

Development of a regeneration protocol for high frequency somatic embryogenesis from explants of grapevines (*Vitis* spp.)

by

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S u m m a r y : An efficient regeneration protocol was developed for leaf explants derived from *in vitro* plantlets of grapevine cv. Seyval blanc. High frequency somatic embryo induction could be obtained on a modified NN69 medium with 20 μM NOA in combination with the synthetic cytokinin TDZ (4 μM). The application of 2.5 mM phenylalanine promoted embryo induction dramatically by reducing the induction period, and by increasing the regeneration rate. Embryos could be induced on almost 80 % of the plated leaf discs, and the regeneration capacity could be maintained for at least two years. Explants of *Vitis thunbergii* and the interspecific hybrid Chancellor could now be regenerated by the application of the regeneration system elaborated for Seyval blanc.

Key words : somatic embryogenesis, cytokinin, amino acid, leaf discs, starting material.

Abbreviations: BAP, 6-benzylaminopurine; LS, Linsmaier and Skoog (1965); NN69, Nitsch and Nitsch (1969); NOA, β -naphthoxyacetic acid; PHE, L-phenylalanine; TDZ, thidiazuron (N-phenyl-N¹-1,2,3-thiadiazol-5-yl-urea)

Introduction

Recent reports demonstrate the capacity of certain grapevine cultivars to regenerate via organogenesis (REISCH *et al.* 1990, STAMP *et al.* 1990) or somatic embryogenesis (MARTINELLI *et al.* 1993, ROBACHER 1993) from different kinds of tissues to intact plants, but often at an unsatisfactory regeneration rate.

The object of this study was the development of a regeneration system with an optimal standardisation for producing embryogenic tissues over long periods on a large scale on the basis of the protocol elaborated for the grapevine cv. Seyval blanc by KRUL and WORLEY (1977), and on the results of CHUPEAU *et al.* (1993) who used TDZ for efficient protoplast development and subsequent plant regeneration of poplar.

Material and methods

For experiments with leaf discs from greenhouse material, woody cuttings were excised in January from field-grown grapevines and cultured in a soil-sand-mixture at ambient temperatures and natural daylight in the greenhouse. At an average shoot length of 30–40 cm fully expanded leaves were surface disinfected with NaOCl-solution (commercial bleach with 12 % active chloride at 1 : 9 parts H₂O containing several drops of "Tween 20") for 25 min and then rinsed 3–4 times with sterile H₂O. Leaf discs (\varnothing 8 mm) were punched out from the midrib, main veins, and the point of petiole insertion on the petiolar sinus and plated abaxial side up on the medium. In earlier studies these areas of the leaf blade had shown a better regeneration capacity in comparison to leaf discs taken

from intercostal fields (unpublished data). Discs from *in vitro* plantlets including the midrib were excised from cultures which had been cultivated at 27 °C in light conditions of 16 h at 50 $\mu\text{E m}^{-2}\text{s}^{-1}$.

In all experiments, for each variation, 6 petri dishes (\varnothing 50 mm) with 6 explants were cultured. The tests with greenhouse-derived starting material and with *in vitro* material were repeated two and three times, respectively.

Callus induction, embryo induction, and embryo germination were carried out on a modified NN69-medium (BM-1). A modified LS-medium (BM-2) was used for the development of rooted plantlets from germinated embryos (HARST-LANGENBUCHER and ALLEWELDT 1993). The media were adjusted to pH 5.8 before sterilisation and solidified with 3 g/l gelrite. Phytohormones were added before autoclaving. Leaf discs were transferred at 4-week intervals, the explants were cultivated under permanent darkness at 28 °C, after germination of the embryos the cultures were transferred to light conditions of 16 h at 50 $\mu\text{E m}^{-2}\text{s}^{-1}$.

Results and discussion

The first experiments with leaf discs from greenhouse grapevines were focused on the effect of different combinations of NOA (auxin component) and two cytokinin compounds, BAP and TDZ, neglecting the leaf position on the shoot (Table). MATSUTA and HIRABAYASHI (1989), testing greenhouse leaf discs of grapevine cv. Koshu-sanjaku, reported better results using TDZ in a concentration of 5–10 μM combined with 2,4-D as the auxin compound (4.2 % embryogenic leaf discs) as compared to the application of 5–10 μM BAP (1.7 %).

Table

Rate of somatic embryogenesis [%] on leaf-discs from greenhouse grown starting material in response to phytohormone combination and concentration after 16 weeks of culture. Data are means of 36 leaf-discs (\pm S.D.)

Concentration of phytohormones [μ M]		NOA		
		5	10	20
BAP	2	0	0	0
	20	0	1.2 \pm 0.3	0
	40	0	0	6.5 \pm 1.8
TDZ	1	0	0	0
	2	0	1.6 \pm 0.4	0
	4	0	0	2.9 \pm 1.1

Due to the low regeneration rate with 10 μ M NOA this concentration was omitted in a further study which examined the effect of leaf position on the shoot: The regeneration rate of older leaves (position 7 from the apex) could be improved with both cytokinin compounds (Fig. 1), in particular with BAP. There are no comparable studies on greenhouse material of grapevine, but STAMP *et al.* (1990) found that older *in vitro* leaves of French Colombard formed less adventitious shoots than younger ones.

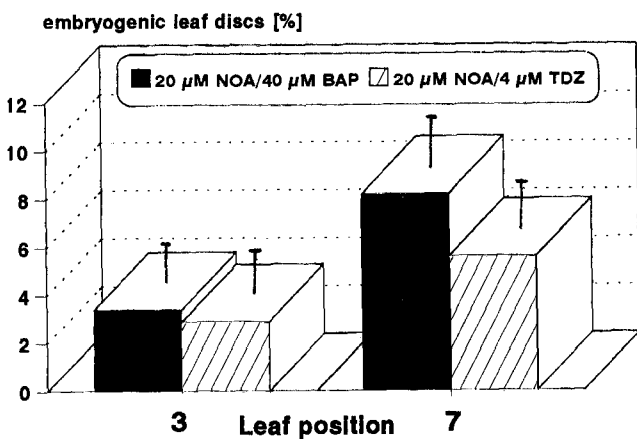


Fig. 1: Influence of the leaf position at the mother plant on somatic embryogenesis on leaf discs of Seyval blanc, 16 weeks after culture initiation. Data are means of 36 leaf discs (\pm S.D.).

Since *in vitro* material seems to be more promising for standardisation than greenhouse material, the most successful hormone combinations were tested further on leaf discs taken from *in vitro* plantlets. Evidently *in vitro* material is to be preferred for an effective regeneration system (Fig. 2) in accordance with the results of STAMP and MEREDITH (1988) with Cabernet Sauvignon. In this case, however, the TDZ treatment on *in vitro* leaf discs was much more effective than the application of BAP. REISCH *et al.* (1990) could induce organogenesis on petioles of Catawba with the same TDZ concentration (4 μ M) on 10 % of the tested explants, but our experiments seem to be the first ones to demonstrate its beneficial effect on the induction of somatic embryos on *in vitro* material.

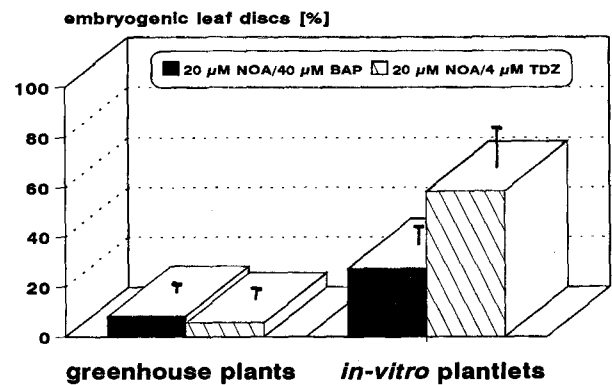


Fig. 2: Effect of the origin of the starting material on somatic embryogenesis on leaf discs of Seyval blanc, 16 weeks after culture initiation. Data are means of all experiments (\pm S.D.).

The effect of amino acids was less pronounced. Leaf discs from greenhouse material which were cultured on induction medium (BM-1 with 20 μ M NOA/4 μ M TDZ) supplemented with 2.5 mM PHE showed an earlier (one week), though not higher embryo induction as compared to untreated explants (data not presented) but on *in vitro* material embryo induction occurred 4 weeks earlier, and in general with a higher regeneration rate on explants treated with PHE (Fig. 3). In the subsequent cultivation on hormone- and phenylalanine-free BM-1 such embryos showed a higher germination rate, and plant recovery was much better than of embryos induced on untreated explants (Fig. 4). Testing the effect of amino acids on induction of somatic embryos on anthers of Cabernet Sauvignon MAURO

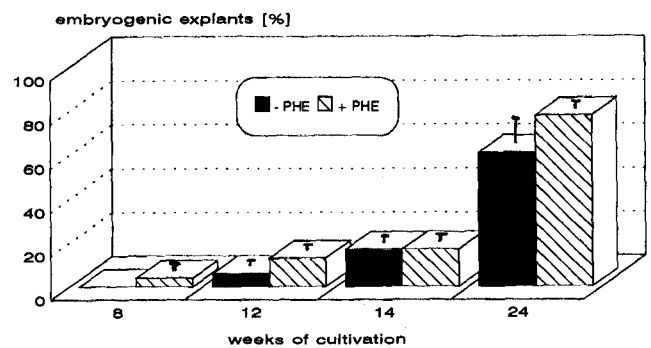


Fig. 3: Effect of PHE-treatment (2.5 mM) on somatic embryogenesis on leaf discs of Seyval blanc. Data are means of 118 leaf discs (\pm S.D.), PHE/phenylalanine.

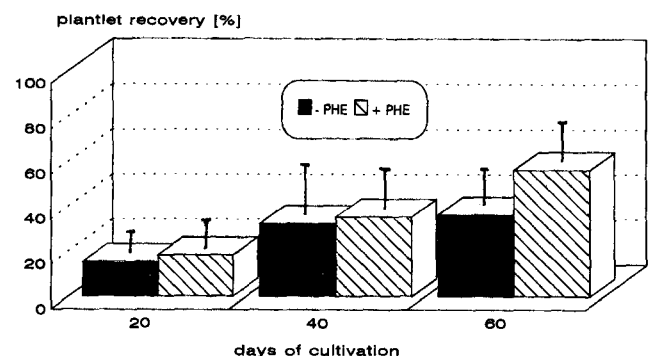


Fig. 4: Effect of PHE-treatment (2.5 mM) on plant recovery of somatic embryos of Seyval blanc. Data are means of 167 somatic embryos (\pm S.D.), PHE/phenylalanine.

et al. (1986) obtained best results when L-glutamine and adenine were used, the application of PHE had no influence on somatic embryogenesis.

The new protocol is summarized in Fig. 5. After the first excision of embryos from the explants the period between one yield and the next could be reduced from 60 d after induction of the first embryos on callus on the initial explants to only 14–21 d in the subsequent embryo excisions. KRUL and WORLEY (1977) observed the first embryo induction on Seyval blanc explants 80 d after culture initiation and obtained a secondary embryo formation 6 months after the first embryo induction. Since these authors used greenhouse plants for their tests the rapid embryo formation in the demonstrated regeneration protocol might be due to the *in vitro* plants used.

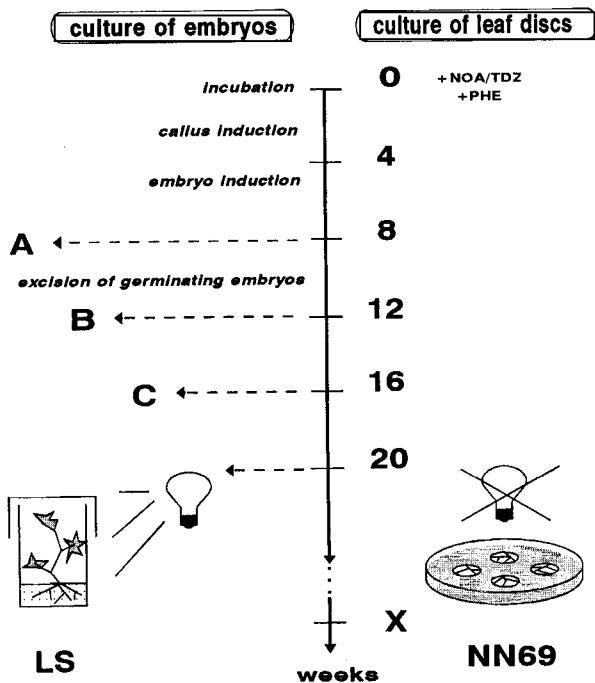


Fig. 5: Regeneration protocol for leaf discs of *in vitro*-grown grapevine Seyval blanc (A, B, C... are generations of embryos excised every 4 weeks from the original explant by a regular transfer on fresh BM-1).

By a regular transfer of the explants to BM-1 embryogenic competence could be maintained for about two years, and the explants are still producing embryos.

The total number of produced embryos has not been determined, because the number of embryos varied in each charge of embryo generation between the subcultures (5–100 embryos/leaf disc), and embryo induction and development did not occur synchronously. From all experiments more than 500 plants have been recovered from somatic embryos. The total output would have been higher but a lot of embryos have been used for other studies.

The regeneration protocol has also been tested on other grapevines, like *Vitis thunbergii* and the interspecific hybrid Chancellor. Both genotypes could not be regenerated using greenhouse material and BM-1 supplemented with NOA/BAP (data not shown), but the application of NOA/TDZ to leaf discs of *in vitro* plantlets resulted in induction of somatic embryos (Fig. 6). Furthermore, total plant

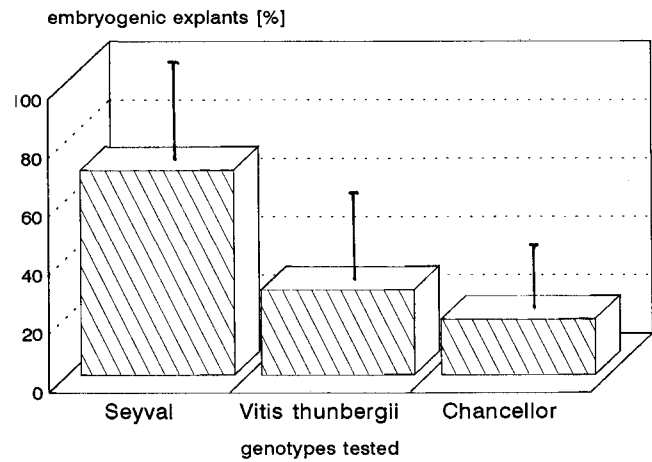


Fig. 6: Regeneration of *Vitis thunbergii* and Chancellor in accordance to the regeneration protocol elaborated for Seyval blanc, 9 weeks after culture initiation. Data are means of 72 leaf-discs (\pm S.D.).

regeneration of protoplasts isolated from embryogenic tissues induced on *in vitro* leaf discs of Seyval blanc was successful when cultivation was carried out in accordance to the demonstrated regeneration system (REUSTLE *et al.* 1994).

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