Vitis **39** (1), 27-29 (2000)

Establishment of embryo suspension cultures of grapevines (*Vitis* L.)

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Summary

Somatic embryos of Seyval blanc, Dornfelder, Regent, Riesling, Müller-Thurgau and Rupestris du Lot originating from leaf discs or anther culture were checked with regard to their ability to be cultivated in suspensions for an induction of secondary embryogenesis. Culture parameters like carbohydrates, phytohormones, pH, intervals of media change, the anti-oxidant dithioerythritol (DTE), and the size of embryos were tested to optimize the suspension culture conditions. In liquid culture, glucose was better utilized than sucrose or maltose. The pH (5.8 - 5.0) and the embryo size mainly influenced the quality of embryogenic suspensions. Medium change proved to be necessary at a pH <4.5 or after a culture period >4 weeks. Browning of media was avoided by addition of 3 or 6 mM DTE. Homogeneous suspension cultures were maintained by fragmentation of embryos according to their developmental stage. From suspensions containing embryos of globulary- and heart-stage (0.5-2.0 mm) 50 % new somatic embryos were obtained whereas further developed embryos (torpedo-stage, >2.0 mm) were not suitable for cultivation in suspension. Best induction of secondary embryos was obtained from Seyval blanc, Dornfelder, Müller-Thurgau and Rupestris du Lot. After transfer to a LS-medium the seedlings of Dornfelder and Müller-Thurgau regenerated to rooted *in vitro* plantlets.

K e y w o r d s : carbon source, DTE, embryo suspension culture, pH, phytohormone, regeneration, *Vitis vinifera.*

Introduction

The production of large amounts of homogeneous embryogenic material is an important prerequisite for many biotechnological experiments, *e.g.* transformation studies. Somatic embryos or embryogenic calli of many cultivars can be induced on different explants (MAURO *et al.* 1986, STAMP and MEREDITH 1988, HARST-LANGENBUCHER and ALLEWELDT 1993, MARTINELLI *et al.* 1993, HARST 1995, REGNER *et al.* 1996). Though the regeneration capacity of mother explants can be maintained over long periods by subculturing on fresh solid medium, embryo yield and quality varies at each subcultivation step. This problem might be solved by induction and propagation of secondary embryos in liquid culture to obtain homogeneous embryogenic tissue. Actually only a few grapevine cultivars were successfully cultivated in suspension culture such as Aripa, Grüner Veltliner, Kober 5 BB (REGNER *et al.* 1996) and Chancellor (KIKKERT *et al.* 1995). Successful embryogenic suspension culture mainly depends on the choice of sugars and phytohormones and their concentrations, the maintainance of pH value during culture, the avoidance of tissue browning, and suitable developmental stages of embryos. This paper is focused on the optimization of the suspension conditions of some grapevines which are of importance for German viticulture and breeding purposes.

Material and Methods

Somatic embryos in different stages of development were used as starting material to establish liquid cultures. Primary somatic embryos were induced on leaf discs of Seyval blanc following the protocol of HARST (1995), and from Dornfelder, Regent, Riesling, Müller-Thurgau and Rupestris du Lot on anther tissue according to HARST-LANGENBUCHER and ALLEWELDT (1993). Primary embryos were harvested from donor material and cultivated in Erlenmeyer flasks in a modified liquid NN69 medium (NITSCH and NITSCH 1969). The medium volume was about 20-fold that of the embryo fresh weight. The embryo suspension cultures were cultivated on a rotary shaker at 70 rpm in permanent darkness at 28 °C.

For cultivation of leaf discs or anther tissue usually sucrose (20 $g \cdot l^{-1}$) is added to the solid medium. Tn suspension culture glucose or maltose were used in equivalent concentrations (36 $g \cdot l^{-1}$ glucose and 18 $g \cdot l^{-1}$ maltose). The effect of sugars was determined by measuring the increase of globulary embryo induction per g fresh weight of embryogenic tissue.

Each medium was supplemented with NOA (5, 10 and 20 -tM) in combination with 4 -tM TDZ. Tn control experiments no phytohormones were added.

The pH adjustment of the basal medium to 5.8 was carried out either prior to autoclaving with NaOH or after warming up the medium at 60 °C with NaOH or with a 0.1 M Tris buffer solution (pH 7.5). To avoid browning of the embryogenic tissue dithioerythritol (DTE, 3 or 6 mM) was added to the medium according to PERL *et al.* (1996).

To maintain homogeneous embryo suspensions at each medium the embryos were assigned at each medium change according to their developmental stage to three fragment

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classes: (1) 0.1-0.5 mm (globular-stage), (2) 0.5-2.0 mm (heart-stage), and (3) >2.0 mm (torpedo-stage) which were removed from the suspension and transferred to hormone-free solid NN69 medium for further germination. The cultivation of torpedo-stage embryos was carried out in Petri dishes in permanent darkness whereas the developed seedlings were subcultured on solid LS medium (LINSMAIER and SKOOG 1965) in culture tubes for plant regeneration. They were cultivated at 27 °C and 16 h light (50-60 µ-tmol·m^{-2}·s^{-1}).

For each of the 7 tested genotypes the experiments were repeated 10 times during a 6-month observation period. Secondary embryogenesis in suspension cultures was determined by the increase of induced globulary embryos per g fresh weight of embryogenic starting tissue.

Results and Discussion

The quality of embryogenic suspensions highly depends on the starting material. Primary globular- and heart-stage embryo originating from donor material are the most suitable developmental stages of somatic embryos for induction of subsequent secondary somatic embryogenesis in suspension culture. More advanced developmental stages failed to produce secondary embryos or did not survive in liquid culture; they cannot be recommended for establishing embryogenic suspensions.

A genotype-specific reaction to suspension culture conditions was observed for all tested varieties except Dornfelder. After harvest of primary embryos from the mother explants and transfer to liquid NN69 medium an adaptation period of about 10 d for Müller-Thurgau and Rupestris du Lot was necessary before determining secondary embryo induction. Finally, a genotype-specific tolerance to the liquid culture conditions was outlined independently from the tested parameters:

Dornfelder > Müller-Thurgau > Rupestris du Lot >>> Seyval > Regent > Riesling.

To optimize suspension culture conditions, the influence of carbon sources, phytohormones and of various medium preparations was examined in detail.

Tnduction and propagation of new embryogenic tissue and culture duration were highly influenced by the carbon source independent of genotype and other supplements to the media. The highest induction rate (29 % secondary embryos) was obtained in media containing glucose. The culture period could be prolonged up to 4 weeks. Using maltose, embryo induction was strongly suppressed to only 2.4 % even two weeks after culture initiation. Embryos in suspensions containing sucrose produced 4.4 % secondary globulary embyros comparable to media containing glucose (Table).

Addition of phytohormones stimulated secondary embryogenesis, 10 µ-tM NOA being the most effective treatment independent of the sugar type or genotype (Fig. 1). The combination of 20 µ-tM NOA and 4 µ-tM TDZ promoted embryo germination of cv. Müller-Thurgau (data not shown).

Browning and finally a total loss of embryogenic tissue was caused by a rapid pH decrease to 3.1 within the first 4 d of cultivation in suspension conditions. Embryos could resist a pH range of 4.9 - 4.5 only during a culture period of 2 d. A frequent control of pH is very important to avoid intolerable pH decreases and to fix the time for medium replacement (Fig. 2 a). This effect was reduced by heating the medium (60 °C) prior to pH adjustment to 5.8 (Fig. 2 b) as well as adding of 0.1 M Tris buffer solution (pH 7.5) (Fig. 2 c). Finally, a medium replacement at 4-week intervals and at a pH range of 5.8 - 5.0 showed highest induction rates of secondary embryos in suspension.

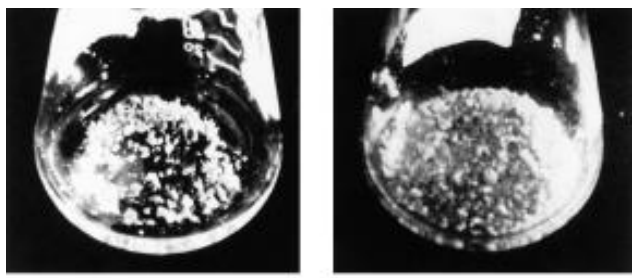
Optimisation of the tested culture parameters does not prevent browning of embyrogenic tissue, whereas browning was reduced or totally avoided by adding DTE (3.0 or 6.5 mM).

A very important point to establish and to maintain a homogeneous embryo suspension is the separation of the embryos according to their developmental stage. At each replacement of medium, embryo separation was carried out by tweezers; shaking has shown not to be suitable for an efficient separation. Shaking of embyrogenic cultures at high rotations (>100 rpm) resulted in abnormal embryo development. Of the three fragment sizes of embryos tested for their capacity to induce secondary embyros, a tissue of 0.1-0.5 mm produced 15 %, while a tissue size of 0.5-2.0 mm produced 50 % of new globulary embryos

Table

Influence of various carbohydrate sources (equivalent concentrations) on the induction of secondary embryogenesis as determined by the increase of induced globulary embryos per g fresh weight of embryogenic tissue during a 4-week culture period

Genotype	Induction of secondary embryogenesis (%) Glucose	Sucrose	Maltose
Seyval blanc	12.1	3.0	2.9
Dornfelder	84.0	6,4	3.2
Regent	4.9	3.0	1.4
Riesling	2.9	1.4	1.0
Müller-Thurgau	47.0	6.0	2.8
Rupestris du Lot	23.1	5.6	3.0
Average	29.0	4.4	2.4



(a) (b)

Fig. 1: Influence of NOA application on quality of embryo suspension cultures in a glucose containing medium of cv. Dornfelder; **(a)** phytohormone-free basal medium; **(b)** medium with 10 mM NOA.

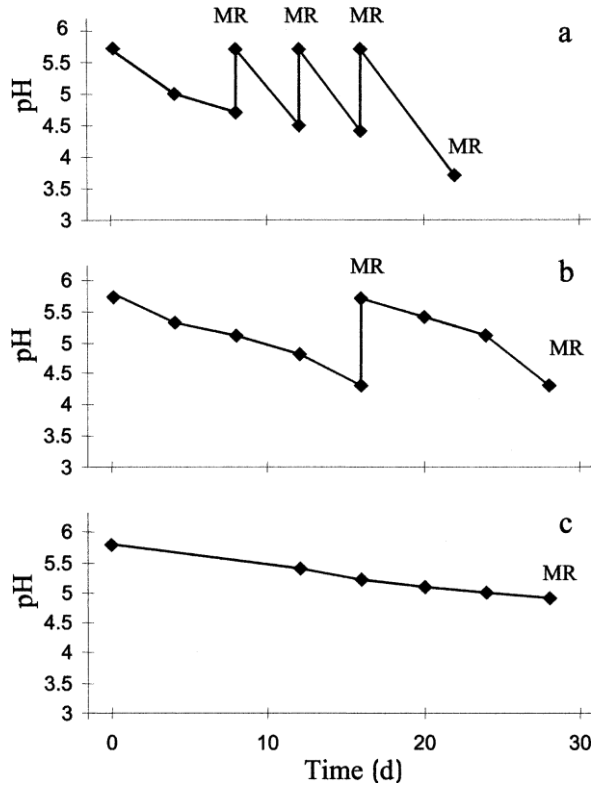


Fig. 2: pH development of a glucose containing medium of embryo suspension and the dates of medium replacement (MR) during 30 d of culture. (**a**) pH adjusted with NaOH at room temperature; (**b**) pH adjusted with NaOH at 60 °C; (**c**) pH adjusted with 0.1 M Tris buffer (pH 7.5) solution at 60 °C.

within 12 weeks. Embryos >2.0 mm were transferred to solidified NN69 medium for further germination. The developed seedlings were transferred to LS medium where they regenerated within 4-6 weeks to rooted *in vitro* plantlets. Within the observation period seedlings of Dornfelder and Müller-Thurgau regenerated to intact plantlets at a rate of 37 and 19 %, respectively.

Conclusion

The results demonstrate that cultures of homogeneous embryos can be obtained by induction of secondary embryos in liquid cultures. Culture conditions can be optimized by addition of 3.6 % glucose and 10 mM NOA to the basal medium as well as by heating (60 °C) and pH adjustment to 5.8 prior to autoclaving (using 0.1 M Tris buffer (pH 7.5) solution). Tn preliminary experiments a medium replacement was necessary for pH values <4.5 or for a culture exceeding 4 weeks.

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Received December 21, 1999