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 programme 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* var. 209 x *J. regia*, 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 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.

Key words Acclimation, Phloroglucinol, iron source, recalcitrant, rooting, temporary immersion system, sucrose

Introduction

Establishment of clonal plantations of highly valuable genotypes is an important requisite in forestry to reduce the risks caused by genetic variability. 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. A reduced number of 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 reduced ability for rooting; chlorosis of microshoots and low survival rates, are amongst the most critical factors that determine the success of walnut micropropagation.

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

A functional micropropagation protocol should be based on the obtention 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; 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 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 close 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 high, 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 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), corrected by McGranahan *et al.* (1987). Other factors 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

Micropropagation protocol was developed for a walnut hybrid (*Juglans major* var. 209 x *J. regia* L.) for wood production. Epicormic branches from *èlite* field growing trees, selected from Bosques Naturales S. A. (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 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, finally the explants were rinsed 3 times before the inoculation in test tubes containing 10 ml of culture *medium*. For the second method, the basal part of epicormic segments, sizing 20-25 cm, 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 the bud breaking, then the softwoods formed were used as starting material, superficially disinfected only with NaOCl (pH was not adjusted), 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*, inoculating a small part of *callus* formed on a culture *medium* composed by meat and yeast extract (5 g/L), glucose (5 g/L), KH_2PO_4 (2 g/L) and agar (7 g/L). Eight genotypes with different behaviour 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 found 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 least 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, sizing more than 20 mm, were inoculated in this culture *medium* and incubated for 5 days in the dark. The expression phase was conducted during 2 weeks under photoperiod conditions, removing IBA from 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 up. To determine what explants were more suitable for initiation, an experiment was conducted: B1, apical segments with 4-6 nodes and approximately 10 mm length; B2, apical segments with 8-10 nodes and approximately 20 mm length; A1, apical segments with 4-6 nodes and approximately 10 mm length cultured in gelled *medium* for 1 week and A2, A1 but cultured for 2 weeks in gelled *medium* were cultured 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 kept. In order to avoid overestimation of the results, the same relation of culture *medium* per explant (10:1) for conventional micropropagation was used. 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 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

Vessel was selected as experimental unit, as a consequence, the measure is the average of all the microshoots in it. The vessels were randomly distributed in growth chamber. Successful experiments were repeated at least 2 times. Bifactorial design were used to discard, or not, 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 $\arcsin\sqrt{x}$.

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, for this reason we have been tuning the protocol since 2005 on the base of the previous experiences. When direct introduction from field growing trees was used, low success was reached, moreover, it is a time consuming process, as the culture *medium* should be continuously renewed due to the profuse releasing of phenolics, being necessary, in some cases, the subculture every 24 h and, in general, not more than 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 them were highly recalcitrant several attempts being necessary, as well as high quantity of starting material; additionally other genotypes, 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 such a purpose we managed 1264 explants in 61 attempts; as a result only 83 segments,

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

belonging to 7 genotypes, were finally *in vitro* established, 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 sizing approximately 20 cm, bearing dormant buds, were introduced 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, with at least 20 mm length, were collected for the initiation (fig 1).

Smaller segments were not suitable because they normally form a big *callus* covering all the explants, avoiding the bud break and shoots growth. 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 compared to 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 during 2012 only 211 explants were managed from which 113 (53.5 %) were lost by different causes as bacterial (30.8 %) and fungi (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 breaking and small shoots formed, emphasizing in the importance of using only healthy shoots with at least 15 mm length as starting material. The advantages of this procedure is based probably on the fact that epicormic sprouts arise from inactive meristems produced during the juvenile phase, thus, 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. Despite that growth was stimulated we did not found any statistical influence on the length of microshoots during multiplication as it had also been

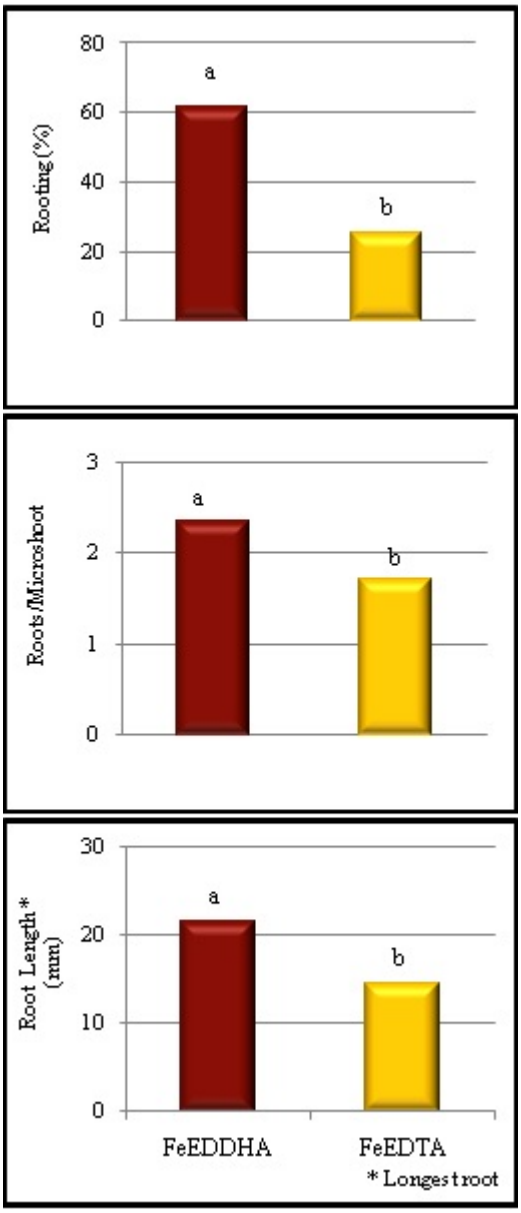

Fig 2 Effects of substitution of FeEDTA by FeEDDHA on rooting of walnut hybrid microshoots ($p \leq 0.05$)

observed in micropropagation of *Rosa hybrida* (Van der Salm e*t al*. 1994). However, the most dramatic results were reached on rooting and acclimation. Microshoots growing in the presence of FeEDDHA had a higher ability to root with higher numerosity 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

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)

number of shoots per 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 as the influence on chemical (pH) and physical (strength) properties of culture *medium*. Van der Salm *et al*. (1994) have not found important differences on pH in culture *medium* using both chelates, but for our conditions great variation were observed. The pH of culture *medium* supplemented with FeEDDHA had 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 values 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 subculture, precisely when the differences between both chelates are more evident. The definitive influence on improving the quality of microshoots replacing FeEDTA by FeEDDHA was observed during acclimation, step in which only 1 % of vitroplants that reached field plantation came from the first one, affected not only by chlorosis but yellowish of leaves, complete defoliation and apical death (fig 3); McGranahan and Leslie (1988) also found that the retention of leaves was important in promoting the explants survival and growth. An important conclusion was that there was none 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.

Phl (mg/L)	*Callus* Weight/ Microshoot (mg)	Nodes/ Microshoot	Microsh. Length (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}	$31,6^{a}$

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

The use of Phl during multiplication

In this process Phl has an important role, once it was not incorporated in 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 the *calli* formation and growth (table 1). Despite Leslie *et al.* (2004) finding that when 1 mM of Phl is added to multiplication *medium* the rooting is promoted,

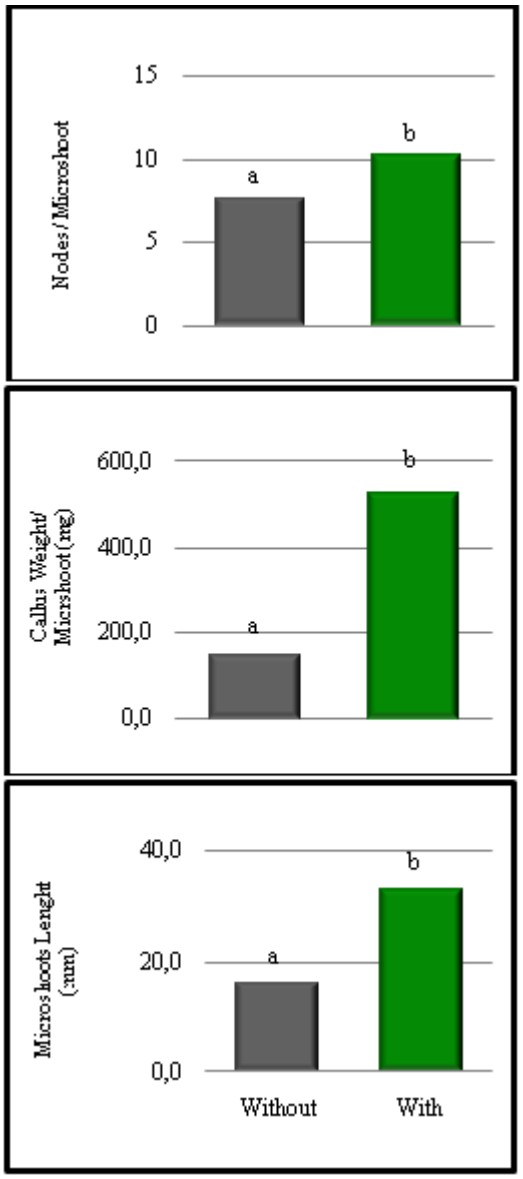

Fig 4 Influence of Phloroglucinol on *calli* formation and walnut hybrid microshoots growth ($p \leq 0.05$)

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 showed a more unstable behaviour of cultures although the auxiliary bud formation was clearly stimulated, especially in a specie that possess a high apical dominance as the walnut hybrid is (data not shown). Like with 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., hence, further experiments should be done under our conditions to determine if Phl dose has some influence on rooting and/or on the results of acclimation.

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; at the same time lignin formation is associated with aging. In walnut, it had been shown that lignin accumulation was lower in the shoot population that did not show visible signs of rooting (Bisbis *et al*. 2003), at the same time Vahdati *et al*. (2004) found in Persian walnut that the stimulation of growth has a negative influence

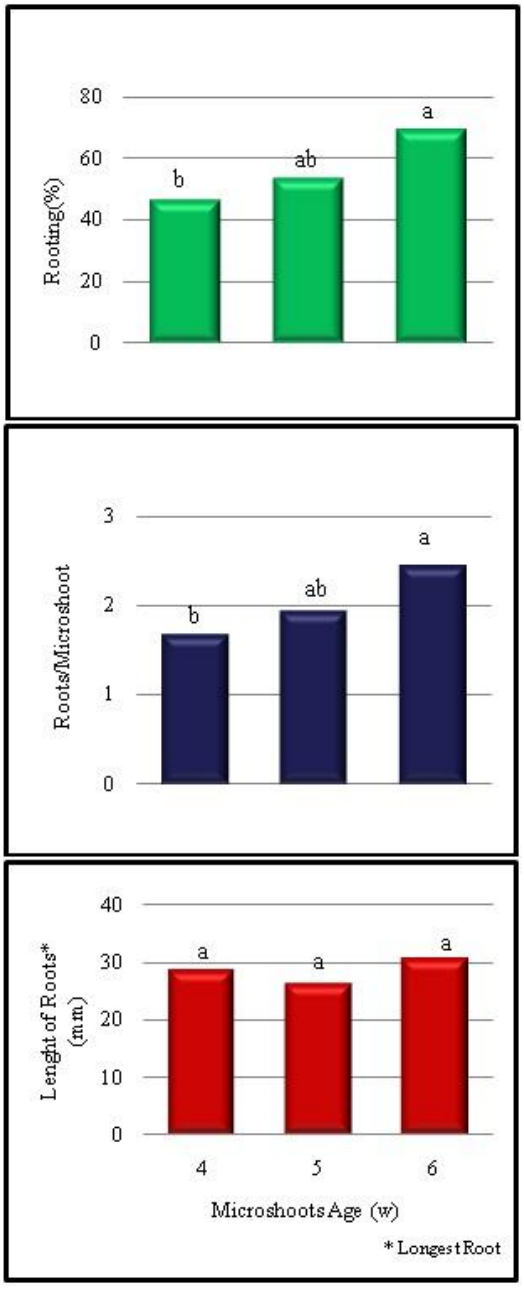

Fig 5 Influence of the length of subculture on rooting of micropropagated walnut hybrids ($p \leq 0.05$)

on root ability, 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) and that 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 a direct answer of root percentage and number of roots formed, regarding the age of the microshoots (fig 5) with independence of genotype; microshoots with 6 weeks are more suitable for rooting than those that have 4 weeks. For some genotypes, as D53, the difference goes from 0 % of rooting, if microshoots during the 4th week of subculture are used for root pre-induction, up to more than 90 % if those have 2 weeks more. These results should be probably influenced by the reduction of the active growth, the aging of microshoots and lignin accumulation, closely related to a higher disposition to rooting.

Dose of sucrose during pre-induction phase

Osmolytes, as sucrose, have a great influence on water potential of culture *medium*, also acts as carbon source, mostly determining the morphogenic answer as well (George, 1993). We did not found any explicit reference to determinate the influence on root pre-induction of different carbon sources and/or concentrations during pre-induction, despite rooting of rooting is divided in two steps; only experiments had been done during expression (Dolcet-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 certainly the dose of sucrose was determinant on rooting and even on 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, growth of radical system is comparatively higher than shoots 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

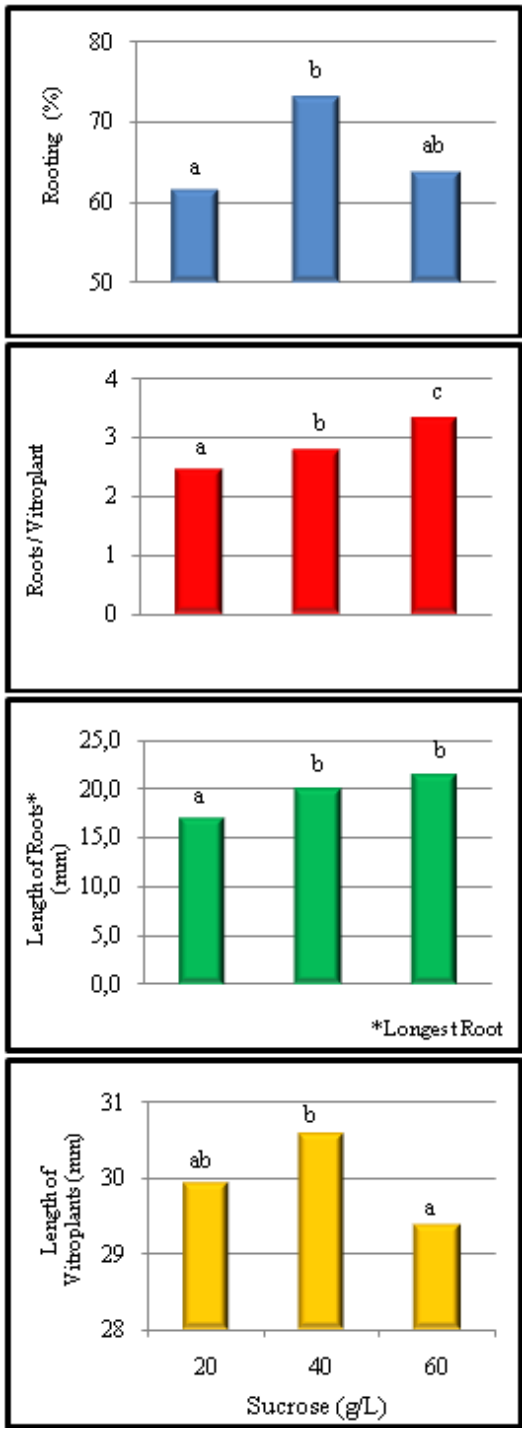

Fig 6 Effects of different doses of sucrose during pre-induction phase on rooting and growth of walnut hybrid microshoots ($p \leq 0.05$)

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 ability for

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

Table 2 Results on rooting and growth of different walnut hybrid vitroplants and sucrose doses ($p \leq 0.05$)

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 prevent the other one from having the same development. Supporting this hypothesis is the finding of Nobel *et al.* (1989) in *Agave lechuguilla* when conditions favouring shoot growing treatment (nitrogen and phosphorus supply) is undertaken the dry weight of roots was approximately constant meanwhile shoot dry weight was increased. Smith *et al*. (1991) also found for several woody species that since lateral roots were not developed, *in vitro* plants maintained vigorous shoot growth, whereas it was temporally suspended while plants initiated roots *ex vitro*.

Although statistical interaction between genotype and sucrose dose were observed, the worst results were obtained for 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 of 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 liquid culture (Roschkel and Pijut, 2007) showing the feasibility to clone black walnut using this system, although we did not succeed when shaking cultures were done. 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. Fitting the immersion time, the volume of culture *medium* and the vessel size we decided to determine what kind of explant was suitable for TIS once abnormal microshoots were obtained when the same explant for traditional micropropagation was used for initiation. 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 could be associated with the utilization of explants with pre-formed basal-*callus* as an expression of adaptation to *in vitro* culture conditions as well as the opening of leaves, that could also contribute to the absorption the components of the culture *medium*. As a consequence, microshoots formed from A2 had a normal appearance similar to that obtained of GM (fig 7C). The results obtained with B2 are, in general, an overestimation because the size of initial explants almost doubled the one under the other treatments; the microshoots obtained with this treatment were curled in the base (fig 7B)

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}

Table 3 Influence of the explant kind on elongation of walnut hybrid microshoots in TISs ($p \leq 0.05$).

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 developed which pointed to the need of previous adaptation and/or a complete formation of *callus* prior to the inoculation in TISs. Despite the genotype used, B1 was the worst explant, with the lower growth as well as with 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, also presented 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, for this reason 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.

Resume of walnut micropropagation and main results obtained

Here some key factors had been analyzed, that helped us to set the bases of 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 as age of microshoots and sucrose dose during root pre-induction also helped us to enhance the root ability for all genotypes used. Vitroplants

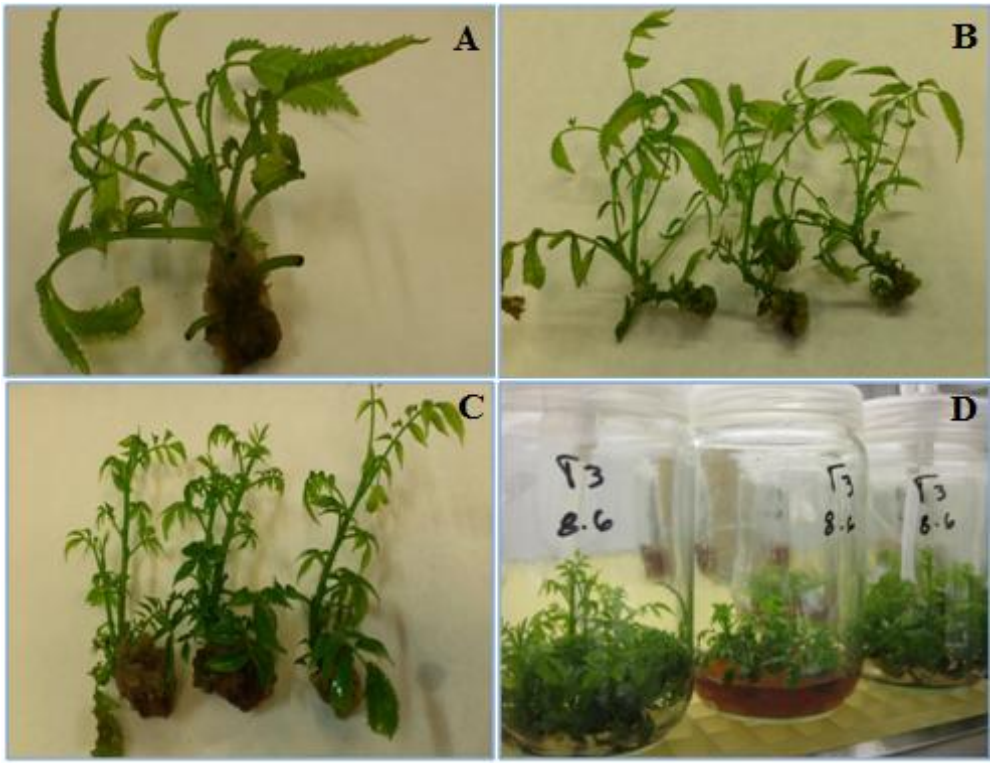

Fig 7 Behaviour of different kind of explants in temporary immersion systems (a) B1, (b) B2 and (c) A2, compared to (d) traditionally micropropagated walnuts in gelled *medium*.

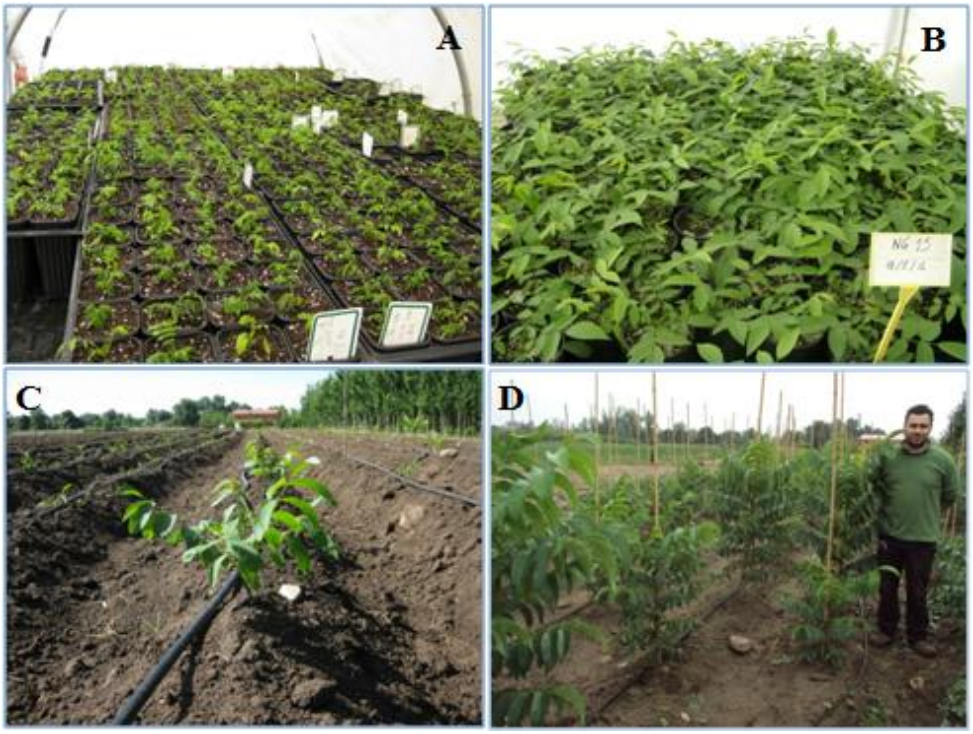

Fig 8 Walnut hybrid vitroplants after transplantation to *ex vitro* conditions (a), 3 months old vitroplants in greenhouse (b); field planted in May, 2012 (c) and the same vitroplants 4 months later (d), photo taken September, 26th, 2012.

acclimated showed a high vigour developing a profuse radical system in few weeks which contributed to obtain 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). In our conditions this protocol had also been used to micropropagate some other walnut species as *J. nigra*, *J. regia* and Vlach, a hybrid Paradox.

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