DOI: 10.14295/holos.v17i1.11726

ACTION MYCORRHIZAL FUNGI AND BIOFERTILIZER IN SOIL CONTAMINATED WITH DIESEL

AÇÃO DE FUNGOS MICORRÍZICOS E BIOFERTILIZANTE EM SOLO CONTAMINADO COM DIESEL

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Artigo recebido em: 30/08/2016 e aceito para publicação em: 16/10/2017.
DOI: http://dx.doi.org/10.14295/holos.v17i1.11726

Abstract: Bioremediation is a technology that uses microorganisms as recuperative agents of soil and other contaminated media. The microbial soil community has great influence on the fate of xenobiotics discarded in the environment. Mycorrhizae, a mutualistic association of fungi with plant roots, have the capacity to recover degraded areas. One of these associations is arbuscular mycorrhizae fungi (AMF), which absorbs water and nutrients for plants, protecting them against pathogens and helping to grow vegetation. This study aims to inoculate AMF in contaminated soil with different concentrations of diesel oil and, in certain samples, to add biofertilizer, verifying the evolution of *Zea mays* (corn) seeds. The development of the seedlings was analyzed through the examination of root length, stem, fresh weight and dry weight, quantification of the bacteria and fungi populations present in the soil. Diesel oil caused toxic effects on seedlings, influencing growth and weight. The biofertilizer addition contributed to the root length and stem in relation to the inoculum of AMF in the development of *Zea mays.* Thus, biostimulation and bioaugmentation acted effectively in the remediation of the soil contaminated with diesel.

Keywords: Mycorrhizal fungi. Biofertilizer. Diesel. Soil.

Resumo: A biorremediação é a tecnologia que faz uso de microrganismos como agentes recuperadores do solo ou outros meios contaminados. A comunidade microbiana do solo possui grande influência no destino de xenobióticos descartados no ambiente. As micorrizas, associação mutualística de fungos com raízes de plantas, possuem a capacidade de recuperar áreas degradadas. Uma dessas associações são os fungos micorrízicos arbusculares (FMAs), os quais absorvem água e nutrientes para as plantas, protegendo-as contra patógenos e auxiliando no crescimento da vegetação. Este estudo tem como objetivo inocular FMA`s em solo contaminado com diferentes concentrações de óleo diesel e, em determinadas amostras, adicionar biofertilizante, verificando a evolução das sementes de *Zea mays* (milho). Considerou-se o desenvolvimento das mudas através da análise do comprimento da raiz, caule, peso fresco e peso seco, quantificação da população de bactérias e fungos presente no solo. O óleo diesel provocou efeitos tóxicos às mudas,

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influenciando no comprimento e peso. A adição de biofertilizante contribuiu para o crescimento radicular e caule em relação ao inóculo de FMA's, no desenvolvimento de *Z.mays*. Assim, a bioestimulação e a bioaumentação atuaram de forma eficaz na remediação de solo contaminado com diesel.

Palavras-chave: Fungos micorrízicos. Biofertilizante. Diesel. Solo.

1 INTRODUCTION

Soil contamination with oil may occur by oil exploration during their production, transportation, storage and refining processes. Besides these aspects, the improper disposal of it or its inadequate treatment in the soil, resulting in serious threats to the environment and health of human beings (YING et al. 2014).

The diesel oil may be obtained by distillation of crude oil and has a high content of polycyclic aromatic hydrocarbons, thus, a fuel normally used by vehicles and machinery toxic to ecosystems (JAGTAP et al. 2013).

To degrade organic and inorganic compounds or minimize the concentration thereof on the Soil, it makes use of bioremediation technique, which is based on the ability of microorganisms acting effectively to mitigate these compounds. This technique depends upon the microbial population in the soil, to be specific, and is affected by environmental conditions and existing hydrocarbons (HESNAWI and ADBEIB, 2013).

The soil bioremediation may be accomplished through the arbuscular mycorrhizal fungi (AMF's), which form symbiotic associations with most species of plants present in the soil. In these associations, exchange occurs between the nutrient fungi (which lodge in the rhizosphere) of plants and roots, thereby benefiting the host plant. These microorganisms may play important ecological roles in inter or intra-specific interactions, as well as perform maintenance of the plant community in ecosystems (SHI et al. 2015).

There is no exact knowledge of the beginning of the appearance of AMF's. It is known that they can result from the symbiosis between an aquatic ancestral microbial or terrestrial zygomycete. AMF's require a host plant to absorb the carbon compounds, establishing a process of symbiosis with the host plant (MOREIRA AND SIQUEIRA, 2006).

According to HRISTOZKOVA et al. (2016) "the community of mycorrhizal fungi and rhizosphere components promotes plant nutrition and improved tolerance

to abiotic stresses. In their study, it was evaluated the contribution of AMF`s about the impact on soil by heavy metals on the development of *Calendula officinali*, which suggested that different species of mycorrhizal fungi can act or adopt explicitly to correct the contaminated soil in any rhizosphere or vegetable, proving that *officinali* is a metal tolerant and AMF`s may be involved in this alternative remediation. Three types of mycorrhizal fungi were tested, which promoted the accumulation of important secondary metabolites as phenols, flavonoids and carotenoids, thereby increasing the capacity antioxidant in their flowers".

Arbuscular mycorrhizal fungi promote plant growth even in adverse environmental conditions. In a study conducted by SELVAKUMAR et al. (2016) "that aimed to propagate the AMF`s using only a single spore inoculation technique in *Sorghum bicolor L.*, of one hundred and fifty inoculants six spores were able to germinate in vitro". It was revealed, so that the spores belonged to *Gigaspora margarita* and *Claroideoglomus lamellosu*. The results showed that the production of species of these fungi can be acquired only through use of spores as inoculum, which is highly reproducible. The isolated cultures AMF`s, can be used as plant growth potential, thus promoting the fungal inoculum".

This study aimed to evaluate the growth and adjustment capacity of the species *Zea mays* in soil contaminated by different concentrations of diesel before and after the application of bioaugmentation as biofertilizers and inoculating mycorrhizal fungi.

2 METHODOLOGY

The experiments were performed in the Microbiology Laboratory of the Faculty of Technology, State University of Campinas.

2.1 Biofertilizer

Microgeo® biofertilizer, 100% natural, (patent # PI0207342 A2-0; by Microbiol Biotechnology), consisting of microorganisms, nutrients, phytorials, indicated for agriculture, livestock and reforestation (D´ANDREA, 2010).

2.2 Fungal Isolates

The fungal isolates used were from the Foundation's culture collection André Tosello, Stored in the plastic bag itself.

2.3 Planting Corn Seeds *(Zea Mays)*

The soil was not autoclaved, no beginning of the experiment, not eliminating native pathogens and AMF'S. It carried out the cultivation of seeds seedlings (*Z. mays*) bags of plant propagation specific to the agricultural area, used for this purpose. The seeds were planted in soil, purchased commercially, which did not have the same fertilizer, and was previously contaminated with diesel, plus a control sample is known (Soil fresh, without an addition of diesel oil). All plantings were done in triplicate. The treatments used are described below:

- (T1) Soil control
- (T2) Control + AMF'S
- (T3) Control + Biofertilizer
- (T4) Control + AMF'S + Biofertilizer
- (T5) 1% Diesel
- (T6) 1% Diesel + AMF'S
- (T7) 1% Diesel + Biofertilizer
- (T8) 1% Diesel + AMF'S + Biofertilizer
- (T9) 2% Diesel
- (T10) 2% Diesel + AMF'S
- (T11) 2% Diesel + Biofertilizer
- (T12) 2% Diesel + AMF'S + Biofertilizer

Soil contamination occurred with the insertion of diesel oil at concentrations of 1 and 2%. An amount was stipulated to 2329.98 g $soil^{-1}$ for each situation and was calculated the percentage of diesel to be dissolved.

After contamination, the soil was divided into three samples with different sachets 776.66 g, respectively. There was the planting of seeds with four offices and four seeds per bag. Inoculation of the fungus was performed by mixing

spores of each fraction to be analyzed at concentrations previously calculated, since in this analysis contained 56.79 g in each sample.

The range of 20 days after planting, weekly watering's biofertilizer were made of 50.0 mL for each part to be analyzed (control and contaminated soil), after the course of this interval, there was the withdrawal of seedlings that have developed.

2.4 Test Of Germination And Growth With Seeds Corn *(Zea Mays)*

To evaluate germination and growth of seeds, the variables were analyzed: the average stem length and fresh root weight and dry weight of the average plant, then developing the same by ISO 11269-1 (1993), and quantification of microorganisms (fungi and bacteria).

The fresh weight was obtained by weighing on an analytical scale plants after its withdrawal from the soil and washing them to remove excess soil, taking care not to occur the breakdown and loss of roots and shoots of plants.

The dry weight was obtained by weighing the seedlings after drying in an oven at 36 ° C during 32 hours, packed in bags of brown paper.

2.5 Quantification of The Microbiota of Soil

The values of the length of stem and root were obtained from the still fresh changes, it was carefully extended and the measured bench with the aid of a slit dividing the operation between the stem and root. Quantification of soil microorganisms was performed using the "pour plate", using the medium Plate Counter Agar (PCA) for bacteria and Sabouraud for fungi, both quantified in Colony Forming Units (CFU) per gram of soil, according to CETESB, 1986.

2.6 Statistical Analyzes

Statistical analyzes were performed for the data collected for microbial length, weight and microbial quantification using of analysis of variance by Friedman, considering $p < 0.05$.

3 RESULTS AND DISCUSSION

To evaluate the damages possible of *Z. mays* for diesel, growth factors such as root and stem were analyzed (Table 1), and the significance between treatments is in Table 2.

Table 1 - Root length and stem length of *Zea mays* in soil contaminated with diesel and with the addition of biofertilizers and AMF's inoculum

Treatments	Root length (cm)	Stem length (cm)
Soil control (T1)	28.83	46.30
Control + AMF'S (T2)	20.56	39.80
Control + Biofertilizer (T3)	30.50	47.80
Control + AMF'S + Biofertilizer (T4)	26.26	42.10
1% Diesel (T5)	21.00	31.10
1% Diesel + AMF'S (T6)	25.00	36.63
1% Diesel + Biofertilizer (T7)	29.33	40.60
1% Diesel + AMF'S + Biofertilizer (T8)	30.00	36.50
2% Diesel (T9)	23.00	24.60
2% Diesel + AMF'S (T10)	26.50	35.40
2% Diesel + Biofertilizer (T11)	28.47	37.23
2% Diesel + AMF'S + Biofertilizer (T12)	24.00	35.40

Table 2 - Significance between treatments, for p value < 0.05, Friedman's analysis

Root length (cm)	Stem length (cm)
T1 – T2	T1 – T5
T2- T3	T1 – T9
T2 – T7	T3 – T5
T2 – T8	T3 – T9
T3 – T5	T3 – T10
T3 – T9	T3 – T12
T3 – T12	T4 -T5
T5 – T7	T4 – T9
T5 – T8	T7 -T9
T8 – T9	-

In all treatments containing diesel concentrations, this caused toxic effects to the species *Z. mays*. With increasing concentrations of diesel, the factors analyzed as root growth, stem growth and weight of the plants suffer negative effects. In *Melilotus albus* plants, the addition of diesel on soil drastically reduced their growth, as observed by HERNÁNDEZ-ORTEGA et al. (2012).

The addition of biofertilizer has encouraged the growth of the roots (30.50 cm) and stem relative the addition of AMF (20.56 cm). The biofertilizer is compound rich in nutrients, which improves the soil quality (JILANI et al. 2007) benefiting the development of *Z. mays*.

Treatment with 1% diesel had a decrease of 27.15% in root length and 32.82% in stem length compared to the control soil. However, treatment with 1% diesel and AMF, the addition ofmycorrhizae assisted the growth of roots and stem. Regarding the root length was increased by 19.04%, as stem length resulted in 17.78%. The same was observed by HERNÁNDEZ-ORTEGA et al. (2012), which AMF in plant *Melilotus albus*, significantly contributed to the increase in total petroleum hydrocarbon degradation.

In treatments that have been added biofertilizers success in the growth of the variables was higher, exceeding the addition AMF's. At the root length increased by 39.66% since the stem obtain 30.54%.

The root length showed greater (higher) sensitivity to diesel about to the stem length. However, when it was added to the bio-stimulators, such as AMF and biofertilizers, root length obtained faster responses.

However, when adding AMF and bio-fertilizer to the soil contaminated with diesel, there were no significant differences about to the Soil with the addition of bio-fertilizer and diesel. Most probably there was competition for sources of carbon between the microbial population contained in biofertilizers and AMF's population.

Concentrations between 1 and 2% diesel no significant differences ($p > 0.05$) compared to the root growth, even with the addition of bio-stimulators.

Treatment with 2% diesel, the addition of biofertilizer to the contaminated soil favors the growth of the root. The same was observed when inoculating AMF's soil with diesel. However, the mixture of soil contaminated with diesel, the AMF's biofertilizer and the increase was not significant ($p > 0.05$). Probably, there was a competition between the microbial population of biofertilizers and the AMF's. The biofertilizer has in its nutrient composition and lives microorganisms, which favor

the nutritional status of plants (VESSEY, 2003). Stem length was less sensitive compared to the root length, since it was not directly in contact with contaminated soil.

Fresh weight and dry weight were also evaluated in soil contaminated with different diesel concentrations, as well as before and after addition of biofertilizer and AMF's inoculum (Table 3), and the significance between treatments is in Table 4.

Table 3 - Quantifications of fresh weight and dry weight (g) of soil contaminated with diesel and adding biofertilizer and AMF`s

Treatment	Fresh weight (g)	Dry Weight (g)
Soil control (T1)	3.12	0.54
Control + AMF'S (T2)	4.86	0.67
Control + Biofertilizer (T3)	5.82	0.64
Control + AMF'S + Biofertilizer (T4)	5.11	0.65
1% Diesel (T5)	2.67	0.37
1% Diesel + AMF'S (T6)	5.70	0.83
1% Diesel + Biofertilizer (T7)	6.18	0.78
1% Diesel + AMF'S + Biofertilizer (T8)	6.73	0.88
2% Diesel (T9)	4.20	0.67
2% Diesel + AMF'S (T10)	4.06	0.48
2% Diesel + Biofertilizer (T11)	6.07	0.50
2% Diesel + AMF'S + Biofertilizer (T12)	2.67	0.50

Table 4 - Significance between treatments, for p value < 0.05, Friedman's analysis

Fresh weight (g)	Dry weight (g)
T1 – T7	T5 – T6
T1- T1	T5 – T7
T5 – T7	T5 – T8
T5 – T8	T6 – T10
T5 – T11	T7 – T10
T7 – T12	T8 – T10
T8 – T10	T8 -T11
T8 – T12	T8 – T12

The fresh weight and dry weight measurements suffered interference with the presence of diesel in the soil. However, with the additions of biofertilizers and inoculum of AMF 's negative effects were reduced. The same was observed by BONNA et al. (2011), which soil contaminated with diesel oil, affect the germination and growth of *Schinus terebinthifolius* seedlings, as well as the reduction of biomass.

By adding biofertilizer to the soil contaminated with diesel, there was an increased weight in treatments. The same occurred when adding the AMF 's inoculum. However, the mixture of biofertilizer and AMF 's favored inoculum increased the fresh and dry weights soil treatments diesel. Quantification of the microbiota, by plating *Pour Plate* and the significance between treatments, for p value < 0.05, Friedman's analysis, are described in the Table 5.

Table 5 - Microbiota of quantification in soil contaminated by diesel and adding AMF 's and biofertilizers

Treatment	**Bacteria (CFU/g) 10^6**	**Fungi (CFU/g) 10^3**
Soil control	4.30	6000.00
Control + AMF`s	0.67	0.11
Control + Biofertilizer	0.90	0.10
Control + AMF`s +Biofertilizer	0.87	0.11
1 % diesel	2.56	8.00
1 % diesel + AMF`s	1.62	1.00
1 % diesel + Biofertilizer	12.70	0.60
1 % diesel + AMF`s + Biofertilizer	0.64	0.10
2% diesel	0.00	0.80
2% diesel + AMF`s	0.01	1.00
2% diesel + Biofertilizer	99.00	0.01
2%diesel + AMF`s + Biofertilizer	15.20	6.00

The treatments that were significant, are listed below, for Bacteria (CFU/g) 10^6, for p value < 0.05, Friedman's analysis:

- T1 – T9

- T2- T11
- T7 – T9
- T7 – T10
- T8 – T11
- T78– T12
- T9 – T11
- T9 – T12
- T10 -T11
- T10 – T12

The treatments that were significant, are listed below, for Fungi (CFU/g) 10^3, p value < 0.05, Friedman's analysis:

- T1 – T3
- T1 – T8
- T1 – T11
- T3 – T5
- T5 – T8
- T11 – T12

The soil control has great microbial density before the diesel application. After application of 1% many bacteria drastically reduced to 40.47% and the population of fungi was reduced to 99.86% affecting microbial growth. The same occurred in the treatment with 2% diesel. The bacterial population did not grow in the presence of diesel, since the fungal population almost 100% of it was damaged.

Regarding treatment with 1% and treatment with 2% diesel application on soil, the addition of biofertilizer was favoring bacterial growth. The addition of AMF's also helped growth, but to a lesser extent. The same was observed by AGAMUTHU et al. (2010) to study soil contaminated with lubricating oil. By adding organic waste was 96% of remediation to add in 1% of lubricating oil application. In applying 2.5% of lubricating oil, it was 89.6% of contaminant removal.

The biofertilizer by features determining factors for microbial growth, favored the development of bacteria and fungi even in the presence of recalcitrant

contaminants, acting in the form of bioaugmentation. Thus, the success of bioaugmentation will depend on the ability of the population to survive in contaminated soil (MROZIK and PIOTROWSKA-SEGET, 2010).

By adding AMF's and biofertilizers to soil contaminated with diesel, there was a relatively minor microbial growth, probably carbon source of competition between mycorrhizal fungi.

Therefore, the addition of AMF's and biofertilizer assisted in *Z. mays* growth even in the presence diesel. However, the study's AMF's and its recovery in degraded soils should be exploited, since it is an effective method, feasible by favoring the environmental balance.

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