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SEED OPTIONS FOR TOXICITY TESTS IN SOILS CONTAMINATED WITH OIL

Thiago Gonçalves Cavalcanti[1], Andrwey Augusto Galvão Viana[1], Thaffarel Pereira Guedes[1], Amanda de Souza Freire[1], Rafael de Almeida Travassos[2] and *Ulrich Vasconcelos[1]
[1]Laboratório de Microbiologia Ambiental, Departamento de Biotecnologia, Universidade Federal da Paraíba – UFPB, Campus I, CEP – 58.051-900, João Pessoa-PB
[2]Laboratório de Farmacobiotecnologia, Departamento de Biologia Celular e Molecular, Universidade Federal da Paraíba – UFPB, Campus I, CEP – 58.051-900, João Pessoa-PB

ABSTRACT

Determination of phytotoxicity is useful as a sequential test by which the microbiological removal of hydrocarbons present in soils is investigated. However, oil can stimulate the growth of some plants and the selection of appropriate seeds may require time. This study investigated the germination index of 11 edible plants seeds exposed to different concentrations of recalcitrant oils. All plants showed symptoms of toxicity and the indices in 9 of them were reduced proportionally to the contaminants concentration. Eudicots were more sensitive than monocots. Subsequently, a sandy soil was contaminated with lubricating oil and different bioremediation strategies were evaluated. Among the 9 selected seeds, 3 were randomly tested before and after 30 days of treatment. There was an increase in germination indices, especially when more than 30% of the contaminant was removed. The evaluated plants were considered good choices for ecotoxicity tests on soils contaminated by hydrocarbons, particularly *C. anguria*.

Keywords: Phytotoxicity, oil hydrocarbons, bioremediation, germination index.

INTRODUCTION

Oil is a generic term often used to denote a complex toxic mixture of aliphatic hydrocarbon, aromatic heterocyclic, salts and a small fraction of metals and organometallic compounds, formed naturally from the anaerobic conversion of organic material deposited in low permeability sediments under specified conditions of pressure and temperature. Petroleum hydrocarbons are a mixture of heterogeneous saturated compounds, aromatics and resins, inevitably introduced into the environment from various sources during all stages of the processing of crude oil and its derivatives, that is, from extraction to storage in retail outlets. Thus, petro derivatives represent one of the most important classes of xenobiotics responsible for negative impacts on the environment, in particular to animal and plant life, to bodies of water and the soil (Drozdova *et al.*, 2013; Sivaraman *et al.*, 2011).

Phytotoxicity is understood to mean intoxication in plants by substances in the environment, which can be absorbed and that accumulate in plant tissues (Araujo and Miller, 2005). The determination of phytotoxicity comprises one of the criteria used to evaluate the bioavailability of toxic compounds, such as petroleum hydrocarbons, needing simple, fast and reliable methods for this (Wang *et al.*, 2002).

In the literature, most studies related to phytotoxicity discuss the toxic effects of pesticides and certain metals, but the number of publications addressing the effect of oil derivative compounds has been growing (Meudec *et al.*, 2007; Hamdi *et al*., 2007; Sverdrup *et al.*, 2003). The phytotoxicity of polycyclic aromatic hydrocarbons (PAH) has been described as a significant negative effect on the metabolism of plants in concentrations that, in the majority of studies, can vary between 10 and 1000 mg / kg (Debiane *et al.*, 2008; Baek *et al.*, 2004). However, concentrations greater than 1000 mg / kg oil have been reported as stimulants of plant growth (Reynoso-Cuervas *et al.*, 2008).

Handbooks suggest that phytotoxicity assays be performed with at least three species, but this number can be reduced to one eudicot and one monocot. These studies seek to identify the most sensitive parameters, these being seed germination and elongation of the radical, being one of the most studied (OECD, 1984).

Most *in vitro* studies are usually carried out over 5 days in the absence of light. This guarantees a specific result for the phytotoxicity of the contaminant rather than the

*Corresponding author e-mail: u.vasconcelos@cbiotec.ufpb.br

product of the photo-oxidative reactions that may have occurred during the experiment (Wieczorek and Wieczorek, 2007). The main advantages of the method relate to the fact that plants grow regardless of seasonality and ease of handling. Furthermore, this can reveal potential candidates for phytoremediation (Reynoso-Cuervas *et al.*, 2008).

The objective of the present study was to identify plants potentially important in studies on the bioremediation of soils contaminated by oil, by determining the germination index, i.e. the ratio between the numbers of germinated seeds to the size of the emerging rootlets exposed to contaminated soil extract and distilled water.

MATERIALS AND METHODS

Soil

A sandy soil without a history of contamination by hydrocarbons was used, having a pH value of 7.9±0.1%, moisture content of 16.1±0.1% and water retention capacity established at 49.9±0.1%. These properties were identified employing the methodology described by EMBRAPA (1979) and Watwood *et al.* (1991).

Contaminants

The study used two types of oil: 1. a mixture of used lubricating oil, obtained from different gas stations in the city of João Pessoa, Paraíba, Brazil; and 2. marine fuel oil MF-380, kindly provided by Dr. Norma Gusmão from the Department of Antibiotics of the Universidade Federal de Pernambuco. The contaminants were characterized by USEPA methods and showed high levels of Total Petroleum Hydrocarbons (TPH), naphthalene, phenanthrene and pyrene (lubricating oil), as well as n-alkanes ranging from C12 to C34, besides fluorene, phenanthrene, pyrene and benzo[a]pyrene, with the polycyclic aromatic hydrocarbons being predominant (MF-380).

Selection of Plants for Ecotoxicity Testing

Eleven types of edible vegetable seeds were tested (Toca do Verde, Canoas, Brazil): 4 monocots and 7 eudicots, chosen randomly, whose characteristics are shown in Table 1.

All seeds were washed three times with distilled water to eliminate excess dye and preservatives. During the process, irregularly shaped seeds were removed. The seeds were dried on filter paper at room temperature for three hours.

The ecotoxicity tests were performed in triplicate according Tiquia *et al.* (1996) with samples from a system containing soil contaminated with lubricating oil mixture or MF-380 oil, in contaminant-ground ratios of 1:50, 1:10 and 1:5, and incubated for 5 days at room temperature. After the incubation period, soil extracts were prepared with 10 g of soil in 100 mL of distilled water. After vigorous agitation, the extract was filtered and approximately 5-10 ml was transferred to Petri dishes, carefully soaked in No. 1 filter paper (Whatman, 90 mm diameter) containing between seven to ten seeds, distributed over the surface of the paper. Control was carried out with distilled water replacing the soil extract. Incubation occurred at 22±1 °C in the absence of light for 5 days (New Ethics, 411D). After this period, the germinated seeds were counted and the size of the roots measured using a caliper. The Germination Index (GI) of the seeds was determined employing the equation (1):

$$I_G = [(S_1 x R_1) / (S_2 x R_2)] \times 100$$

Table 1. Plant characteristics for the ecotoxicity test.

Seeds	Size (mm)	Photoblastism
Monocots		
Allium cepa	2.0±0.1	Neutral
Allium porrum	2.9±0.2	Positive
Allium fistolosum	2.8±0.2	Negative
Zea mays	5.9±0.7	Neutral
Eudicots		
Artemisea dracunculus	1.1±0.2	Positive
Brassica nigra	1.0±.0.1	Positive
Brassica oleracea	1.0±0.1	Negative
Brassica rapa	3.0±0.1	Neutral
Cucumis anguria	5.6±0.8	Negative
Cucumis melo	12.2±1.0	Negative
Cuminum cyminum	4.6±0.5	Negative

Where: S_1- number of germinated seeds in soil extracts, R_1- average root length on soil extract, S_2- number of germinated seeds in control and R_2 mean root length in the control. The level of toxicity was rated as high when GI < 50%, moderate (GI = 50-80%) or null when GI > 80% (Anastasi *et al*., 2009).

For the test to be conducted in the absence of light, it was necessary to know the photoblastism of the seeds. Two Petri dishes for each plant were prepared as described above, soaking the filter paper with distilled water and incubating at 22°C for 5 days in the dark and under artificial light, as described by Cordazzo and Aracama (1998). After this period, the germinated seeds were counted, applying the equation 2:

$$PB= (S_d/ S_l)$$

Where: PB – photoblstism; S_d – the number of germinated seeds in the dark and S_l the number of germinated seeds in the light. The photoblastism was classified as positive if greater than 1, negative if less than 1 and neutral when the value was 1.

Application of Selected Seeds in Ecotoxicity Tests

The test was performed with three selected seeds in soil extracts before and after different bioremediation treatments. Bioremediation was conducted in laboratory-scale at room temperature for 30 days, using transparent polyethylene reactors filled with 200g of sandy soil contaminated with a mixture of lubricating oil at a ratio to soil of 1:40, thus establishing a condition of the total petroleum hydrocarbons contamination of approximately 16,000 mg/kg. Three test conditions were tried: 1- bioaugmentation with a strain of *Pseudomonas aeruginosa* TGC01, to which was added a corresponding suspension of approximately 10^7 CFU/mL; 2- biostimulation, by supplementation with 250 mg.kg of *Gossypium* sp. cake; and 3- association of bioaugmentation with biostimulation treatments. The abiotic loss was obtained in a sterile reactor with a solution of 10% (w/v) silver nitrate. Tests were conducted in duplicate.

RESULTS AND DISCUSSION

In the first part of this study, 17 different species of seeds were tested, of which 6 were excluded since their germination periods exceeded three days, that is, there was insufficient time necessary for their incubation as well as seed photoblastism may be implicated. According to Flores *et al.* (2011), some plants demand light energy for seed germination, particularly dark seeds. Otherwise light seeds usually germinate in absence of brightness. To know such a feature was interesting, given the conditions of absence of light in this test.

A total of six seeds, including grasses and *Solanaceae* did not germinate. This was attributed to the longer time required for these seeds, limiting their use under the conditions of this study, i.e. 5 days. Later it was found that germination would occur in around 10 to 15 days both in the presence and absence of light. In the eleven remaining species (Table 1), there was negative photoblastism in five seeds (*Brassica oleracea*, *Cucumis anguria*, *Cucumis melo*, *Cuminum cyminum* and *Allium fistolosum*), while in three others there was neutral photoblastism (*Brassica rapa*, *Allium Cepa* and *Zea mays*). Three species had positive photoblastism (*Artemisia dracunculus*, *Brassica nigra* and *Allium porrum*).

Seed germination is a complex mechanism that begins with a swelling of the seed and ends with the emergence of the primary root and cotyledon. This process can be influenced by several factors, such as moisture, pH and salinity of the medium. Additionally, the integument of the seed, besides serving as a light filter, participates in the early stages of germination by establishing a permeability barrier and interfering with the water diffusion processes and gas exchanges which exert a constricting effect or mechanical expansion in the embryo (Hamdi *et al.*, 2007).

In the studies on soils contaminated with petro derivates, ecotoxicity tests, such as those which use plants, are fundamental for two reasons: they disclose the need for intervention in these environments; and they indicate the effectiveness of a treatment after the reduction or removal of the contaminant. The results of the eleven tested seed germination rates are shown in Figure 1 and their numerical values in Table 2.

Phytotoxicity categories are assigned based on the germination index: high when the values are set between 0 and 50%, moderate between 50 and 80%. Any value above this is considered null. This may indicate that there are smaller concentrations of contaminants or that the metabolites produced during biodegradation are not toxic (Anastasi *et al*., 2009). As the test relates the number of germinated seeds to the root elongation events in the presence and absence of exposure to hydrocarbons, there is the possibility of obtaining indices greater than 100%, as observed in *C. cyminum* with a MF 380 marine sea oil ratio to soil of 1:50.

For the two types of contaminants tested, there were predominantly lower germination rates of 50%, followed by values classified as moderate phytotoxicity. The eudicots were more sensitive than the monocots. Some seeds, particularly those exposed to MF-380 marine oil, showed symptoms of toxicity relative to the concentration of the contaminant.

Table 2. Germination index of seeds exposed to different oil concentrations.

Plants	Lubricant oil			MF-380 marine oil		
	contaminant:soil ratio					
	1:50	1:10	1:5	1:50	1:10	1:5
C. cyminum	0.0±0.0	13.3±0.5	0.0±0.0	300.0±0.5	50.0±0.5	0,0±0.0
B.oleracea	30.2±0.1	48.3±0.1	49.5±0.1	53.8±0.1	28.6±0.1	40.6±0.1
A. dracunculus	56.8±0.2	44.7±0.2	27.3±0.2	82.9±0.2	52.2±0.2	58.4±0.2
C. anguria	42.2±0.8	0.0±0.0	80.2±0.8	12.9±0.8	0.0±0.0	42.6±0.8
C. melo	76.1±0.1	43.5±0.1	52.2±0.1	45.8±0.1	80.2±0.1	57.5±0.1
B. nigra	64.3±0.1	0.0±0.0	0.0±0.0	35.5±0.1	0.0±0.0	0.0±0.0
B. rapa	39.7±0.1	58.8±0.1	54.7±0.1	42.5±0.1	51.8±0.1	66.2±0.1
A. porrum	12.0±0.1	66.3±0.1	92.0±0.1	67.4±0.1	58.9±0.1	98.9±0.1
A. cepa	63.5±0.1	59.4±0.1	76.1±0.1	76.7±0.1	6.3±0.1	12.7±0.1
A. fistolatum	55.3±0.5	65.4±0.5	7.2±0.5	67.6±0.5	63.5±0.5	6.8±0.5
Z. mays	0.0±0.0	0.0±0.0	0.0±0.0	32.7±0.1	0.0±0.0	49.0±0.1

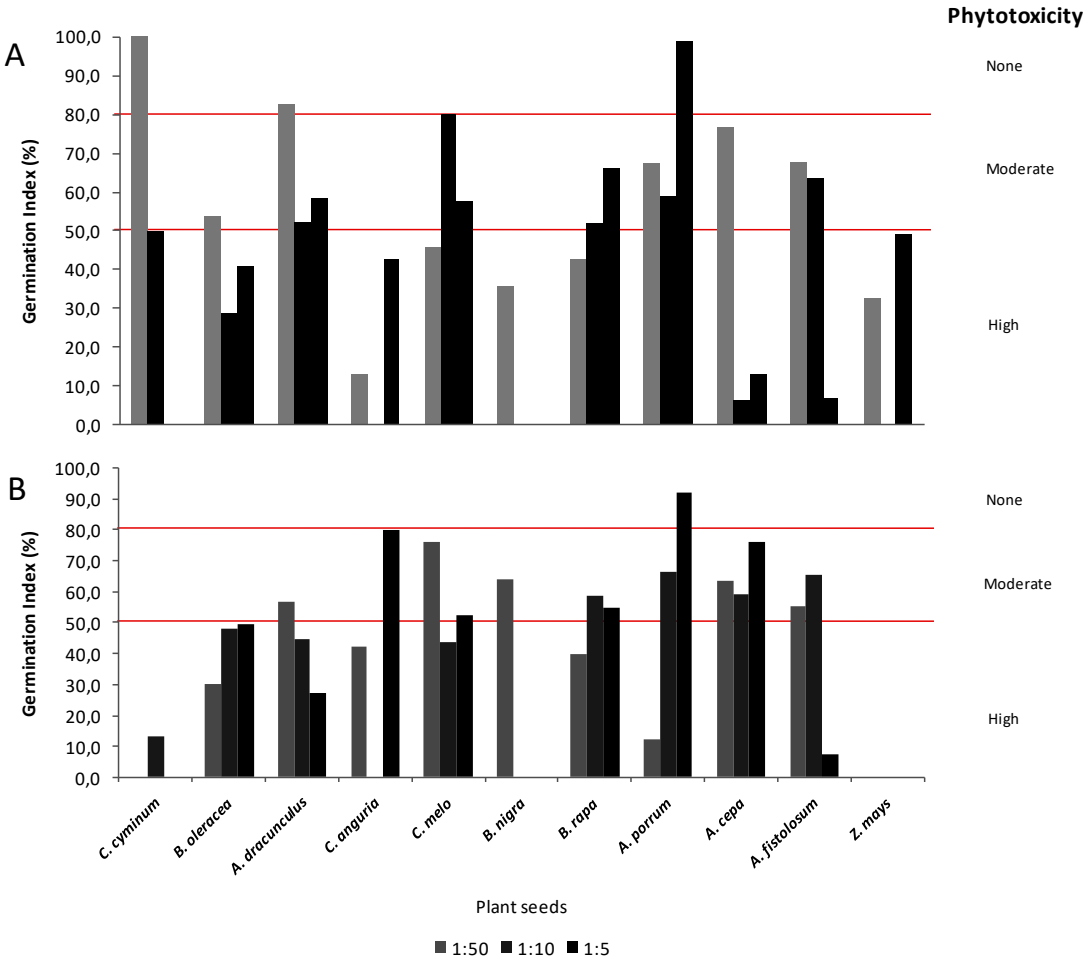

Fig. 1. Germination index of eleven seeds exposed to three different concentrations of MF-380 marine oil (A) and lubricant oil (B). Horizontal lines highlight grades of phytoxicity.

Besides the number of cotyledons, the size and color of the seeds are important characteristics that may influence the test response. Seeds with two small, dark cotyledons, e.g. *B. oleracea* and *B. nigra* are more sensitive than larger lighter seeds with one cotyledon (Tiquia *et al.*, 1996). However, in this study, *Z. mays* and *C. anguria* were also quite sensitive.

Among the eudicots that measured between 1.0 and 4.6 mm, *B. nigra*, *B. oleracea* and especially in *A. dracunculus* and *C. cyminum*, phytotoxicity was proportional to the amount of contaminant oil in the soil. *C. cyminum* provided the best evidence of negative photoblastism, subject to MF-380 marine oil. However, it is noteworthy that although *A. dracunculus* and *B. nigra* exhibited positive photoblastism, and measured about 45 times less than *C. cyminum*, there was a significant decrease in germination and root elongation in the plates containing the contaminated soil extract, suggesting that the plant can serve as a preferred choice among the eudicot plants with small seeds, especially *B. nigra* which reached GI=0% in the two largest concentrations of both contaminants evaluated. This finding may be due to seed size and color, since small, dark seeds are more sensitive to this test (Cordazzo and Aracama, 1998). A different pattern of intoxication symptoms, observed previously, occurred with *B. rapa*. While the mixture of used lubricating oil was highly phytotoxic, surprisingly higher concentrations of MF-380 marine oil reduced the phytotoxicity from high to moderate.

Despite the fact that hydrocarbon oil has been reported to be toxic and an important cause of adverse effects on the metabolism of plants in concentrations in the majority of studies varying from 10 to 1000 mg / kg (Debiane *et al.*, 2008; Baek *et al.*, 2004), concentrations greater than 1000 mg/Kg oil, have been reported as stimulants to plant growth (Reynoso-Cuervas *et al.*, 2008).

Among the larger seeds, measuring between 5.6 and 12.2 mm, germination rates were observed which were not proportionate to the concentration of the oil tested, as were the smaller seeds. This was probably due to the size of these seeds. In these cases, germination seems to be related more on the energy reserves than on environmental factors. Although large, light and containing many starch reserves, the seeds have low moisture content, depending much more on ground water for their development.

It should also be emphasized that the presence of oil may have formed a kind of film around the seeds, preventing water from the soil extract to penetrate their integument (Adam and Duncan, 2002; Storck *et al.*, 2013). Moreover, the presence of volatile compounds, more toxic in the root growth phase, may have contributed to the results. In comparison, the severity of the intoxication in *C. anguria* was more pronounced than in *C. melo.* This feature has already been recognized in a previous study (Vasconcelos *et al.*, 2010). *C. anguria* is a plant whose seeds have two cotyledons and germinate at temperatures in a range between 16.0 and 35.5°C. As the test was conducted at 22.0°C, this confirms the role of seed size in the results.

Among the monocots, except for *Z. mays*, the germination rates ranked phytotoxicity predominantly as moderate. Because it is a light and large seed, it was expected that the *Z. mays* germination rates would be high, but the tested oil concentrations proved very toxic, especially lubricating oil. Corn, when exposed to high concentrations of hydrocarbons, shows symptoms of water stress as well as a modification in calcium content. This is an important indication of environmental pressures having a direct effect on plant growth and development (Dupuy *et al.*, 2015).

Toxicity symptoms were stronger in the two seeds exposed to MF-380 marine oil compared to the mixture of lubricant oil: *A. fistolatum* and particularly *A. cepa*. In this plant, the toxic effect was proportional to the oil concentration, and the germination rate was up to 12 times lower than that obtained with the lowest concentration of oil.

The oil promoted a stimulating effect on *A. porrum*, resulting in an increase of the germination index as the oil concentration increased. This indicates that a low hydrocarbon concentration can cause metabolic stress due to the presence of volatile compounds. On the other hand, higher concentrations may promote conditions for the plant to grow at the expense of morphoanatomic and biochemical adaptations. Thus, plants whose growth is stimulated in the presence of high hydrocarbon concentrations are also good candidates for ecotoxicity tests.

Based on these results, the eleven plants can be used in ecotoxicity tests. It is important to clarify that this selection did not consider the presence of possible toxic metabolites arising from the bioprocess. The sensitivity of these plants to non-metabolized hydrocarbons provides means of choosing the proper species, as well as reduces search time for appropriate seeds.

In order to verify the use of the seeds as biomarkers of contamination by oil and its metabolites, three of the eleven evaluated seeds were randomly selected and exposed to soil extract contaminated by a mixture of lubricating oil before and after microbiological treatment for a period of 30 days (Table 3). Excluding the abiotic losses established at approximately 10% as reported in the literature (Vasconcelos *et al.*, 2011), removal of the TPH, respectively, to the conditions bioaugmentation, biostimulation and the combination of the two techniques was 29.6 ± 3.0%, 31.1±3.1% and 32.5±3.3%.

Table 3. Germination index of seeds before and after bioremediation.

Plants	Microcosms conditions and time (days)					
	BA + BS		BS		BA	
	0	30	0	30	0	30
Artemisia dracunculus	68.2	164.3	68.2	172.5	68.2	65.7
Brassica nigra	58.3	74.7	58.3	184.2	58.3	73.8
Cucumis anguria	21.6	37.6	21.6	51.4	21.6	34.8

BA+BS – association between bioaugmentation with *P. aeruginosa* and biostimulation with cottonseed cake; BS – bioestimulation; BA – biaugmentation. Standard deviation: *A. dracunculus* (±0.2%), *B. nigra* (±0.1%) e *C. anguria* (±0.8%)

In reactors whose contaminant removal was greater than 30%, the germination rates increased in all seeds exposed to the contaminant. *C. anguria* showed more symptoms of toxicity than *B. nigra* and *A. dracunculus*, respectively. In both after 30 days of treatment, the phytotoxicity was classified from moderate to null. This highlights the fact that the conditions that biostimulation was tested with cottonseed cake, as well as in association with bioaugmentation, may serve as an alternative for the removal of hydrocarbons. Additionally, in cases where these indices were higher than 80%, the residual oil concentration or metabolites formed during biodegradation were not considered toxic, suggesting that the longer continuity of the process leads to the mineralization of the contaminant. On the other hand, when only bioaugmentation was employed, oil removal was less than 8.9% under the best conditions, reflecting a moderate toxicity in *B. nigra* and *A. dracunculus* and high toxicity for *C. anguria*. Another study, Bouchez *et al.* (2000) suggests that under bioaugmentation conditions, the introduction of a highly concentrated microbial suspension requires adaptive time to compete first of all with the soil indigenous microbiota. In addition, until equilibrium in microbial density is reached, there may be a delay in the bioprocess of up to 15 days (Mille-Lindblom *et al.*, 2006), coinciding with what was observed in this study, that is, the establishment of the lowest percentage of TPH and removal of its fractions in 30 days.

C. anguria showed the greatest sensitivity among the tested seeds and this result was in agreement with previous research (Santos and Cardoso, 2001; Vasconcelos *et al.*, 2010). It is noteworthy that 30 days is a very short time to promote effective removal in the context of bioremediation of soil contaminated by oil. However, plants have provided indications that they may be used, given biological reduction of approximately 30% of the contaminant, suitable to raise the germination rates to accepted values considering the short period of process.

CONCLUSION

Determination of the phytotoxicity of oil hydrocarbons present in soils complements the test sequence, which investigated the characterization of a contaminant, its microbiological removal, as well as certification with respect to the degree of toxicity of the metabolism products. When a particular soil is affected by a complex mixture of hydrocarbons, the symptoms of physiological and biochemical stress on some plants, related to contaminant concentrations present, can provide important information about the health status of the area, from a biological point of view, and contribute to demonstrate the natural ability of soil recovery. This may signal the need for intervention or liberation, in terms of the processes surrounding bioremediation. Except for *B. rapa* and *A. porrum*, the germination rate in nine seeds was reduced. This provides information on the use of these plants as options for ecotoxicity testing, optimizing the time of selecting appropriate seeds for bioremediation of soils contaminated by hydrocarbons.

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