RESEARCH ARTICLE

Use of a novel nonantibiotic triple marker gene cassette to monitor high survival of *Pseudomonas fluorescens* SBW25 on winter wheat in the field

Lotta Jäderlund[1], Maria Hellman[1], Ingvar Sundh[1], Mark J. Bailey[2] & Janet K. Jansson[1]

[1]Department of Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden; and [2]NERC Institute of Virology and Environmental Microbiology (IVEM), Oxford, UK

Correspondence: Janet K. Jansson, Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, S-750 07 Uppsala, Sweden. Tel.: +46 (0) 18 67 3201; fax: +46 (0) 18 67 3392; e-mail: janet.jansson@mikrob.slu.se

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Abstract

Pseudomonas fluorescens SBW25 was tagged with a triple marker gene cassette containing *gfp*, encoding green fluorescent protein; *luxAB*, encoding luciferase; and *telABkilA*, encoding tellurite resistance, and the tagged strain was monitored in the first Swedish field release of a genetically modified microorganism (GMM). The cells were inoculated onto winter wheat seeds and the GMM cells (SBW25::tgl) were monitored in the field from September 2005 to May 2006 using plating, luminometry and microscopic analyses. Cell numbers were high on all sampling occasions and metabolically active cells were detected on all plant parts. Field results were similar to those obtained in a parallel phytotron study, although the amount of SBW25::tgl detected on shoots was significantly higher in the phytotron than in the field. After winter, cell counts were 100-fold higher on the roots and root-associated soil compared with prewinter measurements, although the cells had a lower relative metabolic activity. The wheat seeds were naturally infested with *Microdochium nivale*, but no treatment resulted in reduction of disease symptoms. No SWB25::tgl cells were ever found in bulk soil or uninoculated plants. The Swedish field trial results complement and contrast with prior field studies performed with the same parent organism in the United Kingdom under different soil, plant and climatic conditions.

Introduction

Pseudomonas fluorescens SBW25 was isolated in 1989 from the phyllosphere of sugar beet in the United Kingdom (Thompson *et al.*, 1995a). This bacterium is an effective colonizer of the rhizosphere and phyllosphere of a number of different plants such as cornflower, pea and wheat (Thompson *et al.*, 1995a, b). In a previous greenhouse study SBW25 was shown to establish on the seeds, roots and first emerging leaves of wheat after inoculation of the seeds (Unge & Jansson, 2001). The SBW25 strain also has plant growth promoting traits, presumably by competitive exclusion of potential pathogens, and has previously been shown to inhibit growth of *Pythium ultimum* (Ellis *et al.*, 2000).

Pseudomonas fluorescens SBW25 has been extensively studied under field (in the United Kingdom), greenhouse (UK and Sweden) and laboratory conditions (Bailey *et al.*, 1995; de Leij *et al.*, 1995a; Thompson *et al.*, 1995b; Lilley & Bailey, 1997; Ellis *et al.*, 2000; Timms-Wilson *et al.*, 2000, 2004; Preston *et al.*, 2001; Turnbull *et al.*, 2001; Unge & Jansson, 2001; Gal *et al.*, 2003). Because considerable data are available about the behavior of genetically modified SBW25 in the field in the United Kingdom, the authors were interested in using this bacterium as the model strain for the first field trial of a genetically modified bacterium in Sweden. The results of this study enable the first comparison of field release data obtained using two genetically modified derivatives of the same parent bacterial strain released as a genetically modified microorganism (GMM) on different crops in two European countries with different soil and climatic conditions.

In order to establish the efficacy of specific inoculants in the field, it is advantageous to have efficient tools for monitoring the specific cells after their release. For example, introduced marker genes can be used to specifically distinguish the cells of interest from indigenous relatives in soil and on plant material (Jansson, 2002, 2003). A combination of two marker genes (*luxAB* and *gfp*) was previously used to

detect both metabolically active and inactive cells of SBW25, respectively (Tombolini *et al*., 1997; Unge *et al*., 1999; Unge & Jansson, 2001). The *gfp* gene, encoding the green fluorescent protein (GFP) (Chalfie *et al*., 1994; Bloemberg *et al*., 1997), fluoresces under blue light illumination, and no additional substrate is needed. The *luxAB* genes, encoding bacterial luciferase, can be used to measure the metabolic activity of the marked organisms because light output from the luciferase reaction is dependent on cellular energy reserves (Meikle *et al*., 1994; Prosser *et al*., 2000). Previous field trials in the United Kingdom with genetically modified SBW25 have been performed with two sets of marker genes to construct the GMM SBW25EeZY6KX. The genes inserted provided the ability to utilize lactose (*lacZY*, lactose permease and β-galactosidase activity), resistance to kanamycin (*kan*^{r}) and catechol 2,3-deoxygenase activity (*xylE*) (Thompson *et al*., 1995b).

Most mini-transposon vectors and other vectors used to integrate marker genes into bacteria rely on antibiotic resistance genes as a means of selection during the tagging process (de Lorenzo *et al*., 1990; Bailey *et al*., 1995, 2000; de Leij *et al*., 1995b; Egan & Wellington, 2000; Ramos *et al*., 2000; Jansson, 2002; Pitkäjärvi *et al*., 2003; Viebahn *et al*., 2003). However, concerns have been raised whether these genes may contribute to the global antibiotic resistance threat in bacteria (Dröge *et al*., 1998; Livermore, 2005; Berger-Bächi & McCallum, 2006) and many countries now restrict the release of antibiotic resistance genes into the environment. Another option is to use genes encoding resistance to heavy metals such as mercury (Herrero *et al*., 1990; Saint *et al*., 1995), or tellurium salts (Summers & Jacoby, 1977; Walter *et al*., 1991; Sanchez-Romero *et al*., 1998). When cultivated on agar containing potassium tellurite oxide (K_2TeO_3), cells having these genes convert TeO_3^{2-} to black metallic tellurium (Te) and they are resistant to tellurite. The resultant colonies form a black precipitate and they can readily be distinguished from background growth.

The aim of this study was to monitor genetically modified *P. fluorescens* SBW25 on wheat plants and in surrounding soil under Swedish field conditions and to determine the fate and persistence of the cells as a model for GMM risk assessment in Sweden. In addition, the GMM strain of *P. fluorescens* SBW25 was monitored in a highly controlled phytotron growth chamber where relative humidity, daylight and temperatures were changed daily to match field conditions and to compare the phytotron results with the results obtained in the field. The GMM was released as a seed inoculum on wheat seeds that were naturally infested with *Microdochium nivale* (causative agent of pink snow mold). *Microdochium nivale* is an economically important plant pathogen in many temperate zones. Disease occurs whenever winter is characterized by heavy snow cover, especially on unfrozen ground, for a longer period of time (Smith, 1981). The hypothesis was that SBW25 would promote wheat plant growth and suppress disease symptoms in the field by indirect means such as induced resistance or competitive exclusion of the pathogen due to its known plant colonization ability (Brown & Beringer, 1983; de Leij *et al*., 1995b; Thompson *et al*., 1995b; Rainey, 1999; Ellis *et al*., 2000; Naseby *et al*., 2001; Unge & Jansson, 2001; Gal *et al*., 2003).

The field trial that is the basis of this study was the first release of a GMM in Sweden, after approval by the Swedish Chemicals Agency (B/SE/05/KEMI-723-573, further information on http://gmoinfo.jrc.it/).

Materials and methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1. *Pseudomonas fluorescens* SBW25 was grown in 0.1 × LB (1 L LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl) at 28 °C. *Escherichia coli* CC118 (λpir) was used to maintain the mini-transposon pUT-plasmid derivatives and it was grown in LB at 37 °C. When needed the growth media were supplemented with K_2TeO_3 (35 μg mL^{-1}), ampicillin (100 μg mL^{-1}) or kanamycin (50 μg mL^{-1}). Growth was measured as $OD_{600\,nm}$.

Mini-transposon vector construction

Cloning procedures were performed according to standard procedures (Sambrook & Russell, 2001). Mini-transposon

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/relevant characteristics	References
Pseudomonas fluorescens SBW25	Sugar beet leaf isolate, root colonizer, PGPR	Thompson *et al*. (1995a)
Pseudomonas fluorescens SBW25::tgl	Tel^R; inserted marker genes; *gfp/luxAB*, *telAB/kilA*	This study
Escherichia coli CC118 (λpir)	*Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE argE(Am) recA1 λpir*	Herrero *et al*. (1990)
Escherichia coli DH5α	Negative control bacterium for fungal inhibition assay	Hanahan (1983)
Paenibacillus brasilensis PB177	Positive control bacterium for fungal inhibition assay	von der Weid *et al*. (2002)
pUTgfplux	Ap^R Km^R; mini-Tn5 delivery plasmid for marker genes: *gfp/luxAB nptII*	Unge *et al*. (1999)
pUTtel	Ap^R Tel^R; mini-Tn5 delivery plasmid for marker genes: *telAB/kilA*	Herrero *et al*. (1990)
pUTtgl	Ap^R Tel^R; mini-Tn5 delivery plasmid for marker genes: *gfp/luxAB telAB/kilA*	This study

vector DNA was purified from the host strain CC118 (λpir) with the QIAprep® Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany) or GenElute™ Plasmid Maxi-Prep Kit (Sigma-Aldrich, St Louis, MO). The P*psbA-gfp/luxAB* DNA fragment was excised from vector pUTgfplux (Unge *et al.*, 1999) using NotI (New England Biolabs, Beverly, MA) and the fragment was purified by gel extraction using the QIAquick® Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). The purified pUTtel vector was cleaved at the unique NotI site, dephosphorylated with Shrimp Alkaline phosphatase (Roche Diagnostics, Basel, Switzerland), extracted with phenol : chloroform and precipitated with ethanol. The cleaved pUTtel vector and the P*psbA-gfp/luxAB* fragment were ligated (DNA ratio 1 : 1) using the Ligation kit Ready-To-Go™ T4DNA Ligase (GE Healthcare, Buckinghamshire, UK), and the ligation mixture was precipitated with ethanol and used in electroporation reactions. *Escherichia coli* CC118 (λpir) cells were made electrocompetent according to Sambrook & Russell (2001) with a modification of using distilled water instead of 10% glycerol, and were electroporated using BIORAD Gene Pulser II (BIORAD, Hercules, CA), with settings at 2.5 kV, 200 Ω, and 25 μF. Immediately after electroporation the cells were resuspended in 1 mL LB medium and incubated at 37 °C for 1 h. This mixture was plated onto LA (LB broth agar) plates supplemented with ampicillin (100 μg mL^{-1}) and K_2TeO_3 (35 μg mL^{-1}) and incubated at 37 °C for 24 h. Black colonies that were green fluorescent under blue light illumination and producing bioluminescence were selected. Transformants were grown overnight in LB containing K_2TeO_3 (35 μg mL^{-1}) and ampicillin (100 μg mL^{-1}). Resulting minitransposon pUTtgl (Fig. 1) was maintained and amplified in CC118 (λpir).

Electroporation of SBW25

Pseudomonas fluorescens SBW25 cells were collected at the exponential growth phase and were made electrocompetent according to standard procedures (Sambrook & Russell, 2001), with the modification mentioned above. The final concentration was adjusted to 10^{10} cells mL^{-1} distilled water. Plasmid pUTtgl, 2.7 μg, was electroporated into the cells using the same settings as for *E. coli* CC118 (λpir). After electroporation the cells were immediately transferred to 1 mL LB and incubated for 3 h at 28 °C. Transformants were selected on LA plates containing K_2TeO_3 (35 μg mL^{-1}).

Characterization of transformants

A southern blot was performed to ensure that only one copy of the transposable element from pUTtgl was inserted into the chromosome of SBW25, using 5′-TACATAACCTTCGGGCATGGCACT-3′ as a probe towards the *gfp* gene.

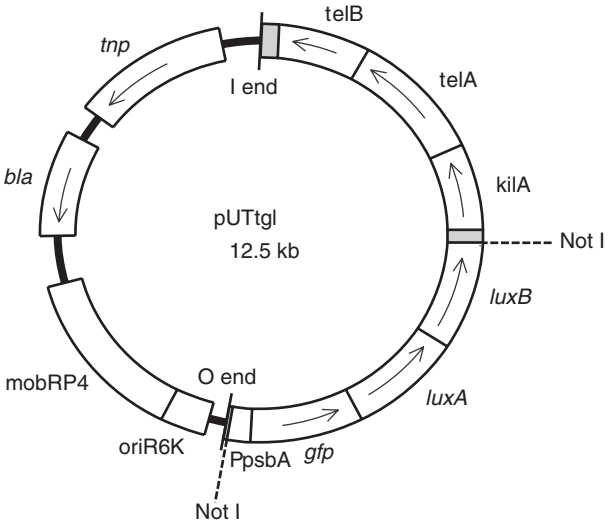

Fig. 1. pUTtgl minitransposon vector used for stable integration of a triple marker gene cassette into the chromosome of recipient bacteria. Marker genes: *gfp*, encoding GFP, *luxAB*, encoding bacterial luciferase, *kilA*, *telAB*, encoding resistance to tellurite. O end and I end indicate insertion sequences that are integrated into the chromosome. NotI; restriction site; *tnp*, transposase; *bla*, ampicillin resistance; mobRP4; transfer region, oriR6K; *Escherichia coli* origin of replication.

Transformants were tested with respect to growth characteristics compared with the SBW25 wt in M9 minimal medium (Sambrook & Russell, 2001). Overnight cultures of each strain were washed in phosphate-buffered saline buffer (PBS 1 L: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; pH = 7.4) and resuspended in triplicate 100 mL flasks, each containing 50 mL M9 medium, to a final concentration of 10^9 cells mL^{-1}. The cultures were incubated at either 28 or 4 °C in different experiments. Samples were taken periodically and growth was determined as $OD_{600\,nm}$.

A selected transformant was also compared with the wild-type (wt) strain for its ability to survive 24 h incubation in cold (4 °C) sterile soil. The same clay loam field soil as that used for the field trial was autoclaved twice (121 °C, 20 min) in 100 mL flasks. Each treatment was established in triplicate flasks containing 10 g sterile soil. Overnight bacterial cultures were washed once in PBS and 3 mL of bacterial suspension (3×10^8 cells mL^{-1}) were added to each flask and incubated at 4 °C for 24 h. CFU were determined from soil samples that were diluted in PBS and inoculated onto nonselective LA plates. The use of nonselective medium and sterile soil enabled the wt *P. fluorescens* SBW25 strain to be monitored, in addition to the GMM variant.

Fungal inhibition assay

A plate assay was performed to determine the possible antagonistic effect of *P. fluorescens* SBW25 towards *M. nivale*. Potato dextrose agar (PDA; Oxoid Ltd, Basingstoke,

UK) plates were inoculated with both bacteria and fungi at a distance of 7 cm from each other. *Pseudomonas fluorescens* SBW25 was inoculated as a 2 cm streak at one end of the agar plate and *M. nivale* was inoculated as an agar-plug (5 mm diameter) from a growing fungal colony at the other end of the plate. After 2 weeks incubation at room temperature the plates were evaluated for evidence of fungal growth inhibition compared with plates that were not inoculated with the bacteria or to control bacterial strains. As a positive control *Paenibacillus brasilensis* PB177 (Table 1) was used (a clear zone of inhibition was seen) and *E. coli* DH5α (Table 1) was used as a negative control (the fungi grew over the whole plate, including the area inoculated with bacteria).

Seed coating

Winter wheat seeds (cultivars Kosack or Tarso for phytotron studies, and Tarso for field trial) naturally infested with *M. nivale* were inoculated with bacteria by seed coating before sowing. The infection levels of the seeds with *M. nivale* were 46% (Kosack) and 34–53% (Tarso) as evaluated in a previous seed hygienic test [International Seed Testing Association (ISTA), www.seedtest.org]. Overnight cultures of SBW25 wt and genetically modified strains were washed and resuspended in PBS. To acclimatize the cells to starvation conditions in soil the cell suspensions were kept in PBS at 28 °C for 24 h before coating the seeds and sowing. The wheat seeds were soaked in the bacterial suspensions (300 mL suspension kg^{-1} seed) for 5 min in 50-mL plastic tubes (phytotron) or 250-mL glass flasks (field trial) during vigorous shaking. The seeds were then placed on absorbent paper for 10 min to remove excess bacterial suspension and then planted directly into soil. For inoculation doses see 'Phytotron experimental conditions' and 'Field trial experimental design' sections.

Phytotron experimental conditions

Before field trial

The phytotron at the Swedish University of Agricultural Sciences (SLU) has growth chambers where air humidity, photoperiod, light intensity, temperature, water and nutrient supply can be independently controlled. The phytotron was used before the field release in order to decide which infection level in percent (*M. nivale*) of winter wheat and cultivar to use in the field trial. Treatments of the seeds were: (1) SBW25::tgl; (2) SBW25 wt; (3) a chemical fungicide, Celest (2 mL kg^{-1} seeds); and (4) PBS. Seeds were coated with bacterial suspensions as described above to a concentration of *c.* 10^7 CFU g^{-1} inoculated seed. For each seed treatment there were four replicate plastic pots (16.5 cm diameter, 11 cm height). To each pot, 0.25 L of nonsterile soil collected from the same site as the eventual field trial was added, and 50 treated seeds were sown in each pot. To cover the seeds 0.5 L of soil was placed on top. Pots were placed randomly in the phytotron chamber and were moved around every second day to avoid interaction between plant development and variations in chamber temperature or other factors. For the experiment before the field trial the climate in the phytotron was set at 8 °C and 16/8 h light/dark cycles.

In parallel to field trial

A second phytotron experiment was performed in parallel with the first 4 weeks of the field trial (i.e. until Day 28). In this experiment temperatures, relative humidity, and light/dark conditions were adjusted to represent field conditions day by day. Wheat cultivar Tarso was used. To evaluate effects of different inoculum doses, seeds were coated with three concentrations of SBW25::tgl; giving *c.* 10^9, 10^8 and 10^6 CFU g^{-1} seed, respectively. Coating and sowing was performed as in the first experiment.

Field trial experimental design

The field release (September 8, 2005 to May 10, 2006) was conducted at the Genetic Garden at the Genetic Centre at the campus of SLU in Ultuna, Uppsala. The same treatments with SBW25::tgl (1), SBW25 wt (2), Celest (3) and PBS (4) were used in the field trial as mentioned above for the phytotron experiment. Inoculation doses for the bacterial suspensions (treatments 1 and 2) resulted in *c.* 10^7 CFU SBW25::tgl g^{-1} seed as confirmed by plate counting. Seed coating was performed as described above and the seeds were hand sown at a distance of *c.* 2.5 cm from each other. Individual treatment plots were 1 m^2 and *c.* 350 seeds m^{-2} were sown. Distances between the rows were 10 cm. The experimental site was divided into three blocks to compensate for natural variation in the soil. All four treatments were performed in triplicates. One replicate plot from each treatment was placed in each block. Statistical analysis was performed using two-way ANOVA. Results from experiments with *P*-values < 0.05 were regarded as significant. The experimental area was surrounded by three guard rows of 1 m width each, two with bare soil surrounding one with untreated wheat in the middle. The air temperature on average from December to April was -2.9 °C, and the soil temperature at 5 cm depth was *c.* -0.1 °C (http://www.geo.uu.se).

Plant growth assay

Possible effects of the different bacterial and chemical treatments (see treatment descriptions above) on growth of winter wheat after sowing of seeds that were naturally infested with *M. nivale* were evaluated by counting the

number of emerged plants after 20 days according to EPPO guidelines (www.eppo.org). In addition, plant height at 20 days was recorded in both the phytotron and field trial experiments.

Sampling of wheat plant material

At each sampling occasion, three plants were collected randomly by hand from each replicate plot of treatments with SBW25::tgl to monitor the fate of the recombinant strain. It was not possible to selectively monitor the wt strain and to distinguish it from the indigenous flora. Therefore, in this study only plant growth properties were assessed in the replicate plots with the genetically modified strain (see 'Plant growth assay' above). The plants were gently shaken to remove loose soil particles and divided into seeds, roots/root associated soil, and shoots with a scalpel. The respective plant portions from three plants per plot were pooled together. When the seed had germinated, the seed residue and a 5-mm base of stem were sampled to represent the former seed area. The phytotron wheat plants were only sampled at the end of the incubation period on Day 28. Plant material samples (seeds, roots/root associated soil, and shoots) were separately homogenized in *c.* 10 mL PBS g^{-1} plant material, using an Ultra turrax T25 Basic (IKA WERKE GmbH, Staufen, Germany) set at 4300 ***g*** for 15–30 s, depending on material roughness, in intervals of 5 s and subsequently analyzed using the methods described below.

Monitoring SBW25::tgl

The number of SBW25::tgl cells colonizing seeds, roots/root associated soil, and shoots of wheat plants at the different sampling dates were determined by counting colonies on selective agar medium. Agar plates (0.1 × LB) containing K_2TeO_3 (35 μg mL^{-1}) were inoculated with diluted plant homogenates from treatments with SBW25::tgl and negative control treatments (i.e. PBS), and incubated overnight at 28 °C. Black bacterial colonies were counted. Background colonies in the negative control treatment consisted mostly of filamentous fungi and were easily distinguished from SBW25::tgl colonies. No tellurite resistant bacterial colonies were detected on control plots that were not inoculated with SBW25::tgl. In addition, to confirm that the GMM strain was specifically being monitored throughout the field trial, colonies were routinely assessed for GFP fluorescence and/or luciferase activity using the assays described below.

Test of dispersion

On one sampling occasion before winter (Day 42 after sowing; October 31, 2005), soil samples and plants were also taken from guard rows and between plots. The smallest distance to the plants was 5 cm, then ranging from 50 cm up to several meters. The soil samples were taken from depths of 2 and 20 cm, sieved (pore size 4 mm) and suspended in 5 mL PBS. Roots, seeds and shoots were separately collected, suspended in 10 mL PBS and homogenized, and SBW25::tgl was monitored as described above.

In order to evaluate the possible spread from the plants to surrounding soil environments after winter, samples were taken 1, 3 and 5 cm from the base of the stem on three plants in each of the three plots treated with SBW25::tgl on Day 240 after sowing (May 10, 2006). The upper 1 cm soil layer was discarded and soil cores of a diameter of 1 cm and a depth of 3 cm were taken at the distances stated above. The soil samples were sieved, 0.5 g soil from each sample were suspended in 5 mL PBS and plated onto LA plates containing K_2TeO_3 (35 μg mL^{-1}). In addition, rhizosphere soil samples from each plant were also plated. Plants were gently shaken to remove soil particles, large adhering particles were removed by tweezers and the remaining soil was rinsed off the roots with 5 mL PBS and plated onto LA plates containing K_2TeO_3 (35 μg mL^{-1}).

Luciferase activity as a measure of metabolic activity

0.5 mL of undiluted plant or soil homogenate was added to a plastic cuvette containing 1 μL of 0.5% *n*-decanal in ethanol. Bioluminescence was measured after 4 min incubation as relative light units (RLU) in a MiniLumat LB 9506 luminometer (Berthold Technologies, Bad Wildbad, Germany). Background values of plant material from PBS treated plants were in the range of 50–200 quanta s^{-1} (except for shoot material where background values varied between 1500 and 4000 quanta s^{-1}), and these values were always subtracted from sample readings.

Microscopic analyses

Plant parts from field sampling Days 1, 4, 7 and 240 were inspected by fluorescence stereomicroscopy for presence of GFP fluorescent SBW25::tgl cells. Before homogenization of plant material, an initial overview of the whole plant was performed using a LEICA stereomicroscope MZ12, with a GFP Plus Fluorescence filter (excitation 480/40 nm, barrier filter 510 nm LP). Seeds with visible GFP-fluorescent areas were further analyzed by epifluorescence microscopy using a Zeiss Axioplan 2 with excitation filter 470/40 nm, emission filter 500 LP and by confocal microscopy. For confocal microscopy, plant parts were embedded in Tissue-Tek® O.C.T. 4583 (Sakura Finetek Europe B.V., Zoeterwoude, the Netherlands) and frozen on dry ice or in a cryostat chamber and sliced using a Leica Jung 2045C cryostat set at −18 to −20 °C. Slices were 15–25 μm thick and were immediately mounted onto microscopic slides and stored at −18 °C

until examined. A drop of Confocal-Matrix ® (Micro-Tech-Lab, Graz, Austria) was added before the coverslip. Confocal microscopy was performed with a Leica TCS SP laser scanning confocal microscope and LEICA CONFOCAL software. A ×40 PL FLUOTAR objective with numerical aperture 0.70 was used with an excitation wavelength at 488 nm (Ar) and emission wavelengths at 500–554 nm (green) and 593–658 nm (red). The resulting images were obtained by merging the outputs of the green and red channels. The confocal picture was exported from the LEICA CONFOCAL software and formatted in Photoshop to meet printing demands.

Results

Marker gene tagging of *P. fluorescens* SBW25

A new mini-transposon vector (pUTtgl; Fig. 1) was constructed and successfully used to insert a triple marker gene cassette containing genes encoding GFP, luciferase and tellurite resistance into the chromosome of *P. fluorescens* SBW25. Nine transformants that exhibited all three phenotypes were obtained and their growth curves were compared in nonselective M9 minimal medium at 28 °C to that of the SBW25 wt strain. All of the transformants had similar growth curves to the wt strain (data not shown) demonstrating that the marker insertion was not detrimental to growth under those conditions. One of the transformants was chosen for the rest of the experiments described below and given the strain designation *P. fluorescens* SBW25::tgl.

Southern blot results confirmed that the marker gene cassette, comprised of the *gfp*, *luxAB* and *telAB/kilA* genes, was only introduced once into the chromosome of *P. fluorescens* SBW25::tgl (data not shown). The integration was confirmed to be stable, judging by bioluminescence and GFP phenotypes after 100 generations in nonselective minimal medium.

Growth of the SBW25::tgl strain in nonselective M9 minimal medium was compared with that of the SBW25::gfplux strain, containing a marker gene cassette encoding GFP, luciferase and kanamycin resistance that was constructed previously (Unge *et al.*, 1999), in order to determine the specific impact of the tellurite resistance marker on growth. The temperature was chosen to be as low as possible to mimic soil winter conditions. In nonselective M9 minimal medium at 4 °C there was only a slight but insignificant reduction in growth of the SBW25::tgl strain compared with the SBW25::gfplux and the SBW25 wt strains at the later sampling periods (Fig. 2). These results suggest that the tellurite resistance marker might have a slight negative impact on growth and survival of the cells after prolonged incubation at cold temperatures in minimal medium. However, when the SBW25 wt and SBW25::tgl strains were inoculated separately into sterile soil at levels of $3 \times 10^8\,g^{-1}$ and incubated 24 h at 4 °C, they were recovered on nonselective LA agar medium at similar levels of 1.8×10^8 and 2.6×10^8 CFU (g sterile soil)$^{-1}$, respectively, and these differences were not significant ($P > 0.05$).

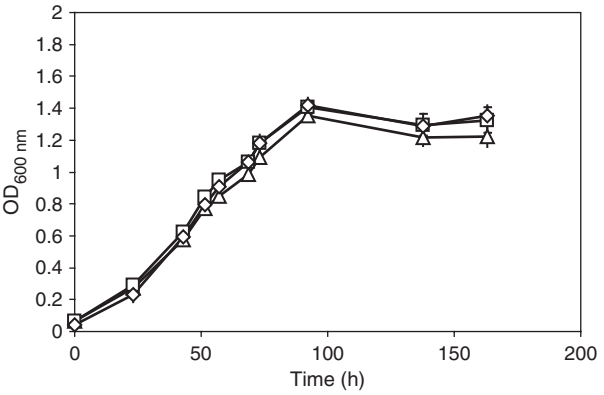

Fig. 2. Comparison of the growth curves of SBW25 wt (◇), SBW25::gfplux (□) and SBW25::tgl (△) in M9 minimal medium at 4 °C. Error bars represent the SD of the mean of triplicate cultures.

Effects on fungal and plant growth

Both the genetically modified and wt strains of *P. fluorescens* SBW25 were tested for antagonism to *M. nivale* on agar medium, but no significant growth inhibition of the fungus was observed (data not shown). In addition, there was no significant improvement in plant growth when the cells were inoculated onto wheat seeds that were naturally infested with *M. nivale* in the phytotron study. No differences in biocontrol efficacy were seen as judged by the number of emerged plants after 20 days. The only significantly positive effect was observed for the chemical control fungicide. Therefore, under the phytotron conditions used in this study, it was concluded that SBW25 was not effective for biocontrol of *M. nivale*. However, *M. nivale* infested seeds were continued to be used (cultivar Tarso) for the subsequent field trial and parallel greenhouse study to determine whether there could be some advantage to the plants through the winter season, such as indirect promotion of plant growth, during disease development in the field.

Effect of inoculation doses

Inoculation of SBW25::tgl at three doses: 10^9, 10^8 and 10^6 CFU g^{-1} seed, had no significant effect on the subsequent number of SBW25::tgl cells or luciferase activity levels on wheat plant parts in the phytotron experiments. After 28 days of incubation cell numbers for all inoculation doses were *c.* 10^8 CFU g^{-1} seed, 10^7 CFU g^{-1} root and 10^6 CFU g^{-1} shoot, respectively.

Field trial

The field trial was initiated September 8, 2005 and concluded May 10, 2006. The number of SBW25::tgl cells on seeds in the field was relatively stable throughout the sampling period (Fig. 3). During the first 24 h period, SBW25::tgl bacterial cells multiplied to reach a maximum value on the seeds of *c.* 5×10^9 CFU g^{-1} seed. After 2 weeks the levels had decreased to *c.* 10^8 CFU g^{-1} seed and they stayed at that level throughout the rest of the sampling period, including over the winter. It is important to note that the definition of 'seed' changed throughout the sampling period, as the seed germinated and eventually became less distinct. At later sampling periods the seed was defined as the plant area of the stem near the base of the roots. Roots emerged at Day 7 and these had the lowest cell counts, always at least one order of magnitude lower than the seeds and at 6, 8 and 10 weeks post sowing the difference was two orders of magnitude. After winter, however, the number of CFU on the roots and root-associated soil had increased 100 times to a level comparable to sampling Day 14. Shoots were sampled at Day 14 for the first time once they had emerged and these also had lower cell counts compared with seeds and after the winter period this difference was 100-fold (Fig. 3a). There were severe disease symptoms due to the long period of snow cover in the field trial. The percent survival of wheat plants ± SD was as follows for each treatment: (1) 57.2 ± 37.6, (2) 38.7 ± 11.7, (3) 31.3 ± 4.5 and (4) 57.2 ± 26.9 and none of these were significantly different due to the large variation. However, the SBW25::tgl bacteria did survive the winter on all remaining plants and plant parts.

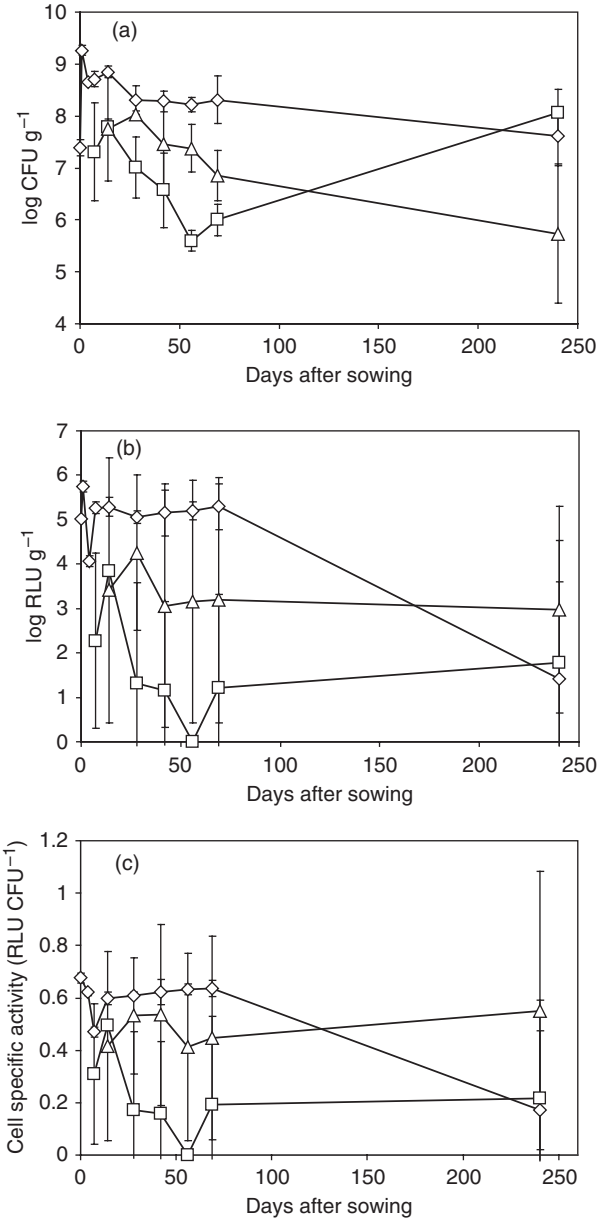

Fig. 3. Monitoring of SBW25::tgl in field samples as CFU g^{-1} (a), RLU g^{-1} (b) and CFU RLU^{-1} (c). Symbols represent numbers per wet weight of seeds (◇), roots/root associated soil (□) and shoots (△). The gap in sampling occasions between Days 69 and 240 is due to the winter season and the inability to sample during that period. Error bars represent the SD of the mean of samples taken from three replicate plots.

Luciferase activity measurements

Luciferase activity, expressed as RLU, was used to estimate the levels of metabolically active SBW25::tgl cells in the samples. Before winter, activity was high and relatively stable on seeds with values of 10^5–10^6 RLU (g wet wt seeds)$^{-1}$ (Fig. 3b). RLU values on shoots and roots were substantially lower and ranged from 0 to 10^4 RLU (g wet wt roots/root associated soil)$^{-1}$ and from 10^3 to 10^4 RLU (g wet wt shoots)$^{-1}$. After winter the activity on the 'seed', i.e. the base of the stem area, had dropped substantially to levels of 10^2 RLU (g wet weight)$^{-1}$. To estimate the metabolic activity per bacterial cell each RLU value was divided by the corresponding CFU value (Fig. 3c). Luciferase activity per CFU on the seeds was relatively high and stable before winter (*c.* 0.6). However, after winter the activity per cell on seeds had dropped dramatically to 0.2. On the shoots the ratio was between 0.4 and 0.5 for all sampling dates, whereas variation in activity was higher on roots (0–0.5).

Dispersion in the field

Before winter on Day 42 (October 31, 2005) no SBW25::tgl cells were found outside the immediate area occupied by the roots/root associated soil of inoculated wheat plants. After winter on the May 10, 2006 sampling date (Day 240) three plant and bulk soil samples were taken from each of the three replicate plots that were inoculated with SBW25::tgl. The GMM cells were found on all plant parts tested and in

the rhizosphere soil of only one out of nine of the plants, but no GMM cells were detected beyond the rhizosphere in any of the bulk soil samples. These results indicate that the SBW25::tgl cells primarily colonized the root tissue during the winter season and did not disperse.

Comparison of phytotron and field trial experiments

A highly controlled growth chamber (phytotron) experiment was performed in parallel with the field trial for the first 4 weeks. Treatments were the same as for the field trial, and night and day temperature, relative humidity and light settings were changed day by day to match field conditions. These results were then compared with those from the field.

Plants from both the field trial and the phytotron experiments were sampled at Day 28 after sowing and the samples were analyzed by counting CFU and measuring luciferase activity. There were no significant differences in colonization capacity or metabolic activity in samples from roots and seeds (Fig. 4a and b). However, SBW25::tgl colonized shoots in the field at elevated average densities compared with the phytotron ($P = 0.021$).

None of the treatments had any significant effect on plant shoot length in both the field trial and the parallel phytotron experiment (Fig. 4c), although treatments with SBW25 (both wt and GMM) seemed to have a slightly positive effect. However, when the phytotron and field trial results were compared with each other, there was a significant difference in plant shoot length ($P = 0.021$). Plants from the phytotron were always at least twice the length of field grown plants (Fig. 4c).

Plant colonization pattern

By fluorescence stereomicroscopy, SBW25::tgl cells were more readily observed on seeds than the other plant parts. Those seeds where visible GFP fluorescence was detected were further analyzed by epifluorescence microscopy and confocal microscopy. GFP-fluorescent bacterial aggregates were most consistently seen on the outer pericarp of the seeds and in the crease of the ventral side of seeds on Days 1 and 4 after sowing (see Fig. 5 for an example). No GFP fluorescent bacteria were visible on plant parts at later sampling dates by stereomicroscopy and were therefore not further analyzed by confocal microscopy.

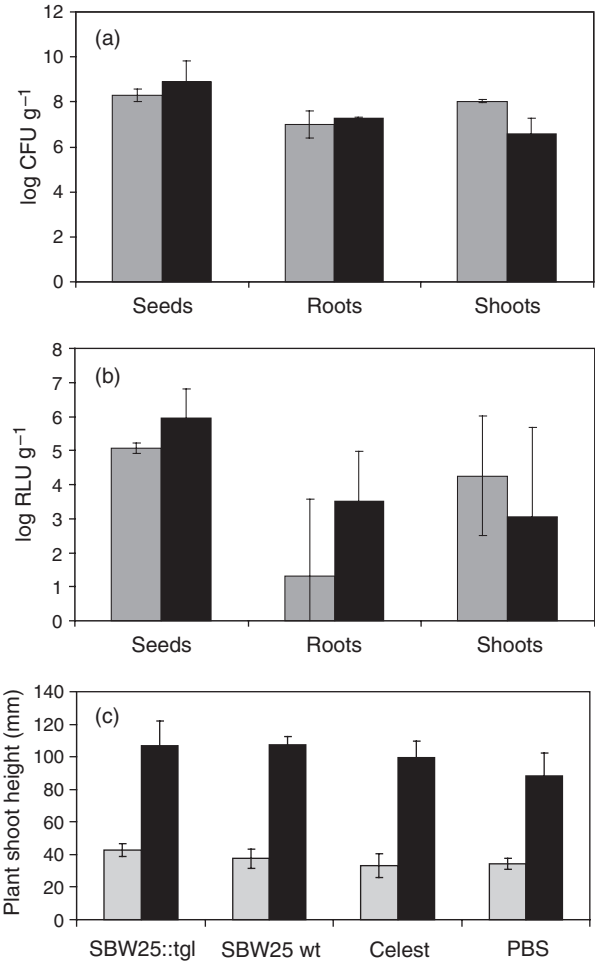

Fig. 4. A comparison between the field trial (light bars) and phytotron (dark bars) experiments with respect to wheat plant colonization by *Pseudomonas fluorescens* SBW25::tgl as CFU g^{-1} (a), RLU g^{-1} (b) and plant shoot height (c) of SBW25::tgl on different plant parts after 28 days. Error bars represent the SD of the mean of triplicate plots (field trial) and four replicate pots (phytotron).

Discussion

Many countries have legislation on the use of GMMs and there is often public concern regarding these microorganisms and their use (Käppeli & Auberson, 1997; Davison, 2002). For these reasons it is preferable to be able to show results performed in green house and laboratory conditions before conducting a field trial (Kim *et al.*, 1997). Nevertheless, the true test is the performance of the GMMs when subjected to natural conditions encountered in the field. In this study, a genetically marked derivative of *P. fluorescens* SBW25 was constructed to be used for the first Swedish field trial with a GMM. Because the potential spread of antibiotic resistance to pathogenic bacteria is an increasing problem in health care and there is concern about release of antibiotic resistance genes into the environment, a marker cassette was constructed using the genes *telAB/kilA*, conferring resistance to K_2TeO_3 for selection and no antibiotic resistance genes were introduced. This vector should be suitable for tagging and subsequent monitoring of other Gram-negative bacteria in future field trials, green house experiments and laboratory studies.

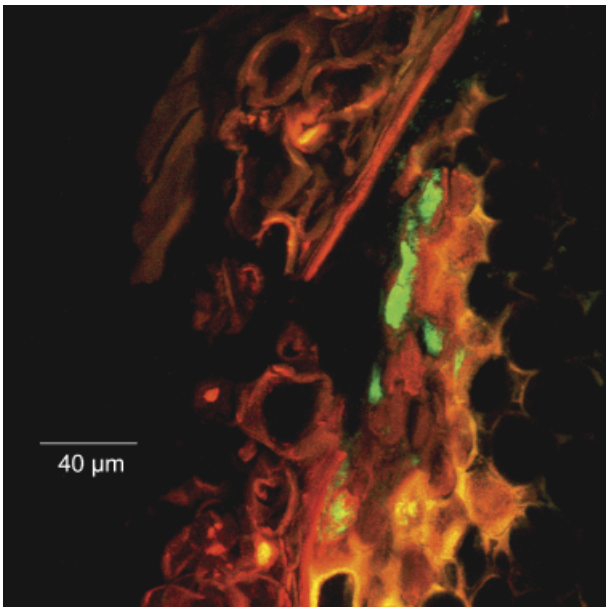

Fig. 5. Representative confocal image of *Pseudomonas fluorescens* SBW25::tgl cells inside the pericarp of a wheat seed, Day 4 after sowing. Image represents a transverse section at the base of the seed, directly beneath the pericarp. SBW25::tgl cells are shown in green and the seed autofluorescence in yellow and red.

None of these introduced markers (tellurite resistance, GFP or bacterial luciferase) are predicted to provide a selective advantage to the introduced GMM strain. This has been confirmed previously for GFP and luciferase markers (Tombolini *et al.*, 1997; Unge *et al.*, 1999; Prosser *et al.*, 2000; Unge & Jansson, 2001) however, no work has previously been done to investigate the effects of *telABkilA* genes on marked cells. Tellurium is a compound used in several industrial processes, but it is otherwise present at low concentrations in nature. Oxyanions of tellurium such as tellurite oxide (TeO_3^{2-}), are highly toxic to most microorganisms (Taylor, 1999). Although bacterial resistance to K_2TeO_3 has been known since the 1930's the mechanisms are still not fully known. Five different Te^R determinants have been discovered and they all appear to be unrelated to one another. This fact together with the low chances of bacteria to encounter this compound in the environment suggests that resistance to tellurite is a secondary effect of another metabolic function (Taylor, 1999). The genes used in this study conferring resistance to K_2TeO_3 originally come from the opportunistic pathogen *Klebsiella aerogenes*, but they have also been found in other bacteria, such as pathogens *Agrobacterium tumefaciens* and *Yersinia pestis* and beneficial microorganisms like *Rhizobium* sp. and *Bacillus subtilis* (Taylor, 1999). Because not much is known about the mechanisms of tellurite resistance it is difficult to predict whether it would provide the bacterium with a selective advantage in soil or plant environments. The tests that were conducted suggest that this is not the case. When growth curves were compared for the SBW25 wt, SBW25::gfplux and SBW25::tgl strains in minimal medium at 4 °C the SBW25::tgl strain that was tagged with tellurite resistance was slightly impaired in its growth compared with the other two strains (Fig. 2). Decreasing fitness with increasing metabolic load has been shown before with *P. fluorescens* SBW25 where different strains with different inserts were evaluated in several environments such as soil and rhizosphere (de Leij *et al.*, 1998). However, the plating efficiency from sterile soil after 24 h incubation at 4 °C showed no significant survival advantage or disadvantage for the SBW25::tgl strain.

SBW25::tgl cells were monitored after inoculation onto wheat seeds and planting in the field for 8 months, from September 2005 to May 2006. After winter, no significant effect of the GMM or wt SBW25 strains on plant shoot height or plant root length (biocontrol, chemical or plant growth promoting) of overwintering plants was evident, although treatments with SBW25 (both wt and GMM) seemed to have a slightly positive effect on wheat shoot length. The winter of the field trial had an unusually high amount of snow, with a continuous layer of snow for several months, from December 2005 to April 2006. There were severe symptoms of snow mold and many of the wheat plants did not survive winter. Therefore, in the field trial not even the chemical control treatment showed a positive effect.

SBW25 survived during the winter on all plant parts that remained. CFU values on shoots were the only ones that decreased during winter. The number of cells on the roots/root associated soil even increased *c.* 100-fold, suggesting that the root and root associated soil zones are the most preferable habitats for overwintering. In a previous field trial with sugar beet in the United Kingdom, SBW25 did not survive winter at detectable levels on sugar beet leaves, although under rare circumstances it was able to colonize resown sugar beets after winter (Bailey *et al.*, 1997). The high survival of SBW25 in this study might be explained by the persistent snow cover that created a milder microclimate near the soil-root interface of metabolically active plant tissue. Alternatively, the difference between results in Sweden and the United Kingdom could be due to the different plant hosts, or to some other environmental factor, such as soil properties.

The highest number of SBW25::tgl cells was consistently found on the seed portions of the plants, which is in agreement with earlier studies in growth chambers (Unge & Jansson, 2001). Cell numbers on the seeds were very stable. Aggregates of SBW25::tgl were seen in the crease at the ventral side of the seeds and on the outer pericarp (Fig. 5). SBW25::tgl was confirmed to reside within outer cells of the seed coat, as previously found in growth chamber studies

(Unge & Jansson, 2001). Cell numbers of SBW25::tgl on plant parts were at approximately the same levels as those found in previous studies (de Leij *et al.*, 1995b; Unge & Jansson, 2001; Timms-Wilson *et al.*, 2004). However, in the field trial performed with sugar beet in the United Kingdom (Thompson *et al.*, 1995b) the cell numbers of SBW25 were lower compared with the Swedish field trial on wheat. One explanation could be that SBW25 is a more efficient colonizer of wheat than of sugar beet, despite the fact that it was originally isolated from the phyllosphere of sugar beet.

When compared with some other plant growth-promoting rhizobacteria (PGPR), the results indicate that SBW25 is a very good survivor and efficient root/rhizosphere colonizer. Field studies (conducted in 1999 and 2000) performed with *Pseudomonas putida* WCS358r on wheat (Viebahn *et al.*, 2003) showed similar cell counts on roots as the Swedish field trial after a couple of days [10^7 CFU (g root)$^{-1}$], but the numbers declined to $< 10^2$ CFU (g root)$^{-1}$ after 100 (1999) and 60 days (2000) respectively. In this study cell numbers of SBW25::tgl were still around 10^8 CFU (g root)$^{-1}$ after winter, 240 days post sowing. Another study (Kropp *et al.*, 1996) tested *Pseudomonas chlororaphis* 2E3 on winter wheat (sowing in September 1994) and after sampling in April 1995 they found 3.7×10^4 CFU (g root dry wt)$^{-1}$, which also is significantly less than numbers of SBW25::tgl counted in this work. Raaijmakers *et al.* (1999) conducted a study with *P. fluorescens* Q2–87 on wheat and found cell numbers of 10^5–10^7 CFU (g root)$^{-1}$ after 3 weeks. After 3 weeks in the field trial SBW25 cell counts on roots were 10^7 CFU (g root)$^{-1}$, comparable with the highest levels of *P. fluorescens* Q2–87.

For this study a phytotron growth chamber was used for comparison to the field studies. To the authors' knowledge this is the first time such a highly controlled approach has been used for direct comparison to a field trial. When comparing these results, the only significant difference detected was a higher number of *SBW25::tgl* cells on wheat shoots in the field compared with the phytotron ($P = 0.021$). This difference may reflect differences in the leaf microclimate of field grown plants. Plants from the phytotron also had significantly longer shoots when measured at Day 20 and this could partly explain why fewer bacterial cells were present per gram plant shoot in the phytotron. Other studies have compared field trial results to those obtained in microcosms with varying results. A large study performed by Kinkel *et al.* (2006) investigated the colonization ability of two ice nucleation-active strains of *Pseudomonas syringae* on leaves of 40 plant species, including wheat. In that study *P. syringae* showed different colonization patterns with different plant species, and differences could also be seen between field and green house grown plants of the same species. In the case of wheat, their results were similar to the ones produced in this study, namely a lower number of *P. syringae* cells on leaves of green house grown plants compared with those in the field. Kropp *et al.* (1996) conducted field and microcosm trials comparing colonization of *P. chlororaphis* 2E3 on wheat, and found no detectable differences.

Inserted genes encoding bacterial luciferase (*luxAB*) enabled measurements of the metabolic activity of the introduced cells. Highest relative luciferase values were found on the seed area of plants before winter. After winter the RLU values of the seeds had decreased to levels similar to shoots and roots. In another microcosm study of SBW25::gfplux on wheat (Unge & Jansson, 2001), RLU values on seeds, shoots and roots during a 70 day period were at least 10-fold higher than the results presented here, despite the same cell number on the plant parts in both studies. This could be due to a higher and more stable temperature (18 °C) in the previous study and perhaps differences between different wheat cultivars. The relative metabolic activity per CFU was highest on seeds sampled before winter. After winter the cells on the shoots had the highest activity per cell, although the total number of cells had decreased.

To examine the possible effects of various inoculation densities, wheat seeds in the phytotron were inoculated with three different bacterial doses. These treatments gave no difference in CFU (g wet plant material)$^{-1}$, RLU (g wet plant material)$^{-1}$ or biocontrol effect after 4 weeks, which means that for the lowest dose the cell density actually increased 100-fold on the seed area. This suggests that irrespective of the cell densities tested in this study a carrying capacity is attained on the plant. Similar results were found by Schmidt *et al.* (2004) who found that inocula of various densities of *P. fluorescens* and *Pseudomonas corrugata* on sugar beet led to the same population densities on the plant. However, other studies investigating different strains and plant species have shown that the resulting population size is dependent on inoculum-density; *P. fluorescens* Q2–87 on wheat (Raaijmakers *et al.*, 1999), *P. aeruginosa* on tomato (Siddiqui & Ehteshanul-Haque, 2001) and *Bacillus* on spring wheat (Kuzmina & Melentev, 2003). This phenomenon seems, therefore, to depend on both the strain and plant species used.

One of the concerns with GMMs is that they may survive for longer periods in soil and on plants and/or disseminate and repopulate new niches. Therefore, it is important to investigate the ability of the GMMs to disseminate to other plants or into the soil outside the rhizosphere area, vertically or horizontally. However, GMMs may also have impaired survival rates due to an increased metabolic burden from expression of inserted genes. For example, Bainton *et al.* (2004) tested the effect of a genetically modified variant of *P. fluorescens* SBW25, tagged with *kan^r-xylE* and *lacZY* genes, against *Pythium ultimum* in pea plants in microcosms. They found that the survival competence of the genetically modified strain decreased with metabolic load, imposed by

the modification, and nutrient poor surroundings. In this field study the cells survived very well but colonization was restricted to the inoculated plant and soil associated with root and plant parts. No SBW25::tgl cells were ever found in uninoculated control plots. In addition, the cells could not be detected in soil or wheat samples of the guard rows, indicating that no horizontal or vertical dissemination occurred. These results are different from those produced by de Leij *et al.* (1995b) in a field study performed in the United Kingdom with another GMM variant of SBW25 on spring wheat. In that study the bacteria were detected in soil up to 45 cm depths with the largest value of $10^{3.55}$ CFU g^{-1} dry soil measured at Day 65 post sowing. This strain variant also spread laterally and was found consistently after 58 days up to 1 m away from the inoculated plots. After 132 days the GMM cells were also found on wheat roots of uninoculated plants growing 2 m from inoculated plants at a level of $10^{1.87}$ CFU (g root)$^{-1}$. This dispersal difference may be explained by different inoculation methods, because the SBW25 cells in the other study were suspended in a 1.25% guar gel before inoculated onto the seeds, or due to differences in soil properties or other unknown environmental conditions. In another study, a strain of *P. corrugata* was marked with Tn7::*lacZY* and applied to wheat seeds before sowing (Choi *et al.*, 2003). In that case, the introduced strain was reisolated up to 4 years after inoculation using an enrichment method. Therefore, dissemination ability is strain specific and depends as well on climatic and physical factors. For these reasons, it would be very interesting to investigate the survival of SBW25::tgl in the Swedish field trial in subsequent years.

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