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Phytotoxicity of Soil Contaminated with Petroleum Derivatives and Biodiesel

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Abstract

The inhibition of root and hypocotyl elongation may reflect toxic substances in low concentrations, which are not sufficient to prevent germination, but may delay or inhibit root and hypocotyl growth. The objective of this study was to evaluate root and hypocotyl growth inhibition in *Cucumis sativus*, *Brassica oleracea* and *Barbarea verna* as a parameter for assessing soils toxicity when contaminated with diesel, lubricant oil and biodiesel. Thus, potential toxicity of contaminants was evaluated according to biodegradation time in soil by examining root and hypocotyl elongation inhibition. Results show that *C. sativus* root is the best indicator for diesel and lubricant oil reduced toxicity after biodegradation. It was also observed that biodiesel increases its toxicity after two months of biodegradation.

Keywords: bioassays, biodegradation, hydrocarbon, hypocotyl, root, seeds.

Fitotoxicidade de Solo Contaminado com Derivados do Petróleo e Biodiesel

Resumo

A inibição no alongamento da raiz e do hipocótilo pode refletir a presença de substâncias em concentrações baixas, que não são suficientes para impedir a germinação, mas podem atrasar ou inibir o alongamento da raiz e do hipocótilo. O objetivo deste trabalho foi avaliar a inibição do crescimento da raiz e hipocótilo de *Cucumis sativus*, *Brassica oleracea* e *Barbarea verna* como um parâmetro para avaliar a toxicidade do solo quando contaminado com diesel, óleo lubrificante e biodiesel. Assim, o potencial tóxico dos contaminantes foi avaliado de acordo com o tempo de biodegradação pela inibição no alongamento da raiz e do hipocótilo. Os resultados mostram que a raiz de *C. sativus* é o melhor indicador para demonstrar a redução da toxicidade de diesel e óleo lubrificante após biodegradação. Foi também observado que o biodiesel aumentou sua toxicidade após 2 meses de biodegradação.

Palavras-chave: bioensaios, biodegradação, sementes, hipocótilo, raiz, hidrocarbonetos.

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INTRODUCTION

The incomplete combustion of fossil fuels originates polycyclic aromatic hydrocarbons (PAHs). Some of these compounds are toxic, mutagenic and carcinogenic (Pashin & Bakhitova, 1979). Nunes-Halldorson *et al*. (2004) compared the toxicity of two PAHs types, toluene e benzene, and found greater toxicity of toluene, which the 10 ppm concentration is sufficient to kill half of Ceriodaphnia population exposed to the pollutant. Thus, less-polluting fuels are being sought. Biodiesel, which is composed of methyl or ethyl esters, offers advantages such as easy biodegradation and low toxicity (Zang *et al*., 2008; Mariano *et al*., 2008; Lapinskiene *et al*., 2006).

Environmental bioremediation is widely used based on the ability of microorganisms to biodegrade these compounds and decrease their toxicity (Vidali, 2001). Several microorganisms possess enzymatic ability to degrade hydrocarbon (Leahy & Colwell, 1990).

The bacteria isolated from genus *Ralstonia*, *Alcaligenes* and *Bacillus* are specially capable of degrading hydrocarbons from petroleum-contaminated site (Plaza *et al*., 2008). However, few microorganisms can degrade complex structure hydrocarbon as PAHs with various aromatic rings (Atlas, 1995). Assessing biodegradation efficiency through chemical analysis does not necessarily shows the real impact each substance has on living organisms (Sisinno *et al*., 2007). Moreover, some compounds when degraded generate intermediate products that can be more toxic than original substance (Nunes-Halldorson *et al*., 2004).

Thus, the use of bioassays to assess toxicity of contaminated soil gained widespread attention in the last 20 years; these tests have proved to be useful for predicting the effect of a complex mixture such as petroleum (Baud-Grasset *et al*., 1993; Banks *et al*., 2005). Plaza *et al*. (2005) used bacteria, protozoa, crustacea and seed development as bioindicators to evaluate remediation of contaminated soil by oil. These authors consider it necessary to combine bioassays with chemical data for a good soil quality assessment.

Plants used in ecotoxicological tests during the early seedling development, when numerous physiological processes occur, are sensitive to presence of toxic substances that interfere with both survival and growth. Therefore, root and hypocotyl elongation inhibition are sub-lethal indicators to assess the toxic potential of various contaminants (Sobrero & Ronco, 2004). Some studies found a decrease of toxicity in seedling development after the biodegradation of the pollutant (Baek *et al*., 2004; Molina-barahona *et al*., 2005; D'Souza *et al*., 2011).

The objective of this study was to evaluate the inhibition of root and hypocotyl growth of *Cucumis sativus*, *Brassica oleracea* and *Barbarea verna* as a parameter to measure the toxicity of soils contaminated with diesel, biodiesel and used lubricating oil according to biodegradation time.

MATERIALS AND METHODS

The sandy soil was obtained in a commercial establishment. Toxicity rates were evaluated by using three different substances: lubricant oil, collected at gas station in the city of Rio Claro-SP, biodiesel (Caramuru) and commercial diesel (B2 - 2% biodiesel) purchased station Petrobras town of Rio Claro-SP. Untreated seeds of *Barbarea verna* (watercress), *Brassica oleracea* (kale) and *Cucumis sativus* (cucumber) - ISLA were used as bioindicator.

Table 1 presents the contaminated samples composition that was prepared according to an adapted Lopes & Bidoia (2009) methodology. In preparing the test control, the volume of contaminant was replaced by distilled water.

The bags were perforated after mixing in order to establish contact with potential biodegrading microorganisms naturally present in soil. The bags were buried in Sao Paulo State University, Rio Claro (22,3968 S, 47,5454 W). A sample of each contaminant was removed at an interval of 30 days and were defined as time zero, one time (30 days), time two (60 days), time three (90 days) and time four (120 days). Except the time zero (T0) that was tested immediately after contamination.

The toxicity test was conducted according to Morales (2004). Seeds were sown in plastic cups containing 50 mL of 50 g of contaminated sand. The tests were performed in triplicates containing 10 seeds each. To the positive control 2.5 mL of 0.05 M zinc sulfate were added to evaluate seed sensitivity. The seedlings were removed and measurement for calculate the toxicity percentage (equation 1) according to Sobrero & Ronco (2004).

Values of root and hypocotyl elongation were subjected to analysis of variance (ANOVA) using the Kruskal-Wallis test. Significant differences were accepted at $p<0,0001$.

Table 1 - Contaminated samples composition

Assays	Contaminant (mL)	Sand (g)	Tween 80® (mL)	Distilled water (mL)
Lubricant oil	52.5	700	1.05	43.75
Diesel	52.5	700	1.05	43.75
Biodiesel	52.5	700	1.05	43.75
Positive control	-	700	1.05	96.25
Negative control	-	700	1.05	96.25

$$\% \text{ inhibition} = \frac{\text{mean negative control - mean sample treatment}}{\text{mean negative control}} \times 100$$

RESULTS AND DISCUSSION

The inhibition percentage in *Cucumis sativus* development is presented in Figures 1 and 2.

Negative numbers in the graph were used in a representative way to demonstrate that cucumber had an unexpected response. Some assays presented a higher growth when compared to control test. However, the test organism should present the highest growth on control tests.

C. sativus root elongation in soil contaminated with diesel fuel significantly reduced the growth at the start of the experiment, but in T3 and T4 the growth reduction was not significant in relation to negative control (Table 2). Root development inhibition caused by diesel in T3 was only 10.04% compared to control (Fig. 1). However, the hypocotyl elongation showed inhibition during all experiment period with no toxicity reduction (Fig 2). The low hypocotyl growth in soil contaminated with diesel coincides with findings by Ogbo (2009).

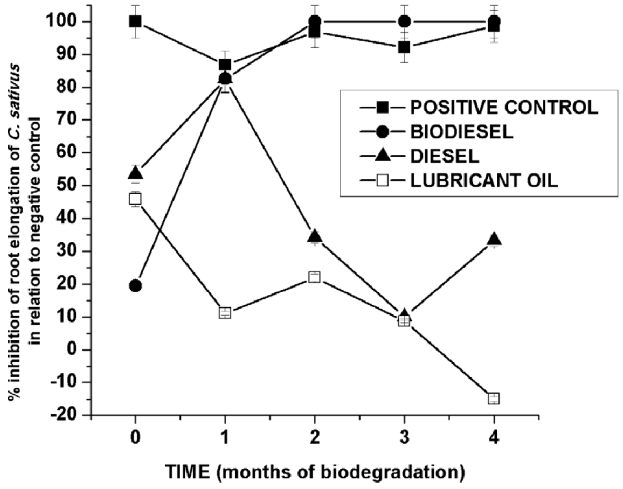

Figure 1. Inhibition percentage of root elongation of *C. sativus* in soil contaminated during biodegradation process.

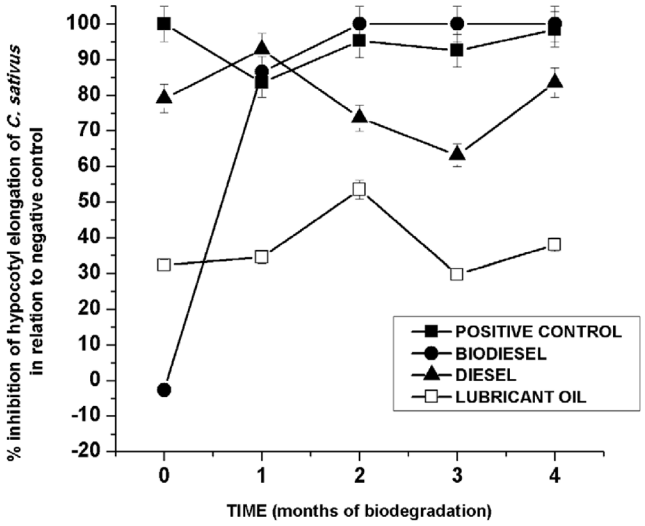

Figure 2. Inhibition percentage of hypocotyl elongation of *C. sativus* in soil contaminated during biodegradation process.

Table 2 - Mean of *Cucumis sativus* root elongation

root *C. sativus*	Time 0	Time 1	Time 2	Time 3	Time 4
Negative Control	4.54 ±2.54[a]	6 ±2.19[a]	4.62 ±1.62[a]	4.68 ±1.32[a]	4.9 ±2.5[ac]
Biodiesel	3.84 ±1.84[a]	1.04 ±1.28[b]	0 ±0[b]	0 ±0[b]	0 ±0[b]
Diesel	0.26 ±0.68[b]	1.05 ±1.09[b]	3.04 ±1.24[c]	4.21 ±1.43[a]	3.27 ±1.7[a]
Lubricant oil	0.75 ±1.22[b]	5.34 ±2.36[a]	3.60 ±1.37[ac]	4.27 ±1.57[a]	5.64 ±2.97[c]

Values correspond to the mean ± standard deviation. Significant differences among treatments within a Time are followed by different letters ($p<0.0001$) according to Kruskal-Wallis test.

Table 3 - Mean of *Cucumis sativus* hypocotyl elongation

hypocotyl *C. sativus*	Time 0	Time 1	Time 2	Time 3	Time 4
Negative Control	4.43 ±2.35[a]	5.98 ±1.61[a]	3.52 ±0.98[a]	1.79 ±0.64[a]	2.16 ±1.08[a]
Biodiesel	3.82 ±1.72[b]	0.8 ±1.148[b]	0 ±0[b]	0 ±0[b]	0 ±0[b]
Diesel	0.07 ±0.19[b]	0.43 ±0.43[b]	0.93 ±0.85[c]	0.66 ±0.59[c]	0.35 ±0.24[c]
Lubricant oil	0.62 ±1.07[b]	5.34 ±2.36[a]	1.63 ±0.8[c]	1.26 ±0.54[a]	1.34 ±0.81[c]

Values correspond to the mean ± standard deviation. Significant differences among treatments within a Time are followed by different letters ($p<0.0001$) according to Kruskal-Wallis test.

Used lubricant oil has no toxic potential in *C. sativus* root development. In T4, toxicity is represented by -15.01%, this means that compared to control test there was no inhibition. The root growth on the contaminant was greater than on control test (Fig. 1). According to Vwioko & Fashemi (2005), low concentrations of used lubricant oil stimulated *Ricinus communis* growth. Table 2 shows the positive effect of the biodegradation process from the contaminated soil with used lubricant oil and diesel, which the growth reduction was not significant in relation to negative control. Soil samples contaminated with biodiesel, affected the root and hypocotyl development of *C. sativus*, reaching 100% inhibition at T2 (Fig. 1 and 2).

Inhibition percentage in the root and hypocotyl development of *B. oleracea* is shown in Figures 3 and 4.

The presence of diesel oil reduced root and hypocotyl growth of *B. oleracea*, especially at the beginning of contamination (Fig. 3 and 4). However, it may be noted that T3 there was a toxicity reduction in soil contaminated with diesel oil. According to Baek (2004) after the bioremediation processes there is a better plant growth.

The kale seedlings development was very sensitive to the used lubricant oil. The root elongation showed high levels of inhibition; despite a lower toxicity demonstrated by the hypocotyl after 3 months of soil incubation (Fig. 4). Thus was observed

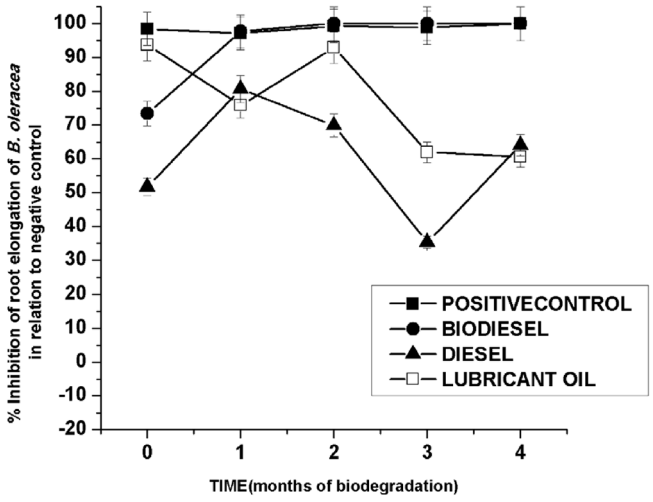

Figure 3. Inhibition percentage of root elongation of *B. oleracea* in soil contaminated during biodegradation process.

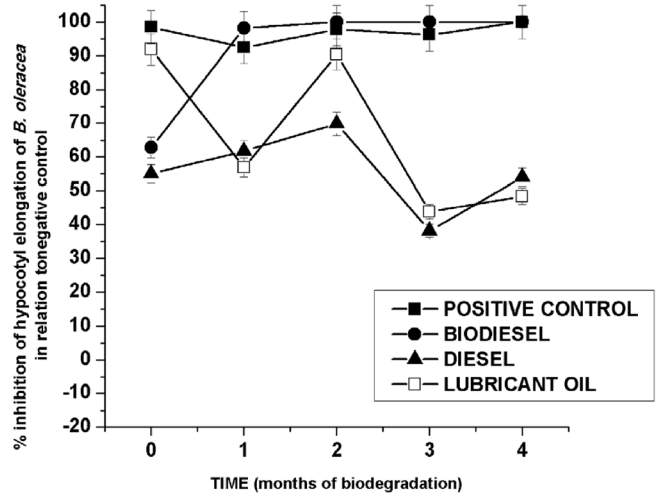

Figure 4. Inhibition percentage of hypocotyl elongation of *B. oleracea* in soil contaminated during biodegradation process.

that the hypocotyl growth mean not was significant in relation to negative control, in other words, the process biodegradation of diesel allowed a better hypocotyl elongation (Table 5).

The inhibitory effect of used lubricant oil for the seedlings development of kale may be related to the large amounts of PAHs present in its composition (Henry, 1998).

The kale showed great sensitivity in freshly contaminated soil with biodiesel, reaching 100% toxicity in T2.

The inhibition percentages in the contaminated soil are presented in Figures 5 and 6.

The root and hypocotyl development of *B. verna* had a high sensitivity for all contaminants, during all periods. Soil contaminated with used lubricant oil showed 100% toxicity for both root and hypocotyl of cress (Fig. 5 e 6). Inhibition caused by diesel oil was above 85% for both parameters and results for biodiesel reached 100% toxicity after 60 days as occurred for cucumber and kale.

Table 4 - Mean of *Brassica oleracea* root elongation

root *B. oleracea*	Time 0	Time 1	Time 2	Time 3	Time 4
Negative Control	2.58 ±2.15[a]	3.2 ±2.23[a]	2.37 ±1.73745[a]	1.37 ±1.11[a]	2.23 ±1.59[a]
Biodiesel	0.68 ±0.71[b]	0.07 ±0.28[b]	0 ±0[b]	0 ±0[b]	0 ±0[b]
Diesel	0.79 ±0.95[b]	0.62 ±0.75[b]	0.71 ±0.99[c]	0.89 ±0.75[a]	0.8 ±0.85[c]
Lubricant oil	0.79 ±0.95[b]	0.77 ±1.02[b]	0.16 ±0.37b[c]	0.52 ±0.61[a]	0.88 ±1.05[c]

Values correspond to the mean ± standard deviation. Significant differences among treatments within a Time are followed by different letters ($p<0.0001$) according to Kruskal-Wallis test.

Table 5 - Mean of *Brassica oleracea* hypocotyl elongation

hypocotyl *B. oleracea*	Time 0	Time 1	Time 2	Time 3	Time 4
Negative Control	2.22 ±1.81[a]	1.84 ±1.47[a]	1.56 ±1.38[a]	0.7 ±0.56[a]	0.82 ±0.64[a]
Biodiesel	0.82 ±0.97058[a]	0.03 ±0.10[b]	0 ±0[b]	0 ±0[b]	0 ±0[b]
Diesel	0.63 ±0.77925[b]	0.70 ±0.88[b]	0.47 ±0.64[c]	0.43 ±0.4[a]	0.38 ±0.39[a]
Lubricant oil	0.18 ±0.42[b]	0.7 ±0.88[b]	0.15 ±0.39[bc]	0.39 ±0.43[a]	0.42 ±0.51[a]

Values correspond to the mean ± standard deviation. Significant differences among treatments within a Time are followed by different letters ($p<0.0001$) according to Kruskal-Wallis test.

The inhibitory effect for *B. verna* is not only due to contaminants, since no pollutant was added to negative control test. However, the species had not achieved 65% germination, which is a minimum rate to ensure the toxic effect of the contaminant in the development of root and hypocotyl (U.S. EPA, 1996), the growth mean was low even in the negative control (Table 6 and Table 7).

C. sativus root growth was the best indicator to evaluate the toxicity reduction of diesel oil and used lubricant oil after 90 days, supporting references that consider *C. sativus* root

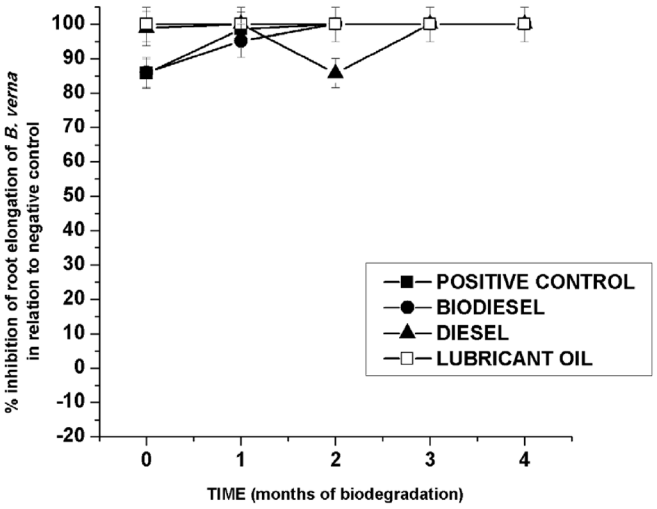

Figure 5. Inhibition percentage of root elongation of *B. verna* in soil contaminated during biodegradation process.

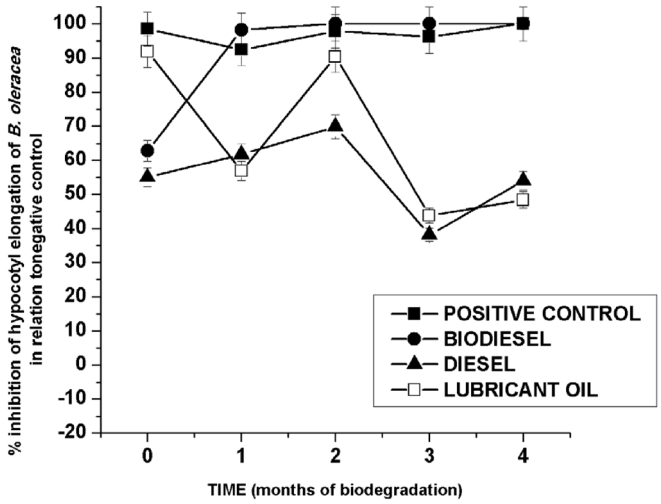

Figure 6. Inhibition percentage of hypocotyl elongation of *B. verna* in soil contaminated during biodegradation process.

Table 6 - Mean of *Barbarea verna* root elongation

root *B. verna*	Time 0	Time 1	Time 2	Time 3	Time 4
Negative Control	1.09 ±0.91[a]	1.18 ±0.88[a]	1.33 ±0.92[a]	0.71 ±0.55[a]	0.35 ±0.65[a]
Biodiesel	0.15 ±0.25[b]	0.05 ±0.13[b]	0 ±0[b]	0 ±0[b]	0 ±0[a]
Diesel	0.01 ±0.07[b]	0 ±0[b]	0.19 ±0.38[b]	0 ±0[b]	0 ±0[a]
Lubricant oil	0 ±0[b]	0 ±0[b]	0 ±0[b]	0 ±0[b]	0 ±0[a]

Values correspond to the mean ± standard deviation. Significant differences among treatments within a Time are followed by different letters ($p<0.0001$) according to Kruskal-Wallis test.

Table 7 - Mean of *Barbarea verna* hypocotyl elongation

hypocotyl *B. verna*	Time 0	Time 1	Time 2	Time 3	Time 4
Negative Control	0.85 ±0.72[a]	0.74 ±0.54[a]	1 ±0.8[a]	0.37 ±0.26[a]	0.22 ±0.41[a]
Biodiesel	0.22 ±0.38[b]	0.05 ±0.12[b]	0 ±0[b]	0 ±0[b]	0 ±0[a]
Diesel	0.01 ±0.07[b]	0 ±0[b]	0.06 ±0.11[b]	0 ±0[b]	0 ±0[a]
Lubricant oil	0 ±0[b]	0 ±0[b]	0 ±0[b]	0 ±0[b]	0 ±0[a]

Values correspond to the mean ± standard deviation. Significant differences among treatments within a Time are followed by different letters ($p<0.0001$) according to Kruskal-Wallis test.

growth a sensitive toxicity indicator (Wang *et al*., 2001). The root growth inhibition of *B. oleracea* and *B. verna* in the presence diesel oil, can be caused by impermeability effect because the oil hydrophobic properties, influencing the absorption of water and nutrients (Adam & Duncan, 2002).

The PAHs in diesel oil may induce a disturbance in plants development (Meudec, 2007). Nevertheless, some PAHs are more soluble than aliphatic hydrocarbons and its toxicity decrease after bioremediation process (Molina-barahona *et al*., 2005). Thus, the *C. sativus* and *B. oleracea* response corroborate with this assertion, because were less inhibited by diesel oil after biodegradation.

The inhibitory effect on the seedlings development in soil contaminated with used lubricant oil may be related to large amounts of PAHs present in its composition (Henry, 1998), besides that there are heavy metals coming from the engine parts as the oil is being used. Thereby the oil concentration increases in soil and pH value decreases, providing an acidic environment that increases the availability of these metals present on oil to the plants (Odjegba & Atebe, 2007; Okonokhua *et al*., 2009). However, certain metals are required in small amounts to ensure improved vegetation growth, because high concentrations of these metals alter functions of plasmatic membrane and reduce cell growth (Wong & Bradshaw, 1982; Janicka-Russak *et al*., 2008). Accordingly, the excess metals may have adversely affected the species development used in this study.

Used lubricant oil changes the soil properties reducing nitrogen and phosphorus concentration in the soil. These nutrients are essential for the microorganisms to degrade the contaminant and reduce toxicity to plants (Okonokhua *et al*., 2009). The *C. sativus* did not present the same inhibition viewed for kale and cress in soil contaminated with used lubricant oil. In this case, after 120 days of incubation, the used lubricant oil seems to stimulate root growth compared to the control test (Fig. 1). This is because after biodegradation, there is an increase in organic matter that can improve soil fertility (Vwioko & Fashemi, 2005).

For contamination with biodiesel, the test organisms showed results in common, all achieved toxicity 100% after 60 days, so it suggests that there was biodegradation after 60 days of soil incubation. The acidity of the biodiesel increases, when it is degraded, because the fatty acid methyl ester molecules are broken down during degradation and the fatty acid chains, thus the acidity generated could affect the process of germination and consequently, the root and hypocotyl development (Lapinskiene *et al*., 2006; Leung *et al*., 2006).

The results with *B. verna* are not conclusive due to low germination in control test. Thus, the high percentage of toxicity cannot be only as an action of the contaminant (Morales, 2004).

Finally, the root elongation in *C. sativus* was the best parameter to evaluate toxicity reduction of diesel oil and used lubricant oil after four months. It was also noticed that biodiesel increases its toxicity after 2 months of biodegradation, thus there was no root and hypocotyl development.

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