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# Selection of Leguminous Trees Associated with Symbiont Microorganisms for Phytoremediation of Petroleum-Contaminated Soil

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**Abstract** Leguminous trees have a potential for phytoremediation of oil-contaminated areas for its symbiotic association with nitrogen-fixing bacteria and arbuscular mycorrhizal fungi (AMF). This study selects leguminous tree associated with symbiotic microorganisms that have the potential to remediate petroleum-contaminated soil. Seven species of trees were tested: *Acacia angustissima*, *Acacia auriculiformis*, *Acacia holosericea*, *Acacia mangium*, *Mimosa artemisiana*, *Mimosa caesalpiniifolia*, and *Samanea saman*. They were inoculated with AMF mix and nitrogen-fixing bacteria mix and cultivated over five oil levels in soils, with five replicates. The decreasing of total petroleum hydrocarbons (TPH) values occurred especially with *S. saman* and its symbiotic microorganisms on highest oil soil contamination. Despite the large growth of *A. angustissima* and *M. caesalpiniifolia* on the highest level of oil, these species and its inoculated microorganisms did not reduce the soil TPH. Both plants were hydrocarbon tolerant but not able to remediate the polluted soil. In contrast were significative hydrocarbon

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decrease with *M. artemisiana* under high oil concentrations, but plant growth was severely affected. Results suggest that the ability of the plants to decrease the soil concentration of TPH is not directly related to its growth and adaptation to conditions of contamination, but the success of the association between plants and its symbionts that seem to play a critical role on remediation efficiency.

**Keywords** Nitrogen fixing · Mycorrhiza · Oil · *Acacia* · *Mimosa* · *Samanea*

## 1 Introduction

The widespread occurrence of sites polluted by petroleum hydrocarbons and the current intensity of oil and derivatives consumption highlight the importance of research on low cost and more environmentally friendly remediation strategies for the recovery of such sites. In these sense, the bio-based technologies have been described as the best alternatives (Khan et al. 2004; Jain et al. 2011).

Phytoremediation is a bio-based technology that uses plant systems as a decontaminating agent in order to remediate soil and water environments impacted by organic (hydrocarbons, pesticides, chlorinated compounds, nitrogen compounds, and explosives) and inorganic (salts, metals, and radionuclides) pollutants (ITRC 2009). Plants and their associated microorganisms can convert toxic compounds into less toxic or non-toxic substances through either biodegradation or biotransformation processes. Phytoremediation technologies can also be used to extract and concentrate hazardous elements (ITRC 2009; Ndimele 2010).

Plant–symbionts systems are capable of transforming petroleum hydrocarbons into products such as alcohols, acids, carbon dioxide, and water, which are generally less toxic and less persistent in environment than the parental compounds (Eweis et al. 1998). Plants can also supply the contaminated soil with aliphatic and nonpolar carbon molecules that facilitate more rapid desorption of polycyclic aromatic hydrocarbons from soil minerals and humins (Nichols and Musella 2009) and even uptake and concentrate these hydrocarbons (Parrish et al. 2006).

Legumes have been assessed in oil-polluted areas (Saggin Júnior et al. 2006; Liste and Felgentreu 2006; Smith et al. 2006; Bento et al. 2007) because of their low implementation cost and high capacity for adaptation to poor, degraded, or contaminated soil. Most of the legumes (Fabaceae) establish symbiotic association with nitrogen-fixers (diazotrophic bacteria) and nearly all species are associated with mycorrhizal fungi (Franco and Balieiro 2000). The plant—diazotrophic bacteria—mycorrhizal fungus symbiosis acquires the ability to incorporate C and N to the soil and improves the efficiency of these organisms in absorbing nutrients and the plant tolerance to environmental stresses (Franco and Balieiro 2000). Moreover, the relationship with nitrogen fixers could favor Fabaceae plants growth in petroleum-impacted soils, where C/N ratio is typically high (Adam and Duncan 2003). Few reports have actually pointed to the great potential of legumes for the remediation of oil-contaminated areas (Kaimi et al. 2007; Dashti et al. 2009; Farias et al. 2009), but some Fabaceae species cannot survive under hydrocarbons high levels (Merkl et al. 2005; Kaimi et al. 2007). Therefore, more studies are necessary to elucidate which Fabaceae species could be really applicable in remediation projects aiming at the degradation or detoxification of petroleum hydrocarbons.

In previous studies, Saggin Júnior et al. (2006) found that soil contamination with oil on five levels (0, 10, 30, 50, and 70 $g kg^{-1}$) affected the germination and growth of leguminous species *Acacia holosericea*, *Mimosa caesalpiniifolia*, and *Samanea saman*, promoting high mortality mainly due to the increase of soil particles hydrophobicity caused by oil. Bento et al. (2007) reported that the addition of hydrogel to petroleum-contaminated soil reduces the problem of hydrophobia, also, the use of hydrogel, mycorrhizal fungi, and diazotrophic bacteria promoted the establishment and growth of leguminous trees even at high levels of oil contamination. Based on these previous results, we designed this study to select tree leguminous associated with symbiotic microorganisms that have the potential to remediate petroleum-contaminated soil conditioned with hydrogel.

## 2 Material and Methods

### 2.1 Soil Preparation, Plant Species, and Experiment Establishment

The experiment was conducted in a greenhouse at Embrapa Agrobiology, Seropédica, Rio de Janeiro

State, Brazil. The soil used as substrate to plant growth was Ultisol ("Argissolo Amarelo" by Brazilian soil taxonomy), which was collected in Pinheiral, Rio de Janeiro State (22°31′17.2″ S and 43°58′51.5″ W; altitude, 469 m) and presented the following characteristics: pH 4.7; Al, 0.8 $cmol_c dm^{-3}$; Ca + Mg, 0.5 $cmol_c dm^{-3}$; P, 5.0 $mg dm^{-3}$; and K, 5.0 $mg dm^{-3}$. The soil was air dried, sieved at 5-mm mesh, and divided into amounts of 3.0 kg each pot (185 pots). After that, the soil was spiked with Cabiúnas heavy crude oil, from Macaé, Rio de Janeiro State, in order to establish the following petroleum concentrations: 0, 10, 30, 50, and 70 $g kg^{-1}$. Soil and oil were thoroughly mixed until an apparent uniform color is reached. The contaminated soil was kept in a greenhouse to be incubated for 30 days for the evaporation of more volatile compounds. After incubation, 5 g of hydrogel Stockosorb 500® (Evonik Stockhausen GmbH, CREASORB, Bäkerpfad 25, 47805 Krefeld, Germany; www.creasorb.com) was applied per pot as a soil conditioner to decrease the hydrophobicity of soil particles promoted by the oil (Bento et al. 2007). The hydrogel was also thoroughly homogenized with the soil–oil mix, which was then irrigated before the leguminous seedlings transplantation.

Seven species of leguminous trees from different origins were tested: *Acacia angustissima*, *Acacia auriculiformis*, *A. holosericea*, *Acacia mangium*, *Mimosa artemisiana*, *M. caesalpiniifolia*, and *S. saman* (Table 1). All these species present the following features: fast growing, resistance to environmental stresses (high temperature, low humidity, and low soil fertility), ability to establish symbiosis with nitrogen-fixing bacteria and with mycorrhizal fungi, and high biomass production (Cole et al. 1996; Durr 2001; Chaer et al. 2011). The seedlings were grown in a nursery for 6 months using Styrofoam trays of 72 cells (100 $cm^3$ per cell) before being used in the experiment.

The inoculation of plants with arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing bacteria was done twice. The first inoculation was done at sowing the plants in the nursery and the second inoculation was made when transplanting the seedlings to pots with soil–oil–hydrogel mix. In both were added 1.6 g per seedling of AMF inoculant containing: 17 spores of *Gigaspora margarita* (A1), 21 spores of *Entrophospora contigua* (A28), 15 spores of *Scutellospora calospora* (A80), 34 spores of *Scutellospora heterogama* (A2), and 25 spores of *Glomus clarum* (A5). All five species of AMF are from the COFMEA—Embrapa Agrobiology Collection of Arbuscular Mycorrhizal Fungi (Embrapa Agrobiology, BR 465, km 07, CEP 23890-000, Seropédica, Rio de Janeiro State, Brazil) and were chosen because they had positive responses to promote the growth of leguminous species in degraded areas (Costa et al. 1997; Chandra and Kehri 2006). In both inoculation moments, the nitrogen-fixing bacteria consortia were carried within 2 mL per seedling of the culture medium. Each consortium of rhizobia applied in this study was comprised of two strains selected by Embrapa Agrobiology for each leguminous tree (Table 1), and all the seven species were also inoculated with the strains L107; L84; S2P9$^{-2}$; F1P6$^{-5}$ (BR 5616); L91 (BR 946); L79; L8R; M16

**Table 1** List of species of legumes tree (Fabaceae family) used in the experiment, the geographic center of origin of species and strains of nitrogen-fixing bacteria inoculated which are selected for each legume species by Embrapa Agrobiology

| Specie | Region of origin | Selected strains inoculated |
|---|---|---|
| *Acacia angustissima* (Mill.) Kuntze | Central America and Southern United States | BR10049 e BR3616 |
| *Acacia auriculiformis* A. Cunn. ex Benth | Australia, Indonesia, and Papua New Guinea | BR3465 e BR3609 |
| *Acacia holosericea* A. Cunn. ex G. Don | Tropical parts of Queensland, New Territories, and West Australia | BR4406 e BR5608 |
| *Acacia mangium* Willd. | Northeast Queensland in Australia, West Papua New Guinea, and eastern Maluku islands | BR3609 e BR6009 |
| *Mimosa artemisiana* Heringer & Paula | Brazil in the states of Bahia, Minas Gerais, and Rio de Janeiro | BR3462 e BR3609 |
| *Mimosa caesalpiniifolia* Benth | Brazil, in the northeast region | BR3407 e BR3446 |
| *Samanea saman* (Jacq.) Merr. | Dry forests from Mexico to Venezuela and Colombia | BR6204 e BR6208 |

(BR3516); 23 (BR3517); 33 (BR3518); 31-1 (BR3519); 52-1 (BR3520), and 27-1 (BR5615) isolated from a refinery's oil-polluted soil (Ferreira et al. 2007).

The experimental design was randomized blocks (DBC) in a factorial 7×5: seven species of leguminous tree and five oil levels, with five replicates, totaling 175 pots with plants. The experimental unit (plot) consisted of one pot with one seedling. As an additional treatment for control of total petroleum hydrocarbons (TPH) analysis, unplanted pots of each oil contamination level were irrigated, fertilized, and placed under the same conditions as the planted pots.

Weekly after planting each pot received 10 mL of Hoagland and Arnon (1950) nutritive solution, modified for reduction in P and N concentrations to use on experiments with nitrogen-fixing bacteria and mycorrhizal fungi ($mg L^{-1}$): N=21.85, P=0.09, K=58.77, Ca=60.12, Mg=14.59, S=51.34, B=0.16, Cu=0.06, Zn=0.02, Fe=1.67, Mn=0.14, Mo=0.002, Na=0.69, and Cl=53.36. The pots were irrigated daily with demineralized water and the experiment was conducted for 101 days.

### 2.2 Petroleum Hydrocarbons Determination

Total petroleum hydrocarbons analysis was carried out at Petrobras Research Center (CENPES). Hydrocarbons were elicited from the polluted soil by pressurized fluid extraction as described on EPA Method 3545A (EPA 2007) and were determined quantitatively on a GC/FID system according to the EPA Method 8015B (EPA 1996). Soil samples were collected before seedlings transplanting and at the end of the experiment. In the first sampling, 1 g soil was withdrawn from each plot after incubation and hydrogel mixing to form a composite sample of each level of oil contamination. In the second (final) sampling this procedure was repeated after roots removal and soil mix and samples were included from the unplanted pots (control). All the samples were stored in inert oil (glass) and kept frozen at −20 °C until further determination. TPH balances in planted pots were compared to the respective TPH concentration measured in unplanted pots at the end of experiment to assess phytoremediation potential of the leguminous trees. Considering that soils were spiked with crude oil, TPH results included alkanes and aromatics (mono and polyaromatics).

### 2.3 Plant Growth and Development Evaluation

Plant growth was evaluated by measuring plant height and stem diameter every 15 days. To equalize the magnitude of data among different tree species, the daily growth rate of each species was estimated by the difference in growth between the first and last assessment, divided by the total number of days.

Chlorophyll content was determined using the portable meter SPAD-502 (Minolta, Osaka, Japan) that allows instantaneous readings of chlorophyll in the leaf without its destruction for chemical analysis. To standardize the measurement, a healthy and vigorous leaf positioned in the middle part of the canopy was chosen and submitted to six chlorophyll readings in 2 days at the end of the experiment (three readings per day). These six chlorophyll content readings were taken at the scale of the device, called SPAD unit (according to Guimarães et al. 1999), and were used to calculate the mean chlorophyll content of each plant (plot).

Shoot was weighed and then dried at 68 °C. After that, it was weighed again and sent to Embrapa Soils to be submitted to nitro-perchloric digestion and nutrient analysis in the tissues by the method of Silva (1999). The roots were washed and then 0.5 g of fine roots were randomly take from each plant and processed for clearing and staining (Koske and Gemma 1989) to assess mycorrhizal colonization. In this evaluation, each sample was assembled on a microscope slide and at least 100 segments of roots were observed under ×200 magnification to assess the colonization by hyphae, vesicles, and arbuscules, according to McGonigle et al. (1990).

### 2.4 Symbionts Assessment

The density of AMF spores in the soil after the experiment was estimated in samples of 50 mL of soil per pot. This sample was wet sieved at 53-µm mesh (Gerdemann and Nicolson 1963) and centrifuged on water (3 min and 3,000 rpm) and sucrose 45 % (2 min and 2,000 rpm). The supernatant was collected, washed thoroughly, and the spores quantified in a stereoscopic microscope at ×40. After counting, spores were mounted on permanent slides for microscopy in order to identify the dominant AMF species based on spore morphology. The nodules in the roots were manually separated, counted, dried, and weighed.

### 2.5 Data Analysis

Data from the assessments described above were tested for homogeneity of variances and normal distribution. The data were transformed into square root of $x+1$ for the variables number and dry mass of nodules and number of AMF spores. The data were subjected to analysis of variance and the means were compared by the Scott Knott test ($P<0.05$) using the statistical program Sisvar 4.6 (Ferreira 2008). For the levels of oil contamination polynomial regressions were established using this software. For shoot dry weight, logarithmic or exponential curves were set using the program TableCurve 3.0 (Systat Software Inc.). For total petroleum hydrocarbons, the control without plants was compared with the legume species treatments using the Student *t* test ($P<0.05$).

## 3 Results and Discussion

### 3.1 Plant Growth Rate and Shoot Dry Mass

*A. angustissima* and *A. auriculiformis* presented daily growth rates in height above 0.2 $cm\,day^{-1}$ up to 50 $g\,kg^{-1}$ of oil in soil, whereas *A. holosericea* had a growth rate above 0.2 $cm\,day^{-1}$ at all levels of oil contamination (Table 2). These three species are highlighted in maintaining the growth rate, even at high levels of contamination by oil, despite the tendency to decrease with increasing contamination. On the other hand, *A. mangium* and *M. artemisiana* showed great reduction in the growth rate in the presence of oil (above 10 $g\,kg^{-1}$), possibly being very sensitive to this pollutant in soil. The species *M. caesalpiniifolia* and *S. saman* showed great variability in growth rates among the levels of contamination, with tendency to have a high growth rate at the highest level of oil contamination. These results pointed to the need for more studies to verify the real growth potential of these last two leguminous species in oil-contaminated soils.

For shoot dry mass (SDM) the adjustments of polynomial regression had the determination coefficients very low, so for this variable exponential or logarithmic curves were adjusted (Fig. 1). SDM results obtained for *A. mangium* and *M. artemisiana* reinforced the hypothesis of high susceptibility of these species to the presence of oil since strong decrease in SDM was observed from oil contamination levels 0 to 10 $g\,kg^{-1}$. *M. caesalpiniifolia* and *S. saman* also displayed marked SDM decrease from 0 to 10 $g\,kg^{-1}$; however, both species presented mass production increase at other levels of oil contamination, making them the largest plants of the experiment at the highest level of oil contamination (70 $g\,kg^{-1}$). Due to this unexpected behavior, adjusting for polynomial regression followed cubic or square-root models. *A. angustissima*, *A. auriculiformis*, and *A. holosericea*, which showed high rates of height growth in almost all levels of contamination, had a small decrease in SDM with

**Table 2** Daily growth rate in height of leguminous trees in soil with different levels of oil contamination

| Levels of oil contamination in soil | Leguminous tree species | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Acacia angustissima* | *Acacia auriculiformis* | *Acacia holosericea* | *Acacia mangium* | *Mimosa artemisiana* | *Mimosa caesalpiniifolia* | *Samanea saman* |
| | Daily growth rate ($cm\,day^{-1}$) | | | | | | |
| 0 $g\,kg^{-1}$ | 0.19 b | 0.36 a | 0.35 a | 0.30 a | 0.21 b | 0.11 b | 0.21 b |
| 10 $g\,kg^{-1}$ | 0.20 a | 0.23 a | 0.28 a | 0.18 a | 0.06 b | 0.22 a | 0.05 b |
| 30 $g\,kg^{-1}$ | 0.23 a | 0.31 a | 0.28 a | 0.10 b | 0.06 b | 0.10 b | 0.08 b |
| 50 $g\,kg^{-1}$ | 0.20 a | 0.31 a | 0.24 a | 0.06 b | 0.00 b | 0.12 b | 0.15 b |
| 70 $g\,kg^{-1}$ | 0.17 b | 0.14 b | 0.22 b | 0.07 c | 0.00 c | 0.34 a | 0.19 b |
| Regression adjustment | NS | NA | Linear | Quadratic | Linear | Cubic | Cubic |
| $R^2$ | – | – | 0.83 | 0.97 | 0.68 | 0.91 | 0.90 |

Same letters in line indicate no significant difference between tree species by Scott Knott 5 %

*NS* regression not significant by *F* test ($P\geq0.05$), *NA* no adjustments to polynomial regression models

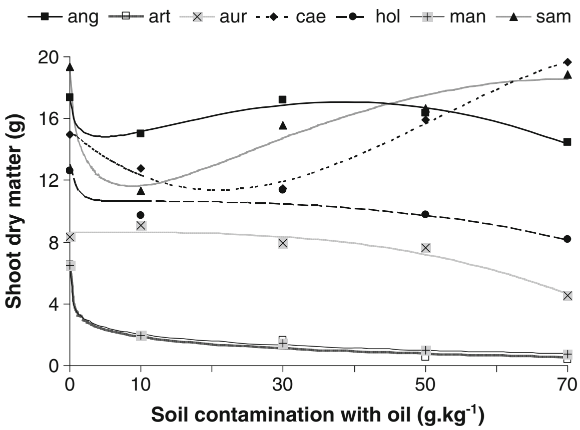



**Fig. 1** Shoot dry mass of leguminous trees species in soils with different levels of oil contamination. ang: *A. angustissima*, $y = 17.64 + 0.89x - 0.07x^{1,5} - 2.90x^{0.5}$, $R^2$=0.96; art: *M. artemisiana*, $y = 3.45 - 0.68\ln(x)$, $R^2$=0.99; aur: *A. auriculiformis*, $y = 8.65 - 1.16E - 05x^3$, $R^2$=0.94; cae: *M. caesalpiniifolia*, $y = 15.18 - 0.40x + 0.012x^2 - 7.72E - 05x^3$, $R^2$=0.99; hol: *A. holosericea*, $y = 10.66 - 7.38E - 06x^3 + 2.36e^{-x}$, $R^2$= 0.94; man: *A. mangium*, $y = 3.51 - 0.64\ln(x)$, $R^2$=0.99; sam: *S. saman*, $y = 19.94 + 1.46x - 0.085x^{1.5} - 6.37x^{0.5}$, $R^2$=0.96, where $e$=2.72

increasing levels of contamination and the species were less influenced by oil in soil. Important part of successful growth of these species in the soil with oil can be attributed to hydrogel as Bento et al. (2007) verified that *M. caesalpiniifolia* and *A. holosericea* grow in soil contaminated by different levels of oil only when hydrogel is used as soil conditioner. Moreover, Saggin Júnior et al. (2006) found that oil contamination greatly affects the leaf area and survival of *S. saman*, *A. holosericea*, and *M. caesalpiniifolia* if no soil conditioner as hydrogel is used.

### 3.2 Chlorophyll Content

The leguminous species with the highest chlorophyll contents were *A. auriculiformis*, *A. holosericea*, *M. caesalpiniifolia*, and *S. saman* (Table 3). These species stand out from the others particularly at the highest levels of oil contamination, suggesting good adaptation to this condition of soil pollution. Therefore, those species with high chlorophyll content possibly had a high rate of atmospheric N fixation either. This chlorophyll–nitrogen relationship is mainly attributed to the fact that 50 to 70 % of total nitrogen in the leaves are in enzymes (Chapman and Barreto 1997) associated with the chloroplasts (Stocking and Ongun 1962).

The species *A. angustissima*, *A. mangium*, and *M. artemisiana* had lower chlorophyll content than the species mentioned in the previous paragraph. This may be due to an intrinsic characteristic of each species, as seems the case of *A. angustissima* that showed enough growth and nodulation, but the lowest chlorophyll content, or due to an inability to adapt to the oil-contaminated soil, as seems the case of *M. artemisiana*, which was the only species that decreased linearly ($y$= 0.37×43.9, $R^2$=0.93) chlorophyll content with increasing level of contamination. The remaining species showed no effect of oil on the chlorophyll content.

### 3.3 Shoot Nutrients

The most significant nutrient results are displayed in Table 4. *M. artemisiana* presented the highest concentrations of phosphorus, copper, and zinc, as well as significant concentrations of potassium and calcium. This species was able to extract nutrients probably because of its high mycorrhizal colonization (see Table 5) but these elements became concentrated because of the low shoot biomass growth imposed by petroleum.

Calcium content was expressively higher in *M. caesalpiniifolia* than in other leguminous. The highest K concentrations occurred in *A. mangium*, followed by *A. auriculiformis* and *M. artemisiana.* Sodium levels were more pronounced in *Acacia* species, mainly *A. auriculiformis*, *A. holosericea*, and *A. mangium*. All the nutrients were low accumulated by *S. saman*.

No general tendency was found for the effect of oil on shoot nutrients content. For P, K, Na, and Zn, in general, no significant regression adjustments were found or settings obtained showed a low coefficient of determination. Quadratic regression adjustments were more frequent for Ca, with the maximum point between 30 and 70 $gkg^{-1}$. Cu contents showed a clear trend of reduction with increasing level of contamination. Since a heavy oil was used in this experiment, copper complexes could be formed with abundant resins and asphaltenes (Grijalva-Monteverde et al. 2005).

### 3.4 Nodules and Mycorrhiza

The highest number of nodules of nitrogen-fixing bacteria was observed in *S. saman* and *A. angustissima* in the absence of oil contamination, but these species nodulations remained prominent even in remaining contamination levels (Table 5). *A. mangium*

**Table 3** Chlorophyll content in leguminous trees species in soils with different levels of oil contamination

| Levels of oil contamination in soil | Leguminous tree species | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Acacia angustissima* | *Acacia auriculiformis* | *Acacia holosericea* | *Acacia mangium* | *Mimosa artemisiana* | *Mimosa caesalpiniifolia* | *Samanea saman* |
| | SPAD unit | | | | | | |
| 0 $gkg^{-1}$ | 15.7 d | 52.3 a | 43.1 b | 29.7 c | 44.5 b | 46.8 b | 45.4 b |
| 10 $gkg^{-1}$ | 15.9 d | 48.9 a | 41.1 b | 27.4 c | 36.8 b | 44.2 a | 45.2 a |
| 30 $gkg^{-1}$ | 14.4 d | 43.9 b | 41.0 b | 27.7 c | 37.5 b | 46.1 a | 52.4 a |
| 50 $gkg^{-1}$ | 14.0 c | 48.1 a | 43.6 a | 31.1 b | 24.9 b | 49.7 a | 52.3 a |
| 70 $gkg^{-1}$ | 20.9 c | 46.9 a | 41.8 a | 28.8 b | 17.2 c | 46.2 a | 50.4 a |
| Regression adjustment | NS | NS | NS | NS | Linear | NS | NS |
| $R^2$ | – | – | – | – | 0.93 | – | – |

Same letters in line indicate no significant difference between tree species by Scott Knott 5 %

*NS* regression not significant by *F* test ($P \geq 0.05$)

and *M. artemisiana* in the highest levels of contamination showed the greatest reduction in nodulation relative to treatment without contamination, following the same reported effect on plant growth. Nodulation of *M. caesalpiniifolia*, *A. auriculiformis*, and *A. holosericea* was also important and little affected by the levels of contamination.

The nitrogen-fixers strains used here are the same studied by Ferreira et al. (2007) and were also applied by Saggin Júnior et al. (2006). Ferreira et al. (2007) evaluated the capability of biological nitrogen fixation of 13 isolates collected in areas contaminated by oil studying their effectiveness and efficiency in *M. caesalpiniifolia*, *A. holosericea*, and *S. saman*. The author reported that five isolates were more efficient and effective than currently recommended strains by Embrapa Agrobiology: isolates 23 (BR3517) and 31-1 (BR3519) for *M. caesalpiniifolia* and L91 (BR 946), F1P6$^{-5}$ (BR 5616) and 27-1 (BR5615) for *S. saman*. Since these five isolates are probably adapted to oil-polluted soils and they were components of the mix inoculated on the leguminous seedlings, they could be responsible for the nodulation of *M. caesalpiniifolia* and *S. saman* be the highest ones at the highest level of oil contamination. The presence of these five isolates could also justify the high height growth and SDM production of these leguminous species at high levels of oil contamination (Fig. 1).

The mycorrhizal colonization of *A. mangium*, *M. artemisiana*, and *M. caesalpiniifolia* surpassed the other species at the highest level of oil contamination, and these species colonization was also highlighted at the other levels of contamination, having more than 48 % of root colonization (Table 5). These results could explain why *M. artemisiana*, even with growth inhibition by oil pollution, was able to extract nutrients from the soil, promoting its shoot concentration. *A. auriculiformis* and *S. saman* had the lowest mycorrhizal colonization, but still with high values, averaging 50 %.

The AMF sporulation in the rhizosphere showed no interaction between tree species and soil contamination. The species *A. angustissima*, *A. auriculiformis*, *A. mangium*, and *M. artemisiana* (166, 132, 119, and 135 spores per 50 mL of soil, respectively) showed greater sporulation than *A. holosericea*, *M. caesalpiniifolia*, and *S. saman* (56, 79, and 57 spores per 50 mL of soil, respectively). The oil contamination has reduced the sporulation, from 315 spores per 50 mL of soil without contamination to about 50 spores per 50 mL of soil with 30, 50, and 70 $gkg^{-1}$ of oil, adjusting a quadratic regression ($y = 0.1163x^2 - 11.706x + 285.26$, $R^2=0.91$). The AMF spores that predominated in the rhizosphere of all seven leguminous tree were *G. clarum*, followed by *S. heterogama* and *G. margarita*. Carrenho et al. (2010) explains the dominance of the genus *Glomus* in different environments by its greater adaptability to different soils under different concentrations of organic matter, liming, texture, among other factors, showing that *Glomus* species are resistant to environmental perturbations. In addition, *G. clarum* is a very promiscuous species regarding the plant host, favoring the growth of many trees species (Pouyu-Rojas et al. 2006).

The use of AMF and nitrogen-fixing bacteria, even if they do not directly degrade soil contaminants, propitiate the establishment and growth of leguminous

**Table 4** Shoot nutrients content in leguminous trees species in soil with different levels of oil contamination

| Levels of oil contamination in soil | Leguminous tree species | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Acacia angustissima* | *Acacia auriculiformis* | *Acacia holosericea* | *Acacia mangium* | *Mimosa artemisiana* | *Mimosa caesalpiniifolia* | *Samanea saman* |
| | | | P ($gkg^{-1}$) | | | | |
| 0 $gkg^{-1}$ | 1.4 a | 1.8 a | 1.3 a | 1.5 a | 1.9 a | 1.4 a | 1.1 a |
| 10 $gkg^{-1}$ | 1.7 b | 1.3 b | 2.3 b | 2.2 b | 4.8 a | 1.7 b | 1.5 b |
| 30 $gkg^{-1}$ | 1.8 b | 2.0 b | 2.1 b | 1.8 b | 3.5 a | 2.0 b | 1.4 b |
| 50 $gkg^{-1}$ | 1.7 a | 1.6 a | 2.4 a | 2.0 a | 2.9 a | 2.0 a | 1.4 a |
| 70 $gkg^{-1}$ | 1.7 a | 2.0 a | 3.3 a | 1.5 a | 2.2 a | 1.7 a | 1.3 a |
| Regression adjustment | NS | NS | Linear | NS | Cubic | NS | NS |
| $R^2$ | – | – | 0.77 | – | 0.76 | – | – |
| | | | K ($gkg^{-1}$) | | | | |
| 0 $gkg^{-1}$ | 4.1 d | 13.0 a | 10.1 b | 15.0 a | 9.4 b | 7.5 c | 4.5 d |
| 10 $gkg^{-1}$ | 6.7 c | 11.6 b | 7.4 c | 16.6 a | 10.3 b | 5.4 c | 3.6 c |
| 30 $gkg^{-1}$ | 4.7 b | 7.1 a | 7.3 a | 7.7 a | 10.0 a | 4.5 b | 3.3 b |
| 50 $gkg^{-1}$ | 3.8 b | 9.6 a | 7.3 a | 11.5 a | 3.8 b | 3.9 b | 3.7 b |
| 70 $gkg^{-1}$ | 4.1 b | 9.6 a | 6.0 b | 9.6 a | 3.0 b | 3.7 b | 3.5 b |
| Regression adjustment | NS | Quadratic | NS | NS | Cubic | Linear | NS |
| $R^2$ | – | 0.78 | – | – | 0.98 | 0.78 | – |
| | | | Ca ($gkg^{-1}$) | | | | |
| 0 $gkg^{-1}$ | 0.43 c | 0.77 b | 0.55 c | 0.59 c | 0.78 b | 1.27 a | 0.41 c |
| 10 $gkg^{-1}$ | 0.67 b | 0.58 b | 0.71 b | 0.57 b | 0.75 b | 1.46 a | 0.64 b |
| 30 $gkg^{-1}$ | 0.65 c | 0.72 c | 0.84 b | 0.40 d | 1.01 b | 1.93 a | 0.66 c |
| 50 $gkg^{-1}$ | 0.70 b | 0.62 b | 0.78 b | 0.59 b | 0.64 b | 1.89 a | 0.71 b |
| 70 $gkg^{-1}$ | 0.63 b | 0.70 b | 0.87 b | 0.85 b | 0.38 c | 1.61 a | 0.72 b |
| Regression adjustment | Quadratic | NS | Linear | Quadratic | Quadratic | Quadratic | Linear |
| $R^2$ | 0.72 | – | 0.70 | 0.93 | 0.85 | 0.98 | 0.64 |
| | | | Cu ($gkg^{-1}$) | | | | |
| 0 $gkg^{-1}$ | 3.1 c | 7.5 b | 5.3 c | 7.7 b | 10.6 a | 6.7 b | 3.0 c |
| 10 $gkg^{-1}$ | 3.7 b | 4.8 b | 5.7 b | 5.9 b | 15.1 a | 6.2 b | 5.7 b |
| 30 $gkg^{-1}$ | 2.8 b | 4.2 b | 5.1 b | 3.7 b | 11.5 a | 5.7 b | 2.7 b |
| 50 $gkg^{-1}$ | 2.9 b | 3.6 b | 4.8 b | 4.1 b | 10.4 a | 4.3 b | 4.3 b |
| 70 $gkg^{-1}$ | 2.3 a | 3.1 a | 3.2 a | 5.9 a | 4.4 a | 3.6 a | 2.9 a |
| Regression adjustment | NS | Linear | Linear | Quadratic | Quadratic | Linear | NS |
| $R^2$ | – | 0.73 | 0.78 | 0.99 | 0.86 | 0.98 | – |
| | | | Zn ($mgkg^{-1}$) | | | | |
| 0 $gkg^{-1}$ | 28 b | 26 b | 23 b | 29 b | 41 a | 21 b | 9 c |
| 10 $gkg^{-1}$ | 34 b | 21 c | 26 b | 27 b | 64 a | 15 c | 10 c |
| 30 $gkg^{-1}$ | 28 b | 21 b | 21 b | 12 b | 55 a | 14 b | 7 b |
| 50 $gkg^{-1}$ | 40 b | 18 c | 29 c | 24 c | 60 a | 21 c | 8 c |
| 70 $gkg^{-1}$ | 39 a | 21 b | 12 b | 30 a | 31 a | 14 b | 7 b |
| Regression adjustment | NS | NS | NS | Quadratic | Quadratic | NS | NS |
| $R^2$ | – | – | – | 0.74 | 0.75 | – | – |
| | | | Na ($mgkg^{-1}$) | | | | |
| 0 $gkg^{-1}$ | 0.61 c | 0.64 a | 0.47 b | 0.72 a | 0.13 c | 0.10 c | 0.08 c |

**Table 4** (continued)

| Levels of oil contamination in soil | Leguminous tree species | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Acacia angustissima* | *Acacia auriculiformis* | *Acacia holosericea* | *Acacia mangium* | *Mimosa artemisiana* | *Mimosa caesalpiniifolia* | *Samanea saman* |
| 10 $gkg^{-1}$ | 0.09 c | 0.65 b | 0.53 b | 0.78 a | 0.15 c | 0.11 c | 0.14 c |
| 30 $gkg^{-1}$ | 0.25 c | 0.80 a | 0.79 a | 0.43 b | 0.17 c | 0.14 c | 0.11 c |
| 50 $gkg^{-1}$ | 0.10 b | 0.67 a | 0.54 a | 0.46 a | 0.44 a | 0.15 b | 0.10 b |
| 70 $gkg^{-1}$ | 0.24 b | 0.44 a | 0.60 a | 0.41 a | 0.42 a | 0.15 b | 0.11 b |
| Regression adjustment | NS | Quadratic | NS | Linear | Linear | NS | NS |
| $R^2$ | – | 0.93 | – | 0.73 | 0.84 | – | – |

Same letters in line indicate no significant difference between tree species by Scott Knott 5 %

*NS* regression not significant by *F* test ($P \geq 0.05$)

in contaminated soil and can stimulate the activity of other rhizospheric microorganisms leading to bioremediation. The synergistic interactions between AMF and nitrogen-fixing bacteria may have a great potential to enhance phytoremediation. A field experiment studied the effects of inoculation of *Glomus caledonium* and *Rhizobium meliloti* on phytoremediation by alfalfa of a soil contaminated with polychlorinated biphenyls (PCBs) (Teng et al. 2010). Planting alfalfa (P), alfalfa inoculated with *G. caledonium* (P+AM), alfalfa inoculated with *R. meliloti* (P+R), and alfalfa co-inoculated with *R. meliloti* and *G. caledonium* (P+AM+R)

**Table 5** Nodule number and percentage of mycorrhizal colonization in leguminous trees species in soils with different levels of oil contamination

| Levels of oil contamination in soil | Leguminous tree species | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Acacia angustissima* | *Acacia auriculiformis* | *Acacia holosericea* | *Acacia mangium* | *Mimosa artemisiana* | *Mimosa caesalpiniifolia* | *Samanea saman* |
| | | | | Number of nodules[a] | | | |
| 0 $gkg^{-1}$ | 230 a | 92 b | 137 b | 105 b | 123 b | 118 b | 292 a |
| 10 $gkg^{-1}$ | 158 a | 88 a | 88 a | 81 a | 50 a | 38 a | 73 a |
| 30 $gkg^{-1}$ | 108 a | 92 a | 41 b | 26 b | 20 b | 50 b | 180 a |
| 50 $gkg^{-1}$ | 144 a | 120 a | 107 a | 35 b | 5 b | 132 a | 186 a |
| 70 $gkg^{-1}$ | 80 a | 45 b | 49 b | 8 b | 4 b | 101 a | 141 a |
| Regression adjustment | Cubic | NA | Cubic | Linear | Linear | Cubic | Cubic |
| $R^2$ | 0.93 | – | 0.86 | 0.84 | 0.86 | 1.00 | 0.65 |
| | | | | Mycorrhizal colonization (%) | | | |
| 0 $gkg^{-1}$ | 71 a | 52 b | 43 b | 48 b | 77 a | 48 b | 52 b |
| 10 $gkg^{-1}$ | 17 c | 43 b | 78 a | 67 a | 62 a | 73 a | 41 b |
| 30 $gkg^{-1}$ | 44 b | 59 b | 78 a | 80 a | 68 a | 84 a | 45 b |
| 50 $gkg^{-1}$ | 79 a | 56 b | 43 b | 66 a | 76 a | 56 b | 49 b |
| 70 $gkg^{-1}$ | 68 b | 60 b | 57 b | 81 a | 83 a | 83 a | 51 b |
| Regression adjustment | Cubic | NA | Cubic | Cubic | NA | Cubic | NA |
| $R^2$ | 0.93 | – | 0.99 | 0.97 | – | 0.95 | – |

Same letters in line indicate no significant difference between tree species by Scott Knott 5 %

*NA* no adjustments to polynomial regression models

[a] Means untransformed by $(x+1)^{1/2}$

decreased significantly initial soil PCB concentrations by 8.1, 12.0, 33.8, and 43.5 %, respectively. Inoculation of alfalfa with AM+R increased alfalfa yield, accumulation of PCBs in the shoots, soil microbial counts, and carbon utilization ability of the soil microbial community. Thus, AMF and nitrogen-fixing bacteria can stimulate the activity of microorganisms directly involved in biogeochemical cycles, which are key drivers of the degradation of many sources of carbon, including petroleum hydrocarbons, as suggest by Santos et al. (2011).

### 3.5 Total Petroleum Hydrocarbons

The initial values of TPH in the soil, before the cultivation of plants, were 9,994, 22,509, 30,680, and 36,093 mg $kg^{-1}$ to the levels of soil contamination of 10, 30, 50, and 70 $gkg^{-1}$ of oil, respectively. The highest TPH decrease values in planted plots occurred in the treatments with *A. angustissima* in oil contamination level 10 $gkg^{-1}$, *A. auriculiformis* at 30 $gkg^{-1}$, *M. artemisiana* at 50 and 70 $gkg^{-1}$, and, especially, *S. saman* at 30, 50, and 70 g $kg^{-1}$ that showed TPH reduction greater than other treatments (Fig. 2).

Although the lowest TPH decrease amounts were observed under the highest level of petroleum contamination, the percentage contribution of leguminous trees and its symbiotic microorganisms for the hydrocarbon degradation was more pronounced right on the most polluted soils. On 50 $gkg^{-1}$ of oil contamination, the results obtained with *M. artemisiana* and *S. saman* were decrease of 10,169 and 9,832 $mgkg^{-1}$, respectively, while in unplanted plots TPH reduction was 6,782 mg $kg^{-1}$, which means that plants and inoculated microorganisms increased the TPH degradation on average 34 %. However, at level 70 $gkg^{-1}$ of oil contamination, the TPH differences among the soils without plants and the ones with *M. artemisiana* and *S. saman* were on average 110 %, that means these two species and its microorganisms decreased TPH more than twice the observed in unplanted control (Fig. 2).

Despite the large growth of *A. angustissima* and *M. caesalpiniifolia* on the highest level of oil contamination, these species did not stand out in reducing the soil TPH on this condition (Fig. 2). Both species were hydrocarbon tolerant, but not able to remediate the polluted soil. In contrast, the results of hydrocarbon decrease on *M. artemisiana* under high oil concentrations were significative, but plant growth was severely affected, which could impair the efficiency of this species on scale field phytoremediation processes. These results also suggest that TPH decrease may be related to the group of microorganisms inoculated to each plant.

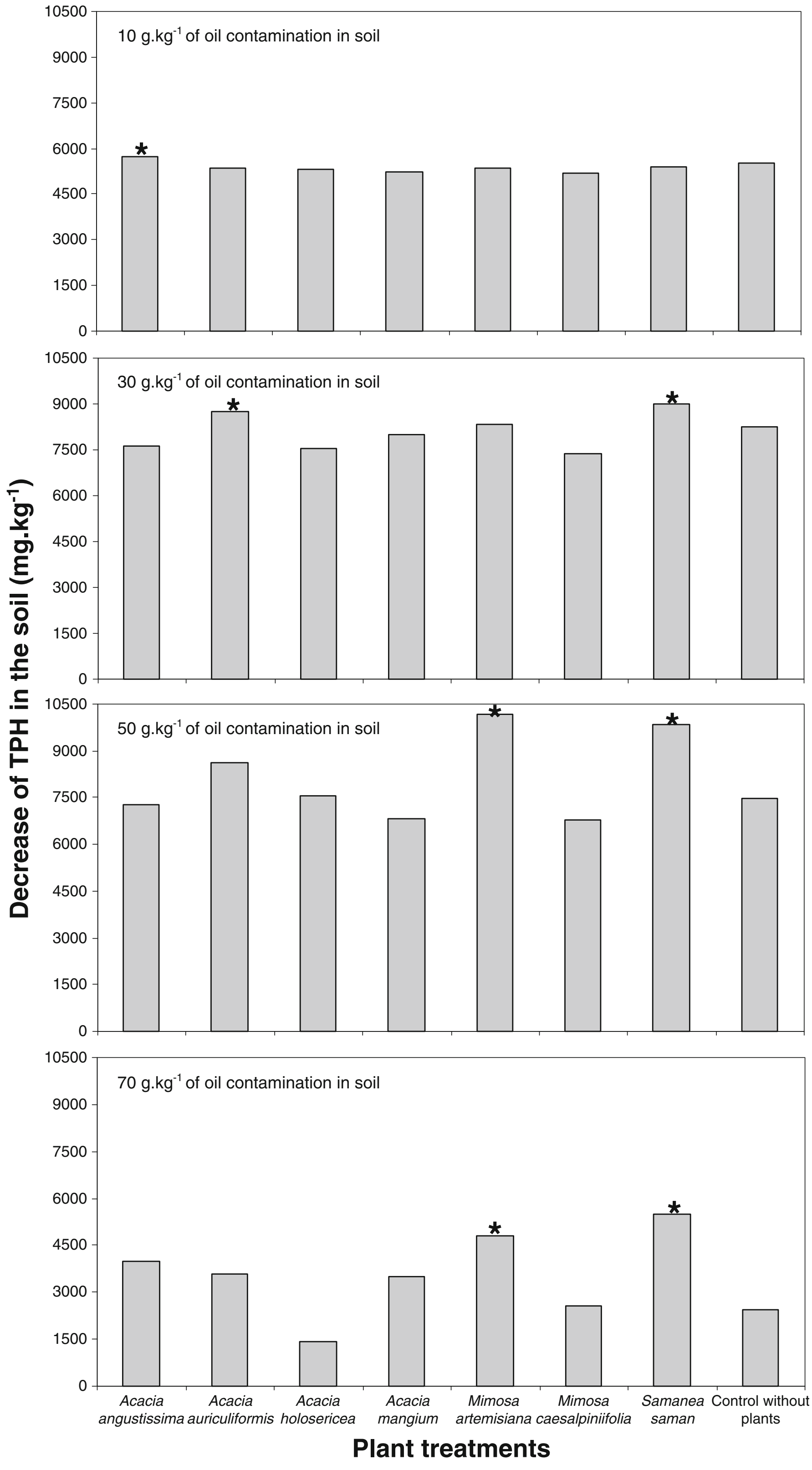
Results show that the ability of the studied plants and its symbiotic microorganisms to decrease the soil concentration of TPH is not directly related to plant growth and adaptation to conditions of contamination. Actually, one of the most important mechanisms in phytoremediation of oil-polluted soils is the plant stimulations to hydrocarbon-degraders growth and activity (Frick et al. 1999; Yateem et al. 2008). Also, the efficiency and adaptation of root symbionts to oil-polluted sites and the success of the association between plants and these symbionts seemed to play critical role on the remediation efficiency since *S. saman* presented the best remediation results (Fig. 2) and successful symbiotic interaction with hydrocarbon-adapted nitrogen-fixing bacteria (Table 5). Therefore, not only the plant species, but also the symbiotic microorganism selection is a critical step to establish phytoremediation processes for the recovery of petroleum-hydrocarbons-impacted soils.

## 4 Conclusions

The results reported in this study show that *A. angustissima*, *A. auriculiformis*, and *A. holosericea* associated to symbiotic microorganisms showed high rates of height growth in almost all levels of contamination, they had a small decrease in SDM with increasing levels of contamination and were the species less influenced by oil in soil. The species *M. caesalpiniifolia* and *S. saman* showed variability in growth among the levels of contamination, but showed a high growth rate and SDM production in the highest level of oil contamination. These five leguminous trees associated with microorganims, possibly with hydrogel soil conditioner, maybe used for revegetation of areas polluted with oil, promoting rapid regeneration of vegetation in tropical environments and restoration of nutrient cycling.

These leguminous trees above, except *A. angustissima*, had elevated chlorophyll content particularly at the highest levels of oil contamination, suggesting good adaptation to this condition of soil pollution and an effective nitrogen-fixing symbiosis. *A. angustissima* showed enough growth and nodulation, but the lowest chlorophyll content. This may be due to an inability to its symbionts to adapt to the oil-contaminated soil or an

**Fig. 2** Decrease of total petroleum hydrocarbons (*TPHs*) in soil with different levels of oil contamination after 101 days of cultivation of leguminous trees species. *Bars marked with an asterisk* are superior to the others on the same level of oil contamination by Student's *t* test ($P<0.05$)



intrinsic characteristic of the species. All these five leguminous trees showed high number of nodules of nitrogen-fixing bacteria in all pollution levels, being above 38 nodules per plant.

All studied leguminous trees in any petroleum contamination conditions showed high mycorrhizal colonization, above 41 %. The only exception was *A. angustissima* at level 10 $gkg^{-1}$ of oil contamination, which suggests an effect of random variability. This suggests that oil contamination in a soil conditioned by hydrogel does not affect the roots infection by mycorrhizal fungi, mainly because the seedlings were transplanted into polluted soil previously colonized. But the AMF sporulation in the rhizosphere showed decrease over 30 $gkg^{-1}$ of oil, indicating that oil pollution also affects the development of this symbiosis.

*S. saman* and its inoculated microorganisms were the most promising combination to be applied in field scale for phytoremediation of oil-polluted soils. *S. saman* had elevated height growth rate and SDM production and decreased TPH in relation to unplanted control. This tree had the highest nodule mass relative to other leguminous, confirming the efficiency of strains BR946, BR5616, and BR5615 for petroleum-contaminated conditions. As *G. clarum*, *S. heterogama*, and *G. margarita* are the dominant AMF spores in all seven tree rhizospheres, a mix of these three species could be used as inoculant for seedlings to oil-contaminated soil.

On the other hand, *A. mangium* and *M. artemisiana* showed great reduction in growth rate and SDM production in the presence of oil (above 10 $gkg^{-1}$). These species had low chlorophyll content that decreased with increasing level of contamination and the greatest reduction in nodulation in relation to treatment without contamination, that suggest a reduction of nitrogen fixation with increased pollution. Nevertheless, these species had a high mycorrhizal colonization and had sporulation in the rhizosphere. *M. artemisiana* also had under high oil concentrations significative decreased TPH related to unplanted control, but its growth was severely affected, these results may be related to the group of microorganisms inoculated to each plant. These results suggest that the abilities of the plants decrease the soil concentration of TPH is not directly related to its growth and adaptation to conditions of contamination, but the success of the association between plants and its symbionts seemed play critical role on remediation efficiency.

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