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Anthraquinones as phytoalexins in cell and tissue cultures of *Cinchona* spec.

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Abstract

The addition of autoclaved mycelia of *Aspergillus niger* and the known phytopathogenic fungus *Phytophtora cinnamomi* to cultured cells of *Cinchona ledgeriana* Moens. caused a marked increase in the anthraquinone content of the plant cells. This finding in combination with the antimicrobial activity of the anthraquinones isolated from calli of *Cinchona pubescens* Vahl. led to the conclusion that anthraquinones are phytoalexins.

Abbreviations

2,4-D : 2,4-dichlorophenoyacetic acid
TLC : thin-layer chromatography
AQ : anthraquinone
DW : dry weight

Introduction

Anthraquinones are produced in tissue cultures of quite a number of Rubiaceae species, the best studied of which are *Morinda citrifolia* L. (Zenk et al., 1975; Leistner, 1975; Inoue et al. 1981), *Galium mollugo* L. (Bauch and Leistner, 1978; Wilson and Marron, 1978; Inoue et al., 1984) and *Rubia cordifolia* L. (Suzuki et al., 1982; 1984; 1985). In 1984 Schulte et al. (1984) reported on the optimization of the anthraquinone production in suspension cultures of 19 Rubiaceae species belonging to the genera *Asperula, Galium, Rubia* and *Sherardia*. By optimization of the medium composition it proved to be possible to get a drastic increase in anthraquinone production by the suspension-cultured cells and anthraquinone concentrations in cultured cells are among the highest known today (Fowler, 1983) for any product.

During the course of our investigations on the tissue culture of *Cinchona* species for studies on the alkaloid biosynthesis therein and eventually the production of these alkaloids by means of a biotechnological exploitation of *Cinchona* tissue cultures we observed that the colour of callus and suspension cultures of both *C. ledgeriana* Moens. and *C. pubescens* Vahl. during their growth cycle turned to yellow orange and sometimes even to dark red. It could be demonstrated that this colouration was due to the production of anthraquinones in the cultures (Mulder-Krieger et al., 1982; 1984). In later studies we were able to isolate a number of anthraquinones from callus material of *C. ledgeriana* and *C. pubescens* which could be identified by the use of various spectroscopic methods (Wijnsma et al., 1984; 1985).

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Although the literature on the chemical constituents of the genus *Cinchona* is very extensive, only one report has appeared indicating that *Cinchona* plants might contain anthraquinones (Covello et al.,1970). Also in our own studies the presence of anthraquinones in healthy *Cinchona* plants could not yet be demonstrated (Wijnsma, unpublished results). Since it is known from the literature (Park, 1977) that anthraquinones exhibit antimicrobial activity towards a range of microorganisms and since the occurrence of anthraquinones in the genus *Cinchona* is only observed in tissue culture we developed the hypothesis that anthraquinones in *Cinchona* might act as stress compounds or phytoalexins. In this paper we will give evidence for this hypothesis by describing the antimicrobial activity of anthraquinone aglucones isolated from calli of *Cinchona pubescens* and the elicitation of the production of anthraquinones by cultured cells of *Cinchona ledgeriana* by the addition of heat-sterilized mycelia of the fungi *Phytophtora cinnamomi* and *Aspergillus niger*, these two criteria, i.e., the antimicrobial activity and the elicitation, defining a compound as phytoalexin (Friend and Rathmell, 1983)

Materials and Methods

Bacteria and Fungi. *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Agrobacterium tumefaciens* (wildtype 4001), *Aspergillus niger* (wildtype), *Phytophtora cinnamomi* (isolated from *C. ledgeriana*) and the yeast *Candida albicans* (ATCC 10235) were used for the tests on antimicrobial activity, mycelia of *P. cinnamomi* and *A. niger* were used for the elicitor studies.

Growth Conditions. Bacteria were grown on CASO-agar or in CASO-broth (30 g/l CASO-broth, Merck Darmstadt) except for *A. tumefaciens* which was grown on a medium containing yeast extract 1 g/l and sucrose 5 g/l. *A. niger* was grown on Sabouraud-agar or -broth (mycological peptone 10 g/l, glucose 40 g/l) and *P. cinnamomi* was grown on malt-agar or -broth (malt-extract 30 g/l) The mycelia for the elicitor treatment were harvested, resuspended in water, homogenized using an Ultra-turrax or a Potter S apparatus and autoclaved for 60 min at 120°C. *C. pubescens* callus cultures were grown as described previously (Wijnsma et al.,1985). *C. ledgeriana* cultures were grown as suspension cultures in B_5 medium supplemented with 2,4-D 1.0 mg/l and kinetin 0.2 mg/l and were routinely subcultured by adding 350 ml of a 7-day-old culture to 1000 ml of fresh medium at weekly intervals. They were kept on gyrotary sha-

kers (140 rpm) at 28°C in the light. For the inoculation of the cultures used for the experiments 10.0 ml of a 7-day-old stock culture was added to 50 ml of medium. To these cultures 1.0 ml of an autoclaved mycelium suspension was added on the day of inoculation containing 70 mg (*P. cinnamomi*) or 32 mg (*A. niger*) mycelium. The experiments were performed at 28°C in the light (*P. cinnamomi*) or in the dark (*A. niger*).
Testing of the Antimicrobial Activity. Mixtures of anthraquinones obtained in the separation of the anthraquinone extract from calli of *C. pubescens* (Wijnsma et al., 1985) were used for the testing. 30 µl of a solution of the anthraquinones was pipetted in a hole (10 mm diam.) punched in the agar (agar-diffusion method). Streptomycin (bacteria), griseofulvin (fungi) and nystatin (yeast) were used as positive controls. Inhibition zones were determined after the appropriate growth period (1 day for the bacteria, 3 - 7 days for the fungi and the yeast).
Analysis of the Cell Cultures. For the determination of the dry weight of the cultures they were filtered on a nylon filter, cells were washed with water and freeze-dried. The freeze-dried cell material was subsequently analyzed for anthraquinone content using a modification of the method described by Zenk et al. (1975) and Schulte et al. (1984). This method was chosen by us because a method for the analytical separation and quantification of the individual anthraquinones is still not available in our laboratory. The cell material was extracted with hot methanol using an Ultra-turrax or a Potter S apparatus. The methanolic solution was then filtered, the cell material on the filter was washed with methanol and the filtrate was diluted to 100.0 ml with methanol. Of these solutions after the addition of 1 drop of 1 M NaOH in the cuvette VIS spectra were recorded from 700 - 400 nm. The maximum at ca. 500 nm was used for the quantitation of the anthraquinone concentrations using a calibration curve obtained with alizarin made under the same conditions (λmax ca. 510 nm). The concentrations of the anthraquinone mixtures used in the antimicrobial activity assay were measured in the same way. Also anthraquinone concentrations in the media were determined in this way, but the media were centrifuged before the spectra were run to remove some cell debris that had passed the filter.

Results and Discussion

Antimicrobial Activity. For the testing of the antimicrobial activity of anthraquinones isolated from callus cultures of *Cinchona pubescens*, fractions containing some 3 - 4 anthraquinones, obtained in the separation by means of preparative thin-layer chromatography of the extract, were used because the amount of pure compounds left after the separation and the recording of the various spectra was too small to be used in the assay. In table 1 the main components of

Table 1. Components of the fractions used in the antimicrobial activity assay.

Fraction	number of AQ's	Identified components
2	4	2,5-(or 3,5-)dihydroxy-1,3,4-(or -1,2,4-)trimethoxyAQ
3	4	2-hydroxy-1,3,4-trimethoxyAQ
4	4	anthragallol-1,2-dimethylether 2-hydroxy-1,3,4,6-tetramethoxyAQ
5	3	none identified
6	4	6,7-dihydroxy-1-methoxy-2-methylAQ
7	3	1,6-(or 1,7-)dihydroxy-2-methylAQ purpurin
8	3	1-hydroxy-2-hydroxymethylAQ
9	4	alizarin-2-methylether 1-methoxy-2,4,5-trihydroxyAQ
10	4	purpurin-1-methylether 1,6-(or 1,7-)dihydroxy-3,7-(or -3,6-dimethoxyAQ

the fractions used in the assay are recorded. They constitute from 60 - 80 % of the total fraction. The testorganisms used in this assay are the ones used in many other laboratories except for *A. tumefaciens* and *P. cinnamomi* which were used in our study because of special interest. The former because of its use in the genetic transformation of plant cells and the latter because it is a known phytopathogen to *Cinchona* trees. From table 2 it is clear that all of the fractions show a high antimicrobial activity towards most of the bacteria, also in most cases the growth of *C. albicans* is inhibited. For the fungi no inhibition of growth was observed in the test. This might have two reasons, the first one being the long "critical time" observed with the growth of fungi and the second one that both fungi are phytopathogens and might have developed ways of overcoming the toxic effects of phytoalexins. The basis of this resistance can be different in nature, it can either be detoxification (Matthews and Van Etten, 1983) or tolerance to high concentrations of phytoalexins (Denny and Van Etten, 1983a,b)

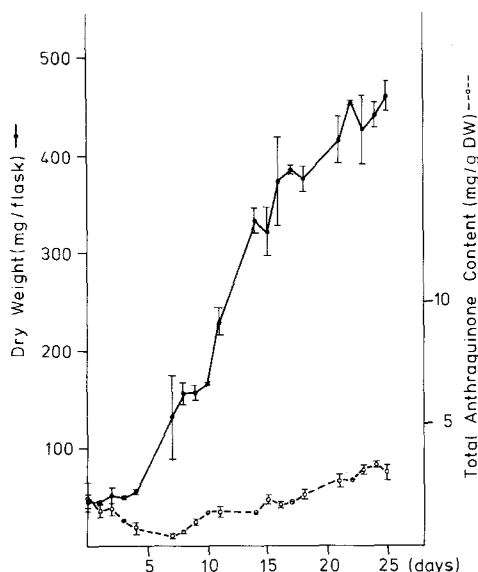
Elicitation of Anthraquinone Production. Addition of 70 mg of autoclaved mycelium of *Phytophtora cinnamomi* to suspension cultures of *Cinchona ledgeriana* showed only little effect on the growth (measured by dry weight) of the cultures (fig's 1a and 1b). The main difference between the two growth curves is the decrease in dry weight (ca. 15 %) after 3 weeks of culture in the presence of the sterilized mycelium. This is propably caused by lysis of cells due to the high concentrations of anthraquinones they contain as the anthraquinone content of the medium rises in this period. About 25 % of the anthraquinone content is found in the medium compared to ca. 5 % for the control culture during the stationary phase of growth.
The effect of addition of the autoclaved mycelia on

Table 2. Results of the antimicrobial activity assay.

Fraction	Concentration (µg/ml)	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*E. coli*	*A. tumefaciens*	*C. albicans*	*A. niger*	*P. cinnamomi*
2	7	+++[a]	++	++	++++	++	+	-	-
3	16	+++	++	+	++++	++	+	-	-
4	28	++	++	+	++++	++	+	-	-
5	43	+++	+++	++	+++	+	+	-	-
6	16	++	+++	+	+++	+	+	-	-
7	50	+++	+++	++	++++	++	++	-	-
8	10	++	+++	-	++++	+	++	-	-
9	7	++	-	+	++++	+	++	-	-
10	12	+++	+++	+	+++	++	-	-	-

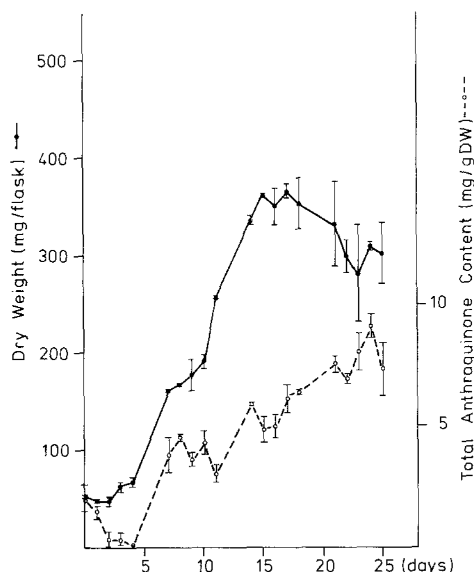
a. -: no inhibition zone
+: inhibition zone of ≤ 12 mm (including the hole)
++: inhibition zone of 13 - 16 mm
+++: inhibition zone of 17 - 22 mm
++++: inhibition zone of 23 mm and more

Figure 1a



Growth and anthraquinone production in the control cell suspension culture of *Cinchona ledgeriana* (CiR1,1 L, 24 h light, 28° C, values ± S.E., n=2)

Figure 1b



Growth and anthraquinone production in the mycelium-treated (*Phytophtora cinnamomi*) cell suspension culture of *Cinchona ledgeriana* (CiR1,1 L, 24 h light, 28° C, values ± S.E., n=2)

the anthraquinone content of the cultures is however substantial (fig's 1a and 1b). After a lag phase of ca. 5 days the anthraquinone content of the mycelium-treated culture shows a sharp rise to 0.5 %. At this time after about 7 days of culture the difference in anthraquinone content between the control culture and the mycelium-treated culture was 900 % which at the end of the experiment decreased to ca. 200 %. The increase in anthraquinone content caused by the addition of sterilized mycelia of a maximum of 900 % when compared to the control culture is not as high as reported for the increase of acridone alkaloid epoxides in *Ruta graveolens* cultures (Eilert et al., 1984) which was about 100-fold and took a shorter period of time (72 h) when compared to the 6 - 7 day period in this experiment. The increase observed in the experiment described here is however much higher than the increase in diosgenin production by *Dioscorea deltoidea* suspension cultures after the addition of autoclaved mycelia of a number of fungi (Rokem et al., 1984). From fig's 2a and 2b it can be seen that addition of 32 mg of autoclaved mycelium of *Aspergillus niger* had a marked effect on the growth of *Cinchona ledgeriana* cells. The maximum dry weight of the mycelium-treated culture is only 40 % of that of the control culture. The anthraquinone content of the mycelium-treated culture on the other hand was raised from a maximum of 0.41 % for the control culture to 1.49 % for the mycelium treated culture.
From these experiments and from the data presented in the literature (Eilert et al., 1984; Rokem et al., 1984) it is obvious that the physiological state of the cells plays an important role in their response to elicitors, especially the rate of their reaction to the elicitors is dependent on this. A better timing of the addition of the elicitor might also in the case of *Cinchona* suspension cultures cause a very rapid (48 - 72 h) increase in anthraquinone content of the cultures.
With the data described in this paper it is demonstrated that anthraquinones are phytoalexins. Future research in our group will be directed towards a better timing of the phytoalexin response in the suspension cultures and towards the demonstration of the accumulation of anthraquinones in *Cinchona* plants after infection.

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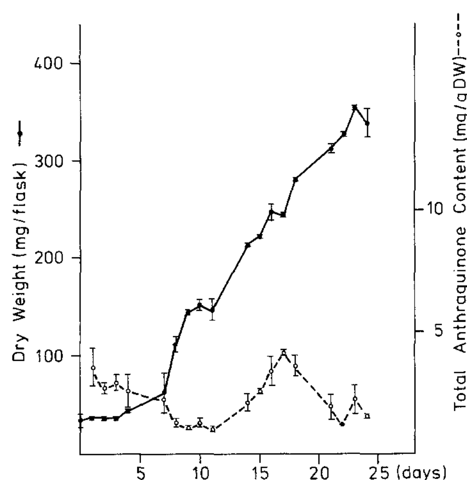
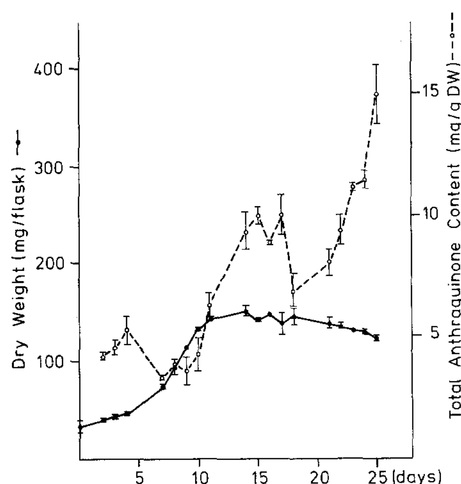
Figure 2a



Growth and anthraquinone production in the control cell suspension culture of *Cinchona ledgeriana* (CiR 1,1 L, 24 h dark, 28° C, values ± S.E., n=2)

Figure 2b



Growth and anthraquinone production in the mycelium-treated (*Aspergillus niger*) cell suspension culture of *Cinchona ledgeriana* (CiR1,1 L, 24 h dark, 28° C, values ± S.E. n=2)

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