

EFFECTS OF PETROLEUM PRODUCTS ON THE SURVIVAL OF BENEFICIAL *PSEUDOMONAS* SPECIES

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ABSTRACT

A comparison of the survival rates of three species of beneficial *Pseudomonas*, isolated from the rhizosphere of tropical plants, was carried out in different concentrations of petroleum products, after 0, 6, and 24 hr exposure times in the products. Percent (%) survival of all the strains in kerosene, lubricating oil (SAE 40) and petrol varied. Of the three products, undiluted lubricating oil was the least toxic to the bacterial cells after a 24-hr exposure time. Cells of all the strains were killed after 6 and 24 hr exposure times in kerosene and petrol. The survival rates of the strains increased when the concentration of the products was reduced to 50 %. All strains were more tolerant of lubricating oil than of kerosene or petrol. In 40 % lubricating oil, about 90 % of cells of *P. aeruginosa* strain PA-PP survived after a 6-hr exposure time. On the contrary, 63 % and 58 % of the cells survived in 40 % kerosene and petrol, respectively. Kerosene was more toxic to *P. putida* strain PP-CA than petrol. The effects of kerosene and petrol on two strains of *P. fluorescens*, Pf-5 and PF-B, were similar. Both products were slightly toxic at low concentrations (0.5-10 %), moderately toxic at moderate concentrations (20-40 %), and highly toxic at high concentrations (60-80 %).

Key words: Petroleum, kerosene, lubricating oil, *Pseudomonas* sp., rhizobacteria, and antagonists.

INTRODUCTION

Current interest in the state of the environment has led to increase studies to evaluate the impacts of mineral oil exploration activities in the oil producing states in Nigeria. The recent awareness in oil producing communities of the numerous problems associated with oil spills has led to several interference on the activities of oil companies in Nigeria. In addition, the consequences of accidental oil spills, and improper disposal of petroleum products, especially petrol, kerosene, and lubricating oil, have been of much concern to the entire citizenry of Nigeria. The most commonly observed petroleum spillage areas are the roadside automobile workshops. Careless handling, improper use and spills resulting from the activities of automobile technicians are consequential since mineral oil spills are known to have long term effects on soils (Odu 1977). An immediate effect of the presence of petroleum products in the soil is a depression in the population of soil microorganisms. Besides the economic and aesthetic damage caused by oil spills, plant and animal life in both terrestrial and aquatic environments are affected as most life forms die rapidly following spillage (Atlas and Bartha 1973). Though reductions in the population of microorganisms were observed in oil-polluted soils, (Jones, 1979), some microorganisms are able to degrade mineral oil (Hill 1977, Naylor *et al* 1982, Okpokwasili and Okorie 1986, Obire 1988).

A review of past literature revealed an absence of information on effects of crude oil or

their products on beneficial rhizobacteria. Several species of rhizobacteria contribute to the natural protection of roots against soil-borne pathogens. The beneficial activities of these bacteria are well documented (Burr *et al.* 1978, Kloepper *et al.* 1980, Janisiewicz and Roitman 1988, Kloepper *et al.* 1988, Loper 1988).

In this paper, findings on effects of three petroleum products on the survival of some soil residents, namely *Pseudomonas aeruginosa* PA-PP, *P. putida* PP-CA, *P. fluorescens* Pf-5 and *P. fluorescens* PF-B are reported. Strains PA-PP, PP-CA and PF-B were isolated from the roots of crops grown in Rivers State, Nigeria. Strain Pf-5 was isolated from the rhizosphere of cotton grown in Texas, U. S. A. All four strains were shown to express different levels of antagonism against *Botryodiplodia theobromae* (Wokoma, unpublished), *Botryodiplodia theobromae* is a common pathogen of several tropical crops including yam, banana, plantain sweet potato, mango cocoa, and citrus fruits (Arinze 1985, Ekundayo and Daniels 1973) and is frequently isolated from crops grown in Nigeria. *P. fluorescens* strain Pf-5 is also an antagonist of *Rhizoctonia solani*, *Fusarium oxysporum*, and *Pythium ultimum* (Howell and Stipanovic 1979, Kraus and Loper 1990, Mojueke 1991). With such significant roles in biological control, factors affecting their survival and proliferation in the soil, particularly in the Niger Delta should be ascertained as these factors would affect their ability to compete with other soil residents.

MATERIALS AND METHODS

Source of petroleum products:

Kerosene, petrol, and lubricating oil (SAE 40) were purchased from a fuel station in Port Harcourt. The lubricating oil was produced and marketed by a foreign oil company while kerosene and petrol were refined products of an indigenous oil company.

Source of bacteria:

The standard culture of *P. fluorescens* strain Pf-5 was obtained from D. C. Gross (Dept of Plant Pathology, Washington State University, Pullman, U. S. A.). It was originally isolated from the rhizosphere of cotton (*Gossypium hirsutum* L.) seedling in Texas, U. S. A. *P. fluorescens* strain PA-PP was isolated from the rhizosphere of pawpaw (*Carica papaya* L.), *P. putida* strain PP-CA from cassava (*Manihot esculenta* Crantz) and *P. fluorescens* strain PF-B isolated from banana (*Musa* sp.). The crops were grown at different locations in Choba in Obio Akpor Local Government Area of Rivers State, Nigeria. The three strains of bacteria isolated from these crops were used as test organisms alongside with the standard strain Pf-5.

Maintenance of the bacterial cultures:

P. fluorescens strain Pf-5 was received as a freeze-dried culture. Placing a chip of the culture into Nutrient Broth medium (Oxoid, England) revived it. After 48 h incubation at ambient temperature (29±2 °C), a loopful of the revived culture was streaked onto plates of *Pseudomonas* selective medium, *Pseudomonas* Agar F (PAF) and incubated at room temperature. The PAF medium contained the following ingredients: 10 g proteose peptone, 10 g tryptone, 1.5 g MgSO₄, 1.5 g K₂HPO₄, 10 ml glycerol, and 15 g agar in 1 L deionised water. After 48-hr incubation on PAF, discrete colonies were transferred to freshly prepared PAF plates and slants. *P. aeruginosa* PA-PP and *P. putida* PP-CA were isolated on PAF medium and identified (Buchanan and Gibbons (1974)). All bacterial cultures were routinely maintained on PAF plates or slants while inocula for laboratory investigations were grown on Nutrient Broth for 24 hr. Unless stated otherwise, all media and glassware were sterilised by autoclaving at 121° C, 1.03-kg cm⁻² for 15 min.

Effects of undiluted petroleum products:

Ten ml of undiluted kerosene, petrol, and lubricating oil or deionised water was pipetted into duplicate test tubes, inoculated with 1 ml of about 10⁶ to 10⁷ colony forming units per ml suspension of 24 hr liquid cultures of each strain. After plugging with cotton wool tubes were incubated at ambient temperature. During incubation, tubes were periodically agitated. After 24 hr, an 0.1-ml aliquot of the suspension was withdrawn from each tube and dispensed onto duplicate PAF plates. A flame sterilised L-shaped glass rod was used to uniformly spread the suspension of the surface of the plates. Viable colonies were counted after 48-hr incubation of plates at ambient

temperature.

Effect of time of exposure in undiluted products:

The effect of exposing the bacterial strains for 0, 6, and 24 hr in undiluted kerosene, petrol and lubricating oil was determined as follows: ten ml of each product or water was placed in duplicate tubes. One ml aliquot of about 10⁶ to 10⁷ colony forming units per ml suspension of 24 hr liquid of each strain was then added to each test tube, plugged with cotton wool, thoroughly mixed and incubated for 0, 6, and 24 hr at ambient temperature. The 0-hr sample was withdrawn immediately after mixing the bacterial cells with the petroleum products, while the 6 and 24 hr samples were withdrawn 6 and 24 hr later. All tubes were periodically agitated during incubation. Viable colonies in each treatment and for each exposure time were determined using the procedure described above.

Sensitivity of strains to 50% dilution of products:

A 50% dilution of each petroleum product was prepared by mixing equal part petrol, kerosene, and lubricating oil with equal part deionised water. Ten ml of each preparation or deionised water was dispensed into duplicate test tubes, and into the test tubes were dispensed one ml suspensions of each strain. After incubation, an 0.1 ml aliquot of each treatment was spread on PAF plates for determination of viable colonies.

Effect of different concentrations of products on survival of strains:

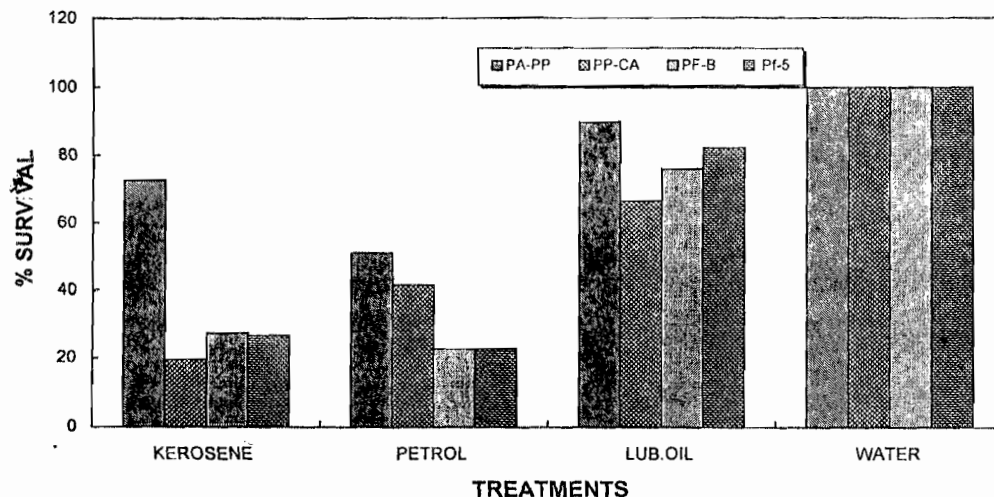
To determine the ability of each strain to survive in different concentrations of the petroleum products, percentages of these products were prepared using deionised water. To obtain a 1.0 % solution, 99 ml water was mixed with 1.0 ml of the product. A 10 % mixture was obtained by mixing 10 ml of a product with 90 ml of water. Using this procedure, other concentrations of each petroleum product were prepared. The percentages of petroleum products tested were 0, 1.0, 10, 20, 40, 60 and 80. All solutions were sterilised after preparation. For each product, duplicate tubes of each concentration were inoculated with a 0.1 ml suspension of about 10⁸ cfu/ml of each bacterial strain. All inoculated tubes were plugged with cotton wool and incubated with frequent agitation at ambient temperature. After 6 and 24-hr incubation periods, 0.1-ml aliquot of each treatment was spread on duplicate plates of PAF and incubated for 48-72 hr, after which viable colony counts were determined.

RESULTS

The percent survival of the strains of *Pseudomonas* is shown in Table 1. A high percentage (80-100 %) of the cells of strains Pf-5 and PF-B survived after a brief exposure (less than 10 min) in the product. None of the cells survived after 6 and 24-hr exposure in kerosene

Table 1. Percent survival of *P. fluorescens* strains PF-5, and PF-B, *P. putida* strain PP-CA and *P. aeruginosa* strain PA-PP in undiluted petroleum products after 0, 6 and 24 hr exposure time.

Petroleum Product	PF-5			PF-B			PP-CA			PA-PP		
	0hr	6hr	24hr	0hr	6hr	24hr	0hr	6hr	24hr	0hr	6hr	24hr
Kerosene	82.0	0.0	0.0	94.4	0.0	0.0	93.0	0.0	1.0	88.7	9.8	1.0
Petrol	100.0	0.0	0.0	97.6	0.0	0.0	97.7	0.0	0.0	96.6	0.0	0.0
Lubricating oil	99.9	35.0	23.1	97.5	28.9	22.8	98.8	29.6	25.0	100.0	33.7	25.8
Water (control)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Figure 1. Percent survival of strains of *Pseudomonas* in 50% petroleum products after 6 hr exposure time.

and petrol, but between 20% and 35% survived in lubricating oil. A similar effect was observed for *P. putida* PP-CA and *P. aeruginosa* PA-PP. However, strain PA-PP was less sensitive to kerosene when compared with other strains.

Although all the bacterial strain survived in 50% dilution of the products, *P. aeruginosa* was most tolerant, with about 70, 50, and 90% of the cells surviving in kerosene, petrol and lubricating oil, respectively (Figure 1). A low percentage (less than 40%) of *P. putida* and *P. fluorescens* survived in 50% dilution of kerosene and petrol, while a moderate percentage (65-80%) survived in lubricating oil.

Low concentrations of the products had very mild effect on *P. aeruginosa*. It maintained a high survival rate at concentrations below 20%. Above this, its survival in kerosene and petrol dropped drastically. A high survival (90-100%) was observed when the cells were exposed to 0.5-40% concentrations of lubricating oil (Figure 2A). This strain was more tolerant of lubricating oil than of kerosene and petrol.

Concentrations of 0.5-20% lubricating oil did not inhibit the two strains of *P. fluorescens* after 6 and 24 hr exposure time. However, this product became inhibitory at concentrations above 20% (Figure 2B and 2C). Kerosene and petrol were more toxic with less than 50% of the bacterial cells surviving at concentrations above 20%. The effects of kerosene and petrol on both

strains of *P. fluorescens* were similar. These products were moderately toxic at moderate concentrations and slightly toxic at low concentrations.

More cells of *P. putida* survived in petrol and lubricating oil than in kerosene at all concentrations tested. While 10% and less concentrations of lubricating oil were not toxic, 0.5-20% concentrations of kerosene were very toxic, killing over 50% of the bacterial cells (Figure 2D). The response of *P. putida* to the three products indicated that lubricating oil was slightly toxic, petrol was moderately toxic, and kerosene was highly toxic.

DISCUSSION

Undiluted kerosene, petrol and lubricating oil were toxic to all the four strains of *Pseudomonas* tested. Accidental spillage of mineral oil and improper disposal of some petroleum products may hinder the multiplication and survival of beneficial rhizosphere colonising bacteria. Consequently, their ability to favourably compete in soils in the presence of other soil-borne microorganisms that are more tolerant of mineral oil may be hindered. When bacterial cells were suspended in different concentrations of the products for different time periods, only a slight increase in inhibition was observed when the exposure time was increased beyond 6 h. This indicates that the toxic effects of the products occurred within few hours of contact with the

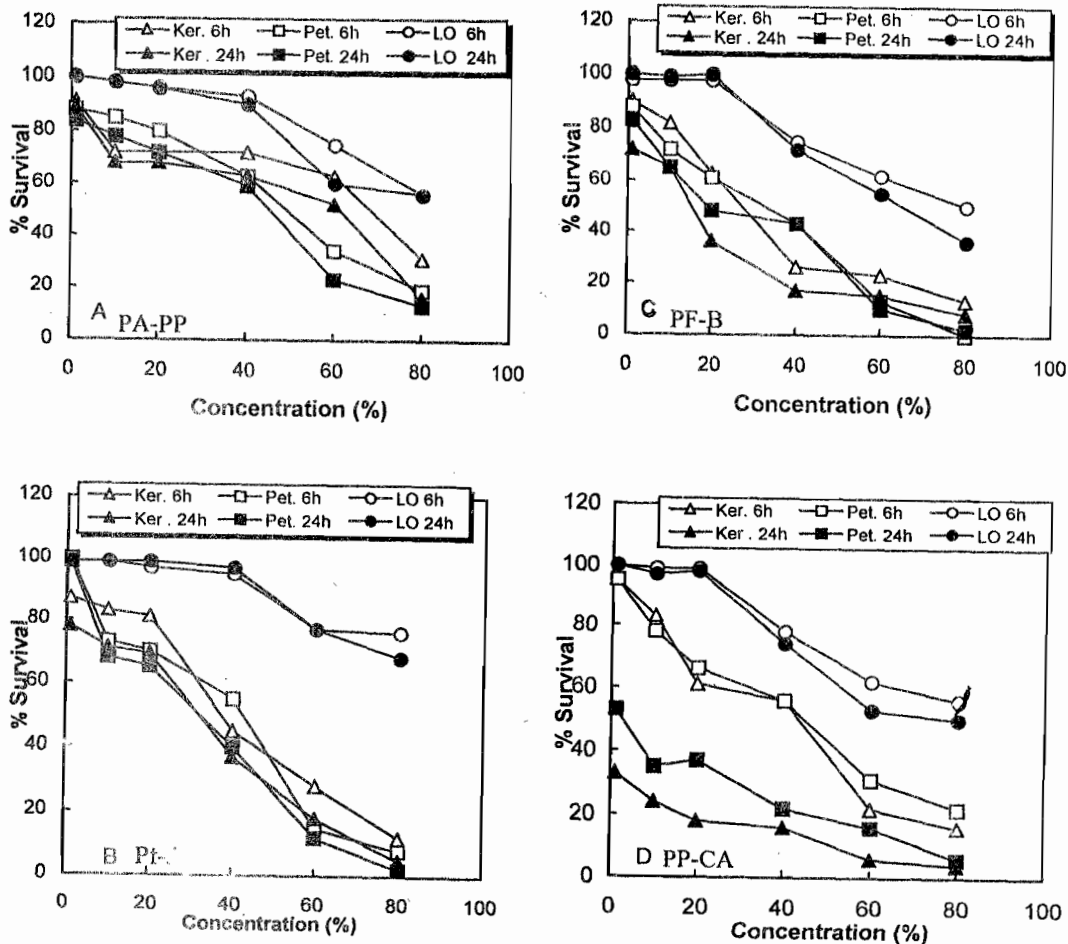


Figure 2. Changes in the population of strains of *Pseudomonas* after 6 hr and 24 hr exposure times in various concentrations of petroleum products, kerosene, petrol and lubricating oil (A - *P. aeruginosa* PA-PP, B - *P. fluorescens* PF-5, C - *P. fluorescens* PF-B, D - *P. putida* PP-CA).

bacterial cells. Cleaning of oil spills may therefore be more effective if it is achieved as soon as such spills are reported since the reaction of microflora to the presence of mineral oil is almost immediate.

The toxic effects of kerosene, petrol and lubricating oil differed. The chemical nature of the three products may have affected their toxicity levels. Lubricating oil was the least toxic with 60-90% of the cells surviving after being suspended for 6 h in 50% lubricating oil. On the contrary, only 20-70% and 20-50% of the cells survived in kerosene and petrol, respectively.

Microorganisms cannot proliferate in dry oil. Lubricating oil retains water and form emulsions more readily than petrol and kerosene, which are lighter fuels (Okpokwasili and Okorie 1988). The existence of a free water phase in lubricating oil-water mixture may have made lubricating oil less toxic to the bacteria since microorganisms live in the free-water phase. Zobel (1969) also reported that short chain alkanes such as kerosene and petrol are more inhibitory to microorganisms than long chain hydrocarbons, which include lubricating oil.

The four strains of *Pseudomonas* tested differed from each other in response to the different concentrations of the three petroleum

products, thus indicating differences in sensitivity among the strains. *P. aeruginosa* PA-PP was least sensitive to the products while *P. putida* PP-CA was more sensitive. These differences in sensitivity may affect their ability to survive in polluted soils. Okpokwasili and Okorie (1988) identified *Pseudomonas* sp. as one of the primary utilizers of mineral oil and Hill (1977) reported the preponderance of *Pseudomonas*, especially *P. aeruginosa* in oil. These observations suggest that though the immediate effect of petroleum is a depression in the population of *Pseudomonas* sp., colonisation of oil-polluted soils could occur since some species of *Pseudomonas* are able to degrade oil (Obire 1988). Furthermore, since brief contacts with the petroleum products did not adversely affect their survival, an immediate soil drench with water may reduce the toxic effect of petroleum products on soil microorganisms. In this study, it was observed that very low concentrations of lubricating oil did not adversely affect the survival of most strains of the bacteria tested. Consequently, if petroleum products are sufficiently diluted before proper disposal, their harmful effect may be minimized. The toxic effect of accidental spills may also be reduced by adequate dilution immediately following a spill.

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