APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1995, p. 3443–3453 Vol. 61, No. 9
0099-2240/95/$04.00+0

Impact of Field Release of Genetically Modified *Pseudomonas fluorescens* on Indigenous Microbial Populations of Wheat

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Received 3 April 1995/Accepted 22 June 1995

In a field release experiment, an isolate of *Pseudomonas fluorescens*, which was chromosomally modified with two reporter gene cassettes (*lacZY* and Kan^r-*xylE*), was applied to spring wheat as a seed coating and subsequently as a foliar spray. The wild-type strain was isolated from the phylloplane of sugar beet but was found to be a common colonizer of both the rizosphere and phylloplane of wheat as well. The impact on the indigenous microbial populations resulting from release of this genetically modified microorganism (GMM) was compared with the impact of the unmodified, wild-type strain and a nontreated control until 1 month after harvest of the crop. The release of the *P. fluorescens* GMM and the unmodified, wild-type strain resulted in significant but transient perturbations of some of the culturable components of the indigenous microbial communities that inhabited the rhizosphere and phylloplane of wheat, but no significant perturbations of the indigenous culturable microbial populations in nonrhizosphere soil were found. Fast-growing organisms that did not produce resting structures (for example, fluorescent pseudomonads and yeasts) seemed to be most sensitive to perturbation. In terms of hazard and risk to the environment, the observed microbial perturbations that resulted from this GMM release may be considered minor for several reasons. First, the recombinant *P. fluorescens* strain caused changes that were, in general, not significantly different from those caused by the unmodified wild-type strain; second, perturbations resulting from bacterial inoculations were mainly small; and third, the release of bacteria had no obvious effects on plant growth and plant health.

Although the potential benefits of genetically modified microorganisms (GMMs) are numerous (11, 24, 36), the use of GMMs on a wide scale is still hampered by a lack of knowledge of the wider ecological impacts that such organisms might have once they are released into the environment. Microorganisms, once released, might not only multiply but might also be subjected to evolutionary selection pressures in the environment which might enhance their ecological fitness (40, 42). Also, the ability of microorganisms to exchange genetic information with related or nonrelated bacteria (32) increases the uncertainty of the fate of GMMs and the effects that they, or the novel genetic elements contained in them, might have on the environment. Therefore, before GMMs can be released into natural ecosystems, a better understanding of the behavior and impact in the environment of GMMs themselves and any novel genetic elements they may contain is needed (37).

Although several release studies with GMMs have been carried out to date, no serious environmental problems associated with these organisms have been identified (11, 19, 24, 26, 30, 36). All of these studies have been concerned with the dissemination, survival, and possible gene transfer of the novel genetic elements to indigenous members of the microbial community. Only for releases of GMMs introduced for their biological control activity has the impact on target plant pathogens been considered (11, 26, 34); however, the effects that these GMMs might have on nontarget, indigenous, microbial populations and ecosystem functioning have not been addressed. All studies to date on possible effects of GMMs on nontarget populations and ecosystem functioning have been conducted in contained experimental environments (35, 38, 44). Biological perturbations caused by functionally improved GMMs in such experimental systems include negative effects on protozoa (5), increased carbon turnover (43), displacement of indigenous *Pseudomonas* populations in the rhizosphere (8), and long-term suppression of fungal populations in soil (36). Only our own studies have so far addressed the impact of a nonimproved bacterium that was chromosomally marked with the gene cassettes *lacZY* and Kan^r-*xylE* on a variety of indigenous microbial populations. The use of two marker gene cassettes instead of one meant that this GMM could be detected with great sensitivity in environmental samples (12), while insertion of the novel genetic elements into the chromosome reduced the risk of these genes being transferred to possible recipient microorganisms in the environment (20, 29). Prerelease experiments with this GMM, carried out in large undisturbed soil cores in the glasshouse, suggested that changes in microbial populations did occur as a result of introductions of the GMM into the rhizosphere and on the phylloplane of wheat but that these perturbations were transient and were never significantly different from perturbations caused by inoculations with the unmodified parental strain (15). However, the microbial population dynamics that occur in experimental systems might differ significantly from those in the field (17). Therefore, results of studies assessing the impacts of GMMs derived from glasshouse experiments might be misleading and should not be a substitute for field experimentation.

Because all studies that were carried out under contained conditions indicated a low risk associated with the release of this GMM into the environment (2, 3), the Secretary of State for the Environment granted permission to release this GMM into the field at Horticulture Research International, Little-

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hampton, England, and at the Institute of Virology and Environmental Microbiology, Oxford, England, during the spring of 1993 as an inoculant of wheat and sugar beet, respectively (4). These were the first releases of free-living (nonsymbiotic) bacteria in the United Kingdom and were unique in that the GMM was marked in two positions on the chromosome. The main aims of the release were (i) to assess the ability of an innocuous GMM, when introduced as a seed coating and spray application, to survive and disseminate in the environment; (ii) to monitor gene transfer of the novel genetic elements to related indigenous microbial populations; and (iii) to determine the impact of deliberately released recombinant microbial inocula on indigenous microbial communities that inhabit the soil, the rhizosphere, and the phylloplane.

Here we report on the impacts of a *Pseudomonas fluorescens* strain modified with two marker gene cassettes and its unmodified parental strain on indigenous microbial populations in the rhizosphere and phylloplane of field-grown wheat. Both the GMM and the parental strain were introduced into the environment as a wheat seed coating and subsequently as a foliar spray, applied at wheat tillering. The results of the field trial are discussed and compared with earlier results obtained in contained environments (15).

MATERIALS AND METHODS

Site and experimental design. The GMM release experiment was carried out during 1993 and 1994 on spring wheat (cv. Axona) at Littlehampton, West Sussex, United Kingdom, in a silt loam soil (Hamble series) with a cropping history of wheat for the previous 4 years. The organic carbon content of the soil was 1.4% (wt/wt), and its pH(H_2O) was 6.0. At the beginning of March 1993, 1.5 months before sowing, the soil received 376 kg of NPK fertilizer (50% N; 25% P; 25% K) per ha, and subsequently in June 1993, 2 months after sowing, 100 kg of urea (46% N) per ha was applied as a soil dressing. The experimental area was situated in the middle of a 3-ha spring wheat field. The experiment itself consisted of 15 completely randomized plots; 5 were treated with recombinant bacteria, 5 were treated with the unmodified wild-type organism, and the remaining 5 control plots were not treated with bacteria (Fig. 1). Each plot was 1.5 m wide and 1.5 m long and was hand sown with 13 rows of approximately 150 wheat seeds. The plots were surrounded by three guard rows and three fallow areas to minimize recombinant spread to the surrounding environment (Fig. 1). Before sowing, the plots were rotovated and raked, and shallow furrows were made into which the seeds were fluid-drilled by hand. The first guard rows were drilled with a manual driller, while the remaining guard rows and the main field were drilled mechanically.

Organisms used. The wild-type organism (SBW25) was isolated from the phylloplane of sugar beet and identified by fatty acid methyl ester analysis as a strain of *Pseudomonas aureofaciens* (LOPAT group IV). Subsequently, the bacterium was identified as a *P. fluorescens* strain by amplified 16S ribosomal restriction analyses (9, 46). Fatty acid methyl ester analyses of the microbial community at the release sites at Littlehampton and Oxford revealed that this group of *Pseudomonas* species was commonly found in soil, rhizosphere, and the phylloplane of both sugar beet and wheat (28, 39).

By site-directed homologous recombination, two different sets of marker genes were introduced into two well-separated (1-Mbp; 15% of the genome), presumed nonessential sites of the chromosome of strain SBW25 to create the GMM, SBW25EeZY-6KX (6). This recombinant strain was similar to the wild-type strain SBW25 with respect to its competitive ability, growth rate, and survival (15, 16). The marker genes (*lacZY* and Kan^r-*xylE*) were chosen to facilitate detection by simple dilution plating onto selective media and most-probable-number methods (12). The *lacZY* genes, for lactose utilization (7, 20, 21), form one of the most widely used metabolic marker systems (21, 28). When organisms with the *lacZY* genes are plated onto media containing the chromogenic substrate 5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside (X-Gal), the *lacZY* genes facilitate cleavage of this substrate, resulting in easily identifiable blue colonies. The Tn*903* kanamycin resistance gene (Kan^r) was isolated on a 1.4-kbp *Bam*HI fragment (Pharmacia, St. Albans, United Kingdom) and conferred resistance to the antibiotic kanamycin. The *xylE* gene originated from the *TOL*(pWW0) catabolic plasmid (45) and enables the conversion of its substrate, catechol, into a yellow compound (2-hydroxymuconic semialdehyde). Except for *xylE* activity, which is part of a large operon that is involved in the degradation of aromatic compounds in most organisms that possess this gene, phenotypic characteristics such as kanamycin resistance and lactose utilization were commonly expressed by the indigenous microbial populations inhabiting the field release site (18).

Culturing. Cells of both wild-type and recombinant *P. fluorescens* were stored at −70°C in 20% (vol/vol) glycerol. When required for experimental use, cells were removed from the freezer and plated onto tryptic soy agar (TSA; 30 g of tryptic soy broth [TSB; Oxoid] and 15 g of technical agar [Oxoid] in 1 liter of water). After incubation for 24 h at 25°C, cells were taken from the TSA plate and inoculated into 500-ml conical flasks, containing 100 ml of sterile TSB. To obtain bacterial cultures in late log phase, flasks were incubated overnight on a rotary shaker (200 rpm; 28°C). Bacterial cells were harvested by centrifugation (5,000 × *g* for 5 min). To remove the culture medium, the bacterial pellet was twice resuspended in sterile 0.25 strength Ringer's solution and centrifuged (5,000 × *g* for 5 min).

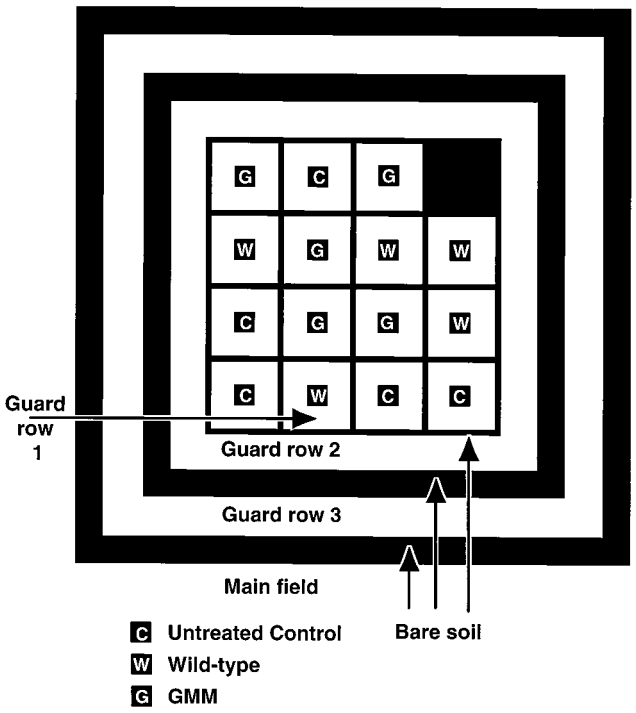

FIG. 1. Field site design for the release of a genetically modified *P. fluorescens* strain at Horticulture Research International, Littlehampton.

Seed inoculation. After removal of the culture medium, the bacterial cells were resuspended in 0.25 strength Ringer's solution to the original culture volume (>10^{10} CFU/ml). Batches (500 g) of wheat seed (cv. Axona; treated with carboxin and thiobendazole) were submerged in 400 ml of inoculum (control seeds were submerged in sterile 0.25 strength Ringer's solution). Seeds were inoculated with bacteria by vacuum infiltration (−0.2 × 10^5 Pa) for 20 min. After inoculation, excess inoculum was removed and each batch of inoculated seeds was suspended to a volume of 500 ml in 1.25% guar gel (Fluid Drilling Ltd., Stratford upon Avon, United Kingdom) held in a 500-ml wash bottle fitted with a wide spout. Subsequently, the seeds were fluid drilled into 2-cm-deep furrows at a rate of 1 seed per cm. The distance between the furrows was 12.5 cm, which meant that each plot (1.5 by 1.5 m) received approximately 2,000 seeds, each of which contained between 10^7 and 10^8 CFU. After drilling, the seeds were covered with soil. In total, between 10^{11} and 10^{12} CFU each of the recombinant and the wild-type *P. fluorescens* SBW25 were initially released into the field by seed inoculation.

Spray application. At tillering (growth stage [GS] 30) (41, 47), the GMM and the unmodified *P. fluorescens* SBW25 were applied to the phylloplane by spraying plants that were already seed inoculated with modified or wild-type *P. fluorescens* SBW25. Bacteria were suspended in the spray solution (0.25 strength Ringer's solution amended with 0.01% [vol/vol] Tween 80). Inoculum (100 ml) containing 3.2 × 10^{10} CFU/ml was sprayed onto the plants of each plot as a fine mist from a pressurized sprayer. The control plots were sprayed with spray solution without bacteria. In total, approximately 1.6 × 10^{13} CFU each of the GMM and the wild-type *P. fluorescens* SBW25 was released by spray application. To minimize drift of GMMs by wind, the inoculum was applied during an almost still evening (Force 2 on the Beaufort scale; wind speed, <4 mph). Also, to minimize drift, the plots were surrounded by a 1-m-high polyethylene sheet, supported by a wooden frame. These screens were left for 1 week after the spray application.

Sampling. At GS 12 (seedling), 22 to 30 (tillering—stem elongation), 50 to 59 (inflorescence emergence), 69 (flowering), 85 (early dough), and 92 (ripening) (42, 43), three wheat plants were removed from each plot to a depth of 30 cm. Plants taken from one plot were pooled and treated as one sample. Subsequently, roots, seeds, hypocotyls, leaves 1, 3, and 5, flag leaves, and ears taken from each

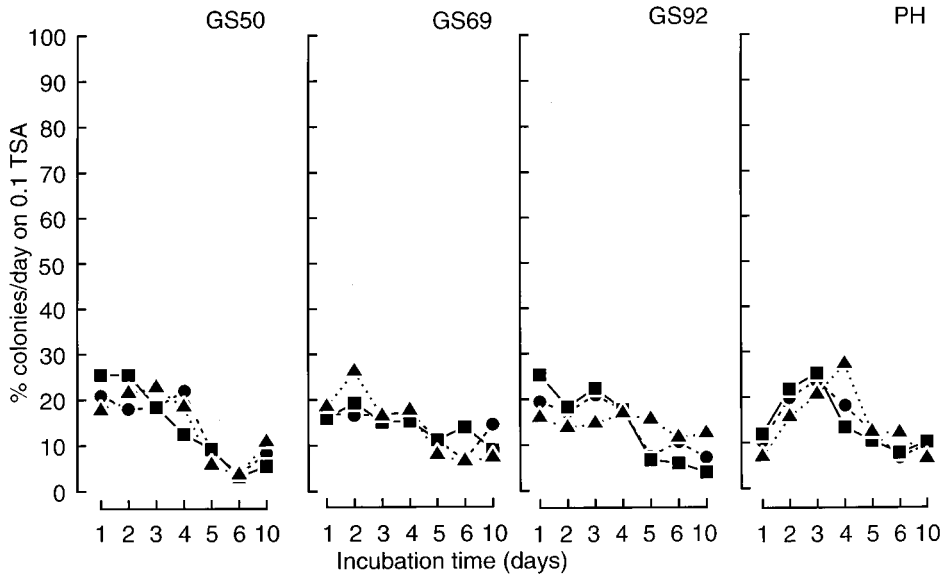

FIG. 2. Bacterial community structures in soil planted with noninoculated wheat (●), wheat inoculated with wild-type *P. fluorescens* SBW25 (■), or wheat inoculated with recombinant *P. fluorescens* SBW25 (▲). The soil was sampled at wheat GS 50 to 59 (inflorescence emergence), 69 (flowering), and 92 (ripening), as well as 1 month after harvest (PH). Data are plotted at each sampling as the percentage of the total CFU count that appeared every day over a period of 6 days and after 10 days of incubation at 25°C on 0.1 TSA. $n = 5$.

plot were processed separately as they became available at the time of sampling. Soil samples were taken separately from each plot with a 1-cm-diameter cork borer to a depth of 10 cm from between the rows of wheat. After mixing of the soil and removal of any root material, 1.2 g (wet weight) (equivalent to 1 g [dry weight]) of each soil sample was transferred to 9 ml of sterile 0.25 strength Ringer's–0.05% agar solution (RA) and vigorously shaken for 20 s on a Whirlimixer (Gallenkamp, Loughborough, United Kingdom). Roots with adhering soil were placed in a bucket of tap water for ca. 3 h to loosen the soil adhering to the roots. Subsequently, most of the soil was carefully removed by hand. The remaining loose soil was removed with two rinses in clean tap water. The roots were then blotted dry, cut into small segments (ca. 2 cm long), and mixed thoroughly by hand. From each root sample, a 1-g subsample was taken and crushed in 9 ml of sterile RA with a sterile pestle and mortar. Phylloplane samples (leaves 1, 3, and 5, flag leaf, and hypocotyl) that were taken from each plot were weighed before they were crushed. No subsamples were taken when samples weighed less than 1 g. Seeds taken from each plot were counted before they were crushed in 9 ml of RA with a sterile pestle and mortar. Numbers of microorganisms were quantified in soil as CFU per gram (dry weight) of soil; in the rhizosphere, as CFU per gram (fresh weight) of plant tissue on the phylloplane; and as CFU per seed on the seed by the methods described below.

Effects on total populations. To estimate different components of the total microbial population, a 10-fold dilution series in RA was prepared from each sample and 0.1-ml aliquots of appropriate dilutions were plated onto the following media: (i) 0.1 strength TSB amended with 15 g of agar (0.1 TSA) for the enumeration of bacterial and actinomycete propagules; (ii) potato dextrose agar (PDA; Oxoid) amended with 100 ppm streptomycin sulfate and 50 ppm rose bengal (PDA^+) for the enumeration of filamentous fungi and yeast propagules; (iii) selective medium (P-1) (25) to enumerate fluorescent pseudomonads (to estimate the numbers of indigenous pseudomonads, the number of GMMs was quantified by spraying plates with catechol [*xylE* activity], allowing accurate estimation of the indigenous fluorescent pseudomonad population, which produced no yellow coloration when sprayed with catechol); and (iv) selective medium (P-2) (25) for the enumeration of *Pseudomonas putida*, a species closely related to the release strain. It was therefore thought that inoculation with *P. fluorescens* SBW25 could displace the indigenous *P. putida* population, because the two populations were likely to occupy the same niche.

Plates were incubated at 25°C. Incubation times for the different media were 10 days for P-1, 0.1 TSA, and PDA^+ and 2 to 3 days for P-2. P-2 is only semiselective and becomes overgrown with bacteria other than *P. putida* after longer periods of incubation (unpublished data). Fluorescent pseudomonad colonies were enumerated by exposing plates to UV light.

Microbial community structures. To characterize the microbial community structure, 0.1 TSA (total bacteria) and P-1 (fluorescent pseudomonads) plates were incubated at 25°C for up to 10 days and colonies were enumerated on a daily basis for six consecutive days and at day 10 (17). For this purpose, plates were examined at low magnification (×6) and each day colonies that were visible were marked and enumerated. In this way, seven counts (or classes) were generated per plate: colonies that were visible after 1, 2, 3, 4, 5, 6, and 10 days. Plates that contained between 5 and 200 colonies were selected for enumeration. When plates became too crowded, the next dilutions were used for enumeration. The number of bacteria in each class was expressed as a percentage of the total count. The different distributions gave insight into the distribution of r- and K-strategists in each sample (17). Characteristics of r-strategists include fast growth in response to enrichment, while K-strategists are characterized by slow growth in response to enrichment (1, 34). Fast growers are defined as bacteria that produced visible colonies at 25°C on 0.1 TSA within 24 h or on P-1 (fluorescent-pseudomonad selective agar) within 48 h (25).

Statistical analyses. All data were analyzed by multinominal analyses of variance (Genstat 5). To normalize data sets, data were $\log_{10}$ or logit transformed before they were analyzed by an F test.

RESULTS

Culturable bacterial populations in soil. The total number of bacterial CFU that could be recovered from soil on 0.1 TSA was not significantly affected by either treatment or time of sampling and stayed at levels of ca. 5×10^7 CFU/g (dry weight) of soil.

Inoculation with either wild-type or recombinant *P. fluorescens* SBW25 did not result in significant changes in the bacterial community structures at any of the samplings (Fig. 2). In general, the population structures in soil were characterized by an even distribution between fast- and slower-growing isolates (Fig. 2). All growth classes contained between 10 and 30% of the total bacterial count. Fast-growing isolates (1, 2, 3, and 4 days) were more dominant ($P < 0.001$) than slowly growing ones (colonies visible after 5, 6, and 10 days) during crop growth. One month after harvest, this pattern changed ($P < 0.001$) in that the percentage of bacteria forming a visible colony after 1 day changed from ca. 20% to ca. 10% (Fig. 2).

Fluorescent-pseudomonad populations in soil. The number of fluorescent pseudomonads that could be recovered from soil on P-1 was not significantly affected by either sampling time or treatment and stayed at levels of ca. 10^6 CFU/g (dry weight) of soil.

The distribution curves of fast- and slowly growing pseudomonads in soil were strongly skewed ($P < 0.001$) toward

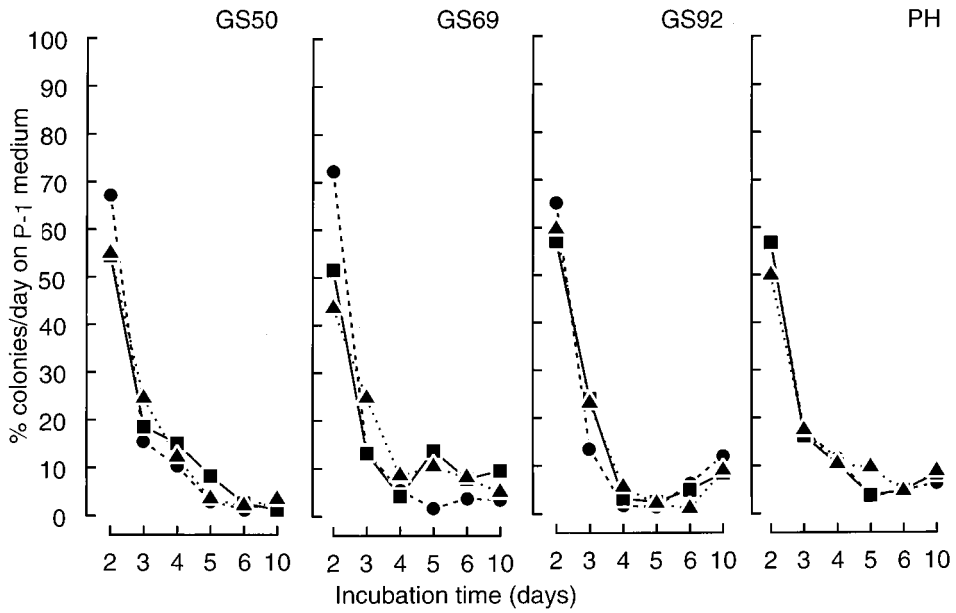

FIG. 3. Fluorescent-pseudomonad community structures in soil planted with noninoculated wheat (●), wheat inoculated with wild-type *P. fluorescens* SBW25 (■), or wheat inoculated with recombinant *P. fluorescens* SBW25 (▲). The soil was sampled at wheat GS 50 to 59 (inflorescence emergence), 69 (flowering), and 92 (ripening), as well as 1 month after harvest (PH). Data are plotted at each sampling as the percentage of the total CFU count that appeared every day over a period of 6 days and after 10 days of incubation at 25°C on P-1. $n = 5$.

Pseudomonas isolates that formed a visible colony on P-1 during a 3-day incubation at 25°C (Fig. 3). This distribution pattern did not change significantly over time and was not significantly influenced by release of wild-type or recombinant *P. fluorescens* SBW25 (Fig. 3).

Culturable bacterial populations in the rhizosphere. Significantly fewer ($P < 0.001$) bacteria could be recovered at the first sampling (GS 12) from the rhizosphere of plants that were inoculated with the wild-type *P. fluorescens* SBW25 than from the other two treatments (Table 1). At the fourth sampling (GS 69), the number of CFU per gram (fresh weight) of root that could be recovered from the inoculated plants was significantly ($P < 0.001$) larger than on the control plants. However, overall, there were no significant differences between the three treatments (Table 1). After the first sampling, a significant ($P < 0.001$) drop in CFU per gram (fresh weight) of root occurred from ca. 10^9 CFU/g to ca. 3×10^8 CFU/g, but bacterial numbers increased subsequently, to ca. 10^9 CFU/g (Table 1).

Significant ($P < 0.001$) differences in the microbial population structure between the control and inoculated plants did occur up to GS 30 (stem elongation). However, these inoculum effects were not consistent; at GS 12 (seedling), the proportion of bacteria that formed a visible colony after 1 day of incubation was 10% higher on the inoculated plants than on the control plants, while at GS 30 (stem elongation), this proportion was 10% lower on the inoculated plants than on the control plants (Fig. 4). Overall, there was no significant treatment effect, and all treatments reverted to a similar population pattern during the later growth stages. During the growing season, the percentage of bacteria that formed a visible colony after 1 and 2 days of incubation decreased ($P < 0.001$), while the percentage of bacterial isolates that formed visible colonies after 3 and 4 days of incubation increased ($P < 0.001$). The percentage of isolates in the rhizosphere that formed a visible colony after 5 days of incubation or more was ca. 5 to 10% of the total culturable bacteria (Fig. 4).

TABLE 1. Culturable bacterial populations in the rhizosphere of wheat in plots given different treatments

Treatment[a]	Population[b] ($\log_{10}$ CFU/g [fresh wt]) at GS[c]:						
	12	30	59	69	85	92	PH
Control	9.05	8.50	8.82	8.79	8.87	9.06	9.00
WT	8.61	8.45	8.74	9.06	8.90	9.18	9.03
GMM	8.95	8.44	8.51	9.03	8.95	9.19	9.13

[a] Control, not inoculated; WT, inoculated with wild-type *P. fluorescens* SBW25; GMM, inoculated with recombinant *P. fluorescens* SBW25.
[b] F_{prob} treatment, not significant; F_{prob} GS, <0.001; F_{prob} interaction, <0.001. Standard error of the difference (SED) = 0.09; $df = 83$.
[c] Populations were monitored at wheat GS 12 (seedling), 30 (stem elongation), 59 (inflorescence emerging), 69 (flowering), 85 (early dough), and 92 (ripening) and 1 month after harvest (PH). $n = 5$.

Fluorescent-pseudomonad populations in the rhizosphere. At GS 69 (flowering), significantly ($P < 0.01$) more fluorescent pseudomonads (measured as CFU per gram [fresh weight] of root) could be recovered from the inoculated plants than from the control treatment (Table 2). Overall, there were no significant treatment effects and fluorescent-pseudomonad numbers stabilized at ca. 3×10^7 CFU/g (fresh weight) of root for the three treatments during the monitoring period (Table 2).

The distribution curve of fast-growing and slowly growing *Pseudomonas* isolates changed ($P < 0.001$) dramatically over time from curves that were skewed toward fast-growing isolates (GS 12; seedling), via a transition phase (GS 50 to 69; inflorescence emergence), to distribution curves that were skewed toward slow-growing *Pseudomonas* isolates at the later growth stages (GS 85 to 92; ripening) and after the crop was harvested (Fig. 5). Although significant ($P < 0.001$) treatment differences did occur, especially during the transition phase (GS 50 to 69), these differences were inconsistent at the different samplings. Overall, no significant differences between treatments were found (Fig. 5).

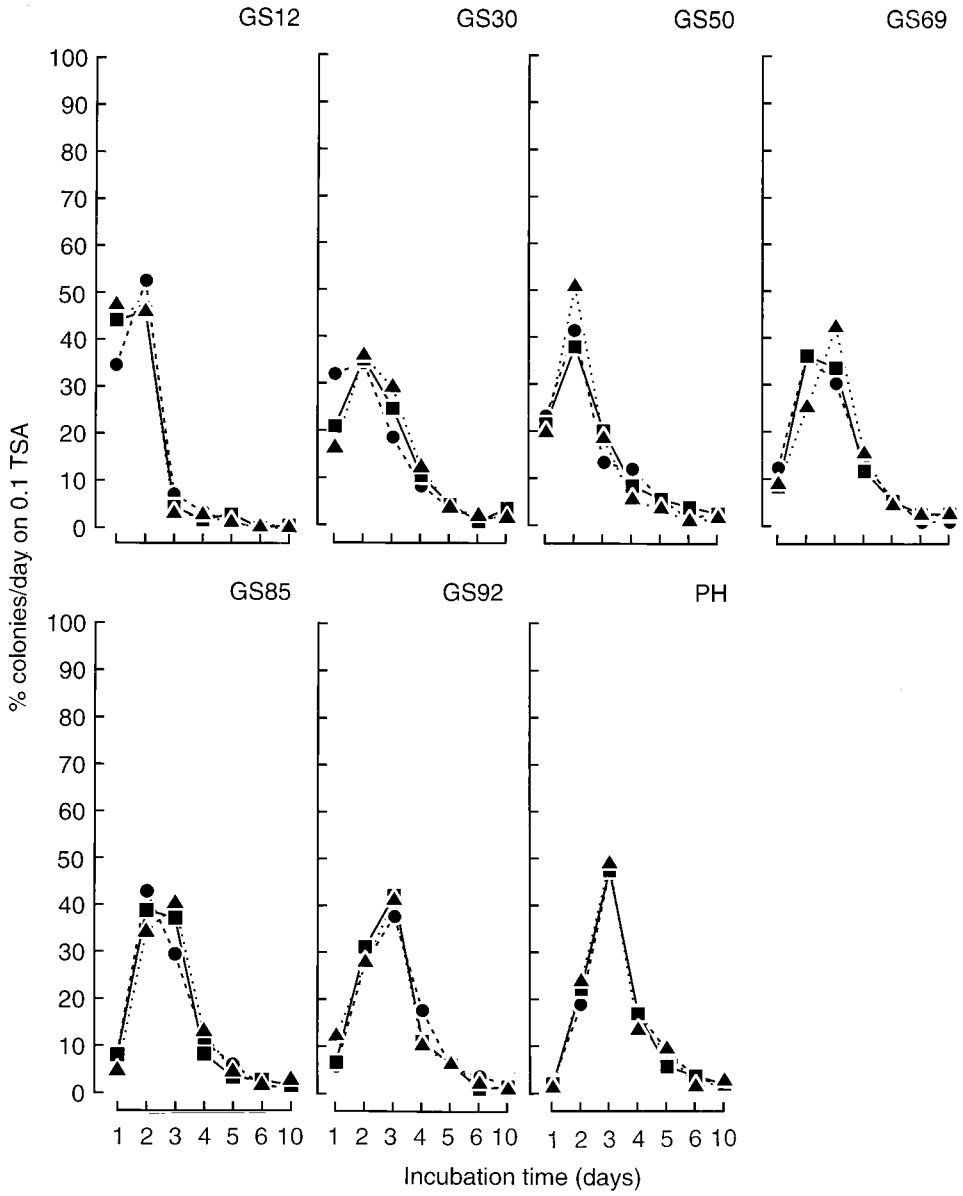

FIG. 4. Bacterial community structures on wheat roots of plants that were either not inoculated (●), inoculated with wild-type *P. fluorescens* SBW25 (■), or inoculated with recombinant *P. fluorescens* SBW25 (▲). The roots were sampled at wheat GS 12 (seedling), 30 (stem elongation), 50 to 59 (inflorescence emergence), 69 (flowering), 85 (early dough), and 92 (ripening), as well as 1 month after harvest (PH). Data are plotted at each sampling as the percentage of the total CFU count that appeared every day over a period of 6 days and after 10 days of incubation at 25°C on 0.1 TSA. $n = 5$.

Culturable bacterial populations on the third leaf to emerge. In general, bacterial development on the third and fifth leaves to emerge, the flag leaf, and the ear were similar. Consequently, to illustrate the bacterial community development on the aerial plant parts and the effect of spray application with either wild-type or recombinant *P. fluorescens* on the indigenous bacterial community, only the bacterial community development of the culturable bacteria and fluorescent pseudomonads on the third leaf to emerge is presented.

The number of bacteria on the third leaf to emerge increased ($P < 0.001$) from ca. 10^5 CFU/g (fresh weight) of leaf at GS 30 to $>10^{10}$ CFU/g (fresh weight) of leaf at the end of the wheat growth cycle (Table 3). The plants that were sprayed with bacteria supported a larger ($P < 0.05$) culturable bacterial

TABLE 2. Indigenous fluorescent-pseudomonad populations in the rhizosphere of wheat in plots given different treatments

Treatment[a]	Population[b] ($\log_{10}$ CFU/g [fresh wt]) at GS[c]:						
	12	30	59	69	85	92	PH
Control	8.14	7.30	7.18	6.95	7.61	7.52	7.17
WT	7.93	7.20	7.51	7.54	7.48	7.55	7.48
GMM	8.24	6.96	6.93	7.34	7.50	7.46	7.53

[a] Control, not inoculated; WT, inoculated with wild-type *P. fluorescens* SBW25; GMM, inoculated with recombinant *P. fluorescens* SBW25.

[b] F_{prob} treatment, not significant; F_{prob} GS, <0.001; F_{prob} interaction, <0.01. SED = 0.17; $df = 83$.

[c] Populations were monitored at wheat GS, 30 (stem elongation), 59 (inflorescence emerging), 69 (flowering), 85 (early dough), and 92 (ripening); $n = 5$.

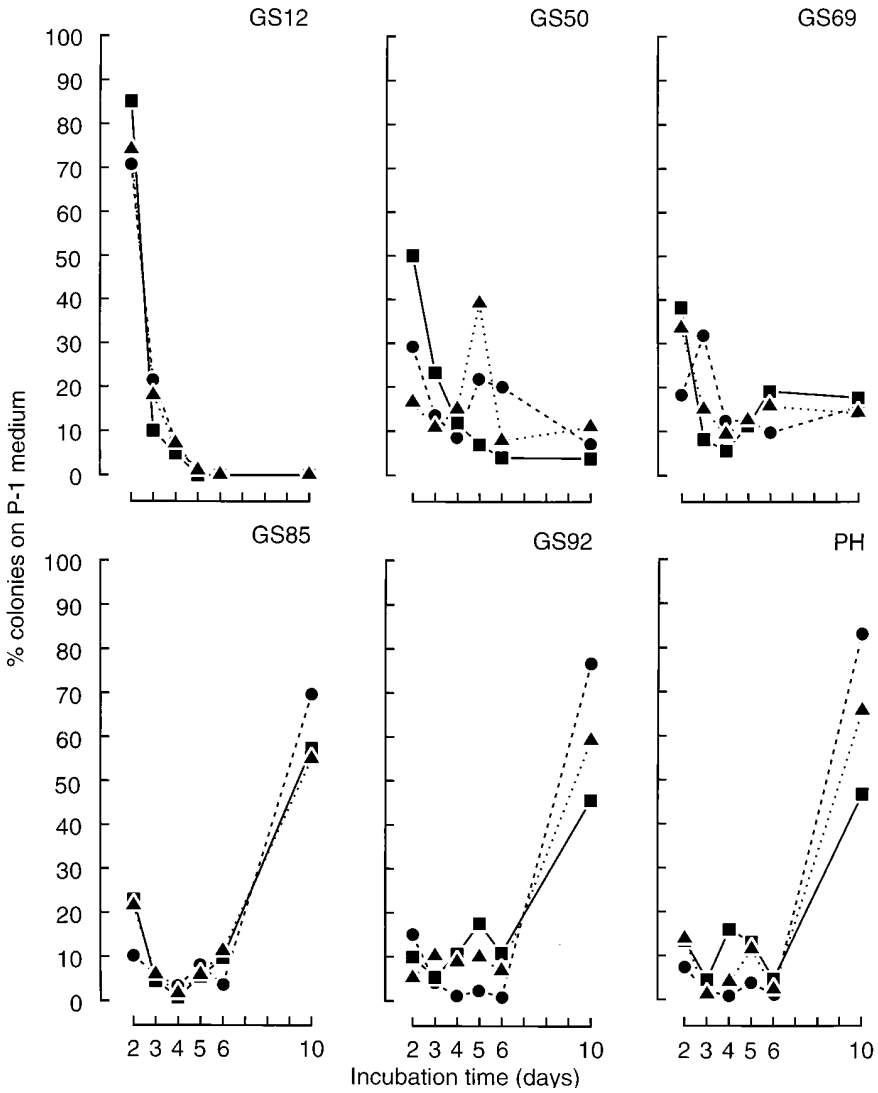

FIG. 5. Fluorescent-pseudomonad community structures on wheat roots of plants that were either not inoculated (●), inoculated with wild-type *P. fluorescens* SBW25 (■), or inoculated with recombinant *P. fluorescens* SBW25 (▲). The roots were sampled at wheat GS 12 (seedling), 30 (stem elongation), 50 to 59 (inflorescence emergence), 69 (flowering), 85 (early dough), and 92 (ripening), as well as 1 month after harvest (PH). Data are plotted at each sampling as the percentage of the total CFU count that appeared every day over a period of 6 days and after 10 days of incubation at 25°C on P-1. $n = 5$.

community than the control plants during the ripening phase (GS 85 to 92) (Table 3).

In general, the distribution curves of fast- and slowly growing bacterial isolates were skewed toward fast-growing isolates; at all sampling, >95% of the culturable population formed visible colonies within 3 days of incubation at 25°C on 0.1 TSA. The distribution of the growth classes changed ($P < 0.001$) with time from a population in which 90% of the isolates formed a visible colony after 1 day of incubation to one in which this proportion was around 40% (Fig. 6). Significant ($P < 0.001$) differences in the bacterial community structure between the control and inoculated plants did occur at GS 69 (flowering), when 70 to 80% of the bacterial community on the inoculated plants consisted of isolates that formed a visible colony within 1 day, while the proportion of such isolates was only 40% on the control plants at GS 69. However, overall, there was no significant treatment effect.

Fluorescent-pseudomonad populations on the third leaf to emerge. In all treatments, fluorescent-pseudomonad populations increased ($P < 0.001$) rapidly from $<10^3$ CFU/g (fresh weight) of leaf at GS 30 (stem elongation) to a stable population of $>10^8$ CFU/g (fresh weight) of leaf between GS 59 (inflorescence emergence) and GS 85 (late dough). Subsequently, populations decreased ($P < 0.05$) slightly, to reach

TABLE 3. Culturable bacterial populations on the third leaf to emerge in plots given different treatments

Treatment[a]	Population[b] ($\log_{10}$ CFU/g [fresh wt]) at GS[c]:				
	30	59	69	85	92
Control	5.04	7.83	9.33	9.68	9.85
WT	4.76	7.66	9.75	10.11	10.27
GMM	5.19	7.66	9.29	10.08	10.13

[a] Control, not inoculated; WT, inoculated with wild-type *P. fluorescens* SBW25; GMM, inoculated with recombinant *P. fluorescens* SBW25.

[b] F_{prob} treatment, not significant; F_{prob} GS, <0.001; F_{prob} interaction, <0.05. SED = 0.19; $df = 60$.

[c] Populations were monitored at wheat GS, 30 (stem elongation), 59 (inflorescence emerging), 69 (flowering), 85 (early dough), and 92 (ripening); $n = 5$.

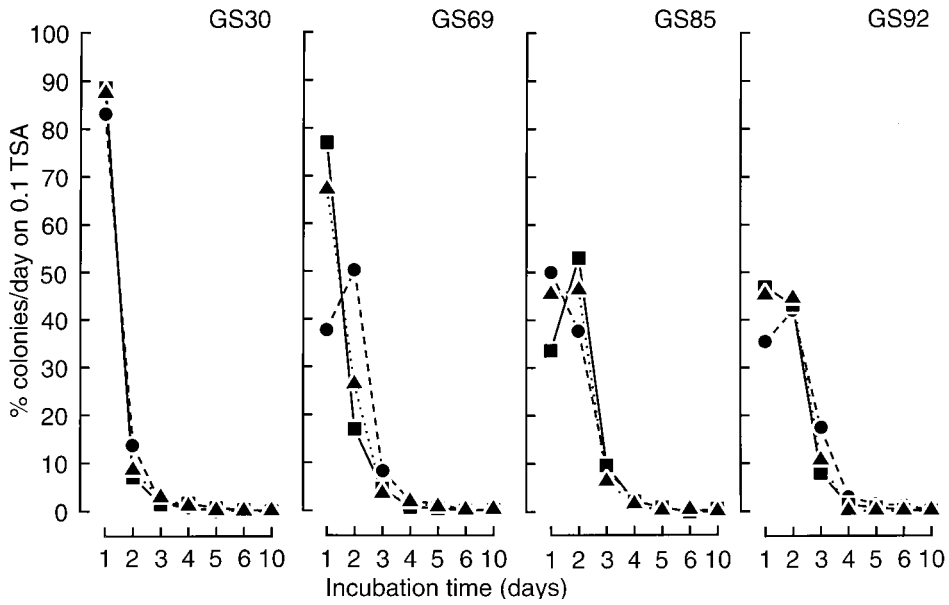

FIG. 6. Bacterial community structures on the third emerging leaves of plants that were either not inoculated (●), inoculated with wild-type *P. fluorescens* SBW25 (■), or inoculated with recombinant *P. fluorescens* SBW25 (▲). The leaves were sampled at wheat GS 30 (stem elongation), 69 (flowering), 85 (early dough), and 92 (ripening). Data are plotted at each sampling as the percentage of the total CFU count that appeared every day over a period of 6 days and after 10 days of incubation at 25°C on 0.1 TSA. $n = 5$.

a level of 3×10^7 CFU/g (fresh weight) of leaf at the time of harvest (Table 4). The leaves sprayed with *P. fluorescens* SBW25 (wild type and recombinant) supported more ($P < 0.05$) indigenous pseudomonads than did the control (Table 4).

The distribution of fast- and slowly growing *Pseudomonas* isolates was highly variable ($P < 0.001$) from sampling to sampling, switching from populations that were dominated by fast-growing isolates (GS 30 and 69) to ones that were completely dominated by slowly growing isolates (GS 85). At harvest (GS 92), an even distribution of fast- and slowly growing isolates was found (Fig. 7).

Effects of wild-type and GM *P. fluorescens* on other microbial populations in the phytosphere of wheat. At 2 weeks after seed inoculation (GS 12; seedling) with wild-type or recombinant *P. fluorescens* SBW25, fewer ($P < 0.05$) yeasts were recovered from the roots, seeds, and hypocotyls of inoculated plants than from the control plants (Table 5). *P. putida* populations were affected in a similar way ($P < 0.05$) by the released bacteria, but no significant perturbations were recorded in the rhizosphere of the wheat seedlings (Table 5). In contrast, filamentous fungal and actinomycete populations were, in general, not significantly affected by the bacterial inocula (Table 5).

After spray application, yeast, *P. putida*, and filamentous fungus populations on the phylloplane were significantly ($P < 0.001$) perturbed by both the wild-type and recombinant *P. fluorescens* SBW25 (Table 6). These effects were most pronounced on the fifth leaf to emerge. Compared with the control treatment, the populations of yeasts, *P. putida*, and filamentous fungi were smaller on the fifth leaf to emerge before the wheat flowered (GS 69) but larger during the first phases of the ripening process (GS 85). At harvest (GS 92), no significant differences between treatments could be found (Table 6).

TABLE 4. Indigenous fluorescent pseudomonad populations on the third leaf to emerge in plots given different treatments

Treatment[a]	Population[b] ($\log_{10}$ CFU/g [fresh wt]) at GS[c]:				
	30	59	69	85	92
Control	2.31	7.57	8.18	7.98	7.38
WT	2.46	8.85	8.31	8.43	7.71
GMM	2.76	8.12	8.07	8.25	7.30

[a] Control, not inoculated; WT, inoculated with wild-type *P. fluorescens* SBW25; GMM, inoculated with recombinant *P. fluorescens* SBW25.

[b] F_{prob} treatment, <0.05; F_{prob} GS, <0.001; F_{prob} interaction, not significant. SED = 0.37; $df = 60$.

[c] Populations were monitored at wheat growth stages 12 (seedling), 30 (stem elongation), 59 (inflorescence emerging), 69 (flowering), 85 (early dough), and 92 (ripening) and 1 month after harvest (PH). $n = 5$.

DISCUSSION

The release of wild-type and recombinant *P. fluorescens* SBW25 resulted in significant but transient perturbations of some of the culturable components of the indigenous microbial community that inhabited the phytosphere of wheat. In terms of hazard and risk assessment, the observed perturbations seemed of minor importance. First, the effects induced by the wild-type *P. fluorescens* SBW25 were, in general, not significantly different from those induced by the GMM. Second, the effects were, in general, small and transient. Third, there were no obvious effects on plant growth or plant health.

The concept of r- and K-strategists for risk assessment purposes might be very useful, since r-strategists are characteristic of environments that undergo rapid changes while K-strategists dominate in stable nonperturbed environments (1, 33). Populations of r-strategists should therefore undergo rapid changes when an environment is perturbed, because r-strategists can respond with large population increases when conditions are favorable, whereas because of their poor competitive abilities and lack of long-term survival mechanisms, their numbers decline rapidly when conditions deteriorate. In contrast, the population size of typical K-strategists is more buffered against perturbation because of their slow growth and ability to

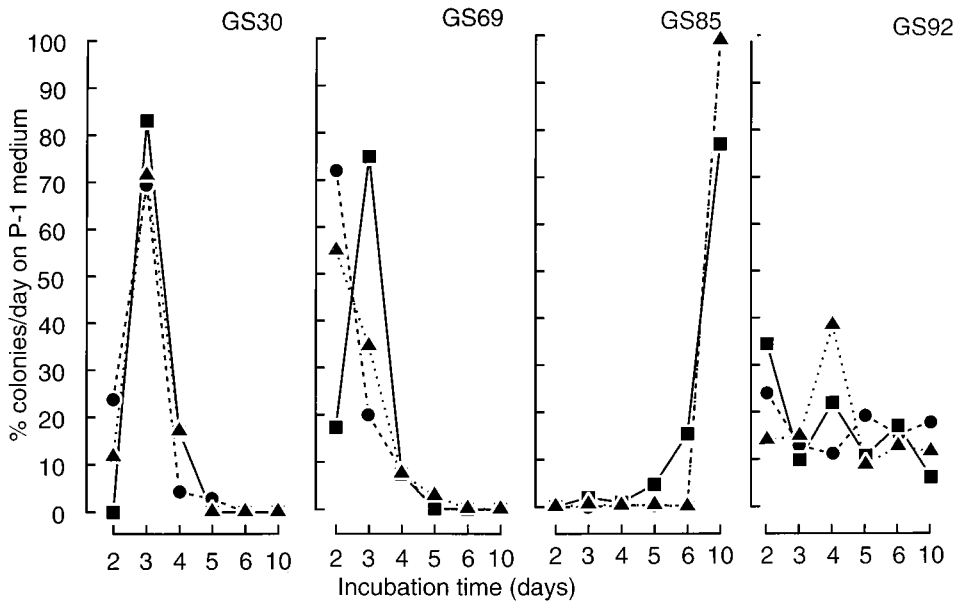

FIG. 7. Fluorescent-pseudomonad community structures on the third leaf to emerge on plots that were either not inoculated (●), inoculated with wild-type *P. fluorescens* SBW25 (■), or inoculated with recombinant *P. fluorescens* SBW25 (▲). The leaves were sampled at wheat GS 30 (stem elongation), 69 (flowering), 85 (early dough), and 92 (ripening). Data are plotted at each sampling as the percentage of the total CFU count that appeared every day over a period of 6 days and after 10 days of incubation at 25°C on P-1. $n = 5$.

form long-term survival structures. Activity in situ cannot be monitored, especially when plating and culturing techniques are used, resulting in more or less stable CFU counts of typical K-strategists, even if large perturbations were to occur. The results of this study are consistent with this hypothesis; organisms such as *P. putida*, rapidly growing bacterial isolates, and yeasts, which all might be regarded as typical r-strategists, not only were sensitive indicators of environmental change (15) but also responded with large population decreases in the presence of the wild-type and recombinant *P. fluorescens* SBW25 during the early stages of wheat development. Presumably, because the introduced organism itself can be regarded as a typical r-strategist, these effects did not last and the indigenous r-strategist populations were restored during the remainder of the growing season.

More difficult to interpret are the changes in fluorescent-pseudomonad community structures that occurred on the roots and the phylloplane during wheat development. Effects of the bacterial inocula on the *Pseudomonas* community structure derived from the rhizosphere occurred during the transition from a *Pseudomonas* population which was dominated by fast-growing isolates (GS 12) to one dominated by slowly growing isolates (GS 85 and 92 and postharvest). On the phylloplane, the fluorescent pseudomonad community structure was in general highly variable. It is unlikely that these differences were the result of a real shift in isolate composition in these habitats in a genotypic sense, i.e., different species. It is more likely that changes occurred because the environmental conditions induced different physiological states (phenotypes) in a genotypically stable fluorescent-pseudomonad community (22, 23). Therefore, not only is the colony developmental time dependent on the bacterial isolate being an r- or K-strategist but also, depending on the type of isolation medium used, other characteristics, such as the physiological state of an organism, might reveal themselves.

Perturbations resulting from introductions of wild-type and recombinant *P. fluorescens* SBW25 did occur in all the monitored habitats, except the soil. These perturbations were most pronounced on the seed and the hypocotyl after seed inoculation and after spray application on the fifth leaf to emerge. Presumably, indigenous microbial populations on seeds, hypocotyl, and the fifth emerging leaves were relatively small at the

TABLE 5. Populations of yeasts, filamentous fungi, *P. putida*, and actinomycetes on roots, seeds, and hypocotyl isolated from wheat seedlings (GS 12) in plots given different treatments

Treatment[a]	Yeast population[b] on:			Filamentous fungus population[b] on:			*P. putida* population[b] on:			Actinomycete population[b] on roots
	Roots	Seeds	Hypocotyl	Roots	Seeds	Hypocotyl	Roots	Seeds	Hypocotyl	
Control	5.37	5.91	5.66	4.64	3.48	3.98	6.72	5.91	4.51	7.71
WT	4.17	2.76	2.78	4.96	3.38	3.95	6.46	5.22	3.65	7.54
GMM	4.62	3.58	3.26	4.92	3.79	3.76	6.70	4.89	3.48	7.55
SED	0.29	0.28	0.85	0.20	0.34	0.34	0.22	0.29	0.18	0.13
F_{prob}	<0.05	<0.001	<0.05	NS[c]	NS	NS	NS	<0.05	<0.05	NS

[a] Control, not inoculated; WT, inoculated with wild-type *P. fluorescens* SBW25; GMM, inoculated with recombinant *P. fluorescens* SBW25.
[b] Populations on roots and hypocotyl are expressed as $\log_{10}$ CFU per gram (fresh weight); populations on seeds are expressed as $\log_{10}$ CFU per seed. $n = 5$; $df = 12$.
[c] NS, not significant.

TABLE 6. Populations of yeasts, filamentous fungi, and *P. putida* isolated from the fifth leaf to emerge on plots given different treatments

GS[a] and probability	Population ($\log_{10}$ CFU/g [fresh wt])[b] on:								
	Yeasts			Filamentous fungi			*P. putida*		
	Control	WT	GMM	Control	WT	GMM	Control	WT	GMM
59	5.97	4.92	4.60	6.00	5.63	5.53	3.99	2.40	3.13
69	5.48	5.97	6.31	6.15	5.84	6.29	2.14	2.26	2.00
85	5.56	6.21	6.49	6.71	7.67	7.65	2.75	3.34	3.97
92	7.07	7.28	6.84	7.50	7.21	7.30	2.17	2.00	2.00
SED		0.30			0.28			0.37	
F_{prob} treatment		NS[c]			NS			NS	
F_{prob} GS		<0.001			<0.001			<0.001	
F_{prob} interaction		<0.001			<0.01			<0.001	

[a] Samples were taken at GS 59 (inflorescence), 69 (flowering), 85 (early dough) and 92 (ripening).
[b] Control, not inoculated; WT, inoculated with wild-type *P. fluorescens* SBW25; GMM, inoculated with recombinant *P. fluorescens* SBW25. $n = 5$; $df = 48$.
[c] NS, not significant.

time of the bacterial applications. The large load of the introduced *P. fluorescens* SBW25 strains, therefore, could effectively colonize these habitats and prevent a population buildup of indigenous microorganisms. In those habitats already colonized by the indigenous microflora (soil, roots, third leaf), perturbations were less pronounced and were often absent, presumably because the numbers and the competitive ability of the introduced *Pseudomonas* strains were not great enough to displace the established microbial populations. The competitiveness of an organism in relation to the indigenous microbial community into which it is released seems to be an important characteristic for determining possible harm to the environment. Only if the competitive ability of the released strains is greater than that of the established microbial populations in the environment would there be reason for concern.

Shortly after application of the microbial inocula, an inhibition of the indigenous microbial populations could often be found. However, such negative effects often progressed to a situation in which the indigenous microbial populations were larger on the inoculated plants than on the noninoculated ones. The reason for this might be that immediately after application of the microbial inocula, the introduced bacteria were active and therefore inhibited indigenous microbial populations. Although the introduced bacteria persisted in the phytosphere of the inoculated wheat plants (14), it is unlikely that activity was maintained during the whole of the growing season. Most probably, a proportion of the introduced inoculum died, releasing nutrients that could be utilized by the indigenous microbial populations for cell maintenance and growth. According to this hypothesis, bacterial populations on the phylloplane later in the season should be larger on the sprayed plant parts (i.e., third leaves) than on parts that were colonized subsequently but did not receive a large bacterial inoculum (i.e., fifth leaves). Results not presented in this paper suggest that this is the case. At growth stages 85 and 92, the fifth leaves taken from plants that were sprayed with either the wild-type or the recombinant harbored similar numbers of culturable bacteria to those on the untreated control. In contrast, for the third leaves of the same plants, significantly more bacteria could be isolated from plants sprayed with bacteria than from the untreated control.

Quantification of the culturable bacterial and fungal populations in the soil, the rhizosphere, and the phytosphere yielded significant population changes, not only as a result of a changing plant environment but also as a result of introductions of unmodified and recombinant *P. fluorescens* SBW25. However, there are a number of criticisms concerning the validity of such an approach. First, it must be assumed that a large proportion of the microbial community present in the environment cannot be cultured on laboratory media (10). As a consequence, perturbations of such viable but nonculturable populations cannot be detected. Second, dilution plating on general isolation media, such as 0.1 TSA and PDA$^+$, will allow the recovery only of those microorganisms that are present in large numbers; recovery of microbial populations that are present in relatively small numbers requires either specific selective media or targeted molecular techniques, such as PCR. Third, quantification of microbial populations by using CFU counts provides no information about the activity of the isolated microbial populations. In the case of *Trichoderma harzianum*, an antagonist of damping-off diseases, it was found that there was an inverse relation between the number of CFU and the activity of this fungus (31). Similar results were obtained with the nematophagous fungus *Verticillium chlamydosporium* (13). Therefore, CFU counts can be misleading, especially those that are derived from propagules produced by filamentous fungi and many gram-positive bacteria which produce large numbers of inactive spores in response to environmental stress. Although microbial population studies can provide valuable information concerning the impact of GMMs on indigenous microbial populations, it must be stressed that microbial population studies on their own are incomplete and should therefore not be used as the only method to assess whether a GMM is environmentally safe. Ideally, microbial population studies should be linked to broader aspects of ecosystem functioning, such as effects on plant growth, plant health, nutrient cycling, and soil structure. Quantification of such effects avoids the problems mentioned above, and their causes can be investigated subsequently by combinations of traditional and molecular techniques.

However, such assessments should not be exclusively used to assess the risks of GMMs. The expression of novel as well as existing traits expressed by released microorganisms should be the focus of concern, not the fact that microorganisms are altered by molecular techniques. Therefore, any introduced organisms, as well as normal agricultural practices, should be evaluated for their impact on ecosystem functioning. The result of the wider application of environmental risk assessments would have the added benefit that the environmental impact of GMMs could be placed into a broader perspective. This would result in a more objective evaluation of risks and benefits connected with future GMM releases.

ACKNOWLEDGMENTS

This work was carried out under contracts PECD 7/8/161 and PECD 7/8/143 of the Department of the Environment Genetically Modified Organism Research Programme.

We thank Mark Bailey for providing us with the chromosomally marked *Pseudomonas* strain.

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