Micropropagation of *Juglans nigra* L. in Liquid Culture

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Figure 1. Black Walnut. A) Plantation. B) Boards. C) Veneer. D) Fruit on the tree. E) Kernels for food consumption. F) Chair made out of black walnut.

Abstract

Black walnut is a valuable hardwood tree species that has great future potential for growing in plantations. Continuous high-grade logging depletes quality trees from natural stands and the shift of public land into reserve programs reduces the market supply of walnut from these resources. Plantations will allow landowners the ability to meet the future market demand for black walnut wood. Rooting of black walnut via conventional stem cutting propagation is very difficult and has prevented the development of a clonal mass propagation system. In order to clonally propagate selected or genetically improved genotypes, a micropropagation, rooting, and acclimatization protocol is needed. Shoot cultures of black walnut from seedlings of selected genotypes were established on a semi-solid DKW medium containing 8.88 µM BA, 0.005 µM IBA, 200 mg l^{-1} casein hydrolysate, and 2 ml l^{-1} Plant Preservative Mixture® (PPM). Nodal explants were initially disinfected using a treatment based on PPM to eliminate external and internal contamination, which is often a problem in establishment of in vitro cultures of black walnut. Nodal sections of in vitro shoots of a selected genotype (Purdue #295) were cultured in a liquid DKW medium containing 8.88 µM BA, 0.005 µM IBA, 200 mg l^{-1} casein hydrolysate, and 2 ml l^{-1} PPM, and incubated on a shaker at 100 rpm under a 16h photoperiod with a light intensity of 55 µmol m^{-2} s^{-1}. Buds broke and developed into clusters of buds, which elongated into microshoots once the cluster grew large enough to not be continuously submerged in the liquid medium. Buds removed from the clusters and placed into separate culture vessels developed into healthy, thick-stemmed microshoots. Buds cultured on semi-solid medium also developed into healthy microshoots.

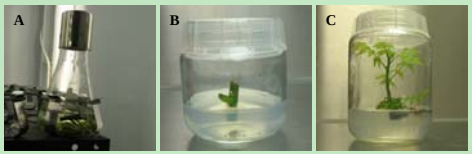

Figure 2. Establishing microshoots from nodal sections. A) 12-hour wash in 0.05% PPM-DKW medium. B) Nodal section in culture vessel after sterilization. C) Elongated shoot culture after 4 weeks on DKW medium containing 8.88µM BA plus 0.005 µM IBA.

Introduction

The continued high demand for black walnut veneer and the high prices on the world market have increased the interest in growing this species in plantations. To date only 13,800 acres in the United States are in black walnut plantations (Shifley, 2004), but land for growing hardwood trees is being shifted into intensive management and is expected to increase considerably by 2050. Furthermore, a reduction in logging on public land, as a result of shifting this land into reserves, can be expected (Alig, 2003). The identification and patenting of elite genotypes of black walnut gives an increasingly good base to establish plantations using selected genotypes that will have a significant increase in yield, as compared to black walnut trees in natural stands (Beineke, 1989).

Clonal propagation of black walnut has proven difficult and grafting of selected genotypes has been used. Since grafting is very labor and cost intensive most plantations are established from seedlings. Those seedlings are half-sib progeny to the elite genotypes and because of the high level of heterozygosity their performance varies. Therefore, clonal propagation via tissue culture needs to be developed.

To take better advantage of the opportunities that tree cultivation offers, genetic transformation of trees is also desirable. Optimization for certain traits by means of traditional breeding can take decades to accomplish or, as in the case of herbicide resistance, is almost impossible. The critical step for successful genetic modification is the development of an efficient in vitro regeneration system. Therefore, an in vitro system needs to be developed for black walnut. Other traits that could be improved via genetic engineering would be: resistance to deer browse, which causes huge financial losses to landowners; the formation of figured wood, which only rarely occurs in natural stands and increases the value of the log; and disease or pest resistance.

Materials and Methods

Micropropagation from juvenile seedlings

Using shoots from the greenhouse of seedlings of selected genotype # 295, the leaves were removed and the shoots washed under running tap water for 30 min. The shoots were cut into sections and washed for 30 sec in 70% ethanol, disinfected for 20 min in 15% bleach solution made with sterile water plus 0.01% Tween 20 on a shaker and washed in sterile water four times for 30 sec. The sections were cut into 1.5 cm long nodal sections and washed for 12 hours on a shaker at 170 rpm in a liquid DKW medium containing 5 ml l^{-1} PPM. The nodal sections were cultured on a semi-solid DKW medium containing 8.88 µM BA, 0.005 µM IBA, 200 mg l^{-1} casein hydrolysate, and 2 ml l^{-1} PPM under a 16 h photoperiod with a light intensity of 55 µmol m^{-2} s^{-1}, cultures were transferred to fresh medium every three weeks.

Establishment of Liquid Cultures

Microshoots were cut into 1.5 cm nodal sections, which were cultured in a liquid DKW medium containing 8.88 µM BA, 0.005 µM IBA, 200 mg l^{-1} casein hydrolysate, and 2 ml l^{-1} PPM, and incubated on a shaker at 100 rpm in a Pyrex 2800 ml Fernbach-Style Culture Flask under a 16 h photoperiod with a light intensity of 55 µmol m^{-2} s^{-1}. To establish cultures 300 ml of liquid medium were used per flask. With increasing size of the shoot cluster, the amount of medium was increased, so that during every rotation the cluster was almost submerged with medium, but still received sufficient exposure to air. The culture medium was exchanged weekly. Buds of the growing cluster were removed and initiated new cultures in separate vessels.

Three centimeter long microshoots growing on a cluster in liquid medium were removed from the cluster and cultured on semi-solid DKW medium as described above.

Adventitious shoot induction

Leaf explants taken from microshoots grown in liquid medium were cut into 1 cm^2 explants along the midrib and the midrib was cut several times to create wounded areas. The explants were placed on a ½ DKW plus ½ WPM with 30 g l^{-1} of sucrose and 8 g l^{-1} Difco-bacto agar, supplemented with 1.0 µM IBA plus 6.8 µM TDZ for 3 weeks in the dark prior to transfer to the light. After 5 weeks in the light the explants were cultured on a ½ DKW plus ½ WPM with 30 g l^{-1} sucrose and 8g l^{-1} Difco-bacto agar, supplemented with either only 0.001 µM or 0.005 µM BS, or 0.001 µM or 0.005 µM BS combined with 8.88 µM BA plus 0.005 µM IBA or 6.8 µM TDZ plus 1.0 µM IBA. Microshoots derived from the leaf explants were then transferred to the standard semi-solid DKW shoot culture medium.

Figure 3. Micropropagation of *Juglans nigra* in BA + IBA-containing liquid medium. A) Culture vessels with newly establishing liquid cultures. B) Close-up of an in vitro nodal section 10 days after initiation into culture. C) Bottom view of a shoot cluster 6 months after initiating liquid culture. D) Side view of a shoot cluster 6 months after initiation into liquid culture.

Results

Micropropagation

Shoot cultures of black walnut were successfully established using the new sterilization method. Forty out of 42 nodal sections broke bud and no fungal or bacterial contamination was detected. The shoot cultures elongated and proliferated successfully.

Liquid culture

Five in vitro nodal sections were cultured in liquid medium and all broke bud within 2 weeks. Collecting buds from greenhouse plant, sterilized them, and directly establishing shoots in liquid culture was attempted, but the breaking of buds always resulted in contamination. Shoots elongated and axillary buds broke immediately giving rise to more shoots, creating a highly branched cluster of shoots. Once the clusters grew large enough to be continuously above the liquid medium, microshoots developed on top of the cluster, which morphologically looked the same as the microshoots grown on semi-solid medium. These microshoots could be transferred to semi-solid medium and continued to grow. The cluster continued to proliferate until its development became limited by the size of the culture vessel. Dividing the cluster proved to be difficult since the center of the cluster became extremely hard and could not be cut with a scalpel. Side shoots, which had developed after being semi-submerged by media, were excised and used to start new cultures in separate culture vessels.

Adventitious shoot regeneration

The leaf explants developed white callus in the dark and turned red and green in the light. The green areas of the callus turned nodular. A week after the nodular callus was moved to the different treatment media, two callus cultures exposed to 0.005 µM BS showed initial regeneration of adventitious shoots. The shoots developed into leaf rosettes and elongated to about 2 cm. At this stage they stopped elongating and were transferred to semi-solid shoot culture medium, but the shoots did not elongate any further.

Conclusions

- The new sterilization method for nodal sections is a first important step towards a micropropagation protocol. While in preliminary tests up to 40% of the nodal sections showed fungal contamination and additionally many nodal sections showed internal bacterial contamination, both problems were solved in this study.
- A novel system for micropropagation of black walnut using liquid culture has been developed. In liquid culture in vitro nodal sections readily break bud and proliferate at high rates. Microshoots originating from liquid medium can be cultured on semi-solid medium. This system gives the opportunity to clonally multiply black walnut in a system that is much less labor intensive than culture on semi-solid medium. The successful growth in a large proliferating cluster opens the opportunity for potential automation of the cultures, if commercialization is desired.
- To make this system a clonal propagation system, an efficient rooting and acclimatization protocol remains to be developed.
- It was demonstrated that adventitious shoots can be regenerated for black walnut from leaf explants, which has not previously been reported in the literature, including one selected genotype Purdue #295.
- These results give a good basis for further investigations into the factors involved in adventitious shoot regeneration of black walnut.

Abbreviations

6-Benzylaminopurine (BA)	Indole-3-butyric acid (IBA)
Homobrassinolide (BS)	Thidiazuron (TDZ)
Driver Kuniyuki Walnut medium (DKW)	Woody Plant Medium (WPM)

Figure 4. Adventitious shoot regeneration from in vitro-grown leaves of *Juglans nigra* on TDZ+IBA-containing media. A) Black walnut microshoots in liquid medium. B) Nodular callus. C) Adventitious shoots elongating on BS containing medium.

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