BIOLOGIA PLANTARUM 35 (3): 401-406, 1993

Differential responses of N-nitrosoamines and aromatic amines in the plant cell/microbe coincubation assay

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Abstract

The plant cell/microbe coincubation assay is based on employing living tobacco cells in suspension culture as the activating system for promutagens and the Ames/*Salmonella* cells as the genetic indicator system. In contrast to aromatic amines (*e.g.* 2-aminofluorene and *m*-phenylenediamine) that were previously reported to be activated to products mutagenic in the *S. typhimurium* strains TA98 or YG1024 by tobacco cells, promutagenic N-nitrosoamines (N-nitrosodimethylamine, N-nitrosomorpholine, N-nitrosopiperidine, N-nitrosomethyl-2-hydroxypropylamine) were not activated to product(s) mutagenic in *S. typhimurium* TA100.

Introduction

Many environmental pollutants, particularly chemicals such as pesticides, components in polluted air, water, soil *etc.*, which come into direct contact to plants, may be absorbed, and metabolically transformed, in some cases to mutagenic products. If these mutagenic products are stable, they may be introduced into the human food chain, and thus induce genetic damage.

Plewa *et al.* (1983) have developed a genetic assay that employed living plant cell cultures to investigate the metabolic activation of non-mutagenic environmental pollutants (promutagens) to mutagenic products. This assay is based on coincubating tobacco suspension cells (as a metabolic activating system), tester strains of *Salmonella typhimurium* (as the genetic indicator organism) together with the tested promutagen. Using this plant cell/microbe coincubation system, Plewa *et al.* (1988, 1993) and Wagner *et al.* (1992) were able to demonstrate the metabolic activation of

Received 25 January 1993, accepted 10 February 1993.
Abbreviations: 2-AF - 2-aminofluorene; 2,4-D - 2,4-dichlorophenoxyacetic acid; NDMA - N-nitrosodimethylamine; NMHPA - N-nitrosomethyl-2-hydroxypropylamine; NMOR - N-nitrosomorpholine; NPIP - N-nitrosopiperidine; mPDA - m-phenylenediamine.

several promutagenic monocyclic and polycyclic aromatic amines to product mutagenic to *S. typhimurium* strains TA98 and YG1024 (see Discussion).

The objective of the experiments reported in this paper was to find out if another group of environmental promutagens, N-nitrosoamines, can also be activated by tobacco suspension cells.

Materials and methods

Chemicals: Highly purified 2-aminofluorene (CAS # 153-78-6) was purchased from *Chemservices Chemical Co.* (West Chester, PA), N-nitrosomethyl-2-hydroxypropylamine was kindly provided by Prof. F. Lijinsky (Frederick, MD), N-nitrosodimethylamine (CAS # 6-75-92), N-nitrosopiperidine (CAS # 100-75-4), N-nitrosomorpholine (CAS # 59-89-2), and sodium azide (26628-22-8) were purchased from *Sigma Chemical Co.* (St. Louis, MO). 2-AF was dissolved in dimethylsulphoxide, all other chemicals in 100 mM potassium phosphate buffer, pH 7.4.

Preparation of the bacterial suspension: *Salmonella typhimurium* strains TA98 and TA100 were provided by Dr. B. Ames, University of California (Berkeley, CA). The bacteria were stored as frozen permanents at -80 °C. Genetic markers were tested in every experiment using the procedures of Zeiger *et al.* (1981).

An overnight culture of *S. typhimurium* was grown from a single colony isolate in 100 ml of Luria broth at 37 °C with shaking (200 rpm). The bacterial suspension was centrifuged for 5 min at 5000 rpm, the bacterial pellet was resuspended and washed in 100 mM potassium phosphate buffer, pH 7.4. The suspension was centrifuged, the supernatant fluid was decanted, and the bacterial pellet resuspended in 10 ml of phosphate buffer. The titer of the bacterial suspension was determined spectrophotometrically at 660 nm and adjusted to 1×10^{10} cells ml^{-1}. The bacterial suspension was placed on ice until use.

Preparation of plant cell suspension: Long term plant cell suspension cultures of tobacco (*Nicotiana tabacum*) cell line TX1 were maintained at 28 °C in MX medium, a modified liquid medium of Murashige and Skoog (1962). For coincubation experiments, a TX1 culture was grown to early stationary phase and the cells were harvested by centrifugation for 3 min at 900 rpm. The supernatant fluid was removed and the cells resuspended and washed in MX^- medium, the liquid culture medium MX that lacked 2,4-D. The fresh mass of the plant cell suspension was adjusted to the requirements of the experimental design (100 or 200 mg ml^{-1}). The plant cell suspension was placed on ice until used ($\leq$ 30 min).

Plant cell/microbe coincubation assay: In the experiments, each reaction mixture consisted of 900 µl of the tobacco cell suspension, 100 µl of the bacterial suspension and the known amount of the promutagens. Concurrent negative controls consisted of plant and bacterial cells alone and both buffer and solvent controls. The positive control for the base substitution tester strain TA100 was sodium azide.

These components were incubated at 28 °C for 1 to 3 h with shaking at 150 rpm. After the incubation, triplicate 100 µl aliquots were added to 2 ml of molten top agar supplemented with 550 µM histidine and biotin. The top agar was poured onto Vogel Bonner minimal medium plates, incubated for 72 h at 37 °C and revertant his^+ colonies were scored.

Results

NDMA, a promutagenic N-nitrosoamine, was titrated (25 to 200 mM) in co-incubation reaction tubes under the conditions described in the methods (100 mg ml^{-1} TX1 cells, 1 h treatment). No increase in the frequency of his^+ revertants above the spontaneous level was observed. Neither increasing the coincubation period from 1 h to 3 h nor increasing the amount of tobacco cells in the coincubation mixture from 100 to 200 mg ml^{-1} led to the conversion of NDMA to product(s) mutagenic in *S. typhimurium* TA100 (Table 1). The positive control consisted of MX^- medium, bacterial cells and 0.2 mM sodium azide.

Table 1. Absence of metabolic activation of N-nitrosodimethylamine (NDMA) by tobacco suspension cells to product(s) mutagenic in *S. typhimurium*.

NDMA [mM]	Mean revertants per plate ± S.E. [1]TX1	[2]MX	[3]TX1	[4]MX
0	79 ± 5.5	90 ± 3.8	160 ± 7.1	162 ± 15.4
25	94 ± 3.7	78 ± 5.9		
50	77 ± 6.3	60 ± 3.4	152 ± 8.2	134 ± 10.7
100	71 ± 5.8	56 ± 1.2	159 ± 15.0	152 ± 5.6
200	100 ± 4.2	52 ± 3.5	161 ± 6.8	142 ± 4.8
[5]NaN_3		504 ± 43		

[1]TX1 - tobacco suspension cells, 100 mg ml^{-1}, 1 h treatment, [2]MX - Murashige and Skoog (1962) revised medium lacking 2,4-D, 1 h treatment, [3]TX1 - tobacco suspension cells, 200 mg ml^{-1}, 3 h treatment, [4]MX - Murashige and Skoog (1962) revised medium lacking 2,4-D, 3 h treatment, [5]0.2 mM NaN_3.

The three other N-nitrosoamines tested; N-nitrosomorpholine, N-nitrosopiperidine, and N-nitrosomethyl-2-hydroxypropylamine (Table 2) were also negative in the tobacco cell/*Salmonella* co-incubation assay.

For illustration, data on the activation of the aromatic amine 2-AF to product(s) mutagenic in *S. typhimurium* TA98 are presented (Table 3). In contrast to the 4 tested N-nitrosamines, with increasing concentration of the aromatic amine in the coincubation mixture, the frequency of the his^+ revertants increased.

Table 2. Absence of metabolic activation of N-nitrosomorpholine (NMOR), N-nitrosopiperidine (NPIP), and N-nitrosomethyl-2-hydroxypropylamine (NMHPA) by tobacco suspension cells to product mutagenic in *S. typhimurium* TA 100.

NMOR [mM]	Mean revertants per plate ± S.E. [1]TX1	NPIPN [mM]	[1]TX1	NMHPA [mM]	[1]TX1
0	75 ± 6.7	0	69 ± 1.7	0	68 ± 4.0
5	84 ± 7.6	1	62 ± 6.5	16	62 ± 4.3
10	95 ± 4.4	2.5	118 ± 8.2	32	75 ± 9.4
25	97 ± 7.9	5	101 ± 5.7	64	70 ± 9.0
50	97 ± 10.8	10	81 ± 7.8	128	84 ± 7.2
100	95 ± 4.7	20	62 ± 5.6		

[1]TX1, tobacco suspension cells, 100 mg m^{-1}, 1 h treatment.

Table 3. Metabolic activation of 2-aminofluorene (2-AF) by tobacco suspension cells to product(s) mutagenic in *S. typhimurium* TA 98.

2-AF µM	Mean revertants per plate ± S.E. [1]MX	[2]TX1
0	13 ± 1.5	15 ± 1.8
12.5	16 ± 0.3	112 ± 7.8
25	23 ± 2.3	154 ± 8.7
50	20 ± 1.8	185 ± 11.8
100	17 ± 1.2	254 ± 6.4
DMSO	12 ± 0.6	

[1]MX, Murashige and Skoog (1962) revised medium lacking 2, 4 - D, 1 h treatment; [2]TX1, tobacco suspension cells, 100 mg ml^{-1}, 1 h treatment.

Table 4. Aromatic amines that were activated and N-nitrosoamines that were not activated by suspension tobacco cells to product(s) mutagenic in *S. typhimurium*.

Aromatic amines	N-nitrosoamines
4-aminobiphenyl[1]	N-nitrosodimethylamine
2-aminofluorene[1]	N-nitrosomethyl-2-hydroxypropylamine
benzidine[1]	N-nitrosomorpholine
2,4-diaminotoluene[1]	N-nitrosopiperidine
m-phenylenediamine[1]	

[1]See Plewa *et al.* (1993) and Wagner *et al.* (1992).

Discussion

A list of aromatic amines that were reported to be metabolized by suspension tobacco cells to product(s) mutagenic in *S. typhimurium* (Plewa *et al.* 1983, 1993, Wagner *et*

al. 1992) and a list of N-nitrosoamines (data in this communication) that were not activated by tobacco cells to mutagenic product(s) in *S. typhimurium* are given in Table 4.

According to a working model (Plewa *et al.* 1991), the activation of the promutagenic aromatic amines by tobacco suspension cells is mediated by intracellular (in the tobacco cells) or by extracellular (in the cultivation medium) peroxidases. Two findings strongly support the assumption of the participation of peroxidases in the oxidative metabolism of aromatic amines in the plant cell/microbe coincubation assay:

(*1*) Diethyldithiocarbamate, a non competitive inhibitor of cell peroxidases, markedly inhibited the activation of *m*-PDA to mutagenic products by tobacco suspension cells (Plewa *et al.* 1991).

(*2*) The activation of 2-AF and *m*-PDA to mutagenic products may proceed also by using the cultivation medium, in which tobacco cells were cultivated for 7 days, instead of tobacco cells (Plewa and Gichner, unpublished). Peroxidases are excreted from tobacco cells into the cultivation medium (Mader and Walter 1986). In contrast cytochrome P-450, another type of enzyme known to metabolize aromatic amines (Bartsch 1981) are bound to microsomes, and thus to the tobacco cells.

The promutagenic N-nitrosoamines are known to be activated to mutagenic products by animal P-450 cytochromes (Preussmann and Stewart 1984). In contrast to aromatic amines that are converted to mutagenic products both by cytochrome P-450 enzymes and by prostaglandin endoperoxide synthetase (animal peroxidases), NDMA as well as NMOR are not activated to mutagenic products by these peroxidases (Robertson *et al.* 1983). The inability of peroxidases to mediate the oxidation of N-nitrosamines may explain why the tested N-nitrosoamines were not activated in the plant cell/microbe coincubation assay.

It is of interest that N-nitrosamines like NDMA were mutagenic after application on intact seedlings of *Nicotiana tabacum* cv. *Xanthi* (Bříza *et al.*,1985), whereas the aromatic amine m-PDA, highly mutagenic in the plant cell/microbe assay, did not evoke somatic mutations in this intact tobacco mutagenicity assay (unpublished). Thus, it seems that neither the mutagenicity assay using intact plants nor the plant cell/microbe coincubation assay are alone capable of monitoring the broad range of environmental mutagens.

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Communicated by J. VELEMÍNSKÝ