Optimisation de la phytoextraction : caractérisation et sélection de bactéries PGPB associées à une plante hyperaccumulatrice de Zn et Cd : Arabidopsis halleri

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Phytoextraction optimization: characterization and selection of PGPB bacteria of soil associated to a Zn and Cd hyperaccumulator : *Arabidopsis halleri*

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Résumé

Les phytotechnologies consistent en l'utilisation de plantes qui, par leur association avec la microflore du sol, ont la capacité de dépolluer les sites contaminés (eau, sol, sédiments). Parmi ces technologies, la phytoextraction, basée sur l'utilisation de plantes hyperaccumulatrices telle qu'*Arabidopsis halleri*, semble être une option pour décontaminer les sols pollués en éléments traces métalliques. Une des voies d'amélioration de la phytoextraction d'*A. halleri* serait l'inoculation du système racinaire de la plante par des bactéries capables de stimuler la croissance végétale (PGPB pour Plant Growth Promoting Bacteria). Dans cette étude, nous proposons un protocole d'échantillonnage des différentes fractions de sol autour de la racine : sols global, rhizosphérique et rhizoplan. Les bactéries cultivables, collectées sur un sol industriel contaminé en Zn et Cd (Auby, France), ont été isolées et analysées pour leurs caractéristiques PGPB : activité 1-aminocyclopropane-1-carboxylate (ACC) désaminase, productions de sidérophores et d'acide indole acétique (IAA). Les bactéries qui présentent trois caractères PGPB ont été préférentiellement sélectionnées pour être inoculées au niveau du système racinaire d'*Arabidopsis halleri*. Les effets de ces bactéries sur les paramètres de croissance de la plante et sa biomasse ou sur le rendement d'accumulation des éléments traces métalliques seront présentés.

Abstract

Phytotechnologies are microbial-assisted techniques that use living plants for the treatment of contaminated sites. Among these, phytoextraction based on hyperaccumulator plants such as *Arabidopsis halleri*, may be an option to remove trace elements in soil. One method to optimize *Arabidopsis halleri* phytoextraction is the inoculation of plant roots by plant growth promoting bacteria (PGPB). In this study, we proposed a protocol for sampling bacteria in three different soil fractions neared root system of *Arabidopsis halleri*: global, rhizospheric and rhizoplan. The cultivable bacteria were isolated for a Zn and Cd contaminated industrial soil (Auby, France) and were isolated and characterized for their PGPB traits : 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, siderophores and Indol-Acetic-Acid (IAA) productions). Bacteria answering positively to the three PGPB tests have been preferentially selected to be inoculated to *Arabidopsis halleri* root system. Bacteria effects on plant biomass and/or accumulating yield will be presented.

Mots-clés : phytoextraction, bactéries PGPB, *Arabidopsis halleri*, bioaugmentation

Key-words : phytoextraction, PGPB bacteria, *Arabidopsis halleri*, bioaugmentation

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1. Introduction

Phytoextraction, a microbial-assisted plant technology usable for the treatment of contaminated sites, exploits natural physiological traits of certain plants able to accumulate high levels of metals in their aerial tissues. The success of phytoextraction is strongly determined by the amount of plant biomass and the concentration of metals in plant tissues. *Arabidopsis halleri*, a hyperaccumulator plant characterized by its exceptional Zn and Cd concentrations in its aerial parts (*Bert et al., 2000*), has a relatively small biomass which limits until now its use for phytoextraction. However, some soil microorganisms described in literature, such as PGPB, are able to significantly increase plant biomass and/or metal uptake through the secretion of specific compounds such as organic acids, siderophores, phytohormones and enzymatic activities such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase *(Zhuang et al., 2007; Lebeau et al., 2008)*. Therefore, they represent a potential valuable tool for the improvement of *A. halleri* mediated phytoextraction.

The aim of this work is to study the cultivable microbial flora associated to the roots of *Arabidopsis halleri* growing on a Zn and Cd polluted soil and to identify valuable bacterial strains that may potentially be involved in plant biomass production and/or plant Zn and Cd accumulation. The effect of selected PGPB bacteria on the *A. halleri* phytoextraction will be studied in a pot experiment.

2. Materials and methods

2.1 Soils sampling

Three different soil samples were collected during the growing season of *Arabidopsis halleri* plants in an industrial site contaminated with Zn and Cd located in the North part of France, Bois des Asturies (Auby, 59, France). Each sample was chosen to reflect a different degree of soil contamination from A to C (respectively ranging from 2700 mg kg^{-1} to 36 000 mg kg^{-1} Zn of FW polluted soil; Niton analyser). Concurrently, physico-chemical properties of each soil were analysed.

For each sample, three sub-fractions of soil were defined and collected independently relatively to their distance to *A. halleri* roots (bulk : G, rhizospheric : R and rhizoplan : RP, soils respectively).

Plants were shaken handly and carefully to collect the bulk soil (50 g of bulk soil were added in 500 ml of a sterile saline solution 0.9% NaCl). Rhizospheric soil was subsequently collected by hand-shaking the roots again during 10 min in 1000 ml of a sterile saline solution 0.9% NaCl to collect the adhering soil. Roots were finally washed and hand-shaked during 10 min in 1000 ml of a 0.9% NaCl sterile saline solution containing 0.01% (vol/vol) Tween 80. The three sub-fractions (G, R, RP) for the three samples (A, B, C) were stored at 4°C.

2.2 Bacterial densities

The three soil suspensions were homogenized at 25°C on an orbital shaker (300 rpm, 90 min) and centrifuged at 25°C (10 min, 150 g) to eliminate soil particles by sedimentation. In order to evaluate bacterial density of each sample, supernatants were serially diluted in a sterile 0.9% NaCl solution until reaching 10^{-8} and were spread in triplicate on LB-agar-cycloheximide (100 mg l^{-1}) media. The agar plates were incubated at 25°C for 72 h. Bacterial densities were expressed as log CFU g^{-1} of dry weight of soil. The log transformed CFU data were statistically submitted to an analysis of variance (Two-way ANOVA).

Bacteria which presented a different morphological aspect were isolated separately on LB-agar medium.

2.3 Characterization of PGPB features of bacteria

Bacteria were grown in 5 ml LB medium (30°C, 120 rpm, 15 h). A 200 µl aliquot was taken from each pure culture for evaluation of Indol-Acetic-Acid (IAA) and siderophore productions and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity.

IAA production was determined by the Salkowski assay (*Gordon and Weber, 1951*). All strains were incubated in LB-Tryptophan (500 µg ml^{-1}) medium at 30°C in the dark with a 120 rpm shaking for 5 d. Culture supernatants were recovered after centrifugation at 6,000 *g* for 10 min. One ml of supernatant was mixed with 150 µl of Salkowski's reagent R1 (12 g $FeCl_3$ in 429 ml H_2SO_4 18M). After a 25°C incubation in the dark for 20 min, absorbance at 535 nm was measured.

IAA concentrations were determined using triplicate standard curves for pure IAA (Sigma-Aldrich, St. Louis, MO) prepared in LB medium. Experiments were performed in triplicate. IAA was expressed as a production rate ($\mu g\ ml^{-1}\ h^{-1}$).

ACC deaminase activity was determinated by the modified method of *Glick et al.,* (1995 a*)*. For this, 10 µl of MM9 minimal pure cultures were incubated into 2 ml NFb-NH_4Cl (1 $g\ l^{-1}$) as the positive control or NFb-ACC modified medium by addition of 1-aminocyclopropane-1-carboxylate (5 mM) as the unique nitrogen source. Cultures were incubated at 30°C, 120 rpm for 5 d and were re-inoculated in the same experimental conditions. Newly cultures grown in NFb-ACC were considered positive for ACC deaminase activity. ACC deaminase activities were determined using turbidimetry at $DO_{600\ nm}$. Bacterial growth in ACC media were compared with bacteria growth in NH_4Cl media and were expressed in ratio $DO_{600\ nm}$ (ACC/ NH_4Cl).

Siderophore production was determined by the method of *Schwyn and Neilands* (1987). Briefly, 2 µl pure bacterial cultures grown in LB were spotted on plates containing agar Chrome Azurol S (CAS). Plates were incubated at 30°C during 5 d and were observed for orange color formation around each colony. Experiments were performed in triplicate. A size-ratio (halo/colony) was calculated. Results were expressed in mm $ml^{-1}\ h^{-1}$.

3. Results

3.1 Soil characteristics

Physico-chemical properties of the three soil samples were analysed and are presented in Table 1

Table 1. Physico-chemical parameters of soils sampled in Auby, France

Characteristics	Unit	Soil sampled		
		A	B	C
Texture sand/silt/clay [a]	$g\ kg^{-1}$	483/634/219	165/693/139	389/492/81
Total metal contents				
Zn	$mg\ kg^{-1}$	> 5000	36749	73289
Cd	$mg\ kg^{-1}$	60	273	241
Mobile metal fraction				
Zn	$mg\ kg^{-1}$	36	149	40
Cd	$mg\ kg^{-1}$	0.262	0.812	0.425
pH, H_20		6.09	6.5	7.02
CEC Metson [b]	$cmol+\ kg^{-1}$	48.8	51.3	29.4
Residual humidity [c]	$g\ kg^{-1}$	40	71	26
Organic carbon [d]	$g\ kg^{-1}$	195.6	251.3	136.7
Organic matter [e]	$g\ kg^{-1}$	391.2	502.5	273.4
$CaCO_3$ total [f]	$g\ kg^{-1}$	3	2	37
Nitrogen total Kjeldahl [g]	$g\ kg^{-1}$	9.79	12.42	6.44

[a] Using method is NF X 31-107 modified
[b] Using method is NF X 31-130
[c] Using method is NF ISO 11465
[d] Using method is NF ISO 14235
[e] Calculated (2 x organic carbon)
[f] Using method is NF ISO 10693
[g] Using method is NF ISO 11261 modified

The three soil samples were different in their physico-chemical parameters. The soil texture of A sample was a clay loam, B corresponded to a medium loam and C to a sandy loam. We observed that the difference between A and B soils depends especially on Zn and Cd total and/mobile contents. Contrary to A and B, C soil sample differed largely in its Zn total content, pH, CEC, $CaCO_3$ total.

3.2 Bacterial densities

Bacterial densities were estimated after a 72 h growth at 25°C of 100 µl of diluted bacterial suspension spread on LB-agar medium. Results are expressed in Figure 1 as the CFU log per gram of dry soil. For

each soil sample, a significant rising gradient of bacterial density was observed when the root distance decreased, the bulk soil exhibiting the lowest population. The degree of pollution seemed also to influence significantly ($p<0.05$). The bacteria density of indigenous flora followed a decreasing gradient from A to C (Figure 1).

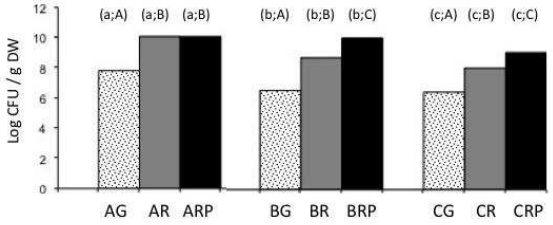

Figure 1. Estimation of bacterial densities for each soil A, B, and C split in bulk (G: white), rhizospheric (R: grey) and rhizoplan (RP: black) soils.

CFU data were submitted to a variance analysis (two-ways ANOVA : pollution and root distance). For each sample, values (pollution factor) with different small letters are significantly different ($p<0.05$). Values (root distance factor) with different capital letters are significantly different ($p<0.05$) (means +/- SD).

3.3 Characterization of PGPB features of bacteria

PGPB tests such as activity ACC deaminase, siderophore and acid indol-acetic-3 productions were performed for 53 strains that presented different morphological aspects in LB-agar medium (Figure 2).

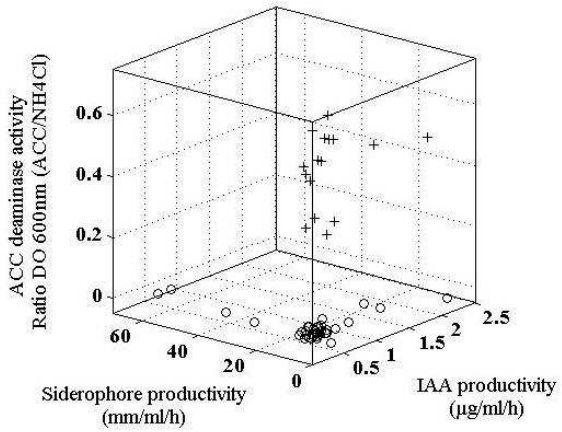

Figure 2. Three-dimensional representation of 53 bacteria strains for their PGPB characteristics : ACC deaminase activity, siderophore and IAA productivities. (+) represents strain able to use ACC as unique nitrogen source, (o) represents strain unable to use ACC

Among them, two strains exceeded 2 µg ml^{-1} h^{-1} for the IAA productivity, three ranged between 1 to 2 µg ml^{-1} h^{-1}, twenty three were comprised between 0.50 to 1 µg ml^{-1} h^{-1} and twenty five were lower than 0.50 µg ml^{-1} h^{-1}.

For the siderophore productivity, two strains exceeded 60 mm ml^{-1} h^{-1}, 2 ranged between 20 to 40 mm ml^{-1} h^{-1}, 16 were comprised between 10 to 20 mm ml^{-1} h^{-1} and 33 were lower than 10 mm ml^{-1} h^{-1}.

For strain ability to use ACC, a $DO_{600\ nm}$ (ACC/ NH_4Cl) ratio equal to 1 indicates an optimal ACC deaminase activity or a 100% capacity in the use of ACC compared to the NH_4Cl optimal nitrogen source. We found five strains with a $DO_{600\ nm}$ ratio above 0.6, therefore believed to be able to use ACC at more 60% of NH_4Cl use. Seven strains ranged between 40 to 60%, four used ACC between 20 to 40 % of NH_4Cl used and thirty seven were unable to use ACC.

In our case, 16 bacteria were able to produce siderophore and IAA, and to use the ACC as the unique nitrogen source in a large range.

Among the 53 bacteria characterized for at least a plant growth promoting trait, eight bacteria were preferentially selected for their marked abilities to produce IAA, siderophore and/or to their ACC deaminase activity (Table 2).

Table 2. Soil bacteria selected on their Plant Growth Promoting characteristics.

Bacteria	PGPB characteristics	IAA productivity	Siderophore productivity	ACC deaminase activity
		$\mu g\ ml^{-1}h^{-1}$	$mm\ ml^{-1}\ h^{-1}$	ratio DO_{600nm} (ACC/NH_4Cl)
IIIG10	IAA +, S +++	0.6	62.9	0
IID11	IAA +, S +, ACC ++	0.4	5.5	0.6
IIC3	IAA +++	2.1	0	0
IE1	IAA +++, S ++, ACC ++	2.3	11.6	0.5
IE4	IAA +, S ++, ACC +	0.8	12.3	0.3
IF10	IAA ++, S +	1.4	8.6	0
IIE8	IAA +, S +, ACC+++,	0.3	7.3	0.7
IA2	IAA +	0.3	0	0

4. Discussion

Our knowledge of plant-microbe-soil interactions is increasing and represents an important environmental interest but it's limited by methods to study bacteria diversity (numerical, taxonomic, and structural) and by the absence of a standard method to sample bulk, rhizospheric and rhizoplan soils. We suggest a protocol that allowed us to obtain after two washes, a root system cleaned without particles of soil although the higher density of *Arabidopsis halleri* root system.

It has been known for a long time that only 1–10% of the bacteria of the soil are found to be cultivable (*Torsvik et al., 1994*). Nevertheless, this method remains highly informative when used to compare different conditions applied to or encountered in the same soil. Consequently, a comparative study between bulk, rhizospheric and rhizoplan cultivable bacteria isolated from a gradually increasing Zn and Cd polluted soil was undertaken. As expected, microbial densities differences were observed for different pollution levels (ranging from $10^7 – 10^6$, $10^{10} – 10^8$, $10^{10} – 10^9$ CFU g^{-1} DW respectively for soil C, B and A). Whatever the level of pollution of the samples, a significant quantitative difference in bacteria populations was found between rhizospheric and bulk soils associated to *A. halleri* ($10^8 – 10^{10}$ CFU g^{-1} DW and $10^6 – 10^8$ CFU g^{-1} DW respectively).

Our results are in good agreement with previous studies showing the positive impact of plant proximity on bacterial population and indicate that the rhizosphere of *A. halleri* sampled in situ enhances the occurrence and the growth of bacteria. As suggested by Aboudrar et *al.*, (2007) this may be explained by an enrichment of carbon concentration generated by plant cell turnover as well as by the large variety of compounds present in the root exudates which may both create a favorable microenvironment for bacterial survival and growth. Interestingly, significant differences were also observed between populations of rhizosphere and rhizoplan affected by a high (soil C) or medium (soil B) level of pollution. The difference was more accentuated when the pollution rate was higher. On another hand, when the pollution level was low (soil A), no significant difference could be observed between rhizosphere and rhizoplan. It has been generally observed that metal pollution influenced bacterial population and community according to its toxicity. Martinez et *al.*, (2009) e.g. used the bacterial enzymatic beta-galactosidase activity as an indicator of biological soil quality. They show that this activity was seriously influenced by Zn, Cd, Pb and Cr heavy metals contaminated soil but the presence of plants seemed to counteract this effect. This could explain the non-significative difference observed between rhizospheric and rhizoplan of the less polluted soil.

It has been reported that some specific bacteria named PGPB (*Plant growth promoting bacteria*) are able to enhance plant growth by atmospheric nitrogen fixation, phytohormone productions, specific enzymatic activities, etc…They also may act in favor of plant protection for diseases by producing antibiotic and substances such as siderophores and chelating agents (*Khan, 2005*). A mechanism most often invoked to explain the various direct effects of plant growth-promoting bacteria on plant is the production of phytohormones, and most attention has focused on the role of auxin (Patten and Glick, 1996). However, in the last few years it has been found that a number of plant growth-promoting bacteria contain the

enzyme ACC deaminase (*Saleem et al., 2007; Glick et al., 2007b*) and this enzyme can cleave the plant ethylene precursor ACC, and thereby lower the level of the phytohormone ethylene in a developing or stressed plant. Siderophores, low-molecular weight iron binding molecules, are synthesized by many microorganisms under low-iron conditions (*Neilands, 1981a*). Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots. Plants may also utilize siderophores synthesized by microorganisms colonizing the rhizosphere; this would be a source of soluble iron for the host plant.

We have chosen to test 3 PGPB traits (IAA and siderophores productions, ACC deaminase activity) generally used in literature. The tests performed allowed us to isolate 53 bacteria exhibiting at least one of these traits and which can be distinguished on the basis of morphological criteria. We have proposed a screening strategy in order to isolate PGPB bacteria for plant assay. We express preliminarily our results as productivity (e.g $\mu g\ ml^{-1}\ h^{-1}$) that was calculated on the base of a fifteen-hour production. We assume that this reflects more efficiently the absolute capacity of a defined strain to impact the considered medium.

5. Conclusion

Some PGPB bacteria isolated from a trace element contaminated soil could be able to modify the plant growth and biomass in a direct way thanks to phytohormones production e.g., IAA and other regulators driven by PGPB such as siderophores and specific enzyme e.g., ACC deaminase. For pot experiment (in progress), 8 bacteria will be inoculated and tested in a bioaugmentation experiment to evaluate their potential impact on phytoextraction of a hyperaccumulator plant *Arabidposis halleri* and more specifically their impact on biomass production and growth of plant . Some of PGPB bacteria exhibit only a single trait at high level (e.g IIIG10, IIC3 and IE1) in order to reveal if only one PGPB trait is sufficient to measure significant effects on plant growth, biomass and/or hyperaccumulation yield.

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