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Methods for studying root colonization by introduced beneficial bacteria

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Abstract – Some free-living rhizobacteria are considered as potential biocontrol and plant growth-promoting agents. Successful application of beneficial bacteria as microbial inoculants requires their presence and activity at the appropriate level, but even more, at the right time and place. Various markers are described in the literature to differentiate introduced bacteria from indigenous microflora and to visualize them. These markers are presented together with the methods currently applied to quantifying bacterial densities and to characterizing the distribution of introduced bacteria. The methods of quantifying bacterial densities are either based on bacterial cultivation or not. Different types of microscopic observations, allowing the characterization of the bacterial distribution and location in the rhizosphere, are also described. The respective advantages and limitations of these markers and methods are discussed.

rhizosphere / bacterization / methodology

Résumé – Méthodes d'étude de la colonisation racinaire par des bactéries bénéfiques introduites. Certaines rhizobactéries libres sont considérées comme des agents potentiels de lutte biologique et de stimulation de la croissance des plantes. Le succès de leur application nécessite la présence et l'activité des bactéries à un niveau suffisamment élevé, mais également au bon moment et au bon endroit. Différents marqueurs permettant la différenciation des bactéries introduites de la microflore indigène ainsi que leur observation dans la rhizosphère, sont décrits dans la littérature. Ces marqueurs ainsi que les méthodes, appliqués pour quantifier la densité et pour caractériser la distribution des bactéries introduites, sont présentés. Les méthodes de quantification de la densité bactérienne sont basées ou non sur la culture des bactéries. Différentes méthodes d'observations microscopiques permettent de caractériser la distribution et la localisation bactérienne dans la rhizosphère. Les avantages et les inconvénients respectifs des marqueurs et des méthodes décrits sont discutés.

rhizosphère / bactérisation / méthodologie

1. INTRODUCTION

The rhizosphere was defined in 1904 by Hiltner [40] as being the volume of soil, influenced by the presence of living plant roots, whose extension may vary with soil type, plant species, age and other factors [34]. Plant roots release an enormous amount of root exudates that may represent up to 10–20% of the photosynthates (see the review by C. Nguyen in the present issue), leading to a significant stimulation of the microbial density and activity. Specific populations are more favored than others in the rhizosphere due to the level of adequation of their metabolic activities with the composition of the root exudates. The structure and diversity of microbial populations in the rhizosphere thus

differ significantly from those of soilborne populations [55, 64, 67]. These quantitative and qualitative variations of the soilborne microflora are described as the rhizosphere effect. This rhizosphere effect varies according to the root exudate composition, which is affected by the plant physiology [26], the stage of plant development [115] and the position on the root system [57].

The microflora associated with the roots affect plant growth and health. Indeed, some bacterial populations are pathogenic, whereas others are beneficial. Beneficial rhizobacteria include both symbiotic and free-living microorganisms. Among the latter, special attention has been given to the fluorescent pseudomonad group. Positive effects on plant growth and health of inoculations of bacterial strains belonging to this

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bacterial group have been reported since the late '70s [7, 18, 36, 46, 56, 116]. However, overall biological control of soilborne diseases achieved by microbial inoculants is often inconsistent [86]. This has been especially illustrated for fluorescent pseudomonads. This inconsistency has been partially associated with inefficient root colonization by the introduced bacteria [54, 116]. Indeed, a clear relationship has been established between suppression of the wheat root disease take-all and that of fusarium-wilts by different strains of fluorescent pseudomonads, and the densities of these bacteria in the corresponding host plants [16, 78]. In order to make biological control more consistent, there is a need for a better knowledge of bacterial traits promoting rhizosphere competence.

Biocontrol of soilborne diseases is ascribed to microbial antagonism and/or to induced resistance of the host plant [25, 108]. Microbial antagonism results from the suppression of saprophytic growth of plant pathogens mediated by antibiotics and siderophores. The concentration of these metabolites in the rhizosphere is expected to be related to the density of active bacteria. Moreover, the synthesis of some of these metabolites (phenazines and pyoverdines) was demonstrated to be regulated by quorum-sensing [75, 94]. Besides the total bacterial density, which is related to the survival kinetics of the introduced strain, the antagonistic metabolites and thus the bacterial cells should be located at the infection courts of the soilborne pathogens. To summarize, the expression of the beneficial effects by the introduced bacteria requires their presence at a density high enough and at the time and location that are favorable for root infection by the pathogens.

The methods required for analyzing bacterial traits involved in the rhizosphere competence and plant-microbe interactions therefore must allow the quantification of bacterial density but also the characterization of the bacterial cell distribution and location. Moreover, these methods should take into account not only culturable, but also total bacterial cells, since the frequency of viable but non-culturable cells would vary according to the environmental conditions [100].

The aim of the present review is to present methods of quantification and characterization of the distribution along the roots of introduced bacteria in the soil and rhizosphere.

2. MARKERS USED FOR TRACKING INTRODUCED BACTERIA

Tracking bacteria introduced into complex environments such as soils requires the ability to discriminate them from the indigenous microflora. Markers used for that purpose should therefore fulfil several prerequisites. These markers should obviously be specific. This specificity must be checked in the environment in which bacteria are introduced. The markers should also be stable in soil and with time. The relative stability of the marker is required both to avoid its loss and/or its transfer to other bacteria. Since the aim of the markers is to perform ecological studies on the introduced bacteria, they should affect as little as possible the behavior of these bacteria. Surprisingly, there are few studies comparing the fitness of the marked and wild-type strains [14, 28, 35, 72, 73, 106]. In the

same way, since the perturbation of the system should be kept as low as possible, the expression of the marker should avoid substrate amendment. More generally, the markers chosen should be easy to track in a wide range of soils, and environmental conditions (pO₂, pH, etc.) favorable for their expression should be considered [43].

In this section different markers are presented together with their properties.

2.1. Serological markers

The primary immunological tool used in environmental microbiology is the antibody. Immunoassays are analytical methods used to detect and/or quantify the antigen-antibody interaction. The conjugation of a signal molecule (fluorochromes, enzymes and radioisotopes) to the antibody is required to visualize the antigen-antibody interaction. Immunological techniques are relevant especially for the detection, enumeration and localization of introduced bacterial strains in the soil and rhizosphere. The critical aspect of serological methods is the specificity of the antibodies used. Polyclonal or monoclonal antibodies may be applied according to their specificity. Monoclonal antibodies are obviously more expensive to raise but are more specific. The specificity of the antibodies, especially polyclonal ones, should be checked to decrease the occurrence of possible cross-reactions. Usually, a high enough specificity may be obtained for fluorescent pseudomonad strains with polyclonal antibodies raised against membrane proteins [35].

2.2. Molecular markers

Various molecular markers such as antibiotic resistance [35, 53, 105], chromogenic (*xyIE*, *gusA* and *lacZ*) [48, 116], luminescent (*luxAB* and *luc*) [50, 60, 79, 80, 81, 102] and fluorescent markers (*gfp* and unstable *gfp*) [13, 58, 71, 95] have been developed and widely applied to studying root colonization.

2.2.1. Antibiotic resistance

Antibiotic resistances have been widely used as markers in microbial ecology. Although various plasmids and transposons have been used [27, 69, 72, 76, 109], most of the studies on bacterial survival kinetics are based on the use of spontaneously occurring antibiotic-resistant mutants. Rifampicin resistance is commonly used as a marker to study survival kinetics of introduced bacteria in the rhizosphere [35, 68, 105]. Stability of rifampicin resistance was checked with *Pseudomonas putida* WCS358 in field conditions [35]. Kanamycin and streptomycin resistance obtained by Tn5 mutagenesis with the suicide plasmid method of Simon et al. [90] was also described as a possible marker [105]. The maintenance of Tn5 in the mutant JM218 was ascertained by comparing bacterial densities of this mutant in root suspensions, estimated by serology, with bacterial density estimated by plate count on King's B medium supplemented with kanamycin [53]. As stressed above, the level of resistance of the indigenous microflora to the antibiotic used as a marker must be determined in order to check the specificity of the marker used. As an example, Wilson et al. [118] have

estimated that the background of naturally kanamycin-resistant bacteria in a Dutch soil was 2×10^4 cfu per gram.

Antibiotic resistance is often used for studies on survival kinetics of introduced bacteria [35, 68, 76, 105] since the corresponding detection method (plate counting, see Sect. 3.1) is quite sensitive, cost effective, reliable and easy to perform. However, possible genetic changes associated with chromosomal-mediated antibiotic resistance may affect several ecologically important traits [14, 28, 63, 91]. Moreover, the use of antibiotic tagged bacteria carries with it the risk of contributing to the indesiderable spread of antibiotic resistance in nature [41].

2.2.2. Chromogenic markers

Several genes encoding metabolic enzymes have been used as markers to detect, quantify and localize introduced bacteria. The *xyIE* gene encodes for a catechol 2,3-dioxygenase catalyzing the formation of hydroxymuconic acid that, reacting with a catechol, forms a yellow semialdehyde derivative. The *gusA* and *lacZ* genes encode for a β -glucuronidase and β -galactosidase, respectively: in the presence of the adequate substrates (5-bromo-4-chloro-3-indolyl- β -D-glucopyranoside and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), they produce a blue pigment [41].

Although the use of these chromogenic markers is simple, not expensive and well defined, several disadvantages have been widely discussed [32, 43, 92]. For example, the *xyIE* gene applicability is restricted due to inactivation of catechol 2,3-dioxygenase by oxygen [85]. The *gusA* and *lacZ* markers have limited application in soil because of the presence of high background (10^5 cfu \cdot g⁻¹ of soil) from the indigenous microflora [33, 118]. Moreover, these chromogenic markers require substrates or reactives to be expressed or detected; the stability of these gene products is usually measured by hours or days [43].

2.2.3. Luminescent markers: *luxAB* and *lux* genes

Another very sensitive approach is the transfer of bioluminescence marker genes to bacteria, providing them with the capability of emitting light. Prokaryotic bioluminescence genes have been cloned from *Vibrio fischeri* and *Vibrio harveyi* [9, 31], while those eukaryotic (*lux* genes) have been cloned by the firefly *Photinus pyralis* [29]. The *lux* operon of *V. fischeri* includes five genes: two genes (*luxAB*) encode the subunits of the luciferase enzyme and the other three (*luxCDE*) encode enzymes involved in the synthesis of the aldehyde substrate (n-decanal) [43].

The requirement for molecular oxygen limits the use and the interpretations of this kind of physiological reporter system, but if oxygen is present, the monitoring of light may result in very useful information on bacterial activity and distribution in different environments such as the plant rhizosphere [70]. The minimum detection limit for fully active cells has been reported as 445 cells per gram of soil [81]. Although eukaryotic *lux* genes show some advantages in comparison with the prokaryotic counterpart (i.e. higher specificity and absence of background in native microflora), the substrate luciferin is expensive and sometimes not readily

taken up by the cells. The stability of the gene product is usually measured by minutes or hours [43].

2.2.4. Fluorescent markers: stable and unstable green fluorescent protein

Another attractive marker system for monitoring bacterial cells in the environment is the green fluorescent protein (GFP). The GFP is a 27 KDa polypeptide which converts the blue chemiluminescence of the Ca⁺²-sensitive photoprotein (aequorin from the jellyfish *Aequorea victoria*) into green light [20]. A series of red shifted GFP mutants, 20–35 times stronger than the wild type, with various excitation and emission wavelengths such as the ECFP (enhanced cyan), EGFP (enhanced green) and EYFP (enhanced yellow), have been recently developed [101].

The advantages and disadvantages of this marker have been extensively discussed by Errampalli et al. [32]. Some of the most relevant advantages are that GFP is extremely stable and resistant to proteases, is easily detectable, does not require exogenous substrate and allows the monitoring of single cells even in real time. Moreover, GFP is continuously synthesized and there is no background in indigenous bacterial populations. However, the interference of soil particles, the variability of GFP expression in different species, the inability to work in anaerobic conditions and the instability of the plasmid should be considered. In order to overcome the latest limitation and reduce the risk of a plasmid transfer to other microorganisms, bacterial strains used are preferentially chromosomally marked. For that purpose, several Tn5 transposon suicide delivery vectors have been developed [95, 98]. The stability of the GFP varies according to the variants and plasmid constructs in the range of hours or days [44, 95].

Recently, Andersen et al. [4] developed a new variant of GFP characterized by its short half-life. The unstable GFP has been constructed by the addition of a short peptide sequence to the C-terminal end of the intact GFP: this modification allows its degradation by bacterial endogenous proteases. Since the GFP produced during bacterial growth does not accumulate, it is possible to perform real-time analysis of the bacterial metabolic activity [58, 93]. However, different levels of proteases may be expressed depending on the microorganisms, the growth phase and environmental factors, and care must be applied in the interpretation of the results [44].

2.2.5. Specific primers and oligonucleotidic probes

Introduced bacteria can be monitored using primers or probes that allow amplification or hybridization of sequences which are strain-specific. Specific probes can be used to hybridize bacterial colonies after in vitro growth [117] or bacterial cells for in situ studies. Probes are usually covalently linked to a fluorochrome such as fluorescein, rhodamine, Texas red, Cy3 and Cy5 [3].

Specific sequences may be introduced by a genetic construction. As an example, a specific primer amplifying across *nptII-lacZ* junctions on the Tn5B20 construct was used to follow the survival kinetics in the soil and rhizosphere of the strain *P. fluorescens* R2f tagged by the *lacZ-nptII* marker gene

[109]. However, as stressed before, genetic constructs may affect the ecological behavior of bacterial strains.

Another strategy consists of identifying sequences specific to the strains in order to design primers and probes. Different approaches have been proposed to develop this identification. One is to compare homologous nucleic acid sequences of ribosomal RNA (rRNA) to sequences available in databases. Since rRNA are present in all living microorganisms in high copy number and are quite stable, oligonucleotidic probes can be applied [3]. They are either species-specific or even strain-specific in some cases [5, 24]. *Pseudomonas* specific primer has been designed by Braun-Howland [15]. This PSMg primer was applied to describing the dynamic of indigenous populations of *Pseudomonas* in soil hot-spots [45] and to characterizing the succession of *Pseudomonas* on barley root in a perturbed environment [96]. Analysis of the 16S rDNA of the *Paenibacillus azotofixans* strain with that of 2000 bacteria also enabled Rosado et al. [84] to identify the presence of three highly variable regions that were used to design primers for studying the kinetics of this bacterial strain in the soil and wheat rhizosphere. Another approach to defining primers and probes is (i) to characterize the diversity of populations belonging or not to the same group by Random Amplification of Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR), in order (ii) to identify discriminating bands, then (iii) to pick them from the gel, and (iv) to re-amplify and test them for specificity.

Monitoring introduced bacteria on the basis of its specific RAPD-PCR pattern has also been proposed but is very time-consuming [21, 51].

3. METHODS TO QUANTIFY DENSITIES OF INTRODUCED BACTERIA

Methods to quantify introduced bacteria can be classified into two major types depending upon whether they are based on the cultivation of the bacteria or not. Obviously, the culture-dependent methods will not allow the detection of viable but not culturable bacteria (VBNC). Since microorganisms introduced into soil can go through different processes (conversion to the non-culturable state and phase changes) doubts have been raised about the representativity of the view given by data yielded with culture-dependent methods of the real processes in soil. Despite this limitation, culture-dependent methods remain widely used, mainly because they are easy to apply. The culture-independent methods can provide a more complete picture of the kinetics of the total number of microbial cells [105]. However, the major limitation of these methods is that they may not allow differentiation between viable and not-viable cells.

In this section, major culture-dependent and culture-independent methods of microbial quantification in the rhizosphere are presented.

3.1. Culture-dependent methods

These methods are based on the suspension-dilution of soil and/or root samples and on inoculation of growing media

(solid or liquid) with adequate dilutions. They require therefore the use of labeled strains (see Sects. 2.1 and 2.2). The culture-dependent methods differ according to the type of marker used giving the specificity to the growing media. This type of method is quite simple to perform, not too expensive and quite sensitive (10^2 – 10^3 cfu per g), but labor-intensive and shows some limitations [44]. This type of method underestimates the number of bacteria present in soil or in the rhizosphere. Bacteria may remain physically attached to the soil particles, may be killed in the dilution medium or may fail to grow on growth media [47]. Some of them may remain aggregated even during the dilution process in such a way that a cfu may be originated by more than one cell. Suspension-dilution can either be plated on solid media or introduced into liquid media with various dilutions in order to determine from which dilution there is no more bacterial growth. This last method, named Most Probable Numbers (MPN), requires the use of probability tables to process data that contribute to reducing the sensitivity of the analysis compared with plating [61].

The most basic method consists of plating mutants resistant to antibiotics on solid growth medium supplemented with the corresponding antibiotic and with an anti-eucaryotic compound such as cycloheximide. This method is widely used, especially for survival kinetics of introduced bacteria and for competition studies between wild-type strains and mutants impaired in specific phenotypes [67, 68, 72, 106].

The sensitivity of this type of plating method may be significantly lowered by combining plating and serological approaches with the immunofluorescence colony-staining (IFC) technique. Detection limits as low as 20 cfu of *Erwinia* spp. per gram of soil have been reported by Van Vuurde and Van der Wolf [111]. The IFC technique, developed by these authors, is based on the use of fluorescein isothiocyanate (FITC)-conjugated IgG antibodies specific to the introduced bacterial strain to discriminate target from non-target colonies. Bacterial colonies remain viable and IFC positive colonies can be subcultured to confirm their identity by other biochemical or molecular methods. Direct IFC has been used by several investigators [52, 53, 63, 77, 110] to track and quantify bacterial strains introduced into soil or onto plants. Since the IFC does not require any alteration in the phenotype or genotype of the wild-type strain, this technique allows the comparison of an unaltered wild-type strain to a genetically modified derivative of the wild-type strain. The main restriction of direct IFC is the necessity of a good quality fluorescent conjugate against the target bacteria. However, outside the medical field, specific conjugates are usually not commercially available. For this reason, Veena and Van Vuurde [113] recently developed an indirect IFC using diluted specific antiserum and commercial conjugate to detect bacterial pathogens on tomato seeds. Indirect IFC is suitable for routine applications with facilities for fluorescence microscopy and does not require much expertise. As for any serological methods, the main limitation is the risk of false positive reactions due to cross-reacting bacteria.

Reporter genes may also be applied for culture-dependent methods. *Lux-luc* tagged bacteria can be detected and enumerated by plate counting, luminometry or scintillation counters and by imaging. Luminometry is an easy and sensitive method that has been applied to evaluating the density of luminescent

bacteria on the root surface [8] and rhizosphere [81]. These two instruments are sensitive but they are not specifically designated for bioluminescence application [17]. Bioluminescent colonies can be counted directly by color photography (autophotography), by exposure to X-ray film, by direct microscopy with a CCD camera enhancement (imaging) or alternatively, if the amount of light emitted is high, it is possible to visualize them by eye [41].

Green fluorescence due to GFP-tagged bacteria can be observed in colonies cultured on agar media under a hand-held long-wave UV lamp. This is a simple and cheap way to enumerate colonies but the potential DNA damage of UV over time may be a limitation [32]. Fluorimetric detection of GFP-labeled bacteria is useful for screening or confirmation of cell growth. A detection limit of 10^3 cells per ml of *P. putida* in soil has been reported by Burlage and Kuo [17]. Recently, Cassidy et al. developed a MPN method to evaluate cell density of GFP-marked *Pseudomonas* in the soil, rhizosphere and rhizoplane [19].

Finally, colonies grown on solid media can be hybridized with specific oligonucleotidic probes [117].

3.2. Culture-independent methods

Culture-independent methods can be distributed into three different categories: serological, molecular and cytological methods.

3.2.1. Serological methods

Among the serological methods, the enzyme-linked immunosorbent assay (ELISA) is a very sensitive immunoassay for the detection of antigens. ELISA is based on direct or indirect sandwich methods. The ELISA method has been used to study and quantify the external and internal root colonization of maize by two *P. fluorescens* strains [11] and the distribution of two diazotrophic enterobacterial strains, *Pantoea agglomerans* and *Klebsiella pneumoniae*, on cereal shoots and roots [82]. The ELISA method is quite sensitive (10^3 cells per ml in pure cultures and 10^4 – 10^5 cells per g of soil) and associated with a standard curve relating the amount of signal given to the direct counts by microscopic enumeration, can provide quantitative information. Disadvantages are related to possible cross-reactivity and non-specific signal production.

3.2.2. Molecular methods: detection of nucleic acids

Detection methods based on nucleic acids extracted from the soil offer the possibility of monitoring specific bacterial genotypes (gene or genomic markers), providing a picture of the dynamics of total numbers of microbial cells [45]. The polymerase chain reaction (PCR), primarily used as a qualitative method to confirm the presence or absence of a specific DNA sequence, has been recently applied to obtaining quantitative information. Up to now, PCR has been the most sensitive method for detection of specific DNA in environmental samples; sensitivities of 1–100 cells per gram of soil have been reported [42].

Before amplification, microbial DNA is extracted from soil. Various methods have been described for this extraction

[65]. All DNA extraction methods present potential bias depending on the soil properties (i.e. humic substances) [107]. The final goal of DNA extraction methods is to obtain DNA with a quality good enough for PCR amplification and for yielding consistent data.

Three PCR methods for quantification have been developed and applied to evaluating bacterial population in the soil or rhizosphere: the most probable number PCR (MPN-PCR), the competitive PCR (C-PCR) and quantitative PCR (Q-PCR).

In the case of MPN-PCR, the quantification of target sequences is based on the serial dilution of the PCR products in order to identify from which dilution the target sequence is no longer detectable by electrophoresis. The detectable limit of the product is calibrated by an external standard and the initial amount of the target molecules is estimated using the dilution factor of the positive sample. This method was first developed by Picard et al. with *Agrobacterium tumefaciens* and *Frankia* spp. [74]. Using this method, Picard et al. were able (i) to detect the *A. tumefaciens* strain when inocula ranged from 10^3 to 10^7 cells, and (ii) to estimate the indigenous populations of *Frankia* spp. at 0.2×10^5 genomes per gram of soil [74]. A detection limit of 10^2 cfu of *Paenibacillus azotofixans* per gram of rhizospheric soil was reported by Rosado et al. [84]. However, as indicated by Jansson and Leser [42], a limitation of the MPN-PCR method is the probabilistic evaluation, based on several dilutions and replicates, which contribute to reducing the precision of the estimation.

In C-PCR, a DNA fragment containing the same primer sequences (internal standard) as the target fragment is allowed to compete in the same tube with the target for primer binding and amplification. Experimentally, PCR reaction tubes containing the target samples are spiked with a dilution series of the competitor fragment. When the molar ratio of PCR products generated from the target and competitor is equal to one, the amount of the target is equal to the competitor. Since the amount of the competitor is known, the amount of the target can be determined. However, this technique is labor-intensive and its accuracy is dependent on an internal competitor, which must possess the same amplification efficiency as the target [42]. Thirup et al. [96] applied this type of PCR to studying the effects of *P. fluorescens* DR54 and the fungicide Ima-zalil on the succession of indigenous *Pseudomonas* spp. and *Actinomyces* on barley roots. Martin-Laurent et al. [66] have quantified with C-PCR the amount of the *atzC* gene known to be involved in atrazine mineralization in a soil treated with this herbicide.

Finally, Q-PCR is the direct measurement of the amount of products generated from different samples by a calibrated instrument. The initial amount of the target molecules in the samples is estimated by the determination of the PCR amplification efficiency defined by the amplification of a known amount of the same target (external standard). Q-PCR has certain limitations, such as accounting for the variation between samples during the reaction and the fact that quantification is only possible during the exponential phase of the amplification [42].

The major advantages of the PCR-based methods are their sensitivity and specificity. Theoretically, a single copy of the target nucleic acid sequence can be detected; normally, at least

10^3 copies of the target are required for PCR methods in nucleic acids isolated from environmental samples. These methods allow one to take into consideration both cultivable and not cultivable organisms, but do not allow differentiation between viable and non-viable organisms.

3.2.3. Cytological method: flow cytometry

Flow cytometry is a cytological tool valid for evaluating root colonization by introduced bacteria. This instrument measures and analyzes the optical properties of hundreds of single cells per second, passing through a focused laser beam. As each cell or particle passes through the flow cytometer, it is monitored by forward scatter (detects each particle according to its size), side scatter (measures simultaneously the size and the shape of the particle) and fluorescence (evaluates the fluorescence intensity) detectors. Flow cytometry allows the detection and quantification of both the individual fluorescent cells within a population and the fluorescent intensity from more than one bacterial group. For environmental samples, bacteria from the particulate matter and an internal standard must be used to quantify the cell number [44]. A limitation of the technique is related to its low sensitivity due to the abundance of fluorescence particles present in most environmental samples. However, the assay is rapid and simple and thousands of cells can be analyzed in a short time, allowing the processing of the data by various statistical procedures.

Flow cytometry can be applied to evaluating the density and characterizing the kinetics of introduced bacteria tagged by fluorescent antibodies [23], GFP [98] and specific oligonucleotidic probes [96]. A detection limit of 10^3 cells per ml has been recorded evaluating the density of fluorescent antibody-tagged *Xanthomonas campestris* in *Brassica oleracea* [23], and of 3×10^4 cells per gram of dry soil in the characterization of the kinetics of the fluorescent oligonucleotidic probe-tagged *Sphingomonas* spp. strain 107 in soil [97].

The use of different dyes or fluorochrome provides an extremely powerful way to characterize the physiological state, activity or degree of viability of bacteria [37, 114], and thus to quantify by flow cytometry the viable and non-viable bacterial cells. Various criteria have been proposed to discriminate the viable from non-viable cells. Impermeability of the bacterial membrane to dyes is the basis of the dye exclusion test. Propidium iodide (PI) and other dyes characterized by the presence of quaternary ammonium groups and two or more positive charges, such as Sytox green, TO-PRO-1 and TO-PRO-3, are membrane-impermeant. Cells retaining these dyes are usually considered as non-viable cells. Moreover, using simultaneously permeant (SYBR green) and non-permeant (PI) dyes makes possible the discrimination of cells with a compromised, slightly damaged or intact membrane [37]. The use of a membrane permeant substrate such as fluorescein diacetate (FDA) and 5-cyano-2,3-ditoly tetrazolium chloride (CTC), that are cytoplasmic enzymatically cleaved to form a fluorescent impermeant product (fluorescein and formazan, respectively) allows the discrimination of cells with intact (retaining the product of the reaction) and damaged membranes (losing the product of the reaction) [112]. Membrane potential (MP) is the most used vitality parameter in microbial flow cytometry. A 100 mV bacterial transmembrane electrical

potential gradient, due to selective permeability and ionic transport, is usually reported. Variations in the MP measurement can be recorded using lipophilic charged dyes that can be accumulated or excluded by the cell [88].

4. METHODS TO CHARACTERIZE DISTRIBUTION AND LOCALIZATION OF INTRODUCED BACTERIA

Distribution and localization of introduced bacteria require the use of fluorescent antibodies, fluorescent markers and oligonucleotidic probes (see Sects. 2.1, 2.2.4 and 2.2.5). These studies are sometimes only possible in gnotobiotic conditions.

Immunolocalization is based on the use of fluorescent signal molecules conjugated to the antibodies; the emission of fluorescent light indicates the presence of a specific antigen. The basic procedure consists of the reaction between a fluorescent specific antibody with the antigen attached to a slide, and of the observation of the sample using a fluorescence microscope. To enhance the signals and the specificity of the reaction, an indirect immunolocalization, using a fluorescent secondary antibody, is usually performed. Simultaneous localization of different antigens can be obtained using antibodies coupled to different fluorochromes. Immunolocalization has been successfully used to study root colonization. Examples include the analysis of the spatial competition between *P. fluorescens* Ag1 and *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) during barley root colonization [49], the cell distribution of *P. fluorescens* DF57 on barley roots [38], the autoecology of the biocontrol agent *P. fluorescens* CHA0 in the rhizosphere of different crops [100] and the endophytic colonization of spruce by *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*) Pw-2R and *P. fluorescens* Sm3-RN [89]. Advantages of immunolocalization are the simplicity of its use and the short time required to obtain results. On the other hand, several problems, such as autofluorescence of the sample, non-specific staining, antigen instability and the inability to check viability have to be considered.

Lux genes have been widely used in the study of bacterial root colonization and activity [50, 60, 79, 83, 103], while *luc* genes have been only recently applied for monitoring activity of *P. fluorescens* 31K3 in forest soil [12] and of *Sinorhizobium arboris* in the *Acacia senegal* rhizosphere [80].

GFP and its derivatives have been applied to characterizing the distribution of the biocontrol agent *P. chlororaphis* MA342 on barley seeds [99], the localization, the viability and the activity of *P. fluorescens* DR54 on the barley rhizosphere [71] and the colonization pattern of *P. fluorescens* WCS365 on tomato roots [13]. The use of different GFP color variants, allowing the simultaneous monitoring of multiple bacterial species, opens new perspectives in the study of complex microbial communities [13]. The visualization of GFP-tagged cells using microscopy assures a single cell detection level.

Fluorescent in situ hybridization (FISH) involves the use of fluorescence-labeled oligonucleotidic probes, constructed on the basis of the 16S rRNA sequence, to target rRNA within morphologically intact cells [2]. The FISH technique can be

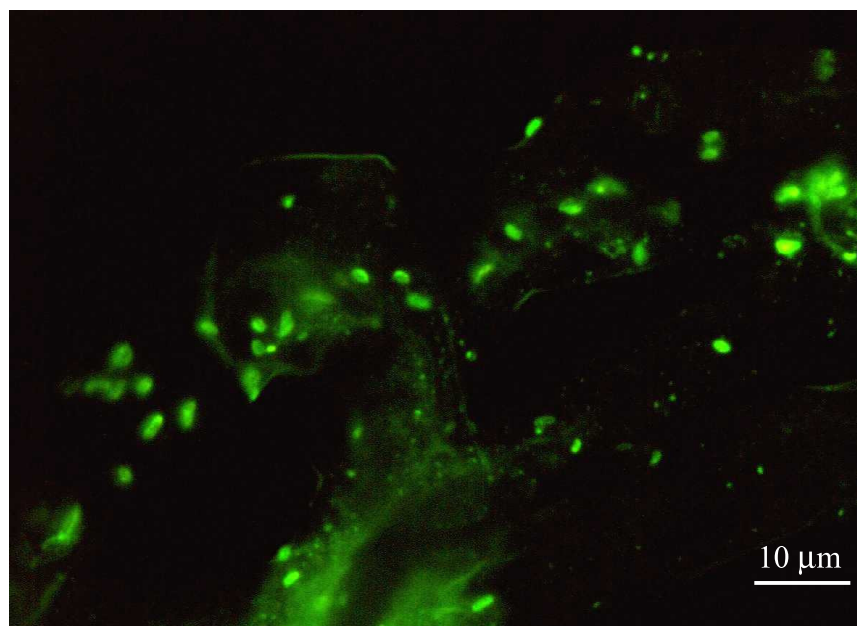


Figure 1. Epifluorescence image of FITC-antibody-labeled *Pseudomonas fluorescens* strain A6RI colonizing root hairs of a 7-day-old root of tomato grown in gnotobiotic conditions.

used to detect all bacterial cells, using a universal probe, or a single population, using a strain-specific probe. The in situ localization of *Azospirillum brasilense* in the wheat rhizosphere was described by Assmus et al. [5], the distribution of *P. syringae* and *Rhodococcus fascians* on the tomato root surface was characterized by Macnaughton et al. [62] and potato tissue infection by *Ralstonia solanacearum* was studied by Wullings et al. [119]. The in situ hybridization method is further detailed in the present issue by Schumpp et al. [87].

Bacteria marked by fluorescent antibodies, fluorescent markers and by oligonucleotic probes, can be detected by direct microscopy using an epifluorescent microscope with an adequate filter kit. The method is simple and the counts are rapid and precise, but the limit of detection is related to the field of view and to the matrix of the sample. Interfacing an epifluorescent microscope with a charged-coupled device (CCD) camera and image analysis software can enhance the sensitivity to a single cell level (Fig. 1). The main problem using direct microscopy is the high background of fluorescence coming from the root, the soil particles and the contaminants.

However, the recent development of confocal laser scanning microscopy (CLSM) has significantly reduced some of these limitations. CLSM is a powerful apparatus for visualizing with high resolution microbial cells labeled by fluorescent antibodies, GFP or oligonucleotidic probes. Because three-dimensional views can be generated, CLSM readily lends itself to digital processing, by which images of thin optical sections can be reassembled into a composite, 3D image. The major advantage of CLSM is that the confocal imaging system allows the detection of signals only from the focused plane, limiting background fluorescence arising from materials such

as plant tissue, soil particles or organic debris. Moreover, by using different fluorescence channels, CLSM allows the simultaneous detection of different bacterial populations and/or secondary metabolites. For all these advantages, there is an increasing use of CLSM to localize introduced microorganisms on plant roots [5, 13, 38, 99]. The limitation in the use of CLSM is the cost of the instrument.

Single-cell distribution of rhizobacteria along plant roots, grown in gnotobiotic conditions, can also be characterized by electron microscopy. The scanning electron microscope (SEM) has been widely used. For example, Chin-A-Woeng et al. [22] described by SEM the spatial-temporal tomato rhizosphere colonization pattern by the biocontrol agent *P. fluorescens* WCS365. More recently, Bacilio-Jimenez et al. [6] observed, by SEM analysis, the presence of two endophytic strains in rice seeds identified as *Bacillus pumilus* and *Corynebacterium flavesces*. The SEM provides an excellent resolution and allows exact localization of microorganisms in relation to the root structure (Fig. 2). However, sample preparation is expensive in time and needs care to avoid the production of artefacts. The transmission electron microscope (TEM) has also been widely applied, for example, to studying (i) the inter- and intracellular colonization of tomato roots by the biocontrol agent *P. fluorescens* WCS417r [30], and (ii) the cell colonization and infection thread formation in sugar cane roots by *Acetobacter diazotrophicus* [10]. Sample preparation for the TEM is much more time-consuming than for the SEM and requires other instruments (i.e. an ultramicrotome) to obtain sections with the adequate thickness.

Information regarding the internal root colonization by two different bacterial strains can be obtained by immunogold labeling. In addition, it has to be stressed that, both by SEM

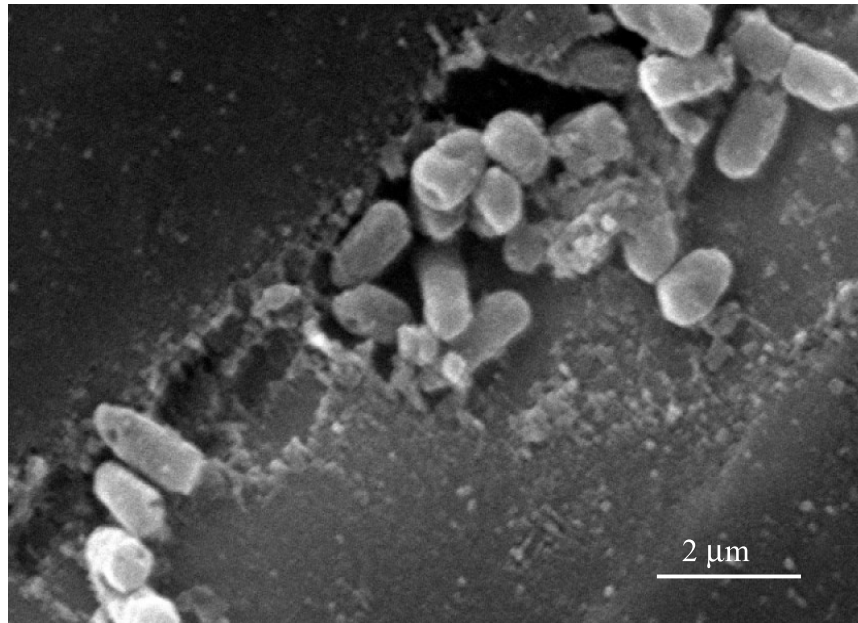


Figure 2. SEM image of *Pseudomonas fluorescens* A6RI colonizing the root surface of a 7-day-old root of tomato grown in gnotobiotic conditions.

and TEM, only very small root samples can be analyzed. To allow the investigation of a more general root bacterial distribution, electron microscopy should be combined with other methods [1].

5. CONCLUSIONS AND PERSPECTIVES

During the present review, different methodologies to quantify and localize introduced bacterial strains have been presented. These methods have their own advantages and limitations. Some only allow the quantification of cultivable bacteria, putting aside the so-called VBNC (culture-dependent methods). Others allow the quantification of the cultivable and non-cultivable cells of the introduced bacterial strain; however, they do not allow the discrimination of the viable and non-viable cells (immunofluorescence and PCR). Taking into consideration the limitations of both types of methods, a combination of culture-dependent methods and serological methods (IFC) or a combination of culture-dependent and molecular methods (colony hybridization) have been proposed. Combining colony counts of an antibiotic-resistant strain with the immunofluorescence technique has been successfully applied to monitoring the distribution and dynamic of bacteria in soil or on the root [39, 49].

Taking into account the advantages and the limitations of the different methods, a polyphasic approach based on the use of different enumeration methods (conventional plate counting, luminometry, fluorimetry, flow cytometry and quantitative PCR) has been proposed by Cassidy et al. [19] to discriminate the total number, and the number of viable and cultivable bacterial cells.

A polyphasic approach could also be proposed to both characterize the localization and the activity of the cells of the introduced bacterial strain. As an example, Lubeck et al. [59], applied the combination of fluorescent antibodies and FISH to studying sugar beet root localization of *P. fluorescens* DR54 by CLSM; this dual staining protocol allowed cellular activity to be recorded in both single cells and microcolonies during the bacterial establishment on the root. Similarly, Unge et al. [103] developed a dual *gfp-luxAB* marker system to monitor simultaneously the cell number and activity of specific bacterial populations. They recently applied this dual marker to characterizing the population size, the metabolic activity and the distribution pattern of *P. fluorescens* SBW25 along wheat roots by luminometry, flow cytometry and CLSM [104].

Over the last few years, there has been an increased interest in microscopic observations of the microflora in the rhizosphere [13, 38, 98], renewing the early studies of Foster [34]. This revival of interest is related to the progress made in the microscopy apparatus and in the molecular and serological markers.

In conclusion, different techniques combining multiple staining/tagging methods should provide more insight into the reciprocal interactions between the plant and the microorganisms in the rhizosphere and about the spatial-temporal colonization pattern and the physiological status of a microbial inoculant along the root.

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