PLANTLET FORMATION IN AMERICAN CHESTNUT
EMBRYONIC TISSUE IN VITRO

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Abstract. -- Axillary shoots developed from mature embryos of American chestnut seeds which were cultured on BAP-supplemented Murashige and Skoog medium. These shoots could be multiplied by subculture on MS medium with reduced BAP concentration. Rooting of the axillary shoots occurred when they were placed on IBA-supplemented medium followed by culture on hormone-free medium.

Additional keywords: Shoot culture, Castanea dentata.

In the past few years, in vitro shoot culture has become a promising technique for vegetatively propagating woody plants. Plantlet formation has been reported in "Nonpareil" almond (Prunus armeniaca L.) x peach [P. persica (L) Batsch] hybrids (Tabachnik and Kester, 1977), apple [Malus sp. Mill. Focke, and M. domestica, Borkh.] (Jones, Hopgood, and O'Farrell, 1977; Lundergan and Janick, 1980), cherry (P. avium L.) (Ivanicka and Pretova, 1980), jojoba (Simmondsia chinensis Link., Schneid.) (Rost and Hinchee, 1980), and plum (P. insititia L.) (Jones and Hopgood, 1979).

The shoot culture technique resulted in axillary shoot formation using seedling buds (Vieitez and Vieitez, 1980a) and plantlet formation using excised embryos (Vieitez and Vieitez, 1980b) of European chestnut (Castanea sativa Mill.). Axillary shoot formation was reported on shoot tips and embryos of American [C. dentata (Marsh) Borkh.], Chinese (C. mollissima B1.) and hybrid chestnuts (McPheeters, Skirvin, and Bly-Monnen, 1980). There have been no reports of complete plantlet formation in American chestnut. This paper reports results using excised American chestnut embryos as the sources for in vitro shoot cultures.

MATERIAL AND METHODS

American chestnut seeds were obtained through Dr. William MacDonald, Forest Pathologist, West Virginia University, from Michigan, West Virginia, and one unknown source. The seeds were either stratified in moist sand in plastic bags for 60 days at 34° F or stored in cans with plastic lids at 34° F. The seeds were sterilized by dipping in 95% ethanol for 1 minute, followed by flaming and cooling in sterile distilled water. The seed coat was cut and peeled away. The embryos were excised and placed radicle-end-down on 30 ml. of nutrient medium in 25 x 150 mm. culture tubes. The tubes were sealed with aluminum foil, and the cultures were placed in a growth chamber at 25 ± 2° C with a T6-hr. photoperiod of 200 f.c. of simultaneous white fluorescent and incandescent light.

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The nutrient media used were those of Murashige and Skoog (MS) (1962) and Vieitez and Vieitez (VV) (1980b). Both media were supplemented with 300 g./l. sucrose and 6.0 g./l. Phytagar. The pH was adjusted to 5.5 - 5.6 using 1N. NaOH prior to filtering and autoclaving. Modifications of and additions to these media, and culture duration, varied according to the stage of culture, as follows:

1. Culture initiation. Both media were supplemented with 1.0 mg./l. of 6-benzylaminopurine (BAP). Cultures were grown for 6 weeks.

2. Shoot multiplication. Nitrates in the MS medium were decreased by half. Only 0.1 mg./l. BAP was added. Cultures were grown for 4 to 6 weeks on 50 ml. of medium in 125 ml. erlenmeyer flasks.

3. Auxin treatment. Only MS medium with nitrates decreased by half was used. Either 1.0 or 5.0 mg./l. of 3-indolebutyric acid (IBA) was added. Cultures were maintained for 1 to 3 weeks on the auxin medium.

4. Rooting. Only MS medium with nitrates decreased by half was used. No growth regulators were added. The culture period depended on the speed of rooting.

RESULTS

Culture Initiation

Although stratification was not necessary for germination and axillary shoot formation, it nearly doubled the percentage of embryos which formed axillary shoots. Of the stratified embryos, 41.1 percent produced axillary shoots. This compares to 23.1 percent of the cold-treated embryos forming shoots (Table 1).

Table 1. -- Axillary shoot formation on excised embryos.

Medium	Seed Source	Seed Treatment	No. of Cultures	% (#) forming axillary shoots	Ave. No. axillary shoots
MS	Unknown	stratified	9	11(1)	2
	Mich.	stratified	2	0	-
	"	cold-stored	1	0	-
	W.Va.	stratified	4	25(1)	3
	"	cold-stored	5	20(1)	6
				Overall	4
VV	Unknown	stratified	6	67(4)	8(2-18)
	Mich.	stratified	3	33(1)	5
	"	cold-stored	3	67(2)	4(3-4)
	W.Va.	stratified	5	100(5)	4(2-5)
	"	cold-stored	4	0	-
				Overall	5

Table 3. -- Partial history of axillary shoot formation by clone.

Clone	Average Number of Axillary Shoots				
	Subculture 1	Subculture 2	Subculture 3	Subculture 4	Subculture 5
Unknown	7	5	5	3	-
Mich.	9	7	3	4	13
WV 1	10	10	5	6	-
WV 2	17	13	7	5	7
WV 3	16	7	3	8	-
Overall	12	8	6	5	10

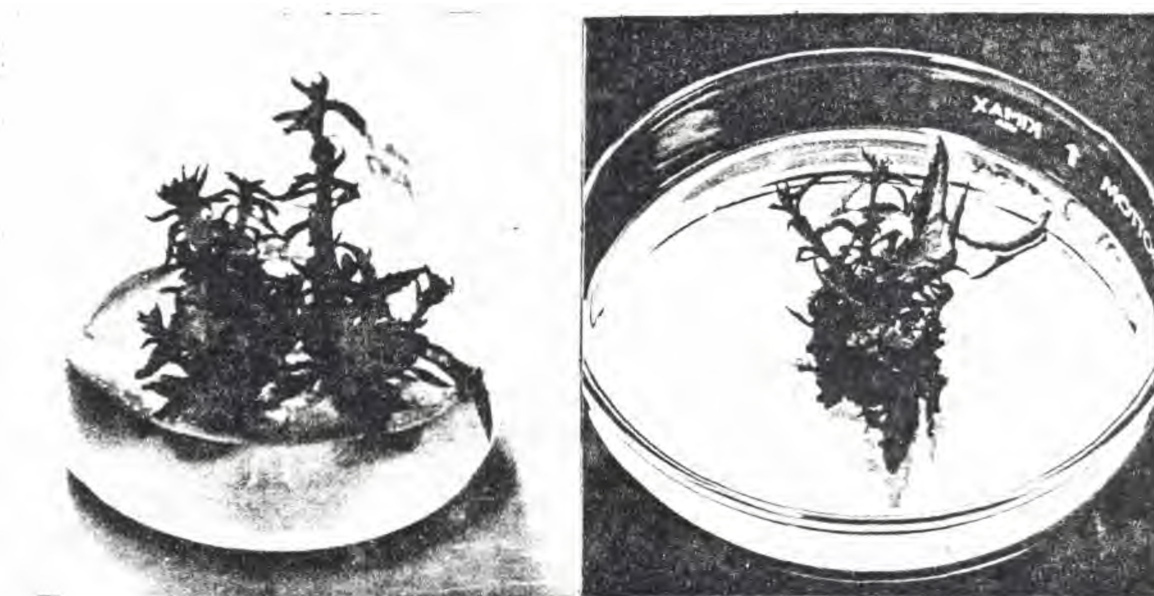

Fig. 1. -- Axillary shoot formation on cultured shoots.

Embryos produced axillary shoots on both of the test media. A greater percentage of cultures formed shoots on the VV medium than on the MS medium. Shoots growing on the VV medium appeared to be less vigorous, with very small leaves. Leaves were well-developed on shoots growing on the MS medium. Shoots on either medium ranged in length from 0.2 to 1.5 cm. The short shoots from either medium failed to elongate when subcultured to the same medium without BAP. The average number of axillary shoots per culture did not vary significantly on the different media (Table 1).

Shoot Multiplication

One hundred percent of the cultures grown on MS medium produced axillary shoots, whereas a lower percentage of cultures formed axillary shoots on VV medium. Shoots on the MS medium ranged in length from approximately 0.2 to 2.5 cm. The VV-grown cultures formed rosettes of numerous shoots less than 0.5 cm. in length (Table 2). When these rosettes were subcultured to VV medium without BAP, shoot elongation did not occur and they eventually become necrotic. When the individual shoots from the rosettes were cultured on BAP-supplemented MS medium, they regained vigor and produced axillary shoots.

Table 2. -- Shoot multiplication

Seed Source	MS medium			VV medium		
	no. of cultures	% forming axillary shoots	Ave. No. axillary shoots	No. of cultures	% forming axillary shoots	Ave. No. axillary shoots
Unknown	1	100	7	3	100	4 - ?[a]
Mich.	1	100	9	3	67	4 - ?[a]
W. Va.	3	100	14(10-17)	3	33	?[a]

[a] The cultures formed a rosette of numerous buds which were not counted.

The average number of axillary shoots was recorded for each culture to determine if they lost their ability to multiply after several subcultures. A Friedman test run on the data after the fourth subculture showed that there was a statistically significant decrease in shoot production. However, the Michigan and West Virginia 2 clones increased shoot production on the fifth subculture. The shoots had been maintained for 8 weeks during this subculture. So it may be that culture duration must be increased for later subcultures in order to maintain adequate shoot production (Fig. 1) (Table 3).

Auxin Treatment and Rooting

The first attempt at rooting shoots was on a medium containing 5 mg./l. IBA. After only 7 days, large callus clumps had formed at the base of the shoots. The callus was cut away prior to subculture to auxin-free medium, but the shoots again regenerated callus tissue at their bases. No rooting occurred using this auxin concentration.

The second test used a medium with 1 mg./l. IBA. Callus proliferation was less than that on 5 mg./l. No rooting occurred on shoots maintained on the auxin medium for 1 or 2 weeks. Of 31 cultures maintained for 3 weeks on the auxin medium, 2 from different clones formed roots. On both shoots, the roots initiated from the shoot rather than the callus tissue. One shoot produced one long tap root. This was subcultured, complete with the agar plug, to a quart jar containing sterile peat: vermiculite (1:1 v/v) soaked with 250 ml. of the rooting medium. It failed to survive the transfer. The second shoot produced three roots which initiated lateral roots (Fig. 2). The agar was washed from the roots, and the plantlet was placed in a 3-inch plastic pot containing vermiculite soaked with 250 ml. of half-strength MS salts. It was placed in a greenhouse propagation bed under mist for 3 weeks prior to transplanting in a sand:soil:peat (1:1:1: v/v/v) mixture in a # 10 can. It is still alive after 4 weeks and is 7.0 cm. in height.

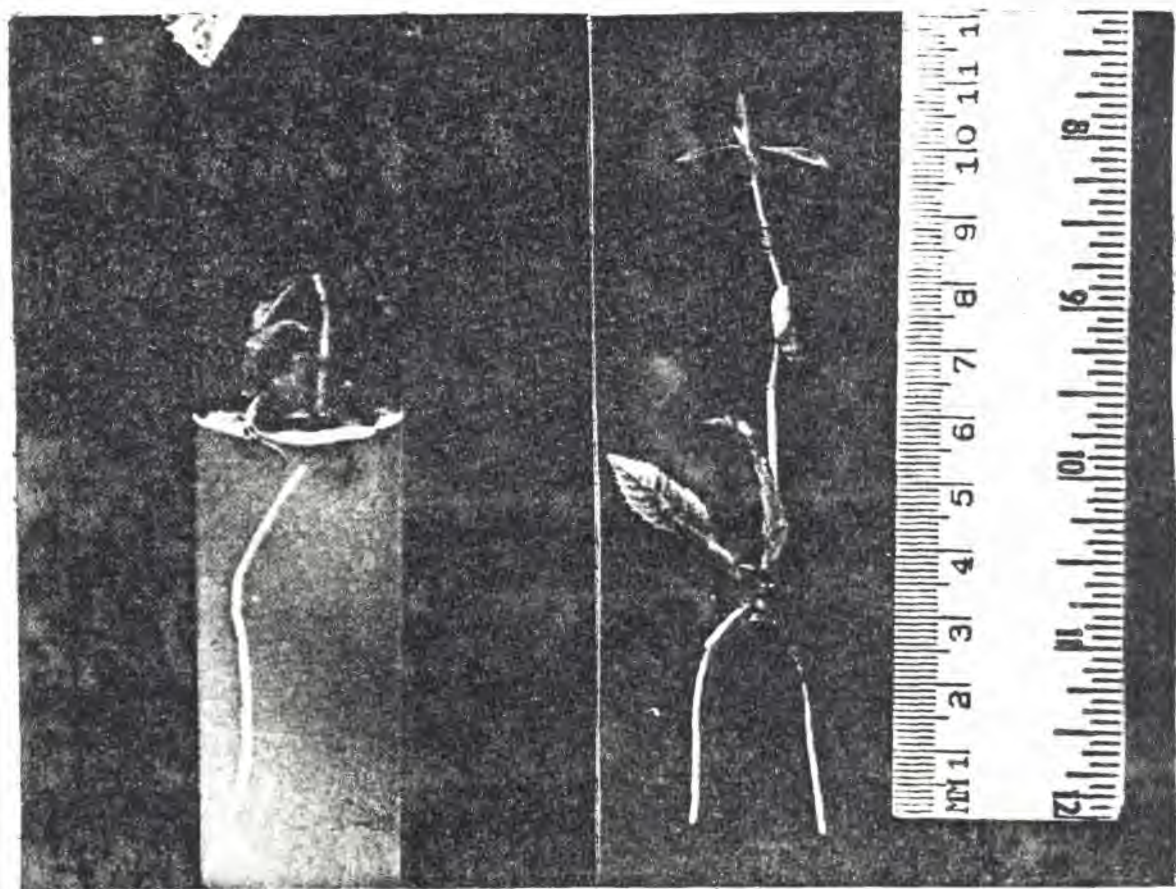

Fig. 2. -- Root formation on cultured shoots.

DISCUSSION AND CONCLUSIONS

This preliminary work shows that excised embryos of American chestnut can be used as a source of tissue for *in vitro* shoot culture. It also demonstrates that root formation can be induced on *in vitro*-grown shoots to form complete plantlets, even after several subcultures. These plantlets can be successfully transferred to soil in pots after a short period under a greenhouse misting system.

This technique, therefore, shows good potential as a means of mass propagation of any blight-resistant clones or hybrids which result from any of the current chestnut breeding programs. In view of the positive results reported with many species, shoot culture is a potential technique for the vegetative propagation of other forest tree species, especially members of the Fagaceae.

The technique still must be tested using buds from mature chestnut trees. A more reliable method of root-induction must be developed. Research on both of these problems is underway in the Division of Forestry of West Virginia University.

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