CHAPTER 35

MICROPROPAGATION OF *JUGLANS REGIA* L.

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1. INTRODUCTION

Micropropagation of *Juglans regia* and hybrids may either be initiated from zygotic embryos, shoots, branches or adult plant scions (Leslie & McGranahan, 1992). Like several other woody species, walnut tree explants obtained from embryonic tissues are easily established *in vitro* and multiplied by direct organogenesis; however, this often happens with little early care. Explants obtained during the fruit production phase can also be multiplied this way (Leslie & McGranahan, 1992). The experience with *Juglans regia* cv. Chandler shows that individuals reproduced *in vitro* by culture of nodal segments have earlier fruit production as compared with those obtained by traditional grafting techniques, besides presenting a stronger rooting system and avoiding incompatibility between rootstock and scions (Hasey et al., 2001; López, 2001).

The major problem associated with *in vitro* introduction of walnut mature material is the endogenous bacterial contamination and phenolic compound exudation. However, the use of renewed material from consecutive pruning or macrografting allows us to develop shoot tips with high growth rates (Claudot et al., 1992; Leslie & McGranahan, 1992), a practice that could reduce such problem in later phases of the culture. The use of annual growth shoot tips (Leslie & McGranahan, 1992), meristems (Meynier, 1984) as well as tips obtained from epicormic sprouts appear to be the best alternatives for introduction of explants from field to *in vitro* conditions (Heile-Sudholt et al., 1986).

McGranahan & Leslie (1988) introduced nodal segments from Sunland, Vina and Chandler cultivars and concluded that their commercial propagation is possible. On the other hand, Navatel and Bourrain (2001) detected differences between genotypes with

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respect to multiplication rates and elongation of internodal zones. Regarding the culture media used, and did not detect any quantitative difference in the multiplication rates, and concluded that larger diameter and more homogeneous microshoots were obtained in the DKW media (Driver & Kuniyuki, 1984). On the other hand, Marques and Dias (2001) reported that sucrose is the most appropriate carbohydrate source for *in vitro* walnut culture. Furthermore, time of cultivation does not affect the multiplication rate of mature material, but has an effect on the incidence of apical necrosis after the third subculture period.

Regarding gelling agents, Saadat and Hennerty (2002) obtained higher shoot proliferation both in MS media plus phytagel and DKW media plus phytagel (2.7 new shoot per explant). Best proliferation results were obtained with 1 mgL^{-1} of benzyl-aminopurine (BAP) plus 0.01 mgL^{-1} of indole-3-butyric acid (IBA).

With respect to the rooting phase, Saadat and Hennerty (2001) obtained higher percentage of rooted microshoots with IBA as compared to naphthalene acetic acid (NAA). They also recommended carrying out the root induction under dark conditions (9 days) in presence of auxins in the culture media, which permit to obtain up to 83% rooted microshoots. On the other hand, Sánchez-Olate (1996) indicates that the best rhizogenic response comes from apical shoot segments 2-cm long, showing a close relationship between auxin concentration and exposure time. Rooting rates of 85% are achieved inducing microshoots in MS media with major nutrients diluted to 25% and in complete darkness. The time of induction will depend on the auxin concentration applied. For a concentration of 3 mgL^{-1} the induction phase should last 7 days while for a concentration of 5 mgL^{-1} the induction phase should be 3 days. However, there are differences in rooting rates depending on the cultivar used (Vahdati et al., 2004).

This work presents the method for micropropagation of mature tissues of *Juglans regia* L. growing in field conditions, and describes the establishment, multiplication, rooting and microplant acclimatization phases.

2. EXPERIMENTAL PROTOCOL

2.1. Explant Preparation

2.1.1. Growing Conditions of Mother Plants

Plants growing in the field. Material showing glabrous shoot tips with 2–4 week growth, 0.5–0.8 cm diameter, 2–5 cm internodes and presence of vegetative leaf buds is collected from selected trees growing during the spring season (Figure 1A). The material must be transferred to the lab using an antioxidant solution (cysteine and ascorbic acid at concentrations of 20 and 5 mgL^{-1}, respectively) and a fungicide solution (Captan and Benomil at concentration of 1 gL^{-1} each).

Greenhouse plants. Juglans regia plants grafted over *Juglans nigra* are maintained in the greenhouse conditions with biweekly applications of a fungicide mixture of Benlate (Dupont®), Kasumin (Hokko Chemical®, Tokyo) and Sanagrícola (Agrocros S.A., Spain), in order to reduce superficial contamination levels. Scion comes from adult trees (25 years), while the rootstock is a 7-year-old plant (Figure 1B).

Figure 1. *A) Walnut mother trees where initial material for in vitro vegetative multiplication is obtained are growing in the field. B) Grafted walnut plant growing in the greenhouse conditions.*

2.1.2. Explant Excision and Sterilization

Excision. Plants growing in the field: Late winter (August) and early spring (September), semi-woody branches 2–4 years old, 20 cm long and having active vegetative leaf buds from mature walnut tree are collected (Figure 2A). They are submitted to a superficial disinfection process with a mixture of fungicides (Captan and Benomil, 1 gL^{-1} each) in a laminar flow chamber and continuous stirring for 20 min. Branches are then washed three times with sterile distilled water and placed on filter paper in the laminar flux chamber until dry. Subsequently, they are left in 200 cc bottles under sterile conditions, with 50 ml water and sealed with translucent plastic bags (Figure 2A). The system is left in cultivation chamber during a 16-hour photoperiod (40–45 $\mu Em^{-2}s^{-1}$) at 25 ± 1°C until vegetative leaf bud shooting.

Greenhouse plants. Nodal segments containing 2 or 3 vegetative leaf buds are extracted from grafted plants maintained in greenhouse. They are transferred to the lab in an antioxidant aqueous solution as explained previously in section 1.1. Sprouts are cut in laminar flux chamber keeping one vegetative leaf bud per knot (Figure 2B) and making superficial washing in solution of Kasumin® (0.5 g L^{-1}) and Captan® (3 g L^{-1}).

2.1.3. Sterilization

Plants growing in the field. Vegetative leaf buds approximately 3 cm long are cut from branches under laminar flux chamber conditions. Superficial asepsis is carried out in hypochlorite solution (1.5 gL^{-1} active chlorine) in continuous stirring for 10 min. They are then washed three times in distilled water and put into an antioxidant

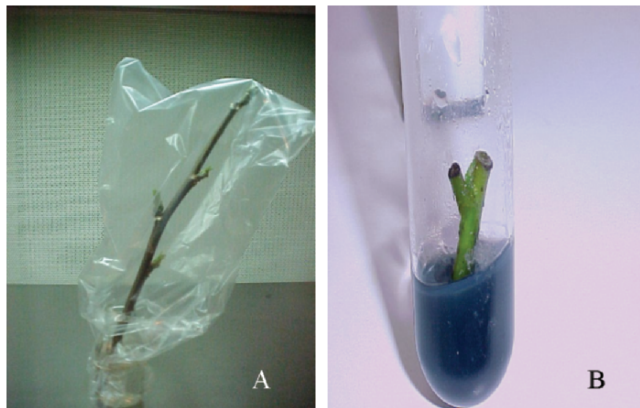

Figure 2. *Type of plant material to be introduced in vitro. A) Branches collected from adult tree growing in field and induced to vegetative leaf bud development. B) Nodal segments obtained from grafted plants growing in greenhouse conditions.*

aqueous solution containing 100 mgL^{-1} polyvinylpyrrolidone (PVP) until transferred to culture media.

Greenhouse plants. The shoot tips excised from the donor plant are dried at temperature of 50°C for 1 hour in a double boiler. Afterwards, they are submerged for 30 min into a mixture of fungicides (Captan and Benomil, 1 gL^{-1} each) in a laminar flux chamber. By this time, the shoot tips are washed in sterile distilled water and transferred into stirred sodium hypochlorite solution (2.5 gL^{-1} of active chlorine) for a 10 min period. Finally, they are washed three times with sterile distilled water. The nodal segments, approximately 3 cm long, and vegetative leaf buds are placed in a Petri dish containing an aqueous solution with 2 gL^{-1} of DIECA (diethyl carbamate acid, tri-hydrated sodium salts, Aldrich®).

2. 2. Culture Medium

2.2.1. Media Composition

Explants must be dried first on sterile filter paper before they can be cultivated in test tubes containing 10 ml DKW (Driver & Kuniyuki, 1984) (Table 1), diluted macro-nutrients at 25% concentration, without hormones and solidified with a 2.5 g L^{-1} gelrite (Phytagel, Sigma®). Prior sterilization, pH must be adjusted to 5.8.

2.2.2. Media Preparation

In order to prepare the DKW-based media variants, powdered media supplied by DUCHEFA Biochemie B.V. were used (Table 1). Stock solutions of major and minor nutrients, vitamins, Na and Fe EDTA, ten times their concentration for easy culture media shall be prepared. The high amount of auxin (1 mg ml^{-1} IBA) was dissolved separately with a few ml of under natured ethanol (70%) before it was

added to the total quantity. After adjusting the pH, the media were heated until all compounds were completely dissolved. After adjusting pH, phytagel was added and the heated media until all compounds were completely dissolved. While stirring carefully, the media was distributed in cultivation bottles. Twenty five ml media were filled into 100 ml culture vessels and 15 ml media were filled into 150 mm long and 25 mm diameter test tubes. After closing with aluminum foil, the media were autoclaved at 121°C, at a pressure of 105 KPa for 20 min.

2.3. In Vitro Shoot Multiplication

Stretched leaf buds are cultivated in test tubes with 10 ml DKW medium containing BAP (1 mgL^{-1}), IBA (0.01 mgL^{-1}), 100 mgL^{-1} PVP, 50 mg L^{-1} streptomycin and solidified with phytagel (2.5 gL^{-1}). Tubes are kept in a growth chamber for a 16-hour photoperiod (40–45 $\mu Em^{-2}s^{-1}$) at 25 ± 1°C. In the establishment phase, 50 mgL^{-1} of streptomycin are added to avoid endogen bacteria to arise. The establishment of proliferation phase is carried out by selecting explants with the best proliferation and lowest contamination rate (Figure 3A). Sub-cultivation of basal and apical tips and entire shoots in fresh culture media occurs every 30 days in order to increase the production of microshoots (Figure 3B) followed by the rooting phase. *Juglans regia* does not present spontaneous rooting but induced one instead, so it is considered to be a recalcitrant species.

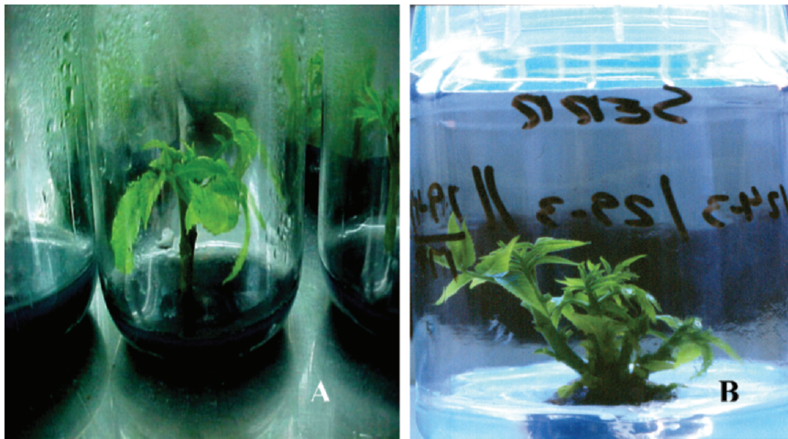

Figure 3. *In vitro introduction of adult walnut plant material on DKW culture medium. A) Nodal segment with vegetative leaf bud activation after 15 days in cultivation. B) Production of multiple shoots after 30 days of culture.*

Table 1. *Formulation of culture medium used for walnut micropropagation based on modified DKW salt augmented with culture stage-specific plant growth regulators.*

Stock	Component	Chemical formula	Stock concentration ($g\ L^{-1}$)	Medium concentration ($mg\ L^{-1}$)
	Major nutrients, 10 × stock, use 100 ml per L medium			
A	Ammonium nitrate	NH_4NO_3	14,16	1416,0
	Calcium chloride-$2H_2O$	$CaCl_2 \times 2H_2O$	1,47	147,0
	Calcium nitrate-$3H_2O$	$Ca(NO_3) \times 3H_2O$	18,11	1811,0
B	Potassium orthophosphate	KH_2PO_4	2,58	258,0
C	Potassium sulfate	K_2SO_4	15,6	1560,0
D	Magnesium sulfate-$7H_2O$	$MgSO_4 \times 7H_2O$	7,4	740,0
	Minor nutrients, 500 × stock, use 2 ml per L medium			
E	Boric Acid	H_3BO_3	6,2	12,4
	Cupric sulfate-$5H_2O$	$CuSO_4 \times 5H_2O$	0,025	0,05
	Manganese sulfate-H_2O	$MnSO_4 \times H_2O$	16,9	33,8
	Sodium molybdate-$2H_2O$	$Na_2MoO_4 \times 2H_2O$	0,25	0,5
	Zinc sulfate-7H2O	$ZnSO_4 \times 7H_2O$	10,6	21,2
	Potassium iodide	KI	0,83	1,66
	Cobalt chloride-$6H_2O$	$CoCl_2 \times 6H_2O$	0,025	0,05
	Amino acids 100× stock, use 10 ml per L medium			
F_1	Glycine		0,1	1,0
	Cysteine		0,1	1,0
	Vitamins 100× stock, use 10 ml per L medium			
F_2	Nicotinic acid		0,1	1,0
	Tiamine HCl		0,1	1,0
	Pyridoxine		0,1	1,0
	Calcium Pantenonate		0,1	1,0
	Biotin		0,001	0,00001
	Myo-Inositol , 500× stock, use 2 ml per L medium			
G	Myo-Inositol		50	100,0
	Iron 100× stock, use 10 ml per L medium			
H	Ethylenediamine tetraacetic acid disodium and iron	Na and Fe EDTA	3,67	36,7

Other additives	Medium Concentration (g L^{-1})
sucrose	30
Polyvinilpyrrolidone	1
Phytagel	2,5

Plant growth regulators add according to culture stage

	BAP(mg L^{-1})	IBA (mg L^{-1})
Establishment	—	—
In vitro shoot multiplication	1	0,01
Rooting	—	4

2.4. In Vitro Rooting

Upon clone establishment, rooting essays are conducted by adding 4 mgL^{-1} IBA to the MS medium (Murashige & Skoog, 1962) during a 3-day darkness induction period, followed by a 27-day manifestation period under a 16-hour photoperiod in DKW medium, where macronutrients are diluted at 25%, mixed with vermiculite:perlite (220/250 v/v) and solidified with phytagel (Sigma®).

Microshoot type and size used are essential to get optimum rhizogenic response. Microshoots more than 2.5 cm long with apical leaf bud have higher rooting rates and the new roots formed are more fibrous (Figure 4).

The rhizogenic capacity of walnut depends on the endogenous content of auxin and the exogenous concentration to be used (Feito et al., 1997; Ríos et al., 1997, 2002, 2005) and is always preceded by basal callus formation. However, their minimum

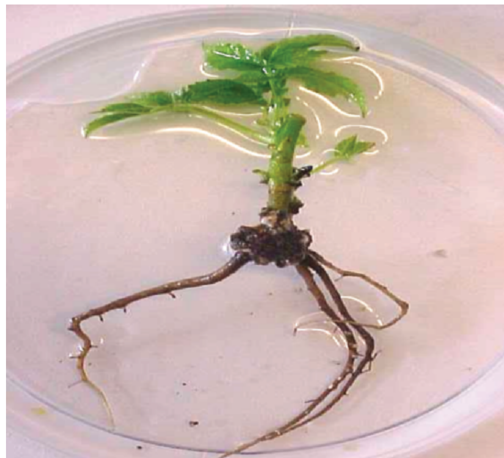

Figure 4. *Induced rooting system in Juglans regia microshoots, after 30 days of culture in a mixture of pearlite/vermiculite substrate.*

formation favors the expression of root primordia, giving place to emergency of a significantly larger number of roots, which assures higher survival rate during the acclimatizing and hardening phases of the new microplants (Sánchez-Olate et al., 1997, 2005).

2.5. Microplant Acclimatization

Upon completion of the rooting manifestation phase, the microplants are extracted from the nutritive medium and washed in running water in order to eliminate all the substrate adhered to their root system. Subsequently, they are planted in polystyrene expanded trays with 84 cavities of 100 cc of sterilized substrate, which consists of a mixture of perlite:vermiculite (1:1, v/v), and maintained under greenhouse conditions (Figure 5). During the first culture week, the plants are irrigated by nebulization using a fogger, at a frequency of 15–min and 10-sec duration, ensuring that humidity is kept close to 100%. Upon observation of apical leaf bud growth, irrigation frequency must diminish (e.g. every 30–45 min for 10 sec, according to the greenhouse conditions) and ventilation must increase. Finally, during the full growth phase, irrigation events become more distanced and the nursery adaptation phase begins gradually.

Figure 5. *Micropropagated plantlets of Juglans regia, 6 weeks after transfer to soil. A) Acclimatized plants. B) Induced root system view after 30 days in greenhouse acclimatization.*

After hardening, the micropropagated plants are transplanted to the nursery if they show sufficient level of lignification. Either growing in containers or rows in the ground, the plants must be kept in nursery for later use in establishment of clonal orchards.

3. CONCLUSIONS

The main problem of propagation of *in vitro* mature material of walnut is the endogenous contamination of initial explants, where the key is the use of antibiotics in the means of cultivation. Tissue oxidation during the superficial asepsis of explants induces a low production of microshoots when obtaining microplants. Difficulties related to material induction are, at a great extent, solved by selecting shoots and nodal segments with semi-woody tissue, which makes them resistant to disinfection products. Although the rate of proliferation of material in the first subcultures is initially low, it will increase over the maintenance period and during the transfer to fresh cultures every 30 days. Finally, successful rooting rate can be reached using both *in* and *ex vitro* treatments, beginning the acclimatization period of microplants under greenhouse conditions (relative humidity of 100% and temperature in the range from 18 to 20°C).

The future appears promising for walnut biotechnology, particularly in commercial production. Although the sale price of *in vitro* walnut is high and has not met yet the consumer expectations, it may be reduced by research in automation, bioreactor, and synthetic seed technologies.

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4. REFERENCES

Claudot, A.-C., Drouet, A. & Jay-Allemand, Ch. (1992) Tissue distribution of phenolic compounds in annual shoots from adult and rejuvenated hybrid walnut trees. Plant Physiol. Biochem. 30, 565–572.

Driver, J.A. & Kuniyuki, A.N. (1984) *In vitro* propagation of Paradox Walnut roostock. HortSci. 19, 507–509.

Feito, I., Gea, M.A., Ríos, D., Albuerne, M., Fernández, B. & Rodríguez, R. (1997) Physiological studies during walnut rooting. Acta Hort. 442, 137–142.

Hasey, J.K., Westerdahl, B.B., Micke, W.C., Ramos, D.E. &Yeater, J.T. (2001) Yield performance of own-rooted "Chandler" walnut versus "Chandler" walnut on Paradox Rootstock. Acta Hort. 544, 489–493.

Heile-Sudholt, C., Huetteman, C.A., Preece, J.E., Van Sambeek, J.W. & Gaffney, G.R. (1986) *In vitro* embryonic and seedling shoot tip culture of *Juglans nigra* L. Plant Cell Tiss. Org. Cult. 6, 189–197.

Leslie, C. & McGranahan, G. (1992) Micropropagation of Persian Walnut (*Juglans regia* L.). In Bajaj, Y.P.S. (Ed), Biotechnology in Agriculture and Forestry 18. High Technology and Micropropagation II. Springer-Verlag. Berlin Heidelberg pp. 137–150.

López, J.M. (2001) Field behavior of self-rooted walnut trees of different cultivars produced by tissue culture and planted in Murcia (Spain). Acta Hort. 544, 543–546.

Marques, D. & Dias, J. (2001) *In vitro* shoot culture on *Juglams regia* L.: repeated subculturing on juvenile and adult material. Acta Hort. 544, 251–256.

McGranahan, G. & Leslie, C. (1988) *In vitro* propagation of mature Persian walnut cultivars. HortSci. 23, 220.

Meynier, V. (1984) Contribution de la culture de méristémes a la micropropagation *in vitro* du noyer. Ann. Rech. Sylvicoles 176, 75–85.

Murashige, T. & Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–479.

Navatel, J. & Bourrain, L. (2001) Plant production of walnut *Juglans regia* L by *in vitro* multiplication. Acta Hort. 544, 465–471.

Ríos, D.G., Sánchez-Olate, M., Gea, M.A., Revilla, M.A. & Rodríguez, R. 1997. Rooting responses in relation with PO, PPO and IAA-o activities on walnut (*Juglans regia* L.) explants. Acta Hort. 442, 241–250.

Ríos, D., M. Sánchez-Olate, M., Gea, M. & Rodríguez, R. (2002) Nuevos sistemas experimentales para el estudio de la rizogénesis en nogal. Agrociencia 17, 221–227.

Ríos, D., Sánchez-Olate, M., Decarli, N., Feito, M. & Rodríguez, R. (2005) Bases moleculares del enraizamiento. In: Sánchez-Olate, M. & Ríos, D. (Eds) Biotecnología vegetal en especies leñosas de interés forestal. Facultad de Ciencias Forestales, Universidad de Concepción, Chile pp. 79–93.

Sánchez-Olate, M. (1996) Bases macromorfológicas y moleculares de la macropropagación de nogal (*Juglans regia* L. cv. Serr). Tesis Doctoral. Universidad de Oviedo, España 203 p.

Sánchez-Olate, M., Ríos, D.G., Gea, M.A., Rodríguez, R.E. & Revilla, M.A. (1997). Parameters affecting the *in vitro* growth and rooting of *Juglans regia* L. Acta Hort. 442, 235–240.

Sánchez-Olate, M., Ríos, D., Revilla, M. & Rodríguez, R. (2005) Factores involucrados en el enraizamiento in vitro de leñosas de interés agroforestal. In: Sánchez-Olate, M. & Ríos, D. (Eds) Biotecnología vegetal en especies leñosas de interés forestal. Facultad de Ciencias Forestales, Universidad de Concepción, Chile pp. 95–104.

Saadat, Y. & Hennerty, M. (2001) Effects of different *in vitro* and *ex vitro* treatments on the rooting performance of Persian walnut (*Juglans regia* L.) microshoots. Act. Hort. 544, 473–480.

Saadat,Y & Hennerty, M. (2002) Factors affecting the shoot multiplication of Persian walnut (*Juglans regia* L.) Sci. Hortic. 95, 251–260.

Vahdati, K., Leslie, C., Zamani, Z. & McGranahan, G. (2004) Rooting and acclimatization of *in vitro*-grown shoots from mature trees of three Persian walnut cultivars. Hortscience (abstract) 39(2).