Luminescence 2003; 18: 346–351
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bio.747

ORIGINAL RESEARCH

Spatial and temporal distribution of a bioluminescent-marked *Pseudomonas putida* on soybean root

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Received 26 February 2003; revised 29 October 2003; accepted 29 October 2003.

ABSTRACT: The ability of rhizobacteria to compete with other microorganisms for root colonization may be critical for its establishment on a root. Over a 6 day period, visualization of the spatial and temporal rhizosphere distribution of a bioluminescent-marked rhizobacterium, *Pseudomonas putida*, strain GR7.4lux, was examined on soybean grown in non-sterile soil conditions. Luminometry technologies showed a rapid root distribution of rhizobacteria where bioluminescence was particularly intense on the seed and upper root parts. The results provide new information on rhizobial root distribution, where, using enrichment broth, 50% of the root tips were still colonized by rhizobacteria up to 6 days after sowing. This suggests that rhizobial enrichment is required to detect low populations at the root tip. Bioluminescent technology represents a promising alternative to previous methods for studying rhizobial growth and distribution on roots. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: spermosphere; rhizosphere; rhizobacteria; luminometry; root distribution

INTRODUCTION

The root colonization process involves multiplication of inoculated rhizobacteria in the zone under the seed exudate's influence, i.e. the spermosphere, then their transfer to the emerging root, and their multiplication and persistence on the developing root system (1–2). One of the first steps is the distribution of inoculated rhizobacteria from the seed to the growing roots, which is controlled by the active motility of rhizobacteria and the passive movement of rhizobacteria by water or vectors (3).

The main techniques for distinguish microorganisms on roots are direct microscopy, immunofluorescence, antibiotic resistance, nutritional specificity, immunogold staining, DNA or RNA hybridization, and the introduction of marker genes (3–6). Some of these techniques required time and intensive labour, and sometimes recovery of the introduced microorganism is fastidious. In contrast, bioluminescence technologies that rely on the light emitted by marked rhizobacteria can be fast and specific, e.g. root colonization of the whole root system was evaluated using X-ray film (7–8), root prints on media (8) and charge-coupled device (CCD) cameras (9–14). Some of these techniques can still be time-consuming, depending on the light intensity produced by the rhizobacteria and the sensitivity of the detection equipment. Hence, there is a need to determine how bioluminescence technologies can be used to understand the dynamics of spatial and temporal root distribution of rhizobacteria, based on active mobility of the introduced bacteria under non-sterile soil conditions.

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Contract/grant sponsor: Natural Sciences and Engineering Research Council, Canada.
Contract/grant sponsor: Laval University, Canada.
Contract/grant sponsor: Auburn University, USA.

MATERIALS AND METHODS

Bacteria and culture conditions

Pseudomonas putida, strain GR7.4, was originally obtained from Allelix Crop Technologies (Mississauga, Ontario, Canada), and was previously reported to have traits related to plant growth-promoting activity (15) and to increase the growth of canola (*Brassica napus*) (16). This strain was subsequently transformed with Tn*5*–*luxAB* to express the bioluminescent phenotype (17) and was designated GR7.4*lux*. *Pseudomonas* Broth F (PBF; 17) and *Pseudomonas* Agar F (PAF; Difco Laboratories, Detroit, MI, USA) were supplemented with kanamycin (50 µg/mL; PAF–RIF) and used to grow GR7.4*lux*.

Inoculation, seeding and root colonization

For this experiment, strain GR7.4*lux* was studied for its ability to colonize seed and root, based on its population and spatial pattern. For each replication, bacteria were grown in PBF for 24 h, washed twice with phosphate

buffer (0.2 mol/L) and suspended in 1% sodium alginate (Sigma, St. Louis, MO, USA). Two soybean seeds were dipped in 2 mL bacterial suspension for 15 min. Plexiglas rhizoboxes were filled with non-sterile field soil, as previously described (18), and two seeds were immediately and aseptically seeded in each rhizobox. The rhizoboxes were wrapped with aluminum foil to keep the roots in the dark and avoid chemiluminescence. The rhizoboxes were individually placed in plastic bags to contain the engineered strain. Every day the bags were opened, and the sides of the bags were sprayed with water to avoid strong evaporation. The plants were not watered to avoid passive movement of bacteria by percolating water. Rhizoboxes were placed in a growth chamber at 20°C by day and 18°C by night, with a 12 h photoperiod. The rhizoboxes were arranged in a randomized complete block design using four replications.

Seeds and roots were observed at 24 h intervals from immediately after inoculation (day 0) to 6 days after seeding. For these seven observation times, the cover of the rhizoboxes was removed and the rhizobox was placed in a humidity chamber. This chamber consisted of a plastic container ($9 \times 20 \times 30$ cm) containing about 50 mL water and 2 ml decanal (Sigma, St. Louis, MO, USA). A glass cover was placed on top of the humidity chamber to keep humidity and decanal vapours inside the chamber. Each rhizobox was photographed with a CCD camera (Photometric Ltd) using a 20 min exposure (18).

After exposure to decanal, the root lengths were measured and seedlings were aseptically dissected into seed and root parts. One seedling per rhizobox was used to make a root print on PAF–RIF medium. The last 2 cm of the roots were then placed on PBF–RIF to confirm root tip colonization by bioluminescence of the resulting bacterial growth. The second seedling was used to determine colony-forming units per seed and root system. Seeds and roots were placed separately in 10 mL 0.2 mol/L phosphate buffer, shaken for 30 min and diluted serially. Bioluminescence was measured with a luminometer (Monolight 2001, Analytical Luminescence Laboratory, San Diego, CA, USA) using a 1 mL sample to which 100 μL of a 100-fold dilution of decanal was automatically injected. The light output was integrated for 10 s. The abbreviation for this variable is lum(RLU). Colony-forming units were determined on PAF–RIF media. Inoculated PAF–RIF plates were incubated at 30°C for 3 days, and bacterial growth was measured to determine the populations of PGPR and length of root colonized. Plates were exposed to decanal vapour to confirm visually the presence of bioluminescent bacteria.

Statistics

The homogeneity of variances was evaluated using Bartlett's test (19). The logarithm 10 of lum(RLU) and CFU were used for statistical analysis to produce homogeneous variances. The analyses of variance and correlation were performed using SAS (20).

RESULTS

Seed colonization

Conventional plating was used to determine seed colonization by strain GR7.4*lux*. After an increase from the inoculation time to day 1, the population remained at about log 9 CFU/seed (Table 1).

The bioluminescent methods were also used to compare seed colonization. Using the CCD camera and without decanal addition, no chemiluminescence from seeds was observed. However, after decanal addition, soybean seed inoculated with strain GR7.4*lux*

Table 1. Rhizobacterial populations and luminometric measurement from seed of soybean inoculated with strain GR7.4*lux* over time, and summary of the analyses of variance

Strain source of variation	Time (days)	Population		Luminometer measurement	
		CFU/seed	SD[a]	Lum(RLU)	SD
		Log		Log	
GR7.4*lux*	0	8.80	0.17	2.69	0.16
	1	9.31	0.19	2.71	0.20
	2	8.83	0.09	2.30	0.04
	3	9.08	0.05	2.50	0.06
	4	8.98	0.04	2.49	0.06
	5	8.94	0.14	2.40	0.05
	6	9.09	0.17	2.46	0.04
Time (T)		**		**	

[a] SD, standard deviation.
** Significance at $p \leq 0.01$.

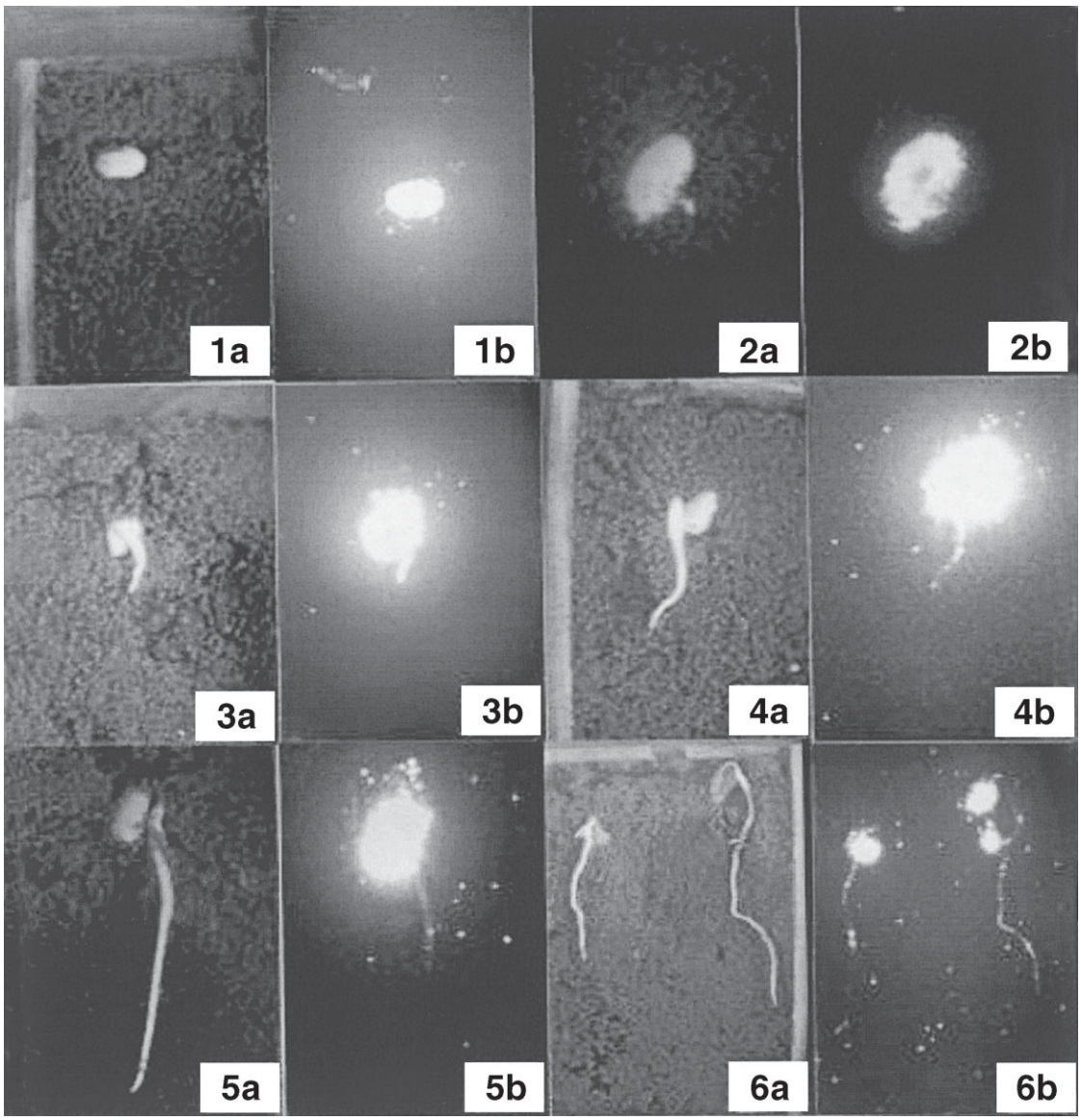

Figure 1. Root distribution of *P. putida* strain GR7.4*lux* on soybean growing in a rhizobox over a period of 6 days. Photographs were taken with a 3 s (a: light) and 1200 s (b: dark) exposure using a CCD camera. Note the colonization of the seed from day 1 to day 6, then the transfer to the root system and the colonization of the upper root system by this bioluminescent PGPR, visualized using a CCD camera.

was visualized using the CCD camera (Fig. 1). Using the luminometer, bioluminescent activity was noted for the 10^{-1}–10^{-6} dilution of the seed washings inoculated with strain GR7.4*lux*. The 10^{-1}–10^{-4} dilutions saturated the luminometer, and only the results from the 10^{-5} and 10^{-6} dilutions were usable. Since these dilutions gave similar results, only the 10^{-6} dilution was selected to present data (Table 1) and for correlation analysis. The maximum lum(RLU) from seeds inoculated with strain GR7.4*lux* was detected with the luminometer 1–2 days after seeding and decreased thereafter. There was a linear relationship between the CFU of strain GR7.4*lux* and lum(RLU) measurements, where lum(RLU) = –0.92 + 0.38(CFU) ($r = 0.46$; $p < 0.01$).

Root colonization

One day after sowing, most roots were still trapped within the seed coat, so measurements of root colonization started on day 2. The conventional dilution-plating technique showed that strain GR7.4*lux* had a mean population of log 5.32 (±0.61)/cm of the total root length, log 4.98 (±0.70) CFU/mg root dry weight and log 5.8 (±0.66) CFU/root system (Table 2). The population increased slightly over time when expressed per root system.

Bioluminescent methods were also used to compare root colonization. Using the CCD camera, soybean root distribution by strain GR7.4*lux* was visualized (Fig. 1).

Table 2. Rhizobacterial population and luminometric measurement from roots of soybean inoculated with strain GR7.4*lux* over time, and summary of the analyses of variance

		Rhizobial density						Luminometer measurement	
Strain/source of variation	Time (days)	Log(CFU/cm total root length)	SD[a]	Log(CFU/mg dry root weight)	SD	Log(CFU)/root system	SD	log(RLU)	SD
								log	
GR7.4*lux*	2	5.65	0.46	5.28	0.30	5.47	0.33	2.77	0.40
	3	5.24	0.16	4.80	0.23	5.62	0.18	4.04	0.31
	4	5.06	0.25	4.99	0.51	5.79	0.32	4.44	0.55
	5	5.40	0.72	4.92	0.78	6.17	0.72	3.54	0.66
	6	5.25	1.14	4.89	1.39	6.17	1.19	3.70	1.13
Time (T)		NS		NS		**		*	

[a] SD, standard deviation; NS, not significant.
*, ** Significance at $p \leq 0.05$ and 0.01, respectively.

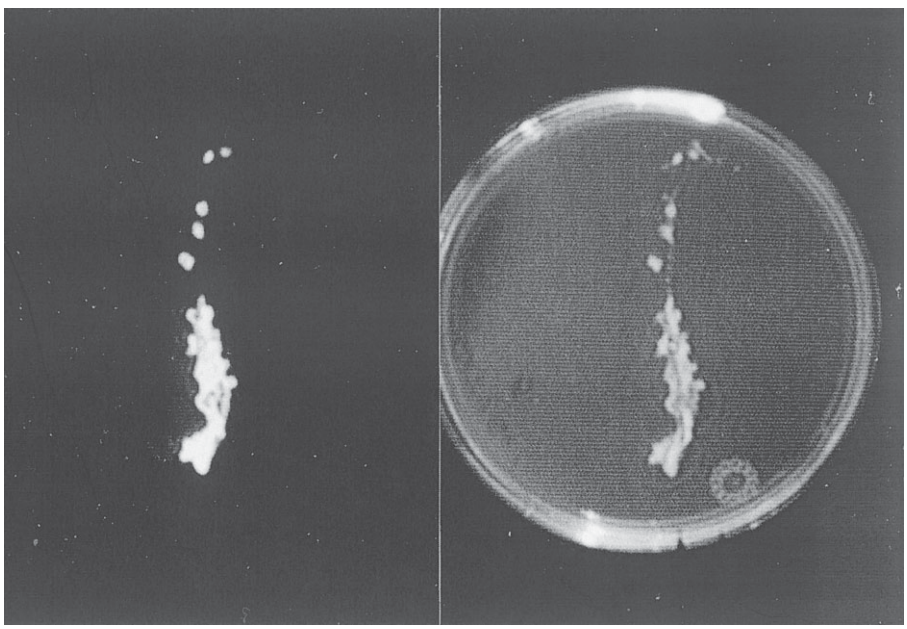

Figure 2. Root distribution of *P. putida* strain GR7.4*lux* on soybean visualized by root print. GR7.4*lux* was a bioluminescent transconjugant detected by isolation on rifampicin and kanamycin-containing medium.

Seed inoculation with strain (GR7.4*lux* resulted in intense *in situ* production of light from the soybean roots. Using root prints, soybean root distribution was also visualized (Fig. 2). The total and colonized lengths of root increased over time, but the root growth and root-colonized rates decreased over time (Fig. 3). Using the luminometer, the 10^{-1} and 10^{-2} dilutions of the root washings showed bioluminescent activity for stain GR7.4*lux*. The first dilution saturated the luminometer and only the results from the 10^{-2} dilution of the root washings are presented (Table 2). The maximum 1 µm(RLU) from the root was detected 4 days after seeding. A linear relationship was present between CFU of strain GR7.4*lux* and the lum(RLU) measurements, where lum(RLU) $10^{-2} = -1.35 + 0.87$(CFU) ($r = 0.68$; $p < 0.001$). Finally, using the root tip, the enrichment in PBF-RIF showed that strain GR7.4*lux* colonized 100% of the root tip up to 4 days after sowing, and 5 and 6 days after sowing, 50% of the root tips were still colonized by this rhizobacterium.

DISCUSSION

Using bioluminescence technologies, it was possible to visualize rapidly the bioluminescent strain GR7.4*lux* surviving on the seed parts, and then transferred to the main root. The conventional dilution-plating technique to determine becterial density was labour-intensive and did not give spatial and temporal root-colonization

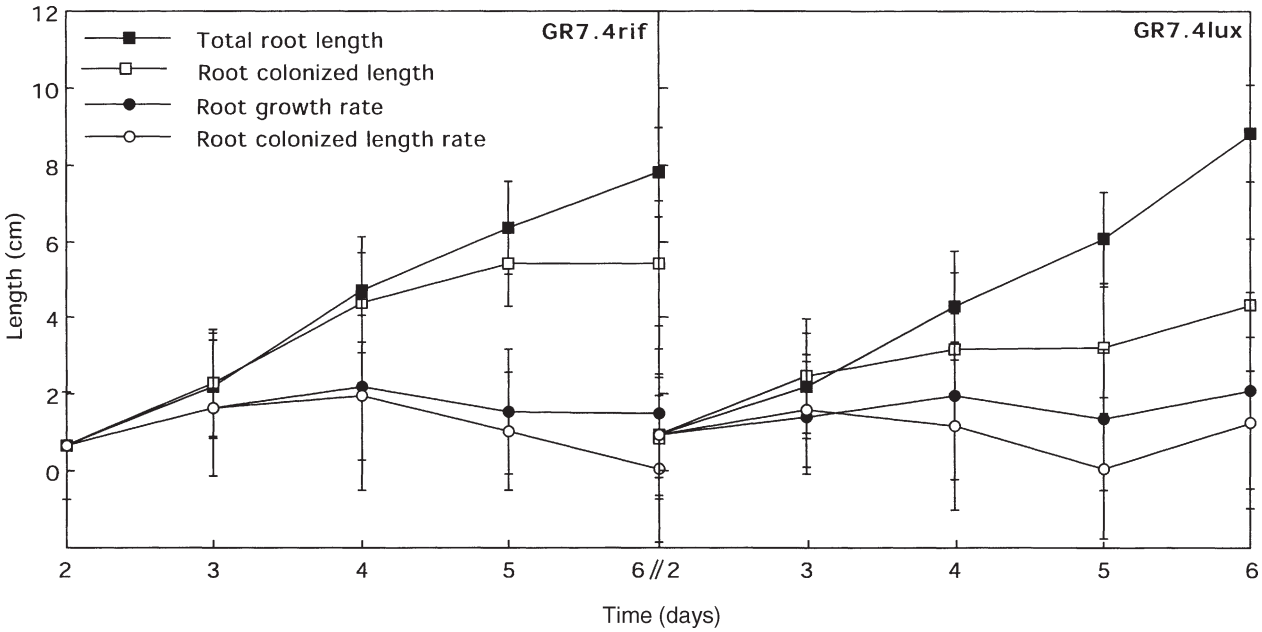

Figure 3. Soybean root growth, root growth rate, and root colonized length and root colonized length rate by strain GR7.4*rif* and GR7.4*lux* over time. The total root growth and root growth rate are the means of eight replicates, whereas the root colonized length and root colonized length rate are the means of four replicates. Error bars are the standard deviations.

patterns. In contrast, the bioluminescence methods that rely on the measurements of light emitted by marked rhizobacteria can be fast and specific, allowing the detection and inspection of the distribution of this rhizobacterium from indigenous microorganisms present in non-sterile soil.

For seed, the maximal bioluminescent activity was recorded within the first day after sowing that corresponded roughly to the maximum seed population. On days 2–6, rhizobacterial populations of the seed remained relatively stable, as well as the bioluminescence activity. For seed, quantification of bioluminescence using the luminometric technologies was performed, but there was weak correlation between the population and bioluminescence activity. Bioluminescence activity has been related to the bioluminescence population in laboratory media (5), in soil and rhizosphere (21) when bacteria were in the exponential growth phase. The weak correlation in this study may reflect the transition from a rich culture medium to the nutrient-limited conditions of the spermosphere environment. Nevertheless, these luminometer measurements can be used to confirm viable and active bioluminescent rhizobacteria.

Seed inoculation, at log 8.8 CFU/seed, provided an effective strategy for introducing rhizobacteria into the rhizosphere with a population density of about log 5 CFU/cm total root length or CFU/mg dry root weight, but 6 CFU/root system. To our knowledge, no standard expression has been suggested in the literature. However, expressing colonization per root system related population to microbial niches of the root surface and avoid the impact of root growth pattern on the microbial phenomena.

For roots, using the CCD camera and the rhizobox system, bioluminescence was observed directly, since the roots were not masked by soil. For strain GR7.4*lux*, rhizobacteria transferred to the roots as soon as they appeared. Root prints gave similar results to those of CCD camera observations, but required 3 days' incubation before visualization of the root colonization pattern. Also, the growth of colonies on agar was mucoid and the colonized length of root was probably overestimated by a few millimeters. Using the luminometer, the maximal bioluminescence activity from the root increased until 4 days after sowing and decreased thereafter. In this case, there was a good correlation between the log(CFU)/root system and bioluminescence activity from the dilution of the root washings, and may reflect the growth and physiological status of strain GR7.4*lux* on roots, as suggested by Marschner *et al.* (22) and Ramos *et al.* (13). So, for the first few days after root emergence, the luminometer measurements can be used to predict bioluminescent rhizobacterial populations on roots.

In root colonization studies, it is also important to determine the presence of rhizobacteria at the root tip, which is often dependent on the detection limit of the techniques selected. The broth enrichment of the last 2 cm of the root confirmed the presence of GR7.4*lux* up to 4 days after seeding, and thereafter 50% of the root tips were still colonized. Both root prints and the CCD

camera failed to detect the presence of strain GR7.4*lux* at the root tips, indicating that these technologies were less sensitive than the broth enrichment. Using CCD camera imaging, Kragelund *et al.* (11), Roberts *et al.* (12) Ma *et al.* (14) were not able to observe bacteria around the root tip. In this regard, this study suggests that root distribution visualized through imaging requires a dense population of bioluminescent rhizobacteria.

CONCLUSION

In studying the root colonization process, it is important to determine densities and localization of the bacterial population and relate them to microbial niches of the root surface. This study demonstrates the usefulness of bioluminescence technologies, such as root prints and CCD camera imaging, to determine the localization of strain GR7.4*lux* in the process of root colonization in non-sterile soil, showing a decrease in rhizobacterial density from the seed part to the root tip. However, the CCD camera imaging method gave more rapid information than the root prints, which required 3 days' growth of the rhizobacteria. Finally, only the broth enrichment technology allowed the detection of the rhizobacteria at the root tip compared to the root prints and CCD camera technologies. Knowledge of the first step of the root colonization can be used to select the best beneficial rhizobacteria–plant association.

Acknowledgements

C. J. Beauchamp acknowledges the Natural Sciences and Engineering Research Council of Canada, and Laval and Auburn Universities for supporting this research.

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