

# Efficient procedure for grapevine embryogenic suspension establishment and plant regeneration: role of conditioned medium for cell proliferation

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**Abstract** An efficient system for the establishment and multiplication of highly prolific embryogenic cell cultures of grapevine (*Vitis* sp.) was developed. Using anther-derived pro-embryogenic masses as starting material, cell suspensions of different grapevine cultivars (Tempranillo, Cabernet-Sauvignon) and rootstocks (Kober 125 AA, Kober 5 BB, 110 Richter) were initiated in liquid medium containing NOA (1.0 mg l<sup>-1</sup>) and BAP (0.25 mg l<sup>-1</sup>) as growth regulators. Conditioned medium was recovered and utilised for establishing new, highly totipotent cell cultures. The suspensions obtained, showed embryogenic competence resulting in somatic embryo induction and subsequent plant regeneration. In this study, a simplified establishment procedure for grapevine embryogenic cell suspension allowing the fast multiplication of embryogenic material is described. Evidence for the promoting effect of the protein fraction derived from conditioned medium, on cell proliferation was found. In bioassays, addition of  $\beta$ -D-GlcY affect cell proliferation suggesting that arabinoga-

lactan proteins are required for growth processes in grapevine cell cultures.

**Keywords** *Vitis vinifera* · Somatic embryogenesis · Cell suspension · Regeneration · Arabinogalactan proteins · Conditioned medium · Cell proliferation

## Abbreviations

AGPs	Arabinogalactan-proteins
BAP	6-benzylaminopurine
$\beta$ -D-Glc-Y	$\beta$ -D-Glucosyl Yariv phenylglycoside
CEPF	Concentrated extra-cellular protein fraction
2,4-D	2,4-Dichlorophenoxyacetic acid
EP	Extra-cellular protein
NOA	Naphthoxyacetic acid
MES	2-( <i>N</i> -Morpholino) ethanesulfonic acid
FM	Fresh medium
CM	Conditioned medium
PEM	Pro-embryogenic masses
PCV	Packed cell volume

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## Introduction

Embryogenic suspension cultures have been found to be a useful system for plant regeneration (Reustle et al. 1995; Taylor et al. 1996; Gupta and Conger 1999). Furthermore, somatic embryo development and synchronisation under liquid conditions were reported as crucial steps in taking full advantage of somatic embryogenesis for high production potential and reduction of unit cost through automation (Tonon et al. 2001). In grapevine, the world's most widely grown fruit crop, somatic embryogenesis represents the best system for plant regeneration and gene transfer technology

(Perl et al. 1996) as well as for cryo-preservation (Dussert et al. 1992; Wang et al. 2004). Somatic embryogenesis has been reported for several *Vitis* species (Rajasekaran and Mullins 1979; Mauro et al. 1986; Stamp and Meredith 1988; Martinelli and Gribaudo 2001). Embryogenic suspension establishment was described by Coutos-Thévenot et al. (1992a) and Jayasankar et al. (1999) and was shown to be the most suitable material for grapevine genetic engineering (Hebert et al. 1993; Kikkert et al. 1996; Vidal et al. 2003). However, embryogenic suspension establishment is often considered as a time-consuming procedure requiring high amounts of embryogenic material to get the suspension started. In addition, growth inhibition and browning of the suspension as well as loss of embryogenic competence of the cells was frequently observed.

van Hengel et al. (1998) postulated secretion of metabolites by cultured cells, contributing to the conditioning of the medium. Beside growth regulators, other classes of molecules have been recently identified as embryo-stimulating factors, especially, those secreted into the culture medium (Massonneau et al. 2005). It was suggested that some extra-cellular proteins such as arabinogalactan proteins (AGP), receptor protein kinases (RPK) and endochitinases were involved in embryogenesis (De Jong et al. 1992; van Engelen and De Vries 1992; Becraft 1998; van Hengel et al. 2002). AGP represent a protein family in which the core protein is linked to arabinogalactan residues. These compounds play an important role in plant development and morphogenesis (Majewska-Sawka and Nothnagel 2000), and were found in the culture media of various plant tissue culture systems (Immerzeel et al. 2004, 2006). For rose cell cultures, AGPs were reported to induce cell proliferation (Kimberly and Nothnagel 1997; Serpe and Nothnagel 1994).

The present study reports an efficient and reproducible procedure for the establishment of embryogenic cell suspension of grapevine using conditioned medium. The procedure results in highly proliferating embryogenic cell suspensions with competence for successful plant regeneration, offering a useful target material for grapevine transformation and the production of transgenic plants. The protocol presented in this paper suggests the presence of extra-cellular proteins, stimulating cell division and leading to a significant increase of callus fresh weight. Experiments were performed to identify some of these extra-cellular substances involved in cell proliferation.

## Materials and methods

### Induction of somatic embryogenesis

Five grapevine varieties were used in this study, the rootstocks 110 Richter (*Vitis berlandieri* × *Vitis rupestris*),

Kober 5 BB (*Vitis berlandieri* × *Vitis riparia*), Kober 125 AA (*Vitis berlandieri* × *Vitis riparia*) and two cultivars of *Vitis vinifera* (cv. Tempranillo and cv. Cabernet-Sauvignon). Embryogenic callus was induced from anther and ovary cultures on solid NN medium (Nitsch and Nitsch 1969) containing 2,4-D (1.0 mg l<sup>-1</sup>) and BAP (0.25 mg l<sup>-1</sup>) according to Mauro et al. (1986). After induction, maintenance and propagation of the embryogenic callus was performed on the same medium as for induction. The embryogenic material was subcultured onto fresh medium in 4-week intervals.

### Procedures for cell suspension establishment and maintenance

Embryogenic callus and proembryogenic masses (PEM) derived from anther-/ovary-culture were used for the initiation of embryogenic suspensions. As grapevine suspension medium, a modified NN69 liquid medium (Nitsch and Nitsch 1969) containing 0.5 g l<sup>-1</sup> MES, 20 g l<sup>-1</sup> maltose and 4.6 g l<sup>-1</sup> glycerol instead of sucrose (Coutos-Thévenot et al. 1992a) supplemented with growth regulators (1.0 mg l<sup>-1</sup> NOA, 0.25 mg l<sup>-1</sup> BAP) was used. The pH was adjusted to 5.8 with KOH before autoclaving (20 min at 120°C). For initiation of suspension, 0.5–2 g fresh weight of fine embryogenic callus (2–3 weeks post subculture) was transferred into a 500 ml Erlenmeyer flask containing 100 ml of medium. Suspensions were incubated in the dark on an orbital rotary shaker (100 rpm) at 24°C, and maintained by weekly exchange of 50% of the old medium with fresh medium.

For cell suspensions establishment, three different approaches were developed and compared:

1. Fresh medium procedure: suspensions were initiated and maintained as described above.
2. Increased volume procedure: suspensions were initiated in 50 ml fresh medium (FM) followed by weekly subculture, removing an increasing volume (10, 20, 30, 40 ml) of old medium and addition of an increasing volume (20, 30, 40, 50 ml) of FM until 100 ml final volume had been reached. Then, 50% of the medium was exchanged by FM in 7-day intervals.
3. Conditioned medium procedure: suspensions were initiated in a mixture of 50 ml conditioned medium (CM) and 50 ml FM, and subcultured in 1-week intervals by exchanging 50% of the medium by FM. The CM was collected from suspensions already established for about 2 months. Therefore, cells were allowed to settle down and 50% of the supernatant was collected and filter-sterilised (0.45 µm) using reusable bottle top filter unit.

Suspensions were initiated with 0.5, 1.0 and 2.0 g of grapevine embryogenic callus and cultivated for 6 weeks.

Fresh weight development of embryogenic callus in suspension was measured weekly by removing the entire liquid medium. For each treatment, three suspensions were initiated and fresh weight of these replicates was determined.

Embryogenic cell suspensions of zucchini (*Cucurbita pepo*) and non-embryogenic suspension of parsley (*Petroselinum crispum*) cultivated in MS medium (5 mg l<sup>-1</sup> 2,4-D, 3 mg l<sup>-1</sup> Kinetin) and Gamborg B5 medium (1 mg l<sup>-1</sup> 2,4-D) respectively were transferred into grapevine suspension medium (modified NN69) and cultivated for about 2 weeks before collecting CM as described before.

#### Concentration of extra-cellular proteins

Seven-day old CM (500 ml) from each grapevine variety was collected and filter-sterilised (0.45 µm). Proteins were concentrated 200-fold by 3.5 kDa cut-off membrane (Membra-Cel<sup>TM</sup> Dialysis Membranes, Serva). Resin (StragaClean<sup>TM</sup> Resin, Stratagene) was added to the concentrated protein fraction and shaken overnight at 4°C before centrifugation at 13,000 g to concentrate the resin-protein binding complex. The resin-protein complex was resuspended in glycine solution (pH 2) to separate proteins from the complex. Samples of concentrated CM (40 µl) from each variety were loaded on a nitrocellulose membrane (Hybond-P, Amersham Biosciences) using a Dot-blot device (Easy-Titer® Elifa System, Pierce, USA). Immunodetection was performed as described by Burnette (1981). AGPs were detected with MAC 207 monoclonal antibody (Carbosource Services, USA) against α-Glc α<sub>1-3</sub> α-Gal α<sub>1-2</sub> Rha carbohydrate chains. This antibody was recognized using a secondary mouse anti-rat antibody conjugated with peroxidase and revealed by chemiluminescent detection (lumilight western blotting kit, Roche Diagnostics).

#### Bioassays with extra cellular protein fraction

1. Concentrated extra-cellular protein fraction (CEPF) of each CM were added to initiate cell suspensions using 0.5 g callus fresh weight in 250 flasks containing 50 ml liquid FM.
2. Arabinogalactan (AG) from larch wood (Sigma-Aldrich, A-9788) was added into the FM at the initiation step for cell suspension and at each weekly subculture.
3. The β-D-GlcY reagent [1,3,5-tris (4-β-D-glycopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene] is a synthetic phenylglycoside which specifically binds to AGP. β-D-GlcY was added to grapevine embryogenic suspensions to determine the effect on cell proliferation. For this, 25 ml of CM were treated with β-D-GlcY (25 µM) for 2 h at room temperature. Half a gram of Tempranillo callus fresh weight was used to

start suspension with 25 ml FM and 25 ml β-D-GlcY treated CM.

#### Regeneration of plants from embryogenic suspensions

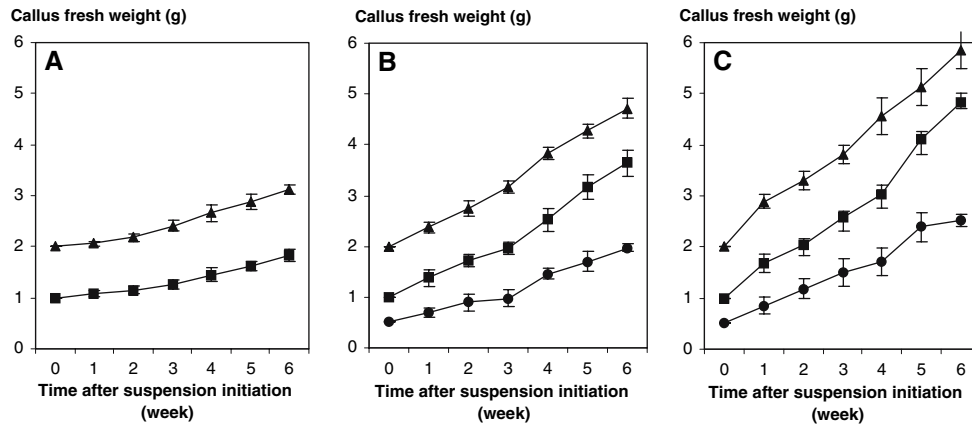
Six weeks after suspension establishment, induction of embryo development was started from the different grapevine varieties. Approximately, 500 µl of packed cell volume (PCV) determined after sedimentation of the cells in a 10 ml sterile pipette, was inoculated in 250 ml flasks containing 50 ml of growth regulator free medium. After 2 weeks of cultivation with a daily transfer of the material in growth regulator free FM, PEM progressively produced embryos at globular, heart and torpedo stage. Following 3–6 weeks development under daily subculture conditions, 25 elongated somatic embryos were transferred to growth regulator free NN69 medium (Nitsch and Nitsch 1969) supplemented with activated charcoal and solidified with 0.3% gelrite. Embryos were adapted stepwise to standard culture room conditions (16 h photoperiod, 60 µmol m<sup>-2</sup> s<sup>-1</sup> photon fluence rate) for conversion and plantlets where transferred to greenhouse conditions. For each variety, 25 somatic embryos were transferred per plate and 20 replicates were tested.

#### Results

##### Establishment of grapevine embryogenic cell suspensions

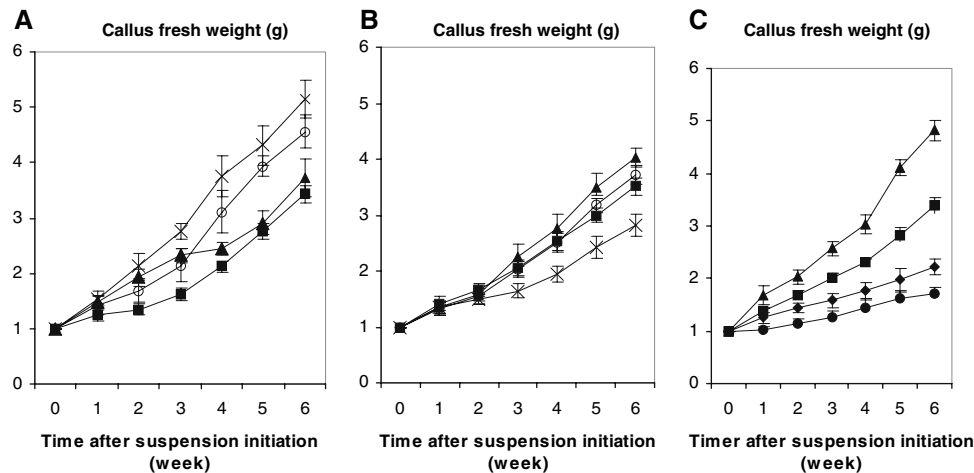
Under standard conditions (FM procedure), when embryogenic callus of grapevine was transferred from solid culture into fresh liquid medium, suspensions were established within 2–3 months. Frequently, cell suspensions turned brown with a dramatic decrease of growth when initiated with 2 g of callus (Fig. 1a). In contrast, the use of less than 1 g of callus for suspension establishment could not initiate cell division and proliferation (data not shown). However, with the increased volume procedure for suspension initiation, PEM started to grow gradually indicating improved growth conditions (Fig. 1b). Suspensions initiated with CM procedure showed vigorous proliferation of fine embryogenic cell aggregates and significant increase of fresh weight was observed (Fig. 1c).

Following the CM procedure, cell suspensions from Tempranillo, Cabernet Sauvignon, Kober 125 AA, Kober 5 BB and 110 Richter could be established within 3–5 weeks (Fig. 2a) using CM of *Vitis vinifera* cv. Tempranillo. A reciprocal experiment was performed using CM of these varieties for the initiation of new embryogenic cell suspensions of Tempranillo (Fig. 2b). Most of the suspensions



**Fig. 1** Efficiency of suspension establishment procedures. Experiments were carried out with embryogenic material of *Vitis vinifera* cv. Tempranillo. Starting inoculum: 0.5 g filled circle, 1.0 g filled square and 2.0 g filled triangle embryogenic tissue. **a** Fresh medium

procedure with 100 ml of fresh liquid medium. **b** Cell suspensions initiated by using increased volume procedure. **c** Cell suspensions initiated with 50% of conditioned medium (CM). Data represent average of three independent replications, bars indicate SD values



**Fig. 2** Effect of conditioned medium (CM) for establishment of grapevine embryogenic cell suspension. **a** Initiation of embryogenic cell suspensions of different grapevine varieties using CM of *Vitis vinifera* cv. Tempranillo. **b** Initiation of embryogenic cell suspension of *Vitis vinifera* cv. Tempranillo using CM collected from suspensions of different grapevine varieties: Kober 125 AA filled square, Kober 5 BB cross mark, Cabernet Sauvignon filled triangle and 110

Richter open circle. **c** Establishment of *Vitis vinifera* cv. Tempranillo embryogenic cell suspension with CM from parsley filled diamond and zucchini filled square suspensions, CM from Tempranillo as positive control filled triangle, FM as negative control filled circle. Data represents average of three independent replications, bars indicate SD values

showed a good multiplication rate and growth remained in an exponential phase. Furthermore, CM derived from embryogenic suspension of zucchini (*Cucurbita pepo*) and non-embryogenic parsley suspensions (*Petroselinum crispum*) could also induce embryogenic suspension establishment and cell proliferation of grapevine (Fig. 2c). This indicates the presence of common compounds in conditioned medium related to cell division processes.

To elucidate if peptides or extra-cellular proteins are responsible for this effect, autoclaving of CM was performed for protein denaturation. Results showed that the promoting effect of CM on biological activity of cell sus-

pensions was lost when autoclaved, resulting in growth performance as obtained with the FM procedure (data not shown).

#### Effect of extra-cellular proteins on grapevine suspensions

Supplementing concentrated extra-cellular proteins to fresh medium resulted in a promoting effect on suspension initiation and cell proliferation (Table 1). Fractionation of concentrated CM proteins by anion exchange chromatography (Mono-Q) yielded more than 100 separate fractions

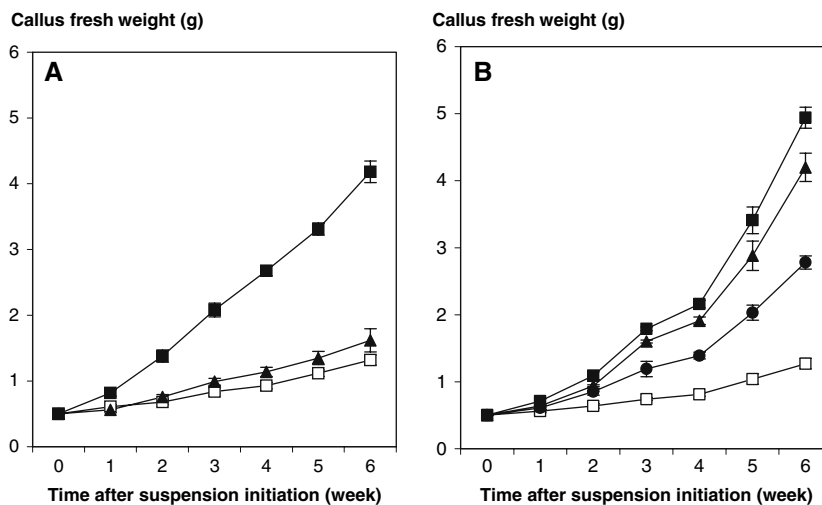
**Table 1** Influence of extra-cellular protein on *Vitis vinifera* cv. Tempranillo cell suspension initiation

Protein fraction tested	Fresh weight after 6 weeks (g)	Weight increase (%) after 6 weeks
FM (control)	1.20 ± 0.11	140
FM + CEPF (Tempranillo)	2.51 ± 0.14	402
FM + CEPF (125AA)	2.28 ± 0.13	356
FM + CEPF (Kober 5BB)	2.78 ± 0.12	456
FM + CEPF (110R)	2.26 ± 0.16	352
FM + CEPF (Cab Sauvignon)	1.78 ± 0.10	256

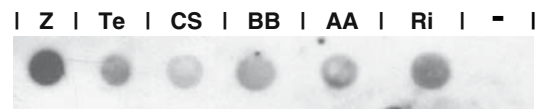
Fresh medium (FM) was taken as a control. Biological effects of protein-derived used media were tested by adding concentrated extra-cellular protein fraction (CEPF) to the medium with standard fresh weight of starting embryogenic material. Three replications were performed for each treatment. Standard errors are indicated as ±

(data not shown), making it difficult to test the activity of each fraction in bioassays.

To investigate the presence of AGP in our embryogenic cell suspension, β-D-GlcY, known to specifically bind and inhibit AGP, was applied to CM before suspension initiation. As shown in Fig. 3a, addition of 25 μM β-D-GlcY results in a prominent inhibition of the cell proliferation. In contrast, supplementing 1 and 2 mg of arabinogalactan from larch to FM demonstrated a stimulating effect on embryogenic cell proliferation (Fig. 3b). Weekly supplementing of arabinogalactan into suspensions did not show any difference of growth compared to arabinogalactan supply at suspension initiation only (data not shown). This points out to a secretion of AGP into the medium by



**Fig. 3** Influence of β-D-GlcY (a) and larch wood arabinogalactan (b) on growth of grapevine cell suspension starting with 0.5 g of embryogenic cells of *Vitis vinifera* cv. Tempranillo. **a** Addition of 25 μM of β-D-GlcY penylglycoside *filled triangle* to CM at the beginning of culture. *Filled square* CM, *open square* FM, **b** initiation of embryogenic cell suspensions of *Vitis vinifera* cv. Tempranillo



**Fig. 4** Dot-blot immuno-detection of arabinogalactan-protein with MAC207, a specific antibody against AGP. Z Zucchini, Te Tempranillo, CS Cabernet-Sauvignon, BB Kober 5BB, AA Kober 125 AA, Ri 110 Richter, - FM

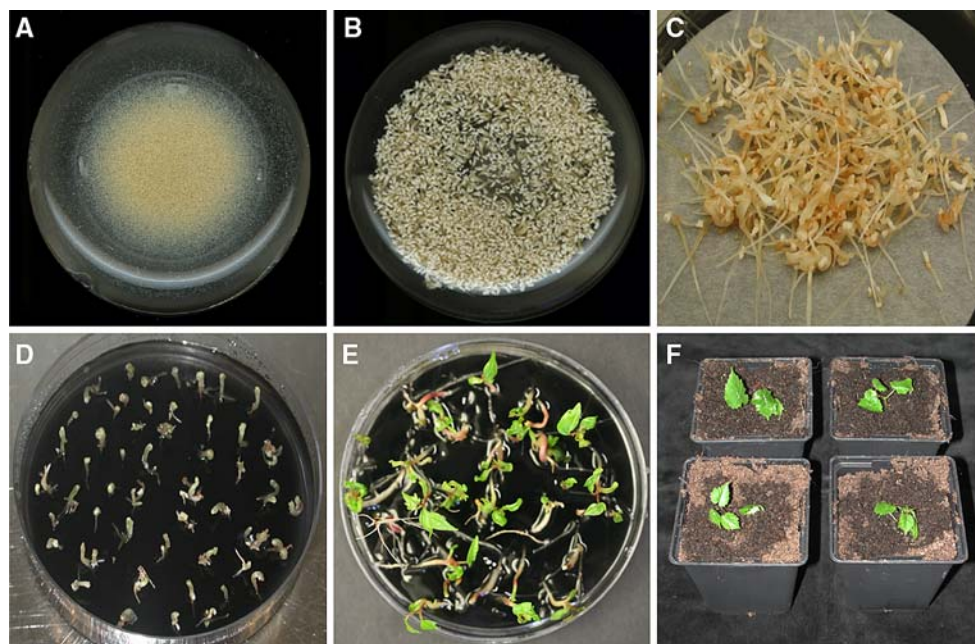
growing cell suspension and to a saturation of the promoting effect of AG/AGP to cell proliferation. We confirmed the presence of AGP in CM by immuno-detection technique. Figure 4 shows the detection of AGP for all grapevine varieties tested. A strong signal was observed in zucchini CM.

### Embryo maturation and plant regeneration

Established grapevine embryogenic cell suspensions were transferred to growth regulator free liquid medium to induce embryo formation and conversion into plantlets (Fig. 5). PEM showed the ability to produce globular embryo-like structures after removal of growth regulators. Heart and early torpedo stages were observed in suspensions after about 3 weeks of a daily fresh medium transfer procedure. A high capacity of synchronised somatic embryo development was observed 5–6 weeks after induction of regeneration. Elongated structures were counted and transferred onto solid medium (Fig. 5). The system produced thousands of somatic embryos per 50 ml of medium.

with 1 or 2 mg of arabinogalactan. Starting inoculum: 0,5 gram of callus fresh weight. *Filled square* CM, *open square* FM, *filled circle* treatment with 1 mg arabinogalactan, *filled triangle* treatment with 2 mg arabinogalactan. Data represent average of three independent replications, bars indicate SD values

**Fig. 5** Plant regeneration process from embryogenic cell suspensions of Kober 5BB. **a** Kober 5BB embryogenic cell suspension, **b** Embryo induction after cultivation in growth regulator free liquid medium for 2 weeks, **c** Mature and elongated embryos cultivated on solid medium for 10 days, **d** Somatic embryos picked separately and transferred to light (16 h light/8 h dark) for conversion, **e** Plantlets obtained from germinated embryos after about 1 month, **f** Regenerated grapevine plantlets transferred to greenhouse conditions



**Table 2** Somatic embryo induction in cell suspensions of grapevine and subsequent conversion to plantlets

Grapevine varieties	Callus production, 6 weeks after suspension initiation		Number of mature somatic embryos	Conversion efficiency	Conversion (%)
	PCV (ml)	FW (g)			
Kober 5 BB	21.04 ± 1.37	21.47 ± 3.01	5,627 ± 608	92/250	36.8
Tempranillo	31.27 ± 2.74	68.18 ± 2.21	10,647 ± 508	29/250	11.7
Cab. Sauvignon	11.95 ± 2.04	10.19 ± 1.34	2,820 ± 180	34/250	13.6

Suspension was started with 0.5 g embryogenic material. Growth of callus was measured by packed cell volume (PCV) and fresh weight (FW) 6 weeks post suspension initiation. Three Erlenmeyer-flasks were used to induce embryo formation. For plant regeneration assay on solid medium, 20 plates were tested in each experimental repetition. Standard errors are indicated as ±

Somatic embryo induction and conversion efficiency strongly differed between the different varieties (Table 2). Elongated embryos transferred onto fresh medium for regeneration, started to form green, shoot-like structures. Depending on the genotype, 11.8 to 36.8% of these embryos could finally be converted into intact plants. Generally, plantlets were obtained from all the varieties tested within about 3 months. However, plant regeneration from Kober 5 BB suspensions was achieved within 6 weeks and the time required for the complete plant development process starting from a proliferating embryogenic suspension was reduced to 8 weeks (Fig. 5).

## Discussion

The establishment and maintenance of friable embryogenic cell lines in suspension is difficult particularly for woody species. Previous reports on grapevine cell suspensions

describe procedures to produce germinating embryos from few varieties (Coutos-Thévenot et al. 1992a; Jayasankar et al. 1999, 2001). A number of studies have been attempted to improve productivity of the system by antioxidant supply or by culturing at low cell density and following frequent subcultures (Coutos-Thévenot et al. 1992a; Perl et al. 1996; Maes et al. 1997). However, the way to initiate liquid medium-based culture is still the main critical point.

Simultaneous embryo development was observed in *Fraxinus* (Tonon et al. 2001) and soybean (Kim et al. 2004), whereas a mixture of asynchronised embryo stages has been reported in grapevine and switchgrass (Jayasankar et al. 1999; Gupta and Conger 1999). Our data demonstrate an improved procedure for the establishment of highly totipotent embryogenic suspensions of different grapevine varieties using conditioned culture medium. The system allows the production of homogeneous suspensions with a good potential for plant regeneration ability. In contrast to

other reports on grapevine and potato (Jayasankar et al. 1999; Vargas et al. 2005) we obtained a high number of embryos suggesting our CM-procedure as being very efficient. Suspension-derived somatic embryos germinated precociously without undergoing a typical dormancy phase as previously reported by morphological and anatomical studies (Jayasankar et al. 1999, 2003).

In the present study, the use of conditioned medium strongly enhanced cell growth after transfer into liquid culture. Our results suggest a beneficial effect of conditioned medium at an intra-specific (*Vitis vinifera*) and inter-specific level (*Vitis* spp.) which so far has not been reported for grapevine. In both cases, conditioned medium components have a potential effect to increase grapevine PEM fresh weight. However, stimulation was completely lost by media autoclaving suggesting that secreted substances could have protein nature and thus are heat-denatured. CM collected from liquid culture of other species (zucchini and parsley) could also perform a stimulating effect on grapevine suspensions. These results support the hypothesis that cell cultures secrete proteins into the medium which are likely genotype-independent and responsible for the promoting effect on the initiation of new cell suspensions.

Several reports indicated that plant glycoproteins secreted into the medium accompany the formation of embryogenic clusters. So far, three different extra-cellular proteins (EP1, EP2, EP3) have already been identified in carrot cell cultures that however do not show any effect on cell division (De Jong et al. 1992). But it's also known, that secreted proteins from cell suspension such as arabinogalactan proteins (AGP) play a major role in cell proliferation and embryogenesis (Becraft et al. 1998; De Jong et al. 1992, 1993; Majewska-Sawka and Nothnagel 2000; Showalter 2001; van Hengel et al. 1998, 2001).

For that reason, we focused our investigation on the presence of AGP in the CM and its effect on grapevine embryogenic suspension. However, AGPs are secreted into the culture media at very low concentrations that do not exceed nanomolar ranges (van Hengel et al. 2001) making purification and direct analysis difficult. Addition of larch wood arabinogalactan ( $2 \text{ mg l}^{-1}$ ) for initiation of grapevine embryogenic suspensions strongly stimulates cell proliferation and production of PEM. Continuous weekly addition of arabinogalactan did not increase the multiplication of the PEM. We suggest that after the initiation process, secretion of AGP from proliferating embryogenic cells is sufficient to continuously stimulate growth of suspension. Also Kaporakis and Alderson (2003) showed the increase of pepper callus mass and viability by AGP addition. This indicates that AGPs are involved in induction of cell division.

Due to their high content of polysaccharide chains (Mau et al. 1995), AGPs do not migrate in SDS-PAGE and thus cannot be detected by Western blot analysis. However, dot-

blot immuno-detection by a specific antibody (MAC207) demonstrated presence of AGP in grapevine and zucchini CM. For zucchini Penell et al. (1989) and Showalter (2001) already reported detection of AGP by dot-blot immuno assays with this antibody. A clear correlation between the amount of AGP in conditioned medium and cell proliferation could not be identified. A high AGP content was detected for zucchini CM; however, the promoting effect of zucchini conditioned medium on grapevine cell suspension development was lower compared to conditioned medium originating from grapevine cell suspensions.

The results support the hypothesis that cell cultures secrete proteins into the medium which are likely genotype-independent and responsible for the promoting effect on the initiation and proliferation of cell suspensions. The amount of secreted AGP seems to depend on the structure of PEM leading to the suggestion that fine material allows more surface contact with liquid medium and provide better cell wall protein release.

Application of  $\beta$ -D-GlcY resulted in a strong inhibition of cell proliferation, indicating the importance of AGP for suspension initiation.  $\beta$ -D-GlcY binds selectively to arabinogalactan side chain by a mechanism that is not fully understood (Jermyn 1978; Guan and Nothnagel 2004) resulting in precipitation of an AGP- $\beta$ -D-GlcY complex. The low proliferation of grapevine cell suspensions in the presence of  $\beta$ -D-GlcY is hypothesised to be due to the perturbation of AGP. Altogether it confirms that extra-cellular AGP in the conditioned media are somehow involved in the induction of grapevine cell proliferation. These are the first results detecting AGP in grapevine embryogenic cell suspension and demonstrating their involvement in the cell proliferation process.

In rose (Serpe and Nothnagel 1994), carrot (Willats et al. 1999), sugar beet (Butowt et al. 1999) and tobacco (Qin and Zhao 2006) cell cultures, application of  $\beta$ -D-GlcY resulted in the arrest of growth and cell division. Similarly, experiments showed that inactivation of AGP alters the progress of cell division and cell wall expansion in undifferentiated cell cultures (Kimberly and Nothnagel 1997; Majewska-Sawka and Nothnagel 2000). Under moderate treatment with  $\beta$ -D-GlcY, the inhibition of proliferation is reversible after washing the cells and growth recovery could be partially achieved (Kimberly and Nothnagel 1997; Majewska-Sawka and Nothnagel 2000). In Arabidopsis cells, higher concentrations of  $\beta$ -D-GlcY induced programmed cell death within 3 days (Majewska-Sawka and Nothnagel 2000).

Glycoproteins splitted by cell wall enzymes might generate molecules like lipochito-oligosaccharides (LCO) homologous with Rhizobium-Nod factors (De Jong et al. 1993; Dyachock et al. 2002). These small molecules could stimulate proliferation of PEM as described in several re-

ports (Oropeza et al. 2001; van Hengel et al. 2002; Matthys-Rochon 2005). van Hengel et al. (2002) suggested that AGPs can be activated by endochitinase and may give rise to several molecules implicated in signalling pathway and controlling proliferation of plant cells. AGPs are substrate of enzymes to produce some components with hormone-like activity or act as a messenger for signal transduction which may contribute in development regulation and cell division (for review, see Showalter 2001).

To initiate embryo maturation and germination, beside the use of hormone free media, a frequent medium change resulted in the highest rate of regenerating embryos. As already reported by Coutos-Thevenot et al. (1992b), accumulation of secreted proteins in the suspension medium completely blocks embryo differentiation. Obviously, with the daily transfer procedure into fresh medium, secreted proteins were diluted to a non-inhibiting concentration, resulting in improved embryo elongation and plant regeneration efficiency. From our experiments it is not clear, if AGP inducing intensive proliferation of embryogenic grapevine suspensions, become inhibitors of embryo differentiation during regeneration steps. However, it seems that cell proliferation and differentiation appear as two distinct morphogenetic pathways which require different environmental conditions. Previous studies proposed that AGPs stimulate somatic embryogenesis (Chapman et al. 2000; Majewska-Sawka and Nothnagel 2000; van Hengel et al. 2002).

Grapevine embryogenic cell suspensions are a useful tool for regeneration but remained difficult to establish. Here we describe a standard procedure, allowing the establishment of embryogenic suspension of grapevine. The improvement of our protocol is related to the fast establishment of productive suspensions yielding mature somatic embryos with competence for plant regeneration. The results of the current study suggest an important role of conditioned culture medium with a significant effect of extra-cellular proteins to promote cell division and effective multiplication with plant regeneration ability. Evidence of the involvement of AGP in the control of cell proliferation is given. However, the molecular mechanism involved remains unknown. Identification of AGP and other secreted components accumulated in conditioned media are currently in process, and may give a better understanding of cell proliferation and differentiation of grapevine embryogenic cell cultures.

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