SCIENTIFIC NOTE

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RHIZOGENIC INDUCTION IN ADULT *Juglans regia* L. cv. SERR TISSUE INDUCED BY INDOLE BUTYRIC ACID AND *Agrobacterium rhizogenes*

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ABSTRACT

The *in vitro* introduction of adult walnut (*Juglans regia* L.) tissue represents an opportunity to clone elite genotypes whose selection occurs in advanced ontogenic states. With the purpose of developing a protocol to allow mass propagation of valuable genotypes from adult material, a comparison was made between two root induction systems of walnut microshoots of the fourth subculture of adult walnut tissue of an *in vitro* introduction program previously reinvigorated through traditional grafting. Rhizogenic induction by indole-3-butyric acid (IBA) and *Agrobacterium rhizogenes* was used. The rhizogenic process was analyzed in two phases for both auxinic (T1: 3 mg L^{-1} IBA; T2: 5 mg L^{-1} IBA) and *A. rhizogenes* inductions (T3: A-477; T4: A-478). The first phase of root induction was during 3 days in the dark while the second phase, root manifestation, was 27 days. Rooting percentage was evaluated and the induced root systems characterized (number, length, diameter, and root insertion zone) in all the procedures. The best rooting results were obtained in T2, although the response obtained with *A. rhizogenes* didn't differ from the T1 response. This appears to be an increasingly interesting methodology for adventitious rhizogenesis in this species.

Key words: rooting, microshoots, adult material, *Agrobacterium rhizogenes*.

INTRODUCTION

The application of walnut regeneration methods by means of *in vitro* culture of embryos has allowed overcoming the barriers for the large scale production of crops, such as low percentage of seed germination and long propagation cycles. In the first case, between two and three months of stratification are required, whereas the propagation cycles are related to obtaining appropriate size patterns for grafting and the development of commercial specimens in a period of two to three years. With respect to the formation of microplants by means of *in vitro* culture, plants with intact roots, shoots, and leaves have been obtained in distinct culture mediums (Leslie and McGranahan, 1992; Driver y Kuniyuki, 1994; Sánchez-Olate *et al.,* 1997; Fernández *et al.,* 2000), finding that roots were more robust and developed than leaves (Kaur *et al.*, 2006). However, there is no record of the utilization of this technique in adult *J. regia* material which is of vital importance for the development of a massive propagation program, taking into consideration that it is a recalcitrant species (Preece *et al.*, 1989; Leslie and McGranahan, 1992; Caboni *et al.*, 1996; Rodríguez *et al.*, 2005) with a reduced morphogenetic capacity in the adult phase due to a complex metabolic and tissular system (Sánchez-Olate *et al.*, 2002). These characteristics make it necessary to search for reinvigoration techniques appropriate to this species, such as the severe pruning applied to *Corylus avellana* L. (Sánchez-Olate *et al.,* 2004) and *Pinus radiata* D. Don (Materán *et al.*, 2008).

In vitro reinvigoration techniques have been applied which allow the establishment of a propagation system on a larger scale from adult material selected for productivity. However, the presence of various phenol endogenous compounds, including allelopathic naphthoquinone called juglone, which interfere with cell growth (Fernández *et al.*, 2000) have made it difficult to obtain successful results. It has been suggested that the presence of polyamines, endogenous juglone, or the continuity of sclerenchymatic cylinders contained in the phloem of plant material inhibits root formation (Günes, 1999). This would explain the fact that only high rhizogenic rates have been obtained from material of embryonic origin (Leslie and McGranahan, 1992). This fact restricts the advantages that elite genotype cloning represents where selection occurs in advanced ontogenic states with a high complexity at both the metabolic and tissular levels (Sánchez-Olate *et al.*, 2002).

Microshoots of micro-propagated *in vitro J. regia* from isolated embryos have been rooted using auxins such as naphthalene acetic acid (NAA) and indole butyric

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acid (IBA) (Ripetti *et al*., 1994), genetic transformation with *Agrobacterium tumefaciens* (McGranahan *et al*., 1988), and inoculation with *A. rhizogenes* (Caboni *et al*., 1996). The latter permits the induction of adventitious roots in the infection zone due to the transfer of genetic information of a portion of (T-DNA) of Ri (Root inducing) plasmid from the bacteria to the plant genome (Strobel and Nachmias, 1988). Caboni *et al*. (1996) used this method to achieve rooting rates between 52 and 68% in embryonic *J. regia* cv. Sorrento microshoots that were successfully transferred *ex vitro.* At this moment, no results using adult material have been reported.

The results obtained with *Agrobacterium* have been related to a synergy between the auxinic (IBA) concentration and the *A. rhizogenes* infection which is expressed by a possible response of living plant cells contiguous to the dead cells infected with the bacteria. The living cells would transmit diffuse signals to other healthy cells that are capable of initiating the rhizogenesis process (Falasca *et al*., 2000). According to Vahdati *et al.* (2002), the *rol* genes derived from the T-DNA of *A. rhizogenes* are involved in changing the following characteristics in the transformed plants: *rol* A: wrinkled leaves, condensed inflorescences, increment in the size of the stigma and large flowers; *rol* B: increments the rooting potential as a result of increasing sensitivity to the tissue auxins, alters the morphology, and increases flower size; *rol* C: reduces internode length, produces flowering abnormalities, and increments ramification; *rol* D: causes dwarfism and early blooming. However, in transgenic 5-year old walnut trees transformed by the *rol* ABC genes, no differences were found in the growth habit of shoots and roots (Vahdati *et al.,* 2002).

The effect of indole butyric acid and two wild strains of *Agrobacterium rhizogenes* on rhizogenic induction in *Juglans regia* microshoots was studied since the aim was to develop a protocol allowing massive propagation from adult material.

MATERIALS AND METHODS

Plant material

Caulinar portions of adult material were used and obtained from epicormic shoots of reinvigorated material by grafting *J. regia* cv. Serr on *J. nigra*. This was maintained in the fourth subculture on a DKW (Driver and Kuniyuki, 1994) proliferation medium with pH 5.8, supplemented with sucrose (3%), benzylaminopurine (BAP) (1 mg L^{-1}), IBA (0,01 mg L^{-1}), and gelled with agar agar (7 g L^{-1}) in photoperiodic environmental conditions of 16 h, 25 ± 1°C during the day, 22 ± 1°C during the night, 60% relative humidity, and light intensity of 40 µE $m^{-2}s^{-2}$, as reported by Sánchez-Olate *et al*. (2002).

Rooting assay

The rhizogenic process was analyzed for auxinic induction (T1: 3 mg L^{-1} AIB; T2: 5 mg L^{-1} IBA) and *A. rhizogenes* induction (T3: A-477; T4: A478) in two phases. The first phase was root induction in the dark for 3 days, and the second 27-day phase was known as root manifestation.

Rooting induced by IBA

Microshoots, 3 cm long, were handled using the rooting methodology described by Ripetti *et al.* (1994), and tested in two concentrations of exogenous IBA (3 and 5 mg L^{-1}) in an MS (Murashige and Skoog, 1962) medium with 25% (MS¼) macronutrients. An induction phase of 3 days was maintained in the dark at a temperature of 25 ± 1 °C during the day, 22 ± 1 °C during the night, and 60% relative humidity. Once the induction phase was finalized, the microshoots were transferred to the root manifestation phase of 27 days in a 16:8 photoperiod in a DKW (Driver and Kuniyuki, 1994) medium with 25% (DKW¼) macronutrients mixed with vermiculite (220/250 v/v), and solidified with gelrite (Phytagel, Sigma®).

Rooting induced by *A. rhizogenes*

Microshoots, 3 cm long, were inoculated in their basal portion with A-477 and A-478, wild strains of *A. rhizogenes* from the Valencia, Spain collection (Dawson *et al.*, 1990). The reactivation of bacterial growth was carried out with an aliquot in a microbiological beaker and by resuspending it in a 2 mL liquid medium of YMB (Yeast Medium Basal) (Hooykaas *et al.*, 1977), and shaking it during 48 h at 300 rpm and 25-27 ºC. Subsequently, 100 µL of aliquots were taken from the initial bacterial suspension to be resuspended in 10 ml capacity tubes containing 2 mL of liquid YMB medium to reapply the initial treatment. Finally, 100 µL of aliquots were cultivated in a solidified medium with agar (8 g L^{-1}) on Petri dishes with 10 mL of medium maintained at 28 ºC during 24 h in inverted position to avoid evaporation.

Once the colonies were developed, a 100 mL Erlenmeyer flask containing 20 mL of liquid YMB medium was inoculated with an inoculation loop by shaking it at 300 rpm at 25-27 ºC for 48 h. Subsequently, the solution was placed on a sterile Petri dish in order to proceed with the inoculation of the microshoots obtained from the proliferative chains. The basal inoculation of the microshoots was carried out by submerging them during 3 min in a bacterial solution after eliminating the basal axillary buds and cutting them in 1 cm lengths to increase the infection area. The microshoots were immediately placed on sterile filter paper arranged in a laminating flow chamber to dry and afterwards cultivate them in glass containers with 25 mL of MS¼ medium during three days

in a dark chamber at a temperature of 25 ± 1 °C during 16 h, 22 ± 1 °C during 8 h, and 60% relative humidity. At the end of the induction phase (3 days in the dark), the microshoots were cultivated in a DKW¼ medium mixed with vermiculite (200/250 v/v), and solidified with gelrite (2,5 g L^{-1} of Phytagel, Sigma®) to which 300 µg mL^{-1} of Cephotaxime (Claforan® 1 g, Roussel Ibérica, S.A.) were added to control bacterial development. The environmental conditions corresponded to a 16 h photoperiod of light during 27 days at a temperature of 25 ± 1 °C during the day, 22 ± 1 °C during the night, and 60% relative humidity.

The experimental design was completely random with four replications. The experimental unit corresponded to a container with four microshoots each measuring 2.5 cm. At the end of the manifestation period the microshoots were extracted and carefully washed to eliminate the substrate adhering to the roots. The treatments were compared by evaluating the percentage of rooting and the induced radicular systems, contrasting them statistically with ANOVA. Significant differences were identified with the Tukey multiple comparison test with 95% probability (Steel and Torrie, 1985).

RESULTS

Results indicated that it is possible to induce adventitious rhizogenesis in microshoots originating from adult material (Table 1) when they are partially reinvigorated by grafting. The rates of rooting and the quality of the resulting radicular system are similar to those obtained in microshoots of embryonic origin (Sánchez-Olate, 1997), but with different responses depending on the inductor used.

As occurs with material of embryonic origin, results showed a close relationship between callogenesis and rhizogenesis. In each treatment, as the percentage of callogenic tissue increased, a smaller number of explants rooted (Table 1). Even with the best rooting results obtained in T2 (Table 1), the response to *A. rhizogenes* (T3 and T4) did not differ from T1. Hence, it could become an increasingly interesting method for adventitious rhizogenesis in this species since observed rhizogenic rates greatly exceeded the results reported by Sierra (2002) in cutting rooting of the same species (12%).

By analyzing the number of roots for each microshoot, T2 generated a significantly greater response with respect to T1, T3, and T4. In the latter two, as occurred in the rooting rate, there were no significant differences (Table 1). On the other hand, major differences occurred in the length of the induced radicular system since the 5 mg L^{-1} application of IBA produced significantly larger roots than in the other treatments for the same manifestation period (Figure 1a and 1b). This led to two hypotheses: a) that the high auxinic concentration forces an accelerated metabolic route toward the synthesis of rhizogenic tissues, or b) that the high auxinic concentration forces a strong initial pulse, the roots are generated in the first hours of manifestation and develop in length over an extended period of time. This second hypothesis coincided with observations made by Ríos *et al*. (2002) in cotyledonal portions of the same species where it was observed that the rhizogenic induction produced by high auxinic concentrations took place in the first few hours after applying treatment. This resulted in a numerous and longer radicular system with respect to the treatments with lesser concentrations of IBA and greater concentrations of other auxins such as indolacetic acid (IAA) and NAA.

With respect to the induction by *A. rhizogenes*, the results indicate that this induction agent seems to be a real alternative to rooting of this species, not only because of its easy application, but the acceptable rooting rates achieved (Table 1) and the radicular systems obtained (Figure 1c and 1d). Furthermore, the greatest area of influence of this induction agent, manifested by the appearance of roots in the whole area treated, generated their better distribution in relation to its insertion in the caulinar portion.

The differences between the materials obtained in both *A. rhizogenes* strains basically rested on the fact that T3 obtained longer roots (Figure 1c). Perhaps the most

Table 1. Rhizogenic evaluation induced by indole-3-butyric acid (IBA) and *Agrobacterium rhizogenes*.

Treatment	Callogenesis	Rhizogenesis	N° of roots	Root length	Root diameter	Root insertion zone[1]
	%	%		mm	mm	
T1	66.7b	50.0a	1.8a	09a	1a	B
T2	50.0a	67.5b	3.1b	18b	2b	MB
T3	90.3c	47.7a	2.3a	09a	2b	SMB
T4	81.3c	50.0a	2.0a	05a	2b	SMB

Different letters show significant statistical differences ($\alpha \leq 0, 05$).
[1] Root insertion zone to the shoot. S: superior zone of the portion in contact with the culture medium. M: medium zone. B: basal zone. T1: 3 mg L^{-1} IBA. T2: 5 mg L^{-1} IBA. T3: A-477. T4: A-478.

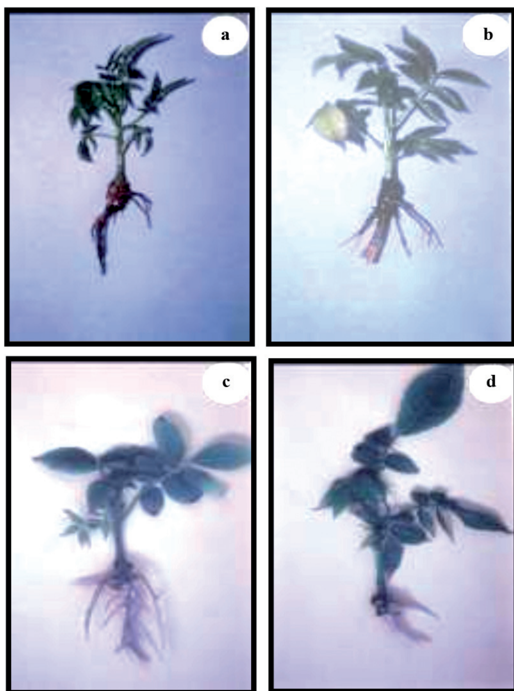

Figure 1. Rooting response in *Juglans regia* L. cv. Serr microshoots. Rooting in T1 and T2 (a and b), T3 and T4 (c and d), respectively.

important difference was that these roots also rapidly produced a high number of secondary roots exceeding the microplants produced with T4 (Figure 1d), characteristic that favors its subsequent acclimatization *ex vitro*.

DISCUSSION

The main problems in the *in vitro* introduction of *J. regia* adult material were related to the permanent appearance of bacterial contamination of endogenous origin called latent contamination (McGranahan *et al.*, 1988), and the exudation of phytotoxic compounds of phenolic origin (Leslie and McGranahan, 1992). However, the use of reinvigorated material through consecutive pruning or macrografting (Claudot *et al.*, 1992; Leslie and McGranahan, 1992) allowed a decrease in the incidence of this problem on the subsequent culture and development of *in vitro* explants (McGranahan *et al.*, 1988), originating synthesis processes of plant growth regulators in quantity and quality similar to that obtained in material of embryonic origin (Sánchez-Olate *et al.*, 2002).

Studies related to this topic concur that the apices portion of the microshoots are the most adequate for the rooting phase, whereas multiplication is better with basal segments of microshoots (Ríos *et al.*, 2002; Sánchez-Olate *et al.*, 2002).

By means of auxinic induction, the rooting rates were greater and the quality of the radicular system gave better results with respect to the number of roots and the zone from which it originated in the microshoot. Rooting was observed at 67.5% compared to 50% attained with *A. rhizogenes*, these values being similar to those obtained by Caboni *et al.* (1996) in embryogenic tissue, condition which can indicate that these bacterial strains have the capacity to improve under organogenic potential imposed by ontogeny (Rodríguez *et al.*, 2005). Infection with *A. rhizogenesis* in the base of the microcuttings was able to induce adventitious radicular systems similar to those obtained in other recalcitrant species (Damiano and Monticelli, 1998; Gutiérrez-Pesce *et al.*, 1998; Hoshino and Mil, 1998; Pérez-Molphe and Ochoa-Alejo, 1998). Abundant adventitious roots were induced in the cut zone, observing characteristics of the transformed roots for the purpose of bacteria plasmid (Tepfer, 1984; Petit *et al.*, 1986; Narasu and Giri, 2000), showing radicular systems distinct from the A-477 and A-478 strains, where the latter appears to have a greater induction capacity than the former, resulting in the particular differences of each strain (Vahdati *et al.*, 2002; Kaur *et al.*, 2006). Furthermore, the transformed roots were able to regenerate transgenic plants or clones that are viable, genetically stable (Narasu and Giri, 2000), and phenotypically normal (Sánchez-Olate *et al.*, 1997). This could be indicating the interaction between endogenous auxins and the *A. rhizogenes* effect (Falasca *et al.*, 2000).

In spite of the observed differences in the rhizogenic rate obtained via IBA or *A. rhizogenes*, the use of the bacterial vector can be a powerful tool to reproduce selected cultivars that are advanced in age, or recuperate high-value cultivars in a state of deterioration, given that the results obtained with this inductor were comparable to those obtained with microshoots of embryonic origin (Ripetti *et al.*, 1994; Caboni *et al.*, 1996).

Finally, considering that the commercial importance of *J. regia* generates a high demand for grafted plants with germoplasma quality, and that the results of the grafting programs are significantly less than the rooting percentages achieved in this study, the use of rhizogenic induction agents can mean plant conversion at higher rates than those achieved via traditional grafting.

CONCLUSIONS

It is possible to attain rooting rates of 50% from *Juglans regia* adult material previously rejuvenated through grafting by using auxinic inducers and *Agrobacterium rhizogenes*. The highest percentage of rooting was obtained in auxinic induction treatments.

RESUMEN

Inducción rizogénica en tejido adulto de *Juglans regia* L. cv. Serr mediada por ácido indol butírico y *Agrobacterium rhizogenes*. La introducción *in vitro* de tejido adulto de nogal (*Juglans regia* L.) representa una oportunidad de clonación de genotipos elite, cuya selección ocurre en estados ontogénicos avanzados. Así, con el objeto de desarrollar un protocolo que permita la propagación masiva de genotipos valiosos a partir de material adulto, se compararon dos sistemas de inducción rizogénica de microtallos de nogal provenientes del cuarto subcultivo de un programa de introducción *in vitro* de tejido adulto de nogal, previamente revigorizado mediante injerto tradicional. Se utilizó la inducción rizogénica por ácido indol-3-butírico (AIB) y *Agrobacterium rhizogenes*. El proceso rizogénico se analizó tanto para inducción auxínica (T1: 3 mg L^{-1} AIB; T2: 5 mg L^{-1} AIB), como para inducción por *A. rhizogenes* (T3: A-477; T4: A-478), en dos fases. Una primera fase de inducción radicular, con una duración de 3 días en oscuridad; y una segunda fase de 27 días, denominada de manifestación radicular. En todos los tratamientos se evaluó porcentaje de enraizamiento y se caracterizaron los sistemas radiculares inducidos (número, largo, diámetro y zona de inserción de raíces). Los mejores resultados de enraizamiento se obtuvieron en T2; sin embargo, la respuesta obtenida con *A. rhizogenes* no difiere de aquella lograda en T1, por lo que pareciera ser una metodología de creciente interés para la rizogénesis adventicia en esta especie.

Palabras clave: enraizamiento, microtallos, material adulto, *Agrobacterium rhizogenes*.

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