

## PROTOCOL FOR MICROPROPAGATION OF *CASTANEA SATIVA*

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### 1. INTRODUCTION

Since the 1970s, natural resources the world over have been progressively re-evaluated in accordance with the principles of sustainable agriculture. The European chestnut, *Castanea sativa* Mill., a hardwood species belonging to the *Fagaceae*, has not been unaffected by this process. It is an important resource in many parts of the world because of its economic and environmental role in many agroforestry systems, and in Europe it has been gaining in value as a source of timber and nut production and due to the contribution of chestnut groves to the landscape (Bounous, 2005).

Like the American chestnut *C. dentata*, *C. sativa* has been plagued for more than a century by ink disease and chestnut blight, caused by the fungi *Phytophthora cinnamomi* and *Cryphonectria parasitica*, respectively. A great deal of research on chestnut focuses on the development of vegetative propagation systems capable of satisfying the demand for elite genotypes that provide both high-quality timber and/or nuts and resistance to these diseases. Since chestnut is a difficult-to-root species, grafting is the most frequent conventional propagation technique, although methods for layering and cutting have recently been improved and are widely used in nurseries to propagate ink-disease-resistant Euro-Japanese hybrids. However, as an alternative to conventional vegetative propagation methods, efforts are being

made to establish reliable *in vitro* regeneration systems that allow clonal propagation. The two major systems are based on embryogenesis or on micropropagation of axillary shoots.

Several studies have shown the potential of somatic embryogenesis of chestnut, not only for clonal propagation but also for genetic engineering programmes (Corredoira et al., 2006). However, although somatic embryogenesis is theoretically more efficient for clonal mass propagation than propagation via axillary shoots, several difficulties need to be overcome in order to make it commercially viable, particularly when cultures originate from adult tissues. By contrast, chestnut can currently be micropropagated from both juvenile and mature material using the axillary shoot development method. In the last years, efforts have been concentrated on the regeneration systems allowing clonal propagation of mature chestnut trees. Large-scale propagation is still in many cases challenging, because it is common for the protocol to require optimization for a specific cultivar; but there are nevertheless several European companies that now produce thousands of plants a year. In this chapter we describe the various steps of the typical protocol for *C. sativa*, with occasional reference to other chestnut species.

## 2. EXPERIMENTAL PROTOCOL

The micropropagation of chestnut via axillary shoots involves four stages: 1) initiation (*in vitro* shoot growth on primary explants); 2) shoot proliferation; 3) shoot rooting; and 4) plantlet acclimatization (hardening). In the interests of efficiency, the rooting and acclimatization stages are combined whenever possible. Before examining each stage in detail, we present the culture media that have been found to be most appropriate for each of the *in vitro* stages.

### 2.1. Culture Media

Of the various culture media that have been assayed for micropropagation of European chestnut, most studies have adopted Gresshoff and Doy mineral medium (GD; 1972) for material of both juvenile and mature origin. Apical necrosis and chlorosis are best avoided, and general appearance improved, with either GD or Murashige and Skoog medium (MS; 1962) with half strength nitrates (Vieitez et al. 1986; Sánchez et al. 1997a; Gonçalves et al. 1998; Ballester et al. 2001). However, in protocols developed for *C. dentata*, Woody Plant Medium (Lloyd and McCown, 1981) has been used both for culture initiation (supplemented with 1 mg/l 6-benzyladenine, BA) and for shoot proliferation (supplemented with 0.2 mg/l BA).

(Xing et al. 1997). The protocols described here for the various stages of micropropagation use GD, modified and/or supplemented as detailed in Table 1.

**Table 1.** Culture media used in the initiation, shoot multiplication and rooting stages of the micropropagation of European chestnut via axillary shoots.<sup>1</sup>

<i>Culture medium</i>	<i>Initiation</i>	<i>Multiplication</i>	<i>Rooting</i>
Macronutrients	GD	GD	1/3 GD
Micronutrients <sup>2</sup>	Modified GD	Modified GD	Modified GD
Fe-EDTA	MS	MS	MS
m-Inositol (mg/l)	10	10	10
Thiamine HCl (mg/l)	1	1	1
Nicotinic acid (mg/l)	0.1	0.1	0.1
Pyridoxine-HCl (mg/l)	0.1	0.1	0.1
Glycine (mg/l)	0.4	0.4	0.4
BA (mg/l)	0.5	0.1-0.2	-
IBA (mg/l)	-	-	3 (5-7 days) 25 (24 h) 1000(1-2 min)
Sucrose (g/l)	30	30	30
Bacto Difco agar (g/l)	7	7	7 <sup>3</sup>
pH	5.6-5.7	5.6-5.7	5.6-5.7

<sup>1</sup> Culture conditions: All cultures are kept in a growth chamber with a 16 h photoperiod (50-60  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , cool white fluorescent lamps) and 25°C day, 20°C night temperatures.

<sup>2</sup> Modified GD (mg/l): 3  $\text{BO}_3\text{H}_3$ ; 10  $\text{MnSO}_4 \text{H}_2\text{O}$ ; 3  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.25  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; 0.25  $\text{MnO}_4\text{Na}_2 \cdot 2\text{H}_2\text{O}$ ; 0.25  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ; 0.75 KI.

<sup>3</sup> Agar used in the root induction medium (5-7 days or 24 h) before transfer to *ex vitro* substrate.

BA, 6-benzyladenine; GD, Gresshoff and Doy; IBA, indole-3-butyric acid; MS, Murashige and Skoog.

## 2.2. Explant Preparation and Culture Initiation

The primary explants from which chestnut shoot cultures are initiated are generally shoot tips and nodes bearing 1 or 2 axillary buds. After excision from the source plant (juvenile or mature tree), they must be sterilized and established *in vitro* on the initiation medium.

### 2.2.1. Juvenile Plant Material

When the initial explants are obtained from juvenile plants, the micropropagation of chestnut is fairly unproblematic. Seedlings obtained conventionally should be grown in the greenhouse or, preferably, in a climate chamber so as to reduce explant contamination rates (Vieitez et al. 1986). Active growing shoots are collected from plants between a few weeks and a few months old and are treated as described below in Section 2.2.2 (steps 5-10). Alternatively, initial explants may be taken from seedlings obtained by *in vitro* germination of zygotic embryonic axes (Vieitez and Vieitez, 1980). In either case, the frequencies of responsive explants, shoot multiplication rates and rooting rates are generally high.

### 2.2.2. Juvenile Parts of Mature Trees

The micropropagation of chestnut from tissues taken from adult trees appears usually to be feasible when these tissues retain physiologically juvenile characteristics, as is the case of basal shoots and stump sprouts (Vieitez et al. 1986; Sánchez and Vieitez, 1991; Sánchez et al. 1997a). In general, cuttings 15-20 cm long are taken in winter from shoots emerging from the base of the trunk, and are stored at 4°C until forced to flush in a climate chamber, primary explants then being taken from the flushed shoots. The use of a climate chamber is both logistically advantageous, allowing primary explants to be obtained throughout the year, and also helps keep contamination rates low (5-15%).

#### Steps

1. Basal shoots or stump sprouts are harvested from selected trees growing in the field in late autumn or winter, and cuttings 15-20 cm long are taken.
2. The cuttings are immersed for 1 h in a 3.4 g/l solution of Cupravit (50% copper oxychloride), left to dry for 24 h, packed tightly in plastic bags, and stored at 4°C for 2-6 months, until use.
3. The cuttings are taken out of cold storage, arranged upright in water or in moistened perlite in trays, and forced to flush (Figure 1A) in a growth cabinet at 24°C and 90% relative humidity (RH) under a 16 h photoperiod (95-100  $\mu\text{mol m}^{-2}\text{s}^{-1}$  provided by cool-white fluorescent lamps).
4. After 15-20 days of flushing, shoots 1-4 cm long are collected as the source of explants (Figure 1B).
5. The collected shoots are stripped of leaves and surface-sterilized by successive immersion for 30 s in 70% ethanol and for 8-10 min in sodium hypochlorite solution (Millipore Chlorine Tablets, 0.6% active chlorine) containing 2 or 3 drops of Tween 80.
6. The shoots are rinsed three times with sterile distilled water.
7. The disinfected shoots are sectioned at 5 mm long shoot tips and nodes (primary explants).

8. The explants are placed upright in 20×150 mm culture tubes containing 15 ml of initiation medium (Table 1).
9. After 24 h, the primary explants are moved to a different site within the same tube to reduce the deleterious effects of exudation and blackening of the medium. Thereafter, the explants are transferred to fresh medium every 2 weeks until 6 weeks after the initiation of culture.
10. Shoots produced on primary explants (Figure 1C) are excised and subcultured to achieve clonal shoot multiplication cultures.

Clones differ in their response to establishment *in vitro*. Although this may be attributed largely to their genetic differences, differences in age among the source trees may also be responsible.

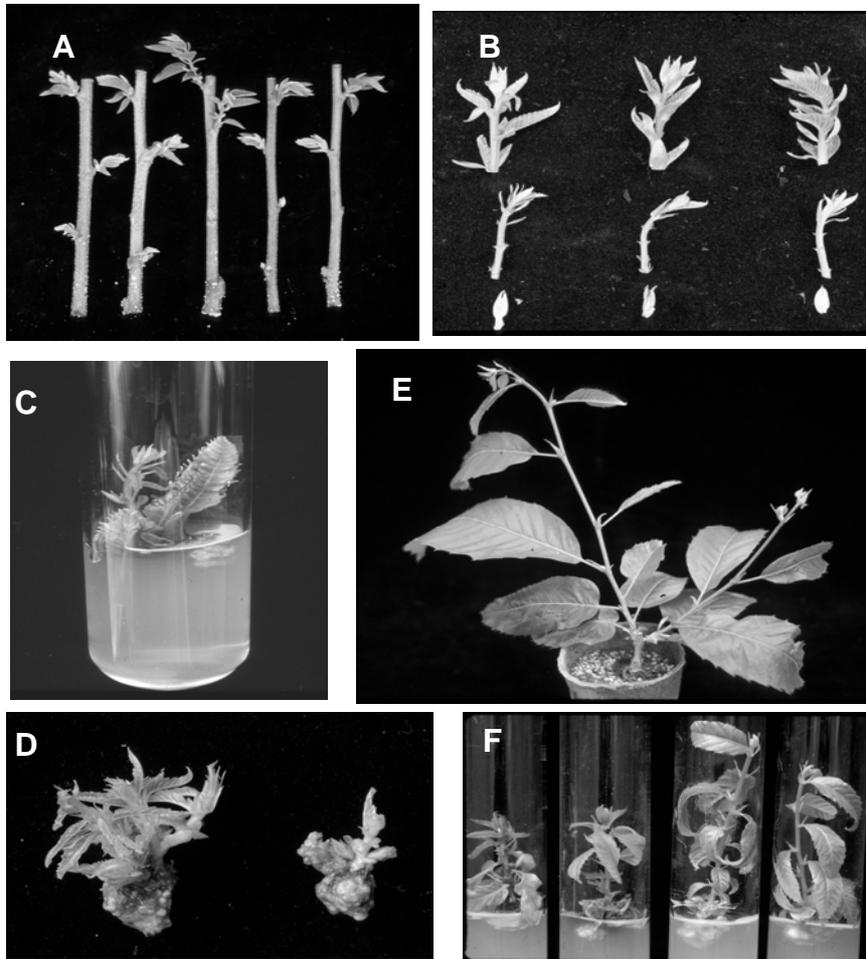
### 2.2.3. Crown Material of Mature Trees

When the material of origin has been taken from the crown of mature chestnut trees, micropropagation has achieved very limited success, shoot multiplication and rooting rates having been poor (Sánchez and Vieitez, 1991). However, the reactivity of mature material can be considerably improved by reinvigorating it before primary explants are excised, either by pre-harvest etiolation (Ballester et al. 1989) or by grafting it onto seedling rootstocks, with or without repeated subsequent spraying with cytokinins (Sánchez et al. 1997b).

Localized etiolation of branches can greatly facilitate the establishment of mature chestnut material *in vitro*, allowing successful explant response rates of up to 79% as against 27% for unetiolated material (Figure 1D). Partial etiolation (blanching) is applied in late May, when 10-15 cm segments of the new year's growth on lower crown branches are stripped of their leaves and wrapped in aluminium foil. In late September, cuttings are taken from the etiolated segments and stored at 4°C until use. These cuttings are forced to flush in a growth cabinet, and the new shoots are used as the source of primary explants as described in steps 5-10 of section 2.2.2.

Successful establishment *in vitro* can also be achieved if 3-4 cm long scions taken directly from the crown are grafted onto 2-week-old seedlings and kept in a growth cabinet or in the greenhouse for 5 weeks, where for the last two weeks they may be sprayed three times a week with 50 mg/l BA solution (Sánchez et al. 1997b). The lateral shoots that emerge during this time are used as the source of primary explants (Figure 1E) as described in steps 5-10 of section 2.2.2. However, serial grafting may be necessary: Giovanelli and Giannini (2000) reported successful establishment *in vitro* after having successively grafted shoots four times onto 10-month-old seedling rootstocks over a 4-year period.

It should be noted that even when mature material is reinvigorated by one of the above techniques, the cultures established generally have lower shoot proliferation and rooting rates than those of shoot cultures derived from juvenile parts of the tree (Figure 1F). Material that retains juvenile characteristics should therefore be used whenever possible (see Section 2.2.2.).



**Figure 1.** Shoot culture initiation from buds of mature chestnut trees. (A) Forced flushing of cuttings taken from basal sprouts of an adult tree. (B) Shoots excised from flushed cuttings (upper), sterilized shoots (middle) and sectioned shoot tip explants to be established in vitro (lower). (C) Shoot development from a primary explant of basal sprout origin one month after culture initiation. (D) Initial cultures of etiolated (left) and unetiolated (right) crown-derived material. (E) Plant grafted onto seedling rootstock, exhibiting new shoots to be used as source of crown-derived explants. (F) Proliferating shoot cultures originated from crown branches (left) and basal sprouts (right), after 4 weeks in multiplication medium.

### 2.3. Shoot Proliferation and Maintenance

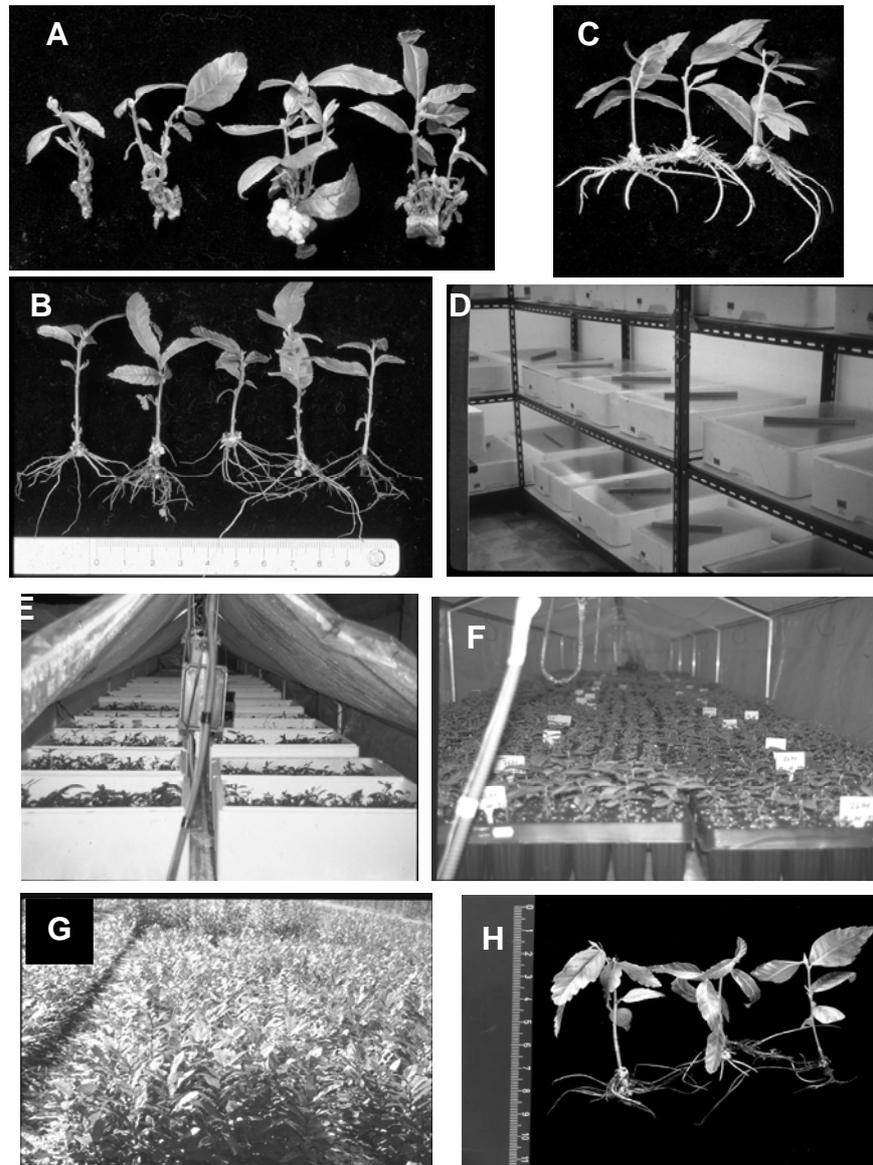
New shoots obtained *in vitro* by 6-8 weeks of culture of primary explants are excised, divided into 0.8-1.0 cm segments (including shoot tips), and subcultured into 500 ml glass jars (9 explants/jar) containing 70 ml of shoot multiplication medium (Table 1). The indicated carbon source of the multiplication medium, 3% sucrose, has been chosen on the basis of comparison of the results obtained with sucrose (Figure 2A), glucose and fructose at concentrations of 1-4%: although 3-4% fructose or glucose afford the greatest number of shoots, these shoots exhibit high hyperhydricity rates and poor growth. A feasible alternative to 3% sucrose is 2% glucose.

Clonal shoot multiplication is effected by subculturing at intervals of 4-5 weeks. *In vitro* stabilization is achieved within 4-8 subculture cycles, and cultures can be maintained for years if healthy, vigorous shoots are always used for subculture. When shoots have not elongated satisfactorily after 3-4 weeks of culture, their length and vigour can be promoted by spending 2-3 weeks more on fresh multiplication medium with a lower BA concentration (0.05 mg/l), giving a 6-7 week multiplication cycle.

Shoot proliferation is influenced by both genotype and the type of explant. Genotype is one of the most important determinants of all relevant parameters (number of shoots, shoot length and overall multiplication rate). Multiplication rates are also influenced by explant type, basal nodal explants (which bear 2-3 axillary shoots) affording higher multiplication rates than shoot tip explants (Sánchez et al. 1997a). Shoot growth on basal segments is more vigorous if their basal callus is retained. Therefore, shoots taken for rooting should have their basal 1 cm removed and this tissue, with its callus, can be recycled to the proliferation stage.

### 2.4. Rooting

Chestnut is a difficult-to-root species, and the rooting and acclimatization of shoots obtained *in vitro* are crucial for mass production of viable plants by micropropagation. As indicated in Table 1, there are three main options for inducing roots on micropropagated chestnut shoots (Sánchez et al. 1997a; Gonçalves et al. 1998): 1) culture for 5-7 days in rooting medium containing 3 mg/l indole-3-butyric acid (IBA); 2) dipping the basal end of the shoots for 1-2 min in 1 g/l IBA solution; and 3) culture for 24 h in rooting medium containing 25 mg/l IBA. All three can afford rooting rates of 80-95%, and although the 24 h treatment generally affords better shoot quality than the quick dip, which option is best will depend on genotype. After one of the three procedures has been carried out, the auxin-treated shoots are transferred either to a substrate mixture (Figure 2B), or to auxin-free root expression medium consisting of basal rooting medium containing 1% activated charcoal (Figure 2C). When possible, the former procedure is clearly



**Figure 2.** Rooting, hardening and cryopreservation of chestnut. (A) Morphology of shoots after 4 weeks of culture in sucrose-supplied multiplication media (from left to right, 1, 2, 3 and 4%). (B) Rooting of auxin-treated shoots following transfer to a mixture substrate. (C) Root development in shoots treated with 25 mg/l IBA for 24 h and subsequent culture for one month in activated charcoal medium. (D) Polystyrene boxes filled with substrate and covered with plastic lids, where IBA-treated microcuttings are transferred for rooting. (E) Plantlets in open polystyrene boxes placed in the acclimatization tunnel. (F) Rooted plants transferred to trays and maintained in the acclimatization tunnel. (G) Micropropagated chestnut plants growing in the outdoor nursery. (H) Rooted plantlets derived from cryopreserved shoot apices.

the more cost-effective: whereas *in vitro* rooting is labour-intensive, rooting in a substrate in the greenhouse allows rooting and acclimatization to be achieved in one step instead of two.

One of the main threats to the survival of plantlets during acclimatization is the occurrence of shoot tip necrosis during rooting (Vieitez et al. 1989; Piagnani et al. 1996), and it is chiefly to reduce necrosis and to promote shoot growth that charcoal is included in the root expression medium (Sánchez et al. 1997a). When shoot tip necrosis does occur, the role of apical dominance is generally taken over by one of the axillary buds, usually the one nearest the apex, and the plantlet survives. The rapid development of an axillary bud can be promoted by the removal of the shoot tip before rooting (Vieitez et al. 1989; Gonçalves et al. 1998).

### 2.5. Hardening

The adaptation of micropropagated chestnut plants to *ex vitro* conditions is generally long and difficult. Survival rates are higher if microcutting rooting has been carried out directly in substrate mixture rather than *in vitro* in root expression medium. Survival depends heavily on genotype, with values ranging from 50% to 90%, and the best period for stimulating rapid stem elongation is February-August. For *C. sativa* × *C. crenata* plantlets, survival rates higher than 80% have been obtained under a range of irradiance and CO<sub>2</sub> availability conditions. However, under high irradiance (300 μmol m<sup>-2</sup>s<sup>-1</sup>) combined with high CO<sub>2</sub> concentration (700 μl/l) growth yields increased consistently as well as the efficiency in inducing an autotrophic behaviour (Carvalho and Amâncio 2002; Carvalho et al. 2005). These authors suggested that these conditions are worth to be tested as an acclimatization protocol for recalcitrant genotypes.

#### *Steps for Direct Rooting and Hardening*

1. Shoots 2.5-5.0 cm long are excised from shoot proliferation cultures.
2. The basal end (1 cm) of the shoots are dipped for 1-2 min in 1 g/l IBA solution in a glass beaker (50 shoots per vessel). Alternatively, the shoots can be cultured for 24 h in rooting medium containing 25 mg/l IBA.
3. The IBA-treated microcuttings are transferred to polystyrene boxes (100 microcuttings/box) filled with a moistened 1:2 (v:v) mixture of pine bark and perlite that has previously been treated with antifungal solution (Figure 2D). The boxes are each covered with a transparent polyethylene lid and are kept in the growth chamber for two months under standard conditions (Table 1), lids with ventilation holes being used during the second month so as to decrease the RH inside the boxes. During this time the microcuttings are watered twice a week, once with antifungal solution and once with water.
4. After 2 months the boxes are placed without lids in plastic acclimatization tunnels in the greenhouse, where they stay for 1 month (Figure 2E). The

- tunnels are provided with mist and fog systems to maintain 90% RH, and during autumn and winter artificial lighting ensures a 16 h photoperiod.
5. The rooted plantlets are transferred to multi-cell trays filled with a 1:2:1 (v:v:v) mixture of pine bark, perlite and peat supplemented with Osmocote<sup>®</sup>, a slow-release fertilizer. The trays are kept in the tunnels for 1 month (Figure 2E), during which time RH is gradually lowered to normal greenhouse conditions.
  6. By the end of the month, average plant height is 25 cm. The plants are removed from the tunnels, transplanted to pots or to trays with larger cells, and kept in the greenhouse for a further 1-2 months.
  7. Still in their pots, the plants are placed outdoors under shade. When the plants have reached a height of 40-50 cm they can be planted out in the field (Figure 2G).

## 2.6. Storage of *in vitro* Cultures

Tissue culture techniques not only offer an opportunity for rapid propagation, they also constitute an approach to genotype conservation. However, the maintenance of large collections in conventional *in vitro* culture systems, which involve subculture at regular intervals, exposes the cultures to increasing risks of contamination and somaclonal variation. In the case of medium-term storage of chestnut cultures, these risks can be reduced by conservation under slow growth conditions (cold storage), which moreover reduces maintenance costs by prolonging subculture periods. For long-term conservation of micropropagated chestnut germplasm, cryopreservation has been used.

### 2.6.1. Cold Storage of *in vitro* Shoot Cultures

Janeiro et al. (1995) reported the possibility of keeping chestnut cultures at 2-4°C for up to 1 year without subculture. Explant necrosis is common following cold storage, but even apparently necrotic explants should spend one subculture cycle in the growth chamber under standard conditions, as some brown cultures can proliferate, especially from lateral buds that remained immersed into the agar throughout cold storage. Also, both the survival rate and the post-storage proliferation capacity of the explants are significantly better if transfer to the cold is effected 10 days after the last subculture rather than immediately after it.

#### *Steps*

1. Nodal segments 10-12 mm long bearing 2-3 axillary buds are excised from shoot multiplication cultures.
2. These explants are placed 6 explants to a jar in 300 ml glass jars containing 50 ml of multiplication medium and are maintained in the growth chamber for

- 10 days under standard growth conditions (Table 1). Most of the length of the explant should be immersed in the medium in order for it to protect lateral buds.
3. After 10 days of subculture, the jars are stored in the dark at 3-4°C in Sanyo Medicoool Cabinets.
  4. The cultures can be kept in the cold for up to 12 months. When removed from the cabinets they are immediately transferred to fresh multiplication medium and kept in a growth chamber under standard conditions (Table 1).
  5. After 1 month of culture, new green shoots will have developed from axillary buds, and shoot multiplication can be carried out as described in Section 2.3.

#### *2.6.2. Cryopreservation of Shoot Tips*

A vitrification procedure involving successive treatments with Loading Solution (LS; Matsumoto et al. 1994) and modified Plant Vitrification Solution (PVS2; Sakai et al. 1990) allows micropropagated chestnut shoot apices to be cryopreserved fairly successfully for subsequent plant regeneration (Vidal et al. 2005). Factors influencing success include the source of the shoot tips (terminal buds are better than axillary buds), the duration of exposure to PVS2 (optimum 120 min), the size of the shoot tip (0.5-1 mm shoot apices isolated from terminal buds), and the composition of the post-cryostorage recovery medium (should include 0.5 mg/l indole-3-acetic acid, IAA, in addition to cytokinin). Depending on genotype, shoot recovery rates of 33-54% have been obtained (recovery being defined as greening, leaf expansion and shoot production), and shoots derived from the cryopreserved material show no significant difference in rooting ability (Figure 2H) from shoots of the same clones that have not been subjected to cryopreservation.

#### *Steps*

1. Terminal shoot buds 1 cm long are excised from shoot cultures 4-5 weeks old that have been maintained in multiplication medium (Table 1).
2. Cold hardening. The terminal buds are transferred to GD medium containing 0.05 mg/l BA dispensed in 9 cm diameter Petri dishes, and are cold-hardened for 2 weeks at 3-4°C under dim light.
3. Shoot apices 0.5-1.0 mm long are dissected from terminal buds.
4. Preculture. The shoot apices are precultured in Petri dishes for 48 h at 3-4°C on GD medium containing 0.2 M sucrose but no BA.
5. Loading. Following preculture, the shoot apices are immersed for 20 min at room temperature in cryoprotectant mixture (LS: 2M glycerol + 0.4 M sucrose) in 2 µl cryotubes.
6. Dehydration. The LS solution is replaced by modified PVS2 (30% glycerol, 15% ethyleneglycol, 15% dimethylsulfoxide, in GD medium containing 0.4 M sucrose). The shoot apices are treated with this mixture for 120 min at 0°C.

7. Cryopreservation. The apices are finally suspended in 0.6 ml of PVS2 in cryotubes, and are plunged into liquid nitrogen (LN).
8. Thawing. For recovery of the cryostored shoot tips, the cryotubes are rapidly warmed for 2 min in a water bath at 40°C.
9. Unloading. The PVS2 solution is drained off and replaced with liquid GD medium containing 1.2 M sucrose. The shoot apices are left in this medium for 10 min, and then for another 10 min in a fresh dose of the same medium.
10. Regrowth. The apices are transferred to sterilized filter paper discs placed in Petri dishes on a recovery medium consisting of GD medium supplemented with 0.5 mg/l BA, 0.5 mg/l IAA and 0.2 mg/l zeatin.
11. Subsequently (24 h, 2 weeks and 4 weeks later), the apices are transferred to fresh recovery medium without paper discs. Standard subculture is carried out 8 weeks after thawing.

Cryopreserved chestnut shoot tips turn blackish-brown within 1 day after thawing, but culture on recovery medium induces surviving tips to resume growth within 2-3 weeks, leaf development becoming evident by the fourth week. Eight weeks after thawing, shoots are an average 2.6-5.0 mm long, depending on genotype. After two one-month periods on multiplication medium (Table 1), these shoots have achieved 2-3 cm and are thus suitable for shoot multiplication or rooting as described in Sections 2.3 and 2.4.

### 2.7. Molecular Marker Analysis

The genetic stability of *in vitro* regenerated plants is an essential requisite to maintain clonal identity. Although shoot tips are in principle genetically stable, micropropagation protocols should include confirmation that true-to-type plants are being produced. Methods for early detection of genetic variation include morphological observations, cytological methods and DNA analysis (Rani and Raina, 2003). In the case of chestnut, screening for DNA polymorphism by RAPD analysis has provided evidence supporting the genetic stability of *Castanea sativa* × *C. crenata* hybrids propagated *in vitro*; after *in vitro* multiplication of axillary shoots for more than 4 years, comparison of RAPD patterns of *in vitro* cultures with those of the plants of origin showed no polymorphism between the former and the latter (Carvalho et al. 2003).

Assessment of genetic stability is also required following cryopreservation (Harding, 2004). For chestnut, the analysis of genetic integrity of cryopreserved shoots has been performed by RAPD analysis (San-José et al. 2005): in experiments on three genotypes using DNA obtained from fresh young leaves, RAPD profiles generated with forty 10-mer primers showed no differences between non-cryopreserved cultures and cultures that had been recovered following cryopreservation.

### *Steps*

1. Young leaves (100 mg) are harvested from individual shoots, and are immediately frozen in LN and stored at -70°C until used for DNA isolation.
2. The frozen leaves are ground to a fine powder in LN in a precooled mortar, and genomic DNA is extracted using a DNeasy Plant Minikit (Quiagen GmbH). The DNA is quantified by spectrophotometry, and its quality is assessed by electrophoresis in agarose gels (1.2% in 0.5× TBE).
3. PCR reactions are carried out in a Biometra Thermocycler using 25 µl of a reaction mixture composed of Tris-HCl (67 mmol/l, pH 8.8), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (16 mmol/l), MgCl<sub>2</sub> (6mmol/l), dNTPs (200 µmol/l each), bovine serum albumin (0.4 g/l), decanucleotide primer (0.4 µmol/l, from kits A and S of Operon Technologies Inc.), 20 ng of genomic DNA, and 0.6 U of BioTaq DNA polymerase (from Bioline).
4. Amplification programme: 1 min at 94°C, followed by 45 cycles of 10 s at 94°C (denaturation), 15 s at 36°C (annealing) and 90 s at 72°C (elongation), and finally 7 min at 72°C. At least two independent PCR runs are performed per sample and selected primer.
5. The amplification products are resolved in agarose gels (1.4% in 0.5×TBE buffer containing 0.15 µg/l ethidium bromide) alongside a 100 bp ladder for size estimation. The bands are visualized under UV light for photography and capture of digitalized images.
6. For each primer, RAPD bands are scored as present or absent by visual inspection of gel photographs. The molecular weight of each fragment can be estimated from digitalized gel images using Bio-Rad Quantity One one-dimensional analysis software.

### 3. CONCLUSION

Micropropagation has been adopted for commercial production of chestnut cultivars and hybrids selected for desirable qualities such as resistance to ink disease. One of the major problems preventing wider use of micropropagation technology has been the difficulty in using explant source material from mature trees. Whenever possible, the material used should be taken from stump sprouts or epicormic shoots, which retain juvenile characteristics. When this is not possible, non-juvenile material can be reinvigorated by techniques involving pruning, grafting or etiolation, although the results are poorer than with juvenile material, especially as regards rooting and plantlet acclimatization. It must be stressed that results in any case depend heavily on genotype, which not only influences shoot proliferation and rooting rates, but also the vigour and acclimatization of regenerated plantlets.

Advances in breeding and genetic engineering programmes create a need for means of preserving valuable new genotypes. Storing chestnut shoot cultures at 3-4°C reduces the risk of contamination and genetic variation by lengthening subculture cycles to up to 1 year, constitutes a convenient method for medium-term storage of germplasm, and facilitates its safe distribution. For long-term storage, cryopreservation of chestnut shoot apices is made feasible by the vitrification procedure.

For confident use of micropropagated chestnut plants, they must be known to be genetically identical to their trees of origin. As yet, relatively little research has been done on verification of genetic stability, but RAPD analyses have found no evidence of genetic variation in chestnut cultures propagated *in vitro* by multiplication of axillary shoots. At present, however, technologies based on molecular markers need to be supported by cytological methods and by morphological observations at field testing level.

#### 4. ACKNOWLEDGEMENTS

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