EVALUATION OF MUTAGENIC POTENTIAL FROM EMISSIONS OF DIRECT COMBUSTION OF DIESEL IN FLAME TUBE FURNACE THROUGH TRAD-SH ASSAY

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Abstract. *Emissions from combustion systems can cause serious risks to human health. Bioassays developed with bioindicators, as genus Tradescantia, are suitable to evaluate mutagenic risks. Tradescantia Stamen-Hair Assay (Trad-SH) is a tool for monitoring the risks imposed to organisms by mutagens. This method is based on the fact that the stamen hair cells of the plants are heterozygous for the color of stamen hair cells, making it possible to detect mutations of the basis of their pigmentation change from blue (dominant) to pink (recessive). This paper reports on the evaluation of mutagenic effects from diesel emissions generated by direct combustion in a flame tube furnace. The combustion test was performed using Brazilian conventional diesel as fuel in a fully instrumented flame tube furnace with continuous gas analyzer and other auxiliary equipment. A fumigation bottle containing Tradescantia samples was exposed to 0.5 L min^{-1} combustion gas line. The gas analysis report showed that the Tradescantia samples were exposed to CO, NO_x, SO_2, THC (total hydrocarbons), CO_2 and O_2 concentrations varying between 0 and 189 ppm, 0 and 14 ppm, 21 and 35 ppm, 0 and 14 ppm, 0.9% and 3.5%, 17.5% and 20.1%, respectively. After exposure to combustion gases both intoxicated (Test) and non-intoxicated (Control) inflorescences were kept in an aeration system. Fully-opened flowers were removed from the stem and the stamens were dissected out for the counting of the number of hairs per stamen. The variance analysis (ANOVA) was applied to compare differences between Control and Test groups. For statistic analysis a considerable increase was observed in the mutagenic events rate in intoxicated flowers, in comparison to the non-intoxicated ones. The difference among the groups was statistically significant ($p<0.001$), and the average numbers of mutant events per 1000 hairs were 46.48 in Group Test 1, 29.89 in Group Test 2 and 35.33 in Group Test 3. The Group Tests were 406.17% (Group Test 1), 261.24% (Group Test 2) and 308.74% (Group Test 3) higher than the Control Group.*

Keywords*: Tradescantia, clone KU-20, Diesel, Mutagenicity, TRAD-SH assay*

1. INTRODUCTION

Biomonitoring can be defined as an indirect experimental method to check the existence of pollutants in a certain area using live organisms that respond with modifications in their life cycle or accumulation of pollutants to stress. (CARRERAS; PIGNATA, 2001). These organisms are usually called bioindicators. When they respond to stress with the accumulation of substances in tissues, they are recognized as resistant organisms and called bioindicators of accumulation. When they are under morphologic, physiologic, genetic and ethologic alterations, they are considered sensitive organisms and called reaction bioindicators (KLUMPP et al.; 2001).

In humans only a minimal fraction of DNA injury may or may not provoke mutations. However, as in *Tradescantia* the great majority of injuries result in mutations, a determined increase in its initial harm (mutations) frequency can indicate the occurrence of mutations in humans in similar proportions (ISHIKAWA, 1992).

In relation to the traditional methods of pollution rates verification, the biomonitoring presents advantages, such as low cost of installation and accompaniment, frequent absence of sophisticated equipment for measurement, efficiency in the monitoring of wide areas and also in long-term monitoring, and viability to evaluate chemical elements present in low concentrations in the studied area (CARRERAS; PIGNATA, 2001).

According to Ma et al. (1994), the TRAD-SH assay was developed by Doctor Arnold H. Sparow and collaborators at the end of 50's and all the 60's. Initially these studies evaluated the genetic effects induced by nuclear radiation. Later the assay was adapted to detect mutagenic agents transported by air and volatile organic components, and then to evaluate the chemical agents in liquid form.

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 heterozygosis for the coloration in the cells and the dominant character "blue" color, and recessive character "rose". 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 (MA et al., 1994).

In an analysis of the mutagenic potential of contaminated atmosphere at Ibirapuera Park in São Paulo, Ferreira et al. (2007) demonstrated that KU-20 clone presents better responses to the mutagenicity of atmospheric gases. The results

with KU-20 clone of *Tradescantia* indicated that the atmosphere of Ibirapuera Park, frequently used by local population for leisure and sporting practices, is potentially mutagenic, while the BNL 4430 clone of *Tradescantia* does not present significant responses.

In this context, the present study aims to evaluate the effect of gases emitted from diesel combustion, estimating the somatic mutation frequency in stamen hairs of *Tradescantia* inflorescences.

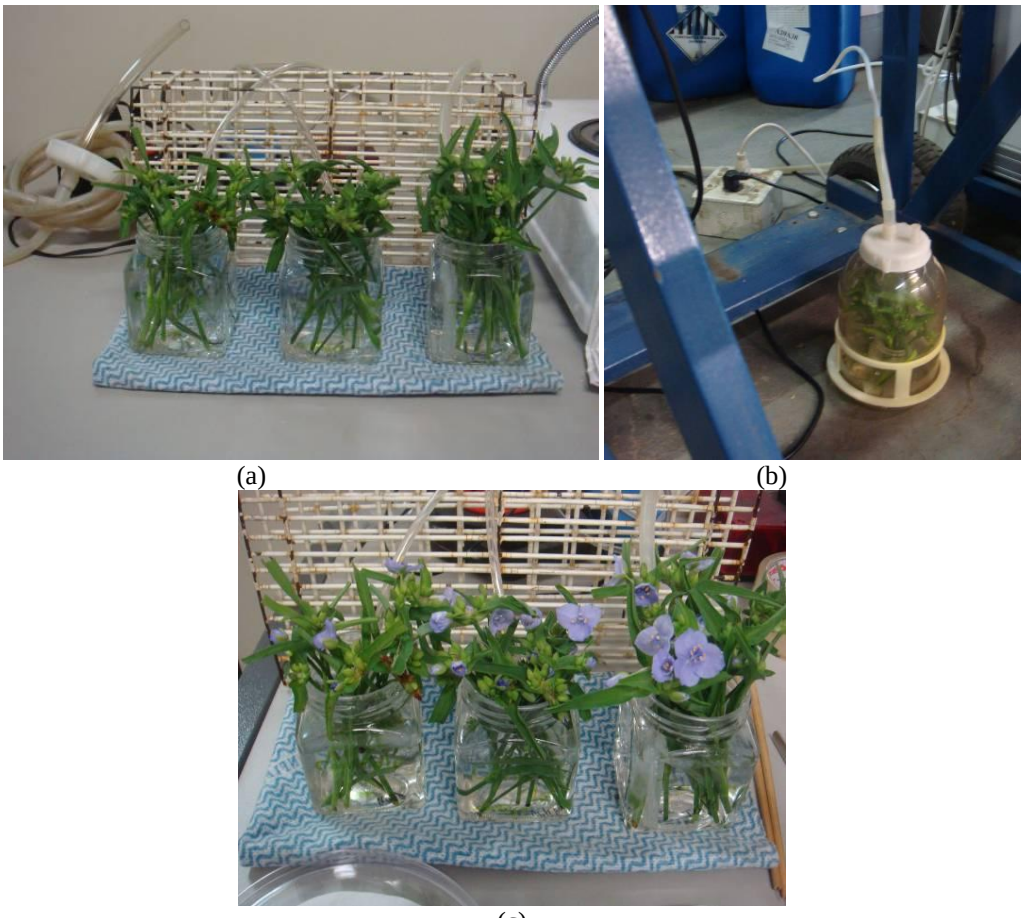
2. MATERIALS AND METHODS

2.1. 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 are not unviable, once, according to Sant'Anna (2003) KU-20 clones do not show adaptations to the polluted environment that protect the plants. Even the clones cultivated in a polluted local do not suffer intense saturation, which might interfere in the response to the intoxication. Therefore, cultivating the control and test groups in the same environment, even if it is not free from pollutants, provides a reliable analysis.

2.2. 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 (Fig. 1a). The group tests were placed in a fumigation bottle (Fig. 1b), where emissions from the burn of the fuel (diesel) of NETeF furnace circulated. They were subjected to intoxication for one hour, and then placed in the aeration systems again, remaining under this condition for approximately four days, while the flowers were opening (Fig. 1c).



(a) (b)

(c)

Figure 1: Intoxication of group test: (a) sample (stems) prepared and placed in the aeration systems before test; (b) sample in fumigation bottle subjected to emissions of diesel burn in furnace and (c) flowers open before dissection and analysis.

2.3. Analysis of Procedure of Mutagenic Events

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 (Fig. 2). 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 derived by mitosis from the apical or subapical 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 (Fig. 2)

After the counting of 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.

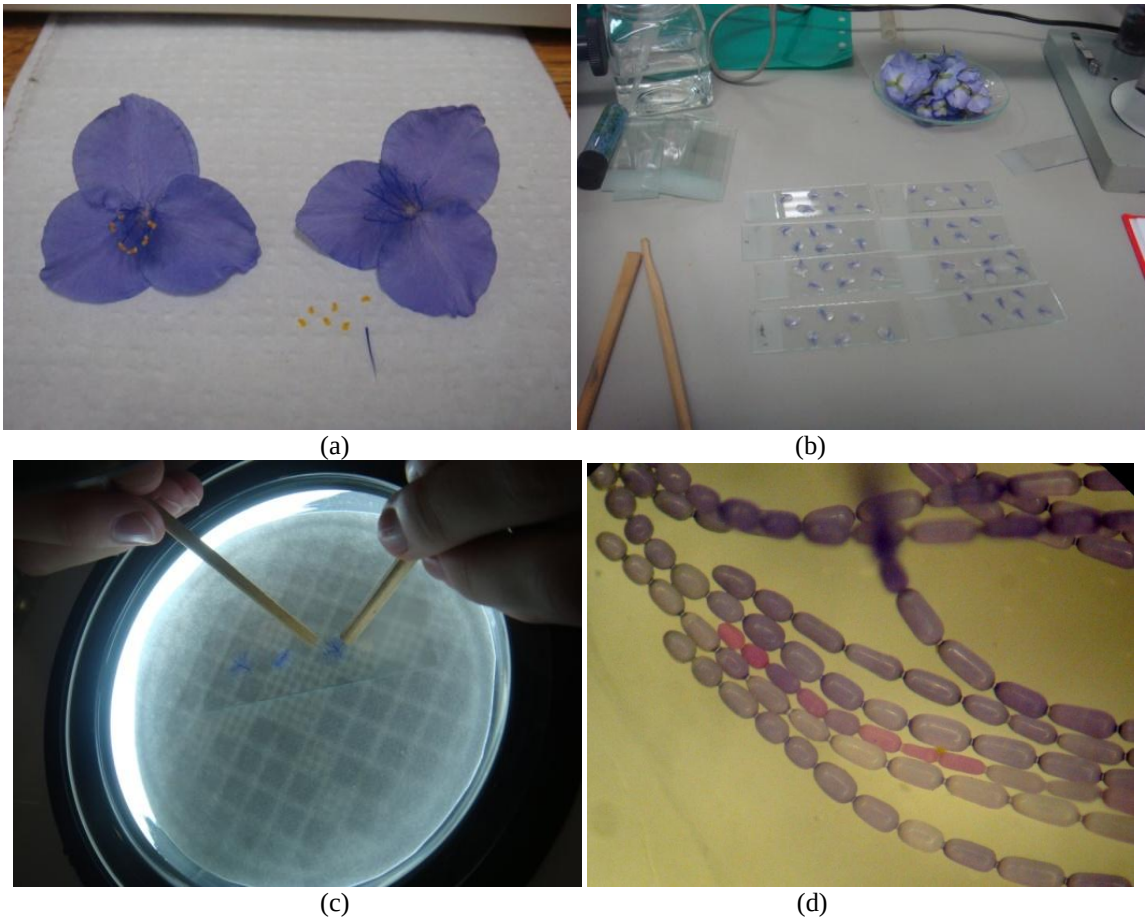


Figure 2: (a) Removal of anthers and pistils; (b) plate preparation; (c) alignment of the hairs for analysis and (d) Mutagenic cells (pink) in stamen hairs.

2.4. Estimate of Number of Stamen Hairs

To estimate the average of stamen hairs per flower a sample with more than 30 flowers randomly picked was used. 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, so the results were multiplied by 2 and the average number of hairs per flower was obtained, considering the number total 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 results, once the mutagenic events are expressed in terms of mutations/1000 stamen hairs.

2.5. 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 multiple comparison (Tukey test) was used to verify if there were significant differences between

the averages of mutation frequencies observed in the studied groups. The procedure described by Callegari-Jacques (2003) was used for the Tukey test.

In the statistical analysis of the results obtained in this study, the following symbology was used.

C	- term of correction C
CV	- coefficient of variation
SE	- standard error (Tukey test)
dl;DL	- degree of liberty
H_0	- hypothesis of nullity
H_1	- alternative hypothesis
k	- number of groups or treatments
n	- number of repetitions
q	- statistics of Tukey test
r	- residue
R^2	- coefficient of determination
s	- standard deviation
s^2	- variance
SRQ	- sum of residual quadrate
STQ	- sum of total quadrates
STrQ	- sum of treatment quadrates (or between then)
t	- statistics of Student t test
y	- quantitative variable
$\bar{y}$	- average
α	- significance level
Σ	- sum

2.6. Sample Intoxication by Gases Emitted from Diesel Burn

A flamotubular calorimetric furnace composed of twelve combustion chambers in four modules was used (Fig. 3). 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 chamber of water flow of 55 mm thickness 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 both inferior and superior parts of the chamber.

Figure 3: Furnace completely instrumented (data provided by Aymer Y. M. Cordoba).

3. RESULTS AND DISCUSSIONS

Three tests of diesel burn were performed in the NETeF furnace under the experimental conditions presented in Tab. 1. The concentrations of O_2, CO_2, CO, THC (Total Hydrocarbon), NO_x and SO_2 emissions were measured in gases analyzers.

Table 1: Experimental conditions adopted in the diesel burn tests in the NETeF furnace.

Experimental Data	Tests		
	T1	T2	T3
Date	17/3/2010	7/4/2010	29/4/2010
Maximum Temperature (^oC)	1112	1022	1256
Medium Temperature (^oC)	989	936	1200
Air Mean Flow in entry (kg/h)	76.9	82.0	73.0
Air/Fuel Relation (kg_{Air}/kg_{Fuel})	17.0	16.1	18.7
Concentration of O_2 (%)	7.49	7.65	3.93
Concentration of CO_2 (%)	13.09	11.37	13.15
Concentration of CO (ppm)	104.69	52.43	125.63
Concentration of THC (ppm)	6.77	0.15	0.99
Concentration of NO_x (ppm)	17.62	0.00	3.21
Concentration of SO_2 (ppm)	13.85	18.33	30.13

Figure 4 presents the profile of emission concentrations generated during the diesel burn for test T1 in function of time.

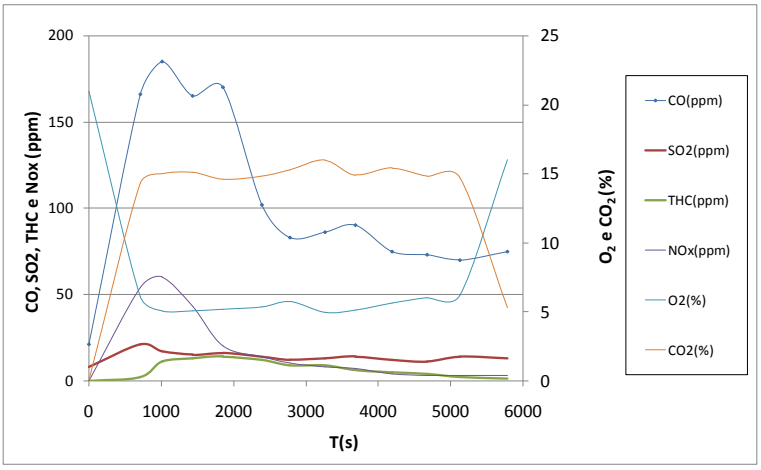


Figure 4: Profile of concentrations of emissions generated in test T1 in function of time.

In the concentration of CO_2 and O_2 gases shown in Fig. 4, it is possible to observe that the system starts a permanent regimen at approximately 1000 s, remaining under this condition until 3000 s. The emissions data described in Tab. 1 are medium values during the period in which the system remained in permanent regimen.

As intoxication tests were performed at different dates, each Group Test had a corresponding Control Group analyzed simultaneously with the intoxicated group to disperse the mutation rate corresponding to the environment where the clones were picked.

To compare the intoxicated groups with a single control, all the three controls were added (CT), and the Analysis of variance (ANOVA) was proceeded to verify if the CT group was not significantly different from the individual control groups (CT1, CT2 and CT3) for the mutation frequencies.

The results of the descriptive analysis are presented in Tab. 2. The average and standard deviation are graphically presented in Fig. 5. Table 3 presents the analysis of variance (ANOVA).

Table 2: Comparison between control groups.

Control Groups	n	Sum	$\bar{y}$	s^2	s	CV
CT1	11	176.66	16.06	132.15	11.50	0.72
CT2	26	233.70	8.99	50.08	7.08	0.79
CT3	20	241.85	12.09	32.24	5.68	0.47
CT (CT1+CT2+CT3)	57	652.20	11.44	64.03	8.00	0.70

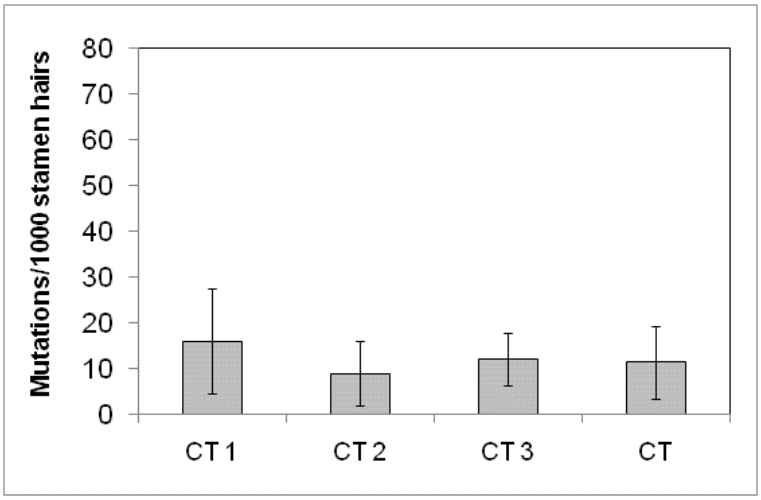


Figure 5: Medium values and respective standard deviation of mutation frequencies in stamen hairs of *Tradescantia* KU-20 inflorescences found in the four control groups.

In Tab. 2 and Fig. 5 it is possible to observe that the control CT1 shows more mutagenic events. However this group also shows the highest dispersion, possibly due to the smaller quantity of samples in relation to the other control groups.

Table 3: ANOVA of comparative experiments between the control groups (Critical value of F at the significance level 5%)

Variation source	SQ	DL	MQ	$F_{calculated}$	p-value	$F_{critical}$
Between groups	399.58	3	133.19	2.16	0.10	2.69
Inside groups	6771.51	110	61.56			
Total	7171.09	113				

Obs.: $R^2 \cong$ 5.57 % of total variation is explained by the variation in the treatment

Table 3 shows that $F_{calculated}$ is lower than $F_{critical}$, and the p-value is higher than the established significance level (0.05 or 5%). Therefore we assumed that are no significant statistical differences between the control groups. It is also possible to verify from R^2 that only 5.57% of the total variation are explained by the variation in treatments, therefore the average of the control groups can be used to compare the three test groups.

After performing the three intoxication tests by diesel burn emissions the mutagenic events of all samples (opened flowers) were counted. The three test groups and the control group were compared using ANOVA. The results are presented in Tabs. 4 and 5 and Fig. 6.

Table 4: Summary Table: comparison between intoxicated groups.

Groups	n	Sum	$\bar{y}$	s^2	s	CV
CT	57	652.20	11.44	64.03	8.00	0.70
T1	10	464.75	46.48	470.20	21.68	0.47
T2	35	1046.20	29.89	30.84	5.55	0.19
T3	20	706.52	35.33	52.86	7.27	0.21

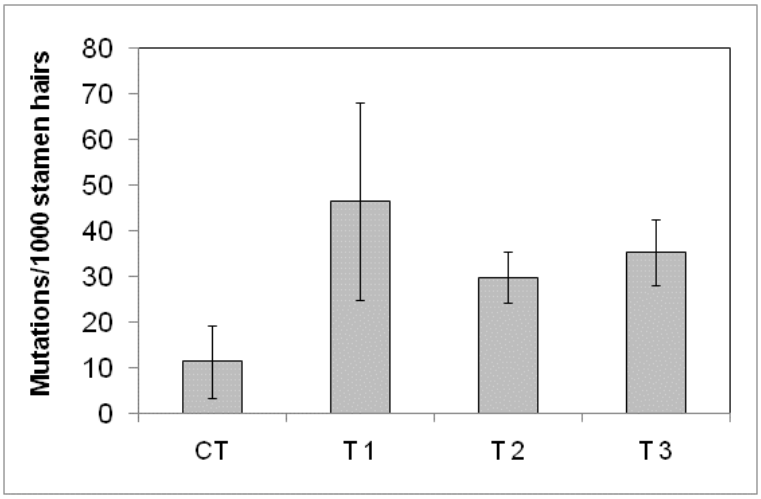


Figure 6: Medium values and respective standard deviation of mutation frequencies in stamen hairs of *Tradescantia* KU-20 inflorescences found in the analyzed groups.

Table 1 and Figure 6 show that test T1 presents the highest values of mutagenic events and also the largest dispersion. This group also contains smaller quantities of samples.

Table 5: ANOVA of comparative experiments between the intoxicated groups (Critical value of F at the significance level 5%).

Variation source	SQ	DL	MQ	$F_{calculated}$	p-value	$F_{critical}$
Between groups (Treatment)	17792.62	3	5930.87	70.90	2.75E-26	2.68
Inside groups (Residues)	9870.17	118	83.65			
Total	27662.79	121				

Obs: $R^2 \approx 64.32\%$ of total variation is explained by the variation in the treatment

Table 5 shows that $F_{calculated} > F_{critical}$ and the p-value is lower than the established significance level (0.05 or 5%). In this case, as p-value < 0.05, it is possible to affirm that the difference between the groups was statistically significant.

Note in Tab. 5 that 64.32% (R^2) of the total variation are explained by the variation in the treatments. In this case the Tukey test was applied to verify which treatment averages differ significantly from the others. The results are presented in Tab. 6.

Table 6: Application of Tukey test.

Treatment	CT	T1	T2	T3
Average	11.44	46.48	29.89	35.33
n	57	10	35	20

MQ(inside)= 83,645		
k	= 5	Table to q(α,k,dlR)= 3.682
dl(inside)	= 118	

Group 1 × Group 2	$n_1;n_2$	$\bar{y}_1 - \bar{y}_2$	**SE**	q_{calc}	$q_{(\alpha,k,dlR)}$	q_{calc}-q_{crit}	**Analysis**
T1 × CT	10;57	35.033	1.862	18.813	3.690	15.12	Averages differ
T1 × T2	10;35	16.584	1.948	8.515	3.690	4.83	Averages differ
T1 × T3	10;20	11.149	2.104	5.300	3.690	1.61	Averages differ
T3 × CT	20;57	23.884	1.412	16.920	3.690	13.23	Averages differ
T3 × T2	20;35	5.435	2.104	2.584	3.690	- 1.11	-
T2 × CT	35;57	18.449	1.166	15.818	3.690	12.13	Averages differ

$q_{calc} > q_{\alpha,k,dlR}$ → (+)Averages differ
$q_{calc} < q_{\alpha,k,dlR}$ → (-) Averages do not differ

By applying Tukey test it was possible to verify that test T1 was different from the others, once the differences between T1 and the other groups were statistically significant. However the averages obtained in tests T2 and T3 did not show significant differences. As expected, the average of the control group was statistically different from that of the intoxicated groups.

According to Freedman (1995), emissions with THC, NO_x, SO_2 and CO concentrations are considered components with high mutagenic potential. Analyzing the results obtained in the three tests, the concentrations of THC, NO_x and CO explain the behavior of the intoxicated groups.

Group T1, which showed the highest mutagenic event rates also showed the highest concentrations of THC and NO_x (Tab. 1) and a high concentration of CO. However test T3 showed higher concentrations of CO (higher than 2.4) and SO_2 (higher than 1.6) than T1. Probably the higher rate of mutagenic events obtained in T1 was due to the high concentration of THC.

4. CONCLUSIONS

Three tests of diesel burn were performed in a flamotubular furnace of NETeF under different operation conditions, and the emission concentrations generated in the process (O_2, CO, CO_2, NO_x, THC e SO_2) were measured in gas analyzers.

Samples of the biosensor (KU-20 clone of *Tradescantia*) were subjected to intoxication in the three tests of diesel burn. The Trad-SH assay preceded the intoxication to evaluate the mutagenic events from emissions generated in the process, comparing them with a control group (non-intoxicated flowers).

The data obtained were statistically analyzed using the Analysis of Variance (ANOVA). The results showed that the differences between the groups were statistically significant and that 64.32% of total variation were explained by the variation in the treatments.

The Tukey test was also applied to identify which treatment averages, taken two by two, were significantly different. The test verified that test T1 was different from the other groups and tests T2 and T3 did not show significant differences.

Test T1, which showed the highest rates of mutagenic events, showed the highest concentrations of THC and NO_x and a high concentration of CO. However the concentrations of CO (higher than 2.4) and SO_2 (higher than 1.6) in T3 were higher than those of T1, leading to the conclusion that the higher rate of mutagenic events obtained in T1 was probably due to the high concentration of THC.

5. ACKNOWLEDGEMENTS

This work is part of the research program on mineral coal combustion of NETeF (Thermal and Fluids Engineering Group - University of São Paulo). The authors are indebted to CNPq (Process 119515/2009-1) and FAPESP (Project 2008/54062-9) for the financial support given to this research.

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