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Micropropagation of valuable walnut genotypes for timber production: new advances and insights

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Abstract - The intensive production of timber from walnuts is mainly hampered by the scarcity of varieties for this purpose. While the hybrid progeny Mj209xRa is considered suitable for timber production in Europe, problems associated with its recalcitrance, the low ability for rooting and the high mortality of acclimated vitroplants, limit the reproduction of elite trees. This research was aimed to assess the influence of two methods for the in vitro introduction of several walnut genotypes, to determine the effects of temporary immersion systems (TIS) on proliferation and rooting, and to reduce the random losses of acclimated vitroplants. Hence, trees from Mj209xRa progeny as well as some common walnuts were used. As an outstanding result, the in vitro establishment of 6 out of 7 trees was obtained. Also important was the improving of quality of microshoots, the multiplication ratios and the rooting using TIS through the management of the kind of bioreactor, the volume of culture media, and the kind of explant inoculated. The direct transplant to field nursery of acclimated vitroplants considerably increased their quality, while mortality was highly reduced. Certainly, these results represent a great contribution to the current micropropagation protocol, especially with the potential introduction of TIS for massive plant production.

Keywords - Temporary Immersion, TIS, acclimation, vitroplant, Ex Vitro management, potted plants.

Introduction

All species of *Juglans* genre are considered highly recalcitrant to tissue culture. This term describes the reduced response of different species and kind of explants (organs, tissues, cells) to be in vitro cultured. During '80 were published the first successful reports on micropropagation of walnuts (Chalupa 1981, Driver and Kuniyuki 1984, McGranahan et al. 1987, Rodriguez et al. 1989); however, few laboratories worldwide have developed the capacity for commercial micropropagation.

In vitro establishment, along with rooting and acclimation, is considered an unpredictable and highly-difficult stage of walnut micropropagation. While most of reports are referred to the use sexual tissue for in vitro initiation, few of them use somatic organs and/or tissues. The recalcitrance along with the releasing of phenolics, and microbial contaminants are among the main factors that impair the success of in vitro establishment from non-sexual starting materials. The individual and combined importance of these factors are highly dependent of the physiological age and the direct exposition of donor plants to climatic elements (George 1993). Thus, explants from adult field-growing trees are less responsive than grafted plants under greenhouse conditions (McGranahan et al. 1987, Stevens and Pijut 2018). The use of juvenile explants also contributes to improve the results of in vitro introduction (McGranahan et al. 1987, Licea-Moreno et al. 2012). Genotype is an additional factor that become the in vitro establishment in a challenge, since this stage is also genotypic dependent (McGranahan et al. 1987, Gotea et al. 2012, Licea-Moreno et al. 2012, Stevens and Pijut 2018).

Proliferation is probably the less problematic stage of walnut micropropagation. However, higher multiplication ratios than those normally obtained are necessaries for commercial micropropagation. Few references exist about the multiplication ratios for walnut. Cornu and Jay-Allemand (1989) found that using semi-solid culture media in 6 weeks the number of bud-clusters is multiplied for 1.5; whereas for hybrid Mj209xRa it is above 3 (Licea-Moreno et al. 2015). Agar and gellan gums, beside to increase the production costs, render low multiplication ratios; meanwhile liquid cultures promote both productivity and proliferation. Stevens and Pijut (2018) have determined that liquid media promoted the multiplication compared with gelled one, although do not offer numerical references regarding its ex-

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tend. Undoubtedly, the use of a rotary liquid system, as the used by Stevens and Pijut (2018), increase the in vitro multiplication of walnut; although it might represent some disadvantages for commercial micropropagation since problems with the optimal use of space and higher establishment costs might arise. The use temporary immersion systems (TISs) might help to overcome these constraints, rendering additionally the same advantages of rotary liquid systems. Licea-Moreno et al. (2012) published the first approaching to the use of TISs in walnut. Although multiplication and elongation were improved, it was suggested that more efforts must be done for the profitable use of TIS in commercial micropropagation of walnut since several abnormalities had arose, as hyperhydricity, excess of callus formation, and the curving of the base of stems of microshoots.

Also scarcer are the references regarding management of vitroplants after acclimation, considered a critical stage, once the cost and the future of new exploitations are highly dependent of the quality of plantation materials (South and Mexal 1984). For the establishment of new exploitations at Bosques Naturales (Spain), potted plants have been used traditionally. However, from the beginning unexplained occasional losses had been affecting random lots. These losses are preceded by abnormalities, characterized by wilting and rolling of leaflet and severe defoliations, amongst the most conspicuous damages, which presumable drive to the death of the more affected plants. It has been determined that these disorders are not associated to clones, although some genotypes are more sensitives than other. Pathogens have also been discarded as a primary cause; whereas, previous observations have allowed to detect that the above-mentioned abnormalities and mortality are associated with the type of substrate as well as the fertilization that have been used.

Aimed to increase the volume of plant produced as well as to address some of problems that impair the micropropagation of élite trees from the hybrid progeny Mj209xRa, some tasks were conducted, as *(i)* the in vitro introduction of new genotypes, *(ii)* the use of TISs, and *(iii)* the assessment of different managements of acclimated vitroplants, and their results are here presented.

Materials and Methods

Plant material

For *in vitro* introduction, three different sources of plant materials were used. The first were sticks collected in May 2017, from grafted plants cultured in greenhouse belonging to the Selection Program of Bosques Naturales S. A. (BN, Spain). These are putative hybrids from the progeny Mj209xRa (*J. major* (Torrey) Heller) var. 209 x *J. regia* L.) and were named with an initial uppercase letter D followed by a number or an uppercase letter. The second source, from CREA (Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Italy), was formed by dormant branches collected in March 2017 from field growing trees from Mj209xRa progeny (2) and one common walnut (*J. regia* L.). These were codified with an initial FD followed by a number. The third was formed by actively growing sticks collected in May 2017 from a grafted common walnut (also under greenhouse conditions) from IndP (Industrial Plants, Bulgaria) that was named as IPW. For the rest of experiments, it is described properly what genotypes were used; however, all of them are from progeny Mj209xRa, selected in BN for their outstanding phenotypes for timber production.

General in vitro culture conditions

The corrected formulation DKW-C (McGranahan et al. 1987) was used for in vitro culture. The micropropagation schedule was the proposed by Licea-Moreno et al. (2012) and Licea-Moreno et al. (2015). In BN, for proliferation, 20 explants were inoculated in Microbox vessels (model O118/120+OD118, SAC 02, Belgium) containing 150 ml of culture medium. Cultures in BN were stored under a 16h/8h photoperiod, with an average light intensity of 50 µmol m^{-2} s^{-1} and a temperature of 24±0.2°C. Whereas in IndPl a 14h/10h photoperiod was used, with an average light intensity of 30 µmol m^{-2} s^{-1}, and temperatures between 23-25°C. For acclimation, the procedure described by Licea-Moreno et al. (2012) and Licea-Moreno et al. (2015) was followed. All the experiments were performed in 2017 and 2018.

In vitro introduction

It was performed in BN facilities. Genotypes from the selection programs of BN (3) and CREA (3) as well as a tree belonging to IndPl were used (Tab. 1). Two procedures were followed, depending of starting material and the season in which these were collected. For dormant materials (Method 1), buds were forced to sprout to obtain suitable shoots for introduction. Those grafted materials that were actively growing (Method 2) were introduced directly. Once explants have passed disinfection step were inoculated in a culture medium supplemented with 4.4 µM of BAP, 0.5 µM of IBA, and 60 mM of Phloroglucinol (PG). Explants contaminated with bacteria and fungi were counted and discarded, as well as, those that do not respond, that finally died.

Putative clean explants were inoculated in a bacterium-indexing medium (BIM) to detect microbial contaminants. Afterward, the presence of colonies growing in culture medium was assessed again. Only clean explants were able to continue with the establishment stage. For more details regarding the *in vitro* introduction process see Licea-Moreno et al. (2012) and Licea-Moreno et al. (2015). Because of the reduced and variable number of explants assessed it was not possible to perform statistical analysis in this stage.

Effects of temporary immersion systems on proliferation and rooting at BN

Two kind of bioreactors were used: Plantform© (Fig. 2a) and handmade temporary immersion bioreactors (TIB) (Fig. 2b). For the construction of TIBs, glass vessels (720 cm^3) were used. Four immersions (2 minutes each), besides four extra aerations, per day were performed. Liquid culture medium, supplemented with 4.4 µM of 6-Benzylaminopurine (BAP), 0.5 µM of Indole-3-Butiric Acid (IBA), and 40 mM of Phloroglucinol (PG), was used. In the previous stage to root pre-induction, 40 and 15 explants were cultured during 6 weeks in Plantforms (450 ml of culture medium) and TIBs (100 and 200 ml of culture medium), respectively. Nodal segments with or without (apical and basal segments were discarded) basal callus were used as explant sources. Both kind of explants bore 2-3 buds each. Cultures in gelled media were used as control. Root pre-induction and root expression stages were performed according to the procedure described by Licea-Moreno et al. (2012) and Licea-Moreno et al. (2015). Clone D15 was used for this experiment. Multiplication ratios, explants with at least a sprouted bud, explants with more than one sprouted bud, and the rooted microshoots were assessed. The multiplication ratios were calculated dividing the number of explants obtained between the initial explants inoculated. A bud was considered sprouted when at least a leaf has been formed. The bioreactor and the vessel for control treatment were considered the basic experimental units; thus, every variable analyzed was the average of the corresponding experimental unit. For each treatment of TIS at least 2 bioreactors were used, whereas control was formed by 5 vessels. The experiment was repeated one more time.

Effects of temporary immersion systems on proliferation at IndPl

Plantform bioreactors were used in this experiment. The cultures were incubated for 7 weeks, instead of the 30-days used for the traditional cultures, in DKW-C gelled media (WM3). Explants bearing apical tips, with approximately 20 mm length, were used. In each bioreactor, 15 explants from 30-days old cultures in WM3-C (for composition, see Tab. 3) were inoculated. A volume of 450 ml of culture media per bioreactor was used. Immersions of five-minutes, with intervals of two-hours, were programmed. Two ways to prepare DKW-C formulation and different hormonal combinations (Tab. 3) were prepared. The number and quality of formed microshoots were assessed. Clone D15 was used for this experiment.

Influence of different management procedures on growth and mortality of acclimated vitroplants

This task was performed at BN facilities. Acclimated vitroplants of clone D117 were used. Three variants were assessed: (1) transplant to pots (3.5L); (2) direct transplant to field nursery using plastic cover and (3) direct transplant to field nursery without plastic cover. For pots, a mix of Gramoflor (80-20 blonde-dark peat mix, recipe 2007, Gramoflor, Germany) with fertilization, as is described below, and a soil revitalizer (Organia Viventia, Fertinagro Nutrientes S.L., Spain) was used. For field nursed plants (variants 2 and 3), fertilization was not applied. The control of weed for covered plants was not necessary (variant 2), while for those uncovered (variant 3), both chemical and mechanical control were applied. The transplant was performed from May 25th to 29th, 2017.

The fertilization schedule used for potted vitroplants was as follow:

- First month: fertigation 2 times/week with equilibrium 1-5-1 (Peters Professional 10-52-10+TE, final dose 600 mg/plant).
- Second month: fertigation 3 times/week with equilibrium 1-1-1 (Solinure FX 20-20-20, final dose 2,700 mg/plant).
- Third month in advance to the end of September: fertigation 3 times/week with equilibrium 1-6-12 (Kristalon Orange 6-12-36+Mg, final dose 1,890 mg/plant).

Although periodical evaluations were made (every 3 weeks), only the data collected during the first week of October 2017 were considered for statistical analysis. The length and diameter (at the stem base) of 50 vitroplants per treatment were then measured; thus, the ratio diameter/height was determined. The number of leaves per vitroplant was also counted. For each variable, the average of 10 vitroplants was determined; hence, 5 replications were considered in a random model.

The general state of vitroplants was also considered, since some disorders in potted vitroplants were observed. These were basically several grades of wilting and leaflet's rolling. To classify these damages a scale was established: 0 – no damage; 1 –few leaflets affected; 2 – less than 50% of leaves and/or leaflets affected and 4 – more than 50% of leaves and/or leaflets affected.

Statistical analysis

Analysis of variance (ANOVA) was used to determine statistical significance, and the Fisher's least significant difference (LSD) was used as a post hoc test for pattern detection. Percentage data were transformed using the formulae arcsin. The software InfoStat (Di Rienzo et al. 2015) was used for calculations.

Results

In vitro establishment

Only 30% of the introduced explants were successfully established (Tab. 1). The main causes of losses were the incapability to respond to in vitro conditions (44.6%), contamination with fungi (16.9%) and bacteria (3.8%). A second screening using BIM revealed that 6 apparently clean explants were contaminated with undetermined microorganisms (Fig. 1e). Only one genotype (FD94) failed to be in vitro established, because of (1) sticks bore mostly floral buds, and (2) the only shoot obtained died after the second subculture. Hence, 6 out of 7 genotypes were successfully in vitro established; however, they responded in different ways. After more than 10 months, two of them (D113 and D116) have already reached the exponential state of multiplication, being incorporated to the next stage of micropropagation (proliferation, stage 2); whereas the other four are still managed as if they were recently in vitro introduced; being necessary to perform frequent changes (2 to 4 weeks) of culture medium to maintain vigorous growths.

Those clean-explants, with positive response to tissue culture showed the same behaviour pattern. The first visible sign of the adaptation was the formation of a basal callus, followed by the sprouting of buds (Fig. 1d). Once in vitro formed shoots from the introducing method 2 have reached a minimum of 10 mm length, bearing healthy and green leaves, were then ready to be separated from the original explant (yellow arrow, Fig. 1d). Most of shoots that reached this state, were able to form healthy and vigorous microshoots (Fig. 1f).

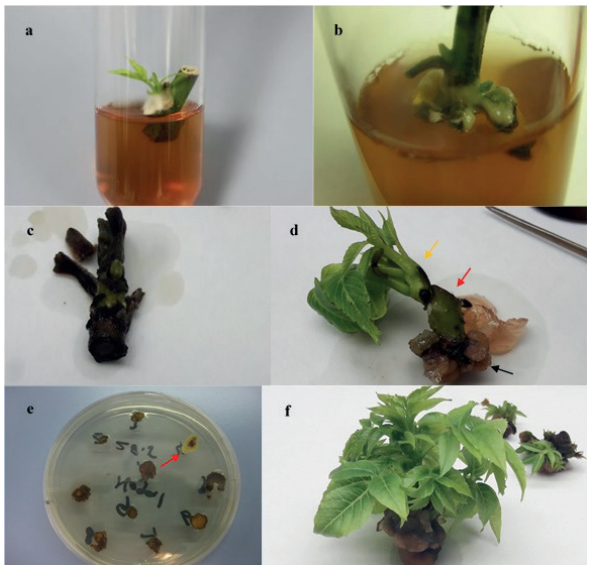

Figure 1 - Selection of clean and viable explants during in vitro establishment. (a) Fungus contamination. (b) Bacterial contamination. (c) Non-responding explant. (d) Responsive explant (red arrow) with basal callus (black arrow), bearing a healthy sprouted bud (yellow arrow). (e) Indexing on bacteria culture medium; with red arrow a contaminated explant. (f) Clean-vigorous microshoot.

Despite both methods were suitable for in vitro establishment, the best results were obtained when sticks bearing dormant buds were used as starting material (Method 1). With this method, 54.3% of explants were not contaminated and/or survived to introduction, whereas when epicormic branches from grafted plants (Method 2) were used, only 24.8% were established. However, losses by con-

Table 1 - Results of in vitro introduction of different walnut genotypes.

Genotype	Origin	Species	Number of Introductions	Introd. Method	Explants	Losses				Established Explants
						Fungi	Bacteria	Death	BIM	
FD94	CREA	*J. regia L.*	1	1	1	0	0	1	0	0 (0%)
FD99	CREA	Mj209 x Ra	3	1	8	1	1	0	2	4 (50%)
FD103	CREA	Mj209 x Ra	5	1	16	2	1	0	4	9 (56%)
IPW	IndPI	*J. regia L.*	1	2	19	1	3	6	0	9 (47%)
D101	BN	Mj209 x Ra	1	2	23	5	0	16	0	2 (9%)
D116	BN	Mj209 x Ra	1	2	33	9	0	19	0	5 (15%)
Z3	BN	Mj209 x Ra	1	2	30	4	0	16	0	10 (33%)
Total					130	22	5	58	6	39 (30%)

tamination were greater for explants from method 1 (45.8%) than those from method 2 (20.9%); being then the main cause of failure for method 2 the death of explants, accounting for 54.3% versus 0% from method 1 (the only explant introduced from tree FD94 were not considered). Outstanding was that releasing of phenolics was not observed, although weekly subcultures were necessary during at least the first month.

Effects of temporary immersion systems on proliferation and rooting at BN

Huge biomass production occurred for both kind of bioreactors (Fig. 2a and b). In general, microshoots obtained from TISs look healthy, with long internodal spaces, especially those cultured in Plantform (Fig. 2d), showing also light hyperhydricity signs in the basal leaves.

When nodal segments with basal callus were used as inoculum, the multiplication ratio and the percentage of sprouted explants in TIB were affected (Tab. 2); although both variables were improved increasing the volume of culture medium from 100 (TIB100) to 200 ml (TIB200) (Tab. 2 and Fig. 2e). The multiplication ratio and the quality of microshoots from nodal segments without callus cultured in TIB (Fig. 2e) were, at least, like the obtained in gelled media (Fig. 2c), regardless the volume of culture medium used. Whereas, microshoots from Plantforms had a lower multiplication ratio compared to the control. The situation was worse when nodal segments with callus were cultured in Plantform. At the end of subculture these microshoots showed wilting and a deep defoliation. That is why the results of this kind of explant are not here presented. A noteworthy result was the promotion of bud sprouting because of the use of TIB, especially when explants were inoculated with 200 ml of culture medium.

Microshoots obtained from TISs were able to be rooted with variable results both in rooting percentage and the quality of vitroplants. The lowest rooting was registered for microshoots from nodal

Figure 2 - Effects of different conditions on multiplication and rooting of clone D15 using temporary immersion systems at Bosques Naturales (a-h) and Industrial Plants (i-k). (a) Plantform bioreactor. (b) Temporary immersion bioreactor (TIB). (c) Microshoots from gelled media (control). (d) Microshoots from Plantform. (e) Microshoots obtained from nodal explants bearing callus, cultured in TIB using 200ml of culture medium. (f) Rooted microshoots from gelled culture media. (g) Rooted microshoots from Plantform. (h) Rooted microshoots obtained from nodal explants bearing callus, cultured in TIB using 200ml of culture medium. (i) Microshoots cultured in Plantform bioreactors. (j) Details of the basal J-shaped of microshoots in WM3. (k) Microshoots cultured in DKW-C2 showing abundant callus formation in the base of stem.

segments without callus cultured in TIB with 200 ml of cultured medium, followed by nodal segments with basal callus in TIB with 100ml of culture medium. Whereas, microshoots obtained from nodal segments with callus inoculated in TIB using 200

Table 2 - Effects of temporary immersion systems (TIS) on proliferation and rooting of clone D15 in Bosques Naturales.

Bioreactor	Explant	Multiplication Rate1	Explant Sprouted (%)	Explants w/ 2 Sprouted Buds (%)	Rooting (%)
Control	Nodal	5a	87.8ab	14.3bc	70.0b
Plantform	Nodal	3a	80.0b	10.0c	55.6c
TIB100	Nodal	5a	85.7ab	28.6ab	63.6c
	Nodal w/callus	2b	50.0c	28.6ab	40.0d
TIB200	Nodal	5a	93.8a	31.3a	33.3e
	Nodal w/callus	4a	86.7ab	33.3a	87.5a

[1] Rounded values. However, for statistical analysis 2 decimal positions were used

ml of culture medium had the highest rooting percentage. At the same time, they had the best quality, since healthy vitroplants with well-developed root systems were obtained (Fig. 2h), even better than the obtained from traditional micropropagation (Fig. 2f). On the other side, rooted microshoots from Plantform (Fig. 2g) and from nodal segments with basal callus cultured in TIB using 100 ml were affected by deep wilting and defoliation.

Effects of temporary immersion systems on proliferation at IndPl

Unexpectedly, no-proliferation of axillary shoots from any treatment was obtained. Thus, low multiplication ratios were observed for all treatments (Tab. 3); however, striking differences were observed. The best treatment was that combining PBA, mT and TDZ in DKW-C formulation (DKW-C2). For this variant, microshoots grew more than those cultured in gelled medium (WM3-control), with robust stems and large leaves. The introduction of GA (WM3) and mT (DKW-C1) improved the quality of microshoots, although not the multiplication ratios. Most of the resulting microshoots from TIS were vigorous, green and healthy, but showing J-shape in the base of stems. The total absence of shoot tip necrosis using TIS is an encouraging result, since apical death appears in cultures in glass jars on semi-solid media at the end of every subculture period.

Influence of different management procedures on growth and mortality of acclimated vitroplants

Although acclimated vitroplants showed a great vigor (Fig. 3a), their direct transplant to field nursery only 6 weeks after the end of *in vitro* culture represented a challenge; however, 92% of them survived. During the first 6 weeks after transplant, the potted plants reached the greatest height, while in average the biggest diameter was for vitroplants with plastic coverture (data not showed). To this point, the evaluations would suggest that the best treatment will be the transplant to pots under green-

Figure 3 - Effects of different managements on the growth of acclimated vitroplants of clone D117. (a) Acclimated vitroplant before transplant (May 25th, 2017). (b&c) Potted vitroplants 3 months after transplant (August 31th, 2017). (d) Potted vitroplant dead after resuming the growth during the spring of 2018. Details of root system and stem showing apical death. (e&f) Vitroplants with plastic cover 3 months after transplant (September 6th, 2017). (g) Vitroplant from field nursery cultured with plastic cover ready to be planted (January 31th, 2018). (h&i) Vitroplants without plastic cover 3 months after transplant (September 6th, 2017). (j) Vitroplant from field nursery cultured without plastic cover ready to be planted (January 16th, 2018). (k-n) Different levels of wilting and leaflets's rolling of potted vitroplants. (k) Grade 0 - not affected vitroplants. (l) Grade 1 - few leaflets affected. (m) Grade 2 – less than 50% of leaves and/or leaflets affected. (n) Grade 4 – more than 50% of leaves and/or leaflets affected.

Table 3 - Effects of different formulation and hormonal combinations on proliferation and on quality of microshoots using TIS in Industrial Plants.

Formulation	BAP (mg/L)	mT (mg/L)	PBA (mg/L)	TDZ (mg/L)	GA (mg/L)	Ratio of Sprouted Buds	Observations
WM3-C (control)	-	-	-	-	-	1	Large leaves, relatively weak shoots as compared to the other treatments.
WM3	-	-	-	-	10	1	Large leaves, more robust shoots than in simple WM3-C.
DKW-C1	0.5	1	-	-	-	1	Similar to WM3.
DKW-C2	-	0.5	1	0.07	-	2	Healthy, large leaves, robust shoots, total lack of axillary shoot proliferation, as in the treatments above.

WM3: DKW dry powder (without Ca(NO)3) formulation according to Licea-Moreno et al. (2015), supplemented with Ca(NO)3x4H2O (8.3 mM); BAP (3.1 µM); PG (40 mM); sucrose (87.6 mM) and Gelrite (1.2 g/L)

DKW-C: stocks solution from the corrected formulation used by Licea-Moreno et al. (2015)

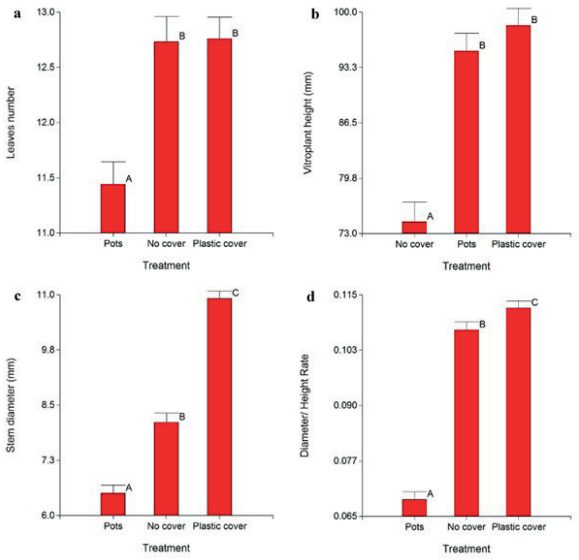

Figure 4 - Effects of different managements on the growth of acclimated vitroplants of clone D117. (a) Leaves formed per vitroplant. (b) Height of vitroplants. (c) Basal diameter of stem. (d) Ratio diameter/height of vitroplant. Treatments with different letters are significantly different (p≤0.05, LSD test).

house conditions. For the 11th week, vitroplants covered with plastic surpassed to those potted for all variables assessed (data not showed). Even, vitroplants without cover, except for height, had greater number of leaves, diameters and diameter/height ratios than the potted plants, as was confirmed at the end of experiment (Fig. 4). Plants nursed under field conditions, both with plastic cover or not, had the best quality, growing in a compensate way, with diameter/height ratios above 0.1, producing plants ready-to-plant with well-developed roots systems and healthy stems (Fig. 3f). Whereas potted ones had the less developed root systems (Fig. 3e), and the lowest stem diameters, which had great influence on the obtaining plants with reduced D/H ratios, below 0.077.

For this assay, the syndrome of wilting and rolling leaflets was also assessed (Fig. 3d and e). Outstanding was the fact that vitroplants that grew in field nursery, with or without cover, were not affected at all by this disorder. On the other side, the potted vitroplants showed a progressive increasing of syndrome from the 7th week in advance. Thus, during the last evaluation, 67% of vitroplants were affected with grades going from 1 to 2. After winter 2018, the mortality of field-nursed vitroplants, both with or without cover, account for less than 8%; whereas, approximately 30% of the potted plants died, most of them after resuming the growth during the next spring (Fig. 3e).

Discussion

Most of reports on walnut micropropagation use sexual materials (seed, embryos) for *in vitro* initiation (Cornu and Jay-Allemand 1989, Revilla et al. 1989, Vahdati et al. 2009, Tuan et al. 2016), which is a great disadvantage if the purpose is to propagate a specific genotype. Microbial contaminations, phenolization, genetic determinism, and the incapacity of somatic tissues to respond to *in vitro* culture, among the main factors, hindering the utilization of nonsexual tissues as starting materials in walnut tissue culture (McGranahan et al. 1987).

Using sticks from adult field-growing trees of the hybrid progeny Mj209xRa, it has been determined that the success of *in vitro* establishment depends of the genotype, as well as the origin and the age of the starting material (Licea-Moreno et al. 2012, Licea-Moreno et al. 2015, Licea-Moreno 2016). In American black walnut, was also determined that the physiological age of the explants essentially determined if microshoots elongated and survived, regardless of cytokinins used (Stevens and Pijut 2018). Although the releasing of phenolics, a typical response of adult and actively growing tissues and organs, was not observed, the use of younger starting materials could have allowed to reduce the mortality of explants, increasing then the success of method 1 regarding method 2. However, both methods were useful to establish in vitro new walnut genotypes. Even with method 2 was possible to introduce genotypes that had been impossible to introduce using method 1, as was the case of clone D101 (see Licea-Moreno 2016).

As in previous reports (Licea-Moreno et al. 2012, Licea-Moreno et al. 2015, Licea-Moreno 2016), the main causes of losses were microbial contaminations, and the dead of explants. McGranahan et al. (1987) mentioned the latent contaminations amongst the main problems for walnut micropropagation. Stevens and Pijut (2018) also registered that microbial contamination often led to the collapse of cultures from *J. nigra*. Although phenolization causes most of fails during in vitro establishment of walnuts (McGranahan et al. 1987, Stevens and Pijut 2018), using as starting materials juvenile shoots forced to sprout under controlled conditions in the laboratory (Method 1) was not observed the releasing of phenolics to culture media. Even, with the direct introduction of explants from sticks collected from grafted plants (Method 2) no-phenolization was neither detected; suggesting that the younger are the explants, the higher are the possibilities to be in vitro established. It is seeming that rejuvenation is the suitablest procedure to reduce the effects of phenolic releasing in walnuts, as have been recommended for several forestall species (Bonga and Von Aderkas 1992), included walnuts (McGranahan et al. 1987).

In gelled media, steady multiplication ratios between 3 and 5 have been obtained for clones from the

Mj209xRa hybrid progeny (Licea-Moreno et al. 2015). However, for commercial propagation higher multiplication ratios would be desirable. Although the positive effects of liquid media on walnut multiplication have been demonstrated both for stationary (Heile-Sudholt et al.1989) and agitated systems (Stevens and Pijut 2018), these might not be suitable for massive plant production. Temporary immersion has been considered appropriated for commercial micropropagation of forestall species as eucalyptus (McAlister et al. 2005), pistachio (Akdemir et al. 2014), chestnut (Vidal et al. 2015) and teak (Quiala et al. 2014). The first known report of the use of TIS for walnut micropropagation was made for some clones of the hybrid progeny Mj209xRa (Licea-Moreno et al. 2012). Despite some abnormalities of microshoots were observed (hyperhydricity, excess of callus on the stems, and the curling of the base of microshoots, among the most frequent) elongation and multiplication were increased, allowing to propagate one clone in TIB. Nevertheless, was suggested that more efforts would be necessaries to take advantage from the use of TIS on walnut micropropagation.

Certainly, the kind of bioreactor, the explant source and the volume of culture medium used had a great influence in the proliferation, rooting and the quality of vitroplants. Although the principle of temporary immersion is valid for both kind of bioreactors, the immersion has physical differences. Thus, the capacity to cover the inoculum with culture medium is bigger in TIB than in Plantform, especially when 200 ml were used. Consequently, the number of sprouted explants and the multiplication ratios seem to be influenced by this factor once lower figures were registered for Plantform regarding TIB. Interestingly, the quantity of explants with at least 2 shoots was increased with the use of 200 ml of culture medium instead 100 ml for TIB, pointing out to the importance to cover the nutritional necessities of plant materials to obtain suitable and proper results. The effects of volume of culture medium on multiplication have been demonstrated for other plant species as sugar cane (Lorenzo et al. 1998) and pineapple (Escalona et al. 1999). Similarly, in eucalyptus using the same quantity of culture medium, the multiplication rate decreased increasing the inoculum (McAlister et al. 2005), suggesting the exhaustion of components of culture media as the main cause. The type of explant also had a great influence either on the multiplication, rooting and the quality of microshoots, confirming thus previous observations made for hybrid Mj209xRa (Licea-Moreno et al. 2012). In chestnut was also determined that the type of explant influenced shoot quality and proliferation (Vidal et al. 2015).

Huge differences were registered in IndPl regarding the experiments in BN with TIS, since low multiplication ratios were obtained, probably because of the use of different culture conditions (explant source, number of explants inoculated, the frequency of immersions, non-extra aeriation provided, incubation conditions, amongst the most important factors). However, some coincidences were also observed, once some treatments promoted the formation of vigorous and healthy microshoots. The J-shape resembles the curling of the base of microshoots reported by Licea-Moreno et al. (2012) when apical explants were used as inoculum, reinforcing the importance of the right selection of initial explant. Noteworthy was the removing of apical death, that has been affecting the culture in gelled media. Similar results have been reported in pistachio, being eliminated the shoot tip necrosis (STN) using RITA bioreactors (Akdemir et al. 2014).

Although the definition of standards of quality for the micropropagated plants was not an objective of this research, was necessary to establish a baseline since was not possible to find any reference about it. It has been stated the quality of nursery plants as the fitness for purpose (Willem and Sutton 1980). Therefore, the "quality" of seedling will vary depending on the objectives (South and Mexal 1984). Hence, the general purpose of any commercial micropropagation protocol, beside render low mortality rates, is to produce healthy and vigorous plants, that growing in a compensate way, able to adapt to the open field conditions. Previous observations have allowed to determine that, under normal conditions, those vitroplants with well-developed root systems and thick stems have the highest probabilities to survive. Thus, only the transplanted plants to field nursery reached and coupled these quality conditions. At the contrary, potted vitroplants, beside to have the poorer growth (D/H ratios far below 0.1), were affected by different grades of defoliation, which might have impaired their development. Thus, while the field nursed vitroplants are ready to pass to commercial plantations, the surviving potted plants must expend other season in nursery to improve their quality. Direct transplant, beside to become a suitable solution that would support the commercial production, has helped to demonstrated that the micropropagation protocol is able to produce high quality vitroplants, as has been previously suggested by Licea-Moreno et al. (2015) and Licea-Moreno (2016). Further experiments will help to determine which of these variables, e. g. diameter, height and D/H ratio, has the greatest contribution on survival and growth under exploitation conditions.

Conclusion

The results here presented might be considered a step forward toward the commercial micropropagation of elite trees from progeny Mj209xRa, and for other walnut genotypes in general. The in vitro establishment of new genotypes is a key factor for the assessment of promising clones with high potential for timber production. There-

fore, both of introduction methods here presented, based on the use of rejuvenated starting materials, have proved their suitability for the successful cloning initiation of outstanding trees. Similarly, the obtaining of good quality and rootable microshoots from TIS is a great achievement regarding previous reported results that might open the door to its use for massive propagation of walnuts, allowing the reduction of unitary costs of produced vitroplants. While, the proposed method to reduce the mortality is also able to produce high quality ready-to-plant vitroplants; however, more efforts must be done to find an optimal protocol to culture acclimated plants in pots.

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