

## Research Note

Regeneration of grapevine (*Vitis* sp.)  
protoplastsG. REUSTLE<sup>1)</sup>, MARGIT HARST<sup>2)</sup> and G. ALLEWELDT<sup>2)</sup>

The availability of a protoplast-to-plant system would offer new perspectives for breeding of disease resistant grapevines.

Grapevine, known to be recalcitrant for tissue culture experiments as many other perennial species, has been successfully regenerated from nodes and meristems from a wide range of varieties, whereas the regeneration of anthers, ovules, or leaf and petiole tissue (STAMP and MEREDITH 1988, CHENG and REISCH 1989, MARTINELLI *et al.* 1993) is restricted to few genotypes. Although intensive efforts have been made during the last decade to develop a regeneration protocol for grapevine protoplasts, only cell division and formation of microcalli could be achieved (LEE and WETZSTEIN 1988, MII *et al.* 1991, REUSTLE and ALLEWELDT 1990, U<sub>I</sub> *et al.* 1990). In our recent experiments, we could now demonstrate that grapevine protoplast regeneration is possible.

**Materials and methods:** For protoplast isolation, embryogenic tissue (somatic embryos in different stages of development), which has been induced on leaf disks of *Vitis vinifera* L. cv. Seyval blanc (HARST and ALLEWELDT 1994) was used as donor tissue. The isolation protocol was carried out according to REUSTLE and NATTER (1994). After over-night digestion and subsequent purification, the derived protoplasts were resuspended in 0.65 M mannitol (pH 5.8). Protoplast density was adjusted to  $0.5 \times 10^6$  cells per ml mannitol. For cultivation, protoplasts were immobilized in Na-alginate (2 %) applying a modified method of KARESCH *et al.* (1991). Protoplasts, embedded in the alginate layers (density of  $0.25 \times 10^6$  per ml) were transferred into petri dishes (5 cm  $\phi$ ) with 2 ml of several media and cultivated at 24-26 °C in the dark.

As culture media a modified CPW-13 medium (FREARSON *et al.* 1973) with 0.54 M glucose and NN-69 medium (NITSCH and NITSCH 1969) with 0.6 M glucose, were used for the initial cultivation step. Each medium was supplemented with 1.0 % PVP-40. During the initializing period, two hormone treatments i) 1 ppm 2,4-D and 0.5 ppm BAP or ii) 4.0 ppm NOA and 0.9 ppm TDZ were tested. As control treatment, a hormone-free variant was used.

After 4 weeks, alginate layers were transferred to hormone-free NN-69 medium (for 4 weeks) containing 0.4 M glucose and 1 % PVP-40, followed by a further subcultivation in the same medium but with reduced glucose concentration (0.2 M).

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Fragments of the alginate layers (1.5-2.0 mm in size) containing microcalli of different types and sizes were then transferred in petri dishes (10 cm  $\phi$ ) onto solidified (0.3 % gelrite) NN-69 medium with 0.2 M glucose.

**Results and discussion:** **Isolation:** With the applied isolation method, yields of  $0.8$  to  $4.7 \times 10^6$  protoplasts per gram material were achieved. The protoplasts varied in size from 10 to 50  $\mu$ m and most of them were rich in cytoplasm.

**Cell division:** Beginning and rate of cell division depended on the media used and on the applied hormones. When CPW-13 medium was used, protoplasts started to divide during the 2nd week of cultivation independent of hormone treatment. Using the NN-69 medium, cell division occurred only after NOA/TDZ application. In contrast to CPW-13 medium, first cell division could be observed at the earliest 4-5 weeks after culture initiation. Furthermore, plating efficiency in CPW-13 medium was higher. Without hormones, protoplasts formed new cell walls within 2 weeks, however, cell division could not be observed.

**Microcallus formation:** Best development of divided protoplasts to microcallus arose in CPW-13 medium with NOA/TDZ as hormone treatment. 6-8 weeks after culture initiation, friable microcalli, consisting of large cells, developed and the microcalli reached sizes of 0.5-1.5 mm. In case of 2,4-D/BAP application, growth of the divided protoplasts was low. Using NN-69 medium with NOA/TDZ as initializing variant, the developed microcalli consisted of smaller cells and the texture remained compact. The positive effect of CPW-13 medium on protoplast division and microcallus formation was already found in earlier studies with grapevine protoplasts (REUSTLE and NATTER 1994). The reduced growth of divided protoplasts in the presence of 2,4-D seems to be due to a genotype-specific sensitivity of Seyval to this hormone.

**Somatic embryogenesis:** Small embryogenic cell aggregates (100 - 200  $\mu$ m) could be observed in the alginate layers at the earliest 8 weeks after cultivation when NN-69 medium with NOA/TDZ was used in the initial cultivation step (Fig. 1). Ten weeks after culture initiation, somatic embryos and embryoides were visible on the alginate layers with the naked eyes (Fig. 2).

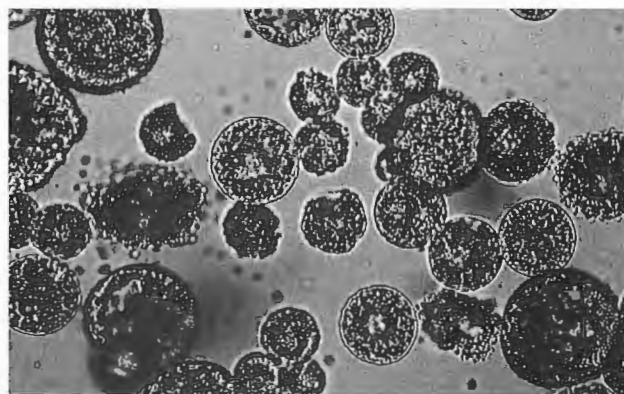


Fig.1: Freshly isolated protoplasts from embryogenic tissue of grapevine

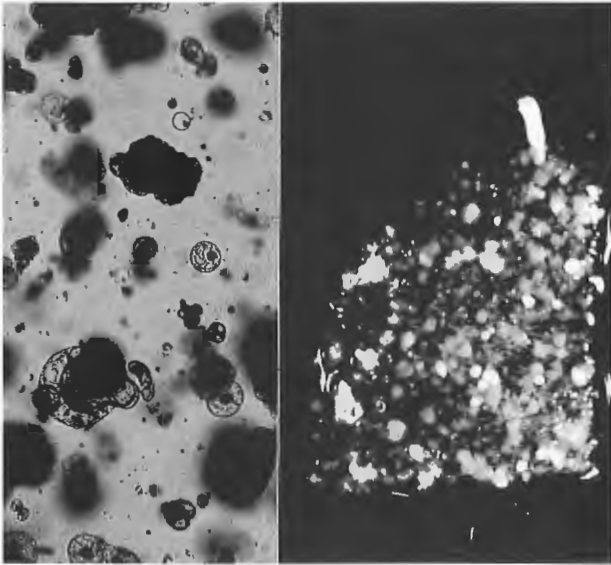


Fig. 2 (left) : Embryogenic cell aggregates, embedded in alginate, approx. 8 weeks after culture initiation.

Fig. 3 (right): Formation of somatic embryos on microcalli of grapevine protoplasts (12-14 weeks after culture initiation).

After transfer of microcalli containing fragments of the alginate layers on solidified NN-69 medium, on up to 40 % of these fragments somatic embryos arose within 4-6 weeks. Only very few of the transferred calli, obtained after 2,4-D/BAP treatment in CPW-13 medium, formed somatic embryos. No embryogenesis could be induced with CPW-13 in combination with the NOA/TDZ treatment.

Obviously, using CPW-13 medium, growth and development of embryogenic cells are suppressed due to the supporting effect of this medium on the growth of non-embryogenic cells. In contrast, using NN-69 medium with NOA/TDZ treatment, development of cells with embryogenic competence seems to be favoured. The suitability of NN-69 medium in combination with NOA/TDZ to somatic embryogenesis induction was found in previous experiments with leaf disks of Seyval blanc as well (HARST and ALLEWELDT 1994).

**Plant regeneration:** After subcultivation on hormone-free NN-69 medium, some of the embryos elongated spontaneously and formed a root and a small shoot (Fig. 3). When the germinated embryos reached a size of 0.5-1.0 cm, they were transferred to solidified (0.3 % gelrite) LS-medium (LINSMAIER and SKOOG, 1965) in culture tubes. Within 4-6 weeks most of them turned green and *in vitro* plantlets developed (Fig. 4).

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Fig. 4: Grapevine plantlet, regenerated from Seyval blanc protoplasts (5 months after culture initiation)

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