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# Somatic embryogenesis from leaf- and petiole-derived callus of *Vitis rupestris*

**Lucia Martinelli** [1], **Paola Bragagna** [1], **Valentino Poletti** [1], **and Attilio Scienza** [1, 2]

[1] Laboratorio Biotecnologie, Istituto Agrario, via Mach 1, I-38010 San Michele all'Adige (TN), Italy
[2] Istituto Coltivazioni Arboree, Università degli Studi di Milano, Italy

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**Abstract**: Somatic embryogenesis from leaf- and petiole-derived calli of *Vitis rupestris* was obtained with an efficiency of 3.2% and 4.2% of plated explants, respectively on two combinations of 6-benzyladenine and 2,4-dichlorophenoxyacetic acid (1/0.1 and 1/1 $mgl^{-1}$) added to MS medium. Embryogenic callus, embryo subcultures and somatic embryogenesis from somatic embryos were obtained either in the presence of 1 $mgl^{-1}$ indole-3-acetic acid or 0.1 $mgl^{-1}$ indole-3-butyric acid added to MS or NN media. Within a 4-month culture, embryo germination occurred at a frequency of 13% of explanted embryos when chilling at 4°C was provided for two weeks and a combination of 6-benzyladenine (1 $mgl^{-1}$) with indole-3-butyric acid (0.1 $mgl^{-1}$) was added to NN medium supplemented with casein hydrolysate (250 $mgl^{-1}$). A higher frequency (51%) was obtained in a longer culture time (9 months) when only indole-3-butyric acid was present in the medium and in absence of chilling.

**Key words:** Germination, grape, *in vitro*, regeneration.

**Abbreviations:** BA = 6-benzyladenine; 2,4-D = 2,4-dichlorophenoxyacetic acid; $GA_3$ = gibberellic acid; IAA = indole-3-acetic acid; IBA = indole-3-butyric acid; MS = Murashige and Skoog (1962); NN = Nitsch and Nitsch (1969); NOA = 2-naphthoxyacetic acid.

## Introduction

Genetic improvement of grape by the adoption of molecular techniques is hindered by the low frequency of regeneration from *in vitro* cultured explants. However, Mullin's recent report on a successful genetic transformation of *Vitis rupestris* somatic embryos (Mullins *et al.* 1990) stresses the importance of improving the *in vitro* performance of grapevine. In grape somatic embryogenesis is usually induced from anthers (Hirabayashi *et al.* 1976; Bouquet *et al.* 1982; Gray and Mortensen 1987; Mauro et al. 1986; Rajasekaran and Mullins 1983; Stamp and Meredith 1988a; ) and ovules (Gray and Mortensen 1987; Mullins and Srinivasan 1976), but rarely from leaves (Hirabayashi 1985; Stamp and Meredith 1988a) and petioles (Reisch and Roberts 1985). In which explants of the latter, on the other hand, regeneration *via* organogenesis has been successfully reported (Cheng and Reisch 1989); Clog *et al.* 1990, and Stamp et al. 1990a, and 1990b). To obtain somatic embryogenesis with a high frequency from tissues such as leaves and petioles which are commonly available throughout the year, would be particularly useful. This paper reports a detailed study of somatic embryogenesis from such explants on *Vitis rupestris* (Martinelli *et al.* 1991). In our protocol somatic embryogenesis has been successfully obtained in established leaf- and petiole-derived callus cultures which have been described as unsuitable material for the induction of regeneration (Krul and Worley 1977).

## Materials and Methods

***Explant source and preparation***: Forty *Vitis rupestris* Scheele leaves with their petioles were harvested from locally cultivated plants in early spring, choosing the first three starting from the shoot tip. They were sterilized for 15 min in a 0.8% v/v sodium hypochlorite solution, and rinsed 3 times in sterile water. Leaves were cut in 40 $mm^2$ square pieces, and petioles in 2 mm long segments. Samples of 261 and 116 fragments were obtained from 40 leaves and petioles, respectively.

***Media preparation and culture vessels***: The pH of the media was adjusted with NaOH at 5.7 before autoclaving for 20 min at 121°C and 1 atm. When needed, filter-sterilized (0.22 µm pore size) IAA was added to the media after autoclaving. For solid cultures, 30 ml medium were dispensed on plastic Petri dishes of 9 cm diameter. For liquid cultures, 50 ml medium were poured in 125 ml flasks closed with a cottonwool plug covered with aluminum foil.

***Callusing, embryogenesis induction and culture***: Leaf explants were placed with their lower surface in contact with MS-based medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 0.8% Difco Bacto-agar, and petiole cuttings were placed horizontally. Explants were incubated at 25+1ÉC in the dark and, after callusing, in a 16 h light photoperiod (60 mol m-2s-1 cool white). Eight combinations of the growth regulators BA and 2,4-D were adopted to optimize culture conditions for callus and somatic embryogenesis induction (the BA/2,4-D combinations

*Correspondence to:* L. Martinelli

were: 0.2/0, 1/0, 0/0.1, 0.2/0.1, 1/0.1, 0/1, 0.2/1 and 1/1 $mgl^{-1}$). Cultures were transferred every four weeks to fresh media.

***Embryonic cultures***: Embryogenic calli were propagated on solid medium containing 0.8% Difco Bacto-agar, while liquid medium was used to induce multiplication and elongation of single embryos. MS- versus NN- (Nitsch and Nitsch 1969) formulations, either full- or half-strength, containing 3% sucrose, were compared, and the requirement of casein hydrolysate (250 $mgl^{-1}$) tested. The effect of two auxins was also tested, by adding IAA (1 $mgl^{-1}$) or IBA (0.1 $mgl^{-1}$) separately to the basic media. Approximately 2700 and 6800 mg of leaf- and petiole-derived embryogenic callus were used to start solid cultures. Two 125 ml flasks with 50 ml of each medium were prepared for liquid cultures. Groups (0.135 g) of small somatic embryos (length 1 - 2 mm) were poured into every flasks. Cultures were incubated at 25°C in dim light (15 mol $m^{-2}s^{-1}$) and media renewed every three weeks. Each 50- ml liquid culture was divided into two flasks; to each of them 25 ml of fresh medium were added. They were shaken continuously at 90 rpm.

***Secondary somatic embryogenesis***: Single somatic embryos were isolated from liquid cultures and placed horizontally on solid half strength MS- or on full strength NN-based media, both containing 0.8% Difco Bacto-agar and supplemented with IAA (1 $mgl^{-1}$) or IBA (0.1 $mgl^{-1}$) respectively. The cultures were incubated at 25°C in dim light (15 mol $m^{-2}s^{-1}$) and moved to fresh medium every four weeks.

***Somatic embryo germination***: A population of 1481 somatic embryos (214 from leaf- and 1267 from petiole-derived callus, respectively) was employed to define the optimal cultural conditions for embryo germination. Embryos were placed with the radicle downward on full- or half-strength NN-based solid media containing 3% sucrose, 250 $mgl^{-1}$ casein hydrolysate and 0.8% Difco Bacto-agar. The effects of BA (1 $mgl^{-1}$), IBA (0.01 and 0.1 $mgl^{-1}$) and combinations of BA/IBA (0/0, 1/0.1 and 1/0.01 $mgl^{-1}$) were tested. Chilling treatment (15 days in the dark at 4°C) versus standard cultural conditions (25°C at 16-h photoperiod, 70 mol $m^{-2}s^{-1}$ cool white light) was also assayed. Samples of 111 chilled and 103 non-chilled somatic enbryos from leaf-derived callus, and of 946 chilled and 321 non-chilled somatic embryos from petiolederived calli were employed (Tab. 1).

***Plantlet culture***: The embryo-derived plantlets were grown in a climate room at 25±1°C with a 16h photoperiod (70 mol $m^{-2}s^{-1}$ cool white light) in Magenta boxes containing 70 ml growth regulatorfree NN-based medium with 1.5% sucrose and 0.8% Difco Bacto-agar.

## Results and Discussion

***Callus induction and culture***: Combinations of BA and 2,4-D appeared convenient for callus induction and culture (Krul and Worley 1977; Bouquet *et al.* 1982; Rajasekaran and Mullins 1983; Stamp and Meredith 1988a; Martinelli *et al.* 1991). All media tested supported a considerable callus formation after a 10 day-induction. Further propagation produced a large amount of soft and friable callus. A regular supply of fresh medium resulted in the establishment of cultures still growing after nearly three years.

***Induction of somatic embryogenesis:*** After a 7-month culture, white globular embryogenic structures differentiated on the surface of leaf- and petiole-derived calli, subcultured on 1/0.1 and 1/1 $mgl^{-1}$ BA/2,4-D respectively, at the rate of 3.2% and 4.2% respectively. Somatic embryogenesis took place after the completion of the callus induction (Martinelli *et al.* 1991). A different developmental pathway was described by Stamp and Meredith (1988a), who obtained a primary embryogenic process taking place directly from the explant rather from the callus.

After removing the embryogenic tissues, the undifferentiated calli were again subcultured on fresh media, and new embryogenic callus reappeared after 10 (petiole callus) and 18 months (leaf callus), suggesting that embryogenic competence was retained.

***Embryonic cultures***: Basic medium composition and casein hydrolysate proved equally effective in supporting embryonic cultures. Both auxins (IAA 1 $mgl^{-1}$ or IBA 0.1 $mgl^{-1}$) supported either the embryogenic callus propagation on solid culture, or the multiplication and elongation of isolated embryos in liquid culture. Normal embryos appear white and clearly polarised, with two cotyledons and a radicle (Fig. 1B). Also fused cotyledons (Stamp and Meredith 1988a) and three cotyledons (Mullins and Srinivasan 1976) were observed. A well-established embryogenic callus (Fig. 1A) and liquid embryogenic cultures are still propagated after more than two years.

***Secondary somatic embryogenesis***: In grapevine secondary embryogenesis has been observed on zygotic (Stamp and Meredith 1988b) as well as on somatic embryos (Krul and Worley 1977; Gray 1989; Vilaplana and Mullins 1989). In our protocol, secondary somatic embryogenesis was induced on single somatic embryos isolated from the liquid cultures. Secondary embryogenesis began on the root/shoot transition zone (Fig. 1C), confirming a gradient of embryogenic competence along the embryo tissues (Krul and Worley 1977). Both media supplemented with 1 $mgl^{-1}$ IAA or 0.1 $mgl^{-1}$ IBA proved suitable.

***Somatic embryo maturation and germination***: Germination of embryos is a limiting step during somatic embryogenesis of grapevine, while germination efficiency is reduced by embryo dormancy (Rajasekaran *et al.* 1982; Mauro *et al.* 1986; Gray 1989). In spite of these drawbacks, we obtained a great number of plantlets from petiole callus embryos. On the contrary, a low frequency of germinating embryos was obtained from leaf-derived callus (6 plants from 214 somatic embryos). Worth stressing, the careful choice of the embryos appeared to be a crucial factor supporting germination, because this occurred only in white well shaped, well polarized embryos, with root and shoot axes, a hypocotyl and two cotyledons (Fig. 1B) (Gianazza *et al.* 1992).

Fig. 1D shows the effect of the growth regulator composition during a 2-month induction of germination in the

**Table 1**: Efficiency (percentage) of embryo germination in chilled and unchilled somatic embryos derived from petiole calli cultured on 6 different growth regulator formulations, during a 9-month culture.

| Growth regulators ($mgl^{-1}$) BA | IBA | Chilling | Number of embryos tested | Germination efficiency during a 9-months culture 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.0 | 0.0 | + | 164 | 2 | 6 | 6 | 6 | 6 | 7 | 7 | 7 |
| | | - | 60 | 0 | 0 | 0 | 7 | 10 | 12 | 12 | 12 |
| 1.0 | 0.0 | + | 174 | 0 | 4 | 5 | 5 | 5 | 5 | 5 | 5 |
| | | - | 101 | 0 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| 0.0 | 0.01 | + | 84 | 0 | 1 | 1 | 2 | 6 | 7 | 7 | 7 |
| | | - | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 0.0 | 0.1 | + | 133 | 0 | 4 | 5 | 6 | 6 | 6 | 6 | 7 |
| | | - | 70 | 5 | 5 | 5 | 8 | 12 | 24 | 42 | 51 |
| 1.0 | 0.01 | + | 303 | 9 | 12 | 13 | 13 | 13 | 13 | 13 | 13 |
| | | - | 47 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1.0 | 0.1 | + | 88 | 1 | 7 | 8 | 9 | 11 | 14 | 14 | 14 |
| | | - | 43 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

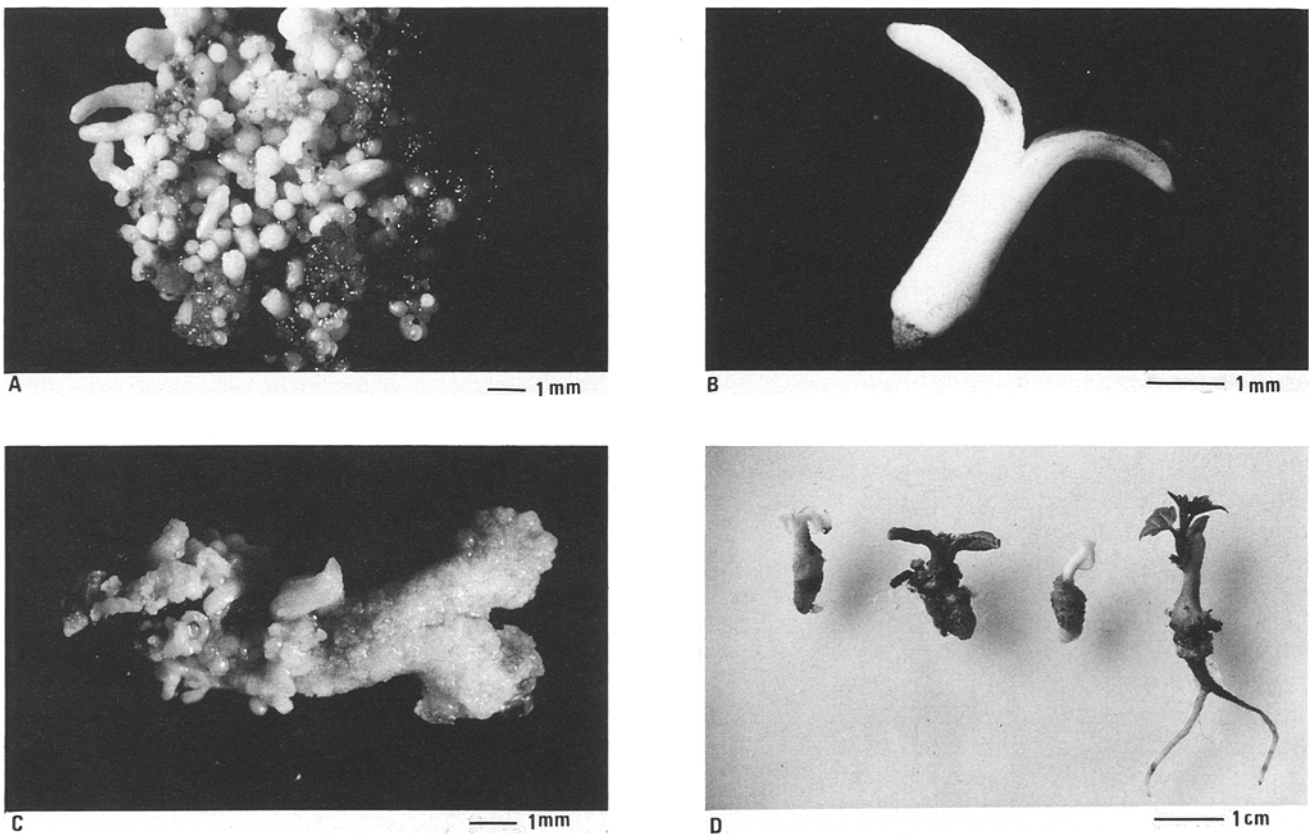


**Fig. 1.** - Main steps typical of somatic embryogenesis of *Vitis rupestris*. A: Embryogenic callus. B: Somatic embryo. C: Induction of secondary somatic embryogenesis on a single somatic embryo. D: Germination of somatic embryo: influence of chilling and four growth regulator combinations; from the left: no growth regulators, BA, IBA, and BA with IBA.

presence of chilling, and Tab. 1 reports the efficiency of embryo germination obtained from petiole-derived callus, during a 9-month culture.
For both chilled and unchilled embryos, the lowest efficiencies were in the presence of BA alone.
When chilling was provided in the presence of BA and IBA combined - at both 1/0.1 and 1/0.01 $mgl^{-1}$ ratios - a large number of plantlets germinated. Within 2 to 4 months, germination efficiency reached desirable levels (8 to 13 % respectively) and stabilized within 7 months (14 and 13 % respectively). When embryos were incubated at a constant temperature of 25°C, no plantlets were obtained with the same combination of growth regulators.
When either BA, or IBA, or no growth regulators were added to the medium, in the presence of chilling, germination efficiency decreased (5 to 7 %) and germination occurred between 2 and 6 months. In the absence of chilling, on the other hand, very high efficiencies (from 12 to 51%) were observed, but germination was dramatically delayed (from 6 to 9 months). Thus, chilling increased the speed of embryo germination. Furthermore, without chilling, embryos frequently produced a green disorganised callusing tissue, and from this tissue, plant regeneration occurred *via* organogenesis rather than embryo germination. Due to this, and in view of the length of the germination process in unchilled cultures, chilling appeared the preferable alternative. However, this treatment, commonly used as a dormancy-breaking strategy on zygotic embryos, already proved effective on somatic embryos (Rajasekaran and Mullins 1979; Rajasekaran and Mullins 1982; Takeno *et al.* 1983; Gray 1989; Mauro *et al.* 1986). Either in presence and absence of chilling, deformations, vitrification and inhibition of germination of somatic embryos were observed in presence of $GA_3$ (Stamp and Meredith 1988b), a growth regulator which has been reported as a stimulator of embryo germination in the absence of chilling (Mullins and Srinivasan 1976; Rajasekaran and Mullins 1979; Stamp and Meredith 1988a).
***Plantlet culture***: The plants germinated from somatic embryos showed a normal phenotype, and in two plantlets only, out of the many obtained in our laboratory, *in vitro* flowering has been observed.

## Conclusions

*Vitis rupestris* proved a good system for establishing a procedure for somatic embryogenesis going from explant to plantlet. This paper constitutes the first detailed report of somatic embryogenesis from established callus culture of leaf and petiole explants. However, embryogenic callus induction from callus cultures appeared the limiting step of the overall process, and the reported levels are susceptible of improvement. Efforts are required to increase the efficiency of embryogenetic induction, and to eliminate the lag time before plant germination. The culture conditions used in this experiment proved, on the other hand, suitable for embryo culture and propagation over long periods of time, and for plant germination which occurred with satisfactory efficiency.
Induction of secondary embryogenesis appears a suitable strategy in the *Agrobacterium*-mediated genetic transformation of grapevine.

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