ANNALS OF SILVICULTURAL RESEARCH
44 (1), 2020: 41-46

https://journals-crea.4science.it/index.php/asr

Technical note

Special Issue: HORIZON 2020 GA 728086 WOODnat
"Second generation of planted hardwood forests in the EU"

Clonal effect on rooting and acclimation rates for *in-vitro* micropropagation in hybrid walnut (*Juglans x intermedia Mj 209*): preliminary observations

Jesus Fernández-Moya[1*], Ricardo Julian Licea-Moreno[1], Ignacio Urbán-Martínez[1], Ruth M. Castro-Fernández[1], Cristina Ramírez-López-Ramallal[1]

Received 3/09/2019 - Accepted 4/01/2020 - Published online 07/02/2020

Abstract - The success of walnut (*Juglans* sp.) planted forests for timber production have been very variable and genetic material is considered as one of the main drivers (together with site selection and forest management) for the success or failure of the plantations, as the performance of the trees from seed material is very variable. Considering the relevance of this genetic material, several clones have been selected and research have been conducted in order to improve micropropagation procedures. The objective of the present study is to analyze the effects of different clones in the rooting and acclimation rates for *in-vitro* micropropagation in hybrid walnut (*Juglans x intermedia* Mj 209). The results show a significative effect of clones on the rooting and the total micropropagation efficiency rates, but not on the acclimation rate. The efficiency rate of D-117 (65%) is considered statistically higher than the one for D-15 (38%), caused by a higher rooting rate of D-117 (73%) compared with D-15 (55%), because acclimation rate (57%) did not show any clone effect. Considering these differences in the micropropagation success, it might be considered (together with other factors) for clone selection to increase the general performance of the plant production units in large-scale propagation.

Keywords - clone selection, hybrid walnut, microprogation, vitroplants, rooting, acclimation.

Introduction

Walnut trees are species of the genus *Juglans* spp. L., traditionally characterized by their highly-valued nuts and timber. Considering the high timber value and the shortage of the species, many walnut forest plantations oriented for timber production have been established during the last decades (Mohni et al. 2009). These plantations have been established with the common species of Persian or European walnut (*J. regia* L.) and Black or American walnut (*J. nigra* L., *J. major* (Torr.) A. Heller, *J. hindsii* (Jeps.) Jeps. ex R.E. Sm.) but also with several hybrids which have been specifically developed for timber production, e.g.: Mj-209xRa y Ng-23xRa (Aletà 2004, Victory et al. 2006, Mohni et al. 2009, Clark and Hemery 2010, Coello et al. 2013).

The success of this kind of planted forests have been very variable and the genetic material is considered as one of the main drivers (together with site selection and forest management) for the success or failure of the plantations (e.g. Aletà et al. 2003, Aletà 2004, Urbán-Martínez et al. 2013, Licea-Moreno 2016). To this respect, Aletà and Vilanova (2006) pointed out the high variability in the performance of the trees from seed material, despite the trees are genetically close between them. Considering the relevance of this genetic material selection for walnut (*Juglans* spp.) planted forests oriented for timber production, there have been two big research groups working in the clone selection in Spain: 1) from the public center IRTA (e.g. Aletà et al. 2003, Aletà 2004) and 2) from the private company Bosques Naturales SA (e.g. Urbán-Martínez et al. 2013, Licea-Moreno 2016). To this respect, the Spanish Register for Forest Reproductive Material includes 29 clones (21 for *J. nigra*, 8 for *J. regia* and 10 for *J. x intermedia*), 9 parents of family (5 for *J. regia* and 4 for *J. x intermedia*) and one seed orchard of *J. regia* (MAPA 2016).

Urbán-Martínez et al. (2013) published a detailed characterization of the selection process of the Bosques Naturales SA clones. At the moment, the *in-vitro* micropropagation plant production is focused in 4 clones (D-15, D-117, D-53 and D-M) selected by their vigour, trunk straightness (based on the classification of MacDonald et al. [2001]) and wood quality. The clone D-15 (commercial name NAT-7-BN] is the one most widely used by the company because it was one of the firsts to be introduced *in vitro*. The original D-15 plus tree is in Cáceres and it had, with 12 years old, 13.5 m height,

1 Bosques Naturales SA, Alcobendas, Madrid – Spain

*Corresponding author: jesusfernandez@bosquesnaturales.com

http://dx.doi.org/10.12899/asr-1929

20.6 cm DBH, 3 m of clear boles and a straightness score of 2/7. Fernández-Moya et al. (in press) analyze the field performance of the planted forests established with the clone NAT-7-BN in their forests at A Coruña, Toledo, Cuenca and Girona. The original D-117 plus tree is in Girona and it had, with 12 years old, 17.3 m height, 29.5 cm DBH, 5 m of clear boles and a straightness score of 7/7. The original D-53 plus tree is in Cáceres and it had, with 13 years old, 18.1 m height, 24.4 cm DBH, 5 m of clear boles and a straightness score of 4/7. The original D-M plus tree is in Cáceres and it had, with 13 years old, 15.2 m height, 22.6 cm DBH, 4 m of clear boles and a straightness score of 7/7.

Despite the interest on walnut clones, traditional vegetative methods are not suitable for massive reproduction of walnut èlite genotypes; becoming tissue culture technologies the most important alternative for cloning, but the high recalcitrance of walnut species hinder their commercial micropropagation (Licea-Moreno 2016). Since the first *in-vitro* procedures were developed for walnut (Driver and Kuniyuki 1984, McGranahan et al. 1987, McGranahan and Leslie 1988, Cornu and Jay-Allemand 1989), a number of modifications have been tested to increase success and extend the methodology to different species and hybrids (e.g., Dolcet-Sanjuan et al. 2004, Leal et al. 2007, Bosela and Michler 2008, Leslie et al. 2009, Vahdati et al. 2009, Toosi and Dilmagani 2010, Licea-Moreno et al. 2012 and 2015, Licea-Moreno 2016). In addition to the problem of introducing field-selected material to *in-vitro* culture, better procedures are needed regarding microshoot multiplication, microshoot rooting and plantlet acclimatization. Poor rooting has been reported as arguably the main factor limiting the establishment of clonal plantations (Woeste and Michler 2011).

As a consequence of these difficulties for the production of clonal plants in the nurseries, there is a shortage of clonal plant material in the market. To this respect, Bosques Naturales SA is one of the key players regarding clonal walnut planted forests in Europe. Since 1998, an experimental micropropagation protocol was developed (Licea-Moreno et al. 2012 and 2015, Licea-Moreno 2016) and the company has been producing around 2,000 vitroplants per year (high quality clonal plantets) in their research facilities. The objective of the present study is to analyze the effects of the different clones in the rooting and acclimation rates for *in-vitro* micropropagation in hybrid walnut (*Juglans x intermedia* Mj 209) using the available data of the annual production of the company Bosques Naturales SA.

Material and Methods

Experimental design: clones used and data collection

The present study is based on the analysis of the work during the 2017 plant production campaign (from 22/December/2016 to 6/November/2017) in the Bosques Naturales SA facilities in Madrid (*in-vitro* laboratory) and Galicia (nursery for acclimation). Hence, there is not a fixed and pure experimental design for this work and the available data of the annual production of the company is used for the analysis.

Within the pool of clones registered by Bosques Naturales SA (all of them of the hybrid walnut - *Juglans x intermedia* Mj 209), 4 clones were selected for the analysis: D-117, D-15, D-53 and D-M. All the selected clones are registered at the Spanish Register for Forest Reproductive Material (MAPA 2016). These clones come from a selection of of walnut èlite genotypes and introduced *in-vitro* in 2008/2009. More details of the selection and *in-vitro* introduction procedures have been previously published (Licea-Moreno et al. 2012, Urbán-Martínez et al. 2013, Licea-Moreno et al. 2015, Licea-Moreno 2016).

The data collection is derived from the daily/weekly control registered in the facilities. To this respect, the micropropagation is performed by batches of plants. There were 40 batches analysed: 16 from D-117 (2,356 explants), 14 from D-15 (1,455 explants), 5 from D-53 (345 explants) and 5 from D-M (299 explants).

In-vitro *micropropagation protocol*

The specific protocol developed by Bosques Naturales for walnut (*Juglans* sp.) *in-vitro* micropropagation has been published in detail in Licea-Moreno et al. (2012 and 2015) Licea-Moreno (2016). This protocol is divided into several stages: multiplication, elongation, pre-induction, rooting and acclimation. The present study is focused on the final stages of the protocol, which are described with more detail as follows.

Pre-induction stage.

This consists in 5 days of total darkness immersed in a medium (6 ml per explant) containing: 30 g L-1 of saccharose, stock solutions: 50% of macronutrients and 100% of micronutrients and vitamins from prepared DKW formula, 119 mg L-1 of FeEEDHA and considerable higher dose of IBA, 10 mg L-1.

Table 1 - ANOVA results of the effect of clones on the rooting rate (%), acclimation rate (%) and efficiency (%) of hybrid walnut (*Juglans x intermedia* Mj209) in vitro micropropagation.

Variable	F - value	P – value
Rooting rate	9.016	0.000138
Acclimation rate	1.05	0.383
Efficiency	2.567	0.0701

Rooting stage.

Microshoots from radical pre-induction stay for 2 to 3 weeks, depending on genotype, in a preparation of vermiculite (instead of agar), intermediate granulometry 0.5-3 mm, soaked with medium made off with 16.5 g L-1 of glucose, 119 g L-1 FeEDDHA and stock solutions: 50% of macronutrients and 100% of micronutrients and vitamins from prepared DKW formula. Cristal vessel are used with 10 explants per vessel with 80 g of vermiculite and 100 mL of medium, although this relation must be adjusted depending on each type of vessel.

Acclimation stage.

Finally, acclimation phase takes place for those explants that have developed roots in previous stage preferably. It will last in this stage for 4 weeks, first 2 weeks guaranteeing in a greenhouse a minimum of 75% of relative humidity (rh) and a lighting of 150 µmol m-2 s-1, the following 3rd and 4th week 70% of rh and 150 µmol m-2 s-1. To transplant the explants to pots for this stage, it is preferable to use thin black and blonde peat (50%-50%) mixed with one part of vermiculite.

Statistical analysis

Rooting rate (%), acclimation rate (%) and efficiency (%) were analyzed, defined as follow:

Rooting rate (%) = rooted microshoots / initial explants

Acclimation rate (%) = acclimated vitroplants / rooted microshoots

Efficiency (%) = acclimated vitroplants / initial explants

An ANOVA was performed to test the effect of clones on each of these variables and a Tukey's HSD test was also done when this effect was identified as significant. General Linear Models were used in all the cases as the diagnostic checking performed showed that the data respect the model assumptions. A significance level of 0.1 is considered if the contrary is not stated. All the statistical analyses were performed using R (R Core Team 2019).

Results and Discussion

Rooting stage

The results show a significative effect of clones on the rooting rate in the micropropagation process for hybrid walnut (Fig. 1, Tab. 1). The model was considered as adequate after a normality analysis of the residuals (Shapiro test p-value=0.5764) and their graphical diagnosis (Fig. 2). The rooting rate of D-117 [73% ± 7 (C.I. 90%)] is considered statistically similar than the one for D-53 [93% ± 5 (C.I. 90%)] and D-M [88% ± 6 (C.I. 90%)], while the rate for D-15 [55% ± 9 (C.I. 90%)] is significatively lower (Fig. 1).

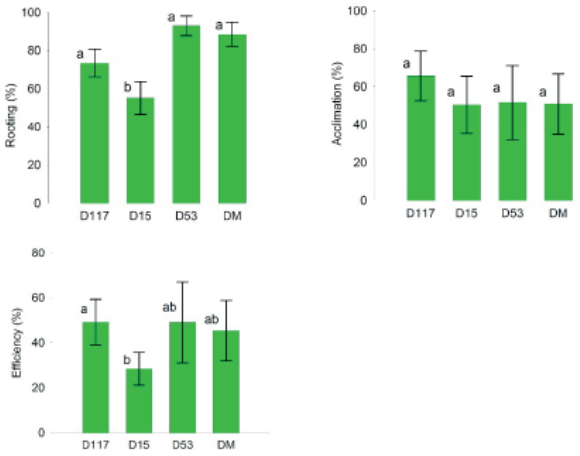

Figure 1 - Effect of clones on the rooting rate (%), acclimation rate (%) and efficiency (%) of hybrid walnut (*Juglans x intermedia Mj209*) in vitro micropropagation.

The effect of different clones on the rooting rate is a common pattern which has been detected either for walnut species (*Juglans* spp.) (Chenevard et al. 1995, Dolcet-Sanjuan et al. 1996, Scaltsoyiannes et al. 1997, Vahdati et al. 2004, Sharifian et al. 2009, Payghamzadeh and Kazemitabar 2011) and other hardwoods (Bennett and Mccomb 1982, San José et al. 1988, Juncker and Favre 1989, Yu et al. 2001), while Tetsumura et al. (2002) did not find a significant effect on the rooting of *J. regia* explants. To this respect, the rooting rates reported for the different clones (Fig. 1) are within the range reported

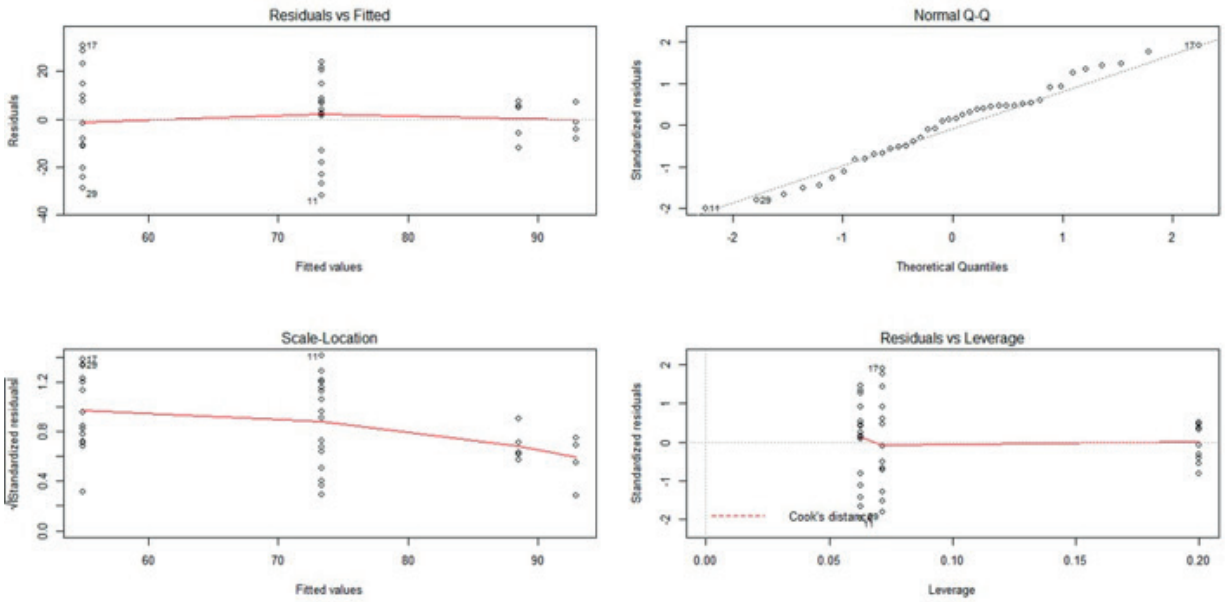

Figure 2 - Graphical evaluation of the residuals of the statistical model analyzing the effect of clones on the rooting rate (%) of hybrid walnut (*Juglans x intermedia Mj209*) in vitro micropropagation.

for the *Juglans* spp. species by other authors (Chenevard et al. 1995, Dolcet-Sanjuan et al. 1996, Scaltsoyiannes et al. 1997, Tetsumura et al. 2002, Vahdati et al. 2004, Sharifian et al. 2009).

Acclimation stage

Notwithstanding the clone effect on the rooting rate, the results do not show a significative effect of clones on the acclimation rates in the micropropagation process for hybrid walnuts (Tab. 1, Fig. 1). Hence, regardless the clone considered, the acclimation rate is 57% ±7 (C.I. 90%). This poor acclimation results have been previously considered as a key issue for the success in the operational micropropagation of walnut, as the acclimatization of micropropagated walnut is reported to be a difficult procedure because of rapid desiccation of plantlets or their susceptibility to diseases due to high humidity and difficult rooting, for a review see Payghamzadeh and Kazemitabar (2011). As oppose as the results from this study, the clonal effect on the acclimation rates of walnut vitroplants has been previously reported by other authors (e.g. Dolcet-Sanjuan et al. 1996, Frossard et al. 1997). Indeed, Frossard et al. (1997) report how *J. regia* clones are more easily adapted to soil conditions than *J nigra* x *J regia* hybrid clones. Improving this acclimation stage is considered as a priority for the commercial walnut micropropagation and there have been many authors reporting several measures to improve the acclimation rates (e.g. Jay-Allemand et al. 1992, Heloir et al. 1994, Voyiatzis and Mc Granahan 1994, Dolcet-Sanjuan et al. 1996, Frossard et al. 1997). To this respect, the acclimation rates of the 2018 campaign are higher than 65% on average, even though the data is not shown in this work due to big differences in the experimental conditions.

Efficiency

Based on the rooting and acclimation rates, the results show a significative effect of clones on the micropropagation efficiency for hybrid walnuts (Tab. 1, Fig. 1). The model was considered as adequate after a normality analysis of the residuals (Shapiro test p-value=0.3744) and their graphical diagnosis (Fig. 3). The efficiency rate of D-117 [65%±15 (C.I. 90%)] is considered statistically higher than the one for D-15 [38%±10 (C.I. 90%)], while the high variability of the clones D-53 [50%±17 (C.I. 90%)] and D-M [51%±13 (C.I. 90%)] cause that they cannot considered as different of any of them (Fig. 1). Considering these differences in the micropropagation efficiency, clones might be selected in order to improve the general performance of the plant production units in large-scale propagation, as suggested by other authors (e.g. Scaltsoyiannes et al. 1997). However, multiplication rates are not considered in the present study and it is a key issue in order to be considered for this selection in addition to other key issues such as field performance (e.g. Aletà et al. 2003, Urbán-Martínez et al. 2013). To this respect, D-15 and D-117 show a better multiplication rate (data not shown) which partially explains why they are the two clones more used in this study (and in the company's commercial production).

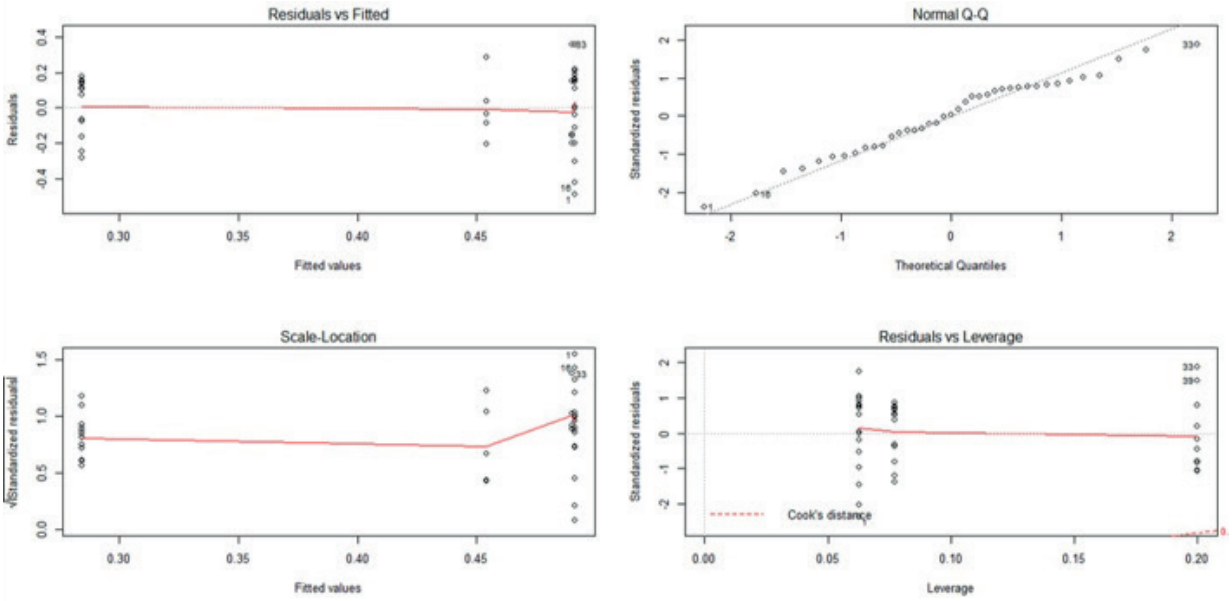

Figure 3 - Graphical evaluation of the residuals of the statistical model analyzing the effect of clones on the efficiency rate (%) of hybrid walnut (*Juglans x intermedia Mj209*) in vitro micropropagation.

Conclusion

The results show a significative effect of clones on the rooting and the total micropropagation efficiency rates of the hybrid walnut (*Juglans x intermedia* Mj 209), but not on the acclimation rate. The efficiency rate of D-117 [65%±15 (C.I. 90%)] is considered statistically higher than the one for D-15 [38%±10 (C.I. 90%)], caused by a higher rooting rate of D-117 [73%±7 (C.I. 90%)] compared with D-15 [55%±9 (C.I. 90%)], because acclimation rate [57% ±7 (C.I. 90%)] did not show any clone effect. Considering these differences in the micropropagation success, it might be considered (together with other factors) for clone selection to increase the general performance of the plant production units in large-scale propagation.

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