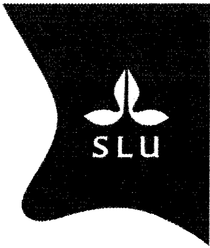

Institutionen för Mikrob
Lotta Jäderlund

Kemikalie inspektionen
Ink. 2009-05-15
Sak nr 723 Löp nr 573-05

2009-05-12

SLUTRAPPORT

Kemikalieinspektionen
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Diarienummer: 723-573-05

Fältförsök med genmodifierad *Pseudomonas fluorescens* SBW25 (jordbakterie), slutrapport maj 2009

Härmed bifogas en slutrapport enligt mall från Kemikalieinspektionen samt en resultattabell från provtagningar både under och efter fältförsöket. Inga genmodifierade bakterier (SBW25::tgl) hittades vid några av de provtagningar som gjordes under 2008. I vår analys av effekter på mikrobiella samhällsstrukturer fann vi inga större effekter av den genmodifierade bakterien. Vår slutsats är att risken för skada på övriga mikroorganismer i fältförsöket är låg.

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Janet K. Jansson, professor

Uppsala, 2008-05-12

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Final report to release a genetically modified micro-organism (GMM)

Risk assessment of non-resident microorganisms used for biocontrol of fungal pathogens

Notification number: 723-573-05

Name of the GMM: ***Pseudomonas fluorescens*** **SBW25::tgl**

Name and address of the notifier:

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Summary

The environmental effects on indigenous microorganisms of a non-pathogenic, plasmid free bacterium called *Pseudomonas fluorescens* SBW25::tgl have been evaluated in a field trial. The bacterium was genetically modified in order to make possible tracking and monitoring in the soil and plant environments. One chromosomal marker cassette containing *telABkilA* (encoding resistance to the potassium tellurite) and *gfp/luxAB* (encoding the green fluorescent protein and bacterial luciferase) has been inserted. The field trial was initiated at the 8th of September 2005 and samplings of plants were performed at seven different time points until the last sampling date of May 10th 2006. The number of SBW25::tgl cells on seeds or plants (shoots, roots, rhizosphere soil) were assessed at each time point using selective plating with potassium tellurite oxide and the metabolic activity of the GMM cells were investigated by luminometry for activity of the *luxAB* genes. The *gfp* gene was used to visualize cells on plant surfaces. A molecular method called T-RFLP (terminal restriction fragment length polymorphism) was used to evaluate the possible impact of the SBW25::tgl cells on resident bacterial and fungal communities. These multivariate data were evaluated using nMDS (non-metric multidimensional scaling).

The GMM was found in high numbers on all plant parts throughout the 8 months period, but only minor impacts were found on native micro flora due to bacterial seed inoculation, and these were only detected at day 4 and 14 post sowing. The largest differences in microbial community structures were between different sample types (roots, seeds or rhizosphere soil) and sampling occasions. No GMM cells were found in untreated plots and guard rows during the field trial (September 8 2005 – May 10 2006). However, at a sampling in November 2007 low numbers of GMM cells were detected in all plots, and also in guard rows. This was probably due to dead plant material on the site which the SBW25::tgl bacteria might have used as a carbon source. No GMM bacteria were detected on 7 sampling occasions in 2008 when the plant material was removed and the field site was covered with a plastic tarpaulin.

The novel non-antibiotic resistance marker gene cassette was successfully used to monitor GMM *P. fluorescens* SBW25 in the field for 8 months. Even though some spread has occurred the risk of damage to the microbial ecology of the field is low since no persistent impacts have been found by molecular methods due to bacterial inoculation of wheat plants. We consider the overall environmental risk low when it comes to the released GMM.

Introduction

The plant growth promoting bacterium *Pseudomonas fluorescens* SBW25, first isolated from the phyllosphere of sugar beet in the UK (Thompson, Ellis & Bailey, 1995), has been extensively studied both in field trials and laboratory studies. It has been shown to be an excellent colonizer of both rhizosphere and phyllosphere of different plants such as pea (Naseby *et al.*, 2001), sugar beet (Thompson *et al.*, 1995a), wheat (de Leij *et al.*, 1995a; Jäderlund *et al.*, 2008a; Unge & Jansson, 2001) and potato and tomato (Jäderlund, 2008). The strain SBW25 has biocontrol abilities towards the fungus *Pythium ultimum* (Ellis, Timms-Wilson & Bailey, 2000), and this is thought to be related to its success as plant colonizer (Rainey, 1999). It has also been shown to interact synergistically with arbuscular mycorrhizal fungi to inhibit snow mould on wheat plants (Jäderlund *et al.*, 2008b). Several previous studies have been performed to study the environmental impacts of strain SBW25 on other microorganisms, for example a field trial in the UK where the effects of a marker gene tagged SBW25 on indigenous culturable microbial populations on wheat were investigated (de Leij *et al.*, 1995b). In this study SBW25 was tagged with marker genes for lactose utilization (*lacZY*), resistance to the antibiotic kanamycin (Tn*903*) and conversion of the substrate catechol into a yellow compound (*xylE*). The results of this investigation were that some of the culturable populations associated with the plants were disturbed to some extent, but these effects were transient. There were no differences between the effects of the wild-type strain and the GMM (de Leij, *et al.*, 1995b). Another study performed in green house with the same GMM on sugar beet showed some changes in community diversity, but in this study the GMM had less impact on the bacterial community than the wild-type (Thompson *et al.*, 1995a). It has been proposed that the release of bacterial inoculants (genetically modified or not) may pose a threat to the environment (van Elsas & Migheli, 1999) and each GMM that is about to be released requires rigorous risk assessment, for both human and environmental safety.

This field trial was performed in order to determine the survival and metabolic activity as well as the plant colonization ability of SBW25 on winter wheat. For this reason a novel non-antibiotic marker gene cassette was constructed (Jäderlund, *et al.*, 2008a), containing the *gfp* gene (encoding for the green fluorescent protein) (Chalfie *et al.*, 1994), the *luxAB* gene (used to measure the metabolic activity of the GMM strain) (Meikle *et al.*, 1994) and the *telABkilA* genes (conferring resistance to potassium tellurite oxide which was used as selective substance in agar plates)(Herrero *et al.*, 1990). Combining the tellurite pUT/miniTn5 vector with the *gfp/luxAB* genes provides a useful tool for monitoring released bacteria in environmental field trials with a degree of predictability that can hardly be found in indigenous bacterial isolates. This marker gene cassette was successfully used in the field trial to monitor the GMM strain on plants (Jäderlund, *et al.*, 2008a).

Another aim of this study was to determine the possible effects of the wild-type or GMM strains on indigenous bacterial and fungal populations. This was performed by using the T-RFLP method (Terminal Restriction Fragment Length Polymorphism), which is a powerful tool for assessing the diversity of complex bacterial communities (Artursson *et al.*, 2005; Hjort *et al.*, 2006; Liu *et al.*, 1997). The method is based on comparison of different 16S rRNA fragments. These fragments are amplified by means of PCR with one or both of the primers labeled with a fluorescent marker in order for the sizes of the restriction fragments to be easily determined and quantified with sequencing. The DNA fragments are cleaved with restriction enzymes and the exact length of each fluorescently marked terminal fragment is measured by electrophoresis. Each sample will give a finger-print of its microbial community

and these patterns may then be compared using multivariate statistics like non-metric multidimensional scaling (nMDS)(Kruskal, 1964a; Kruskal, 1964b). Our hypothesis was that the SBW25 cells would cause only short-term changes to the indigenous microbial communities on the wheat plants, and that there would be no significant differences between the effects of the GMM and wt strains. For this study we used T-RFLP as a molecular fingerprinting approach to determine the impact of the wild-type and GMM strains on the bacterial and fungal communities, by targeting bacterial 16S rRNA genes and the fungal ITS region, respectively.

Objectives

The objective of the field trial was to determine the survival, activity, plant colonization and possible dispersal of genetically modified plant growth promoting bacterium *Pseudomonas fluorescens* SBW25 in a field trial with winter wheat. This was performed using a novel non-antibiotic marker gene cassette for tagging the bacterium. Another objective was to examine the impact of wild type and GMM strains of *P. fluorescens* SBW25 on indigenous bacterial and fungal communities on wheat seeds, roots and rhizosphere soil.

Experimental design

Each treatment plot was 1 m^2 and the experimental site was divided into three blocks to compensate for natural variation in the soil. Winter wheat seeds (cultivar Tarso) were treated with the following treatments: 1) SBW25::tgl (GMM), 2) SBW25 wild-type, 3) Celest (a chemical fungicide) and 4) phosphate buffer (PBS). All four treatments were performed in triplicates. One replicate plot from each treatment was placed in each block. 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. For bacterial treatments (treatments 1 and 2) seeds were soaked in bacterial suspensions resulting in approximately 10^7 CFU SBW25::tgl g^{-1} seed as confirmed by plate counting. The seeds were naturally infested with *Microdochium nivale* and were inoculated with bacteria by seed coating before sowing. Seeds were hand sown with approximately 350 seeds m^{-2} and distances between the rows were 10 cm (Jäderlund, *et al.*, 2008a). At each sampling occasion, six plants were collected randomly from each replicate plot. Three plants were used for plating and measurements of metabolic activity and the other three were divided into seeds, roots and rhizosphere soil for the T-RFLP analyses. Statistical analysis regarding activity and survival was performed using 2-way ANOVA. Results from experiments with p-values < 0.05 were regarded as significant. T-RFLP profiles were evaluated using non-metric multidimensional scaling (nMDS) (Jäderlund, 2008).

The release

The field release (8 September 2005 – 10 May 2006) was conducted in the Genetic Garden at the Genetic Centre at the campus of SLU in Ultuna, Uppsala. The air temperature on average from December to April was -2.9 °C, and the soil temperature at 5 cm depth was approximately -0.1 °C (http://www.geo.uu.se). Plants were sampled and analyzed for GMM cell numbers and metabolic activity at the following dates; 9th, 12th and 22nd of September, 6th and 20th of October, 23rd of November and 10th of May 2006.

Results of the experiment

Cell numbers of SBW25::tgl were high on all plant parts throughout the experiment (September 2005 – May 2006). Cell numbers on the seeds (or the lowest part of the stem when seeds had germinated) were relatively stable throughout the sampling period, varying between 10^8 and 10^9 CFU/g plant material (Jäderlund, *et al.*, 2008a). GMM levels on shoots before winter were in the range of 10^6-10^7 CFU/g plant material. Roots and root associated soil were the plant part where GMM numbers varied the most during samplings, ranging from $10^{5.5}$ – 10^8 CFU/g plant material. After winter the CFU counts on shoots seemed to decline and the numbers of CFU on roots (including rhizosphere soil) had increased a 100-fold compared to the measurements in November, indicating that the root and rhizosphere zones are more protected and preferable habitats for the SBW25::tgl cells during winter. Metabolic activity of the GMM measured by luminometry was detected on all plant parts, but with greater variations compared to CFU numbers (Jäderlund, *et al.*, 2008a). The wheat seeds were naturally infested with *Microdochium nivale*, but no treatment resulted in reduction of disease symptoms. This winter had an unusually high amount of snow, with a continuous layer of snow for several months, which is favorable for snow mold. Plants showed severe symptoms of snow mold and not even the chemical fungicide showed a positive effect in the field trial (Jäderlund, *et al.*, 2008a).

The T-RFLP technique was used to detect changes in bacterial and fungal populations associated with wheat plants due to seed inoculation with GMM and wild-type SBW25. One effect that was noticeable was the differences in relative abundance of the TRF that represents the inoculated strain and other *P. fluorescens* (TRF 206) on seeds at the earliest days of sampling. TRF 206 had a higher relative abundance in seeds treated with the wild type strain compared to seeds treated with the GMM strain at the 9th of September. At day 4 (12th of September) the differences between the bacterial treatments and the PBS control treatment were still detected, but at later sampling dates no significant differences between treatments were found for the bacterial community compositions on seeds. For roots and rhizosphere soil the differences between the different treatments were minor.

Regarding the effects on bacterial community structure detected by T-RFLP on the different plant parts (seeds and roots) and rhizosphere soil these results were analyzed using nMDS. The largest differences were due to time and plant part sampled, and only a few changes were detected when comparing wheat plants treated with GMM, wild-type strain or phosphate buffer control. Differences between bacterial community structures from plants treated differently in respect to bacterial inoculation (GMM, wild-type or phosphate buffer control) were detected by nMDS on roots and in rhizosphere soil at day 4 (September 12) and on seeds at day 14 (September 22). At both these sampling dates the bacterial community structures from plants treated with the wild-type strain was slightly different than the community structures from GMM or buffer control treated wheat plants. No additional treatment differences were noticed at later samplings, the last one was performed 8 months post sowing (May 10), whereas the differences due to plant part seemed to be larger with time. No changes between treatments were noticed in the fungal community structures when the data were analyzed with nMDS (Jäderlund & Jansson, unpublished data).

Post-release monitoring

Monitoring programme

Post release monitoring of soil from the field site was performed in order to determine possible dispersal from the treated plots to the soil and survival in the soil. Selective plating using potassium tellurite oxide was used together with measurements of metabolic activity of the SBW25::tgl cells. The first post release monitoring was performed in October 2006 and no GMM cells were found at that occasion. The next sampling was carried out in November 2007 and at that time point GMM cells were found in the soil, even though no metabolic activity could be measured. This finding led to sampling occasions with shorter time intervals during 2008 to investigate the extent and range of the dispersal. During 2008 seven monitoring samplings were performed (March, May, June, July, August, September and December) with an extended sampling area, as far as two metres outside of the experimental site guarded by a fence.

Results of the post-release monitoring

After plants were removed a first monitoring was performed in October 2006 and the experimental site was treated with RoundUp to minimize weed growth. At this sampling no SBW25::tgl were found in the soil near the plants, on wheat plants in the guard row around the treated plots or in the soil in the bare soil guard rows. When the site was sampled a year later in November 2007, weed had been growing on the site. The site had been treated with RoundUp and the dead plant material had not been removed which might have served as an energy source for the GMM bacteria. At the sampling in November 2007 SBW25::tgl was detected in soil from all plots, not just the plots treated with the GMM, and at both 2 and 20 cm depths. Also, soil samples from the guard rows contained low levels of the GMM. The GMM numbers found in 2007 was approximately 1% of the indigenous population of *P. fluorescens*. The field site was cleaned from dead plant material and in order to minimize the weed growth and to keep the area free of falling leaves a plastic sheet was placed on the field site. At the samplings in 2008 the sampling area was increased in order to detect any possible dispersion in addition to the sampling points inside the fenced field site. No GMM cells were found in any of the samples taken in 2008.

Risk assessment after finalized post-release monitoring programme

The findings in November 2007 were probably due to dead plant material that was not removed from the field site. This hypothesis has been strengthened since no GMM cells have been detected after the plant material was removed and the field area was covered with plastic tarpaulins. The SBW25 strain is known to colonize a variety of plants (Jäderlund, 2008) and probably take advantage of released plant nutrients. Our results from the sampling in November 2007 suggest that SBW25 may spread through contact between different plants, which also has been shown in a field trial with SBW25 in the UK in 1993 (Thompson *et al.*, 1995b). Limited spread was expected, for example by heavy rain or transport via vertebrates or other small animals. *P. fluorescens* has a worldwide distribution in soil, water and plant environments. SBW25 is an innocuous member of the natural phytosphere microbial community in the UK. It is not a pathogen against plants, animals or humans. There will not be any effects on human or animal health.

In laboratory experiments it was evaluated that the growth of SBW25::tgl was slightly reduced compared to the wild type but this has not been tested in the field due to the lack of a technique to specifically detect the wild type strain. However, the relative abundance of TRF

*Hha*I 206 (representing *P. fluorescens*) could be calculated in the field on seeds treated with either GMM, wild-type or buffer control. After 1, 14 and 28 days of field incubation the levels of TRF *Hha*I 206 in GMM treated seeds were lower than in the seeds treated with the wild-type strain (Jäderlund, 2008). This implies that the GMM strain has lowered seed colonization ability compared to the wild-type bacteria. No differences could be seen on roots or rhizosphere soil. The reason for this difference between SBW25 GMM and wild-type is still unclear, but it is probable that it could be due to a heavier metabolic load to carry extra inserted genes and to express these genes, which has previously been shown with other genetically modified variants of this strain (de Leij *et al.*, 1998). The genes inserted in this study will not confer any competitive advantages over the wild type or any other indigenous microorganism.

According to the T-RFLP data there will not be any persistent changes in the microbial flora of soil and plants. The minor effects on the indigenous bacterial flora due to different treatments that were detected using nMDS were only found after day 14 after sowing. This implies that there have not been any irreversible effects on microbial community structures or biogeochemical cycles. Even though spread have occurred no persistent impacts will be likely on bacterial or fungal communities. The overall environmental risk of the release of SBW25::tgl is low.

Conclusion

With this method only minor and transient impacts on bacterial community structures could be noticed. The GMM strain was slightly reduced in growth in the laboratory and the data from the TRF specific to *Pseudomonas spp.* indicates a similar behaviour on wheat seeds the first days after inoculation. No dissemination to bulk soil was detected until Nov 2007 (more than 2 years post release) when the GMM could be detected at low levels in bulk soil from all treated plots, guard rows and fallow areas (Jäderlund, 2008). Even though spread has occurred the environmental impact is low, as detected by molecular methods. When dead plant material was removed from the field site the GMM numbers decreased under the detection limit. We consider the overall environmental risk low when it comes to the released GMM.

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Field trial SBW25::tgl September 2005 - May 2006, Sampling of soil. Number of SBW25::tgl cells/g soil detected on selective agar plates.

Sowing September 2005, plants removed October 2006, Round-Up treatment, field site covered with plastic tarpaulin since November 2007.

	Sample	2005		2006		2007	2008							
		Sep	Oct	May	Oct	Nov	Mar	May	Jun	Jul	Aug	Sep	Dec	
		number of GMM cells (CFU/g)					*(corresponding luminescence values were always 0)*							Monitoring
GMM treated plants	1. Soil before sowing	0	-	-	-	-	-	-	-	-	-	-	-	Pre
	2. 2 cm depth, 3 cm from plant	-	0	0	0	-	-	-	-	-	-	-	-	During
	3. 20 cm depth, 3 cm from plant	-	0	0	0	-	-	-	-	-	-	-	-	
	13. 3 cm depth – 1 cm from GMM inoculated plant	-	-	0	-	-	-	-	-	-	-	-	-	
	14. 3 cm depth – 3 cm from GMM inoculated plant	-	-	0	-	-	-	-	-	-	-	-	-	
	15. 3 cm djup – 5 cm from GMM inoculated plant	-	-	0	-	-	-	-	-	-	-	-	-	
	1A 2 cm depth	-	-	-	-	1100	0	0	0	0	0	0	0	Post
	1A 20 cm depth	-	-	-	-	450	0	0	0	0	0	0	0	
	1B 2 cm depth	-	-	-	-	1350	0	0	0	0	0	0	0	
	1B 20 cm depth	-	-	-	-	650	0	0	0	0	0	0	0	
	1C 2 cm depth	-	-	-	-	400	0	0	0	0	0	0	0	
	1C 20 cm depth	-	-	-	-	450	0	0	0	0	0	0	0	
Control	16. 3 cm depth – 1 cm from control plant	-	-	0	-	-	-	-	-	-	-	-	-	During
	17. 3 cm depth – 3 cm from control plant	-	-	0	-	-	-	-	-	-	-	-	-	
	18. 3 cm depth – 5 cm from control plant	-	-	0	-	-	-	-	-	-	-	-	-	
Wheat guard row	10. Wheat shoots, roots, and seeds from guard-row	-	0	0	0	-	-	-	-	-	-	-	-	
Between plots	4. 2 cm depth, between plots	-	0	0	0	900	0	0	0	0	0	0	0	During and post
	5. 20 cm depth, between plots	-	0	0	0	800	0	0	0	0	0	0	0	
Soil samples from guard rows	6. 2 cm depth, wheat guard row	-	0	0	0	1100	0	0	0	0	0	0	0	
	7. 20 cm depth, wheat guard row	-	0	0	0	700	0	0	0	0	0	0	0	
	8. 2 cm depth, bare soil guard row	-	0	0	0	1150	0	0	0	0	0	0	0	
	9. 20 cm depth, bare soil guard row	-	0	0	0	350	0	0	0	0	0	0	0	

Outside field site	11. 2 cm depth bare soil guard row outside field site area	-	0	0	0	750	0	0	0	0	0	0	0	
	12. 20 cm depth bare soil guard row outside field site area	-	0	0	0	350	0	0	0	0	0	0	0	
	19. 2 cm 1 m West, outside of outer guard row	-	-	-	-	-	0	0	0	0	0	0	0	Post
	20. 20 cm 1 m West, outside of outer guard row	-	-	-	-	-	0	0	0	0	0	0	0	
	21. 2 cm 1 m North, outside of outer guard row	-	-	-	-	-	0	0	0	0	0	0	0	
	22. 20 cm 1 m North, outside of outer guard row	-	-	-	-	-	0	0	0	0	0	0	0	
	23. 2 cm 1 m East, outside of outer guard row	-	-	-	-	-	0	0	0	0	0	0	0	
	24. 20 cm 1 m East, outside of outer guard row	-	-	-	-	-	0	0	0	0	0	0	0	
	25. 2 cm 1 m South, outside of outer guard row	-	-	-	-	-	0	0	0	0	0	0	0	
	26. 20 cm 1 m South, outside of outer guard row	-	-	-	-	-	0	0	0	0	0	0	0	