SCIENTIFIC NOTE

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# COMPARISON OF ROOT INDUCTION IN MATURE FILBERT (*Corylus avellana* L.) EXPLANTS BY *Agrobacterium rhizogenes* AND INDOLBUTIRIC ACID

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## ABSTRACT

From *in vitro* cultured adult material of *Corylus avellana* L. cv. Negretta, adventitious rooting of microshoots was evaluated. Rhizogenic induction mediated by two strains of wild-type *Agrobacterium rhizogenes* (A477 and A478) and indolbutyric acid (IBA) were compared under two light conditions (16:8 h photoperiod and complete darkness). The results indicate that in the 16:8 h photoperiod induction, the rooting rate with IBA (90%) was significantly higher than that obtained with the strain A477 of *A. rhizogenes* (67.7%), while with the strain A478 no statistically significant difference was obtained for the same variable (75%). On the other hand, under complete darkness, rooting mediated by IBA (90%) significantly surpassed the results obtained with both strains of *A. rhizogenes* (40 and 20%, for A478 and A477, respectively). In terms of the morphological variables of the resulting root system, induction mediated by IBA, with a 16:8 h photoperiod, generates a significantly higher number of roots (19 roots per microshoot) than that obtained with *A. rhizogenes* (mean 3.7 roots per microshoot), producing significant differences when comparing the results with the strain A478 (5 roots per microshoot) to those of the strain A477 (2.4 roots per microshoot). Induction under complete darkness does not have any effect on root number, independent of the rhizogenic inductor employed. Root length did not present significant differences among treatments, except in the presence of *A. rhizogenes* A477 and darkness.

**Key words**: rooting, *Agrobacterium rhizogenes*, European hazelnut.

## INTRODUCTION

Studies by Sánchez-Olate *et al.* (2004a) on the micropropagation of *Corylus avellana,* report a good response to the stimulation of cuttings for the development of vegetative buds and the feasibility of using cultured epicormic shoots and *in vitro* multiplication. Nevertheless, general standards cannot be established that would allow for ensuring the success of subsequent rooting and acclimatation of the seedlings (Preece *et al.,* 1989), given that the response varies according to the genotype, ontogeny, the position of the material used and mainly the cultivation and *in vitro* manipulation conditions (Leslie and McGranahan, 1992; Kozai *et al*., 2005; Hazarika, 2006). This obliges the study of techniques that can allow for improving the characteristics of the plant, with emphasis on the radicular system for its subsequent transfer to the field (Gonçalves *et al.*, 1998).

*Agrobacterium rhizogenes* is a common soil organism in capable of infecting a plant through a wound and the insertion and expression of codifying genes for indolacetic acid (AIA) from its t-DNA, causing an abundant proliferation of secondary roots (Ercan *et al*., 1999). This natural or induced phenomenon represents a possible alternative for the *in vitro* production of plants. The underlying mechanism of the formation of adventitious roots is the transfer of various bacterial genes to the plant's genome, specifically the t-ADN portion of the Ri plasmid ("Root Inducing") (McAfee *et al.*, 1993; Ercan *et al.*, 1999; Li and Leung, 2003). The symptoms observed with *A. rhizogenes* are the increased sensitivity of cells to the effect of auxins, as well as the production of these (Ercan *et al*., 1999). Thus, using this technique, Caboni *et al*. (1996) reported rooting rates of between 52 and 68% in walnut microshoots (*Juglans regia* L. cv. Sorrento), which were then transferred successfully to *ex vitro* conditions. Studies by Caro *et al.* (2000) with *Prosopis chilensis* (Molina) Stuntz reported that the roots obtained in these cultures are characterized by a high degree of genetic stability, which allows for the generation of complete and viable plants.

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The application of *A. rhizogenes* has been described as a feasible means to increase the rhizogenic response in many forest species, among them those belonging to genera *Pinus*, *Larix* and *Eucalyptus* (Caro *et al*., 2000). Given that genetic improvement programs are slow and that the induction of specific genes through crossbreeding has proven difficult, the use of *A. rhizogenes* can be a successful alternative as a rapid and direct route for the introduction and expression of specific genes for rooting (Huang *et al.,* 1991).

On the bases of this information, the objective of this study was to evaluate the rhizogenic response induced by *A. rhizogenes* and indolbutyric acid (IBA) under two light conditions in microshoots of *Corylus avellana* cv. Negretta of adult origin. This type of material is recalcitrant to adventitious rooting by conventional methods, because of which new rooting methodologies could facilitate the production of plants with genotypes of interest, tested in the field before producing it commercially.

## MATERIALS AND METHODS

### Vegetal material

Microshoots of proliferative chains were used, originating from vegetative buds of *C. avellana* cv. Negretta from cuttings and epicormic shoots, according to the methodology described by Sánchez-Olate *et al.* (2004a). The culture medium used for rhizogenic induction was MS2 (modified Murashige and Skoog medium) (Sánchez-Olate *et al*., 1997), supplemented with 2.5 mg $L^{-1}$ benzyl amino purine (BAP), 0.01 mg $L^{-1}$ of 3-indole acetic acid (AIA), 0.1 mg $L^{-1}$ of gibberellic acid ($GA_3$) and 30 g $L^{-1}$ of sucrose. The pH was adjusted to 5.8 and 7 g $L^{-1}$ of agar-agar was added. Once the mixture was obtained, it was sterilized in an autoclave at 120 °C and 0.101 MPa 20 min.

### Bacterial culture

The bacterial strains used were the wild strains A477 and A478 of *A. rhizogenes*, from the collection at the Universidad de Valencia, Spain (Universidad de Valencia, 1990). Bacterial growth was reactivated by suspending an aliquot of the bacterial solution in 2 mL of YMB liquid medium (yeast medium bacterial) for its multiplication (Hooykaas *et al*., 1977). This suspension was agitated for 48 h at 300 rpm and a temperature of 27 ºC. Subsequently aliquots of 100 µL were gathered and re-suspended in 2 mL of YBM liquid medium in tubes with 10 mL of volume. Finally, to develop bacterial colonies, aliquots of 100 µL were cultivated in Petri dishes with 10 mL of YMB medium solidified with 8 g $L^{-1}$ agar Difco®, at 28 ºC for 24 h maintained in an inverted position to avoid evaporation.

### Inoculation of microshoots

Once the colonies were developed, the bacteria were seeded in 20 mL of YMPB liquid medium using a microbiological loop. The solution was then placed in sterile Petri dishes to proceed with the inoculation of microshoots with a length of 2.5 cm from the better treatments obtained in the preliminary tests (Sánchez-Olate *et al*., 2004a). The basal inoculation was made by submerging these microshoots in bacterial (A477 and A478) and auxinic (IBA 3 mg $L^{-1}$) solutions for 3 min, after eliminating the axillary buds from the base of the microshoots and making lengthwise cuts of 0.5 cm to increase the area exposed to infection. Immediately after inoculation and before placing the microshoots in the culture medium, they were dried with sterile filter paper.

The microshoots were subsequently cultivated in medium without growth regulators (MS) for 3 days. During this period two lighting conditions were applied (photoperiod of 16:8 h and complete darkness), with temperatures of 25 ± 1 ºC during the day and 22 ± 1 ºC during the night and a relative humidity of 60% in the growth chamber. In the photoperiod of 16:8 h, the light intensity of the chamber was 40 µmol $m^2$ $s^{-1}$. The microshoots were then placed in a MS medium with macronutrients reduced to 25% of their original concentration, mixed with vermiculite sterilized in an autoclave (200/250 v/v) and solidified with 2.5 g $L^{-1}$ of Phytagel (Sigma). Some 300 µg $mL^{-1}$ of the antibiotic Cefotaxima (Claforan®) was added to this mixture to control bacterial development. The evaluation of the rhizogenic response was made at the end of the 30-day induction period. At the end of this period the percentage of rooting and number and length of roots, were evaluated.

### Statistical analysis

Six treatments were evaluated: T1: complete darkness plus A478; T2: complete darkness plus A477; T3: complete darkness plus 3 mg $L^{-1}$ de IBA; T4: photoperiod of 16:8 h plus A478; T5: photoperiod of 16:8 h plus A477 and T6: photoperiod of 16:8 h plus 3 mg $L^{-1}$ of IBA. The experimental unit was a glass container with four explants with homogeneous macro-morphological characteristics, with four replications per experimental unit. A completely random design was used with a factorial arrangement (2 x 3), making a descriptive univariate analysis (ANOVA) with a probability of 95%. Significant differences were identified using the Tuckey multiple comparisons test (P = 0.05) with Kramer correction (Steel and Torrie, 1985).

## RESULTS AND DISCUSSION

The highest percentages of rooting were the treatments that used auxinic induction (90%), with no significant differences between treatments T3 and T6, independent of the light conditions used (Table 1). Neither were there

significant differences between these treatments and the treatment T4, strain A477 with a photoperiod of 16:8 h, but with a lower percentage of rooting (75%) compared to auxinic induction under equal light conditions (Table 1). However, the greatest number of roots was observed in treatments that used auxinic induction with a photoperiod of 16:8 h, with an average of 19 roots per microshoot. Compared to the percentage obtained in T6 (90%) there were significant differences between the treatments. The root lengths reached in the treatments with auxinic induction (22.2 and 38.4 for T3 and T6, respectively) did not present significant difference from the induction of A477 in complete darkness, which presented more elongation of the microshoots with 75.5 mm (Figure 1).

The results obtained could be influenced by the genotype of the bacterial strain, the species to be induced and the culture conditions. Thus, the differences found among the strains used were dependent on the type of strain, which according to Ercan *et al.* (1999) is an important factor in the induction of adventitious roots. These results are in agreement with what was reported by Dobigny *et al*. (1995), who working with two potato cultivars (*Solanum tuberosum* L.) and four distinct strains of *A. rhizogenes,* found that two of them (2659 and 2659 GUS) strongly induced the formation of adventitious roots, while strains 15834 and 8196 GUS provoked low to nil response in rhizogenic induction.

Another important factor is the culture medium, which significantly influences the formation of adventitious roots (Giri and Narasu, 2000). Mediums with a high saline concentration, like MS, induce the formation of roots that benefit from the application of growth regulators such as auxins, the percentage of rooting under these conditions being significantly higher, independent of the light conditions used in the induction stage. This effect of the MS medium has been observed in *Nothofagus alpina* (Sánchez-Olate *et al*., 2004b), where the stressing effect of the saline medium results in spontaneous rhizogenic expression when the microshoots are changed to a reduced macronutrient medium.

**Table 1. Rooting evaluation in microshoots of *Corylus avellana* in the different applied treatments, including 16 microshoots per treatment.**

| Treatment | Rooting | Number of roots per explant | Root length |
|---|---|---|---|
| | % | | mm |
| T1 | 40.0a | 4.3a | 20.0a |
| T2 | 20.8b | 4.7a | 75.5b |
| T3 | 90.0c | 4.8a | 22.2a |
| T4 | 75.0cd | 5.0a | 17.2a |
| T5 | 67.7d | 2.4b | 19.0a |
| T6 | 90.0c | 19.0c | 38.4a |

Different letters in the column indicate significant differences according to the Tukey test (P = 0.05).
T1: complete darkness plus strain A478 of *Agrobacterium rhizogenes*; T2: complete darkness plus strain A477; T3: complete darkness plus 3 mg $L^{-1}$ IBA; T4: photoperiod of 16:8 h plus strain A478; T5: photoperiod of 16:8 h plus strain A477; and T6: photoperiod of 16:8 h plus 3 mg $L^{-1}$ IBA.

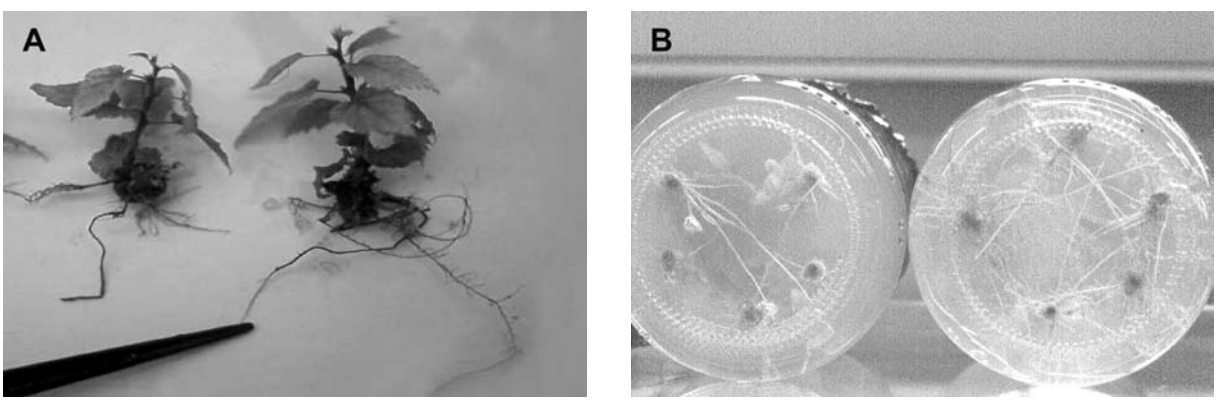


**Figure 1. Rooting response of *Corylus avellana* microshoots. (a) Root length; (b) *in vitro* rooting, after 30 d culture in treatment T6 (photoperiod 16:8 h and 3 mg $L^{-1}$ indolbutyric acid).**

The results obtained at this stage of the research do not allow for viewing the inoculation of microshoots of *C. avellana* with the studied bacterial strains and culturing conditions as an alternative for improving rhizogenic induction. However, given the experiences reported by Banerjee *et al*. (1998) and Li and Leung (2003), where inoculating vegetal material with *A. rhizogenes* and culturing it in a IBA supplemented medium resulted in an increase in the percentage of rooting and the number of roots, it is recommendable to research the combined use of those inductive agents in subsequent tests. In this sense, Ercan *et al*. (1999) reported that the symptoms observed with *A. rhizogenes* are the result of an increase in the sensitivity of the cells to the effect of auxins rather an increase in auxin production. This is in accord with what was reported by Caro *et al*. (2003), who, working with *P. chilensis,* reported high percentages of rooting in the treatments that used induction mediated jointly by *A. rhizogenes* and auxins. Despite the high rates of rooting reported by these authors, significant differences were not found between the treatments that used induction mediated separately by bacteria or auxin. In the case of *C. avellana*, the differences are highly significant, because of which the use of *A. rhizogenes* would presumably be advantageous only if the differences in its favor result in higher survival and growth rates once plants are transferred to the field.

## CONCLUSIONS

The highest percentage of rooting was obtained in treatments with auxinic induction, independent of the light conditions used in the induction stage. In general there were no significant differences among the treatments in the number of roots. Root lengths did not present significant differences among the treatments, except in darkness with strain A477 where shorter roots were observed than in the other treatments.


## RESUMEN

**Comparación de inducción rizogénica en explantes adultos de avellano europeo (*Corylus avellana* L.) por *Agrobacterium rhizogenes* y ácido indolbutírico.** A partir de material adulto de *Corylus avellana* L. cv. Negretta cultivado *in vitro*, se evaluó el enraizamiento adventicio de microtallos, comparándose la inducción rizogénica mediada por dos cepas silvestres de *Agrobacterium rhizogenes* (A477 y A478) y ácido indolbutírico (IBA), bajo dos condiciones de luminosidad (fotoperíodo de 16:8 h y oscuridad completa). Los resultados muestran que en la inducción bajo fotoperíodo de 16:8 h, la tasa de enraizamiento con IBA (90%) fue significativamente mayor a la obtenida con la strain A477 de *A. rhizogenes* (67,7%), mientras que con la cepa A478 no hubo significancia para la misma variable (75%). Por otra parte, bajo oscuridad completa el enraizamiento mediado por IBA (90%) superó significativamente los resultados obtenidos con ambas cepas de *A. rhizogenes* (40 y 20%, para A478 y A477, respectivamente). En cuanto a las variables morfológicas del sistema radicular resultante, la inducción por IBA bajo fotoperíodo de 16:8 h, generó un número de raíces significativamente mayor (19 raíces por microtallo) que las obtenidas con *A. rhizogenes* (3,7 raíces promedio por microtallo), produciéndose diferencias significativas al comparar los resultados de la cepa A478 (5 raíces por microtallo) con aquellos de la cepa A477 (2,4 raíces por microtallo). La inducción bajo oscuridad completa no produjo efectos en el número de raíces, independiente del inductor rizogénico utilizado. Por otro lado, la longitud de raíces no presentó diferencias significativas entre tratamientos, excepto en presencia de *A. rhizogenes* A477 y oscuridad.


**Palabras clave***:* enraizamiento, *Agrobacterium rhizogenes*, avellano europeo.

## LITERATURE CITED


Banerjee, S., L. Rahman, G.C. Uniyal, and P.S. Ahuja. 1998. Enhanced production of valepotriate by *Agrobacterium rhizogenes* induced hairy roots cultures of *Valeriana wallichii* DC. Plant Sci. 131:203-208.

Caboni, E., P. Lauri, N. Tonelli., G. Falasca, and C. Damiano. 1996. Root induction by *Agrobacterium rhizogenes* in walnut. Plant Sci. 118:203-208.

Caro, L., P. Marinangeli, N.R. Curvetto, y C. Hernández. 2000. *Agrobacterium rhizogenes* vs. inducción auxínica para la rizogénesis *in vitro* de *Prosopis chilensis* (Mol.) Stuntz. Multequina (Mendoza) 9:47-53.

Caro, L., P. Marinangeli, N.R. Curvetto, C. Hernández, and N. Santecchia. 2003. *Agrobacterium rhizogenes* v/s auxinic induction for *in vitro* rhizogenesis of *Prosopis chilensis* and *Nothofagus alpina*. Biocell 27(3):311-318.

Dobigny, A., A. Ambrose, R. Haicour, C. David, L. Rossignol, and D. Sihacharkr. 1995. Transformation of potato using mannopine and cucumpine strains *Agrobacterium rhizogenes*. Plant Cell Tiss. Organ Cult. 40:225-230.

Ercan, G., S. Yuce, K. Turgut, and M. Taskin. 1999. *Agrobacterium rhizogenes*-mediated hairy root formation in some *Rubia tinctorum* L. populations grown in Turkey. Turk. J. Bot. 23:373-377.

Giri, A., and M.L. Narasu. 2000. Transgenic hairy roots: recent trends and applications. Biotechnol. Adv. 18:1-22.

Gonçalves, J.C., G. Diogo, and S. Amancio. 1998. *In vitro* propagation of chestnut (*Castanea sativa* x *C. crenata*): effects of rooting treatments on plant survival and anatomical changes during adventitious root formation. Sci. Hortic. (Canterbury, Engl.) 72:265-275.

Hazarika, B. 2006. Morpho – physiological disorders in *in vitro* culture of plants. Sci. Hortic. (Canterbury, Engl.) 108:105-120.

Hooykaas, P.J.J., P.M. Klapwjik, M.P. Nuti, R.A. Schilperoort, and A. Rorsch. 1977. Transfer of the *A. tumefaciens* ti plasmid to avirulent *Agrobacteria* and *Rhizobium* ex planta. J. Gen. Microbiol. 98:477-484.

Huang, Y., A. Diner, and F. Karnosky. 1991. *Agrobacterium rhizogenes* mediated genetic transformation of a conifer: *Larix deciduas. In vitro* Cell Dev. Biol. Plant 27:201-207.

Kozai, T., F. Afreen, and S. Zobayed. 2005. Photoautotrophic (sugar-free medium) micropropagation as a new micropropagation and transplant production system. 316 p. Springer, Dordrecht, The Netherlands.

Leslie, C., and G. McGranahan. 1992. Micropropagation of Persian Walnut (*Juglans regia* L.). p. 137-150. *In* Bajaj, Y.P.S. (ed.) Biotechnology in agriculture and forestry. High Technology and Micropropagation II. Springer-Verlag, Heidelberg, Germany.

Li, M., and D. Leung. 2003. Root induction in radiate pine using *Agrobacterium rhizogenes.* Electron. J. Biotechnol. 6(3):254-270.

McAfee, B., E. White, L. Pelcher, and M. Lapp. 1993. Root induction in pine (*Pinus*) and larch (*Larix*) ssp. using *Agrobacterium rhizogenes*. Plant Cell Tiss. Organ Cult. 34:53-62.

Preece, J.E., J.W. Van Sambeek, C.A. Huetteman, and G.R. Gaffney. 1989. *In vitro* studies with walnut (*Juglans*) species. p. 159-180. *In* Phelps, J.E. (ed.) The continuing quest for quality. Proceed. 4th Black Walnut Symp., Carbondale, Illinois, USA.

Sánchez-Olate, M., D. Ríos, M. Gea, R. Rodríguez, and M. Revilla. 1997. Parameters affecting the *in vitro* growth and rooting of *Juglans regia* L. Acta Hort. (ISHS) 442:235-240.

Sánchez-Olate, M., D. Ríos, R. Rodríguez, M.E. Materán, y G. Pereira. 2004a. Duración del efecto revigorizante de podas severas de plantas adultas de avellano europeo (*Corylus avellana* L.) cv. Negretta sobre el cultivo *in vitro*. Agric. Téc. (Chile) 4:338-346.

Sánchez-Olate, M., D. Ríos, M. Pedraza, G. Pereira, H. Castellanos, y R. Escobar. 2004b. Propagación *in vitro* de *Nothofagus procera* ((Poepp. & Endl.) Oerst.) a partir de embriones aislados. Bosque 25(1):123-28.

Steel, R.G.D., y J.H. Torrie. 1985. Bioestadística: Principios y procedimientos. 2ª ed. 622 p. McGraw-Hill, Bogotá, Colombia.

Universidad de Valencia. 1990. Spanish type culture collection. 3ª ed. 316 p. Universidad de Valencia, Facultad de Ciencias Biológicas, Valencia, España.