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

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Received February 1, 1989; Revised version received September 15, 1989 - Communicated by W. Barz

**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), Kinetin (0.2 mg/l) and sucrose (20g/l). The cells were grown in 250 ml erlenmeyer flasks each containing 40 ml of medium under continuous illumination (1000 lux) at 25°C on gyrotary shakers (120-150 rpm). CGS were induced by replacing kinetin by benzylaminopurine (0.2 mg/l). The cells in CGS induction medium were subcultured every
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
spontaneous 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 preceding section.
After four months of biomass accumulation samples of the
different clones were used for alkaloid-induction experiments.

Histological studies

CGS were fixed in FAA (ethanol 40%,formaldehyde 4%,
propionic acid 3%, acetic acid, 3% in water v/v) for 18-24 hours,
and embedded in Pogopese 100 S (Giego chemicals, New
York). Sections (7-10 mm) 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
Wijasma 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 10254
plates (Merck). Detection of alkaloids was performed by
observing the autofluorescence after spraying with 5N H2SO4
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 2,4-D, 0.2 mg/l 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 2,4-D. For
each concentration of 2,4-D 4 culture flasks were
inoculated. After 28 days, two flasks of each
collection 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:

\[ R = \frac{3(W_2^{1/3} - W_1^{1/3})}{t_2 - t_1} \]

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-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-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 2,4-D to
medium with lower (including zero)
concentrations of auxin) favours alkaloid
production. Omission of the cytokinin, however,
resulted in a substantial decrease of the alkaloid
content. In the presence of 0.2 mg/l BAP alkaloid