Plant Cell Reports (1986) 5:423–426

Plant Cell Reports

Effects of nutritional and hormonal factors on growth and production of anthraquinone glucosides in cell suspension cultures of *Cinchona succirubra*

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Received June 12, 1986 / Revised version received August 25, 1985 – Communicated by F. Constabel

ABSTRACT

Cell suspension cultures of Cinchona succirubra were found to produce anthraquinone glucosides. The effects of nutritional and hormonal factors on growth and anthraquinone production were investigated in order to study the enzyme-catalyzed glucosylation of these metabolites.

Abbreviations:

AQ: Anthraquinone
2,4-D: 2,4-dichlorophenoxyacetic acid
NAA: 1-naphthaleneacetic acid
IAA: 3-indole-acetic acid
HPLC: High Performance Liquid Chromatography
TLC: Thin Layer Chromatography

INTRODUCTION

Most species of the Rubiaceae are known to accumulate a variety of AQs when cultured in vitro, among which Morinda citrifolia (Inoue et al., 1981; Leistner, 1975; Zenk et al., 1975; 1984), Galium mullugo (Bauch and Leistner, 1978; Inuoe et al., 1984; Wilson and Marron, 1978), Rubia cordifolia (Suzuki et al., 1982; 1984; 1985) and Cinchona spp. (Mulder-Krieger et al., 1982; 1984; Wijnsma et al., 1984; 1986) are the most documented. Although AQ formation in cultured tissues seems to be regulated both by nutritional and hormonal factors (Harkes et al., 1985; Schulte et al., 1984), however, no generalization could be made regarding the optimum production of these metabolites (Ibrahim, 1986). Furthermore, it is not known whether the AQs of Cinchona cultures are synthesized in the free or bound form, since most reports indicate that AQs are present as aglycones (Mulder-Krieger et al., 1982; 1984; Verpoorte et al., 1985; Wijnsma et al., 1984; 1986).

Preliminary studies in our laboratory indicated that cell suspension cultures of C. succirubra produce AQs in the form of glucosides. This prompted us to study the nutritional and hormonal requirements for optimal growth and production of these metabolites with the aim to characterize the enzyme-catalyzed glucosylation of AQs. During the progress of this work, Wijnsma et al. (1986) reported the isolation and characterization of the AQ aglycones from callus cultures of C. pubescens (syn. C. succirubra).

MATERIALS AND METHODS

Culture conditions. A callus culture was initiated from stem segments of 6 week-old seedlings on B5 medium (Gamborg et al., 1968) supplemented with 2% sucrose (w/v), 100 ppm m-inositol, 5% coconut water (v/v), 100 ppm phloroglucinol, 1 ppm 2,4-D, 0.1 ppm kinetin and 0.7% agar. Culture growth was maintained on the same medium excluding phloroglucinol (control medium) and was subcultured at 4- weekly intervals. Suspension cultures were initiated by transferring the callus tissue to one-liter nipple flasks containing 100 ml of the control medium. The flasks were allowed to rotate centripetally around an axle connected by a universal joint to a variable-gear motor at 2 rpm. A 20% inoculum (ca. 1.5 g fr. wt.) was transferred into fresh medium at 15-day intervals. Culture growth was determined by measuring fresh and dry weights, protein content of the cells, as well as the pH and conductivity of the medium.

Extraction of AQs. Cells were harvested by filtration using suction. They were extracted twice, with hot 75% methanol (v/v) and the extracts were reduced in vacuo at 30 °C to an aqueous residue which contained the AQ glucosides and trace amounts of aglycones. The aqueous residue

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was hydrolyzed with 2N HCl for 15 min at 80 °C and the hydrolyzate was extracted with chloroform. After evaporation of the organic layer, the remaining residue was dissolved in ether and partitioned against 1M NaOH. The aqueous layer was acidified with HCl and extracted with ether. The latter extract contained the AQ aglycones.

Estimation of AQ content. The absorbance of the methanolic extracts of either AQ glucosides or their aglycones was measured using an LKB (Ultrospec 4050) spectrophotometer at 435 nm. AQ content (relative to alizarin) was calculated using a molar extinction coefficient of 5500, considering that differences in the molar extinction coefficient of different AQs did not exceed 5% (Zenk et al., 1975).

Characterization of AQs. The major AQ aglycones were characterized by co-chromatography with authentic samples (where available) on silica plates and by HPLC, as well as their color in visible and UV-light, before and after exposure to ammonia vapor. HPLC of the aglycones was performed on a Waters-Millipore solvent delivery system using a μ-Bondapack C18 column (3.9 mm x 30 cm), 436 nm filter and 1% AcOH in 70% aqueous MeOH as solvent at a flow rate of 1 ml/min. The TLC solvent systems used for the glucosides were: A, benzene-MeOH-ethyl formate-formic acid (15:5:5:1) and B, ethylacetate-MeOH-water (77:13:10); whereas those for the aglycones were: C, chloroform-MeOH-ammonia (85:14:1); D, toluene-diethylether-EtOH (70:25:5) and E, toluene-MeOH (9:1).

RESULTS AND DISCUSSION

Culture growth

C. succirubra cells in culture grow as a homogeneous suspension yellow-orange in color, the intensity of which was almost proportional to AQ content of the culture. Early exponential-phase cells appeared isodiametric (Fig 1A) which, on continued growth, became mostly elongated and tandemly joined at their narrow ends (Fig. 1B). It is interesting to note that culture growth did not respond to conventional gyrotary shaking and required a large air-to-liquid ratio, as well as slow rotation (2 rpm) of flasks. These conditions were satisfied by the use of the set-up described in the Methods section.

Figure 2 shows the changes with time in growth parameters and AQ production of cells, cultured in the control medium. The formation of AQ paralleled cell growth and reached its maximum (ca. 30 μmol/l) after approx. 11 days in culture. AQ production was almost concomitant with maximum protein content. The parallelism between cell growth and metabolite production in C. succirubra is similar to that of many cultured tissues (Tabata, 1977).

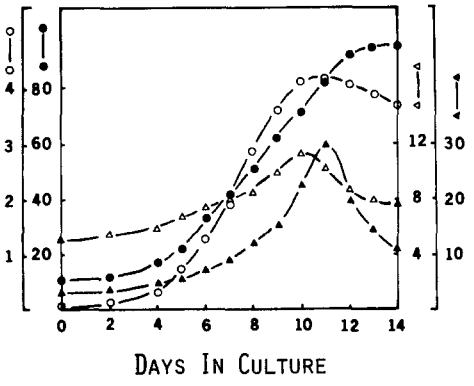

Figure 2: Changes in growth parameters and AQ production of C. succirubra suspension culture. ●—●, fresh weight (g/l); o—o, dry weight (g/l); △—△, protein content (mg/l); ▲—▲, total AQ content (μmol/l).

Characterization of AQs

Acid hydrolysis of the AQ glucosides revealed the presence of alizarin and emodin as the major constituents of C. succirubra; both of which amounted to 80% of the total AQs. The remaining 20% consisted of a number of di- to tetrahydroxy AQs. Whereas hydrolysis with β-glucosidase resulted in the release of the sugar residue which was identified as D-glucose on TLC (Hansen, 1975) suggesting their presence as β-glucosides. All of the AQ constituents of this culture were found to be bound as their corresponding β-O-D-glucosides. These results are in contrast with previous reports of Cinchona spp. (Mulder-Krieger et al., 1982; 1984; Verpoorte et al., 1985; Wijnsma et al., 1984; 1986) where AQs were reported as the aglycones. However, due to the difficulty in the separation of individual glucosides, either by TLC (Berg and Labadie, 1984) or HPLC, the latter compounds were characterized as their corresponding aglycones, by co-chromatography with reference compounds using HPLC. The fact that alizarin and emodin are biogenetically derived from shikimate and acetate, respectively does

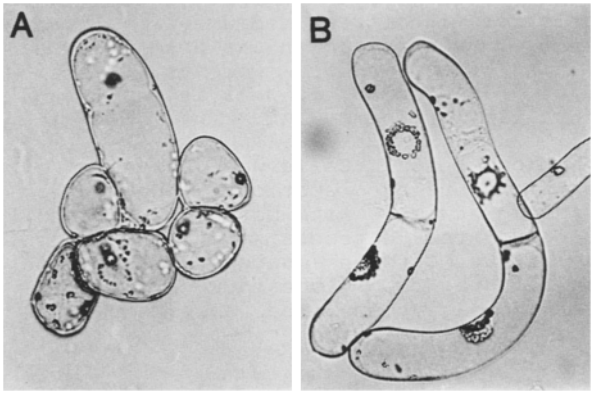

Figure 1: Early (A) and late (B) exponential phase cells of C. succirubra suspension, cultured in control medium.

not exclude the possibility of their co-occurrance in cultured tissues since AQs are considered as stress metabolites in Cinchona cultures (Wijnsma et al., 1985).

Effect of nutritional factors

1.Sucrose: Various concentrations of sucrose (2 to 10 %, w/v) were used to test their effect on growth and AQ production in the control medium. Table 1 shows that optimum cell growth was observed with 2% sucrose. Increasing sugar concentration resulted in a progressive decrease in cell yield, although it increased the formation of AQs. The latter was more pronounced when metabolite production was calculated on cell dry weight basis (200%) than per liter of medium (33%), since an increase in sugar concentration above 2% resulted in decreased cell growth (Table 1). However, when sucrose concentration was raised above 8% both parameters were severely inhibited. These results compare with those reported for C. ledgeriana where medium (2%) and high (4%) sucrose concentrations were reported to stimulate both cell growth and AQ formation (Harkes et al., 1985). Furthermore, the effect of sucrose on cell growth and AQ production in C. succirubra compares well with that of M. citrifolia (Zenk et al., 1975) and R. cordifolia (Suzuki et al., 1984).

Table 1: Effect of sucrose concentration on growth and AQ production.

Sucrose Concn.	F.W. g/l	D.W g/l	AQ µmol/l	AQ µmol/g D.W.
2%	96.7	4.8	8.6	1.8
4%	79.2	4.2	11.4	2.7
6%	68.9	3.8	11.4	3.0
8%	64.9	3.1	11.2	3.6

Cells were grown in the control medium as described in the Methods section. Values are average of 3 determinations; S.E.<10%.

2.Medium composition: The effect of the different media B5, MS, White's and Heller's (supplemented with 2% sucrose, 1 ppm 2,4-D and 0.1 ppm kinetin) was studied on growth and AQ production (Table 2). Cells grown in MS or B5 media showed similar growth rates as well as AQ content. On the other hand, those grown in White's or Heller's media exhibited a decline in cell growth but an increase in AQ production, whether the latter was determined per gm dry weight or per liter of medium.

When the macro- and microelements of the control medium (B5) were diluted 2- and 4-fold, AQ production was increased by 55% and 100%; although cell yield was decreased by 23% and 38%, respectively (Table 2). These results indicate that the use of nutritient-limited media (Heller's or White's) or dilution of the control medium resulted in stimulation of AQ production. Most studies have shown that decreasing nitrogen and/or phosphorus increased the production of most phenolic metabolites (for review, see Ibrahim, 1986) including AQs (Harkes et al., 1985; Suzuki et al., 1984; Zenk et al., 1975). This is in contrast with the nutrient-rich media which support prolific cell growth and protein synthesis. Very recently, Wijnsma et al. (1985) suggested that the formation of AQs in Cinchona cultures may play a role as stress metabolites (phytoalexins), since they are not normally produced in the intact tissues. The results reported here support this view.

Table 2: Effect of medium composition on growth and AQ production.

Medium	F.W. g/l	D.W. g/l	AQ µmol/l	AQ µmol/g D.W.
B5	95.0	4.2	8.4	2.0
1/2 B5	83.5	3.7	10.4	2.8
1/4 B5	67.5	3.0	11.2	3.7
MS	104.0	4.4	7.6	1.7
White	97.0	3.6	11.6	3.2
Heller	103.2	3.7	12.3	3.3

Values are averages of 3 determinations, S.E.<10%.

Effect of growth regulators

The effect of different concentrations of growth regulators, both individually and in combination, was tested on cell growth and AQ production in B5 medium.

In the absence of auxin, either benzyladenine or zeatin was more effective than kinetin (over a concentration range of 0.025 and 0.25 ppm) in the formation of AQs, although zeatin slightly inhibited cell growth as compared with the two other cytokinins (Table 3).

On the other hand, increasing concentrations of NAA (1.25 to 12.5 ppm) or IAA (2.5 to 25 ppm), in the absence of cytokinin, enhanced both cell growth and AQ production; whereas increasing concentrations of 2,4-D (0.25 to 2.5 ppm) inhibited both parameters (Table 3). These results show that whereas cell growth was stimulated by 2,4-D, AQ production was enhanced in the presence of IAA and, to a lesser extent, by NAA. This is in agreement with other cultured tissues where 2,4-D was shown to stimulate cell growth and NAA to enhance AQ formation (Bauch and Leistner, 1978; Harkes et al., 1985; Mulder-Krieger et al., 1982; Zenk et al., 1975). However, it is interesting to note that the addition of any of the cytokinins to the auxins used did not affect either cell growth or AQ production (Table 3). This seems to indicate that AQ production is regulated by the type and concentration of auxin rather than the cytokinin used.

No major differences in the AQ composition of the different treatments were observed, except for the NAA- or IAA-supplemented cultures which contained increased amounts of hydroxy-AQs.

Table 3: Effect of growth regulators on growth and AQ production.

Addn.	Concn. ppm	F.W. g/l	D.W. g/l	AQ µmol/l	AQ µmol/g D.W.
K	0.1	124.6	4.4	5.6	1.27
BA	0.1	115.0	4.6	9.1	1.98
Z	0.1	82.1	4.0	8.6	2.15
2,4-D	0.25	130.9	4.5	8.1	1.80
	1.0	85.0	4.3	4.9	1.14
	2.5	57.2	4.0	3.4	0.85
NAA	1.25	82.5	4.0	11.9	3.00
	5.0	88.5	4.1	12.6	3.10
	12.5	124.5	4.3	12.4	2.88
IAA	2.5	39.4	2.6	15.2	5.85
	10.0	65.3	2.9	16.2	5.59
	25.0	64.1	2.8	19.7	7.04
2,4-D, Z	1,0.1	111.4	4.4	6.7	1.52
NAA, Z	5,0.1	89.2	3.9	13.2	3.38
IAA, Z	10,0.1	75.0	3.1	17.1	5.52

Cytokinin concentration was varied between 0.025 and 0.25 ppm; data shown for 0.1 ppm (K, kinetin; BA, benzyladenine; Z, zeatin). Values are averages of 2 determinations; S.E.<10%.

Glucosylation of AQs

A cell free extract from 7-day-old cultures, grown in the control medium under conditions of optimum AQ production, utilized a number of AQ aglycones in the presence of UDPG with the formation of their corresponding glucosides, as indicated by autoradiography. However, the same protein fraction also catalyzed the glucosylation of other phenolic compounds, including flavonoids and phenylpropanoids. The rate of glucosylation of AQs was measured during a culture growth and maximum glucosyltransferase activity was found in 10-day old cultures. The partially purified (ca. 150-fold) enzyme preparation catalyzed the glucosylation of AQs at a pH optimum of 7.0 in Histidine/HCl buffer and required SH-groups, but not Mg^{+2}, for activity. Further work on the purification and characterization of the enzymes involved in glucosylation of AQs is in progress.

ACKNOWLEDGEMENTS

This work was supported in part by operating grants from NSERC and FCAR, for which we are grateful.

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