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Fluorescent pseudomonads occuring in *Macrotermes subhyalinus* mound structures decrease Cd toxicity and improve its accumulation in sorghum plants

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Abstract

Cd-tolerant bacterial strains of fluorescent pseudomonads, mostly belonging to *Pseudomonas monteillii*, were isolated from termite mound soil (*Macrotermes subhyalinus*, a litter-forager and fungus-growing termite), in a Sudanese shrubby savanna, Burkina Faso. Such large mounds appeared as sites of great bacterial diversity and could be considered as hot spots of metal-tolerant fluorescent pseudomonads. Microbial isolates were inoculated to Sorghum plants (*S. bicolor*) in glasshouse experiments with soil amended with $CdCl_2$ (560 mg Cd kg^{-1} soil). Microbial functional diversity was assessed at the end of the experiment by measurement of *in situ* patterns of catabolic potentials. All the bacteria isolates significantly improved the shoot and total biomass of sorghum plants compared to the control. Results concerning root biomass were not significant with some strains. Arbuscular mycorrhiza (AM) was greatly reduced by $CdCl_2$ amendment, and fluorescent pseudomonad inoculation significantly increased AM colonisation in the contaminated soil. The bacterial inoculation significantly improved Cd uptake by sorghum plants. Measurement of catabolic potentials on 16 substrates showed that the microbial communities were different according to the soil amendment. Soils samples inoculated with pseudomonad strains presented a higher use of ketoglutaric and hydroxybutiric acids, as opposed to fumaric acid in soil samples not inoculated. It is suggested that fluorescent pseudomonads could act indirectly in such metabolic processes by involving a lower rate of degradation of citric acid, in line with the effect of small organic acid on phytoextraction of heavy metals from soil. This is a first contribution to bioremediation of metal-contaminated sites with soil-to-plant transfer, using termite built structures. Further data are required on the efficiency of the bacterial strains isolated and on the processes involved.

Keywords: Heavy metals; Termite; Phyremediation; Fluorescent pseudomonads; Arbuscular mycorrhizal fungi

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1. Introduction

Agricultural practices such as agrochemical usage, long-term application of urban sewage sludge and human industrial activities cause accumulation of metals or metalloids (i.e. arsenic, cadmium, copper, zinc, nickel, mercury). They generally affect aquatic and terrestrial ecosystems and induce potential health risks due to soil-to-plant transfer of metals (Kabata-Pendias, 1992; Khan et al., 2000). Cadmium is one important element for food-chain contamination and is an extremely significant pollutant (Pinto et al., 2004).

Various physico-chemical and biological remediation technologies have been developed during the last decades (Mulligan et al., 1999, 2001a; Wu et al., 2006). In many cases, these processes of heavy metals remediation are usually expensive and labour extensive (Khan, 2005). Hence, alternative and cheaper technologies have to be developed to recover the degraded land, particularly in developing countries. One of these innovative technologies includes plants to clean up metal-contaminated sites by extracting metals from the soil and concentrating them in the aboveground biomass (Salt et al., 1995; Brooks, 1998). This technology emerged as an ecological-friendly alternative to conventional methods (Arduini et al., 2006). However, metal-accumulating plants have to be cultivated for a relatively long time with a number of growth cycles to remove metal-enriched biomass (Felix, 1997; Wenzel et al., 1999). The interactions of heavy metals with microorganisms have therefore been of increasing interest because of their potential to directly remove directly heavy metals from polluted soils and to improve the transfer of metals to aboveground biomass of higher plants (Guo et al., 1996; Burd et al., 2000). The metals lead to the establishment of tolerant microbial populations usually belonging to *Bacillus*, *Arthrobacter* and *Corynebacterium* genera of gram-positive bacteria and *Pseudomonas*, *Alcaligenes*, *Ralstonia* and *Burkholderia* genera of gram-negatives (Kozdrój and van Elsas, 2001; Ellis et al., 2003). It has also been assessed that arbuscular mycorrhizal fungi (AMF) frequently reduced plant uptake and/or decreased the phytotoxic effects of soil heavy metals (Hildebrandt et al., 1999; Chen et al., 2003a) even if they could enhance uptake of toxic metals in other situations (Weissenhorn et al., 1995).

Innovative techniques based on microbial inoculants need to screen, identify and select bacterial and/or fungal isolates for their potential application in bioremediation of contaminated sites. Large quantities of viable microbial inocula have to be produced after that, which requires an appropriate technical knowledge. In addition, the survival of inoculated bacteria was usually not sustained in subsequent crops and it implied repeated inoculant application. The high cost and technical requirements of this process could limit its use especially in developing countries.

It has recently been demonstrated that termite mound materials, used as natural microbial inoculants, promoted plant growth and increased nutrient supplies from organic and inorganic soil amendments through their effects on soil microorganisms (Duponnois et al., 2005). Among different mound materials tested, fluorescent pseudomonads have only been detected in the termite mounds of *Macrotermes subhyalinus* (a litter-forager termite) (Duponnois et al., 2005, 2006). Although numerous studies have reported that fluorescent pseudomonads could bend heavy metals efficiently (Minz et al., 1996; Khan, 2005; Piotrowska-Seget et al., 2005), the potentialities of these bacterial species inhabiting the termite mounds of *M. subhyalinus* are unknown.

The objectives of this study were to determine (i) the proportion of cadmium-tolerant bacteria among the total fluorescent pseudomonad population in the termite mound and (ii) the potentialities of some fluorescent pseumonads isolates to increase Cd uptake by plants and reduce its toxicity in sorghum plants.

2. Materials and methods

2.1. Screening for Cd-tolerant fluorescent pseudomonads

Soil samples from five termite mounds were collected in a Sudanese shrubby savanna in Burkina Faso near the village of Yaktenga (50 km north of Ouagadougou). They were crushed and passed through a 2 mm-sieve before use. The chemical and microbiological analyses have been reported in a previous study (Duponnois et al., 2005). 10 g fw per sample were placed in Erlenmeyer flasks containing 100 ml of 0.1 M magnesium sulfate ($MgSO_4$, $7H_2O$) and shaken at 180 rpm for 30 min. Serial 10-fold dilutions of homogenized suspensions were then plated onto King's B medium in order to detect and count fluorescent pseudomonads under UV light (King et al., 1954). This medium was amended with 2, 4 and 6 mM Cd added as $CdCl_2$. The plates were incubated at 28 °C for 48 h prior to counting. The isolates of fluorescent pseudomonads were randomly selected (10 bacterial strains called KRM1 to KRM10) from the King's B plates amended with 6 mM Cd, purified, subcultured on the same nutrient agar medium as before and cryopreserved at −80 °C in glycerol 60%-TSB (Tryptic Soy Broth, 3 g L^{-1}. Difco laboratories) culture [1/1, volume in volume (v/v)].

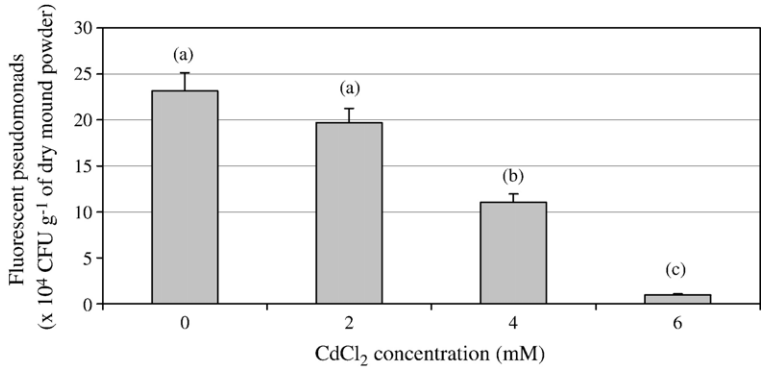

Fig. 1. Number of Cd tolerant fluorescent pseudomonads on King's B agar medium contaminated with increasing $CdCl_2$ amendments. Error bars represent standard errors. Data of columns indexed by the same letter are not significantly different according to the Newman–Keuls test ($p<0.005$).

2.2. Bacterial inoculum

The phylogenetic analysis of fluorescent pseudomonads showed that most of them belonged to the *Pseudomonas monteilii* species (Duponnois et al., 2006). The bacterial isolates were grown in 0.3% (m/v) TSB liquid medium for 3 days at 28 °C on a rotary shaker. Then, they were centrifuged (2400 *g*, 20 min) and suspended in 0.1 M magnesium sulfate. The final concentration of the bacterial suspension was about 10^8 CFU mL^{-1} after enumeration on a plate count agar medium (King's B medium). This suspension was used as inoculum.

2.3. Glasshouse experiment

Seeds of sorghum (*Sorghum bicolor* L.) were surface-sterilized with 1% NaOCl for 15 min and rinsed with demineralised water. They were pre-germinated for 2 days in Petri dishes on humid filter paper at 25 °C in the dark. The germinating seeds were used when rootlets were 1–2 cm long.

The soil used was collected from a non-cultivated area near Ouagadougou (Burkina Faso). Its chemical and physical characteristics were as follows: pH (H_2O) 5.6; clay, 4.6%; fine silt, 0.0%; coarse silt, 0.8%; fine sand, 25.5%; coarse sand, 69.1%; total carbon, 0.204%; total nitrogen, 0.04%; soluble phosphorus 0.043 mg kg^{-1}; total phosphorus, 0.116 mg kg^{-1}. One part of the soil was autoclaved (140 °C, 40 min) and the other was not. Cd was added as chloride. The soil was treated with a single dose of 560 mg Cd kg^{-1} soil. The salt ($CdCl_2$) was dissolved in 1 L distilled water and mixed thoroughly with the soil (both autoclaved and non-autoclaved) according to Kozdrój (1995).

Sorghum plants were individually grown in 0.1 L pots filled with the autoclaved or non-autoclaved soil and amended with Cd. Immediately after planting, the young seedlings were inoculated with 5 ml bacterial suspension. Control treatments without bacteria received 5 ml of 0.1 M magnesium sulfate. In order to determine the Cd toxicity and its effect on sorghum growth, a control treatment was prepared using both disinfected and non-disinfected soils but without $CdCl_2$ amendment and without bacterial inoculation.

1 month after microbial inoculations, the plants were harvested and their root systems gently washed under running tap water. The oven dry weight (1 week at 65 °C) of the shoot was measured. In order to quantify the colonization of arbuscular mycorrhizal fungi, the roots were cleared and stained according to the method of

Table 1
Effect of $CdCl_2$ amendment (560 mg Cd kg^{-1} soil) on plant growth after 1-month culture in a disinfected and non-disinfected soil

Treatments	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)	Total biomass (mg dry weight)
Disinfected soil			
$-CdCl_2$	208.3 (6.31)[(1)]b[(2)]	191.4 (15.5)b	399.7 (19.1)b
$+CdCl_2$	24.9 (1.68)a	34.1 (3.81)a	58.9 (4.21)a
Non-disinfected soil			
$-CdCl_2$	200.6 (12.94)b	97.9 (10.58)b	298.5 (20.14)b
$+CdCl_2$	24.8 (2.24)a	9.9 (0.83)a	34.6 (2.76)a

[(1)] Standard error of the mean. [(2)] Data in the same column and for each factor followed by the same letter are not significantly different according to the Newman–Keuls test ($p<0.05$).

Table 2
Effect of bacterial inoculation and soil disinfection on plant growth after 1-month culture in a soil amended with $CdCl_2$ (0.5 mM kg^{-1} of soil)

Factor [A]	Shoot biomass (mg dry weight)	Root biomass (mg dry weight)	Total biomass (mg dry weight)
Soil disinfection			
Des.	33.2 (1.21)[1]a[2]	42.9 (1.79)b	76.1 (2.53)a
Non-des.	43.4 (2.09)b	28.4 (1.45)a	71.8 (2.99)a
Microbial inoculation			
Control	24.8 (1.32)a	23.3 (3.58)a	48.1 (3.89)a
KRM1	31.7 (1.92)b	29.3 (3.11)a	60.9 (4.22)b
KRM2	29.9 (2.03)b	29.4 (2.49)a	59.4 (3.18)b
KRM3	34.7 (3.70)b	31.2 (3.78)ab	65.8 (6.28)b
KRM4	47.6 (3.59)c	40.6 (3.99)bc	88.2 (6.16)cd
KRM5	38.3 (6.72)b	33.5 (4.12)a	71.8 (7.51)bc
KRM6	45.1 (3.97)c	41.6 (3.91)bc	86.7 (6.49)cd
KRM7	37.1 (3.09)b	47.3 (4.95)c	84.3 (6.41)cd
KRM8	43.2 (3.36)bc	36.3 (2.73)bc	79.5 (4.48)c
KRM9	43.5 (3.67)bc	48.3 (5.49)c	91.8 (6.05)d
KRM10	40.9 (5.42)b	39.9 (4.49)bc	80.7 (6.47)cd
Soil disinfection (SD)	S	S	NS
Microbial inoculation (MI)	S	S	S
Interaction (SD × MI)	S	NS	NS

S: significant ($p<0.05$), NS: not significant ($p<0.05$). [2] Standard error of the mean. [3] Data in the same column and for each factor followed by the same letter are not significantly different according to the Newman–Keuls test ($p<0.05$).
[A] Values are means of 18 replicates for microbial inoculation and 90 replicates for disinfection effect. Bacterial isolate factor is for all physical soil treatment combined; the disinfection factor is for all bacterial isolates combined.

Phillips and Hayman (1970). They were cut into 0.5 cm short pieces and placed on a slide for microscopic observations at 250× magnification (Brundrett et al., 1985). The entire root system was observed for each plant. The extent of mycorrhizal colonization was expressed as (the number of mycorrhizal root pieces)/(number of non-mycorrhizal root pieces) × 100. Then, for each plant, root pieces were put together and their dry weight was measured (1 week at 65 °C).

The soil from each pot was carefully mixed and kept at 4 °C for further analysis. Microbial functional diversity in soil treatments with non-autoclaved soil was assessed by measuring the patterns of *in situ* catabolic potential (ISCP) of microbial communities (Degens and Harris, 1997). These measurements were made from 3 soil samples randomly chosen in each microbial treatment. Fifteen carboxylic acids (glutamic acid; tartaric acid; gluconic acid; citric acid; ketobutyric acid, uric acid; oxalic acid;

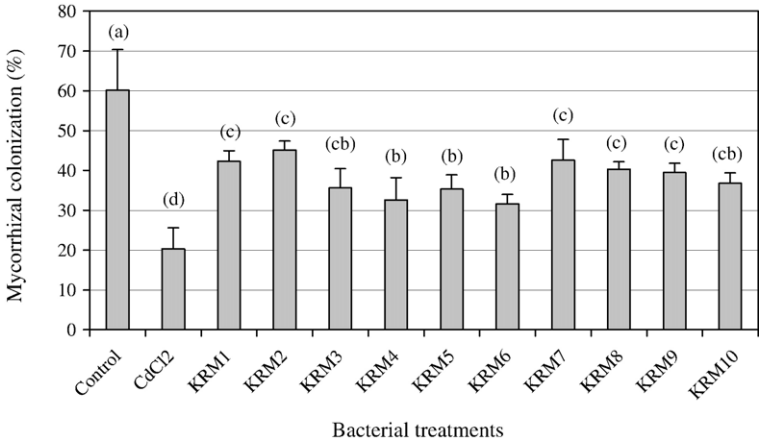

Fig. 2. Effect of bacterial isolate inoculation on Cd shoot content per plant after 1-month culture in non-disinfected soil amended with $CdCl_2$ (560 mg Cd kg^{-1} soil). Error bars represent standard errors. Data of columns indexed by the same letter are not significantly different according to the Newman–Keuls test ($p<0.005$).

succinic acid; gallic acid; mannose; ascorbic acid; quinic acid; ketoglutaric acid; hydroxybutyric acid; fumaric acid) and one carbohydrate (D-glucose) were screened for differences in SIR responsiveness between soil treatments. Their concentrations providing optimum SIR responses were 100 mM for carboxylic acids and 75 mM for glucose (Degens and Harris, 1997). 1 g equivalent dry weight soil was mixed with each substrate suspended in 2 ml sterile distilled water (West and Sparling, 1986) in 10 ml bottles. CO_2 production from basal respiratory activity in the soil samples was also determined by adding 2 ml sterile distilled water to 1 g equivalent dry weight of soil. Bottles were closed immediately after the addition of the substrate solutions to soil samples and kept at 28 °C for 4 h. CO_2 fluxes from the soils were measured using an infrared gas analyzer (IRGA) (Polytron IR CO_2, Dräger™) in combination with a thermal flow meter (Heinemeyer et al., 1989). Results were expressed as μg CO_2 g^{-1} soil h^{-1}.

For the analysis of leaf Cd contents, 50 mg of dried leaves (1 week at 60 °C) were ground in a porcelain capsule, ashed (2 h at 450 °C) in a muffle furnace and cooled down. At ambient temperature, the ash was suspended in 2 ml HCl 6 N and then in 5 ml distilled water. The suspension subsequently filtered through a quantitative filter paper. Then they were analysed by atomic absorption spectrophotometer flame using a Varian Spectra AA200FS analytical system.

2.4. Statistical analysis

The data were treated with one- and two-way analysis of variance. Means were compared using the Newman and Keuls test ($p<0.05$). The percentages of mycorrhizal colonization were transformed by arcsin(sqrt) before statistical analysis.

Between-Group Analysis (BGA, Dolédec and Chessel, 1987, 1989; Culhane et al., 2003) was used to analyse the SIR responses in soil samples amended with cadmium and innoculated with various pseudomonads strains. Three groups of samples were considered: control (Cd−), Cd amendment (Cd+), and Cd amendment with fluorescent pseudomonad inoculation (Bact). BGA is a multivariate analysis technique derived from principal components analysis (PCA). The aim of PCA is to summarize a data table by searching orthogonal axes on which the projection of the sampling points (rows of the table) have the highest possible variance.

From a theoretical point of view, BGA is the particular case of PCA with respect to instrumental variables (PCAIV, Rao, 1964; Lebreton et al., 1991) where the instrumental variables table is reduced to just one qualitative variable. This variable defines groups of rows in the data table, and BGA consists of the PCA of the table of the means by groups. This table has a number of rows equal to the number of groups, and the same number of columns as the original table. The aim of this analysis is to separate the groups. This is also the aim of discriminant analysis (also called canonical variates analysis), but while discriminant analysis is limited to tables that have a high number of samples compared to the number of variables, BGA can be used even when the number of rows is less than the number of variables. BGA can thus be considered as a robust alternative to discriminant analysis when the number of samples is low.

A Monte Carlo test (permutation test) can be used to check the significance of the differences between groups. This method consists in performing many times a random

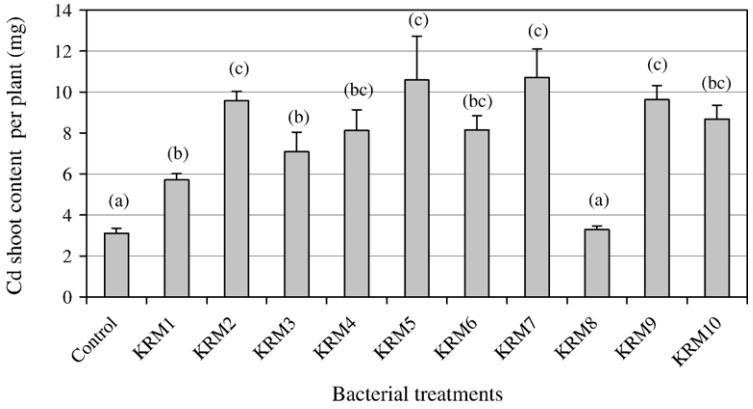

Fig. 3. Effect of bacterial isolate inoculation on root mycorrhizal colonization after 1-month culture in non-disinfected soil amended with $CdCl_2$ (560 mg Cd kg^{-1} soil). Error bars represent standard errors. Data of columns indexed by the same letter are not significantly different according to the Newman–Keuls test ($p<0.005$). (Control: soil without $CdCl_2$ amendment).

permutation of the rows of the table (but not of the qualitative variable defining the groups), followed by the re-computation of the between-class inertia. By comparing the between-class inertia obtained in the normal analysis with the between-class inertia obtained after randomisation, we get an estimation of the probability of meeting a situation similar to the observed situation, without differences between groups (i.e., a significance test of the differences between groups).

Computations and graphical displays were made with the free ADE-4 software (Thioulouse et al., 1997), available on the Internet at http://pbil.univ-lyon1.fr/ADE-4/.

3. Results

In the range from 2 mM to 6 mM $CdCl_2$, the percentages of Cd-tolerant fluorescent pseudomonads varied from 85.1% to 4.2% (Fig. 1). After 1-month culture, the addition of $CdCl_2$ has significantly decreased the shoot and root growth of sorghum plants in both the disinfected and in the non-disinfected soil (Table 1).

For all microbial inoculations combined, there were no significant differences between the total biomass of sorghum plants in both the disinfected and in the non-disinfected soil (Table 2). However, root biomass was significantly higher in the disinfected soil whereas shoot biomass was higher in the non-disinfected soil (Table 2).

For both disinfected and non-disinfected soil treatments combined, all the bacterial isolates have significantly improved the shoot and total biomass of sorghum plants compared to the control (Table 2). However, with four of them (KRM1, KRM2, KRM3 and KRM5) no significant effects have been recorded on root biomass (Table 2).

No mycorrhizal structures have been detected on the roots of sorghum plants growing in the disinfected soil. In the non-disinfected soil, mycorrhizal colonization by indigenous AM fungi was significantly reduced by $CdCl_2$ amendment and decreased from 60.2% (non-Cd amended soil) to 20.3% (Cd amended soil without bacterial inoculation) (Fig. 2). Fluorescent pseudomonad inoculation significantly increased AM colonization indexes in the contaminated soil as they ranged from 31.6% (KRM6) to 45.1% (KRM2) (Fig. 2).

In the control treatment, the metal concentration in the aboveground biomass was 3.11 mg per plant (Fig. 3). The bacterial inoculation significantly improved Cd uptake by plants excepted for the KRM8 fluorescent pseudomonad isolate (Fig. 3). Metal contents per plant ranged from 5.7 (KRM1) to 10.7 mg per plant (KRM7) (Fig. 3). In addition, measurements of Cd concentrations in sorghum shoot were significantly higher in the bacterial treatments (from 97 mg kg^{-1} to 189 mg kg^{-1}) than in the control (90 mg kg^{-1}).

BGA of the SIR responses with respect to the three soil types appeared on Fig. 4. The map of soil samples (Fig. 4B) showed that the three types (Cd−, Cd+, Bact) were clearly separated, which showed that the microbial communities were different according to the soil amendment. The Monte Carlo test was significant ($p=0.025$). The map of the 16 substrates (Fig. 4A) showed that, on the first axis, the use of

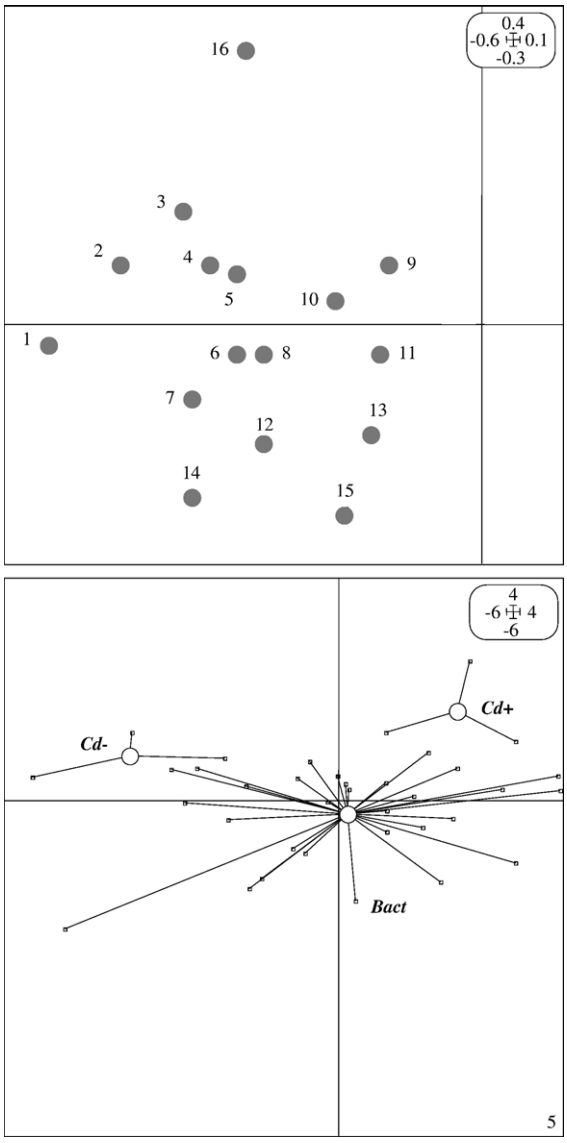

Fig. 4. Between-Group Analysis (BGA) of the Substrate Induced Respiration (SIR) responses with respect to the bacterial treatments (Bact) in a $CdCl_2$ amended soil (Cd+). The control treatment (Cd−) was without $CdCl_2$ amendment. 1: glutamic acid; 2: tartaric acid; 3: gluconic acid; 4: glucose; 5: citric acid; 6: ketobutyric acid, 7: uric acid; 8: oxalic acid; 9: succinic acid; 10: gallic acid; 11: mannose; 12: ascorbic acid; 13: quinic acid; 14: ketoglutaric acid; 15: hydroxybutyric acid; 16: fumaric acid.

glutamic acid was higher in the control samples (left part of the figure). Conversely, CO_2 production was globally lower in soil samples amended with Cd (right part of the figure). The second axis opposed fumaric acid (up) to ketoglutaric and hydroxybutyric acids (down). Soil samples inoculated with pseudomonad strains presented a higher use of these two acids, while fumaric acid use was higher in soil samples not inoculated with pseudomonad strains.

Cd concentrations in the aboveground biomass of sorghum plants were negatively correlated with SIR responses with glutamic acid ($r=0.52$, $p=0.02$), citric acid ($r=0.38$, $p=0.02$), fumaric acid ($r=0.32$, $p=0.05$) and gluconic acid ($r=0.39$, $p=0.02$).

4. Discussion

It has been previously assessed that many soil bacteria were intrinsically resistant to high concentrations of heavy metals (Gadd, 1992; Landmeyer et al., 1993; Angle et al., 1993). For instance, a single 5 mg Cd g^{-1} introduction into the soil reduced the number of heterotrophic bacteria (from 10^7 cfu g^{-1} to 10^6 cfu g^{-1} of dry soil). Enumeration of metal tolerant bacteria from contaminated soils have been realized in numerous studies (Kandeler et al., 2000; Ellis et al., 2003; Dell'Amico et al., 2005). Piotrowska-Seget et al. (2005) have showed that about 50% of the culturable bacteria isolated from sandy-loam soil contaminated with heavy metals were Cd-tolerant. This high percentage should be explained by the impact of long-term metal exposure involving the establishment of tolerant bacterial populations. Moreover, Piotrowska-Seget et al. (2005) have counted the Cd metal tolerant bacteria using a nutrient agar medium amended with 0.5 mM Cd. In the present study, the termite mound structure has never been contaminated with Cd and almost all the *P. monteilii* isolates were tolerant to a higher Cd concentration (2 mM). Hence *M. subhyalinus* mound structure could be considered as a natural hot spot of metal-tolerant bacteria, especially for the fluorescent pseudomonad group. This study confirmed that termite mounds could be sites of great bacterial and fungal diversity (Meiklejohn, 1965; Mohindra and Mukerji, 1982; Holt, 1998; Rouland-Lefevre et al., 2002). Higher microbial diversity recorded in termite structures, was generally attributed to higher levels of organic matter and a better supply of nitrogen (Mohindra and Mukerji, 1982), as well as to higher moisture levels and higher substrate availability (Abbadie and Lepage, 1989).

Fluorescent pseudomonad inoculation significantly increased the total biomass of sorghum plants and it seemed it was very effective in protecting plants from growth inhibition caused by Cd contaminants. Hence, *P. monteilii* isolates could be considered as Plant-Growth Promoting Rhizobacteria (PGPR). PGPR stimulate plant development directly by providing bio-available phosphorus for plant uptake, sequestering trace elements (Iron) for plants by siderophores, producing plant hormones (Glick et al., 1999). It is known that gram-negative copiotrophic organisms as pseudomonads are strongly stimulated by root exudates (Kozdrój and van Elsas, 2000). Pseudomonads were found to be efficient in bioaccumulation of heavy metals in polluted conditions (Hussein et al., 2001, 2005). Such organisms are producing anionic biosurfactants (i.e. rhamnolipids in *P. aeruginosa*) removing metals from contaminated soils (Mulligan et al., 2001b).

It has also been also reported that soil microorganisms could directly contribute to plant establishment by immobilizing heavy metals in rhizosphere and by reducing subsequent plant uptake (Chanmugathas and Bollag, 1987). In general, with bacterial inoculation alterations of metal concentration in plant tissues are not very significant (Wu et al., 2006). In the present study, bacterial inoculation increased the Cd content of the shoots. Although soil Cd content has not been measured, as the loss of Cd through water lixiviation has not been controlled, this result suggested that bacterial inoculants increased the Cd bio-availability.

In addition, these bacterial isolates significantly increased AM formation and acted as Mycorrhiza Helper Bacteria (MHB). It has been previously reported that a MHB, isolate of *P. monteilii* (isolate HR 13), stimulated AM colonization of *Acacia holosericea* seedlings by *Glomus intraradices* (Duponnois and Plenchette, 2003). In that study, it has also been reported that bacterial inoculant alone had no effect on the growth of Australian *Acacia* species. Hence, in the present study, positive bacterial effects on the plant growth could result from two main mechanisms: (i) the higher development of AM fungi enhanced supply of essential nutrients from the soil to the host plant and reduced phytotoxic effects (Hetrick et al., 1994; Hildebrandt et al., 1999; Joner and Leyval, 2001; Chen et al., 2003a) and (ii) bacterial inoculant enhanced Cd bio-availability and plant Cd uptake. These data support previous studies in which the use of indigenous AM fungi and MHB, inoculated together, is recommended in order to restore metal contaminated soils (reviews in Leyval et al., 1997; Khan, 2002, 2004).

The patterns of *in situ* catabolic potential (ISCP) of microbial communities showed that Cd amendment affected their functional abilities as it has been found in numerous studies (Kandeler et al., 2000; Ellis et al., 2003).

The mechanisms controlling the mobility of metals in soil are generally subjected to organic compounds that play

an essential role by chelating metals and, consequently, influence their solubility in the soil horizons (Brynhildsen and Rosswall, 1989; Krishnamurti et al., 1997). Among the organic compounds in soil solution, the low-molecular-weight organic acids belonging to the non-volatile aliphatic category (e.g. citric and oxalic acids) are of special interest because of their ubiquitous occurrence and high metal-complexing capacity (Stevenson, 1967; Wildung et al., 1979). Such organic acids enhance the mobilisation of metals through weathering and chelation (Grayston et al., 1996; Chen et al., 2003b). Furthermore, low molecular weight organic molecules may carry metal ions and increase cell membrane permeability (Pizzeghello et al., 2000; Pinto et al., 2004). Chelating agents, such as EDTA and citric acid have been used as a viable technology for mobilizing lead (Wu et al., 1999) and zinc (Luo et al., 1992). The role of citric acid on the availability, accumulation and detoxification of Cd was studied by Chen et al. (2003b). They concluded that this organic acid converted the cadmium into more easily transported forms and stimulated its transportation from root to shoot. In the present study, the SIR responses with citric acid were negatively correlated with the Cd amounts in the shoots. Hence, microorganisms, able to catabolize this organic acid were less abundant. It suggested that fluorescent pseudomonad inoculants acted indirectly in these biological processes by involving a lower rate of degradation of citric acid, as it has been described by Evangelou et al. (2006) on the effect of small organic acids on phytoextraction of Cu and Pb from soil with tobacco plants.

In conclusion, this study confirmed that microbial communities that inhabit Macrotermitinae-built structures are of great genetic and functional diversity that could be used for different topics such as phytoremediation processes. The processes involve interactions between the rhizosphere microflora and plant roots, a key question that need to be better understood (Kozdrój and van Elsas, 2000). Further use in technologies employing plants to treat metal-contaminated sites requires more information such as its efficiency on other heavy metals.

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References

Abbadie L, Lepage M. The role of subterranean fungus comb chambers (Isoptera, Macrotermitinae) in soil nitrogen cycling in preforest savanna (Côte d'Ivoire). Soil Biol Biochem 1989;8: 1067–71.

Angle JS, Chaney RL, Rhee D. Bacterial resistance to heavy metals related to extractable and total metal concentrations in soil and media. Soil Biol Biochem 1993;25:1465–6.

Arduini I, Ercoli L, Mariotti M, Masoni A. Response of miscanthus to toxic cadmium applications during the period of maxium growth. Environ Exp Bot 2006;55:29–40.

Brooks RR. Geobotany and hyperaccumulators. In: Brook RR, editor. Plants that hyperaccumulate heavy metals. Wallington, UK: CAB International; 1998. p. 55–94.

Brundrett MC, Piche Y, Peterson RL. A developmental study of the early stages in vesicular-arbuscular mycorrhizal formation. Can J Bot 1985;63:184–94.

Brynhildsen L, Rosswall T. Effects of cadmium, copper, magnesium, and zinc on the decomposition of citrate by a *Klebsiella sp*. Appl Environ Microbiol 1989;55:1375–9.

Burd GI, Dixon DG, Glick RR. Plant growth promoting bacteria that decrease heavy metal toxicity in plants. Can J Microbiol 2000;46: 237–45.

Chanmugathas P, Bollag J. Microbial role in immobilization and subsequent mobilization of cadmium in soil suspensions. Soil Sci Soc Am J 1987;51:1184–91.

Chen BD, Tao HQ, Christie P, Wong MH. The role of arbuscular mycorrhiza in zinc uptake by red clover growing in a calcareous soil spiked with various quantities of zinc. Chemistry 2003a;50:839–46.

Chen YX, Lin Q, Luo YM, HE YF, Zhen SJ, Yu YL, et al. The role of citric acid on the phytoremediation of heavy metal contaminated soil. Chemistry 2003b;50:807–11.

Culhane AC, Perriere G, Considine EC, Cotter TG, Higgins DG. Between-group analysis of microarray data. Bioinform 2003;18: 1600–8.

Degens BP, Harris JA. Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biol Biochem 1997;29:1309–20.

Dell'Amico E, Cavalca L, Andreoni V. Analysis of rhizobacterial communities in perennial Graminaceae from polluted water meadow soil, and screening of metal-resistant, potentially plant-growth promoting bacteria. FEMS Microbiol Ecol 2005;52:153–62.

Dolédec S, Chessel D. Rythmes saisonniers et composantes stationnelles en milieu aquatique I-Description d'un plan d'observations complet par projection de variables. Acta Oecol 1987;8:403–26.

Dolédec S, Chessel D. Rythmes saisonniers et composantes stationnelles en milieu aquatique II-Prise en compte et élimination d'effets dans un tableau faunistique. Acta Oecol 1989;10:207–32.

Duponnois R, Plenchette C. A mycorrhiza helper bacterium enhances ectomycorrhizal and endomycorrhizal symbiosis of Australian *Acacia* species. Mycorrhiza 2003;13:85–91.

Duponnois R, Paugy M, Thioulouse J, Masse D, Lepage M. Functional diversity of soil microbial community, rock phosphate dissolution and growth of *Acacia seyal* as influenced by grass-, litter- and soil-feeding termite nest structure amendments. Geoderma 2005;124: 349–61.

Duponnois R, Assigbetse K, Ramanankierana H, Kisa M, Thioulouse J, Lepage M. Litter-forager termite mounds enhance the ectomycorrhizal symbiosis between *Acacia holosericea* A. Cunn. Ex G. Don and *Scleroderma disctyosporum* isolates. FEMS Microbiol Ecol 2006;56:292–303.

Ellis RJ, Morgan P, Weightman AJ, Fry JC. Cultivation-dependent and -independant approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl Environ Microbiol 2003;69: 3223–30.

Evangelou MWH, Ebel M, Schaeffer A. Evaluation of small organic acids on phytoextraction of Cu and Pb from sol with tobacco (*Nicotiana tabacuum*). Chemistry 2006;6:996-1004.

Felix H. Field trials for in situ decontamination of heavy metal polluted soils using crops of metal-accumulation plants. Z Pflanzenernaehr Bodenkd 1997;160:525–9.

Gadd GM. Metals and microorganisms: a problem of definition. FEMS Microbiol Lett 1992;100:197–204.

Glick BR, Patten CL, Holguin G, Penrose DM. Biochemical and genetic mechanisms used by plant growth-promoting bacteria. London, UK: Imperial College Press; 1999.

Grayston SJ, Vaughan D, Jones D. Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. Appl Soil Ecol 1996;5:29–56.

Guo Y, Georges E, Marschner H. Contribution of an arbuscular mycorrhizal fungus to the uptake of cadmium and nickel in bean and maize plants. Plant Soil 1996;184:195–205.

Heinemeyer O, Insam H, Kaiser EA, Walenzik G. Soil microbial biomass and respiration measurements: an automated technique based on infrared gas analysis. Plant Soil 1989;116:77–81.

Hetrick BAD, Wilson GWT, Figge DAH. The influence of mycorrhizal symbiosis and fertilizer amendments on establishment of vegetation in heavy metal mine spoil. Environ Pollut 1994;86:171–9.

Hildebrandt U, Kaldorf M, Bothe H. The zinc violet and its colonization by arbuscular mycorrhizal fungi. J Plant Physiol 1999;154:171–9.

Holt JA. Microbial activity in the mounds of some Australian termites. Appl Soil Ecol 1998;9:183–7.

Hussein H, Krull R, Abou El-Ela SI, Hempel DC. Interaction of the different heavy metal ions with immobilized bacterial culture degrading xenobiotic water compounds. Proceedings of the Second Int Water Association. World Water Conf, Berlin, Germany, 15–19 October; 2001.

Hussein H, Farag S, Kandil K, Moawad H. Tolerance and uptake of heavy metals by *Pseudomonads*. Proc Biochem 2005;40:955–61.

Joner EJ, Leyval C. Time course of heavy metal uptake in maize and clover as affected by root density and different mycorrhizal inoculation regimes. Biol Fertil Soils 2001;33:351–7.

Kabata-Pendias A. Trace metals in soils in Poland — occurrence and behaviour. Soil Sci 1992;140:53–70.

Kandeler E, Tscherko D, Bruce KD, Stemmer M, Hobbs PJ, Bardgett RD, et al. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biol Fertil Soils 2000;32:390–400.

Khan AG. The handing of microbes (Chapter 13). In: Wong MH, Bradshaw AD, editors. The restoration and management of derelict land: modern approaches. Singapore: World Scientific Publishing; 2002. p. 149–60.

Khan AG. Co-inoculum of vesicular-arbuscular mycorrhizal fungi (AMF), mycorrhiza-helper-bacteria (MHB), and plant-growth-promoting rhizobacteria (PGPR) for phytoremediation of heavy metal contaminated soils. Proceeding fifth int conf on environmental geochemistry in the tropics, March 21–26, 2004, Haiko, Hainan, China. Nanjing, PR China: Inst Soil Sc, Chinese Acad Sc; 2004. p. 68.

Khan AG. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. J Trace Elem Med Biol 2005;18:355–64.

Khan AG, Kueck C, Chaudhry TM, Khoo CS, Hayes WJ. Plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. Chemistry 2000;41:197–207.

King EO, Ward MK, Raney DE. Two simple media for the demonstration of pyocyanine and fluorescein. J Lab Clin Med 1954;44:301–7.

Kozdrój J. Microbial responses to single or successive soil contamination with Cd or Cu. Soil Biol Biochem 1995;27:1459–65.

Kozdrój J, van Elsas JD. Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. Soil Biol Biochem 2000;32:1405–17.

Kozdrój J, van Elsas JD. Structural diversity of micro-organisms in chemically perturbed soil assessed by molecular and cytochemical approaches. J Microbiol Methods 2001;43:197–212.

Krishnamurti GSR, Cieslinski G, Huang PM, Van Rees KCJ. Kinetics of cadmium release from soils as influenced by organic acids: implication in cadmium availability. J Environ Qual 1997;26:272–7.

Landmeyer JE, Bradley PM, Chapelle FM. Influence of Pb on microbial activity in Pb contaminated soils. Soil Biol Biochem 1993;25: 1465–6.

Lebreton JD, Sabatier R, Banco G, Bacou AM. Principal component and correspondence analyses with respect to instrumental variables: an overview of their role in studies of structure–activity and species–environment relationships. In: Devillers J, Karcher W, editors. Applied multivariate analysis in SAR and environmental studies. Kluwer Acad Pub; 1991. p. 85-114.

Leyval C, Turnau K, Haselwandter K. Effect of heavy metal pollution on mycorrhizal colonization and function: physiological, ecological and applied aspects. Mycorrhiza 1997;7:139–53.

Luo YM, Christie P, Baker AJM. Metal uptake by Thlaspi caerulescens and metal solubility in a Zn/Cd contaminated soil after addition of EDTA. Proceedings of the Fifth International Conference on the Biogeochemistry of Trace Elements, Vienna, Austria, vol. 2. ; 1992. p. 882–3.

Meiklejohn J. Microbiological studies on large termite mounds. Rhod, Zambia and Malawi. J Agric Res 1965;3:67–79.

Minz D, Rosengerg E, Ron EZ. Cadmium binding by bacteria: screening and characterization of new isolates and mutants. FEMS Microbiol Lett 1996;135:191–4.

Mohindra P, Mukerji KG. Fungal ecology of termite mounds. Rev Ecol Biol Sol 1982;19:351–61.

Mulligan CN, Young RN, Gibbs BF. On the use of biosurfactants for the removal of heavy metals from oil contaminated soil. Environ Prog 1999;18:50–4.

Mulligan CN, Young RN, Gibbs BF. Remediation technologies for metal-contaminated soils and ground-water: an evaluation. Eng Geol 2001a;60:193–207.

Mulligan CN, Yong RN, Gibbs BF. Surfactant-enhanced remediation of contaminated soil: a review. Eng Geol 2001b;60:371–80.

Phillips JM, Hayman DS. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 1970;55:158–61.

Pinto AP, Mota AM, de Varennes A, Pinto FC. Influence of organic matter on the uptake of cadmium, zinc, copper and iron by sorghum plants. Sci Total Environ 2004;326:239–47.

Piotrowska-Seget Z, Cycon M, Kozdroj J. Metal-tolerant bacteria occurring in heavily polluted soil and mine soil. Appl Soil Ecol 2005;28:237–46.

Pizzeghello D, Sessi E, Muscolo A, Albuzio A, Nardi S. High and low apparent molecular size humic substances affecting plant metabolism. 19th International Meeting of the International Humic Substances Society, Toulouse; 2000. p. 919–22.

Rao CR. The use and interpretation of principal component analysis in applied research. Sankhya 1964;26:329–59.

Rouland-Lefevre C, Diouf NM, Brauman A, Neyra M. Phylogenetic relationships in Termitomyces (Family Agaricaceae) based on the nucleotide sequence of ITS: a first approach to elucidate the evolutionary history of the symbiosis between fungus-growing termites and their fungi. Mol Phylogenet Evol 2002;22:423–9.

Salt DE, Blaylock M, Kumar NPDA, Dushenkov V, Ensley BD, Chet I, et al. Phytoremediation : a novel strategy for the removal of toxic

metals from the environment using plants. Bio/Technology 1995;13:468–74.

Stevenson FJ. Organic acids in soil. In: McLaren AD, Peterson GH, editors. Soil biochemistry. New York: Marcel Dekker, Inc.; 1967. p. 119–46.

Thioulouse J, Chessel D, Dolédec S, Olivier J. ADE-4: a multivariate analysis and graphical display software. Stat Compass 1997;7: 75–83.

Weissenhorn I, Leyval C, Belgy G, Berthelin J. Arbuscular mycorrhizal contribution to heavy metal uptake by maize (*Zea mays* L.) in pot culture with contaminated soil. Mycorrhiza 1995;5:245–51.

Wenzel WW, Adriano DC, Salt D, Smith R. Phytoremediation: a plant-microbe-based remediation system. In: Adriano DC, et al, editor. Bioremediation of contaminated soils. Agronomy monographsMadison, WI: ASA, CSSA and SSSA; 1999. p. 457–508.

West AW, Sparling GP. Modifications to the substrate substrate-induced respiration method to permit measurements of microbial biomass in soils of differing water contents. J Microbiol Methods 1986;5:177–89.

Wildung K, Gries EM, Oehr P. Nickel complexes with soil microbial metabolites — mobility and speciation in soils. ACS Symp Ser 1979;93:181–200.

Wu J, Hsu FC, Cunninham SD. Chelate-assisted Pb phytoextraction: Pb availability, uptake and translocation constrains. Environ Sci Technol 1999;33:1898–904.

Wu SC, Cheung KC, Luo YM, Wong MH. Effects of inoculation of plant growth-promoting rhizobacteria on metal uptake by *Brassica juncea*. Environ Pollut 2006;140:124–35.