Identification and Measurement of Endogenous Formaldehyde in *Datura innoxia* Mill. Callus Tissues by OPLC, HPLC and MALDI MS Techniques

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Abstract

The purpose of the present study was to determine the presence of measurable amount of endogenous formaldehyde (HCHO) in callus tissue cultures of *Datura innoxia* Mill. applying different analytical methods. The effect of HCHO deprivation with dimedone - as abiotic stress - was also investigated on the growth and endogenous HCHO level in *D. innoxia* callus cultures. In our work, HCHO in dimedone adduct form (formaldemethone), has been isolated and characterised by overpressured layer chromatography (OPLC), high pressure liquid chromatography (HPLC), and matrix assisted laser desorption / ionization mass spectrometry (MALDI MS). Endogenous HCHO deprivation with dimedone affected the cell proliferation in *D. innoxia* callus tissues as well.

INTRODUCTION

Different biological samples - originated from plant, animal and human tissues and fluids - contain analysable amounts of endogenous HCHO. A high level of HCHO and accumulation of different fully-N-methylated substances were found in young tree leaves and in other plant tissues containing rapidly dividing cells (Szende et al., 1995). It has been demonstrated that the formation of HCHO is probably linked to the enzymatic transmethylation of histamine (Huszti et al., 1986). In these cases HCHO can originate mainly from methylation reactions. The presence of HCHO in pig liver, in human blood and in urine has also been examined (Sárdi and Tyihák, 1994).

It is known that there is a correlation between different stress situations and endogenous HCHO levels. The effect of biotic (e.g. virus infection) and abiotic (e.g. heat shock) stress conditions led to increases in measurable HCHO levels (Burgyán et al., 1982). These findings indicate that HCHO is a basic substance of biological processes (Tyihák et al., 1996).

Organized and unorganised plant tissue cultures are good subjects for studying growth, cell proliferation, primary and secondary metabolism and the effects of different circumstances during sub cultivation (Szőke et al., 1982). Since tissue cultures can be cultivated under reproducible and well-controlled conditions they are often used for studying biochemical aspects of tissues. Because of the importance of Solanaceae plants and their comparatively well examined metabolism, we established a *Datura innoxia* Mill. callus tissue culture as a good subject for our investigations and for studying the role of HCHO in biotransformation.

The aim of this paper is to demonstrate the presence of HCHO in *D. innoxia* callus tissues using different analytical methods and the effect of HCHO deprivation with dimedone as an abiotic stress condition on the growth and endogenous HCHO level of the cultures.

MATERIALS AND METHODS

Establishment and Cultivation of Callus Tissues

The test material used in our experiments was secondary callus tissue isolated from shoots of *Datura innoxia* organized culture. The tissues were grown in the dark at 25°C on AMS solid medium (pH= 5.6; 0.7% agar) containing 30 g l^{-1} of sucrose (Reanal), 5.0 mg l^{-1} of Ca-pantothenate (Reanal), 4.43 g l^{-1} of Murashige and Skoog basal medium (Sigma), 1.0 mg l^{-1} of kinetin (Fluka) and 1.0 mg l^{-1} of 2,4-D (BDH Chemicals).

Besides the control, four different dimedone concentrations were applied. The dimedone solutions (1 ppm, 10 ppm, 100 ppm and 1000 ppm) were made with distilled water at room temperature and added into the culture medium.

Callus tissues were reweighed every two weeks during the six weeks of the cultivation period. The dry weight was measured after freeze-drying.

The fresh callus tissues were frozen in liquid nitrogen, powdered and treated with 0.2 % solution of dimedone in methanol (e.g. 0.25 g tissue powder and 0.7 cm^3 of 0.2 % dimedone solution). This suspension was centrifuged (500 *g* for 5 minutes at 4 °C). The clear supernatants were used for OPLC, HPLC, and MALDI MS determination.

OPLC Separation

OPLC separations of formaldemethone were carried out using a chloroform - methylenechloride eluent mixture (35:65, v/v), silica gel 60 F_{254} chromatoplates with sealed edges and a Chrompres 25 OPLC instrument (Tyihák et al., 1996). Quantitative analysis was performed densitometrically (Shimadzu Cs 930 scanner, Shimadzu Co., Kyoto, Japan) at λ=260 nm. Formaldemethone was identified using an authentic standard.

HPLC Separation

HPLC separation of formaldemethone was made using a Gynkotek M 480 pump, TOSOH 6040 UV detector (260 nm) and Rheodyne 8125 injector with a 20 µl loop. The column used was a Chrompack ChromSpher C-18 (150x4.6 mm; 5µm). The applied mobile phase was methanol - 0.01 M HCl (76:24, v/v; pH=2.63). The chromatograms were recorded through an EF 2102 ADDA converter (Elektroflex GM, Szeged, Hungary) by personal computer.

MALDI MS Analysis

The MALDI mass spectrometer used in this work was a Finnigan LASERMAT 2000 (Finnigan MAT Ltd., Hemel Hempstead, UK). The matrix material was α-cyano-4-hydroxy cinnamic acid (ACH) (Sigma). The sample (0.5 µl) was diluted with methanol in the ratio 1:100, and then it was mixed with 0.5 µl α-cyano-4-hydroxy cinnamic acid (ACH) matrix (2 mg/mL in 60 % methanol/ 40 % water) directly on the disposable sample slide. The droplet was allowed to dry naturally before analysis. The sample preparation for the authentic formaldemethone is mentioned below, but the diluted supernatant was substituted for the standard solution ($2 \cdot 10^{-5}$ M).

Test Material

Formaldemethone (1,1′,3,3′ - tetraketo -5,5,5′,5′ - tetramethyl - 2,2′-dicyclohexyl - methane) was prepared for identification purposes - as standard material - by adding formaldehyde solution (20 mL of 38 % solution) to a solution of dimedone (5 g) in hot ethanol (30 mL) and water (5 mL). The white precipitate was filtered and recrystallized from aqueous ethanol (m.p. 191 °C). The suspension was centrifuged (1000 *g* for 10 minutes at 20 °C) and the clear supernatants were used for HPLC separation and MALDI MS analysis.

Statistical Analysis

Data of the cultures, represented by six individuals, were evaluated and summarized each time; standard deviation (± SD) was calculated. Confidence limits were

added at $p<0.05$.

RESULTS AND DISCUSSION

Identification and Measurement of Endogenous HCHO

Dimedone is known to be a specific reagent for aldehydes. In the case of HCHO, the reaction product (formaldemethone) can be used for identification and quantitation of this simplest aliphatic aldehyde. The proton donor ability of dimedone plays an important role in the collection of HCHO from different labile bindings (e.g. methylol-arginine and so on) (Tyihák and Szőke, 1996). It is important to remark that during all our investigations, the applied dimedone concentrations were in excess.

Various methods for the determination of HCHO (colorimetric, thin-layer chromatographic, gas chromatographic, HPLC) have been reported (Sárdi and Tyihák, 1994). However a simple, quick and sensitive method is needed.

We have found that MALDI MS analysis - just like HPLC - can be applied for the identification of formaldemethone from different biological samples. The MALDI MS method mainly differs from classical mass spectrometry in the application of matrix material. The matrix molecules absorb the energy impulse of a laser and transfer some of this to the sample. The matrix has got dissociable protons (for example from carboxylic groups), which can be given to the sample molecules. As a result of fragmentation less ionisation the components of the mixture can be analysed in the presence of each other without difficult sample clean up (László et al. 1998). We have determinated the presence of formaldemethone both in the test solution and in unpurified tissue extract by OPLC, HPLC and MALDI MS (Fig. 1 and 2).

Effect of HCHO Deprivation on *D. innoxia* Callus Tissues

We have measured endogenous HCHO levels during subcultivation for six weeks by OPLC and HPLC methods. During this time the callus tissues were grown in dimedone free - as control - and dimedone containing culture media.

In the controls the fresh weight increased during the cultivation period, but there was a decrease of HCHO level with the aging of the cultures until the sixth week (Table 1 an 2). This is in accordance with previous observations that the endogenous HCHO level changes with aging (Tyihák and Szőke, 1996).

Dimedone in the culture medium influenced characteristically the growth of tissues. The callus cultures grown in the dark were brownish coloured, parenchymatic and with no signs of differentiation (Fig. 3). The tissues were soft, but became more compact with increased dimedone content of the culture medium. It could be observed especially in cultures cultivated on 1000 ppm dimedone containing medium, as these tissues were much more compact than the control (Fig. 3). The fresh weight increased at 1, 10 and 100 ppm dimedone concentrations compared to the controls (Table. 1). After 2 and 4 weeks at 10 ppm dimedone concentration a significant growth increase could be detected (Table 1). At that time the measurable HCHO level was low in these callus tissues (Table 2). After six weeks the fresh weight at 1000 ppm dimedone concentration almost reached that of the control, while in the case of 2 and 4 weeks old cultures there was a strong inhibition of cell proliferation at the same dimedone concentration (Table 1).

In the four week old tissue cultures, an increase of HCHO level could be detected at the 1000 ppm dimedone concentration. The lowest formaldehyde level was detected after six weeks (Table 2). It is supposed that in the control tissues (young, dimedone-free tissues) there is HCHO of normal energy level, while in stressed tissues there is a possibility of the formation of excited HCHO (H^*CHO), which is a very toxic substance (Tyihák et al., 1994).

The tissues - in spite of HCHO deprivation - in the case of all applied dimedone concentrations contained HCHO in similar concentration ranges showing that HCHO is not a side product, but a basic component of biological processes. There is a dynamic relationship between enzymatic methylation and demethylation processes (Fannin and

Bush, 1992) and in these transmethylation reactions special HCHO precursors and acceptors play a crucial role (Anthoni et al, 1991; Blunden et al., 1992).

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Tables

Table 1. Effect of different dimedone concentrations on the fresh weight of *D. innoxia* callus tissues.

Age of cultures (weeks)	Dimedone concentration in culture medium (ppm)				
	0 (Control)	**1**	**10**	**100**	**1000**
	Fresh weight of callus tissues (g)				
2	5.09 ± 0.83	10.31 ± 0.76	11.51 ± 0.58	7.75 ± 0.32	3.02 ± 0.27
4	15.36 ± 0.38	17.19 ± 0.42	19.55 ± 1.04	17.75 ± 0.46	6.22 ± 0.45
6	16.12 ± 1.90	17.54 ± 0.81	17.93 ± 0.87	17.34 ± 0.64	14.70 ± 0.69

Table 2. Effect of different dimedone concentrations on the endogenous HCHO content of *D. innoxia* callus tissues.

Age of cultures (weeks)	Dimedone concentration in culture medium (ppm)				
	0 (Control)	**1**	**10**	**100**	**1000**
	Endogenous HCHO content (μg/g)				
2	17.2 ± 1.65	14.3 ± 1.24	11.2 ± 0.85	12.1 ± 0.96	11.0 ± 0.78
4	18.0 ± 1.61	13.4 ± 1.08	12.1 ± 0.57	12.3 ± 1.14	17.0 ± 0.93
6	6.3 ± 0.56	5.6 ± 0.49	4.6 ± 0.34	5.8 ± 0.25	6.2 ± 0.51

Figures

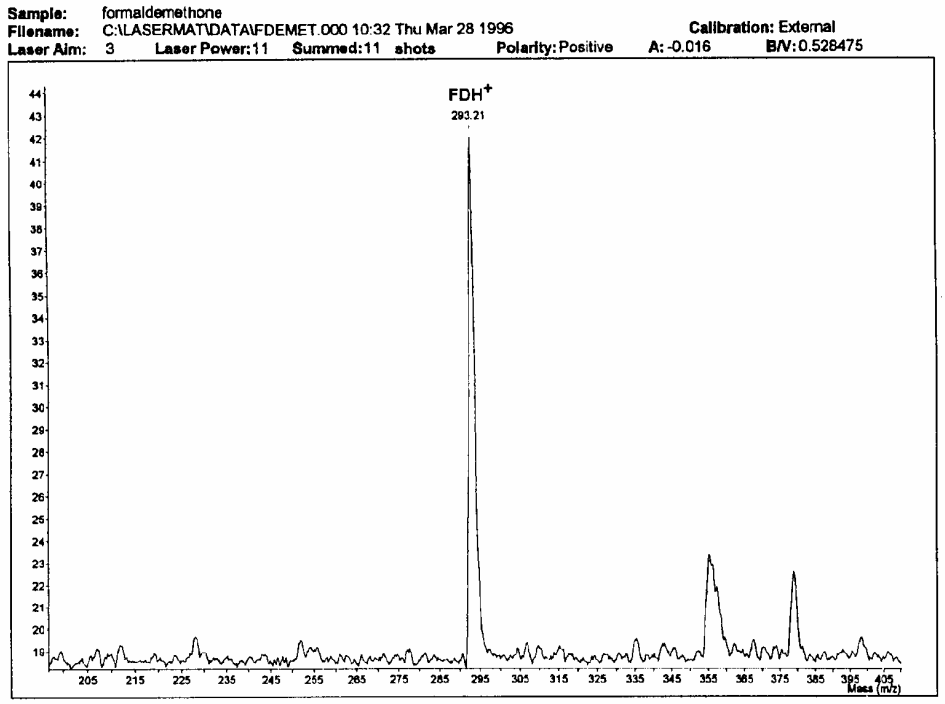

Fig. 1. MALDI MS spectrum of formaldemethone test solution (FDH^+:protonated formaldemethone).

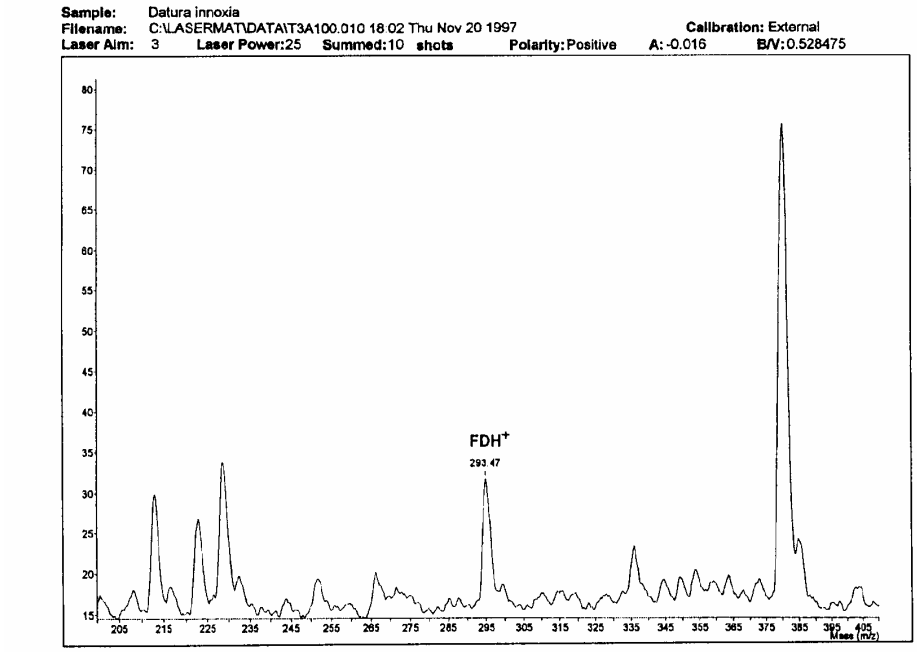

Fig. 2. MALDI MS spectrum of *D. innoxia* callus tissue extract (six weeks cultivation in the dark on 1000ppm dimedone containing culture medium, FDH^+:protonated formaldemethone).

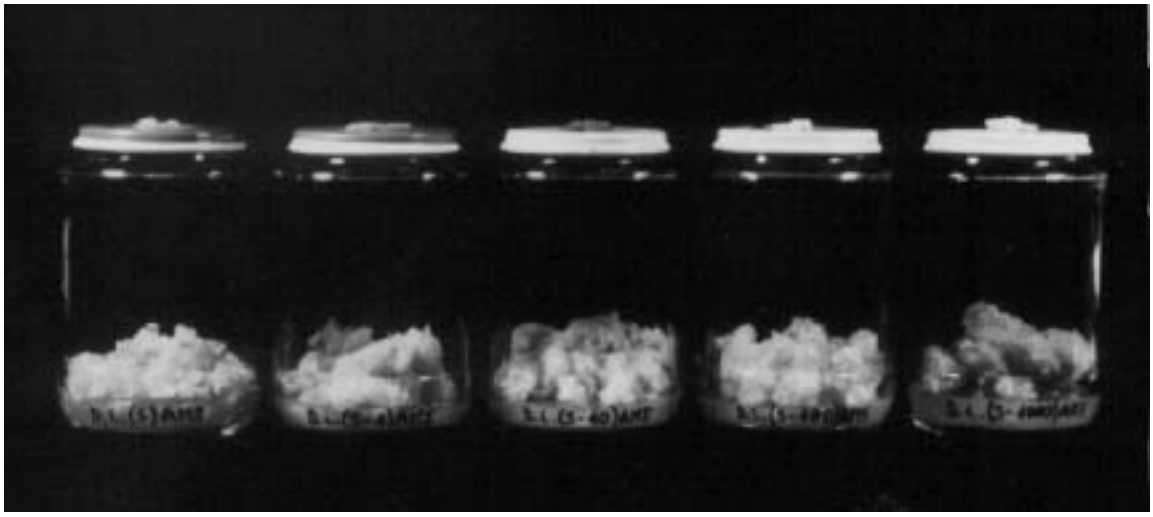

Fig. 3. *D. innoxia* callus tissues after six weeks cultivation in the dark (from left to right: 0 ppm, 1 ppm, 10 ppm, 100 ppm and 1000 ppm dimedone in culture medium).