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Genotoxicity testing of food additives by employing *Vicia* MN assay

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Abstract
In the present study genotoxic effect induced by two food preservatives viz. ammonium acetate and zinc acetate was evaluated in the root meristem cells of *Vicia faba.* Genotoxic property was evaluated by scoring micronucleus in the root tip cells of treated roots. Roots were treated with 1, 2.5. 5, and *7.5* g/l of ammonium acetate and zinc zcetate solutions at room temperature in dark for 6, 12, 18 and 24h. The dosages selected for evaluation were below LD50 dose of the compounds. The increase in the concentration and period of treatment of each compound resulted in increase in the frequency of micronuclei. The results indicate that ammonium acetate and zinc acetate induce genotoxic effect in the dose range tested.

Keywords: Genotoxicity; ammonium acetate, zinc acetate, *Vicia faba*; micronucleus

INTRODUCTION

Food additives are substances added to food to preserve flavor or enhance its taste and appearance. With the increasing use of processed foods since the 19th century, there has been a great increase in the use of food additives of varying levels of safety. There has been significant controversy associated with the risks and benefits of food additives. Some artificial food additives have been linked with cancer, digestive problems, neurological conditions, heart disease or obesity.

Ammonium acetate (E 264) is the ammonium salt of acetic acid. It is produced commercially by oxidation of acetaldehyde. It is used as food preservative, acidulant, flavor enhancer in condiments, bottled sauces, snack food, bread cakes etc. and antimicrobial agent and is highly effective against bacteria and fungi. There are no reported mutagenic, carcinogenic or birth defect risks, but the sparse data available on these aspects of ammonium acetate cannot rule out such effects.

Zinc acetate (E 650) is used as a dietary supplement and in lozenges used to treat the common cold. In chewing gum, zinc acetate is a breath freshener and plaque inhibitor. It is also used in wood preserving, manufacturing other zinc salts, polymers, manufacture of ethylene acetate, as a dye mordant, and analytical reagent. Zinc acetate can also be used to treat zinc deficiencies. As an oral daily supplement it is used to inhibit the body's absorption of copper as part of the treatment for Wilson's disease. Zinc acetate is also sold as an astringent in the form of an ointment, a topical lotion; or combined with an antibiotic such as erythromycin for the topical treatment of acne. Zinc acetate did not show any mutagenic activity in an assay with 5 strains of *Salmonella*, in the presence and absence of the S9 activation system. In the in vitro cytogenetic CHO assay, dose-dependent positive responses of zinc acetate were obtained in the presence and absence of the S9 activation system. Zinc acetate produced dose-related positive responses in the L5178Y mouse lymphoma assay and an in vitro cytogenetic assay with Chinese hamster ovary cells, but was negative in the *Salmonella* mutation assay and did not induce unscheduled DNA synthesis in primary cultures of rat hepatocytes (1).

Since there is no clear cut understanding on the genotoxicity of ammonium acetate and zinc acetate and their widespread uses as food additive, we have evaluated the genotoxicity of both these compounds in the root meristem of *Vicia faba.*

MATERIALS AND METHODS

Primary roots of *Vicia faba* were used as test material in the present experiment. Ammonium acetate (E 264, CH_3COONH_4, CAS Registry Numbers: 631-61-8) and Zinc acetate (E 650, $CH_3COO)_2Zn.2H_2O$, CAS Registry Numbers: 5970-45-6) purchased from local chemist. Four different dosages i.e. 1, 2.5, 5 and 7.5 g/l of both the compounds were selected for treatment. Solutions of the test compounds were prepared in glass distilled water.

Roots (1-2 cm long) were treated for 6, 12, 18 and 24 h simply by suspending them in the test solutions containing the chemical in separate glass jars. In each experiment, negative controls (distilled water) and positive controls (0.20% EMS solution) were included. After treatment, roots were washed thoroughly in running tap water and the root tips were cut and fixed in a mixture of ethyl alcohol and glacial acetic acid (3:1 v/v) for 24 h. The fixed roots were then transferred into 70 % ethyl alcohol and stored at 4°C in a refrigerator for future use. All the treatments were carried out simultaneously and under the same condition.

Before slide preparation the root tips of were hydrolyzed with a solution of 1N HCl (1 part) and 45% acetic acid (9 parts). The root tips were stained with 1.5 % carmine in 45% acetic acid. After removing the root caps from the well stained root tips, 1 mm of the meristematic zones were cut in a drop of 45% acetic acid on a clean slide and squashed under cover-slip by exerting thumb pressure on it. 5000 cells from five root tips (1000 from each root tip) were scored for micronuclei under 100 x magnifications. All experiments were repeated at least three times. The data shown represent the mean ± SE.

The significant differences between means of control and the

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treated groups were assessed by student's t-test. Two way analysis of variance (ANOVA) test was adopted for testing significant differences, if any, amongst concentrations of test compounds as well as between treatment durations

RESULTS

The genotoxic effects as shown by induction of micronuclei in the root tip cells of *Vicia faba* following exposure to 1, 2.5, 5 and 7.5 g/l of ammonium acetate and zinc acetate for 6, 12, 18 and 24 h are presented in Tables 1 and 2. In the root tip cells of control roots a low frequency of micronuclei were observed (0.218%). Treatment of roots with 0.2% ethyl methane sulphonate (EMS) led to statistically highly significant ($p<0.01$) increase in the frequency of micronuclei. Treatment of roots with ammonium acetate and zinc acetate resulted in increase in the frequency of micronuclei in the root tip cells. The frequency increased concomitantly with the increase in concentration of the test compounds as well as period of treatment reaching at maximal after 24 h of treatment. The two lowest concentrations (1 and 2.5g/l) of both the compounds did not induce statistically significant increase in the frequency of micronuclei. Statistically significant increases in the frequency of micronuclei were observed at the two higher concentrations (5 and 7.5g/l). Similarly the frequency of micronuclei also increased with increase in the period of treatment and reached at peak after 24 h treatment.

Table 1. Frequency of micronuclei observed in root tips of *Vicia faba* after treatment with different concentrations of ammonium acetate at different periods of exposure.

Chemical / concentration	Frequency (%) of micronuclei [a]				Mean ± SE
	6 h	12 h	18 h	24 h	
Control	0.20 ± 0.048	0.21 ± 0.038	0.20 ± 0.036	0.23 ± 0.020	0.218 ± 0.010
EMS	1.18 ± 0.07**	1.50 ± 0.05**	2.03 ± 0.13**	2.95 ± 0.35**	1.915 ± 0.38**
1 g/l	0.20 ± 0.008	0.24 ± 0.013	0.25 ± 0.030	0.29 ± 0.017	0.245 ± 0.018
2.5 g/l	0.24 ± 0.020	0.25 ± 0.030	0.29 ± 0.020	0.32 ± 0.035	0.275 ± 0.025
5 g/l	0.27 ± 0.054	0.30 ± 0.049*	0.32 ± 0.055*	0.42 ± 0.088*	0.328 ± 0.032*
7.5 g/l	0.31 ± 0.048	0.36 ± 0.066*	0.47 ± 0.058*	0.52 ± 0.082*	0.415 ± 0.040*

[a] 1000 cells per root tip and total 5000 cells have been scored in each case
*, ** differ significantly from the control in Student's t-test

Table 2. Frequency of micronuclei observed in root tips of *Vicia faba* after treatment with different concentrations of zinc acetate at different periods of exposure.

Chemical / concentration	Frequency (%) of micronuclei [a]				Mean ± SE
	6 h	12 h	18 h	24 h	
Control	0.20 ± 0.048	0.21 ± 0.038	0.20 ± 0.036	0.23 ± 0.020	0.218 ± 0.010
EMS	1.18 ± 0.07**	1.50 ± 0.05**	2.03 ± 0.13**	2.95 ± 0.35**	1.915 ± 0.38**
1 g/l	0.20 ± 0.027	0.22 ± 0.026	0.22 ± 0.033	0.24 ± 0.018	0.220 ± 0.008
2.5 g/l	0.23 ± 0.025	0.25 ± 0.027	0.28 ± 0.058	0.35 ± 0.022*	0.278 ± 0.026
5 g/l	0.28 ± 0.033	0.30 ± 0.039	0.33 ± 0.033*	0.39 ± 0.043*	0.325 ± 0.023*
7.5 g/l	0.36 ± 0.027*	0.40 ± 0.033*	0.45 ± 0.031*	0.51 ± 0.037*	0.430 ± 0.032*

[a] 1000 cells per root tip and total 5000 cells have been scored in each case
*, ** differ significantly from the control in Student's t-tes

Fig.1 compares the trends in the increase in frequency of micronuclei (mean of all the treatment periods) induced by different concentrations of ammonium and zinc acetate. A strong positive correlation was recorded between concentration of test compounds and percent frequency of micronuclei. Two-way analysis of variance (ANOVA) test revealed that there exist significant differences between treated groups only (Table 3).

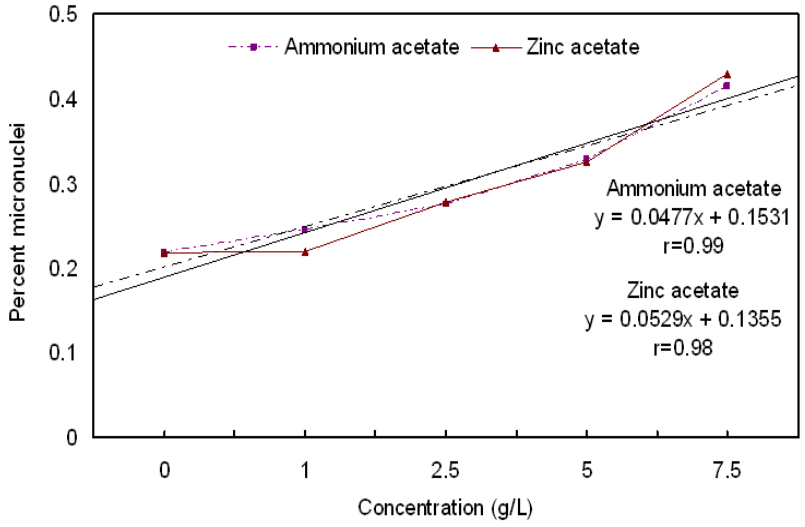

Fig 1. comparison of trends in the induction of micronuclei in the root tip cells of *Vicia faba* after treatment with ammonium and zinc acetate

Table 3. Two-way analysis of variance (ANOVA) of micronuclei showing significant variation between treatments as well as durations of treatment.

Sources of Variation	df	Mean Square		F-value	
		Ammonium acetate	Zinc acetate	Ammonium acetate	Zinc acetate
Between periods	3	0.165	0.118	2.43	2.63
Between treatment	4	0.680	0.612	10.03**	13.66**
Residual	12	0.067	0.044		

** significant at <0.01

DISCUSSION

In the present study genotoxic effect of two food additives viz. ammonium acetate and zinc acetate was evaluated in the root meristem cells of *Vicia faba*. No data on the genotoxicity of these compounds are available. On the other hand, many food additives have been tested for their genotoxic effects in different test systems and positive results were obtained (2-9). Food preservatives sodium benzoate and sodium sulfite was found to inhibit DNA synthesis and induce chromosomal aberrations in *Vicia faba* root meristems (10). Sorbic acid and its potassium salt induced chromosome aberration and sister chromatid exchange in Chinese hamster cells (11). Sodium and potassium sorbate has induced sister chromatid exchange and 6-thioguanine resistant mutation in Chinese hamster cells in vitro (11-12). Sodium nitrite induced chromosomal aberrations in Chinese hamster fibroblast cells and was mutagenic in the *Salmonella* microsome test system (13-14). Rencuzogullari et al. (15-16) have reported decrease in mitotic index in *Allium cepa* and human lymphocytes, due to the effect of sodium metabisulphite. Sodium phosphate (E 339) induced significant reduction in the frequency of dividing cells and induced chromosomal aberrations in dose dependent manner in pot Marigold root tips (17).

Mutagenicity testing based on plant bioassay has been in existence for many years. *Vicia faba* offers a good experimental model for in vivo evaluation of genotoxicity of substances and complex mixtures. The screening of micronuclei (MN) is a more rapid method than the screening of chromosomal aberrations and commonly used as a single parameter for the recording of genotoxic damage. MN formation in dividing cells is considered a true indicator of mutagenic effect (18). The induction of micronuclei is the manifestation of chromosome breakage or whole chromosome lagging during cell division due to spindle abnormalities (19-21). The chromosome lagging is induced by a weak C-mitotic effect and they indicate a risk of aneuploidy. Micronuclei formation were recorded by many investigators following treatment with different food additives (22-26). Therefore, in the present study genotoxicity of two food preservatives ammonium acetate and zinc acetate was evaluated by scoring micronuclei in the root meristem of *Vicia faba.* Induction of the formation of MN by ammonium acetate and zinc acetate in appreciably higher frequencies clearly indicate their mutagenic effect. The high frequency of micronuclei as observed in the present study also suggests that these compounds are clastogens that induce chromosome breaks and/or aneugens that induce lagging chromosomes.

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