PERGAMON

Phytochemistry 50 (1999) 775–779

PHYTOCHEMISTRY

Effects of chloride ion on acutumine and dechloroacutumine production by *Menispermum dauricum* root culture

H.A.A. Babiker, Y. Sugimoto *, T. Saisho, S. Inanaga

Arid Land Research Center, Tottori University, 1390 Hamasaka, Tottori 680-0001, Japan

Received 12 May 1998; revised 13 August 1998

Abstract

The effects of chloride ion on the production of acutumine and dechloroacutumine, by *Menispermum dauricum* root culture, were studied. The chloride ion content in the medium plays a key role in the production of both alkaloids. A low chloride medium promoted production of dechloroacutumine and suppressed that of acutumine. Production of the two alkaloids during the 60 day culture period was closely associated with root biomass. Both alkaloids accumulated in the roots and a relatively small proportion was exuded into the medium. The intact plant produced very low amounts of both alkaloids. On the average, cultured roots contained 22- and 75-fold more acutumine and dechloroacutumine, respectively, than intact plants.

Keywords: *Menispermum dauricum*; Menispermaceae; Root culture; Culture filtrate; Alkaloid; Dechloroacutumine; Acutumine

1. Introduction

Tissue cultures are renowned for their ability to produce a variety of secondary metabolites. The preponderance and relative abundance of individual metabolites are, largely, modulated by the composition of the culture medium (Whitaker, Hobbib, & Steward, 1986). Acutumine **1**, a chlorine containing benzylisoquinoline alkaloid, was reported as a major constituent in *Sinomenium acutum* (Goto & Sudzuki, 1929), *Menispermum canadense* (Doskotch & Knapp, 1971) and *M. dauricum* (Tomita et al., 1967). Research in our laboratory (Sugimoto et al., 1996) showed that *M. dauricum* root culture is a rich source of **1**. Recent work, employing the same cultures, led to the isolation of a new benzylisoquinoline alkaloid, dechloroacutumine **2**, with the same basic structure as **1**, but without a chlorine substituent. The dechlorinated alkaloid was postulated to be the immediate biosynthetic precursor of **1** (Sugimoto et al., 1998). Investigations on the biosynthetic pathway of **1** may provide useful information about the biosynthesis of chlorinated plant products, and in particular about the chlorination step. In order to perform biosynthetic studies, optimization of the medium for the production of **1** and **2** is necessary.

The present report describes the effects of chloride ion concentration and the length of the culture period on the growth of *M. dauricum* roots and their production of **1** and **2**.

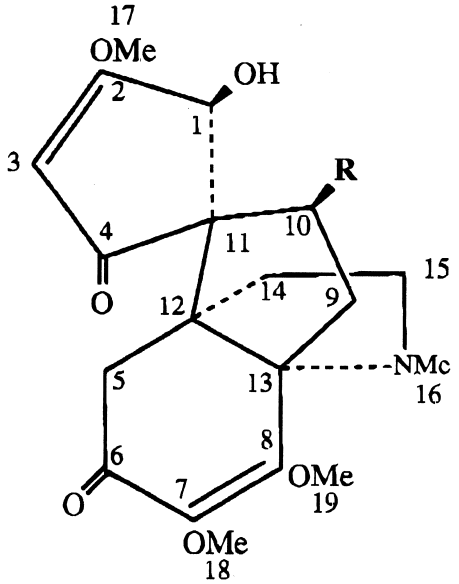
2. Results and discussion

2.1. Effect of chloride ion concentration on the growth of *M. dauricum* roots and on the production of **1** and **2**

The accumulation of **1** and **2** by *M. dauricum* roots was modulated by the chloride ion concentration (Table 1), where it consistently increased with chloride ion concentration reaching a maximum at 6 mM. An increase in concentration of chloride ion to 8 and 10 mM decreased the production of **1**, albeit not significantly. Production of **2** was maximum at a chloride ion concentration of 0.04 mM. An increase of chloride ion to 2 mM or more significantly suppressed production of **2**. Exudation of **1** and **2** into the medium was relatively small (1–20% of the total amount produced). For a given chloride ion concentration exuda-

* Corresponding author. Tel.: +81-857-21-7035; Fax: +81-857-29-6199; E-mail: sugimoto@center.tottori-u.ac.jp

0031-9422/99/$19.00
PII: S0031-9422(98)00605-0



1 R = Cl
2 R = H

tion of **1** was, invariably, higher than that of **2**. Exudation of **1** and **2**, albeit not consistently, appeared to follow their production by the roots. Replacement of $CaCl_2$ in the medium by $CaBr_2$ or CaI_2 resulted in poor root growth. Furthermore, no brominated or iodinated products were detected (data not shown).

2.2. Growth and production of **1** *and* **2** *by M. dauricum root culture in chloride-enriched and chloride-deficient media*

The influence of chloride ion on the growth of *M. dauricum* roots, accumulation in the roots, and exudation of **1** and **2** into the medium are shown in Figs. 1 and 2. Roots of *M. dauricum* grew, accumulated and exuded **1** and **2** throughout the 60 day culture period in both media. Roots displayed a higher growth rate in the chloride-enriched medium. Maximum root biomass (330 mg dry wt.) was achieved 45 days after subculturing in the chloride-enriched medium whereas in the chloride-deficient medium maximum root biomass (310 mg dry wt.) was attained 50 days after culturing. Accumulation of **1** and **2** in the roots closely followed root biomass production ($r = 0.84$ to 0.89, $P < 0.05$). In the chloride-enriched medium, the accumulation of **1** and **2** displayed a slow initial rate followed by a surge at 30 to 50 days after subculturing and then a decline on extension of the culture period to 55 days or more (Fig. 1). Similar amounts of **1** and **2** were produced during the first 25 days of root growth. On extension of the culture period to 30 days or more, production of **1** exceeded that of **2**. However, significant differences were only realized at 30 to 50 days after culturing. In the chloride-deficient medium, roots accumulated low and almost similar concentrations of **1** and **2** during the first 35 days of growth (Fig. 2). Forty days after subculturing, the accumulation of **1** and **2** increased by 88 and 173%, respectively. Extension of the culture period to 60 days had little effect on accumulation of **1**. However, accumulation of **2** consistently increased with time, reaching a peak (1310 μg/flask) at 55 days and then declined, albeit not significantly. Both **1** and **2** were exuded into the medium. With few exceptions, noted during the early stages of growth, the amount exuded was proportionally small when compared to the total amount produced (Figs. 1 and 2). Exudation of **1** was higher in the chloride-enriched medium, while that of **2** was maximum in the chloride-deficient medium.

The relationship between the production of **1** and **2** in the two media suggests that the concentration of chloride ion is a key factor in determining the relative proportions of **1** and **2**. Increasing the concentration of the chloride ion in the medium promoted production of **1** and suppressed that of **2**.

Table 1
Effect of chloride ion concentration on cultured *M. dauricum* root growth and the production of acutumine **1** and dechloroacutumine **2**

Cl^- concentration (mM)	Growth index (N-fold)	Acutumine **1** content (μg/flask)		Dechloroacutumine **2** content (μg/flask)	
		cultured roots	filtrate	cultured roots	filtrate
0.04	20.1 ± 0.13	280.4 ± 11.40	30.5 ± 4.78	529.1 ± 20.03	21.2 ± 1.86
2[a]	19.9 ± 0.03	302.8 ± 37.58	61.9 ± 17.72	131.1 ± 25.94	10.2 ± 2.57
4	20.4 ± 0.02	317.8 ± 36.68	16.3 ± 11.80	179.6 ± 9.59	1.8 ± 1.16
6	16.9 ± 0.12	357.9 ± 2.78	45.6 ± 1.26	135.5 ± 10.0	3.5 ± 0.26
8	16.5 ± 0.52	264.7 ± 23.50	38.9 ± 4.39	102.8 ± 13.68	4.6 ± 0.21
10	20.7 ± 0.18	210.9 ± 6.70	16.5 ± 0.87	166.8 ± 8.11	3.2 ± 0.20

[a]Concentration of the chloride ion in the standard Gamborg's B5 medium. Excised root (ca. 0.15 g) placed in 100 ml flask containing 25 ml medium. Each value represents the mean of three replicates ± S.E.

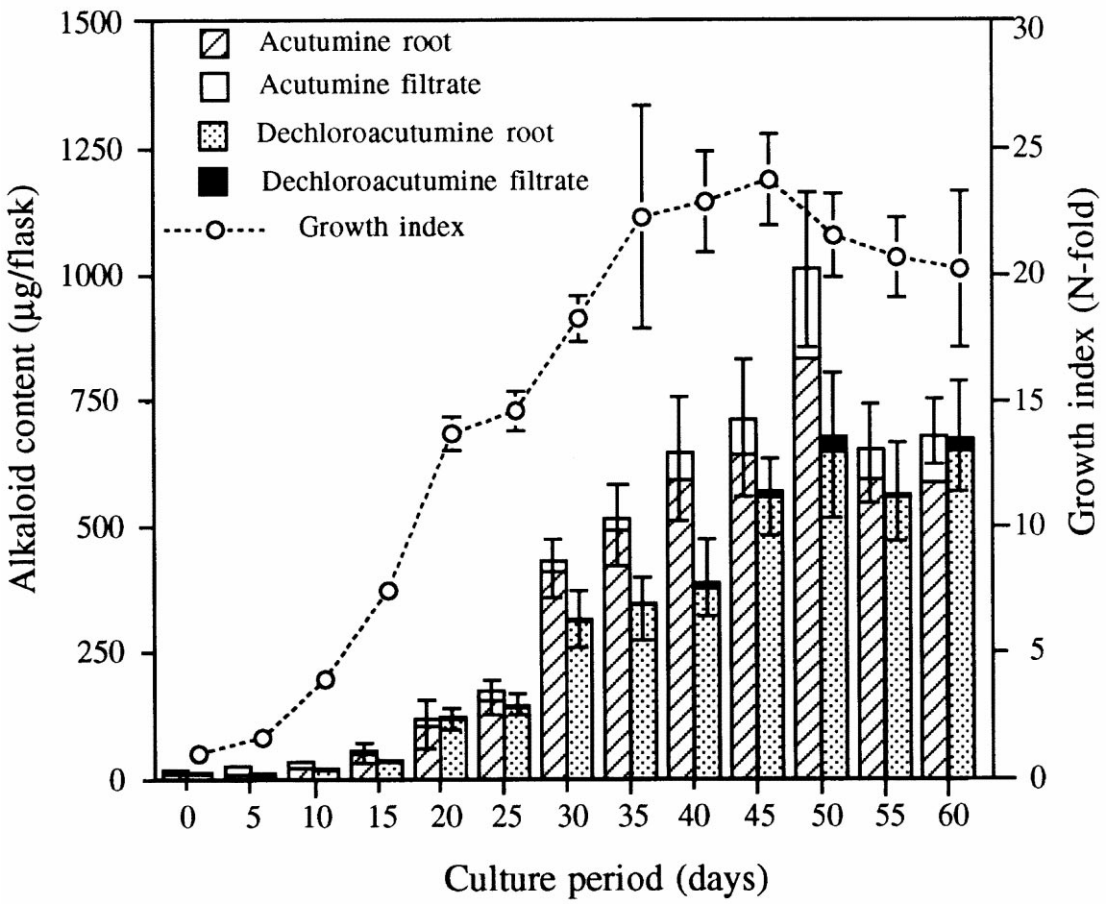


Fig. 1. Time course of *M. dauricum* root growth and production of acutumine (**1**) and dechloroacutumine (**2**) in chloride-enriched medium. Excised root (ca. 0.15 g) placed in 100-ml flask containing 25 ml of the medium for 60 days.

Intact *M. dauricum* plants contained very little amount of **1** and **2** (1.3–4.5% of that produced by root culture). The chlorinated alkaloid **1** was found in amounts varying between 3–18 μg/50 mg dry wt. in the leaves, petioles, stems, main and lateral roots of the intact plants (Fig. 3). However, the dechlorinated alkaloid **2** was detected only in the root system. The limited distribution of **2** may be due to restricted biosynthesis, differential translocation and/or metabolism.

Concurrent production of **1** and **2** by cultured *M. dauricum* roots, increased production of **1** in the chloride-enriched medium and the attendant decrease in the accumulation of **2**, together with the structural similarities of the two alkaloids (Sugimoto et al., 1998), suggest that **2** might be the immediate precursor of **1**. However, possible involvement of subtle physiological changes in the chloride-mediated production of **1** and **2** cannot be ruled out and further work is needed to ascertain the biosynthetic relationship between the two alkaloids.

3. Experimental

3.1. Plant materials

M. dauricum plants were grown in a green house at the Arid Land Research Center, Tottori University, Japan. For studies involving tissue cultures, *M. dauricum* roots were obtained from established cultures (Sugimoto, Yoshida, Uchida, Inanaga, & Yamada, 1994).

3.2. Culture conditions

Excised *M. dauricum* roots (ca. 0.15 g) were grown in a modified B5 medium containing 3% sucrose and 7.5 μM NAA (Gamborg, Miller, & Ojima, 1968). The roots, placed in 100-ml flasks containing 25 ml of the respective culture medium, were maintained in the dark at 27°C on a rotary shaker (70 rpm) until harvest.

3.3. Alkaloid extraction and analysis of roots and culture filtrate

The alkaloids **1** and **2** were extracted from both the roots and the culture filtrate and further purified as described previously (Sugimoto et al., 1998). Briefly, harvested *M. dauricum* roots were freeze-dried and powdered. The powder (50 mg) was soaked in MeOH overnight and filtered. Soaking in methanol was repeated twice and the combined filtrates were evaporated to dryness. The residue, dissolved in 6 ml of 3% citric acid, was made alkaline (pH 10) with aq. NH_4OH, and loaded onto an Extrelut column (Merck Art. 15372). The column was allowed to stand for 10 min prior to elution with $CHCl_3$ (3.5 ml × 2). The combined $CHCl_3$ eluates were evaporated to dryness. The residue, dissolved in MeOH, was analyzed for **1** and **2** by HPLC, using a column of ODS-3 (4.6 × 150 mm) eluting with 60% MeOH with 0.2% NH_4OH. The flow rate was 0.3 ml/min. A short pre-column (4.6 × 30 mm) was placed between the injector and the separation column. The column effluent was monitored by UV at 245 nm. For the determination of **1** and **2** in the culture filtrate, the latter was acidified with 3% citric acid and extracted with $CHCl_3$. The aq. layer, made alkaline (pH 10) with aq. NH_3, was extracted with $CHCl_3$ (3 ml × 3). The combined $CHCl_3$ extracts were evaporated to dryness and dissolved in MeOH (1 ml). Concentrations of **1** and **2** were determined by HPLC.

3.4. Effects of chloride ion on the growth of M. dauricum roots and the production of 1 and 2

Excised *M. dauricum* roots were cultured in the modified B5 medium which was further supplemented with KCl (2–8 mM). The roots were allowed to grow for 55 days prior to harvest and assessed for their content of **1** and **2**.

3.5. Effects of chloride-enriched medium on the growth of M. dauricum roots and the production of 1 and 2

Excised *M. dauricum* roots were cultured for 60 days in the modified B5 medium. The chloride content of the medium was adjusted to 6 mM. Culture conditions

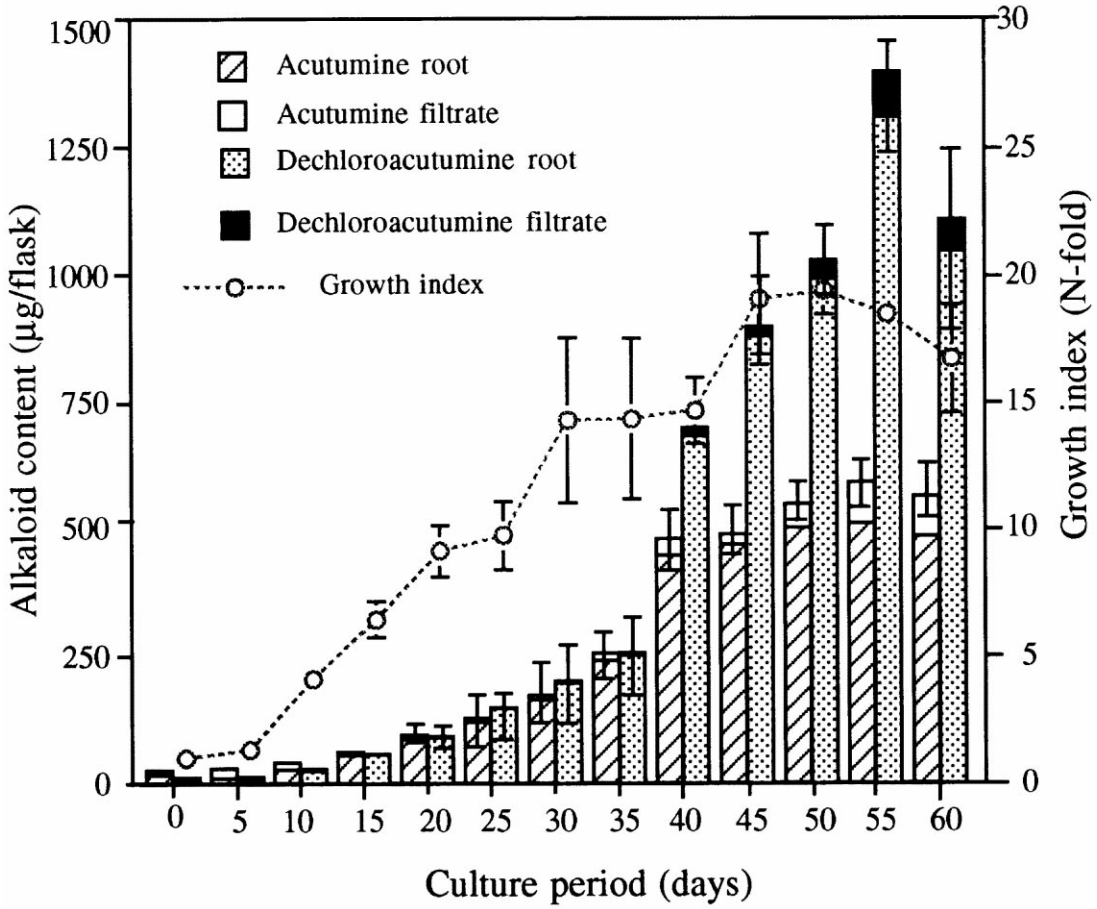


Fig. 2. Time course of *M. dauricum* root growth and production of acutumine (**1**) and dechloroacutumine (**2**) in chloride-deficient medium. Excised root (ca. 0.15 g) placed in 100-ml flask containing 25 ml of the medium for 60 days.

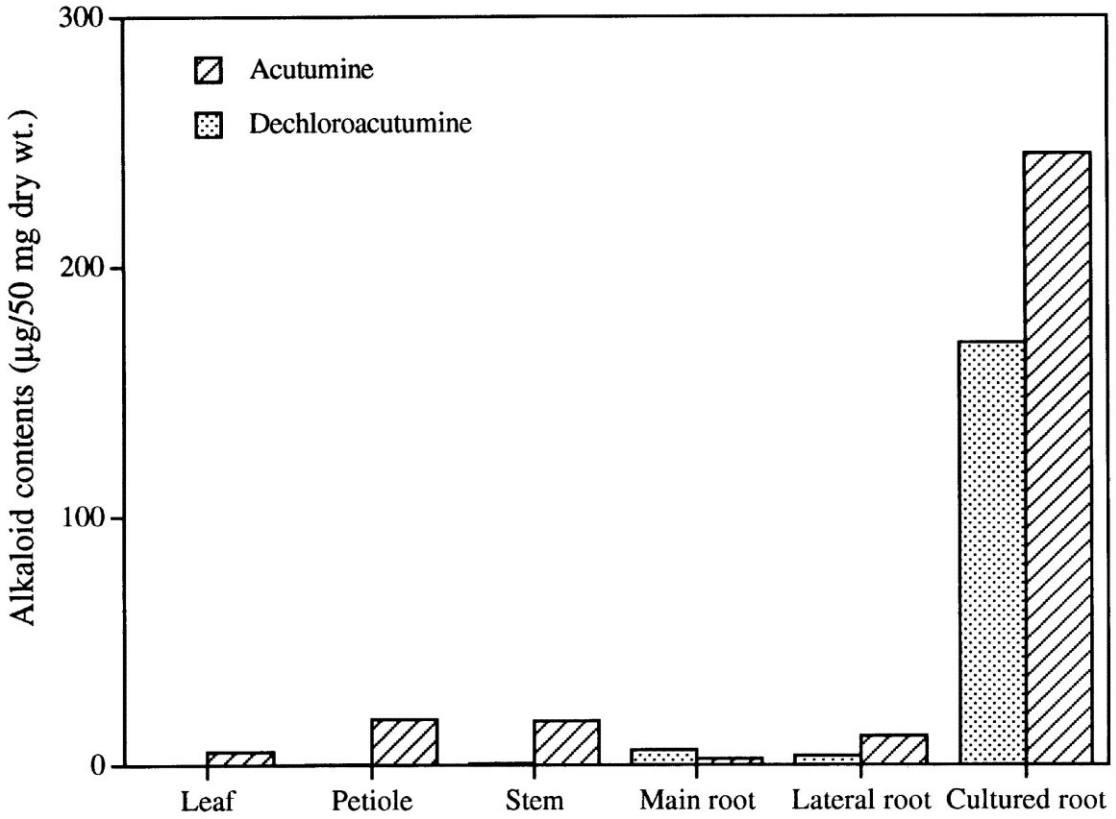


Fig. 3. Production of acutumine (**1**) and dechloroacutumine (**2**) by intact *M. dauricum* plants and root culture.

and alkaloid assessments were as described in Section 3.3.

3.6. *Effects of chloride-deficient medium on the growth of M. dauricum roots and the production of* **1** *and* **2**

A chloride-deficient medium, based on Gamborg's B5 medium, was prepared as described previously (Sugimoto et al., 1998). Briefly, $CaCl_2$, the main source of chloride in Gamborg's B5 medium, was replaced by $Ca(NO_3)_2$. The medium was further supplemented with sucrose and NAA as specified before. Excised *M. dauricum* roots were cultured in the medium and allowed to grow for 60 days prior to harvest. Alkaloid extraction and analyses were performed as previously described.

3.7. *Analysis of* **1** *and* **2** *from intact plant*

Whole plants were harvested and separated into leaves, petioles, stems and main and lateral roots. The plant parts were freeze-dried and powdered. Alkaloids were extracted and analyzed as described for the cultured roots.

Acknowledgements

The authors would like to express their thanks to Professor A.G.T. Babiker (Tottori University) for his critical reading of the manuscript. This work was supported, in part, by a grant from the Kampou Science Foundation to Y.S.

References

Doskotch, R. W., & Knapp, J. E. (1971). *Lloydia, 34,* 292.
Gamborg, O. L., Miller, R. A., & Ojima, K. (1968). *Experimental Cell Research, 50,* 151.
Goto, K., & Sudzuki, H. (1929). *Bulletin of the Chemical Society of Japan, 4,* 220.
Sugimoto, Y., Yoshida, A., Uchida, S., Inanaga, S., & Yamada, Y. (1994). *Phytochemistry, 36,* 679.
Sugimoto, Y., Uchida, S., Inanaga, S., Kimura, Y., Hashimoto, M., & Isogai, A. (1996). *Bioscience, Biotechnology and Biochemistry, 60,* 503.
Sugimoto, Y., Inanaga, S., Kato, M., Shimizu, T., Hakoshima, T., & Isogai, A. (1998). *Phytochemistry, 49,* 1293.
Tomita, M., Okamoto, Y., Kikuchi, T., Osaki, K., Nishikawa, M., Kamiya, K., Sasaki, Y., Matoba, K., & Goto, K. (1967). *Tetrahedron Letters,* 2421.
Whitaker, R. J., Hobbib, C. G., & Steward, L. A. (1986). *Journal of the American Chemical Society, 317,* 347.