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Improving chestnut micropropagation through axillary shoot development and somatic embryogenesis

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Abstract [Review article]
The effects of the mineral media and the carbon source on the proliferation capacity of different *Castanea sativa x C. crenata* cultivars were the focus of the research reported here. Using the commercial cultivar Marigoule, the addition of riboflavin to the rooting medium did not improve the rooting rates recorded. It did, however, positively affect the survival of regenerated plantlets after weaning. By measuring certain physiological parameters, the beneficial effect of high concentrations of CO_2 on the acclimatization of chestnut regenerated plantlets was recorded. However, general protocols for large-scale micropropagation of specific cultivars could not be defined.

Our research has determined for the first time the developmental window in which somatic embryogenesis induction is possible from ovaries, ovules and/or zygotic embryos in *C. sativa.* Induction is possible between the 2nd and the l0th weeks post-anthesis, giving an overall frequency of 4.5%. Somatic embryogenesis induction in chestnut was not possible from mature tissues; however, embryogenesis was achieved using leaf tissue from shoot multiplication cultures. This indicates, for the first time, that material from explants other than zygotic chestnut embryos is competent for somatic embryogenesis.

The effect of thidiazuron on the ability of different seedling explants of chestnut to induce multiple shoots was also evaluated: cotyledonary node explants, which contain preformed meristematic tissue, were the only responsive explants.

Keywords: Axillary shoots, *Castanea sativa, C. sativa x C. crenata,* chestnut, micropropagation, somatic embryogenesis

1 Introduction

The two chestnut species, *Castanea sativa* (European chestnut) and *C. dentata* (American chestnut), have, for a long time, suffered from ink disease and chestnut blight. Ink disease is the result of attacks by the fungus *Phytophthora cinnamomi,* and chestnut blight by *Cryphonectria parasitica.* A substantial body of research on the chestnut still focuses on the development of vegetative propagation systems capable of satisfying the demand for elite genotypes, and more specifically, genotypes resistant to both diseases. As an alternative to conventional vegetative propagation methods, efforts are being made to establish reliable *in vitro* regeneration systems that allow the clonal propagation of these materials.

In vitro tissue techniques have been applied to chestnut regeneration since the 1980's (VIEITEZ and VIETIEZ 1980a, 1980b; VIEITEZ *et al.* 1983). However, certain points need to be addressed in order to apply the technique more widely. Bearing in mind that *in vitro* establishment in chestnut is possible from both juvenile and mature material (VIEITEZ and VIEITEZ 1980a, SÁNCHEZ *et al.* 1997), points such as the selection of culture medium, carbon source, rooting stage and acclimatisation period need investigation.

In addition, several papers have shown the potential of somatic embryogenesis not only as an alternative clonal system for chestnut micropropagation (VIEITEZ *et al.* 1990, VIEITEZ 1995, CARRAWAY and MERKLE 1997, XING *et al.* 1999) but also as a tool in genetic engineering programmes (CARRAWAY *et al.* 1994, SEABRA and PAIS 1998). For these purposes, the development of adventitious shoots would be an alternative. However, systems based on adventitious shoot regeneration (SAN-JOSÉ *et al.* 1984) have not yet proved to be reliable. The use of meristematic tissues of apical or axillary buds has been reported as a target tissue that is useful for genetic transformation in species recalcitrant for somatic embryogenesis and/or adventitious shoot regeneration (MORRE *et al.* 1998, SAN-JOSÉ *et al.* 2001).

In this paper we summarise the work carried out by the Working Group 1 "Tree Physiology" within the scope of the COST G4 Action "Multidisciplinary Chestnut Research". We focus on three aspects of *in vitro* chestnut tissue culture:

1) optimising micropropagation protocols based on axillary shoot development;
2) defining protocols for induction, maturation and germination of somatic embryos; and
3) inducing multiple shoot formation from cotyledonary nodes.

2 Material and methods

2.1 Micropropagation through axillary shoot development

Effect of basal medium: In order to select the best basal medium, axillary shoots of five different clones were induced to proliferate on the following media: HELLER (1953) (H), MURASHIGE and SKOOG (1962) with half-strength nitrates (MS 1/2N), GRESSHOFF and DOY (1972) (GD), Lepoivre (QUOIRIN and LEPOIVRE 1977) (Lp), BLAYDES (1966) (Bl), SCHENK and HILDEBRANDT 1972) (SH), DRIVER and KUNIYUKI (1984) (DKW) and P24 (TEASDALE 1992). All media were supplemented with 0.2 mg/l BA. After 4 weeks of subculture under photoperiodic conditions (16 h light under 30 $\mu mol.m^{-2}.s^{-1}$, 25 °C temperature), the following parameters were recorded: number of shoots per explant (NSH), number of subculturable segments per explant (NS), percentage of responsive explants (R) and multiplication coefficient (mc). Data were analysed by analysis of variance.

Effect of carbon source: To study the effect of different carbon sources on the proliferation of chestnut, explants from the *C. sativa x C. crenata* cultivars Verdesa, Torcione, SP431 and SP125 were cultivated on GD basal mineral medium supplemented with 0.2 mg/l BA and 0.0876M of sucrose, fructose, glucose or sorbitol. After a 4-week subculture period, the number of shoots produced per explant was recorded.

Rooting: The valuable commercial cultivar, Marigoule *(C. sativa x C. crenata),* was used for rooting experiments. Two methods were tested: a) The use of BdR (BOURRAIN *et al.* 1998) medium supplemented with 2 mg/l IBA for seven days and subsequent transfer of the shoots to an auxin-free MS medium (macronutrients diluted 1/4) plus vermiculite; and b) Riboflavin technique: BdR medium supplemented with 2 mg/l IBA, 30 g/1 sucrose, and 2 mg/l riboflavine diluted in a few drops of ethanol. The shoots were kept in the same medium for the whole rooting period. Four weeks after the beginning of both experiments, the percentage of rooting and the mean number of roots per rooted shoot were recorded.

Acclimatisation: Rooted shoots of the clone MI *(C. sativa x C. crenata)* were transplanted to plastic pots filled with 200 cm^3 peat:perlite (1:2 v/v). The plantlets were acclimatised over the fo1lowing four weeks in controlled chambers and subjected to CO_2 concentrations of either 350 or 700 μl^{-1}. The dry weight of leaves, stem and roots were recorded. Leaf and root areas were obtained by computer image analysis.

2.2 Somatic embryogenesis

Induction from embryonic tissues: Chestnut burs were harvested weekly from four *C. sativa* trees at two different sites in Austria during July and August for two consecutive years. After surface sterilisation, the cupula was removed and the seeds were sectioned and separated into several tissues. Explants were cultured on solid induction P24 medium with 0.8% agar, 3% sucrose, 1 mg/l 2,4-D and 0.1 mg/l BA. After three weeks, the cultures were transferred to P24 medium supplemented with 0.2 mg/l BA but no 2.4-D.

Induction from leaf sections: Leaf and internode explants from stock shoot cultures of juvenile origin (clone 12, *C. sativa x C. crenata*) were used as explants. The two to three uppermost internodes (2–3 mm in length) and proximal leaf of unfurled expanding leaves were cultured on MS basal medium, supplemented with different concentrations of BA in combination with IAA or NAA. The cultures were transferred after six weeks to a medium supplemented with 0.1 mg/l BA plus 0.1 mg/l NAA for a further four weeks.

2.3 Induction of multiple shoot formation from cotyledonary nodes

Chestnut embryonic axes were aseptically germinated for 14 days in MS basal medium supplemented with 0.1 mg/l thidiazuron (TDZ) or 1 mg/l BA. Then the hypocotyl, epicotyl and cotyledonary node explants were cultured on MS 1/2N medium containing 0.01 mg/l NAA in combination with different TDZ concentrations. After four weeks of culture, the explants were transferred for a further eight weeks to media with low concentrations of BA. The number of explants forming callus, shoot buds and shoots longer than 5 mm were recorded every four weeks (SAN-JOSÉ *et al.* 2001).

3 Results and discussion

3.1 Micropropagation through axillary shoot development

The responses of five chestnut clones to different mineral media are shown in Table 1. According to the ANOVA results (data not shown), the model

$$X_{ijk} = \square + C_i + M_j + C^*M_j + \varepsilon_{k(ij)}$$

(X = the observed value of each explant affected by the factor clone C; culture medium M; and their interaction M*C) is highly significant for all parameters evaluated, both for main factors (clone and medium) and for the interaction media-clones. But the value for this interaction is comparatively much less important than the media and clone values. The media comparison results indicate that the GD medium was the best for the variable NSH, but MS (1/2 nitrates) was the most valuable mineral medium for the variable NS. Most of the shoots used for the experiment responded to the culture conditions, regardless of the medium composition used, and the multiplication coefficient (the most useful parameter for evaluating multiplication capacity) mainly ranged from two to five. In addition to the measurable parameters, the quality of the shoots should be also taken into consideration, as the success of the rooting step clearly depends on the physiological state of the shoots produced in the multiplication stage.

The results indicate that it is very difficult to recommend a mineral medium for general application; nevertheless, GD and MS (1/2 N) media appear to be the most suitable for multiplication.

Table 1. Effect of six mineral media on the proliferation of five chestnut clones. Data were recorded at the end of the multiplication stage. NSH: number of shoots per explant; NS: number of segment of explant; RE: % of responsive explants; MC: multiplication coefficient. Within each clone and column, values followed by the same letter are not significantly different at the 5% level. Student-Newman-Keuls test.

Clone	Medium	NSH	NS	%RE	MC
CHR39	MS	3.33 b	4.60 a	100.00 a	4.60 a
(Castanea crenata	GD	3.65 b	3.21 b	100.00 a	3.21 b
x C. sativa)	Hm	3.21 b	3.46 b	98.33 a	3.40 b
	Lp	4.31 a	4.10 a	93.33 ab	3.82 ab
	Bl	2.28 c	2.41 c	88.33 b	2.12 c
	SH	3.60 b	3.30 b	93.33 ab	3.07 b
CHR121	MS	2.05 c	3.55 c	100.00 a	3.55 ab
(Castanea crenata	GD	3.55 a	5.21 ab	98.33 a	5.13 ab
x C. sativa)	Hm	2.83 b	4.31 bc	100.00 a	4.31 ab
	Lp	2.91 b	5.46 a	98.33 a	5.36 a
	Bl	2.20 c	3.38 c	93.33 b	3.15 b
	SH	2.81 b	4.40 bc	95.00 ab	4.18 ab
CHR155	MS	2.31 b	4.58 a	98.33 a	4.47 a
Castanea sativa	GD	2.71 ab	4.41 ab	95.00 a	4.20 a
	Hm	2.68 ab	4.45 ab	100.00 a	4.45 a
	Lp	3.13 a	5.01 a	100.00 a	5.01 a
	Bl	2.23 b	3.71 b	98.33 a	3.65 a
	SH	2.56 b	4.66 a	98.33 a	4.50 a
CHR162	MS	3.11b	7.25a	100.00a	7.25a
(Castanea mollissima	GD	3.00 b	4.61 c	93.33 a	4.30 a
x C. sativa)	Hm	3.36 b	4.83 c	98.33 a	4.74 a
	Lp	4.15 a	6.10 b	98.33 a	5.99 a
	Bl	2.95 b	4.66 c	98.33 a	4.58 a
	SH	3.60 b	5.33 bc	93.33 a	4.97 a
Marigoule	GD	4.0	4.2	95.00	4.0
(C. sativa x C. crenata)	DKW	4.3	4.4	97.00	4.3
	P24	3.2	3.3	75.00	2.5

The results obtained when four carbon sources were tested on shoot multiplication cultures of four chestnut cultivars are shown in Figure 1. The proliferation capacity of axillary shoots varied according to cultivar. Compared to fructose and glucose, a sucrose-containing medium seemed to stimulate the proliferation of Torcione, while there was little difference between the sugars in the cultivars Verdesa SP431 and SP125. When sorbitol was used as the main carbon source, the proliferation rate was significantly reduced in all cultivars. Here again, not only is the number of shoots produced important, but also the quality of the usable shoots. In this regard, sucrose was highly beneficial for the cultivars SP431 and SP125, whereas glucose had a promotive effect in Torcione and fructose in Verdesa.

There were no conclusive results on the rooting of the commercial cultivar Marigoule, as the two methods defined in Material and methods produced similar data, namely, 86% rooting rate using the BdR system and 90% with the riboflavin method. The percentage of plantlets of good quality (vigour, expanded leaves, healthy appearance) that developed after the acclimatisation phase was higher in the riboflavin medium than with the BdR technique (72% vs 64%), but the data were not significantly different. Furthermore, the BdR technique required a secondary medium, increasing the costs of the process.

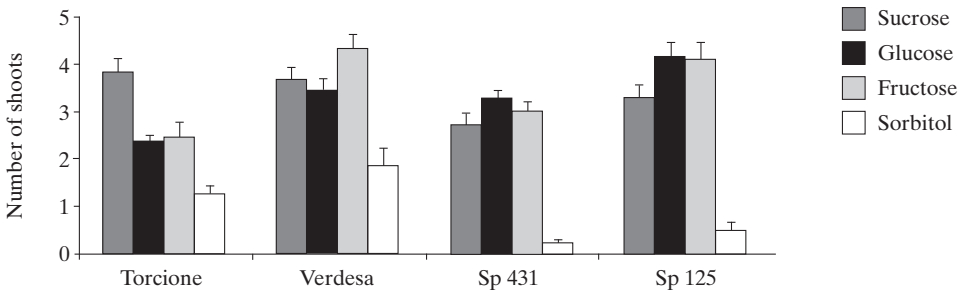

Fig. 1. Influence of carbon source on the proliferation of axillary shoots of three *C. sativa x C. crenata* cultivars grown on GD medium supplemented with 0.2 mg/l BA and 0.0876M of sucrose, fructose, glucose or sorbitol. 20 shoots were used per treatment and the experiment was repeated at least twice. Bars give the standard deviation.

In the acclimatization studies, the elevated CO_2 concentration did not affect the survival rates of the plantlets but gave rise to a significant increase in their relative growth, the shoot/root ratio and the leaf area ratio (see data in GONÇALVES *et al.* 1999). WOLFE (1995) reports that temperate tree saplings show either little response or an increase in shoot/root ratio at high CO_2 levels. We recorded similar findings with the chestnut. The shoot/root ratio showed a less developed root system for the plants under the elevated CO_2, in spite of the greatest diameter of their roots. This was confirmed by the root system analysis. Although the results achieved are promising, more significant gains could be expected using a higher light intensity, since the saturation light level of chestnut plantlets is near 400 μmol $m^{-2}s^{-1}$ (GONÇALVES *et al.* 1999).

As mentioned in the Introduction, *in vitro* chestnut regeneration was achieved many years ago (VIEITEZ and VIEITEZ 1980a). Large-scale propagation of chestnut species using *in vitro* techniques is still difficult. Nevertheless, around 50 000 plants per year are produced *in vitro* by a European company, which is the largest number of *in vitro* chestnut plants produced world-wide (GARCÍA-NIMO 1998). We were not, however, able to define a single, general protocol for chestnut micropropagation, which means that to micropropagate a specific cultivar, the protocol will probably have to be adapted to the characteristics of each cultivar.

3.2 Somatic embryogenesis

For the first time it has been possible to define the developmental window for *Castanea sativa* during which somatic embryogenesis (SE) may be induced. In total, 142 SE cell lines out of more than 3000 juvenile explants were initiated during 1998 and 1999, corresponding to an overall frequency of 4.5% (5.1% for ovaries, 3.0% for ovules and 27% for zygotic embryos). In Table 2, the frequencies of SE induction are shown from explants obtained 1 to 10 weeks post-anthesis (WPA). The embryogenic induction rate is related to the size (and water content) of the original zygotic embryo. It was demonstrated that SE induction was possible from the second to the tenth WPA, regardless of the climatic conditions of the year. The ability for proliferation via repetitive SE differed among cell lines, and direct formation of secondary embryos was frequently observed at the hypocotyl region of primary somatic embryos. The inclusion of BA in the culture medium promoted indirect secondary embryogenesis from cortical parenchyma cells, indicating a multicellular origin. This may explain the occurence of polycotyledonary embryos, fused embryoids and other anomalous forma-

tions of somatic embryos, which were frequently observed. The fresh weight was recorded at 4-, 8-, and 12-week intervals (data not shown), and the highest fresh weight was reached after eight weeks. Cell line cultures can be cold stored (at 10 °C) without subculture for at least three months, regardless of the cell line and the culture medium. In maturation assays, the positive effect of increasing the agar concentration (up to 1.1% instead of the standard concentration of 0.8%) was observed, giving a mean number of somatic embryos of 11.3 per original cluster. Five somatic-embryo-derived plants are currently growing in the greenhouse.

Table 2. Frequencies of embryogenic induction in relation to both the water content and size of the explant during two consecutive years. a1: percentage of embryo induction from ovaries; a2: percentage of embryo induction from ovules; a3: percentage of embryo induction from zygotic embryos; b: number of explants; c: percentage (%) of moisture was calculated on a fresh weight basis (FW-DW)/FWx100; DW dry weight, determined after drying at 104 ºC for 24 hours.; d: length x width; e: mean value.

Date	Weeks Post-anthesis	Explant Ovary		Ovule		Embryo		Moisture content[c] %	Size index[d] of cupula x[e]	Size index[d] of embryo x[e]
		n[b]	a1	n[b]	a2	n[b]	a3			
28.06.98	2	20	0		–				93.63	
13.07.98	4	43	16.3		–				157.0	
20.07.98	5	45	8.9		–				237.1	
27.07.98	6	21	19.1	33	0	1	0		269.5	13.5
03.08.98	7	20	5.0		–	1	0		346.3	53.0
10.08.98	8	9	11.1	7	0	18	27.8	82.2	331.7	61.3
17.08.98	9		–	28	7.2	17	41.2	81.6	423.9	160.0
24.08.98	10		–	2	0	17	47.1	81.7	571.2	254.0
21.06.99	1	52	0		–		–		39.0	
28.06.99	2	39	0	85	0		–		85.1	
05.07.99	3	60	0	364	0.8		–		164.6	
12.07.99	4	49	2.0	400	4.0		–		294.4	
19.07.99	5	57	5.3	501	7.8	13	30.8	81.6	339.0	6.3
26.07.99	6	48	8.3	589	2.7	17	58.8	81.6	374.3	11.0
02.08.99	7	28	0	455	0	19	10.5	80.3	432.1	40.5
09.08.99	8		–	56	0	22	13.6	81.5	460.7	150.5
16.08.99	9		–		–	24	25.0	81.4	579.4	262.3
23.08.99	10		–		–	23	21.7	74.2	802.6	432.5

Somatic embryogenesis induction was also possible from leaf explants taken from *in vitro* shoot cultures of a hybrid *C. sativa x C. crenata.* The induction rate was very low (0.5%) and was achieved when the explants were cultured on MS medium supplemented with 1 mg/l BA + 1 mg/l NAA. Somatic embryos were subcultured on half-strength MS medium supplemented with 0.1 mg/l BA + 0.1 mg/l NAA, where new secondary embryos developed along with a nodular embryogenic callus. Somatic embryos and embryogenic calli were maintained by repetitive embryogenesis for two years. Cotyledonary stage somatic embryos (somatic embryos with two cotyledons higher than 5 mm) were cultured on 6 different maturation media for further maturation and germination. After four weeks of culture on a maturation medium and the application of a chilling treatment (4 °C) for eight weeks, the somatic embryos were transferred to the germination medium (MS + 0.1 mg/l BA). The conversion rate into plantlets was 6.3%, but an additional 33.3% of the embryos produced only shoots.

This is the first report demonstrating that explants other than zygotic embryos are able to produce embryogenic chestnut cultures. Somatic embryogenesis induction in hybrids *C. sativa x C. crenata* (VIEITEZ *et al.* 1990, VIEITEZ 1999) as well as in *C. dentata* (CARRAWAY and MERKLE 1997) has previously been reported. In both cases, the original explant was derived from immature zygotic embryos. In many forest tree species, the only response to embryogenic induction occurs with explants derived from juvenile tissue. In this report, the SE induction was achieved from juvenile tissues of *C. sativa,* but attempts to induce SE from adult material failed. In addition, the developmental window during which SE induction is possible has been defined for this chestnut species. Furthermore, the results reported in our work demonstrate, for the first time in the chestnut, that the induction of SE is also possible in tissue other than zygotic embryos. We found that leaf sections of *in vitro* clonal material responded to the embryogenic stimuli. Induction of SE from leaf sections has been reported for other members of the Fagaceae (CUENCA *et al.* 1999).

3.3 Induction of multiple shoot cultures from cotyledonary nodes

The objective of this work was to act on cotyledonary node meristematic tissue before its organisation into axillary buds. The cotyledonary nodes of preconditioned germinated plantlets showed a high ability to develop shoot buds when cultured on media supplemented with TDZ. These buds were developed in the cotyledon axil zone, generally in compact clusters (see SAN-JOSÉ *et al.* 2001 for details). The methodology developed would be useful for efficient genetic transformation programmes since it means that there is a reasonably high probability of a large number of transformed cells producing transformed buds.

Some attempts have been carried out to transform chestnut via genetic engineering (SEABRA and PAIS 1998, XING *et al.* 1999), but the results were disappointing, mainly due to the poor regeneration obtained. However, the results reported in this paper and those reported by SAN-JOSÉ *et al.* (2001) open up new possibilities for using preconditioned, cotyledonary nodes as a material with a high capacity for regeneration.

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