Original article

# Reinvigoration treatments for the micropropagation of mature chestnut trees

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**Summary** – Crown material from five adult chestnut trees was given different reinvigoration treatments, such as 6-benzylaminopurine (BA) applications (spray or pulse) to forced cuttings, and juvenile grafting alone or combined with BA sprays, then used for the establishment in vitro. The in vitro performance, in terms of establishment, multiplication and rooting, of both untreated and treated material was compared. Grafting alone or in combination with BA spray greatly increased the in vitro reactivity of crown-derived explants. By combining in vivo pretreatments and a horizontal reculturing system, crown-derived microshoots exhibited maximum rooting rates, similar to those found for cultures from basal shoots of the same tree in previous work.

**chestnut / horizontal reculture / micropropagation / mature trees / partial rejuvenation**

**Résumé – Traitements de rajeunissement de châtaigniers adultes.** Pour faciliter l'établissement in vitro du châtaignier, différents traitements, tels que des applications de 6-benzylaminopurine (BA, pulvérisation ou trempage de 2 h), le greffage sur porte-greffe juvénile, seul ou combiné avec des pulvérisations de BA, ont été appliqués au matériel prélevé dans la couronne de cinq châtaigniers adultes. On a comparé le comportement durant l'établissement, la multiplication et l'enracinement in vitro, du matériel non traité (témoin) et du matériel rajeuni. Durant l'établissement in vitro, la réactivité du matériel témoin a été relativement faible pour tous les clones (variations entre 0 et 22 %). Le greffage, seul ou combiné avec des pulvérisations de BA, augmente significativement la réactivité des explants provenant de la cime des arbres, atteignant 94 % pour le clone HV. En ce qui concerne la phase de multiplication, les meilleurs résultats ont été obtenus avec les microboutures dérivées du matériel provenant de la couronne, pulvérisé avec BA (C+S), ainsi qu'avec ceux des greffes pulvérisées avec BA (G+S). Dans les deux cas, on a obtenu des valeurs bien supérieures à celles du témoin. Un comportement similaire a été observé dans leur aptitude à l'enracinement. Ces résultats montrent que les traitements C+S et G+S induisent un certain rajeunissement du matériel adulte. En combinant les prétraitements in vivo avec un système de culture répétée des explants in vitro en position horizontale (recyclage), les microboutures dérivées de la couronne présentent un taux de multiplication

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significativement supérieur à celui des explants en position verticale et/ou horizontale non recyclés. De plus, avec ce système on a obtenu des taux d'enracinement (63 et 28 % pour les clones 431 et HV, respectivement) similaires à ceux précédemment trouvés dans des cultures dérivées de rejets de la base du même arbre.

**arbres adultes / châtaignier / micropropagation / reculture horizontale / rajeunissement partiel**

## INTRODUCTION

One of the limiting factors for the micro-propagation of recalcitrant mature trees is the loss of morphogenetic capacity of explants as the tree ages. In vitro cloning of mature chestnut trees has been successfully accomplished from material retaining physiologically juvenile characteristics, such as basal shoots and stump sprouts (Biondi et al, 1981; Vieitez et al, 1983; Chauvin and Salesses, 1988). In contrast, it is well known that the micropropagation of material from the crown of mature trees still remains very difficult. In a previous paper, we reported for five chestnut clones the morphogenetic capacity greater for basal shoot-derived explants than for crown branch-derived ones. Shoot cultures were established from both sources, but the in vitro performance of crown explants, in terms of reactivity, multiplication and rooting rates was very poor (Sánchez and Vieitez, 1991). For this reason, the success in micropropagating a selected chestnut tree is, to a large extent, a function of the availability of juvenile or reinvigorated material.

Although mature phenotypic characteristics are stably maintained and transmitted once maturity has been attained, they are reversible under certain conditions (Hackett, 1985). Therefore, as the morphogenetic response of explants in vitro is greatly influenced by their maturation state (Hackett and Murray, 1993), it is interesting to obtain reinvigorated or partially rejuvenated material from a desirable mature tree by several experimental approaches. According to Pierik (1990), a majority of the treatments used cause an increase in vigor and rooting

(reinvigoration), with true rejuvenation being difficult to achieve. The rooting capacity of mature material was improved in different woody species by treatments including severe pruning (Howard et al, 1989), serial rooting of cuttings (Morgan et al, 1980), juvenile grafting (Franclet, 1981; Ballester et al, 1990), spraying with cytokinins (Bouriquet et al, 1985), partial etiolation (Ballester et al, 1989) as well as by inducing epicormic shoots in crown branches of mature trees (Vieitez et al, 1994) or stem sections (Evers et al, 1993). In vitro rejuvenation methods, such as meristem culture, serial micrografting and reculture of the same original explant, among others, have also been successful.

In this study, different reinvigoration pre-treatments were applied to crown material of five chestnut clones and the micropropagation ability (in vitro establishment, multiplication and rooting) was compared to untreated controls. In addition, an in vitro rejuvenation method was assayed in the established cultures.

## MATERIALS AND METHODS

Crown cuttings of five mature chestnut trees, referred to as HV, 431, A2, A3 and P1 were collected during the rest period (December–January) and stored at 4 °C for 3 months, before being forced to flush or grafted. The HV and 431 trees, aged 30 and 15 years, respectively, are *Castanea sativa* Mill × *C crenata* Siebeld and Zucc hybrids resistant to *Phytophtora cambivora* and *P cinnamomi*. The other three clones were obtained from natural stands of healthy *C sativa* Mill trees aged 50 (A2), 40 (A3) and 80 (P1) years. A diagram of the material and different

treatments used for in vitro establishment of cultures is shown in figure 1.

## Spray treatment

In March the cuttings from all five trees, collected the previous December, were separated for each clone in two different sets, placed in water and forced to flush in a growth cabinet. During the flushing period, one set of cuttings of each clone was sprayed with a sterilized 222 µM solution of 6-benzylaminopurine (BA) three times a week, and the other set was used as a control. After 2 weeks, the new shoots that developed from both untreated and BA treated cuttings were collected, and used as the source of initial explants (C and C+S). These shoots were sterilized, subdivided into 5 mm shoot tips and nodes bearing one or two axillary buds and established in vitro.

## Pulse treatment

To stimulate the in vitro response of crown explants, a subset of the sterilized shoot tips and nodes derived from unsprayed cuttings of HV and 431 trees were placed for 2 h in Petri dish plates (eight explants per plate) containing a filter-sterilized solution of 111 µM of BA. After this pulse treatment, explants (C+P) were transferred to in vitro culture conditions.

## Juvenile grafting

In April, scions (3–4 cm long) bearing two to three buds were taken from stored January cuttings of HV, A3 and 431 trees and grafted onto 2-week-old seedlings, which were obtained by germinating seeds of the HV tree. Grafting was performed as per Vieitez and Vieitez (1981) by
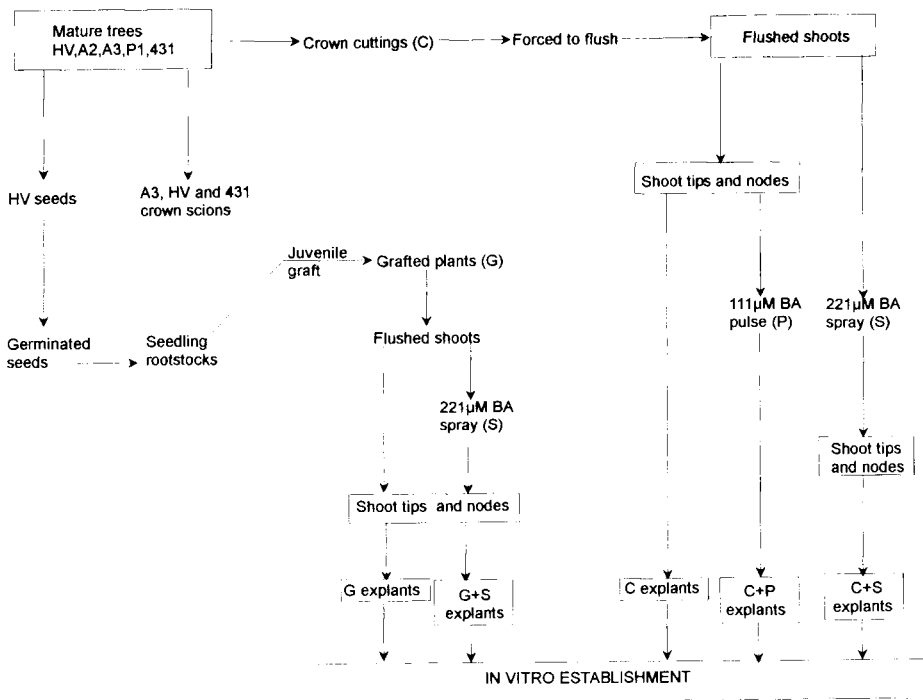


**Fig 1.** Diagram outlining the different treatments applied to the starting material for the in vitro establishment of the cultures used in this study.

removing the seedling epicotyls and inserting the scion into the split hypocotyl. The grafts were kept for 5 weeks in a growth chamber to force flushing of lateral buds. Three weeks later, some grafts were sprayed with 222 µM BA solution three times a week for 2 weeks. Newly grown shoots from unsprayed and sprayed grafts were used as the source of initial in vitro explants (G and G+S).

## In vitro culture

Micropropagation procedures were carried out as described elsewhere (Vieitez et al, 1983; Sánchez and Vieitez, 1991). Briefly, all shoots used for initial explants were stripped of leaves and surface-sterilized by successive immersion for 30 s in ethanol and 10 min in 12% commercial bleach (40 g·L$^{-1}$ of active chlorine), followed by three rinses in sterile distilled water. Sterilized explants from all sources were placed vertically in $20 \times 150$ mm test tubes containing 15 mL of establishment medium which consisted of Gresshoff and Doy's medium (1972) supplemented with 2.22 µM BA, 30 g·L$^{-1}$ sucrose and 6 g·L$^{-1}$ Difco agar. The pH of the medium was adjusted to 5.5–5.6 before autoclaving at 121 °C for 20 min. One day after implantation in vitro, the explants were moved to a different place within the same tube to reduce the negative effect of the blackening of the medium probably due to phenol oxidation and exudation. All explants were then transferred to a fresh medium every 2 weeks to overcome the negative effect of this exudation. After 6 weeks, the newly developed shoots, cut into 8 mm long shoot tips and nodal segments, were subcultured vertically on a fresh medium with 0.89 µM BA (multiplication medium) to start the shoot multiplication stage. Unless otherwise stated, subculturing was carried out every month except for HV explants, which were transferred, after 3 weeks on BA, for 2 more weeks on a medium containing 0.46 µM of zeatin, giving a 5 week multiplication cycle.

For rooting, the base of the shoots was dipped in 4.9 mM indole-3-butyric acid (IBA) solution for 2–3 min, and transferred to fresh medium without BA and with macronutrients reduced to one-third strength (rooting medium). Shoot multiplication and rooting experiments were carried out after subculturing for at least 1 year.

All cultures were grown under cool-white fluorescent lamps delivering 30 µmol m$^{-2}$ s$^{-1}$ during a 16 h photoperiod, with day/night temperatures of 24/20 °C.

## Recycling of the same horizontal explant

The influence of repeated culture of the same explant (recycling) placed horizontally on the media on multiplication rates and rooting ability of shoots obtained from multiplication cultures was investigated on clones HV and 431. Microshoots (20–25 mm long) were harvested from shoot proliferating cultures which were initiated from C+S explants for clone 431 or G+S explants in the case of clone HV. Shoots were decapitated and placed horizontally in glass jars (six per jar) containing 70 mL of multiplication medium. At the end of 4 (clone 431) or 5 weeks (clone HV) all the new axillary bud-derived shoots were harvested and used for multiplication or rooting experiments. Once the shoots were harvested (first cycle), the original explant was recycled on a fresh medium (second cycle). In successive recultures, the shoots were developed from axillary buds that failed to elongate in the original explant or from the axillary buds located in the stump of previously harvested shoots. Each original explant was recycled four times in the case of clone HV and up to eight times for clone 431. The reculture period of the clone 431 was reduced to 2 weeks in the third and successive recultures due to the fast shoot growth, while a 5 week period was maintained in clone HV. Rooting capacity was evaluated in shoots taken from successive reculture cycles.

## Data collection and statistical analysis

At the end of the establishment stage (initial cultures), the in vitro reactivity (defined as the percentage of surviving explants with shoot development), the number of shoots greater than 8 mm per responsive explant, and the length of the tallest shoot per explant were recorded. In shoot multiplication experiments, the shoot mean number, the tallest shoot as well as the number of 8 mm nodal and shoot tip segments produced per explant were evaluated. In rooting experiments, the percentage of rooted shoots, the number of

roots per rooted shoot and the longest root length for each rooted shoot were assessed.

In in vitro establishment experiments, the number of initial explants is shown in table I. For each clone and treatment, 18 replicates were used in shoot multiplication and rooting experiments, and the experiments were repeated four times. The multiplication data and the rooting percentages of recycling experiments were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test at $P < 0.05$ to compare means. Arcsine transformation was applied to rooting percentage data prior to analysis. Non-transformed data are present in the tables and figures. The significance of differences among reactivity percentages (table I) and among rooting percentages (table II) were analyzed by the test of independency (G-test) (Sokal and Rolf, 1981).

# RESULTS

In all five clones there were no differences in the sprouting capacity between BA sprayed and untreated crown cuttings forced to flush in the growth cabinet. Most buds just swelled and remained green for about 1 week, then became brown or necrotic; however, 10–35%, depending on the clone, broke, and grew to a maximum size of 1 cm, but failed to elongate further. BA treatment did not improve the bud breaking of crown cuttings forced to flush. In contrast, when grafting was carried out, vigorous shoots, 20–25 cm long, were obtained and used as the source of G and G+S explants (fig 2). The success of grafts performed with scions

**Table I.** Results obtained after 6 weeks establishment in vitro culture of primary explants derived from crown branches of five chestnut trees given different treatments.

| Clone | Treatment | Explants cultured | Infected (%) | Reactivity (%) | Shoots per reacting explant | Length of longest shoot (mm) |
|-------|-----------|-------------------|--------------|----------------|-----------------------------|-------------------------------|
| P1 | C | 11 | 9.1 | 10.0 [a] | 2.0 ± 0.0 | 11.0 ± 0.0 |
| | C+S | 9 | 11.1 | 25.0 [a] | 2.0 ± 0.7 | 12.0 ± 2.8 |
| A2 | C | 24 | 8.3 | 18.2 [a] | 1.8 ± 0.4 | 7.5 ± 0.3 |
| | C+S | 21 | 14.3 | 33.3 [a] | 1.8 ± 0.3 | 8.3 ± 0.3 |
| A3 | C | 25 | 8.0 | 21.7 [a] | 1.6 ± 0.4 | 8.6 ± 0.4 |
| | C+S | 21 | 14.3 | 38.9 [ab] | 1.6 ± 0.4 | 8.3 ± 0.4 |
| | G | 15 | 6.7 | 57.1 [bc] | 1.6 ± 0.2 | 8.8 ± 0.4 |
| | G+S | 16 | 12.5 | 85.7 [c] | 1.7 ± 0.1 | 9.9 ± 0.8 |
| HV | C | 18 | 5.6 | 0 [a] | – | – |
| | C+P | 20 | 10.0 | 0 [a] | – | – |
| | C+S | 21 | 14.3 | 33.3 [b] | 1.0 ± 0.0 | 8.3 ± 0.2 |
| | G | 16 | 6.3 | 33.3 [b] | 1.2 ± 0.1 | 10.2 ± 1.3 |
| | G+S | 60 | 10.0 | 94.4 [c] | 1.4 ± 0.1 | 12.7 ± 0.6 |
| 431 | C | 22 | 0.0 | 13.6 [a] | 1.3 ± 0.3 | 11.0 ± 0.7 |
| | C+P | 13 | 7.6 | 16.7 [ab] | 1.5 ± 0.4 | 10.0 ± 0.7 |
| | C+S | 17 | 11.8 | 26.7 [ab] | 1.5 ± 0.5 | 10.3 ± 1.7 |
| | G | 18 | 5.8 | 50.0 [bc] | 1.5 ± 0.2 | 10.5 ± 1.4 |
| | G+S | 18 | 11.1 | 75.0 [c] | 1.7 ± 0.2 | 13.2 ± 1.1 |

C: control; G: grafting; P: 111 μM BA pulse; S: 221 μM BA spray. Values are mean ± SE. [abc] Percentage values followed by the same letters for the same tree are not significantly different at 0.05 level (R × C test of independence using the G-test).

**Table II.** Effect of different reinvigoration treatments applied to starting material of five chestnut trees, on both the shoot multiplication stage (segment number) and the rooting stage (rooting %) of stabilized shoot cultures derived from the treated material. Shoot cultures derived from untreated crown branches were used as control (C).

| Treatment | Clone P1 | | Clone A2 | | Clone A3 | | Clone HV | | Clone 431 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Multiplication | Rooting | Multiplication | Rooting | Multiplication | Rooting | Multiplication | Rooting | Multiplication | Rooting |
| C | $2.5 \pm 0.1$ [a] | $8.2$ [a] | $1.8 \pm 0.1$ [a] | $0$ [a] | $1.7 \pm 0.1$ [a] | $8.9$ [a] | * | * | $2.8 \pm 0.1$ [a] | $16.9$ [a] |
| C+P | – | – | – | – | – | – | * | * | $2.9 \pm 0.1$ [ab] | $19.3$ [ab] |
| C+S | $2.7 \pm 0.1$ [a] | $13.3$ [a] | $2.2 \pm 0.1$ [b] | $18.9$ [b] | $2.1 \pm 0.1$ [b] | $22.8$ [b] | $2.6 \pm 0.1$ [a] | $2.7$ [ab] | $3.1 \pm 0.1$ [b] | $30.4$ [b] |
| G | – | – | – | – | $1.7 \pm 0.1$ [a] | $9.7$ [a] | $2.8 \pm 0.1$ [a] | $0$ [a] | $3.0 \pm 0.1$ [ab] | $21.6$ [ab] |
| G+S | – | – | – | – | $2.1 \pm 0.1$ [b] | $13.7$ [ab] | $3.2 \pm 0.1$ [b] | $9.2$ [b] | $3.1 \pm 0.1$ [b] | $31.3$ [b] |

G: grafting; P: 111 µM BA pulse; S: 221 µM BA spray; *: no available material (table I); –: treatment not performed. Values are mean±SE. [ab] Within each clone and column, values followed by the same letters are not significantly different at 0.05 level using least significant difference (LSD) test (segment number) or G-test (rooting %)

**Fig 2.** Shoot development from a HV crown scion (arrow) grafted onto a seedling hypocotyl of chestnut, 6 weeks after grafting.

of clones A3, 431 and HV was 30, 43 and 55%, respectively.

The effect of the different treatments applied to the starting material on in vitro establishment is shown in table I. Contamination rates did not exceed 15%, with the highest values for explants taken from material that had been sprayed with a BA solution (C+S and G+S). Within each clone, the percentage of surviving explants with shoot development (reactivity) depended on the treatment. The in vitro response of C explants was relatively low in all tested clones, with A3 being the most responsive clone (21.7%). Untreated HV explants were totally unreactive. The reactivity of C explants (clones HV and 431) was not improved by a BA pulse whereas for HV C+S explants it was significantly increased.

Moreover, the percentage of reactive explants was also increased (not significantly) in C+S explants for the other four clones. In grafted material (G explants), the reactivity was significantly greater than in the controls (C explants) and was further increased with a BA spray (G+S explants). For clone HV, G+S explants exhibited a reactivity significantly higher than those observed from grafted plants. Within each clone, the number of shoots per explant and the longest shoot length were not affected by any treatment.

In shoot multiplication stage, pretreatments of starting material had a greater effect on the number of segments than on the shoot number. Pretreatments had no effect on shoot numbers of A2 and A3 clones, whereas in clones P1 and HV the significant best values were found in C+S or G+S cultures, respectively (data not shown). Shoot multiplication rates, in terms of mean number of 8 mm nodal and shoot tip segments produced per explant is shown in table II. Generally, treatments including spray application (C+S and G+S explants) afforded, in a significant way, the best mean segment number, except in P1 and 431 cultures. The length of the tallest shoot was not affected by treatments, ranging from 10 to 20 mm depending on the genotype (data not shown). The rooting capacity of microshoots derived from different pretreatments was very low (table II). Significantly higher rooting values in comparison to controls were obtained when either C+S (A2, A3 and 431) or G+S (HV and 431) shoots were used, whereas no effects on root number and longest root length were observed.

Figure 3 shows the effects of explant orientation and recycling on shoot multiplication of clone 431. The average number of shoots produced in explants cultured horizontally once (H1) was significantly greater than in vertically placed explants (V1). Production of high numbers of shoots was maintained by recycling the same horizon-
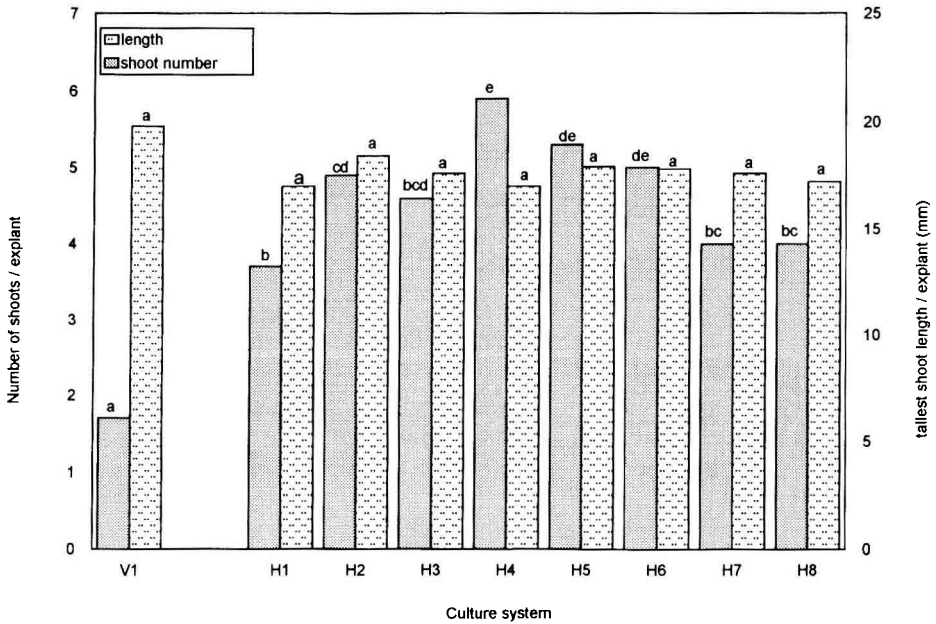
**Fig 3.** Effects of explant orientation and recycling the same horizontal explant on shoot production of the BA spray-treated 431 clone. V: vertical; H: horizontal. For each variable, bars with the same letters are not significantly different at 0.05 probability level using least significant difference (LSD) test.

tal explant. Within the horizontal reculture system, the number of shoots was significantly increased in most of the subsequent reculture cycles. The tallest shoot length was not affected by explant orientation or by reculture number. This horizontal reculture procedure was also applied to shoots of clone HV derived from G+S explants with similar results (figure 4).

The rooting performance of 431 and HV clone shoots harvested from vertical and horizontal culture systems was evaluated. Explant orientation during the first multiplication cycle of the explant did not affect the rooting capacity of the shoots produced in vertical (9 and 30% for clones HV and 431, respectively) and horizontal (9 and 26% for clones HV and 431, respectively) cultures. However, reculture of the horizon-

tally placed explants had a significantly positive effect ($P < 0.01$) on rooting percentage of shoots produced. In 431 material, the rooting frequency was increased nearly twofold from the first (26%) to the second reculture (51%), and then leveled off. The highest rooting percentage (62.8) as well as the greatest root number (2.6) were achieved in shoots harvested from the sixth reculture, being significantly higher than material derived from the first horizontal cycle. Average longest root length varied from 10 to 18.4 mm, but was not significantly affected by shoot orientation or reculture number. Similar trends were seen in clone HV, whose rooting capacity is very poor. In this clone, rooting frequency and longest root length were significantly increased from the first (9% and 9 mm, respectively) to the fourth

**Fig 4.** Shoot multiplication of HV cultures, after one (top) or three (bottom) reculture cycles of the same horizontally placed explant. Stock shoot cultures derived from material previously reinvigorated by grafting plus BA spray treatment.

horizontal reculture (28% and 18 mm, respectively). Mean root number ranged from 1.3 to 1.8, but no significant differences were found among different treatments.

## DISCUSSION

The data presented here show that suitable reinvigoration methods have been developed to successfully micropropagate mature chestnut trees from crown material. These results confirm that the in vitro response of explants taken from the crown of recalcitrant trees is very poor and, when juvenile material is not available, reinvigoration methods need to be applied in order to in vitro clone and maintain selected genotypes.

It should be emphasized that no rooted plantlets were obtained from untreated material in two of five tested clones.

In the present study, the BA spray treatment did improve the in vitro reactivity of crown-derived explants, especially those derived from the very recalcitrant HV tree, allowing them to be established in vitro. On the contrary, explants exposed to short duration pulses of BA were not reactivated. However, Read (1985) obtained microshoot proliferation in several species, either by spraying the donor plant with cytokinins, including the same cytokinin in the medium, or by soaking the explants in cytokinin prior to establishment of cultures. Pulse treatment has also been shown as an alternative treatment to the incorporation of the cytokinin into the medium for the induction of adventitious buds in conifers (Von Arnold et al, 1988; Martinez Pulido et al, 1992). These reports indicate that the concentration, exposure time and mode of application are important factors influencing the morphogenetic response of explants. Therefore, the observed different response of chestnut explants to the BA applications (spray versus pulse) may be due to the fact that the BA spray was repeated six times and to the different concentration used in pulse and spray treatments. The application frequency of sprays has been reported to have an important effect on the rejuvenation of *Picea abies,* even more than the concentration factor (Bouriquet et al, 1985). The application of BA in a water–ethanol solution to *Eucalyptus ficifolia* trees induced bud break from buds in the lignotuber as well as from latent buds in the upper trunk region (Mazalewsky and Hackett, 1979). BA spraying of the parent tree also improved the micropropagation of *Platanus × Acerifolia* (Donkers and Evers, 1985), *Pinus taeda* (Timmis, 1985) and *Pinus pinaster* (Dumas, 1987). It is significant that the initial BA-induced reinvigoration of chestnut explants was maintained through out the multiplication stage and affected the rooting capacity, which was

similar to that of G+S shoots. Reynord (1983) also reported that the foliar sprays of BA applied to *Pseudotsuga menziesii* were as effective as several successive grafts. If a gradual rejuvenation results from conditions that stimulate multiplication of juvenile cells (Greenwood, 1995), in this case the BA, a more juvenile status could then take place in the developed shoots. Therefore, cytokinins would act as a rejuvenating agent for mature trees (Franclet, 1981; Kelly, 1988).

Chestnut crown cultures from three mature trees were successfully established in vitro after a single grafting. Grafting has been reported previously as a suitable method for inducing bud break and subsequent in vitro establishment of crown material taken from one chestnut tree, but no further data about multiplication and rooting have been recorded (Ballester et al, 1990). Rejuvenation of *Sequoiadendron giganteum* has been reported by Monteuuis (1991) in one of 300 grafted apex after only one grafting and subsequent in vitro meristem culture. As in other woody species (Doorenbos, 1965; Struve and Lineberg, 1988), chestnut juvenile grafting causes a great increase in both vigor and growth of shoots, indicating an apparent partial rejuvenation. It should be emphasized that our grafting system fulfils the requirements for achieving a successful reinvigoration: the high vigor of the juvenile rootstock (Franclet, 1979), the proximity of scion to the seedling root (Chaperon, 1979; Paton, 1984) as well as the small size of the scion (Stoutmyer and Britt, 1961; Franclet, 1979).

A combination of both treatments, grafting and BA spray, proved to be more effective for the in vitro establishment of chestnut crown material than either BA spray or grafting alone, which agree with preliminary results in one clone of chestnut reported by Ballester et al (1990). This treatment was not successful for mature cultivars of walnut trees (McGranaham et al, 1987). The reac-

tivities of explants derived from grafted plus BA sprayed material used in the present work were similar to those of basal shoot explants of the same clones, whereas the shoot multiplication rates and rooting capacities were not improved to the same extent as reactivity (Sánchez and Vieitez, 1991). Therefore, the degree of reinvigoration depends on both the treatment being used and the characteristic being evaluated, supporting the quantitative aspect of the rejuvenation as was suggested by Hackett (1985). These results show that both treatments, grafting plus BA spray, and BA spray alone, induce a partial rejuvenation from a mature phase to a more physiologically juvenile state. Moreover, the ex vitro behavior and other characteristics of this material have not been evaluated. According to Fontanier and Jonkers (1976), a true ontogenetical rejuvenation occurs when an adult meristem produces plants completely juvenile.

Because of the different morphogenetic responses to in vivo treatments exhibited by the clones HV (more recalcitrant) and 431 (more responsive), microshoots of both clones were selected for testing the effect of recycling the same horizontal explant. The morphogenetic response of clone 431 and HV material obtained by spraying with BA and grafting plus BA spray treatments, respectively, was highly enhanced by the horizontal reculture system. It should be emphasized that this method provided not only the greatest proliferation rates, but also the best rooting frequencies, which were similar for clone 431 (62.8%) or even higher for clone HV (28.4%) than those reported for microshoots originating from the base of the same trees (51.4 and 19.4% for clones 431 and HV, respectively; Sánchez and Vieitez, 1991). Moreover, for clone 431, the length of the tallest shoot was similar in the different reculture cycles even though the reculture period was reduced to 2 weeks after the second cycle. Therefore, this method provides a more rapid and efficient

micropropagation system since more rootable shoots are obtained from each reculture cycle in a shorter period. A similar reduction of the reculture period and a high production of vigorous shoots has also been reported for oak, after the first reculture cycle (Vieitez et al, 1994). The horizontal position has been found to improve the micropropagation efficiency of juvenile and mature *Quercus robur* (San-José et al, 1988; Vieitez et al, 1994) and other woody species (Zimmerman and Fordham, 1989; McClelland and Smith, 1990). The possible mechanism of rejuvenation (repeated drastic pruning) during this reculture system applied to the micropropagation of *Q. rubra* and *Q. robur* has been discussed by Vieitez *et al* (1993, 1994).

By combining in vivo and in vitro rejuvenation treatments, microshoots of chestnut derived from the crown exhibit a similar behavior to those of basal origin, indicating a high level of rejuvenation for at least two parameters, multiplication and rooting capacity. Based on the concept of Greenwood (1995), we assume that a gradual rejuvenation of chestnut has been achieved through out the different treatments instead of an abrupt reversion to the juvenile state. Recent reports show that differences in gene expression exist between juvenile and mature material (Hutchison et al, 1990; Murray et al, 1994; Sánchez et al, 1995). Further investigations at the molecular genetic level must be carried out to better understand the rejuvenation process.

## ACKNOWLEDGMENTS

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