Micropropagation of *Cephalotaxus harringtonia*

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Additional index words. Japanese plum yew, tissue culture, anticarcinogen

Abstract. Shoot proliferation of Japanese plum yew [*Cephalotaxus harringtonia* (Forbes) K. Koch] derived either from a seedling or from a mature clone was achieved in a medium based on Murashige and Skoog salts supplemented with vitamins and casein hydrolysate in the presence of 10% (v/v) coconut water. Rooting was unsuccessful from microcuttings proliferated on agar-based medium, but, when cultured in liquid medium on membrane rafts, detached shoots rooted under mist in greenhouse conditions with or without auxin treatments. Rooted microcuttings successfully acclimated to greenhouse conditions.

The Japanese plum yew is an ornamental *Taxus*-like plant native to eastern Asia that is a source of several alkaloids, including harringtonine and homoharringtonine, which have shown antitumor activities (Cloonley et al., 1983; Perdue, 1970; Smith et al., 1976). *Cephalotaxus harringtonia* has been difficult to propagate by cuttings and exhibits very slow growth in callus cultures (Delfel, 1980; Delfel and Rothfus, 1977; Delfel and Smith, 1980; Heinstein, 1985), but suspension cultures have been initiated and maintained in medium supplemented with coconut water (Westgate et al., 1991a, 1991 b). This paper reports the protocol developed to achieve rapid propagation of selected clones of *C. harringtonia* through tissue culture.

Shoot propagation. Preliminary studies were carried out with a single plant of a high alkaloid clone (#6) provided by N.E. Delfel, Northern Regional Laboratory, ARS/USDA, Peona, Ill., and sent to Purdue Univ. in 1982. This clone was difficult to propagate by air layering and, after treatment with 1%, 3%, or 8% indolebutyric acid (IBA), only a single root was observed after 4 weeks when girdled stems were treated with 3% IBA. When 6-benzylaminopurine (BA) or benzylamino-9-(2-tetrahydropyranyl)-9H-pyridine (PBA) was sprayed, each at 1000 ppm alone or in combination, on shoots of this plant, budbreak increased (Table 1). In the following year, actively growing shoot tips from this plant were explanted on Woody Plant Medium (Lloyd and McCown, 1980) or Murashige and Skoog (MS) basal medium (1962) supplemented with 0.3 μM (0.1 $\text{mg}\cdot\text{liter}^{-1}$) thiamine $\cdot\text{HCl}$; 2.4 μM (0.5 $\text{mg}\cdot\text{liter}^{-1}$) pyridoxine $\cdot\text{HCl}$; 0.9 mM (100

$\text{mg}\cdot\text{liter}^{-1}$) *i*-inositol; 4 μM (0.5 $\text{mg}\cdot\text{liter}^{-1}$) nicotinic acid; 26.6 μM (2.0 $\text{mg}\cdot\text{liter}^{-1}$) glycine; 1 g casein hydrolysate/liter; 87.6 mM (30 $\text{g}\cdot\text{liter}^{-1}$) sucrose; 6 g agar/liter (Sigma, St. Louis); with 0.4 to 44.4 μM (0.1 to 10 $\text{mg}\cdot\text{liter}^{-1}$) BA and 0.4 to 49.2 μM (0.1 to 10 $\text{mg}\cdot\text{liter}^{-1}$) 6-(γ , γ -dimethylallylamino) purine (2iP) with pH adjusted to 5.7. In general, axillary budbreak was sporadic in response to BA or 2iP and growth was not maintained in culture (data not presented), even with the addition of charcoal to the medium.

Lack of success with the mature clone led us to investigate the potential for somatic embryogenesis. Seeds of *C. harringtonia* received from U.S. Agri Corp. (Newport Beach, Calif.) were disinfested with 0.5% NaOCl [10% household bleach (v/v)] for 30 min, and rinsed three to five times in sterile distilled water. Zygotic embryos were excised from the surrounding endosperm and explanted on basal medium consisting of the basal MS medium described above with 0 or 4.4 μM (1 $\text{mg}\cdot\text{liter}^{-1}$) BA and 0.4 or 4.5 μM (0.1 or 1.0 $\text{mg}\cdot\text{liter}^{-1}$) 2,4-dichlorophenoxyacetic acid (2,4-D). Embryos were grown at 26°C under a 16-h photoperiod provided by cool-white fluorescent lamps. The lamps provided a photosynthetic photon flux density (PPFD) (400–700 nm) of 45 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. Twenty-seven out of 180 embryos greened and swelled, and one of these embryos (in basal medium alone) germinated. All of the 27 embryos were transferred to medium with 10% coconut water and 1 mg 2,4-D/liter. None of the explants produced somatic embryos, but the single germinated embryo started to produce new shoots and continued to proliferate when maintained in this medium. After 1 year of subculturing, the original explant had produced enough shoots to start a series of experiments designed to optimize the shoot multiplication medium.

Two factorial experiments were established to determine the effect of 2,4-D and BA on the seedling clone on MS medium containing coconut water. Our results indicated maximum

proliferation with 1 mg 2,4-D/liter, with no effect due to BA (data not presented). Consequently, a factorial experiment was conducted with or without 2,4-D and BA using basal medium with either full- or half-strength salts. The experiment was evaluated after 6 weeks, then all axillary shoots were transferred to the same treatment for two additional subcultures. Maximum proliferation of seedling-derived shoots was obtained with 1.0 mg 2,4-D/liter with full-strength salts (Table 2). BA had negligible effects on proliferation. The response increased with subculturing; by the third culture, there was a greater than four-fold increase in proliferation in response to 1.0 mg 2,4-D/liter with full-strength salts, suggesting a reversion to juvenility (Mullins et al., 1979).

To determine if the mature clone would react similarly to 2,4-D and coconut water, 48 shoots (≈ 1 mm long) from clone 6 were explanted onto basal media supplemented with 0 or 1.0 mg 2,4-D/liter and 0% or 10% coconut water. The explants were subculture every 4 weeks. Shoot multiplication was greatest with 10% coconut water, but the effect of 2,4-D was negligible (Table 3). This result contradicted our earlier findings (see Table 2), and we repeated the 2,4-D-coconut water factorial experiment using microcuttings proliferated from stock cultures of clone 6 as well as the seedling clone maintained on 2,4-D-coconut water medium. Microcuttings were subculture every 4 weeks. After three subcultures, shoot multiplication for both clones was greatest with 10% coconut water and was inhibited by 2,4-D (Table 4). The seedling clone was more proliferative than clone 6. These results confirm that *C. harringtonia* shoots proliferate in response to coconut water. The conflicting results with 2,4-D are unexplained. The response of shoots to coconut water is intriguing in light of the necessity of coconut water for cell suspension (Westgate et al., 1991a, 1991 b). Shoots of *C. harringtonia* have continued to proliferate for 7 years in basal medium containing coconut water (Fig. 1).

Rooting. Conventional techniques to induce in vitro rooting of microcuttings produced on agar-based medium were unsuccessful. Unsuccessful treatments included transfer to basal medium without coconut water or growth regulators and the addition of IBA (1 to 10 $\text{mg}\cdot\text{liter}^{-1}$) and phloroglucinal (126 $\text{mg}\cdot\text{liter}^{-1}$). Subsequently, rooting experiments were carried out at the Univ. of Delaware from shoot cultures received from Purdue Univ. Microcuttings derived directly from agar-based proliferation medium did not root after treatment with 2500 μM IBA whether grown under aseptic laboratory or septic greenhouse conditions (data not presented).

Table 1. Effect of BA (1000 ppm) and PBA (1000 ppm) on axillary budbreak in situ of *Cephalotaxus harringtonia*.

Treatment ^a	Total budbreaks per shoot
Control	2.0
BA	6.5
PBA	9.0
BA + PBA	15.5

^aTwo shoots per treatment.

Received for publication 24 Mar. 1993. Accepted for publication 13 Sept. 1993. Journal Paper no. 13,728 of the Purdue Univ. Agricultural Experiment Station, West Lafayette, Ind. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

Table 2. Effect of 2,4-D, BA, and salt strength on proliferation of seedling Microcuttings of *Cephalotaxus harringtonia* subcultured every 6 weeks. All media contained 10% coconut water.

2,4-D (mg·liter ⁻¹)	Mean no. axillary shoots/microcutting ± SE ²					
	1/2× MS ¹			1× MS		
	BA (mg·liter ⁻¹)			BA (mg·liter ⁻¹)		
	0	0.5	1.0	0	0.5	1.0
<i>First subculture</i>						
0	1.9 ± 0.4	2.1 ± 0.3	2.6 ± 0.3	3.4 ± 0.4	3.0 ± 0.3	3.0 ± 0.4
0.1	1.6 ± 2.0	3.1 ± 0.5	3.3 ± 0.4	3.5 ± 0.3	3.6 ± 0.4	3.8 ± 0.2
1.0	3.0 ± 0.3	3.1 ± 0.3	4.0 ± 0.7	3.0 ± 0.7	3.4 ± 0.5	3.8 ± 0.6
<i>Second subculture</i>						
0	2.0 ± 0.3	2.9 ± 0.5	3.9 ± 0.8	5.0 ± 0.9	5.0 ± 0.7	3.8 ± 0.7
0.1	2.0 ± 0.3	4.0 ± 0.7	4.3 ± 0.7	5.0 ± 0.8	5.3 ± 0.6	5.6 ± 0.7
1.0	6.3 ± 0.7	4.8 ± 0.4	7.9 ± 1.2	6.8 ± 1.0	7.4 ± 1.3	6.4 ± 0.9
<i>Third subculture</i>						
0	2.2 ± 0.4	3.5 ± 0.7	5.1 ± 1.4	6.5 ± 1.4	8.1 ± 2.9	4.4 ± 0.9
0.1	1.7 ± 0.4	4.8 ± 0.9	6.2 ± 0.9	7.7 ± 1.5	7.7 ± 1.4	7.9 ± 1.3
1.0	16.3 ± 2.1	13.3 ± 2.1	16.9 ± 3.4	26.2 ± 3.5	25.4 ± 4.1	19.4 ± 3.4

Sub- culture	Main effects							
	2,4-D (mg·liter ⁻¹)			BA (mg·liter ⁻¹)			MS	
	0	0.1	1.0	0	0.5	1.0	1/2× MS	1× MS
1	2.7 ± 0.2	3.2 ± 0.2	3.4 ± 0.2	2.7 ± 0.2	3.1 ± 0.2	3.4 ± 0.2	2.7 ± 0.2	3.4 ± 0.1
2	3.8 ± 0.3	4.4 ± 0.3	6.6 ± 0.4	4.5 ± 0.4	4.9 ± 0.3	5.3 ± 0.4	4.2 ± 0.3	5.6 ± 0.3
3	5.0 ± 0.6	6.0 ± 0.5	19.6 ± 1.4	10.1 ± 1.3	10.5 ± 1.3	10.0 ± 1.2	7.8 ± 0.8	12.6 ± 0.2

¹Ten microcuttings/treatment.

²MS = Murashige and Skoog (1962) salts.

Table 3. Effect of 2,4-D and coconut water on proliferation of mature microcuttings of *Cephalotaxus harringtonia*, subculture every 4 weeks.

Coconut water (%)	2,4-D (mg·liter ⁻¹)	Mean no. axillary shoots/microcutting ± SE ²				
		Subculture				
		1	2	3	4	5
0	0	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
	1.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
10	0	0.9 ± 0.3	2.6 ± 0.5	7.4 ± 0.8	13.5 ± 1.7	15.6 ± 1.8
	1.0	0.4 ± 0.2	3.3 ± 0.6	7.6 ± 1.0	10.1 ± 1.4	12.4 ± 1.6

²Ten microcuttings/treatment.

Microcuttings were cultured on membrane rafts (Sigma) in GA7 (Magenta Corp., Chicago) vessels containing 50 ml of MS basal medium plus vitamins, as described previously for shoot propagation, but containing half-strength salts (1/2×), 1.2 μM (0.4 mg·liter⁻¹) thiamine-HCl, without agar and supplemented with 5 μM (1 mg·liter⁻¹) IBA. After 4 to 6 weeks, microcuttings (1 to 5 cm long) were transferred to GA7 vessels containing either 75 cm³ fine vermiculite or a membrane raft, each containing 41 ml of basal medium supple-

mented with 25 μM (5 mg·liter⁻¹) IBA. Laboratory cultures were maintained at 25 ± 3°C under a 16-h photoperiod (PPFD = 50 μmol·s⁻¹·m⁻²) provided by cool-white fluorescent lamps. After 8 to 10 weeks, individual microcuttings were cut away from basal callus and dipped in

a 2500-μM (500 ppm) IBA (K-salt) or a 1000-μM (500 ppm) 2,3,5-triodobenzoic acid (TIBA) solution, inserted in a soilless greenhouse mix [2 Redi-Earth (W.R. Grace, Cambridge, Mass.) to 1 fine vermiculite (v/v)] and placed under intermittent mist {10 to 15 sec every 15 light-sum-accumulation units [1 unit = (J·cm⁻²)/10], or every 15 rein, whichever was sooner from 7:00 AM to 5:00 PM daily}. Microcuttings were evaluated for rooting and root growth after 9 weeks, at which time they were potted and placed on a greenhouse bench.

When microcuttings were subculture either onto membrane rafts or into vermiculite for 8 to 10 weeks, they subsequently rooted under greenhouse conditions whether non-treated or dipped in 2500 μM IBA or 1000 μM TIBA (Table 5, Fig. 2). Longer microcuttings rooted better than shorter ones; overall, 92% of microcuttings >2 cm long rooted compared to 31% <2 cm long. Of the microcuttings that

Table 5. Rooting of microcuttings of *Cephalotaxus harringtonia* in a soilless greenhouse mix under intermittent mist after culture on membrane rafts or vermiculite.

Expt.	Culture medium ²	Growth regulator ¹	No. cuttings	Rooted cuttings (%) ³
1	Vermiculite	IBA	24	46
		TIBA	24	8
2	Vermiculite	None	12	25
		IBA	12	58
		TIBA	12	25
	Membrane raft	None	24	54
		IBA	24	62
		TIBA	24	62

¹Microcuttings were cultured in liquid medium on membrane rafts 8 to 10 weeks before final subculture in vermiculite or recultured to membrane rafts. All cuttings were rooted in soilless greenhouse mix [2 Redi-Earth :1 vermiculite (v/v)].

²IBA (2500 μM) and TIBA (1000 μM) applied as dips.

³Data collected after 9 weeks under intermittent mist.

Table 4. Effect of 2,4-D, coconut water, and explant source on axillary shoot proliferation of *Cephalotaxus harringtonia*, subculture after 4 weeks.

Coconut		Mean no. axillary shoots/ microcutting $\pm$ SE ²		
water	2,4-D	Subculture		
(%)	(mg·liter ⁻¹)	1	2	3
<i>Seedling clone</i>				
0	0	0.4 $\pm$ 0.2	1.3 $\pm$ 0.2	1.8 $\pm$ 0.3
	1.0	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2	0.0 $\pm$ 0.0
10	0	2.0 $\pm$ 0.6	5.3 $\pm$ 0.6	9.8 $\pm$ 1.0
	1.0	0.4 $\pm$ 0.2	2.5 $\pm$ 1.0	3.9 $\pm$ 1.1
<i>Mature clone</i>				
0	0	0.8 $\pm$ 0.2	0.7 $\pm$ 0.2	0.8 $\pm$ 0.2
	1.0	0.4 $\pm$ 0.2	0.4 $\pm$ 0.3	0.2 $\pm$ 0.2
10	0	1.2 $\pm$ 0.2	2.5 $\pm$ 0.3	4.7 $\pm$ 0.6
	1.0	1.1 $\pm$ 0.3	1.9 $\pm$ 0.3	2.3 $\pm$ 0.6

²Ten microcuttings/treatment.

Fig. 1. Proliferating shoots of *Cephalotaxus harringtonia*. Scale divisions = millimeters.

Fig. 2. Rooting of *Cephalotaxus harringtonia* after proliferation on membrane rafts. (left) Rooted microcutting after 9 weeks in mist. (right) Rooted microcuttings after 7.5 months in the greenhouse.

rooted, 85% (Expt. 1) and 93% (Expt. 2) survived greenhouse conditions (Fig. 2).

Successful rooting of microcuttings of *C. harringtonia* after culturing in liquid medium may be due to leaching of inhibitors (Hackett, 1988). Increased leaf area was not responsible for improved rooting, unlike suggestions by Jarvis and Shaheed (1986), McCown (1988), Rappaport (1940), and Reuveni and Raviv (1980), because leaf size of membrane-derived shoots ($15.8 \pm 4.0 \times 1.4 \pm 0.1$ SD; $n=30$) was not different than that of agar-derived shoots ($16.2 \pm 2.6 \times 2.0 \pm 0.1$ SD; $n=30$).

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