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MORPHOGENESIS

Improved germination of somatic embryos and plant recovery of European chestnut

Elena Corredoira · Silvia Valladares · Ana M. Vieitez · Antonio Ballester

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Abstract For the mass production of chestnut trees with selected, hybrid, or genetically engineered genotypes, one potentially desirable propagation strategy is based on somatic embryogenesis. Although methods exist for the initiation of embryogenic cultures of *Castanea sativa* from immature zygotic embryos or leaf explants, the embryos produced have had low rates of conversion into plantlets. This study explored the possible benefits for somatic embryos that have already undergone maturation and cold treatments, of (a) partial slow or fast desiccation, and (b) of the addition of plant growth regulators or glutamine to the germination medium. Germination response was evaluated in terms of both conversions to plantlets and through embryos developing only shoots (shoot germination) that could be rooted following the micropropagation protocols developed for chestnut. Two or 3 wk slow desiccation in sealed empty Petri dishes resulted in a slight reduction in water content that nevertheless increased total potential plant recovery, shoot length, and the number of leaves per plantlet. However, best results were achieved by 2 h fast drying in a laminar flow hood, which reduced embryo moisture content to 57–58% and enhanced the potential plant recovery and quality of regenerated plantlets. Plant yield was also promoted by addition of 0.44 μM benzyladenine and 200–438 mg/l of glutamine to the germination medium, and plantlet quality (as evidenced by root, shoot, and leaf growth) by the further addition of 0.49 μM indole-3-butyric acid.

E. Corredoira · S. Valladares · A. M. Vieitez (✉) · A. Ballester
Plant Physiology, Instituto de Investigaciones
Agrobiológicas de Galicia, CSIC,
Apartado 122,
15080 Santiago de Compostela, Spain
e-mail: amvieitez@iiag.csic.es

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Introduction

European chestnut or sweet chestnut (*Castanea sativa* Mill.), a hardwood species belonging to the Fagaceae, is important on an international scale because of its distribution and its economic and environmental role in many agroforestry systems. In Europe, sweet chestnut woods have been gaining in value not only as sources of nuts and timber but also because of their contribution to the landscape (Bounous 2005). Since the early twentieth century, however, *C. sativa* populations have been ravaged by ink disease (caused by *Phytophthora cambivora* and *P. cinnamomi*) and by chestnut blight (caused by *Cryphonectria parasitica*). As an alternative to conventional vegetative propagation of chestnut, which is beset by numerous difficulties, efforts are being made to establish reliable *in vitro* regeneration protocols based on somatic embryogenesis. Somatic embryogenesis is an important biotechnological tool not only for clonal propagation of selected genotypes but also for cryostorage of valuable germplasm and genetic engineering (Corredoira *et al*. 2004a, b; Polin *et al*. 2006). The transfer of antifungal genes (Collada *et al*. 1992; García-Casado *et al*. 2000) or antimicrobial genes (Powell *et al*. 1995; Liang *et al*. 2002) into chestnut cells, followed by the generation of transgenic plants by somatic embryogenesis, would constitute a biotechnological alternative to conventional breeding efforts for developing blight- and/or ink-resistant trees.

The feasibility of initiating embryogenic cultures from immature zygotic embryos has been shown for *C. sativa* and Euro-Japanese hybrids (Vieitez *et al*. 1990; Vieitez 1995; Sauer and Wilhelm 2005; Corredoira *et al*. 2006) and

also for *C. dentata* (American chestnut; Merkle *et al*. 1991; Carraway and Merkle 1997; Xing *et al*. 1999). For *C. sativa*, the induction of somatic embryos on leaf explants has also been reported (Corredoira *et al*. 2001, 2006). The relatively poor germination of somatic embryos and their low rates of development into functional plants are limiting steps for the widespread use of somatic embryogenesis in European chestnut improvement programmes. To improve these stages of the overall plant regeneration process, more research into embryo maturation and germination is required.

For many species, a salient feature of the later stages of the development of zygotic embryos is desiccation, which plays a role in the transition between embryo maturation and germination (Kermode 1990) and appears to redirect the metabolism toward germination (Wetzstein *et al*. 1989). However, the seeds of certain forest hardwood species, including chestnuts and oaks, fail to withstand water loss and are presumed to be highly susceptible to desiccation injury. Nevertheless, a positive effect of partial drying of recalcitrant seeds on germination has been reported (Tompsett and Pritchard 1998). Partial desiccation also improved the germination of embryonic axes excised from chestnut seeds (Corredoira *et al*. 2004b). The germination of the somatic embryos of a number of species is also best when preceded both by culture on a maturation medium and by further pregermination treatments such as partial desiccation or cold storage. For example, the germination rate of mature somatic embryos of several conifer species is improved by slow drying under high relative humidity (≥95%) before the transfer to germination medium (Malabadi and van Staden 2005; Salaj *et al*. 2005; Tremblay *et al*. 2005; Vágner *et al*. 2005). In the case of chestnut, the germination success rate of somatic embryos matured on a medium of high osmotic potential was markedly improved by cold treatment but remained low (Vieitez 1995; Corredoira *et al*. 2003; Andrade and Merkle 2005). Meanwhile, slow removal of 14% of water content failed to increase the rate of conversion into plantlets (Corredoira *et al*. 2003). However, a detailed study on the influence of partial desiccation on germination response of matured, cold-treated somatic embryos of chestnut has not been attempted.

Organic nitrogen supplement is a critical component of culture media for somatic embryogenesis, not only for somatic embryo induction but also for proper embryo maturation, as it generally promotes the accumulation of storage reserves (Schmidt *et al*. 2005). Glutamine (3 mM) was found to be necessary for the proliferation of chestnut embryogenic cultures, as in its absence, proembryogenic masses and embryo development ceased after four to five successive subcultures (Vieitez 1995). In our laboratory, glutamine has accordingly been included routinely in the proliferation medium of embryogenic lines but not in the germination medium. However, the effect of glutamine in the germination medium on germination rates and the quality of the plants generated has not yet been investigated in different embryogenic systems of chestnut.

The aim of this study was to improve the germination and plant recovery ability of European chestnut somatic embryos from different embryogenic lines. The inclusion of different plant growth regulators and/or glutamine in the germination medium was investigated. A further objective was to evaluate the effect of partial desiccation (slow and fast drying) of somatic embryos as a pregermination treatment that might enhance plant recovery from somatic embryos previously subjected to a cold storage period.

Materials and Methods

Maintenance of embryogenic lines and culture conditions. Somatic embryos were taken from three embryogenic lines (HV-Z2, CI-3, and CI-9) that had been initiated from immature zygotic embryos of *C. sativa* (Vieitez 1995; Corredoira *et al*. 2006) and had been maintained for more than 5 y by secondary embryogenesis with sequential subculture at 6-wk intervals on a proliferation medium consisting of MS (Murashige and Skoog 1962) mineral salts (with half-strength macronutrients) and vitamins, 438 mg/l (3 mM) glutamine, 7 g/l agar (Sigma A-1296), 30 g/l sucrose, 0.44 μM 6-benzyladenine (BA), and 0.54 μM 1-naphthaleneacetic acid (NAA). The pH of all media was adjusted to 5.7 before autoclaving at 121°C for 20 min. Unless otherwise indicated, all cultures were incubated under a 16-h photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 50–60 μmol m^{-2} s^{-1}) with 25°C light/20°C dark temperatures.

Standard procedure for maturation and germination of somatic embryos. In all experiments, white opaque cotyledonary somatic embryos with two cotyledons were isolated from the embryogenic lines and cultured in Petri dishes (ten to a dish) containing 25 ml of maturation medium, which consisted of basal medium (proliferatiom medium minus BA and NAA) with 3% maltose instead of sucrose. After 4 wk of culture in maturation medium, the embryos were stored at 4°C for 2 mo., after which they were transferred to germination medium (basal medium with 0.44 μM BA) and grown under photoperiodic light conditions for 8 wk. The germination ability of the three embryogenic lines under these conditions was determined using four replicates, with 30 somatic embryos per replicate, resulting in a total of 120 embryos per genotype.

Effect of plant growth regulators in the germination medium. In this experiment, HV-Z2 embryos of two size classes (2–5

and 6–8 mm) were isolated from proliferation medium, cultured for 4 wk on maturation medium, stored for 2 mo. at 4°C, and then placed on germination medium devoid of plant-growth regulators (PGR; control) or containing 0.44 μM BA alone or in combination with 0.54 μM NAA or 0.49 μM 3-indolebutyric acid (IBA). Non-cold-stored embryos were also germinated on 0.44 μM BA medium to confirm the effect of pregermination chilling treatment for this genotype. Each treatment was applied to a total of 100 embryos in four replicates with 25 somatic embryos per replicate.

Effect of partial drying of somatic embryos. Matured somatic embryos of lines CI-3 and CI-9 were subjected to cold storage for 2 mo., transferred aseptically to sterile open Petri dishes (12 embryos to a dish), dehydrated in a laminar flow cabinet at 25°C for 0, 1, 2, 3, or 4 h, and then placed on germination medium including 0.44 μM BA and 0.49 μM IBA. During the whole 4-h desiccation period, the total fresh weight of two samples of 12 somatic embryos was monitored, after which these samples were dried at 85°C for 24 h and reweighed. Embryo water content after each desiccation time was calculated from these data as a percentage of fresh weight. In a further experiment, the cold-stored CI-3 embryos were either slowly dried inside sealed empty Petri dishes for 1–3 wk in darkness or were dried for 2 h as above; the partially desiccated embryos were then placed on germination medium containing 0.44 μM BA and 0.49 μM IBA, and moisture contents were determined as above. In both experiments, each treatment was applied to four replicates with 15 somatic embryos per replicate.

Effect of glutamine. Following maturation and 2 mo. of cold storage, embryos of lines CI-3 and HV-Z2 were cultured on germination medium supplemented with 0, 50, 100, 200, 438, or 600 mg/l of glutamine, which had been added to the medium before autoclaving. Each treatment was applied to four replicates with 15 embryos per replicate.

Data recording and statistical analysis. After 8 wk of culture in the germination medium, the numbers of germinated embryos exhibiting plantlet conversion (both root and shoot development longer than 5 mm), root-only germination or shoot-only germination were recorded. The lengths of the roots and shoots, and the numbers of leaves per regenerated plantlet were also determined. Germination and conversion frequencies were analysed by contingency table χ^2 analysis. Shoot and root lengths, and leaf number data were analysed by analysis of variance (ANOVA).

Results and Discussion

After 8 wk of culture in the germination medium supplemented with 0.44 μM BA, the conversion rates of the three embryogenic lines were as follows: 16% in line CI-3, 17% in line CI-9, and 19% in line HV-Z2. However, all these lines also produced partially germinated embryos exhibiting only shoot development; these shoots can be multiplied and rooted, increasing the potential plant regeneration efficiency (Corredoira *et al*. 2003). Plantlets were obtained after auxin treatment (122.5 μM IBA for

Table 1. Effects of 2 mo. of cold storage pretreatment and of plant growth regulators in the germination medium, on germination response of somatic embryos of chestnut line HV-Z2

Cold storage	PGRs in the germination medium (μM)	Root only		Shoot only		Conversion (shoot+root)				Total potential plant recovery (%)
		Percent	Length (mm)	Percent	Length (mm)	Percent	Root length (mm)	Shoot length (mm)	No. of leaves	
No	BA 0.44	36.0±2.8	15.5±0.8	4.0±1.6	6.0±2.1	4.0±2.3	12.0±0.0	7.5±0.4	0.8±0.5	8
Yes	PGR-free	9.0±2.5	17.1±2.2	3.0±1.0	27.0±15.8	19.0±5.5	77.0±10.7	29.0±1.2	1.6±0.2	22
Yes	BA 0.44	23.0±2.5	13.4±2.4	17.0±3.0	19.4±4.1	18.0±1.2	25.1±3.9	24.0±3.7	1.2±0.2	35
Yes	BA 0.44+ NAA 0.54	6.0±4.7	17.7±10.2	11.0±3.0	25.9±5.8	23.0±3.4	40.0±2.3	24.5±1.7	1.6±0.1	34
Yes	BA 0.44+ IBA 0.49	9.0±1.0	27.4±6.5	15.0±2.5	25.5±1.5	22.0±1.2	40.0±3.2	36.3±2.4	2.5±0.5	37
		$P<0.01$	ns	$P<0.001$	ns	$P<0.001$	$P<0.001$	$P<0.001$	ns	$P<0.05$

Data were recorded after 8 wk on germination medium. Total potential plant recovery: conversion % + shoot-only development %. Values are means ± standard errors of four replicates with 25 somatic embryos per replicate. Germination and conversion frequencies were analysed by contingency table (χ^2) analysis, and shoot and root length and leaf data by ANOVA.
ns not significant

24 h) in HV-Z2 and CI-3 microshoots, where 95% and 82% rooting frequencies were achieved, respectively.

Effects of plant growth regulators in the germination medium. Embryo size had no significant influence on germination response, and in Table 1, the data for the two size classes have been pooled. Andrade and Merkle (2005) similarly reported that morphological characteristics such as the number of cotyledons had no effect on the germination and conversion rates of somatic embryos of American chestnut.

In keeping with the behavior of other chestnut genotypes (Vieitez 1995; Corredoira *et al*. 2003), cold storage significantly increased the plantlet conversion of HV-Z2 embryos germinated on the standard germination medium (basal medium plus 0.44 μM BA; Table 1). Among the cold-treated embryos, the frequency of conversion among those grown on PGR-free medium did not differ significantly from those observed among the other treatments, but the percentage of germinants with only shoot development was significantly lower than with BA in the medium ($P<0.001$). Shoot length on PGR-free medium was similar to shoot length on the media with PGRs for converted embryos, and their roots were about twice as long on the PGR-free medium as on any other medium. Higher total potential plant recovery rates (conversion % + only-shoot development %) were achieved in embryos germinated in BA-containing media. However, the inclusion of an auxin together with BA in the medium resulted in longer roots than with BA alone, and shoot and leaf development were better when the auxin was IBA than with NAA (Table 1). This combination (0.44 μM BA and 0.49 μM IBA) appears to give rise to the most vigorous plantlets and was used in all subsequent experiments in this study.

BA has previously been reported to be beneficial for the *in vitro* germination of zygotic embryonic axes of chestnut, especially for the development of shoots from axillary meristems of cotyledonary nodes (Viéitez and Viéitez 1980; San-José *et al*. 2001). Similarly, plant recovery rates were promoted by including BA in the recovery medium of cryopreserved embryonic axes (Corredoira *et al*. 2004b). For the germination of somatic embryos produced from different embryogenic systems, media have been used that either likewise contained low levels of BA (Corredoira *et al*. 2003; Sauer and Wilhelm 2005) or else were PGR-free (Andrade and Merkle 2005). In the related species *Quercus suber*, shoot elongation frequency was increased by applying BA to the germinated embryos but not by including both BA and 3-indoleacetic acid (González-Benito *et al*. 2002).

Effect of partial drying of somatic embryos. The moisture content of CI-3 embryos, 84.3% on a fresh-weight basis before partial desiccation by laminar air flow, fell with increasing desiccation time to 35% after 4 h (Table 2). The frequency of conversion into plantlets (Fig. 1*A*) was not increased by desiccation treatment, and both conversion and root germination were suppressed completely by the 4-h treatment ($P<0.05$). However, after this desiccation period, 13% of embryos were able to show shoot-only germination, which significantly increased ($P<0.01$) after 2–3 h desiccation (Fig. 1*A*). The shoot length of germinated embryos and the number of leaves per plantlet were

Table 2. Effects of various periods in a laminar flow hood on the moisture contents of somatic embryos of chestnut lines CI-3 and CI-9, and on the root length (RL), shoot length (SL), and number of leaves (NL) of germinating embryos exhibiting root-only development, shoot-only development, or plantlet conversion after 8 wk on germination medium

Desiccation time (h)	Moisture content (%)	Root-only	Shoot-only	Conversion (shoot + root)		
		RL (mm)	SL (mm)	RL (mm)	SL (mm)	NL
LINE CI-3						
0	84	7.3±0.8	9.3±1.3	9.3±1.1	9.5±2.6	0.8±0.3
1	70	11.3±6.3	15.7±1.7	12.6±3.9	14.8±1.4	2.1±0.3
2	57	7.5±1.8	16.7±1.7	10.8±1.1	18.9±3.1	3.6±1.3
3	46	9.8±0.4	15.4±0.6	14.3±0.8	13.3±3.3	1.3±0.9
4	35	–	10.4±6.3	–	–	–
		ns[a]	$P<0.01$	ns	ns	ns
LINE CI-9						
0	89	10.1±0.8	9.4±4.4	22.8±6.5	11.9±1.9	0.6±0.5
2	58	13.1±1.1	14.2±1.4	18.7±2.3	15.3±1.4	1.1±0.2
3	49	13.6±1.6	11.4±1.6	13.8±4.7	12.3±5.0	0.3±0.1
		ns[a]	ns	ns	ns	ns

Values are means ± standard errors of four replicates with 15 somatic embryos per replicate.
ns not significant
[a] *F* test

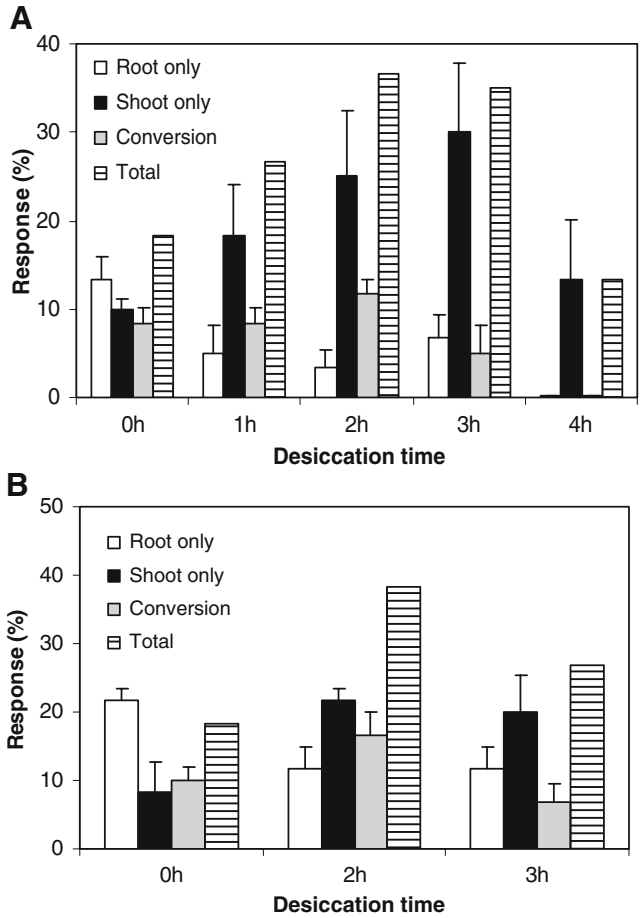

Figure 1. Effect of various periods in a laminar flow hood on the frequency that somatic embryos of lines CI-3 (*A*) and CI-9 (*B*) developed roots only, shoots only, or both roots and shoots (conversion). Total potential plant recovery: conversion % + shoot only %. *Columns* indicate means and *bars* the standard errors of four replicates with 15 somatic embryos per replicate.

enhanced, and the total potential plant recovery was greatest when 2-h desiccation had reduced water content to about 57% (Fig. 1*A*; Table 2). Similar trends in moisture content and germination response were observed when CI-9 embryos were subjected to 2 or 3 h desiccation (Fig. 1*B*, Table 2); the total plant recovery was significantly greater ($P<0.05$) when moisture content was reduced to 58% by 2 h desiccation. In this study, 2 h desiccation produced the best results by improving the shoot germination and the potential plant recovery, as well as the quality of the regenerated plantlets. The apparently beneficial effect of this treatment is consistent with previous studies with zygotic embryonic axes of chestnut, the germination rate of which increased from 71% for freshly excised axes to 100% for axes that had had 31–37% of their water content removed by air drying in a laminar flow hood (Corredoira *et al.* 2004b).

When CI-3 embryos were slowly dried in sealed empty Petri dishes for 1–3 wk, water loss was very slight (Table 3). Slow drying nevertheless significantly increased ($P<0.001$) the proportion of embryos with shoots and the shoot length and leaf number on converted embryos, and the root growth of converted embryos was considerably less variable after 2 or 3 wk of slow drying than after 1 wk or among undried embryos (Fig. 2; Table 3). However, even the slow drying time affording the largest number of embryos with shoots produced fewer than did 2 h drying in a laminar flow cabinet (Fig. 2), and we accordingly consider this latter treatment as preferable.

Under our conditions, a slight reduction of embryo moisture content occurred after 2–3 wk of desiccation in empty plates, and this resulted in an improved germination response and vigor of regenerated plantlets (shoot length and leaf number). Slow drying for 4–6 d, resulting in a 12–15% loss of fresh weight, has been reported to stimulate the germination of *Ocotea catharinensis* somatic embryos more than 2 h rapid desiccation in a flow chamber (which reduced fresh weight by 57%) (Viana and Mantell 1999). Slow drying for 5 d has also been used for germination of pecan somatic embryos (Wetzstein *et al.* 1996), and Black

Table 3. Effects of 1–3 wk slow drying or 2 h fast drying on the moisture content of somatic embryos of chestnut line CI-3, and on the root length (RL), shoot length (SL), and number of leaves (NL) of germinating embryos exhibiting root-only development, shoot-only development, or plantlet conversion after 8 wk on germination medium

Desiccation time	Moisture content (%)	Root only	Shoot only	Conversion (shoot + root)		
		RL (mm)	SL (mm)	RL (mm)	SL (mm)	NL
None	88	16.7±7.7	5.6±1.9	25.0±14.0	6.4±2.7	0.3±0.3
1 wk	86	7.8±2.8	12.3±2.2	29.5±16.9	8.2±3.5	0.7±0.6
2 wk	86	15.5±1.8	11.9±2.2	15.7±1.3	12.9±2.4	0.4±0.2
3 wk	85	20.9±6.6	10.5±2.0	17.7±1.6	17.9±1.7	1.1±0.5
2 h	54	8.0±5.2	11.7±1.7	14.5±2.5	18.5±2.2	2.4±0.7
		ns[a]	ns	ns	$P<0.05$	$P<0.05$

Values are means ± standard errors of four replicates with 15 somatic embryos per replicate.
ns not significant
[a] *F* test

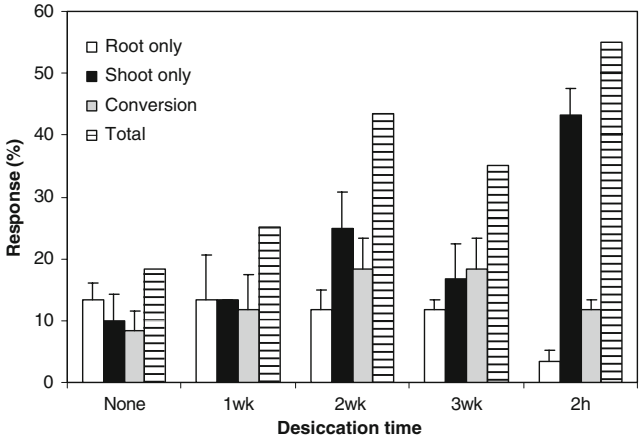

Figure 2. Effect of 1–3 wk slow drying or 2 h fast drying on the frequency that somatic embryos of line CI-3 developed roots only, shoots only, or both roots and shoots (conversion). Total potential plant recovery: conversion % + shoot only %. *Columns* indicate means and *bars* the standard errors of four replicates with 15 somatic embryos per replicate.

et al. (1999) found that reducing the initial water content of wheat embryos from 73% to 69% of fresh weight was sufficient to induce desiccation tolerance, which they tentatively attributed to the triggering of tolerance mechanisms by the reduction in turgor and alteration of osmotic balance. That a degree of desiccation can trigger rapid biochemical changes is supported by the increase in plant regeneration achieved by partial desiccation of rice embryogenic callus, which was related to differences in soluble protein patterns as early as 1 d after desiccation treatment (Rance *et al*. 1994); water-stress-induced proteins may be playing a key role in water stress tolerance and improvement of somatic embryo maturation (Malabadi and Nataraja 2006).

It should be noted that the slow desiccation of somatic embryos in empty Petri dishes causes not only water stress but also starvation stress. There is a correlation between somatic embryogenesis and environmental stresses including starvation (Von Aderkas and Bonga 2000). The withdrawal of sucrose from nodular callus of date palm for 2 wk has been reported to have promoted embryogenesis (Veramendi and Navarro 1996), although no effect on subsequent embryo germination was observed. Under more severe starvations conditions, the conversion into plantlets of somatic embryos of *Camellia sinensis* obtained from embryogenic cultures maintained by the temporary immersion method is reportedly improved by 4 wk without immersion (Akula and Akula 2005). Starvation in empty wells for a period of 5 d also enhanced the production and increased the germination rate of carrot somatic embryos, especially embryos with anomalous morphology (Lee *et al*. 2001). To explain the improvement of somatic embryo production and quality by starvation stress, it has been hypothesized that starvation and water loss stimulate the tissue synchronization toward the development of existing embryos instead of the proliferation of undifferentiated cells and/or additional proembryos (Veramendi and Navarro 1996; Lee *et al*. 2001).

Murch *et al*. (1997) proposed that stress conditions lead to an increased availability of energy and reducing power that is used in the induction of stress adaptation mechanisms of regenerative growth (Von Aderkas and Bonga 2000). Thus, shoot formation *in vitro* has been associated with increased activity of the pentose phosphate pathway and increased reducing power (Thorpe 1993) and, in androgenesis the starvation of pollen, though associated with partial degradation of the cytoplasm, does not destroy the integrity of mitochondria, which accordingly maintain capacity to convert reserves into energy (Von Aderkas and Bonga 2000). In the present study, the positive effect of starvation and/or desiccation on the number of germinating chestnut embryos with shoots may be an example of stress-promoting morphogenesis. This is especially true in the apical meristem, where the differentiation of axillary cotyledonary meristems may be involved.

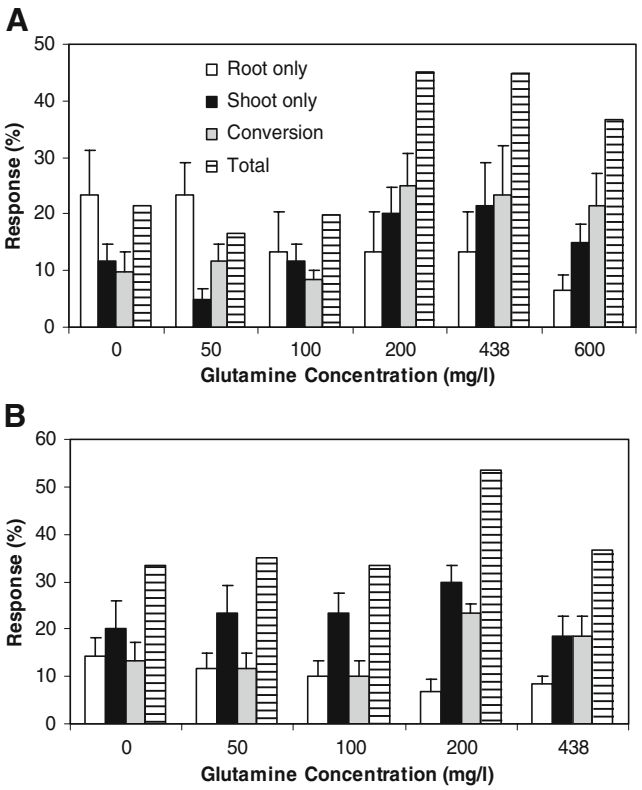

Figure 3. Effect of the concentration of glutamine in the germination medium on the frequency that somatic embryos of lines CI-3 (*A*) and HV-Z2 (*B*) developed roots only, shoots only, or both roots and shoots (conversion). Total potential plant recovery: conversion % + shoot only %. *Columns* indicate means and *bars* the standard errors of four replicates with 15 somatic embryos per replicate.

Table 4. Effects of the concentration of glutamine in the germination medium on the root length (RL), shoot length (SL), and number of leaves (NL) of somatic embryos of chestnut lines CI-3 and HV-Z2 with root-only development, shoot-only development, or plantlet conversion after 8 wk on germination medium

Glutamine (mg/l)	Root only	Shoot only	Conversion (shoot + root)		
	RL (mm)	SL (mm)	RL (mm)	SL (mm)	NL
LINE CI-3					
0	22.7±9.7	10.0±2.3	18.5±8.2	10.4±3.8	0.4±0.2
50	33.1±9.2	5.0±1.7	25.0±15.9	9.0±1.5	0.3±0.3
100	15.2±7.4	11.2±2.9	28.3±14.3	5.8±0.8	0.0±0.0
200	22.5±10.5	13.8±2.1	46.7±0.9	15.3±1.8	0.8±0.3
438	24.8±6.5	13.3±2.8	30.2±5.3	13.8±1.2	0.6±0.4
600	31.9±12.8	9.4±1.8	25.3±4.9	12.8±3.0	0.5±0.4
	ns[a]	ns	ns	ns	ns
LINE HV-Z2					
0	10.0±3.4	18.2±2.5	22.3±3.1	20.1±3.1	1.6±0.8
50	11.3±1.7	17.4±2.2	25.4±5.5	23.2±4.0	1.6±0.4
100	10.0±0.8	17.6±3.1	17.2±6.6	20.3±4.1	1.4±0.3
200	11.0±4.9	26.3±2.7	26.4±6.3	28.8±5.1	2.8±1.1
438	33.8±10.9	25.5±4.3	28.8±4.2	27.3±4.7	2.1±0.6
	ns[a]	ns	ns	ns	$P<0.05$

Values are means ± standard errors of four replicates with 15 somatic embryos per replicate.
ns not significant
[a] *F* test

Effect of glutamine. The inclusion of 200 mg/l of glutamine in the germination medium increased both the numbers of CI-3 and HV-Z2 embryos converted into plantlets ($P<0.01$ for CI-3) and the numbers developing only shoots ($P<0.05$ for CI-3); in CI-3 line, similar results were obtained with 438 mg/l of glutamine, although at higher concentrations, the effect began to decline (Fig. 3). The quality of regenerated plantlets, in terms of shoot and root lengths and the number of leaves, was also enhanced for both lines by 200 or 438 mg/l of glutamine (Table 4), although differences were only significant ($P<0.05$) for the number of leaves on HV-Z2 plantlets.

There is some inconsistency on the use of glutamine in different chestnut embryogenic systems. For European chestnut and its hybrids, it has been found advantageous to include 3 mM (438 mg/l) of glutamine in the embryo proliferation medium (Vieitez 1995; Corredoira *et al*. 2003), and a similar concentration, 3.4 mM (500 mg/l), has been used for American chestnut (Andrade and Merkle 2005). By contrast, Sauer and Wilhelm (2005) reported that glutamine had no significant beneficial effect on secondary embryogenesis. Robichaud *et al*. (2004) found that the addition of 25 mM of glutamine (3.7 g/l) to the medium used for maturation of American chestnut somatic embryos increased dry weight/fresh weight ratios and starch content but did not increase the subsequent germination ability, whereas 25 mM (3.8 g/l) L-asparagine treatment significantly enhanced the germination rate. The present results show that the glutamine supplement currently used for proliferation and maturation of chestnut somatic embryos should also be included in the germination medium but at lower concentration 200 mg/l. Future experiments should determine whether it is desirable to use filter-sterilized glutamine because autoclaving forms pyroglutamic acid, which has been reported to be detrimental to the growth and maturation of soybean somatic embryos (Schmidt *et al*. 2005).

Plant acclimatization. Plantlets obtained from somatic embryos converted in BA (0.44 μM) and IBA (0.49 μM)

Figure 4. Somatic embryo derived plants 1 y after transplanting to soil.

germination medium (PGRs experiment) were transferred to pots containing a 3:1 mixture of commercial substrate (Pinot®) and perlite, and were placed in a growth chamber at 25° C and 90% RH under a 16-h photoperiod (95 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps). Approximately 77% (23 out of 30) survived and resumed growth within 4–12 wk of transplantation. Surviving plants were moved to the greenhouse and allowed to grow for 1 y (Fig. 4).

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