Environmental Microbiology (2006) **8**(5), 871–884 doi:10.1111/j.1462-2920.2005.00973.x

Numbers and locations of native bacteria on field-grown wheat roots quantified by fluorescence *in situ* hybridization (FISH)

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Summary

Native bacteria, *Pseudomonas* and filamentous bacteria were quantified and localized on wheat roots grown in the field using fluorescence *in situ* hybridization (FISH). Seminal roots were sampled through the season from unploughed soil in a conservation farming system. Such soils are spatially heterogeneous, and many roots grow slowly through hard soil with cracks and pores containing dead roots remnant from previous crops. Root and rhizosphere morphology, and contact with soil particles were preserved, and autofluorescence was avoided by observing sections in the far-red with Cy5 and Cy5.5 fluorochromes. Spatial analyses showed that bacteria were embedded in a stable matrix (biofilm) within 11 μm of the root surface (range 2–30 μm) and were clustered on 40% of roots. Half the clusters colocated with axial grooves between epidermal cells, soil particles, cap cells or root hairs; the other half were not associated with visible features. Across all wheat roots, although variable, bacteria averaged 15.4×10^5 cells per mm^3 rhizosphere, and of these, *Pseudomonas* and filaments comprised 10% and 4%, respectively, with minor effects of sample time, and no effect of plant age. Root caps were most heavily colonized by bacteria along roots, and elongation zones least heavily colonized. *Pseudomonas* varied little with root development and were 17% of bacteria on the elongation zone. Filamentous bacteria were not found on the elongation zone. The most significant factor to rhizosphere populations along a wheat root, however, was contact with dead root remnants, where *Pseudomonas* were reduced but filaments increased to 57% of bacteria ($P < 0.001$). This corresponded with analyses of root remnants showing they were heavily colonized by bacteria, with 48% filaments ($P < 0.001$) and 1.4% *Pseudomonas* ($P = 0.014$). Efforts to manage rhizosphere bacteria for sustainable agricultural systems should continue to focus on root cap and mucilage chemistry, and remnant roots as sources of beneficial bacteria.

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Introduction

A major limitation for the prediction and manipulation of the rhizosphere in agricultural systems is the lack of information from field-grown roots (Bowen, 1980; Jones *et al.*, 2004). This is particularly true of rhizosphere microbial ecology. Most information about organisms in the rhizosphere comes either from model systems where roots are grown in a simple medium and organisms of interest are applied, or from soil systems in controlled or field environments, and organisms are washed from the roots and identified on a selective medium, or by DNA analysis. Few studies have quantified native rhizosphere microorganisms of field-grown roots *in situ*. Electron and light microscopy have been used for *in situ* locations of bacteria on field-grown roots; however, stains such as osmium tetroxide and 4,6 diamidine-phenyl indole (DAPI) bind indiscriminately to bacteria, and it is not possible to distinguish different populations beyond differences in morphology (Foster, 1986; Gochnauer *et al.*, 1989; Liu *et al.*, 2001).

Fluorescence *in situ* hybridization (FISH) offers the opportunity to identify and localize rhizosphere organisms native to roots growing in the field. A DNA probe, conjugated to a tag that can be visualized with a microscope, is hybridized to rRNA in chemically fixed cells (DeLong *et al.*, 1989; Amann *et al.*, 1995). To date, FISH has been applied to roots grown in media and soil in controlled environments (e.g. Assmus *et al.*, 1995; Eller and Frenzel, 2001; Briones *et al.*, 2002), and both native and applied organisms have been quantified either directly on the roots or on rhizosphere biofilm and soil isolated from roots. In this study we quantify and localize bacteria in general, and *Pseudomonas* in particular, on wheat roots growing in the field.

Pseudomonas species have long been considered important rhizosphere colonizers (Sørensen *et al.*, 2001).

Their impact on plant growth is variable and *Pseudomonas* isolates from the rhizosphere reapplied to plants can inhibit, promote or have no effect on plant growth (Gerhardson *et al*., 1985; Lugtenberg and Dekkers, 1999). Growth promotion may be through inhibition of a pathogen, or production of a plant-growth stimulant. Inhibition may be due to production of a compound toxic to the plant (Bolton *et al*., 1989). These effects depend on densities and locations of *Pseudomonas* cells on the roots, and this information is not known for roots in any field system.

We focus on wheat roots grown in a conservation cropping system where seeds are direct-drilled into the soil without ploughing. *Pseudomonas* from the rhizosphere of direct-drilled wheat can inhibit wheat root growth when applied in agar at 10^7 cells per ml (Simpfendorfer *et al*., 2002). Roots in unploughed soils grow 60% more slowly compared with those in ploughed soil, bend twice as often, and half their length is in contact with dead roots remnant from previous crops (Watt *et al*., 2006). Slow-growing roots in hard soil accumulate *Pseudomonas* within 1 cm of the root tips, according to culture studies (Watt *et al*., 2003). Here we set out to (i) quantify *in situ* numbers of total bacteria and *Pseudomonas* on field-grown wheat roots and (ii) test whether factors such as time of year, position along roots and contact with dead remnant roots alter *Pseudomonas* numbers. We compare these results of native bacteria on roots in the field to previously reported controlled environment and culturing studies. One unexpected result of preliminary FISH labelling of the field-grown roots was very obvious colonization by filamentous bacteria. Consequently, these are also quantified and localized in this study.

Results

Optimization of confocal analysis

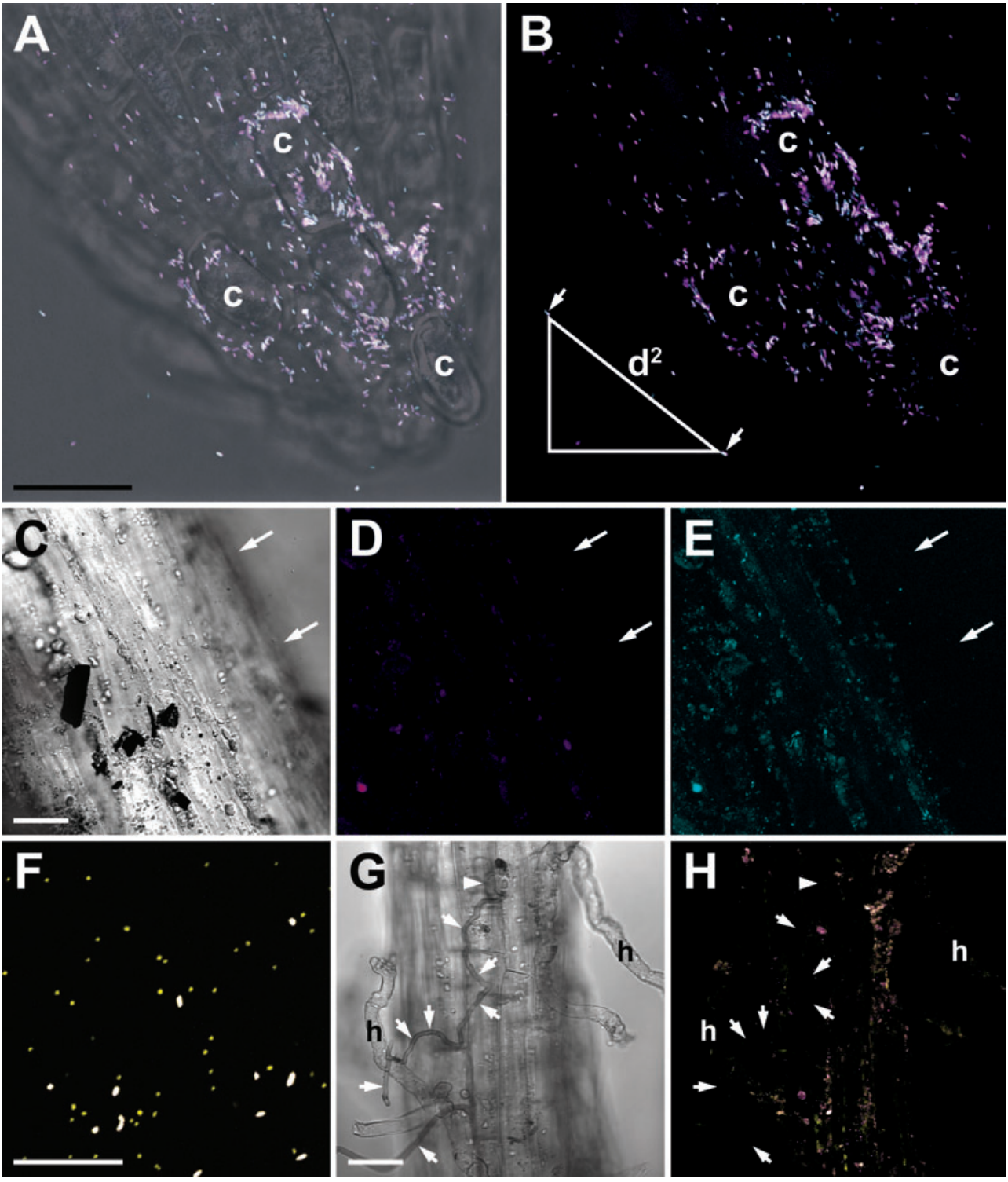
Fluorochromes with high molar extinction coefficients that are excited by wavelengths between green and orange, such as fluorescein, Cy3 and ROX, were not used on field-grown roots because soil autofluorescence was too high to reliably count labelled bacteria (Fig. 1C–E). Reliable counts of labelled bacteria on field-grown roots were only achieved when samples were excited at 633 nm (Fig. 1D), where soil and root autofluorescence at longer wavelengths was much less. We were able to detect Cy5- and Cy5.5-labelled cells in the same preparation following illumination with 633 laser (Fig. 1F). Cy3-labelled probes were very visible on agar-grown roots where interference from soil particles was not a problem (Fig. 1A and B). Autofluorescence from root apices was minimal in both agar and field-grown roots (compare Fig. 1A and B with Fig. 2B and C). Some field-grown root surfaces had non-specific fluorescence from the probes (binding of oligonucleotide or fluorochrome) or autofluorescence from the root surfaces even in the long red wavelengths, and these tended to be older roots (Fig. 2I and J). Fungal hyphae did not bind the probes (Fig. 1G and H). Filamentous bacteria were labelled by the EUB338 probe, and fine and thick filaments were quantified when present (Fig. 2I–K).

The confocal settings were a compromise between the largest area possible on the root and the maximum number of pixels per bacterium. Thus, in images captured for analysis, the pixel dimensions were 0.133 μm by 0.133 μm, and the minimum particle size counted as a bacterium during image analysis was 0.4 μm (> 3 pixels depending on digital zoom). The confocal settings were also chosen to optimize contrast and minimize background. They had a large influence on particle size, and analysis of the dye-impregnated calibration beads showed that they enlarged particles by a factor of 1.79. Thus, it was important to maintain confocal settings across samples as particles of less than three pixels were cut off during analysis.

Spatial arrangement of bacteria within the rhizosphere

Bacteria were bound to the root within a mean depth, *z*, of 11 μm from the root surface (range 1.7–31 μm). Agar-

Fig. 1. A and B. Apex of a wheat seminal root grown on agar with applied *Pseudomonas* sp., and hybridized with EUB338-Cy5 (magenta) and PSE227-Cy3 (cyan) probes. (A) Composite of the brightfield image of the root (grey) with root cap cells (c) visible on the apex surface, and the fluorescence images of the bacteria. Note bacteria retained to left of tip in mucilage. (B) Fluoresence images of the bacteria. White cells are the *Pseudomonas* cells that have both EUB338-Cy5 (magenta) and PSE227-Cy3 (cyan) probes. Bacteria tend to surround cap cells (c). Distances between two bacterial cells (arrows) with coordinates (x, y) and (x^1, y^1) were calculated from d^2 as described in the text.
C–E. Surface of a field-grown root processed through hybridization steps without probes with fluorochromes (control) and viewed with a confocal microscope as for labelled samples. (C) is brightfield image. Dark soil particles are scattered on the root surface. Arrows indicate edge of the root. (D) Root excited with Cy5 excitation. (E) Root excited with Cy3 excitation. Note many more bright spots similar in size to bacterial cells, on Cy3-excited surface compared with Cy5-excited surface (E) that are due to autofluorescence.
F. Suspension of cultured *Pseudomonas* sp. (rod-shaped cells) and *Acinetobacter* sp. (round cells) mounted on a slide and viewed with confocal microscopy after hybridization with EUB338-Cy5.5 (yellow) and PSE227-Cy5 (magenta) probes. Image is a composite of yellow and magenta fluorescence images. All cells have bound the EUB338 probe (yellow), and the *Pseudomonas* cells have also bound the PSE227 probe (magenta), such that they appear white.
G and H. Surface of a field-grown wheat root viewed with a confocal microscope after hybridization with EUB338-Cy5.5 (yellow) and PSE227-Cy5 (magenta) probes. (G) is brightfield image and (H) is composite of fluorescence images. Probes have bound to bacteria on the root surface. Arrows surround a fungal hypha that has not bound either of the bacterial probes. Arrowhead points to fungal vesicles within the root. h, root hair
Bars = 40 μm for all images except for (F), where bar = 20 μm.



grown roots clearly showed that bacteria were retained by root cap mucilage despite sample processing (Fig. 1A and B). The *Pseudomonas* and filamentous bacteria were both at varying distances from the root surfaces, and we did not determine whether the different populations were more associated with the inner or outer regions of colonization.

Approximately 40% of all rhizospheres observed had half the bacteria clustered into 15% of the rhizosphere volume (Table 1), such that 60% did not have clustered

Table 1. Clustering of bacteria in the rhizosphere of wheat roots from the field.[a]

	Rhizospheres with clustered cells (% total observed)	Cells in clusters (% total rhizosphere cells)	Volume rhizosphere with clustered cells (% total rhizosphere volume)	Cells per cluster volume ($\times 10^5$ mm^{-3})	Features associated with clusters (% total clusters observed)				Cluster volume (mm^3)	Distances between cells in clusters (μm)		
					None visible	Soil	Root hairs	Axial grooves	Cap cells	Mean	Smallest	Largest
Bacteria non-filamentous	37	52 (19–86)	12 (0.1–37)	73.6 (9.57–244)	42	21	7	28	2	5.57×10^{-5} (1.23×10^{-7} to 1.54×10^{-4})	84 (64–110)	0.88 (0.38–1.45)	170 (118–204)
Pseudomonas	43	63 (30–100)	15 (1–37)	24.4 (9.92–60.3)	41	31	10	7	10	5.44×10^{-5} (3.22×10^{-6} to 1.6×10^{-4})	87 (73–103)	1.05 (0.66–1.44)	165 (119–213)

a. Groups of cells judged by eye to be clustered compared with overall cells were outlined with analysis software and volumes of clusters and their cells were compared with the total rhizosphere volume and cell numbers (see *Experimental procedures* for additional information). Mean and range in brackets presented.

cells that were noticed by eye. Bacteria were five times denser within clusters than outside, while *Pseudomonas* were 17 times denser within clusters than outside. The average rhizosphere volume observed in a microscope field was 4.6×10^{-4} mm^3 (range 2.5×10^{-5} mm^3 to 1.3×10^{-3} mm^3), and the average cluster volume was approximately one-tenth of this volume. Bacteria within clusters were as close as 1 μm and as far apart as 213 μm. Distances between the centres of clusters ranged widely between 10 μm and 162 μm and did not vary between axial and radial directions on roots. Approximately 60% of clusters colocated with features listed in Table 1. Although bacteria were observed in axial grooves between epidermal cells (Fig. 2F and G), they were never observed in radial grooves. Approximately 40% of all clusters did not colocate with a physical, recognizable feature on the root surface, and thus likely were initiated by chemical factors, or by physical features that were no longer bound to the root surface.

Temporal, positional and root type impacts on rhizosphere bacterial densities

The mean densities of bacteria in the rhizospheres of current crop wheat roots, sampled at three times of the season, are presented in Table 2. Numbers were generally lower in early winter for bacteria and *Pseudomonas*, but neither filamentous bacteria nor *Pseudomonas* changed as a percentage of total bacteria through the season. Across all samples, bacteria ranged widely from 0 to 85.9×10^5 cells per mm^3 rhizosphere. Although filamentous bacteria and *Pseudomonas* on average accounted for 4% and 9%, respectively, of the bacteria across all three sampling dates, these percentages ranged widely from 0% to 42% for filaments (0–9.89×10^5 cells mm^{-3}) and from 0% to 56% for *Pseudomonas* (0–6.05×10^5 cells mm^{-3}).

Remnant roots from previous crops or weeds were often present in samples, either separated from the current crop wheat roots, or still bound after sample preparation (Fig. 2H). We compared these remnant roots and current crop roots when harvested at the same time (Table 3). Remnant roots had 1.8 times more bacteria than current roots, significant at the 80% probability level (Table 3). Remnants, however, had 23 times more filamentous bacteria than current roots (Fig. 2I), but five times fewer *Pseudomonas* than current roots. Thus, the percentage of filamentous bacteria was 10 times higher ($P < 0.05$), and the percentage of *Pseudomonas* was 18.6 times lower ($P < 0.05$), on remnants than on current roots. Overall mean values of both root types masked these differences.

We re-analysed the results from different sampling times, and grouped them into five different positions along

Table 2. Densities of bacteria in the rhizospheres of wheat roots of current crop sampled from the field at three times, and quantified using FISH.[a]

Sampling time	Bacteria $\times 10^5$ cells mm^{-3}			% Filamentous bacteria	% *Pseudomonas*
	Total	Filamentous	*Pseudomonas*		
Overall mean	15.4	0.573	1.46	3.7	9.4
Early winter	5.33	0.0888	0.632	1.5	11.2
Mid-winter	20.7	0.790	1.35	3.8	6.5
Spring	16.5	0.660	2.13	4	12.9
SED	7.35	0.769	0.629	3.7	6.4
F-pr	0.201	0.718	0.135	0.979	0.986

a. Numbers per sample date were compared by one-way ANOVA and means, SED and probabilities of difference between harvest dates presented. Percentages are of total bacteria. Remnant roots not included. $n = 33$ for all samples; 9 for early winter; 12 each for mid-winter and spring.

roots (Fig. 2A and H): the root cap, elongation zone, young root hairs, mature region and mature region in contact with remnant root (Fig. 3). Bacteria were most abundant on the root cap and least abundant on the elongation zone (Fig. 3A; see images in Fig. 2B–E). Filamentous bacteria and *Pseudomonas* were a similar percentage of total bacteria at the root cap (12%) (Fig. 3B) and *Pseudomonas* numbers varied little along roots (Fig. 3A). On the elongation zone, *Pseudomonas* comprised 17% of all bacteria present and filamentous bacteria were absent. Regions close to remnant roots, however, were dominated by filamentous bacteria (57% of all bacteria). The large impact of contact with remnant roots on filamentous bacteria in the rhizosphere corresponds with the high number of filaments on remnants (Table 3).

Discussion

The use of FISH to quantify bacteria on field-grown roots

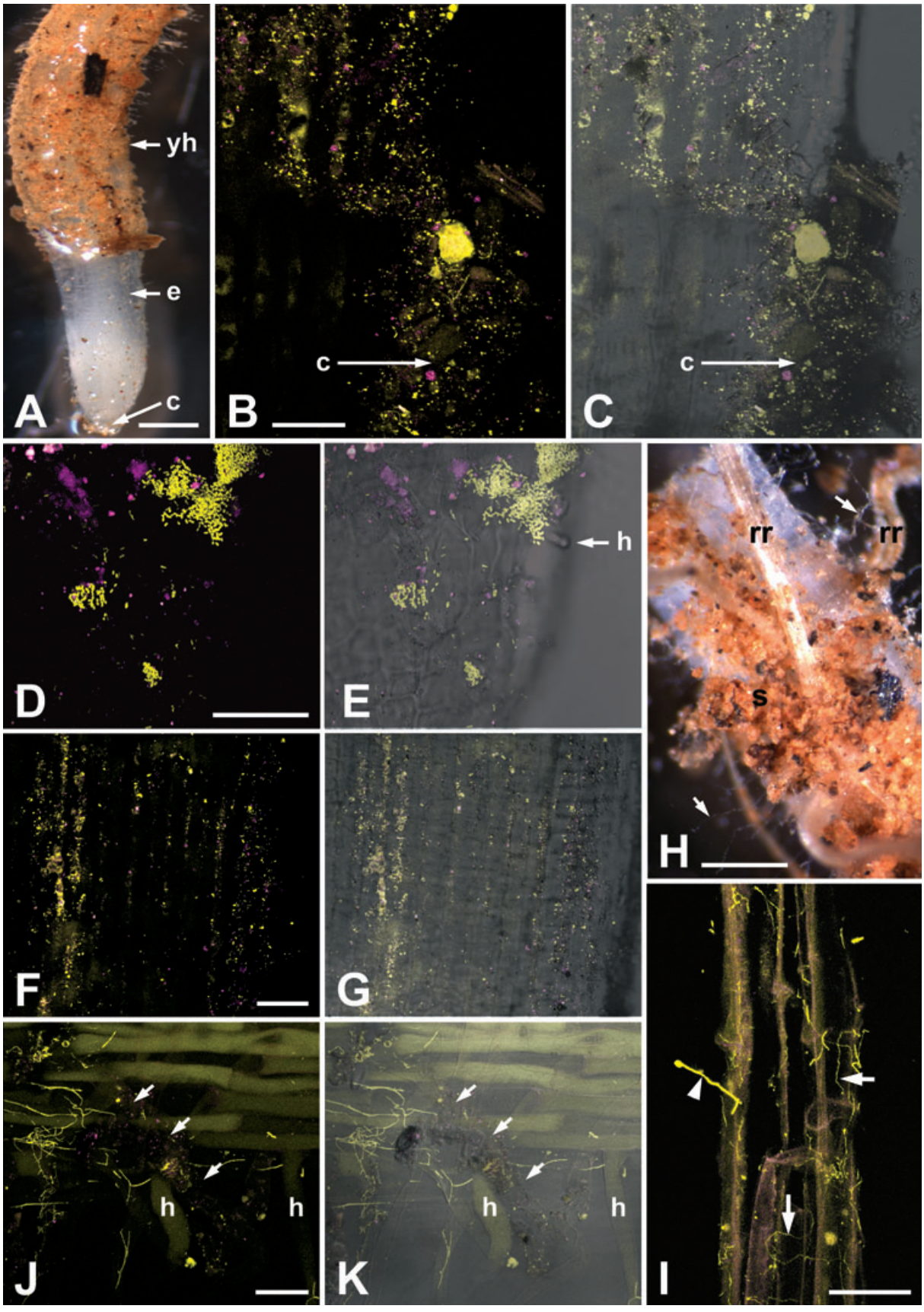
The overwhelming advantage of FISH for rhizosphere studies, compared with other microbiological methods, is the ability to identify, localize and quantify bacteria *in situ*. The distinctive morphology of the filamentous bacteria makes them particularly obvious on roots and amenable to study with FISH. Fluorescence *in situ* hybridization is most powerful when samples are well defined, and spatial features that alter bacterial communities are present in a microscopic field of view. We paid close attention to sampling seminal roots at all sampling times, and selecting common tissue types such as apices to minimize sample variation. Features within microscopic fields of view such as dead root remnants were also noted, allowing us to correlate these with bacterial populations. Some field studies of rhizosphere bacteria using culturing or DNA extraction methods have also sampled carefully (e.g. Marschner *et al*., 2001). The area observed on the root surface with microscopy is very small compared with the entire surface of a root, or root system. For example, the average rhizosphere volume observed was approximately 0.64% of the rhizosphere associated with 10 mm of root if the root diameter is 0.5 mm and the rhizosphere depth is 11 μm, and 0.02% of the rhizosphere associated with 10 mm of root if the rhizosphere depth is 1 mm. Thus, it is nearly impossible to sample sufficient area to overcome sample variability and thereby to use FISH to distinguish gross field treatments (Kent and Triplett, 2002), but it is possible to identify variation when samples and features on samples are well defined.

Fluorescence *in situ* hybridization has a number of limitations for work with field-grown plants. The first is interference from soil autofluorescence (e.g. Briones *et al*., 2002). By using Cy5 and Cy5.5 that emit in the far-red, we avoided most soil autofluorescence but we were only able to reliably study *Pseudomonas* and single cells versus filamentous cells as a subset of total bacteria, at any one time. Second, bacteria are likely underestimated

Table 3. Densities of bacteria quantified by FISH in the rhizospheres of current crop wheat roots and remnant dead roots from previous crops or weeds.[a,b]

Root type	Bacterial cells $\times 10^5$ mm^{-3}			% Filamentous bacteria	% *Pseudomonas*
	Total	Filamentous	*Pseudomonas*		
Overall mean	25.5	9.09	0.759	35.6	3.0
Current crop	20.7	0.79	1.35	3.8	6.5
Dead root remnants	38.3	18.2	0.256	47.5	1.4
SED	15.5	8.81	0.43	8.15	4.63
F-pr	0.268	0.059	0.018	< 0.001	0.014

a. Current crop and dead remnant roots were sampled at the same sample time, in mid-winter.
b. Numbers were compared by one-way ANOVA and means, SED and probabilities of difference between the two root types presented. Percentages are of total bacteria. $n = 26$ for all samples; 13 each for current crop and dead root remnants.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due to the image resolutions and confocal settings. The smallest bacterial cell quantified was > 3 pixels or > 0.4 μm diameter. Cells < 0.4 μm diameter very likely were present but may have been quite inactive and had low ribosomal number (Christensen *et al*., 1999). Numbers per rhizosphere were underestimated by deriving volumes from projected surface areas that did not account for root curvature and other features that altered topology, and from stacks of images that masked any bacteria overlapping in the *z* dimension. In future an increase in the scanning resolution, and the size of screen used to capture the images should be used with software to account for true volume.

The third limitation to FISH is that cells observed in a sample are only those into which probes have penetrated and have sufficient ribosomal number to produce a visible fluorescent signal. In a very thorough review of the use of FISH across different environmental samples, Bouvier and Del Giorgio (2003) found the EUB338 probe bound to 1–100% of cells in samples, depending on a number of factors including sample source and preparation. EUB338 bound to 35% of DAPI-stained cells from the rhizoplane of rice grown in a soil microcosm (Eller and Frenzel, 2001). Further, EUB338 does not bind to all bacterial cells, notably the *Planctomycetes* and *Verrucomicrobia* (Daims *et al*., 1999). Finally, perhaps the most important limitation of FISH and other microscopic methods (Foster, 1986) is that the observed cells and their spatial arrangement are those still remaining after sample preparation. Given that the roots are maintained in aqueous solution and the hybridization step has salt, ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS), it is very likely that the *Pseudomonas*, filamentous and other bacteria observed are bound to or within root and bacterial mucilage by polymer bonds (Marshall, 1980). This layer has been characterized using electron microscopy, biochemical and histochemical analyses. It is comprised of root cap and bacterial muco-polysacharides that are resistant to hydration, and therefore loosening of bacteria, after drying (Watt *et al*., 1993). However, still very little is known about what determines the types, numbers and positions of organisms embedded in this matrix, or 'biofilm', on soil-grown roots (Morris and Monier, 2003). These organisms, and the metabolites and signals involved in their regulation, are possibly retained very close to the root in this biofilm, and as such are resistant to washing by soil water movement. The development and processes that occur in this matrix are likely central to root–microbe interactions and should continue to be the focus of research.

Overall bacterial numbers and comparisons with reports in the literature

We compared our results with a selection of reports in the literature of roots exposed to either bacteria native to the growth medium, or to bacteria inoculated onto the seed or the medium (Table 4). We converted our units of bacteria per rhizosphere volume to compare with other studies despite the differences that arise from using root or soil weights to express bacteria (Duineveld and van Veen, 1999). Overall, we found our FISH numbers for bacteria similar to or slightly lower than other studies with FISH on soil-grown plants in pots, and the total bacteria, *Pseudomonas* and filamentous bacteria within ranges determined by culturing from soil-grown roots. Fluorescence *in situ* hybridization may target viable cells with high ribosomal numbers that are easily cultured on selective media. Alternatively the close agreement between FISH and culture results indicate that the rhizosphere, particularly at the root surface, may indeed have metabolites and signals that favour *Pseudomonas* and filamentous bacteria.

The mean *Pseudomonas* numbers obtained here with FISH are approximately 10- to 100-fold lower than the

Fig. 2. A. Apex of a wheat seminal root from the field viewed with a dissecting microscope with root cap (c), elongation zone (e) and the region where root hairs are starting to emerge from the surface (yh). These regions were sampled for bacteria in the rhizosphere (Fig. 3). Bar = 0.3 mm.
B and C. Flank of a seminal root apex harvested from the field, with cap (c) and adhered soil, and hybridized with EUB338-Cy5.5 (yellow) and PSE227-Cy5 (magenta) probes. (B) A composite image of EUB338-Cy5.5 fluorescence and PSE227-Cy5 fluorescence images. (C) Brightfield (grey) image added to (B) to show the root surface structure.
D and E. Flank of a seminal, branch root apex harvested from the field and hybridized with EUB338-Cy5.5 (yellow) and PSE227-Cy5 (magenta) probes. Hair (h) is emerging close to the tip. Bacteria (yellow) are distinct from *Pseudomonas* bacteria (white/magenta). (D) shows fluorescence images only; (E) shows composite image with brightfield view of root and fluorescence images of bacteria.
F and G. Expansion zone of wheat root hybridized with EUB338-Cy5.5 (yellow) and PSE227-Cy5 (magenta) probes. (F) Fluorescence images only. (G) Composite of brightfield (grey) view of root surface and fluorescence images with bacteria. Bacteria are aligned along the axial grooves overlying the epidermal cells.
H. Wheat seminal root from the field with a dead root remnants (rr) associated, and viewed with a dissecting microscope. Arrows point to root hairs of the seminal root. S, soil. Bar = 0.25 mm.
I. Dead root remnant hybridized with EUB338-Cy5.5 (yellow) probe. Thicker (arrowhead) and finer filamentous bacteria (arrow) that have bound the EUB338 probe are present over the surface.
J and K. Wheat root surface, mature with root hairs (h), and pieces of remnant root (arrows), hybridized with EUB338-Cy5.5 (yellow) and PSE227-Cy5 (magenta) probes (J shows brightfield view with fluorescence images). Filamentous bacteria with bound EUB338 probe run along root hairs, possibly originating from remnants.
Bars = 40 μm for all images except (A) and (H).

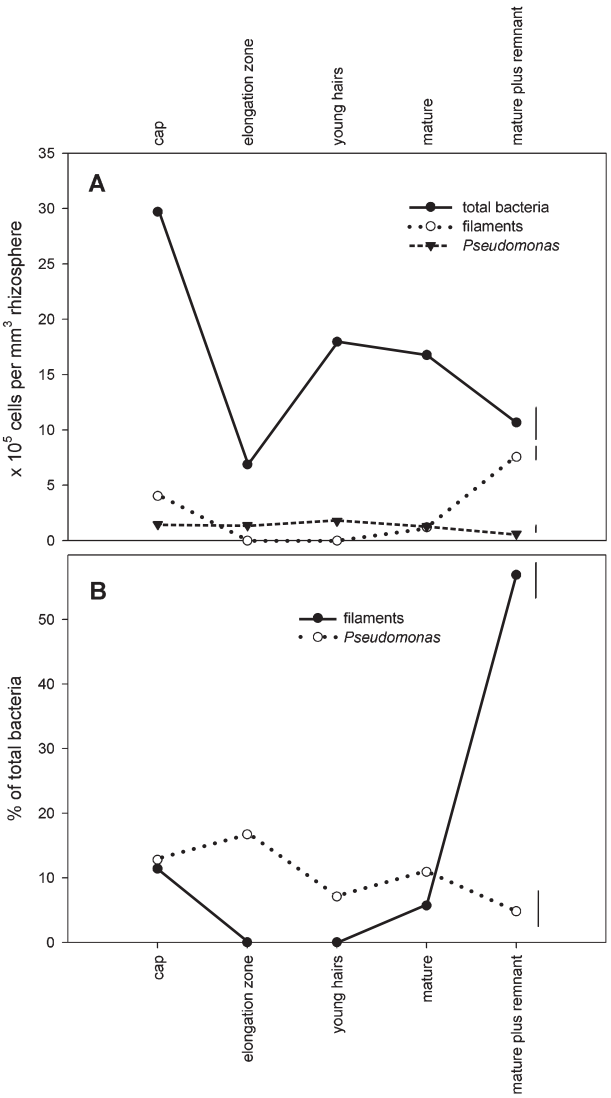

Fig. 3. Numbers of bacteria at different positions on roots of field-grown wheat, identified and quantified with FISH (A), and per cent *Pseudomonas* and filaments of total bacteria (B). Values analysed with a one-way ANOVA for position and means and SED (bar) for position presented. $n = 6–15$ roots for each position. Points joined for clarity.

numbers of *Pseudomonas* recovered from, or quantified directly on, roots of plants initially inoculated with those strains (Table 4). Inoculated cells generally have a very poor survival rate on roots, even in controlled environments (see Loper *et al*., 1985). Our *Pseudomonas* numbers within clusters (Table 1), however, are a similar density to inoculants surviving on roots. Thus, the percentage of any one species of inoculated *Pseudomonas*, such as a biocontrol agent or a deleterious strain producing a toxin to plants, is likely to constitute a minor portion of the native population unless it proliferates with a specific rhizosphere substrate, either from the current wheat root or on dead remnants that contact wheat roots.

Spatial analysis and possible factors regulating bacterial distribution on the root

Fewer than half the rhizospheres observed had significantly clustered bacteria along the length and width of the root. Forty per cent of these clusters colocated with either axial grooves on the epidermal surface, soil particles, root hairs or cap cells, features reported in the literature as associated with bacterial clusters, mainly from cultured roots and bacteria. More than half the rhizospheres in the field did not have noticeably clustered cells, and only 50% of those clustered were over-recognized features, while the other 50% did not colocate with a recognized physical and structural feature. This would suggest that the often reported, but not quantified, clustering of bacteria in axial grooves in controlled environments does not occur as frequently in the field.

Clusters observed that did not colocate with a recognized feature were possibly triggered by either local or diffusible exudates from roots or other organisms such as *N*-acyl-L-homoserine lactone (AHL) molecules used by bacteria to sense population density (quorum sensors). *N*-acyl-L-homoserine lactones move as far as 70 μm to reach native bacteria in soil-grown tomato rhizospheres (Steidle *et al*., 2001). Results reported here show that bacteria within and outside clusters are located well within 70 μm from each other, and they are embedded in a matrix that may retain such signals, facilitating exchange and protection from degradation which can occur very rapidly in some soil (Wang and Leadbetter, 2005). Quantification of *in situ* cell-to-cell clustering is important for future rhizosphere studies as it provides the distances that organisms and molecules must travel in the rhizosphere to interact (Watt *et al*., 2006).

Impact of position along root and contact with dead root remnants

The high number of bacteria on root caps (Fig. 3) supports an important role for the root cap in initially stimulating rhizosphere bacteria (both filamentous and *Pseudomonas*) upon arrival of the tip in a volume of soil (Gochnauer *et al*., 1990; Hawes *et al*., 1998). The elongation zone had the lowest numbers of bacteria and no filaments, indicating high spatial variability along apices, and that it is necessary to divide apices into caps and elongation zones. Our numbers were highly variable, but suggested that *Pseudomonas* were stimulated by the root cap, and remained embedded in the mucilage of the cap into the elongation zone, while filaments were not.

Most striking was the abundance of filamentous bacteria where wheat roots contacted dead root remnants. These may have included pleomorphic bacteria capable

Table 4. Comparison of bacterial numbers in the rhizosphere quantified in this study with a selection of studies reported in the literature using other methods.

Reference	Organisms	Bacterial cells or colony-forming units				Plant and growth conditions	Quantification method
		Per cm root	Per g soil	Per cm^3 root	Per mm^2 root		
This study[a]	Total bacteria *Pseudomonas* Filamentous	2.6×10^5 2.5×10^4 1.0×10^4	4.42×10^7 4.2×10^6 1.7×10^6	1.3×10^8 1.3×10^7 5.0×10^6	1.7×10^4 1.6×10^3 6.3×10^2	Wheat seminal roots, field	FISH, confocal microscopy
Briones *et al.* (2002)	Bacteria			4.0×10^8		Rice, flooded in soil in pots	FISH, confocal microscopy
Lübeck *et al.* (2000)	Bacteria				1.8×10^5	Sugar beet, soil in pots	FISH, confocal microscopy
Watt *et al.* (2003)	Bacteria	2×10^6 (base) to 2×10^7 (tip)				Wheat seminal roots, field	DAPI (bacteria)
	Pseudomonas	1×10^4 (base) to 1.5×10^5 (tip)					*Pseudomonas (*culturing)
van Vuurde and Schippers (1980)		2×10^4 (tip) to 2×10^5 (base)				Wheat, soil, pot	Culturing
Simpfendorfer *et al.* (2002)	Aerobic bacteria *Pseudomonas* Filamentous			8.6×10^7 8.0×10^6 8.0×10^6		Wheat, field	Culturing
Gochnauer *et al.* (1989)	Total viable bacteria *Pseudomonas* Filamentous			10^9 7.5×10^7 (sheathed roots) 3.9×10^7 (bare roots) 0 (sheathed roots) 3.1×10^5 (bare roots)		Maize, field	Culturing
Persello-Cartieaux *et al.* (2001)	*Pseudomonas* inoculated into growth medium	4×10^4 to 10^6				*Arabidopsis*, phytagel, soil	Culturing, microscopy of GFP-labelled bacteria
Chin-A-Woeng *et al.* (1997)	*Pseudomonas* inoculated onto germinated seed	10^2 (tip) to 10^6 (base)				Tomato, sand	Electron microscopy

a. Bacteria were expressed in a range of units by estimating root and rhizosphere diameter and volume, and dry weights to compare with other published values.

of converting from single cells to filaments, or members of the actinomycetes (Thirup *et al*., 2001, and references therein). In these regions of contact with root remnants, the wheat root epidermis and root hairs were still associated with the root suggesting that they were immature. Van Vuurde and Schippers (1980) found actinomycetes on the mature regions of seminal roots of young wheat plants, but not on the young root regions, and Gochnauer and colleagues (1989) found abundant filamentous bacteria (up to 30% of viable rhizoplane bacteria) on the mature regions of maize roots that were shedding their cortices and soil sheaths. In these two examples, the rhizosphere filamentous bacteria appear associated with root ageing.

The filamentous bacteria on the relatively young wheat roots were very localized, and indicate strong influence of the previous season's roots on the rhizosphere organisms of new crop roots (Fig. 2J and K). Such close association and sharing of soil organisms between current and previous seasons' roots is supported by the ability of some fungal diseases to infect new roots season to season, and how rhizobia infect newly planted legumes. It also offers an opportunity to encourage biocontrol or other beneficial organisms to inoculate new crop roots (Bowen, 1980). Kluepfel (1993) tracked inoculants through the soil profile in the field and found inoculant mostly where root tissue from previously inoculated wheat plants were. It may be possible to encourage specific actinomycetes on remnants so they can inoculate the new wheat, and hence offer protection from deleterious organisms or other growth inhibitor. In a recent quantification of root-remnant contact in the field (Watt *et al*., 2006), at least half of the current wheat crop root length was in contact with remnant roots in direct-drilled and cultivated soils, and this was dramatically increased after a perennial pasture crop, suggesting contact and soil organisms can be regulated by selecting different preceding crops.

Dead root remnants had high numbers of bacteria, a high abundance of filaments and low level of *Pseudomonas*. Actinomycetes are traditionally associated with decomposition of complex organic compounds. Thirup and colleagues (2001) followed native *Pseudomonas* and actinomycete populations by quantitative polymerase chain reaction (PCR) on barley roots grown in soil. Actinomycete DNA steadily increased in rhizosphere soil at the base of the barley roots until plant harvest (60 days), then dropped initially, but then increased steadily on the dead remnants to 120 days from planting, when measurements stopped. *Pseudomonas* DNA also increased during plant growth but dropped to low levels after harvest and did not increase. These results support our finding of filaments with high rRNA on dead roots remnant from previous seasons. The root remnants we observed were at least 7 months old.

Conclusions

High spatial variability between roots and positions along roots makes FISH, and indeed other currently available microscopy methods for *in situ* bacteria, of limited value for studies of gross field treatments on the rhizosphere of a root system, but very valuable to quantify factors directly influencing local differences at the millimetre scale along roots. Fluorescence *in situ* hybridization and confocal microscopy reveal bacteria embedded within water-stable polymers very close to the root, where solutes can move quickly between roots and organisms relative to those farther from the root (Watt *et al*., 2006). Filamentous bacteria changed more as a percentage of the bacteria than did *Pseudomonas*, suggesting that these populations either are morphologically flexible, having switched from single cells to filaments, or are predicable actimomycete successors, and thus are more readily manipulated in agricultural systems. This study confirms that key areas for regulating the rhizosphere are the chemical nature of the root cap, expansion of the elongation zone, contact with remnants and chemistry of the polymer matrix that possibly retains specific organisms and compounds close to the root.

Experimental procedures

Field-grown roots

Roots of wheat (*Triticum aestivum* L. cv. Janz) were sampled from the surface 20 cm of soil of unploughed plots (seed direct-drilled) that were part of a larger rotation and tillage trial in south-east New South Wales (NSW), Australia. The soil is an acidic, red-brown earth (Kandosol). Wheat was sown in May, 2003. Nitrogen was applied to the surface of the soil at sowing, and fertilizer containing phosphorus (20 kg ha^{-1}), nitrogen (18 kg ha^{-1}) and sulfur (14 kg ha^{-1}) was applied with the seed. The crop sown the preceding year was wheat. Roots were sampled three times during the season, from at least three plants from three randomly spaced plots at each sampling. We sampled seminal roots at each sampling, ensuring that both apices and more mature tissues were included in each sample. Sampling dates were in early June when seedlings had just emerged and leaf 1 was developing, early July when leaf 3 was developing and late September when plants were mature with tillers. Roots were carefully exposed with a shovel and by hand, and 1- to 2-cm-long pieces were excised with a blade and forceps and placed directly in 1 ml of sterile fixative on ice in an eppendorf tube (details below). No attempt was made to shake off adhering soil or dissociate roots of the current wheat crop from dead root remaining from previous seasons.

Agar-grown roots for method development

Seedlings of wheat (cv. Janz) were grown on agar (1% w/v in quarter-strength Hoaglands solution) to develop methods of preparing tissue to minimize root damage, probe labelling,

and to quantify bacteria. At 3–5 days from primary seminal root emergence, plates were opened and roots (which were growing along the agar surface) were inoculated at their tips with 10 μl of a suspension of *Pseudomonas* sp. (approximately 10^8 cells ml^{-1}), and incubated for 1–2 days at 20°C. Roots were then gently lifted from the agar surface and pieces 1–2 cm long excised and immediately fixed in sterile paraformaldehyde as described below.

Preparation of tissue to preserve root and rhizosphere structure

Fixation, dehydration and hybridization of roots were modified from Hugenholtz and colleagues (2001) to optimize probe binding, and preserve root and rhizosphere morphology and contact with soil. Roots were fixed in sterile, filtered 4% paraformaldehyde in phosphate-buffered saline (PBS) (7 mM Na_2HPO_4, 3 mM $NaHPO_4$ and 130 mM NaCl, pH 7.2), on ice for 3 h, gently transferred with forceps to 1 ml of PBS on ice, and then transferred to 1:1 (v/v) PBS:ethanol and maintained at –20°C until hybridization of probes. Before hybridization, root pieces > 500 μm diameter were placed in a drop of 50% ethanol in PBS on a piece of dental wax under a dissection microscope, and a tangential slice of the root surface and associated rhizosphere was cut by hand with a double-edged razor blade. The slice was returned to 50% ethanol in PBS until progressed through dehydration steps. Root pieces less than 500 μm diameter were processed and viewed whole. Samples were dehydrated on ice by exchanging the ethanol solution from 50% to 75% (3 min) and then 75% to 100% (3 min). The 100% ethanol was then drawn off and tubes were left open on the bench for 30 min to allow residual ethanol to evaporate. Hybridization solution was added. One millilitre of hybridization solution comprised of 180 μl of 5 M NaCl, 20 μl of 1 M tris-HCl, pH 7.2, 300 μl of formamide (30%), 499 μl of purified water (Milli-Q), 1 μl of 10% SDS, followed by up to three oligonucleotide probes (described below; 15–30 μl each). Tubes were gently inverted to mix, incubated for 2 h at 48°C, then immediately transferred to a 46°C water bath for 15 min. Root pieces were then removed with forceps, excess solution wicked away from the corner of each root piece with a tissue, and then immediately mounted in the antifade solution, Citifluor (Citifluor, London, UK) on a gelatin-coated slide. Before placing a coverslip on top, root pieces were observed with a dissecting microscope and the rhizosphere was oriented upwards. Samples were generally viewed the same day, although probe binding and brightness were stable for at least 4 days when slides were maintained at 4°C.

Oligonucleotide probes

Probes were purchased from Thermo Electron (Germany, http://www.thermohybaid.de), hydrated to 500 ng μl^{-1} in sterile, filtered Milli-Q water, and kept at –20°C until used at a working concentration of 50 ng per μl of water. A probe, PSE227 (5′-AAT CCG ACC TAG GCT CAT C-3′), was designed to target all *Pseudomonas* spp., using the database and software ARB, and tested at a range of formamide concentrations according to Hugenholtz and colleagues (2001). An *Acinetobacter* sp. (strain RA3957, provided by Dr Alan Richardson, CSIRO Plant Industry, Canberra), with a single-base difference in the centre of the PSE227 binding region, was used to validate PSE227. PSE227 was also tested on suspensions of *Pseudomonas* spp. isolates, SS266 and SS51, and *Serratia* spp. isolates, SS301 and SS305 (selected from the rhizospheres of wheat, Simpfendorfer *et al*., 2002; and obtained from NSW Agriculture, Wagga Wagga, Australia). The identity and probe binding sites of these isolates were confirmed by comparative analysis of their 16S rRNA genes. After we confirmed that PSE227 was specific to our *Pseudomonas* isolates, we tested mixtures of isolates with probe PSE1284 (also designed to bind to *Pseudomonas* but targeted to positions 1284–1304 of the 16S rRNA, 5′-GAT CCG GAC TAC GAT CGG TTT-3′; Gunasekera *et al*., 2003) and EUB338 (designed to target most bacteria, 5′-GCT GCC TCC CGT AGG AGT-3′; Amann *et al*., 1990) at different formamide concentrations to verify that 30% was optimum for the three probes.

Fluorochromes and minimizing soil autofluorescence

With laser excitation wavelengths from 457 nm to 543 nm, autofluorescence from field-grown samples swamped signal from fluorochromes. Hence we used 633 nm excitation, with emission collected between 650 and 685 nm to localize Cy5, and between 675 and 694 nm to localize Cy5.5. This meant that only the bacterial probe (EUB338, generally conjugated to Cy5.5) and the *Pseudomonas* probes (PSE227 plus PSE1284, both conjugated to the same fluorochrome, generally to Cy5) could be used together. Some samples had only the EUB338 probe or the PSE227 and PSE1284 probes conjugated to Cy5. Despite the greatly reduced sample autofluorescence with red excitation, control samples carried through the hybridization procedure without probes still had a few fluorescing spots that appeared as bacteria. We subtracted these counts from samples with probes.

Observation and quantification of bacteria on roots

Samples were viewed with a Leica SP2 confocal microscope (Leica Microsystems, Sydney, Australia) and a water immersion objective (×63), at zoom 1 or 2 digital magnification. Three images were captured for each field of view: the Cy5 channel, the Cy5.5 channel and the transmitted light (bright-field or DIC) channel to show morphological features of the root surface (Fig. 1A). The length and width of the square field of view was 0.238 mm for zoom 1, and 0.119 mm for zoom 2, such that the longest length of root that could be analysed at any one time was < 0.337 mm. Laser intensities and image settings (gain = 800; off set = –15) were kept constant for all samples to standardize the brightness and apparent size of bacteria across samples. To calibrate the pixel size and determine the bacterial sizes under the settings used, images of a mixture of 0.1- and 0.5-μm-diameter beads impregnated with dyes including the Cy5 range (TetraSpeck beads, Molecular Probes) were analysed as for bacterial samples.

Images were analysed with analySIS™ software (Soft Imaging Systems). Hand-counts from images of suspensions

Table 5. Operations applied to images to quantify bacteria labelled by FISH per volume of rhizosphere using image analysis software, analySIS™.

1. Brightfield and fluorescence images imported into analysis software.
2. Images calibrated. Important to check pixels per μm as this will determine the minimum number of pixels to be counted as a 'particle'. A particle = a bacterium.
3. Projected surface areas outlined by hand on images. Area of root and rhizosphere outlined on brightfield image. Areas with clustered bacterial cells, as judged by eye, outlined on fluorescence images. These outlines from the fluorescence images are layered onto the corresponding brightfield image to identify features on the root that colocate with the bacterial clusters.
4. Fluorescence images converted from colour to intensity.
5. Lower and upper limits to intensity adjusted by eye to represent labelled bacteria. Settings vary slightly depending on sample and probe.
6. Filaments measured by tracing a line along the length of each.
7. Bacteria separated (if necessary) using a function in the software that draws lines around individual bacteria. Settings kept constant across all samples.
8. Measurements (bacterial number, *x*, *y* coordinates and diameter) and projected areas are determined. Minimum pixel number per bacterium is defined based on pixels per μm. Measurements and areas are used for bacteria per volume and distances between bacteria, as defined in the text.

of bacteria in solution and soil, and bacteria on agar-grown roots were used initially to develop settings within the software to quantify bacteria and their spatial arrangement. The standard settings applied to each sample are listed in Table 5. Each field of view was analysed individually as settings needed to be adjusted slightly by eye because of sample-to-sample variation. The three types of measurements are listed below.

Bacteria per rhizosphere volume

For each field of view, a series of images was taken from the upper to lower-most point that bacteria were observed on the root surface (depth of colonization, *z*). The regions of maximum intensity, threshold 25, from each image were added together to give a final image for analysis and a value for *z*. The brightfield image was used to outline the projected root surface area with analySIS™, which was multiplied with *z* to determine rhizosphere volume. Counts within a given volume were determined as in Table 5.

Clustering

Working first with the single maximum fluorescence image ('stack'), groups of closely spaced bacteria were judged by eye and outlined by hand in analySIS™. The outlined areas were multiplied by *z* to determine rhizosphere volumes with closely spaced bacteria. Bacteria within the volumes were quantified. The per cent of total rhizosphere volume with closely spaced bacteria was compared with the per cent of bacteria in clustered volumes to estimate clustering within a field of view. To determine morphological features associated with the closely spaced bacteria, the file with the outlined regions of clustered bacteria was overlaid onto the brightfield image, and recognizable features within the outlines were recorded.

Distances between bacteria

The following form of the Pythagorian equation was used within Excel to determine the distances *d* between two bacteria with coordinates (x, y) and (x^1, y^1) (Fig. 1B):

$$d = [(x^1 - x)^2 + (y^1 - y)^2]^{1/2}$$

Statistical analyses

Numbers of bacteria were analysed by one-way ANOVA for probability of effect of sample time, type of root and position along roots using GENSTAT (IACR, Rothamsted, UK). Means, standard error of differences (SED) and probabilities of differences are presented in Tables 2 and 3. To indicate the variation within sample types, ranges are presented in the text.

Acknowledgements

We are grateful to Linda Blackall, Gene Tyson and Mark Fegan for help with the FISH technique and identification of the field isolates, to John Kirkegaard and Geoff Howe for management of the field experiment, and to Peter Thrall for advice with spatial analysis. Peter Janssen, Margaret McCully and Ulrike Mathesius provided valuable comments for this article. This study was funded by the Australian Grains Research and Development Corporation.

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