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Effect of auxin on cytodifferentiation and production of quinoline alkaloids in compact globular structures of *Cinchona ledgeriana*

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Abstract

Fine cell suspension cultures of Cinchona ledgeriana produce only very low amounts of quinoline alkaloids. These cultures formed self-propagating compact globular structures (CGS) on medium containing 2,4-D and BAP. These CGS could be induced to produce significant amounts of quinoline alkaloids by replacing 2,4-D by low amounts of 1-NAA, which was accompanied by histological changes of the CGS. A few high producing CGS clones could be selected. The stability of this trait was studied over a period of about one year of culture in maintenance medium.

Abbreviations: BAP = Benzylaminopurine; 2,4-D = 2,4-Dichlorophenoxyacetic acid; 1-NAA = 1-Naphthylacetic acid; CGS = compact globular structures

Introduction

In cultures of Cinchona ledgeriana significant production of the four major quinoline alkaloids quinine, quinidine, cinchonine and cinchonidine was found in some morphologically organized cultures. Staba and Chung (1981) have reported the production of quinoline alkaloids (0.45% of dry weight) in leaf organ cultures of Cinchona ledgeriana, whereas root organ cultures failed to produce any quinoline alkaloids. Anderson et al (1982) established leaf organ and root organ cultures of Cinchona ledgeriana and reported quinoline alkaloid production in amounts up to 0.03% of dry weight. In callus cultures of Cinchona ledgeriana Mulder-Krieger et al. (1982a,b) found a total alkaloid content up to 0.006% of dry weight. Harkes et al. (1985) found only small amounts of quinoline alkaloids, up to 0.013% of dry weight, when cells of a fine suspension culture of Cinchona ledgeriana were plated on different solid media for the optimization of growth and alkaloid production. In fine suspension cultures of unorganized cells of Cinchona ledgeriana, Anderson et al. (1982) reported alkaloid contents of 0.04% of dry weight. Robins et al. (1986) and Rhodes et al. (1986) reported stable quinoline alkaloid production of up to 0.0143% of fresh weight in suspension cultures of smooth, green beads of Cinchona ledgeriana. In this report we describe the initiation of self-propagating green compact globular structures (CGS) in a fine cell suspension culture from Cinchona ledgeriana and the effect of auxin on growth, structural organisation and alkaloid production of the CGS. Moreover, we describe experiments aimed at elucidating possible stable differences with respect to the production of quinoline alkaloids among various clones each derived from a single CGS.

Materials and Methods

Initiation and maintenance of cultures of compact globular structures.

Cell suspension cultures either derived from callus of roots, cotyledons or hypocotyls of seedlings of Cinchona ledgeriana were maintained in the basal medium of Gamborg et al (1968) containing 2,4-D (1mg.l^{-1}), Kinetin (0.2 mg.l^{-1}) and sucrose (20g.l^{-1}). The cells were grown in 250 ml erlenmeyer flasks each containing 40 ml of medium under continous illumination (1000 lux) at 25°C on gyrotary shakers (120-150 rpm). CGS were induced by replacing kinetin by benzylaminopurine (0.2 mg.l^{-1}). The cells in CGS induction medium were subcultured every

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seven days. In 8 - 10 weeks compact green globules (3-6 mm in diameter) were formed. These CGS were collected and maintained in CGS induction medium, under the same conditions as described for the cell suspension cultures.
They were routinely subcultured every 14 days by inoculating 2.0 g of biomass in each flask containing 40 ml of fresh medium. The medium was refreshed seven days after each transfer. Biomass accumulation took place by self-propagation of the CGS, i.e. secondary globular structures were formed by spontanous separation of lobes from the mother globules.

Clonal propagation of compact globular structures.

A cell line initiated from callus of hypocotyl tissue of several seedlings was used to make a new stock culture of CGS. Five months after initiation of this stock line , which was of course non-isogenic, 12 globules were taken as starting material for 12 different clones. The CGS chosen were all green and smooth and varied between 4 and 6 mm in diameter. They were grown in maintenance medium as described in the preceeding section. After four months of biomass accumulation samples of the different clones were used for alkaloid-induction experiments.

Histological studies

CGS were fixed in FAPA (ethanol 40%,formaldehyde 4%, propionic acid 3%, acetic acid, 3% in water v/v) for 18-24 hours, and embedded in Pegoperse 100 S (Glyco chemicals, New York). Sections (7-10 µm) were stained with safranine O, and counterstained with Astra Blue FM 0.5% in 2% tartaric acid (Chroma, Stuttgart) and mounted in Eukitt (Kindler, Freiburg).

Extraction and analysis of quinoline alkaloids.

Extractions of CGS cultures were performed according to Wijnsma et al. (1987) . The final quinoline extracts were analyzed on a HPLC system as described in Smith(1984). The alkaloids were detected by UV monitoring at 254 nm and by their autofluorescence (ex = 365 nm; em = 455 nm). Peak areas were calculated with the aid of an integrator (Shimadzu RF 530) and corrected for the recovery factor (recovery of the added internal standard dihydroquinine). A qualitative analysis was carried out by TLC using 0.25 mm thick silicagel F254 plates (Merck). Detection of alkaloids was performed by observing the autofluorescence after spraying with 5N H_2SO_4 and quenching at 254 nm.

Results

Structural organization of the CGS

CGS cultures could be derived from either roots, cotyledons or hypocotyls of seedlings of Cinchona ledgeriana. In maintenance culture with a high auxin/cytokinin ratio (1.0 mg.l^{-1} 2,4-D, 0.2 mg.l^{-1} BAP) continuous growth of the cultures resulted from the mitotic activity of a peripheral layer of dense cytoplasmic cells. The central part of the globules consisted of parenchymatic tissue and concentric zones of small groups of tracheary elements. The extent of lignification of these groups of tracheary elements increased towards the periphery of the globules, as could be judged from their staining properties. As the globules grew older, the meristematic zone was split into separate parts by intervening vacuolated cells. From these parts lobes were formed which finally spontaneously separated from the mother globule.
No quinoline alkaloids could be detected in CGS subcultured in maintenance medium. This observation prompted us to study in some detail the effect of the hormonal composition of the medium on the alkaloid production by the CGS.

Effect of auxin on alkaloid production by CGS.

In order to investigate the effect of the concentration of 2,4-D on growth and alkaloid production, CGS were subcultured in maintenance medium with 0.0; 0.1; 0.2 and 1.0 mg.l^{-1} 2,4-D. For each concentration of 2,4-D 4 culture flasks were inoculated. After 28 days, two flasks of each concentration were taken and samples from each flask were fixed and processed for light microscopy. The rest of the material was extracted and analyzed for quinoline alkaloid content. At 42 days the rest of the culture was harvested and processed the same way as the CGS harvested at 28 days. Fresh weight was determined every time the medium was refreshed by actually weighing the CGS under aseptic conditions. Since the CGS grow by a kind of peripheral meristem, we used the equation of Singer (1985a,b) for the relative growth rate R:

$$R = \frac{3(W2^{1/3} - W1^{1/3})}{t_2 - t_1}$$

in which W_1 = fresh weight at t_1, W_2 = fresh weight at t_2 and $t_2 - t_1$ = time span of growth period. An experiment of similar design was performed in which CGS were subcultured in maintenance medium with 0.0; 0.1; 0.2; 1.0 mg.l^{-1} of 1-NAA instead of 2,4-D, and with neither cytokinin nor auxin. Before the CGS were transferred from the stock culture to these media, however, they were first grown for 14 days in maintenance medium plus 1 mg.l^{-1} 1-NAA in order to wash out and to replace the 2,4-D present in residual medium from the stock line and in the tissues.
The effects of the above described treatments on the relative growth rates of the CGS cultures and on alkaloid production by these cultures have been summarized in table 1, 2 and 3 respectively. As can be inferred from table 1, there was no clear correlation between 2,4-D concentration in the medium and relative growth rates of the CGS cultures. The same holds true when 2,4-D was replaced by 1-NAA. In medium with 1-NAA, however, the relative growth rates were substantially lower than in the medium with 2,4-D. This can be explained by assuming that the CGS transferred from maintenance medium containing 1 mg.l^{-1} 2,4-D to medium with lower (including zero) concentrations of 2,4-D, still contained sufficient levels of residual 2,4-D as to maintain near maximum growth, whereas pretreatment of the CGS with 1-NAA substantially lowered the level of residual 2,4-D. Obviously, 1-NAA was not effective in maintaining growth of the CGS.
From tables 2 and 3 we may infer that removal of residual 2,4-D by pretreatment of the CGS with 1 mg.l^{-1} 1-NAA and subsequent incubations of the CGS in medium lacking 1-NAA (or with low concentrations of auxin) favours alkaloid production. Ommission of the cytokinin, however, resulted in a substantial decrease of the alkaloid content.In the presence of 0.2 mg.l^{-1} BAP alkaloid

production was progressively suppressed by increasing 1-NAA concentrations. These results confirm the data from some previous experiments performed in our laboratory, in that replacement of 2,4-D by a low concentration of 1-NAA (0.1 $mg.l^{-1}$) decreased the growth of the CGS and at the same time induced alkaloid production. A detailed histological study of these CGS revealed that cells in the meristematic zone had differentiated into non-dividing chloroplast containing parenchymatous cells, i.e. the meristematic zone had lost its activity. In addition, structures which are reminescent of vascular tissue had developed in a peripheral zone adjacent to the original meristematic layer, i.e. they contained tracheary and sieve elements with some cambium-like tissue in between. The peripheral part of these CGS, containing the vascular-bundle like structures and the parenchymatous cells derived from the original meristematic zone could be peeled off.

Table 1 : RELATIVE GROWTH RATES

AUXIN/BAP	$R_{2,4\text{-}D}$	R_{NAA}
0.0/0.0[a]	--	0.16
0.0/0.2	0.35	0.13
0.1/0.2	0.36	0.15
0.2/0.2	0.18	0.14
1.0/0.2	0.52	0.04[b]

R = growth rate, mean of two values, time span = three weeks. [a] :hormone concentration in $mg.l^{-1}$; [b] : single value. -- = not done

Separate alkaloid determinations revealed that from the total quinoline alkaloid content, 90% was present in the peripheral part and 10 % in the rest, representing 38 % of the total dry weight.

Table 2:RESULTS OF 2,4-D EXPERIMENT ([BAP] = 0.2 $mg.l^{-1}$) production of quinoline alkaloids

a	b								c	
2,4-D	Cd_1	Cd_2	C_1	C_2	Q_1	Q2	Qd_1	Qd_2	T_{28}	T_{42}
0.0	--	8	--	27	16	4	--	3	16	42
0.1	--	--	24	17	7	5	6	7	37	29
0.2	--	--	--	14	--	3	--	3	--	20
1.0	--	--	--	--	--	--	--	--	--	--

a :Concentration of 2,4-D in $mg.l^{-1}$; b :Alkaloid content in $\mu g.g^{-1}$ dryweight at t_{28} days (1) and at t_{42} days (2);c :T_{28} and T_{42} = Total quinoline alkaloid content at t_{28} and t_{42} days respectively Cd = Cinchonidine; C = Cinchonine; Qd = Quinidine; Q = quinine. -- = not detectable

In the present experiments a rough screening of sections from CGS revealed that, in all cases in which the growth of CGS cultures was decreased, the mitotic activity of the peripheral meristematic zone had stopped, whereas differentiation of vascular bundle like structures was most conspicious in those CGS which were grown in the presence of 0.2 $mg.l^{-1}$ BAP and low or zero concentration of 1-NAA, and which had a relatively high content of quinoline alkaloids.

Table 3:RESULTS OF NAA EXPERIMENT Production of quinoline alkaloids

a	b								c	
N/B	Cd_1	Cd_2	C_1	C_2	Q_1	Q_2	Qd_1	Qd_2	T_{28}	T_{42}
0.0/0.0	--	--	56	1	4	5	15	2	75	8
0.0/0.2	--	--	63	132	9	34	26	47	98	218
0.1/0.2	7	--	64	110	--	30	14	24	85	164
0.2/0.2	--	--	40	59	--	22	10	13	50	94
1.0/0.2	--	--	28	22	2	15	9	4	39	41

a :Concentration of 1-NAA/BAP in $mg.l^{-1}$; for b and c see Table 2

Clonal propagation of CGS

After four months of subculturing, six well growing CGS clones selected from the twelve we started with, were transferred to alkaloid-induction medium (maintenance medium with 0.2 $mg.l^{-1}$ BAP and 0.1 $mg.l^{-1}$ 1-NAA) after pretreatment with 1 $mg.l^{-1}$ 1-NAA. After three weeks on this medium the CGS were analyzed for quinoline alkaloid content. Four months after this first screening this experiment was repeated in order to investigate the stability of the CGS clones with respect to production of quinoline alkaloids. The results from the first and second screening are summarized in table 4.

Table 4 : QUINOLINE ALKALOIDS IN CLONES AFTER INCUBATION WITH 0.1 $mg.l^{-1}$ 1-NAA AND0.2 $mg.l^{-1}$ BAP.

Clone nr.	Cd[a] b/c	C b/c	Q b/c	Qd b/c	Total b/c
2	--/--	--/--	--/--	--/--	--/--
6	--/nd	--/nd	Tr[d]/nd	Tr/nd	--/nd
8	197/52	185/52	71/23	56/15	509/142
9	258/29	468/46	31/10	169/38	926/123
10	--/--	--/--	Tr/--	Tr/--	--/--
12	--/nd	--/nd	--/nd	--/nd	--/nd

a = alkaloid content in $\mu g.g^{-1}$ dry weight. Cd = cinchonidine; C = cinchonine; Q = quinine; Qd = quinidine. b = first screening/c = second screening; [d] = Traces found by fluores cence detection; nd = not determined

Again four months later the two productive clones (clones 8 and 9) were screened for their ability to produce alkaloids and this time only trace amounts of quinoline alkaloids could be detected (< 10 $\mu g.g^{-1}$ dry weight).

We may conclude from these data that it is possible to select high-producing CGS clones, but that this trait was unstable, i.e. when these clones were kept on maintenance medium for about one year, they almost had lost the ability to produce quinoline alkaloids on induction medium.
Interestingly, when we calculated the ratios of the 6stereoisomeric pairs of quinoline alkaloids
(C + Qd / Cd + Q = 2S,3R/2R,3S), we found that the high-producing clone 8 had a 2S,3R/2R,3S ratio of 0.9 whereas the high-producing clone 9 had a ratio of 2.2 in both the first and the second screening. Hence, it appears that the ratios between the stereoisomeric pairs is a constant trait of the individual clones. This is in contrast with the methoxylation rate (quinine + quinidine / cinchonine + cinchonidine) which was 0.33 and 0.37 in clone 8 and 0.28 and 0.64 for clone 9.

Discussion

In general cell cultures from Cinchona ledgeriana are difficult to initiate and to maintain (Scragg et al. 1988). Nevertheless, we were able to establish cell lines from Cinchona ledgeriana which, however, failed to produce significant amounts of quinoline alkaloids. If we manipulated these cultures by replacing kinetin for BAP, self-propagating organized structures were formed, which we designated as compact globular structures. The question whether or not these structures are similar to the green-brown aggregates described by Robins et al. (1986) cannot as yet be answered, since no anatomical details are presented by these authors.
Cultures of CGS could be maintained in a B5 medium containing 1 $mg.l^{-1}$ 2,4-D and 0.2 $mg.l^{-1}$ BAP. In this maintenance medium the CGS did produce only trace amounts of quinoline alkaloids. Removal of residual 2,4-D by pretreatment of the CGS with 1 $mg.l^{-1}$ 1-NAA and subsequent incubation of the CGS in medium lacking auxin induced production of quinoline alkaloids up to 926 $\mu g.g^{-1}$ dry weight, whereas production was progressively suppressed at increasing 1-NAA concentrations.
Relatively high production of quinoline alkaloids on induction medium was correlated with a substantial decrease in growth of the CGS cultures due to loss of mitotic activity in the peripheral meristematic zone of the CGS and concurrent formation of vascular bundle like structures in a peripheral zone adjacent to the original meristematic layer. Although we found that the highest quinoline alkaloid level was present in the peripheral most differentiated part of the CGS, the question whether or not the vascular bundle like structures and/or the parenchymous tissue derived from the original meristem are the main centres of alkaloid production remains open for further investigations. High productivity and the observed tissue differentiation may be concurrent but unrelated phenomena. It was possible to select high producing CGS clones. This trait, however, turned out to be unstable and was lost when the selected clones were kept on maintenance medium for about one year. It is still possible that more extensive selection experiments could yield stable high producing CGS clones, but at present we doubt whether induction, selection and maintenance of the CGS cultures from Cinchona ledgeriana is a good alternative for establishing a stable high producing in vitro culture system. On the other hand, at present it seems that formation of CGS from cell suspension cultures of Cinchona ledgeriana and subsequent induction of quinoline alkaloids is the only way to obtain a producing in vitro culture. Therefore, we focus now on the question whether the cell suspension cultures of Cinchona ledgeriana retain their ability to form CGS over long times, and whether newly initiated CGS have always a relatively high capacity to produce alkaloids independent of the age of the stock culture in which they were induced. In this respect, it is important to examine in more detail at which developmental stage of newly initiated CGS maximal production can be induced. Moreover, if the system works in principle, cell-suspension lines could be selected in which still higher producing CGS populations can be induced.

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