Adventitious Shoot Regeneration and Micropropagation of Black Walnut

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Abstract

Black walnut (*Juglans nigra* L.) is a very valuable species for growing in plantations. Continuous high logging depletes quality trees in natural stands, and the shift of public land into reserves reduces the market supply from these sources, which will not be able to meet the future market demand. In- and ex vitro rooting of black walnut is very difficult and has prevented the development of a clonal mass propagation system. To clonally propagate selected genotypes and potentially transgenic black walnuts, a micropropagation, rooting, and acclimatization protocol is needed.

As the first objective, shoot cultures of black walnut were established from seedlings and will be established from grafts of mature selected genotypes. A new disinfection treatment based on PPM was developed, eliminating external and internal contamination, which is often a problem with in vitro culture of black walnut. To optimize the plant growth regulator concentration and amino-nitrogen source for shoot multiplication and elongation, different concentrations of BA (2.2, 4.4, 8.8, 13.2, or 17.6 µM) and CH (0, 0.2, 0.4, or 0.6 g l^{-1}) will be tested. The second objective is to develop a highly efficient adventitious shoot regeneration system by testing different explant sources (leaves, petioles, and internodal sections), culture media (DKW, MS, WPM, or ½ DKW plus ½ WPM), and plant growth regulator (TDZ plus IBA, TDZ plus Kinetin, BA, and IBA) combinations for several genotypes. For one unselected genotype, a ½ DKW plus ½ WPM medium supplemented with 6.8 µM TDZ plus 1 µM IBA lead to adventitious shoots on 60% of the leaf explants. For the selected genotype #55 (Purdue One) adventitious shoots were regenerated on leaf explants at a low rate (10%) on ½ DKW plus ½ WPM medium supplemented with 3.4 µM TDZ plus 0.5 µM IBA. Increasing the root induction efficiency of microshoots, by optimizing sucrose concentration (20, 30, 40, or 50 g l^{-1}), basal medium (MS or DKW) and plant growth regulator concentration (NAA or IBA at 16, 32, or 64 µM) for the selected genotypes, and comparing the effect of culture in the dark or in the light during root induction, is the third objective of this study. For acclimatization, the standard protocol of acclimatizing in vitro rooted shoots in the laboratory will be compared to acclimatizing plantlets in the greenhouse, and the acclimatization of shoots by direct rooting in the greenhouse.

Figure 3: Adventitious shoot regeneration from greenhouse-grown leaves of *Juglans nigra* on TDZ+IBA-containing media. A) Black walnut seedlings in the greenhouse. B) Emerging adventitious shoots. C) Adventitious shoots on leaf explant after 3 weeks in the dark and 5 weeks in the light. D) Adventitious shoot 3 weeks after being sub-cultured to zeatin shoot culture medium.

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.

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.

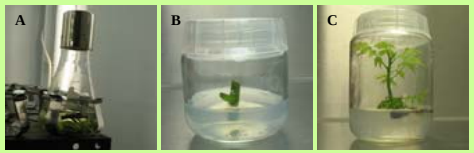

Figure 2: Establishing microshoots from nodal sections. A) 12-hour wash in 0.05% PPM-DKW medium. B) Nodal section in culture vessel after sterilization. C) Elongated shoot culture after 4 weeks on DKW medium containing 8.88µM BA plus 0.005 µM IBA.

Materials and Methods:

Micropropagation from juvenile seedlings

Using shoots from the greenhouse, leaves were removed and the shoots washed under running tap water for 30 min. Shoots were cut into sections and washed for 30 sec in 70% ethanol, disinfected for 20 min in 15% bleach solution plus 0.01% Tween on a orbital shaker and rinsed 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 Driver and Kuniyuki walnut medium (DKW) (1984) containing 0.05% Plant Preservative Mixture (PPM) (Plant Cell Technology Inc.).

To develop a protocol for the micropropagation of black walnut, the optimal concentration of plant growth regulator and casein hydrolysate (CH) needs to be determined to multiply and elongate shoots. For each of the four genotypes we will culture six explants obtained from greenhouse seedlings on a DKW medium supplemented with 0.2 g l^{-1} CH, 0.005 µM indole-3-butyric acid (IBA) plus 2.2, 4.4, 8.8, 13.2, or 17.6 µM 6-benzylaminopurine (BA) (Scaltsoyiannes et al., 1997). The BA concentration shown to be most effective for shoot multiplication and elongation will be used to determine the optimal CH concentration. Casein hydrolysate concentrations of 0, 0.2, 0.4, 0.6g l^{-1} will be tested. The optimized shoot culture medium will be used to establish shoot cultures from selected mature genotypes (2-year-old grafts).

Adventitious shoot induction

Leaf explants from greenhouse seedlings were surface washed in running tap water for 5 min, sterilized for 10 min in 10% bleach solution and rinsed four times with sterile water. The leaves were cut into 1 cm^2 explants along with the midrib, and the midrib was cut several times to create wounded areas. The explants were placed on a ½ DKW plus ½ Woody Plant medium (WPM) (Lloyd and McCown, 1981) with 30 g l^{-1} of sucrose and 8 g l^{-1} Difco-bacto agar, supplemented with 0, 0.5, or 1.0 µM IBA plus 0, 6.8, or 13.6 µM thidiazuron (TDZ) for 3 weeks in the dark and then cultured in the light at 24 ± 2ºC under a 16h photoperiod (80 µmol m^{-2} s^{-1}).

To determine the optimal basal medium for adventitious shoot induction, leaf explants will be cultured on a Murashige and Skoog (1962) medium (MS), WPM, DKW, or ½ DKW plus ½ WPM medium supplemented with 3.4, 6.8, or 13.6 µM TDZ plus 0, 0.5, 1.0, or 1.5 µM IBA.

Based on the basal medium found to be most effective, explants from seedlings (leaf, petiole, and internodal sections) will placed on a factorial of 0, 3.4, 6.8,13.6 or 27.2 µM TDZ plus 0, 0.5, 1.0 or 1.5 µM IBA, and 0.05 µM IBA plus 0, 4.7, 9.3, or 14.0 µM Kinetin plus 2.3, 4.6 or 6.8 µM TDZ plus 0, 4.44 or 8.88 µM BA.

Results

Micropropagation

Shoot cultures of black walnut were successfully established using the new sterilization method (Fig 2 A). Forty out of 42 nodal sections broke bud and no fungal or bacterial contamination was detected. The shoot cultures elongated successfully (Fig. 2 C).

Adventitious shoot induction

Of the six genotypes that were cultured on various combinations of TDZ and IBA, three genotypes (D,E,F) showed adventitious shoot regeneration. The leaf explants developed white callus in the dark and turned red and green when cultured in the light. The green areas of the callus turned nodular. After five weeks adventitious shoot meristems were visible on the surface of the callus (Fig. 3 B, C), which expanded and formed small rosettes (Fig. 3 D). On 6.8 µM TDZ plus 1.0 µM IBA, genotype D regenerated shoots on 60% of the explants; being the highest rate for all genotypes. Genotype F showed 10% shoot regeneration on the same medium as well as on 6.8 µM TDZ plus 0 µM IBA. This was also the only plant growth regulator combination on which genotype E responded, regenerating shoots on 10% of the explants (Fig. 5).

The leaves from seedlings of the selected genotype "Purdue One" showed adventitious shoot development after one week in the dark and 8 weeks in the light on medium supplemented with 3.4 µM TDZ plus 0.5 or 1.5 µM IBA. For both combinations only one explant showed adventitious shoot regeneration (Fig 4 A, B). Embryo-like structures formed on one explant after 1 week in the dark and 5 weeks in the light on a 13.6 µM TDZ plus 1.0 µM IBA containing medium (Fig 4 C).

Figure 4: Adventitious shoot regeneration from greenhouse-grown seedling leaves of *Juglans nigra* "Purdue One" on TDZ+IBA-containing media. A) and B) Adventitious shoot after 3 weeks in the dark and 6 weeks in the light C) Embryo-like structures.

Discussion

The new sterilization method for nodal sections is the first important step towards a micropropagation protocol. In preliminary trials up to 40% of the nodal sections showed fungal contamination and additionally many nodal sections showed bacterial contamination, both problems were solved in this study. This allows us to establish a larger number of nodal sections to test other factors for micropropagation. It was demonstrated that adventitious shoots can be regenerated for black walnut from leaf explants, which has not been reported in the literature, for several genotypes, including one selected genotype (Purdue One). These results give a good base for further investigations of the factors involved in adventitious shoot

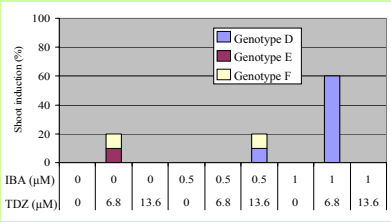

Figure 5: Shoot induction with TDZ and IBA for three genotypes of *Juglans nigra* from young greenhouse leaves after 11 weeks

References

Alig, R. J. (2003) US landowner behavior, land use and land cover changes, and climate change mitigation, *Sil. Fen.* 37(4): 511-527.

Beineke, W. F. (1989) Twenty years of black walnut genetic improvement at Purdue University, *NJAF* 6: 68-71.

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

Lloyd, G.; McCown, B. (1981) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. *Comb. Proc. Intl. Plant Prop. Soc.* 30:421-427

Murashige, T.; Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Plant. Physiol.* 15:473-497.

Scaltsoyiannes, A., Tsoulpha, P., Panetsos, K. P. & Moulalis, D. (1997) Effect of genotype on micropropagation of walnut trees (*Juglans regia* L.), *Sil. Gen.* 46(6): 326-332.

Shifley, S. R. (2004) The Black Walnut Resource in the United States. In: Michler, C. H.; Pijut, P. M.; Van Sambeek, J.; Coggeshall, M.; Seifert, J.; Woeste, K.; Overton , R.; Ponder, F., Jr., eds. 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. pp. 168-176.