

## Effect of Co-enrichment, Soybean Rhizosphere and P-hydroxybenzoic Acid, on Microbial Metabolic Diversity and P-HBA Degradation

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**Abstract:** In agro ecosystems, considerable amounts of phenolic acids may reach the soil or the rhizosphere. These compounds were found to be inhibitors of some plants. Soil microorganisms decompose many of these compounds, but some appear to be stabilized against microbial degradation. Little is known about the effect of the *p*-hydroxybenzoic acid (*p*-HBA) on the microbial communities and its mineralization kinetics. The addition of *p*-hydroxybenzoic acid significantly decreases the counts of bacterial CFU recovered from either bulk soil or rhizosphere soil. Application of *p*-hydroxybenzoic acid to the rhizospheric soils in a repeated low concentration or in a non-repeated high concentration caused a significant increase in the number of these bacteria compared with those recorded in control treatment. As the *p*-HBA dose increased, the maximum Average Well Color Development decreased. The substrates used in Biolog microplate were slowly utilized by microbial populations extracted from soils treated with the *p*-HBA either for bulk or rhizospheric soil. The Shannon biodiversity indexes of the two soils were not significantly different for all *p*-HBA treatment, indicating a similar level of biodiversity. The utilization patterns of *p*-HBA as a sole source of carbon showed a high significant increase between the maximum WCD for communities extracted from rhizosphere soil as compared with those of bulk soil. The cumulative percentage of mineralization and the mineralization rates of treated rhizospheric soil were higher than those of bulk soil. The maximum cumulative percentage of mineralization was observed for the two soils with the repeated application of the low dose of *p*-HBA. In our experimental conditions, we conclude that, the repeated application of *p*-HBA in a week concentration to the soybean rhizosphere can enrich the rhizospheric soil with the *p*-HBA utilized bacteria.

**Key words:** Metabolic diversity, bacterial enumeration, soybean, rhizosphere, enrichment, *p*-hydroxybenzoic acid utilized bacteria, Biolog, mineralization kinetics.

### INTRODUCTION

In agro ecosystems, a large amount of litter is turned over during the cropping season, fallow period and land preparation. This release a flush of phenolic acids, amounts exceed very much the quantities released in root exudation<sup>[28]</sup>. These compounds were found to be major germination inhibitors of some plants; (e. g., *p*-hydroxybenzoic acid inhibited germination and seedling growth of lettuce seeds)<sup>[7]</sup>. Soil microorganisms rapidly decompose many of these compounds, but some appear to be stabilized against microbial degradation through sorption by organic matters and clay minerals in soils<sup>[15]</sup>. Numerous studies have been conducted to investigate the utilization of

phenolic acids as carbon sources by microorganisms<sup>[12,19,23,24]</sup>. Induction and/or selection of phenolic acid-utilizing bacteria within the bulk soil and rhizosphere have been observed when soils are enriched with individual phenolic acids at concentration  $\geq 0.25 \mu\text{mol/g}$  soil. In previous studies, additions of such concentrations of *p*-hydroxybenzoic acid to soil-microbe or soil-microbe-plant systems resulted in the induction of bacteria capable of utilizing this compound as a sole source of carbon<sup>[2,20]</sup>.

The continual presence of the same and related plant hosts in natural plant communities not only favours plants pathogens and rhizosphere antagonistic microorganisms but also changes microbial diversity<sup>[8,9,13]</sup>. More recently, Mohamed<sup>[16]</sup>, found that

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repeating culture of soybean in a short period (6 weeks) for four cultures significantly change CFU of bacterial enumeration, metabolic and genetic diversity. Rhizospheric strains isolated after 4<sup>th</sup> culture were more competitive than those isolated from the 1<sup>st</sup> one. Furthermore, populations of the 4<sup>th</sup> rhizosphere were more capable of utilizing phenolic acids as a sole source of carbon, especially coumaric and *p*-hydroxybenzoic acid as compared with those of the 1<sup>st</sup> rhizosphere. Interestingly, this was obtained without any addition of phenolic acids to soil.

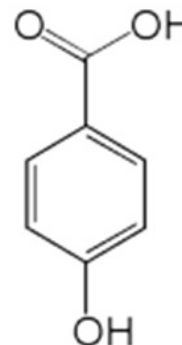
In the ecological approach such as biofertilization and phytoremediation, selected microorganisms should be able to function in the same environmental niche. Thus suitable places for isolation of such ecologically adapted microbes would be the surface of seeds and roots of the plants susceptible to do their function in question<sup>[17]</sup>. Effect of co-enrichment plant rhizosphere and a degradable compound on the biodegradation of such compound was little studied. Rouchaud *et al.*<sup>[18]</sup>, studied soil biodegradation of 25% chlorfenvinphos in cauliflower, Brussels sprouts and Chinese cabbage crops. They found that differences in the rates of soil biodegradation between fields in different areas were attributed to differences in the number of years of preceding monoculture and continuous insecticide treatment. It is concluded that continuous monoculture with soil insecticide treatment generated adapted microbial populations and higher rates of chlorfenvinphos biodegradation.

In this context, our study aims to evaluate the effect of co-enrichment plant rhizosphere and *p*-hydroxybenzoic acid additions on: (1) metabolic microbial diversity (2) enumeration of *p*-hydroxybenzoic acid degraded bacteria (3) utilization of these bacteria to *p*-hydroxybenzoic acid as a sole carbon source (4) mineralization rate of this compound.

## MATERIALS AND METHODS

**Soil and Plant:** The soil studied was a clayed eutric cambisol (clay 46.7%, silt 47.4%, and sand 5.9 % ; 41g organic matter kg<sup>-1</sup>, pH 7.5 ) recovered from 0-15cm layer in the INRA Experimental farm situated in Bretennières, 21, France. Soybean seeds (*Glycine max* L. Merrill) Essor, were sterilized in hypochlorite solution (70% active chlorine) for 20 min, rinsed several times with sterile water. Four seeds were sowed in pots containing 300g soil to have finally two plants per pot which considered as a replicate. Three replicates were done. Plants were grown in a chamber with day/night conditions of 11/13 h at 18/15°C and lighting at 240mE μ<sup>-1</sup>.

### *P*-hydroxybenzoic Acid (*p*-HBA)



*P*-hydroxybenzoic acid or 4-hydroxybenzoic acid, is a phenolic derivative of benzoic acid. It is a white crystalline powder that is slightly soluble in water and chloroform, but soluble to extremely soluble in alcohol, acetone and ether; melts at 215°C. It contains both hydroxyl and carboxyl group at para-position, which react with either acid or alcohol. *P*-Hydroxybenzoic acid is used for the preparation of biocides, antiseptics and bacteriostatic agents. It is used as chemical intermediate or synthetic drugs, pharmaceuticals, dyes and plasticizers.

***P*- Hydroxybenzoic Acid Treatments:** Stock solutions of *p*-hydroxybenzoic acid were prepared in ethanol 95%. Solutions were diluted in distilled water and added to soils in order to maintain soil moisture at 80% of water holding capacity. Treatments of *p*-hydroxybenzoic acid were as follow:

- 1- No addition of *p*-hydroxybenzoic acid, control (c).
- 2- 1mMolar in soil solution repeated every six weeks for 4 times (r1)
- 3- 10 mMolar in soil solution applied one time at the first of experiment (10)
- 4- 10 mMolar in soil solution repeated every six weeks for 4 times (r10). Every treatment was applied to bulk soil (B) (soil in pots without plants) and soil cultivated with soybean plants (rhizospheric soil, R) and maintained at the same conditions described above. Each treatment was replicate three times. After six weeks (one cycle), roots were lifted out from the pots. Bulk or rhizospheric soils of each pot were mixed with or without appropriate solution of *p*-hydroxybenzoic acid according to the previous treatments. This was repeated for four cycles.

**Soil and Soil Rhizosphere Recovery:** At the final of the 4<sup>th</sup> cycle, the plants were lifted out from the pots and root systems of two plants (considered as a replicate) were transferred to 11 Erlenmeyer flask

containing 50ml sterile distilled water. The flask was shaken for 10 min at 170 rpm on a rotary shaker. Soil suspension was recovered in a sterilized bottle and a second soil extraction was performed in the same way. The two 50ml soil suspensions were mixed and considered as rhizosphere soil suspension. Remaining soil in planted pots and bulk soil were mixed and stocked at 4°C for the radioactivity measurements.

Samples were taken for the counts of total and *p*-hydroxybenzoic acid degraded bacteria and for metabolic studies.

**Total and *P*-hydroxybenzoic Acid Utilized Bacterial Counts:** Serial dilutions of soils and rhizospheric soils were plated on Tryptone Soybean Agar (TSA) medium diluted at 1/10 for the total bacterial counts. Three plates of each appropriate dilution were incubated in the dark at 20°C. After 6 days, Colonies Forming Units (CFUs) were counted.

*p*-hydroxybenzoic acid utilized bacteria were counted by the method described by Catroux *et al.*<sup>[6]</sup>. Buffered phosphate solution containing per 1l: KH<sub>2</sub>PO<sub>4</sub>: 2.27g; Na<sub>2</sub>HPO<sub>4</sub>· 12H<sub>2</sub>O: 11.84g was adjusted at pH 7. Medium containing per 1l of phosphate solution: NH<sub>4</sub>Cl: 1g; MgSO<sub>4</sub>· 7H<sub>2</sub>O: 0.5g; CaCl<sub>2</sub>: 10mg; FeSO<sub>4</sub>· 7H<sub>2</sub>O: 1mg; glucose: 10mg; yeast extract: 10mg; agar: 15g were sterilized at 120°C during 20 min. Stock solution of *p*-hydroxybenzoic acid (1%) was prepared in phosphate solution and sterilized by filtration. Solution was added to medium in order to maintain *p*-hydroxybenzoic acid concentration at 400 ppm. Three plates of each appropriate dilution were incubated for 6 days in the dark at 20°C. Ten ml of a fresh reactive solution containing 1% FeCl<sub>3</sub> and 1% K<sub>3</sub>Fe(CN)<sub>6</sub> was poured directly on agar plates. After ten min, the medium color turn green-blue and bacterial colonies which rounded by clear zone were counted as *p*-hydroxybenzoic acid utilized bacteria.

**Biolog Profiles of the Communities:** Biolog MT plates were used (Biolog, Hayward CA, USA). MT plates were set up to characterize the bacterial community using carbon substrates more adapted to the soil and rhizosphere bacteria than the Biolog GN substrates. These substrates were malic acid, D,L-lactic acid, oxalic acid, L-serine, arginine, linolenic acid, oleic acid, stearic acid, β methyl D-glucoside, D-trehalose and *p*-Hydroxy benzoic acid and they were chosen according to previous studies. The MT plates (0.3 mg of each carbon substrate per well) were prepared as reported by Campbell *et al.*<sup>[5]</sup> and slightly modified by Mohamed<sup>[16]</sup>. For all amino acids, sugars and long chain fatty acids, individual carbon source stock solutions were prepared aseptically and each well

was filled by dispensing 60 μl of a 0.5% (w/v) solution. The carboxylic acids (oxalic and lactic acids) were prepared from 2% stock solutions except, for malic acid which was prepared as 1% (w/v) stock solution and 30 μl dispensed into each well. In order to check the sterility of carbon sources added to MT microplates, one microplate was inoculated with sterile de-ionized water and incubated together with the test microplates. One well was left blank, as a control. Each set of carbon sources (10), including the blank were replicated three times in a single 96 well MT plate. The plates were then inoculated with 150 μl of the 10<sup>-4</sup> dilution of the suspension of rhizospheric soils and incubated at 20°C for 170 hours. Color development was measured after 0, 24, 48, 74, 78, 93, 98, 117, 122, 126, 141, 150, 165, and 170 hours, at 590nm using a Microplate reader (Thermomax, Molecular Device, USA). Initial ODs were measured immediately after inoculation and were subtracted from subsequent readings.

Average Well Color Development (AWCD) was determined for each plate<sup>[5,11]</sup>. Shannon biodiversity indexes were calculated for Maximum AWCD as described by Staddon *et al.*<sup>[22]</sup>. The same procedure was used for the *p*-hydroxybenzoic acid preparation and color development measurements except, more dilute solution was prepared at 0.4% (w/v) and 75 μl was added into each well<sup>[16]</sup>.

**Mineralization of *P*-hydroxybenzoic Acid by Radioactivity Measurements:** *p*-hydroxybenzoic acid (analytical grade purity > 99%) and <sup>14</sup>C-ring labelled *p*-hydroxybenzoic acid (specific activity 11.5 mCi/mmol; 99% radiochemical purity) were purchased from Sigma (USA). Equivalent of 50g dry soil were placed in bottles of 250 ml volume. Each bottle received 5 ml of a solution containing 20mg of non-radioactive *p*-hydroxybenzoic acid and 200000 DPM of *p*-hydroxybenzoic acid ring UL <sup>14</sup>C (Sigma, USA). Samples were moistened to 80 % of WHC and incubated at 20 ± 0.5°C in the dark for 11 days in closed jars<sup>[21]</sup>. <sup>14</sup>CO<sub>2</sub> resulting from mineralization of <sup>14</sup>C labelled *p*-hydroxybenzoic acid was trapped in 5 ml of 0.2M NaOH solution placed in the jars. These traps were changed after 1, 2, 3, 4, 6, 11 days and analyzed for radioactivity content by liquid scintillation counting using ACSII (Amersham) scintillation fluid (Packard Tri. Carb 460C). Percentages of mineralized compound from the initial added dose were calculated and Kinetics of mineralization of <sup>14</sup>C ring-labeled *p*-hydroxybenzoic acid for different treatments was obtained.

**Statistical Analysis:** Variance analysis for Total and *p*-hydroxybenzoic acid utilized bacterial counts, AWCD for the Top Ten at 170H, Shannon biodiversity indexes values, well color development at 170H for *p*-hydroxybenzoic acid utilization as a sole carbon source and the cumulative percentages of mineralization of *p*-HBA from the initial added dose after 11 days of incubation were performed using StatView Version 5 (SAS Institute Inc., USA) with the Fisher's PSLD test at the probability level of 5%.

## RESULTS AND DISCUSSION

**Total and *P*-hydroxybenzoic Acid Utilized Bacterial Counts:** Results for total and *p*-HBA utilized bacterial enumeration and their levels of significance are given in Tables (1, 2). The numbers of total bacterial colonies recovered from non-treated-rhizospheric soil was two log more than those of bulk soil. Adding the *p*-hydroxybenzoic acid reduced the difference between the numbers of bacterial CFU recorded in rhizospheric soil and bulk soil to be ranged between 1.2-1.6 log. The addition of *p*-hydroxybenzoic acid significantly decreases the counts of bacterial CFU recovered from either bulk soil or rhizosphere soil (Table 1).

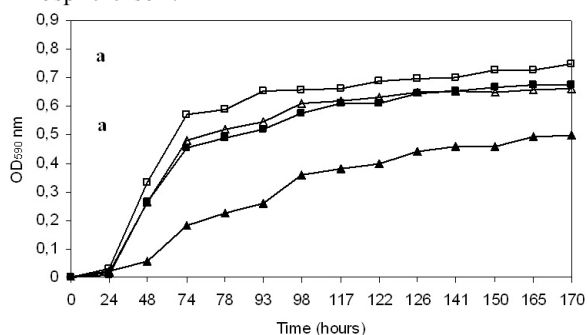
*p*-hydroxybenzoic acid utilized bacteria were not detected in all bulk soil treatments. In the contrary, these bacteria were countable in all planted soil treatments (Table 1). In the rhizospheric soils, *p*-hydroxybenzoic acid utilized bacterial counts in treatments Rr1 and R10 were significantly more than those recorded in Rc. Repeated the high dose of *p*-hydroxybenzoic acid (Rr10) causes a significant decrease in the counts of these bacteria as compared with those recorded in Rr1 and R10 but not in Rc (Table 2).

**Metabolic Microbial Diversity:** The rate of substrate utilization (color development) for each *p*-HBA treatment was initially more rapid in bulk than in rhizosphere soils (Fig. 1a, b). However, whatever the treatment of *p*-HBA, no significant difference was observed between the maximum AWCD (170H) of bulk soil microbial communities as compared with those of rhizospheric soil, Table 3. As the *p*-HBA dose increased, the maximum AWCD decreased with a high significant level for bulk soil treatments. In the contrary, the observed reduction in maximum AWCD was not significant in rhizospheric soil treatments, Table 3. The substrates were slowly utilized by microbial populations extracted from soils treated with the repeated high dose of *p*-HBA (Br10 and Rr10 treatments) and had much lower AWCD after 170H with an indication of longer lag times also (Fig. 1a, b). The Shannon indexes of each community were reported in Table 4. The Shannon biodiversity indexes were not significantly different for all *p*-HBA treatments either in bulk or rhizospheric soils, indicating a similar level of biodiversity. In the contrary, the biodiversity levels

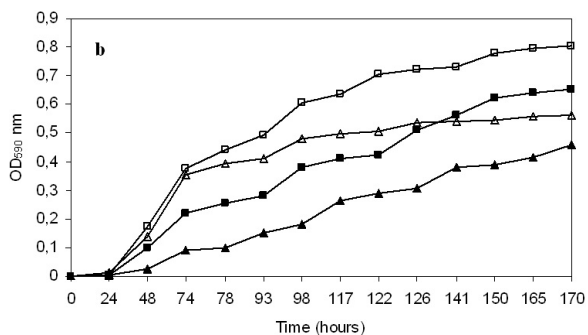
were significantly more for each of *p*-HBA treatment in bulk soil when compared with those of rhizospheric soil, except for the control treatment.

**Utilization of *P*-HBA as a Sole Carbon Source:** The rate of substrate utilization (Well Color Development WCD) for each *p*-HBA treatment was less in bulk than in rhizosphere soil (Fig. 2a, b). Regardless of the treatments of *p*-HBA, the utilization patterns of *p*-HBA as a sole source of carbon showed a high significant (a probability of less than 0.0001) difference between the maximum WCD for communities extracted from bulk (i.e. with an average of 0.206) and rhizosphere soil (i.e. with an average of 1.063).

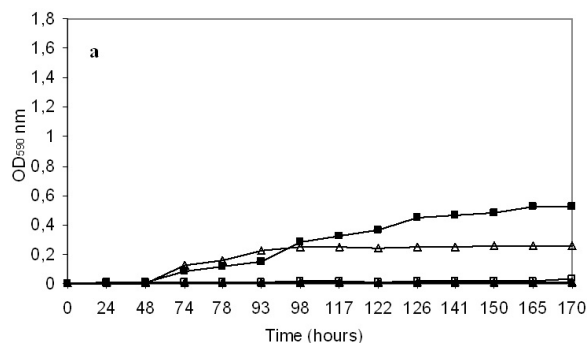
Slow utilizations levels were observed for communities extracted either from non- or repeated 10 mMolar treated rhizosphere soil while bulk soil showed no utilization levels for communities delivered from these two treatments. The maximum utilization rate was higher for communities extracted from soil of r1 treatment than in the other treatments (Fig. 2a, b). No significant difference was observed between 10 and r10 treatments in bulk soil while there was a high significant difference between the two treatments in rhizosphere soil.



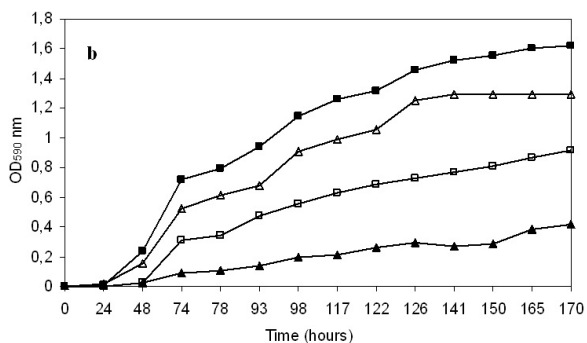
**Fig. 1a:** AWCD of the Top Ten substrates for the bulk soil treatments (a), the control c (□), Br1 (■), B10 (▲) and Br10 (▲).



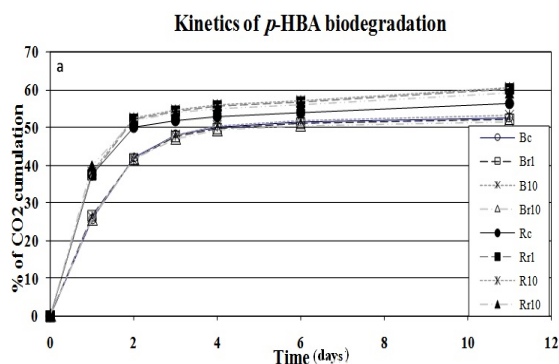
**Fig. 1b:** AWCD of the Top Ten substrates Br10 (▲) and for the rhizospheric soil treatments (b), c (□), Rr1 (■), R10 (▲) and Rr10 (▲).



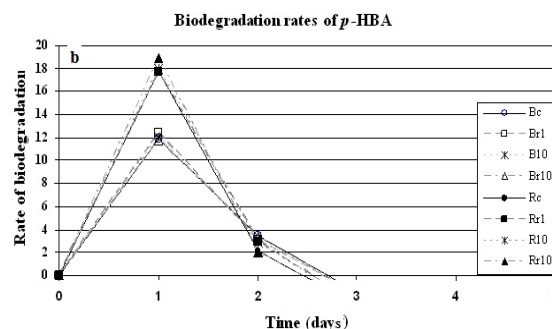
**Fig. 2a:** The utilization of *p*-HBA as a sole source of carbon for the communities extracted from the bulk soil treatments (a), the control c (□), Br1 (■), B10 (▲) and Br10 (▲).



**Fig. 2b:** The utilization of *p*-HBA as a sole source of carbon for the communities extracted from the rhizospheric soil treatments (b), c (□), Rr1 (■), R10 (▲) and Rr10 (▲).



**Fig. 3a:** Kinetics of mineralization of <sup>14</sup>C ring-labelled *p*-HBA from the bulk and rhizosphere soil treatments.



**Fig. 3b:** Mineralization rates of <sup>14</sup>C ring-labelled *p*-HBA from the bulk and rhizosphere soil treatments.

***P*-hydroxybenzoic Acid Mineralization Kinetics:** The Mineralization kinetics of *p*-hydroxybenzoic acid was determined by measuring <sup>14</sup>CO<sub>2</sub> trapped in NaOH after 1, 2, 3, 4, 6 and 11 days of incubation. The obtained results indicated that each of the cumulative percentage of mineralization and the mineralization rates of treated rhizospheric soil were higher than those of treated bulk soil. These percentages were 37.53, 37.36, 37.86 and 39.7 for Rc, Rr1, R10 and Rr10 (treated rhizospheric soil) respectively and 25.7, 26.69, 26.14 and 25.49 for Bc, Br1, B10 and Br10 (treated bulk soil) respectively, of the initial added radioactivity after one day of incubation (Fig. 3a). Also the data presented in Figure 3b showed that the mineralization rates of treated rhizospheric soil ranged from 17.68 to 18.85 while those of treated bulk soil ranged from 11.74 to 12.34. After 11 days of incubation, the cumulative percentage of mineralization of treated rhizospheric soil varied from 56.27 to 60.35 and 51.38 to 53.01 for treated bulk soil, of the initial added radioactivity. The maximum cumulative percentage of mineralization was observed for the two soils with the repeated application of 1mM *p*-HBA (r1 treatment), with a high probability (0.0088) for this treatment when compared with the control of the rhizospheric soil. While the cumulative percentages of mineralization were not significantly different for all *p*-HBA treatments in bulk soil, they were significantly different in rhizospheric soil with a probability of 0.0291. The obtained results revealed that the differences between the parameters of *p*-HBA mineralization kinetics of treated rhizospheric soil and those of treated bulk soil were attributed to the rhizospheric effects more than the repeated application of *p*-HBA.

**Discussion:** Induction, selection and stimulation of phenolic acid utilizing bacteria within the bulk and rhizosphere soil have been observed when soils are enriched with individual phenolic acids at concentrations  $\geq 0.25 \mu\text{mol g}^{-1} \text{soil}^{[2,20]}$ . However, since

**Table 1:** Effect of *p*-hydroxybenzoic acid on total and *p*-hydroxybenzoic acid utilized bacterial counts (CFU g<sup>-1</sup> dry soil).

Treatments of <i>p</i> -HBA	Total bacterial counts		<i>p</i> -hydroxybenzoic acid utilized bacteria	
	Bulk soil	Rhizosphere soil	Bulk soil	Rhizosphere soil
c	9.7x10 <sup>6</sup> (6.4x10 <sup>5</sup> )	1.03x10 <sup>8</sup> (1.4x10 <sup>8</sup> )	<10 <sup>2</sup> (BD)	2.6x10 <sup>3</sup> (1.3x10 <sup>3</sup> )
r1	3.4x10 <sup>6</sup> (2.9x10 <sup>5</sup> )	1.5x10 <sup>7</sup> (2.7x10 <sup>7</sup> )	<10 <sup>2</sup> (BD)	1.9x10 <sup>4</sup> (4.9x10 <sup>3</sup> )
10	5.7x10 <sup>5</sup> (4.9x10 <sup>4</sup> )	9.3x10 <sup>6</sup> (1.6x10 <sup>6</sup> )	≥10 (BD)	1.4x10 <sup>4</sup> (1.9x10 <sup>3</sup> )
r10	9.3x10 <sup>4</sup> (1.3x10 <sup>4</sup> )	2.3x10 <sup>5</sup> (5.7x10 <sup>5</sup> )	<10 <sup>2</sup> (BD)	1.03x10 <sup>4</sup> (5.2x10 <sup>3</sup> )
Probability	<0.0001*	<0.0001*		0.0039*

Data inside parentheses indicate standard error (SE).

BD: Below detection limit.

\* indicates a significant difference at the 5% level (*P*<0.05).

c = No addition of *p*-hydroxybenzoic acid, control.

r1 = 1mMolar in soil solution repeated every six weeks for 4 times.

10 = 10 mMolar in soil solution applied one time at the first of experiment.

r10 = 10 mMolar in soil solution repeated every six weeks for 4 times.

**Table 2:** Level of significance for total and *p*-hydroxybenzoic acid utilized bacterial counts between *p*-hydroxybenzoic acid treatments in bulk and rhizosphere soils (values of probability (*P*) from the PLSD Fisher test).

Treatments of <i>p</i> -HBA	c and r1	c and 10	c and r10	r1 and 10	r1 and r10	10 and r10
<b>Bulk soil:</b>						
Total bacterial counts	<0.0001*	<0.0001*	<0.0001*	0.0004*	0.0002*	0.3685
<i>p</i> -HBA utilized Bacterial counts	-	-	-	-	-	-
<b>Rhizospheric soil:</b>						
Total bacterial counts	<0.0001*	<0.0001*	<0.0001*	0.2060	0.1871	0.9487
<i>p</i> -HBA utilized Bacterial counts	0.0026*	0.0194*	0.6902	0.2065	0.0015*	0.0104*

\* indicates a significant difference at the 5% level (*P*<0.05).

**Table 3:** Maximum AWCD and probability values between bulk and rhizospheric soil communities as affected by *p*-HBA treatments (5% significance level).

<i>p</i> -HBA treatments	Maximum AWCD (Bulk soil)	Maximum AWCD (Rhizospheric soil)	Probability
c	0.747(0.031)	0.804(0.204)	0.6543
r1	0.674(0.015)	0.652(0.033)	0.8598
10	0.661(0.018)	0.560(0.052)	0.4351
r10	0.498(0.031)	0.461(0.123)	0.7711
Probability	0.0007*	0.3099	

Data inside parentheses indicate standard error (SE).

\* indicates a significant difference at the 5% level (*P*<0.05).

**Table 4:** Biodiversity Shannon indexes of the bulk and rhizospheric soil communities as affected by *p*-HBA treatments.

<i>p</i> -HBA treatments	Shannon index (Bulk soil)	Shannon index* (Rhizospheric soil)	Probability
c	2.081 (0.027)	1.958 (0.118)	0.143
r1	2.140 (0.041)	1.832 (0.058)	0.0014*
10	2.087 (0.033)	1.776 (0.051)	0.0013*
r10	2.072 (0.007)	1.808 (0.043)	0.0044*
Probability	0.4255	0.3787	

Data inside parentheses indicate standard error (SE).

\* indicates a significant difference at the 5% level (*P*<0.05).

filed soils frequently contain individual phenolic acids at concentrations well below  $0.1 \mu\text{mol g}^{-1}$  soil<sup>[3,4,14,26,27]</sup>, the actual importance of such induction and/or selection under field conditions remains uncertain. The research presented here attempts to determine to which extent the co-enrichment plant rhizosphere and *p*-HBA may influence the bacterial communities and enhance the degradation of this compound.

In non-treated soils, bacterial numbers were in average of  $9.7 \times 10^6 \text{ g}^{-1}$  dry bulk soil while they were  $1.03 \times 10^9 \text{ g}^{-1}$  dry rhizospheric soil. In opposition with the weak microbial activity in bulk soil, the rhizosphere is the place of an intense microbial life<sup>[10]</sup>. Counts the micro-organisms present in 1 g of rhizospheric soil (R) and in 1 g of the same bulk soil (S), may always give a ratio of R/S higher than 1. This ratio expresses the rhizosphere effect, and it varies on average from 2 to  $20^{[11]}$  and it may reached to  $47.5^{[16]}$ . Our results demonstrate that the rhizosphere effect was more obvious for non-treated soils than the treated ones since it was a little more than 100 for non-treated soils and decreased in treated ones to give a range from 16 to 44. This may be due to a toxicity effect of the *p*-hydroxybenzoic acid on bacterial communities and this means that some bacteria from the soil microflora are not tolerant to this compound. This effect was more obvious on rhizospheric than bulk soil communities. While *p*-hydroxybenzoic acid utilized bacteria were countable in all treatments of rhizospheric soil, they were not detected in bulk soil. Our results were partially in agreement with which was observed by Blum and Shafer<sup>[2]</sup>, Shafer and Blum<sup>[20]</sup>, since they found that the addition of such compound stimulated the numbers of bulk and rhizosphere soil bacteria. The detection of the *p*-hydroxybenzoic acid utilized bacteria in the non-treated rhizospheric soils indicated that these bacteria were adapted to the rhizosphere of soybean and a slight enrichment, in the number of these bacteria, might be occurred through the repeated culture of soybean. Mohamed<sup>[16]</sup>, found that, after the repeating culture of soybean, the populations of the 4<sup>th</sup> rhizosphere were more capable of utilizing phenolic acids as a sole source of carbon, especially coumaric and *p*-hydroxybenzoic acid as compared with those of the 1<sup>st</sup> rhizosphere. Adding the *p*-hydroxybenzoic acid to the rhizospheric soils caused a significant increase in *p*-hydroxybenzoic acid utilized bacterial numbers suggesting that enrichment in the number of this group of bacteria has occurred. The repeated addition of *p*-hydroxybenzoic acid at concentration of 10 mMolar in soil solution for 4 times (Rr10) caused a decrease in the numbers of these bacteria allowing us to conclude that the addition of such compound to a threshold concentration may enrich the soils with these bacteria. From a practical point of view, increasing the number

of *p*-hydroxybenzoic acid utilized bacteria can reduce or eliminate the observed phytotoxicity of such compound<sup>[25]</sup>. More importantly, Blum *et al.*<sup>[3]</sup>, noted that there was an inverse relationship between phytotoxicity and rhizosphere bacterial populations that could utilize phenolic acids as a sole source of carbon when cucumber seedlings were grown in soil treated with phenolic acids.

We used ten substrates to study the Biolog profiles of bulk and rhizospheric soils communities since they were described by Campbell *et al.*<sup>[5]</sup> as the Top Ten substrates which discriminate the soil communities. The profiles of the four communities either for bulk or rhizospheric soil confirmed the results obtained for bacterial enumeration since the maximum AWCD decreased for the two soils as the *p*-HBA dose increased. This may be due to the effect of *p*-HBA doses on bacterial populations since the bacterial number decreased by a little more than two log for the two soils in the r10 treatment as compared by the control treatment.

Biodiversity estimated by the Shannon index was similar for all communities of *p*-HBA treatments either for bulk or rhizospheric soil. In the contrary, the biodiversity indexes were significantly more in bulk soil treatments when compared with those of rhizospheric soil except for the control treatment. However, the rate of *p*-HBA utilization as a sole source of carbon for each treatment was less in bulk than in rhizosphere soil. This may be explained by the fact that, the less diversity observed after the application of *p*-HBA in rhizospheric soil were more effective in the utilization of this compound.

Among all communities, the highest utilization rate of *p*-HBA was observed for communities extracted from soils treated with 1mM *p*-HBA in soil solution (r1). This was more obvious in rhizospheric communities. These results were in agreement with the enumeration data of *p*-HBA utilized bacteria since the number of these bacteria was also the highest for this treatment among all rhizospheric communities. This may encourage us to think that the repeated application of a week concentration such 1mM *p*-HBA in soil solution may lead to enrich the rhizosphere with this group of bacteria.

The Mineralization kinetics of *p*-hydroxybenzoic acid clearly demonstrates that the cumulative percentages of mineralization and the mineralization rates of treated rhizospheric soils were higher than those of treated bulk soils. This could be explained by the presence of more than 3 log of *p*-hydroxybenzoic acid utilized bacteria in rhizospheric soils, even in non treated rhizospheric soil, while these bacteria were below detected level in all bulk soil treatments. These differences between the two soils may be due to the

rhizospheric enrichment effects more than the different application treatments of *p*-HBA since no significant differences were observed for all *p*-HBA treatments in bulk soil. In addition, the maximum cumulative percentage of mineralization was significantly higher for the repeated application of 1mM *p*-HBA (r1 treatment) when compared with control in the rhizospheric soil.

In conclusion, there was a high coherence between results from the enumeration of *p*-HBA utilized bacteria and the utilization of this compound as a sole source of carbon and its mineralization kinetics. Such coherence allows us to conclude that, in our experimental conditions, the repeated application of *p*-HBA in a week concentration (i.e. 1mM in soil solution) to the soybean rhizosphere can enrich the rhizospheric soil with the *p*-HBA utilized bacteria.

#### ACKNOWLEDGEMENTS

The authors wish to thank G. Catroux for valuable discussions on rhizospheric enrichment strategies and his helpful aid for the work scheme and methodology. The authors also gratefully acknowledge the technical assistance of C. Catroux for the radioactivity measurements.

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