Distribution of *Pseudomonas* sp. populations in relation to maize root location and growth stage

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Abstract – The distribution of *Pseudomonas* sp. populations of the maize rhizosphere in relation to root location and growth stage was investigated using an agar plate method on TSA medium and on a selective medium S1, and a molecular approach on the 16S rDNA followed by restriction enzyme analysis ARDRA. Maize was cultivated on soil for 15 and 30 days. The root system was divided into three parts: the root tip, the root hair and the ramification zones. After 15 days of plant growth, *Pseudomonas* sp. were only localized on the root tips and on the roots close to the plant stem. No *Pseudomonas* were detected in the maize root hair zone. After Day 30, *Pseudomonas* sp. were recorded on the root tips, but also on the medium part of the root system. After amplification and restriction with the endonuclease *Hinf*I, the 212 bacteria isolated from the different root parts were distributed into 5 major ARDRA ribotypes. The evolution of these 5 groups was analyzed. At Day 15, the 5 major ARDRA ribotypes are well represented, whereas all the isolates are gathered into only 3 major ribotypes at Day 30. The ribotypes ARDRA 1 and ARDRA 3 at 15 days persist and the number of strains belonging to these ARDRA ribotypes increases until Day 30. In contrast, the number of strains belonging to ribotype ARDRA 2 decreases and ribotype ARDRA 4 disappears. Ribotype ARDRA 1 is present in all root sections (except in the root hair zone at Day 15) and during all the growth stages. The diversity of the isolates at Day 30 appeared to be lower than at the first growth stage in all root zones. These shifts along the root system were related to a variation of the nature and the quantity of maize exudates along the root system and during growth.

rhizosphere / maize / *Pseudomonas* / diversity / 16S rDNA / ARDRA

Résumé – Distribution des populations de *Pseudomonas* en relation avec la zone racinaire et le stade de développement du maïs. Nous avons déterminé le nombre total de bactéries cultivables sur milieu TSA ainsi que la densité des *Pseudomonas* sur milieu sélectif S1, ceci dans différents compartiments racinaires de maïs et en sol nu, à deux dates de récolte. Il en ressort que les populations de *Pseudomonas* ont une densité plus élevée dans la rhizosphère de maïs que dans le sol nu. De plus, ces densités augmentent fortement entre 15 et 30 jours, au niveau de la zone d'élongation et des poils absorbants. Les différentes souches isolées nous ont permis de constituer un soucheur de 212 souches caractérisées par la technique ARDRA (amplification de la région 16S de l'ADNr et restriction enzymatique par l'enzyme *Hinf*I). Les souches analysées se répartissent en 5 groupes ARDRA. La structure des populations de *Pseudomonas* sp. a été modifiée selon les zones racinaires étudiées et au cours du temps.

rhizosphère / maïs / *Pseudomonas* / diversité / 16S rDNA / ARDRA

1. INTRODUCTION

The rhizosphere is the volume of soil adjacent to and influenced by the plant [25]. Roots are known to excrete several forms of organic materials. The amounts and composition of these organic materials are different according to plant species and cultivars, and change during plant development [5, 21, 33]. As a result, the bacterial communities in the rhizosphere, which can use these organic materials as a

substrate, would differ in composition and density [5, 8]. This may result in the buildup of a microflora specific to a particular plant species and genotype as well as to stages in the plant development.

With the growing interest in the introduction of plant growth-promoting microorganisms into the rhizosphere, it becomes of particular importance to characterize better the bacterial species naturally associated with the root system. Among the non-symbiotic rhizobacteria, much attention has

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been given to *Pseudomonas* sp. [29]. Specific *Pseudomonas* sp. strains are able to increase crop yields significantly and this increase is related to the promotion of plant growth and to the plant protection against pathogenic microorganisms [12, 31]. In order to develop effective strategies for the delivery and maintenance of bacterial inoculants in association with the root system, it is essential to demonstrate whether there are differences in populations supported by different portions of the root system. To date, few studies address the diversity of bacterial populations isolated from the different root system portions [7, 13, 50].

In this study we aimed to obtain a better understanding of root part effects on the total bacterial community in soil and to determine the structure between populations of *Pseudomonas* sp. associated with maize roots. *Pseudomonas* sp. populations isolated at two plant growth stages from distinct morphological portions of the maize root system and from bulk soil were compared. The *Pseudomonas* species are characterized by an extraordinary nutritional versatility that favors the ability to colonize highly different habitats, among which are soil and plant rhizospheres [4, 30]. Knowledge of the structure of bacterial populations and how it changes with time is essential to understanding the mechanisms concerning the introduction of plant growth-promoting microorganisms into the rhizosphere.

2. MATERIALS AND METHODS

2.1. Isolation of *Pseudomonas* strains

Maize plants (*Zea mays*) were grown under non-sterile conditions. Seeds (cv. DEA, Pioneer France, weight 0.28–0.30 g, not treated with fungicide) were sown in circular-section pots (17 cm diameter, 21 cm height) containing a mixture of an agricultural soil and sand (2:1). The composition of the soil was as follows: 42.6% silt; 12.8% clay; 44.6% sand; pH = 6.5. This soil was first sieved (5 mm) and dried, together with the sand (2 mm), in a greenhouse (20 °C). The pots received the same amount of dry sand-soil mixture (4 kg) and were flooded overnight. The medium weight of the pots after draining gave the amount of water corresponding to 100% of the soil water holding capacity. The pots were maintained in the greenhouse for 6 weeks (to ensure microbial reactivation) and regularly checked for distilled water adjustment to 70% of the soil water holding capacity. Maize seeds (1 per pot) were sown at a depth of 2 cm after this 6-week period. The control treatment consisted of pots without plants, stored in the same conditions. The pots were then transferred to an environmental growth chamber (photoperiod 16 h, temperature 19 °C night and 22 °C day, relative humidity 65%, PPFD 350 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 30 days. Three plants were collected randomly at each sampling, i.e. 15 and 30 days of plant growth; corresponding to the end of the germination and elongation stages. The pots without plants, as controls, were also collected at the same time.

At each sampling, the root systems were removed from the pots without disturbing the soil. The seminal roots were divided into three portions: the ramification zone with secondary roots, the root hair-elongation zone and finally, the root tips. For each zone, the roots and the adhering soil were placed

in flasks containing 100 ml of PBS (8 g NaCl, 1.44 g Na_2HPO_4 , 0.24 g KH_2PO_4 per liter, pH = 7.2). After dispersion of the adhering soil, the root portions were removed. The flasks were placed on an orbital shaker (120 rpm) for 10 min and centrifuged (750 rpm, 10 min, 5 °C). For the controls, 10 g of fresh weight of soil were also dropped into a flask containing 100 ml of the phosphate buffer. Serial dilutions of these suspensions were plated on 0.1 $\times$ Trypsic Soy Agar (TSA) amended with 12 g $\cdot\text{l}^{-1}$ agar for the enumeration of total culturable bacteria and on S1 (18 g agar, 10 g saccharose, 10 ml glycerol, 2.3 g K_2HPO_4 , 5 g Caseine, 1 g NaHCO_3 , 1 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ per liter) for the enumeration of *Pseudomonas* sp. [16]. S1 and 0.1 $\times$ TSA plates were incubated at 26 °C for 72 h.

Bacterial isolation was always performed with samples diluted to the same level, i.e. one-hundred-fold dilutions from S1 plates with 30–100 colonies. Isolates were subjected to single-colony isolation on S1 medium and cryopreserved at $-80\text{ }^\circ\text{C}$ in 40% glycerol.

2.2. DNA extraction

Bacterial cells were grown for 24 h at 26 °C in YG medium (yeast extract 5 g $\cdot\text{l}^{-1}$, glucose 10 g $\cdot\text{l}^{-1}$). One-and-a-half ml of bacterial cell suspension was centrifuged (3000 rpm) for 10 min. The supernatant was discarded and DNA from the bacterial pellet was extracted by a rapid method using Proteinase K/CTAB and phenol/chloroform [23]. The DNA was resuspended in 50 μL of sterile distilled water and stored at $-20\text{ }^\circ\text{C}$ until use.

2.3. ARDRA

DNA coding for the 16S rRNA of each isolate was amplified with primers R (5'-CTACGGCTACCTTGTTACGA-3') and F (5'-GAGAGTTTGATCCTGGCTCAG-3'). These primers were designed on the basis of the conserved bacterial sequences at the 5' and 3' ends of the 16S rRNA gene (Eurogentec, France; positions 27f and 1492r, respectively, on *Escherichia coli* rDNA), which allowed amplification of almost the entire gene. PCR reactions were done in a total reaction volume of 25 μL containing 2.5 μL of Taq polymerase buffer 10X, 200 μM of each dNTP, 1.5 mM MgCl_2 , 0.5 μM of each primer, 0.625 U Taq polymerase and 1 μL of DNA template. The thermal cycling parameters (Gene cycler, Biorad) used were an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1.30 min, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min.

5 μL of the amplified 16S was analyzed by 0.8% agarose (Quantum, France) gel electrophoresis in TBE (Tris-borate-EDTA) buffer. Gels were stained using ethidium bromide (10 mg $\cdot\text{l}^{-1}$) and photographed under U.V. light.

2.4. Restriction digest and electrophoresis

5 μL of the amplified 16S was digested with the endonuclease *Hinf*I, or the mix *Alu* I/*Msp*I according to the manufacturer's instructions (Appligene, France). The restriction products were analyzed by 8.0% acrylamide gel electrophoresis

Figure 1. Densities of culturable bacteria in the ramification zone, root hair zone, apex and control soil of 15-day-old (white) and 30-day-old (grey) maize. Results are expressed in log CFU per gram soil dry weight. Means ($n = 3$) with different letters are significantly different at $P < 0.05$.

(PAGE) [41] and mini protean III Cells (Biorad, France). Phage X174-DNA digested by *Hae*III was used as a size standard. Gels were stained using ethidium bromide and photographed under U.V. light.

2.5. Statistical analysis

The log-transformed CFU data were analyzed by analysis of variance (ANOVA one way, STATBOX software, Grimmer Logiciels, 1997, Paris FRANCE).

3. RESULTS

3.1. Colonization of maize root by total culturable bacteria and *Pseudomonas* sp.

After 15 days of plant growth, most bacteria were recorded on the root tips and on the root hair-elongation zone (log CFU close to 8) (Fig. 1). Total growing bacteria showed similar population densities in the two other zones: the bacterial densities supported by the ramification zone and bulk soil are not significantly different (log CFU close to 7) but significantly lower than the density measured at the root tip and root hair-elongation zone. At Day 30, culturable bacteria showed the same distribution along the root system as that observed at the first sampling date. Most bacteria were also recorded on the root tips and on the root hair-elongation zone and the densities observed in these two zones were significantly higher than those supported by the ramification zone and bulk soil.

Pseudomonas isolation was also performed with samples diluted to the same level, i.e. one-hundred-fold dilutions from S1 plates. *Pseudomonas* sp. grown on the specific S1 medium followed a different colonization pattern. After 15 days of plant growth, most *Pseudomonas* sp. were localized only on the root tips and on the roots close to the plant stem (respectively, log CFU close to 8 and 6). At the first sampling date, no *Pseudomonas* were detected in the maize root hair zone and in control soil. After 30 days of plant growth, most *Pseudomonas* sp. were recorded on the root tips, but also on the medium part of the root system; the root hair-elongation zone. An uneven distribution of *Pseudomonas* sp. was observed in the maize root system during plant growth

Figure 2. Densities of *Pseudomonas* sp. in the ramification zone, root hair zone, apex and control soil of 15-day-old (white) and 30-day-old (grey) maize. Results are expressed in log CFU per gram soil dry weight. Means ($n = 3$) with different letters are significantly different at $P < 0.05$.

(Fig. 2). Moreover, at each sampling, no *Pseudomonas* was detected in control soil.

3.2. Bacterial community waves by molecular analysis

Total DNA was extracted from each strain isolated on S1 medium. The percentage of amplification was very high (about 95%). A total of 212 colonies were isolated and analyzed.

After amplification, no differences were seen between the different isolates. PCR amplification of 16S rDNA of each of the 212 isolates resulted in a fragment (data not shown) of about 1500 pb. After restriction with the mix *Alu*I/*Msp*I, one major pattern was obtained. 95% of the strains belong to the same *Alu*I/*Msp*I ribotype. The endonuclease *Hinf*I was then used. Restriction analysis of the amplified DNA of each sample with the enzyme *Hinf*I allowed us to distinguish 5 ribotypes named ARDRA 1 to ARDRA 5 with a sixth group containing all the complex patterns different from the 5 ARDRA ribotypes. These 5 major ribotypes (ARDRA 1 to ARDRA 5) contained 96% of the isolates and the complex patterns are weakly represented. The number of strains belonging to ARDRA/*Hinf*I ribotypes, isolated at the two sampling dates from the different parts of the root system, are presented in Table I.

3.3. Distribution of *Pseudomonas* populations according to growth stage and along the root system

In the bulk soil, there are very few isolates which are distributed into 3 different ribotypes belonging to the 5 major ARDRA ribotypes. The number of isolates were higher in the different root parts of the maize than in the bulk soil.

During growth, the distribution of the ARDRA ribotypes changes for each root zone. At Day 15, the 5 major ARDRA ribotypes are well represented, whereas all the isolates were gathered into only 3 major ribotypes at Day 30 (Tab. I). The ribotypes ARDRA 1 and ARDRA 3 at 15 days persist and the number of strains belonging to these ARDRA ribotypes increases until Day 30. In contrast, the number of strains belonging to ribotype ARDRA 2 decreases and ribotype ARDRA 4 disappears (respectively, 1.3% and 0% at Day 30 instead of 16% and 10% at Day 15). The ribotype ARDRA 4 disappears despite the higher number of total culturable bacteria at the second growth stage. Ribotype ARDRA 1 is

Table I. Number of strains (relative distribution in brackets) belonging to ARDRA/*Hinf*I ribotypes at the two sampling dates and from the different root zones.

Ribotype	Root	Tips	Root hair	Zone	Ramification	Zone	Control	Soil	Total in	Rhizosphere soil
	Day 15	Day 30	Day 15	Day 30	Day 15	Day 30	Day 15	Day 30	Day 15	Day 30
ARDRA 1	4 (12.5)	40 (49.4)	0 (0)	38 (92.7)	1 (6.25)	18 (48.7)	1 (33.33)	0 (0)	5 (10)	96 (60.4)
ARDRA 2	2 (6.2)	0 (0)	1 (50)	0 (0)	5 (31.25)	2 (5.4)	0 (0)	0 (0)	8 (16)	2 (1.3)
ARDRA 3	3 (9.4)	21 (26)	0 (0)	2 (4.9)	2 (12.5)	4 (10.8)	0 (0)	0 (0)	5 (10)	27 (17)
ARDRA 4	3 (9.4)	0 (0)	0 (0)	0 (0)	2 (12.5)	0 (0)	0 (0)	0 (0)	5 (10)	0 (0)
ARDRA 5	17 (53.1)	18 (22.2)	0 (0)	1 (2.4)	6 (37.5)	12 (32.4)	1 (33.33)	0 (0)	23 (46)	31 (19.5)
Diverse	3 (9.4)	2 (2.4)	1 (50)	0 (0)	0 (0)	1 (2.7)	1 (33.33)	0 (0)	4 (8)	3 (1.8)
Total 212	32 (100)	81 (100)	2 (100)	41 (100)	16 (100)	37 (100)	3 (100)	0 (100)	50 (100)	156 (100)

present in all root sections (except in the root hair zone at Day 15) and during all the growth stages (Tab. I). Moreover, its proportion increases at Day 30 and represents 60.4% of the patterns.

By dividing the number of different patterns by the total number of strains, the diversity in the root tips for samples was 0.19 at Day 15 and 0.05 at Day 30. The diversity of the isolates at Day 30 appeared to be lower than at the first growth stage. Similar results were obtained by analyzing the results in the other root zones.

We also observed a variable repartition of the same ribotype along the 4 zones and the 5 ribotypes were highly represented in the root tip part of the maize root and in the ramification zone. Moreover, over time, the number of strains belonging to ribotype ARDRA 1 showed an increase.

4. DISCUSSION

Rhizosphere soil represents a region of intense microbial activity which is maintained at a high level because of the release of root exudates by plant roots [6]. Exudation is known to display quantitative and qualitative variations between different plant species, such as oil rape and maize [42], grass species [36], pea and tomato [48]. It has long been hypothesized that the diversity of microorganisms present in different plant rhizospheres, and especially their functional abilities, may be linked to these variations in exudates [17, 18, 29]. But exudation patterns also undergo changes with plant age and location along the root system. The preferential sites of exudation along the root system have long been identified; these differ depending on plant species and experimental design. Among the most frequently studied sites are the root apices of primary and lateral roots, the area just behind the root cap, the root hair zone and the point of emergence of lateral roots [8]. These exudation sites produce exudates which differ in terms of both quantity and composition, as is the case in maize [42], annual grass [26] and pea [46]. These spatial and temporal variations of carbon sources occurring within a root system could be the factors which probably influence the composition and structure of associated rhizosphere bacterial communities. As far as we know, there is a paucity of studies dealing specifically with this type of ecological frame.

So, the aim of this experiment was to study temporal changes in the diversity of *Pseudomonas* sp. populations in bulk and rhizosphere soils originating from distinct root portions along the maize root system. We hypothesized that differences between root compartments or developmental stages would be sustained by distinct rhizodeposition pools. The description of the *Pseudomonas* sp. population was carried out using a combination of two methods: an agar plate method on a selective medium S1, and a molecular bacterial population profiling method (16S rDNA) followed by restriction enzyme analysis ARDRA.

At Day 15, most *Pseudomonas* were recorded on the root tips and on the ramification zone. Chiarini et al. made the same observation in their study (sampling after 15 days of plant growth) concerning maize root colonization by *Burkholderia cepacia* [7]. At this stage of maize growth (the germination growth stage), a higher concentration of exudates is found in the ramification zone, presumably due to seed storage compounds [22]. Schilling et al. analyzed soluble root exudates of maize for distinct root parts [42]. They found that the basal part of roots (the ramification zone) delivered the highest contents of sugars while the root tip was characterized by the highest contents of carboxylic acids. Amino acids were present in both locations in the same proportions. The root apices of primary and lateral roots are also known to be major sites of rhizodeposition. The root tips were the first parts of roots which came into contact with microbes of the bulk soil. *Pseudomonas* sp. seemed to be able to utilize mucilage. In spite of the complex composition of these compounds: a matrix of polysaccharide fibrils [14, 15, 20], this association of polymers seemed to be compatible with the fast development of high *Pseudomonas* sp. densities. But some authors also claim a rapid leakage of low-molecular compounds at the root tip [21]. This assumption has been recently accredited by a study using bacterial sensors of sucrose along the root system of an annual grass, *Avena barbata* [26]: sucrose was apparently most abundant in soil near the root tip. Moreover, this zone is characterized by a great number of dead cells: 4000 to 21 000 dead cells per day (i.e. 4 $\mu\text{g C} \cdot \text{d}^{-1}$).

At Day 30 (the elongation growth stage), a different distribution of *Pseudomonas* sp. was observed in the maize root system. Most *Pseudomonas* sp. were recorded on the root tips but also on the medium part of the root system; the root

hair zone. This result is in accordance with a study in which bioluminescence markers were used to detect *Pseudomonas* sp. in the rhizosphere [10]. A high level of luminescence was found in the root hair zone. This zone, just behind the apex, is thought to be an active source of rhizodeposition [8]. Indeed, carbon and nitrogen concentrations are generally high in the vicinity of root tips [34, 35, 45]. Recently, Chiarini et al. [7] observed that *Burkholderia cepacia* tended to colonize preferentially the terminal portion of roots in mature plants (after 70 days of growth). Since the apex can be considered as a moving source of nutrients, carboxylic acids and amino acids exuded at this location are likely to be found in the root hair-elongation zone, provided they have not been degraded by microorganisms of the apex.

Of the various nucleic acid techniques used to estimate microbial community composition and diversity in complex habitats, the most useful is the determination of the sequences of 16S RNA (rRNA) genes [24, 47]. These molecules are composed of highly conserved regions and also of regions with considerable sequence variation [49]. This molecular technique proved to be very effective for discriminating between *Pseudomonas* species [1, 28, 39, 40]. The choice of the restriction enzyme used in this study was driven by the studies of Laguerre et al. [28] and Scortichini et al. [40], who showed that it was possible to discriminate between strains isolated at different times from different geographical areas and sites only with *Hinf*I.

In our study, we observed for each ribotype that the percentage of repartition was different along the root system. The ribotype ARDRA 1 became dominant whatever the zone considered. We also observed that the *Pseudomonas* population structure was modified along the root system and between samplings. Rhizodeposits could be one of the factors responsible for the observed repartition of ARDRA ribotypes. It has been hypothesized [9, 50] that changes in root exudates' composition along roots affected the rhizosphere bacterial communities. The rhizosphere parameter often suggested as supporting these genetic modifications between root compartments is the organic carbon released by living roots [3]. Only a few studies specifically focused on the effect of quantitative and qualitative variation of organic carbon on bacterial communities [19, 27]. But it is unrealistic to think that competition for nutrients (rhizodeposits) was the sole factor. For instance, spatial variation of pH along maize roots has been measured [37]. Root colonization and rhizosphere competence are also known to imply numerous microbial properties such as antibiotics synthesis, motility, chemotaxis, amino acids and vitamin B1 prototrophy, and the presence of the O-antigen of lipopolysaccharide, among the most studied factors [4, 32, 38].

Changes in genetic structure over time have often been described. Di Cello et al. [11] showed that populations of *Burkholderia cepacia*, isolated from the maize rhizosphere at five stages of development (between 3 to 18 weeks after planting), displayed a decreasing diversity over time, using ARDRA and RAPD. In our case, the diversity estimated by dividing the number of different patterns by the total of strains [44] also showed a decrease in each root zone over time. Indeed, the diversity of the isolates at Day 30 appeared to be lower than at the first growth stage.

Our aim was to measure the influence of time and maize root location on *Pseudomonas* population structure. We observed an uneven distribution of *Pseudomonas* cells between different root system portions. We hypothesized that potential differences could be assigned to differences in the availability of carbon resources. Indeed, it is conceivable that, among other factors, root exudates may play an important role, as it is known that spatial variation in soluble carbon sources along roots coincides with different trophic groups of bacteria [2, 43]. At the genetic level the factor which probably alters the genetic diversity of *Pseudomonas* is also thought to be organic carbon, the availability and nature of which are known to vary with root location and over time. The present work sheds more light on the dynamics of a single bacterial species in the rhizosphere of maize and on the transformations it undergoes during plant development. More studies are required to understand the specific role of rhizodeposits (mucilage and exudates) in bacterial diversity.

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