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ScienceDirect
Bioresource Technology 99 (2008) 6379–6385
BIORESOURCE TECHNOLOGY

In vitro evaluation of germination and growth of five plant species on medium supplemented with hydrocarbons associated with contaminated soils

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Received 20 February 2007; received in revised form 26 November 2007; accepted 26 November 2007
Available online 25 January 2008

Abstract

The effect of a hydrocarbon mixture (HCM) of three polycyclic aromatic hydrocarbons (PAH) and Maya crude oil on germination, growth and survival of four grasses (*Bouteloua curtipendula, Cenchrus ciliaris, Echinochloa crusgalli and Rhynchelytrum repens*) was studied and compared to a control (*Festuca arundinacea*) under *in vitro* conditions. The species were cultured on MS medium with different HCM initial concentrations. Germination was not affected for any assayed concentration; however, the length of the stems and roots decreased when HCM increased and the survival of the four species also diminished. Except for *F. arundinacea*, a direct link between hydrocarbon concentration and plant survival was observed. *In vitro* studies are clean and easy to handle techniques allowing isolation of the plant activity from that derived from associations with microorganisms in non-sterile cultures. To our knowledge, this is the first work towards phytoremediation assisted by *in vitro* plant cultivation.

Keywords: Polycyclic aromatic hydrocarbons (PAH); Maya crude; Phytoremediation; *Poaceae*

1. Introduction

The polycyclic aromatic hydrocarbons (PAH) have been a focus of attention by the scientific community due to their impact on public health and the environment. Some PAH compounds as chrysene, benz[*a*]anthracene, dibenz[*a*,*h*]anthracene and benzo[*a*]pyrene are mutagens and carcinogens (Ferreira, 2001) and most of them remain in soils as persistent contaminants. The accumulations in soil of PAH are due to many anthropogenic sources such as town gas sites, coking plants, solid fuel domestic heating, aircraft exhaust, car exhaust and forest fires. In Mexico, several hydrocarbon activities such as extracting, pumping, refining and transport are particularly intense. PAH are difficult to remove from soil due to their recalcitrant nature and, apart from naphthalene, they are practically insoluble in water and slow to degrade (Kottler and Alexander, 2001).

Many methods have been used for the clean up of PAH contaminated soil sites. These include removal, incineration and removal followed by thermal desorption, all of which are expensive options but, depending on the final use, sometimes necessary. Biological methods have been investigated as more financially acceptable options; bacteria are able to degrade different xenobiotics under aerobic and anaerobic conditions. Fungi were also studied regarding the ability to metabolize various xenobiotics (Martens and Zadrazil, 1998). Plants have shown to be a promising biotechnological alternative to clean up contaminated soils as well. Phytoremediation has been proposed as a feasible

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doi:10.1016/j.biortech.2007.11.074

option or complementary technique to treat soil polluted by mineral and organic compounds (Macek et al., 2000). However, relatively few plant species have been studied for phytoremediation purposes. Biodiversity is needed and more species are required. Further geobotanical surveys and plant screenings are necessary since these could lead to the identification of additional species with potential value for phytoremediation (Díez et al., 2006).

To achieve maximum PAH reduction, or even elimination, and to successfully establish stable vegetation cover, we should consider numerous criteria as, e.g. root length which determines the capacity to acquire water and nutrients. Plants should be chosen to provide maximum root surface area (Merkl et al., 2005). Plants should be native to the area to which they are used and they should be tolerant to weather and soil conditions. As cost is an important factor, plants that require little attention are preferable.

In this study, we evaluated, under *in vitro* conditions, the effect of a known mixture of three different PAH (phenanthrene, pyrene and benzo[*a*]pyrene) and Maya crude oil on germination, growth and survival of four different grasses compared to a control, tall fescue (*Festuca arundinacea* Schreb.). Assays under *in vitro* sterile conditions allowed us to select potential plants for phytoremediation and distinguish unequivocally between the plant activity responses and those derived from native microorganisms, always existing in soils.

2. Methods

2.1. Plants

Seeds of "tall fescue" (*Festuca arundinacea*) were obtained from the wholesale food market of Mexico City and were kept at room temperature. The seeds of "Side oats grama" (*Bouteloua curtipendula* cvs. Tenius Gould & Kapadia) were collected from different wild plants located at 4.5 km of highway Queretaro-Huimilpan, Mexico. The seeds of "Buffel grass" (*Cenchrus ciliaris* L.), "Barnyard grass" (*Echinochloa crusgalli* var. *crusgalli*) and "Natal grass" (*Rhynchelytrum repens*), were collected from a water channel of Access II of Benito Juarez Industrial zone at Queretaro City, Mexico. The four species were identified at the herbarium of Biology faculty of Universidad Autonoma de Queretaro. The seeds were separated and kept at room temperature for subsequent desinfestations and culture.

2.2. Culture preparation

2.2.1. Seed disinfestation

Prior to disinfestation, damaged and small seeds were removed. Selected seeds were placed into a filter paper envelope, then submerged into powder soap solution (60 g soap L^{-1} water) for 20 min with constant agitation, and rinsed with tap water for 10 min. Under laminar flow hood the envelope with seeds was submerged into 70% (v/v) ethanol for 30 s, follow by immersion in 0.6% (v/v) sodium hypochlorite with 0.1 mL of Tween-20 for 20 min. with constant agitation. The envelope was washed five times with 150 mL deionized sterilized water. The envelope was opened under sterile conditions and stored in sterile empty Petri dishes.

2.2.2. Culture tubes

Murashige and Skoog (MS) culture medium was prepared from micro and macro nutrient stock solutions (Murashige and Skoog, 1962), pH was adjusted with 0.1 M HCl to 5.8 and 2 g L^{-1} Phytagel was added (agar substitute, Sigma Chemical Co., St. Louis, MO, USA), and then boiled. MS medium (10 mL) was added to each culture tube (diam. 2.5 cm, height 15 cm), prior to sterilizing for 15 min. at 1.2 kg cm^{-2}).

2.2.3. Polycyclic aromatic hydrocarbons (PAH)

Phenanthrene (PHE) (purity >96%), pyrene (PYR) (purity >98%) and benzo[*a*]pyrene (BaP) (purity >97%) were purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. The substances were dissolved in dichloromethane (DCM) (Mallinckrodt Baker SA de CV EDOMEX, Mexico).

2.2.4. Maya crude oil

The Maya crude oil was obtained from Instituto Mexicano del Petroleo (IMP). Elemental analyses (% weight): carbon, 83.96; hydrogen, 11.80; oxygen, 0.34; sulfur, 3.51; nitrogen, 0.36 (Leahy and Colwell, 1990). Volatile oil compounds were eliminated by heating for 5 days at 70 °C until constant weight (Díaz-Ramirez et al., 2003).

2.3. Bioassays

In vitro germination of five plant species was evaluated with and without hydrocarbons. Test substances were dissolved in DCM; the PAH blend ratio was: PHE, PYR and BaP (1:1:1; w/w/w); Maya crude oil and PAH blend (1:1; w/w), afterward identified as hydrocarbon mixture (HCM), was preserved in a stock DCM-solution. For each of the test concentrations used, dilutions from the stock DCM-solution were made adding the requisite amount of DCM. The HCM initial concentrations were 500, 1000, 2000, 3000, 4000 to 5000 mg (kg of gel)$^{-1}$. Pure DCM was used for the control samples; controls without solvent were also included. HCM solution (0.5 mL) was added to culture tubes just before hardening of the medium, shaken and maintained at room temperature for three days; one seed was aseptically placed into each culture tube containing semi solid MS medium supplemented with the HCM test concentrations, the culture tube was closed with a plastic cap and sealed with parafilm. Sealed culture tubes were kept at 25 °C, with 16 h photoperiod for 20 d for germination studies and up to 40 d for growth and survival.

2.4. Analytical methods

2.4.1. Plants

In order to evaluate dose/response of bioassays of 6 HCM concentrations for five species, after 40 d culture, we sampled plants destructively. Five replicates per HCM initial concentration were analyzed. The root and stem length of the plants inside the culture sealed tubes were measured with a graduated 30 cm transparent plastic rule and vernier were placed over the surface of culture tubes for estimating root and stem growth. Germination was evaluated daily for 20 d as positive or negative; it was positive if a visible cracking of the seed coat with measurable root or shoot production was observed (Maila and Cloete, 2002). Survival was evaluated as positive or negative; it was positive if the plant was comparable to controls without HCM or DCM.

2.5. Data analyses

Statistical analysis was carried out by using NCSS-2000, version 2001 (Copyright 2001 by Jerry Hintze). Analysis of variance (ANOVA) was performed by comparing tests with $\alpha \leqslant 0.05$. The experimental design was randomized complete block.

3. Results and discussion

3.1. Germination

Germination is undoubtedly an important stage in plant growth and is particularly sensitive to contaminants (Maila and Cloete, 2002). All the species showed different germination time-patterns and maximum germination was achieved after 10 days of culture. After 10 d, non germinated seeds were discarded (data not shown). Germination (%) of the five assayed species in the presence of DCM (0.5 mL in each tube), and with or without HCM are shown in Table 1. The control (*F. arundinacea*) showed the greatest germination (97%) after 10 days, as compared to the other species: *C. ciliaris*, 51%; *B. curtipendula*, 39%; *E. crusgalli*, 36% and *R. repens*, 21%. There were no significant differences ($P \geqslant 0.95$) between the control germination (without HCM), and with DCM or HCM; germination of *R. repens* was significantly higher with DCM as compared to media with or without HCM. This result could be explained because the solvent could remove the waxy layer of the pericarp and thereby facilitate water imbibition. Subbaiah (1982) observed that pre-soaking of cashew (*Anacardium occidentale* L.) seeds in organic solvents for 2 h hastened and partially synchronized germination.

Table 1
In vitro mean germination of five plant species cultured with or without 0.5 mL contaminants per tube after 10 days

	Germination (%)[a]		
	With DCM	Without HCM	With HCM[b]
F. arundinacea	98 ± 1.4	97 ± 2.3	96 ± 3.1
C. ciliaris	51 ± 1.2	51 ± 1.7	49 ± 4.2
B. curtipendula	40 ± 0.9	39 ± 1.8	40 ± 2.1
E. crusgalli	35 ± 1.6	36 ± 1.1	35 ± 3.1
R. repens	33 ± 2.7	21 ± 2.5	23 ± 1.2

[a] Means and standard deviation values of five replicates.
[b] Values correspond to an initial concentration of 500 mg of HCM kg^{-1}.

Once seeds germinated and a reliable control was assured, we decided to evaluate the germination in the presence of the HCM at different initial concentrations (from 500 to 5000 mg kg^{-1}). The *in vitro* germination of the five assayed species in the presence of 500–5000 mg kg^{-1} of HCM at 10 d culture is shown in Fig. 1. Although germination was monitored during 45 d (results not shown) for all plant species, the result did not change after 10 d culture. The presence of HCM (even at the highest concentration, 5000 mg kg^{-1}) did not affect germination. This is in contrast to data reported by Adam and Duncan (2002), showing a negative effect of PAH on germination with 22 different species including grasses, legumes, herbs and commercial crops. Even though *in vitro* conditions probably increased the availability of hydrocarbons, germination was unaffected. Many reports involve freshly contaminated soils or soaking seeds into PAH solutions. Synthetic addition of PAH to soil, was often restricted to one or two PAH, e.g., Nichols et al. (1997), who added phenanthrene and pyrene, Wetzel et al. (1997) who added anthracene and pyrene, and Dzantor et al. (2000) and Liste and Alexander (2000) who added pyrene. Regarding the control (*F. arundinacea*) responses to hydrocarbons, Huang et al. (2005) studied the effect of total petroleum hydrocarbons in soil on the germination of *F. arundinacea*; their results showed that the germination was severely depressed; instead, Smith et al. (2006) evaluated different grasses and legumes in freshly PAH contaminated soil and observed that the germination was unaffected, in agreement with our results. Differences found could be attributed to: (i) different germination methodologies, (ii) germination in soils deals with humic matter and hydrocarbon weathering, and (iii) *in vitro* cultures provide the best germination conditions.

3.2. Growth

Some studies have suggested a link between poor germination and subsequent poor growth in hydrocarbon contaminated soil (Chaîneau et al., 1997); nevertheless, some others have shown exactly the opposite: germination to be unaffected but subsequent growth to be significantly diminished (Li et al., 1997). In our work, the germination was the same with and without hydrocarbons, but the subsequent growth (evaluated as stem length, after 40 d culture) of the different species under all assayed concentrations was depressed (Fig. 2), even at the lowest HCM concentration (500 mg kg^{-1}). *F. arundinacea* displayed the greatest growth in all assayed concentrations and it was confirmed as a good control for the study (Fig. 2). In previous studies, petroleum hydrocarbons have been reported

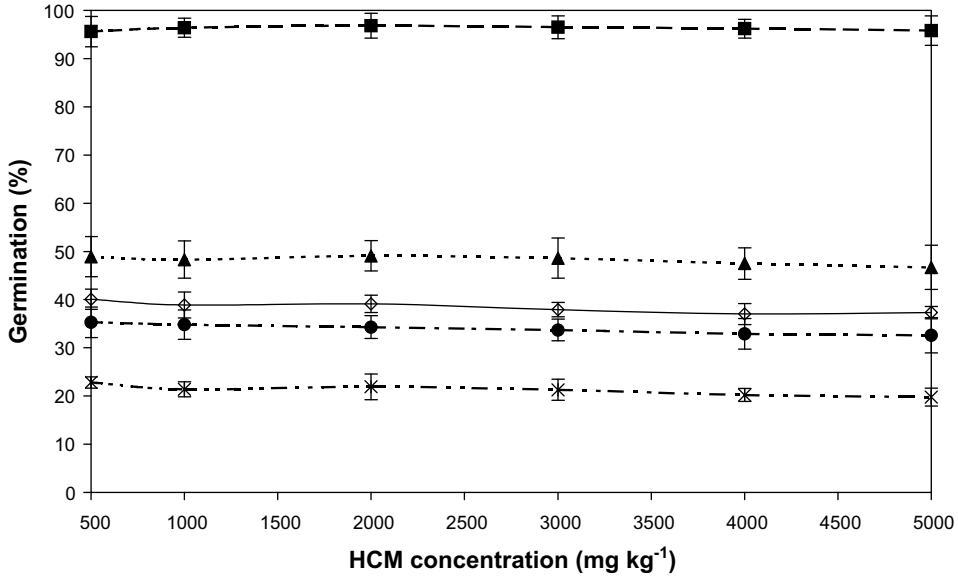

Fig. 1. *In vitro* germination of five plant species with different initial concentrations of hydrocarbon mixture (HCM), at 10 d culture. *Festuca arundinacea* (■), *Cenchrus ciliaris* (▲), *Bouteloua curtipendula* (O), *Echinochloa crusgalli* (□) and *Rhynchelytrum repens* (X). Error bars represent standard deviations of five replicates for all bioassays.

stimulate the growth of plants (Lin and Mendelssohn, 1996). Lin et al. (2002) observed that low fuel dosages (7000 and 14,000 mg kg^{-1} dry soil) enhanced growth in *Spartina alterniflora*; in our experiments (Fig. 2), *C. ciliaris* displayed slight growth stimulation at 1000 mg kg^{-1} but then growth was reduced at higher concentrations. Shann and Boyle (1994), used *E. crusgalli* to study final removal of four soil contaminants (phenol; 2,4-dichlorophenol; 2,4-dichlorophenoxiacetic acid; 2,4,5-trichlorophenoxiacetic acid). The study was performed in serological bottles with artificially contaminated soil; the best removal results were obtained with monocotyledons (including *E. crusgalli*). In contrast, our monocotyledons (*E. crusgalli* and *R. repens*) exhibited poor growth at all concentrations, reaching zero over 3000 mg kg^{-1}. These two species were most difficult to germinate even in the absence of hydrocarbons. Even when the growth of the stems was zero, it does not mean that they did not germinate, but that the plants survived.

B. curtipendula and *C. ciliaris* showed similar stem length at 2500 mg kg^{-1} as in the control (see Fig. 2); however, from 3500 mg kg^{-1} to 4000 mg kg^{-1} only *C. ciliaris* was similar to *F. arundinacea*, suggesting that both plant species (*B. curtipendula* and *C. ciliaris*) could be meaningful candidates for phytoremediation. Nevertheless, since one of the most important characteristic for phytoremediation is the plant root surface, we decided to study the growth of roots.

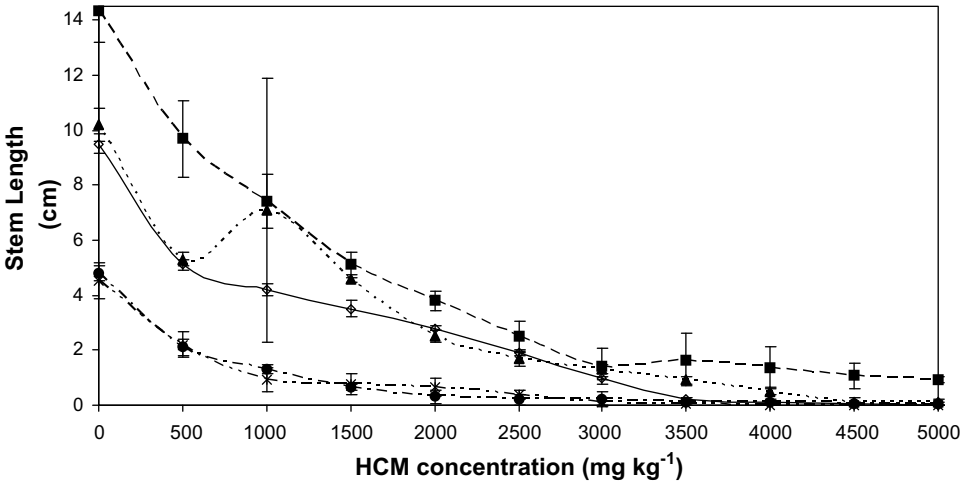

Fig. 2. *In vitro* stem length of five plant species evaluated at different initial hydrocarbon mixture (HCM) concentrations, after 40 d culture. *Festuca arundinacea* (■), *Cenchrus ciliaris* (▲), *Bouteloua curtipendula* (O), *Echinochloa crusgalli* (●) and *Rhynchelytrum repens* (X). Error bars represent standard deviations of five replicates for all bioassays.

Graminoids are usually selected for phytoremediation because of their intensive growth, colonization ability and widely branched roots that offer an extensive surface for microbial colonization and plant-microbial interaction (Hutchinson et al., 2001; Glick, 2003). Such advantages may be counterbalanced by the possible toxic effect of the contaminant altering root biomass and also expected morphological changes. Alterations in the root morphology directly influence the water and nutrient uptake and thus affect plant growth and phytoremediation. Merkl et al. (2005) observed a significant decrease of root biomass of different graminoid species grown in crude oil-contaminated soil. The root growth of our five species was altered by the presence of the hydrocarbons, measured as the root length decrease as shown in Fig. 3. Over 3000 mg kg^{-1} there were no significant ($P \geqslant 0.95$) changes in any of the species. The sensitivity of all species to the presence of the hydrocarbons was different and again, the most sensitive were *E. crusgalli* and *R. repens*. Sverdrup et al. (2003) evaluated the effect of 8 polycyclic aromatic compounds on germination and early life-stage growth of three terrestrial plants in a greenhouse. Their results show that seedling growth was more sensitive than germination for all substances and sensitivity differed among the tested species. Since the hydrocarbons in the medium had a negative effect on root development and probably this result altered morphology and ability to acquire water and nutrients from the medium we decided to study the impact induced by the HCM in plant survival.

3.3. Survival assays

While extensive plant mortality may result immediately after a contaminant spill, plant recovery is generally expected to occur as the toxicity of residual petroleum hydrocarbons is reduced by natural processes (e.g., evaporation, photooxidation and biodegradation). The successful use of phytoremediation will depend, in part, on identifying the concentration that will allow successful plant establishment in contaminated soil (Lin and Mendelssohn, 1998). Our five plant species grew at different HCM concentrations but mortality differed among. We identified the most phytotoxic concentration for each plant species. The survival response, evaluated as the number of plants survived of 100 seeds planted, of the five species at the different HCM concentrations assayed is shown in Fig. 4. The horizontal dotted line in Fig. 4 indicates that below the line, more than 50% of the population in study has died. Once again, *F. arundinacea* is an excellent control, even though at 5000 mg kg^{-1} more than a half of the initial population survived. By comparison 66% of the initial population of *C. ciliaris* and *B. curtipendula* survived at 2500 and 2000 mg kg^{-1}, respectively. *R. repens* did not survive when HCM concentration was higher than 2500 mg kg^{-1}, and the same was observed with *E. crusgalli* at 3000 mg kg^{-1}. In order to survive, plants probably adsorbed the contaminant onto a cell surface or accumulated into the cell. In previous studies, phenanthrene and pyrene accumulated in the roots (Gao and Zhu, 2004). Many contaminants become bound to the root surface and are not translocated; therefore the water and nutrients could not pass through the plant and provoked plant death. As Fig. 4 shows, there is a direct link between the HCM concentration and plant survival: *C. ciliaris* and *B. curtipendula* are the two species that better tolerated the presence of hydrocarbons while *R. repens* and *E. crusgalli* were the more sensitive. It is clear that certain plants are able to interact with hazardous organic compounds through degradation or accumulation (Banks et al., 2003), even more, plants may indirectly contribute to the

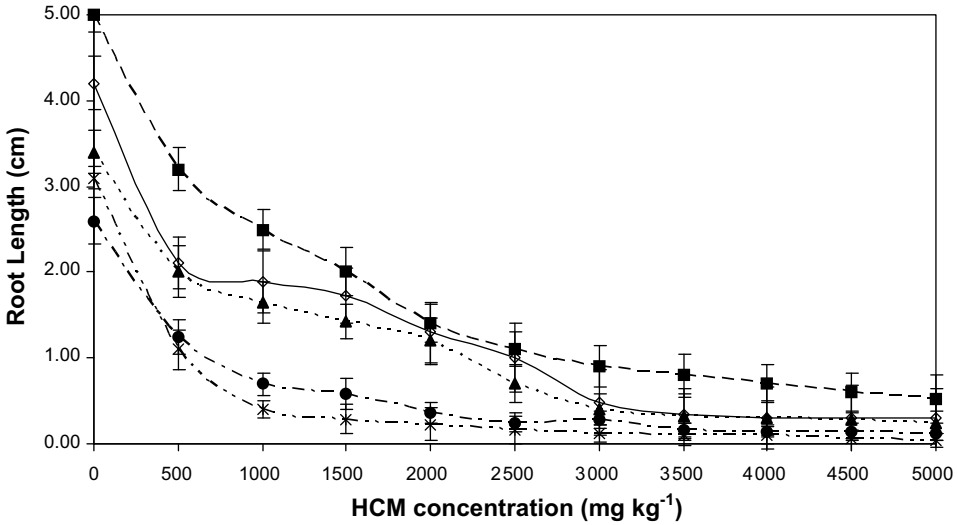

Fig. 3. *In vitro* root length of five plant species evaluated at different initial hydrocarbon mixture (HCM) concentrations, after 40 d culture. *Festuca arundinacea* (■), *Cenchrus ciliaris* (▲), *Bouteloua curtipendula* (O), *Echinochloa crusgalli* (●) and *Rhynchelytrum repens* (X). Error bars represent standard deviations of five replicates for all bioassays.

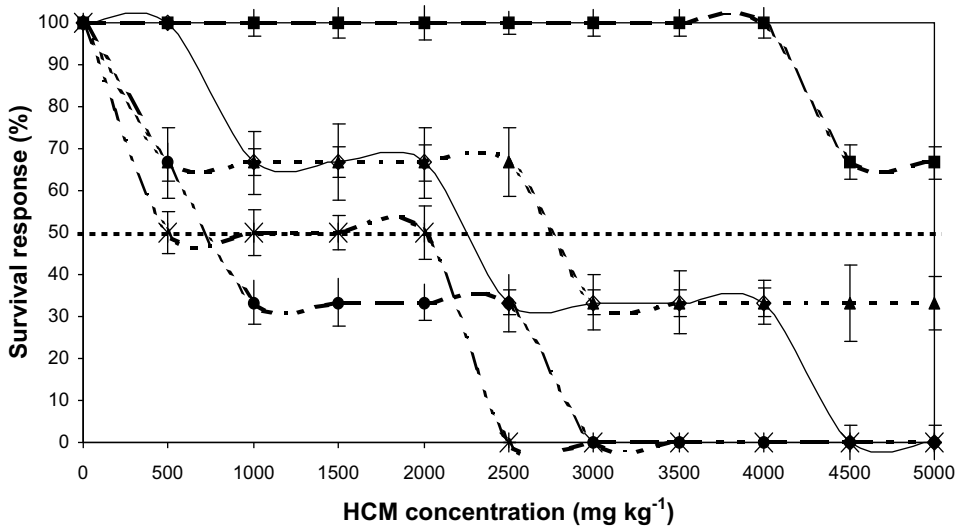

Fig. 4. *In vitro* survival response for the five plant species at the end of 40 d culture. *Festuca arundinacea* (■), *Cenchrus ciliaris* (▲), *Bouteloua curtipendula* (O), *Echinochloa crusgalli* (●) and *Rhynchelytrum repens* (X). Error bars represent standard deviations of five replicates for all bioassays.

dissipation of contaminants in vegetated soil, and soil adjacent to the root contains increased microbial numbers and populations (Liste and Alexander, 2000), but under *in vitro* conditions there are no microorganisms able to assist plants to remove the contaminants from culture medium and responses are exclusively due to plant activity.

4. Conclusions

The presence of the HCM does not affect seed germination of any of the assayed plant species but the subsequent growth, estimated by stem and root length, was reduced. Plant survival responses were different among species. *F. arundinacea* germinated and grew well over a wide range of HCM concentrations; *C. ciliaris* and *B. curtipendula* were tolerant to hydrocarbons and *R. repens* and *E. crusgalli* were the more sensitive plants. Plant *in vitro* cultures are useful systems with practical advantages: (i) the plants can grow under laboratory conditions, independent of the weather; (ii) are clean, easy for measuring and monitoring systems that often grow more rapidly in comparison to plant-soil conditions; (iii) resulting effects are only attributable to plants and not to combined plant-microbial rhizospheric effect; and, (iv) are valuable techniques for selecting potential plants for phytoremediation of soil contaminated with hydrocarbons or heavy metals. The use of plant in the remediation of contaminated soils is certainly promising. However, *in vitro* plant response to HCM may not accurately predict plant reaction in soil.

Acknowledgements

We thank Herbarium of Universidad Autonoma de Queretaro, Leticia Buendía and Juan Orozco for their invaluable help. This work was funded by CONACyT, fellowship no. 172716 and PEMEX-Refinación.

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