,4-D increased the frequency of callus formation, however, did not produce embryogenic structures.

The fact of obtaining embryogenic calli in no more than 1/3 of the grape genotypes studied only, may find its reason in the various requirement of hormones or, perhaps, in the suboptimal development of the excised anthers. Pearl (1995) claimed that the optimal period for the embryogenic ability within the anther tissues is close to the first mitotic division of the pollen grains. However, in our experiments the right time of isolating the anthers was not checked according to phases of microsporogenesis but to morphological attributes.

Plant regeneration from embryogenic callus

One of the four grape varieties which produced embryogenic calli, *Chardonnay* was studied closely to trace the course of plant regeneration. On the hormonefree, solid medium used to induce embryos, first embryogenic



Figure 2c – germinating embryo.



Figure 2d – plant regenerated ready to be planted.

aggregations appeared in the third week as torpedoed, later as plantlets with growing cotyledons (Figure 1 and 2b). At the fourth week, a mass of embryos started to germinate. The Petri dishes were exposed to light, so a part of the embryos developed cotyledons and started root growth within one week (Figure 2c). At that phase the germs of 5–10 mm length were transferred into singular containers. The plants developed within 4–6 weeks true leaves and a branching root system, they were ready to be planted and acclimated (Figure 2d). The 53% of the plants developed normally whereas 47% had fasciated cotyledons and their growing point was blind. Many papers are dealing with the problem, how those anomalies of somatic embryos could be prevented and counteracted (Pearl and Eshdat, 1998). From that point of view, our results obtained with the variety *Chardonnay* indicate the favourable

efficiency of the method applied as for the ratio of successfully raised plants.

The development of embryos induced from calli raised either in liquid or in solid media did not show different speed of development. However, embryos grown on a liquid medium were different morphologically from those grown on solid medium. In spite of being put subsequently on a solid medium, the ratio of normally developing plantlets was 28 % only. Consequently, solid media should be preferred in the whole process of plant regeneration started with embryo induction.

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