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Towards scaling-up the micropropagation of *Juglans major* (Torrey) Heller var. 209 x *J. regia* L., a hybrid walnut of commercial interest

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Abstract: As a result of the program for quality-wood production conducted by Bosques Naturales SA, several walnut *èlite* genotypes have been selected. Whereas it is important to establish an efficient clonal propagation system for such hybrids, doing it has been hampered by the well-known recalcitrance of walnut to *in vitro* manipulation. As a few general micropropagation protocols are available, they must be adapted to the specific genotypes and conditions of each laboratory. Here, we present some recent findings that have allowed us to improve the current micropropagation strategies for *Juglans major* (Torrey) Heller var. 209 x *J. regia* L., a hybrid of commercial interest. We specifically investigated the influence of phloroglucinol (Phl) during multiplication; the benefits of using FeEDDHA instead of FeEDTA; the influence of subculture length; the optimal doses of sucrose for root pre-induction and the use of a Temporary Immersion System (TIS) to promote elongation. Phl was a key factor to promote basal-*calli* formation and to increase microshoot length. There was no statistically significant interaction between genotype and iron source used, a relevant finding taking into consideration that substitution of FeEDTA by FeEDDHA was a determinant for rooting and for survival. Despite genotype-specific effects on *in vitro* behaviour, there was a high influence of sucrose concentration during root pre-induction. Microshoots growing in TISs were taller than those multiplied in gelled culture media. More than 90 % of the vitroplants that survived the first 4 weeks under *ex vitro* conditions could reach nursery and field plantation. These results allowed us to clone successfully 8 *èlite* genotypes since 2010.

Introduction

Establishment of multi-clonal plantations of highly valuable genotypes is an important requisite in forestry to reduce the risks caused by the genetic variability of seed orchards. Micropropagation is the best choice for massive multiplication of *èlite* trees, however, due their high recalcitrance only a few woody species are commercially propagated.

Juglans are considered amongst the most recalcitrant genera for *in vitro* culture. Few walnut species and/or varieties are mass micropropagated, most of them used as rootstocks or fruit trees. Low survival percentage during *in vitro* establishment; high influence of genotype, mainly characterized by long stationary phases, as well as a reduced ability for rooting; chlorosis of microshoots and low survival rates during acclimation, are amongst the most critical factors that determine the success of walnut micropropagation.

In a walnut selection program, associated to the accelerated annual propagation of several highly valuable trees for wood production, a standardized and functional micropropagation protocol is needed. However, the high genotypic determinism and the well-known recalcitrance of walnuts hinder the obtaining of a common system for all them.

A functional micropropagation protocol should be based on the obtaining of healthy and highly reactive microshoots as a manifestation of their "right" physiological state: an adequate mineral composition of culture medium is important to reach this goal. Microshoots that had been multiplied in the corrected DKW formulation (McGranahan *et al*. 1987) had a healthy appearance except for the pale green colour of the leaves. A similar disorder was observed by Najafian Ashrafi *et al.* (2010) in Persian walnuts. As chlorosis might have been caused by iron deficiency we decided to replace FeEDTA by FeEDDHA, considered a more stable and more suitable chelate for field growing fruit trees (Pestana *et al*. 2003) and for micropropagation. Moreover, FeEDTA is photosensitive, provoking chlorosis by iron deficiency (Van der Salm *et al*. 1994).

Once survival to *ex vitro* conditions was greatly enhanced using rooted microshoots, our work addressed the improvement of the rooting ability for all clones. The root formation is closely related with the utilization of quality microshoots. Microshoots good for rooting were defined as those that came only from apical explants that reached more than 20 mm in height, with at least 8 nodes and green and healthy leaves. A key factor to obtaining a quality walnut microshoot is the formation of a big basal callus in the first 2 weeks of every subculture. In this process Phl has an important role. Phl is used *in vitro* as growth regulator, promoting the multiplication of apple (Rustaei *et al*. 2009) and *Ficus religiosa* L. (Siwach and Rani Gill 2011) and improving the rooting of almond (Ainsley *et al.* 2001).

Here a compilation of the main results on walnut hybrid micropropagation since 2005 is presented. The introduction of Phloroglucinol (Phl) on multiplication and the substitution of FeEDTA by the more stable chelate FeEDDHA are the most important changes introduced in the culture medium proposed by Driver and Kuniyuki (1984) and corrected by McGranahan *et al.* (1987). Other factors, such as the influence of sucrose dose during root pre-induction phase and the length of subculture prior to rooting were also assessed. The use of TISs to promote the elongation of microshoots from highly recalcitrant genotypes was also investigated.

Materials and Methods

Plant material and in vitro *introduction*

Our micropropagation protocol was developed for the walnut hybrid (*Juglans major* var. 209 x *J. regia* L.) for wood production. Epicormic branches from *èlite* field growing trees, selected from Bosques Naturales SA (Spain) orchards, were used for *in vitro* culture initiation. Two different methods were followed for introduction. For the first one, epicormic branches were collected during late spring and beginning of summer in 2008 and 2009; segments bearing at least 1 dormant bud were washed for 10 minutes with sterilized water and detergent. Afterwards they were dipped for 60 seconds in alcohol (96 %), followed by a 20 min treatment with NaOCl (1%, pH were adjusted approximately to 7.5) and some drops of Tween 20 and finally the explants were rinsed 3 times before inoculation in test tubes containing 10 ml of culture medium. For the second method, the basal part of epicormic segments, 20-25 cm long, collected in late winter and early spring of 2011 and 2012, were introduced in sterilized water and stored under thermo and photoperiod conditions in the laboratory to promote bud breaking. Then the softwoods formed were used as starting material, superficially disinfected only with NaOCl (pH was not adjusted), while the rest of the procedure was kept the same. Before being incorporated in the general micropropagation schedule, microshoots successfully introduced were tested to be free from endogenic actively-growing *bacteria* by inoculating a small part of callus formed on a culture medium composed of meat and yeast extract (5 g/L), glucose (5 g/L), KH_2PO_4 (2 g/L) and agar (7 g/L). Eight genotypes that behaved differently through all the phases of micropropagation were used. Non recalcitrants were considered to be those that were easily introduced, whose average size of microshoots was above 35 mm and had a high ability (>80 %) for rooting (DA, D53 and D15). Recalcitrants were those that had some problems for *in vitro* establishment, microshoots were between 25 and 34 mm high and/or had an average of 50 up to 80 percent of root induction (DM, DN, D49 and D51). In this group were some genotypes that could be included in the first one if some particular characteristics were considered (like DM, with little microshoots (<20 mm) but a high ability for rooting). High recalcitrants were those that needed several attempts during introduction and had low rooting percentages, usually less than 50 % (G3 and D48).

Culture medium and general conditions

DKW (McGranahan *et al.* 1987) formulation was used in all phases of micropropagation. For

multiplication 8 microshoots were inoculated in 380 ml vessels with 80 ml of culture medium supplemented with 1.0 and 1.5 mg/L of BAP for introduction and multiplication, respectively. Two doses of agar (Industrial Agar 707469, Pronadisa, Spain) were used, 5.5 or 6 g/L, depending if the iron source was FeEDDHA or FeEDTA. The introduction culture medium was additionally supplemented with 75 mg/L of Phl. Microshoots grew under a 16/8 photoperiod (PPFD 45-50 µm. m^{-2}. s^{-1}) in a growth chamber with a temperature of 24± 0.2ºC.

Substitution of FeEDTA as iron source

An equivalent dose (119 mg/L) of ethylenediamine di-2-hydroxy-phenyl acetate ferric (FeEDDHA, Duchefa Biochemie) was used instead of the less stable iron source FeEDTA. The influence of FeEDDHA on multiplication, rooting and survival were assessed.

Use of Phl during multiplication

The effects of several doses of Phl (0, 25, 50 and 75 mg/L) during multiplication were assessed. *Calli* weight and length and nodes per microshoots were measured.

Rooting conditions

Rooting was divided into two separated steps: pre-induction and expression as were recommended by McGranahan *et al*. (1987). For pre-induction the macroelements were reduced to 50 % and IBA (5 or 10 mg/L) was used as hormone. Microshoots, more than 20 mm long, were inoculated in this culture medium and incubated for 5 days in the dark. The expression phase was conducted for 2 weeks under photoperiod conditions (16 h light), with IBA being removed from the culture medium and replacing agar by vermiculite.

Length of subculture prior to root pre-induction

The influence of different ages of microshoots (4, 5 and 6 weeks) during the phase pervious to root pre-induction was assessed. Rooting percentage, number of roots, length of the longest root and height of microshoots were measured.

Dose of sucrose during pre-induction phase

Three concentrations of sucrose (20, 40 and 60 g/L) were used as carbon source in pre-induction culture medium. The effects of each were assessed at the end of the expression phase. Rooting percentage, number of roots, length of the longest root and height of microshoots were measured.

Temporary immersion systems (TIS)

A TIS, similar to that proposed by Lorenzo *et al.* (1998) and Escalona *et al*. (1999), was built. To determine what explants were most suitable for initiation, an experiment was conducted: B1, apical segments with 4-6 nodes and approximately 10 mm in length; B2, apical segments with 8-10 nodes and approximately 20 mm in length; A1, apical segments with 4-6 nodes and approximately 10 mm in length cultured in gelled medium for 1 week and A2, which is equal to A1 but is cultured for 2 weeks in gelled medium prior to inoculation in TIS. Length of microshoots and nodes/microshoots were assessed. Microshoots growing in gelled medium were used as control. After some previous assays, it was established that 45 seconds of immersion per day was enough and the dose of BAP was reduced 50%, the other conditions were the same. In order to avoid overestimation of the results, the same relation of culture medium per explant (10:1) for conventional micropropagation was used. For TIS, fifteen explants were inoculated in vessels with 750 ml volume.

Acclimation

Rooted microshoots were potted in a mix of peat (90 %) and vermiculite (10 %). Temperature ranged from 32 (day) to 16ºC (night). The illumination was at a maximum of 300 µm. m^{-2}. s^{-1} during the first 3 weeks and was kept below 500 µm. m^{-2}. s^{-1} the next 4-5 weeks. Relative humidity went from 60 up to 90 % .

Experimental conditions

Each vessel was selected as experimental unit, and consequently all data are averages obtained from all the microshoots in the vessel. The vessels were randomly distributed in a growth chamber. Successful experiments were repeated at least 2 times. A Bifactorial design was used to discard, or retain, a hypothetic genotypic answer. Statistical significance was determined by means of ANOVAs and post-hoc tests were performed to know the differences between the different levels of each factor. Percentage data were transformed with arcsin√x.

Results and Discussion

In vitro introduction

The fact that our starting material for *in vitro* introduction are branches collected from single-selected trees, highly hampered the performance of standardized experiments. Therefore we have, since 2005, based the protocol on previous experiences. When direct introduction from field grown trees was used, low success was reached.

Moreover, it is a time consuming process, as the culture medium should continuously be renewed due to the profuse releasing of phenolics. This necessitated, in some cases, the subculture every 24 h and, in general, at least every 2 days during the first 5 to 6 weeks. Only a few trees (DA, D15 and D53) could be routinely introduced using this procedure. However, most of genotypes were highly recalcitrant and several attempts were necessary, as well as a high quantity of starting material. Additionally other trees, like G27, were not able to be *in vitro* cultured after 3 years using this method. During the summer of 2008 we tried to introduce 30 *èlite* trees. For that purpose we managed 1264 explants in 61 attempts. Only 83 segments, belonging to 7 genotypes, were finally established *in vitro*, the other 1181 (93.4 %) were discarded for fungi and *bacteria* contamination, for death as a consequence of the high release of phenolics to culture medium and for their inability to respond to *in vitro* conditions. Due to the limited success of this method, we found it necessary to use a more efficient alternative that would include both rejuvenation and growing under controlled conditions of starting material prior to initiation as was recommended by Bonga and Von Aderkas (1992). Hence, segments of epicormic branches approximately 20 cm long, bearing dormant buds, were placed in tap water and stored under controlled conditions (light and temperature) simulating the beginning of spring. The bases of the sticks were removed and the water was replaced weekly. Despite the genotypic influence, after 3 weeks bud sprouting was observed and healthy shoots formed. When these reached at least 20 mm in length, they were collected for initiation (fig 1). Smaller segments were not suitable because they normally form a big callus covering the explants completely, which prevented bud break and shoot growth.


Fig 1 *Forcing softwood formation of walnut hybrid from dormant bud under controlled conditions*

During 2011 and 2012 we had 16 walnut hybrid genotypes introduced successfully; despite the microbiological contamination being still present, the losses associated to this factor were less than those obtained with the other method. However, the most important achievement was that phenolization was completely removed probably due to the use of rejuvenated explants. Moreover, it allowed the reduction of the number of subcultures and the manipulation of explants, facilitating *in vitro* establishment. As a result in 2012 only 211 explants were managed from which 113 (53.5 %) were lost by different causes such as bacterial (30.8 %) and fungal (20.4 %) contamination and only 2.4 % of them died later. Finally, we were able to introduce 8 out of 11 genotypes: 5 walnut hybrids, 1 Persian walnut, 1 black walnut (*J. nigra* L.) as well as the Paradox clone Vlach. The 3 genotypes that did not respond to *in vitro* culture had problems associated to poor bud break and formation of small shoots. This emphasizes the importance of using only healthy shoots with a length of at least 15 mm as starting material. The advantages of this procedure are based probably on the fact that epicormic sprouts arise from inactive meristems produced during the juvenile phase. Therefore it is expected that these explants would be established more easily *in vitro* than more adult meristems (Van Sambeek *et al.* 2002).

Substitution of FeEDTA as iron source

As a first result the microshoots growing in a culture medium supplemented with FeEDDHA had greener leaves and were more vigorous than the ones cultured with FeEDTA (fig 3B). Although growth was stimulated we did not find any statistical influence on the length of microshoots during multiplication as has also been observed in micropropagation of *Rosa hybrida* (Van der Salm e*t al*. 1994). Noteworthy results were reached on rooting and

acclimation. Microshoots growing in the presence of FeEDDHA had a higher ability to root with a higher number and length of the roots than did the ones growing in the presence of FeEDTA (fig 2). The influence of different iron sources on pear micropropagation was also studied and although FeEDDHA increased the number of shoots per
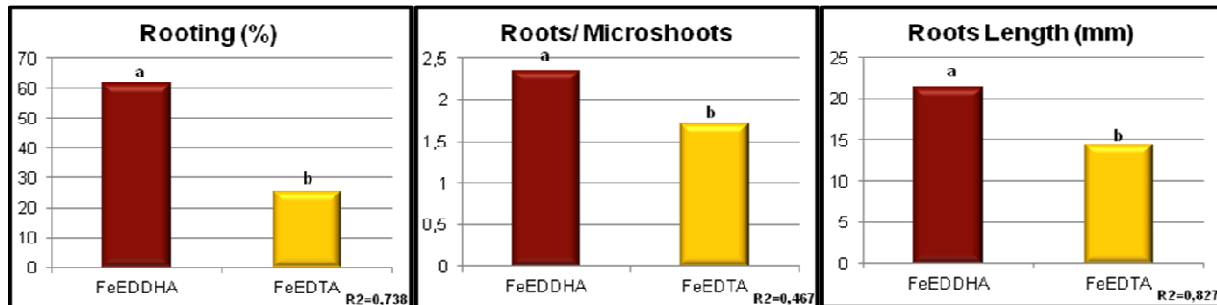
Fig 2 *Effects of substitution of FeEDTA by FeEDDHA on rooting of walnut hybrid microshoots (p≤0.05)*

explant and fresh mass production, FeEDTA was more suitable than FeEDDHA for rooting (Sotiropoulos *et al.* 2006). If the behaviour of a plant is a manifestation of its metabolic state, with an important influence of nutrition, we assumed that these results are a probable consequence not only of a higher and better availability of iron but of the other mineral components (Sotiropoulos *et al.* 2006) as well. Furthermore, the influence on chemical (pH) and physical (strength) properties of the culture medium has to be considered. Van der Salm *et al.* (1994) did not find important differences in pH in culture medium using both chelates, but under our conditions great variations were observed. The pH of culture medium supplemented with FeEDDHA showed a slight reduction after sterilization, kept stable from the beginning to the second week, on the third week the pH fell down to 4.5, recovering its higher value a week later; at the end it rose up to 6.6 approximately. On the other hand, when FeEDTA is used, the pH drops down to 5.1 after autoclaving, increasing steadily to above 7.0 at the end of the subculture cycle. This high value of pH may be the cause of the decay of microshoots at the end of the subculture, precisely when the differences in pH effect between both chelates are more evident.

The definitive influence on improving the quality of microshoots by replacing FeEDTA by FeEDDHA was observed during acclimation. After acclimation only 1 % of the vitroplants that reached field plantation come from cultures grown on FeEDTA. These were affected not only by chlorosis but also by complete defoliation and apical death (fig 3). McGranahan and Leslie (1988) also found that the retention of leaves was important in promoting the explant survival and growth. An important conclusion was that there was no statistical interaction between genotype and the iron source used. It seems to show that FeEDDHA is more suitable for micropropagation of walnut hybrid than FeEDTA.

Fig 3 *Abnormalities of walnut hybrid microshoots growing in culture medium supplemented with FeEDTA: chlorosis (A, B, and C), defoliation (A and C) and apical death (D)*

The use of Phl during multiplication

In this process Phl has an important role, without it in the culture medium, the microshoots failed to grow, probably as a consequence of the formation only of little *calli* (fig 4). The incorporation of 25 mg/L was enough to promote *calli* formation and growth (table 1). Despite the Leslie *et al.* (2004) finding that when 1 mM of Phl is added to multiplication medium the rooting is promoted, we did not observe any important advantage of increasing the Phl dose to 75 mg/L (table 1). On the other hand, some experiments conducted with higher doses (800 µM) showed a more unstable behaviour of cultures although the auxiliary bud formation was clearly stimulated, especially in species that possesses as high an apical dominance as the walnut hybrid (data not shown). Like with the iron source, there was no interaction between genotype and Phl dose, another important result if we take into account that our main goal is to set up a commercial walnut micropropagation protocol. As Sharifian *et al.* (2009) showed that the effect of Phl on rooting is different for each genotype of *J. regia* L., further experiments should be done under our conditions to determine if the Phl dose has some influence on rooting and/or on the results of acclimation.
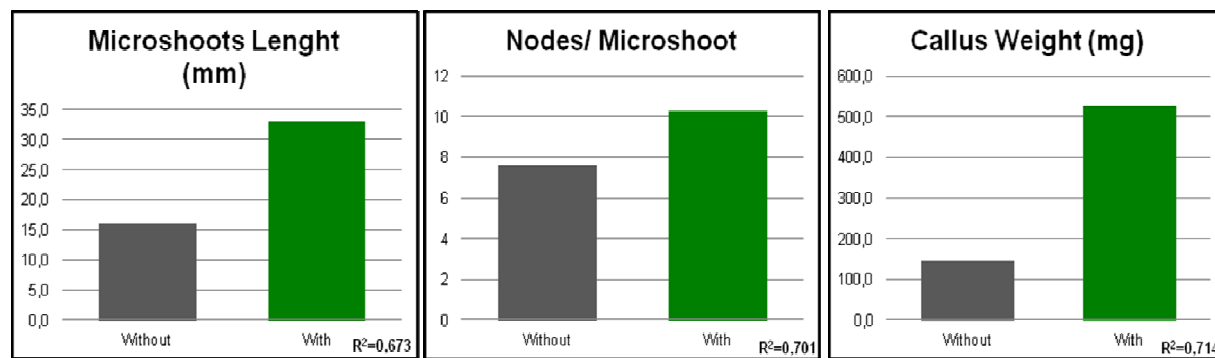
Fig 4. *Influence of Phloroglucinol on calli formation and walnut hybrid microshoots growth (p≤0.05): gray=Without Phloroglucinol; green= with Phloroglucinol.*

Table 1 *Effects of different doses of Phloroglucinol on calli formation and growth of in vitro walnut hybrid microshoots (p≤0.05)*

| Phl (mg/L)	Callus Weight/ Microshoot (mg)	Nodes/ Microshoot	Length of Microshoots (mm)
0	145,4[b]	8[b]	16,2[b]
25	371,1[a]	10[a]	27,3[a]
50	526,2[a]	10[a]	32,9[a]
75	593,5[a]	11[a]	31,6[a]

Influence of the length of subculture prior to root pre-induction

It is has been stated that rooting has a close relationship with lignin accumulation. Fu *et at.* (2011) found changes in phenylalanine ammonia lyase (PAL) activity during rooting of peony trees as well as differences in the content of total phenolics between easy and difficult-to-root genotypes. Santos Macedo *et al.* (2012) also demonstrated that a significant increase in the accumulation of total phenolics, flavonoids and lignin is observed preceding root initiation in IBA-treated olive explants. At the same time lignin accumulation is associated with aging. In nature, the amounts of acid-insoluble Klason lignin increase with the age of micropropagated regenerants of *Morus nigra* L. (Ďurkovič *et al.* 2012). *In vitro* gardenia microshoots also show an increase in lignin content during adventitious root formation from day 0 to day 20 of induction (Hatzilazarou *et al.* 2006). In walnut lignin accumulation was lower in the shoot population that did not show visible signs of rooting (Bisbis *et al*. 2003). Vahdati *et al*. (2004) found in Persian walnut that the stimulation of growth has a negative influence on rooting

ability, a process that is probably associated with an actively vegetative state, once 3 weeks-old microshoots were used. Other important elements are that auxins only accumulate in the basal part of walnut shoots where new roots will be formed (Falasca *et al*. 2000). The timing for root formation in elm was significantly delayed in the apical part in comparison with the basal part where the first roots appeared (Malá *et al.* 2005). For these reasons and based on the fact that BA (6-benzylaminopurine) concentration strongly affected the deposition of lignin in the vascular cells (Quiala *et al.* 2012), we hypothesized that rooting ability is highly influenced by the age of microshoots. In fact, we found that rooting percentage and number of roots formed improved with the age of the microshoots (fig 5) independently of genotype. Microshoots that are 6 weeks old are more suitable for rooting than those that are 4 weeks old. For some genotypes, for instance D53, the difference goes from 0 % of rooting, in microshoots after the 4th week of subculture to more than 90 % 2 weeks later. These results are be probably influenced by the reduction of the active growth, aging and lignin accumulation in the microshoots.
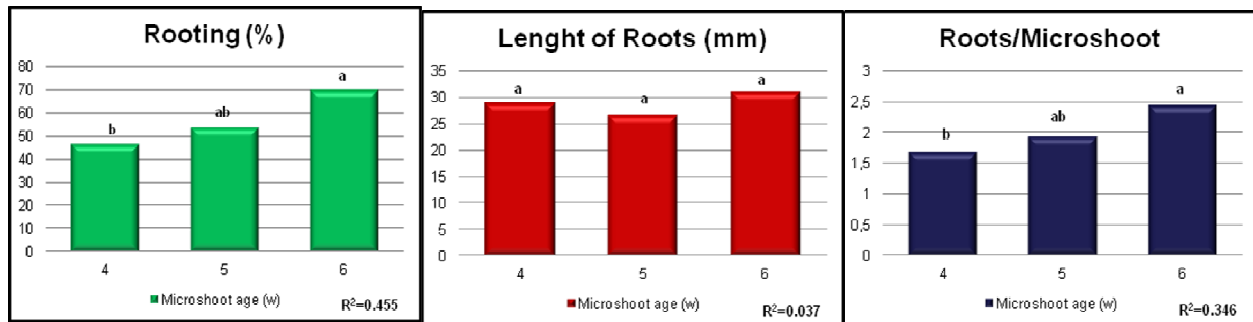
Fig 5 *Influence of the length of subculture on rooting of micropropagated walnut hybrids (p≤0.05)*

Dose of sucrose during pre-induction phase

Osmolytes, like sucrose, have a great influence on water potential of the culture medium. They also act as carbon source and help in determining the morphogenic response (George 1993). We did not find any reference that dealt specifically with the influence of different carbon sources and/or concentrations during pre-induction. Of the two rooting phases experiments with carbon sources have only been done during the expression phase (Dolcet-
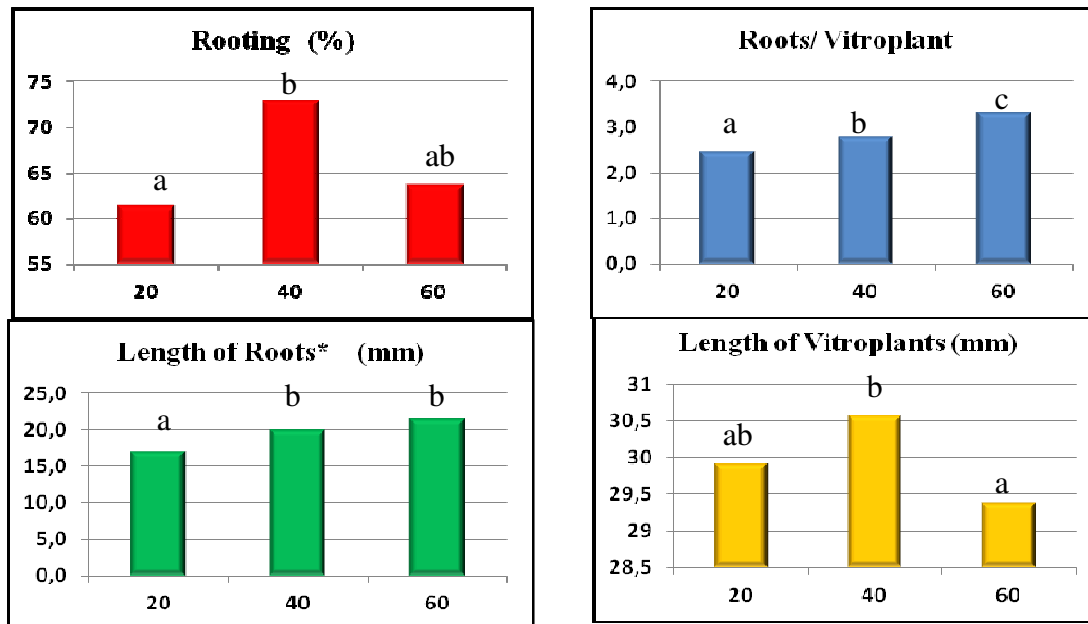
Fig 6 *Effects of different doses of sucrose (g/L) during pre-induction phase on rooting and growth of walnut hybrid microshoots (p≤0.05), * longest root*

Sanjuan *et al.* 2004). Based on our previous experience, and the recommendation of McGranahan *et al.* (1987) to use 53 mg/L of sucrose, we decided to assess the influence of 3 doses in this phase. Like for the other factors there was a genotypic influence and the dose of sucrose determined the rooting rate and the length of microshoots. As our goal is to seek generalities towards the establishment of a commercial walnut micropropagation protocol we will not analyse the genetic contribution. The dependent variables were affected in different ways but the most positive influence was obtained with 40 and/or 60 g/L of sucrose (Fig 6). Although rooting percentage was statistically similar for both doses, the number of roots was higher when 60 g/L was used. Microshoots were smaller with this dose than with 40 g/L, due, probably, to the fact that shoot and root growth are opposite and energy-consuming processes. Hence, under some conditions, when a particular treatment is favoured, for instance rooting, root growth is comparatively higher than shoot development. More details can be seen in Table 2, where, into each genotype group, a clear contraposition between these two variables is observed. Vahdati *et al.* (2004) also obtained similar results for "Vina" walnut, although they did not find statistically significant differences among doses. These results may suggest that the higher the size of the microshoots the lower the ability for rooting. However, a more precise explanation would be that as they seem to be sharing growth resources, conditions that boost the growth of one of these two systems prevents the other one from maximun development. Supporting this hypothesis is the finding of Nobel *et al.* (1989) in *Agave lechuguilla* where conditions that favour shoot growth (high nitrogen and phosphorus supply) the dry weight of roots was approximately constant while shoot dry weight was increased. Smith *et al.* (1991) also found for several woody species that as long as lateral roots did not develop, *in vitro* plants maintained vigorous shoot growth, whereas shoot growth was temporarily suspended while plants initiated roots *ex vitro*.

Table 2 *Results on rooting and growth of different walnut hybrid vitroplants and sucrose doses (p≤0.05)*

Gentoype	Sucrose (g/L)	Rooting (%)	Roots/ Vitroplant	Length of Roots* (mm)	Length of Vitroplants (mm)
DM	20	69,0[cd]	1,8[a]	15,7[bc]	18,7[a]
DM	40	90,9[ef]	2,8[bcd]	19,0[cd]	19,2[a]
DM	60	98,7[f]	4,7[e]	26,9[ef]	18,65[a]
DA	20	73,1[cd]	2,6[bc]	18,3[c]	32,44[b]
DA	40	81,3[de]	3,0[cd]	19,1[cd]	30,84[b]
DA	60	45,3[a]	2,1[ab]	15,1[b]	31,58[b]
D51	20	54,7[ab]	2,1[a]	11,7[a]	35,79[de]
D51	40	57,4[bc]	2,2[ab]	12,7[ab]	37,08[e]
D51	60	59,5[bc]	2,9[cd]	15,3[b]	35,08[cd]
D53	20	75,7[d]	3,2[cd]	22,3[de]	32,75[bc]
D53	40	73,0[cd]	3,4[d]	29,2[f]	35,2[cde]
D53	60	74,3[cd]	3,6[d]	28,5[f]	32,27[b]

*Longest root

Although a statistical interaction between genotype and sucrose dose was observed, the worst results were obtained with 20 g/L, except for clone DA (table 2). Both doses, 40 and 60 g/L, seem to be more suitable for rooting and growth, in general, though some extra experiments should be done when abnormal behaviour is observed for a new particular genotype.

Temporary immersion system (TIS)

The advantages of TISs have been demonstrated on micropropagation for banana (Alvard *et al.* 1993), sugarcane (Lorenzo *et al.* 1998), pineapple (Escalona *et al.* 1999), *Eucalyptus* (McAlister *et al.* 2005) and teak (Quiala *et al.* 2012). However, there is only one reference on micropropagation of walnut using a liquid culture

(Roschkel and Pijut 2007). This shows the feasibility to clone black walnut using this system, although we did not succeed with shake cultures. The main goal was to know if our walnuts could be successfully cultured in TISs and if there was a positive influence on elongation of highly recalcitrant genotypes. A former assay gave us the first input regarding the necessity of reduction of the dose of BAP since 1.5 mg/L promoted the formation of hyperhydric microshoots. After correcting the immersion time, the volume of culture medium and the vessel size we decided to determine what kind of explant was suitable for TIS. In a first approach, abnormal microshoots were obtained when the same explant kind that was used for traditional micropropagation was inoculated in TISs. The growth was highly influenced by the explant source, affecting both length and number of nodes, as well as the proportion of suitable microshoots for rooting (table 3). The best option was to use explants cultured for at least 2 weeks in gelled medium (A2), which had a pre-formed basal-callus as well as opened leaves, that could contribute to the absorption of the components of the culture medium. As a consequence, microshoots formed from A2 had a normal appearance similar to that obtained from GM (fig 7C).

Table 3 *Influence of the explant kind on elongation of walnut hybrid microshoots in TISs (p≤0.05)*

Explant Kind	Microshoot Length (mm)	Microshoot >20 mm (%)	Microshoot >25 mm (%)	Nodes/ Microshoot	Normal Shoots (%)
GM	20,9[c]	61,3[c]	28,8[d]	-	100,0[a]
B1	20,0[c]	37,8[e]	24,9[e]	7[c]	94,8[a]
B2	31,1[a]	82,5[a]	59,8[a]	8[b]	96,9[a]
A1	24,3[b]	55,2[d]	44,8[c]	9[a]	98,5[a]
A2	31,1[a]	67,7[b]	56,3[b]	11[a]	98,5[a]

The results obtained with B2 are, in general, an overestimation because the size of initial explants was almost double that of the one under the other treatments. The microshoots obtained with this treatment were curled in the base (fig 7B) which is an important limitation to root induction and transplantation to *ex vitro* conditions. The quality of microshoots from A1 are similar to A2 regarding their morphology although they certainly are less
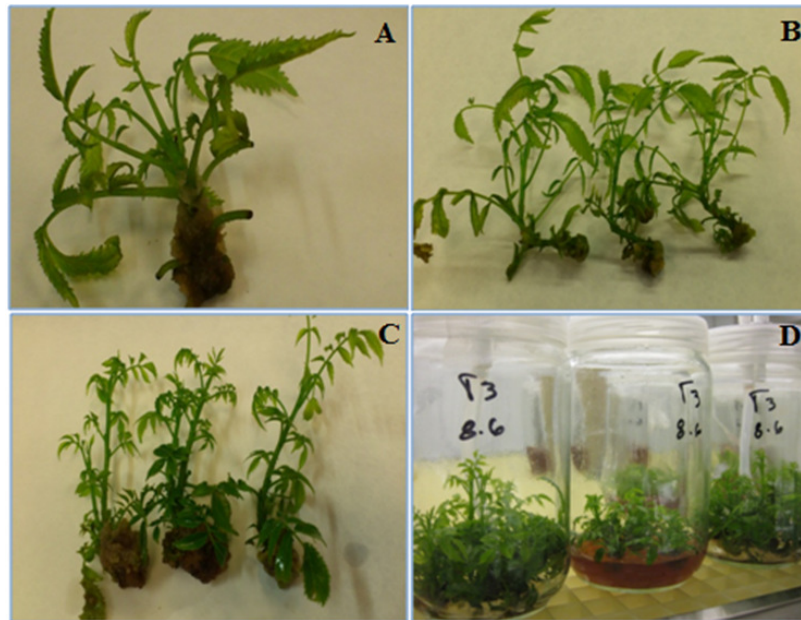

Fig 7 *Behaviour of different kinds of explants in a temporary immersion system (A) B1, (B) B2 and (C) A2, compared to (D) traditionally micropropagated walnut hybrids in gelled medium*

developed. This point to the need of previous adaptation and/or a complete formation of callus prior to the inoculation in TISs. Despite the genotype used, B1was the worst explant, with the lowest growth as well as a profuse formation of *calli*, in some cases covering almost all the stem (fig 7A). Hyperhydricity is a physiological disorder commonly associated to the use of liquid culture, including in TISs (Etienne and Berthouly 2002). Although there were no statistical differences for the percentage of normal shoots, some degree of hyperhydricity appeared in TISs. Therefore further experiments (volume of culture medium, use of growth retardants, extra aeration, time and number of immersion per day) should be done to set more suitable conditions for walnut in TISs.

Resumption of walnut micropropagation and main results obtained

Some key factors were analyzed, that helped us to set the bases for a commercial protocol for walnut hybrid micropropagation at Bosques Naturales S. A. The introduction of Phl and the replacement of FeEDTA by FeEDDHA were the most important changes that allowed us to produce suitable microshoots for rooting and acclimation. Other factors, such as age of microshoots and sucrose dose during root pre-induction, also helped us to enhance rooting ability for all genotypes used.

Acclimated vitroplants showed a high vigour developing a profuse radical system in a few weeks which contributed to obtaining high survival percentages in the green house and field plantations. Similar results were obtained by Dolcet-Sanjuan et al. (2004) with selected genotypes of *J. regia* and several walnut interspecies hybrids. The applications of these results allowed us to clone up to 8 genotypes from 2010 to 2012 (fig 8). This protocol has also been used to micropropagate some other walnut species as *J. nigra*, *J. regia* and Vlach, a hybrid Paradox.
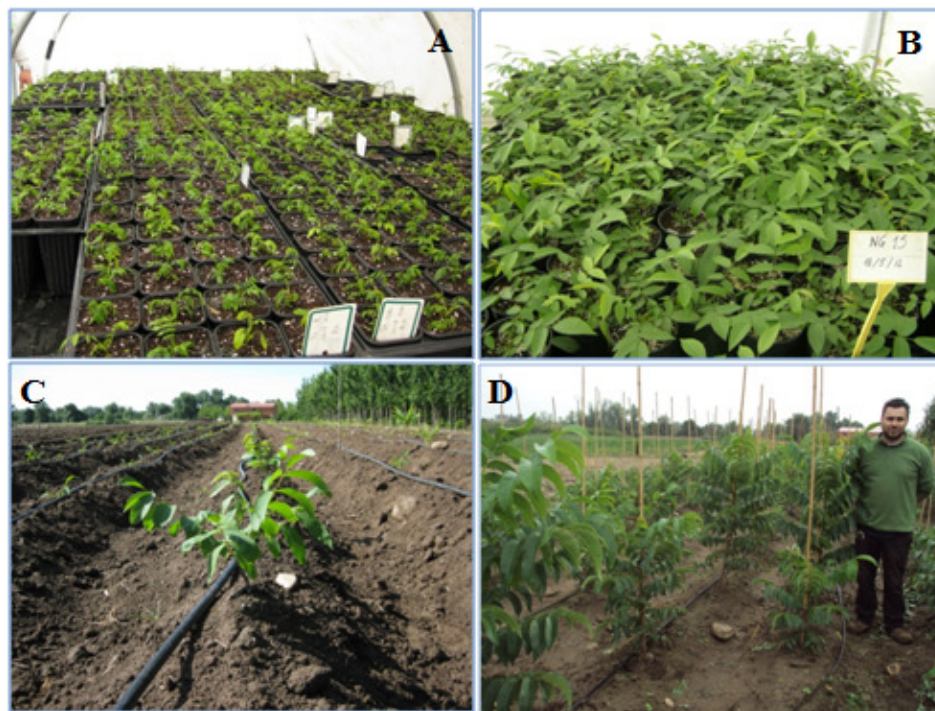
Fig 8 *Walnut hybrid vitroplants after transplantation to ex vitro conditions (A), 3 months old vitroplants in greenhouse (B); field planted in May, 2012 (C) and the same vitroplants 4 months later (D), photo taken September, 26ᵗʰ, 2012*

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