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Research Note

An improved method for embryogenic suspension cultures of 'Richter 110' rootstock

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Abbreviations: CP: Chée & Pool medium; MC: Monte Carlo simulation method; MS: Murashige and Skoog medium; NOA: naphthoxyacetic acid; Sm: Supplementary material.

Introduction: Grapevine (*Vitis* spp.) is one of the most important and widely grown fruit species. A highly efficient, well-synchronised regeneration system based on somatic embryogenesis is essential for gene transfer, *in vitro* clonal selection and mutation experiments as well as for the production of viroid and virus free plants. Successful somatic embryogenesis has been reported on solid media for various *Vitis* species and *V. vinifera* genotypes (Carimi *et al*. 2012, Martinelli and Gribaudo 2009). Establishment of embryogenic cell suspensions, initiated mainly from anther-derived embryogenic calli, have also been published (Vidal *et al*. 2009, Xu *et al*. 2014). The aim of the present study was to improve the efficiency of establishment and maintenance of *Vitis berlandieri* x *V. rupestris* 'Richter 110' rootstock cell suspensions. Based on the described method a well-synchronised, highly efficient multi-step regeneration system was also developed.

Material and Methods: Anther-derived embryogenic callus cultures of the *V. berlandieri* x *V. rupestris* 'Richter 110' rootstock were induced and maintained on solid MSE medium as desribed by Zok *et al*. (2010). Eight different media were used to study their effectiveness for cell suspension initiation and growth. Embryogenic cell suspensions were started in 30 mL medium in 125 mL Erlenmeyer flasks. Six different maintenance methods were compared in MSM1 medium differing in initial cell densities and maintenance method. The cultures were incubated in the dark on an orbital rotary shaker (120 rpm) at 25 ± 1 °C in three or five replicates (Sm_Tab. 1.).

Five weeks after the initiation of embryogenic cell suspensions somatic embryo differentiation was started in hormone-free D1 (half-strength MS) and D2 (full-strength MS) media both supplemented with 18 $g \cdot L^{-1}$ maltose, 4.6 $g \cdot L^{-1}$ glycerine and 1 $g \cdot L^{-1}$ lactalbumin hydrolysate (pH 5.8) with different initial cell densities. During culture maintenance the whole amount of the medium was replaced weekly with fresh medium. After four weeks the cultures were diluted to different densities (Sm_Tab. 2) in D1, D2 or D1ac (D1 medium supplemented with 1.0 $g \cdot L^{-1}$ activated charcoal). Treatments were carried out in three replications.

Seven weeks after the initiation of embryo differentiation cultures were transferred to hormone-free solid MSC medium (half-strength MS with 10.0 $g \cdot L^{-1}$ sucrose, 2.0 $g \cdot L^{-1}$ activated charcoal, 6.0 $g \cdot L^{-1}$ agar, pH 5.8) and incubated in a light room at 25 ± 1 °C with a 16/8 h photoperiod (50 $\mu M m^{-2} \cdot s^{-1}$). Cultures were subcultured monthly to the same medium. After two months the total weight of the embryos on each plate and the weight of 4 × 100 embryos were recorded. For plant regeneration 25 embryos were plated in eight replications on MSC medium. After the development of the first leaves plantlets were transferred to MSEM medium (Zok *et al*. 2010).

Statistical analysis: Repeated measures of ANOVA model were applied to compare the media effect on fresh mass increase, the maintenance method effect on pH and relative increase of mass. In order to estimate the expected number of embryos in terms of 1.0 g embryogenic cell aggregate, we applied the Monte Carlo (MC) method to simulate the situation as if the experiment were executed for the whole amount of initial 1.0 g embryogenic cells (see Sm).

Results and Discussion: Repeated measures ANOVA was significant (Wilk's unexplained variance; fresh mass increase: $\lambda = 0{,}03$; $p < 0.001$; pH: 0.16; $p < 0.001$; relative increase of mass: 0.007; $p < 0.001$). Both within-subject effects (time) and between-subject effects (media; maintenance method, respectively) were highly significant. Results of Games-Howell's post hoc test is represented on Figure and Sm_Fig. 1 (see Sm). Our results indicate that MS medium alone can be used both for the induction and maintenance of 'Richter 110' embryogenic calli as well as for the establishment of embryogenic cell suspensions. The greatest increase in fresh weight of embryogenic cell suspensions was observed in MSM1 and CP2 media both containing maltose (18 $g \cdot L^{-1}$) as carbohydrate source and 3.34 µM NOA (Figure). The "dynamic" maintenance mode applied in treatments I.–IV. where the density was readjusted to the initial value each week resulted in the greatest increase in fresh weight. By the end of the 5th week the cultures in treatment I. exhibited a fresh mass increase of more than 40-fold (Sm_Fig. 1).

Cultures with various densities (0.5-8.0 mg cell$\cdot mL^{-1}$) established in liquid D1 (half-stregth MS) medium formed synchronised embryos in the globular stage by the 4th week (Sm_Fig. 2). These densities were readjusted after four weeks. The cultures with the lowest density (0.5 mg cal-

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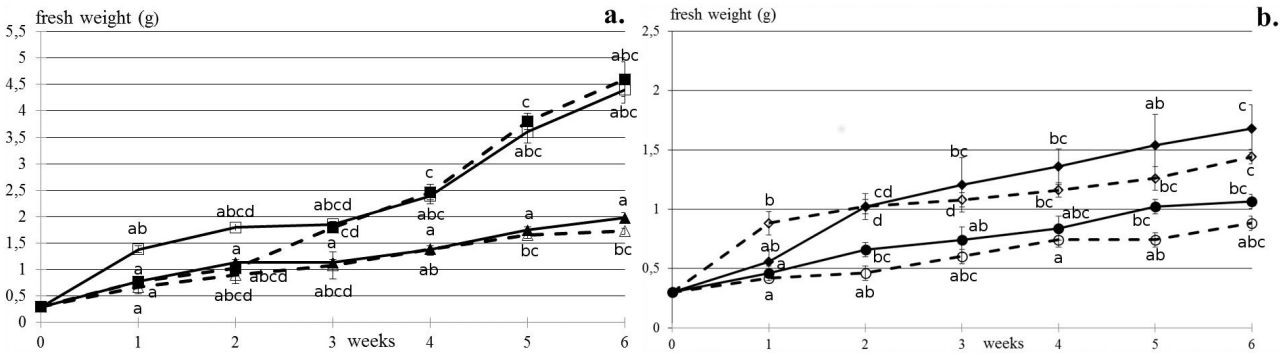

Figure: Effect of media with different components on the fresh weight increase of 'Richter 110' embryogenic cell suspensions. **a**: MSM1 (■), MSM2 (△), CP1 (▲), CP2 (□). **b**: MSB1 (○), MSB2 (◆), MSB3 (●) and MSB4 (◇). Data represent means of three independent repeats, bars indicate SD values. (see also Sm_Tab. 1). Different letters indicate significant differences in media effect recorded weekly by Games-Howell's post hoc test ($p < 0.05$).

lus·mL^{-1}) formed asynchronised globular-, heart-, torpedo-shaped or cotyledonary embryos seven weeks after the initiation of embryo differentiation cultures. At higher densities (1.0 or 2.0 mg·mL^{-1}) the cultures formed synchronised cotyledonary embryos. At 4.0 mg·mL^{-1} cell density torpedo-shaped and early cotyledonary stage embryos were produced, while at 8.0 mg·mL^{-1} only globular and torpedo-shaped embryos were observed. When cultures were maintained at 8.0 mg·mL^{-1} cell density and diluted after four weeks to 1.0 mg·mL^{-1}, mostly torpedo-shaped and cotyledonary embryos were formed. Cultures maintained at 0.5, 1.0 and 2.0 mg·mL^{-1} densities differentiated approximately 1.0–1.6 million embryos from 1.0 g cell cultures as calculated by the MC method (Sm_Tab. 3. and Sm_Fig. 3). The culture density affects not only the amount of differentiating embryos, but also their stage of development and the synchronisation of the cultures (Martinelli and Gribaudo 2009). These data show that to achieve full synchrony, it is essential to use low cell density.

When the 'Richter 110' cell suspensions (1 mg·mL^{-1} density) were not diluted, the globular embryos failed to exhibit further differentiation. As the cultures were readjusted to higher cell densities after four weeks the embryo development continuously decreased. In D1ac medium (containing activated charcoal) smaller cotyledonary embryos with diverse morphology developed. Depending on the medium used, the cultures produced approximately 1.2-2.2 million embryos from 1.0 g starting material after four weeks. Seven weeks after initiation of embryo differentiation 200 embryos grown in D1ac medium were transferred to solid MSC medium. Approximately 82 % of them regenerated into entire plants (Sm_Fig. 4).

Successful regeneration and genetic transformation of 'Richter 110' through somatic embryogenesis has already been published (Carimi *et al*. 2012, Geier *et al*. 2008, Zok *et al*. 2010), but we have limited knowledge on establishment of synchronized suspensions cultures of this rootstock (Ben Amar *et al*. 2007). The most frequently applied method of maintaining cell cultures includes replacement of 50-75 % of the medium every 1-2 weeks (de la Torre *et al*. 2012, Vidal *et al*. 2009), but the weekly re-adjustment of the initial density (Jayasankar *et al*. 1999) are rarely employed. The published protocols resulted in approximately a 3-10-fold fresh weight growth for different rootstock and *V. vinifera* cultivars (Ben Amar *et al*. 2007, de la Torre *et al*. 2012). In our experiments approximately 40-fold fresh weight increase was observed after five weeks of growth for 'Richter 110' started with low cell densities. We improved the production of well synchronized 'Richter 110' embryos without sieving the cell cultures, or daily transfer into fresh medium or fractionation of the cell population as previouly published (Jayasankar *et al*. 1999, Ben Amar *et al*. 2007, Colova (Tsolova) *et al*. 2007). To the best of our knowledge it is the highest growth and embryo differentiation rate for embryogenic suspension cultures of 'Richter 110'. Our results suggest that the greatest increase in fresh weight could be achieved using this dynamic maintenance method, during which the density of the culture was re-adjusted to the initial cell density weekly by adding fresh medium. Our result may contribute to an efficient grapevine transformation and virus elimination method.

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