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Transparent soil microcosms allow 3D spatial quantification of soil microbiological processes *in vivo*

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Keywords: plant roots, Rhizobia, confocal, Nafion, refractive index matching, lettuce, image analysis, PGPR, *Pseudomonas fluorescens*, GFP

Abbreviations: PGPR, plant growth promoting rhizobacteria; RI, refractive index; GFP, green fluorescent protein; CFU, colony forming units; FISH, fluorescent in situ hybridization; T-RFLP, terminal restriction fragment length polymorphism.

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The recently developed transparent soil consists of particles of Nafion, a polymer with a low refractive index (RI), which is prepared by milling and chemical treatment for use as a soil analog. After the addition of a RI-matched solution, confocal imaging can be carried out in vivo and without destructive sampling. In a previous study, we showed that the new substrate provides a good approximation of plant growth conditions found in natural soils. In this paper, we present further development of the techniques for detailed quantitative analysis of images of root-microbe interactions in situ. Using this system it was possible for the first time to analyze bacterial distribution along the roots and in the bulk substrate in vivo. These findings indicate that the coupling of transparent soil with light microscopy is an important advance toward the discovery of the mechanisms of microbial colonisation of the rhizosphere.

Plant growth promoting rhizobacteria (PGPR) enhance plant health and yield via complex interactions with the roots and soil.[1-3] Rhizobacteria can offer the plant protection from pathogenic microorganisms by outcompeting them and through the promotion of plant growth via the release of plant hormones.[4] They can also aid plant uptake of nutrients via the rhizosphere, for example by releasing iron-scavenging siderophores.[4,5] The spatial and temporal heterogeneity of soil and the rhizosphere undoubtedly influences the communities and function of bacteria which inhabit niches where nutrients are available in soil.[6] However, studying the interactions between soil bacteria and their physical habitat is currently very challenging partly due to the lack of conventional laboratory techniques and protocols. Light microscopy cannot be used to observe soil in depth because soil is opaque. X-ray imaging techniques are suitable for studying the soil structure but cannot simultaneously resolve microorganisms.[7] Although many molecular methods can be used to identify the structure of soil microbial communities,[8] most do not provide insight into their spatial arrangements. In contrast, recent applications of FISH (fluorescent *in situ* hybridization) have proved successful to analyze spatial distribution of microorganisms in soil, but the method is not suitable to study dynamic processes because samples need to be fixed prior to imaging.[9]

Previously, we published a study describing a new transparent soil analog for imaging plant roots using optical microscopy.[10] It consists of a matrix of solid particles of the low refractive index (RI) ionomer, Nafion, water with plant nutrients and air. Transparent soil can be saturated with a RI matched liquid to reveal biological structures within. Further to this work, we have applied transparent soil to the observation of PGPR spatial interactions with roots and soil particles non-destructively, in vivo and in situ. Quantitative analysis methods were developed to study the spatial distribution of PGPR *Pseudomonas fluorescens* SBW25 in transparent soil, on the surface of *Lactuca sativa* (lettuce) roots and in the surrounding transparent soil, in relation to the pore geometry. The effect of substrate parameters on the colonisation of roots was also tested by varying the substrate particle size. The aims were to measure the effect of plants and substrate on the abundance of PGPR both on root and on the surrounding particles. After inoculation of the transparent substrate with a culture of GFP-tagged *P. fluorescens*, one day old *L. sativa* seedlings were added to the microcosms. The microcosms were sealed and incubated for 5 d allowing the plants to grow and the bacteria to colonise the roots. The transparency of the substrate allowed images to be captured on a 3D grid using confocal microscopy, thus sampling the microbial abundance at points along the roots and in the bulk soil at 2 distances from the root (supplementary information, **Fig. S1**).

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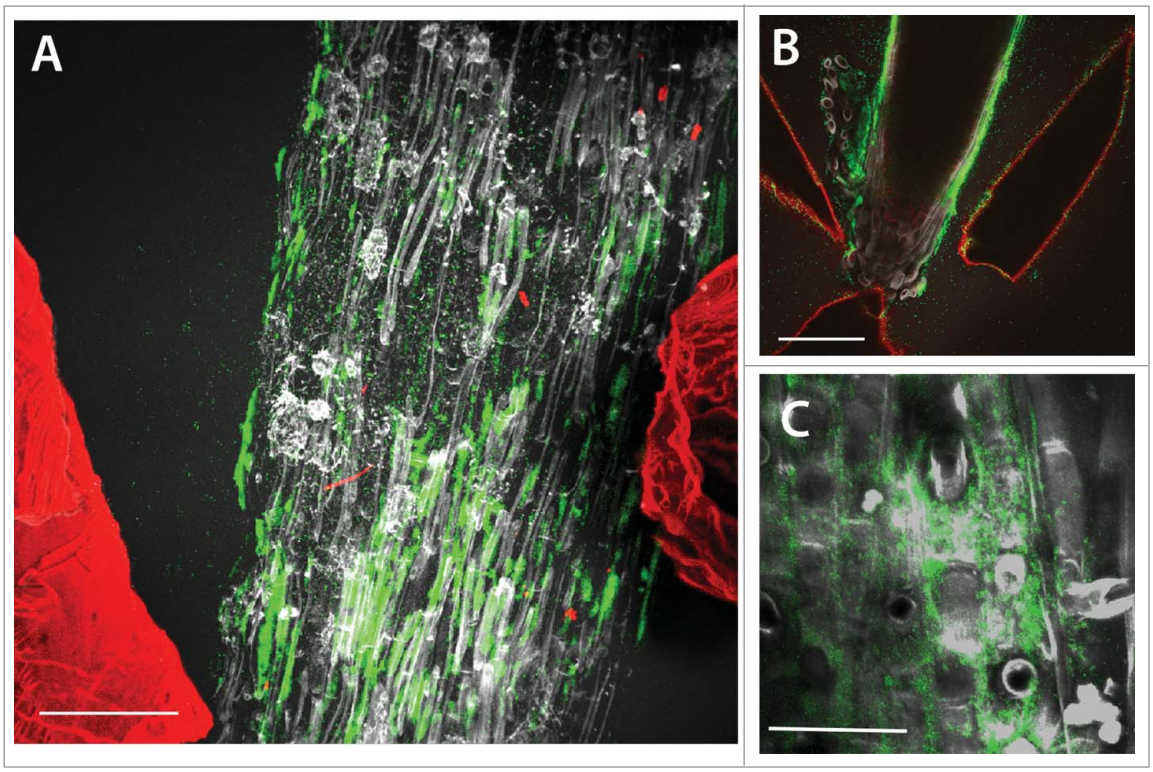

Figure 1. Maximum projection confocal images of GFP-labeled *Pseudomonas fluorescens* colonies (green) on the surface of lettuce root tissues (gray) in situ in transparent soil with Nafion particles from the substrate labeled with sulphorhodamine B fluorescent dye also visible (red). (**A**) The majority of the bacterial fluorescence is associated with the root tissue. Scale bar = 150 μm. (**B**) Bacteria are present on the root tip and in this case also the surfaces of Nafion particles in close proximity to the root have bacterial fluorescence associated with them. Scale bar = 150 μm. (**C**) At higher resolution, bacterial colonisation was predominantly observed in the intercellular junctions of root epithelial cells. Scale bar = 45 μm.

Fluorescent labeling with a range of fluorophores allowed discrimination of bacteria (GFP), root tissue (calcofluor) and the surfaces of solid Nafion particles (sulphorhodamine-B) (**Fig. 1**), which facilitated image analysis (**Fig. 2**).

Bacteria were most abundant on the root surfaces, or rhizoplane, and on the surfaces of Nafion particles (**Fig. 1**). Colonisation on the root surface was concentrated in the intercellular junctions of the root epidermal cells (visual observation in 3 samples, e.g., **Fig. 1C**), which was similar to observations of field-grown wheat roots.[11] Watt et al. quantified the fraction of the volume of soil occupied by *Pseudomonas* spp. found in wheat rhizospheres. Results showed that on average 15%[11] of the soil volume was occupied by *Pseudomonas* spp. We did not characterize the colonisation of lettuce root by *Pseudomonas* spp. in soil, however, the overall mean rhizosphere volume occupied by *P. fluorescens* in the present study is of the same order of magnitude (10%) as those measured by Watt et al. Further studies comparing rhizosphere colonisation with the same plant and bacterial species in both soil and transparent soil would allow a more accurate comparison of the 2 substrates for this application. Bacterial fluorescence was detected in the pore spaces of the substrate, although at a lower level than on the surfaces (**Fig. 1 and 2A**). Image analysis also revealed that the abundance of bacteria in positions with no roots (**Fig. 2Bi**, positions A1–3 and B1–3), was constant and independent of image position, particle size and whether a plant was present or not in the chamber. This may indicate that the effect of the plants on soil microbial abundance could be limited to the substrate directly adjacent (i.e. <1.5 mm) to the root. Along the X axis (horizontal), in samples with plants, the number of discrete bacterial aggregates and the average size of the aggregates was greater on the root (position R) than at 1.5 mm (position A) and 3 mm (position B) from the root, and there was no significant difference in bacterial abundance or aggregate number between positions A and B (**Fig. 2Bii**). In samples with no plants, there was no difference in bacterial abundance along the X axis (horizontal positions). Along the Y axis (vertical), the number of bacterial aggregates was lower at the root tip (position 1, **Fig. 2Bii**) than the 2 positions further from the tip (position 2 and 3, **Fig. 2Bii**) but when the percentage area of the image with bacterial fluorescence was used to quantify abundance, there was no difference along the roots (data not shown). In samples with no plants present, the average size of bacterial aggregate was lowest at position 1 and highest at position 3, therefore the points closest to the surface of the substrate had the largest bacterial aggregates (**Fig. 2Biii**). This could be due to a higher concentration of dissolved oxygen closer to the surface, which has been observed in sludge with better bacterial flocculation at high dissolved oxygen concentrations.[12]

Several studies have described the distribution of PGPR on the surface of plant roots with a range of, and sometimes contrasting

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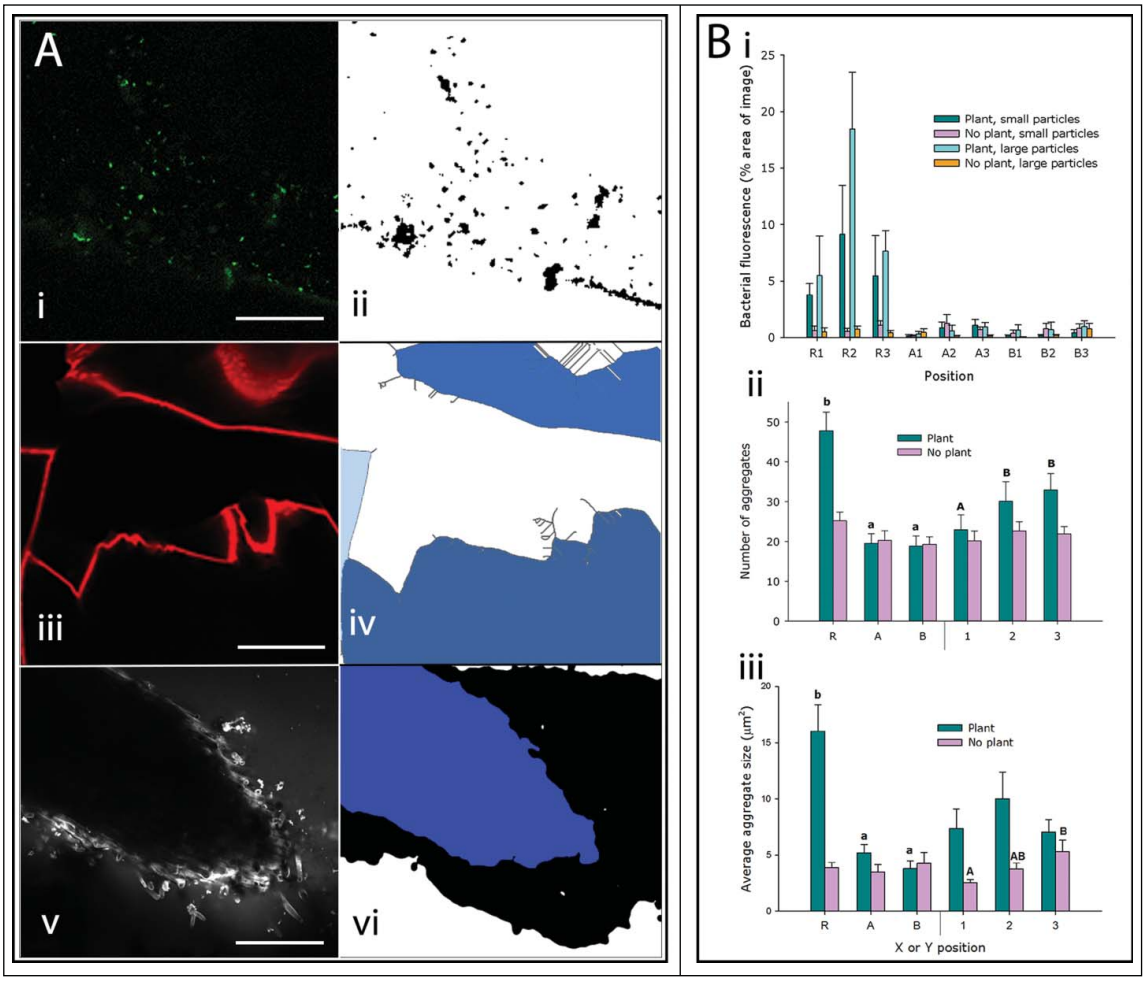

Figure 2. Quantification of *Pseudomonas fluorescens* in the rhizosphere. (**A**) Bacteria, Nafion particles and roots were processed sequentially to allow quantification. (i-ii) Bacterial fluorescence before and after processing with a median filter and thresholding facilitated measuring the bacterial abundance. Scale bar = 40 μm. (iii-iv) Original images of particle surfaces were processed and skeletonised. Gray lines in (iv) represent skeleton of particle surfaces in (iii). It was then possible to select the volumes inside particles (shown here in blue) to measure them to correct for available area (pore space). Scale bar = 200 μm. (v-vi) Example image of a section of lettuce root before and after the application of a median filter and subsequent thresholding were applied. This allowed the selection of the internal volume of the root for measurement (shown in blue). Scale bar = 200 μm. (**B**) Quantification of bacterial distribution in transparent soil with small (500–850 μm) and large particles (850–1200 μm). The positions R1 to B3 represent a 3 × 3 grid of points on and around the roots, where R is on the root and A and B are at intervals perpendicular to the root. 1 is the root tip and 2 and 3 are closer to the shoot. See **Figure S1** for schematic. (i) There was higher bacterial abundance in images that include a section of plant root. At all other positions, there was a consistent area of bacterial fluorescence as a proportion of the area of backgound in images without plant roots. These values were corrected for available area. (ii) Number and (iii) average size of bacterial aggregates at the 3 horizontal (X) positions (R, A & B) and at the 3 vertical (Y) positions (1, 2 and 3) in samples with or without plants. Letters above the bars indicate the results of Fisher's protected LSD tests.

results. High bacterial abundance was found on the root tips[11,13-15] and at root branching zones.[13] Yet other studies reported an absence or scarcity of bacterial colonisation at the root tips[16-20] perhaps caused by the high turnover of mucilage and border cells at the root apex.[20] It is likely that the choice of the technique used to determine bacterial numbers along the root has a strong influence on bacterial count estimates. Methods based on colony forming units (CFU) are inaccurate because they rely on taking samples and this is difficult on the root tip, and only bacteria that grow well in lab cultures can be quantified. Microscopy techniques such as SEM are usually limited to detect bacteria embedded within the mucilage,[16] and methods that requires fixing of samples, e.g., FISH, are susceptible to perturbation for example when washing the roots prior to imaging.[11] The method described in the current study involved the addition and removal of liquids to and from the substrate. Although fluxes of water are common in soil due to rainfall or irrigation, the filling of soil samples by the matching liquid has the potential to induce anaerobic stress in the plant and bacteria over long periods. This effect was minimised by using fresh aerated solutions and by limiting the length of time during which the substrate was saturated. There are numerous non-destructive methods to image in soil, e.g., X-rays, Neutron and Magnetic Resonance Imaging.[21-23] These do not rely on filling samples in liquid, but the methods are not able to resolve many micro-organisms, and imaging of biological processes such as gene expression or cell division is not possible. Molecular methods are developing rapidly, but currently these are

either destructive,[9] or unable to resolve spatial or temporal processes e.g., T-RFLP.[24]

The rhizosphere hosts large and diverse bacterial communities that establish sophisticated modes of interactions with plant roots. To date, it has been difficult to characterize such interactions because observation of roots and bacteria in depth and over time has been limiting.[25] The model system described here overcomes many previous technological limitations. It combines the ability to grow biological organisms in a physically complex soil-like environment with optical microscopy[25] and to detect multiple fluorescent signals in situ. The application of transparent soil microcosms is not limited to the study of roots and soil bacteria and it holds potential for studying the function of other soil organisms. Future developments could see the introduction of a diversity of microorganisms such as mycorrhizal fungi, nematodes, small invertebrates, or the incorporation of bacterial communities composed of several functional types (e.g., predators and prey). Exploiting this potential now requires exploring, testing and analyzing biological activity in transparent soil microcosms to better understand the benefits and limitations of the technology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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