Rooting of American Chestnut Microcuttings[1]

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Abstract

A procedure for micropropagation of American chestnut (Castanea dentata (Marsh.) Borkh.) seedlings was devised and three factors affecting *in vitro* rooting were investigated. A two-step rooting procedure is utilized with a short term exposure to the auxin, IBA (indole-3-butyric acid), called the pulse treatment followed by transfer to an auxin-free medium called the rooting treatment. Optimum rooting was attained with an IBA concentration of 369 μM, a sucrose concentration of 4% (w/v) and half strength WPM basal medium concentration. This rooting regimen should enhance vegetative propagation techniques needed for rapid multiplication of selected disease-resistant trees.

Index words: micropropagation, chestnut, IBA, sucrose, basal medium

Significance to the Nursery Industry

Selected resistant American chestnut trees would be desirable for urban planting, nut production and possibly timber plantations. Breeding programs involving hybridization with the Chinese chestnut and backcrossing to the American chestnut are in progress (1). The introduction of selected resistant American chestnut will rely on rapid and reliable vegetative propagation techniques for screening programs and distribution of the superior clones.

This work shows that the American chestnut can be successfully propagated using micropropagation techniques. It should be noted, however, that the source tissue used was derived from juvenile plants and adaptations to the procedure presented here may be necessary for success with plant material taken from a selected mature tree.

(**Ed. note:** This paper reports the results of research only, and does not imply registration of a pesticide under amended FIFRA. Before using any of the products mentioned in this research paper, be certain of their registration by appropriate state and/or federal authorities).

Introduction

The American chestnut, *Castanea dentata* (Marsh.) Borkh., relinquished its role as one of the most important forest tree species of the United States in the early 1900's (2). Chestnut blight, a fungal disease, decimated the population. Originally comprising 25% of the trees in the Eastern forests, the decline was dramatic and relatively fast, spanning only about 50 years (1900–1950). The pathogen, *Cryphonectria parasitica* (Murr.) Barr (formerly *Endothia parasitica* (Murr.) And.), is thought to have been introduced to North America in the late 1800's on Asiatic chestnut tree stock or wood products (10).

Blight-resistant American chestnut trees are being developed for re-establishment in the forest and urban environments (1, 2). Conventional breeding programs underway are hampered by difficulties with vegetative propagation of *C. dentata* and interspecific hybrids (1, 2); however, vegetative propagation success is possible (8). The use of auxin treatments greatly enhanced progress in the rooting of cuttings, but even then, mature chestnuts are difficult to propagate vegetatively (3).

In vitro culture systems have been utilized by a few groups to try to overcome this problem. Most research has been conducted with *Castanea sativa* Mill., the European chestnut. Some tissue culture work has been done on the American chestnut but not dealing with the rooting of microcuttings (5, 6, 9). Thorough reviews of tissue culture work with *Castanea* are offered by Hansman (4) and Vieitez (13).

In vitro multiplication has been successful for many juvenile clones of *C. dentata* and its hybrids; however, rooting has proven difficult. In this paper, the results of investigations leading to the optimization of various factors for an *in vitro* rooting system will be presented.

Materials and Methods

American chestnut seeds collected from a tree growing in an isolated group were germinated in the greenhouse in February. The apical 4–7 cm (1.5–2,75 in) of the shoots including the shoot tip and three developed nodes and internodes were excised from the greenhouse-grown seedlings in mid-July. The leaves and stipules were removed and the defoliated shoots were placed in a 1% commercial bleach solution (0.05% NaOC1) with three drops Tween 20/1. In the laboratory, the shoots were soaked for 10 minutes in a 10% commercial bleach solution (0.5% NaOC1) plus three drops Tween 20/1. After the bleach soak and in a laminar flow hood, the shoots were rinsed with sterile water, rinsed with 70% ethanol for 30 sec., and finally rinsed three times with sterile water to remove the ethanol. The shoots were then cut into the 3 nodal sections and the tip. These were cultured on 10 ml woody plant medium (WPM) (11) sup-

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plemented with 0.54 μM naphthaleneacetic acid (NAA) and 1.3 μM benzyladenine (BA), 2% sucrose (w/v), and 0.65% (w/v) agar in culture tubes. The pH was adjusted with KOH to 5.6 prior to adding agar. All cultures were kept under a 16 hr light (34 μmol sec^{-1} m^{-2})/8 hr dark photocycle at 20–24°C (70–75°F) in Conviron growth cabinets (Controlled Environments, Winnipeg, Canada). Swelling of the axillary buds and growth of the shoot tip were noted within two weeks. The macronutrient, and vitamin components of WPM will be referred to as the basal medium.

After 6 weeks on the initial medium, shoots from axillary buds and shoot tips were approximately 6 cm (2.4 in) tall with little branching. Nodal sections were subcultured onto multiplication medium, that differed from the initiation medium with BA (0.85 μM) and agar (0.6% w/v). Every 5 weeks shoots were excised from their callused nodal bases and the leaves were removed. These shoots were subdivided into 1–3 node explants for continued multiplication. The explants were laid on their sides with the proximal end slightly depressed into the medium. The culture vessels were either baby food jars with a volume of 170 ml capped with Magenta b-caps (Magenta Corp., Chicago IL) or Magenta GA7 and GA7-3 vessels 500 ml and 300 ml in volume, resp. Baby food jars were filled with 25 ml of medium upon which 3 explants were placed. The GA7 and GA7-3 vessels were filled with 50 ml of medium upon which 5 explants were placed. None of the lids on the vessels were sealed with parafilm.

After 7 months of continuous culture, stable multiplication rates were attained, and experiments to develop a rooting strategy were begun.

General rooting procedure. Based on previous work with other chestnut species and non-related woody species, the following procedure for *in vitro* rooting of American chestnut was used and experimentally modified and will be referred to as the general rooting procedure. Shoots from 4-week old multiplication cultures were used for all rooting experiments. The shoots were approximately 2 cm (0.75 in) long and had 3–4 nodes and the shoot tip. All leaves were removed except for 1–2 small leaves at the tip. If not removed, the leaves often grew very large when in contact with the medium and forced the shoot base into the air.

To initiate rooting, the base of the cutting was exposed to IBA (indole-3-butyric acid) at various concentrations. This exposure will be referred to as the *pulse treatment.* The standard pulse treatment lasted 5 days under conditions described for multiplication. The standard pulse medium consisted of half strength WPM basal medium, 4% (w/v) sucrose, 0.44 μM BA, and 123 μM IBA. The pH was adjusted to 5.6 with KOH. No agar was used. The shoots were held upright in Magenta GA7-3's containing 50 ml of liquid medium by being inserted into drilled holes in the top of a Magenta b-cap fit into the GA7-3.

After the pulse treatment, each cutting was transferred to an individual glass culture vial containing 15 ml of the standard rooting medium which was the same as the pulse medium except that it was solidified with 0.6% (w/v) agar and contained no growth regulators. The cultures were incubated in 24 hour darkness at 22°C (72°F). This treatment will be called the standard *rooting treatment.*

Three factors were investigated for optimizing the general rooting procedure: IBA concentration in the pulse treatment, sucrose concentration in the rooting treatment, and basal medium concentration during both treatments. All other conditions were as described above as standard conditions.

IBA concentration. Three IBA concentration (49, 123, or 369 μM) were used during the pulse treatment. Each IBA concentration was represented by 30 shoots with all 90 shoots in a completely randomized design.

Sucrose concentration. Three sucrose concentrations (1%, 2%, or 4% (w/v)) in the rooting treatment were investigated. Each sucrose concentration was represented by 30 shoots with all 90 shoots in a completely randomized design.

Basal medium concentration. Two basal medium concentrations (full- or half-strength) were used in both the pulse and rooting treatments. Each basal medium concentration was represented by 30 shoots with all 60 shoots in a completely randomized design.

Results and Discussion

The bases of the cuttings became slightly swollen during the pulse treatment and continued to swell with some callus growth during the rooting treatment. Roots were evident about 2 weeks after the beginning of the pulse treatment. More roots developed in the following weeks, and roots reached a length of 10 cm (4 in) in 4 weeks. Apical necrosis was evident on some shoots at 2 weeks, and the number of shoots affected increased with time. Necrosis was generally limited to the shoot tip, rarely exceeding the top node and therefore 2 nodes and axillary buds remained viable. These buds produced the shoots that allowed survival of the rooted cutting.

The highest concentration of IBA investigated (369 μM) gave the highest percentage rooting and the greatest number of roots per shoot (Table 1 and Fig. 1). Higher concentrations of IBA were investigated in preliminary experiments with *C. dentata* but over-production of callus and increased tip necrosis resulted.

Sucrose concentration in the rooting medium was positively correlated with rooting percentage (Table 2). The number of roots per shoot also increased with sucrose concentration (Fig. 2) and there were significant differences in root number among all three sucrose concentrations. Higher sucrose concentrations should be investigated.

A decrease in the basal medium concentration enhances both the percentage rooting (Table 3) and the number of roots per shoot (Fig. 3). Preliminary experiments (data not shown) indicated, however, that if no basal medium (just agar and sucrose) is used during the rooting treatment no rooting occurs and the microcutting dies.

Table 1. Effects of IBA concentration on the percentage rooting of American chestnut microcuttings.

Treatment	Percentage of shoots with roots	
	3 weeks	4 weeks
49 μM IBA	6.7a[z]	51.7a
123 μM IBA	66.7b	93.3b
369 μM IBA	63.3b	93.3b

[z]Values within a column not followed by the same letter or letters are significantly different using a chi-square analysis.

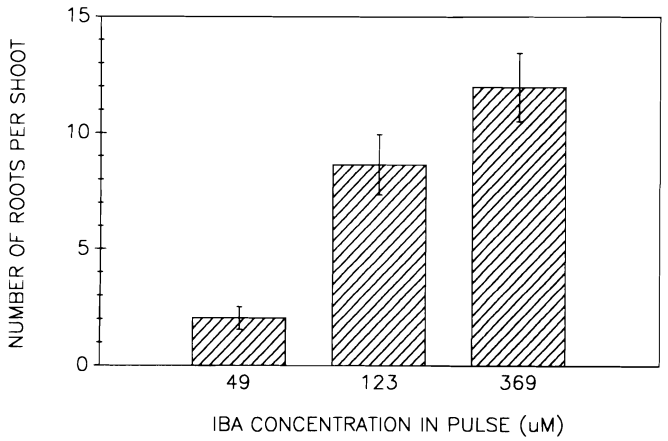

Fig. 1. Influence of IBA concentration in the pulse on the number of roots per shoot at 4 weeks. Each bar represents the mean ± S.E. of 30 shoots except 49 μM BA which had 29 shoots.

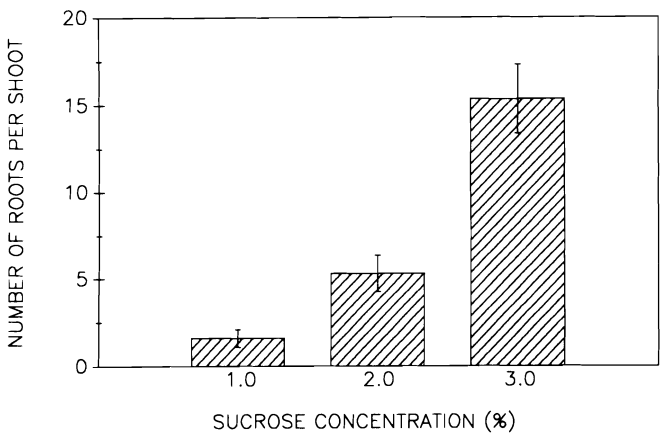

Fig. 2. Effect of sucrose concentration in the rooting medium on the number of roots per shoot at 4 weeks. Each bar represents the mean ± S.E. of 30 shoots.

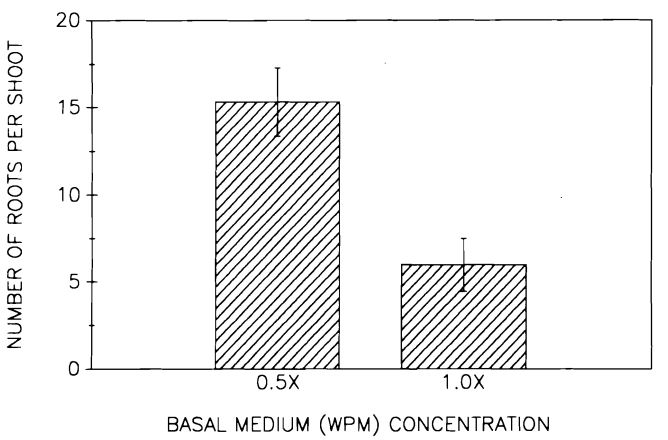

Fig. 3. Effect of woody plant basal medium concentration in the pulse and rooting media on the number of roots per shoot at 4 weeks. Each bar represents the mean ± S.E. of 30 shoots.

The sucrose and basal medium concentrations are appropriate and the IBA pulse concentration should be at least 123 μM.

In vitro rooting of American chestnut is significantly affected by the 3 factors investigated here. The effects of these 3 factors on *in vitro* rooting have been studied for other plant species (7, 12, 14) and the results were similar to those presented for the American chestnut.

Table 2. Effects of sucrose concentration on the percentage rooting of American chestnut microcuttings.

Treatment	Percentage of shoots with roots	
	3 weeks	4 weeks
1% sucrose	6.7a	23.3a
2% sucrose	40.0b	56.7b
4% sucrose	73.3c	80.0c

[z]Values within a column not followed by the same letter or letters are significantly different using a chi-square analysis.

Table 3. Effects of basal medium concentration on the percentage rooting of American chestnut microcuttings.

Treatment	Percentage of shoots with roots	
	3 weeks	4 weeks
Full basal medium	33.3a[z]	63.3a
Half basal medium	73.3b	80.0a

[z]Values within a column not followed by the same letter or letters are significantly different using a chi-square analysis.

Rooted microshoots have successfully acclimated to greenhouse conditions.

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