Supplementary information

Materials and methods

Transparent soil microcosms

Transparent soil technique developed recently [21] were adapted to create microcosms where rhizosphere processes can be studied quantitatively. Pellets of the precursor form of Nafion® (Ion Power Inc., New Castle, DE, USA) were used as the starting material for the engineering of the transparent soil substrate. The size of the Nafion pellets was reduced by freezer milling from an initial pellet size of 3 – 4 mm. The particles obtained by freezer milling were sieved regularly during milling and particles larger than 1200 µm were replaced in the freezer milling machine. Different categories of particle size distribution were obtained by sieving and two different categories were used: 500–850 and 850–1200 µm. The resulting particles were chemically treated to give them an anionic charge and the exchange sites were saturated with cations from Murashige & Skoog (M&S) Basal Medium (Sigma-Aldrich Co.) for plant nutrition (Figure S1). A generic protocol for the preparation of transparent soils is available in this document.

Pseudomonas fluorescens SBW25 marked with a mini-Tn*7*–GFP gentamycin-resistance cassette (PrrnB P1 gfp.ASVa, [22]) was used in the transparent soil microcosms. Bacteria were cultured in Luria–Bertani (LB) medium [23] with 1 mg L^{-1} gentamycin at 28 °C at 200 rpm to provide inocula grown to contain ~ 6×10^7 cfu ml^{-1}.

Lactuca sativa (lettuce, var. capitata, Seed Parade, UK) seeds were surface sterilised by washing in 10% bleach (Domestos, Unilever UK Ltd.) for 20 minutes followed by four washes in sterile dH_2O. The seeds were sown in Petri dishes containing 7 g L^{-1} phytagel (Sigma-

Aldrich Co.) and half-strength M&S Basal Medium for pre-germination one day prior to transfer to transparent soil (Figure 1B).

The microcosms were set up in standard purpose-built glass chambers constructed using a microscope slide and long cover glass. The slide and cover glass were assembled into a chamber using a 4 mm spacer placed on the bottom and side edges with an opening at the top of the chamber (Figure 1C). The spacer, slide and cover glass were sealed using Araldite® glass and ceramic adhesive (Huntsman International LLC). Sterile half-strength M&S medium was added to the sterilised Nafion particles to an approximate gravimetric water content of 35%.

50 µl of bacterial suspension was added per gram of hydrated transparent soil which was then mixed to evenly distribute the bacteria. The transparent soil containing plant nutrients and bacteria was then added to the 3D microcosms and compacted by gentle tapping of the chambers. One pre-germinated seedling was added to each transparent soil chamber by placing it in a small hole at the surface of the transparent soil. Controls consisted of samples where only bacteria were present. The glass chambers were covered with aluminium foil to exclude light from the transparent soil and they were placed in a growth room at 20 °C with 16 hours light: 8 hours darkness. Each treatment was replicated four times. In total, 16 samples were used, 4 samples with small Nafion particles with and without plants, and 4 samples with large Nafion particles with and without plants.

Transparent Soil microscopy

Imaging was carried out 5 days after plants and bacteria had been transferred to the transparent soil microcosms. On the day before imaging, all samples were saturated with half-strength M&S medium containing 1 mg ml^{-1} calcofluor (Sigma-Aldrich Co.) to stain the

root tissue (Figure S1B). Immediately before imaging, this solution was removed and replaced with pure Percoll (Sigma-Aldrich Co.) containing 1 μg ml^{-1} sulphorhodamine B (Sigma-Aldrich Co.). The Percoll was used to match the refractive index of the liquid solution with that of the particles (n = 1.34 [24]). The sulphorhodamine B was used for staining the surface of the particles. All draining and addition of liquids was performed using sterile needles and syringes.

27 positions distributed on a 3 by 3 regular grid in the XY plane were imaged in each soil sample (Figure 1D). The origin of the grid was defined at the tip of the root. Three positions were defined along the root (1 to 3), and three positions were also defined perpendicular to the root, R (root axis), A and B. Positions along the roots were regularly spaced by 750 μm. Each position on the grid is therefore identified by a unique ID. For example the root tip is labelled R1, and B3 indicates the furthest position from the root tip (Figure S1D). At each position on the XY plane, three images were acquired. The first image represented the upper surface of the root, and the last image was 90 μm further down in the Z direction, with one image taken in between (Figure S1D). In samples with no plants, a reference position was chosen at a position similar to samples with roots and the same grid system was used for acquiring the images.

Images were taken with a Leica TCS SP2 confocal laser scanning microscope using a 20× / 0.50 NA water dipping objective lens so that the whole diameter of the roots could be observed in a single image. Imaging was carried out at a resolution of 1849 pixels mm^{-2} producing images 1024 × 1024 pixels. GFP fluorescence was excited by illumination with the 488 nm wavelength line of an argon laser and detected between 500 and 530 nm. To image the sulforhodamine B on the Nafion particle surfaces, a 561 nm laser was used for excitation

and light was collected between 580 and 620 nm. To image the calcofluor signal from the lettuce roots, a 405 nm laser was used for excitation and the emitted light was collected between 430 and 470 nm. 2 × line averaging was used throughout. In order to examine the spatial arrangements of bacterial colonies on the root surface, high resolution imaging of the root surface was carried out. High resolution images were obtained at position R2 (60 images in Z stack with a total depth of 97 µm) using a 63× / 0.90 NA water dipping objective lens.

Image processing and analysis

Images were analysed using the open source program Fiji [25]. Images obtained with GFP fluorescence (Figure 2Ai-ii) were processed using a median filter with a 9 × 9 pixel window. A fixed threshold (min. 14, max. 255) was then applied to the images resulting in a binary image representing the fluorescence from bacterial aggregates (Figure 2Aii). In order to quantify these aggregates, images were processed with a despeckle filter and an edge tracing algorithm was used to delineate the perimeter of aggregates [25]. This enabled a number of measurements to be carried out. The measurements included: number of fluorescent bacterial aggregates per image, average aggregate size and area occupied by bacterial fluorescence. At the imaging resolution of 1849 pixels mm^{-2}, individual bacterial cells could neither be resolved nor counted from the images. All measurements were therefore based on the number of pixels with detectable GFP fluorescence above a threshold value.

Images obtained using sulphorhodamine B of particle surfaces were used to quantify the size and geometry of the Nafion particles (Figure 2Aiii-iv). Firstly a fixed threshold was obtained by finding the best visual match between threshold images and the original data.

Thresholded images were followed by the removal of white pixels. A median (window = 5) filter was applied to remove gaps in the particle edges and the edges of the particles were segmented by fixed thresholding. A skeletonisation of the binary image was then applied to delineate the edges of the particles and a growing region algorithm (Magic Wand function in Fiji) was applied to segment the interior of the particles (Figure 2Aiv). A different approach was developed to measure the perimeter of the Nafion particles. The thresholded images of the Nafion particles were processed by morphological erosion followed by a Gaussian filter. The areas of high pixel intensity were then identified using an edge tracing algorithm (Analyse Particle function in Fiji).

Images obtained using calcofluor to stain root tissues were used to quantify root size and geometry (Figure 2Av-vi). A Gaussian filter was applied followed by thresholding to create a binary image where the inside of the root could be selected using the selection tool in FIJI (Figure 2Avi). In some cases the calcofluor was not entirely removed from the soil solution and residual background fluorescence in the soil solution was observed (Figure 2Av). When the background signal from residual calcofluor was too high, root cross sections were traced manually.

Quantification of bacterial spatial arrangement

The quantitative traits used to characterise bacterial colonisation include bacterial abundance calculated as the quotient between area of fluorescence and area of the pore space (%), number of aggregates per image and average aggregate area (μm^2). The mean and standard error for each of these traits were calculated for each sample and at each position described in Figure S1D. The 3 images obtained in the Z direction at each position were treated as pseudoreplicates of the XY position. To analyse the effect of the particle size

and the presence of plants on the bacterial abundance at each position, a general analysis of variance (ANOVA) was performed. Data were square root transformed to satisfy requirement for normality. Post-hoc Fisher's least significant difference (LSD) tests were applied after analyses of variance to provide means ranking. Statistical analyses were carried out using Genstat, 14th edition (VSN International Ltd.).

Protocol: Preparation of Nafion particles to make transparent soil for soil biological experiments

Day 1

Particle size reduction

Raw material: Precursor form Nafion, pellets (Ion Power Inc.)

Reduce size by freezer milling. Use dry particles. Pre-cool in a polystyrene tub by immersing mill tubes in liquid N_2 for at least 5 minutes. Freezer mill performance will vary from machine to machine but we used a SPEX SamplePrep 6850. The desired particle size can be obtained by sieving with the appropriate mesh sizes and several rounds of milling can be used to achieve the desired particle size range. The desired particle size range will depend on the soil type.

Day 2

Ensure conversion of Nafion to anionic form

Solutions

- 15% KOH / 35% DMSO / 50% dH_2O
- 15% Nitric acid (enough for 2 washes)

1. Heat water bath to 80 °C
2. Add KOH solution to particles and place in water bath
3. Heat particles in solution for 5 hours
4. Remove solution and replace with dH_2O. Leave for 30 minutes at room temperature
5. Remove dH_2O and rinse the particles thoroughly with fresh dH_2O
6. Add 15% nitric acid to particles at room temperature and leave for 1 hour
7. Rinse with dH_2O and replace with fresh nitric acid solution and leave overnight

Day 3

Purification of Nafion particle surfaces

Solutions

- 1 M sulphuric acid
- 3 wt % hydrogen peroxide

1. Remove nitric acid solution and rinse particles thoroughly with dH_2O.
2. Replace with 1 M sulphuric acid solution at room temperature.
3. Heat the solution to 65 °C and incubate for 1 hour
4. Allow to cool down to room temperature

5. Remove sulphuric acid, add deionised water and heat to 65 °C
6. Maintain at this temperature for 1h
7. Allow to cool down to room temperature
8. Wash the particles with deionised water multiple times
9. Add 3 wt % hydrogen peroxide solution
10. Heat the solution to 65 °C and incubate for 1 hour
11. Allow to cool down to room temperature
12. Rinse with dH_2O multiple times

Day 4

Nutrient solution titration.

Any plant or microbial nutrient solution that contains cations can be used. We have used Murashige & Skoog Basal Medium (Sigma-Aldrich) at a concentration of 4.4 gL^{-1} in dH_2O (roughly 20 × the volume of particles to be treated is required.) and MSR medium [1] at stock concentration. Note that the nutrient solution used may affect the final refractive index of the particles.

1. Add nutrient solution to Nafion particles in a Duran bottle
2. Put bottle in shaker at 30 °C for 30 minutes
3. Remove nutrient solution and test pH
4. Repeat this procedure until pH is neutral
5. Rinse Nafion thoroughly with dH_2O
6. Autoclave the particles immersed in fresh dH_2O
7. Add fresh, sterile nutrient medium to the particles before use.

Note: Refractive index matching

In this study, Percoll was used as the solution for refractive index matching. It is a commercially available (Sigma-Aldrich) transparent colloid suspension, normally used for density gradient centrifugation of cells and other particles. It is a silica solution with covalently linked silane and is impermeable to biological membranes. Although some light scattering occurs with Percoll because of the colloidal particles, it was deemed to be the most suitable RI matching liquid for this study because of its impermeability to membranes. It would be suitable for imaging at multiple time points to reduce the stress on the plant. We found that by adding pure percoll to our samples immediately before imaging, a good refractive index match could be achieved. This included the liquid already present in the substrate matrix and so some dilution of the percoll occurred. This will clearly vary depending on the moisture content of samples and therefore should be tested before setting up an experiment.

We have also used sorbitol solutions in other cases, especially where root imaging was carried out at only one time point, because it is will exert an osmotic stress on plants and microorganisms in the substrate. We found that a sorbitol concentration of 20% wv^{-1} in dH_2O was appropriate for matching with Nafion particles in samples with a gravimetric water content of approximately 35%.

References

1. Strullu, D.G. and C. Romand, *Méthode d'obtention d'endomycorhizes à vésicules et arbuscules en conditions axéniques.* Les Comptes Rendus de l'Académie des Sciences, 1986. **303**: p. 245-250.