Research Article

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Bacteria from *Jatropha curcas* rhizosphere, degrades aromatic hydrocarbons and promotes growth in *Zea mays*

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Abstract: The rhizosphere is one of the most important reservoirs of microorganisms. Because of the microbial metabolic activities, these can be used for various biotechnological, agricultural and environmental purposes. In this study we evaluated five genetically related bacterial strains; *Pseudomonas aeruginosa* N7B1 (MG457074), *Pseudomonas* sp. (MG457075), *Pseudomonas* sp., Bf1 (MG457076) and *Pseudomonas aeruginosa* F23 (MG457077), isolated from *Jatropha curcas* rhizosphere, capable of growing and degradating benzene and phenanthrene. The hydrocarbon degradation by these strains was quantified by gas chromatography coupled to mass spectrophotometry. The *Pseudomonas aeruginosa* N7B1 strain removed 84% of phenanthrene and 45% of benzene in a seven-day period, while the other strains showed a lower hydrocarbon degradation capacity. Another biotechnological feature of these strains is maize growth promotion, in a substrate enriched with 0.5% of phenanthrene and 1.0% of benzene. *Pseudomonas aeruginosa* N7B1 and *Pseudomonas aeruginosa* F23 showed an increase in root and shoot fresh and dry weight, plant height and root length variables. These results open the possible use of these strains as bioinoculants to promote the growth of maize plants in phenanthrene and benzene polluted soils.

Keywords: *Pseudomonas;* Phenanthrene; Benzene,; Growth promotion; Biodegradation

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1 Introduction

Nowadays, great concern has been expressed over the accumulation of Polycyclic Aromatic Hydrocarbons (PAH´s), which are pollutants that include aromatic rings arranged in linear, angular, or clustering forms (Li et al. 2014; Chen et al. 2015). PAH´s have teratogenic, carcinogenic, and mutagenic properties and can pose a huge threat to human health through bioaccumulation in the food chain (Moscoso et al. 2012). This usually occurs in industrial sites and results from the incomplete combustion of organic materials such as coal, oil, and wood. Most PAH´s are recalcitrant in the environment due to their high resistance to nucleophilic attack and low bioavailability (Yuan et al. 2002; Zhang et al. 2006; Cheng et al. 2016).

The elimination of PAH´s using biological candidates, such as microorganisms, is generally preferred because of their ecological nature and profitability. However, a few bacterial genera are capable of degrading polycyclic and heterocyclic aromatic hydrocarbons, particularly anthracene, phenanthrene, and dibenzothiophene (Smalla et al. 2001). It has been suggested that during biodegradation, some microorganisms consume aromatic com-

pounds through a series of intrinsic pathways and use them as energy sources, thereby detoxifying contaminants (Gałązka and Gałązka 2015). Muratova et al. (2015) studied phenanthrene catabolism by *Ensifer meliloti*, *Pseudomonas kunmingensis*, *Rhizobium petrolearium*, and *Stenotrophomona* sp., in interaction with *Medicago sativa*; the results indicate that plants are actively involved in phenanthrene rhizospheric degradation. Jin et al. (2016) reported a 98.5% degradation phenanthrene rate by *Pseudomonas* sp., JPN2 after 10 days of incubation at an initial concentration of 100 mg/L. de Lima et al. (2016) reported *Pseudomonas veronii* 1YdBTEX2 and *Pseudomonas veronii* 1YB2 ability to degrade benzene. Some studies have shown that plants release organic compounds that change the physicochemical and biological properties of the soil, which probably facilitates the chemotactic bacteria attraction to promote plant growth and pollutant biodegradation (Olson et al. 2003; Chaudry et al. 2005; Haichar et al. 2008; Hartmann et al. 2009; Glick 2010; Uroz et al. 2010).

Plants release organic compounds, including terpenes, flavonoids and some components derived from lignin, with chemical structures similar to those of PAH´s; these chemicals can induce PAH-degrading gene expression in rhizospheric microorganisms (Sun et al. 2010). Once attracted, PAH degrading rhizosphere bacteria can improve PAH´s plant tolerance and additionally result in a faster recovery of soil health (Escalante-Espinoza et al. 2004; Barrutia et al. 2011). Toyama et al. (2011) reported that the increase of phenolic compounds in root exudates has been associated with a greater degree of benzene [a] pyrene degradation in *Phragmites australis* rhizosphere.

Because of the rhizospheric microorganisms' ecological role and plant-microbe interaction above cited, this study aim was to evaluate the degradation capacity of phenanthrene and benzene by four *Pseudomonas* strains, to quantify the polyphenol oxidase activity and to compare the growth-promotion in maize under phenanthrene and benzene polluted conditions.

2 Materials and Methods

2.1 Origin, isolation and 16S rRNA molecular identification of the bacterial strains

Bacterial strains isolated from *Jatropha curcas* L. rhizosphere, were isolated from plant samples collected in Chiapas, Mexico. Approximately one gram of root was placed into tubes containing 9 ml of 10 mM $MgSO_4.7H_2O$, which were shaken vigorously in order to suspend the rhizosphere microorganisms. From this suspension, 200 µL were inoculated on Baz semisolid medium (0.2% Azelaic acid, 0.02% L-citrulline, 0.04% K_2HPO_4, 0.04% KH_2PO_4, y 0.02%, $MgSO_4.7H_2O$), and incubated at 28°C for one week, this procedure was repeated twice. After that, bacteria were reseeded and incubated at 28°C for 72 h on solid culture medium supplemented with cycloheximide BAc (100 mg/mL) enriched with phenanthrene 0.5% and benzene 1.0% as only carbon source. The bacterial colonies that were capable of growing on PAH´s and showed different morphology, were purified in BAc medium, the purity of isolated bacterial colony was verified in PY medium. Pure colonies were stored in 70% glycerol at -70°C.

The next procedure with the isolated bacteria was molecular identification by amplification of 16S rRNA gene, using rD1 and fD1 oligonucleotides (Weisburg et al. 1991). PCR conditions were as follows: initial denaturation for 5 min at 94°C; followed by 30 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 60°C, and 1 min of elongation at 72°C; followed by a final 5 min elongation at 72°C. The acquired 970-1000 bp portions of the 16S rRNA gene sequences were deposited in GenBank with MG457074 (*Pseudomonas aeruginosa* N7B), MG457075 (*Pseudomonas* sp.), MG457076 (*Pseudomonas* sp., bf1) and MG457077 (*Pseudomonas aeruginosa* F23) accession numbers.

2.2 Biodegradation kinetics in Bushnell Haas Broth medium (BHB)

Previous to biodegradation kinetics assays, bacterial strains were grown in PY (Peptone Yeast Extract) medium for 24 h at 29°C and shaken at 200 rpm in order to produce inoculum biomass. Then bacterial strains were adjusted to an optical density of 0.2 (10^6 CFU/mL) to 600 nm, and inoculated into flasks containing 200 mL of Bushnell Haas Broth medium (BHB), its composition in g/L is: 0.2 $MgSO_4 \cdot 7H_2O$, 0.02 $CaCl_2.2H_2O$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.05 $FeCl_3$, and phenanthrene 0.5% and benzene 1.0% as a carbon source. As a positive control, 5.0 g of sodium succinate was used as soluble and biodegradable carbon source. Three independent experiments were carried out with three repetitions each.

2.3 Determination of bacterial growth by viable count

Bacterial strains were cultured in BHB media for 168 h at 29°C at 200 rpm, bacterial growth during the aromatic

compounds degradation process was determined by plate count method after decimal dilutions, 100 µL of the dilutions was inoculated in Petri dishes with PY culture medium, incubated at 29°C for 48 h; measurements were performed at 0, 24, 48, 72, 96, and 168 h. Three independent experiments were carried out with three repetitions each. The results were analyzed by means comparison with Tukey test, with a significance level of P≤0.05, using "Statistical Analysis System" (SAS) software.

2.4 Evaluation of enzymatic activity

The polyphenol oxidase activity was determined using a reaction mixture with 100 mM catechol in 200 mM sodium acetate phosphate buffer solution pH 6.2, and the enzymatic extracts of the aliquots taken every 24 h for 7 days. Oxidation of catechol, was measured spectrophotometrically by an increase of absorbance at 420 nm for 2 min.

2.5 Extraction of aromatic hydrocarbons from biodegradation assays in liquid bacterial cultures

The benzene and phenantrene degradation was quantified at 0, 24, 48, 72, 96 and 168 h. From liquid bacterial cultures aliquots of 5 mL were taken, and after that a solid phase extraction technique (SPE) was used in its cartridge mode with C18. The extraction columns were pre-activated with 5 mL methanol, 5 mL hexane and 5 mL mili-Q water, 50 mL of the aqueous sample was passed through the SPE tubes at a pressure of 25 psi and flow rate of 1-2 mL/min. Then 5 mL of mili-Q water was added. Elution of PAHs was performed with 10 mL hexane. The extract was concentrated to dryness under a high argon flow of 99.997% purity (INFRA®) and the extract was resuspended with 1 mL of acetonitrile. The concentrated extract was stored in a 2 mL amber borosilicate vial with screw cap and PTFE septum, cooled to 4°C until chromatographic analysis.

2.6 Polycyclic Aromatic hydrocarbons analysis by Gas Chromatography coupled to Mass Spectrometry (GM-MS)

A gas chromatograph (Thermo Trace GC Ultra) coupled to an ion trap mass spectrometer (ITQ 900) (Thermo Fisher Scientific Inc, Austin, TX USA) was used for the determination of AHs. The operating conditions of GC-MS were as follows: 2 µl of sample was used per analysis. The injection was in splitless mode with an autosampler (TriPlus Autosampler). The temperature of the injector was 250°C and the transfer line was 270°C. The temperature of the ion source was 200°C. The components were separated in a capillary column; model TR-5MS, 5% phenylmethylsiloxane (30 m long x 0.25 mm internal diameter) with 0.25 µm thick stationary phase. The oven temperature ramp was at an initial temperature of 70°C held for 1 min, followed by a gradient of 15°C/min to reach 180°C maintained for 1 min, subsequently temperature increments of 5°C/min to 270°C maintained for 15 min (running time: 42 min). The carrier gas was ultra-high purity helium (INFRA) (99.9999%). The mass detector operated in selective ion monitoring mode (SIM).

2.7 Growth promoting of *Zea mays* with *Pseudomonas* strains PAH´s biodegraders

The *Pseudomonas aeruginosa* N7B1 (MG457074), *Pseudomonas* sp. (MG457075), *Pseudomonas* sp., bf1 (MG457076), and *Pseudomonas aeruginosa* F23 (MG457077) strains were grown in PY medium for 24 h, 29°C at 200 rpm, then the bacterial cultures were adjusted to an optical density of 0.5 to 600 nm. The corn seeds were disinfected with a 10% hypochlorite solution for 15 min; excess chlorine was removed with washes of sterile distilled water. The seeds were subsequently incubated for 72 h, and maize seedlings were planted in pots containing sterile vermiculite with benzene at 1.0% and phenanthrene 0.5% respectively. Maize plants were inoculated with one milliliter of the bacterial culture, additionally plants were watered with Farheus nutritional solution every third day during four weeks under greenhouse conditions. The following variables were quantified: height, fresh weight and dry weight of roots and aerial part, stem diameter, root length. Experiments were independently performed in triplicate.

2.8 Statistical analysis

All data were expressed as a mean of randomized block design experiment under greenhouse conditions. Significant differences in height, root, length, stem diameter, aerial fresh weight, aerial dry weight, root fresh weight, and root dry weight were tested by a one-way analysis of variance (ANOVA) to compare all treatments over the control. The test was followed by Tukey's post-hoc test (where P<0.05). The statistical analysis was carried out using the "Statistical Analysis System" (SAS).

3 Results

3.1 Bacterial growth in benzene and phenanthrene

Figure 1 shows the kinetic growth with benzene and phenanthrene as the only carbon source. All bacterial strains tested, *Pseudomonas aeruginosa* N7B1 (MG457074), *Pseudomonas* sp. (MG457075), *Pseudomonas* sp., bf1 (MG457076), and *Pseudomonas aeruginosa* F23 (MG457077), showed typical growth and were capable of growing in both benzene 1.0 % and phenanthrene 0.5%; they showed similar behavior with respect to the control treatment (with sodium succinate).

After 48 h of culture, *Pseudomonas aeruginosa* N7B1 and *Pseudomonas* sp. reached an exponential growth with a magnitude order 10^{11} CFU/mL higher than the exponential growth reached by *Pseudomonas* sp., bf1, and *Pseudomonas aeruginosa* F23, with a magnitude order of 10^{9} CFU/mL. However, between treatments (benzene, phenanthrene, and sodium succinate), there were not statistical differences in the growth of all bacterial strains tested. All bacterial strains finished their growth after 96 h of culture.

3.2 Enzymatic activity

Polyphenol oxidase activity was observed at different times intervals during bacterial growth on benzene and phenanthrene. The maximum enzymatic activity was observed in cell-free extracts of *Pseudomonas aeruginosa* F23 (MG457077) and *Pseudomonas aeruginosa* N7B1 (MG457074) cultured at 72 h for benzene and 48 h for phenanthrene with (Figure 2). Whereas with *Pseudomonas* sp. (MG457075), and *Pseudomonas* sp., bf1 (MG457076) strains, the highest enzymatic activity was observed at 72 h with both substrates (Figure 2).

3.3 Polycyclic Aromatic hydrocarbons analysis by GM-MS

The bacterial strains *Pseudomonas aeruginosa* N7B1 (MG457074), *Pseudomonas* sp. (MG457075), *Pseudomonas*

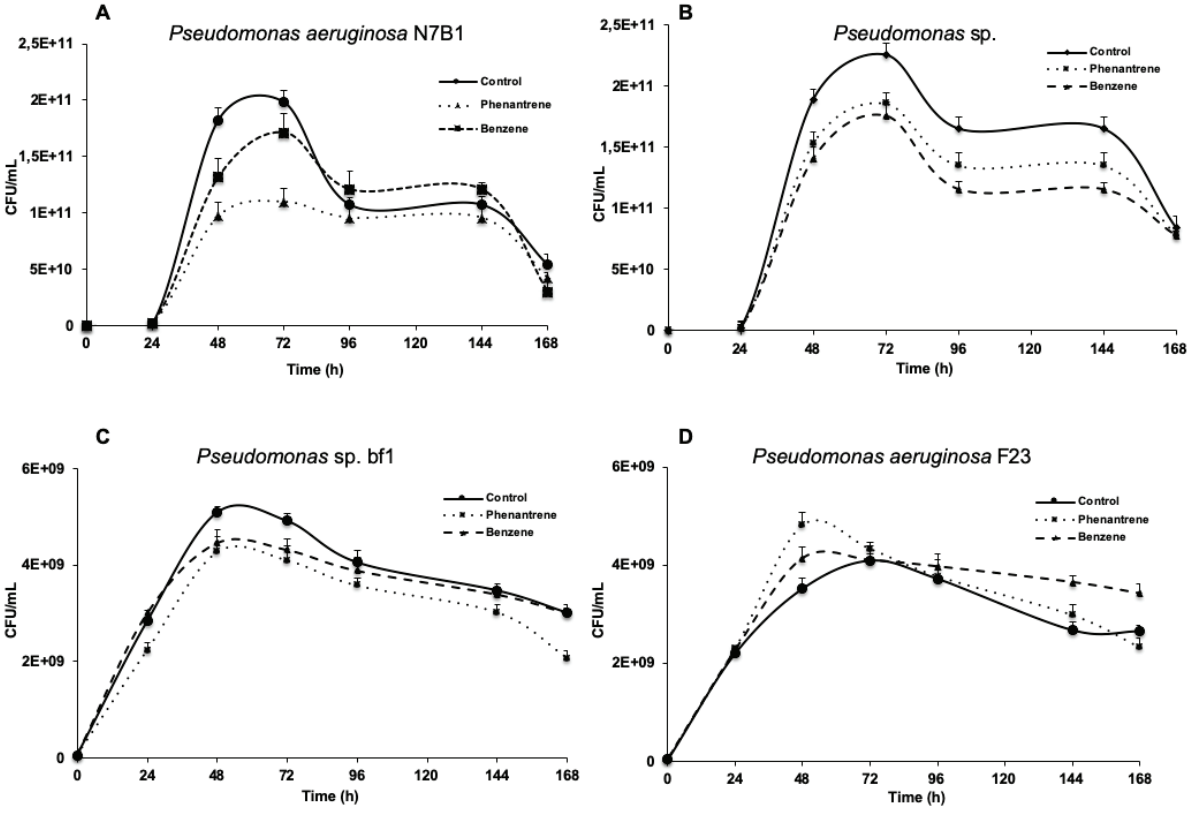

Figure 1: Microbial growth curves of *Pseudomonas* strains in BHB medium with phenanthrene 500 ppm and benzene 1000 ppm A) *Pseudomonas aeruginosa* N7B1, B) *Pseudomonas* sp. C) *Pseudomonas*sp bf1, and D) *Pseudomonas aeruginosa* F23

sp., bf1 (MG457076), and *Pseudomonas aeruginosa* F23 (MG457077) grew in the presence of benzene and phenanthrene. Figure 3 shows the biodegradation percentage of PAHs, figure 3A is observed that after 168 h, *Pseudomonas aeruginosa* N7B1 degraded 84% phenanthrene and 45% benzene; *Pseudomonas aeruginosa* F23 degraded 64% phenanthrene and 45% benzene (Figure 3D); *Pseudomonas* sp., degraded 59% phenanthrene and 34% benzene (Figure 3B); and *Pseudomonas* sp., bf1 52% phenanthrene and 45% benzene (Figure 3C).

3.4 Pseudomonas strains inoculation and aromatic hydrocarbons effect, on maize growth

After inoculation of all four Pseudomonas strains in maize plants grown in the presence of benzene 1.0% and phenanthrene 0.5%, it was observed that *Pseudomonas aeruginosa* F23 has the best growth promotion effect (see Table 1). In the presence of benzene and inoculated with *Pseudomonas aeruginosa* F23, an increase of 16% in height, 26% stem diameter, 6% root length, 40% foliage fresh weight, 108% foliage dry weight, 188% root fresh weight, and 116% root dry weight, was observed compared to maize plants that were only treated with benzene (Table 1).

Also, in maize plants treated with phenanthrene and inoculated with *Pseudomonas aeruginosa* F23 the best growth promoter effect was observed. According to evaluated variables, it increased 20% highness, 30% stem diameter, 35% root length, 21% foliage fresh weight, 32% foliage dry weight, 94% root fresh weight and 47% root dry weight, with respect to maize plants that were treated with phenanthrene exclusively (Table 2).

4 Discussion

Studies on rhizosphere are being focused on exploration of plant-growth promoting bacteria that additionally can degrade organic recalcitrant compounds. Plant-microorganism interactions are happening in the rhizosphere, where there is a liberation of radical exudates, which

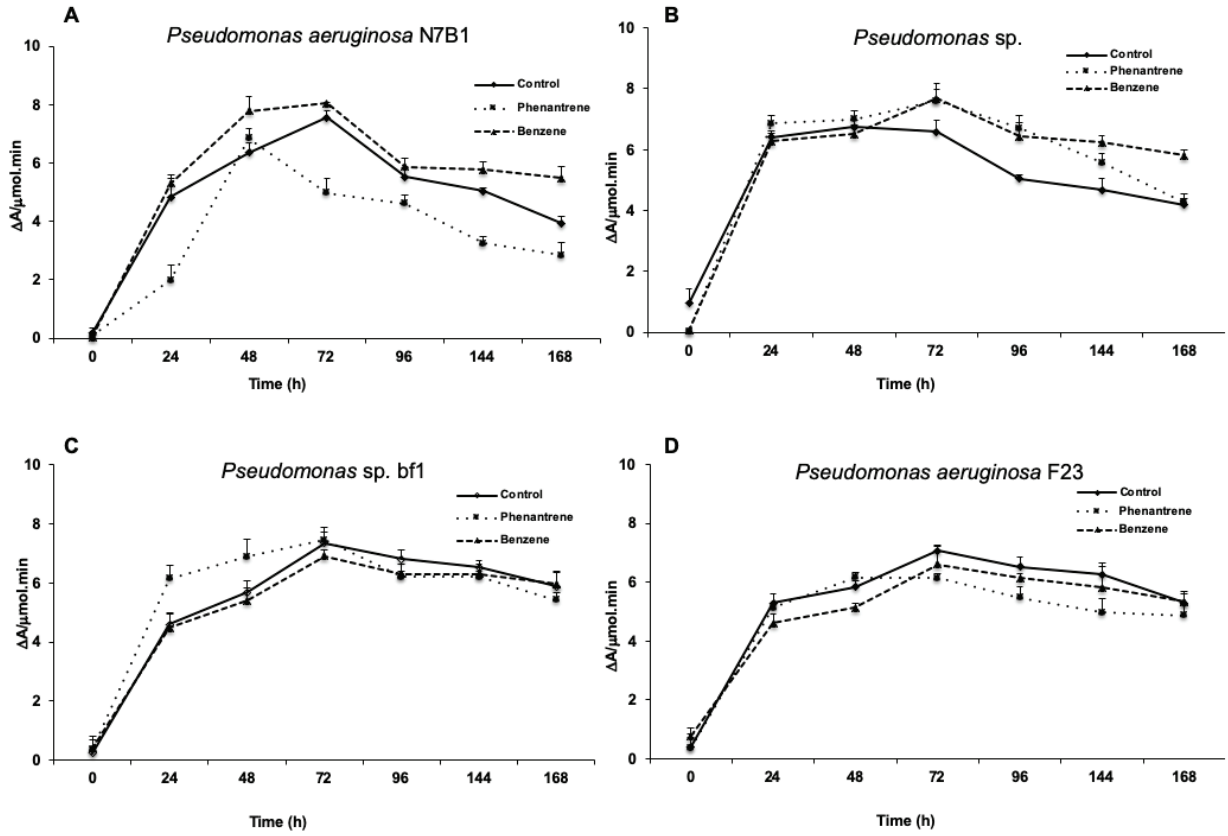

Figure 2: Polyphenol oxidase activity in cell-free extracts of *Pseudomonas* strains grown in BHB medium at 500 ppm phenanthrene and 1000 ppm benzene concentrations. A) *Pseudomonas aeruginosa* N7B1, B) *Pseudomonas* sp., C) *Pseudomonas* sp bf1, and D) *Pseudomonas aeruginosa*F23.

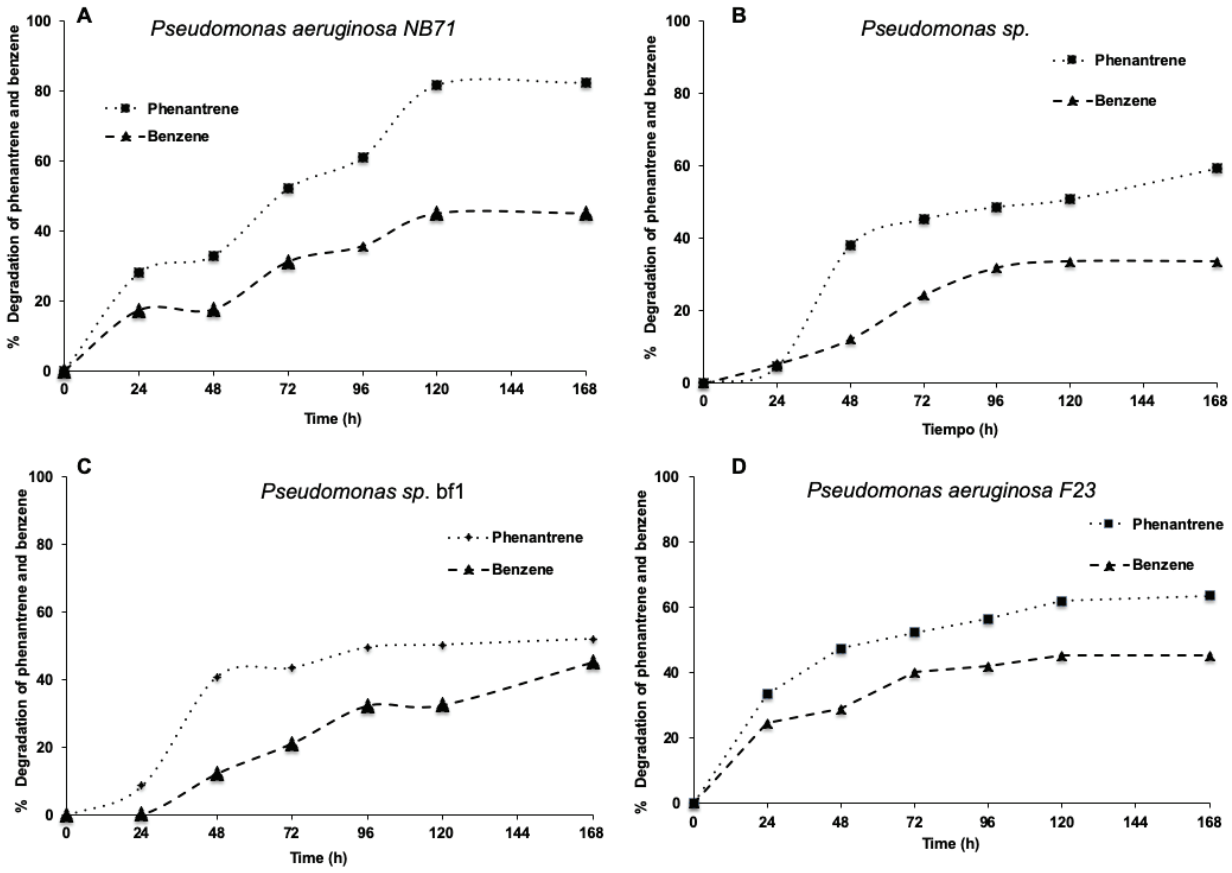

Figure 3: Percentage of biodegradation by *Pseudomonas* strains in 500 ppm phenanthrene and 1000 ppm benzene, quantified by mass-coupled gas chromatography A) *Pseudomonas aeruginosa* N7B1, B) *Pseudomonas* sp., C) *Pseudomonas* sp bf1, and D) *Pseudomonas aeruginosa* F23

Table 1: Pseudomonas strains inoculation and aromatic hydrocarbons (with benzene 1000 ppm) effect, on maize growth

Treatments	Height (cm)	Root length (cm)	Stem diameter (mm)	Aerial fresh weight (g)	Aerial dry weight (g)	Root fresh weight (g)	Root dry weight (g)
Benzene	67.30 b	40.10 a	0.46 b	3.60 b	0.62bc	3.35 b	1.18 c
P. aeruginosa N7B1	72.40 ab	42.00 a	0.54 ab	3.95 ab	0.64bc	2.77 b	1.15 c
Pseudomonas sp	73.80 ab	41.00 a	0.65 a	4.89 ab	0.80 b	9.27 a	1.89 b
Pseudomonas sp bf1	67.80 b	40.10 a	0.50 b	3.63 b	0.51 c	3.71 b	1.21 c
P. aeruginosa F23	78.10 a	42.70 a	0.58 ab	5.19 a	1.29 a	9.65 a	2.56 a

Means with the same letter are not statistically different (Tukey p = 0.05)

stimulate growth and microbial activity (Chaudry et al. 2005; Leigh et al. 2006).

In this study, four bacterial strains belonging to *Pseudomonas* genus isolated from *Jatropha curcas* rhizosphere, were capable of growing and biodegrading PAH´s. They reached a maximum growth rate at 72 h in BHB medium (Figure 1). The microbial growth of *Pseudomonas aeruginosa* N7B1 (MG457074), *Pseudomonas* sp. (MG457075), *Pseudomonas* sp., bf1 (MG457076), and *Pseudomonas aeruginosa* F23 (MG457077) strains in benzene 1.0% and phenanthrene 0.5% presence was very similar between them and with respect to the control, which had sodium succinate, a three carbons source easily metabolizable by bacteria in the rhizosphere. These results agree with those

Table 2: Pseudomonas strains inoculation and aromatic hydrocarbons (with phenanthrene 500 ppm) effect, on maize growth

Treatments	Height (cm)	Root length (cm)	Stem diameter (mm)	Aerial fresh weight (g)	Aerial dry weight (g)	Root fresh weight (g)	Root dry weight (g)
Phenantrene	65.44 c	37.33 bc	0.42 c	3.17 a	0.49 a	3.68 b	1.02 c
P. aeruginosa N7B1	63.00 b	32.88 c	0.63 a	3.95 a	0.69 a	5.88 ab	1.63 b
Pseudomonas sp	63.13 ab	38.25 bc	0.47bc	2.81 a	0.53 a	5.40 ab	1.82 ab
Pseudomonas sp bf1	73.91 a	44.55 ab	0.57 ab	3.81 a	0.67 a	5.72 ab	1.75 a
P. aeruginosa F23	78.55 bc	50.55 a	0.55 ab	3.84 a	0.65 a	7.14 a	1.50 bc

Means with the same letter are not statistically different (Tukey p = 0.05)

reported by Marcon et al. (2007), where the ability to use toluene, xylene, phenanthrene, naphthalene, and pyrene as the only carbon source was evaluated.

In recent years, interest in studying degradation of PAHs by Pseudomonas genus has increased, because of its biodegradation capacity through biosurfactants production (Yañez-Ocampo Wong-Villarreal 2013). Wong et al. (2016) reported that strains isolated from the rhizosphere of *Jatropha curcas* have the capacity to produce biosurfactants that contribute toe biodegradability, as well as growth in the presence of benzene and toluene (Wong-Villarreal et al. 2015).

In this study, the *Pseudomonas aeruginosa* F23 (MG457077) strain isolated from *Jatropha curcas* rhizosphere had the capacity to remove 84% phenanthrene and 45% benzene (Figure 3A); Yuan et al. 2002 reported that *Pseudomonas fluoresens* and *Haemophilus* spp., degrade 70-100% anthracene, acenaphthene, fluorene, phenanthrene, and pyrene; however, the origin of these strains is a petrochemical residues polluted environment, the reason why they are adapted to the presence of these compounds. de Lima et al. (2016) reported that Pseudomonas veronii 1YdBTEX2 and 1YB2 contain a 2-hydroxy muconic-dehydrogenase component for a single catabolic pathway for benzene degradation.

There are also reports of *Pseudomonas* sp., JPN2 strain degrading 98.52% phenanthrene at 100 ppm initial concentration after 10 days of incubation (Jin et al. 2016). Although the degradation percentage of these strains with respect to *Pseudomonas aeruginosa* F23 (MG457077) strain is higher, the degradation rate is 10 times higher and the performance time lapse is shorter. More relevant data from these rhizosphere isolates is that they come from uncontaminated sites, so they are not adapted microorganisms to these compounds presence. Nevertheless, they had the ability to remove up to 84% phenanthrene and 45% benzene (Figure 3A). Other relevant characteristics of these *Pseudomonas* strains are the production of biosurfactant, indoleacetic acid, phosphate solubilization, and metal resistance (Wong-Villarreal et al. 2015).

Due to the degradation and plant growth promotion characteristics of *Pseudomonas aeruginosa* N7B1, *Pseudomonas* sp., *Pseudomonas* sp., bf1, and *Pseudomonas aeruginosa* F23 strains, were inoculated in maize plants, being *Pseudomonas aeruginosa* N7B1 (MG457074) and *Pseudomonas aeruginosa* F23 (MG457077) which had an effect on fresh and dry weight increment in roots and shoots, plant height and root length of maize plants increase. Therefore, native strains of *Jatropha curcas* rhizosphere identified as *Pseudomonas aeruginosa* N7B1 and *Pseudomonas aeruginosa* F23 have a clear potential as bio-inoculants to improve the maize plants productivity in the presence of phenanthrene and benzene.

5 Conclusions

The isolated bacteria from *Jatropha curcas* plants rhizosphere grew in the presence of hydrocarbons. After seven days, the *Pseudomonas aeruginosa* N7B1 strain eliminated 84% of phenanthrene and 45% of benzene, and showed the highest capacity of the evaluated strains. The *Pseudomonas aeruginosa* F23 and *Pseudomonas aeruginosa* N7B1 strains had a growth promoting effect in maize plants that grew in the presence of 1.0% benzene and 0.5% phenanthrene, this could be observed in the increase of the values of the agronomic variables evaluated. These results open the possibility of using these strains as biofertilizers in soils contaminated with aromatic hydrocarbons.

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Conflict of interest: The authors declare that they have no conflict of interest.

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