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Research Note

A rapid method for somatic embryogenesis and plant regeneration from cultured anthers of *Vitis riparia*

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K e y w o r d s : Vitis riparia, tissue culture, somatic embryogenesis.

Somatic embryogenesis has been reported from numerous *Vitis* genotypes (for literature see Stamp and Meredith 1988; Martinelli *et al.* 1993; Robacker 1993). However, regeneration of plants from these cultures usually occurs at a low level. We developed a relatively rapid method using *Vitis riparia* cv. Gloire de Montpellier to produce plants from anthers via somatic embryogenesis, in less than five months from initiation of cultures. A regeneration rate of up to 60% was obtained when a short-time liquid culture was included between culturing phases on a solid medium.

Materials and methods: Dormant cuttings of *V. riparia* cv. Gloire de Montpellier were collected in November 1992 and stored at 4 °C for 3 months. After storage, two-node cuttings 15-20 cm long were rooted in soil in the greenhouse. Flower buds were continuously available from 25 to 56 d after planting. Buds were harvested shortly prior to anthesis. They were surface sterilized in 1 % NaOCl for 15 min and rinsed in sterile distilled water.

E m b r y o g e n i c c a l l i i n i t i a t i o n : Translucent yellow anthers, which were used for initiation of embryogenic calli, were dissected aseptically from the flower buds and placed on a solid medium which consisted of MS medium (Murashige and Skoog 1962) supplemented with 1.1 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BA (amended with 0.8 % Noble-Agar (Difco)). The pH was adjusted to 6.4 with 1 M KOH. A FeNa-EDTA stock solution (746 mg Na-EDTA and 0.556 mg $FeSO_4 \cdot 7H_2O$ in 100 ml distilled water) was sterilized separately and was added to the sterilized medium (5 ml l^{-1}) immediately before use. This procedure prevented the formation of toxic substances resulting from breakdown of carbohydrates during autoclaving because of catalyzation by FeNa-EDTA (Schenk *et al.* 1991). 108 anthers were placed on Petri dishes (9 cm diameter) and incubated at 28 °C in the dark.

E m b r y o g e n i c i n d u c t i o n : After 30 d, those anthers which formed calli, were transferred to fresh plates of the same medium and incubated as before. After another 30 d, calli that appeared to be viable, were transferred to an induction medium (half strength MS salts plus vitamins and containing 10 g l^{-1} sucrose and 0.6 % Noble-Agar) and incubated under the same conditions as before.

E m b r y o g e r m i n a t i o n : Globular and heart-stage embryos from solid medium were placed in a 25 ml liquid MS medium in 100 ml Erlenmeyer flasks and incubated in an orbital shaker at 27 °C in the dark. After 3 weeks of subculturing weekly in a fresh liquid medium. Parallel with this experiment embryos were germinated on solid MS medium at same temperature in dark. Embryos with cotyledons and primary roots were placed individually in test tube, on a solid half strength MS medium and incubated in a culture room at 25-27 °C in continuous light, provided by cool-white fluorescent tubes (Tungsram F25/36W).

P l a n t r e g e n e r a t i o n : Plantlets with sufficiently developed roots were placed in the greenhouse in culture tubes with caps for 1 d and for 2 d without cap to adapt to natural light. They were then planted to soil in 9-cm-diameter pots and covered with a plastic cover for 3 d. Other plantlets with poorly developed roots were transferred to the following rooting medium: half strength MS salts and vitamins supplemented with 0.1 mg l^{-1} NAA and gelled with 0.8 % agar. After 4 weeks, they were planted to soil as described above.

Results: After the first 30 d period 34 anthers out of 108 formed calli. Half of them stayed viable for further 30 d. These were transferred to an induction medium. 2-3 weeks after this last subculture, primary embryos and secondary embryos appeared in large numbers on the surfaces of 6 calli.

Embryos developed asynchronously. Globular, heart- and torpedo-shaped embryos were present at the same time as embryos with cotyledons and primary roots. In early developmental stages they were transferred to a fresh induction medium, where secondary embryos were formed. After additional subculturing, the formation of secondary embryos declined. The embryos were inclined to embryomorphogenesis rather than to formation of secondary embryos. After 2-3 subcultures, they completely lost their ability to produce new secondary embryos. When embryos germinated on solid medium were exposed to light, most of them were unable to regenerate into plants because they failed to produce meristems. They formed abnormal cotyledons, which often were fused (Figure, a) or

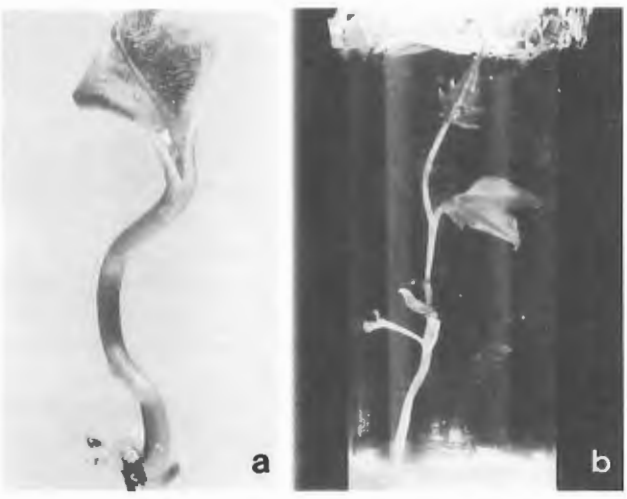

Figure: a) Abnormally developed embryo; b) normally developed plantlet from embryo germinated in liquid medium.

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showed other abnormal developments. Morphological classification of this abnormalities was described previously (GOEBEL-TOURAND *et al.* 1993). Disorganized cell proliferation on their surface of cotyledons was also observed. When solid culture was interrupted by a 3-4 weeks liquid culture, normal shoot and root development occurred (Figure, b) and high percentage of embryos regenerated plantlets (Table). These embryos contrary to germinated on solid medium had two well developed cotyledons and meristem.

Of the planted plantlets 80 % developed normal plants with well-organized shoots and root systems. They did not differ from conventionally propagated plants.

Table

Plant regeneration in different germination systems

Germination System	Total number of embryos	embryos developed to normal plantlets
solid medium	45	4
liquid medium	52	32

Discussion: Under conventional tissue culture conditions, in many instances, development of grapevine embryos was stopped in the heart-stage, and plantlets were not formed. Daily subculturing of embryos in a fresh medium was used successfully to obtain full embryo development (COUTOS-THEVENOT *et al.* 1992) but it was very time consuming procedure. The removal of cotyledons of Cabernet Sauvignon embryos (MAURO *et al.* 1986) increased the regeneration rate, but this procedure was not easily applicable for a large number of embryos. Dehydration of mature, well developed grape embryos also increased plant regeneration up to 34 % (GRAY 1989). With our technique, using *V. riparia* cv. Gloire de Montpellier, more than 60 % of the embryos produced normal plantlets when a 3-4 weeks liquid culture was included between culturing phases on solid medium. Less than 10 % regeneration was obtained when embryos were continuously cultured on a solid medium. The role of subculture in liquid medium for improving regeneration was not clearly evident, but the liquid culture apparently stimulated the embryos that had passed through heart- and torpedo-stages to regenerate normal plants at a significantly higher frequency. Presumably inhibitor compounds, extracellular macromolecules (COUTOS-THEVENOT *et al.* 1992) and/or free polyamine (FAURE *et al.* 1991), that accumulated slowly in the solid medium, blocked regeneration of normal plantlets. In liquid culture, however those inhibitors possibly were diffused and eliminated by weekly replacement of the medium. The fact that the new embryo formation was slowed and arrested after 2-3 generations indicated that long term embryogenic cultures should be maintained in liquid culture.

The advantages of our method over the existing ones are: (1) rapidity: in 5-6 months plants can be planted in soil; (2) simplicity: no daily changes of the medium or removal of cotyledons are necessary; (3) high rate of plant regeneration.

Although plant regeneration via organogenesis from leaves was reported from *V. berlandieri x V. riparia* hybrids (CLOG *et al.* 1990), to our knowledge, our paper is the first report of somatic embryogenesis and *in vitro* plant regeneration from *V. riparia*. This method, modified toward more efficient secondary embryogenesis, may open the way to propagate grapevine rootstocks *in vitro* in large quantities. Further studies are needed to obtain somatic embryos from other cultivars with this method. Of further interest, we were able to obtain somatic embryos of a species that does not have naturally zygotic embryos (GALET 1988).

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