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QUININE AND QUINIDINE PRODUCTION BY CINCHONA LEAF, ROOT AND UNORGANIZED CULTURES

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Key Word Index—*Cinchona ledgeriana*; *C. succirubra*; Rubiaceae; alkaloids; tissue cultures; propagation.

Abstract—Serially propagated *Cinchona ledgeriana* and *C. succirubra* (Rubiaceae) leaf, root and unorganized suspension cultures established from germinated seeds were studied for quinine and quinidine production. Leaf organ cultures were grown and subcultured in Murashige and Skoog's Revised Tobacco Medium supplemented with benzyladenine; root organ cultures were grown on the same medium supplemented with indolebutyric acid; and unorganized suspension cultures were grown on the same medium supplemented with 2,4-dichlorophenoxyacetic acid and benzyladenine. On a dry weight basis, leaf organ cultures of *C. ledgeriana* contained 0.06% quinine and 0.05% quinidine and of *C. succirubra* contained 0.04% quinine and 0.04% quinidine. No quinine and quinidine were detected in either root organ or unorganized suspension cultures.

INTRODUCTION

Quinidine is extensively used for cardiac arrhythmias, and quinine as an anti-protozoan or as a beverage bitter. Plant cell and tissue cultures are suggested as an industrial source of biochemicals [1–3], and specifically for the cinchona alkaloids [4, 5]. Root organs [6], leaf and stem organs [7–9], and embryoids [10] are examples of some plant tissue types potentially capable of producing secondary metabolites. Recently, aseptic tissue cultures were established for the purpose of propagating *C. ledgeriana* [11].

RESULTS

Plants

The alkaloid content of 1-yr-old *Cinchona ledgeriana* Moens et Trimen and *C. succirubra* Paron et Klotzsch greenhouse-grown plants is reported in Table 1. Our HPLC analysis of Ecuadorian cinchona bark for alkaloids (quinine—2.17%; quinidine—0.17%; cinchonine—1.33%; cinchonidine—1.58%) was in close agreement with that of the supplier (quinine—2.60%; quinidine—0.10%; cinchonine—1.70%; cinchonidine—2.50%. Berlex Laboratories Inc., Wayne, NJ 07470). The total alkaloids by our extraction method was 7.10% as compared to the supplier's figure of 9.20%.

Tissue cultures

Leaf organ cultures grew rapidly as compared to suspension cultures and root cultures. Leaf organ cultures appeared as green leaves with shoots, the root organ cultures as brown fine hair clusters, and the unorganized suspension cultures as greenish brown aggregates. The tissue cultures required subculturing every 4 weeks with weekly medium replacement. The 6-weeks growth index (final tissue fresh wt/inoculum fresh wt) for *C. ledgeriana* and *C. succirubra* tissue cultures is reported in Table 1.

Leaf organ cultures contained *ca* 50% of the alkaloids of 1-year-old cinchona plants and *ca* 10% of the alkaloids of cinchona bark (Table 1). Tissue cultures and cinchona bark [17] contain *ca* 90 and 10% water, respectively. Root organ cultures and unorganized suspension cultures did not contain the four major cinchona alkaloids when they were analysed by HPLC (Table 1); however, TLC analysis showed them to contain four Dragendorff positive spots near the solvent front.

Analytical. Thin layer chromatography (TLC). Two-dimensional TLC was more rapid than one-dimensional TLC (Fig. 1A). One-dimensional resolution of the cinchona alkaloids was obtained by developing the TLC plates in solvent system I (R_f values: quinine—0.40, quinidine—0.42, cinchonine—0.32, cinchonidine—0.33), air-drying, and then developing 4 times in solvent system II (R_f values: quinine—0.41, quinidine—0.56, cinchonine—0.65, cinchonidine—0.50). The alkaloid spots appeared orange when sprayed with modified Dragendorff reagent [12], and orange-pink when oversprayed with potassium iodoplatinate reagent [13]. However, cinchonidine gave a purple-blue color. Colors were intense and lasted *ca* 3 hr.

Gas chromatography (GC). Separation and identification of quinine–quinidine from cinchonine–cinchonidine was possible in the system described, but not their resolution from each other. Retention time for cinchonine and cinchonidine was 2.62 min and that for quinine and quinidine was 3.98 min.

High performance liquid chromatography (HPLC). The retention times for theophylline (int. standard), quinine, quinidine, cinchonine and cinchonidine were 14.20, 26.70, 23.30, 16.10 and 19.00 min, respectively (Fig. 1B).

DISCUSSION

Of the *ca* 40 cinchona species known, *C. ledgeriana* (yellow bark) contains the highest average quinine content (7%) with selected clones containing *ca* 15% quinine [20]. *C. succirubra* (red bark) contains 5–7% total alkaloids and 2–3% quinine. The bark of many cinchona plants contains little or no quinine [17].

Table 1. Alkaloid analysis of *Cinchona* plants and tissue cultures

Biological material	Wet wt (g)	Dry wt (g)	Total alkaloid (mg)	Alkaloids (%)*			
				Cn	Cd	Qn	Qd
Plants (1-year-old)							
C. ledgeriana							
Leaf–stem		3.4	76.00	0.18	0.35	0.09	0.03
Root		0.70	10.0	0.08	0.11	0.15	0.06
C. succirubra							
Leaf–stem		2.4	35.0	0.10	0.06	0.07	0.01
Root		0.6	11.0	0.27	0.10	0.13	0.05
Ecuadorian cinchona							
Bark (age unknown)		10.0	710.0	1.33	1.58	2.17	0.17
Tissue cultures†							
C. ledgeriana							
Leaf organ (VI passage; G.I. = 21.40)	355.3	24.7	220.0	0.09	0.25	0.06	0.05
Root organ (II passage; G.I. = 6.12)	26.0	2.8	41.0	—	—	—	—
Unorganized suspension (IV passage; G.I. = 8.29)	61.9	7.0	78.0	—	—	—	—
C. succirubra							
Leaf organ (IV passage; G.I. = 20.10)	153.1	10.3	82.0	0.08	0.22	0.04	0.04
Root organ (II passage; G.I. = 5.26)	8.0	0.9	12.0	—	—	—	—
Unorganized suspension (IV passage; G.I. = 7.04)	57.2	6.2	65.0	—	—	—	—

* Cn—Cinchonine; Cd—cinchonidine; Qn—quinine; Qd—quinidine.

† All tissue cultures analysed and for which the Growth Index (G.I.—final tissue fresh wt/inoculum fresh wt) reported were 6 weeks old.

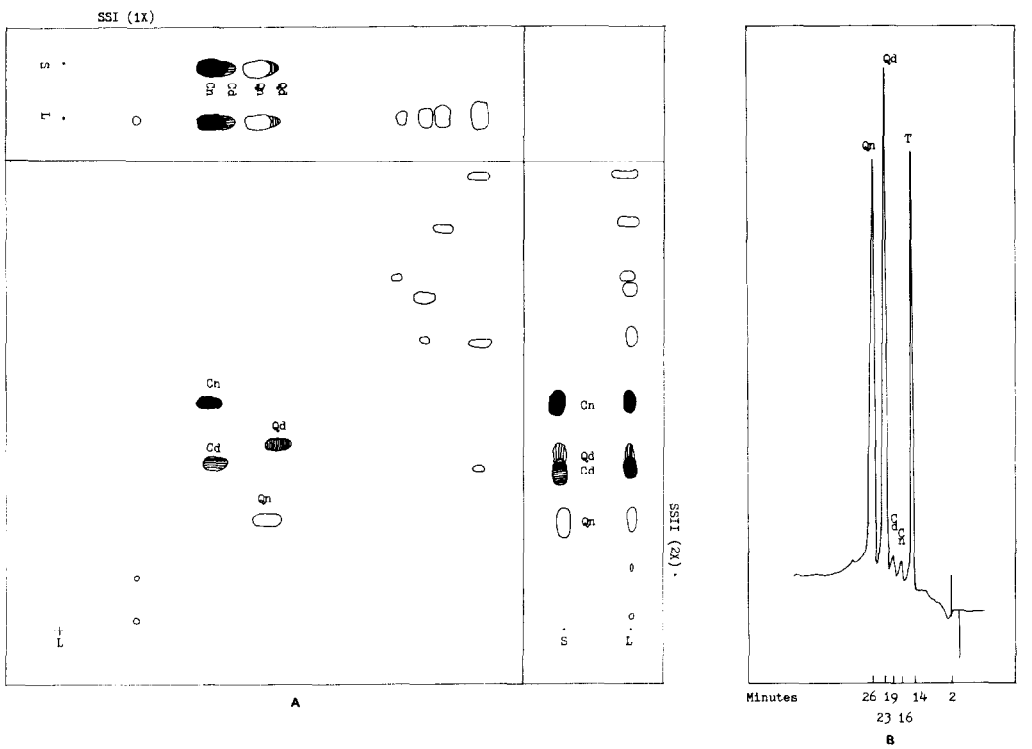

Fig. 1. TLC and HPLC of cinchona alkaloid standards and/or cinchona leaf organ extract. Qn—quinine, Qd—quinidine, Cn—cinchonine, Cd—cinchonidine, T—theophylline, S—standard mixture. (A) Two-dimensional TLC. L—*C. ledgeriana* leaf organ culture extract (6 week). SSI—$CHCl_3$–MeOH–NH_4OH, (24:6:0.05); SSII—Et_2O–$NHEt_2$ (17:1). Detection: modified Dragendorff reagents and potassium iodoplatinate reagent. (B): HPLC. Column. C_{18}-μBondapak (30 cm × 4 mm). Amount of alkaloid standards used—5 μg, theophylline—7.5 μg. Gradient solvent system: 25 min initially with 2% aq. HOAc–MeOH (19:1) to 2% aq. HOAc–MeOH (4:1) at a continuous flow rate of 1.5 ml/min. UV detection: 254 nm.

The trace amounts of alkaloids detected in our cinchona root cultures support the early observation of Moens [19] that the roots do not significantly influence alkaloid production. In his study, scions of high-alkaloid *C. ledgeriana* grafted on low-alkaloid *C. succirubra* roots maintained their high capacity to produce alkaloids. The quinine–quinidine alkaloids in the roots of our young plants may have been translocated from the shoots rather than produced *de novo* in the roots.

Although cinchona alkaloids principally accumulate in trunk–bark tissue, they are present in higher concentration in the limb-bark of *C. micrantha* Ruiz et Pavpn [17]. They are also found in *C. ledgeriana* heartwood [18] and occur in the leaf tissue of *Olea europaea* [19] and other plant species [18]. Optimal accumulation of the cinchona alkaloids in the plant occurs between 8 and 20 years [17]. It was therefore reasonable that the alkaloid content of our young leaf organ cultures (6 weeks old) was *ca* 10% of a reference cinchona bark and *ca* 50% of 1-year-old control cinchona plants.

Tissue culture techniques have been developed to propagate ornamental tropical foliage plants [21]. Semi-hard wood cinchona plant cuttings, treated with various growth regulators, have been vegetatively propagated [20]. *In vitro* culture techniques have been applied by Hunter [11] to *C. ledgeriana* apical meristem and seedlings. Browning and necrosis of tissue culture propagated seedlings were reduced and growth was enhanced by the addition of phloroglucinol to modified Murashige and Skoog's medium [11]. The mean height of shoots obtained from various tissue culture media by Hunter was *ca* 28 mm in 44 days. The height of our serially propagated *C. ledgeriana* and *C. succirubra* leaf organ cultures was *ca* 120 mm in 42 days. It is known that cinchona trees require acid soil [17]. The pH of our tissue culture media decreased to *ca* 4.0 in 7 days and it was necessary to replace them with fresh media (pH 6.0) to obtain rapid growth.

EXPERIMENTAL

Tissue cultures. C. ledgeriana and *C. succirubra* leaf, root and unorganized suspension tissue cultures were established from seeds supplied by Dr. S. K. Chatterjee, West Bengal, India. Aseptic cotyledon seedlings were transferred onto solid (1% agar) Murashige and Skoog's Revised Tobacco Medium (RT) [16] containing either benzyladenine (BA; 5 ppm), indolebutyric acetic acid (IBA; 3 ppm) or a mixture of 2,4-dichlorophenoxy-acetic acid (2,4-D; 1 ppm) and BA (5 ppm). Approximately 3 months later, the cultures were transferred into 250-ml flasks containing 50 ml of the appropriate RT liquid medium and maintained on a gyrotory shaker (80 rpm) at 25°. The concn of 2,4-D was reduced from 1.0 to 0.5 ppm in the liquid medium. Cultures that contained BA and 2,4-D were grown under a 16-hr day-light cycle (500 f.c., Fluorescent Plant-Grow Bulbs, 40 W, cool-light, Sears, Roebuck). Cultures containing IBA were grown in the dark. All liquid cultures required subculturing every 4 weeks; however, weekly medium replacement is recommended for more rapid growth.

Alkaloid extraction. Cinchona tissue cultures, plant roots, and plant aerial parts were oven dried at 40° for 24 hr and powdered in a mortar. Each gram of powdered material, including the Ecuadorian bark, was then treated with 1 ml of 50% trichloroacetic acid in MeOH for 10 min followed with 3 ml of 15 N NH_4OH (14). The air-dried material was next Soxhlet extracted with benzene for 24 hr and this cooled benzene fraction was extracted 3 × with 2 N HCl. The combined acid extracts were made basic to pH 12 with 15 N NH_4OH and extracted 4 × with $CHCl_3$. After drying (Na_2SO_4), the $CHCl_3$ was removed *in vacuo* to obtain the total alkaloids. Appropriate concns of the extracts were dissolved in glass-distilled MeOH, filtered through 0.2 μm Millipore (Bedford, MA 01730) and assayed by either TLC, GC or HPLC.

Analysis. Standards: Cinchonine, cinchonidine and quinidine sulfate dihydrate were obtained from Aldrich. Quinine hydrochloride and theophylline were obtained from Sigma. Standard solns of the free bases were prepared as 3 μg/μl in MeOH.

TLC: Semi-purified tissue culture and plant extracts and standard materials were chromatographed on Si gel GF (250 μm) Uniplates (Analtech, Newark, DE). For one-dimensional TLC, the plates were developed once at 25° using solvent system I ($CHCl_3$–MeOH–NH_4OH, 24:6:0.05) [15], air-dried, and developed 4 × with solvent system II (Et_2O–Et_2NH_2, 17:1) [15]. For two-dimensional TLC, solvent system II was used twice. After drying 3 hr at 45° to ensure removal of the diethylamine, the plates were examined under both short and long wavelength UV and sprayed sequentially with modified Dragendorff reagent [12] and potassium iodoplatinate reagent [13].

GC: GC analysis of the extracts was done isothermally at 240° on a 3% OV-17 glass column (2 m × 2 mm) with a Model 1200 chromatograph (Varian, Walnut Creek, CA).

HPLC: HPLC analysis was done with a C_{18}-μBondapak (30 cm × 4 mm) column (Waters Associates, Milford, MA 01757) and a linear gradient from 2% aq. HOAc–MeOH (19:1) to a 2% aq. HOAc–MeOH (4:1) in 25 min at a continuous flow rate of 1.5 ml/min. Also used were Model 110A pumps, Model 153 UV-254 nm detector, and Model 420 microprocessor (Beckman Instrument Inc., Fullerton, CA).

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