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

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Table 1

Media used for the establishment and maintenance of embryogenic cell suspensions

Medium	Basic components of the medium	PGRs*	Carbon source	Maintenance method**
MSM1	MS	3.34 µM NOA	18 g·L^{-1} maltose	I, II, III, IV, V, VI
MSM2	MS	4.52 µM 2,4-D	20 g·L^{-1} sucrose	II
CP1	CP	4.52 µM 2,4-D	18 g·L^{-1} maltose	II
CP2	CP	3.34 µM NOA	18 g·L^{-1} maltose	II
MSB1	Gamborg B5 macroelements, MS microelements and vitamins	4.52 µM 2,4-D	60 g·L^{-1} sucrose	II
MSB2	Gamborg B5 macroelements, MS microelements and vitamins	3.34 µM NOA	18 g·L^{-1} maltose	II
MSB3	Gamborg B5 macroelements, MS microelements and vitamins	4.52 µM 2,4-D	18 g·L^{-1} maltose	II
MSB4	Gamborg B5 macroelements, MS microelements and vitamins	4.52 µM 2,4-D	20 g·L^{-1} sucrose	II

*PGRs: plant growth regulators. **Methods: I: Cell density was adjusted weekly to 5.0 mg·mL^{-1}, II: to 10.0 mg·mL^{-1} and III: to 20.0 mg·mL^{-1} with fresh medium and 30 % of the old medium was removed. IV: Cell density was adjusted weekly to 10.0 mg·mL^{-1} during maintenance and fresh medium was added to maintain the initial cell density. V: Cell density was not adjusted weekly, dilution involved doubling the volume weekly. Initial cell density was 10.0 mg·mL^{-1}. VI: Cell density was not adjusted (no dilution) and maintenance involved the weekly exchange of 50 % (15 mL) of the used medium to fresh medium. Initial cell density was 10.0 mg·mL^{-1}. MSM1 and MSM2 are Murashige and Skoog (1962) based media, CP1, CP2 are Chée and Pool (1987) based media. MSB1-MSB4 media were prepared with Gamborg's B5 macroelements (Gamborg *et al*. 1968) supplemented with MS microelements and vitamins. Each medium contained 4.6 g·L^{-1} glycerine, 4.52 µM 2,4-dichloro-phenoxy-acetic acid (2,4-D) or 3.34 µM naphtoxy-acetic-acid (NOA), and carbon sources (sucrose or maltose) (Mauro *et al*. 1995, Jayasankar *et al*. 1999). The pH was adjusted to 5.8 before autoclaving of the media.

References

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Table 2

Cell densities during embryo differentiation

Initial medium and density		Density and medium applied for dilution after four weeks	
Medium	Cell density (mg·mL^{-1})	Medium	Cell density (mg·mL^{-1})
D1	0.5	D1	0.5
	1		-, 0.5, 1, 2, 4, 8
	2		2
	4		4
	8		1, 8
D2	0.5	D2	0.5
	1		1
	2		2
	4		4
	8		8
D1	1	D1ac	1

- : no dilution

Table 3

Estimated number of 'Richter 110' embryos obtained from 1.0 g embryogenic cell suspension formed in different media with different densities and their stage of development

Medium	Initial cell density ($mg \cdot mL^{-1}$)	Readjusted cell density in the fourth week ($mg \cdot mL^{-1}$)	Number of embryos obtained from 1 g cell aggregates*	Embryo developmental stage
D1	0.5	0.5	1 671 127±338 934	globular-heart-torpedo-cotyledonary
D1	1	1	1 387 808±114 032	cotyledonary
D1	2	2	1 056 491±148 305	cotyledonary
D1	4	4	515 596±60 680	torpedo-early cotyledonary
D1	8	8	415 363±87 726	globular-torpedo
D1	8	1	471 381±163 747	torpedo-early cotyledonary
D1	1	-	-	globular
D1	1	0.5	1 875 270±161 274	cotyledonary
D1	1	2	1 234 604±157 579	cotyledonary
D1	1	4	1 404 178±229 056	early cotyledonary
D1	1	8	1 489 222±401 013	torpedo-early cotyledonary
D1→D1ac	1	1	2 247 738±304 040	cotyledonary
D2	0.5	0.5	707 106±88 673	globular-heart-torpedo-cotyledonary
D2	1	1	743 301±88 724	globular-heart-torpedo-cotyledonary
D2	2	2	717 782±120 609	globular-heart-torpedo-cotyledonary
D2	4	4	283 809±62 280	globular-heart-torpedo
D2	8	8	310 063±32 895	globular-heart-torpedo

*Mean and standard deviation was calculated by Monte Carlo simulation method. ±: SD; - : no dilution.

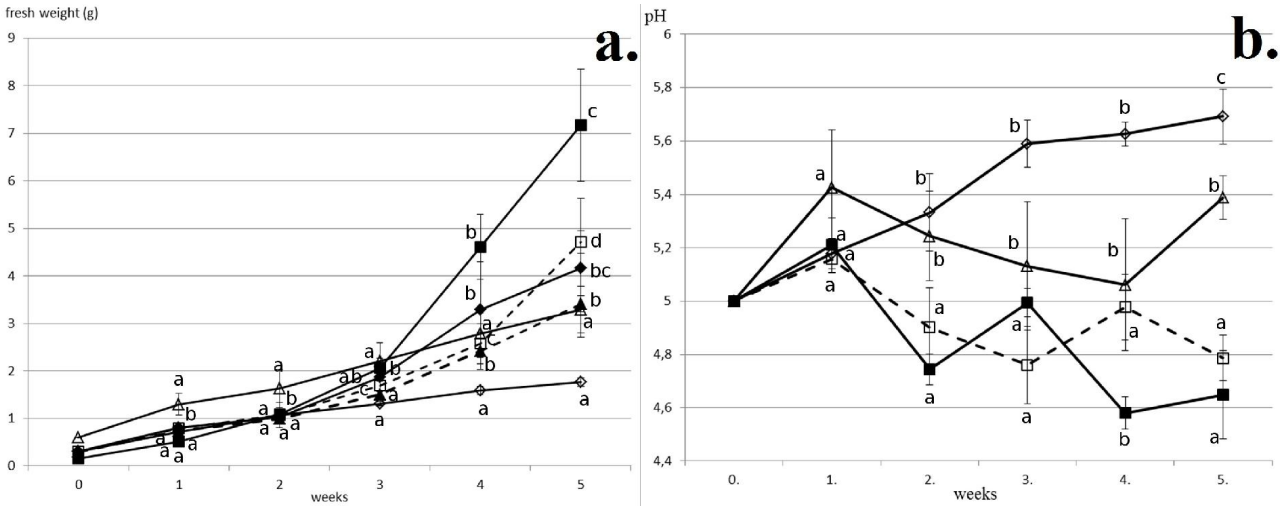

Fig. 1: Effect of different maintenance methods on the fresh weight (**a**) and pH (**b**) of 'Richter 110' embryogenic cell suspensions in MSM1 medium during the 5 weeks after initiation. **a:** I: Cell density was adjusted weekly to 5.0 $mg \cdot mL^{-1}$ (■), II: to 10.0 $mg \cdot mL^{-1}$ (□) and III: to 20.0 $mg \cdot mL^{-1}$ (△) with fresh medium and 30% of the old medium was removed. IV: Cell density was adjusted weekly to 10.0 $mg \cdot mL^{-1}$ during maintenance and fresh medium was added to maintain the initial cell density (◆). V: Cell density was not adjusted weekly, dilution involved doubling the volume weekly (▲). Initial cell density was 10.0 $mg \cdot mL^{-1}$. VI: Cell density was not adjusted (no dilution) and maintenance involved the weekly exchange of 50 % (15 mL) of the old medium for fresh medium (◇). Initial cell density was 10.0 $mg \cdot mL^{-1}$. Data represent the means of five independent replications, bars indicate SD values. **b**: Alteration of the pH value of embryogenic cell suspensions in MSM1 medium during the 5 weeks after initiation. I: (■), II: (□), III: (△) and VI: (◇). In all cases, the cultures were established in 30 mL of fresh medium in 125 mL Erlenmeyer flasks. Data represent means of five replications, bars indicate SD values. Different letters indicate significant differences in media effect recorded weekly by Games-Howell's post hoc test ($p < 0.05$). The pH dropped after autoclaving from the initial 5.8 value to 5.0. The pH of the media was measured from the removed part of medium (method I., II., III. and VI:) weekly using a Radelkis OP-115 pH meter.

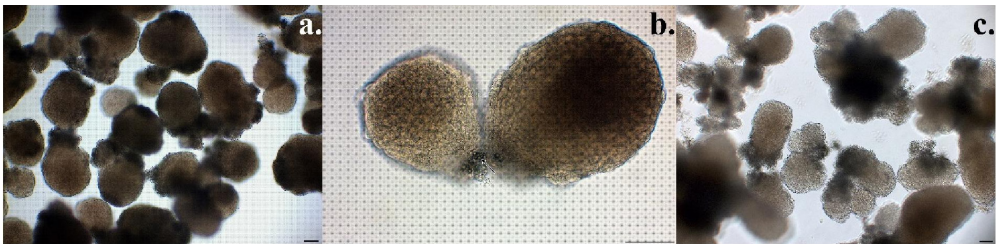

Fig. 2: Synchronised 'Richter 110' embryos in the 4th week of cell cultures established in D1 medium with an initial density of 1.0 mg·mL^{-1} (**a**, **b**). Non-synchronised 'Richter 110' embryos attached to cell aggregates in the 4th week, in D2 medium with an initial cell density of 1.0 mg·mL^{-1} (**c**).

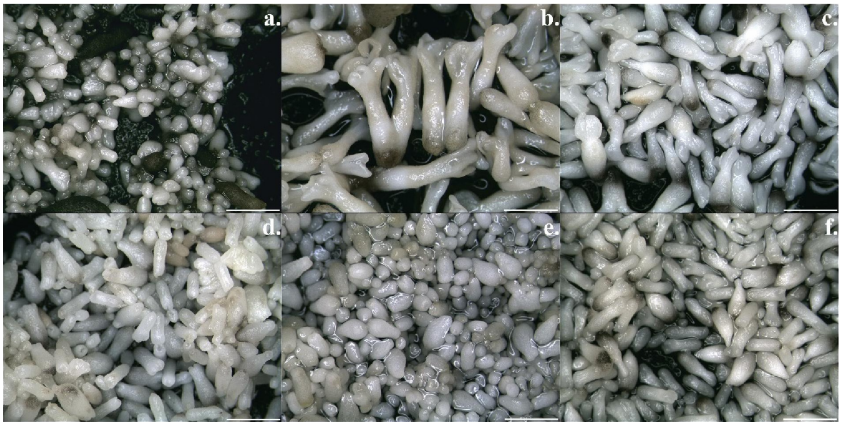

Fig. 3: 'Richter 110' embryos developed in D1 medium at various densities during the 4th week on solid MSC medium. Density was adjusted to **a**: 0.5 mg·mL^{-1}, **b**: 1.0 mg·mL^{-1}, **c:** 2.0 mg·mL^{-1}, **d**: 4.0 mg·mL^{-1}, **e**: 8.0 mg·mL^{-1}, and readjusted after four weeks to the same value, **f**: the initial 8.0 mg·mL^{-1} density was diluted to 1.0 mg·mL^{-1} after four weeks. Bars represent 2.0 mm.

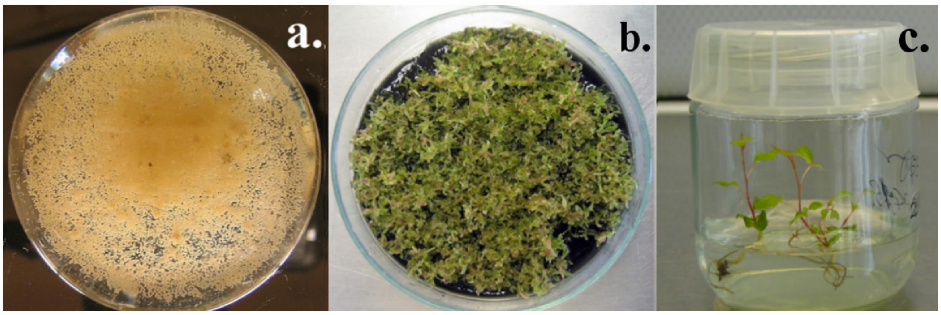

Fig. 4: The regeneration process of 'Richter 110'. Embryo differentiation in hormone-free liquid D1 medium in the 4th week. **a**: Green plantlets in the 8th week on solid MSC medium; **b**: Entire plants on solid MSEM medium (**c**).

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 defined by ln((weight_i-weight_0)/weight_0)). Normality of residuals was proved by Shapiro-Wilk's test ($p > 0.05$). Sphericity and homogeneity of variances were both slightly violated (Levene's; fresh mass increase: $0.05 > p > 0.001$; pH: $0.05 > p > 0.01$; relative increase of mass: $p > 0.04$), therefore we used Greehouse-Geisser's degree of freedom correction ($\varepsilon = 0.55$; 0.55 and 0.46, respectively) for within-subject effects detection and Games-Howell's post hoc test for pairwise comparisons. 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 (fresh mass increase: $F_{time}(2.8;44.15) = 262.15$; $p < 0.001$); $F_{media}(7;16) = 26.60$; $p < 0.001$; pH: $F_{time}(2.2;34.9) = 7.21$; $p < 0.01$); $F_{maintenance\ method}(3;16) = 84.33$; $p < 0.001$; relative increase of mass: F_{time} (1.9;44.6) = 1256.95; $p < 0.001$); F $F_{maintenance\ method}$ (5;24) = 86.86; $p < 0.001$).

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 of embryogenic cells. Having the means and standard deviations calculated from counted numbers of embryos on the plates (with sample sizes 12) together with the different ratios of the initial and fourth week dilution densities as the simulated sample sizes (N), we generated random samples of normal distribution 100 000 times. We also used a correction term for the standard deviations which was calculated as the ratio of the standard deviations of a sample with size N and 12, taken both from the very same distribution. Finally, we calculated the 95 % confidence interval for the expected number of embryos in terms of 1.0 g embryogenic cell in each case of media and density pair.