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ORIGINAL ARTICLE

A standardized method for the sampling of rhizosphere and rhizoplan soil bacteria associated to a herbaceous root system

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Abstract Plants-microorganisms interactions play a fundamental role in terrestrial ecosystems and various methods have been reported for plant-associated bacteria extraction. However, these methods exhibit notable variations and lack of some procedural details that may impact the interpretations of results. We propose here a standardized and detailed protocol for the independent extraction of bulk, rhizosphere and rhizoplan soil fractions. This protocol was applied to the sampling of different polluted soil fractions collected in the vicinity of *Arabidopsis halleri* dense root system. It allowed us to determine the cultivable bacterial densities in each fraction and to confirm the existence of a bacterial gradient linked to roots distance, with a higher amount of bacteria in the rhizospheric area. We suggest to use this unified procedure as a common basis for soil sampling and bacterial communities analysis from other roots systems.

Keywords Soil sampling · Bulk soil · Rhizosphere · Rhizoplan · Bacteria

Introduction

There has been considerable renewed interest in soil microorganisms and their key role in various terrestrial ecosystems. Indeed, soil bacterial communities have been shown to largely contribute to complex processes such as biogeochemical cycles, plant nutrition and health or soil structure and fertility (Nealson and Stahl 1997, Gyaneshwar et al. 2002, Jeffries et al. 2003, Lynch and Bragg 1985). The most important challenge is to unravel the diversity of these communities and to understand their dynamics, particularly regarding their potential interactions with associated plants. The careful monitoring of plant surrounding micro-flora is necessary to evaluate the mutual impact plant and bacteria may have on each other. This implies to first distinguish bulk soil from rhizosphere and/or rhizoplan fractions in order to evaluate, and if possible, isolate their respective bacterial contents.

Rhizosphere - or more appropriately the rhizospheric area - was first defined by L. Hiltner (1904) as the volume of soil influenced by plant roots and their exudates. It is classically distinguished from bulk soil, which corresponds to the area located outside of the rhizosphere, therefore non-adhering to roots and not under its influence. The rhizospheric area forms a hot-spot of microbial abundance and activity due to the presence of plant exudates and rhizodeposits (Kamaludeen and Ramasamy 2008, Zhuang et al. 2007). This microenvironment is a dynamic niche containing complex microbial communities and it may participate in a variety of beneficial interactions with plants such as water and nutrient uptake and may as well contribute to plant growth and health (Canbolat et al. 2006, Yang et al. 2009, Bais et al. 2006). This zone can be separated in two distinct fractions: rhizosphere *sensu stricto* and rhizoplan.

Rhizoplan is defined as the thin layer of soil covering the roots and strongly adhering to them (Cleyet-Marel and Hinsinger 2000, Seguin et al. 2005). It forms an interface between roots and rhizosphere, which corresponds to the rest of the rhizospheric area. Therefore, the term rhizosphere refers to the distal fraction of rhizospheric area that is

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adjacent to rhizoplan, still under roots influence, but without direct contact with them. It is thus not surprising that bacterial diversities of bulk soil, rhizosphere and rhizoplan significantly differ from each other and it may be of considerable importance to precisely identify the strains belonging to each fraction. However, a totally defined methodology for microbial extraction in the rhizospheric area does not exist. This may be attributed to the difficulty to define exactly the zone influenced by root exudates, which depends on plant and its root system (Angle et al. 1994). Consequently, it is difficult to compare rhizospheric population between different studies.

Our aim here is to suggest a standardized and detailed protocol for the sampling of bulk, rhizosphere and rhizoplan soil fractions. We state that it will represent a valuable tool facilitating bacterial analyses comparisons. For this purpose, we have used the trace elements hyperaccumulator plant *Arabidopsis halleri* as a model. As a matter of fact, *Arabidopsis halleri* possesses a dense root system from which soil particles are difficult to collect and especially from a clay soil, these two elements forming the most difficult sampling conditions. We assume that the proposed protocol will work efficiently for most types of plants and soils that may be encountered.

Materials and methods

Soils sampling

Arabidopsis halleri plants and their associated soil samples were collected during plant growing season as a 20 cm² by 30 cm depth lump from a metallicolous grassland highly contaminated with zinc and cadmium (Le Bois des Asturies) located in Auby (59, France). Four different soil samples (S1 to S4) were chosen to reflect different degrees of Zn contamination (2,703, 6,078, 16,100 and 36,200 ppm g^{-1} of fresh mass respectively) as well as different soil textures (namely clay loam, medium loam and sandy loam). Plants and soils were immediately transferred in polyethylene bags to avoid excessive desiccation during transport and were stored thereafter at 4 °C. Each sample was separated into three fractions: bulk soil (B), rhizospheric soil (R) and the rhizoplan fraction (RP) as illustrated on Fig. 1.

In order to collect bulk soil, plants were vigorously shaken by hand for 10 min, paying attention to the roots integrity. The actual limit for shaking and thus for sampling for this soil fraction was considered as reached when roots non-adhering soil particles were completely removed (Fig. 1, Step 1).

Rhizosphere soil was afterwards collected by hand-shaking roots for 10 min in one litre of a sterile 0.9 % NaCl solution to remove the adhering soil (Fig. 1, Step 2).

To sample rhizoplan fraction, roots were washed and hand shaken a second time for 10 min in one litre of a sterile 0.9 % NaCl solution containing Tween 80 (0.01 % v/v) (Fig. 1, Step 3).

To estimate the quantity of rhizospheric and rhizoplan soil sampled, flasks containing NaCl solutions were weighed before and after sampling to monitor mass gain. For bulk soil, the fraction sampled was obtained dry and its amount was much higher than for other soil fractions. For this reason, 100 g of bulk collected soil were added to one litre of a sterile 0.9 % NaCl solution, an amount that was found to be nearly equivalent to those obtained by washing.

The three soil suspensions were then incubated to homogenize bacterial content on an orbital shaker (300 rpm, 90 min, 25 °C) before being centrifuged (150 *g*, Eppendorf 5810-R, 25 °C, 10 min) in 200 mL sterile tubes to concentrate soil particles in the pellet. Supernatants were subsequently roughly filtered on 1 mm sieves to eliminate remaining residuals in suspension.

Bacterial density

To evaluate the cultivable bacterial density, 1 mL of each supernatant was serially diluted in a sterile NaCl 0.9 % solution (9 mL) until reaching 10^{-8}. The number of colony forming units (CFU) was determined by spreading of 100 μL for each dilution in triplicate on Luria Broth (LB)-agar Petri-dishes (90 mm), supplemented with cycloheximide (100 mg L^{-1}). The agar plates were incubated at 25 °C for 72 hours before CFU counting.

Results and discussion

Angle et al. (1996) reviewed for the first time the different strategies to sample rhizospheric soil and gave some recommendations concerning different steps of the process. For root collection, they suggested to collect as soon as possible the global root system. In the soil preparation step, he recommended shaking roots with caution and if root system weighed more than 5 g, he advised to suspend it in a large volume of liquid. Finally, he proposed to macerate 10 min at 180 rpm the soil suspension previously obtained. However, since then, most publications still contain notable sampling protocol variations. In this paper, we briefly review various methods that have been used until now to sample bulk, rhizosphere and/or rhizoplan soil fractions and we propose a standardized and step-by-step detailed protocol including Angle's recommendations (1996) to sample the metalliferous clay soil fractions surrounding *Arabidopsis halleri*'s dense roots.

Timonin (1946) was one of the first who shook roots to sample bulk soil and separate it from rhizospheric soil. This

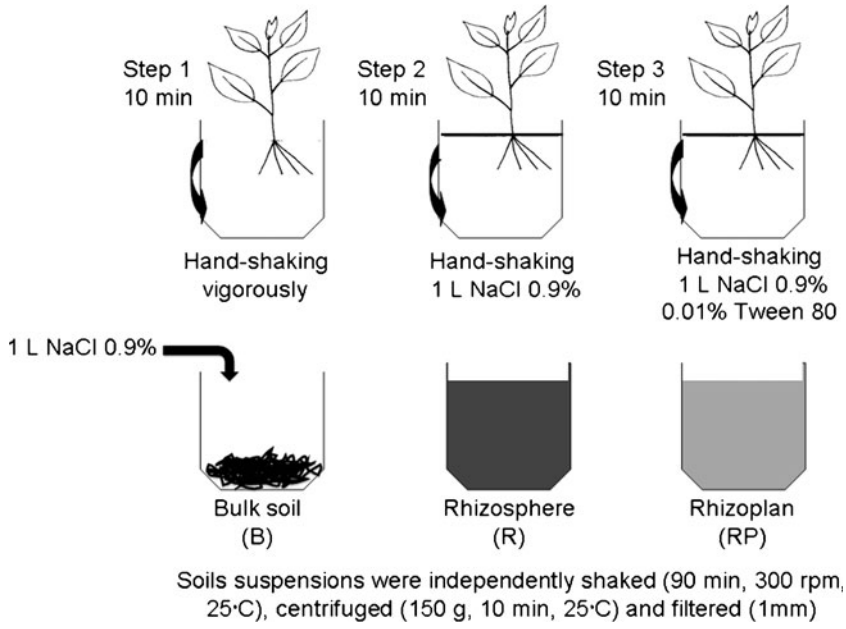

Fig. 1 Standardized protocol suggested for sampling bulk, rhizosphere and rhizoplan soils

is actually done by vigorously hand-shaking (Table 1), assuming that mucilage and exudates will maintain rhizospheric soil adhering to root system. The result of this step largely depends on roots nature (e.g. pivoting or booklet roots) and soil type (e.g. sandy or clay soils) but also relies on the operator's way of shaking (e.g. shaking time and strength applied). As said by Luster et al. (2009), because soil texture and actual soil moisture strongly influence the amount of soil adhering to the root system, the results should be compared with caution. For example, Turpault (2006) suggested to collect from dry soil, which appears not suitable for microbial analysis. For that reason, most protocols include a washing step. The introduction of a washing step of the roots to collect adhering rhizospheric soil was first suggested in 1992 (Jiang and Sato 1992, Tedla and Stanghellini 1992). This procedure seemed to be appropriate for the isolation of rhizospheric bacteria only (Angle et al. 1996). Since then, rhizospheric fraction is subsequently sampled, washing the roots in a flask containing a sterile solution (e.g. phosphate buffered saline (PBS) or physiological buffers). However, immersion time and solution volume are still often subject to variations between authors (Table 1). We suggest here that shaking should last 10 min and that one litre of a sterile 0.9 % NaCl solution should be used to rinse correctly the roots. NaCl was preferred to PBS since phosphate ions were suspected to be potentially able to form a precipitate through their interaction with the high amount of Zn and Cd divalent cations present in soil samples.

Rhizoplan is generally sampled through a second wash in a buffer solution that helps to scatter the thin layer of soil remaining attached to the roots after the first washing step (Harley and Waid 1955). The solution used for this purpose may be identical or different of the one used for rhizospheric sampling (Table 1) but the reasons for introducing or not some modifications remain often unclear. Moreover, authors are not unanimous on the impact of a supplementation of washing with detergents such as Tween (Zeng et al. 2006, Brown and Winsley 1969). The only widely accepted point concerns the shaking step, which seems to be effective only if lasting several min (Angle et al. 1994). We recommend that the shaking should last 10 min in one litre of a sterile 0.9 % NaCl solution containing Tween 80 as a detergent. Tween 80 was preferred to Triton X100 on the basis of its higher hydrophile-lipophile balance (HLB).

Using the protocol described above, four different soil samples (S1 to S4) corresponding to different pollution levels were investigated and bacterial densities of their respective bulk, rhizospheric and rhizoplan fractions were estimated. The results are expressed as log CFU per gram of dry weight soil (Fig. 2). The values found in bulk soil samples (ranging between 10^6 and 10^7 CFU g^{-1} DW) are slightly inferior and opposite to those generally encountered. Indeed, bulk soil bacteria density is considered to be close to 10^7 CFU g^{-1} DW in sandy bulk soil and to 10^8 CFU g^{-1} DW in a clay bulk soil (Taylor et al. 2002). However, since Auby soil is highly polluted with Zn and Cd, such a discrepancy may be explained by the variable impact of different pollution levels on the endogenous flora, an observation that has been already reported elsewhere (Vasquez-Murrieta et al. 2006).

A root gradient could be observed with a higher amount of bacteria in the rhizospheric area as compared to bulk sample. Indeed, we observed a significant increase of bacterial density with root proximity (6,37 x 10^7 to 1,17 x 10^{10} CFU g^{-1} DW respectively for bulk and rhizoplan soils S1; 3,45 x 10^6 to 1,01 x 10^{10} CFU g^{-1} DW respectively for bulk and rhizoplan soils S2; 2,64 x 10^6 to

Table 1 Examples of recent procedures used for sampling bulk, rhizosphere and rhizoplan soil fractions. The references are presented from 1996 to 2009

Soil	Protocol	Reference
Rhizosphere	Carefully shaking roots, using an inoculation needle to remove large clumps of attached soil. 2.5 g of soil (fresh weight) were placed into 99 mL of an appropriate solution, or 5 g in 95 mL or more than 5 g in a larger volume. The soil suspensions were shaken and macerated (10 min, 180 rpm).	(Angle et al. 1996)
Rhizosphere/Bulk	Carefully removing all roots by hand (bulk soil). Fine roots and soil were gently shaken for 1 min in a plastic container to separate the soil aggregates from the roots (rhizosphere).	(Gobran and Clegg 1996)
Rhizosphere/Bulk/ Rhizoplan	Bulk was remaining after picking out the roots from the core. Rhizospheric soil was the soil still adhering to the roots after gentle shaking and was added in 20 mL of a sterile physiological solution by vigorous Vortex agitation (High speed, 20 sec). The rhizoplan was obtained after the second washing in 20 mL of a sterile physiological solution.	(Marilley et al. 1998)
Rhizosphere/Bulk	Roots were shaken vigorously to separate soil not tightly adhering to the roots. 3 g of soil or plant roots with firmly adhering soil were re-suspended in 9 mL of distilled water and treated in a Stomacher 400 blender (Seward) for 1 min at high speed. After centrifugation (2 min, 500 *g*), the supernatant was collected and the resulting pellet was re-suspended in 9 mL of distilled water followed by Stomacher blending and low-speed centrifugation. This step was repeated once. The supernatants of the three centrifugation steps were combined before centrifugation at high speed (10,000 *g*, 30 min) to collect the microbial pellet.	(Smalla et al. 2001)
Rhizosphere/Bulk	Roots were gently shaken by hand. A sample of 10 g bulk soil or roots was dropped into a flask containing 100 mL phosphate buffer. After dispersion of the adhering soil by gentle shaking, the flasks were placed on an orbital shaker (120 rpm, 10 min) and centrifuged (100 *g*; 10 min).	(Baudoin et al. 2003)
Rhizosphere/Bulk	1 g of roots with adhering soil or fresh soil re-suspended in 10 mL of a sterile 0.8 % NaCl by vigorous shaking for 3 min.	(Angelo-Picard et al. 2004)
Rhizosphere/Bulk	Plants were shaken carefully to remove the bulk soil. The soil still adhering to the roots was separated from the roots by moderate agitation in 50 mL of a sterile 0.9 % NaCl solution during 5 min and then centrifuged (8000 *g*, 10 min).	(Gremion et al. 2004)
Rhizosphere/Bulk	Hand-shaking roots: roots were placed in a flask containing 100 mL of a PBS buffer and were gentle shaken. Soil suspensions were shaken (120 rpm, 10 min) and centrifuged (150 *g*, 10 min).	(Benizri et al. 2005)
Rhizosphere	Same protocol as Angle et al. 1996	(Dell'Amico et al. 2005)
Bulk	3 g of soil in 30 mL sterile buffer solution MOPS. Soil suspension was then shaken (150 rpm, 20 min) and centrifuged (500 *g*, 5 min).	(Lock and Janssen 2005)
Rhizosphere/Bulk	Hand-shaking roots in 100 mL of a sterile saline solution 0.9 % NaCl or hand-shaking 10 g of fresh bulk soil. Shaking fresh soil suspension for 30 min and centrifuging (750 *g*, 10 min).	(Aboudrar et al. 2007)
Rhizosphere	Roots were excised and loosely adhering soil was removed. Each root was weighted, blended and re-suspended in phosphate-buffered saline solution.	(Cavaglieri et al. 2007)
Rhizosphere	Soaking the roots in 25 mL of a phosphate buffer saline solution during 30 min.	(Braud et al. 2009)
Rhizosphere/ Rhizoplan	Root was shaken gently to remove loosely attached soil. Adhering soil (1 g) was rinsed in 9.0 mL phosphate-buffered saline solution (PBS). The root was subsequently washed with PBS containing 0.01 % (vol/vol) Tween 20, rinsed twice with PBS, then immersed in 9.9 mL PBS and incubated on an orbital shaker (185 rpm, 30 min).	(Han et al. 2009)
Rhizosphere	Sample by gentle shaking off the soil that adhered to roots. 1 g of soil was placed into 9 mL autoclaved Milli Q ultra pure water and shaken during 1 hour.	(Mijangos et al. 2009)

1,1 x 10^9 CFU g^{-1} DW respectively for bulk and rhizoplan soils S3; 1,15 x 10^7 to 1,63 x 10^{10} CFU g^{-1} DW respectively for bulk and rhizoplan soils S4). This result is statistically confirmed at $p<0.05$ (Fig. 2). Therefore, cultivable bacteria amount in rhizosphere is a thousand fold higher than in bulk soil. Moreover, rhizoplan soils contain up to 100 fold more cultivable bacteria than rhizospheric soil. The only modulation to this tendency is the most clayed soil, in which the separation between rhizosphere and rhizoplan is probably more difficult to achieve.

This concurs with previous studies since a similar effect was already reported for an unpolluted soil (Ridder-Duine et al. 2007) where the bacterial density was 10 to 100 fold higher in rhizosphere as compared to bulk soil. It was further confirmed on nickel-polluted soil, since it was found a rhizospheric bacterial density 20 fold higher than in bulk soil (Aboudrar et al. 2007). Bacterial amounts we observed in the rhizospheric area are moreover in good agreement with those observed on a highly Zn polluted soil (Dell'Amico et al. 2005). The large difference we could generally observe between rhizospheric and rhizoplan bacterial densities suggested that the second root washing step is an evidence to maximize the extraction of bacteria.

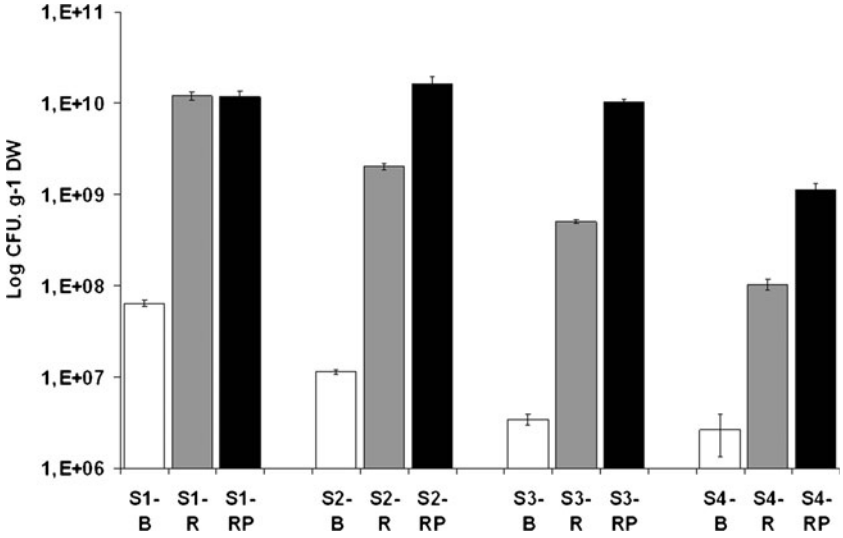

Fig. 2 Estimation of bacterial densities based on triplicates for S1 to S4 soils splitted in bulk (B: white), rhizospheric (R: grey) and rhizoplan (RP: black) soils. CFU data were analysed by analysis of variance (one-way ANOVA). For each sample, values with different small letters are significantly different ($p<0.05$) (means +/- SD)

Conclusion

Our knowledge of plant-microbe-soil interactions has increased over the years but remains impaired, or at least limited, by the availability of efficient methods to study bacterial diversity as well as by the absence of a detailed standard method to sample bulk, rhizospheric soil and rhizoplan. This limit is probably due to the lack of a clear definition for rhizosphere and rhizoplan themselves.

We are conscious that the standardization of the sampling protocol will not solve alone all sampling problems. Discrepancies observed between studies can also partially depend on the variation in roots system volumes and adhering soils amounts as well as on the nature of soils themselves, all parameters that may be widely different for each plant and soil tested. Each of them may deeply impact the recovery of bacterial fractions and the clear assignation of specific fraction content. They also significantly complicate the interpretation of generated results, particularly impairing comparison of bacterial content between apparently related ecosystems. However, the existence of a unified protocol may be considered as a decisive step forward for the comparative analysis of samples, without which any conclusion appears difficult to draw.

The detailed soil sampling procedure presented here may contribute to clarify this situation and allowed us to fix a methodological limit of separation between bulk, rhizospheric and rhizoplan soil fractions. We believe that it represents a valuable complement to the review of Luster et al. (2009) that summarized the main models and methodologies used for soil characterization, but still lacks the definition of a precise sampling protocol for subsequent microbial analysis of the different soil fractions. Moreover, these authors underlined the fact that the existing tools rather referred to artificial conditions.

The fact that bacterial density values obtained here with a complex soil surrounding *Arabidopsis halleri*'s dense root system are in good agreement with preceding studies may be interpreted as a sign that this unified protocol could also be efficient on the vast majority of soils associated to other plants.

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