Isolation and culture of grapevine protoplasts

G. Reustle and G. Alleweldt

Bundesforschungsanstalt für Rebenzüchtung Geilweilerhof, D-6741 Siebeldingen, F. R. Germany

Summary: Methods were established for isolation of protoplasts from different organs and tissues of grapevine plants grown *in vitro*. Cell division could not be induced in protoplasts from leaves, shoot tips and petioles of cv. Optima, whereas stem and root protoplasts showed division activity. Protoplasts derived from stems continued developing and formed microcalli and calli. In experiments using stem protoplasts of several varieties, root and stem protoplasts divided in all cases; stem protoplasts of 4 varieties (Riesling, Kerner, Optima, Vidal) could be regenerated to callus. In leaf protoplasts, cell division could be induced only in case of cvs Vidal and Rupestris du Lot, however without formation of callus.

Key words: Protoplast, isolation, variety of vine, leaf, shoot, root, in vitro culture, methodology, cell division, cell wall formation, yield, viability, callus regeneration.

Introduction

For some objectives of plant improvement, tissue culture, e. g. meristems and embryos, are used with the advantage of the ease to regenerate whole plants.

Protoplasts are also the object of intensive investigations. They are useful for basic studies on plant physiology and in particular, they offer new alternatives in plant breeding. They are helpful for effective selection of somaclonal variation at the cellular level, for somatic hybridization and for genetic transformation via direct DNA uptake.

A prerequisite for the use of protoplast systems is the regeneration to plants (Krul 1989). Regeneration from protoplasts is possible for many plant species and in the case of potato, protoplast techniques e. g. fusion, are well established and already integrated in breeding programs (Puite *et al.* 1988).

In spite of many investigations with grapevine protoplasts (Shimizu 1985; Wright 1985; Barbier and Bessis 1987 a, 1987 b), plant regeneration has not been reported and only few scientists have obtained callus from protoplasts of grapevine tissue (Skene 1975; Brezeanu and Rosu 1984; Yamakawa *et al.* 1985; Lee and Wetzstein 1988). The presented results describe the isolation procedure for grapevine protoplasts and the effect of the donor material on cell division and callus formation of protoplasts.

Material and methods

The protoplast isolation method was developed using leaf material of *in vitro* grown grapevines, cv. Optima. *In vitro* cultures were established on LS-medium (Linsmaier and Skoog 1965) supplemented with 0.01 ppm NAA and 0.03 ppm BAP. Culture conditions were 14 h photoperiod, 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity and 24-26 °C.

In some experiments, donor plants were cultured in medium with reduced ammonium concentration (150 mg/l) and without hormons. Furthermore, donor plants were incubated at reduced growth conditions (10 $\mu E \cdot m^{-2} \cdot s^{-1}$, 8 °C) for 2-6 weeks prior to isolation.

In a further attempt, leaves were preconditioned according to Haberlach *et al.* (1985) in order to induce cell division of protoplasts.

Abbreviations: NAA, 1-naphtaleneacetic acid; BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; MES, 2 (N-morpholino)ethanesulfonic acid: BSA, bovine serum albumin: PVP, polyvinylpyrrolidone.

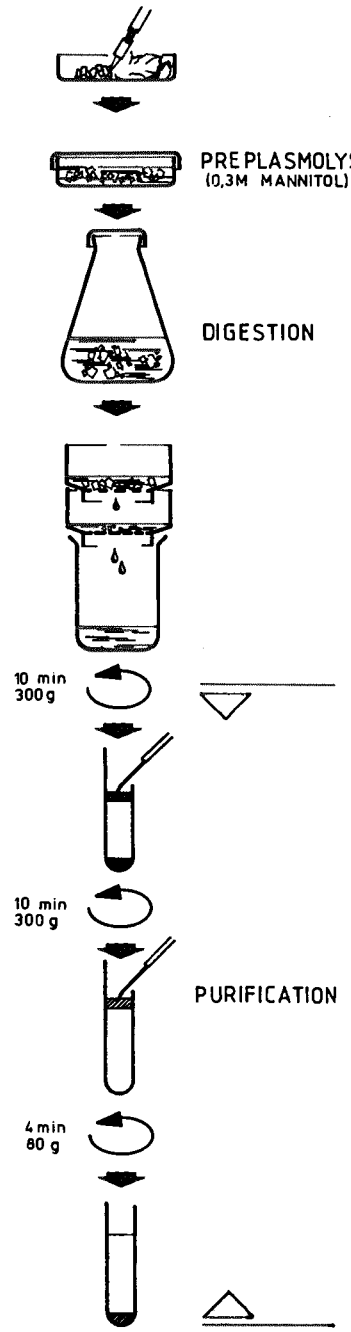

Fig.1: Isolation procedure.

Isolation

Prior to isolation procedure (Fig. 1), donor plants were cultured in darkness for 24 h.

Plant material was cut into small pieces and incubated for 15 min in 0.3 M mannitol for preplasmolysis. Afterwards, the material was transferred to an enzyme solution for digestion. The solution contained a combination of three different cellulases: cellulase *Aspergillus niger* (0.2-0.8 %), cellulase *Penicillium funiculosum* (0.2-0.8 %) and cellulase *Trichoderma viride* (0.4-1.6 %) (De Filippis and Ziegler 1985) and Macerozyme R-10 (0.1-0.5 %). The mixture was supplemented by BSA (0.5 %), MES-KOH (20 mM), $CaCl_2$ (1 mM), VKM-salts (1/10 strength), according to Binding and Nehls (1977) and 0.54 M sucrose or 0.6 M mannitol. Cellulase concentration and duration of digestion depended on the used material.

After digestion, the protoplast suspension was sieved (100 μm and 50 μm) to eliminate large undigested pieces. To purify the solution from cell debris and broken cells two flotation steps (300 *g* for 10 min) in 0.6 M sucrose followed by a sedimentation step (80 *g* for 4 min) in wash-solution (VKM-salts, 18.7 g/l NaCl) were carried out. Finally the protoplast pellet was resuspended in VKM-culture medium (Binding and Nehls 1977).

Cell density was adjusted to 2-5 • 10^5 protoplasts/ml medium. Yield was estimated in a counting chamber, viability and cell wall formation were detected using fluorescein diacetate and calcofluor white, respectively.

Culture

Protoplasts were cultured in darkness at 26 °C. The culture medium was supplemented with different substances (BSA, PVP-40, amino acids) and 1 ppm 2,4-D and 0.5 ppm BAP. Treatments of subculture were carried out, depending on the development of protoplasts and the intensity of browning of the medium.

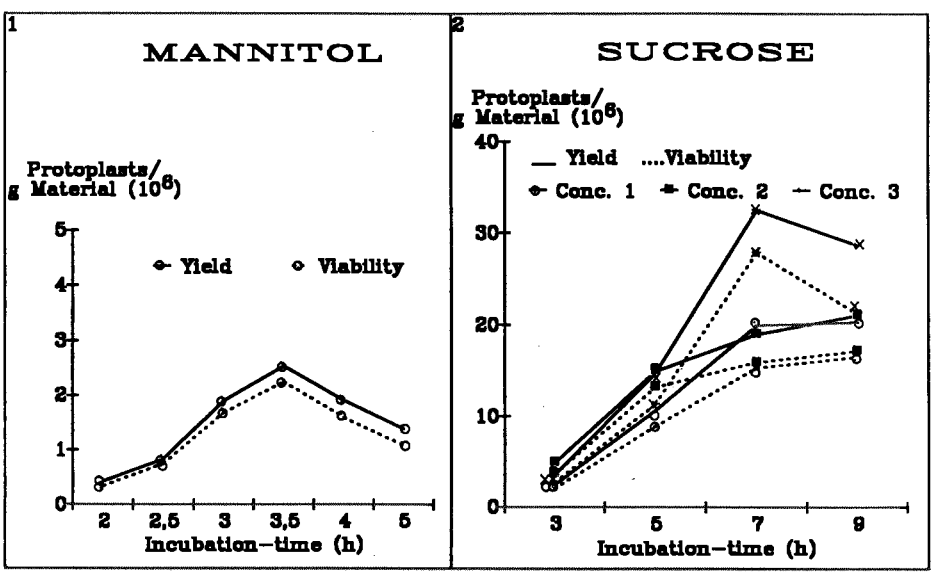

Fig. 2: Effect of osmoticum, enzyme concentration and incubation time on yield and viability of leaf protoplasts (enzyme concentration see Table 1).

Table 1: Effect of incubation time and enzyme concentration on viability and cell wall formation (CWF) of leaf protoplasts after 14 d of culture

Duration of Isolation	Conc.1 Viability %	Conc.1 / CWF* %	Conc.2 Viability %	Conc.2 / CWF %	Conc.3 Viability %	Conc.3 / CWF %
3 h	37,4	17,4	68,9	28,3	40,9	10,9
5 h	31,7	13,5	33,1	18,9	33,9	13,9
7 h	21,0	9,2	10,5	3,4	19,7	7,9
9 h	13,8	7,9	8,4	5,0	9,7	3,8

	Conc.1	Conc.2	Conc.3
cellulase (total concentration)	0,8 %	1,6 %	3,2 %
macerozyme R-10	0,1 %	0,2 %	0,4 %

*) cell wall formation;

Results and discussion

Isolation

Besides enzyme concentration and time of digestion, the release of protoplasts from leaf material was influenced by the osmotic substances, mannitol and sucrose, used (Fig. 2).

Mannitol resulted in a typical release of protoplasts depending on time of isolation. An optimum of yield was obtained after 3-4 h of digestion. Longer incubation time resulted in a reduction of yield and viability of protoplasts (Fig. 2.1).

With sucrose as osmoticum (Fig. 2.2), different cellulase concentrations as well as different durations of digestion were tested. Only the highest concentration (3.2 % cellulase, 0.4 % Macerozyme R-10) in combination with the longest duration of digestion (9 h) resulted in a reduced yield and viability of protoplasts. Furthermore protoplast yield was higher with sucrose than with mannitol as osmoticum after digestion using the same enzyme concentrations and durations of digestion.

This phenomenon may be the result of procedural differences since one centrifugation step was omitted when using sucrose. Another explanation could be a direct influence of sucrose on stability of protoplast membranes or on harmful substances in the digestion solution.

A measurement of the protoplast viability is their development in culture. Protoplasts, isolated with different cellulase concentrations and incubation times using sucrose as osmoticum (Fig. 2.2) were cultured and viability and cell wall formation were investigated after 14 d of cultivation. As shown in Table 1, viability as well as cell wall formation were distinctly influenced by incubation time, whereas the effect of enzyme concentration was not clear.

The influence of the intensity of isolation on protoplast development was demonstrated with these experiments. Moreover it is shown, that a short incubation time with an intermediate enzyme concentration is preferable to a longer duration of digestion with lower enzyme concentrations.

Culture

Different factors influencing protoplast culture were tested but few improved the protoplast development (Table 2).

To prolong viability of protoplasts, the addition of BSA (directly from the beginning of cultivation) and PVP-40 (added after 1-2 weeks of cultivation) was important to eliminate harmful phenolics.

Damaging effects could be observed with sucrose as osmoticum in the culture medium when compared to glucose, mannitol and sorbitol containing media.

Table 2: Effect of different factors on the development of leaf protoplasts

	reaction
Growth Conditions of the Donor Plants	
reduced Ammonium concentration	0
growth on hormonefree medium	0
reduced growth conditions	-
Preconditioning of the Leaves	
for 3 days	0
for 7 days	0
Culture Conditions	
reduced temperature (18°C)	0
light intensity (50 $\mu E \cdot m^{-2} \cdot s^{-1}$)	-
Protoplasts Density	
> $1 \cdot 10^6$ or < $1 \cdot 10^5$	-
$2\text{-}5 \cdot 10^5$	+
Culture Medium	
Bovine Serum Albumin (2,5 g/l)	+
Polyvinylpyrrolidon (5 g/l)	+
Glucose, Sorbitol, Mannitol as Osmoticum	0
Sucrose as Osmoticum	-
Amino Acids (Alanin, Glutamine Acide, Cystein)	0

+: improved viability and cell wall formation;
0: no clear reaction;
-: accelerated death of the cultures;

Neither different growth conditions of donor plants and the preconditioning of leaves (Haberlach *et al.* 1985) nor the tested culture conditions and media could induce cell division.

Optimal conditions for leaf protoplast cultivation resulted in cell wall formation starting approximately at the 3rd d of culture and in few cases initial cell division occurred. Divisions and further development could not be observed. Cultivation of leaf protoplasts was not possible for longer than 4-5 weeks.

Donor material

As shown in different plant species, division capacity of protoplasts depends on the kind of donor material used for isolation (Potrykus *et al.* 1977; Vasil and Vasil 1979; Binding *et al.* 1981; Lenee and Chupeau 1986).

To examine the regeneration capacity of protoplasts from different tissues and organs of grapevine (leaves, shoot tips, petioles, stems, roots and callus), isolations under under suitable conditions were performed (Table 3).

Table 3: Enzymatic treatments for protoplast isolation from different tissues and organs of grapevine

Donor Material					
Leaves	Shoot tips	Petioles	Stems	Roots	Callus
Enzymatic Treatment					
conc.1 3h	conc.2 5h	conc.1 7h	conc.2 16h	conc.2 8h	conc.2 8h

	conc.1	conc.2
Cellulase *Aspergillus niger*	0,4 %	0,5 %
Cellulase *Penicillium funiculosum*	0,4 %	0,5 %
Cellulase *Trichoderma viride*	0,8 %	1,0 %
Macerozyme R-10	0,2 %	0,5 %

Differences in development between protoplasts from young and older leaves could not be found. Most of the protoplasts died within the first few days of culture followed by a phase of

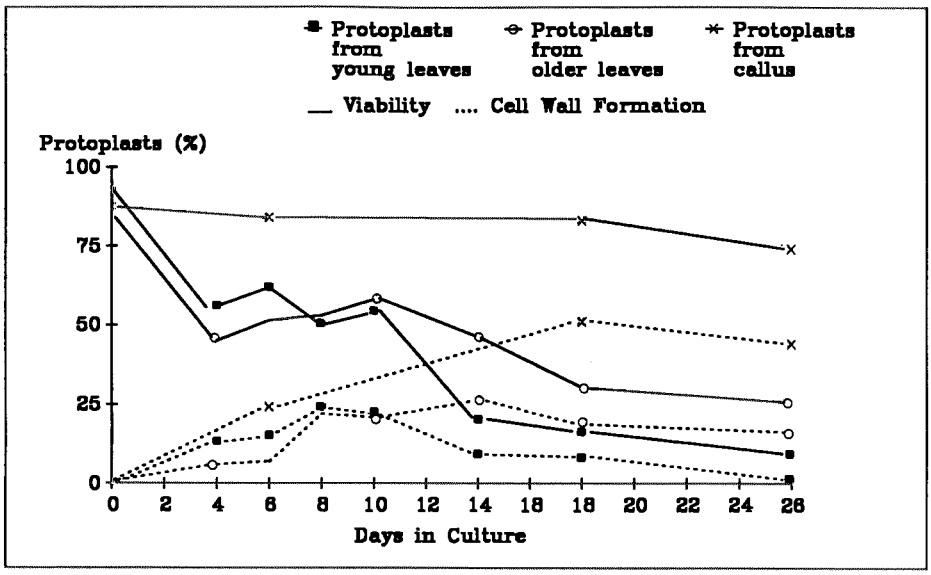

Fig. 3: Process of viability and cell wall formation of protoplasts from different starting material.

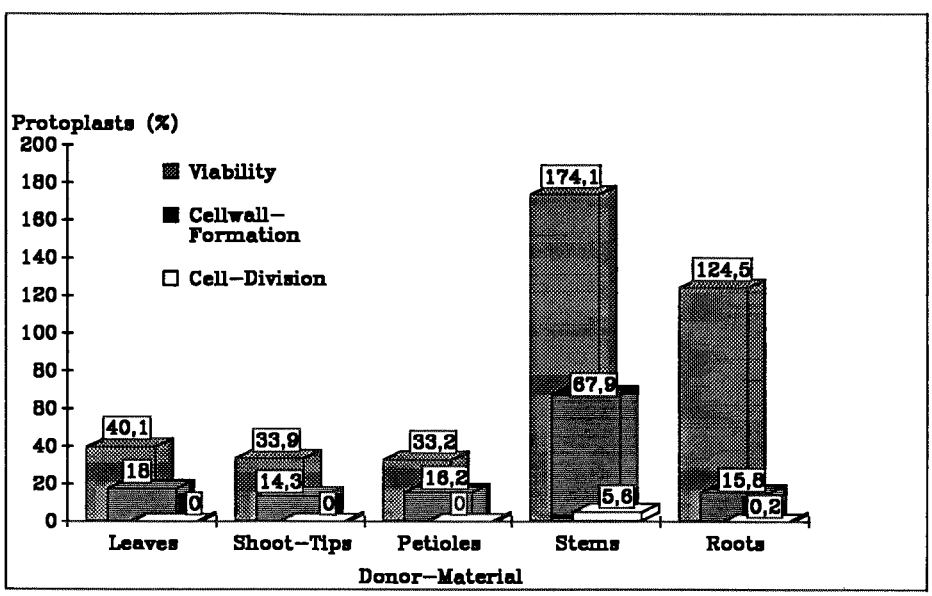

Fig. 4: Development of protoplasts derived from different organs of grapevine after 14 d of culture.

stabilization of the rate of viable protoplasts. After 10-12 d, the rate of viability decreased drastically and could not be stopped by changing or supplementing the medium. In contrast, protoplasts derived from callus suspensions showed high viability and intensive cell wall formation for a duration of 3 weeks (Fig. 3), but no cell division occurred.

The development of protoplasts from petioles and shoot tips was similar to the development of those isolated from leaves. After 14 d of culture, only 33-40 % of the cells were viable and 14-18 % had formed a new cell wall (Fig. 4). In contrast, protoplasts derived from stems and roots showed an intensive development. Cell wall formation started during the first few days and after 3-5 d first cell divisions could be observed.

In case of stem and root protoplasts due to evaporation of medium (10 % in 14 d) and proliferation of cells by cell division, density of viable cells increased. In comparison, the division activity of root protoplasts (0.2 %) was much lower than that of stem protoplasts (5.6 %).

Protoplasts with high division activity are supposed to be of cambial origin. Isolation experiments with stems showed that a reduced incubation time led to protoplasts with reduced division activity (results not shown). The short time of digestion (8 h, Table 3) in which root protoplasts were obtained could be an explanation for the reduced division activity of these protoplasts.

The higher regeneration capacity of stem protoplasts was confirmed in further observations. Only cultures of stem protoplasts showed further divisions and formed microcalli and calli, whereas the cultures of root protoplasts did not show further development and turned brown in the 3rd week of cultivation. The low division activity of root protoplasts was probably responsible for this behaviour.

Different cultivation techniques – liquid culture, solid culture, cultivation in special Biomembrane containers – as well as suitable methods of subcultivation were tested to establish a successful and easy regeneration system for grapevine protoplasts. Changing or supplementing the media to reduce harmful phenolics in the cultures was not effective. Moreover, the cultivation of protoplasts in solid medium could not stimulate protoplast development. The only successful method was cultivation in liquid medium on a solid reservoir-medium. Using this technique, stem protoplasts formed microcalli and within 8-10 weeks after isolation, visible calli developed.

Finally, regeneration capacity of protoplasts from tissue of different cultivars was tested (Table 4). Stem protoplasts from 4 of a total of 8 varieties tested formed callus. In the other cases, either the division activity was too low or the production of phenolics was too high (Rupestris du Lot) and the cultures died. Except of Vidal and Rupestris du Lot, leaf protoplasts did not divide, whereas root protoplasts of all tested varieties showed first division but did not form callus.

Table 4: Regeneration capacity of protoplasts from tissues and organs of different varieties

Varieties	Leaves	Roots	Stems
Optima	CW	D	C
Riesling	CW	D	C
Kerner	CW	/	C
Müller Thurgau	CW	/	D
Orion	CW	/	D
Vidal	D	D	C
Seyval	-	D	D
Rupestris du Lot	D	D	D

/: not tested;
-: no reaction;
CW: protoplasts showed cell wall formation;
D: protoplasts showed first division;
C: protoplasten formed callus;

These results show the importance of the donor material for regeneration experiments of protoplasts. In the case of grapevine, stems seem to be an appropriate material to obtain protoplasts with high division capacity.

Further regeneration of the achieved calli to plants is presently being attempted. Toward this goal, these calli are held on different media and under different culture conditions. In particular, donor material which could yield protoplasts with a high regeneration capacity is used. For improved starting material, suspensions of embryogenic callus could be useful. With 'recalcitrant' grapevine cultivars the production of embryogenic suspensions may be possible. Embryogenic suspensions of several important varieties have been reported by BESSIS and LABROCHE (1985), MULLINS (1987) and STAMP and MEREDITH (1988).

Conclusion

Isolation methods for protoplasts from different organs and tissue of grapevine were established. Cell division could not be induced in protoplasts from leaves, shoot tips, petioles and callus, wehreas root and stem protoplasts showed high division activity. However, stem protoplasts formed microcalli and calli after cultivation in liquid medium on a solid reservoir-medium. Protoplasts from stems of 4 varieties could be regenerated to callus.

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Transformation of *Vitis vinifera* by *Agrobacterium* based vectors

J. V. POSSINGHAM, N. S. SCOTT, K. G. M. SKENE and T. J. BARIBAULT

C.S.I.R.O. Division of Horticulture, GPO Box 350, Adelaide, 5001, Australia

A b s t r a c t : Stable transgenic grapevine callus can be generated by infection *in vitro* of grape tissues with *Agrobacterium tumefaciens* based vectors. These include a range of both Biovar I and Biovar III types either in cointegrate or binary forms. The neomycin phosphotransferase (NPTII) gene has been used as a selectable marker to identify those calli with resistance to the antibiotic kanamycin. The presence of this gene in the calli has been demonstrated by Southern blotting and enzymic analysis. These experiments show that Biovar I type *Agrobacterium* vectors are suitable for grapevine transformation and that the nopaline synthase promoter sequence (pNos) is active in grapevine cells. In similar experiments disarmed *A. tumefaciens* vectors were used in a grapevine system in which plant regeneration is achieved through adventitious bud formation. Kanamycin tolerant plants occur at a low frequency and grow slowly under the selection pressure.

An alternative marker gene coding for the enzyme beta-glucuronidase (GUS) is being used to optimize the transformation of grapevine. This gene is not present in most plants, including grapevine, and when it is used as a marker, individual transformed cells can be identified histochemically (JEFFERSON *et al.* 1987)*. We have used this system to identify transformed grapevine cells in regenerating tissue and to compare the pathway of shoot regeneration in transformed grapevine with the model tobacco leaf disc system.

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