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HIGH BERBERINE-PRODUCING CULTURES OF *COPTIS JAPONICA* CELLS

FUMIHIKO SATO and YASUYUKI YAMADA

Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

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Key Word Index—*Coptis japonica*; Ranunculaceae; biosynthesis; cultured cells; regulation; selection; small cell aggregate-cloning; alkaloid; berberine.

Abstract—The highest berberine content of unselected *Coptis* cells cultured under the best conditions for berberine production (darkness, high aeration, 3% sucrose and White's basal medium) was about 5% on a dry wt basis. Fluoromicroscopy showed that cultured *Coptis* cells had heterogeneous characters; therefore, selection was used to establish a high berberine-producing culture of *Coptis* cells. When small cell aggregates were cloned, high berberine-producing cell lines were produced. Repeated cloning, however, was needed to obtain stable cell lines that produced large amounts of berberine. The highest berberine production in a selected cell line was 13.2% on a dry wt basis (1.39 g/l. culture). The average production was 8.2% (0.90 g/l. culture).

INTRODUCTION

Berberine is a useful antibacterial agent and stomachic and its anti-inflammatory effect has been reported recently [1]. It takes 5–6 years to produce *Coptis* roots, the raw material for berberine production. In contrast, cultured *Coptis* cells have been shown to produce moderate amounts of berberine (2–4% on a dry wt basis) in a short period (3 weeks) [2]. However for industrial use, this yield is too low.

The general concept of the totipotency of plant cells prompted us to investigate the regulation of metabolism in useful-compound production by cultured cells. For example, light inhibits growth and berberine production in cultured *Coptis* cells, whereas high aeration stimulates both [2]. Cultured plant cells, however, show heterogeneity and special characters because the cell culture itself generates genetic variations [3]. Cultured plant cell strains that produce large amounts of useful metabolites have been established by the selection of cells [4–7]. We have investigated both metabolic regulation and the selection of cell lines for the production of large amounts of berberine in cultured *Coptis* cells.

RESULTS

Effect of the composition of the medium on growth and berberine production

The effects of media commonly used for cell growth and for berberine production were investigated (Table 1). Linsmaier-Skoog [8], B5 [10], Schenk-Hildebrandt [13] and Nitsch [14] basal media stimulated growth. Growth in White [11], Heller [12] and Eriksson [15] basal media was poor, but the berberine content of the *Coptis* cells was highest in White medium. When we compared berberine production of *Coptis* cells cultured in these basal media to the total amount of berberine in a culture, B5 and White basal media were the best. Accordingly, our studies of the metabolic regulation of berberine production used White basal medium.

Effect of carbon sources on growth and berberine production

Of the carbon sources used, sucrose was the most effective for cell growth and berberine production (Table 2). Both fructose and glucose promoted growth, but berberine production was low. When we varied the sucrose concentration in the medium, *Coptis* cells grew well at all the concentrations (1%, 3%, 6%, 10%) used. Increases in the dry wt of *Coptis* cells cultured in media with different concentrations of sucrose ranged from 390% to 450%. Except for the 10% sucrose medium, the sucrose concentration in the medium had little effect on berberine production. Production, based on the dry wt of the cells, ranged from 3.8% to 5.2% (0.23–0.30 g/l. of medium); in the 10%-sucrose medium it was 3.5% on a dry wt basis (0.20 g/l. of medium). The optimal concentration of sucrose for both cell growth and berberine production was 3%.

Cloning with small cell aggregates

It is essential to use homogeneous cells in investigations of metabolic regulation. Many researchers have based their investigations on the belief that cultured plant cells are homogeneous [16, 17], but fluoromicroscopy has shown that *Coptis* cells produce varied amounts of berberine. In addition to the variations in berberine production induced by the physiological condition of the cells, we considered that there are also differences caused by genetic variations in the cells.

The probability that cultured cells are heterogeneous encouraged us to try to select high berberine-producing cell lines by cloning small cell aggregates. We investigated the effect of tyrosine, a precursor of berberine, as a selective agent for high berberine-producing cell lines, too. Tyrosine was added only to the medium used for cloning.

Small, fine cell aggregates of *Coptis* that we plated in agar media grew slowly, colonies, 1–2 mm in diameter, being detected after 1–2 months of culture. Some colonies (*ca* 6%) formed roots although no root formation took

Table 1. Effect of the basal medium on growth and berberine production in cultured *Coptis* cells

Medium*	Growth (g dry wt/ l. culture)	Berberine production (mg/l. culture)	
		Cell	Medium
L.S. [8]	6.09	109	53
M.S. [9]	5.28	125	45
B5 [10]	6.60	200	73
White [11]	4.77	208	64
Heller [12]	4.93	144	60
S.H. [13]	7.24	157	60
Nitsch [14]	5.89	129	67
Eriksson [15]	4.81	113	59

*L.S.: Linsmaier–Skoog, M.S.: Murashige–Skoog, S.H.: Schenk–Hildebrandt.

Each flask was inoculated with 1.36 g (dry wt) of *Coptis* cells/l. of medium then cultured for 3 weeks in the dark.

Table 2. Effect of carbon sources on the growth and berberine production of cultured *Coptis* cells

Sugar	Growth (g dry wt/ l. culture)	Berberine production (mg/l. culture)	
		Cell	Medium
Sucrose	5.75	160	137
Fructose	5.43	76	65
Glucose	4.57	85	35
Maltose	3.59	109	61
Starch	3.61	65	56

Each flask was inoculated with 1.12 g (dry wt) of cultured *Coptis* cells/l. of medium then cultured for 3 weeks.

place during the usual liquid subculture. White colonies also appeared at low frequency (*ca* 6%). The addition of tyrosine to the medium inhibited colony formation by one tenth; only yellow, undifferentiated colonies that grew slowly were produced. Undifferentiated colonies of homogeneous appearance were selected and transplanted, first to slant cultures then to liquid cultures. Even though only undifferentiated colonies were selected, some of the cell lines formed roots. Those that did were removed before transplantation so that only undifferentiated colonies were subcultured selectively.

The berberine contents of the cell lines cloned are shown in Table 3. The lines varied in their contents on a dry wt basis and in the total amount of berberine produced. This variability was preserved after nonselective subculture. The berberine content of the first culture after cloning correlated well with the content of the second subculture. Addition of tyrosine to the cloning medium produced an increase in the berberine contents of cell lines that grew very slowly. Some lines (34, 231, 63T and 64T) produced more berberine, both on a dry wt and total content basis, than did the nonselected cell line.

Small cell aggregates from the third subculture after the first cloning were cloned. These aggregates came from cell lines that produced large and moderate amounts of berberine. Variations in the berberine productivity of these cell lines isolated by repeated cloning are shown in Fig. 1. Although lines isolated from the second cloning showed greater homogeneity than those from the first cloning, berberine productivity did not increase when lines (64T) with high productivity were used. Subcell lines isolated from a moderate berberine-producing line (156) had the highest productivity. In the second cloning, an addition of tyrosine to the cloning-medium had no effect on the selection of high berberine-producing lines.

Stability of berberine productivity in cell lines isolated by cloning

The cell lines isolated from the first cloning produced large amounts of berberine, and except in one line this productivity decreased after the sixth nonselective subculture (Table 4). Only the productivity of 64T was stable. Therefore, to establish lines with higher and more stable productivity, cells were repeatedly cloned. After repeated cloning some lines had very stable productivity, even after the 27th nonselective subculture (Fig. 2). Fluctuations in berberine production and in the growth of *Coptis* cells during nonselective subcultures (third to fifth) may be caused by a factor in the transplant method. Conditioned media may inhibit the growth of *Coptis* cells, thus transplantation of cells to a fresh medium should maintain high berberine production and growth.

Compositional stability of cell lines isolated by cloning

The compositional stability of the isolated cell lines was determined during nonselective subculture. HPLC showed that the *Coptis* cells contained at least five berberine type alkaloids. The main one was berberine; 55–75% of the total alkaloids in the peak. Other prominent alkaloids were jateorrizine and coptisine. The ratio of these alkaloids remained the same during culture (2–4 weeks) except in the lag periods. Therefore, we were able to use alkaloid composition as an index of the quality of the cell lines.

Lines isolated from the second cloning had this characteristic alkaloid composition (Fig. 3), but 156-1 contained almost all berberine. In the 64T–14T line the amount of coptisine present was relatively large, and in the ABA line jateorrhizine was high. The alkaloid compositions of these twice-cloned cell lines were much more stable than those of ABA cell lines selected visually without cloning and that of the nonselected cell line.

DISCUSSION

High berberine-producing cultures of *Coptis* cells have been established by selection as have been high vitamin B_6 [7], high alkaloid [4, 5] and high anthocyanin [18, 19] producing cell lines. The average productivity of one selected cell line (156-1) was 8.2% of the cell on a dry wt basis and 0.90 g/l. of L.S. medium during 27th subculture (the highest production was 13.2% and 1.39 g/l. of medium). The former value was higher than the value for the natural product (*ca* 5% on a dry wt basis). Selection rather than metabolic regulation is essential at an early stage to establish a high berberine producing culture because cultured *Coptis* cells are heterogeneous.

Whereas variation is induced and heightened by cell culture and high metabolite-producing cell lines can be

Table 3. Berberine contents of *Coptis* cell lines isolated by cloning

Line No.	Growth (g dry wt/ l. culture)	Berberine* production (mg/l. culture) Cell	Medium	Berberine* content (dry wt. %) in cell
3	6.28	91	94	1.5
5	9.88	206	80	2.3
20	10.08	214	—	2.1
27	6.12	274	164	4.5
30	10.28	379	26	3.7
34	7.88	585	139	7.4
37	7.84	172	112	2.2
102	8.48	170	80	2.0
104	8.40	352	60	4.2
153	8.28	178	86	2.2
156	8.20	385	85	4.7
160	10.60	318	33	3.0
200†	12.28	410	62	3.3
202†	8.72	273	152	3.1
231†	9.08	664	116	7.3
1T‡	8.32	152	80	1.8
2T‡	10.80	355	29	3.3
3T‡	7.92	122	59	1.6
5T‡	8.96	294	52	3.3
6T‡	9.60	476	110	5.0
63T†‡	7.76	412	88	5.3
64T†‡	8.08	586	78	7.3
81T†‡	9.76	532	81	5.5
Nonselected cell line	8.40	437	8	5.2

*Fractions contained small amounts of coptisine and palmatine.
†Cell lines grew very slowly during cloning.
‡Cell lines isolated on a medium containing tyrosine.

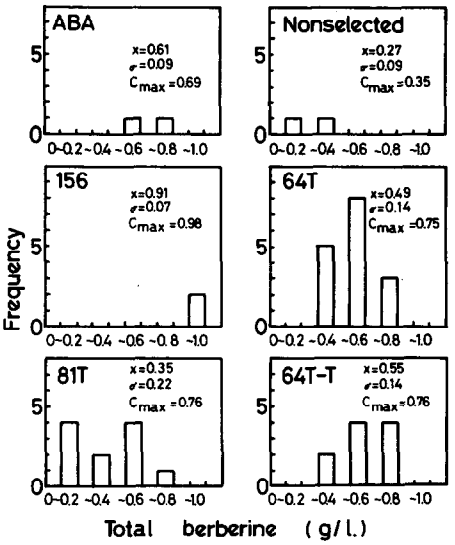

Fig. 1. Distribution of berberine production in *Coptis* cell lines isolated from a second cloning. Lines that had produced large (64T, 81T) and moderate (156) amounts of berberine in the first cloning were recloned. Subcell line 64T–T was isolated from line 64T on a medium containing tyrosine. The ABA and unselected cell lines were not cloned. $\bar{x}$ = average value; σ = standard deviation; C_{max} = maximum value.

Table 4. Stability of berberine productivity in cell lines isolated by cloning

Line No.	Berberine* production (mg/l. culture) 1st†	6th†	Berberine* content (dry wt. %) 1st†	6th†
231	780	440	7.3	4.2
63T‡	500	532	5.3	4.1
64T‡	664	600	7.3	6.6
81T‡	612	404	5.5	4.0
Nonselected	444	476	5.2	4.2

*Fractions contained small amounts of coptisine and palmatine.
†Subculture number.
‡Cell lines isolated on a medium containing tyrosine.

isolated, nonselective subculture usually decreases the production of a metabolite [7, 18]. By repeated cloning, however, some cell lines with stable productivity could be obtained (Fig. 2). Yamamoto *et al.* [19] also reported that repeated selection increased the stability of pigment production.

Repeated cloning of clones that accumulate the maximum amount of metabolites has been shown to increase

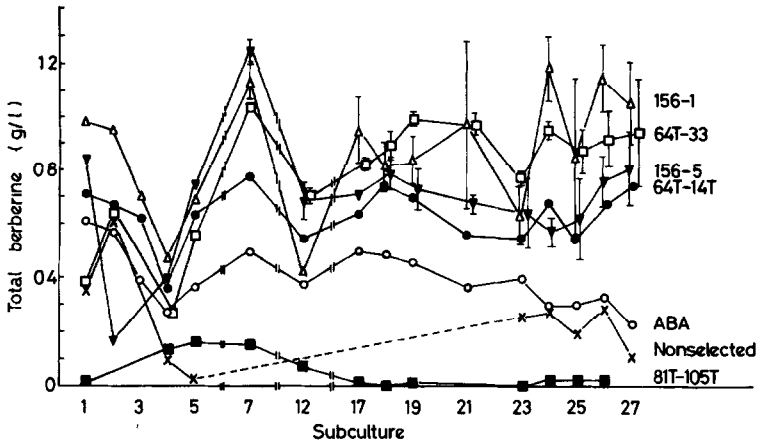

Fig 2 Stability of berberine productivity of cell lines isolated from a second cloning Lines isolated from a second cloning were subcultured unselectively △, 156–1, □, 64T–33, ▼, 156–5, ●, 64T–14T, ○, ABA, ■, 81T-105T, ×, unselected cell line The bars represent the standard deviation of the mean values

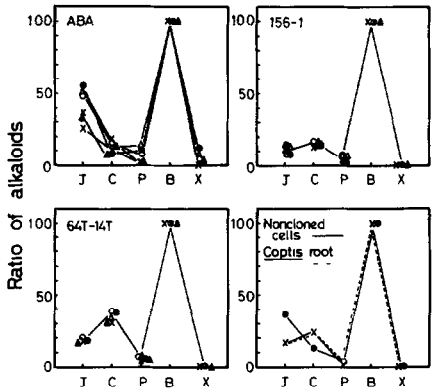

Fig 3 Changes in the composition of berberine alkaloids in various clones during subculture Subculture 1 x—x, 3 ○—○, 4 △—△, and 5 ●—● J, jateorrhizine, C, coptisine, P, palmatine, B, berberine, ×, undetermined compound The dotted line represents the composition of berberine alkaloids in *Coptis* roots

productivity [19, 20] The highest berberine-producing cell lines of *Coptis*, however, was isolated from a line that produced a moderate amount of berberine in the first measurement Thus in order to establish a high metabolite-producing cell line by selection, it is necessary to clone a number of cell lines to get the best one

The time consuming step in the selection of a cell line with high and stable productivity is the repeated cloning of clones Selection agents (such as *p*-fluorophenylalanine for the selection of phenolics-producing cell lines [21]) improve the efficiency in selecting high metabolite-producing cell lines Tyrosine may act as a selection agent in the berberine production of *Coptis* cells

We established high berberine-producing cell lines by repeated selection The quantitative productivity of berberine fluctuated, however, when there were changes in physiological conditions and in the nutrients in the culture (Fig 2). Therefore, in addition to clonal selection, improvements in culture conditions and metabolic regulation are needed to obtain a high and stable berberine-producing cell line

EXPERIMENTAL

Plant materials Cultured *Coptis* cells derived from small fragments of rootlets of *Coptis japonica* Makino var dissecta (Yatabe) Nakai (in Japanese, *Seribaworen*) were cultured in the dark at 26 ± 1° in liquid Linsmaier-Skoog basal medium The hormonal combination used was 10 μM NAA and 0 01 μM BA This was our unselected cell line A cell line grown on Linsmaier-Skoog medium with 1 μM ABA and selected visually was the ABA cell line

Cloning with small cell aggregates The suspension-cultured cells consisted of aggregates of various sizes Therefore, the suspensions were first filtered through a 500 μm sieve then through a 250 μm one Small, fine cell aggregates of 1–30 cells were collected from the filtrate on a 64 μm nylon sieve These aggregates were suspended in liquid medium then mixed with melted agar-medium after which the cells were plated in Petri dishes at a density of about 10^4 cells/ml (*ca* 1000 aggregates) Cloning experiments were carried out with Linsmaier-Skoog medium

Colonies appeared after 1–2 months of culture Those that looked homogeneous and were without root differentiation were transplanted to slant cultures Thereafter, the cell lines were cultured in liquid media After two subcultures of each line in liquid media, the berberine content was measured Cloning was repeated with cell lines that produced large or moderate amounts of berberine Tyrosine (0 1 % w/v), a precursor of berberine, was added to the media as a selective agent Cell lines selected from media with tyrosine have the letter 'T' appended to their numerical designations

Measurement of growth In the metabolic regulation experiments, about 1 5 g of cultured *Coptis* cells was inoculated in the media (75 ml of medium per 300 ml flask) and cultured for 3 weeks Cells were harvested, dried in a freeze drier and weighed For the first cloning, each sample (about 0 5 g) was cultured in 100 ml flasks that contained 25 ml medium, for the second each line (about 0 3 g) was cultured in 10 ml medium (50 ml flask) for 3 weeks

Measurement of berberine content. Berberine alkaloids were extracted with 90% MeOH from the freeze-dried cultured cells. The MeOH extract was subjected to TLC, or to HPLC, and the berberine content determined by densitometry (TLC) or spectrophotometry (HPLC). TLC: as described previously [2]; HPLC: μ Bondapack C_{18} (Waters Co., Ltd. 30 cm × 3.9 mm), MeCN–H_2O (7:13) containing 0.005 M sodium octanesulfonate (as paired ions) and 1% AcOH (1.0 ml/min). For the selection procedure, we used another solvent (MeOH–H_2O, 13:7 with pick B_7 reagent; Waters Co., Ltd.), but the berberine fraction obtained contained small amounts of coptisine and palmatine.

The berberine measured as berberine hydrochloride ($C_{20}H_{18}ClNO_4 \cdot \times H_2O$) contained about 91% berberine anhydride. The berberine, coptisine and palmatine in the MeOH extract were identified with authentic samples by GC/MS analysis after the hydrogenation of each alkaloid.

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