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Editorial note

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

In memory of Gaetano Castro

This special issue of ASR is dedicated to Gaetano Castro, researcher at the Research Centre for Forestry and Wood at Casale Monferrato, recently passed away, who, as an expert in wood technology, participated in the work of the H2020 European project "WOODnat - Second generation of planted hardwood forests in the EU".

The research activity of our friend Gaetano mainly concerned wood and derived products, from investigations to support the genetic improvement of poplar to the harvesting techniques of forest plantations and the technological qualification of wood-based panels. In particular, he effectively and competently managed the operational connection of research in poplar breeding and cultivation with the wood industries. Known nationally and internationally for his research on the quality of wood and for his expertise about standardization in wood technology, recently nominated Chairman of ISO/TC 89/SC3 'Plywood', Gaetano was not only a brilliant researcher, but also an IT expert: particular mention deserves, for example, the creation of a software for the macroscopic identification of the wood of the main tree species in Italy.

His sympathy and contagious cheerfulness, as well as his kindness and positive attitude, made him appreciated by all those who had the opportunity to work with him. All of us will be missing not only the competence of a colleague but also the simple, genuine and real smile of a friend.

Piermaria Corona

ASR Editor in Chief

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Preface

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

Challenging the market uptake of European Walnut in Europe

Elias Cueto[1*]

The importance of wood as a resource grows rapidly in the context of an economic transition towards circular economy. In technical terms, it is a renewable material which can store CO_2. From a wider point of view, wood is part of the natural and traditional landscape as well as a source for richness and employment at rural areas.

Despite of the positive impact of forests, the market promotes fast growing species and intensive forest management models. Those choices lead to a cost-effective supply of biomass entering into the market, but they also conditioned the regression of traditional species which are part of the traditional landscape. One of those species is European Walnut (*Juglans regia* L.), which suffered a significant regression in the last decades.

Fighting against market rules is a worthless effort in our globalized world. For this reason, the market uptake is an unescapable challenge for all European companies, and especially for the European SMEs. Scientifics, researchers, and technicians can contribute to that challenge by enabling improved productive processes, as knowledge and technology and the key factors for competitiveness in Europe.

This special issue summarizes up to three years of research and development focusing on walnut trees for wood production in Europe. The efforts have been performed within the contest of the Project "WOODnat: Second generation of planted hardwood forests in the EU". This is an Innovation Action which has been granted by the European Union in the framework of H2020 Programme, making possible to develop innovative experiments and trials.

By means of this publication, the Partners of the Consortium are happy to present their results to the community. The work performed includes a wide spectrum of researches: from genetics of walnut plants to large format digital printing; from innovative forestry models to growing edible fungi.

The breadth of these studies is appealing by itself for any curious lector, but most of all, it shows how many innovative approaches can be adopted for improving the performance of the European forests in a globalized and competitive world.

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Second generation of walnut planted forests in EU

Jesús Fernández Moya[1] and Ignacio Urbán Martínez[1*]

Walnut planted forests were the origin of the WOODnat project (2016-2019), an Innovation Action which granted by the European Union in the framework of H2020 Program.

WOODnat has been focused overall the walnut value chain with the aim to increase the use of quality walnut hardwood produced under sustainable management practices in planted forests, involving the different stakeholders. WOODnat integrates a series of innovations that would help to promote a second generation of walnut planted forests in Europe: from nurseries to industry and market, including sustainable forest management.

This Special Issue includes some of the innovations derived from WOODnat, which can be divided in 3 categories depending on the beneficiaries of these innovations: forest managers, nurseries and transformation industries.

Innovations for nurseries

Selecting the right plant source is one of the main issues for the success once one has decided to establish a new walnut plantation in a site. The management experience in walnut planted forests shows how a good plant material from a seed orchard planted in relatively homogeneous site turn out in a forest where differences between trees are much larger than expected both in shape and size.

To this respect, the main advantage of clonal planted forests vs. plantations from seeds might not be a higher performance of clones (i.e. elite trees) in terms of higher growth but straight shape and the homogeneity between the trees within a single plantation. The relevance of using clonal material to ensure homogeneity is higher when the sylvicultural scheme is changed and the tree density is reduced to follow a silvo-arable approach, there is a need of ensuring that, as your plantation has fewer trees, they need to be of much better quality. The use of clones is highly recommended when walnut is planted under agro-silvo-pastoral approaches.

However, 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. As a consequence, nowadays there is shortage of clonal plant material in the market and different strategies have been used to overcome these problems, as it is discussed in WOODnat WP 5 and the papers within this Special Issue, Licea-Moreno et al. (2020) reports some new advances and insights regarding micropropagation of valuable walnut genotypes for timber production. Fernández-Moya et al. (2020) analyze the clonal effect on rooting and acclimation rates for in-vitro micropropagation in hybrid walnut.

Thanks to the WOODnat innovations, the company Bosques Naturales has increased their production capacity from a research scale (< 4,000 plants/year) to a commercial scale (> 10,000 plants/year); which would allow European landowners to access to a much better plant quality for the establishment of new planted forests.

Innovations for forest managers

Most of the walnut planted forests in Europe are characterized by their small scale. Based on the analysis performed by Pelleri et al. (2020) within WOODnat, around 85% of walnut forest managers (representing 58% of the planted area) owns plantations smaller than 5 ha. This big amount of small-scale walnut planted forests might not have a size enough to be worthwhile for the forest owners to hire the services of a forest consultancy specialized on walnut.

However, this big amount of forest managers would be presumably very interesting in acquire a free technical guidance via remote/automated platforms, manuals, guidelines, etc. Similarly, these small-scale

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forest owners might not have the machinery, equipment, personnel and/or experience necessary to achieve their objectives and might need to contact some enterprises to provide the necessary products or services. A smartphone APP and a web platform [woodnat.azurewebsites.net] has been developed within WOODnat combining the 2 abovementioned needs, creating a platform which will allow the establishment of a community of people interested on walnut.

Hence, small-scale landowners will have free access to a platform which offer them (1) access to technical guidance for their plantations, (2) direct relationships with other landowners with walnut planted forests and (3) a wide range of companies offering products and services (either generalist or specific for walnut). On the other hand, the companies providing products/services would be interested in the access to this community as a potential source of clients.

In addition, a silvicultural guide has been published (Fernández-Moya et al. 2019) as a result of the WOODnat project. The objective of this guide is to be a silvicultural manual analyzing the considered as the better practices to be applied for walnut plantation management oriented to timber production, taking into account scientific literature and the experience in the sector of the companies who compound the WOODnat consortium. This guide tentatively answers the questions any forest manager would have about walnut planted forests. Applied with common sense, combined with new information as available and modified when needed, these guidelines should be useful tools for the management of walnut planted forests under different cultivation systems and circumstances.

Within this Special Issue, Pelleri et al. (2020) perform a characterization of the walnut planted forests in Italy and Spain and analyze different forest management alternatives to be used in the new planted forests to be established. De la Parra et al. (2020) analyze the potential sprouting suppression and mushroom production after inoculation of *Juglans x intermedia* stumps with edible fungi inoculum. Fernández-Moya and Urbán-Martínez (2020) show a statistical model to estimate crown competition factor and crown diameter in hybrid walnut planted forests to be used in thinning planning.

Innovations for transformation industries

Walnut (*Juglans* sp.) timber has been traditionally highly appreciated and mainly used for furniture, flooring and paneling.

In the WOODnat project we analyzed what the properties and the volume disposal for are the next years in the first generation of walnut planted forest in order to answer the main economic question about the market of the wood produced in the different walnut planted forest models

On this three years a solution to small diameters trees obtained by thinning in the plantations was not totally completed, but one of the most important problem to this type of wood were the white color, because the heartwood did not start, within this Special Issue, Cueto (2020) analyzes the potential use of digital printing for changing the color of European Hybrid Walnut to be more close to the market possibilities.

WOODnat project was a new effort in the way to make the walnut planted forest as a profitable model for the Europe rural development.

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International project report
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"Second generation of planted hardwood forests in the EU"

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 25^{th} to 29^{th}, 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).

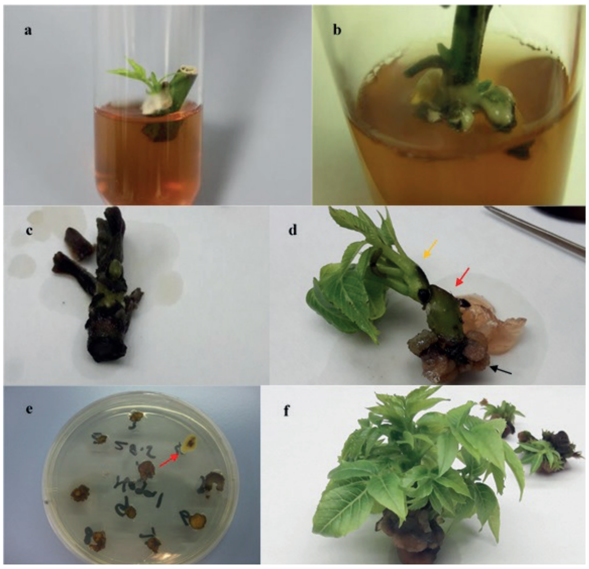

Figure 1 - Selection of clean and viable explants during in vitro establishment. (a) Fungus contamination. (b) Bacterial contamination. (c) Non-responding explant. (d) Responsive explant (red arrow) with basal callus (black arrow), bearing a healthy sprouted bud (yellow arrow). (e) Indexing on bacteria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

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.

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

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.

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-

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)

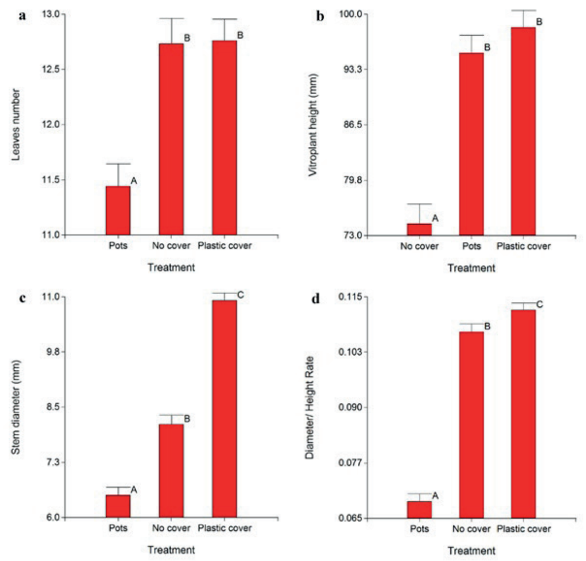

Figure 4 - Effects of different managements on the growth of acclimated vitroplants of clone D117. (a) Leaves formed per vitroplant. (b) Height of vitroplants. (c) Basal diameter of stem. (d) Ratio diameter/height of vitroplant. Treatments with different letters are significantly different ($p \leq 0.05$,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

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"Second generation of planted hardwood forests in the EU"

The walnut plantations (*Juglans* spp.) in Italy and Spain: main factors affecting growth

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Abstract - Walnut tree species (*Juglans* spp.) are commonly used for high-quality wood production in plantation forestry. In this paper, the most relevant walnut plantations in Italy and Spain have been reviewed and analysed under a geographic and technician management point of view. Between 2016 and 2019 a total of 96 plantations (15 - 25 years old) were visited distributed in the North-western part of the Mediterranean basin. A statistical analysis (linear model no interaction and PCA) was then performed to evaluate the relative importance of some environmental and management variables for walnut trees in analysed plantations. Results highlighted a variable situation with many different adopted planting schemes across the regions as well as a not standardised spatial layout and management type (thinning). Lower densities and smaller trees were adopted in Italy with about 200 trees ha^{-1} versus 330 trees ha^{-1} in Spain. In addition to the age of the plantation as one of the most influencing parameters also the plantation density and the average crown diameter were highly statistically significant. Overall, the interesting potentiality of walnut for timber production with active management in suitable areas was detected as the focal point for a successful timber production from walnut trees.

Keywords - planted forests, agroforestry, timber, wood quality, H2020 Woodnat project.

Introduction

Forest plantations have globally increased during recent decades in response to the growing demand for timber, pulp, energy and other goods (Ares and Brauer 2005, Evans 2009, Cambria and Pierangeli 2012, FRA 2015). Around 7% of global forest area is currently covered by planted forests (291·10^6 ha) and fulfil almost 65% of the global demand of timber products representing a consistent reduction of the human pressure over the natural forests (Evans 2009, EFIATLANTIC 2013). About 50% of planted forests are currently owned by public bodies while approximately 33% belongs to small landowners (Carle 2013). At European level planted forests are the 8% of the whole forested area (83·10^6 ha) and showing an annual increment of +1.11% between 1990 and 2015 (FRA 2015). To this respect, the European Commission Regulation 2080/92 played a key role with approximately 1,000,000 ha of afforestation distributed in small lands between 1992 and 1999 in the 15 countries of the European Union (IFD 2001).

Walnut species (*Juglans* spp.) are among of the most popular and widely-used forest tree species in tree farming activities, characterized by marketable nuts and high timber quality. Walnut forest plantations oriented for timber production have been often established during the last decades all over the world (Mohni et al. 2009). The Persian (or European) walnut (*J. regia* L.), Black (or American) walnut (*J. nigra* L., *J. major* (Torr.) A. Heller, and *J. hindsii* (Jeps.) Jeps. ex R.E. Sm.) probably are the most used walnut trees in both pure or mixed stands (Nichols et al. 2006, Clark et al. 2008). However also several hybrids have been specifically developed for timber production, e.g. Mj-209xRa and Ng-23xRa (Aletà et al. 2003 , Aletà 2004, Victory et al. 2004, Mohni et al. 2009, Clark and Hemery 2010, Coello et al. 2013, Bernard et al. 2018). Despite the relevance that walnut timber has historically had within the forest sector, just a small proportion of the forested area in Europe is currently characterised by the presence of such valuable tree species. The species is also not usually included in the forestry statistics in the EU due to its relative (spatial and numerical) scarcity, owing to a complicate, time-consuming and biased estimation. According to Spiecker et al.

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(2009) around 2.4% of the planted forests associated to the ECR 2080/92 (i.e. 24,000 ha) corresponded to *Juglans* spp. stands, mainly established in abandoned agricultural lands and located in the Southern Europe and around the Mediterranean basin (i.e. Northern Italy, Southern France, Spain). The use of *Juglans* spp. trees has been relatively different according to the environmental variability they were planted. Most of the walnut planted forests belonging to small scale forest owners and the EU Regulation 2080/92 has been determinant for their expansion, relatively wide planted forests were established by medium size enterprises with more than 100 ha each.

Among all the European countries, Italy, Spain and France currently are the most active in cultivation of *Juglans* spp. trees. Concerning Italy, approximately 100,000 ha were afforested within the framework of the ECR 2080/92 between 1992 and 1999 with 70% realised with valuable or semi-valuable broadleaves such as *Juglans* spp., *Prunus avium*, *Fraxinus* spp. and *Populus* spp. (IFD 2001). Romano and Cesaro (2016) estimated that more than 200,000 ha were afforested with valuable broadleaves in former agricultural lands between 1994 and 2013; however just around 25% of the considered surfaces included walnut species. In France, walnut plantations represent 6% of the afforested area within the framework of EC 2080/92 (Spiecker et al. 2009). Hence, considering that 45,147 ha were planted between 1992 and 1999 within this program (IFD 2001), walnut trees were used in more than 2,700 ha, mainly as unique tree species in pure stands. In Spain most walnut plantations were established in large areas (100-600 ha each), mainly by private companies. For instance, 600 ha were planted by Foresta Capital S.L., around 300 by Bosques Naturales S.A. and 120 ha by Valor Forestal S.A. In addition to these major companies, many other relatively big (approx. more than 5 ha but less than 100 ha) private plantations were censed with different business models which started after the big companies (García-Martín et al. 2011).

The objective of this paper is to summarize and evaluate the most relevant walnut plantations (pure or mixed) in Italy and Spain and to evaluate the effect of a wide range of variables at plantation level and single-tree level on tree diameter growth. Overall 96 First Generation Planted Forests (FGPF) between 15 and 25 years-old were visited between 2016 and 2019 and analysed to derive indication on management type, productivity and issues related to the cultivation of this tree.

Materials and Methods

Tree-level and stand level data

To make an evaluation of walnut's FGPF, different areas where walnut is traditionally cultivated were sampled across Italy and Spain. Overall, 79 plots were established in Italy and mainly for European walnut plantations, realized in small and medium farms located in four Regions: Piedmont, Lombardy (North Italy), Tuscany (Central-West Italy) and Marche (Central-East Italy). Plantations have been selected below 600 m above the sea to avoid failed stands, very likely in the analysed environments. Concerning Spain, only 17 hybrid walnut were considered (*Juglans x intermedia* Carr.). The sampling mainly included actively managed stands owned by an important forest enterprise (Bosques Naturales) and geographically distributed in the regions of Galicia (North West Spain), Girona (North East Spain), Toledo, Cuenca and Cáceres (Central Spain). Intensive management included the application of irrigation, fertilization, weeding and thinning trials was manly applied in Spain only. According to Buresti Lattes and Mori classification (2007, 2008) different plantation types have been retrieved in Italy (pure, mixed, polycyclic, etc.) and mainly European walnut plantations have been detected. Conversely, only 17 pure walnut plantations have been sampled in Spain.

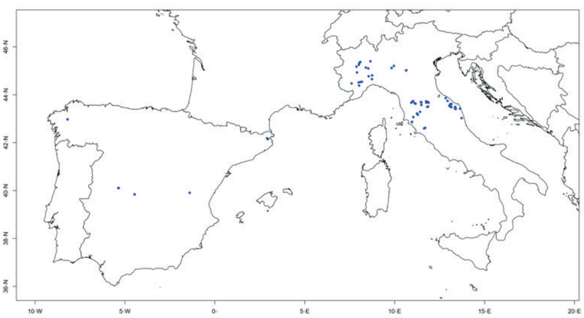

Figure 1 - Spatial distribution across Italy and Spain of the 96 sampled stands.

A sample made by 30 walnut trees was measured and included within a representative plot of the stand, averaging the measurements to calculate unbiased estimates. The main parameters surveyed for each tree were: diameter at breast height (DBH), total height, crown insertion, diameter at 2.6 m, stem diameter at crown insertion, crown diameter, stem quality classes, sanitary conditions (general, leaf, stem, root damages). The plantation age was determined interviewing the owners, reading documentation or by means of coring trees in the proximity of collar. Stem quality was also evaluated attributing a stem quality class to the first logs up to 2.6 m and according to stem quality classification already existing at European level for other species such as *Quercus* spp. and *Fraxinus* spp. (EN standards, Nosenzo et al. 2012). Stem ovality was also considered and estimated measuring orthogonal DBH values.

The phytosanitary conditions of walnut stands represented the most time-consuming effort. In accordance with a qualitative damage scale, 4 single-tree levels were considered: 0: healthy tree; 1: slight damage to the crown and/or the stem; 2: damaged collar and/or stem necroses; 3: whole tree decline with slow or stopped growth, overshadowed tree. Single trees were observed and evaluated according to this scale along random transects in each stand. A synthetic rating was then assigned to the whole stand considering the most frequent damage class among the observed trees. The damage classes roughly mirror a criterion aimed to emphasize main differences in damage: class 1 was associated with mainly quantitative damage, consisting in a lower height and diametric increment; class 2 included trees with mainly qualitative damage, since stem necroses induce discolorations and alterations of the underlying wood; class 3 often involved the loss of entire trees, combining quality and quantity damage. More in detail, during the monitoring the major walnut adversities were recorded with respect to the tissues affected. All the recorded adversities were previously reported for years in several surveys (Belisario 1996, Fernández-Moya et al. 2019). The damage class frequencies were calculated and statistically processed in the (χ2).

Concerning biomass and quantitative data, timber volume was calculated by using the mean diameter at various stem section ($S_{1.3}$, $S_{2.6}$, S_{crown_ins}, S_{hd10}) and using the total height for the final portion. Other parameters such as mean annual increment (MAI) and mortality were calculated from raw data. For all the plantations, a characterization of site quality classes of walnut using DBH and age, has been realized according to Cisneros et al. (2008).

Statistical analysis

Among all the descriptive numeric data (i.e. Latitude/Longitude of the trial, number of trees per hectare, average diameter, height, volume, etc.), 20 variables were used to derive insights on the structure of the surveyed plots. In addition to a Principal Component Analysis (PCA) a correlation matrix was built, using the Spearman correlation method in order to include non-linear relationships. To avoid biases in PCA calculation due to different units between the different variables, a correlation matrix was used in place of the classical covariance matrix. Afterwards the mean annual diametric increment (MAI_{dbh}) was selected as target variable in a modelling procedure, aimed at evaluating whose environmental/management parameters were detectable as the most relevant drivers for walnut growth, within the surveyed plots. A multivariate linear model (LM) was built, with no interaction between terms in order to generate a robust and simple but informative output:

$$MAI_{dbh} = i + \beta_1 \cdot x_1 + \beta_2 \cdot x_2 + \beta_3 \cdot x_3 + \cdots \beta_n \cdot x_n + \varepsilon$$

Once the model was fitted, a stepwise procedure (backward direction) was implemented, to remove not useful parameters and to maximize the concentration of explained variance into few non-correlated variables. Then the relative importance of each predictor (x_1, x_2, x_3...x_n) was tested, decomposing the proportion of deviance explained by each. Finally, the derived coefficients (β_1, β_2, β_3... β_n) were used as indicator in order to understand whether the significant coefficients were directly (positive) or inversely (negative) influencing MAI_{dbh} values. All the statistical analyses were compiled in R language (R core Team 2019)

Results

The investigated plantations

Small scale plantations were dominant in the database for a mean surface 2.7±2.4 ha and a total surface of 210.1 ha (Tab. 1). Only 12 of them (15%) were more than 5 ha wide, covering a total of 89 ha (42%) while only 2 of them (3%) were more than 10 ha (10%). This situation is somehow different when considering the consistent amount of medium size (5-100 ha) planted forests in Spain. Regarding the used species, almost all the Italian plantations were characterized by European walnut trees (75 plantations), while three were established with black walnut and one mixed European walnut and hybrid walnut. Concerning Spain only the hybrid walnut was used. Then different plantation types have been adopted in Italy with pure (mono-specific) and mixed (multi-specific) plantations with nurse trees including N-fixing (trees and shrubs). Medium-size and big pure plantations were sampled in Spain (mean surface 11.0±9.1 hectares with a total surface of 186.9 hectares).

Italian plantations showed an average age around 20 years with the pure and pure-with-nurse-trees plantations slightly older than the mixed plantations. The younger and more recent type were the polycyclic plantations with 13.8 years in average. Overall, younger sites were visited in Spain for an average age around 14.5 years.

Table 1 - Number surface and age of the sampled plots per walnut (*Juglans* spp.) plantation types in Italy and Spain.

Italy (IT) and Spain (SP) stand type	Sampled stands	Mean surface ha ± SD.	Total surface ha	Mean age yrs ± SD
Italy	79	2.66 ± 2.4	210.07	20.0 ± 4.7
IT mixed	20	2.94 ± 2.7	58.77	18.0 ± 2.4
IT mixed with nurse trees	13	3.29 ± 2.3	42.83	18.7 ± 3.5
IT polycyclic	5	3.33 ± 2.4	16.63	13.8 ± 4.0
IT pure	34	2.22 ± 2.3	75.44	22.1 ± 4.9
IT pure with nurse trees	7	2.34 ± 2.4	16.4	22.6 ± 4.2
Spain	17	11.00 ± 9.1	186.95	14.5 ± 3.1
Pure	17	11.00 ± 9.1	186.95	14.5 ± 3.1

Mensurational parameters

Walnut planting densities were very variable across Italy and according to the different layout and management types (thinning in particular). Lower densities were adopted in Italy than in Spain with about 200 trees ha^{-1}. An average spacing between 5 and 6 metres was adopted in the pure plantations while wider distances (from 8 to 10 metres) were used in the mixed and polycyclic ones. Conversely in Spain, densities were generally higher than Italy and around 330 trees ha^{-1} with distances of 5-6 meters everywhere. Smaller DBH values were detected in Spanish sampled plantations than Italians, with mean value of 17.3 cm against 19.5 cm for Italy whose value came from data ranging between 17.2 cm in mixed plantation and 21.3 cm in pure and polycyclic plantation (Tab. 2). The volume per hectare of walnut trees was, on average, around 20.2 m^3 ha^{-1} in Italy, highly variable again among the plantation types. However higher values were found in some pure plantations: for example, in Piedmont, a volume up to 104 m^3 ha^{-1} has been estimated in a pure 23 years old plantation. In Spain walnut volumes reached 29.6 m^3 ha^{-1} on average and up to 56.6 m^3 ha^{-1} in a pure plantation in Gerona (17 years-old, 6 m spacing). The average marketable timber volume per tree in Italy was 0.118 m^3 with superior volume in younger polycyclic plantation and around 0.144 m^3 while, in Spain, single-tree volume reach and average value of 0.09 m^3 (±0.037) in pure plantation with similar age. The relationship between the average DBH values and age is shown in Figure 2 and compared to the site quality curve for walnut plotted using the methodology applied by Cisneros et al. (2008). According to our data 19 plots were referred to class I, 47 were close to class II and 30 allocated in class III. Concerning growth trends, an average MAI_{dbh} value of 1.0 cm yr^{-1} was observed for Italy with difference among planting types. The best MAI_{dbh} have been observed in polycyclic plantation with value of 1.6 cm. yr^{-1} and with a maximum of 1.9 cm yr^{-1} in a polycyclic plantation in Lombardy (10 years-old) where walnut trees have been planted with a rectangular layout admixed with poplar trees and Short Rotation Systems (SRC). In Spain a MAI_{dbh} of 1.22 cm yr^{-1} was measured on average with e maximum of 1.65 cm yr^{-1} in a plantation near a Coruña (Boimorto/Arzúa).

Overall, poor stem quality was detected in Italy. Only 30.2% of the logs is included in the first two stem classes suitable for industrial transformation (Tab. 3). The best wood quality in Italy have been observed in the polycyclic plantation with 67% of trees belonging to the A and B classes, while the worst stem quality was for pure plantations, with only 7%. In Spain the use of selected material (i.e. hybrid *Juglans with walnut*) and the choice of appropriate sites and management, brought to superior results than Italy with 78% of tree suitable for the industrial transformation.

Table 2 - Number of trees, DBH, volume and MAI per country and per plantation types for the sampled walnut plantations.

Italy (IT) and Spain (SP) stand type	Walnut n trees ha^{-1}		Walnut DBH cm		Walnut vol. m^3 ha^{-1}		Tree volume m^3		DBH MAI cm	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
IT mixed	179.9	124.4	17.2	5.9	13.4	11.9	0.091	0.068	1.0	0.3
IT mixed with nurse trees	138.9	66.2	18.2	7.2	12.7	11.7	0.105	0.081	1.0	0.4
IT polycyclic	93.4	33.5	21.3	7.1	14.7	15.3	0.144	0.113	1.6	0.4
IT pure	221.6	78.5	21.1	5.8	26.4	19.7	0.136	0.098	1.0	0.3
IT pure with nurse trees	260.4	76.6	19.2	5.9	27.2	23.5	0.109	0.074	0.9	0.3
Average Italy	192.8	97.4	19.5	6.3	20.2	17.8	0.118	0.088	1.0	0.4
SP pure hybrid walnut	328.6	26.0	17.3	2.5	29.6	12.3	0.090	0.037	1.22	0.2
Average Spain	328.6	26.0	17.3	2.5	29.6	12.3	0.090	0.037	1.22	0.2

Table 3 -Stem quality classes per plantation types in Italy and Spain.

Italy (IT) and Spain (SP) stand type	Stem quality classes				
	A %	B %	C %	D %	A+B%
IT mixed	8.3	22.2	32.0	27.0	30.5
IT mixed with nurse trees	11.3	18.5	24.1	38.2	29.7
IT polycyclic	28.7	38.7	23.3	7.3	67.3
IT pure	6.7	21.0	35.4	29.7	27.6
IT pure with nurse trees	0.5	15.2	31.0	48.1	15.7
Average Italy	8.7	21.5	31.5	30.6	30.2
SP pure hybrid walnut	35.9	42.4	16.9	1.6	78.3
Average Spain	35.9	42.4	16.9	1.6	78.3

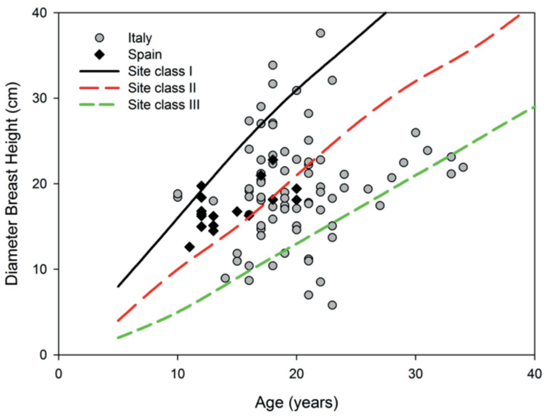

Figure 2 - Characterization of site quality of walnut (*Juglans* sp.) of the Woodnat database from Italy and Spain, according to the site classes defined by Cisneros at al. (2008).

Phytosanitary conditions

The results of surveys for damage class in Italy and Spain did not show significant differences as such. Although the walnut Italian pure stands appeared more damaged than the others plantation types and with respect to Spanish pure stands (Tab. 4). The samples were too heterogeneous to give a significant chi-square value ($\chi^2 = 17.42$, $p = 0.0577$). Instead, grouping class 0 and 1 with no or slight damage and class 2 and 3 with marked damage, the chi-square test become significant ($\chi^2 = 11.88$, $p = 0.0431$), owing mainly to a marked deviation of the observed frequencies of Italian pure stands from the expected ones. As regards the observed pests and diseases in Italian stands, the most incident was anthracnose by *Ophiognomonia leptostyla* on leaves and apical branches, followed by the bacterial shallow bark canker by *Brenneria nigrifluens* on the trunk. The feared *Geosmithia morbida*, pathogenic agent of the Thousand cankers disease recently reported in Italy, was never recorded in the analysed plots, whereas the bacterial blight by *Xanthomonas arboricola* pv. *juglandis*, the downy leaf spot by *Pseudomicrostroma juglandis* and the pustule canker by *Juglanconis juglandina* were seldom observed. Among non-specific pests, the goat moth (*Cossus cossus*) and the leopard moth (*Zeuzera pyrina*) were rarely recorded on trunks, and the fall webworm (*Hyphantria cunea*) on leaves as well; among specific pests, only aphids (*Panaphis juglandis* and *Chromaphis juglandicola*) were recorded with low or no incidence. Frost cracks or mechanical damage were sometimes observed in confined conditions.

Statistical structure of the investigated variables and influence on MAI_{dbh}

The results of the correlation analysis and a graphical plot of PCA are summarized in Figure 3 and Table 5. Overall many variables were highly inter-correlated showing a statistically significant (p.value ≤ 0.05) correlation value with correlation

Table 4 - Number of Italian and Spanish stands included in damage classes (see text for their definition).

Italy (IT) and Spain (SP) stand type	Damage class				Total
	0	1	2	3	
IT mixed	45%	50%	5%		20
IT mixed with nurse trees	15%	77%	8%		13
IT polycyclic	40%	60%			5
IT pure	26%	41%	26%	7%	34
IT pure with nurse trees	28%	44%	28%		7
SP pure	47%	47%	6%		17
Total	33%	50%	15%	2%	96

Table 5 - Spearman correlation values (lower the diagonal) and p.values (upper the diagonal) between the 20 parameters included in the PCA. Above the diagonal p.values have been coded according to the following rules: '***' ≤ 0.001; 0.001 < '**' ≤ 0.01; 0.01 < '*' ≤ 0.05; n.s.> 0.05. Variables are: Latitude [1], Longitude [2], Number of trees per hectare [3], Number of walnuts per hectare [4], Average DBH of walnut trees [5], Ovality of the stem [6], Total height of walnut trees [7], Crown depth of walunt trees [8], Average diameter at 2.6 m [9], Length of the pruned stem [10], Quality of trees [11], Average crown diameter [12], Timber volume [13], Volume of the first marketable log [14], Sanitary condition [15], Failure percentage [16], Total volume per hectare [17], Mean annual volume increment [18], Age [19], Mean annual increment of DBH [20].

	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]
[1]	1	n.s.	n.s.	***	n.s.	n.s.	n.s.	**	n.s.	n.s.	***	**	n.s.	n.s.	***	**	**	***	***	***
[2]	0.04	1	*	***	**	n.s.	n.s.	n.s.	**	**	*	**	**	***	*	*	n.s.	n.s.	n.s.	n.s.
[3]	-0.02	-0.25	1	*	***	n.s.	n.s.	n.s.	***	***	n.s.	***	***	***	n.s.	n.s.	*	n.s.	*	*
[4]	-0.44	-0.41	0.25	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	*	***	***	n.s.	n.s.
[5]	0.08	0.30	-0.42	-0.17	1	n.s.	***	***	***	***	**	***	***	***	n.s.	n.s.	***	***	*	***
[6]	-0.12	0.13	-0.11	0.03	-0.12	1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
[7]	-0.02	0.19	-0.20	-0.19	0.80	-0.12	1	***	***	***	***	***	***	***	n.s.	n.s.	***	***	n.s.	***
[8]	-0.28	-0.03	-0.08	0.04	0.44	0.03	0.60	1	***	**	***	*	***	***	n.s.	n.s.	***	***	n.s.	***
[9]	0.07	0.28	-0.43	-0.19	0.94	-0.10	0.76	0.47	1	***	***	***	***	***	n.s.	n.s.	***	***	*	***
[10]	0.12	0.29	-0.47	-0.16	0.97	-0.10	0.71	0.29	0.93	1	**	***	***	***	n.s.	n.s.	***	***	**	***
[11]	-0.38	-0.22	-0.12	0.09	0.32	-0.07	0.42	0.53	0.37	0.27	1	**	***	***	*	***	***	***	***	***
[12]	0.30	0.32	-0.52	-0.44	0.78	-0.14	0.58	0.25	0.76	0.79	0.31	1	***	***	n.s.	n.s.	***	**	n.s.	***
[13]	-0.02	0.27	-0.36	-0.13	0.94	-0.07	0.82	0.67	0.92	0.87	0.44	0.71	1	***	n.s.	n.s.	***	***	*	***
[14]	0.04	0.34	-0.35	-0.16	0.97	-0.14	0.78	0.46	0.93	0.92	0.33	0.74	0.94	1	n.s.	n.s.	***	***	**	***
[15]	0.48	-0.25	0.05	0.08	0.04	-0.11	0.04	-0.06	0.06	0.08	-0.23	0.06	0.03	0.01	1	n.s.	n.s.	n.s.	***	**
[16]	0.31	0.25	0.06	-0.20	-0.02	0.05	-0.13	-0.16	-0.06	-0.02	-0.37	0.04	-0.07	-0.04	0.07	1	*	**	n.s.	n.s.
[17]	-0.31	-0.07	-0.21	0.51	0.67	-0.10	0.57	0.55	0.65	0.64	0.47	0.33	0.73	0.66	0.06	-0.22	1	***	n.s.	***
[18]	-0.47	-0.09	-0.16	0.53	0.58	-0.13	0.53	0.55	0.56	0.54	0.57	0.27	0.65	0.57	-0.05	-0.27	0.95	1	n.s.	***
[19]	0.41	0.10	-0.22	0.01	0.26	0.15	0.12	-0.02	0.25	0.28	-0.38	0.16	0.23	0.27	0.41	0.16	0.16	-0.10	1	***
[20]	-0.38	0.18	-0.22	-0.07	0.60	-0.24	0.55	0.36	0.56	0.55	0.60	0.52	0.58	0.57	-0.29	-0.20	0.47	0.63	-0.53	1

coefficients ranging between -0.21 and 0.93. Most of the silvicultural parameters (e.g. volume of the first marketable log and average diameter at breast height of walnut trees) were highly correlated and as shown by the PCA with overlapping red arrows. Overall, the most influencing and isolated drivers were the latitude, the number of walnuts per hectare, the quality of trees and the mean annual volume increment. A wide group of 7 highly correlated variable was then recognised. Those were the average DBH of walnut trees, the total height of walnut trees, the average diameter at 2.6 m, the length of the pruned stem, the average crown diameter, the timber volume and the volume of the first marketable log. Finally, low importance was given to the number of trees per hectare, the stem ovality, the phytosanitary condition and the mortality.

Concerning the statistical model, the main result was that after the stepwise procedure all climatic variables were removed by. Then 91.6% of the total variance was obtained with 11 variables (Tab. 6). The age of the plantation was the most influencing parameters, explaining 25.17% of the variance of the model with a negative effect. The crown diameter was highly significant as well, but with a positive effect on MAI_{dbh}. Other significant parameters were the longitude (negative) and the latitude (positive) of the site explaining environmental and climat-

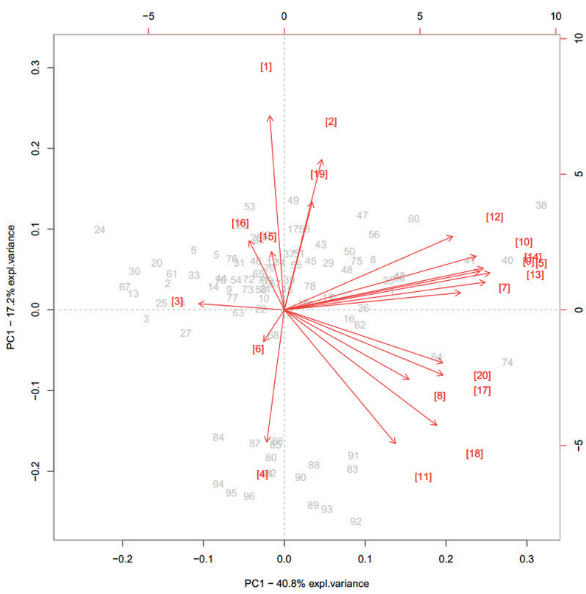

Figure 3 - PCA plot of the surveyed plantations. The length of each red arrow expresses the importance of each measured variable. While uncorrelated variables are expressed by orthogonal arrows, opposite or overlapped arrows describes correlation values of -1 and 1 respectively. Variables are: Latitude [1], Longitude [2], Number of trees per hectare [3], Number of walnuts per hectare [4], Average DBH of walnut trees [5], Ovality of the stem [6], Total height of walnut trees [7], Crown depth of walunt trees [8], Average diameter at 2.6 m [9], Length of the pruned stem [10], Quality of trees [11], Average crown diameter [12], Timber volume [13], Volume of the first marketable log [14], Sanitary condition [15], Failure percentage [16], Total volume per hectare [17], Mean annual volume increment [18], Age [19], Mean annual increment of DBH [20].

Table 6 - Deviance analysis of the statistical model where the current DBH increment has been modelled (R2: 0.916). Asterisks have been added to p.values according to the following rules: '***' ≤ 0.001; 0.001 < '**' ≤ 0.01; 0.01 < '*' ≤ 0.05; n.s.> 0.05.

Predictor	Sum of Squares	Prop. of variance	DF	F value	Pr(>F)		Effect
Age	1.0412	25.17%	1	70.5136	3.75E-12	***	(-)
Crown diameter	0.7080	17.11%	1	47.9526	1.82E-09	***	(+)
Longitude	0.0890	2.15%	1	6.0285	0.0166	*	(-)
Latitude	0.1663	4.02%	1	11.2603	0.0012	**	(+)
Trees per ha	0.1608	3.89%	1	10.8893	0.0015	**	(-)
Quality	0.1523	3.68%	1	10.3167	0.0020	**	(+)
Soil texture	0.3201	7.74%	10	2.1676	0.0302	*	none
Soil Depth	0.1752	4.23%	2	5.9316	0.0042	**	50-100 cm less than others
Morphology	0.1240	3.00%	3	2.798	0.0465	*	Wide valley better than others
Plantation type	0.1330	3.21%	4	2.2519	0.0723	.	Pure with nurse trees better than others
DBH ovality	0.0487	1.18%	1	3.2969	0.0737	.	(-)
Residuals	1.0188	24.63%	69				

ic factors and highly related to the walnut species used in the plantation (i.e. mainly the hybrid versus the European). Then the soil texture and soil depth were statistically significant too but with low differences within them. Indeed, while no evidences were derived for the texture of the soil, only the middle class of soil depth (i.e. between 50 cm and 100 cm) was acknowledged as a negative factor for growth when compared to deeper soil. Concerning the planting site, the morphology was the last statistically significant predictor with the plantations established in wide valleys showing higher MAI_{dbh} values than the others. Even if not statistically significant, also the plantation type and the ovality of the stem were included in the LM by the stepwise procedure. In this case a slight within-variable difference was observed for the plantation type with the pure with nurse trees plantations showing some positive influence on the target variable. A high leverage value was associated to just few observations, and overall the Shapiro test p value was 0.186 acknowledging a normal distribution of regression residuals.

Discussion

The analysis of the main mensurational parameters highlighted different results between countries, walnut species and plantation types. The best performances were obtained in Spain where the hybrid walnut species was mainly used. As expected, age, crown size and total number of trees per hectare were among the most relevant drivers, explaining 25.0%, 17.1% and 3.9% of the whole variance.

MAI_{dbh} was negatively correlated with the plantation age and stem densities. The lack of a regular/correct management and thinning, due to low price of small assortment and unfavourable market conditions, have negatively affected the growth performance of the main part of plantations. The statistical model also confirmed the importance of both geographical and management parameters on growth trends (Balandier et al. 2000, Paris et al. 2005). Even if simple, the performed LM was able to respect the general assumptions of statistical modelling, with the additional advantage of being informative without any data management or statistical transformation, generating unbiased estimates of coefficients (Marchi 2019).

The analysis of FGPF has pointed out the two different choices in the two countries: although with pure plantations predominant in Italy, mixed plantations have had a remarkable development too showing interesting results often superior to walnut monoculture under the wood quality. It is well known that the use of different species/genotypes increases the resilience of the plantation reducing the risk of pest and disease damages; unfortunately, the wood produced by these nurse trees, with the first thinning, often found strong difficulty in the placing on the market. As ancillary result, the recent developed of polycyclic plantation have achieved further progress in the design of mixed plantations allowing to realize innovative plantation more profitable under the economic and environmental point of view. Inter-specific and intra-specific competition can cause a strong reduction of DBH growth on walnut trees (Mohni et al. 2009, Fernández-Moya et al. 2019) and early thinning are necessary to maintain a stable diametric growth. Then latitude and longitude demonstrated that the plots at higher latitudes showed superior MAI_{dbh} in comparison to the plots situated in the South and Mediterranean areas of the two Countries. Among these the northern regions of Spain (Galicia) and Italy (Piedmont and Lombardy) were better than central Italy (i.e. Tuscany) and Catalugna. These results probably agree with the walnut requirement in rainfall with an optimum at 700 mm of total annual precipitation with more than 125 mm during the vegetative period (May-September) which characterise almost all the sampled plots This mainly occurs in Oceanic or Continental areas rather than in strictly Mediterranean area (Gonin et al. 2014, Mohni et al 2009, Fernández-Moya et al. 2019). Concerning longitude, this parameter is more connected to the species used for planting than to ecological features. While hybrid walnut has been planted in all the Spanish sampled plantations and in one plantation in Lombardy, the European walnut was used in almost all the other areas. Indeed, the hybrid walnut is acknowledged to be able to overcome the European one in term of growth (Mohni et al. 2009, Fernández-Moya et al. 2019). This is also reflected by our survey and in some experimental plantations included in this investigation (Pelleri et al. 2013). Spanish pure plantations using first hybrid walnut derived by seed and then clones have permitted to obtain interesting results in term of wood production and stem quality. For wood production several hybrids have been obtained during the last 100 years. Among them, the progeny of the mating between *J. major* var. 209 x *J. regia* (Mj209xRa) seems to be the most suitable for the European conditions. This seed progeny is characterized by its high vigour, a remarkable apical dominance and an outstanding growth (Aletà et al. 2003, Clark and Hemery 2010). The use of suitable clones permits to reduce heterogeneity inside the plantation in term of growth and stems quality (Urbán-Martínez et al. 2018, Licea-Moreno 2016). For specific site conditions the selection of suitable walnut clones permit to obtain a superior DBH homogeneity as showed in Figure 4 and where a comparison between Italian (pure or mixed plantation) and Spanish pure plantation is reported.

Concerning soil and environmental characteristics, the soil depth, texture and morphology were detected as important and statistically significant factors influencing walnut MAI_{dbh}. Soil depth explained 4.3% of variance while 8.4% was associated to soil texture. Results confirmed the requirement of fertile and deep soils (≥ 80-100 cm) characterized by a loamy texture. According to literature, the tolerance of clay would be lower in relation to higher amount of rainfall (Oliver et al. 2008, Mohni et al. 2009, Fernández-Moya et al. 2019) which probably occurred in analysed stands close to high mountain chains. Regarding morphology walnut trees prefers wide valley and plain areas not interested by frequent late frost. In our surveys, best results have been found in wide valley where frost is less likely to occur. In the same way the plantation types are often acknowledged as interesting parameter. Many experimentations in Italy demonstrated the use of nurse trees (especially N-fixing trees and shrubs) to reduce the management costs (pruning in particular) in comparison with walnut monoculture. Then higher increment rates, due to the intercropping with Nitrogen fixing nurse trees were shown on poor soil (Bianchetto et al. 2013, Marron and Epron 2019, Loewe-Muñoz et al. 2019). The recent developed of polycyclic plantation stimulated progresses in the design of mixed plantation more profitable under the economic and environmental point of view (Buresti Lattes and Mori 2016, Buresti Lattes et al. 2017, Pra et al. 2019).

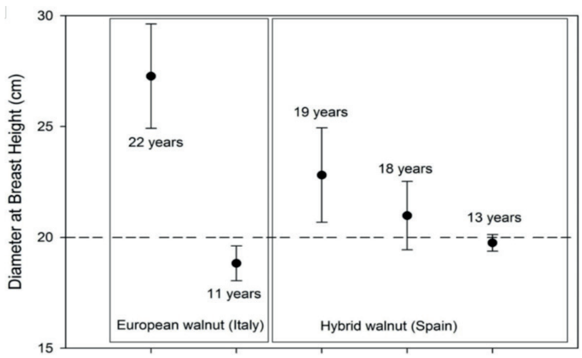

Figure 4 - Difference on mean DBH ± standard deviation homogeneity among different plantations in Italy and Spain.

The stand density was a further indicator of site management, given its dependence to thinning and/or planting densities. As expected, this factor was positively correlated with number of walnut trees and negatively correlated with walnut MAI_{dbh}, DBH, crown diameter, and timber volume. Medium and high-density plantation not managed in suitable way caused a decrease of DBH growth and of wood quality (Carle 2013). A low stocking density of 70-120 final crop trees per hectare have been most widely adopted in many countries, but not always actively managed (Mohni et al. 2009). In fact, these final densities are obtained in late postponing or avoiding the precommercial thinning in order to permit an easier placement of the assortments into the market but reducing the growth and the quality of the final crop trees. Stem quality explained 3.7% of variance which was also positively correlated with average crown diameter, and with all volume factors (timber volume, volume of first log, total volume per hectare). The quality of trees was influencing MAI_{dbh} with and increased growth rate was where more A class trees were observed for a more valuable marketable volume (Nozenzo et al. 2012). This was also connected to crown diameter, with MAI_{dbh}, positively associated to a good development of walnut due to a suitable management system application. Finally, the Spanish pure stands resulted more healthy than Italian pure stands; however, the former are composed by hybrid walnut genotypes and the latter by European walnut. This fundamental genetic difference may have importance in connection with the respective site pedoclimatic conditions (Pollegioni et al. 2009). In addition, the provenance of Italian material was often unknown and heterogeneous.

Conclusions

This survey has pointed out the interesting potentiality of walnut for timber production; both European walnut and hybrid walnut are able to obtain interesting growth rate in diameter some time superior to 1.5 cm yr. This growth rhythms will permit to obtain suitable assortments for industrial transformation (>40 cm) in 25-30 years. On the other hand, the results of the survey conducted stressed the importance of favourable site conditions (deep soils, loamy texture, morphology, etc.) as well as the need to apply correct planting designs and correct management systems. The failure of many Italian plantations realised under the support of the EU 2080/92 regulation can be attributed to the lack of knowledge at national level. Financial funding was often the main aim of farmers, especially in marginal areas and converting part of their farms to wood production.

Pure walnut plantations have often obtained interesting results due to the use of hybrid walnut, the selection of clones suitable for different environmental situations and adoption of suitable management system. This type of pure plantation, in Mediterranean area, needs high intensity management characterized by watering, repeated weed control per year, pruning of all the trees and often precommercial thinning. Pure plantations are often one of the best options in favourable site conditions and, under certain aspects, the use of only one specie is simpler to manage in comparison to mixed plantation and, generally, make it possible to produce a greater timber quantity of a specific valuable tree species. Mixed plantations have had a remarkable development in Italy showing interesting results, often superior to walnut monoculture under the wood quality, and sometime in growth, in not suitable site condition. It is well known that the use of different species/genotypes increases the resilience of the plantation reducing the risk of pest and disease damages; unfortunately, the retrieved timber from the first thinning is not easily placed in the market. For these reasons the polycyclic plantations, where walnut is intercropped with poplar clones or SRC, have been developed solving the problem of precommercial thinnings with the earlier production of valuable assortments (poplar for plywood and SRC for biomass production) requested from the industry.

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Research paper

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"Second generation of planted hardwood forests in the EU"

Estimation of crown competition factor for hybrid walnut (*Juglans x intermedia*) Mj209xRa planted forests in Spain

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Abstract - Many walnut (*Juglans* spp.) planted forests oriented for timber production have been established during the last decades. These plantations usually have a relatively low initial density (250-400 trees ha^{-1}) and 1 or 2 thinnings are needed for valuable timber production (75-150 trees ha^{-1} for final harvesting). Hence, forest managers need to design when to perform the thinnings and how to do it. Analyzing the trees crown area is a very helpful and easy-to-use tool to evaluate the competence between trees and design the thinnings. The present study proposes two statistical models to estimate Crown Diameter (CD) and Crown Competence Factor (CCF) as a function of Diameter at Breast Height (DBH) for hybrid walnut (*Juglans x intermedia*) Mj209xRa planted forests, within a DBH range between 5 cm (min) and 33 cm (max). CD and DBH were measured in 702 trees at the Bosques Naturales SA walnut planted forests in Cuenca, Girona and A Coruña (Spain). The CCF model is a tool to evaluate the Crown Competition Factor as a function of measured DBH in a plantation, and, accordingly, decide if a thinning should be done or not yet and design it.

Keywords - crown diameter, crown competition factor, *Juglans* spp., planted forests, thinning, walnut.

Introduction

Walnut trees are species of the genus *Juglans* spp., 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.: Mj209xRa and Ng23xRa (Aletà 2004, Victory et al. 2004, Mohni et al. 2009, Clark and Hemery 2010, Coello et al. 2013).

Walnut planted forests have been usually established with a relatively low density (around 250-400 trees ha^{-1}) and 1 or 2 thinnings are needed for valuable timber production and around 75-150 trees ha^{-1} are harvested at the end of the rotation period (Cisneros et al. 2008, Mohni et al. 2009, Coello et al. 2013, Fernández-Moya et al. 2019). Thinning is regarded as a key silvicultural activity when managing planted forests oriented to the production of high-value timber, aiming to reduce stands density and produce large-diameter trees. To this respect, forest managers must deal with several main questions when designing a thinning: *(i)* It is necessary to apply thinning?; *(ii)* How many times would the forest be thinned along the rotation period?; *(iii)* When the thinning should be done?; *(iv)* How many trees should be removed?; and *(v)* Which particular trees should be removed? (West 2006, Kerr and Haufe 2011).

Walnut does not readily react to canopy opening if it is grown in dense plantations where excessive lateral competition and reduction in a crown's functionality is already evident (Clark 1967, Hemery et al. 2005, Marchino and Ravagni 2007). In such situations, the trees demonstrate small diameter growth for many years after thinning. Therefore, thinning must be undertaken before lateral competition influences diameter growth, with the aim of maintaining trees with crowns free from competition and able to grow with large and constant diameter increments. However, estimating when this lateral competition starts to influence tree growth is difficult to manage in the practice and, consequently, some silvicultural tools need to be designed in order to estimate when to perform the thinnings based on easy-to-measure parameters.

The method of crown competition factor (CCF) is proposed as a method for thinning design (Krajicek et al. 1961, USDA 1981, Schlesinger 1988 a, b, Mohni et al. 2009). This factor is an objective method to evaluate the optimal stocking density and to plan thinning regimes. According to this method, when CCF reach a certain value (100-110%), the stand

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Table 1 - Location, climate and soil attributes in the studied hybrid walnut (*Juglans x intermedia*) Mj209xRa planted forests of Bosques Naturales SA in Girona, Cuenca and A Coruña (Spain). Location data is shown in geographic coordinates. Climate data was obtained from https://sig.mapama.gob.es/geoportal/, except summer precipitation that was obtained from www.worldclim.org. Soil attributes data represents the mean and the confidence interval (95% probability) from the soil samples taken at each site.

	Attributes	Girona	Cuenca	A Coruña
Location	Latitude	42.1878	39.9148	42.9805
	Longitude	2.8943	-1.4078	-8.1944
	Altitude (m a.s.l.)	125	1030	440
Climate	Papadakis classification	Continental Mediterranean	Temperate Mediterranean	Temperate Mediterranean
	Mean annual rainfall (mm)	700	500	1400
	Duration of dry period (months)	3	3-4	2
	Summer precipitation (mm)	119	79	162
	Mean annual temperature (ºC)	14	12	12
Soil	Number of soil samples	15	7	16
	pH	8.4 (8.2 / 8.5)	8.6 (8.5 / 8.7)	5.5 (5.8 / 5.2)
	Electrical conductivity (mmhos/cm)	0.16 (0.12 / 0.19)	0.11 (0.09 / 0.13)	0.09 (0.1 / 0.08)
	Organic matter (%)	1.2 (1.1 / 1.3)	0.6 (0.4 / 0.8)	7.8 (9.1 / 6.5)
	C/N ratio	6.3 (5.7 / 6.9)	6.5 (4.7 / 8.3)	9.3 (11.8 / 6.9)
	Sand (%)	37 (31 / 44)	64 (56 / 72)	46 (43 / 50)
	Silt (%)	45 (34 / 55)	30 (20 / 41)	11 (2 / 19)
	Clay (%)	18 (8 / 28)	6 (0 / 11)	43 (32 / 53)
	Carbonates (%)	25 (18 / 33)	12 (10 / 14)	
	Active CaCO3 (%)	18 (9 / 27)	5 (4 / 7)	
	CIC (meq/100g)	18.2 (15.7 / 20.8)	16.5 (11.7 / 21.3)	14.8 (21.6 / 7.9)
	K (meq/100g)	0.35 (0.30 / 0.40)	0.46 (0.35 / 0.57)	0.65 (0.98 / 0.31)
	Ca (meq/100g)	22.1 (19.4 / 24.9)	16.2 (12.1 / 20.3)	2.3 (3.1 / 1.5)
	Mg (meq/100g)	0.9 (0.7 / 1.0)	0.8 (0.5 / 1.1)	0.4 (0.5 / 0.3)
	Na (meq/100g)	0.3 (0.1 / 0.5)	0.2 (0.1 / 0.3)	0.2 (0.3 / 0.2)
	P (mg/kg)	73 (0 / 152)	18 (10 / 26)	53 (82 / 23)
	Fe (mg/kg)	10 (7 / 12)	8 (5 / 11)	96 (132 / 60)
	Cu (mg/kg)	2 (1 / 3)	1 (1 / 2)	2 (2 / 1)
	Mn (mg/kg)	17 (12 / 22)	5 (3 / 6)	46 (65 / 27)
	Zn (mg/kg)	1.0 (0.7 / 1.3)	0.5 (0.3 / 0.7)	1.0 (0.7 / 1.3)

should be thinned. This CCF is a relatively easy to use method with the following steps: *(i)* estimate a mean Diameter at Breast Height (DBH) for each stand or plantation; *(ii)* calculate the crown diameter or the crown area based on a statistical model; *(iii)* calculate the proportion of crown area by surface [divide the crown area (in m^2) of each tree by 10,000 (m^2 ha^{-1})]; *(iv)* estimate the total CCF by multiply the proportion of crown area calculated above by the tree density (trees ha^{-1}); and *(v)* analyze the results. If CCF is higher than a certain value (100-110%) a thinning should be designed to reduce the crown competence (USDA 1981). Several authors have published 110% as a CCF limit value for walnut (USDA 1981, Mohni et al. 2009) while Schlesinger (1988 a, b) refers to 100%.

In addition, the CCF model allow forest managers to design the initial layout of a plantation and/or estimate when the thinning is going to be needed. Hence, using the models to perform projections of the trees DBH under different scenarios, we might establish an initial density adequate for the specific conditions. The difference in the mean DBH of the trees to be thinned is especially relevant when considering the market potential interest, making a difference between thinning as a management investment if DBH is low and "commercial thinning" as an intermediate income when DBH is big enough. However, market prices and interests are very variable and fluctuates a lot depending on final-consumer demands. Indeed, during recent years the market demand has been lower than expected and these "commercial thinnings" that were regarded as an intermediate small income by many forest managers had turned into investments needed in order to achieve in the future the objectives fixed for the final harvest at the end of the rotation period. Hence, the expected market price is a key issue regarding the initial establishment of a planted forests as the idea of a thinning regarded as an income or as an investment would directly influence the decision about the initial tree density planted at establishment.

To estimate crown area, several statistical models have been proposed to estimate walnut crown diameter (CD) as a function of DBH in order to evaluate tree competence for different walnut species: *Juglans regia* L. (Hemery 2000, Hemery et al. 2005, Montero and Cisneros 2006) and *Juglans nigra* L. (USDA 1981, Bechtold 2003). However, there are no published statistical models to estimate CD and/or CCF for hybrid walnut (*Juglans x intermedia*) Mj209xRa to our knowledge. This paper aims to made public the statistical models used for that purpose by the Spanish company Bosques Naturales SA.

Material and Methods

Study area and data collection

The study was conducted in the Bosques Naturales SA company hybrid walnut (*Juglans x intermedia*) Mj209xRa planted forests in various locations in Spain, described in Table 1. Girona and Cuenca sites have calcareous soils relatively similar (Table 1), even though soils in the planted forest in Cuenca are generally deeper while soils in Girona are shallow at some sites, with toxicity due to excess of active lime. Soils is A Coruña are generally more acidic with a deep 40 cm layer rich in organic matter (umbric horizon).

The management of the planted forests is relatively similar in the different locations. Plantation density at establishment is 333 trees ha^{-1} (5 x 6 m spacing). Weeding is done by using herbicides combined with mechanical methods and tillage between plantation rows during the first years (3-5 years) and with mechanical methods and tillage accompanied by sheep grazing in older plantations. Pruning is done according to tree height up to a total clean bole of 4 m (5 m in A Coruña). Fertirrigation is used in Cuenca and Girona according to each site needs and in A Coruña, where irrigation is usually unnecessary, regular fertilization is done complemented with liming (1000 kg ha^{-1} each 2 years). More details of the management of the plantations can be read in Fernández-Moya et al. (2019).

Crown diameter (CD) and Diameter at Breast Height (DBH) were measured in 808 trees (675 in Girona, 27 in Cuenca and 106 in A Coruña). Mean DBH of the measured trees was 20 cm with a range between 5 cm (minimum) and 33 cm (maximum). For CD estimation, two crown diameters (i.e. four radii) were measured, the direction of the first diameter was selected at random, and the second was at right angles to it (Hemery et al. 2005). Measures were taken during the vegetative period (with leaves) between July and September, using measuring tape. The criterion to establish the crown perimeter projection on the ground was a visual estimation.

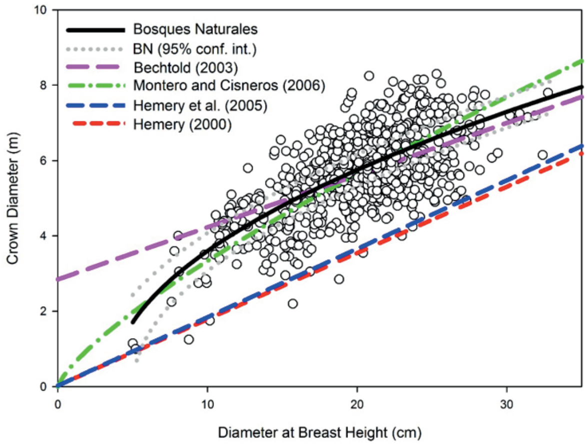

Figure 1 - Comparison between different regression models for estimating Crown Diameter as a function of Diameter at Breast Height in walnut (*Juglans* spp.) planted forests. Bosques Naturales refer to the model detailed in the present paper (Table 2, Figure 1) for hybrid walnut (*Juglans x intermedia*) Mj209xRa in Spain and BN (95% conf. int.) refers to the confidence intervals (95%) of this model. Bechtold (2003) is fitted for black walnut (*J. nigra L.*) in USA. Montero and Cisneros (2006) is fitted for common walnut (*J. regia L.*) in Spain and Hemery (2000) and Hemery et al. (2005) are also fitted for common walnut in England.

Estimation of Crown Competition Factor

Crown Competition factor (CCF) (Krajicek et al. 1961) is calculated as the per cent ratio between Maximum Crown Area (MCA) – calculated based on crown diameter measurement – and the surface (A) of land occupied by trees:

$$CCF = \Sigma\,(MCAi)/A \times 100 \quad (1)$$

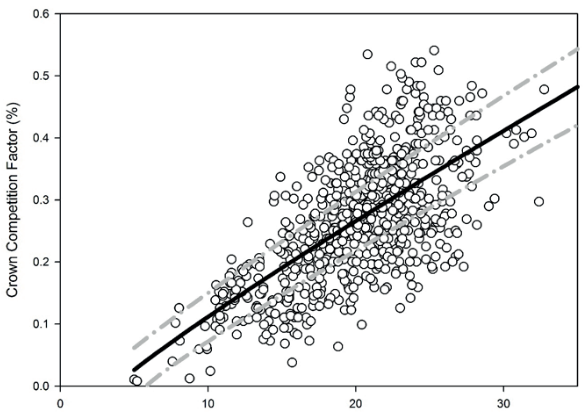

Figure 2 - Regression model for estimating Crown Competition Factor as a function of Diameter at Breast Height in hybrid walnut (*Juglans x intermedia*) Mj209xRa planted forests of Bosques Naturales SA in Girona, Cuenca and A Coruña (Spain). DBH range between 5 cm (minimum) and 33 cm (maximum). See Table 3 for details about the model parameters. Grey lines represent confidence intervals (95%).

Table 2 - Model parameters of the regression for the estimation of Crown Diameter (CD, [m]) as a function of Diameter at Breast Height (DBH, [cm]) in hybrid walnut (*Juglans x intermedia*) Mj209xRa planted forests of Bosques Naturales SA in Girona, Cuenca and A Coruña (Spain). DBH range between 5 cm (minimum) and 33 cm (maximum). Model structure: $CD = (b_0 + b_1 \cdot DBH)(1/\lambda)$, where $\lambda=2$.

Parameter	Estimate	Standard Error	p-value
b_0	-7.13535	1.2076	< 0.0001
b_1	2.012019	0.0628	< 0.0001

Table 3 - Model parameters of the regression for the estimation of Crown Competition Factor (CCF) as a function of Diameter at Breast Height (DBH) in hybrid walnut (*Juglans x intermedia*) Mj209xRa planted forests of Bosques Naturales SA in Girona, Cuenca and A Coruña (Spain). DBH range between 5 cm (minimum) and 33 cm (maximum). Model structure: $CCF = (b_0 + b_1 \cdot DBH)(1/\lambda)$, where $\lambda=1.1$.

Parameter	Estimate	Standard Error	p-value
b_0	-0.0536611	0.0117863	< 0.0001
b_1	0.0143371	0.0005768	< 0.0001

Hence, CCF of the forest stand can be regarded as the sum of each tree individual CCF which is the measure used in the present paper to be modeled as a function of individual trees DBH.

Statistical analysis

All the statistical analyses were performed using R (R Core Team 2018). Different model types were tested to fit the regression between either individual trees CD and CCF with DBH, within the general framework of the Generalized Linear Models (GLM), choosing the best fitting one based on the Akaike Information Criterion (AIC). The gamma and the gaussian family were compared. Different link functions were also tested: power links with lambda between -2 and 2, with 0.1 intervals. Finally, the possible random effect of the three sites (Girona, A Coruña and Cuenca) was analyzed compared the GLM with an analogous Generalized Linear Mixed Model (GLMM). Model efficiency (EF) was calculated as a pseudo-R^2 measurement, as:

$$EF\ (\%) = \text{model deviance} \cdot 100 / \text{null deviance} \quad (2)$$

Definition of planted forests

The term "planted forests" has been used along this document according to the FAO definition: "A planted forest is defined as a forest that at maturity is predominantly composed of trees established through planting and/or deliberate seeding. Planted forest includes but is not limited to plantation forest" (see http://www.fao.org/forestry/plantedforests/67504/en/).

Results and Discussion

Estimation of Crown diameter and Crown Competition Factor

A GLM with gaussian family and power link function (lambda=2) and no random effect resulted as the best model for estimating individual tree Crown Diameter (CD) as a function of DBH (Tab. 2, Fig. 1), with an efficiency of 52.6% within a DBH range between 5 cm (minimum) and 33 cm (maximum). Within the core DBH range of the model (10-30 cm) this model is relatively similar to the ones fitted for *Juglans nigra* L. (Bechtold 2003) and *Juglans regia* L. (Montero and Cisneros 2006), but a little different from those fitted for *Juglans regia* L. by Hemery (2000) and Hemery et al. (2005) (Fig. 1). A more detailed review of the model proposed by Hemery (2000) shows that this model also underestimate the original data within the DBH range between 10-30 cm, considering that the model is fitted for a DBH range up to more than 90 cm. This might be explained because it uses a simple regression model (straight line) and initial values for the ratio between crown diameter and stem diameter reduces as stem diameter increases, dropping by about 60% from 10 to 70 cm DBH (Hemery et al. 2005).

A GLM with gaussian family and power link function (lambda=1.1) and no random effect resulted as the best model for estimating individual tree Crown Competition Factor (CCF) as a function of DBH (Tab. 2, Fig. 2), with an efficiency of 57.7%. The proposed model is very similar to the one published by USDA (1981) for *J. nigra* L. within the DBH range between 10-20 cm but the differences outside this range are noticeable. Hence, the individual tree CCF for a black walnut of DBH of 10, 15 and 20 cm are 0.12, 0.20 and 0.31%, respectively, while the values for these DBH are 0.11, 0.19 and 0.27%, respectively, for the hybrid walnut (Fig. 2). However, a tree CCF of 0.59% is proposed by USDA (1981) for a black walnut with 30 cm DBH while the proposed model for hybrid walnut estimates 0.41% for that tree (Fig. 2).

Application of the methodology for the design of thinnings

Some recommendations have been published as general rules for thinnings schedules in walnut planted forests. Cisneros et al. (2008), in a scheme with initial plant densities lower than 400 trees ha^{-1}, propose the first thinning corresponding with a mean DBH higher than 20 cm, and a DBH higher than 35 cm where initial plant densities are lower than 200 trees ha^{-1}. Coello et al. (2013) proposed a first thinning when the trees would have a DBH of 20 cm, in a scheme with an initial walnut density of 185 trees ha^{-1}.

A more detailed approach such as CCF can be used to design thinnings for a specific plantation (Krajicek et al. 1961, USDA 1981, Mohni et al. 2009). Hemery et al (2005) summarizes how the crown diameter and the ratio between crown and stem diameters can be used in forest management, mainly to estimate optimal stand density at various stages in the stand's development and design thinnings. Besides these uses of the crown diameter data, Krajicek et al. (1961) proposed the CCF method for the thinning design based on crown area measurements, which is explained in detail for *J. nigra* L. in USA by the USDA (1981) and proposed to be used in Europe by Mohni et al. (2009). There have been some experiences with the application of the CCF in walnut planted forests in Italy, where thinnings of 30-35% of the number of trees are proposed for reducing the CCF from 110% to 70% (Frattegiani and Mercurio 1991, Mercurio and Minotta 2000)

The proposed CCF model (Tab. 3, Fig. 2) would help to design a specific thinning scheme for a hybrid walnut planted forest. This model is a tool to estimate individual tree CCF as a function of measured DBH. The CCF of a stand is calculated as the sum of the individual tree CCF or multiply the individual CCF from the average DBH by the stand's density. Hence, if the stand's CCF would be higher than 100 - 110%, it should be thinned (USDA 1981, Schlesinger 1988 a, b, Mohni et al. 2009).

As an example about the application of this methodology, for a planted forest with 333 trees ha^{-1} (5x6 m wide layout – very common in the current plantations in Spain), a mean CCF of 0.33% per each tree would be needed to reach a CCF of 110%, which corresponds with a mean DBH of 24 cm (21-28 cm [95% Confidence Interval]). Similarly, to reach a CCF of 100% in the same planted forests, a mean CCF of 0.30% per each tree would be needed, which corresponds with a mean DBH of 22.4 cm (19.4-26 cm [95% Confidence Interval]) (Fig. 2). Taken into account that walnut is considered as species with a poor response to late thinnings, when excessive lateral competition and reduction in a crown's functionality is already evident (Marchino and Ravagni 2007); this difference about the use of a CCF limit value of 100 (proposed by Schlesinger 1988 a, b) or 110 (proposed by USDA 1981, Mohni et al. 2009) might be relevant regarding the trees' response to the thinning. To this respect, more research is needed in order to what is the best CCF limit value for the thinning design in walnut planted forests.

Conclusion

The proposed models are easy to use tools to estimate individual trees Crown Diameter and Crown Competition Factor as a function of Diameter at Breast Height in hybrid walnut (*Juglans x intermedia*) Mj209xRa planted forests. These models are helpful for the thinning design for a specific plantation and for the planification of the initial layout of a new plantation, to estimate the tree size that the trees would have in the scheduled thinnings, depending on the stand's density.

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Research paper

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"Second generation of planted hardwood forests in the EU"

Sprouting suppression and mushroom production after inoculation of *Juglans x intermedia* stumps with edible fungi species.

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Abstract - Removal of stumps and suppression of sprouts after harvesting by conventional methods, such as using heavy machinery or herbicides, alters the physico-chemical characteristics of soil, may cause environmental damage and can be very costly. In this study, the performance of inoculation with edible fungi as a biological alternative for stump degradation, has been examined in walnut plantations of five Spanish provinces. Stumps were inoculated with two species of edible fungi: *Pleurotus ostreatus* (Jacq. Ex Fr.) P. Kumm and *Lentinula edodes* (Berk) Pené. Compared with untreated controls, the two biological treatments resulted in a significant and evident reduction of the sprouting probability, which was stronger than the result obtained with chemical treatments. Inoculated stumps also produced edible sporocarps, averaging 15.58 g per stump during the first year. This article constitutes the basis for the development of a sustainable, environmentally friendly and cost-effective product, which is a bioeconomy-based solution for stump degradation in intensive plantations.

Keywords - stump degradation, saproxylic species, logistic models, walnut, nature-based solutions.

Introduction

After harvesting a tree plantation, a large amount of woody debris remains in place, mostly stumps and medium/small-sized branches. This is regarded as a nuisance by forest managers, because removing wood debris and stumps is costly. If the branches and stumps are left untouched in place, naturally occurring decay processes by rot fungi may take a long time, typically more than 25 years (Onofre et al. 2001). It is even worse for sprouting broadleaf tree species, as these stumps can easily become trees again (Hamberg and Hantula 2018). Coppice forests are based on this sprouting ability either from roots or from stumps, *e.g.* holm oak forests managed for firewood production. However, if the main objective is producing timber, managers usually have to clear stumps and other debris to efficiently start a new production cycle. In addition, in conifer forest regeneration areas, sprouting of spontaneous broadleaf species (like birch, rowan or aspen) can compete with conifer seedlings (Hamber et al. 2011, Harding and Raizada 2015, Hershenhorn et al. 2016).

Detached branches from logged trees can be shredded and used to produce compost or biomass that will generate energy, thus yielding some economic revenue. Alternatively, branches can be shredded *in situ* and left for natural decay, which will enrich the soil in organic matter. While the latter would not generate a direct economic revenue, it may have an impact on the growth and health of trees planted in future cycles (Franklin et al. 1997).

Managing the stumps requires a different approach, because they are frequently removed to facilitate the establishment of the next rotation. Stump removal using heavy machinery is expensive (Coder 2003, Andrade et al. 2012). As an example, the cost of mechanical stump removal in a *Eucalyptus* plantation in its third rotation could be US $1,400.00 per hectare (Pavan et al. 2010), and annual cost for extraction of stumps from a hectare of broadleaf forest in Spain could vary from 772.11 to 1282.5 € ha^{-1} (TRAGSA 2019). In addition, this treatment can result in deleterious ecological effects such as alteration of soil structure and litter disturbance (Coder 2003). This may cause a reduction in soil productivity by depleting soil nitrogen, carbon and sulphur availability, although possibly increasing phosphorous availability (Hope 2007), with up to 10 years needed to restore the original soil condition.

The use of chemical and biological approaches

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has been proposed as an alternative to mechanical stump removal. Chemical herbicides have been commonly used to avoid stump sprouts and root suckers, but their use is heavily restricted. This situation encourages the development of environmentally friendly methods for preventing stumps from sprouting and for accelerating their decay (Green 2003). Biological stump degradation has been proposed as an alternative solution. This technique consists of inoculating stumps with mycelium or spores from wood decay fungi. Biochemical activity of fungal enzymes degrades wood and prevents or reduces sprouting (Dumas et al. 1997, Becker et al. 2005, Bellgard et al. 2014), resulting in the improvement of physic-chemical characteristics of the soil and nutrient enrichment, while increasing the biodiversity and richness of microorganisms (Tian et al. 2010).

Species of wood-decay fungi occur naturally in temperate forests, but they can be locally scarce. Fungi spores or mycelium need to reach the stump and colonise it, which can take years to happen (Becker et al. 2005). For this reason, the process of stump decay can be unreliable and often take too long if left to develop naturally. Stump degradation can be accelerated by controlled inoculations, with either a single species or a mix of species with complementary wood-decay features. By placing actively growing mycelium over the surface of a stump, the colonization is largely guaranteed and the process is accelerated. Previous experiences have demonstrated the efficiency of this approach, mostly focused on sprouting suppression, being *Chondrostereum purpureum* the most extensively used species (Lygis et al. 2012, Hamberg et al. 2011, 2015, 2016 and 2018). Other species used include *Pycnoporus sanguineus* (Andrade et al. 2012), *Phellinus gilvus* (Da Silva et al. 2010), *Laetiporus sulphureus* or *Trametes versicolor* (Alonso et al. 2007). The list is surprisingly short considering the high number of wood decay species that can be found in nature (Buée et al. 2009). It is also surprising the lack of studies focused on edible species, while many wood decay fungi (e.g. *Pleurotus ostreatus*, *Lentinula edodes*, *Flammulina velutipes*) produce edible mushrooms which could be harvested and generate income as non-wood forest products.

In this study, we benefit from previously described methods for stump inoculation with wood decay fungi, exploring novel applications resulting in synergistic outcomes on wood decay, sprout suppression and production of edible fungi. Specifically, the effect of stump inoculation with edible wood decay fungi in walnut plantations (*Juglans x intermedia Carr.*) was assessed and compared to natural degradation and to an alternative chemical treatment. The aim of this study was to provide useful information for sustainable forest management, by using a nature-based approach for stump degradation while also generating a product, i.e. mushrooms, that could contribute to profitability.

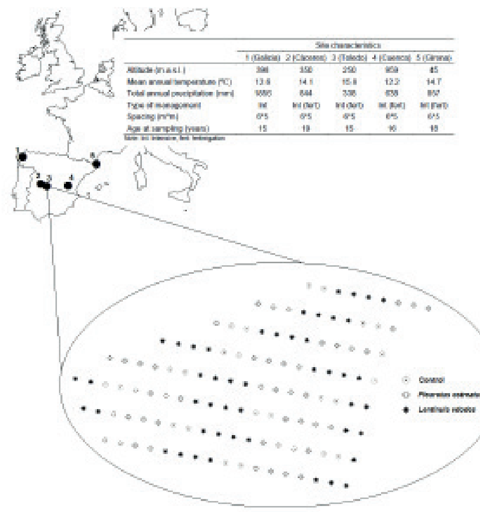

Figure 1 - Location of different walnut plantations.

Materials and methods

Experimental design and data collection

The study was carried out in five plantations, owned by Bosques Naturales company, across Spain (Fig. 1). In each plantation, walnut trees were harvested resulting a total of 910 stumps (120 in Cuenca, 120 to Cáceres, 130 in Girona, 120 in Toledo and 420 in Lugo).

Two stump degradation treatments were applied: a chemical treatment (Q) in 144 stumps, by using herbicides [Roundup], and a biological treatment (B) with two species, B1 with *Pleurotus ostreatus* (Jacq.) P. Kumm. in 363 stumps, and B2 with *Lentinula edodes* (Berk.) Pegler. in 168 stumps. In addition, an untreated control was also considered in 235 stumps.

The two fungal species used in this study were white-rot decay fungi of the *Basidiomycota phylum*, within the Agaricales order. *Pleurotus ostreatus* is one of the most cultivated species in the world (Sánchez 2010). It grows naturally on the surface of stumps and trunks of softwoods such as poplar, beech or willow. Also known as oyster mushroom, it has also been used industrially for mycoremediation purposes. *Lentinula edodes* is an emerging cultivated species, known and appreciated in Asia for centuries. Also known as shiitake, it grows in group, on the wood in decomposition of deciduous trees, as the shii, chestnut, oak, maple, beech, poplar, hornbeams and blackberries. Those species were chosen due to their properties among wood-decay

Table 1 - Transect types and treatments applied by site.

Site	Transect type	Number of transects	Treatment
Lugo	2, 3	42	*Pleurotus ostreatus* biological treatment
			Lentinula edodes biological treatment
			Chemical treatment
			Control
Girona	2	13	*Pleurotus ostreatus* biological treatment
			Chemical treatment
			Control
Cuenca	1	12	*Pleurotus ostreatus* biological treatment
			Control
Cáceres	1	12	*Pleurotus ostreatus* biological treatment
			Control
Toledo	3	12	*Pleurotus ostreatus* biological treatment
			Lentinula edodes biological treatment
			Control

fungal species, also known as lignicolous fungi, and the production of edible fruiting bodies.

Commercial mycelium of both species was obtained from a spawn company. The ability of the strains to grow on walnut wood was assessed in a laboratory trial. It consisted of preparing wood shavings, which were soaked, drained, sieved and mixed with cereal oat bran, which is a common practice in commercial mushroom production to supplement wood based substrates with some source of nitrogen and other nutrients. The mix, together with lime powder was sterilised in an autoclave at 121ºC for two hours. Then, for each of the two assayed species (*Pleurotus* and *Lentinula*), 24 substrate packages (1-kilogram wet weight) were inoculated with 50 grams of mycelium under sterile conditions. The ratio between dry and drained wet wood shavings was 1:4. To test the ability of each fungal strain on walnut wood, a trial was set to grow the mycelium under close to natural conditions. Packages with inoculated walnut wood shavings were incubated for three months at 20ºC and then transferred to a fructification room, were temperature was moderate (16ºC) and humidity high (>70%). The plastic bags, in which the substrate was incubated, were removed and the contents were placed on racks over water containers. Mushroom fructification started readily after the mycelium was exposed to low CO_2 concentration.

After the laboratory trial showed the suitability of the commercial inoculum, we applied the treatment to the stumps in the field. A cross-sectional disc (5-10 cm thick) was cut in each stump using a chainsaw and the mycelium was inoculated between the stump and the cross-sectional discs. Finally, the stump was covered with a biodegradable plastic bag and soil to protect the inoculated stump from external conditions.

The stump degradation treatments were applied to rows of 10 aligned stumps forming a transect. Within each transect, treatments were applied to contiguous groups of stumps, hereafter referred to as blocks. There were three different types transects, depending on the sequence of treatments applied (Fig. 2). Transect type 1 consisted of four stumps inoculated with *Pleurotus* and six controls. Transect type 2 consisted of four stumps inoculated with *Pleurotus*, four with the chemical treatment and two controls. Transect type 3 consisted of four stumps inoculated with *Pleurotus*, four stumps inoculated with *Lentinula*, and two controls. Not all transect types were installed in all sites. Type 1 was installed in Cáceres and Cuenca, type 2 in Lugo and Girona and type 3 in Lugo and Toledo. The resulting design is shown in Tab. 1.

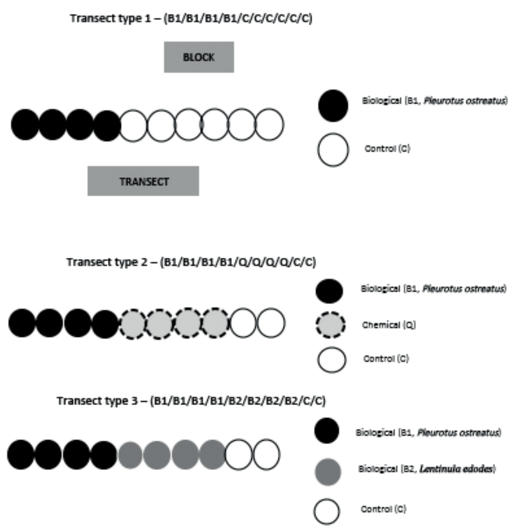

Figure 2 - Transect types considered in the study.

Table 2 - Logistic regression models used in the analysis.

Model	Equation
1	$log(\omega) = log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 I_{CU} + \beta_2 I_{LU} + \beta_3 I_{GE} + \beta_4 I_{TO} + \beta_5 I_{B1}$
2	$log(\omega) = log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 I_{GE} + \beta_2 I_{B1} + \beta_3 I_{Chemical}$
3	$log(\omega) = log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 I_{TO} + \beta_2 I_{B1} + \beta_3 I_{B2}$

Note: ω is the odd of sprouting, $\omega = \varrho/(1 - \varrho)$, and p is the probability of sprouting. Dummy variables represent treatments and sites, where: ICU is 1 if the stump belongs to Cuenca. ILU is 1 if the stump belongs to Lugo. IGE is 1 if the stump belongs to Girona. ITO is 1 if the stump belongs to Toledo. IB1 is 1 if the stump was treated with *Pleurotus ostreatus*. IChemical is 1 if the stump was treated with chemical. IB2 is 1 if the stump was treated with *Lentinula edodes*.

For each stump, quantitative and qualitative characteristics were recorded to assess the stump degradation efficiency after applying the treatments several months later. Data on whether the stump sprouted was collected. For those that sprouted, the viability of the sprouts (0 not viable, 1 viable) was recorded and the proportion of the area of the stump covered by sprouts was estimated (0 for 0% coverage, 1 for 25%, 2 for 50%, 3 for 75% and 4 for 100%) in order to know their origin and distribution, what could be related to the efficiency of the treatment. The number of all sprouts and their height (in meters) were also recorded. Treatment quality indicated the state of treatment and was measured by observing the success of inoculation, whether protection and initial conditions were maintained and observing the presence of damage. It was categorized by 0 for bad quality, 1 for ordinary and 2 for a good quality treatment.

In addition, in the stumps that received the biological treatment in Lugo, Toledo and Girona, the number of fruiting mushrooms and their dry weight in each stump was recorded during the fruiting session (weekly for 10 weeks from October to December in 2018).

Statistical analysis

First, descriptive statistics were computed to summarize the main characteristics of the sprout degradation capacity, such as the proportion of stumps sprouting and their viability, the mean height of sprouts and the mean number of sprouts, and the main characteristics of mushroom production, including mean number of sporocarps and mean weight.

Logistic regression models were fitted to estimate the probability of sprouting as a function of the treatments. Because the details of the design, i.e., the stumps were clustered into blocks and transects, different model structures and random effects configurations were tested: transect (E1), transect and block within transect (E2) and block within transect (E3). The final models were chosen based on the best statistical performance (based on the AIC criterion [Akaike information criterion]) with a reasonable biological interpretation. Since the experimental design was unbalanced and not all treatments were applied in all sites, three logistic models were fitted to: a) compare the effect of the B1 (biological treatment with *Pleurotus ostreatus*) treatment vs control in all 5 sites (Tab. 2, Model 1); b) compare the effect of the B1, Q (chemical treatment) and control treatments in Lugo and Girona (Tab. 2, Model 2) and c) compare the effect of the B1 (biological treatment with *Pleurotus ostreatus*), B2 (biological treatment with *Lentinula edodes*) and control treatments in Toledo and Lugo (Tab. 2, Model 3). All the models include two covariates, a factor for treatment and a factor for site effects, to account for differences in sprouting among sites. Site was considered as a fixed effect because there was a relatively small number of sites, which makes it impossible to estimate a variance component for exact location (Crawley 2002) and to avoid fitting a three-level hierarchical models (as transects and blocks are nested within site).

The probabilities of sprouting in stumps by treatments were calculated with the formula where was the odds estimated from the logistic models and *p* was the probability. Since all stumps with chemical treatment in Lugo resprouted, the effect of the treatment on the regrowth was estimated separately in Girona, due to the singularity of the logistic model. All the statistical analyses were performed in RStudio, with package “lme4”, (R version 3.5.3, RStudio version 1.1.463).

Table 3 - Number of sampled stumps with sprouts by treatment and site.

Site	Treatment	Re-sprouts		Total
		0	1	
Cáceres		24	96	120
	B1	11	37	48
	C	13	59	72
Cuenca		3	117	120
	B1	3	45	48
	C	0	72	72
Lugo		150	270	420
	B1	95	73	168
	B2	52	68	120
	C	3	82	85
	Q	0	47	47
Girona		23	107	130
	B1	8	43	51
	C	1	23	24
	Q	14	41	55
Toledo		15	105	120
	B1	12	36	48
	B2	3	45	48
	C	0	24	24

Note: B1 is the *Pleurotus ostreatus* treatment, B2 is the *Lentinula edodes* treatment, Q is the chemical treatment, C is control treatment.

Results

Descriptive statistics

Out of the 910 stumps analysed in the study 24% did not sprout. The proportion of stumps that did not sprout was 35% for the B1 treatment (biological treatment with *Pleurotus ostreatus*), 33% for the B2 (biological treatment with *Lentinula edodes*), 14% for chemical treatment, and 6% for Control (Tab. 3). Lugo was the site with highest percentage of non-sprouting stumps (35%). The quality of the treatment was good in 85% of the treatments. The distribution of the sprouts across the stump area was relatively homogeneous: 24% of the stumps had no area occupied by shoots, 18% were distributed by 25% of the stump area, 21% by half, 17% by 75%, and 20% were distributed by the entire stump area.

Despite the sprouting, 25% of the sprouts turned out to be not viable in Cuenca, and 13% in Lugo. The site with the lowest number of sprouts was Lugo, with a mean number of 1.9 sprouts per stump, followed by Cáceres. The shortest shoots appeared in Lugo, with an average of 0.6 meters (Tab. 4).

Effect of the treatments on the probability of sprouting

According to the AIC, a random effect of block nested within transect was best for models 1 and 3, and a random effect of transect was best for model 2 (Tab. 5).

Model 1

There was a clear effect of the biological treatment B1 (biological treatment with *Pleurotus ostreatus*) on the probability of stump sprouting after harvest compared to the control ($p<0.0001$, Tab. 6). The odds of sprouting in the untreated control stumps were 17.41 times larger than in stumps treated with *Pleurotus ostreatus* (95% CI for the odds ratio, 6.86 to 44.17). Interestingly, the probability of sprouting in control treatments was almost 100% in all locations (Tab. 7).

Table 4 - Mean number and mean height of the sprouts by stump by treatments.

Treatment	Mean n sprouts	Mean height (m)
Pleurotus ostreatus biological treatment	1.91	0.91
Lentinula edodes biological treatment	1.48	0.60
Control	3.93	1.66
Chemical treatment	2.97	1.63

Note: B1 is the *Pleurotus ostreatus* treatment, B2 is the *Lentinula edodes* treatment, Q is the chemical treatment, C is control treatment.

Table 5 - Considered random effects configurations for each model.

Model	Random effects	AIC
1.1	Transect	523.5
1.2	Transect + treatment in transect	505.7
1.3	Treatment in transect	503.7
2.1	Transect	160.7
2.2	Transect + treatment in transect	162.5
2.3	Treatment in transect	162.7
2.4	Transect (Girona dataset)	122.6
3.1	Transect	391.6
3.2	Transect + Treatment in Transect	391.2
3.3	Treatment in transect	389.2

Note: nc: not converge; AIC: Akaike Information Criterion.

Model 2

There was not a significant difference in the odds of sprouting between the control and B1 (biological treatment with *Pleurotus ostreatus*) treatments, nor between B1 and Q treatments in Lugo and Girona, but there was a marginal difference between the effects of the control and chemical treatments (p=0.0983, Tab. 8). However, there was a difference in the probability of sprouting between Girona and Lugo (p=0.00585).

The results of the model for Girona alone indicate that, compared with the control, the treatments resulted in a decrease in the probability of sprouting and that the effect was greater with Q than with B1. However, the effect was not significant for B1 treatment (p=0.19) and only marginally significant for Q treatment (p=0.0524) (Tab. 9).

Model 3

There was strong evidence that both biological treatments decreased the probability of sprouting compared with the control (p<0.0001 for both B1 [biological treatment with *Pleurotus ostreatus*] and B2 [biological treatment with *Lentinula edodes*]). The odds of sprouting in the control treatment were 99.4 (95% CI for the odds ratio, 20.9 to 472.3) and 22.7 (95% CI for the odds ratio, 5.00 to 103.2) times greater as large as those in stumps treated with *Pleurotus ostreatus* and *Lentinula edodes*, respectively (Tab. 10). The *Pleurotus ostreatus* treatment was more effective preventing sprouting than the *Lentinula edodes* treatment (p<0.0001). The odds of sprouting in stumps treated with *Lentinula edodes* were 4.37 times as large as in stumps treated with *Pleurotus ostreatus* (95% CI for the odds ratio, 2.33 to 8.21).

In summary, results showed evidence for the effect of biological treatment on reducing the probability of sprouting in walnut stumps. Both biological treatments showed better performance than chemical and control treatments, albeit *Pleurotus ostreatus* was more efficient than *Lentinula edodes*.

Mushrooms yields

There were 1157 records in Lugo, 109 in Girona and 864 in Toledo (Fig. 3). The number of sporocarps in a single stump ranged between 0 and 43. The mean number of sporocarps per stump was 0.47, but it was very different depending on the site (0.52 in Lugo, 1.79 in Girona and 0.23 in Toledo). The mean weight of mushroom per stump was 15.58 g, but ranged between 18.25 g in Lugo, 107.24 g in Girona and 0.43 g in Toledo. Week 46 (second week of

Table 6 - Solution for the biological and control treatment model comparing the effect of the *Pleurotus ostreatus* (B1) and control treatments across all sites (model 1.3, table 2).

Fixed effects	Estimate	Std.Error	z value	Pr(>lzl)	Significance codes
(Intercept)	3.2313	0.5050	6.399	<0.0001	
site Cuenca	3,1581	0.8833	3.576	0.0004	***
site Lugo	-0.3370	0.4751	-0.709	0.4781	
site Girona	1,8598	0.7353	2.529	0.0114	*
site Toledo	1,1452	0.6728	1.702	0.0887	.
treatment B1	-2.8573	0.4749	6.016	<0.0001	***

Note: Std. Error is the standard error of the estimate, z value is the value of the Z test, Pr(>lzl) is the P-value associated with the test. Reference level for place is Cáceres and for the treatment is the control. The estimate for site Cuenca is the difference in the log odds of sprouting in Cuenca, site Lugo is that for Lugo, site Girona is that for Girona, site Toledo is that for Toledo. The estimate for treatment B1 is the difference in the log odds between treatment B1 and the control. Significance codes: 0 '***'; 0.001 '**'; 0.01 '*'; 0.05 '.'; 0.1 ''; 1.

Table 7 - Estimated probabilities of sprouting in stumps treated with B1 by sites.

Site	*Pleurotus ostreatus* treatment	Control
Cáceres	0.5924	0.9620
Cuenca	0.9716	0.9983
Lugo	0.5092	0.9476
Girona	0.9032	0.9939
Toledo	0.8204	0.9876

November) was the most productive, with a mean number of mushrooms of 1.73 and a weight of 67.28 g (Fig. 3).

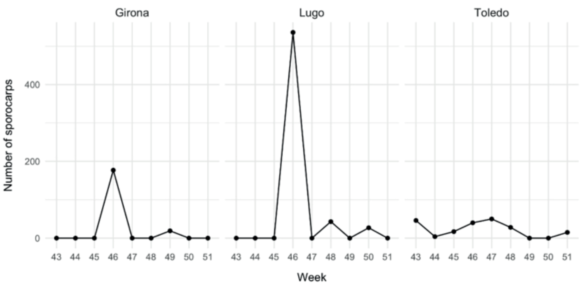

Figure 3 - Number of sporocarps picked by site and week.

Discussion

In this study, the effect of stump inoculation with edible wood decay fungi in walnut plantations has been assessed. Our results prove the great potential of these fungi for stump degradation and how they provide two beneficial effects: *i)* sprouting reduction and *ii)* production of edible mushroom. This article underpins wood decay fungi as a more efficient technique for stump degradation than natural degradation or chemical treatment.

Our findings show that biological stump degradation can be considered as a viable alternative for the elimination of stumps and the reduction of sprouts after logging a stand. In our studied plantations, all the trees that were not subjected to either biological or chemical treatment, did not die and continued growing as coppice. In forestry, most of the experiences of stump degradation have been carried out through chemical or heavy mechanization (Alonso et al. 2007). Mechanical extraction of stumps is estimated to cost over 700 € ha^{-1} for hardwood species in Spain (TRAGSA S.A. 2019). Regarding chemical treatments, herbicides can kill the stump but would never contribute to its degradation. Environmental concerns have led to a global reduction of the chemical herbicides used in forests (Dumas et al. 1997, Becker et al. 2005). Although in this paper, biological stump degradation has been only evaluated from an ecological point of view, future studies including a wide array of forest stand characteristics (species, density, age, etc.), may help to define appropriate forest management prescriptions in which biological stump degradation could be a viable economic and ecological alternative.

Our results showed that fungal inoculation reduced probability of a stump sprouting, the number of sprouts per stump and their height compared to an untreated control. Specifically, an untreated stump was 17.41 times more likely to resprout than inoculated peers. However, this general pattern was not uniform across tested sites, with Lugo and Cáceres displaying the lowest sprouting frequency found among the inoculated stumps, 0.51 and 0.59 respectively. This contrasted with much higher sprouting rates in Toledo (0.82), Girona (0.90) and Cuenca (0.97), where almost all stumps sprouted, and inoculation meant no advantage over control. These results can be explained by site-specific conditions regarding the plots themselves, and by intrinsic limitations of the experiments carried out

Table 8 - Solution for the model comparing the effects of the *Pleurotus ostreatus* (B1), chemical and control treatments in Girona and Lugo (model 2.1, table 2).

Fixed effects	Estimate	Std. Error	z value	Pr(>lzl)	Significance codes
(Intercept)	4.7951	1.0171	4.715	<0.0001	***
site Girona	-1,9984	0.7251	-2.756	0.0059	**
treatment B1	-1,0938	0.8185	1.336	0.1814	
treatment Q	-1.3373	0.8090	-1.653	0.0983	

Note: Std. Error is the standard error of the estimate, z value is the value of the Z test, Pr(>lzl) is the P-value associated with the test. Reference level for treatment is the control and Lugo for the site. treatment B1 is difference in the log odds of sprouting between the *Pleurotus ostreatus* (B1) treatment and the control, treatment Q is the difference in the log odds of between the chemical treatment and the control. Significance codes: 0 '***'; 0.001 '**'; 0.01 '*'; 0.05 '.'; 0.1 ''; 1.

Table 9 - Solution for the model comparing the effects of the *Pleurotus ostreatus* (B1), chemical and control treatments in Girona (model 2.4).

Fixed effects	Estimate	Std. Error	z value	Pr(>lzl)	Significance codes
(Intercept)	3.210	1.039	3.090	0.0020	**
treatment B1	-1.448	1.093	-1.325	0.1853	
treatment Q	-2.076	1.070	-1.940	0.0524	.

Note: Std. Error is the standard error of the estimate, z value is the value of the Z test, Pr(>lzl) is the P-value associated with the test. Reference level for treatment is Control (C). Treatment B1 is the difference in the log odds of sprouting between the *Pleurotus ostreatus* (B1) treatment and the control, treatment Q is the difference in the log odds of between the chemical treatment and the control. Significance codes: 0 '***'; 0.001 '**'; 0.01 '*'; 0.05 '.'; 0.1 ''; 1.

at each location. In line with this hypothesis, the site with highest rainfall (and hence soil moisture), provided better conditions for stump colonization by fungi. Site-specific conditions influenced the behaviour of the inoculated fungi, which ultimately related to site humidity. While in Lugo, all the stumps treated with chemical treatment sprouted, in Girona (with the driest conditions) chemical treatments was marginally more effective than the two biological treatments. The reason in Girona may have been a result of the biological treatment not having enough time to develop the mycelium and begin to degrade the stump. In this sense, since there is an increasing pressure from society against using herbicides and for using environmentally beneficial alternatives (Bellgard et al. 2014), results of this study demonstrate the potential of *Pleurotus ostreatus* and *Lentinula edodes* as a nature-friendly and sustainable, as well as efficient alternative.

Wood rot fungi species possess a powerful set of enzymes for wood decomposition. They evolved adapting to access an abundant source of food which is nonetheless very stable chemically. From the vast number of naturally occurring species, only a few have been tested in stump inoculation experiments, most of them non edible. In this paper, *Pleurotus ostreatus* and *Lentinula edodes* saprophytic edible species, have shown their ability to reduce the resprouting of walnut species. These species, together *Pycnoporus sanguineus* and *P. chrysosporium* (Kerem et al. 1992, Sik and Unyayar 1998, Pu et al. 1998, Kodrík 2001, Poiting et al. 2003, Bari et al. 2015) were analysed previously to assess the potential of fungi in stump degradation of other tree species like *Quercus rubra* L. (Oriaran et al. 1989, Labosky et al. 1991), *Betula papyrifera* Marshall (Tai et al. 1990), *Paulownia elongata* S.Y. Hu (Tan et al. 1998), *Fagus sylvatica* L., *Pinus sylvestris* L. (Poiting et al. 2003) and *Picea glauca* (Moench) Voss (Roy et al. 2010). In *Eucalyptus* species, Silva et al. (2010) evaluated in vitro Eucalyptus sawdust degradation with several white-rot fungi and found that the most efficient decay was achieved with *Pycnoporus sanguineus* and *Phellinus gilvus*. Negrão et al. (2014) found a great ability of *Pycnoporus sanguineus* and *Lentinus bertieri* to degrade *Eucalyptus* hardwood. In *Eucalyptus* plantations in Brazil (*E. saligna*, *E. grandis*, *E. urograndis*, *E. urophylla*), *Pycnoporus sanguineus*, *Ganoderma* sp. and *Peniophora* sp. (Ferraz et al. 1998, Alonso et al. 2007), *G. applanatum*, *Lentinus bertieri*, *Xylaria* sp., *Lentinula edodes*, *Pycnoporus sanguineus* (Negrão et al. 2014) or *Gloeophyllum trabeum* (Aguiar et al. 2013) have been used as biological control agents.

Other researchers have evaluated the effect of fungi species in stump resprouting capacity. Bellgard et al. (2014) in *Salix fragilis* L. and *Salix cinerea* L. and Hantula et al. (2012) studied the efficiency of *Chondrostereum purpureum* to control sprouting in *Betula pendula* Roth, *Betula pubescens* Ehrn., *Populus tremula* L. and *Sorbus aucuparia* (L.) Crantz. The latter, found that the proportion of dead stumps with the best isolates exceeded 80% in

Table 10 - Solution for the model comparing the effects of the *Pleurotus ostreatus* (B1), *Lentinula edodes* (B2) and control treatments in Lugo and Toledo (model 3.3)

Effects	Estimate	Std. Error	z value	Pr(>lzl)	Significance codes
(Intercept)	3.4289	0.7445	4.605	<0.0001	***
site Toledo	2.4205	0.3985	6.074	<0.0001	***
treatment B1	-4.5990	0.7953	-5.783	<0.0001	***
treatment B2	-3.1235	0.7721	-4.045	<0.0001	***

Note: Std. Error is the standard error of the estimate, z value is the value of the Z test, Pr(>lzl) is the P-value associated with the test. The control treatment was the reference level for the treatment test and Lugo was the reference level for site. Site Toledo is the difference in the log odds of sprouting betwee Toledo and Lugo, treatment B1 and treatment B2 difference in the log odds of sprouting between the *Pleurotus ostreatus* and *Lentinua edodes*, and control treatments, respectively. Significance codes: 0 '***'.

Betula pendula Roth and *pubescens* Ehrn.

Becker et al. (2005) assessed the effect of *Chondrostereum purpureum* to prevent the resprouting of *Alnus rubra* Bong. in Canada, finding that all the stumps died and there were not any sprouts 2 years after inoculation. Other studies (Dumas et al. 1997) evaluated the efficiency of the same fungi species in *Populus tremuloides* Michx and *Populus grandidentata* Michaux, and found a decrease of 63% in the resprout and a 39% in the stump mortality one year after inoculation.

On the other hand, other researchers have evaluated the success of biological stump degradation in terms of losses of degraded mass. Abreu et al. (2007) reported stumps mass losses of 22.6%, 16.2% and 11.3% thanks to the action of *Pycnoporus cinnabarinus* (Jacq.) Fr., *Pleurotus ostreatus* and *Schizophyllum commune* Fries, respectively, after 8 weeks of incubation in *Eucalyptus*. Calonego et al. (2010) showed mass losses of 34.3% after 12 weeks caused by *Pycnoporus sanguineus* in *Eucalyptus grandis* W.Hill. Finally, Boyle (1998) showed losses of 31.8% in *Acer sp.* and *Betula sp.* by the action of *Lentinula edodes* and *Pleurotus sajor caju* (Fr.). Our results did not record enough information to estimate mass losses because stump degradation was evaluated only several months after treatment. Further research focused on time degradation effects must be carried out in the future to improve the accuracy and efficiency of the biological technique.

Alternatively, limitations in the experimental design could be affecting the results of the study. The statistical analysis carried out allowed us to discuss the differences among treatments and to compare the treatments and the control in each site. The comparison between treatments in all sites was not possible due to the unbalancedness of the design, which may limit the ability to generalize the results. However, results showed an important site effect that was important to try to elucidate which factors may be causing those differences in effectiveness. Further studies must take into account this consideration in each site in order to conclude in this sense.

Moreover, longer-term studies could improve the results, allowing monitoring of the biological degradation of stumps and providing additional data to fully evaluate it as an alternative from ecological and economic points of view. Additional factors to be considered in future could be the date of the inoculation process as well as the humidity and temperature conditions after treatment. These factors could enable the establishment of different scenarios to predict the uncertainty (Herrero et al. 2019). Variables linked to degradation process are very important in the current context of climate change, since alterations in the climatic conditions are expected in the Mediterranean area. Increasingly irregular precipitation accompanied by extreme rainfall events (IPCC 2013), would likely affect the outcome of stump treatment with wood decay fungi.

The economic and social relevance of edible mushroom resources has increased in the last decades. The performance of alternative biological controls, specifically inoculation with edible fungi, has also provided income to forest owners, who manage their plantation with a new output with an important social and economic impact.

Conclusions

Our article shows the first results on stump degradation in walnut plantations and shows evidence of ability of biological treatment to reduce the probability of sprouting in walnut stumps. Biological treatments with *Pleurotus ostreatus* and *Lentinula edodes* showed better performance than control treatments. Moreover, *Pleurotus ostreatus* was more efficient than *Lentinula edodes*. These results provide useful information for sustainable forest management, by using a close to nature approach for stump degradation while also generating a product, *i.e.* mushrooms. This could contribute to profitability to the walnut chain, one of the most important and profitable hardwood species widely cultivated for timber and nut production.

Our findings support the stump inoculation of rot fungi as an alternative nature-based solution for stump removal after logging, with clear enhancement of stump degradation and sprouting reduction. This paper raises also awareness on the sustainability of resources with a big social and environmental prospective impact. Biological stump degradation, which constitute a toxic-free technique, demonstrates the potential of these two fungi species as biocontrol agents with valuable applications in forest management.

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Technical note

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

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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 specificall 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,

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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)

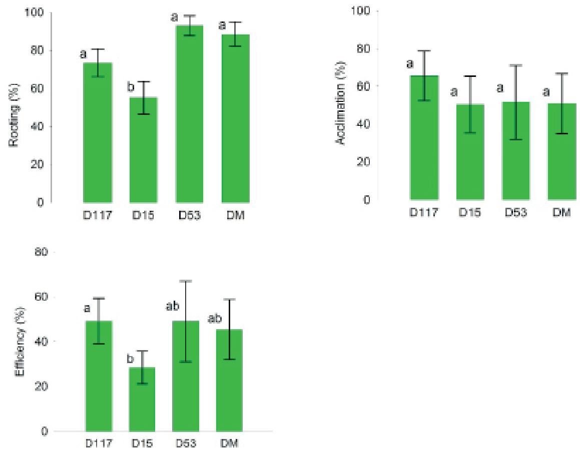

Figure 1 - Effect of clones on the rooting rate (%), acclimation rate (%) and efficiency (%) of hybrid walnut (*Juglans x intermedia Mj209*) in vitro micropropagation.

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

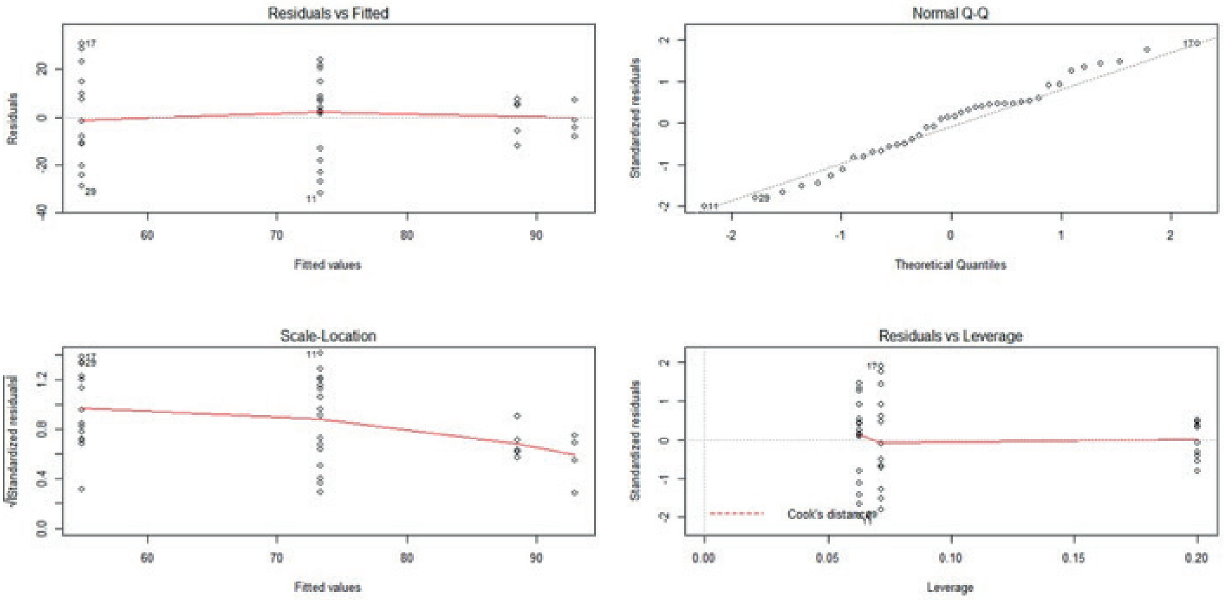

Figure 2 - Graphical evaluation of the residuals of the statistical model analyzing the effect of clones on the rooting rate (%) of hybrid walnut (*Juglans x intermedia Mj209*) in vitro micropropagation.

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 efficienc , 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).

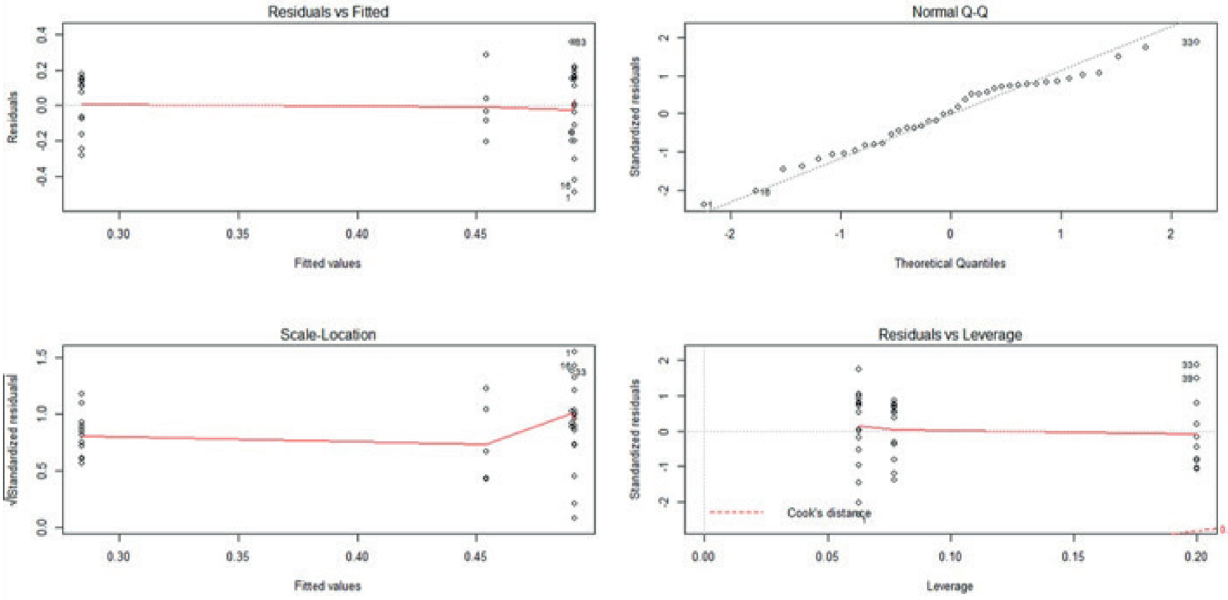

Figure 3 - Graphical evaluation of the residuals of the statistical model analyzing the effect of clones on the efficiency rate (%) of hybrid walnut (*Juglans x intermedia Mj209*) in vitro micropropagation.

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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Technical note

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"Second generation of planted hardwood forests in the EU"

Changing the colour of European Hybrid Walnut by means of digital printing

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Abstract - In terms of market, the colour of Walnut timber and veneer is the one from *Juglans Regia* L.. That colour is darker than the one from Hybrid European Walnut . This fact is a barrier for market success of Hybrid European Walnut plantations. There are different methods for modifying the colour such as vaporising, dyeing, thermal modification, etc. Digital printing is an innovative technology that can be used for changing the colour of wooden surfaces. Using transparent inks makes possible to maintain the grain and figure while the colour is modified. In addition to this, digital printing makes possible not just to apply a flat colour but a texture. The review details the colour coordinates allowing that transformation under a certain printing device. It also explains further opportunities of that technology.

Keywords - digital printing, colour modification, European Hybrid Walnut.

Introduction

One of the challenges that timber and veneer products made with young Hybrid Walnut suffer is that they are too light coloured and too unsaturated. For that reason, they do not look as Walnut products. Therefore, the market price of Hybrid Walnut products is lower, and using this species as a source of material becomes unfeasible in economic terms.

In order to overcome this challenge, two industrial Companies (Seistag and Losan) have worked on colour modification of wooden products such as veneer and timber. The collaboration was possible thanks to Woodnat Project, which is an Innovation Action under H2020 that has been supported by REA (GA- 728086). The research focuses on changing the natural colour to reach a new colour that fits to the standard likes of the market. This paper provides an overview of that research and shows the results and methodology for digitally printed wooden surfaces carried out by Seistag.

Figure 1 - Sliced logs after the vaporising process.

In order to dark the natural wood of young Hybrid Walnuts, during Period 1 of Woodnat Project it was studied the process of vaporising. The vaporising process applied to hybrid Walnut is based on the values traditionally applied to vaporizing beech. This process is applied to the logs provided by a partner Company. Those logs were sliced to produce veneer after the vaporising (Fig. 1).

According to the experiences developed by Losan, the vaporising process contributes to: *(i)* improve the technical quality of the veneer for the slicing process and, *(ii)* change the colour of the material reaching a darker and warmer tone. Nevertheless, the colour change due to vaporising does not reach the target of Walnut Regia colour which is the standard in the current market.

Considering those results, two independent research lines have been carried out during Period 2 of Woodnat Project. On one hand, Losan continued to study the colour modification using dyeing. On the other hand, Seistag worked with the same aim, but using digital printing.

In addition to this work of adding colour, both Companies enlarged the focus of the study to consider the opposite process, which is reducing the saturation. This action transforms the original col-

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Figure 2 - Color modification obtained by the described processes.

our of the wood into a grey surface, which is also a valuable decorative effect for the current market (Fig. 2). Both processes, dyeing and digital printing, share the definition of the challenge: reaching a certain target colour by means of modifying the natural colour. Therefore, colour coordinates modification is the basis for both researches. Nevertheless, each process is developed under different circumstances. The modification of colour based on dyes takes advantage of the CIELab coordinates of selected dyes. After the selection, those dyes oght to formulated considering their interaction. Meanwhile, the colour modification by means of digital printing uses four standard inks, which are combined in a well-known colour space (CMYK) in order to fill the gap between the original colour and the targeted one.

In addition to the technical difference both technologies provide different opportunities:

(i) Dyeing changes the inside of the wood while digital printing works on its surface, this means that dyed veneers can be sanded while digitally printed cannot; *(ii)* Dyeing makes possible a complex interaction with the different chemical nature of each part of the wood, so more complex final colours are possible. Meanwhile, digital printing interaction is just a transparency, which is a simpler effect in terms of colour, but more complex in terms of grain; *(iii)* Digital printing can add different colour in different places while dyeing adds the same colour for all the surface of the wood. Due to this issue, dyed veneer is suitable for higher accuracy, while digitally printed veneers makes possible gimmicky effects.

Method

Colour modification

Transforming young wood from Hybrid Walnut into old Walnut Regia by means of digital printing included a two-step research. *(i)* The first to be done is calibrating the change of colour for the average tone of wood. The result of this process is a plain tone to be printed in order to change light coloured Walnut into dark Walnut (Pastore et al. 2004) *(ii)* Later, a complex colour map with sapwood, grains and different changes can be added to the flat colour. By means of this, the printing is not a mere varnish layer over the wood, but a complex effect of transparency that visually melts with the wood surface.

Results

Regarding the first step, changing the colour, the work done includes the following stages: *(i)* Measuring the average starting colour in CIELab by means of a colorimeter (95, 2, 13). In this phase, the method and filters applied to the spectrophotometer are critical for and accurate and meaningful measurement; *(ii)* Measuring the average target colour in the same manner (37, 18, 20); *(iii)* Calculating the colour transformation in CIELab coordinates. In this phase the simple approach of Delta E76 calculation (Euclidean) demonstrated to be precise enough for the purpose as the process starts at a very high value for Delta E (Δ = 84, 29) (Kawasumi et al. 1999); *(iv)* Establishing the printing mode and CMYK coordinates that reproduce the colour neutralization. This work is done by means of iterative experimental tests where the result of each test is measured according to the effective Delta E76 achieved (37, 62, 61, 56). The process stopped when reaching a Delta E of enough accuracy (Δ < 5) (Fig. 3).

For this task, the printing device and inks are critical to the results as the results refer to that specific device. The device compromises the sum of veneer type, ink, head printers and printing mode. EFI Vutek UVI printer and 3M inks with 4 levels of grey were used during the experimental development.

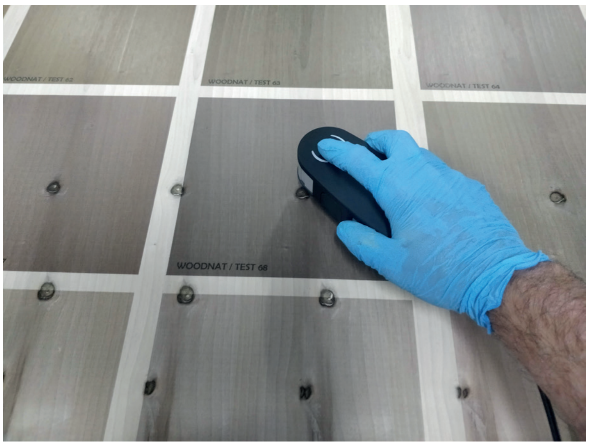

Figure 3 - Measuring the average starting color in CIELlab.

Figure 4 - Final color modification results.

Figure 5 - Transparent overlap of luminosity added layer.

The above-mentioned method is focused on reaching a target colour of *Regia* Walnut wood, but it can be also applied to reach any other targeted tone as for example a grey surface (62,51,40,30). In this case, the expected result of printing is to reduce the last two values of the targeted CIELab coordinates. The lower their value is the greyish the wood is. Nevertheless, both values should keep in a similar level (ca. ±2) to ensure the absence of any dominant tone such as greenish or blueish.

Further transformation of wood surface by means of digital noise

Once the targeted colour has been reached, the process of adding extra effects might start. Those effects allow: *(i)* to produce a richer colour surface, *(ii)* to speed up the printing process without banding, and *(iii)* to add grain, fibre or figures to the final surface. This process is based on complex maps of colour to modify the average CMYK pattern (Lucassen et al. 2008). (Fig. 4).

In order to achieve the desiderated effect with minimal efforts, the colour modification (a flat colour) is stored in a separated layer while two other layers are added to achieve complexity: *(i)* The first layer is an effect of noise with the form of colour variations along wooden planks. It is added by means of a transparent overlap of colour blending; *(ii)* The second layer is an effect of noise with the form of wooden fibres. Other patterns such as knots or cracks can also be considered. This layer is added by means of a transparent overlap of luminosity, blending between 20 to 35% depending on the pattern (Fig. 5).

Measuring the productivity and the ink consumption per square meter has been an important part of the research. The ink consumption was measured using EFI Software based on the analysis of the images showing a consumption of inks between 3,82 and 13,80 ml/sqm. The timing showed even higher deviations, from 4:10 to 23:37 minutes per square meter. That time depended heavily on the printing

Figure 6 - Final results of Walnut surface modification.

Figure 6 - Final results of Walnut surface modification.

mode. Adding noise has demonstrated to allow less consumption and higher productivity rates (Donevski et al. 2015) By means of these techniques, the cost of the process has been reduced and a cost-effective technical process to transform the image of the wood surface has been achieved (Fig. 6).

This method for changing the surface of wooden panels has also been used as a creative tool. By means of this, innovative effects such as woodcarving and arabesques have been produced using plain Hybrid Walnut wooden surfaces(Fig. 7).

These results defined a new field of experimentation, a field which does not refer to technical research but to product innovation. It is important to note that this line of development is not constrained to the specific challenge of Hybrid Walnut, as it can also be applied to other species and wooden products increasing the potential impact of the work performed by Seistag.

Conclusions

It has been demonstrated that the technology of digital printing is suitable to transform the original colour of wooden surfaces into a targeted colour.

Within Woodnat Project, Seistag has developed a technical method to perform that transformation with enough accuracy in an easy manner.

Moreover, the company developed technology to speed up the printing process and reduce the consumption of ink by means of overlapping digital noise to reduce the lack of quality, which is associated to low-cost printing.

The market uptake of the technology is currently under evaluation. Good results have already been achieved (stgnature.com).

Aknowledgments

The experience developed by Seistag has been possible thanks to the collaboration of: *(i)* Bosques Naturales, who provided the logs from European Walnut Hybrids; *(ii)* Losan, who produced the veneered boards needed for colour measurement and digital printing; *(iii)* Talendis, who provided access to the printing device, and; *(iv)* REA, who granted this research among the Innovation Action under Grant Agreement 728086.

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Supplementary material

Special Issue: HORIZON 2020 GA 728086 WOODnat
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WOODnat photogallery

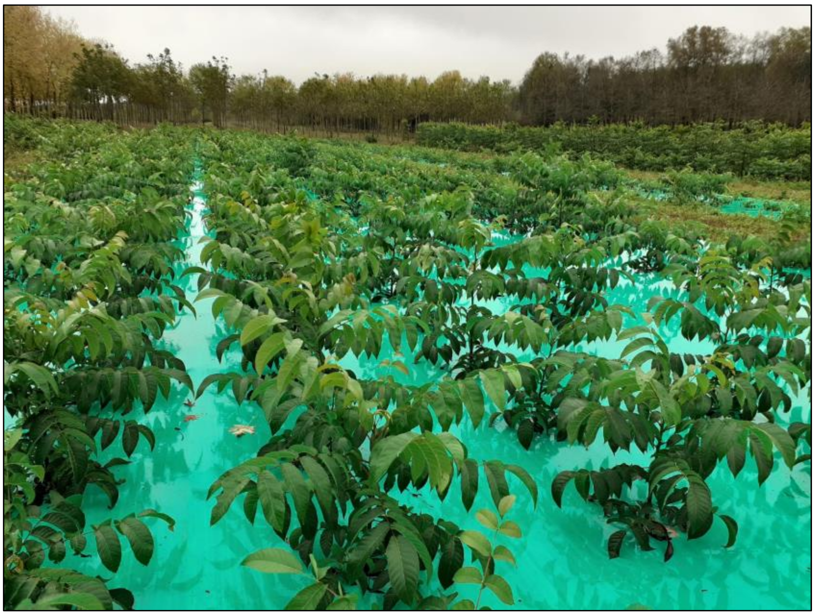
◄ **Picture 1** - Micro propagated plants at Bosques Naturales Lab in Madrid Spain (photo Pelleri).

Picture 2 – Seedling of walnut in a ► nursery pot in Galicia, Spain (photo Urbán).

◄ **Picture 3 -** Pots trial in Galicia, Spain (photo Urbán).

◀ **Picture 4 -** Commercial plants produced using innovative pot (Air-Pot®) in Galicia, Spain (photo Urbán).

Picture 5 - Nursery of hybrid walnut to produce bare root commercial material (photo Urbán). ▼



◀ **Picture 6 –** Pure hybrid and common walnut plantation in Galicia, Spain (photo Chiarabaglio).

◄ **Picture 7 –** Pure hybrid and common walnut plantation in Galicia, Spain (photo Chiarabaglio).

Picture 8 - Forest management ► unit of Bosques Naturales in Galicia. Pure hybrid walnut fourteen years old plantation (photo Urbán).

◄ **Picture 9 –** Pure Hybrid walnut plantation in Galicia, Spain (photo Urbán)

◀ **Picture 10 –** Forest management unit of Bosques Naturales in Galicia. Pure hybrid walnut fourteen years old plantation (photo Urbán).

Picture 11 - Pure walnut plantation with nurse trees during thinning (photo Bidini). ▼

Picture 12 – Pure walnut plantation with nurse trees (see picture 11), 10 years after thinning (photo Pelleri) ▼

◀ **Picture 13 –** Walnut tree near Empoli, Italy (photo Pelleri).

◀ **Picture 14 –** - Polycyclic plantation near Lodi, Italy (photo Pelleri).

Picture 15 - Polycyclic plantation near Lodi, Italy (photo Pelleri). ▶

◀ **Picture 16 –** Polycyclic plantation near Lodi, Italy, after poplar harvesting (photo Pelleri).

◀ **Picture 17 –** Hybrid walnut in polycyclic plantation near Mantova, Italy (photo Pelleri).

Picture 18 - Walnut in polycyclic ▶ plantation near Lodi, Italy (photo Bidini).

◀ **Picture 19 –** Linear walnut plantation near Empoli, Italy (photo Pelleri).

◀ **Picture 20 –** Walnut-alder-pumpkins (Agroforestry system) near Casale Monferrato, Italy (photo Pelleri).

Picture 21 – Hybrid walnut- corn Agroforestry system in Galicia, Spain (photo Urbán). ▼

▲ **Picture 22** - Hybrid walnut-corn (Agroforestry system) in Galicia, Spain (photo Urbán).

Picture 23 – Agroforestry ▶ system in the forest management unit of Galicia (photo Urbán).

◄ **Picture 24 –** Herd of sheep for vegetation control in Galicia, Spain (photo Urbán).

Picture 25 – Thinning operations in a pure hybrid walnut plantation near Toledo, Spain (photo Pelleri). ▼

Picture 26 - Thinning operations in a pure hybrid walnut plantation. Trees were felled and a Biological Stump Degradation treatment with lignicolous fungi species was applied (photo Cuesta). ▼

◄ **Picture 27 –** Cross-sectional disc (5 cm-10 cm thick) obtained by chainsaw on each felled (photo da la Parra).

◀ **Picture 28 –** Inoculated stump with the mycelium to be degraded with biological treatment. (photo da la Parra).

Picture 29 - The Biological ▶ Stump Degradation happened between the stump and the cross-sectional discs on the felled trees (photo Cuesta).

◀ **Picture 30 –** Biological treatment was covered with a biodegradable plastic bag and land to protect the effect (photo da la Parra).

▲ **Picture 31** - Walnut shoots found in control plot with no treatment after the experiment (photo Cuesta).

Picture 32 – Pleurotus ostreatus emerged after Biological Stump Degradation (photo Cuesta) ▼

◀ **Picture 33 –** Logs from first thinnings of hybrid walnut planted forest (photo Urbán)

Picture 34 – Boule, typical ▶ air-drying system for big natural walnuts (photo Santonja)

▲ **Picture 35** - Boards produced in the trials with hybrid walnut logs (photo Urbán).

Picture 36 – Veneer trial at Tabu company in Cantù, Italy (photo Pelleri). ▼

◀ **Picture 37** – Veneer quality check at Tabu company in Cantù, Italy (photo Pelleri)

Picture 38 – Walnut ▶ veneer sheets with different thermo treatments (photo Groutel)

Picture 39 - Some examples ▶ to enhance small assortment of walnut (photo Urbán).

◀ **Picture 40 –** Project technical visit to an experimental walnut plantation near Cremona, Italy (Photo Bidini).

◀ **Picture 41 –** Walnut in polycyclic plantation near Mantova, Italy (photo Bidini).

Picture 42 - Polycyclic plantation ▶ near Casale Monferrato, Italy (photo Pelleri).

◄ **Picture 43 –** Big walnut boards near Bosques Naturales Office in Madrid, Spain (photo Groutel).

Picture 44 - Woodnat staff at the general assembly in Madrid, Spain (photo Groutel). ▼