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Effect of polyvinylpyrrolidone and activated charcoal on formation of microcallus from grapevine protoplasts (*Vitis* sp.)

by

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Summary: The effect of the addition of polyvinylpyrrolidone (PVP-40) to grapevine protoplast cultures (*Vitis* sp. cv. Vidal blanc) at different stages of development (day 0, 7 and 14 of cultivation) and the influence of activated charcoal (AC) in several concentrations (0, 0.1, 0.5 and 1.0 %) on protoplast growth were studied. Both additives were investigated using different modified culture media (CPW-13, V/KM, MS-P). The application of 0.5 % PVP could not prevent browning of the culture media but reduced it to a low level. Although the effect of PVP on plating efficiency was not clear, the formation of microcalli from grapevine protoplasts was remarkably improved when adding PVP at "day 0" or "day 7" of cultivation. AC could prevent browning of the media in all concentrations tested but at the same time inhibited plating efficiency. Conversely, formation of microcalli occurred only with 0.5 and 1.0 % AC, but at very low frequencies. A superior suitability of one of the culture media tested for grapevine protoplast culture could not been found.

Key words: grapevine protoplasts, plating efficiency, microcallus, regeneration.

Introduction

To improve breeding efficiency in grapevine, conventional breeding procedures have to be supplemented by new technologies (Alleweldt and Possingham 1988) like protoplast techniques as somatic hybridization of protoplasts (symmetric and asymmetric fusion) and gene transfer. A prerequisite for the utilization of these strategies is the regeneration of protoplasts to plants, which is still not successful in grapevine.

Until now only microcalli from grapevine protoplasts have been obtained (Lee and Wetzstein 1988; Reustle and Alleweldt 1990; Ui *et al.* 1990; Mii *et al.* 1991). One of the factors inhibiting protoplast growth is browning of the culture media due to the accumulation of phenolic compounds released from the protoplasts into the surrounding media. The addition of chemicals to eliminate or reduce harmful substances produced by the explants is often used in anther culture experiments (Johansson 1985) but less for protoplast culture (Saxena and Gill 1986; Ui *et al.* 1990). The aim of our studies was to verify the possible use of polyvinylpyrrolidone (PVP) and activated charoal (AC) as "browning control" in grapevine protoplasts cultures, in the sense of improving protoplast growth.

Materials and methods

Plant material: *In vitro* plants of Vidal blanc (French hybrid) were cultivated in glass vessels (Weck, 0.5 l) on LS-medium (Linsmaier and Skoog 1965) at 24-26 °C, 100 $\mu E m^{-2} s^{-1}$ light intensity and 14 h photoperiod. Plants were subcultured every 6 weeks. Prior to protoplast isolation, plants were kept in dark for 24 h. Stems of 6-week-old plants were used as source for protoplasts, due to their high dividing competence which has been found in earlier studies (Reustle 1989).

Protoplast isolation: Stems (1.5-2.5 g) were placed in a petri-dish with little V/KM sucrose-solution (V/KM-S) containing 0.55 M sucrose and 1/10 strengh V/KM-salts (Binding and Nehls 1977) and cut into small pieces. After incubation for 1 h in V/KM-S for preplasmolysis, the solution was replaced by approx. 15 ml of an enzyme mixture containing the cellulases *Aspergillus niger* (0.5 %), *Penecillium finiculosum* (0.5 %) and Celluslysin (1.0 %), Macerozyme R-10 (0.2 %), BSA (0.5 %), $CaCl_2$ (1 mM), MES-KOH (20 mM), sucrose (0.42 M) and V/KM-salts (1/10 strengh). Digestion was carried out over night (16 h) at 24 °C on a rotary shaker (50 rpm). The obtained protoplast suspension was filtered through 100 and 50 µm sieves and floated twice in V/KM-S by centrifugation (300 *g* for 10 min). The obtained protoplast layers were collected using a Pasteur Pipette and the purified protoplasts were finally resuspended in wash-solution containing 1/10 strengh CPW-salts (Frearson *et al.* 1973), 1 mg/l thiamin HCl, 1 mg/l glycine, 100 mg/l myo-inositol, 0.4 M mannitol and 18.1 mg/l NaCl. The protoplasts were sedimented by centrifugation (80 *g* for 4 min) and the pellet was diluted in 2 ml of culture medium. Protoplast yield was calculated in a counting chamber (Thoma) and viability measured using 10 µl of 0.01 % fluorescin diacetat (FDA) per 100 µl of protoplast suspension.

Protoplast culture: Protoplasts were cultured in 24-well multidishes as a bilayer system, previously described by Pellow and Towill (1986): For the lower layer each well was filled with 250 µl of solidifying (0.3 % gelrite) RM-medium containing MS-macrosalts, halfstrengh MS-microsalts, NN69-vitamines (Nitsch and Nitsch 1969), 100 mg/l caseinhydrolysate, 0.025 M mannitol, 0.05 M sucrose, 1 ppm NAA and 0.5 ppm BAP.

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For the upper layer, protoplasts were diluted in culture medium to a density of 1 x 10^6 cells/ml and mixed with the equal volume of 1.2 % agarose (Sigma, type VII), molten in the same culture medium so that the final protoplast density was 5 x 10^5 protoplasts/ml. As soon as the lower layer was solidified, each well was stratified with 250 µl of the protoplast containing medium. Three different culture media were tested. They were (1) modified V/KM medium according to BINDING and NEHLS (1977) with 0.54 M glucose, (2) modified CPW-13 medium according to FREARSON *et al.* (1973) with 0.52 M glucose and (3) an MS-based medium (MURASHIGE and SKOOG 1962) with 0.4 M glucose. Each medium was supplemented with 1 ppm 2.4-D and 0.5 ppm BAP.

PVP treatment: PVP-40 (Sigma) was diluted in distilled water at a concentration of 25 % and autoclaved at 110 °C and 0.5 bar for 30 min. 10 µl of the stock solution were added to each well to the bilayer-cultures (final concentration: 0.5 % PVP) at culture initiation (day 0) and 7 and 14 d after initiation. The control was cultured without supplementing PVP.

AC treatment: AC (Sigma, neutralized) was added in different concentrations (0.1; 0.5; 1.0 %;) to the RM-medium before autoclaving and plated in the wells of the multidishes as lower layer. For the control no AC was used in the RM-medium.

Performance of the experiments: Experiments were carried out from February to May, known to be a "suitable season" for grapevine protoplast culture (unpublished results). In a first set of experiments, PVP treatments were tested with each culture medium, one after another each in 3 repeated experiments (Tab. 1). In the second set, PVP treatments and tests of culture media were carried out simultaneously (3 repeated experiments), to find alternating effects of both factors (Tab. 2). In a third set (Tab. 3), activated charcoal was tested. First of all, AC concentrations were applied to each culture medium variant (2 repeated experiments, expts. 1 and 2), one after another. Finally (expt. 3) AC treatments and culture media were tested simultaneously.

Statistical analysis: Protoplast response was assessed in (i) terms of the plating efficiency (PE) and expressed as percentage of the initially cultured protoplasts that divided (after 14 d of culture) and in (ii) rate of wells in which microcalli, visible to naked eyes, were proliferated after 30 d (for PVP-variants) and 45 d (for AC-variants) of culture respectively.

The results are presented as means from 3-10 replications (well in the multidish) of the different treatments of each experiment. In case of PE, means were subjected to t-test analysis.

Results

With the applied procedure, protoplast yield changed between 1.1 x 10^6 and 4.4 x 10^6 protoplasts/g donor material with viabilities of 70-80 %.

In general, formation of new cell walls started at day 2 to 4 of culture and first cell division occurred at the beginning of the second week. If further growth of dividing protoplasts occurred, the media were covered with microcalli after 30 d of culture. Simultaneously to cell division, the beginning of the browning process could be observed. Apart from PVP and AC treatments, intensity and rapidity of browning varied in the media tested (V/KM > CPW-13 > MS-P) and moreover changed from experiment to experiment.

Table 1

Effect of PVP on plating efficiency and formation of microcallus of grapevine protoplasts

Plating efficiency at day 14 (%)

	CPW-13 medium			V/KM medium			MS-P medium		
Expt.	1	2	3	1	2	3	1	2	3
PVP-addition									
control	2.8[a*]	4.1[a]	1.2[b]	3.5[b]	2.6[b]	2.6[a]	0.8[a]	0.2[b]	4.1[a]
day 0	5.4[a]	5.6[a]	1.8[a]	4.8[a]	4.0[a]	3.3[a]	0.6[a]	0.9[a]	3.2[a]
day 7	2.9[b]	4.6[a]	2.4[a]	3.9[b]	4.7[a]	2.9[a]	0.6[a]	0.3[b]	2.3[b]

Wells with microcallus at day 30 (%)

	CPW-13 medium			V/KM medium			MS-P medium		
Expt.	1	2	3	1	2	3	1	2	3
PVP-addition									
control	0	0	0	0	0	0	0	0	0
day 0	86	100	0	100	82	0	0	0	92
day 7	27	89	0	100	100	0	0	0	79
day 14	38	11	0	0	36	0	0	0	0

* : different indices indicate significant differences at 5 % level

The addition of PVP to the protoplast cultures could not prevent browning of the media, but intensity and rapidity of the browning process were reduced. However, no clear correlation of browning and growth of protoplasts could be found.

In comparison to the control (no PVP treatment), a significant increase in plating efficiency (PE) was found in CPW-13 medium (expts. 1 + 3), V/KM medium (expts. 1 + 2) and MS-P medium (expt. 2) when PVP was added at day 0 (Tab. 1). Application of PVP at day 7 resulted in a significant increase of PE only in one experiment with CPW-13 and V/KM medium. Concerning further growth of divided protoplasts, no microcallus has been achieved without PVP whereas application at day 0 and day 7 yielded in intensive microcallus formation. With the application of PVP at day 14, formation of calli was reduced or failed.

The experiments in which the effect of PVP in different media was tested, led to the following results (Tab. 2): (i) PE was more distinct from experiment to experiment than between the media tested. (ii) Concerning PE, no difference between the media could be found, when PVP was added at day 0. The control and PVP-addition at day 7 provided tendentious and significantly higher PEs in CPW-13 medium compared to V/KM and MS-P medium respectively. (iii) No positive effect of any PVP treatment on PE was found, whereas the formation of microcallus was clearly improved by PVP addition. (iv) Microcallus developed in all experiments, when PVP was added at day 0 or day 7. (v) Formation of microcallus failed or occurred scarcely, when no PVP treatment or addition at day 14 (not shown) was applied.

The addition of AC to the culture system hampered the browning process clearly. However, no positive effect could be achieved on the development of the protoplasts (Tab. 3). On the one hand, PE was significant or at least tendentiously higher without AC compared to all AC treatments. On the other hand, visible microcalli occurred only, when 0.5 and 1.0 % AC was added, except of MS-P in

Table 2

Interaction of media and PVP-addition on plating efficiency and formation of microcallus of grapevine protoplasts

Plating efficiency at day 14 (%)									
	control			PVP at day 0			PVP at day 7		
Expt.	1	2	3	1	2	3	1	2	3
CPW-13	7.6^{a*}	15.6^{a}	1.1^{a}	3.6^{a}	11.4^{a}	3.0	5.1^{a}	4.9^{a}	3.1
V/KM	6.1^{b}	9.6^{b}	2.7^{a}	3.4^{a}	8.2^{a}	mc	3.9^{b}	4.8^{a}	mc
MS-P	6.7^{ab}	14.1^{ab}	nt	2.9^{a}	8.6^{a}	nt	4.3^{ab}	5.5^{a}	nt

Wells with microcallus at day 30 (%)									
	control			PVP at day 0			PVP at day 7		
Expt.	1	2	3	1	2	3	1	2	3
CPW-13	63	100	0	100	100	67	100	100	100
V/KM	0	0	0	100	100	100	100	100	100
MS-P	0	0	nt	63	100	nt	100	100	nt

* : different indices indicate significant differences at 5 % level; nt: not tested; c: microcalli

Table 3

Effect of activated charcoal (AC) on plating efficiency of grapevine protoplasts

Plating efficiency at day 14 (%)									
	CPW-13 medium			V/KM medium			MS-P medium		
Expt.	1	2	3	1	2	3	1	2	3
AC conc.(%)									
0	7.5^{a*}	1.5^{a}	5.7^{a}	7.3^{a}	4.4^{a}	5.8^{a}	6.6^{a}	1.1^{ab}	8.8^{a}
0.1	0.7^{c}	0.5^{b}	2.6^{b}	7.0^{a}	3.1^{ab}	5.0^{a}	0.1^{c}	0.4^{b}	2.1^{b}
0.5	0.8^{c}	1.5^{a}	0.9^{b}	2.5^{b}	1.1^{c}	1.5^{b}	0.2^{bc}	0.8^{a}	0.5^{b}
1.0	1.2^{b}	1.8^{a}	0.6^{b}	1.2^{c}	2.1^{b}	0.8^{b}	0.3^{b}	1.2^{a}	0.4^{b}

* : different indices indicate significant differences at 5 % level

expt. 3 (data not shown). In any case, in contrast to the PVP-treated cultures, microcalli could be observed at the earliest after 45 d of culture also in a very low frequency.

Discussion

Since experiments were initiated to regenerate grapevine protoplasts, results varied from experiment to experiment. In spite of standardized growth conditions and propagation steps for donor plants, responses of protoplasts on several treatments were superposed sometimes by the influence of the donor material.

More reliable results were obtained by intense selection of donor plants on "protoplast division suitability". Nevertheless, still then, unknown factors influenced plating efficiency and complicated interpretation of the results. The intense formation of microcallus in most of the experiments on the one hand and the very low activity of protoplasts in few experiments on the other hand, shown in our report, illustrate this problem.

To prove the influence of media compositions on plating efficiency and microcallus formation, different basic media were used in this study. Although the compositions of the media tested were strongly different concerning salts, vitamines and sugars, the response of protoplasts showed no differences when compared directly in the same experiment (Tabs. 2 + 3). No adequate comparison of culture media with grapevine protoplasts has been reported in the literature so far. LEE and WETZSTEIN (1988) obtained up to 70 % plating efficiency with B5 medium (GAMBORG *et al.* 1968) supplemented with the same concentration of growth regulators as used in our study. MII *et al.* (1991) achieved similar results with B5 medium, whereas UI *et al.* (1990) successfully used MS-based medium. KATSIRDAKIS and ROUBELAKIS-ANGELAKIS (1992) discovered specific requirements of grapevine protoplasts for cell wall regeneration and cell elongation concerning osmoticum, osmolality, pH-value and growth regulators. However, THEODOROPOULOS and ROUBELAKIS-ANGELAKIS (1990) could find very little response of grapevine protoplasts to application of different hormone concentrations (2,4-D/BAP) and to coconut milk, whereas coconut meat and glutamine had a positive effect on survival rate of protoplasts. Obviously, several basic media can be used successfully for grapevine protoplasts, however, possibly special additives are of more influence on protoplast growth.

Browning of culture media is the result of oxidation of mono- and di-phenoles, released from tissue, e.g. protoplasts, into the surrounding medium (SAXENA and GILL 1986). Accumulation of these substances likely leads to toxic concentrations and finally to dying protoplasts. Conversely, synthesis of metabolites gives evidence for active protoplasts possibly starting regeneration process. Furthermore, released compounds could be of stimulating nature for cell growth and regeneration. The intention of our studies was to allow stimulating effects of metabolites but prevent the accumulation of phenolic compounds to toxic concentrations by application of PVP at different stages of protoplasts development. In accordance to SAXENA and GILL (1986), the addition of PVP to the protoplast medium was positive for the development of grapevine protoplasts, although the effect on cell division (PE) was not very clear. Application of PVP in time seems to be a prerequisite for microcallus formation. It could be clearly shown that application of PVP after 14 d of culture could not prevent inhibitory effects on protoplast growth. As illustrated in experiment 3 with MS-P medium (Tab. 1), in spite of a tendentious or significantly higher PE of the control, microcallus occurred only in the PVP-variants "day 0" and "day 7". Consequently, supplementing PVP to the initial culture medium is essential for microcallus formation from grapevine protoplasts.

Activated charcoal is known to be a very effective compound to absorb potentially harmful phenolics in culture media, produced by the tissues (WEATHERHEAD *et al.* 1979). FRIDBOURG *et al.* (1978) suppose the absorbtion of substances by AC, which are necessary for cell growth. WEATHERHEAD *et al.* (1978, 1979) found the absorption of growth regulators (auxins and cytocinins) and of thiamine, nicotinic acid and inositol. However, UI *et al.* (1992) could improve the rate of cell division of grapevine protoplasts when 0.2 % AC was added to the culture media. In our experiments, the effectiveness of absorption of the applied concentrations was high enough to avoid browning process of the media. The negative effect of AC on plating efficiency could be due to a direct inhibitory effect on the one hand and to the absorption of substances which are essential for growth on the other hand. So, using the described culture systeme, AC has to be considered as an unsuitable additive to support protoplast division. Nevertheless, for further growth of divided protoplasts, AC seems to be helpful due to the elimination of harmful substances.

The results describe an effective system for the production of micro- and macrocallus from grapevine protoplasts. Beside using cv. Vidal blanc, the method was applied to several other grapevine genotypes. While Seyval blanc gave similar results, Riesling and Kober 5BB showed a reduced microcallus formation. Distinct suitability of genotypes for protoplast culture as well as genotype-specific requirements to growth conditions and culture media could therefore be responsible.

The achievement of high quantities of microcalli is a prerequisite for further experiments to develop a protoplast-to-plant system. First experiments with the obtained calli were carried out to induce morphogenetic processes but so far, depending on the applied growth regulators, only proliferation, sometimes greening and in few cases rooting of the calli could be achieved. Neither embryogenesis nor shoot formation could be observed till now.

Concluding from the obtained results, PVP is a helpful additive for grapevine protoplast culture in early application. The importance of basic media seems to be inferior for microcallus formation and is superposed by the influence of the donor material. From this experience our studies recently started are focused on the use of genotypes and donor tissue (embryogenic callus, somatic embryos) with higher embryogenic and regenerating competence.

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