Plant Cell Reports (1991) 9:496–499

Plant Cell Reports

Effect of carbon dioxide and ethylene on berberine production and cell browning in *Thalictrum minus* cell cultures

Y. Kobayashi, H. Fukui, and M. Tabata

Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan

Received August 16, 1990/Revised version received November 8, 1990 – Communicated by G.C. Phillips

ABSTRACT

Cultured cells of Thalictrum minus L. (Ranunculaceae), transferred from culture flasks to a bubble column bioreactor, produced little berberine and turned dark brown, even when supplied with sufficient oxygen. This phenomenon was ascribed to the removal of CO_2 from the culture medium by bubbling air, and could be reproduced in flask cultures artificially deprived of CO_2. The induction of cell browning by exogenously administered ethylene suggested that CO_2 probably acts antagonistically against endogenously generated C_2H_4. The physiological damage caused by forced aeration could be prevented by adding 2 % CO_2 to the air in the bioreactor.

INTRODUCTION

Large-scale culture of plant cells in bioreactors often fails to keep the productivity of secondary metabolites as high as that in suspension cultures in flasks (Wagner and Vogelmann 1977). As pointed out by Smart and Fowler (1981), one of the major problems is the mode of aeration, especially the supply of sufficient oxygen for biosynthesis as well as for cell growth (Breuling et al. 1985, Kobayashi et al. 1989). In flask cultures on a shaker, oxygen can be supplied adequately by increasing the ratio of the surface area of culture medium in direct contact with air to the medium volume, whereas in bioreactor systems the oxygen is supplied by bubbling air into the medium. In the latter case, the oxygen supply increases with an increase in the rate of air flow, while the amount of CO_2 in the medium tends to decrease. However, cultured cells in shaking flasks usually grow in an environment where the CO_2 concentration may become more than one hundred times as high as that in the atmosphere (ca. 0.03 %). This is comparable to the high CO_2 concentrations observed in the rhizosphere, where it is frequently between 0.1 to 5 % (De Jong and Schappert 1972) and may become as great as 20 % (Norstadt and Porter 1984). This paper reports that an excessive removal of CO_2 is a major limitation in culture systems of Thalictrum minus for berberine production, and describes the influence of CO_2 on the generation of ethylene and the browning of cells.

MATERIAL AND METHODS

Cell suspension culture

A cell line of T. minus L. var. hypoleucum Miq. (Nakagawa et al. 1984) has been maintained as a suspension culture in "growth medium", i.e. LS medium (Linsmaier and Skoog, 1965) containing 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D), by subculturing every two weeks.

For induction of berberine production, 3 replicates of 17-d-old cells (1 g fresh wt = 75 mg dry wt) of the stock culture were transferred to 30 ml of "production medium", i.e. LS medium containing 100 μM 1-naphthaleneacetic acid (NAA) and 10 μM benzyladenine (BA), in a 100 ml Erlenmeyer flask. The cultures were agitated on a reciprocal shaker at a speed of 100 strokes/min at 25 °C in the dark.

Effect of CO_2 deprivation from the culture system was examined by using a two-tier culture vessel consisting of two vertically connected flasks (Street, 1977). Cells (1 g fresh wt) were inoculated in the upper flask (30 ml medium) connected by a duct to the lower flask containing a 20 % KOH solution (50 ml).

Culture of immobilized cells

T. minus cells (17-d-old) were entrapped in Ca-alginate beads according to the method of Kierstan and Bucke (1977) with minor modifications, employing a similar device used by Vorlop and Klein (1987). Cells (8 g fresh wt) suspended in 1.5 % alginate (100 ml) were dripped from nozzles (1 mm in diameter) into a 50 mM $CaCl_2$ solution; the Ca-alginate beads (ca 2 mm in diameter) formed were left in the solution for 3 h at 25 °C in the dark, then washed with 30 ml of LS basal medium.

Beads containing a total of 7 g cells were inoculated into 200 ml of the production medium in a bubble column bioreactor (Fig. 1)

Offprint requests to: M. Tabata

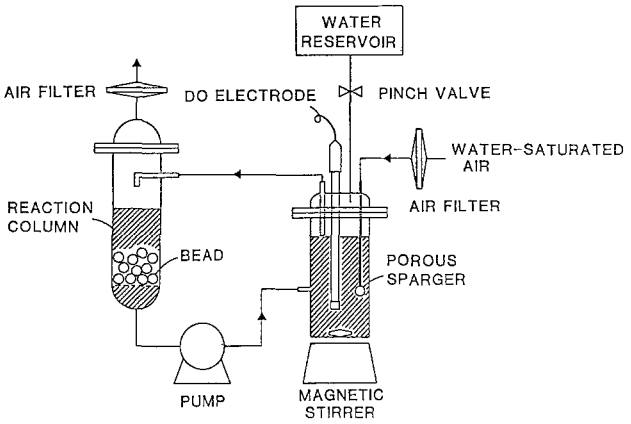

Fig. 1. Diagram of the bubble column reactor system used for berberine production by immobilized cells of Thalictrum minus.

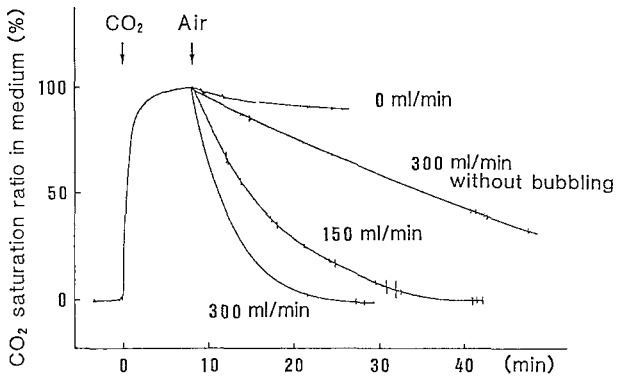

Fig. 2. Relationship between increasing air flow rate and the removal of CO_2 from the culture medium.

and incubated under the same conditions as above. The flow rate of CO_2 was adjusted with a gas mixing unit (Tokyo Rika Kikai CO., Japan).

Quantitative analysis of berberine

The quantitative analysis of berberine was carried out by HPLC as described elsewhere (Nakagawa et al. 1984), using SEP-PAK C_{18} instead of Amberlite XAD-2 for the separation column.

Determination of CO_2 and C_2H_4 concentration

The CO_2 concentration in the headspace of the culture vessel was determined by GC using a Shimadzu gas chromatographic apparatus (model GC-7A) with a thermoconductivity detector; stainless steel column (6 m X 3 mm), Gaskuropack 54 60/80 (Gasukuro Kogyo Inc., Japan), temperature: 70°C, carrier gas: helium (30 ml/min).

The C_2H_4 concentration was determined by GC using a Hitachi gas chromatographic apparatus (model 163) with a flame ionization detector; stainless steel column (2 m X 2 mm): Gaskuropack 54 60/80, temperature: 70°C, carrier gas: nitrogen (30 ml/min). The identity of C_2H_4 was confirmed by the mercury perchlorate test (Warner and Leopold 1969).

Measurement of cell browning

Freeze-dried cells were extracted in EtOH under argon gas to remove berberine and alcohol-soluble phenolics, and the residual powder in a tablet (15 mm in diameter) was used for quantitative estimation of cell browning by measuring the reflectance at 420 nm using a Shimadzu Dual-wavelength TLC scanner (model CS-900).

Determination of phenolic contents

Phenolic contents were determined by the method of Swain and Hills (1959) using Folin-Denis reagent. Freeze-dried cells were refluxed in EtOH for 2 h under argon gas, thrice, and the extract was used for the quantitative determination of EtOH soluble phenolics (free phenolics). The residue was then refluxed in 1N NaOH for 16 h under argon gas and the extract was used for determination of the EtOH insoluble but 1N NaOH soluble phenolics content (bound phenolics). The residue was then used for the determination of lignin-like compounds using acetyl bromide according to the method of Fukuda and Komamine (1982) with a slight modification.

RESULTS AND DISCUSSION

Table 1 shows the influence of aeration on cell growth, berberine production, and cell browning in two different bioreactor systems. In the liquid-gas two phase bioreactor system (Kobayashi et al. 1988), the immobilized cells of T. minus secreted berberine into medium with good yield (163 mg/l). In the bubble column bioreactor system (Fig. 1), however, the immobilized cells secreted only 28 mg/l of berberine and turned brown; although the value of K_La (volumetric O_2 transfer coefficient) was kept at 20 h^{-1},

Table 1. Growth, berberine yield, and cell browning of Thalictrum minus cultures in different aeration systems (culture period : 15 d).

	Bioreactor	
	Liquid-gas two phase system	Bubble column system
Flow rate of air(ml/min)	100	300
K_La^* (h^{-1})	N.D.	20
Cell growth(gDW/L)	2.69	2.66
Berberine(mg/L)	163.0	28.3
Relative cell browning	1	1.88

* Volumetric O_2 transfer coefficient
N.D. = not determined

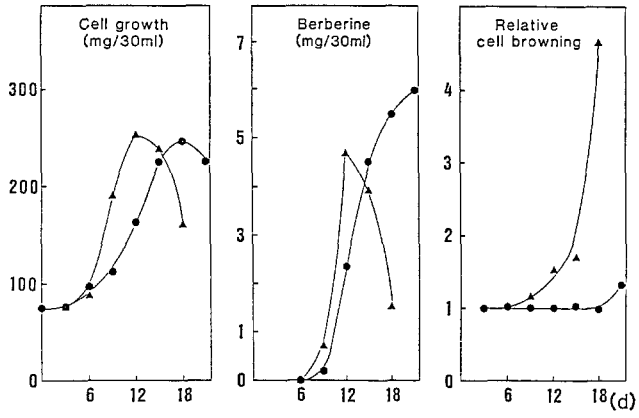

Fig. 3. Time-courses of cell growth, berberine production, and cell browning in cultures with a normal CO_2 level (--●--) and those with a low CO_2 level (--▲--) in a two-decked flask culture system.

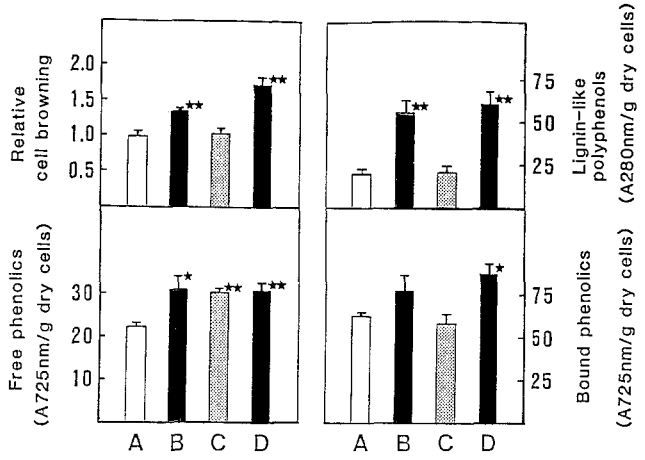

Fig. 4. Effects of incorporating a CO_2 adsorbent (20 % KOH) and adding Ethrel, on cell browning and contents of phenolics: (A) control culture, (B) culture treated with Ethrel, (C) culture with CO_2 adsorbent, (D) culture with both Ethrel and CO_2 adsorbent. (* : $P < 0.05$, ** : $P < 0.01$)

which should be sufficient for berberine production (Kobayashi et al. 1989). The major difference between the two reactors is in the mode of aeration; oxygen is supplied to immobilized cells by exposing gel beads to air in the former system, whereas it is done by bubbling in the latter. Under the bubbling condition, the oxygen supply will increase with the increasing air flow rate, as indicated by a linear increase in K_La value (data not shown), but the deprivation of CO_2 from the medium will be accelerated (Fig. 2).

The possibility that the loss of CO_2 would seriously affect the physiology of cells in the bubble column bioreactor was examined by inoculating T. minus cells in the upper chamber of a two-tier culture vessel, with the lower chamber containing 20 % KOH for trapping CO_2. The removal of CO_2 caused rapid browning and early death of cells as well as a drastic fall in the berberine-producing activity after 12 d of culture in the flask shake cultures (Fig. 3). As shown in Table 2, the addition of 20 % KOH to the lower vessel reduced the CO_2 concentration by 78.3 % of the control 12 d after inoculation, while bringing a 2.63 times increase over the control in the concentration of C_2H_4.

Table 2. Effect of CO_2 adsorbent (20 % KOH) on the level of CO_2 and C_2H_4 within the culture vessel after 12 d.

	CO_2 (%)	C_2H_4 (ppm)
Control culture	3.41 ± 0.26*	2.88 ± 0.67
Culture with CO_2 adsorbent	0.74 ± 0.07	7.58 ± 0.26

* Mean ± S.E.

It is known that a high concentration of CO_2 inhibits the ability of C_2H_4 to prolong the storage life of fruits (Isenberg 1979) and vegetables (Smock 1979). Accordingly, the effects of C_2H_4 and CO_2 on cell browning were examined by adding 200 ppm Ethrel (2-chloroethylphosphonic acid), a generator of C_2H_4 (Yang 1969), to the medium, or by pouring a 20 % KOH solution into the lower flask at the tenth d of culture. After 5 d of incubation, these cultures were harvested for measurements. As expected, cell browning as well as the formation of phenolics was promoted by the addition of Ethrel (Fig. 4). Unexpectedly, the removal of CO_2 by itself hardly affected either cell browning or the formation of polyphenols except for free phenolics. However, the effects of Ethrel were intensified by removing CO_2 from the culture system.

Cell browning, a frequently observed phenomenon in plant tissues, is thought to be brought about through the metabolism of phenolic compounds (Luh and Phithakpol 1972,

Table 3. Effect of CO_2 addition on berberine production and cell browning in the bubble column bioreactor system (culture period : 21 d).

	Flow rate of CO_2 (ml/min)	
	6	0
Flow rate of air (ml/min)	300	300
Cell growth (gDW/L)	2.81	2.71
Berberine (mg/L)	382.8	110.6
Relative cell browning	1	1.82

Mayer and Harel 1979). As for T. minus cell suspension cultures, the high level of cell browning observed after 5 d of culture in the presence of exogenous C_2H_4 appears to be correlated with an increase in the content of cell wall-bound phenolics (Fig. 4). Similar results were reported by Ke and Saltveit (1988) for russet spotting in iceberg lettuce, where cell browning appeared to be caused mainly by the action of C_2H_4 in phenolic metabolism.

Even though manipulation of the endogenous C_2H_4 level is difficult, a supplement of 2 % CO_2 to the air in the bubble column reactor proved to be effective not only in preventing cell browning, but also in supporting berberine production (Table 3). These results clearly indicate the important role of CO_2 in secondary metabolism, and the necessity of adjusting its atmospheric concentration level when scaling up plant cell cultures.

REFERENCES

Breuling M, Alfermann AW, Reinhard E (1985) Plant Cell Rep. 4: 220-223.

De Jong E, Schappert HVJ (1972) Soil Sci. 113: 328-333.

Fukuda H, Komamine A (1982) Planta 155: 423-430.

Isenberg FMR (1979) Hort. Rev. 1: 337-394.

Ke D, Saltveit ME Jr. (1988) Plant Physiol. 88: 1136-1140.

Kierstan M, Bucke C (1977) Biotech. Bioeng. 19: 387-397.

Kobayashi Y, Fukui H, Tabata M (1988) Plant Cell Rep. 7: 249-253.

Kobayashi Y, Fukui H, Tabata M (1989) Plant Cell Rep. 8: 255-258.

Linsmaier EM, Skoog F (1965) Physiol. Plant. 18: 100-127.

Luh BS, Phithakpol B (1972) J. Food Sci. 37: 264-268.

Mayer AM, Harel E (1979) Phytochem. 18: 193-215.

Nakagawa K, Konagai A, Fukui H, Tabata M (1984) Plant Cell Rep. 3: 254-257.

Norstadt FA, Porter LK (1984) Soil Sci. Soc. Am. J. 48: 783-789.

Smock RM (1979) Hort. Rev. 1: 301-336.

Smart NJ, Fowler MW (1981) Biotechnol. Lett. 3: 171-176.

Street HE (1977) in "Plant Tissue and Cell Culture" (Street HE, ed.), Blackwell Scientific Publications, Oxford, London, Edinburgh, Melbourne pp. 61-102.

Swain T, Hills WE (1959) J. Sci. Food Agric. 10: 63-68.

Vorlop KD, Klein J (1987) In "Methods in Enzymology" (Mosbach K, ed.) Vol. 135, Academic Press, pp. 259-268.

Wagner F, Vogelmann H (1977) In "Plant Tissue Culture and Its Biotechnological Application" (Barz W, Reinhard E and Zenk MH, eds.) Springer-Verlag, Berlin, Heidelberg, New York pp.245-252.

Warner HL, Leopold AG (1969) Plant Physiol. 44: 156-158.

Yang SF (1969) Plant Physiol. 44: 1203-1204.