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Somatic embryogenesis from grapevine cells. I-Improvement of embryo development by changes in culture conditions

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Abstract

In conventional culture conditions without auxin, somatic embryos arising from suspension cultures of grapevine rootstock 41B (*Vitis vinifera* cv. Chasselas × *Vitis berlandieri*) are arrested at the heart stage of development. Starting from indications that inhibitors excreted in the culture medium could be responsible for this arrest, new culture conditions based on daily subculturing embryos in fresh medium have been successfully used to obtain full embryo development. From this technique, a microassay was devised for screening small amounts of extracellular molecules as potential inhibitors of embryonic development. Our results show that extracellular macromolecules of molecular weight higher than 10 kDa are likely involved in the inhibition of caulinary meristem initiation. However, other factors obviously cooperate to inhibit embryo development in conventional culture conditions.

Abbreviations: CH76 – cv. Chardonnay clone 76 (*Vitis vinifera*), NOA – 2-naphthoxyacetic acid

Introduction

Somatic embryogenesis of grapevine has been described in several instances. The first description of regeneration of plantlets by the route of somatic embryogenesis was reported for an ancient clone of grapevine (*Vitis vinifera* L. cv. Cabernet Sauvignon) in liquid culture conditions (Mullins & Srinivasan 1976). Formation of adventitious embryos was also obtained in solid agar medium culture conditions from callus of 'Seyval', a French hybrid of grape (Krul & Worley 1977). More recently, other cultivars have been shown to be competent for somatic embryo production and different explants like leaves, anthers, zygotic embryos and ovaries were used for somatic embryogenesis induction (Bouquet et al. 1982; Rajasekaran & Mullins 1983; Bessis & Labroche 1985; Mauro et al. 1986; Gray & Mortensen 1987; Lebrun & Branchard 1987; Stamp et al. 1988a, 1988b). In liquid medium, it is possible to obtain a large number of somatic embryos, but in many cases, the yields of plantlets produced were low and most embryos were abnormal (Rajasekaran & Mullins 1979; Srinivasan & Mullins 1980; Bessis & Labroche 1985; Mullins 1986). This hampers the possibility of obtaining transgenic plants by somatic embryogenesis after genetic transformation of grapevine cells in suspension culture.

We have obtained interesting responses with some grapevine cultivars like 41B (*Vitis vinifera*

cv. Chasselas × *Vitis berlandieri*) that showed a good competence for producing globular and heart-stage embryos in suspension culture. However, further embryo development was blocked at these stages and few embryos developed into plantlets. For other cultivars like cv. Chardonnay clone 76 (*Vitis vinifera*), the cells showed a low competence for embryo production in liquid culture.

Reasons for the arrest of embryo development are not precisely known. One possibility could be the shortage of essential elements necessary for the transitions from heart to torpedo stage. For example, specific extracellular glycoproteins have been shown to promote somatic embryo development in carrot (De Vries et al. 1988a, 1988b). A second and better documented possibility would be the accumulation of inhibitory factors in the medium. Ethanol, acetaldehyde (Perata et al. 1988), ethylene (Vain et al. 1989a, 1989b; Sinska 1989) and specific glycoproteins (De Vries et al. 1988a, 1989) have been shown to perturb the development of somatic embryos. These studies and some others (Halperin 1967; Hari 1980; Huang et al. 1990) pointed out that high cell population density in embryogenic cultures is a negative parameter for the expression of somatic embryogenesis.

In order to develop an efficient system for regeneration of grapevine via somatic embryogenesis, we investigated the possibility that inhibitory factors released by cells into the medium could be responsible for the blockage of somatic embryogenesis in this species. The basic strategy used was to maintain the embryogenic suspensions at low cellular density by daily subcultures in fresh medium in order to avoid the accumulation of extracellular inhibitors. This strategy was adapted to volumes of cell suspension as low as 500 µl, allowing test of small amounts of extracellular macromolecules.

Material and methods

Plant material and embryogenesis in conventional culture conditions

The initial cell suspensions derived from the rootstock 41B (*Vitis vinifera* cv. Chasselas × *Vitis berlandieri*) and the cv. Chardonnay clone 76 (*Vitis vinifera*) were supplied by Moët et Chandon (Epernay, France). These three-year-old cell suspensions were established and subcultured every week according to Lebrun & Branchard (1987) in 250 ml Erlenmeyer flasks with 80 ml of medium (5 µM NOA) and inoculated with 5 mg ml^{-1} (fresh weight) of cells. Suspension cultures were grown on an orbital shaker (110 rpm) at 21°C in the dark.

The culture medium used to obtain the development of somatic embryos was a modified Murashige & Skoog (1962) liquid medium (half concentration of macroelements; 18 g l^{-1} maltose and 4.6 g l^{-1} glycerol instead of sucrose; 1 g l^{-1} casein hydrolysate) without auxin (medium termed GMo). The pH was adjusted to 5.8 with NaOH and the medium autoclaved for 20 min at 120°C. The undifferentiated cells cultured with NOA were successively filtered through nylon screens of 500 and 200 µm pore size. Cell clusters retained on the 200 µm filter were washed 3 times and resuspended in 30 ml of GMo medium. The packed cell volume (PCV) was determined after sedimentation (1xg) of the cells in a conical graduated tube. The embryogenic suspension cultures were inoculated at the density of 1 µl PCV ml^{-1} in 250 ml Erlenmeyer flasks with 80 ml of GMo medium. These flasks were placed at 26°C under continuous light (OSRAM L 36W/20 cool white fluorescent tubes) on an orbital shaker (80 rpm).

After 21 days of culture, the embryos were transferred to a solid agar medium (half-strength Murashige and Skoog macro and micro nutrients; 20 g l^{-1} sucrose; 7 g l^{-1} Bacto-agar Difco; pH 5.8) without any growth regulators, to obtain plantlets. The conditions of culture were 25°C, light intensity of 27 µmol m^{-2} s^{-1} (Mazdafluor A9 TFRS 40/BI fluorescent tubes) with a 16-h photoperiod.

Embryogenesis under daily subculture conditions

For a daily transfer of embryos to a new medium, the culture medium was removed by aspiration. About 10 ml of GMo were added and PCV was determined after sedimentation (1xg) of the embryos in a conical graduated tube.

Eighty µl of sedimented embryos were resuspended in 80 ml of fresh GMo medium.

Histological analysis

For histological analysis, embryos were fixed in 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5, for 7 h, rinsed in buffer and post-fixed in 1% osmium tetroxide for 1 h. After several washes in the same buffer, they were dehydrated to 100% ethanol, impregnated with propylene oxide and embedded in Spurr's resin (Spurr 1969). Specimens were then cut to 2 µm. The removal of the epoxy resin was performed by exposure to bromine vapors for 2 min (Yensen 1968), followed by 4 washes (2 min each) in absolute acetone and 2 washes (2 min each) in absolute methanol. Sections were classically rehydrated and double stained with methylene blue-safranin.

Obtaining and assaying extracellular macromolecules

Extracellular macromolecules were collected according to the technique of De Vries et al. (1988a). After 12 days of culture in GMo medium in conventional conditions, 1 l of medium was concentrated 200–500 fold by pressure ultrafiltration using Amicon YM 10 (10 kDa cut-off) membranes. The concentrate was extensively dialyzed against 10 mM phosphate buffer, pH 6.5, using the same ultrafiltration technique. Amounts of total proteins were determined by Bradford assay (Bradford 1976). Samples were sterilized by filtration (0.2 µm pore size) before addition to cell cultures. For the assay of macromolecule effect on embryo development in microtitration wells, the proembryogenic masses (PEMs) were inoculated in GMo medium at 1 µl of PCV ml^{-1}. Aliquots of 500 µl of this suspension were dispensed in 24-well microtitration plates (Nunc) and cultured at 26°C on an orbital shaker (130 rpm). After 4 days, embryos were daily subcultured by removing the medium by aspiration and replacing it by 500 µl of fresh GMo medium. Every day, extracellular macromolecules were added to the fresh medium at different protein concentrations. Embryos subcultured without addition of macromolecules were supplied with the same volume of 10 mM phosphate buffer, pH 6.5. Embryos at the different stages of development were counted after 10 days of culture as described by Coutos-Thevenot et al. (1990) and the percentage of the different embryo stages was estimated by reference to the total number of embryos (100 embryos counted).

Results and discussion

Improvements introduced by daily subculturing 41B and CH76 embryos in fresh medium

In conventional culture conditions, the 41B cell line displays a high capacity for somatic embryogenesis after auxin removal. The PEMs produced numerous globular embryos after 4 days of culture (Fig. 1A). After 10 days, most of these embryos reached the heart stage. However, between day 10 and day 20 (Fig. 1B) there was no further development of these embryos, which remained at globular and heart stages (Table 1).

After 4 days of continuous culture, PEMs from the 41B cell line were subcultured daily, leading to further embryo development. After 6 daily subcultures, most of these embryos had an elongated axis, two well developed cotyledons and an emerging root (Fig. 1C). After 20 days of culture, 91% of the embryos had developed into plantlets (Table 1; Fig. 1D) with green cotyledons and root elongation. Under these conditions, 96 dissected plantlets (among 100 examined) exhibited one shoot apical meristem and the first leaf primordia. These results were confirmed by a light microscopy study of the control and daily subcultured embryos. Only subcultured embryos developed well-structured root and shoot apices, two cotyledons and vascular bundles (Fig. 2A). By contrast, conventionally cultured embryos were blocked at the globular or heart-shaped stage, without meristem, vascular bundles and cotyledons (Fig. 2B). After transfer to solid agar medium, 80% (mean of 5 independent experiments) of the cotyledonary embryos exhibited the capacity to develop into normal plants (Table 1) and 20% developed large cotyledons without appearance of shoot apex. No plants could be obtained from control embryos grown without subculturing.

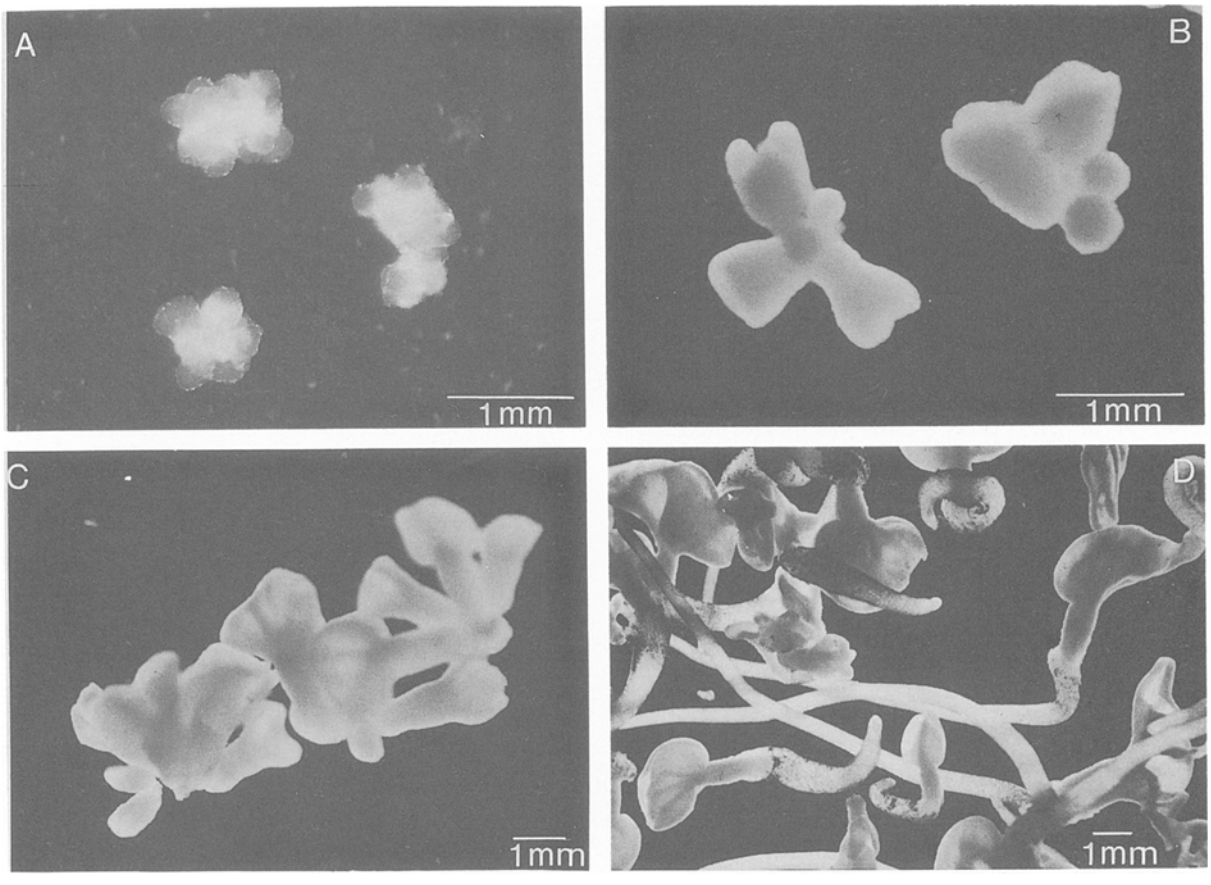

Fig. 1. Time course of embryo development in suspension cultures of grapevine rootstock 41B in different culture conditions. (*A*) Morphology of PEMs after 4 days of culture in GMo medium. (*B*) Morphology of embryos after 20 days of conventional culture. (*C*) Morphology of 10-day-old embryos, 6 days after the beginning of subcultures. (*D*) Morphology of 20-day-old embryos, after 16 days of subculture.

Table 1. Proportion of different embryogenic stages produced by 41B cell line as a function of culture conditions.

Transfer conditions	Stages (%)				
	Globular	Heart	Torpedo	Plantlets	Developed plants
20 days without transfer	40	60	0	0	0
4 days without transfer +16 daily transfers	0	1	8	91	80
10 days without transfer +16 daily transfers	17	68	15	0	0

Globular, heart and torpedo embryos as well as plantlets were counted after the end of the culture in liquid medium (20 or 26 days). The percentage of developed plants was determined 40 days after transfer of embryos to solid agar medium.

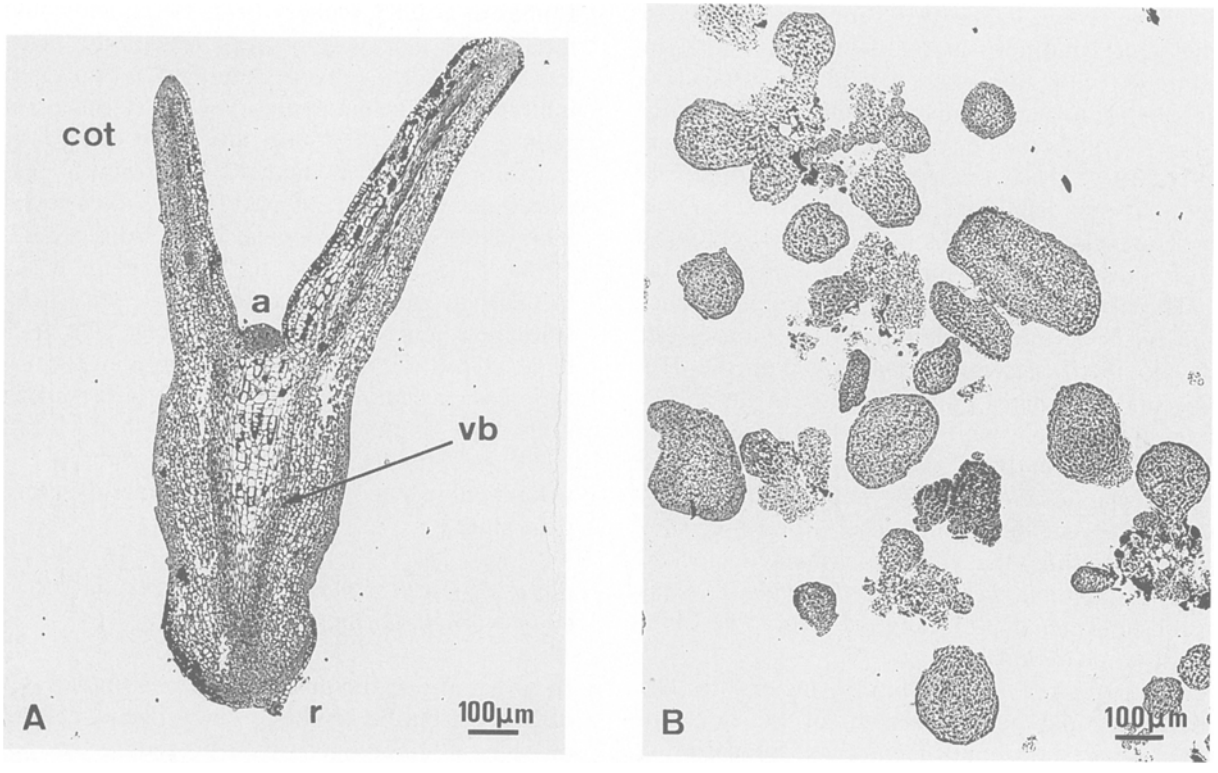

Fig. 2. Histological study of grapevine rootstock 41B embryos. (*A*) Daily subcultured embryos after 14 days of culture (10 days of daily transfers); cot: cotyledon, a: shoot apex, r: root tip, vb: vascular bundles. (*B*) Control embryos after 14 days in conventional culture conditions.

In the CH76 cell line, the development of embryos after auxin removal was more limited. After 10 days, some abnormal globular structures were observed in the suspension culture and many PEMs showed necrosis. Even after 20 days (Fig. 3A) there was no evidence of normal embryo development. In the CH76 line the competence to develop somatic embryos was thus strongly reduced compared to that of the 41B cells.

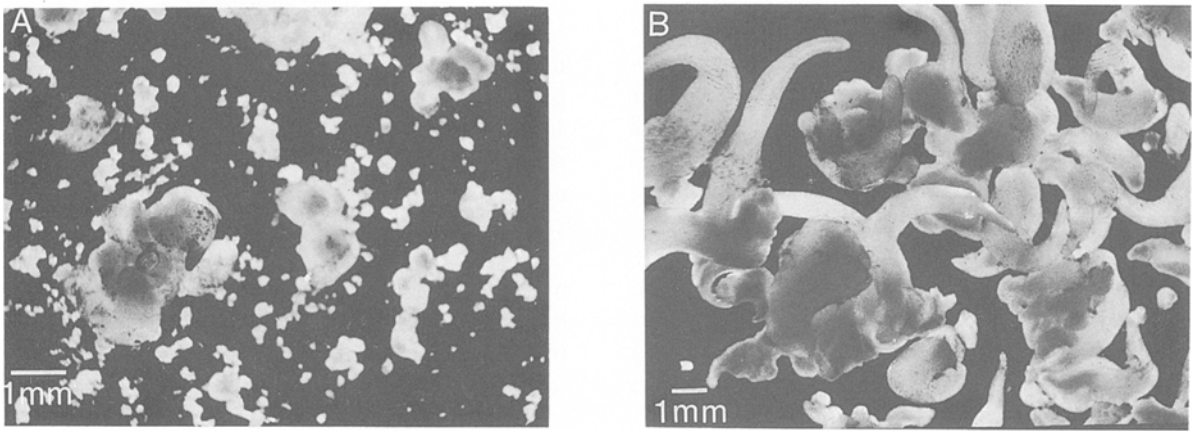

Fig. 3. Comparison between the morphology of embryos from CH76 line in conventional culture conditions or daily subculture conditions. (*A*) Morphology of abnormal structures produced after 20 days in conventional culture conditions. (*B*) Morphology of 20-day-old embryos under daily transfer conditions starting at day 4.

The CH76 cell line was subcultured daily in the same conditions as for the 41B cell line. In contrast with the non-subcultured suspension (Fig. 3A), a large number of embryogenic structures were developed after 20 days of culture (Fig. 3B). These embryos were abnormal, although some exhibited cotyledon, hypocotyl and root development. Plants were not obtained from these abnormal subcultured embryos.

These results demonstrate that a simple daily subculture at low population density induces full development of somatic embryos from the 41B line and stimulates embryogenesis in the CH76 line. In addition, we observed that subculturing should start early in order to be effective in modifying the embryogenic pattern. If the first subculture was performed after 10 days of continuous culture, the embryos remained blocked and no plantlets were obtained (Table 1). This result indicates that blockage of embryo development is not reversible.

Accumulation of inhibitory factors in the medium could be responsible for the developmental arrest in control embryos. Subculturing introduces several important modifications in gas concentrations, extracellular pH values, ethanol and acetaldehyde concentrations and extracellular amounts of macromolecules as referenced below. Daily opening of the culture flasks could be responsible for decreased concentrations of ethylene and CO_2, which accumulate with time. These gases are known to regulate many aspects of plant growth including somatic embryogenesis (Yang & Hoffman 1984; Sinska 1989; Vain et al. 1989a, 1989b). Extracellular pH is another important parameter that is modified by the subcultures. In our 41B cell line of grapevine under conventional culture conditions, the extracellular pH decreased from 5.8 to 4.1 after 4 days and then increased gradually from 4.1 to 4.9 during the following 8 days of culture. In contrast, in subcultured suspensions, the external pH value was maintained around 5.8 after day 4 by the daily supply of fresh GMo medium. Such external pH changes could be important in regard to the distribution of growth regulators like auxin between the intra and extracellular compartments as known in *Acer pseudoplatanus* cells, Zucchini membrane vesicles or crown gall cell suspensions (Leguay & Guern 1975; 1977; Lomax et al 1985; Rubery 1977, 1987), and could modify the embryogenic response of the cells. Ethanol or acetaldehyde, described as important inhibitors of somatic embryogenesis in carrot cells (Perata et al. 1988), are likely diluted by transfers to new medium. More recently, extracellular glycoproteins have been shown to inhibit embryo development (De Vries et al. 1988a). This is the case for example for a 52/54 kDa glycoprotein that inhibits embryo development in carrot cells at heart stage (De Vries et al. 1989). Extracellular glycoproteins, which are also accumulated in the medium during grapevine embryogenesis (unpublished data), could participate in inhibition of embryo development when not diluted by the subculture procedure.

Assay of effect of macromolecules on somatic embryogenesis in microtitration wells

In order to test the influence of macromolecules obtained from an inhibited embryogenic culture on somatic embryogenesis, a procedure was devised to study embryo development in microtitration wells. Control embryos grown in microtitration wells without medium changes displayed a developmental pattern identical to that of non-subcultured embryos grown in Erlenmeyer flasks and were blocked at heart stage (Fig. 4A). In contrast, embryos subcultured by daily replacement of their medium by 500 µl of fresh medium had already developed to the torpedo stage at 10 days (Fig. 4B).

The macromolecular fraction representing a mixture of polysaccharides, glycoproteins and proteins with a molecular weight higher than 10 kDa was tested, with a protein basis as a reference of quantity, for its ability to perturb embryo development. Usually, GMo medium of 12-day-old conventional embryogenic cultures contains 3 to 6 µg protein ml^{-1}. The extracellular macromolecules released by the blocked embryos were added daily at different concentrations to the medium of daily subcultured somatic embryos from the 41B line and their effect was scored.

Embryos treated with a macromolecular fraction corresponding to 3 µg ml^{-1} of proteins showed an abnormal caulinary apex with a pro-

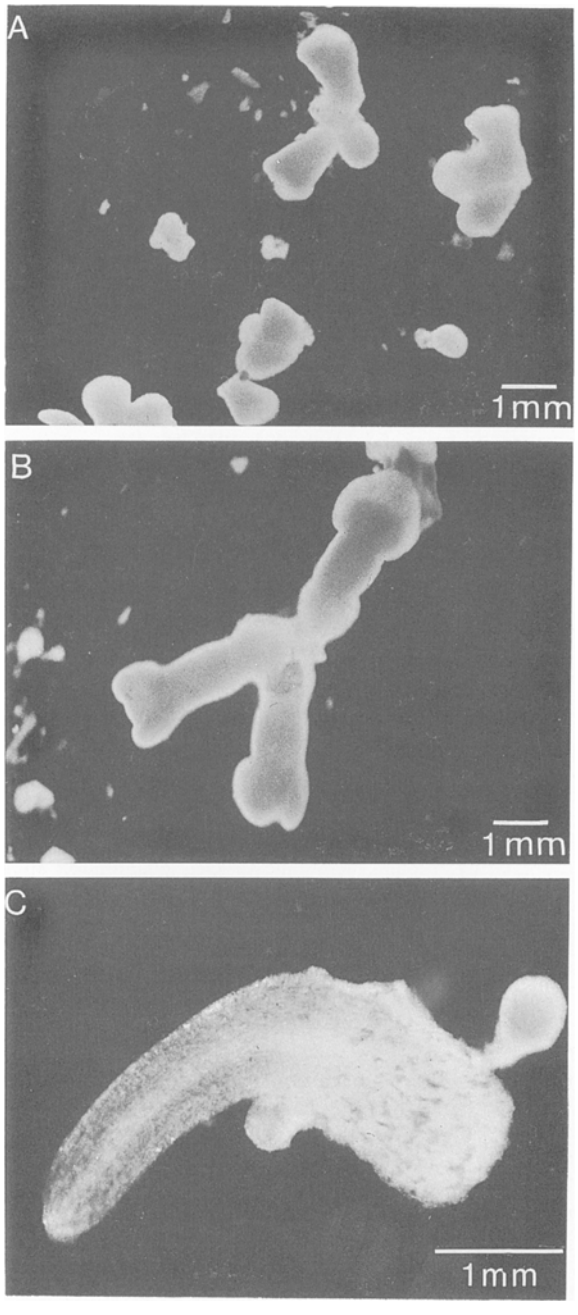

Fig. 4. Efficiency of subcultures in microtitration wells and effects of extracellular macromolecules on grapevine rootstock 41B embryo development. Observations were made after 10 days of culture. (*A*) Control embryos grown without subculturing. (*B*) Embryos subcultured from day 4 to day 10. (*C*) Embryos subcultured as in B and treated every day by the extracellular macromolecular fraction (3 μg protein ml^{-1}) of a 12-day-old embryo conventional culture.

liferation of secondary embryos (Fig. 4C). Mainly due to this formation of abnormal secondary embryos, the total number of embryos produced by the cultures increased as a function of increasing protein concentration (Fig. 5A), whereas the percentage of cotyledonary embryos decreased (Fig. 5B). However, the percentage of embryos with developed roots increased with the concentration of macromolecules (Fig. 5B). These results indicate that the macromolecular fraction dramatically disturbs embryo development, leading to a marked inhibition of the caulinary meristem development. They call for a characterization of the properties and biological activities of the components of this fraction with, according

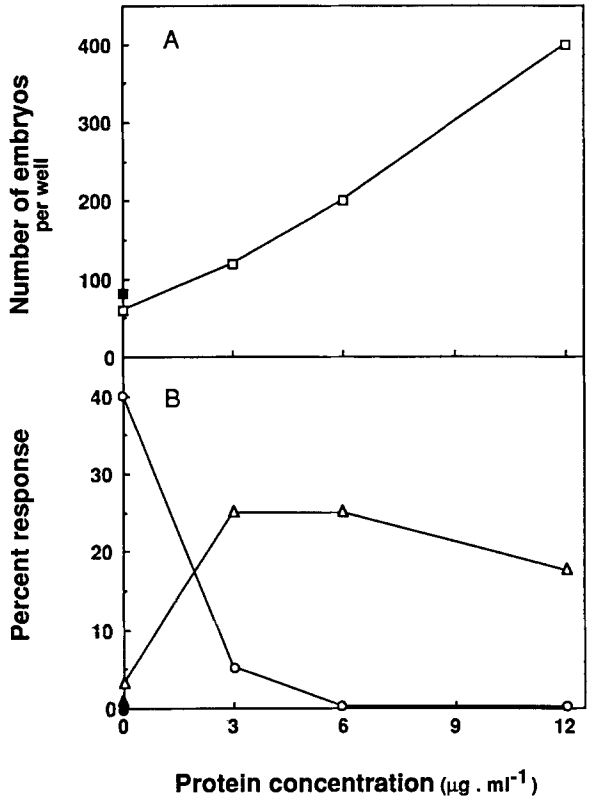

Fig. 5. Activity of extracellular macromolecules on the development of subcultured grapevine rootstock 41B somatic embryos. Embryo stages were counted after 10 days of culture in microassay culture conditions. Number of embryos represents the mean of two independent experiments each with four replicates. (*A*) Number of embryos in conventional culture conditions (■) or after daily subcultures (□). (*B*) Percentage of embryos with developed cotyledons in conventional culture conditions (●) or after daily subcultures (○), percentage of embryos with developed roots without subculture (▲) or after daily subcultures (△).

to the recent results concerning the model carrot systems, a special interest for extracellular glycoproteins (De Vries et al. 1988a, 1989; Sterk et al. 1991). However, the macromolecular fraction does not fully block embryo development since root growth is increased. These observations suggest that other factors cooperate with external macromolecules to inhibit embryo development in conventional culture conditions.

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References

Bessis R & Labroche C (1985) Callogenèse et régénération chez le Pinot, le Chardonnay et le Gamay. In: Moët-Hennessy (Ed) Amélioration de la Vigne et Culture *in vitro* (pp 127–134). Paris (France)

Bouquet A, Piganeau B & Lamaison AM (1982) Influence du génotype sur la production de cals, d'embryoïdes et de plantes entières par culture d'anthères *in vitro* dans le genre *Vitis*. C.R. Acad. Sci. Paris Ser. III 295: 569–574

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 249–254

Coutos-Thevenot P, Jouanneau JP, Brown S, Petiard V & Guern J (1990) Embryogenic and non-embryogenic cell lines of *Daucus carota* cloned from meristematic cell clusters: relation with cell ploidy determined by flow cytometry. Plant Cell Rep. 8: 605–608

De Vries SC, Booij H, Janssens R, Vogels R, Saris L, LoSchiavo FF, Terzi M & Van Kammen A (1988a) Carrot somatic embryogenesis depends on the phytohormone controlled presence of correctly glycosylated extracellular proteins. Genes & Development 2: 462–476

De Vries SC, Booij H, Meyerink P, Huisman G, Wilde DH, Thomas TL & Van Kammen A (1988b) Acquisition of embryogenic potential in carrot cell suspension cultures. Planta 176: 196–204

De Vries SC, Booij H, Cordewener J, Van Angelen FA, de Jong A, Van Kammen A, LoSchiavo F, Schellekens G, Sterk P & Terzi H (1989) Developmental mutants and extracellular proteins in carrot somatic embryogenesis. In: ADEBIO (Ed) International Symposium of Biotechnology for Major Crops (pp 22–23). Toulouse (France)

Gray DJ & Mortensen JA (1987) Initiation and maintenance of long term somatic embryogenesis from anthers and ovaries of *Vitis longii* '*microsperma*'. Plant Cell Tiss. Org. Cult. 9: 73–80

Halperin W (1967) Population density effects on embryogenesis in carrot cell cultures. Exp. Cell Res. 48: 170–173

Hari V (1980) Effect of cell density changes and conditioned media on carrot cell embryogenesis. Z. Pflanzenphysiol. 96: 227–231

Huang B, Bird S, Kemble R, Simmonds D, Keller W & Miki B (1990) Effects of culture density, conditioned medium and feeder cultures on microspore embryogenesis in *Brassica napus* L. cv. Topas. Plant Cell Rep. 8: 594–597

Krul WR & Worley JF (1977) Formation of adventitious embryos in callus cultures of 'Seyval', a French hydrid grape. J. Amer. Soc. Hort. Sci. 102: 360–363

Lebrun L & Branchard M (1987) Embryogenèse 'indéfinie' chez *Vitis* sp. cultivé *in vitro*. In: OIV (Ed) 3ème Symposium International sur la Physiologie de la Vigne (pp 38–41). Bordeaux, (France)

Leguay JJ & Guern J (1975) Quantitative effects of 2,4-dichlorophenoxyacetic acid on growth of suspension-cultured *Acer pseudoplatanus* cells. Biochim. Biophys. Acta 497: 329–333

Leguay JJ & Guern J (1977) Quantitative effects of 2,4-dichlorophenoxyacetic acid on growth of suspension-cultured *Acer pseudoplatanus* cells. II. Influence of 2,4-D metabolism and intracellular pH on the control of cell division by intra-cellular 2,4-D concentration. Plant Physiol. 60: 265–270

Lomax TL, Mehlhorn RJ & Briggs WR (1985) Active auxin uptake by zucchini membrane vesicles: quantitation using ESR volume and ΔpH determinations. Proc. Natl. Acad. Sci. USA 82: 6541–6545

Mauro MC, Nef C & Fallot J (1986) Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet-Sauvignon. Plant Cell Rep. 5: 377–380

Mullins MG & Srinivasan C (1976) Somatic embryos and plantlets from an ancient clone of the grapevine (cv. Cabernet-Sauvignon) by apomixis *in vitro*. J. Exp. Bot. 27: 1022–1030

Mullins MG (1986) Progress in the regeneration of grapevines *in vitro*. Vignevini 12: 29–32

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Perata P, LoSchiavo F & Alpi A (1988) Ethanol production and toxicity in suspension-cultured carrot cells and embryos. Planta 173: 322–329

Rajasekaran K & Mullins MG (1979) Embryos and plantlets from cultured anthers of hybrid grapevines. J. Exp. Bot. 30: 399–407

Rajasekaran K & Mullins MG (1983) The origin of embryos and plantlets from cultured anthers of hybrid grapevines. Amer. J. Enol. Vitic. 34: 108–113

Rubery PH (1977) The specificity of carrier-mediated auxin transport. Planta 135: 275–283

Rubery PH (1987) Auxin transport. In: Davies PJ (Ed) Plant

hormones and their role in plant growth and development (pp 341–362). Martinus Nijhoff Publishers, Dordrecht

Sinska I (1989) Interaction of ethephon with cytokinin and gibberellin during the removal of apple seed dormancy and germination of embryos. Plant Sci. 64: 39–44

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31–43

Srinivasan C & Mullins MG (1980) High-frequency somatic embryo production from unfertilized ovules of grape. Scientia Hort. 13: 245–252

Stamp JA & Meredith CP (1988a) Proliferative somatic embryogenesis from zygotic embryos of grapevine. J. Amer. Soc. Hort. Sci. 113: 941–945

Stamp JA & Meredith CP (1988b) Somatic embryogenesis from leaves and anthers of grapevine. Scientia Hort. 35: 235–250

Sterk P, Booij H, Schellekens GA, Van Kammen A & De Vries SC (1991) Expression pattern of carrot EP2 gene encoding an extracellular lipid transfer protein. The Plant Cell 3: 907–921

Vain P, Flament P & Soudain P (1989a) Role of ethylene in embryogenic callus initiation and regeneration in *Zea mays* L. J. Plant Physiol. 135: 537–540

Vain P, Yean H & Flament P (1989b) Enhancement of production and regeneration of embryogenic type II callus in *Zea mays* L. by $AgNO_3$. Plant Cell Tiss. Org. Cult. 18: 143–151

Yang SF & Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35: 155–189

Yensen J (1968) Removal of epoxy resin from histological sections following halogenation. Stain Technol. 43: 344–346