EVALUATION OF THE MUTAGENIC POTENTIAL OF EMISSIONS GENERATED IN THE COMBUSTION OF GLYCERIN AS AN ALTERNATIVE FUEL

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Abstract. *This paper evaluates the use of crude glycerin, a by-product of the manufacture of a Brazilian biodiesel, as a sustainable alternative of energy generation. The mutagenic effects from emissions generated by the direct combustion of glycerin and diesel in a flame tube furnace were evaluated using the Trad-SH assay. The emissions from the combustion tests, such as sulphur dioxide (SO_2), nitrogen oxides (NO_X), total hydrocarbons (THC), carbon monoxide (CO) and carbon dioxide (CO_2) were quantified through gases analyzers. Tradescantia plants were exposed to those emissions in a fumigation chamber for 30-40 min. The analysis of variance (ANOVA) and the Tukey test were used to compare differences between six test groups (three intoxicated with emissions from glycerin combustion and three from diesel combustion) and one non-intoxicated control group (resuting from the merger of three similar control samples). Due to the complexity of the process, the groups were not test in replicate, but each groups was composed by 10 flower samples. Comparing the six groups, only one glycerin group showed statistical differences, presenting a higher mutation rate. However, the difference between the mutagenic events in glycerin tests may be due to the complexity of the burning process and also to the impurities present in the material. The results have showed that the crude glycerin has a mutagenic potential similar to that of diesel and may be used to replace it without causing further damage to the organisms.*

Keywords*: glycerin, diesel, combustion emissions,* Tradescantia*, Trad-SH.*

1. NOMENCLATURE

The following symbols were used in the statistical analysis of the results.

CV	- coefficient of variation	s^2	- variance
DL	- degree of liberty	SE	- standard error (Tukey test)
F	- statistics of ANOVA	SQ	- sum of squares
k	- number of groups or treatments	y	- quantitative variable
MQ	- Mean squares	$\bar{y}$	- average
n	- number of repetitions	*Greek Symbols*	
q	- statistics of Tukey test	α	- significance level
s	- standard deviation	Σ	- sum

2. INTRODUCTION

In comparison with traditional methods, the use of biosensor plants – plants that respond to pollutants with no visible effects, presenting molecular, cellular, physiological and biochemical alterations (De Temmerman et al., 2004) – to evaluate pollution levels has presented additional advantages, such as low cost of installation, absence of sophisticated instruments for measurement, efficiency in the monitoring of large areas and for long periods of time, and viability to evaluate chemical elements present in low concentrations (Guimarães et al., 2000; Carreras and Pignata, 2001).

Although the genotoxic effects observed in plants cannot be extrapolated directly to human populations, the results of experiments with plants can be taken into consideration for the analysis of atmospheres, due to the high sensitivity of these organisms, even in situations of low air contamination levels. Therefore, it is possible to affirm that a pollutant that does not present any detectable damage to the most sensitive species of plants will not affect other organisms, including humans (Guimarães et al., 2000).

The *Tradescantia* stamen hair assay has been one of the most appropriate tests to detect genetic effects of chemicals and ionizing radiation and study of variations in the frequencies of spontaneous somatic mutations (Imai et al., 1991; Ma et al., 1994; Ichikawa and Wushur, 2000). The assay is also highly efficient to determine the genotoxicity of liquid and gaseous environmental agents (Ma et al., 1996; Mohammed and Ma, 1999).

The Trad-SH assay was initially applied to evaluate the genetic effects induced by nuclear radiation (Nayar and Sparrow, 1967; Sparrow et al., 1972) and subsequently adapted to detect mutagenic agents carried in the air and volatile organic compounds and then to evaluate chemical agents in liquid form (Ma et al., 1994). In humans a tiny fraction of DNA lesions may or may not induce mutations, but in *Tradescantia*, the great majority of lesions result in mutations. Thus, an increase in the initial damage frequency (mutations) in Tradescantia may be indicative of mutation occurrence in humans in the same proportion (Ichikawa, 1992). Once Tradescantia has high sensibility to chemical and physical environmental agents, the plants need only to be exposed to a contaminated environment to produce some effects, with no need of collecting, concentrating or altering the air composition.

Due to many inherent characteristics, the Trad-SH assay is considered one of the most viable assays to detect mutations that either occur spontaneously or are induced by low concentrations or doses of toxic agents (Underbrink et al., 1973; Takahashi & Ichikawa, 1976). The utility of this biosensor has been tested by rigorous environmental and laboratorial experiments (Van't Hof and Schairer, 1982).

The atmosphere in an urban area is usually contaminated by pollutants from stationary and mobile sources, mainly from the combustion of fuels. The effects of urban pollution have been long studied with the use of biomonitors, and the Trad-SH has been a good alternative to delimit mutagenic risks in contaminated environments (Guimarães et al., 2000; Ferreira et al., 2007).

In the late 90s, BNL 4430 *Tradescantia* clone was used to analyze the conditions of Sao Paulo city. However, several authors have confirmed that the Trad-SH assay using clone KU20 inflorescences is a better alternative as an urban biosensor for toxicity analysis, since it presents better response and greater sensibility to the toxicity of atmospheric gases, in comparison to BNL 4430 clone (Ferreira et al., 2007).

By cultivating KU20 clone in Caucaia do Alto and São Paulo, Guimarães et al. (2000) observed that mutations in the stem hairs increased rapidly and significantly when inflorescences were subjected to polluted urban atmosphere. They also observed that regardless of the samples being cultivated in situ or in a non-polluted place, KU20 clone did not show any adaptation to the urban environment that might reduce the mutagenic responses. Therefore the Trad-SH assay with KU20 clone proved more advantageous than the micronucleus assay (Trad-MCN) with *Tradescantia pallida*, which becomes less sensitive when grown in contaminated places (Guimarães et al., 2000; Guimarães et al., 2004).

Among the air pollutants generated by the combustion of fossil fuels, SO_2 is considered one of the most toxic to plants and can be absorbed by both roots and stomata. Nitrogen oxides (NO_X) are also potentially toxic because they are absorbed by the stomata and inside the leaves, react with water and are dissociated into nitrate and nitrite, acidifying the internal spaces. Hydrocarbons present in the emissions must also be considered components of high mutagenic potential (Freedman, 1995).

The global demand for energy has been increasing and the only way to supply this need is using alternative fuels. The production of biodiesel from raw vegetable or animal material inevitably results in the co-production of glycerin (1,2,3-propanetriol), also known as glycerol. Due to its properties, glycerin is commonly used by food, cosmetic and pharmaceutical industries (Carmines and Gaworski, 2005; McNeil et al., 2011). Approximately 100 kg of glycerin are produced per ton of biodiesel, and the industries absorption capacity is limited, leading to a search for new ways of using the compound (McNeil et al., 2011). Many authors have worked with glycerin as fuel, proving the possibility of employing it for this purpose. However, the use of alternative fuels requires studies to quantify their toxic effects to the environment and humans and compare them with those traditionally used.

From this perspective, this paper verifies the importance of the use of crude glycerin, a co-product of the manufacturing process of biodiesel, as an alternative and sustainable energy source. It is essential to evaluate its mutagenic risk in comparison with the fuels traditionally used on an industrial scale, such as diesel. In this study, the emissions from the combustion of diesel and crude glycerin are evaluated and the Trad-SH test, using KU20 clones of *Tradescantia*, is applied to evaluate the ability of the fuels to induce mutations.

3. MATERIALS AND METHODS

3.1 Materials

A crude glycerin from bovine tallow, a by-product from the manufacture of a Brazilian biodiesel, and a Brazilian commercial diesel were used in this study. Table 1 shows the chemical composition of the fuels.

Table 1. Ultimate analysis and high calorific value of crude glycerin and diesel.

Analysis as received	*Crude glycerin*[(i)]	*Diesel*[(ii)]
Ultimate analysis		

Carbon (%)	50.3	86.5
Hydrogen (%)	9.7	13.2
Oxygen (%)	37.4	0.3
Nitrogen (%)	2.6	-
High heating value (MJ/kg)	25.5	45.19

[(i)]Crnkovic et al., 2012; [(ii)] Rose and Cooper, 1977.

3.2 Gases Emitted from the Burning of Fuels

A flamotubular calorimetric furnace composed of twelve combustion chambers in four modules was used for the fuels combustion. Each module is 1 meter long and constituted by a central tube of 305 mm internal diameter, superimposed by another tube with internal diameter of 415 mm, forming a 55-mm thick chamber of water flow between the two tubes. The module is individually constituted by three calorimetric chambers of 328 mm length. Each chamber is crossed by two tubes of 25 mm internal diameter and has two tubes of 19 mm internal diameter soldered in their inferior and superior parts. The concentration of the emitted gases (O_2, CO_2, CO, THC, NO_X and SO_2) were measured in analyzers Horiba Enda 1400 and Tecnomotor.

3.3 Mounting of the Intoxication System

For the intoxication tests, the stems were picked in the yard of NETeF some hours before intoxication and kept in aeration systems. The test groups were placed in a fumigation bottle with emissions from the burning of the fuels (diesel or crude glycerin). They were subjected to intoxication for 30-40 min (according to the duration of the combustion) and then placed in the aeration systems again, remaining under this condition for approximately four days, while the flowers were opening.

The combustion tests were performed in a flame tube furnace in three stages, and on the same day two combustion tests were conducted – one with diesel and one with glycerin. For each pair of tests (diesel and glycerin) one control group was evaluated (non-intoxicated samples of *Tradescantia*), and comparing the three control groups no significant statistical differences were found. A control medium (C_M) was obtained from the average of the three individual controls of each pair of tests in order to compare the mutagenic responses of the samples with the natural variation observed in the environment.

From the three tests a total of seven groups was obtained – six intoxicated groups, resulting from the intoxication with diesel emissions (D1, D2 and D3) and crude glycerin (G1, G2 and G3) and a control group (C_M).

3.2 Planting of KU-20 Clone Biosensor of *Tradescantia*

For this study the KU-20 clones of *Tradescantia* were cultivated in a flower bed in the yard of NETeF – USP. Although it is a place with vehicle circulation, the tests were not unviable, once according to Guimarães et al. (2004), KU-20 clones do not show adaptations to the polluted environment that protects the plants. Even the clones cultivated in a polluted place do not suffer intense saturation, which might interfere in the response to the intoxication. Therefore, the cultivation of the control and test groups in the same environment, even if it is not free from pollutants, provides a reliable analysis.

3.4 Estimate of Number of Stamen Hairs

A sample with more than 30 flowers randomly picked was used to estimate the average of stamen hairs per flower. Three stamens were removed and the plates were prepared as in the analysis. Using a microscope, the number of hairs in each stamen was counted. Each flower had six stamens, therefore the results were multiplied by 2 and the average number of hairs per flower was obtained, considering the total number of samples used.

For this estimate 33 flowers were used and an average of 368 stamen hairs/flower was obtained. This average number was considered in the analysis of the results, once the mutagenic events are expressed in terms of mutations/1000 stamen hairs.

3.5 Analysis of Mutagenic Events

In the *Tradescantia* stamen hair mutation assay (Trad-SH), the main cells are the mitotic cells of the stamen hair under development in young inflorescences. The assay is based on the coloration heterozygosis of cells (dominant character = “blue” and recessive character = “pink”). Once all the cells have an allele to the blue color and another to the pink color, a simple mutation causes a phenotypic alteration of easy perception (Nayar and Sparrow, 1967; Ma et al., 1994; Rodrigues et al., 1997).

For the analysis of stamen hairs, the plates are prepared by first removing the anthers and pistils and then the six stamens, one by one. Each stamen is placed on a plate with a drop of water. Using a colony counter and a pair of needles to comb the hairs of each stamen, they are aligned to facilitate the analysis. After the preparation of the plates, they are taken to the microscope and observed in the least magnification against a white background to reveal the true color of the cells.

The cells of a stamen hair originate from a single epidermal cell of the filament. All the cells in each hair are generated by mitosis from the apical or sub apical cells, therefore a pink mutant cell may divide repeatedly and give rise to a string of pink cells. This is considered a single mutation event, once it originates from a single mutation. Two or more pink mutant cells separated by blue normal cells are considered distinct mutagenic events.

After the counting of the mutagenic events, the results must be expressed in terms of mutant events per 1000 stamen hairs, i.e., the result must be multiplied by 1000 and divided by the average of number of stamen hairs per flower.

3.6 Statistical Analysis of Data

The Statistical Analysis was performed using the tool of excel Data Analysis. The significance level was adjusted to 5%. The analysis of variance (ANOVA) was applied to test for significant differences between the analyzed groups. The analysis of multiple comparisons (Tukey test) was used to verify if there were significant differences between the averages of the mutation frequencies observed in the studied groups. The procedure described by Callegari-Jacques (2003) was used for the Tukey test.

4. RESULTS AND DISCUSSION

A total of three pairs of tests were performed and the experimental conditions adopted and the average results of the emissions generated are presented in

Table 2.

Table 2. Experimental conditions adopted in the combustion tests with diesel and crude glycerin.

Experimental Data		*Test 1*		*Test 2*		*Test 3*	
		D1	*G1*	*D2*	*G2*	*D3*	*G3*
Process Parameters	*Average Temperature (°C)*	763.4	736.7	1100.3	961.60	966.30	863.50
	Equivalence Ratio (λ)	1.61	1.92	1.15	1.14	1.23	1.23
Emissions Generated	*Concentration of O_2 (%)*	7.54	9.47	2.50	2.10	3.96	3.98
	Concentration of CO_2 (%)	8.90	7.71	11.82	13.07	10.98	11.33
	Concentration of CO (ppm)	76.00	39.00	410.00	344.00	163.00	109.00
	Concentration of THC (ppm)	23.00	17.00	96.00	104.00	71.00	28.00
	Concentration of NO_X (ppm)	81.00	8.00	92.00	47.00	102.00	31.00
	Concentration of SO_2 (ppm)	58.00	16.00	93.00	20.00	77.00	32.00

Two process variables were evaluated, i.e., equivalence ratio and average temperature of combustion.
The equivalence ratio (λ) is defined by equation

$$\lambda = \frac{AF_{act}}{AF_{Th}} \tag{1}$$

where AF_{act} is the actual air-fuel ratio and AF_{Th} is the theoretical air-fuel ratio.

The air–fuel ratio (AF) is determined by equation

$$AF = \frac{m_{air}}{m_{fuel}} \tag{2}$$

where m_{air} is the mass of the air and m_{fuel} is the mass of the fuel.

According to Table 2, when the equivalence ratio (λ) increases, the temperature decreases. This result was expected because with the increase of λ, there is an increased amount of air mass entering the process, particularly N_2

present in higher amount than O_2, reducing the flame temperature and considerably the heat exchange and the radiation efficiency of the equipment. Regarding the combustion reaction gases (O_2, CO_2, CO), in function of the increase of ʎ, there is an increase in O_2, and a decrease in CO_2 and CO, characterizing a better combustion. In the THC concentrations there was a decrease with ʎ increasing. The emission of SO_2 and NO_X did not show a default behavior with ʎ variation.

The results of the mutagenic responses of the groups subjected to gaseous emissions from the combustion of diesel and glycerin are shown in Table 3. Figure 1 presents the average and standard deviation of all groups, and the rates of mutations, measured by the number of pink cells, are expressed in terms of mutations/1000 stamen hairs.

Table 3. Summary Table – comparison between groups intoxicated with gaseous emissions from diesel and glycerin and control group.

Groups	*Intoxication time (min)*	*n*	Σ	$\bar{y}$	s^2	*s*	*CV*
D1	30	10	189.57	18.96	11.23	3.35	0.18
G1	30	10	196.68	19.67	15.04	3.88	0.20
D2	30	10	236.97	23.7	14.97	3.87	0.16
G2	34	10	222.75	22.27	10.23	3.2	0.14
D3	40	10	218.01	21.8	7.24	2.69	0.12
G3	42	10	341.23	34.12	11.48	3.39	0.10
C_M	0	10	54.5	5.45	5.47	2.34	0.43

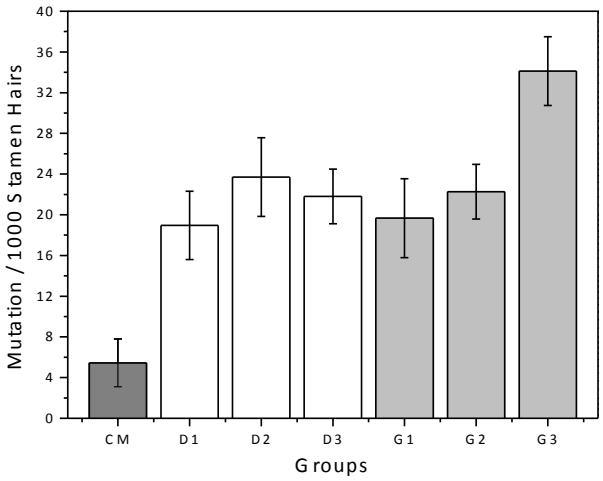

Figure 1. Average values and respective standard deviations of mutation frequency in stamen hairs of clone KU-20 found in all groups.

The analysis of variance (ANOVA) was applied in order to statistically compare the samples and the results are presented in Table 4. According to Callegari-Jaques (2003), ANOVA is a robust statistical method wich provides reliable results even with considerable heteroscedasticity, since the sample sizes are similar, as in this study.

Table 4. Analysis of variance (ANOVA) of the intoxicated and control groups.
(Critical value of F in the significance level of 5%).

Variation source	*SQ*	*DL*	*MQ*	F_{STAT}	*p-value*	F_{TABLE}
Between groups	4293.48	6	715.58	66.2	2.56×10^{-25}	2.25
Inside groups	680.95	3	10.81			
Total	4974.43	9				

Based on the ANOVA it is possible to either accept or reject a null hypothesis of the groups' similarity from the calculation of F (symbol of the statistical test of comparison of variances). When the F stat (calculated) is higher than the F table, the null hypothesis is rejected and the statistics determines that there is no difference between the groups. The ANOVA also implies that when the p-value is below the significance level, the null hypothesis is rejected. Table 4 shows that the F_{STAT} (66.2) is superior to the F_{TABLE} (2.25) and p-value is higher than the significance level (0.05 or 5%).

The Tukey test was applied to determine which samples differ from each other statistically. Table 5 shows the data of the Tukey analysis.

Table 5. Application of Tukey test.

Treatment	*G3*	*D2*	*G2*	*D3*	*G1*	*D1*	C_M
Average	34.12	23.7	22.27	21.8	19.67	18.96	5.45
n	10	10	10	10	10	10	10

MQ (inside)= 12.367		
k	7	q_{TABLE} = 4.310
DL(inside)	63	SE = 1.040

Group 1 × Group 2	$\bar{y}_1 - \bar{y}_2$	q_{STAT}	q_{STAT} - q_{TABLE}	*Analysis*
G3 × D2	10.427	9.376	5,72	Averages differ
G3 × G2	11.848	10.654	7,09	Averages differ
G3 × D3	12.322	11.08	7,54	Averages differ
G3 × G1	14.455	12.998	9,59	Averages differ
G3 × D1	15.166	13.637	10,28	Averages differ
G3 × CM	28.673	25.783	23,27	Averages differ
D2 × G2	1.422	1.279	- 2,94	-
D2 × D3	1.896	1.705	- 2,49	-
D2 × G1	4.028	3.622	- 0,44	-
D2 × D1	4.739	4.262	0,25	Averages differ
D2 × CM	18246	16.407	13,24	Averages differ
G2 × D3	0.474	0.426	- 3,85	-
G2 × G1	2.607	2.344	- 1,80	-
G2 × D1	3.318	2.983	- 1,12	-
G2 × CM	16.825	15.129	11,87	Averages differ
D3 × G1	2.133	1,918	- 2,26	-
D3 × D1	2.844	2.557	- 1,58	-
D3 × CM	16.351	14.703	11,42	Averages differ
G1 × D1	0.711	0.639	- 3,63	-
G1 × CM	14.218	12.785	9,37	Averages differ
D1 × CM	13.507	12.146	8,68	Averages differ

$q_{STAT} > q_{TABLE}$ → (+) Averages differ.
$q_{STAT} < q_{TABLE}$ → (–) Averages do not differ.

The Tukey test (Table 5) showed that among the intoxicated groups, only group G3 has an average of pink mutations that differs from the average of the other groups. As expected, all samples intoxicated with gaseous emissions generated by diesel and crude glycerin burning have showed a higher average frequency of mutagenic events than the control group.

Evaluating the diesel emissions, group D2 has shown a mutation rate statistically higher than that of group D1, however both rates are similar to that of D3. According to Table 2, group D2 was subjected to gaseous concentrations higher than that observed in D1, but very close to that of D3, except the concentration of CO, which was higher than that of all other groups. Since the different groups (D1 and D2) are similar to D3, it has been assumed that the difference between them occurred due to a natural variation in both gases composition and plants physiology.

Analyzing the groups intoxicated in the glycerin combustion, group G3 was subjected to the highest concentrations of SO_2 (Table 2) and longer exposure time (Table 3). Group G2 was subjected to the highest concentrations of CO, THC and NO_X, but showed an average of mutagenic events similar to that of G1 and lower than the one of G3. Comparing groups G1 and G2 with all the groups, the ones intoxicated with diesel emissions were subjected to higher concentrations of SO_2, and group D2, subjected to a higher concentration of CO, showed rates of pink mutations higher than that of group D1.

Group D3 was subjected to experimental conditions (temperature and ʎ) and exposure time to toxic emissions (40 min) close to the conditions adopted for G3. However, the mutagenic events of D3 are quite lower (4.7 times lower) than those of group G3 and similar to those of the other groups.

According to the literature, the gaseous emissions of NO_X, SO_2, CO and THC are considered toxic and mutation inductors (Clark and Vigil, 1980; Arey et al., 1992; Freedman, 1995; Grant and Briggs, 2002). The increase in the time of exposure to toxic emissions was also evaluated and showed evidences that it causes an increase in the number of pink mutations (Oliveira et al., 2007).

This study has not allowed concluding which variables caused more damage to group G3. However it is possible to state that glycerin is a product with impurities and a more heterogeneous composition in comparison to diesel.

Since the combustion system simulates a real machine, the process of burning fuel in a furnace is an unstable process, whose measurements are influenced by variations within the furnace during firing. This study has taken into consideration the difficulty and lack of details of the combustion process of crude glycerin, since the material's composition is not fully known.

6. CONCLUSIONS

The present study has evaluate the efficiency of the application of Trad-SH assay to determine a possible mutagenic potential of gases emitted by the burn of different fuels, by exposing seedlings in chambers of flux of gaseous agents.

Diesel and glycerin are fuels of considerable mutagenic effects, since both have showed an increase of DNA injury in plants higher than that observed in the control group.

Applying ANOVA and Tukey tests, only group G3 differed from the others, showing a superior rate of mutagenic events. Analyzing the experimental data of all groups, it was not possible to determine a variable that could explain the increase in the pink mutations frequency observed in group G3. Thus, it has been assumed that the combustion process of glycerin is difficult and complex, since the fuel has an unclear composition, and that some unidentified factor occurred exceptionally in this test (G3) without a recurrent character.

It can be stated that glycerin is a viable fuel option, since it is a waste material generated in the production of biodiesel and has potential to induce mutations similarly to diesel.

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