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Optimization of horse chestnut (*Aesculus hippocastanum* L.) somatic embryo conversion

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Abstract Factors affecting conversion of horse chestnut (*A. hippocastanum* L.) somatic embryos into plantlets were evaluated. Anther filament derived embryogenic tissue developed bipolar structures with two cotyledons and a well-developed shoot and root apical meristem upon auxin omittance from the culturing medium. The impact of carbohydrate type (glucose, fructose, sucrose and maltose) and concentration (3 and 6%) on somatic embryo maturation and conversion were evaluated. Although conversion frequencies were high for all treatments, overall quality of regenerated plantlets was poor. Increasing the carbohydrate concentration in the maturation medium did not increase conversion of somatic embryos or quality of regenerated plantlets in terms of shoot height. On the contrary, addition of PEG (polyethylene glycol) in maturation media had a beneficial effect on shoot quality of regenerated plantlets. Sucrose was a superior carbon source when PEG was included in the maturation medium, in terms of conversion rate (65.7%) as well as of shoot quality of plantlets (43.8% of plantlets had shoots >2 cm). Clonal fidelity of the different development stages of somatic embryogenesis and of converted plantlets was assessed by flow cytometry and no major ploidy changes were found.

Abbreviations

BA	N^6-benzyladenine
CV	Coefficient of variation
IBA	4-[3-Indolyl]butyricacid
MOPS	3-N-[Morpholino]propanesulfonicacid
MS	Murashige and Skoog
PEG	Polyethylene glycol
WPM	Woody plant medium

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Introduction

Horse chestnut (*Aesculus hippocastanum* L.) is one of the most popular ornamental trees in Europe due to its high tolerance to air pollution and wide phenotypic variation. It is native only to the Balkan-peninsula, but has been planted throughout Europe and the US as an ornamental and industrial shade tree. However, since 2002 the number of horse chestnuts with bleeding cankers has increased and the disease became severe in some parts of Europe, especially in the Netherlands (Schmidt et al. 2008). Propagation technologies offer the prospect for accelerated delivery of tolerant genotypes. Approaches for clonal tree production include vegetative cuttings, organogenesis (shoot multiplication) and somatic embryogenesis.

Somatic embryogenesis is a preferred method for automated mass propagation and serves as explant for cryostorage of valuable germplasm. Several methods for successful induction and proliferation of somatic embryos of horse

chestnut from anther filament cultures (Ćalić et al. 2005; Capuana and Debergh 1997; Jörgensen 1989; Kiss et al. 1992; Radojevíc 1978) and from stem explants (Gastaldo et al. 1994) have been reported. Other explants, like primary leaves of plantlets taken from mature seeds (Dameri et al. 1986), immature embryos (Radojević 1988) and cotyledon fragments (Profumo et al. 1990) were also used for induction of horse chestnut somatic embryos, but these involve juvenile material of unknown genotypes. The use of stamen filaments or stem tissue from an adult plant may guarantee relative genetic stability in the commercial production of plants that come from such embryos (Gastaldo et al. 1994). The frequency of embryos that germinated and converted into plantlets was for each of these protocols very low. Low conversion rates of somatic embryos into plantlets might be associated with aberrant embryogenesis and the absence of a well-organized shoot meristem (Moon et al. 1994; Suhasini et al. 1996; Wetzstein et al. 1993). Also, asynchronous development, as was reported for horse chestnut somatic embryogenesis by Radojevic (1988) and Capuana and Debergh (1997), may have a negative impact. In many species however, low conversion rates were attributed to poor somatic embryo quality caused by a lack of maturation and desiccation tolerance (Etienne et al. 1993). Attempts to promote the conversion of somatic embryos of horse chestnut, by increasing ABA levels in the maturation medium or by desiccation treatments involving air drying, failed to increase conversion rates higher than 10% (Capuana and Debergh 1997). Partial desiccation can also be induced using a proper concentration and type of carbohydrate as osmoticum in the culture medium. This approach increased the maturation and germination of somatic embryos of a number of tree species including *Quercus robur* (Sánchez et al. 2003) and *Castanea sativa* (Corredoira et al. 2003). Also polyethylene glycol (PEG) was shown to be an important osmoticum in the maturation stage of somatic embryos (Linossier et al. 1997; Misra et al. 1993; Shoji et al. 2006; Svobodová et al. 1999; Tremblay and Tremblay 1995; Walker and Parrott 2001). PEG is a non-plasmolysing osmoticum as its large molecules are not able to pass through the cell wall, leading to a restriction of water uptake, a reduced turgor pressure and a more negative intracellular osmotic potential (Misra et al. 1993).

The aim of this work was to study histological changes associated with the induction and development of horse chestnut somatic embryos and to test treatments to improve the maturation in order to obtain a more efficient conversion of horse chestnut somatic embryos. Therefore the effects of carbohydrate type and concentration were studied and the suitability of adding PEG to the maturation medium was also evaluated. Finally, flow cytometric analysis was used to verify the ploidy stability of the *A. hippocastanum* L. somatic embryogenesis process.

Materials and methods

Plant material and culture conditions

Flower buds of horse chestnut (*Aesculus hippocastanum* L.) were collected at stage 3–4 (buds 2–3 mm long and completely closed) and surface sterilized according to Capuana and Debergh (1997). The same adult tree of *A. hippocastanum* was sampled as by Capuana and Debergh (1997). Anther filaments were cultured in Petri dishes on MS (Murashige and Skoog 1962) mineral salts supplemented with 2 mg l^{-1} thiamine, 5 mg l^{-1} nicotinic acid, 10 mg l^{-1} panthothenic acid, 100 mg l^{-1} myo-inositol, 200 mg l^{-1} casein hydrolysate, 2% sucrose, 1% Difco-bacto agar and 2 mg l^{-1} 2,4-D according to Capuana and Debergh (1997). The pH of the medium was adjusted to 5.7 before autoclaving at 120°C for 30 min. Cultures were incubated in the dark at 23 ± 1°C. After 1 month, calli appeared on the filaments, and these were subcultured for an extra month on the same fresh medium. The calli were, according to Capuana and Debergh (1997), subsequently grown at a 16 h photoperiod and transferred to the same medium devoid of 2,4-D, supplemented with 400 mg l^{-1} of filter-sterilized glutamine and 50 g l^{-1} PEG (PEG 4000, Roth, Karlsruhe; MG = 3,500-4,500 g mol^{-1}). Cultures were incubated at 23 ± 1°C under cool white fluorescent tubes (OSRAM 31, 36 W) providing a photosynthetic active radiation of 40 µmol m^{-2} s^{-1}.

Histological analysis

Embryogenic and non-embryogenic calli and somatic embryos at different stages (globular, heart, torpedo and cotyledonal stage) were fixed in FAA (formaldehyde: acetic acid: 70% ethanol 1:1:18). After a dehydration process through gradual ethanol/butanol series the samples were embedded in paraffin. Longitudinal sections of 12 µm were cut on a rotary microtome. Sections were dewaxed with xylol and hydrated in ethanol series. The sections were stained with safranin and Fast green according to Johansen (1940), mounted in Canada balsam and observed under a light microscope (Leica DM IRB microscope).

Maturation of somatic embryos

Experiment 1: type and concentration of carbohydrate

Early cotyledonal stage somatic embryos of 8–10 mm with two cotyledons were isolated from the embryogenic lines and cultured horizontally in Petri dishes containing 25 ml of maturation medium. The basal medium consisted of WPM salts and vitamins (woody plant medium, McCown and Lloyd 1981) supplemented with 4 mg l^{-1} thiamine,

4.5 mg l^{-1} nicotinic acid, 900 mg l^{-1} myo-inositol, 0.8% Difco-bacto agar, and 400 mg l^{-1} of filter-sterilized glutamine, at pH 5.7. The monosaccharide's glucose and fructose and the disaccharides sucrose and maltose were added to the basal maturation medium at two concentrations, 3 and 6%. Each treatment was applied to 50 somatic embryos distributed ten per dish and in five replicates. The cultures grew on these media for 4 weeks under the same growth conditions as for calli, mentioned above.

Experiment 2: type of carbohydrate and addition of PEG

In a second experiment polyethylene glycol (PEG 4000, Roth, Karlsruhe; MG = 3,500–4,500 g mol^{-1}) at 0 or 50 g l^{-1} was combined with 3% glucose, fructose, sucrose or maltose as carbohydrate source. All other conditions were the same as for experiment 1.

Cold treatment and conversion of somatic embryos

The effect of various maturation media was evaluated in terms of subsequent somatic embryo germination and plantlet conversion ability. After 4 weeks of culture on diverse maturation media, somatic embryos were transferred to basal medium supplemented with 3% sucrose and stored at 4°C for 8 weeks in darkness. Initial experiments showed that minimal 8 weeks of cold treatment were necessary for conversion of *A. hippocastanum* somatic embryos (data not shown). Similar results were found for other recalcitrant woody species f.e. for *Castanea sativa* (Corredoira et al. 2003) and for *Castanea dentata* (Andrade and Merkle 2005) somatic embryos. After this, embryos were transferred into glass vessels (375 ml) containing 100 ml germination medium (five somatic embryos per vessel with ten replications). Germination medium consisted of WPM salts and vitamins (Lloyd & McCown 1981) supplemented with 3% sucrose, 0.8% agar, 0.2 mg l^{-1} BA (N^6-benzyladenine) and 0.02 mg l^{-1} IBA (4-[3-indolyl]butyric acid), at pH 5.7. For germination, embryos were oriented with their radicle ends inserted into the gelled medium. Culture conditions for germination were the same as for maturation. After 6 weeks of culture in germination medium, performance was evaluated in terms of percentage of embryos developing only roots or shoots or both roots and shoots (plantlet conversion) longer than 5 mm. The length of the shoot of regenerated plantlets was also determined.

Flow cytometric analysis of ploidy level

Nuclear suspensions from zygotic embryos, somatic embryos and converted plantlet leaves were prepared. Young leaf material of *Nicotiana tabacum* L. was used as

internal reference standard (2C = 11.7). Approximately 100 mg of embryo material or fresh leaf tissue was chopped with a sharp razor blade in 900 μl chopping buffer (45 mM MgCl$_2$, 20 mM MOPS (3-N-[Morpholino]propanesulfonic acid), 30 mM sodium citrate, and 0.1% Triton-X 100, 2.0%), modified from Galbraith et al. (1983). The suspension of nuclei was filtered through a 40 μm filter to remove fragments and large tissue debris. Then, 1 mg ml^{-1} of RNase A (Sigma–Aldrich; Steinheim, Germany) and 0.5 mg ml^{-1} of propidium iodide (Sigma–Aldrich; Steinheim, Germany) were added to the samples. Samples were analyzed within a 10 min period in a Beckman-Coulter Epics Altra flow cytometer (Beckman-Coulter; Mijdrecht, Netherlands). At least 10,000 nuclei were analyzed per sample. The nuclear genome size of *A. hippocastanum* was estimated according to the following formula:

$A.\,hippocastanum$ 2C nuclear DNA content (pg)

$$= \frac{A.\,hippocastanum\,G_0/G_1\;\text{peak mean} \times 11.7}{N.\,tabacum\,G_0/G_1\;\text{peak mean}}$$

Conversion of mass values into base-pair numbers was done according to the factor 1 pg = 978 Mbp (Doležel et al. 2003).

Statistical analysis

Factorial analysis of variance (ANOVA) was performed on the data with the General Linear Model procedure (SAS Inc, USA). When significant differences occurred, means were separated by the Tukey Studentized Range Test at $P < 0.05$. Homogeneity of squared deviations from group means was calculated with Levene's test.

Results and discussion

Histological analysis of somatic embryogenesis

After 1 month culturing, calli appeared on the filaments. Subsequent sub-culturing for an extra month on fresh medium produced two types of callus. Embryogenic callus had a compact structure and a yellowish colour, while non-embryogenic callus appeared to be less dense and whitish in colour (Fig. 1a), similar to the work of Capuana and Debergh (1997). Histological sections showed that non-embryogenic callus was composed of unorganized isodiametric cells (Fig. 1b) while embryogenic callus developed regions of small cells with dens cytoplasm (Fig. 1c). Continuous division within those embryogenic calli resulted in the formation of pro-embryos 1 week after transferring to a medium without 2,4-D (Fig. 1d, e, f). Initially, the pro-embryos were connected to the callus and detached easily at

Fig. 1 Histological analysis of calluses and pro-embryos on (anther)filaments of *A. hippocastanum*. **a** Formation of embryogenic (*EC*) and non-embryogenic (*NEC*) callus; **b** non-embryogenic callus; **c** embryogenic callus; **d**,**e** Cluster of pro-embryo's; **f** detail of globular embryos with multicellular attachment to the callus. SE = somatic embryo. Scale *bar* = 1 mm
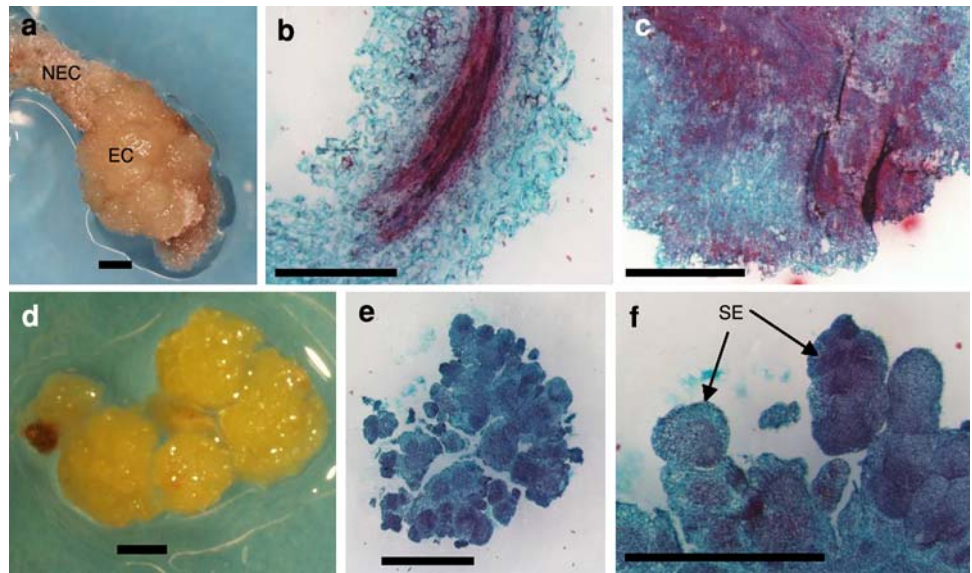
a later stage of development. Cell differentiation and organogenesis began with the formation of a protoderm covering a globular primordium. These globular structures showed sustained cell divisions giving rise to heart and torpedo stage somatic embryos. Finally, cotyledonal stage somatic embryos developed 3 weeks after transfer (Fig. 2a, b, c, d). Procambial cells and polarity was observed at all stages of embryo development (Fig. 2e, f, g, h). Closely-resembling patterns of development were also found in other species, including soybean (Santos et al. 2006) and cowpea (Ramakrishnan et al. 2005). Development of somatic embryos was asynchronous and continued during embryonic callus cultivation. Occasionally, irregular embryos with abnormal cotyledon shapes, single, cone-shaped cotyledons or more than two cotyledons were observed (data not shown).

Histological observations clearly showed that vascular systems were individualized and that the shoot and root apex were well established. We therefore concluded that low plantlet conversion rate cannot be attributed to aberrant embryo development.

Maturation and conversion of somatic embryos

Maturation is a key phase preceding embryo germination. The level of conversion of somatic embryos to plantlets is dependent on culture conditions that induce the maturation of somatic embryos (Quesada et al. 2004). Subsequently,

Fig. 2 Morphological and histological analysis of somatic embryos of *A. hippocastanum*. **a** Late globular stage embryo; **b**,**e** Early heart stage embryo; **c**,**f** Torpedo stage embryo; **d**,**g** cotyledonal stage embryo; **h** detail of shoot apex of a cotyledonal stage embryo. SA = shoot apex; CT = cotyledons; PC = procambium. Scale *bar* = 1 mm
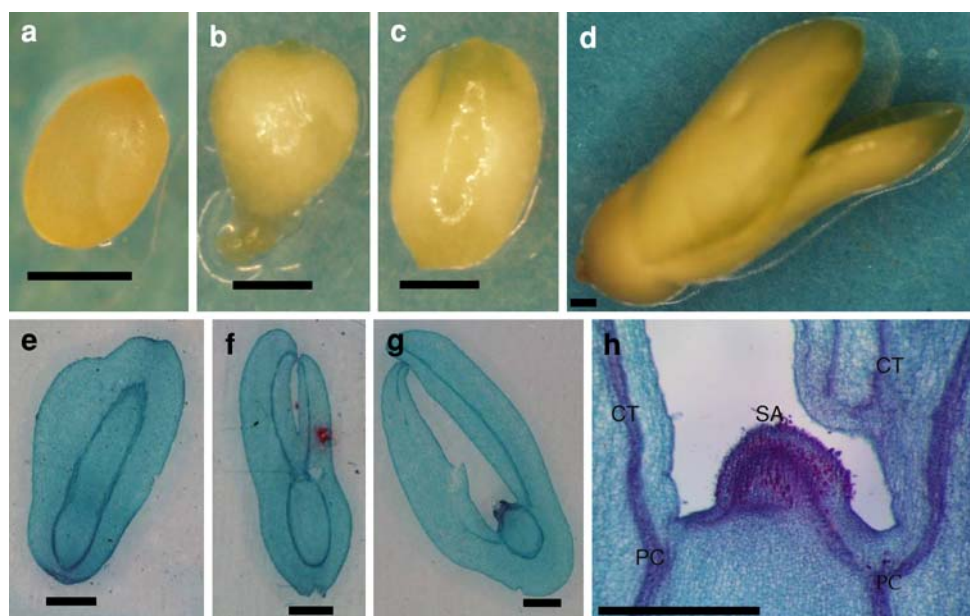
germination of somatic embryos depends on the degree of embryo maturation. Mature embryos typically show a regular morphology and contain sufficient storage molecules that are remobilized during germination (Arnold et al. 2002).

Experiment 1: type and concentration of carbohydrate

It is well-known that sugar not only serves as a carbon source for the growth of plant cells, but also as an osmoticum. Therefore, the effects of sugar type (glucose, fructose, sucrose and maltose) and concentration (3 and 6%) on somatic embryo maturation and germination were examined. All maturation media promoted the formation of large, thick, green cotyledons. In general, no recurrent embryogenesis was observed during maturation and cold storage periods. Germination response, however, was significantly affected by the carbohydrate treatments (Table 1).

Only carbon source had a significant effect ($P < 0.0001$) on the percentage of somatic embryos showing only root development (Table 1). At both concentrations, the sucrose treatment yielded the highest percentage of somatic embryos forming only roots while addition of fructose significantly reduced this percentage.

Increasing the carbohydrate concentration from 3 to 6% significantly enhanced the percentage of somatic embryos forming only shoots ($P = 0.0009$). Also carbohydrate type had a significant effect on the percentage of somatic embryos showing only shoot development (Table 1). Especially for the carbon source applied at 6%, sucrose yielded a significant lower percentage of somatic embryos forming only shoots compared to fructose (2.2 compared to 36.0%, respectively), while no significant differences with maltose and glucose treatments (24.3 and 18.0%, respectively) were found.

Overall conversion frequencies (both root and shoot development) of somatic embryos were very high, ranging from 54.0 to 88.9% (Table 1). Concentration of carbohydrate had no significant effect on conversion of somatic embryos. On the other hand, Sánchez et al. (2003) showed that high carbohydrate levels in the maturation medium significantly increased plantlet recovery frequency of oak somatic embryos. Carbohydrate source applied at 3% affected the conversion rate of *A. hippocastanum* with a significant higher conversion on fructose (88.9%) compared to sucrose (56.7%). No significant differences were found for conversion frequencies at a carbohydrate concentration of 6%. Overall quality of converted plantlets was, however poor, with a percentage of shoots larger than 2 cm between 0.0 and 12.5% (Table 1). The shoot height of regenerated plantlets was not significantly affected by the kind of maturation treatment.

Increasing the concentration of carbohydrate in the maturation medium did not improve the conversion of somatic embryos nor the quality of regenerated plantlets. Although conversion frequencies were very high for all

Table 1 Effect of type and concentration of carbohydrate in maturation media on the germination and conversion of *A. hippocastanum* somatic embryos

Maturation Medium		Germination response			
Carbon source	Concentration	Root only (%)	Shoot only (%)	Conversion (shoot + root)	
				%	Shoots >2 cm (%)
Glucose	3%	20.00ab	6.00a	66.00ab	5.83a
Fructose		4.44b	6.67a	88.89a	0.00a
Sucrose		40.00a	3.33a	56.67b	6.67a
Maltose		12.00b	12.00a	72.00ab	0.00a
Glucose	6%	24.00A	18.00AB	54.00A	12.50A
Fructose		0.00B	36.00A	64.00A	5.00
Sucrose		24.44A	2.22B	71.11A	2.78A
Maltose		8.57AB	24.29AB	64.29A	10.00A
Source of variation	df				
Carbon source	3	<0.0001**	0.0076*	0.1041NS	0.6401NS
Concentration	1	0.2573NS	0.0009*	0.1416NS	0.2635NS
Interaction	3	0.4649NS	0.0549NS	0.0725NS	0.6829NS

Data were recorded after 6 weeks on germination medium following 4 weeks on maturation media and 2 months cold treatment on basal medium

For each concentration, treatment means with a different letter differ significantly according to Tukey ($P < 0.05$). Two factorial (factor = carbon source (glucose, fructose, sucrose and maltose) and concentration (3 and 6%). *F*-test: *NS*, *, **: not significant or significant at $P < 0.05$ or $P < 0.0001$, respectively

treatments, overall quality of regenerated plantlets, in terms of shoot height, was poor. Moreover, plants smaller than 2 cm did not show active growth after conversion. This could be a consequence of low quality somatic embryos and the limited accumulation of sufficient storage material.

Experiment 2: type of carbohydrate and addition of PEG

PEG as a non-permeating osmoticum in maturation media has been used to improve somatic embryo germination and conversion. In this experiment we analysed the effect of PEG on somatic embryo maturation (Table 2).

Carbon source significantly affected the percentage of somatic embryos showing only roots, but an interaction between carbon source and addition of PEG was found (Table 2).

The addition of PEG to the maturation medium significantly increased the percentage of somatic embryos forming only shoots and decreased the conversion frequencies (Table 2). Moreover, a significant interaction between carbon source and addition of PEG on conversion frequencies was found.

Irrespective of the carbohydrate source, maturation on medium with PEG resulted in a significant lower percentage of converted somatic embryos than maturation on PEG-free medium. The quality of regenerated plantlets, in terms of shoot height higher than 2 cm, was, however, significantly enhanced by PEG. Also, carbohydrate source significantly affected the shoot quality of converted somatic embryos when PEG was included in the maturation medium. The highest percentage of high quality shoots (in terms of shoot height) was 43.8% and was found for the combination of sucrose and PEG (Fig. 3) while total conversion was 65.7%. The beneficial effect of PEG on somatic embryo maturation is consistent with previous studies on conifer somatic embryos (Misra et al. 1993; Shoji et al. 2006; Svobodová et al. 1999; Tremblay and Tremblay 1995), rubber tree (Linossier et al. 1997) and *Glycine max* (Walker and Parrott 2001). According to Svobodová et al. (1999), treatment with PEG 4000 (3.75%) for 6 weeks accelerated maturation of embryos and led to an enhanced synthesis of proteins and lipids in Norway spruce. Secondary embryogenesis was greatly reduced and somatic embryos matured to the torpedo stage when rubber tree embryo maturation medium was supplemented with PEG (Linossier et al. 1997).

We can conclude that maturation of somatic embryos on media containing PEG promoted the shoot quality of regenerated plantlets in *A. hippocastanum*. Moreover, sucrose is the preferred carbon source when PEG is included in the maturation medium, in terms of conversion frequencies as well as the shoot quality.

Flow cytometric analysis of ploidy level

The ploidy stability during different steps of somatic embryogenesis was assessed using flow cytometry. Nuclear

Table 2 Effect of type of carbohydrate and PEG in maturation media on the germination and conversion of *A. hippocastanum* somatic embryos

| Maturation Medium		Germination response			
Carbon source	PEG	Root only (%)	Shoot only (%)	Conversion (shoot + root)	
				%	Shoots > 2 cm (%)
Glucose	0 g l^{-1}	20.00ab	6.00a	66.00ab	5.83a
Fructose		4.44b	6.67a	88.89a	0.00a
Sucrose		40.00a	3.33a	56.67b	6.67a
Maltose		12.00b	12.00a	72.00ab	0.00a
Glucose	50 g l^{-1}	15.00A	22.50A	55.00A	17.70AB
Fructose		6.67A	33.33A	55.56A	15.63AB
Sucrose		5.71A	25.71A	65.71A	43.81A
Maltose		22.22A	28.89A	44.44A	12.96B
Source of variation	df				
Carbon source	3	0.0391*	0.5979NS	0.1730NS	0.0123*
PEG	1	0.1155NS	<0.0001**	0.0025*	<0.0001**
Interaction	3	0.0053*	0.7937NS	0.0261*	0.1420NS

Data were recorded after 6 weeks on germination medium following 4 weeks on maturation media and 2 months cold treatment on basal medium

For each concentration of PEG, treatment means with a different letter differ significantly according to Tukey ($P < 0.05$). Two factorial (factor = carbon source (glucose, fructose, sucrose and maltose) and concentration of PEG (0 and 50 mg l^{-1}). *F*-test: NS, *, **: not significant or significant at $P < 0.05$ or $P < 0.0001$, respectively

Fig. 3 Plantlets regenerated from horse chestnut somatic embryos after 6 weeks on germination medium following 2 months cold treatment and a 4 week maturation period on sucrose 3% + PEG 50 g l^{-1}

suspensions from zygotic embryos, somatic embryos with two or three cotyledons, pro-embryos and converted plantlet leaves were analysed. The histograms of *A. hippocastanum* nuclei (Fig. 4) showed distinct G_0/G_1 peaks with coefficients of variation (CV) between 1.8 and 4.3% (Table 3). The internal reference standard, *N. tabacum*, presented a mean CV value of 1.06% (data not shown). Galbraith et al. (2002) suggested a CV value of less than 5% as the acceptance criterion that reflects the quality of the applied methodology. Our estimated DNA content values (Table 3) are in general very close to the previously published data for *A. hippocastanum* (2C = 1.2 pg; Bennett and Leitch 2005), except the DNA value in the leaves of converted somatic embryos. Similar to *Quercus suber* L. (Loureiro et al. 2005) and *Eucalyptus globulus* Labill. (Pinto et al. 2004), leaf tissue may contain compounds that affect the flow cytometric analysis. Favre and Brown (1996) stated that browning and tanning substantially interfere with propidium iodide staining. The morphological difference between cotyledonal somatic embryos with two and three cotyledons did not reflect major differences in DNA content. The results reported here indicate that all the samples analyzed have the same ploidy level and that no polyploidy occurred in pro-embryos and in (converted) somatic embryos. With regard to chromosomal instability in plant callus culture, polyploidy
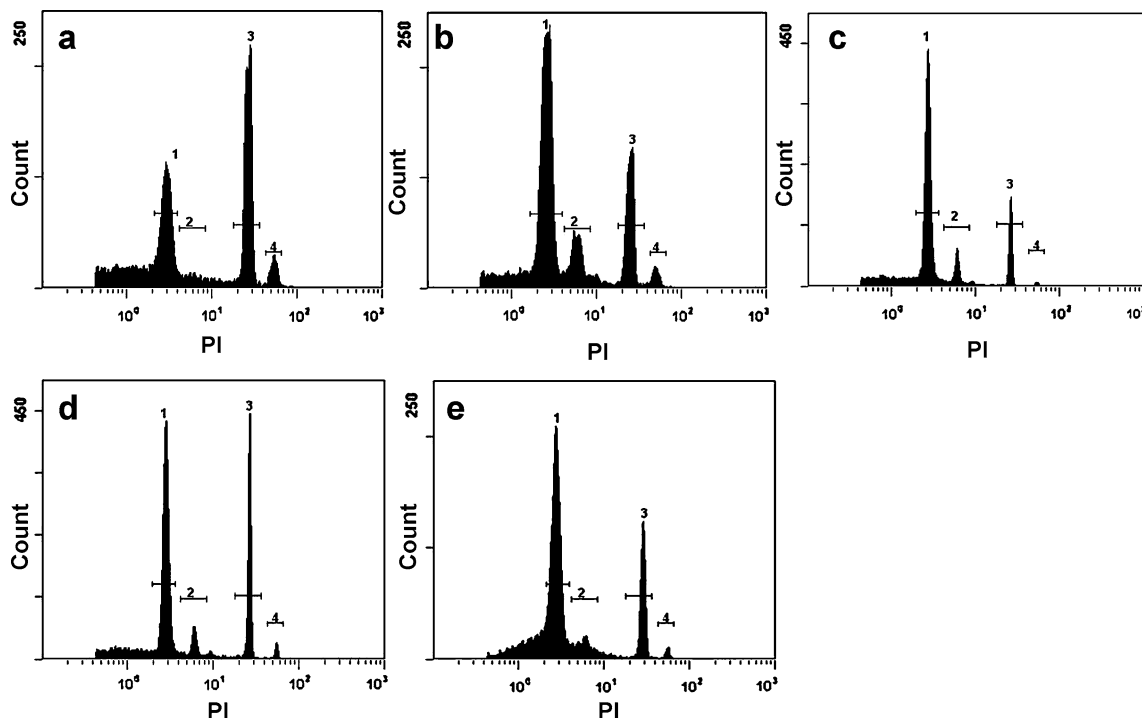
Fig. 4 Histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from *Aesculus hippocastanum* L. and *Nicotiana tabacum* L. (as an internal reference standard): **a** Zygotic embryo; **b** somatic embryo with two cotyledons; **c** somatic embryo with three cotyledons; **d** cluster of pro-embryos; **e** leaves of a converted somatic embryo. In all flow cytometry histograms four peaks were observed: *peak 1* nuclei at G_0/G_1 phase of *Aesculus hippocastanum* L., *peak 2* nuclei at G_2 phase of *Aesculus hippocastanum* L., *peak 3* nuclei at G_0/G_1 phase of *Nicotiana tabacum* L., *peak 4* nuclei at G_2 phase of *Nicotiana tabacum* L

Table 3 Estimated DNA content of zygotic and somatic embryo material determined by flow cytometric analysis of propidium-iodide-stained nuclei using *N. tabacum* as internal reference standard

Plant material source	Nuclear DNA content (pg/2C)	1C genome size (Mbp)[a]	CV (%)[b]
Zygotic embryo	1.19	581.91	3.4
Somatic embryo—2 cotyledons	1.22	596.58	4.3
Somatic embryo—3 cotyledons	1.20	586.80	1.8
Cluster of pro-embryos	1.22	596.58	2.0
Leaves of a converted somatic embryo	1.09	533.01	2.9

[a] 1 pg DNA = 978 Mbp (Doležel et al. 2003)

[b] CV = coefficient of variation of the *A. hippocastanum* G_0/G_1 peak

is the most commonly observed somaclonal variation (Geier 1991). However, ploidy stability during somatic embryogenesis has been reported in *Bambusa balcooa* (Gillis et al. 2007) and *Quercus suber* L. (Loureiro et al. 2005).

These findings clearly show that the somatic embryogenesis protocol used, did not induce major genetic changes in the somatic embryos and this is a first indication for a 'true-to-type' micropropagation protocol of *A. hippocastanum*. The flow cytometry measurements do not exclude somaclonal variations that do not involve large changes in DNA content. Further experiments are necessary to determine whether the *A. hippocastanum* somatic embryos are genetically stable.

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