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Research Note

Somatic embryogenesis from leaves of *Vitis* x *Muscadinia* hybrids

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The regeneration of the grape was achieved using organogenesis or embryogenesis from various types of explants including stems, leaves, petioles, ovules, anthers (Gray and Meredith 1992) and recently from protoplasts (Reustle *et al.* 1994) but, like most woody species, the grape is still considered as recalcitrant to regeneration because procedures are restricted to only few varieties. Despite of the importance for grape improvement (Olmo 1986), there are very few results about regeneration of *Muscadinia rotundifolia* and its hybrids with *Vitis vinifera*. So far, regeneration of the muscadine grape was achieved by somatic embryogenesis from leaves (Robacker 1993) and zygotic embryos (Gray 1992) but not by organogenesis. Previously, we reported a method for high-frequency adventitious bud formation and plant regeneration from leaves of *Vitis* x *Muscadinia* hybrids (Torregrosa 1994). But, all attempts to induce somatic embryogenesis by anther culture have been unsuccessful (Torregrosa and Bouquet, unpubl.). Using the procedures described by Stamp and Meredith (1988) for *V. vinifera*, we induced somatic embryogenesis from zygotic embryos excised from mature open pollinated seeds of these hybrids. But, this has many disadvantages with respect to the genetic characterization of the regenerated plants. Here we report the possibility to induce somatic embryogenesis and regenerate plants from leaves of *Vitis* x *Muscadinia* hybrids.

Materials and methods: Plant materials used in this study were (i) VRH 8715 : *Vitis vinifera* (Ugni blanc x Cabernet Sauvignon) x *Muscadinia rotundifolia* cv. Carlos, (ii) VRH 8773 : *V. vinifera* (Cabernet Sauvignon x Alicante Bouschet) x *M. rotundifolia* cv. NC 184-4 and (iii) VMH 1 : VRH 8773 x 140 Ru (*V. berlandieri* x *V. rupestris*). Preliminary experiments showed that leaves taken from rooted plantlets have poor regenerative capacities and, in contrast, that explants from axillary shoot proliferation medium present better competence (Torregrosa 1994). Consequently, explants were removed from 45-day-old proliferating nodal cultures on MS/2 modified medium supplemented with 4.4 µM BAP. Procedures used to culture and propagate VM or VR hybrids by axillary budding have previously been described (Torregrosa and Bouquet 1995). Immature leaves (0.5 to 4 mm in length) were excised from the youngest part of axillary shoots. They were placed with their abaxial side on the surface of the medium. The 55 mm Petri dishes contained 15 ml of medium. Twenty explants were plated on each Petri dish (240 to 500 total explants per treatment).

Cultures were maintained at 25 ± 1 °C in darkness and were observed after 45 and 90 d for necrosis, callogenesis and embryogenesis. The basal medium used to initiate callus formation and proliferation consisted of mineral salts of the MS medium (half strength), solidified with 6 $g.l^{-1}$ agar, supplemented with sucrose 25 $g\ l^{-1}$ and other organic components as previously described (Torregrosa and Bouquet 1995). To determine the best combination of growth regulators, the auxins NOA, 2,4-D and 2,4,5-T (at 5 or 10 µM), combined with BAP (at 1.1 or 2.2 µM) were used. All media were adjusted to pH 5.8 with KOH (0.5 M) and sterilized for 30 min at 115 °C. During the induction period, explants were maintained on the same medium without transfer. When embryogenic tissues were detected, entire explants were transferred 1 month later to a proliferating medium consisting of a modified MS/2 medium supplemented with amino acids (1 $g\ l^{-1}$ casein hydrolysat, 100 $mg\ ml^{-1}$ glutamine, 10 $mg\ ml^{-1}$ phenyl-alanine and 2 $mg\ ml^{-1}$ glycine) and 5 µM 2,4-D / 1.1 µM BAP. Then, embryogenic callus were fragmented and subcultured, every 2 months, on the same medium or on a medium supplemented with only 1.2 µM 2,4-D, according to the differentiation level of the callus.

Results and discussion: When cultured on media containing appropriate auxin concentrations, leaf explants manifested strong growth and callogenesis during the first month. When NOA was applied, callus initiated from petiole stubs or principal veins, and the general appearance of the lamina was conserved. Small and rapidly necrosed roots emerged from callus localized in petiole stub area. Callus initiated with NOA did not manifest any other organogenic or embryogenic competence. The two other auxins manifested better capacities in inducing embryogenesis. Like Rajasekaran and Mullins (1981), who tested the efficiency of several auxins on internodes of *V. vinifera* x *V. rupestris* hybrids, we noted that 2,4,5-T was very phytotoxic. With 5 µM 2,4,5-T, explants produced unorganized callus and very few embryogenic structures, but only in the case of VRH 8715. With 10 µM 2,4,5-T, explants turned black in a few days. Many of them did not produce any callus and consequently could not initiate any embryogenic tissues. When 5 µM 2,4-D were added, callogenesis was possible; it affected entire explants, which lost their typical leaf character. Callus was friable, initially yellow to grey and became progressively brown. The use of 10 µM 2,4-D did not increase callogenesis and caused necrosis, resulting in a decrease of embryogenesis (Table).

Transfer of the explants to a fresh medium did not influence significantly necrosis and callogenesis, nor the regeneration ability. Callogenesis decreased progressively during the second month of culture to stop completely in the third when callus became brown to dark. We did not observe any embryogenic formations during the first month in culture. Most embryogenic formations were observed after 6-10 weeks. Embryogenesis decreased during the third month but, without changing the medium, embryos or

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Table

Effect of auxins on somatic embryogenesis

Auxin [μM]	Explants with necrosis (%)		Embryogenic response (%)	
	VRH 8715	VMH 1	VRH 8715	VMH 1
2,4-D [5]	37[a]	3[a]	4.8[a]	5.3[a]
2,4-D [10]	71[b]	8[b]	3.1[a]	0.9[b]
2,4,5-T [5]	86[b]	94[c]	0.7[b]	0.0[b]
2,4,5-T [10]	94[c]	99[d]	0.0[b]	0.0[b]

After 90 d in culture data were collected from 240 explants of each treatment. Means with the same letter are not significantly different. Gabriel's multiple comparison procedure was made by genotype (p = 0.05).

embryogenic callus continued to emerge and proliferate from the original callus over a 6 months period. With leaves of VRH 8715, embryogenesis was initiated from callus and embryogenic structures emerged as white to yellow typical nodular embryogenic callus bearing globular embryos easy to differentiate (Fig. a, b and d). In contrast, VMH 1 did not manifest great capacity to produce proliferating embryogenic callus. It produced single differentiated embryo or somatic embryo-like structures consisting of cluster of linked embryos difficult to propagate and differentiate (Fig. c). With VRH 8715, we could induce indefinite embryogenic proliferation (for over 2 years), without loss of regenerative ability, subculturing periodically embryogenic calluses on the same medium used for induction. Like GRAY (1992), we noted that somatic embryos present very frequently, during their development, morphological abnormalities consisting in cotyledonary fusions.

To convert somatic embryos into viable plantlets, direct transfer of embryogenic callus to growth regulator-free medium, showed poor efficiency. We obtained the best plant recovery rate when actively proliferating entire embryogenic callus were transferred on proliferating medium supplemented by 5 μM filter-sterilized IAA and 1.1 μM BAP. On this medium, globular embryos developed rapidly into white well-shaped embryos, which began to elongate to 3-10 mm in length after 6 - 8 weeks. Single well-differentiated embryos were carefully selected and placed horizontally under standard photoperiod on micropropagation medium supplemented with 1.1 μM BAP, for 2 weeks. These culture conditions stimulated germination leading to an enlargement of greening cotyledons and shoot growth, but root emergence and development remained moderate. In the case of VRH 8715, 10 % of the selected embryos germinated. Transplanted in culture tubes on a growth regulator-free medium, 95 % of these germinated embryos converted to vigorous plantlets with normal phenotypic characteristics.

Thus, the efficiency of the method depends on the use of (i) immature leaves from axillary shoot proliferation as explants, (ii) 5 μM 2,4-D / 1.1 μM BAP combination and (iii) adapted procedure of germination. It is the first report of somatic embryogenesis from *Vitis* x *Muscadinia* hybrids. This procedure proved to be highly efficient and convenient as we obtained regeneration with all VM and VR hybrids tested and because we used *in vitro* explants, which are easily available. Consequently, this method appears to be very helpful and suitable for genetic engineering of *Vitis* x *Muscadinia* hybrids.

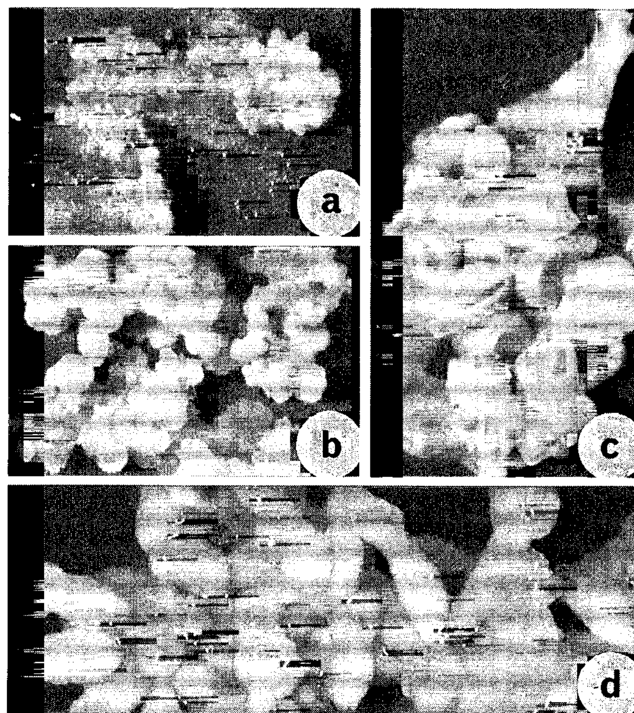

Figure: Somatic embryogenesis from VM and VR hybrids. *(a)* Embryogenic callus of VRH 8715 arising from initial brownish callus. *(b)* Typical embryogenic callus of VRH 8715. *(c)* Cluster of embryo-like structures of VMH 1 emerging directly from the callusing explant. *(d)* Well differentiated somatic embryos of VRH 8715.

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