

Physiological and biochemical changes in seeds and seedlings of red clover submitted to diesel oil

Fernanda Reolon Tonel¹, Patrícia Marini¹, Caroline Leivas Moraes², Juliana de Magalhães Bandeira¹, Leopoldo Mario Baudet Labbé², Francisco Amaral Villela² & Dario Munt de Moraes¹

¹ Universidade Federal de Pelotas, Instituto de Biologia, Departamento de Botânica, Programa de Pós-graduação em Fisiologia Vegetal. Campus Capão do Leão, s/n, caixa postal 354, CEP: 96010-900, Capão do Leão, RS, Brazil. fernandareolon@yahoo.com.br; marinipati@gmail.com; bandeira_jm@hotmail.com; moresdm@ufpel.tche.br

² Universidade Federal de Pelotas, Departamento de Fitotecnia, Faculdade de Agronomia Eliseu Maciel. Caixa Postal 354, CEP: 96010-970 - Capão do Leão, RS, Brazil. caroline.moraes@gmail.com; lmbaudet@ufpel.edu.br; francisco.villela@ufpel.edu.br

Recebido em 23.V.2013. Aceito em 04.XII.2013

ABSTRACT – The aim of this study was to verify the seed and seedling tolerances of red clover exposed to increasing diesel oil concentrations, through physiological and biochemical changes, in order to indicate this species for use in possible phytoremediation of contaminated areas. Therefore, the substrate was moistened with different diesel oil concentrations (0; 0.1; 0.2; 0.3% v/v) and the seeds were placed to germinate, and the following were later assessed: first germination count (FGC), germination speed index (GSI), electrical conductivity (EC), initial growth, and activity of hydrolytic enzymes. The germination, the FGC, and the GSI decreased as the diesel oil concentrations increased, as well as seed α -amylase and phosphatase enzyme activity. There was a decrease of EC in both incubation periods in the highest diesel oil concentration. The physiological and biochemical changes caused by diesel oil in red clover seeds and seedlings demonstrate that the germination and early growth of this species is harmed.

Key words: germination, phytoremediation, plant growth, *Trifolium pratense* L.

RESUMO – **Alterações fisiológicas e bioquímicas de sementes e plântulas de trevo vermelho submetidas ao óleo diesel.** Objetivou-se verificar a tolerância de sementes e plântulas de trevo vermelho expostas a diferentes concentrações de óleo diesel, através das alterações fisiológicas e bioquímicas provocadas por este composto, visando indicar esta espécie como possível fitorremediadora de áreas contaminadas. Para isso, o substrato foi umedecido com diferentes concentrações de óleo diesel (0; 0,1; 0,2; 0,3% v/v) e as sementes foram semeadas colocadas para germinar, sendo posteriormente avaliadas quanto à germinação, primeira contagem de germinação (PCG), índice de velocidade de germinação (IVG), condutividade elétrica (CE), crescimento inicial e atividade de enzimas hidrolíticas. A germinação, a PCG e o IVG reduziram com o aumento das concentrações de óleo diesel, assim como a atividade das enzimas α -amilase e fosfatase nas sementes. Houve redução da CE em ambos os períodos de incubação na maior concentração de óleo diesel. As alterações fisiológicas e bioquímicas causadas pelo óleo diesel em sementes e plântulas de trevo vermelho demonstram que a germinação e o crescimento inicial desta espécie são prejudicados.

Palavras chave: crescimento vegetal, fitorremediação, germinação, *Trifolium pratense* L.

INTRODUCTION

Oil is an important fossil fuel for the world's economy. However, one of its derivatives, diesel oil, could represent a considerable environmental problem, as accidents that occur while transporting

this compound has been one of the main causes of several types of environmental pollution (Andrade *et al.*, 2010; Aguiar *et al.*, 2012).

Over the past few years, the maritime industry and the naval hub have become essential sectors for the state of Rio Grande do Sul, specially in the

city of Rio Grande, where a series of oil platforms are located, characterizing the revitalization of the naval industry in the country. However, the growing development of this activity leads to more chances of oil product contamination such as leakages in fuel storage tanks and pipelines, as well as the dumping of these residues in the soil and accidental spills (White Jr. *et al.*, 2006; Spinelli *et al.*, 2012).

The numerous leaks of oil products registered in Brazil have motivated the development of new techniques aimed, mainly, at decontamination, in order to reduce the leakage effects on the ecosystem (Marin *et al.*, 2006). Oil is a complex mixture leading to the treatment of contaminated areas by its derivatives, i.e. diesel oil. It is also a moderately volatile fuel containing several phytotoxic compounds, which can be introduced into the environment and remain in the soil (Jacques *et al.*, 2007), contaminating groundwater and interfering in normal plant development (Andrade *et al.*, 2010).

Due to the fact that environmental accidents caused by these elements occur in coastal regions, such as southern Rio Grande do Sul, appropriate attention should be given for the recovery of riparian vegetation in these damaged areas, since the contaminated soil becomes sterile, damaging the ecosystem. A possible solution for this situation would be the introduction of seeds and seedlings to revegetate these areas. Scientific studies of native and pioneering plants, which survive in polluted environments, may be used to accelerate the recovery of these degraded areas. In this context, to reach an efficient and low cost cleaning of a large scale of pollutants, the phytoremediation technique has been considered an emerging technology that uses plants to remediate soils contaminated by chemical substances deriving from oil (Andrade *et al.*, 2010; Soleimani *et al.*, 2010).

Most revegetation programs have used seedlings although some studies indicate the use of species through direct sowing on the fields as a feasible alternative, mainly due to lower costs. Thus, studying the response of plant species sown in soil contaminated with diesel oil, from germination to plant development, is extremely important; as this can be indicated for recovery plans with direct sowing.

Red clover is a species originating from North America and well adapted to the climate and soil conditions of Rio Grande do Sul state. The purpose of this study was to verify the tolerance of red clover seeds and seedlings exposed to increasing

diesel oil concentrations, through physiological and biochemical changes, in order to indicate this species for putative use in the phytoremediation of contaminated areas.

MATERIAL AND METHODS

The study was carried out at the Seeds Physiology Laboratory in the Botany Department of the Universidade Federal de Pelotas (UFPEL). The red clover seeds (*Trifolium pratense* L.) used were bought locally. Standard viability and vigor tests were conducted according to the Seeds Analysis Rules (BRASIL, 2009), as described below:

Germination test (G): carried out with 200 seeds (four sub-samples of 50 seeds) for each repetition, in a total of four repetitions. Transparent plastic boxes (Gerbox[®]) were used, with two blotting paper sheets as the substrate, which were previously moistened with different diesel oil concentrations (0, 0.1, 0.2, 0.3% v/v) from gas stations and, afterwards, the red clover seeds were sown and kept in a germinator at 25°C. The results were expressed in germination percentage.

First germination count (FGC): carried out with the germination test, in which the first count for the red clover was performed four days after sowing (DAS). The results were expressed in percentages of normal seedlings.

Germination speed index (GSI): carried out with the germination test, in which daily counts were performed after radicle protrusion from the seed tegument until the number of seedling remained steady. The last counting day for this test was the same as indicated for the germination test (10 days). The calculation for the germination speed index was undertaken according to Maguire (1962), using the following formula: germination speed index = $G_1/N_1 + G_2/N_2 + \dots + G_i/T_i$ where G_1 , G_2 , and G_i refer to the number of germinated seedlings in each day, and, T_1 , T_2 , T_i refer to the DAS.

Aerial part length (APL) and seedling root length (RL): were obtained through the average of 40 seedlings by repetition. At the end of the germination test, the length was measured using a millimeter ruler and the results expressed in mm seedling⁻¹.

Total dry mass (TDM): obtained at the end of the germination test, the dry mass was obtained gravimetrically, in a forced ventilation oven 70 ± 1°C until reaching a constant mass and the results were expressed in mg seedling⁻¹.

Electrical conductivity (EC): four sub-samples of 25 seeds by repetition were used. Firstly, the

seed mass was determined, which were soaked for 60 minutes in different diesel oil concentrations (0, 0.1, 0.2, and 0.3% v/v). Immediately afterwards, the seeds were washed in distilled water, placed in 80 mL beakers of deionized water and kept in a germinator at 25°C. After the incubation periods of three and 24 hours, the recipients were removed from the germinator, smoothly agitated and the samples and deionized water were read using a Digimed CD-21 Conductivimeter. To find the electrical conductivity solution value with the seeds, the conductivity value read in the conductivimeter was subtracted from the deionized water value reading, dividing the value obtained by the dry mass of the 25 seeds, with the results expressed in $\mu\text{S cm}^{-1} \text{g}^{-1}$ of seeds, using the method described by Krzyzanowski (1999).

Determination of the activity of acid phosphatase and α -amylase enzymes: determined according to the method presented by Aoac (1965), with some adaptations. The extractions were carried out in red clover seeds from a germination test, zero and 10 days after sowing (DAS), where 1000 mg of seeds (zero) and seedlings (10 days) were macerated by a mortar, using 20 mL of potassium acetate buffer (50 mM, pH 5.0) and centrifuged at 4000 rpm for 20 minutes, at 4°C. Then, the supernatant was removed and placed in test tubes, which were stored in a refrigerator at 4°C until the analyses were performed. For acid phosphatase, 0.2 mL of the extract, 0.8 mL of potassium acetate buffer (50 mM, pH 5.0), and 0.1 mL of p-nitrophenyl phosphate (0.018 M) were added in all test tubes. The tubes were incubated at 30°C for five minutes. Afterwards, 1 mL of sodium hydroxide (0.5 N) was added and read in

a spectrophotometer at 400 nm, with the activity expressed in $\mu\text{mol p-NPP hydrolysis min}^{-1} \text{g}^{-1}$ of fresh mass (FM). For the determination of α -amylase the extract was put at 70°C for 20 min. After that, a 15 minute centrifugation was carried out in order to have the supernatant. Then, in each test tube, 0.2 mL of the extract, 0.8 mL of buffer solution, 1 mL of starch solution, and 1 mL of $\text{I}_2 + \text{KI}$ were added. The reading was executed in a spectrophotometer at 620 nm and the results were expressed in μg of hydrolyzed starch $\text{min}^{-1} \text{g}^{-1}$ FM.

The experimental design used was completely randomized, with four replications and the data related to the measured variables were subjected to the variance analysis with subsequent polynomial regression.

RESULTS AND DISCUSSION

The red clover seeds exposed to different diesel oil concentrations (0, 0.1, 0.2, and 0.3% v/v) decreased in germination percentage according to the increase in diesel oil concentrations (Fig. 1a). These results concluded that the substrate contaminated with this oil product, in these concentrations, was sufficient to characterize the toxic effect in the seeds reducing their germination. Additionally, inhibitory effects on the germination may be attributed to physical limitations induced by petroleum hydrocarbons, complex substances which form a hydrophobic layer around the seed and probably act as a physical barrier reducing both water absorption and oxygen transfer to the seed (Adam & Duncan, 2002). This physical impedance explains the decrease observed in the variables of the first germination counts and germination speed index (Fig. 1b and 1c).

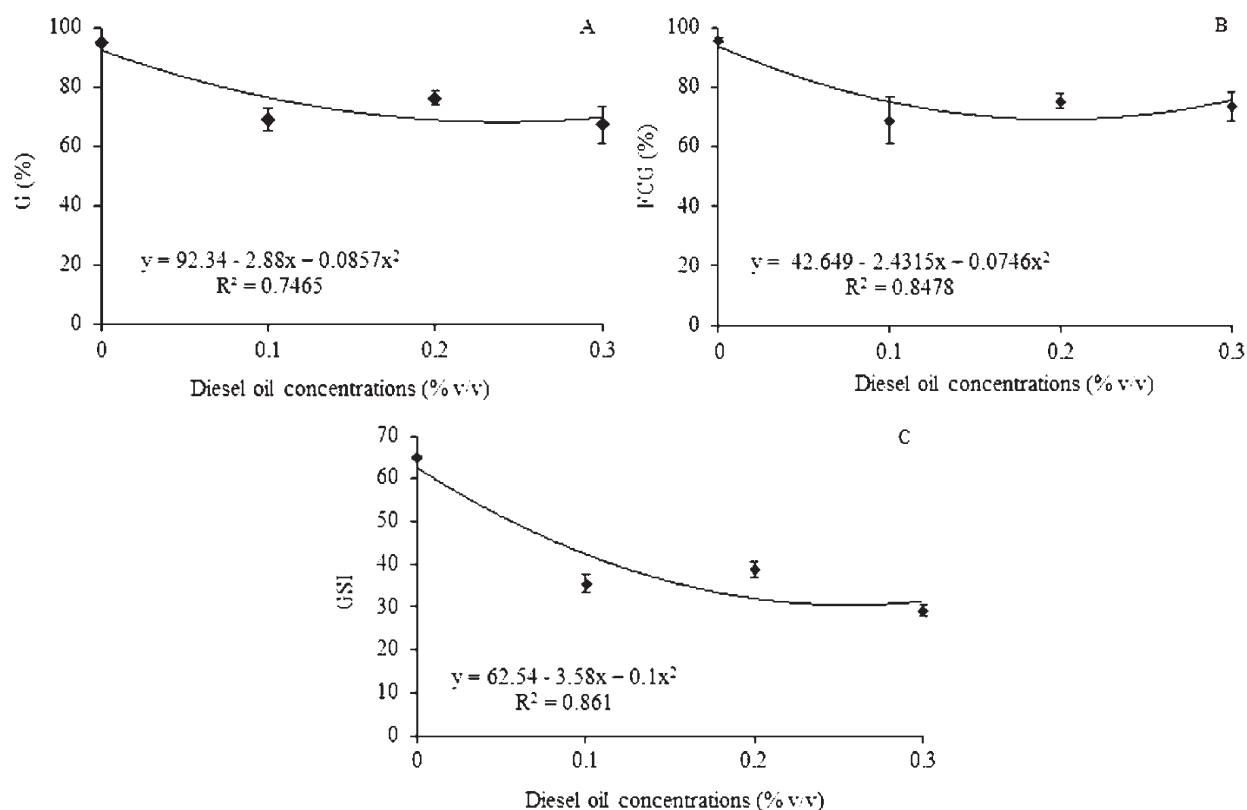


Fig. 1. (A) Germination test (G), (B) first germination count (FGC) and (C) germination speed index (GSI) of red clover seeds (*Trifolium pratense* L.), under different diesel oil concentrations (0, 0.1, 0.2, and 0.3% v/v). The bars represent the standard error of four replications.

Studies have reported a decrease in seed germination (Smith *et al.*, 2006), and inhibition of biomass production (Merkl *et al.*, 2004b) in the presence of petroleum derivative compounds. Other research using different petroleum concentrations (0, 1:1, and 1:3 v/v) in *Agropyron*, sunflower, clover, and safflower seeds have reported no inhibitory effect or delay in the germination rate of these cultures (Besalatpour *et al.*, 2008). However, according to the conclusions by Adam & Duncan (2002), the diesel oil volatile fraction reduces germination. However, the response of the seed germination is quite likely to change in the same species and even in the same family showing a differential sensitivity to diesel fuel contamination (Besalatpour *et al.*, 2008).

The electrical conductivity in a three-hour-incubation resulted in a quadratic regression, since at 0.1% diesel oil concentration there were higher conductivity values, characterizing higher leaching of solutes (Fig. 2a). Through this result it is possible to conclude that the lowest concentration of this compound (0.1% v/v) caused destabilization of the cellular membranes, possibly due to the compound acting as a type of solvent. On the other hand, in 24 hours of seed imbibition the regression curve decreased in a linear way. However, in both incubation periods it was possible to check significant differences between the treatments, with a decline in conductivity values at the highest diesel oil concentration (Fig. 2b).

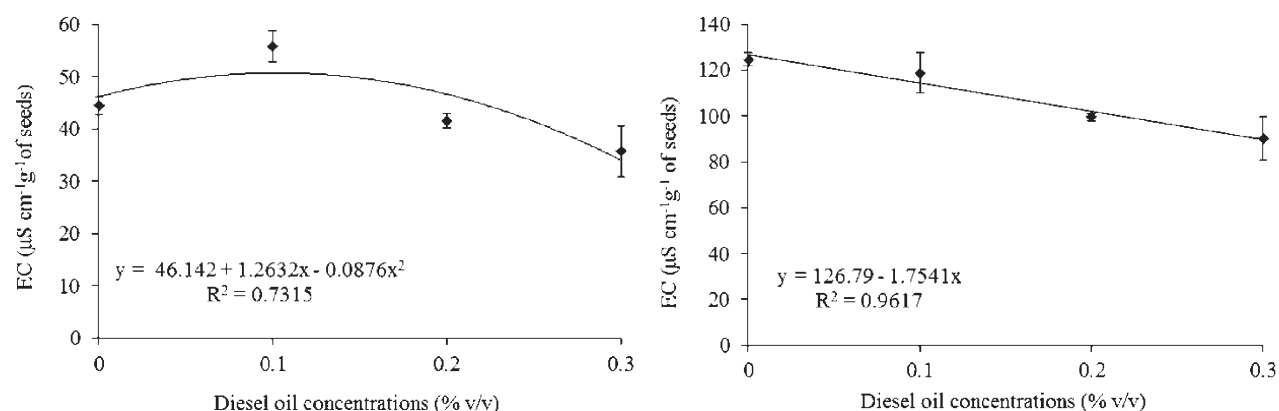


Fig. 2. Electrical conductivity (EC) of red clover seeds (*Trifolium pratense* L.), under different diesel oil concentrations (0, 0.1, 0.2, and 0.3% v/v), after (A) three and (B) 24h of soaking. The bars represent the standard error of four replications.

A physical barrier with a hydrophobic layer, which this oil product possibly created around the seeds, explains the lowest release of exudates from seeds during the highest diesel oil concentration. This fact may have hindered the release of ions through the tegument of seeds, harming the input

of water and, consequently, delaying and reducing germination, directly decreasing growth of the aerial part and roots, as well as the total seedling dry mass that were submitted to these diesel oil concentrations (Fig. 3).

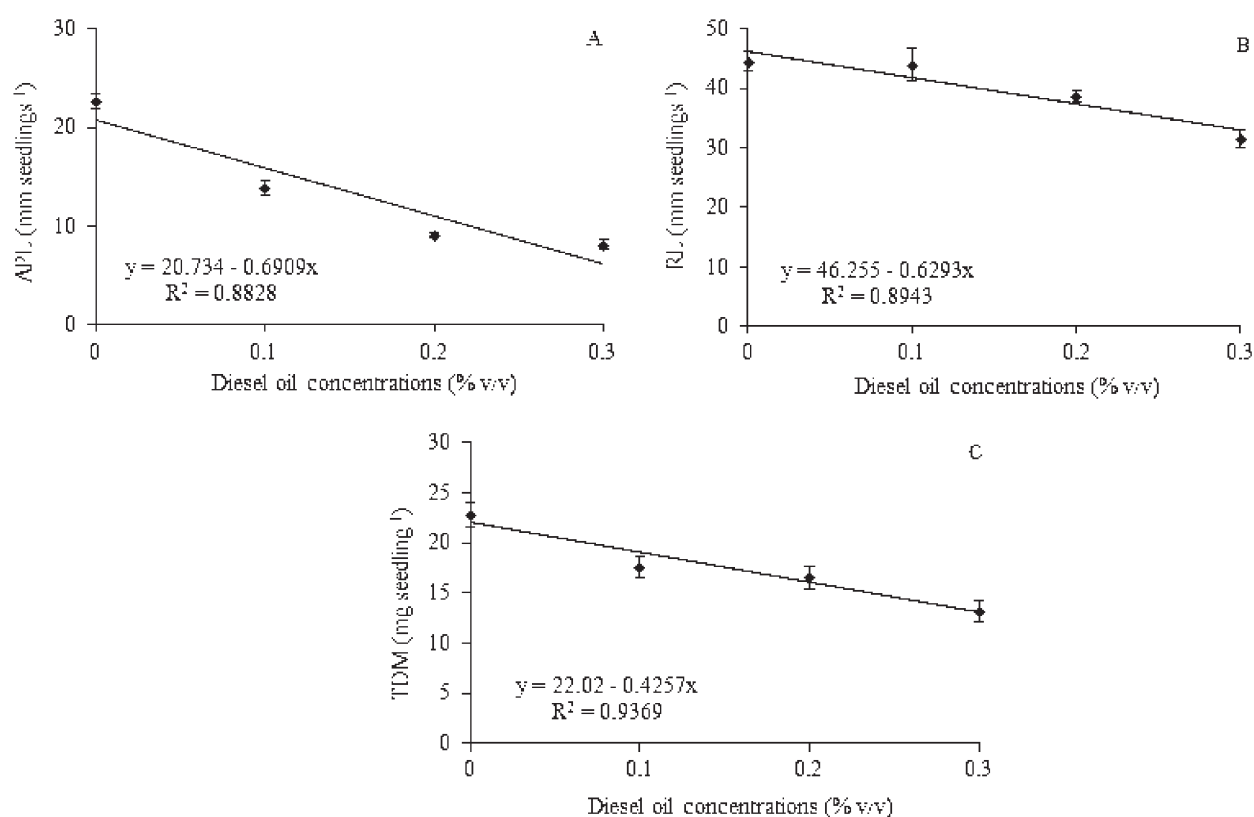


Fig. 3. (A) Length of aerial part (APL), (B) root (RL), and (C) seedling total dry mass (TDM) of red clover seeds (*Trifolium pratense* L.), under different diesel oil concentrations (0, 0.1, 0.2, and 0.3% v/v). The bars represent the standard error of four replications.

In studies undertaken with grassy plants, such as *Panicum maximum* L. and *Brachiaria bryzantha* (A. Rich.) Stapf in order to remediate soil contaminated with petroleum hydrocarbons to check their effect on the seed germination, it was noticed that water and nutrients absorption changes when oil film covered the roots (Hernandez-Valencia & Mager, 2003), leading to biomass decrease due to the decline of transported processors. Also, these decreases in growth caused by diesel oil may be due to reduction of cell division in the meristem root zone and cell lengthening suppression caused

by the plasticity decrease of the cell wall (Sharma & Dubey, 2005). Moreover, the decline of some enzyme activities involved in the embryo growth, such as the α -amylase enzyme activity in time zero (0 days) where the activity of this enzyme statistically decreased in relation to the control (Fig. 4a), showing that as there is an increase in diesel oil concentrations, there is lower water absorption and, consequently, there was a smaller starch degradation performed by this enzyme, characterizing smaller preparation of reserves destined to embryo axis growth.

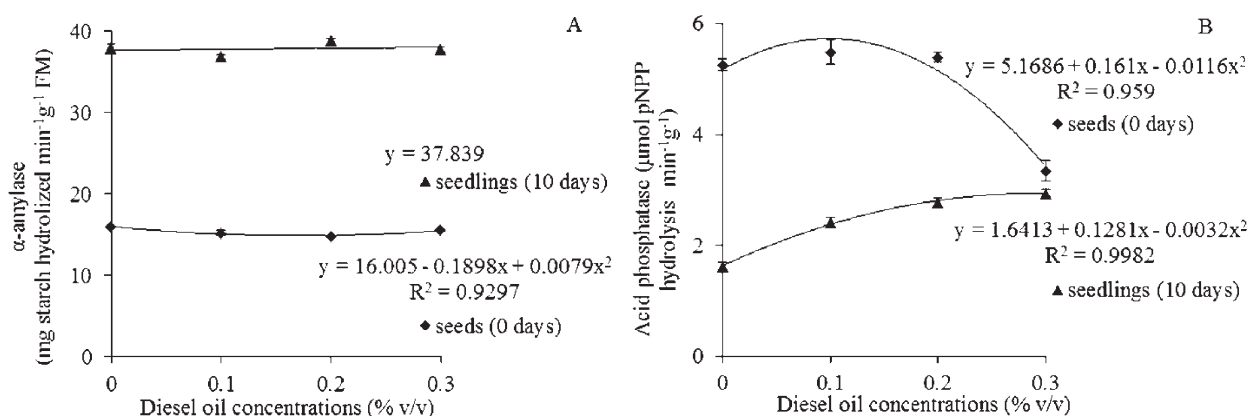


Fig. 4. (A) Activity of α -amylase enzyme and (B) activity of acid phosphatase in seeds (0 days) and seedlings (10 days) of red clover seeds (*Trifolium pratense* L.), under different diesel oil concentrations (0, 0.1, 0.2, and 0.3% v/v). The bars represent the standard error of four replications.

A similar response was found for the activity of the acid phosphatase enzyme (0 days), which is present in ester hydrolysis reactions and is also involved in keeping cell phosphate, as its activity may affect the metabolism of phosphate in seeds (Santos *et al.*, 2005). There was a decline in the activity of the acid phosphatase enzyme in seeds with the increase of diesel oil concentration (Fig. 4b). This response probably happened due to the lack of water absorption which occurred due to the rise of an oil layer around the seed, which affected the breakage of reserves used to form the seedling and consequent vegetation establishment. On the other hand, there was an increase in the activity of the acid phosphatase enzyme in all diesel oil concentrations 10 days after sowing (Fig. 4b). This can be justified by the greater need to reverse the negative effect caused by the diesel oil in seeds in order to release energy as phosphate for the embryo growth. The same trend was not observed in α -amylase enzyme in this period (10 DAS), in which it was not

possible to check significant differences between the treatments. The selection of plant species is an important factor in the phytoremediation process of contaminated areas, since to be successful this technique needs the selected species to be able to germinate, get established and flourish in the conditions of that contaminated environment (Coutinho & Barbosa, 2007). The results presented above are particularly necessary to assess the contaminant toxic effect in the species studied, which will be used for phytoremediation, before carrying out the experiment at field scale.

CONCLUSIONS

The physiological and biochemical changes caused by diesel oil in red clover seeds and seedlings demonstrate that germination and early growth of this species was harmed, showing that this species is not the most indicated for phytoremediation of contaminated areas with this oil derivative.

ACKNOWLEDGEMENTS

The authors thank the Programa de bolsas de Pós-Doutorado (PNPD-CAPES/FAPERGS and DOCFIX - CAPES/FAPERGS) for the financial support, to Dr. H. D. Laughinghouse IV and R. M. Fischer for reviewing the English text.

REFERENCES

- Aoac - Association of official agricultural chemists. 1965. Official methods of analysis. 10 ed. Editorial Board. Washington. 909p. Disponível em: <http://www.aoac.org/> Acesso em 20.03.2013.
- Adam, G. & Duncan, H. 2002. Influence of diesel fuel on seed germination. *Journal Environmental Pollution*, 120:363-370.
- Andrade, J.A., Augusto, F. & Jardim, I.C.S.F. 2010. Biorremediação de solos contaminados por petróleo e seus derivados. *Eclética Química*, 35:17-43.
- Aguiar, C.R.C., Lopes, B.C., Barbosa, M.V.D.B., Balieiro, F.C. & Gomes, M.M. 2012. Fitorremediação de solos contaminados por petróleo. *Revista Trópica – Ciências Agrárias e Biológicas*, 6:3-9.
- Besalatpour, A., Khoshgoftarmanesh, A.H., Hajabbasi, M.A. & Afyuni, M. 2008. Germination and Growth of Selected Plants in a Petroleum Contaminated Calcareous Soil. *Soil & Sediment Contamination*, 17:665-676.
- Brasil. 2009. Ministério da Agricultura, Pecuária e Abastecimento. Regras para análise de sementes. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Brasília, 395p.
- Coutinho, H.D. & Barbosa, A.R. 2007. Fitorremediação: Considerações gerais e características de utilização. *Silva Lusitana*, 15:103-117.
- Hernández, I. & Mager, D. 2003. Fitorremediar suelos contaminados con un crudo de petróleo liviano. *Bioagro*, 15:149-155.
- Jacques, R.J.S., Bento, F.M., Antonioli, Z.I. & Camargo, F.A.O. 2007. Biorremediação de solos contaminados com hidrocarbonetos aromáticos policíclicos. *Ciência Rural*, 37:1192-1201.
- Krzyzanowski, F.C., Vieira, R.D. & França Neto, J.B. 1999. Vigor de sementes: conceitos e testes. Associação Brasileira de Tecnologia de Sementes, Londrina, 218p.
- Maguire, J.D. 1962. Speed of germination-aid in selection aid evolution for seedling emergence and vigor. *Crop Science*, 2(2):176-177.
- Marín, J.A., Moreno, J.L., Hernández, T. & García, C. 2006. Bioremediation by composting of heavy oil refinery sludge in semiarid conditions. *Biodegradation*, 17: 251-261.
- Merkel, N., Schultze-kraft, R. & Infante, C. 2004b. Phytoremediation in the tropics – The effect of Crude Oil on the Growth of Tropical Plants. *Bioremediation Journal*, 8:177-184.
- Santos, C.M.R., Menezes, N.L. & Villela, F.A. 2005. Modificações fisiológicas e bioquímicas em sementes de feijão no armazenamento. *Revista Brasileira de Sementes*, 27:104-114.
- Soleimani, M., Afyuni, M., Hajabbasi, M.A., Nourbakhsh F., Sabzalian, M.A. & Christensen, J.H. 2010. Phytoremediation of an aged petroleum contaminated soil using endophyte infected and non-infected grasses. *Chemosphere*, 81:1084-1090.
- Sharma, P. & Dubey, R.S. 2005. Lead toxicity in plants. *Brazilian Journal Plant Physiology*, 17:35-52.
- Smith, M.J., Flowers, T.H., Duncab, H.J. & Alder, J. 2006. Effects of polycyclic aromatic hydrocarbons on germination and subsequent growth of grasses and legumes in freshly contaminated soil with aged PAHs residues. *Environmental Pollution*, 141:519-525.
- Spinelli, A.C.O.C., Kato, M.T., Lima, E.S. & Gavazza, L. 2012. Bioremediation of a tropical clay soil contaminated with diesel oil. *Journal of Environmental Management*, 113:510-516.
- White Jr., P.M., Wolf, D.C., Gregory, J.T. & Reynolds, C.M. 2006. Phytoremediation of alkylated polycyclic aromatic hydrocarbons in a crude oil-contaminated soil. *Water, Air, and Soil Pollution*, 169:207-220.

