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Plant regeneration of grapevine (Vitis sp.) protoplasts isolated from embryogenic tissue

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Summary. Protoplasts with high embryogenic competence could be isolated from leaf-disk-derived embryos and embryoids of *Vitis sp.* cv. Seyval blanc. After a 4-week induction treatment in NN-69 medium supplemented with 4.0mg/l naphthoxyacetic acid (NOA) and 0.9mg/l thidiazuron (TDZ) and subsequent subcultivation in hormone-free medium, 38.5% of the developed microcalluses showed somatic embryogenesis. In contrast, only few formed somatic embryos after induction in CPW-13 medium with either 1.0mg/l 2,4-dichlorophenoxyacetic acid and 0.5mg/l benzylaminopurine treatment (13.8%) or NOA/TDZ treatment (1.4%). Up to 30% of these embryos germinated and about half of them regenerated into typical *in vitro* grapevines when transferred onto LS-medium in culture tubes.

Abbreviations: BAP, benzylaminopurine; BSA, bovine serum albumin; 2,4-D, 2,4-dichlorophenoxyacetic acid; MES, 2-[N-morpholino]-ethanesulfonic acid; NOA, naphthoxyacetic acid; PCR, polymerase chain reaction; PVP, polyvinylpyrrolidone; TDZ, thidiazuron;

Introduction

Although transgenic grapevines have been successfully regenerated from transformed petiole derived embryos (Martinelli and Mandolino 1994) and embryogenic suspensions (Torregosa *et al.* 1994), the use of protoplasts would be more promising. Different transformation methods could be used, selection might be easier and additional techniques like somatic hybridization (protoplast fusion) and selection of somaclonal variants on the protoplast level could be applied. The prerequisite for a successful application of protoplast technology, is the availability of methods for plant regeneration. However, neither organogenesis nor embryogenesis of protoplasts have yet been reported, although considerable work in this field has been done with leaves, stems or callus of different genotypes as donor material for the production of protoplasts (Skene 1975; Shimizu 1985; Wright 1985; Lee and Wetzstein 1988; Reustle and Alleweldt 1990; Ui *et al.* 1990; Mii *et al.* 1991).

Observations by Hartmann *et al.* (1992), Matthews *et al.* (1991) and Vasil *et al.* (1990) that in other plant species protoplasts with high regeneration ability could be obtained from embryogenic tissue and the availability of such tissue due to the work of Harst (1995) led us to develop the following protocol which is, to our knowledge, the first one to describe the isolation of protoplasts from embryogenic grapevine tissue and the regeneration of these protoplasts into plants.

Materials and methods

Donor material. Somatic embryogenesis was induced on leaf disks of Vitis sp. cv. Seyval blanc (Harst 1995). For protoplast isolation, embryos and embryoids were harvested from leaf disks and subcultured on solidified hormone-free NN-69 medium (Nitsch and Nitsch 1969).

Protoplast isolation. 1.5-2.5g of the embryogenic tissue were placed in a Petri-dish with 5.0ml of enzyme solution (pH 5.6) containing the cellulases of Aspergillus niger (0.5%, Calbiochem) and Penicillium finiculosum (0.5%, Calbiochem), Cellulysin (1.0%, Calbiochem), Macerozyme R-10 (0.2%, Serva), BSA (0.5%), $CaCl_2$ (1 mM), MES-KOH (20mM), sucrose (0.42M) and 1/10 strength V/KM-salts (Binding and Nehls 1977). The plant material was cut into small pieces

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using a razor blade and then filled up with the enzyme solution to a final volume of 15ml. Digestion was carried out overnight (16h) at 24°C in darkness on a rotary shaker (50rpm). The resulting protoplast suspension was filtered through sieves of 100µm and 50µm and purified by two flotation steps (10min at 300g each) in V/KM-S (1/10 strength V/KM salts, 20% sucrose) and a final sedimentation (4min at 80g) of the protoplasts in "washing-solution" containing CPW-13 substances (Frearson et al. 1973), 18.1g/l NaCl but without sucrose (Reustle and Natter 1994). The protoplast pellet was resuspended in 2ml of 0.65M mannitol and protoplast yield was calculated using a counting chamber (Thoma). By adding 0.65M mannitol, density of protoplasts was adjusted to $5.0x10^5$ cells per ml.

Protoplast culture. Protoplasts were embedded in Na-alginate and cultivated in several liquid media. For the immobilization in alginate, the protocol described by Karesch et al. (1991) was adapted to the requirements of grapevine protoplasts as follows: The protoplast-mannitol-suspension was mixed with the same volume of 2% Na-alginate (Sigma) in 0.55M mannitol (pH 5.8). Aliquots of 0.75ml of these mixture were placed in Petri dishes (ø 5cm) onto 5ml of solidified medium containing 0.3% Gelrite, 20mM $CaCl_2$ and 0.6M mannitol. After 2 hours of incubation, 2ml of liquid medium containing 10mM $CaCl_2$ in 0.62M mannitol (pH 5.8) were added to each Petri dish to complete the gelling process. Within two to three hours, the alginate-gels were transferred with a spatula into new Petri dishes with 2ml of culture medium. Cultivation was carried out at 24-26°C in permanent darkness.

Induction of embryogenesis. Either CPW-13 medium (Frearson et al. 1973) or NN-69 medium, supplemented with 0.54M and 0.6M glucose respectively, were used as basic media for the initial cultivation step. During the initializing period (4 weeks), two hormone treatments i) 1.0mg/l 2,4-D and 0.5mg/l BAP or ii) 4.0mg/l NOA and 0.9mg/l TDZ were tested. As control treatment, a hormone-free variant was used. After four weeks, alginate-gels were transferred to hormone-free NN-69 medium (for 4 weeks) containing 0.4M glucose. A further subcultivation step for another four weeks was carried out in the same medium but with reduced glucose concentration (0.2M). During the first eight weeks of cultivation, each medium was supplemented with 1.0% PVP-40, to prevent browning of the cultures. Developed calluses, 1.5-2.0mm in size, were transferred in Petri dishes (ø 10cm) onto solidified (0.3% Gelrite) NN-69 medium with 0.2M glucose and cultured under dimmed light (16h photoperiod, 10µmol $m^{-2}s^{-1}$). Induced embryos were harvested and subcultured on the same medium until germination. After having reached a size of 0.5-1.0cm the germinated embryos were transferred in culture tubes with hormone-free solidified (0.3% Gelrite) LS-medium (Linsmaier and Skoog 1965) and cultured under light (16h photoperiod, 100µmol $m^{-2}s^{-1}$).

Analysis of the results. Ten successive experiments were carried out. The number of replications (alginate-gels) of the variants in each experiment varied from 5 to 8 depending on protoplast yield. The development of protoplasts was observed using an inverted microscope (Zeiss). Beginning and intensity of cell division were observed. After 12 weeks of cultivation, microcallus formation frequency was calculated. Within five months of cultivation of the transferred callus on solidified hormone-free NN-69 medium, developing somatic embryos were harvested. Calluses exhibiting somatic embryogenesis were assessed as rates of the total number of transferred calluses.

Results and discussion

Isolation

The composition of the enzyme mixture as described above was found to be suitable for enzymatic digestion of embryogenic grapevine tissue. Yields of $0.8\text{-}4.7x10^6$ protoplasts per gram material were achieved. The size of protoplasts varied from 10-50µm, and most of them were rich in cytoplasm (Fig.1a).

Cell division

The timing and frequency of cell division depended on the media used and on the applied phytohormone combinations. Media without hormones (CPW-13: IM-1 and NN-69: IM-4), produced protoplasts which formed new cell walls within two weeks. However, no cell division occurred. Using CPW-13 medium, both hormone treatments (2,4-D/BAP: IM-2; NOA/TDZ: IM-3), initiated cell divisions within the first and second week of cultivation. Frequency of cell division reached more than 30% with IM-3 application and almost 20% with IM-2 treatment. In NN-69 medium with 2,4-D/BAP application (IM-5) no cell division was found, whereas division frequency reached 20% in maximum after NOA/TDZ application (IM-6). However, first cell divisions in IM-6 took place at the earliest 4 to 5 weeks after culture initiation. The improving effect of CPW-13 medium on the division rate of grapevine protoplasts was already known from earlier experiments (Reustle and Natter 1994). Skene (1975) observed dividing protoplasts of the cultivar Sultana after application of 2,4-D (0.1mg/l) or NAA (1-4mg/l) to the culture medium. Mii *et al.* (1991) found different phytohormone requirements for *Vitis labruscana* cv. Niagara (0.2mg/l 2,4-D and 0.02mg/l BAP) and *Vitis thunbergii* (2.0mg/l NAA and 0.2mg/l BAP) for optimum cell division.

Microcallus formation

Each of the ten successive experiments resulted in microcallus formation when IM-3 was applied. Seven of the experiments provided microcallus in the IM-6 variants whereas only two led to microcallus after IM-2 application. In the case of IM-3, 6-8 weeks after culture initiation, friable microcalluses, consisting of large cells, developed and reached sizes of 0.5-1.5mm. Related to the initial plated protoplasts 4.8±2.0% formed microcallus (Table 1). Compared to the IM-3 variants, growth of protoplasts was reduced after IM-2 treatment whereas initiation with IM-6 resulted in compact microcallus which remained small. Related to the experiments which yielded microcallus, formation frequency of IM-2 and IM-6 variants achieved 1.9±0.6% and 2.4±1.9%

respectively. Lee and Wetzstein (1988) observed microcallus consisting of numerous cells after six weeks of culture. They reached plating efficiencies up to 70% related to the initial cultivated protoplasts. Mii *et al.* (1991) obtained up to 70% of protoplasts which showed division, and most of them formed microcallus of 1-2mm in size already after 20 days of cultivation.

Somatic embryogenesis

Small embryogenic cell aggregates (Fig.1b) could be observed in the alginate-gels after 8 weeks of cultivation when NN-69 medium with NOA/TDZ (IM-6) was used in the initial cultivation step. Ten to twelve weeks after culture initiation, somatic embryos and embryoids were visible on the alginate-gels with the naked eye (Fig.1c). As indicated in Table 1, an average of 38.5±12.2% of the transferred calluses of this induction variant showed somatic embryogenesis. Only 13.8±8.7% and 1.4±1.2% of the transferred calluses, which were obtained after treatment in IM-2 and IM-3 respectively, formed somatic embryos. In the case of IM-6 most of the somatic embryos arose during four to six weeks of subcultivation of the calluses on solidified medium. In contrast, up to 12 weeks of subcultivation were necessary until the first embryos could be observed in the IM-3 variant. In the case of IM-6 treatment, the regeneration process might probably be explained by direct differentiation of protoplasts into somatic embryos without any preceding callus phase whereas after application of IM-3, cell proliferation seems to have happened before the onset of differentiation. The suitability of NN-69 medium with the phytohormone combination NOA/TDZ for induction of somatic embryogenesis was already known from previous experiments with leaf disks of Seyval blanc (Harst 1995). Srinivasan and Mullins (1980) suggest a positive effect of auxins with oxyacetic acid side chains on the initiation of grapevine callus with regenerative competence. Krul (1989) presumed that a shift to a less potent auxin is required for continued cell division and differentiation. This agrees with our observations with IM-6, where cell divisions and embryogenic cell formations occurred after transfer of the protoplasts into hormone-free medium.

Plant regeneration

As shown in Table 1, 31.0% of the embryos derived from IM-2 elongated spontaneously and formed a root and a small shoot. 16.4% of embryos from IM-3 and 15.8% from IM-6 started conversion to plants. After transfer of the germinated embryos into culture tubes cotyledons developed and turned green. As observed also by Goebel-Tourand *et al.* (1993) in regeneration experiments with somatic embryos of *Vitis*, an important heterogeneity of form and growth of the embryos occurred. Those which showed normal cotyledons (50-60%) developed into typical *in vitro* grapevines (Fig.1d) whereas plant recovery from abnormally developed embryos was strongly reduced. Conversion frequency (from embryo to plant) was 16.4% after IM-2, 10.7% after IM-3 and 7.7% after IM-6 treatment (Table 1). According to the investigations of

Table 1. Effect of several induction treatments on microcallus formation, frequency of somatic embryo formation and plant regeneration. Results of 10 successive experiments.

Media-indications[a]	IM-1	IM-2	IM-3	IM-4	IM-5	IM-6
Microcallus formation frequency (%±SD)[b]	0	1.9±0.6	4.8±2.0	0	0	2.4±1.9
No. of transferred calluses	0	1028	5138	0	0	4175
Embryo formation frequency (%±SD)[c]	-	13.8±8.7	1.4±1.2	-	-	38.5±12.2
Germinating embryos (%)	-	31.0	16.4	-	-	15.8
Frequency of embryo conversion (%)	-	16.4	10.7	-	-	7.7
No. of regenerated protoclones[d]	-	14	7	-	-	119
Regeneration rate (%)[e]	0	1.1×10^{-4}	0.8×10^{-4}	0	0	13×10^{-4}

a: IM-1: CPW-13, hormone-free; IM-2: CPW-13, 1.0mg/l 2,4-D, 0.5mg/l BAP;
IM-3: CPW-13, 4.0mg/l NOA, 0.9mg/l TDZ; IM-4: NN-69, hormone-free;
IM-5: NN-69, 1.0mg/l 2,4-D, 0.5mg/l BAP; IM-6: NN-69, 4.0mg/l NOA, 0.9mg/l TDZ;
b: related to the experiments which showed microcallus formation;
c: recorded 8 month after culture initiation;
d: regenerates derived from different calluses;
e: related to the total number of plated protoplasts;

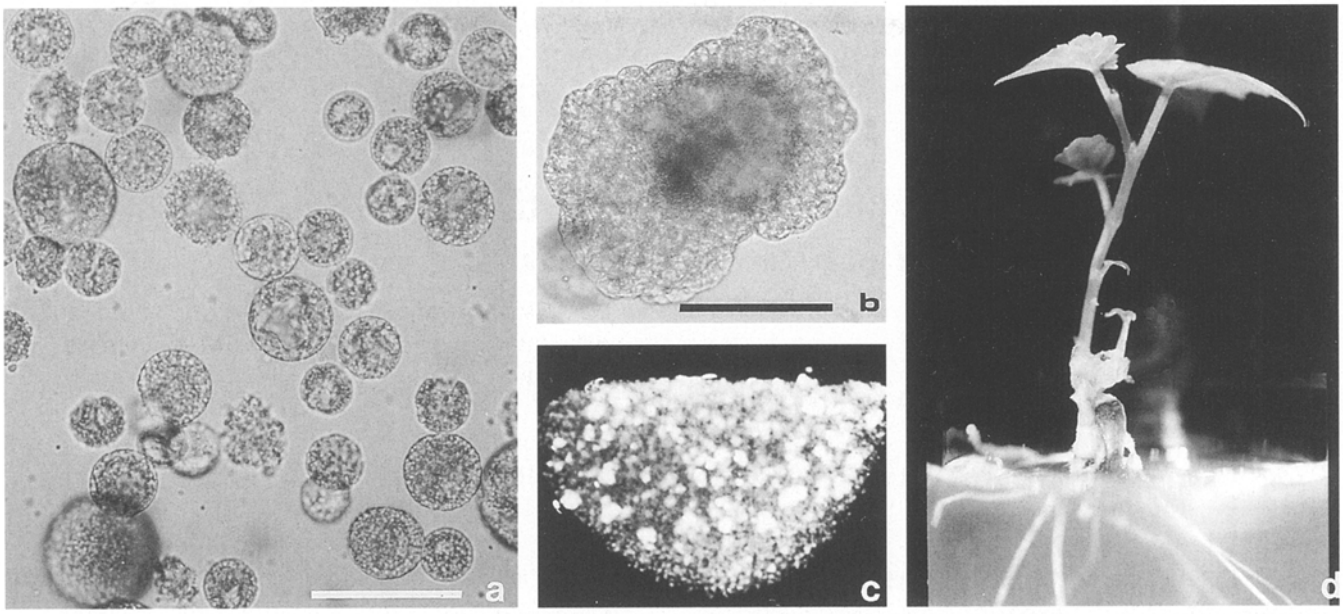

Fig.1. Regeneration of grapevine protoplasts: a) isolated protoplasts (bar = 50μm); b) embryogenic microcallus (bar = 100μm); c) embryogenic structures and embryos on the alginate-gel; d) in vitro grapevine regenerated from a protoplast.

Coutos-Thevenot *et al.* (1992) with suspension cultures of a grapevine rootstock extracellular macromolecules may be involved in the inhibition of embryo conversion. With the achieved regeneration rate of $1.1x10^{-4}$ (IM-2), $0.8x10^{-4}$ (IM-3) and $13x10^{-4}$ (IM-6), 140 protoclones were obtained so far (Table 1). To test for somaclonal variation, chromosome analysis and PCR-analysis are in progress. After transfer of the protoclones into the greenhouse, ampelographic measurements are to give information on the dimension of genetic variation. The results demonstrate that the main factor for a successful induction of somatic embryogenesis of grapevine protoplasts is the use of embryogenic tissue as donor material. It seems that previous regeneration experiments with grapevine protoplasts were unsuccessful because too much importance was attributed to the optimization of the plating efficiency. This led to a selection of media supporting frequent and fast cell division. As already proposed by Hartmann *et al.* (1992) to explain results obtained with protoplast cultures of *Abies alba*, an antagonism between proliferation and organization might exist. The cessation of grapevine protoplast regeneration at the stage of microcallus formation in previous experiments could be attributed to such a phenomenon.

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