MICROPROPAGATION OF MATURE RED MAPLE (*ACER RUBRUM* L.)

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Abstract.--Micropropagation of mature red maple was achieved from axillary buds of both dormant and greenwood branch cuttings. Axillary bud break and multiple shoot formation occurred on MS medium containing 0.01 mg/L thidiazuron (TDZ), as benzyladenine (BA) proved to be inhibitory to shoot proliferation and elongation. Low light intensity and culture system were critical to successful micropropagation, as shoot cultures maintained at 150 ft-c on microporous polypropylene membrane "rafts" produced a 10-fold increase in the number of shoots suitable for rooting when compared to cultures maintained on agar-solidified medium at 450 ft-c. For most genotypes tested, roots were visible in as little as six days on shoots given an auxin pretreatment in vitro. Rooting of shoots and establishment of plantlets in soil occurred at an overall frequency of 77%.

Keywords: Acer rubrum L., micropropagation, tissue culture, plantlets.

INTRODUCTION

Red maple (Acer rubrum L.) is the most widely distributed hardwood species in the Eastern United States, being found in virtually all states east of the Mississippi River as well as Southern Ontario. In coastal regions, its range spans from east Texas to Newfoundland. Red maple is one of the most frequently planted landscape trees in municipalities in the United States, and is especially appreciated for its fall color. Based on fall color, several commercial cultivars (e.g., 'Red Sunset', 'October Glory' and 'Autumn Flame') are well-known. In the Southern United States, red maple is abundant in river bottom hardwood forests. Red maple can become an important component of pulpwood furnishes for paper mills in this region, especially during dry summers.

Of all hardwood species present in the Southern United States, red maple is perhaps the most outstanding fiber source for the manufacture of bleached grades of paper. In bleached grades, used primarily for writing and printing papers, the emphasis is on the optical properties (such as opacity) and printing properties (such as smoothness). In general, the paper properties of smoothness and opacity increase as the coarseness (fiber mass in milligrams per 100 meter length) of the papermaking fibers decrease. Fiber coarseness, in turn, is strongly influenced by fiber length (Clark, 1978). At a fiber length of 0.82 mm (Isenberg, 1951), red maple has the shortest fiber (and, consequently, the lowest coarseness) of all the major hardwood species used for pulpwood in the Southern United States. Given the recent emphasis on paper quality, and, that virtually all new paper manufacturing capacity in the South is in bleached grades, red maple is a species eminently suited for the goals and objectives of the fine paper manufacturers.

Red maple is a good candidate for vegetative propagation for several reasons. Red maple cannot be easily grown from seed using conventional nursery practices developed for many other hardwood species (R. Heeren, personal communication). Part of the problem stems from the fact that it is one of the few species that seeds in the late spring. Because the seed is fleshy and does not store well, it must be collected, processed, sown, and seedlings grown, all in one season.

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Vegetative propagation would be one way to avoid this logistical problem. In addition, experience with red maple seedlings grown in an open nursery bed has shown that they tend to be tall and thin, with poor root collar diameters, and poor survival after establishment. Vegetative propagation may be one way to produce higher quality planting stock by setting cuttings of larger caliper, and, thereby increasing root collar diameter over seedlings.

In addition to logistic and planting stock quality considerations, the lack of genetically improved material suggests that vegetative propagation may represent the fastest and most cost-effective way to exploit the best individuals currently available. Finally, red maple is a species amenable to vegetative propagation, as propagation by rooted cuttings is used commercially to produce large numbers of the cultivars noted above (MacDonald, 1989).

Red maple micropropagation was undertaken as a means of rejuvenating mature trees in the field and producing stock for test plantings. Red maple micropropagation has been previously reported for a specific commercial cultivar 'Red Sunset' (Welsh, 1986; McClelland and Smith, 1990), and for hybrids between red and silver maple (Acer x freemanii; see Kerns and Meyer, 1986). However, published procedures employed only greenwood cuttings collected from grafted individuals. In particular, no information was available on how field-grown material would respond in culture, or on how the timing of collection would influence the ability to establish cultures and induce multiple shoot proliferation. In addition, the number and quality of microshoots suitable for rooting produced by cultures grown on published media formulations was lower than desired. For these reasons, an effort was undertaken to develop a more versatile and efficient micropropagation system for red maple which could be extended to a large number of genotypes from throughout its range.

METHODS

Plant Material and Culture Initiation

Explants (dormant, axillary buds or greenwood scions) were collected from locations in Virginia, South Carolina, and New Jersey. Dormant buds were collected from sexually mature trees ranging in age from 20 to 40 years. Greenwood scions (for establishment of cultures from nodal segments) were collected from young saplings or seedlings. Dormant buds were collected from the period of July-February, and nodal segments from greenwood cuttings were used to establish cultures from April-May. Dormant buds were removed from branches and rinsed under tap water to remove surface dirt. (Note: axillary buds from mature trees are often three-lobed; only the center lobe was sterilized, as the two outer lobes are flower buds.) With the outer bud scale still attached, dormant buds were given a treatment 10% Wavicide (a hospital disinfectant containing 2% glutaraldehyde; Wave Energy Systems, Cedar Grove, NJ)) for 10 min. Under aseptic conditions, buds were then treated with 20% household bleach (1.1% sodium hypochlorite) for 20 min, employing three drops of Tween 20 per 100 mL solution as a wetting agent. Following three rinses with sterile water, the outer bud scales were removed under aseptic conditions, and the buds were treated with 2.5 % bleach for 10 min, rinsed three times with water and then treated 0.1% $HgCl_2$ for 5 min. After another three rinses with sterile water, the remaining bud scales were removed and the shoot tips were treated with 1% household bleach for 5 min, rinsed three times, and were plated onto culture media. For greenwood explants, branch segments (1 cm in length) containing a single node (i.e., two axillary buds) were treated with 20% bleach for 20 min, rinsed three times with sterile water, soaked for 1 h in sterile water, and were plated onto culture media.

All culture media was solidified with 0.7% bacto agar, and cultures were maintained at 26°C under 1,200 $\mu W/cm^2$ illumination (cool white fluorescent) on a 16 h photoperiod. For both

dormant buds and greenwood cuttings, the media contacting the explants darkened very soon after initiation. To minimize any deleterious effects of this dark exudate, the explants were transferred to a different part of the plate that had no visible darkening. Transfer was done three days after initiation, and again after six or seven days. After this time, cultures were transferred to fresh media every four-six weeks.

Shoot Elongation and Root Formation (Best Way)

Shoot cultures were routinely initiated and maintained on MS medium containing 0.01 mg/L thidiazuron as the only growth regulator. For the production of long shoots suitable for rooting, cultures were transferred from agar-solidified medium to microporous polypropylene membrane "rafts" (Sigma Chemical Co., M-1049) placed over liquid medium of the same composition. After four to six weeks, microshoots (1-2 cm) were harvested for rooting. Cultures from which shoots were harvested were returned to agar-solidified media for continued growth.

Shoots were set for rooting in one-third strength WPM media (macro- and microelements) containing 0.1 mg/L indolebutyric acid (IBA) and 1% sucrose. Roots typically were visible in as little as six days. Rooted shoots were set in a soil-less mix containing equal parts peat, perlite and vermiculite, and were kept in a plexiglass "fog box" at 100% relative humidity. Plantlets were kept under high humidity at 2,700 µW/cm2 under a 16 h photoperiod until shoot growth resumed, usually within four weeks or less. After this time, plantlets could be maintained under the ambient conditions of a greenhouse. Planting stock for field testing was produced by transplanting plantlets (4-5 cm) to 10 in ^{3} Leach tubes in the spring, growing the plantlets all summer in a greenhouse, and hardening them to outdoor conditions in September. Plantlets (30-50 cm) with well-developed root systems were overwintered at 4°C prior to establishment in the field.

RESULTS AND DISCUSSION

Establishment of shoot cultures in red maple was influenced by the season in which explants were collected (see Table l). Shoot proliferation from nodal segments taken from greenwood cuttings collected in the spring proceeded rapidly (three weeks), and was induced in over 80% of the explants cultured on MS media containing 0.01 mg/L TDZ. In addition, every genotype cultured from nodal segments collected in April and May could be established in culture. Previously published reports on micropropagation of 'Red Sunset' or red x silver maple hybrids have capitalized on this observation, and report exclusively on the use of nodal explants taken from greenwood cuttings collected in spring or early summer from grafted individuals. Our findings show that grafting is an unnecessary step, as greenwood cuttings can be collected from the field and established in culture with little difficulty. The rapid rate of shoot proliferation from explants collected in the spring means that plantlets could be established in soil from a mature tree in as little as five months.

Table 1. Effect of collection date on the establishment of shoot cultures in red maple.

		Genotypes, No.		Time To Shoot
Month	Genotype Origin	Collected	Established In Vitro	Proliferation, Wks.
February	VA	4	2	12
May	VA, NJ	4	4	3
July	SC	5	2	8
September	VA, NJ	5	3	8
December	VA	4	2	12

On the other hand, explants from greenwood cuttings that had set bud in July were more difficult to establish in culture and took up to eight weeks. Axillary buds had to be explanted from these cuttings, as culture establishment from the nodal segments was unsuccessful. Although axenic cultures could be established from excised buds from greenwood cuttings, subsequent shoot elongation and proliferation was not as rapid and vigorous as from actively growing greenwood cuttings. Indeed, even though four of the five genotypes were established in axenic culture, after six months only two of the genotypes went on to produce rapidly proliferating shoot cultures. Seasonal variation in the rate and extent of shoot proliferation from bud explants of woody perennials has been previously documented, and is thought to be related to the depth of internal dormancy (Fukui et al., 1990).

Multiple shoot proliferation from axillary dormant buds was also slow. At the various times that cultures were initiated from this explant (September, December and February) 8-12 weeks were required before multiple shoot formation was observed. Unlike greenwood cuttings, no seasonal effect on shoot proliferation was observed in cultures established throughout the dormant season. However, not every genotype examined could be established in culture, mainly due to losses associated with contamination, or from damage caused by the sterilization treatment used. In many hardwood species (e.g., oak, aspen and sweetgum) there exists a ramified layer of cortex tissue subtending the apical meristem, as well as numerous layers of leaves surrounding the meristem. Both of these features, which can protect the apical meristem from damage due to the sterilizing agents, are missing in dormant red maple buds. Without the protection afforded by numerous leaves and cortical tissue, establishing axenic cultures from dormant buds can be difficult.

Thidiazuron (TDZ) has been reported to be critical to shoot proliferation in tissue cultures of *Asimina* freemanii (Kerns and Meyer, 1987), and this was also observed in red maple (see Table 2). When used alone or with low concentrations of BA, a concentration of TDZ at 0.01 mg/L was near optimal, as 0.1 mg/L TDZ caused excessive basal callus formation at the expense of shoot proliferation. As the BA concentration was increased to 2 mg/L in the presence of 0.01 mg/L TDZ, shoot proliferation was again inhibited and replaced by basal callus was formation. At 0.001 mg/L TDZ, no shoot proliferation was observed (result not shown). New shoot formation from single-node shoots placed vertically on TDZ-containing medium followed two pathways. First, two new shoots elongated rapidly from the two axillary buds. Second, several adventitious shoots differentiated more slowly from the callus that formed at the base of the original shoot. As adventitious shoots appeared, their axillary meristems would, in turn, elongate. The result was that at the end of a six week period, it was not unusual to have up to eight new shoots (i.e., two axillary; two adventitious, with their four axillary shoot beginning to elongate) from a single shoot. Therefore, micropropagation of red maple using this procedure yields a mixture of axillary and adventitious shoot types. Although the possibility exists of off-types being produced via an adventitious pathway, no phenotypic variants have been observed among the hundreds of plantlets produced to date.

Table 2. Effect of benzyladenine and thidiazuron on shoot proliferation in red maple (three genotypes; four week evaluation).

		Number of New Shoots/Shoot			
		BA, mg/L			
		0	0.1	1.0	2.0
TDZ, mg/L	0	30% Rooting	0.2 ± 0.1	0.13 ± 0.05	Callus
	0.01	4.1 ± 1.5	3.5 ± 1.2	3.5 ± 1.3	Callus
	0.1	Callus	Callus	Callus	Callus

Used alone, the widely-utilized cytokinin BA did not support shoot proliferation. Instead, at increasing concentrations, BA stimulated the formation of large amounts of basal callus from the cut end of excised shoots. At lower concentrations (0.01-0.025 mg/L), the ineffectiveness of BA was underscored by the observation that shoots rooted quantitatively (results not shown). Inclusion of low concentrations of cytokinin to in vitro rooting treatments can produce higher quality plantlets by promoting shoot health during rooting; this should not be overlooked as a means of increasing plantlet survival, especially in difficult-to-root species (K. Louis, personal communication, 1993).

Red maple shoot cultures maintained under light regimes typically used for woody plant tissue cultures (i.e., 1,200 µW/cm2 cool white fluorescent, 16 h photoperiod) had a decidedly red coloration of both stem and foliage. Cultures exhibiting red coloration did not proliferate well, but more importantly, did not produce the long (greater than 1 cm) high quality shoots needed for rooting. A reduction in light intensity (effected by the use of three layers of cheesecloth) by two thirds (i.e., down to 400 p.W/cm^2) greatly reduced the red coloration of the cultures and increased the number of shoots suitable for rooting in some genotypes (see Table 3). Although the increase in shoot elongation was not observed in all genotypes, the improvement in the overall appearance of the cultures was such that shoot cultures of all genotypes were routinely maintained at the reduced light intensity.

Table 3. Effect of light intensity on the shoot elongation for two red maple genotypes (4 week evaluation).

	Number of Long Shoots/Culture*	
Genotype	1,200 - - - Light Intensity, µW/cm2 - - -	400
'Jordan Pocosin'	13.7 ± 4.0 a	8.8 ± 3.0 a
'Jillcott'	4.2 ± 0.8 a	1.0 ± 0.4 b

* Within a row, means followed by common letters are not significantly different as determined by t-test (p = 0.05).

Enhanced growth rates of shoot cultures of woody species can often be achieved by the use of liquid culture. However, liquid culture often leads to shoot vitrification and reduced survival upon transfer to soil. A recent development in culture systems that offer the growth advantages of liquid culture, while minimizing vitrification, are microporous, polypropylene membrane "rafts" (Sigma Chemical Company, 1990). In this system, shoot cultures are placed on a porous, polypropylene support over a liquid media that allows for the diffusion of substances that might be inhibitory to growth, as well as the effusion of nutrients such that nothing becomes limiting to growth.

Table 4 shows the results obtained using the membrane raft system. Again, improvement in growth rate and shoot elongation with the membrane raft culture system was genotype dependent. In genotypes in which the membrane system improved growth rate and shoot elongation, it was not possible to maintain the cultures on the raft system for extended subculture intervals as vitrification would develop. However, the extent of vitrification could be controlled by alternating between the raft system for one cycle (4 weeks), followed by a four week cycle on agar before once again undergoing transfer to the rafts. Coupling the reduced light intensity with the membrane rafts could, with some genotypes, greatly increase the production of shoots suitable for rooting.

Table 4. Effect of culture system on shoot elongation of red maple (4 weeks).

	Shoot Elongation, No. Shoots > 1 cm		Growth Rate, gm	
Genotype	Agar	Membrane Raft	Agar	Membrane Raft
'Jillcott'	0	3.6 ± 1.1	0.31 ± 0.04	1.45 ± 0.48
'Jordan Pocosin'	8.8 ± 3.0	9.8 ± 2.6	ND	ND

Rooting and transfer to soil of red maple microshoots was remarkably easy. As noted, several media (WPM or MS) containing either no growth regulators, low concentrations (0.01-0.25 mg/L) of BA, or IBA (0.1 mg/L) promoted rooting, usually within as little as six days. All genotypes rooted easily, and no genotypic effects on root formation were observed. The ease of rooting implies that physiological rejuvenation has occurred (Howard et al., 1989). The overall rooting and survival in soil of twelve genotypes in which planting stock has been produced averaged 77%. After one season's growth in a greenhouse, and overwintering, several small demonstration plantings have been established near Franklin, VA.

CONCLUSION

Micropropagation of mature red maple is one way in which selected trees can be rapidly propagated for evaluation of growth potential. The procedure described avoids the time-consuming step of grafting, and identifies spring and early summer as the optimum times for culture establishment. However, the procedure can also be extended to material collected throughout the year, although culture establishment after bud set in the summer should be avoided. The procedure developed was successfully applied to a wide variety of genotypes, and the remarkable ease of root formation suggested that some type of rejuvenation had occurred. Procedures for red maple micropropagation departed from those used for many Southern hardwoods in that BA, the most widely used cytokinin, was inhibitory to shoot proliferation, while TDZ was critical to success. Reduction in light intensity, although not crucial to successful micropropagation, dramatically improved the quality of shoots set for rooting. In hardwood micropropagation, when excessive shoot pigmentation or extensive basal callus formation is observed, consideration should be given to an investigation of alternative growth regulators and light regimes.

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