

Mutagenic Action of Sugarcane Vinasse in the *Tradescantia Pallida* Test System

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Abstract

The soil is a very complex and dynamic system and its contamination has become one of the main environmental concerns. Sugar-ethanol mills, for example, have a high hazard potential, mainly soil contamination, due to the production of different residues. The use of vinasse, a by-product of ethanol production, as fertilizer is a technically and economically feasible alternative. However, excessive quantities have been used, causing adverse changes in the soil. The present study was aimed at evaluating the toxic potential of vinasse in the *Tradescantia pallida* test system using the micronucleus test (Trad-MCN). Ten plants were exposed to ultrapure water as the negative control (NC), in MMS as the positive control (PC), raw vinasse (RV), and vinasse diluted to 50% (C1), vinasse diluted to 25% (C2), vinasse diluted to 12.5% (C3). The medium buds of each plant were used to assess the mutagenic effects based on the presence of micronuclei in tetrads. The results revealed that RV and C1 had a mutagenic potential compared to that of NC. The alterations observed suggest damage to the DNA of the test organism, which may not be reintegrated to the cell nucleus. The findings suggest caution in the application of this product to the soil despite this being an economically viable practice.

Keywords: Trad-MCN; Ethanol production; Agroindustrial waste; Fertiligation

Introduction

The human exposure to mutagenic agents, effective in causing damage to the genetic material, is currently a great concern worldwide. These agents are closely associated with the development of cancers and can potentially compromise future generations by causing DNA damage to germinative cells, which may be passed on to future generations. The discovery of the vulnerability of the genetic material to environmental stresses has led to a new research field, Toxicological Genetics, in which specialists focus on the study of lesions and alterations induced by chemical compounds and/or physical agents to DNA [1]. Studies in this research area can lead to precise information on the exposure and risks to cell integrity, and thus an effective prevention of health problems.

The agricultural use of organic waste from industrial, urban and agricultural resources is an interesting alternative to disposal, enabling the recycling of nutrients in ecosystems [2]. In this context, it includes the vinasse or stillage, a by-product of the sugarcane industry, residue of ethanol production obtained after distillation of fermented grape, residue known to have high organic content which classifies it as a good fertilizer.

The incentive for the use of ethanol as a fuel, because this has lower pollution potential resulted in a stimulus to the sugarcane agroindustry, gaining great prominence in agricultural activities of socio-economic expression. Consequently, the wastes generated by this activity also increased, in particular the vinasse. For every liter of ethanol are produced 10 to 18 liters of vinasse [3] resulting in a problem for proper disposal.

Species of *Tradescantia* have been often used to assess the mutagenic potential of various substances in the air, soil, and water capable of inducing chromosome alterations due to the presence of clastogenic agents [4], or chromosome losses as a result of aneugenic activity, thus causing genotoxic damage [5].

The micronucleus test using *Tradescantia* (TradMCN) is considered a valuable tool by many researchers due to the simplicity of the methodology and sensitivity of the plant exposed to the genotoxic agents [6]. The test was developed by Ma and collaborators in 1976

from the Brookhaven National Laboratory initially using the clone 4430 as test organism and later adapted to the species *T. pallida*.

Tradescantia pallida, also known as Purple Heart, is native to the Gulf of Mexico. It is easily adaptable to different climate conditions [7] and environments, and exhibits year-round growth in the field and greenhouse in tropical and subtropical regions. The relatively small size of plants and genetic material consisted of six pairs of relatively large and easily observable chromosomes make this plant a suitable organism for cytogenetic studies [4]. Its inflorescences develop sequentially, allowing the exposure to the test substance in all stages of development [8].

Studies conducted by Mielli et al. [9] with young plants of *T. pallida* and the clone 4430 exposed to water and different concentrations of As_2O_3 exhibited similar responses. These results support those reported by Suyama et al. [10] with plants exposed to X-rays, suggesting that both are suitable for micronucleus bioassays. The same was not observed in experiments conducted in the field, which showed that *T. pallida* is more sensitive than clone 4430 [9].

Thus, the present study was aimed at evaluating the toxic potential of vinasse samples from a sugar-ethanol mill using *T. pallida* as test-organism in mutagenicity tests.

Materials and Methods

Test substance: sugarcane vinasse

The sugarcane vinasse was collected in the Santa Lúcia Mill,

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located in the city of Araras, São Paulo, Brazil and maintained in a cold storage chamber (4°C) at the Department of Biochemistry and Microbiology of the Institute of Biosciences, UNESP (São Paulo State University), Rio Claro, São Paulo, Brazil, until use. Two vinasse samples were collected from two different crops (crop 1 and crop 2), from different years.

Vinasse samples were sent for analysis to the laboratory TASQA, Paulínia, São Paulo, Brazil, where the following parameters were measured: pH, non-filterable residue, hardness, electric conductivity, nitrate, nitrite, kjeldahl nitrogen, sodium, calcium, potassium, magnesium, sulfate, phosphate, biochemical oxygen demand (BOD), chemical oxygen demand (COD) and metals by atomic absorption spectrophotometry (ICP) (As, Ba, Cd, Cu, Cr, Hg, Mo, Ni, Pb, Se, Zn).

Concentrations of vinasse

Vinasse was diluted in ultrapure water to three concentrations: vinasse diluted to 50% (C1), vinasse diluted to 25% (C2) and vinasse diluted to 12.5% (C3). Raw vinasse (RV) was also tested. These dilutions were tested based on the dose of vinasse applied in the field according to the guideline P4.231 of the Environmental Sanitation Technology Company (CETESB). Two bioassays were carried out with the two samples.

Bioassays

Plants of *T. pallida* were cultivated in standardized plots and watered weekly until flowering. At that time, plants were cut and brought to the Department of Biology, Institute of Biosciences, UNESP, Rio Claro, São Paulo, Brazil and immediately exposed to different treatments during 24 h.

The positive control (PC) consisted of 0.154 µL methyl methanesulfonate (MMS) in 20 mL of water, and the negative control (NC), ultrapure water. Plants were exposed to different treatments (PC, NC, RV, C1, C2, C3) in a beaker. After exposure, plants were allowed to recover in ultrapure water for 8 h.

Micronucleus test with *Tradescantia* (Trad-MCN)

For the evaluation of mutagenicity, 10 young inflorescences (all flower buds closed), of approximately 6 to 8 cm were used. After exposure, four clusters were selected. Each cluster consisted of three buds (small, medium, and large). The medium bud (right or left side) was placed on a slide and macerated with a razor blade to remove remnants of inflorescence. The material was then stained with a drop of acetic carmine, cover slipped, and then quickly passed over a flame.

After this procedure, 10 slides per treatment were analyzed under light microscope, totaling 300 tetrads observed per slide and 3000 per treatment.

The statistical analysis was carried out with the non-parametric Mann-Whitney test and $p > 0.005$.

Results

Chemical characterization of raw vinasse samples

The results of the physico-chemical and metal analysis of vinasse are presented in Table 1.

In general, vinasse had a low pH, and high BOD, COD, and potassium levels. In the crop 1, the concentration of potassium was

approximately 12 times higher than that of the crop 2. In the crop 1, levels of calcium, magnesium and sodium were also higher, while in the crop 2, nitrate and kjeldahl nitrogen were higher.

In the crop 2, levels of barium, copper, chrome, nickel, and zinc were also higher.

Mutagenicity analysis

The results found by observing tetrads carrier micronuclei (Figure 1) in the two bioassays were very similar. In the first bioassay (crop 1), a significant micronucleus index was obtained for the RV and C1 treatments when compared to NC, similar to the obtained for the second bioassay (crop 2) (Table 2 and Figure 2).

Parameters	Crop 1	Crop 2	Method
Arsenic (mg/L)	<LQ	<LQ	SM21 3120 B
Barium (mg/L)	0.41	<LQ	SM21 3120 B
Cadmium (mg/L)	<LQ	<LQ	SM21 3120 B
Lead (mg/L)	<LQ	<LQ	SM21 3120 B
Copper (mg/L)	0.35	0.76	SM21 3120 B
Chromium (mg/L)	0.04	3.56	SM21 3120 B
Total sulfur (mg/L)	1219	1681	SM21 3120 B
Mercury (mg/L)	0.0019	<LQ	EPA 7470 A
Molybdenum (mg/L)	0.008	<LQ	SM21 3120 B
Nickel (mg/L)	0.03	<LQ	SM21 3120 B
Selenium (mg/L)	<LQ	<LQ	SM21 3120 B
Zinc (mg/L)	1.66	<LQ	SM21 3120 B
Ammonia NH3 (mg/L)	<LQ	<LQ	USEPA 4405-55-001
Calcium (mg/L)	719	671	SM21 3120 B
Electrical conductivity (µS/cm)	13530	15110	SM21 2510 B
BOD (mg/L)	5046	7941	SM21 5210 B
COD (mg/L)	13380	25,225	SM21 5220 B
Hardness (mg CaCO3/L)	2493	276	
Total phosphate (mg/L)	1.3	207	SM21 4500-P C
Magnesium (mg/L)	237	264	SM21 3120 B
Nitrate (mg/L)	1.3	1.49	SM21 4500-NO2F
Nitrite (mg/L)	0.008	0.033	SM21 4500-NO2B
Ammoniacal nitrogen (mg/Kg)	276	-	SM21 4500-H+B
Kjeldahl nitrogen (mg/Kg)	267	171	SM21 3120 B
pH (mg/L)	3.9	4.37	SM21 4500NORG 3
Potassium (mg/L)	2056	3401	SM21 3120 B
Sodium (mg/L)	50.2	114	SM21 3120 B
Sulfate (mg/L)	710	2993	SM21 4500 SO4 E
Non-filterable Residue (mg/L)	2765	1800	SM21 2540 D

Table 1: Physico-chemical analysis of raw vinasse.

LQ. limit of quantification; BOD. Biochemical Oxygen Demand; COD. Chemical Oxygen Demand; EPA. Environmental Protection Agency; SM21. Standard methods for the examination of water and wastewater 21:2005; USEPA. United States Environmental Protection Agency.

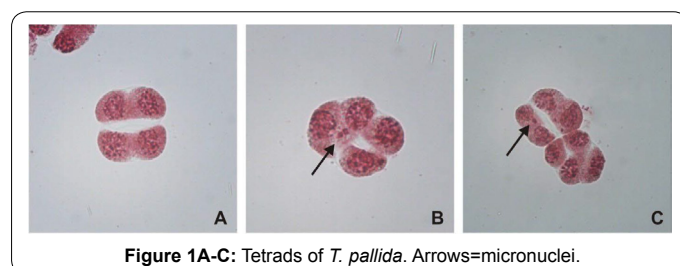


Figure 1A-C: Tetrads of *T. pallida*. Arrows=micronuclei.

	Crop 1	Crop 2
NC	1.9 ± 1.52	1.4 ± 1.35
PC	7.1 ± 2.99*	9.7 ± 4.69*
RV	4.8 ± 3.46*	6.7 ± 4.32*
C1	4.7 ± 2.06*	4.3 ± 2.50*
C2	2.2 ± 2.35	1.9 ± 2.10
C3	1.2 ± 1.13	0.8 ± 0.92

Table 2: Mutagenicity frequency (average and standard deviation) in tetrads of *T. pallida* after exposure to different dilutions of vinasse. (NC. Negative control; PC. Positive control-MMS; RV. Raw vinasse; C1. vinasse diluted to 50%; C2. vinasse diluted to 25%; C3. vinasse diluted to 12.5%. *Statistically significant).

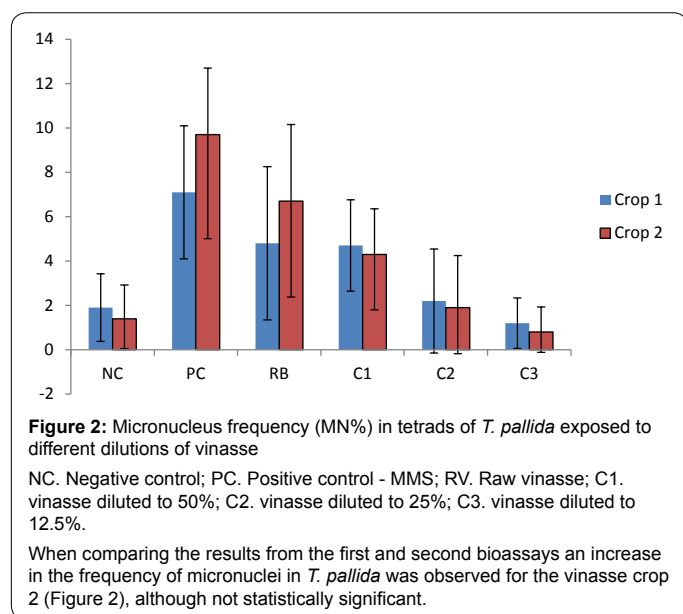


Figure 2: Micronucleus frequency (MN%) in tetrads of *T. pallida* exposed to different dilutions of vinasse

NC. Negative control; PC. Positive control - MMS; RV. Raw vinasse; C1. vinasse diluted to 50%; C2. vinasse diluted to 25%; C3. vinasse diluted to 12.5%.

When comparing the results from the first and second bioassays an increase in the frequency of micronuclei in *T. pallida* was observed for the vinasse crop 2 (Figure 2), although not statistically significant.

Discussion

The analysis of micronuclei in different organisms is a widely used method for the assessment of the mutagenic potential. A compound capable of inducing the formation of micronuclei can be considered a clastogenic or aneugenic agent. The clastogenic action of a compound can be demonstrated by the presence of micronuclei resulted from chromosome breaks during cell division. The aneugenic action, on the other hand, occurs when the mitotic fuse is inactivated, resulting in the loss of entire chromosomes, which are absent in the main nucleus of the cell.

Yamamoto and Kikuchi [11] demonstrated that the type of action of the mutagenic agent, clastogenic or aneugenic, could be determined based on micronucleus size. For these authors, clastogenic agents induced small MN, while aneugenic compounds, larger MN. Although the method was satisfactory for several bioassays, it was not reliable in many cases. Combes et al. [12] concluded that micronucleus size was not enough to determine the clastogenic and aneugenic potential of a compound. Fenech [13] reported that because the size of chromosomes in human cells and other cell types are heterogeneous, the correlation between micronucleus size and type of action of the mutagenic agent is unfeasible. For this author, a small micronucleus can be a fragment from a large chromosome or an entire small chromosome.

The results obtained in this study showed that the vinasse samples tested had a mutagenic potential however, it was not possible to discriminate aneugenic and clastogenic modes of action, as MN of

various sizes were observed. These observations corroborate the statements of the aforementioned authors [12,13].

The vinasse is a residue produced in many countries from different raw materials such as sugarcane in South America, beets and fruits in Europe, corn in North America [14]. Thus, the chemical properties of the residue depends on the raw material (biomass) used for the production of ethanol [15]. The chemical composition of sugarcane vinasse also varies depending on the each plant used in the production of ethanol as well as the subsequent distillation process. For this feature, two samples of vinasse were used obtained from two different crops. Despite this feature, the results observed by Trad-MN test showed no statistically significant differences in the action of vinasse on the genetic material of the organism used when comparing the two samples. The chemical analysis showed some differences between the samples observed mainly in the quantification of potassium, calcium, magnesium, sodium, nitrate, kjeldahl nitrogen and the metals, barium, copper, chrome, nickel, and zinc. Because it is a complex mixture is not possible to say which of the detected elements were responsible for the induction of micronuclei observed in *T. pallida*.

In a series of studies on the role of certain nutrients in meiosis, Steffensen [16-18] reported that the presence of micronuclei in the microspores of *T. paludosa* was considered an indication of chromosome break. According to Xavier et al. [19], large quantities of phosphorus, calcium, and nitrogen present in certain compounds that may promote alterations in the genetic characteristics of plants. One could suggest that a synergistic effect of the elements found in vinasse could cause the observed changes. Also, according to Lerda [20] and Matsumoto et al. [21], this can also be due to the exposure of cells to cadmium, copper, lead, chrome, and nickel, which increase the clastogenic and aneugenic effect on certain plants. The chemical analysis of vinasse samples did not reveal large quantities of these elements. However in the crop 2, large quantities of chrome and phosphate were observed, which could have influenced the results.

Studies conducted by Souza et al. [22] using landfarming soil samples from an oil refinery, rich in PAH and metals, biorremediated with vinasse, demonstrated that the latter potentialized the clastogenic effect of the soil, possibly making metals available.

Genotoxicity studies using *A. cepa* as test system have demonstrated the genotoxic and mutagenic potential of vinasse combined with the soil. However, a decrease in mutagenic potential was observed after bioprocessing by millipedes [23]. Using the same test system, Pedro-Escher and Fontanetti demonstrated that samples of raw vinasse were mutagenic and genotoxic to the system tested. However, after dilution in water, the mutagenic effect was not observed. The results revealed that *T. pallida* was more sensitive than *A. cepa*, supporting the importance of an application of different tests of genotoxicity and mutagenicity to determine the mutagenic action of different agents.

It should be pointed out that the application of this technique reduces the time needed to perform toxicological assays based on conventional chronic exposure and is sensitive to even substances present in low concentrations. Preliminary genotoxicity and mutagenicity studies could be applied to determine the toxicity of different products, as they can identify the effects of excessive use of vinasse, and permanent damage to DNA can be passed on to future generations. This study points to greater care in the use of this waste as fertilizer in agricultural fields. Although this practice is economically viable, the indiscriminate use of this residue in the soil can cause damage to DNA from different organisms.

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