# Micropropagation as a Nursery Technique for Chestnut Hybrid Clones

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ABSTRACT. Hybrid chestnuts selected for their resistance to ink disease are mainly propagated by layering. However, this method has several disadvantages that lead to poor efficiency. The objective of the present research was to develop an easy micropropagation technique for application in commercial nurseries for several chestnut hybrid clones resistant to ink disease *(Phytophthora* spp.) and selected for wood and nut production. These clones would be mass propagated for commercial purposes and for use as stock plants for conventional propagation by cuttings or stooling.

Hybrid chestnuts selected for their resistance to ink disease are mainly propagated by layering. However, this method has several disadvantages that lead to poor efficiency, being characterized by: 1) an unproductive period of at least five years for stump formation; 2) low level production per ha; 3) high cost due to the numerous man-hours required; and, 4) regular plant quality. Micropropagation could solve the problem of production, especially since demand has been increased considerably in recent years.

The objective of the present research was to develop an easy micropropagation technique for application in commercial nurseries for several chestnut hybrid clones resistant to ink disease *(Phytophthora* spp.) and selected for wood and nut production. These clones would be mass propagated for commercial purposes and used as stock plants for conventional propagation by cuttings or stooling.

## MATERIALS AND METHODS

The material examined consisted of a collection of several interspecific chestnut hybrid clones from forest and nut selections (11, 13, J. Fernandez, unpublished data).

**Initiation and micropropagation.** Shoot cultures used **in** this study had been established 8 mo earlier from young field-grown stump shoots. In the multiplication of plants by tissue culture, several stages have been distinguished (0, I, II, Ma and Bib) (2). Culture techniques and suitable environmental conditions for stages I, II and Ma were based on the experience provided by studies of Vieitez et al. (9). For initiation of cultures, axillary buds and shoot apices were disinfected and established. For multiplication Heller (4) medium + 1 mM SO4(NH4) was used. Immediately prior to rooting, shoots were subcultured to Murashige and Skoog (MS) medium (7) with half-strength nitrates. Shoots prepared prepared by a single cycle in MS (half-strength nitrates) appeared to possess a nutritional balance that was optimal for root differentiation (10). In order to save costs in plant tissue propagation, we maintained cultures as clusters of shoots in the elongation stage. For multiplication and elongation of shoots, 0.2 mg/I of BAP was added to the culture medium. Cultures were maintained in a growth chamber at 25 C with a 16-h photoperiod.

The quality of the *in vitro* produced shoots has been tore important for rooting success than the treatments iven during the rooting stage *in vivo* (5). For most plants, longation can be obtained by transferring cultured hoots from the propagation medium (stage II) to an ppropriate elongation medium (stage Ina) (6). Based on he experience provided by Maene and Debergh (6) and C.auvin and Salesses (1), we studied the possibility of saving manual labor by inducing elongation. This was accomplished by adding liquid culture media to established, exhausted cultures, instead of transplanting the tissues to fresh medium. For this study, we focused our attention on both the characteristics of elongated shoots and root development. We used the same basal elongation medium in the solid and in the liquid phase (MS, half-strength nitrates).

**Rooting of shoots.** In a commercial micropropagation system, rooting should preferably happen *in vivo* (2, 3), due to economical and phytopathological reasons (5). Rooting of shoots was achieved in either a hormone-free medium or a sterilized substrate. The lower leaves were removed from the microcuttings and the bases of elongated, excised shoots were dipped into a solution of IBA (1 g/l) and placed in culture Murashige and Skoog (7) medium with half-strength macronutrients (9) or into a moist, sterilized substrate (the percentage water in the substrate was 66.6%) in polystyrene trays. Microcuttings were immediately watered and covered with a transparent plastic sheet to maintain moisture and kept for 4-8 wk in growth chamber under the same conditions as for the earlier phases. Rooting substrate consisted of a mix of perlite and composted pine bark (Dermont) (2:1). Use of this substrate was based on our previous experience.

**Greenhouse and field procedures.** After 8 wk of hardening-off with progressively reduced humidity under a fog system, plants were transferred to a greenhouse and grown under a mist system. After the first week, the plants were sprayed weekly with a fungicide (Benlate 2 g/l) to reduce disease problems. In spring 1990 and 1991, plantlets were established in a field for bare root cultivation to study and compare the survival and growth of plants produced by different propagation techniques (micropropagation and stooling) from the same genetic material.

**Data analysis.** All experiments were repeated at least twice with 30 replicates per treatment. After 4 wk, the multiplication rates of different clones were recorded and the quality of the elongated shoots and the rooting ability from the two elongation systems were compared. The rooting quality of both rooting systems *(in vitro* and direct rooting) was assessed. Data were processed by analysis of variance with SAS (8) using the GLM method. Comparison of means was made using a Student-Newman-Keuls (SNK) test with a 0.05 level of significance.

## RESULTS AND DISCUSSION

**Multiplication of roots.** There were important differences with regard to the multiplication rates of the different interspecific clones (Table **1).** These behavioral differences were observed both in the *in vitro* culture stages and in the subsequent *ex vitro* development (greenhouse and nursery culture). These differences were probably greatly influenced by the genotype, and they also may have appeared in other published reports on chestnut (1, 9). The clonal effect on the number of shoots per culture was significant at P=0.05. Also, each clone behaved differently, depending on the number of replications with respect to multiplication and rooting rates (data not shown).

**Table 1.** Multiplication rates of different interspecific clones.

| | Multiplication Rates | |
|---|---|---|
| Clone | Number of Shoots/Culture | Length of Tallest Shoots (cm) |
| HS | 4.81 a | 2.09 bcde |
| 90.043 | 3.22 b | 1.26 fg |
| 90.025 | 3.17 bc | 1.80 defg |
| 156 | 2.92 bc | 2.35 bcd |
| 90.015 | 2.85 bcd | 1.82 defg |
| 111 | 2.67 bcde | 2.77 ab |
| 16 | 2.63 bcde | 1.69 defg |
| CA-118 | 2.57 bcdef | 1.98 cdef |
| 100 | 2.48 cdef | 2.82 ab |
| CA-15 | 2.38 cdefg | 1.44 efg |
| 2 | 2.36 cdefg | 3.22 a |
| 3237 | 2.32 cdefg | 2.73 ab |
| 19 | 2.27 cdefg | 1.43 efg |
| 2003 | 2.25 cdefg | 2.60 abc |
| 10 | 2.20 cdefg | 1.64 defg |
| 2034 | 2.17 defg | 1.70 defg |
| RH-13 | 2.09 efg | 2.31 bcd |
| X | 2.04 efg | 2.08 bcde |
| MARA | 1.85 fg | 1.08 g |
| 130 | 1.75 g | 1.55 defg |
| 2671 | 1.67 g | 1.42 efg |

**Elongation of roots.** Despite the good results obtained by Maene and Debergh (6) and Chauvin and Salesses (1), we do not advise the addition of liquid culture medium to established, exhausted cultures, as opposed to transferring the clusters of shoots to fresh medium. With addition of liquid medium, the elongated shoots produced in liquid medium were thinner, had smaller leaves and their green color was much lighter. More importantly, the shoots thus elongated gave poorer rooting results than the ones elongated on solid medium. Therefore, the average number of roots per rooted shoot was lower and the roots were thinner and less branched.

**Rooting of shoots.** Roots that developed in agar media were not effective in the soil environment. They were weaker and less branched than roots produced *in vivo.* Plants directly rooted in substrate had a higher number of roots per rooted shoot and this system led to improved acclimatization of plantlets, as it enabled acclimatization and rooting to occur simultaneously. Similar results were obtained by Webster and Jones (12).

**Greenhouse and field procedures.** Excellent acclimatization was obtained in a humidity chamber by progressive reduction of relative humidity, accomplished first with a fog system and later on with an intermittent mist system that misted for one second every 30 min. Although initial growth of the plantlets in the greenhouse was very slow, after about 4 wk, vigorous new growth appeared along with considerable increase in the size of the newly developed leaves. At least 70% survival after acclimatization was recorded.

The increased growth in the nursery of plants from micropropagation and from stooling was evidenced by the total dry weight of plantlets cultured for 1-2 yr in the nursery (Figures 1 and 2). Plants from micropropagation had better growth and were well balanced with an improved ratio between the root and aerial portions than plants from stooling. During the second growing season, height and weight increments were very high.

After 1 yr, nursery survival was above 90% and their size was large enough for forestry use. More than 3,000 micropropagated plantlets have been obtained. In general, the differences between *in vitro* propagated and traditionally propagated plants are in favor of the *in vitro*-derived plants.

## ACKNOWLEDGMENTS

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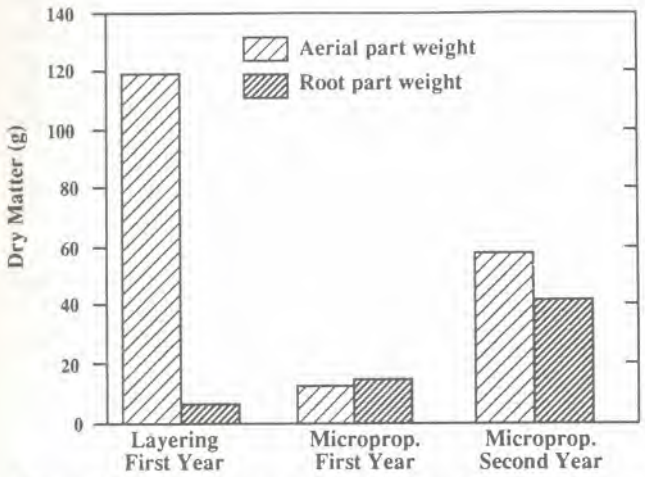


**Figure 1.** Comparison of dry weights between aerial and root parts of chestnut plants (gm) from two vegetative propagation systems, stooling and micropropagation.

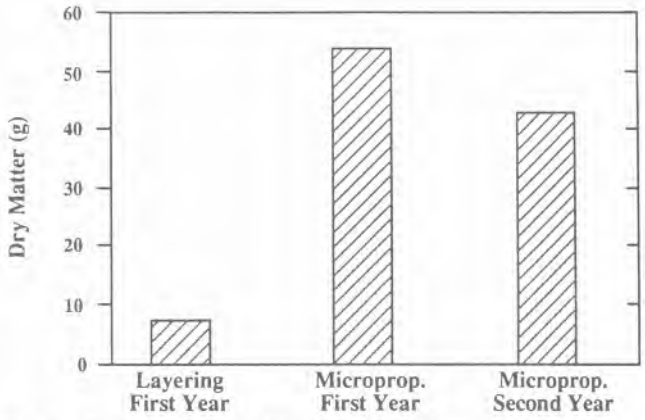


**Figure 2.** Percentage of total plant dry weight accounted for by roots with two vegetative propagation systems, stooling and micropropagation.