Walnut Tissue Culture: Research and Field Applications

Jose M. Lopez[1]

ABSTRACT—Vitrotech Biotecnologia Vegetal began researching propagating *Juglans regia* (English walnut) and various *Juglans* hybrids by tissue culture in 1993 and has operated on a commercial scale since 1996. Since this time, more than one and a half million walnuts of different species have been propagated and field planted. Tissue cultured walnuts on their own roots have proven to be more vigorous and productive than conventionally propagated walnuts. Also, tissue culture is an efficient tool to help breeders select and test possible superior genotypes.

WALNUT: A DIFFICULT TREE TO PROPAGATE

Most of the species of the genus *Juglans* are difficult to propagate by conventional vegetative propagation using rooted cuttings, budding, and grafting (Coggeshall and Beineke 1997). Walnut trees are propagated by commercial nurseries by grafting onto seedling rootstocks both for *J. regia* (English walnut) nut production as well as clonal selections of *J. nigra* (black walnut for timber production. Variability with different conditions (climatic, type of plant material, and technique) also affects the success of budding and grafting. Results may be quite variable from year to year. Budding and grafting are still widely used and there are a large number of commercial nurseries around the world that are propagating and selling walnuts with this procedure.

Cuttings have been tried many times with variable success (Coggeshall and Beineke 1997). In fact, the most important limitation to commercial propagation has been the variability of the results. McKenna (1997) got good results with rooted cuttings, but the trees were not able to be commercially deployed. Some investigators indicate that viability of propagated cuttings has been worse than seedlings.

Walnut has proven to be a genus that is also hard to propagate by tissue culture (Rodriquez and others 1989, Preece and others 1989). Attempts have been made by many different researchers (Driver and Kuniyuki 1984; McGranahan and Leslie 1988; Rodríguez and others 1989; Gruselle and Boxus 1990; Gautman and Chalupa 1990; McGranahan 1992; Jay-Allemand and others 1993; Bourrain and Navatel 1994, 1995). The difficulties have been related to: internal contamination of plant material and susceptibility of explants to damage caused by disinfectant substances, oxidation of the explants, maladaptability to the culture media, poor proliferation, poor elongation and rooting rates, difficult acclimatization (stage IV) of the rooted propagules and complications when growing small plantlets in the nursery. Despite this, Vitrotech Biotecnologia Vegetal began the research in this field in 1993 and walnut commercial propagation by tissue culture in 1996. Since then this company has propagated around one and a half million trees of different species of this genus.

WALNUT PROPAGATION: WHAT TO GET AND HOW?

Commercial tree propagation usually starts with the best method to get the maximum profit from the plant material. This consists of grafting the selected clones/varieties/cultivars onto selected clonal rootstocks using scions that have desirable traits for yield, fruit or timber quality, resistance to diseases and/or adverse climatic conditions. The investigator can also use rootstocks selected for good soil adaptability, vigour or resistance to soil pathogens. In the case of walnuts, this has not been available, because the lack of a propagation method for rootstocks, or when selections have been made, no scientific tests could be performed because no replicates of the selected clone were available.

[1]General Manager (JML), Vitrotech Biotecnologia Vegetal, N-340 Km 631, 30840 Alhama de Murcia (Spain). JML is corresponding autor; to contact call +34 968 633231 or e-mail at jose.m.lopez@vitrotech.es

Citation for proceedings: Michler, C.H.; Pijut, P.M.; Van Sambeek, J.W.; Coggeshall, M.V.; Seifert, J.; Woeste, K.; Overton, R.; Ponder, F., Jr., eds. 2004. Black walnut in a new century, proceedings of the 6th Walnut Council research symposium; 2004 July 25-28; Lafayette, IN. Gen. Tech. Rep. NC-243. St. Paul, MN: U.S. Department of Agriculture, Forest Service, North Central Research Station. 188 p.

When Vitrotech started commercial propagation of *J. regia* by tissue culture, we found that no rootstocks were available for their propagation, or the ones accessible were not tested thoroughly. Under such circumstances we decided to try the propagation of the varieties on their own roots. We started with walnut tissue cultures obtained from other researchers around the world (UC Davis, Burchell Nursery, INRA, and CTIFL) and propagated some varieties/clones on their own roots.

Most of the initial production of our company was based on English walnut nut cultivars, though some clones of *J. hindsii*, paradox (*J. hindsii* x *J. regia*), and other hybrids (*J. major* x *J. regia, J. nigra* x *J. regia*) were also propagated. Throughout Europe and most of the world (except California) *J. regia* cultivars were grafted onto *J. regia* seedlings. Our company thought that a tissue cultured walnut tree on its own roots should not have less productivity than a grafted tree. In addition, most of the walnut cultivars come from mother trees grown on their own roots (seedlings), that could show us that those cultivars can be good root systems (or, at least, not worse than the average of seedlings).

AGRONOMIC FEATURES OF MICROPROPAGATED WALNUTS

We were surprised to find that the performance of self-rooted walnut cultivars was better than expected. In most cases, they were superior to performance with grafted trees. Prunet and Ginibre (2000) described that self-rooted *J. regia* 'Lara' were more vigorous than trees of the same cultivar grafted onto seedling rootstocks of *J. regia* and *J. nigra* x *regia* hybrids, as well as clonal *J. regia* rootstocks that had been selected for their excellent vigour and propagated by tissue culture.

Hasey and others (2001) found that self-rooted *J. regia* 'Chandler' was much more vigorous than Chandler grafted onto Paradox rootstock (Table 1). This was a not expected, as Paradox hybrid is more vigorous than conventional *J. regia* rootstocks of which Chandler is considered as a medium-vigor cultivar (Table 1). Cumulative yield over 5 years on self-rooted Chandler reached close to three times that of conventional grafted Chandler onto Paradox (Table 2). In addition, self-rooted Chandler trees were supporting a higher nematode population on their roots than grafted trees (causing nematode root lesions) and some of them died back. Hasey and others (1999) mentioned that it was striking to observe the higher vigor of those self-rooted Chandler, even with a high nematode population on their roots. They explained that a similar situation was found when vigorous Paradox rootstocks showed more tolerance to nematodes than *J. hindsii* rootstocks. One more interesting observation was that several grafted Chandler onto Paradox exhibited crown galls (*Agrobacterium tumefaciens*), while none of the self-rooted Chandler did.

With our own experience, we have found that self-rooted walnuts of different species and cultivars have shown outstanding vigor, even with very difficult growing conditions (Fig. 1). On a farm located in Murcia province, Spain, soil pH ranged from 8.2 to 9.3. Active calcium content reaches between 23% and 30%. The soil was extremely rocky, and soil preparation was very poor. Climatic conditions were good (no late or early frost,

Table 1.—Trunk circumference (cm) and trunk cross sectional area measured at 60 cm from 1998-2001 for trees planted in 1991 (Hasey and others 2001).

	Circumference (cm)				Trunk Cross Section Area			
Treatment	1998	1999	2000	2001	1998	1999	2000	2001
Own-rooted	64.3 a	68.1 a	71.4 a	74.6 a	340 a	381 a	419 a	457 a
On Paradox	41.4 b	45.1 b	49.4 b	51.7 b	146 b	173 b	208 b	227 b

Means followed by the same letter in a column are not significantly different (LSD $P \leq 0.05$)

Table 2.—Yield in kgs/tree from 1995-2001 (Hasey and others 2001).

Treatment	1995	1996	1997	1998	1999	2000	2001
Own-rooted	10.8 a	23.4 a	43.2 a	29.1 a	41.9 a	33.2 a	46.4 a
On Paradox	4.2 b	7.9 b	14.2 b	10.1 b	19.8 b	19.7 b	31.6 a

Means followed by the same letter in a column are not significantly different (LSD $P \leq 0.05$)

Figure 1.—Summer 2001 photograph of a self-rooted walnut tree (*Juglans regia*) planted on a farm located in Murcia (Spain) in 1997.

maximum temperatures around 35-40º C and minimum around -5º C), with a long vegetative season (from mid-March to the end of October). Water quality was excellent. The height of the plants planted on the farm ranged from 4 to 20 cm (Fig. 2). The main planting was done between February and April, 1997. At the second year, trees of some of the more vigorous cultivars (*J. regia* 'Sunland' and 'Serr') reached more than 3.5 m. Table 3 shows average diameters per cultivar found in September 2001. Fruit bearing was also very precocious. Some trees produced their first female flowers during their second leaf in the field. Female flower production increased since then, and is still increasing. However catkin production was very delayed compared to conventional grafted trees. First catkins were seen at the sixth leaf, depending on the cultivar (Frutos 2003). Yield has not been recorded yet, as all the original trees on this farm, including pollinators, were self rooted, so most of the possible production has been lost because the lack of pollen.

A further and clear advantage of the use of self-rooted cultivars over grafted trees onto seedling rootstocks is the higher homogeneity of the plantations due to using clonal rootstocks of the same cultivar. Some of the stronger defenders of the use of rootstocks could say that by using self-rooted cultivars an unknown clonal root system is being utilized, which could be very risky. However, our evidence shows that more than 20 different clones on their own roots have shown a better performance than the original mother tree, and no further problems have been found regarding adaptability to different soils than those already present on

Figure 2.—Typical in-leaf tissue-cultured walnut plants when planted at an irrigated farm located in Murcia (Spain).

Table 3.—Trunk diameters (cm) of the different treatments ± SE. Different letters in the same row indicate differences at 95% level (Lopez 2001).

Age/cultivar	Sunland	Serr	Chandler	Sorrento	Vina
2nd leaf	10.94±0.50a	9.88±0.28a	---	8.01±0.25b	7.01±0.26b
3rd leaf	18.68±0.33a	17.88±0.20a	13.85±0.22b	12.67±0.47b	---

the different species/clones. In some cases this adaptability has been clearly better when using self-rooted trees with many different clones.

What is the reason for such improved behavior? Several possible explanations can be considered:

1. Root architecture of self-rooted walnut trees is completely different than walnut seedlings. Instead of the usual main taproot, self-rooted trees exhibit a fibrous root structure. We have counted more than 20 primary roots with all of them coming directly from the crown (Fig. 3). Anchorage is very good and no guides are used with most of our trees. Such root structure may provide for excellent water and nutrient absorption. At the Murcian farm mentioned before, we observed that, in the same conditions, grafted or seedling trees showed leaf chlorosis (due to the limiting soil conditions relative to pH and calcium content) much easier than self-rooted trees. Also, vigor of self-rooted trees was much higher than the vigor of grafted trees.

2. The lack of a graft union seems also to improve the growth of the trees. Olson and Walton (2001) found that self-rooted Chandler (SRCh) walnut trees are more vigorous than SRCh scions grafted onto SRCh rootstocks with similar root architecture. In fact, SRCh were the most vigorous of any of the treatments when Chandler scions were grafted to Paradox, *J. regia* seedlings, or clonal Paradox. Thus, it was concluded that graft unions limit the vigor of the walnut trees. Prunet and Ginibre (1999) also reported that self-rooted Lara trees were more vigorous than Lara grafted onto very vigorous clonal *J. regia* rootstocks propagated by tissue culture. These differences cannot be explained by the root architecture (similar on the treatments), but by the presence/absence of the graft union.

3. Homogeneity of the plant material in terms of genetics. Root systems have a strong influence on the vigor of the tree. Vigorous rootstocks are commonly used to increase productivity or to decrease it (dwarfing rootstocks). This feature is especially important when dealing with trees, as most of the research in breeding with walnuts has been looking for very vigorous scions that must be grafted on seedling rootstocks of unknown and heterogeneous growth capabilities. Once selected scions are shown to be very vigorous and productive on their own roots, it can be assumed that micropropagated selections of walnuts must result in more homogeneous and vigorous trees than the same selections grafted onto seedling rootstocks.

Figure 3.—Root architecture of a 3-year-old walnut tree (*J. regia* cv. *Vina*).

4. Disease tolerance/resistance. No evidence has shown that tissue cultured walnuts of any clone/cultivar are resistant to any soil disease. As stated before, Hasey and others (2001) said that nematode root lesion had affected SRCh trees less than expected, and they assumed that was due to their superior vigor. The same authors found, while in the same block, some grafted trees on Paradox rootstock were affected by crown gall and not one of the self-rooted trees showed this disease. The reason was that soil diseases on grafted trees grown in a nursery row were much more difficult to control than when the trees are grown in pots (case of the tissue cultured self-rooted). Also, digging usually causes injuries to the roots that can be the entry points for such pathogens.

OUTLOOK OF THE USE OF TISSUE CULTURED WALNUTS

Commercial walnut propagation by tissue culture has been successfully achieved by our company. This does not mean that all the walnut plant material is ready to be propagated.

Walnut tissue culture requires research to be performed on almost every new clone/variety to be propagated. Differences with tissue culture behavior between species are greater than with other vegetative propagation methods. *J. nigra* has shown to have very different requirements than *J. regia*, and its performance *in vitro* is more difficult. This is just one illustration, but shows the trend of the entire genus.

Other limitations to walnut mass propagation by tissue culture are the need of more and longer tissue culture stages, which involves larger facilities and more staff to produce the same amount of plants than for other species. The great influence on the process of supposedly less important factors, which with walnuts can limit the production to levels sometimes below what can be considered to be commercially viable, also affect production costs.

In the case of *J. regia* cultivars used for nut production, the use of self-rooted cultivars is of high interest particularly on those situations where no specific rootstock needs are required. These are mostly on sites where *J. regia* seedlings are used as rootstock. There is some advantage in price, but the main benefits are found with the performance of the trees.

When a specific rootstock is needed because the presence of particular soil condition, it is desirable to use clonal material coming from superior selections. Under those circumstances, performance of the trees will be better than those grafted onto seedling rootstocks. Tissue culture is also a determinant tool to make a good rootstock selection, because there is a reliable method for the necessary propagation of the original selected trees in order to get enough repetitions to perform field tests in different conditions. This is the case of most of the California walnut orchards that commonly require the use of Paradox rootstocks because they are more tolerant than *J. regia* to *Phytophtora* species, nematodes, and water logging. Paradox rootstocks in California are also more vigorous when compared to other species of the genus, but in this case, the results of the trials performed to compare self-rooted trees with trees grafted on Paradox have shown that self-rooted trees are more vigorous.

The use of clonal rootstock is justified by a specific requirement (mostly related with adaptability to soil conditions or tolerance/resistance to soil diseases), and when such requirement cannot be obtained by the use of self-rooted trees. The cost of a grafted tree onto a clonal rootstock propagated by tissue culture will be clearly higher than the one of a self-rooted tree. If the grafted tree is not providing a commercial advantage then there is no reason to use more expensive walnut trees.

The use of tissue-cultured walnuts for timber production seems to be as favorable as for nut production, or probably more, because vigor is usually a more desirable feature for timber production. Selected clones can improve both productivity and timber quality, and can allow the utilization of plant material adapted to specific soil or climatic conditions, or resistant/tolerance to either soil or air borne pathogens. Uniformity of plantations will be greater, and phytosanitary treatments can be applied more effectively. In the case of self-rooted trees, the lack of graft union may avoid the presence of defects to the wood that could reduce the price of the timber, although selected clonal rootstocks can be used if necessary. Particularly, *J. nigra*, native to the eastern USA, where soil diseases are not a problem, and most of the clonal selections are based on the features of the scion, propagation of the same by tissue culture to produce self-rooted clonal trees is a feasible possibility when looking for increased productivity, shortened rotations and higher quality timber. In the same way as for nut producing trees, when clonal rootstocks provide desirable features, tissue culture is the only propagation method available for them.

LITERATURE CITED

Bourrain, L.; Navatel, J.C. 1994. Micropropagation of the walnut tree *Juglans regia* L. Part 1: *In vitro* production. Infos-Ctifl. 98: 40-46.

Bourrain, L.; Navatel, J.C. 1995. Micropropagation of the walnut tree *Juglans regia* L. Part 2: Acclimatization in the greenhouse and nursery performance. Infos-Ctifl. 112: 28-33.

Coggeshall, M.V.; Beineke, W.F. 1997. Black walnut vegetative propagation: the challenge continues. In: Van Sambeek, J.W., ed. Knowledge for the future of black walnut: 5th Black walnut symposium; 1996 July 28-31; Springfield, MO. Gen. Tech. Rep. NC-191. St. Paul, MN: U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station: 70-77.

Driver, J.A.; Kuniyuki, A.H. 1984. In vitro propagation of Paradox walnut rootstock. HortScience. 19(4): 507-509.

Frutos Tomas, D. 2003. Diferenciacion floral en vergel de los cultivares de nogal (*Juglans regia*, L.) enraizados in vitro. Actas de Horticultura nº 39. X Congreso Nacional de Ciencias Horticolas, Pontevedra, España: 271-273.

Gautam, R.; Chauhan, J.S. 1990. A physiological analysis of rooting in cuttings of juvenile walnut (*Juglans regia* L.). Acta Horticulturae. 284: 33-41.

Gruselle, R.; Boxus, P. 1990. Walnut micropropagation. Acta Horticulturae. 284: 45-52.

Hasey, J.; Westerdahl, B.; Micke, W.; Ramos, D.; Yeager, J. 2001. Yeild performance of own-rooted 'chandler' walnut versus 'chandler' walnut on paradox rootstock. In: Germain, E.; Calvi, D., eds. Proceedings, 4th International walnut symposium; Bordeaux, France. Acta Horticulturae. 544: 482-489.

Hasey J.; Westerdahl, B.B.; Lampinen, B.D. 1999. Long-term performance of own rooted 'chandler' vs 'chandler' on paradox rootstock: the walnut research reports annual proceedings. Sacramento, CA: The Walnut Marketing Board of California: 87-92.

Hasey J.; Westerdahl, B.B.; Lampinen, B.D. 2001 Long-term performance of own rooted 'chandler' vs 'chandler' on paradox rootstock: the walnut research reports annual proceedings. Sacramento, CA: The Walnut Marketing Board of California. 87-92.

Jay-Allemand, C.; Peng S.; Capelli, P.; Cornu, D. 1993. Micropropagation of hybrid walnut trees: some factors involved in rooting. Acta Horticulturae. 311: 117-124.

Lopez, J.M. 2001. Field behaviour of self-rooted walnut trees of different cultivars produced by tissue culture and planted in Murcia (Spain). In: Germain, E.; Calvi, D., eds., Proceedings, 4th International walnut symposium. Bordeaux, France: Acta Horticulturae. 544: 543-546.

McKenna J.; Sutter, E. 1997. Root formation in walnut hybrid rootstoocks (*Juglans hindsii* X *J. regia*). In: Altman, A.; Waisel, Y., eds. Biology of root formation and development. Basic Life Sciences. 65: 85-90.

McGranahan, G. 1992. Micropropagation of persian walnut (*Juglans regia* L.). In: Bajaj, Y.P.S., ed. Biotechnology in agriculture and forestry, high-tech and micropropagation II. Berlin, Heidelberg: Springer-Verlag. 5(18): 136-150.

McGranahan, G.; Leslie, C.A. 1988. *In vitro* propagation of mature persian walnut cultivars. HortScience. 23(1): 220.

Olson, B.; McKenna, J.; McGranahan, G.; Walton, J.; Bertagna, N. 2001. Walnut rootstock trial 2001. The walnut research reports annual proceedings, Sacramento, CA: The Walnut Marketing Board of California: 99-100.

Preece, J.E.; Van Sambeek, J.W.; Huetteman, C.A.; Gaffney, G.R. 1989. Biotechnology: *In vitro* studies with walnut (*Juglans*) species. In: Phelps, J.E., ed. The continuing quest for quality: 4th Black walnut symposium; 1989 July 30-August 2; Carbondale, IL. Indianapolis, IN: Walnut Council: 159-180.

Prunet, J.P.; Ginibre T. 2000. Comportement agronomique des nouveaux portegreffes CTIFL *Juglans regia*. Travaux de Recherche et d'expérimentation. Station Expérimentale de Creysse.

Rodríguez R. Revilla A.; Albuerne M.; Pérez C. 1989. Walnut (*Juglans* spp.). In: Bajaj, Y.P.S., ed. Biotechnology in agriculture and forestry. Berlin, Heidelberg: Springer-Verlag: Trees II. 5: 99-126.