TITLE	**IMPACT OF WILD TYPE AND GENETICALLY MODIFIED *PSEUDOMONAS FLUORESCENS* ON SOIL ENZYME ACTIVITIES AND MICROBIAL POPULATION STRUCTURE IN THE RHIZOSPHERE OF PEA.**
AUTHORS	**D.C. NASEBY AND J.M. LYNCH**
ADDRESS	**SCHOOL OF BIOLOGICAL SCIENCES, UNIVERSITY OF SURREY, GUILDFORD, SURREY, GU2 5XH, U.K.**
FAX	**(44) 01483 259721**
E-MAIL	J.LYNCH@SURREY.AC.UK
SHORT TITLE	**IMPACT OF GMMs IN THE RHIZOSPHERE**
KEY WORDS	**FUNCTIONAL IMPACT, *PSEUDOMONAS FLUORESCENS*, SOIL ENZYMES, GMM, MICROBIAL POPULATIONS.**
SUBJECT CATEGORY	**PLANT SOIL INTERACTIONS.**
TEXT PAGES	**26**
TABLES	**5**
FIGURES	**1**
CORRESPONDING AUTHOR	**PROFESSOR J.M. LYNCH**

Abstract

The aim of this work was to determine the impact of wild type along with functionally and non-functionally modified *Pseudomonas fluorescens* strains in the rhizosphere. The wild type F113 strain carried a gene encoding the production of the antibiotic 2,4 diacetylphloroglucinol (DAPG) useful in plant disease control, and was marked with a *lacZY* gene cassette. The first modified strain was a functional modification of strain F113 with repressed production of DAPG, creating the DAPG negative strain F113 G22. The second paired comparison was a non-functional modification of wild type (unmarked) strain SBW25, constructed to carry marker genes only, creating strain SBW25 EeZY-6KX. Significant perturbations were found in the indigenous bacterial population structure, with the F113, (DAPG+) strain causing a shift towards slower growing colonies (K strategists) compared with the non-antibiotic producing derivative (F113 G22) and the SBW25 strains. The DAPG+ strain also significantly reduced, in comparison with the other inocula, the total *Pseudomonas* populations but did not affect the total microbial populations. The survival of F113 and F113 G22 were an order of magnitude lower than the SBW 25 strains. The DAPG+ strain caused a significant decrease in the shoot to root ratio in comparison to the control and other inoculants, indicating plant stress. F113 increased soil alkaline phosphatase, phosphodiesterase and aryl sulphatase activities compared to the other inocula, which themselves reduced the same enzyme activities compared to the control. In contrast to this, the β-glucosidase, β-galactosidase and N-acetyl glucosaminidase activities decreased with the inoculation of the DAPG+ strain. These results indicate that soil enzymes are sensitive to the impact of GMM inoculation.

Introduction

The effect of genetically modified micro-organisms (GMMs), released as biocontrol agents, on soil nutrient cycling/soil enzyme activities and indigenous microbial populations is poorly described in the literature (Naseby and Lynch 1997a). Thus a more comprehensive knowledge of the consequences of such releases on the rhizosphere must be provided before they can be utilised safely (Smit *et al* 1992).

De Leij *et al* (1995) reported transient perturbations in the indigenous microbiota with the introduction of wild type and genetically marked *Pseudomonas fluorescens* to the rhizosphere of wheat plants in field experiments, but did not find differences between the two respective inoculants. Other authors working on indigenous populations and ecosystem function in contained experimental systems also found such transient perturbations (Seidler 1992, Stotzky *et al* 1993 and Whipps *et al* 1996). Perturbations have been recorded by several authors with the introduction of functionally modified GMMs, including displacement of indigenous populations (Bolton *et al* 1991), suppression of fungal populations (Short *et al* 1990), reduced protozoa populations (Austin *et al* 1990) and increased carbon turnover (Wang *et al* 1991). Population dynamics of micro-organisms with different life-style strategies, for example r (rapid colony formation) and K (slow colony formation) strategies, can be utilised in ecological studies (Andrews and Harris 1986, De Leij *et al*, 1993a and Panikov 1994). However, methods requiring microbial growth can be hampered by the non-culturability of many micro-organisms (Colwell *et al* 1985). Molecular genetic methods are useful tools for assessing the ecology and population genetics of targeted

microbial populations or communities (Mills 1994, Morgan 1991 and Van Elsas and Waalwijk, 1991), but such methods do not provide an insight into ecosystem function as a whole

Measurement of soil enzyme activities may be useful for gaining a greater understanding of the nature of perturbations caused to ecosystem function. Soil enzyme measurements have been successfully used by Mawdsley and Burns (1995) to assess perturbations caused by the introduction of a *Flavobacterium* species, and by Naseby and Lynch (1997b) with the inoculation of a *Pseudomonas fluorescens* strain. However, Doyle and Stotzky (1993) did not find such perturbations in non-rhizosphere soil with the addition of an *Escherichia coli* strain. Jones *et al* (1991) did not find an effect with addition of a GMM on either nitrogen transformations or populations of nitrogen transforming micro-organisms. Both the work of Jones *et al* (1991) and of Doyle and Stotzky (1993) did not include plants in the soil systems and thus are not ecologically relevant to soil-plant-microbe interactions.

The aim of the experiments reported here was to investigate the effect of inoculation of the pea rhizosphere with *Pseudomonas fluorescens* strains having either functional or non-functional modifications, on both the indigenous microbiota and soil enzyme activities. The non-functionally modified strain and its wild type comparison were the same as used previously in our studies (De Leij *et al* 1995a&b). The functional gene in a different *P. fluorescens* strain encoded the production of the antibiotic 2,4-diacetylphloroglucinol, useful in the control of damping off diseases (Fenton *et al* 1992). The wild type DAPG producing strain, which was previously released in field

experiments (Moenne-Loccoz *et al* 1997) was compared to its genetically modified non producing derivative in large numbers in the pea rhizosphere to assess the impact of the antibiotic production and its deletion in the rhizosphere.

Materials and Methods.

Soil description

The soil used was a sandy loam of the Holiday Hills series, taken from Merrist Wood Agricultural College (Surrey), and had been under permanent pasture for at least 15 years. The analysis of the soil, conducted at the University of Surrey, was: pH 5.4, particle ratio 10:9:81 clay: silt: sand respectively, and organic matter content 1.6% by weight.

Microcosm

Coarsely sieved (6 mm) loose soil (250 g) was placed in experimental microcosms consisting of 210 mm high acetate cylinders, slotted between the top and base of plastic 90 mm diameter Petri dishes creating semi-enclosed microcosms.

Bacterial strains and treatments

Two strains of *Pseudomonas fluorescens* were used with different modifications. Strain SBW25, a wild type strain isolated from the phytosphere of sugar beet, and its genetically modified derivative (strain SBW25 EeZY-6KX), containing the marker genes *lac*ZY (lactose utilisation), kanamycin resistance and *xyl*E (catechol degradation); (Bailey *et al* 1995). Strain F113 which produces the antibiotic 2,4 diacetylphloroglucinol (DAPG), and was marked with a *lac*ZY gene cassette, and a

DAPG negative derivative (strain F113 G22) produced by Tn5 mutagenisis (Shanahan *et al* 1992b).

The bacteria were grown on full strength tryptone soya agar (Oxoid) for 4 days at 30^{o}C. The bacteria were suspended in 10 mL of sterile quarter strength Ringer's solution using disposable plastic plate spreaders to scrape off the bacterial mat and the colony forming units (c.f.u.) were determined. Control plates (without bacteria) were also flooded with quarter strength Ringers solution and surface scraped with spreaders. The resulting suspensions containing $6x10^{9}$ c.f.u./mL were subsequently used to imbibe pea seeds (*Pisium sativum* var. Montana), at a ratio of one seed per mL, for 8 hours (stirred every 30 minutes) resulting in between 2 and 4 x 10^{8} c.f.u. per pea seed.

Experimental design

Each treatment was replicated seven times. Each microcosm consisted of eight imbibed seeds, planted at a depth of approximately 1 cm below the soil surface. Thirty mL of water was added to each microcosm before they were placed in a random design into a growth chamber (Vindon Scientific) set at a 16 hour photoperiod with a day/night temperature regime of 21^{o}C/15^{o}C respectively. The relative humidity was maintained at 70%.

Sampling and analysis

After 21 days growth the microcosms were harvested, after which soil closely associated with the plant roots (rhizosphere soil) was collected by shaking soil closely associated with the roots over a 2 mm sieve and stored over-night at 4^{o}C. Subsequently each sample was assayed for soil acid and alkaline phosphatase, phosphodiesterase, aryl sulphatase, β glucosidase, acid β galactosidase and N-acetyl glucosaminidase by the methods of Naseby and Lynch (1997b).

Shoot and root fresh weights were measured and a 1g root sample from each replicate was macerated in 9 mL of sterile quarter strength Ringers solution using a pestle and mortar. Filamentous fungi and yeast populations were quantified by plating a ten fold dilution series of each root macerate onto 10% malt extract agar containing 100 ppm streptomycin and 50 ppm rose bengal. Plates were incubated at 20^{o}C for 5 days before enumeration. P1 medium (Katoh and Itoh, 1983) was used for the enumeration of indigenous, fluorescent *Pseudomonas*. To enable quantification of introduced *P. fluorescens* strains, this medium was amended with 50 ppm X-Gal upon which recovered *lac*ZY modified *Pseudomonas* could be identified as blue colonies. P1 plates were incubated at 25^{o}C and enumerated after 5 days growth. The sum of the indigenous and introduced *Pseudomonas* populations were calculated and described as total *Pseudomonas* populations. Tryptone soya agar (10%) was used for the enumeration of total culturable bacteria and for determining the population structure in terms of r (rapid colony formation) and K (slower colony formation) strategists (De Leij *et al* 1993a). For this purpose, plates were incubated at 25^{o}C for 7 days and enumerated on a daily basis. The number of colonies appearing was plotted against time since inoculation.

Statistical analysis

Data was analysed using SPSS for Windows (SPSS inc.) by means of a one way ANOVA and subsequently differences between treatments (multiple comparisons) were determined using least significant differences between means. Data from the r and K population structure curve were transformed into $\log^{it}$ and analysed by means of a one way ANOVA, significant differences were determined using least significant differences between means.

Results and Discussion

The semi-enclosed microcosm used in this study required little maintenance, and with the use of pea as the model rhizosphere, produced large amounts of rhizosphere soil (> 20 g) from a large compact root mass, creating an ideal system for rhizosphere study.

The non-functional modifications to the SBW25 strain (lactose utilisation, kanamycin resistance and catechol degradation) are designed not to give the modified derivative a selective advantage, nor to have an effect on the ecosystem into which the organisms were released. These modifications are marker genes, introduced to enable the reisolation and identification of the released bacteria from the environment and hence to study its ecology (Drahos *et al* 1986). Both the parent and derivative F113 strains have the *lac*ZY gene cassette inserted for ecological study. The deletion of DAPG production in the F113 G22 strain is a functional modification, and allows the assessment of the impact of an antimicrobial-producing inoculum in comparison with a non-producer. These genetic differences are designed to have an effect on the ecosystem and indeed Shanahan *et al* (1992b) has shown that the wild type has an inhibitory effect *in vitro* on both bacteria and fungi and has isolated DAPG from soil (Shanahan *et al* 1992a). DAPG production has also been shown in the rhizosphere of microcosm grown plants (Keel *et al*, 1992 and Maurhofer *et al*, 1995).

Plant growth

Plant growth measurements (Table 1) were used to assess the potential impact of the different inocula on crop productivity. As neither shoot nor root weights showed significant differences between treatments, yet the F113 (DAPG+) inocula resulted in smaller shoot weight and greater root weights, the results were converted into shoot to root ratio, to take both into account together. The conversion into shoot/root ratio has been used extensively in the past (Clark and Reinhard, 1991) and has been suggested to be an indicator of plant stress, whereby the lower the shoot/root ratio (or higher the root/shoot ratio) the more stressed the plant. It should be recognised however, that such stressed plants may be more effective in acquiring water and nutrients as a result of the expanded root system and thus this is a positive adaptive response to such stresses.

As the F113 (DAPG+) strain produced a significantly smaller shoot/root ratio than the control, the F113 G22 and the SBW25 treatments, it can be deduced that this strain has attributes that cause stress in pea plants, that have been deleted by the Tn*5* insertion to create the F113 G22 strain. The Tn*5* insertion was aimed at preventing the production of the DAPG antibiotic and thus the plant stress is a result of the DAPG production. Either the antibiotic affected the plant directly or the indigenous microbial community structure in the rhizosphere, which in turn caused plant stress by an increase in detrimental micro-organisms and/or a reduction in beneficial populations (protective or stimulatory organisms).

Microbial populations

It is conceivable that the inoculation of a micro-organism into the environment will cause the largest effects on other micro-organisms, especially upon populations of a similar nature to the released strain, i.e. the indigenous *Pseudomonas* populations. Thus, it was necessary to perform an extensive study of total microbial populations and a more specific analysis of the effect on *Pseudomonas* populations.

The F113 G22 treatment had a significantly greater (by approximately 0.35 log units) effect on indigenous fluorescent *Pseudomonas* population (Table 2) than the SBW25 GMM and the F113 (DAPG+) treatments (SBW25 WT is not genetically marked and not distinguishable from the indigenous population). This suggests that the F113 G22 strain promotes the indigenous population. However, the mechanisms of these differences vary, the WT and GM SBW25 inocula resulted in a smaller indigenous *Pseudomonas* populations caused by competitive exclusion and the F113 by repression caused by antibiotic production. The introduced *Pseudomonas* populations (Table 2) provide evidence for the competitive exclusion caused by the SBW25 strains. The SBW25 GMM strain resided in numbers much greater than the two F113 strains ($p<0.05$), yet the total *Pseudomonas* population was similar to the SBW25 WT and F113 G22 treatments. Evidence for F113 (DAPG+) strain repressing the indigenous *Pseudomonas* population directly by the production of the antibiotic comes from the higher indigenous population with the inoculation of the F113 G22 (DAPG-) strain.

The total *Pseudomonas* populations (Table 2), which is the sum of the introduced and indigenous populations, with both the WT and GM SBW25 inoculants were much

greater than the control ($p<0.05$). This indicates that the introduced bacteria not only displaced a proportion of the indigenous population (lower indigenous *Pseudomonas* population), they also occupied a wider niche or colonised the rhizosphere more intensely than the displaced indigenous populations. The seeding effect of the high inocula gave the SBW25 strains an advantage in early root colonisation over indigenous populations (both *Pseudomonas* and other micro-organisms). However, the F113 strains were inoculated at similar intensities to the SBW25 strains and the F113 G22 did not displace the indigenous populations. It is, therefore, plausible that the F113 G22 strain had an additive effect on the total *Pseudomonas* population by occupying a niche distinct from that of the indigenous *Pseudomonas* populations. Evidence for this hypothesis comes from the reduced indigenous *Pseudomonas* population with introduction of F113 (DAPG+), where the inocula did not form a larger population to compensate for the reduced indigenous *Pseudomonas* population. This was shown with the resulting total *Pseudomonas* population which was similar to the control with the F113 (DAPG+), and significantly ($p<0.05$) smaller than the other three inocula.

The use of X-gal in the *Pseudomonas* isolation media to identify the *lac*ZY marked strains meant that other bacteria, capable of growing on the P1 media, that can utilise lactose as a carbon source could also be easily enumerated. Large numbers of background lactose utilising bacteria have been found in other studies, indeed De Leij *et al* (1993b) found that 5% of the cultural bacterial population in a specified soil were both lactose utilisers and kanamycin resistant. The SBW25 GMM treatment resulted in a significantly lower (by more than half a log unit) lactose utilising population (Table 2) than the control and the two F113 strains, which had comparable population sizes.

However, the SBW25 GMM inoculum did not result in a significantly smaller population than the wild type, which was also significantly smaller than the two F113 strains. Therefore, the reduction in the population of lactose utilising bacteria is a general effect of the SBW25 strains (on competitive or grounds other than lactose utilisation) and not the genetic modification (*lac*ZY insert) *per se.* This conclusion is also supported by the fact that both the F113 strains have *lac*ZY inserts and did not reduce the lactose utilising populations.

The numbers of fungi were not significantly affected by any of the bacterial inocula (Table 3). The *in vitro* effects reported by Shanahan *et al* (1992b), whereby the F113, (DAPG+) inhibited the fungi and the non-producer did not, were not apparent *in vivo* in this study. This is also contrary to the work of Short *et al* (1990) who found that fungal populations were suppressed by a strain of *Pseudomonas putida* inoculated into soil.

As the total bacterial and yeast counts (Table 3) did not change it is possible that they were not affected by the inocula. However, components of the bacterial community (fluorescent *Pseudomonas*) were affected and other workers have found such effects, for example Austin *et al* (1990) also found negative effects upon protozoa populations with a *Pseudomonas* inoculation. These results highlight the difficulty created when microbial counts are expressed on a log scale, as the counts can be highly variable for isolations from such heterogeneous environments as the rhizosphere. Differences of 0.3 and above on a log scale were required for statistical significance, which is a difference of over 100% on a linear scale.

Bacterial community structure

The controls, both the SBW25 and the F113 G22 inocula resulted in similar colony formation profiles (Figure 1), with most colonies (approximately 70%) emerging between 36 and 60 hours after inoculation, producing a large broad peak of colony formation over this time before steadily declining. The F113 (DAPG+) strain resulted in a colony formation profile with a marked difference to the other treatments. A large narrow peak (consisting of approximately 55% of the total colonies) over a single observation time point (60 hours after inoculation) was found whilst significantly less ($p=0.05$) colonies, than with the four other treatments, were visible 36 hours after inoculation ($p=0.05$). After the 84 hour time point the colony emergence for the F113 DAPG+ treatment was similar to the other treatments. Thus, the colony emergence with the F113 treatment was slower initially and was therefore displaced towards K strategy. This effect was not found with both the SBW25 inocula and the F113 G22 (DAPG-), thus it is the DAPG producing property of the F113 strain that caused the effect.

There are two possible mechanisms for the shift in colony forming distribution, the first, as proposed by Hattori (1982 & 1983) is a change in the physiological state of the bacterial cells. This hypothesis suggests that starved cells or stressed cells will take longer to form colonies than non-stressed cells. If it is assumed that the antibiotic production caused some stress in the indigenous bacterial community, then the shift towards later colony formation can be explained in terms of the physiological state of

the bacterial cells. The alternative hypothesis proposed by De Leij *et al* (1993a, is that there was a shift in the composition of the bacterial populations towards a slower growing community (towards K strategy). De Leij *et al* supported this hypothesis by inoculating selected colonies isolated from soil onto fresh plates and recording the colony forming distribution again, finding a similar profile to the original isolation. However, this work used the inoculation of a non-functionally modified organism and the effect of a functionally modified bacteria (especially modified in antibiotic production) may be somewhat different. During this experiment a number of colonies taken from the F113 treatment were re-inoculated onto fresh plates. The colony formation was approximately equally distributed between colonies appearing after a similar time period to the initial soil isolates and others appearing 24 hours earlier than their respective parent colonies. It is therefore likely that the mechanism for the distribution shift is some combination of the two theories.

Soil enzyme activities

The alkaline phosphatase activity (Table 4) was significantly higher with the inoculation of the F113 DAPG+ strain than with the other three inocula ($p<0.05$), whilst the control was intermediate in activity. The F113 (DAPG+) treatment also produced the highest phosphodiesterase activity (Table 4), which was significantly greater than the control, SBW25 WT and F113 G22 treatments. However, statistical significance could not be shown in comparison to the SBW25 EeZY-6KX. Therefore, the alkaline phosphatase and phosphodiesterase activities were increased by the presence of the DAPG-producing strain whereas the other inocula reduced the activity

with respect to the control. This infers that the effect of increased activity is caused by the production of DAPG, as the effect was not found with the inoculation F113 G22. This subsequently infers that the general effect of inoculation upon these enzymes is to reduce the activity.

In previous work (Naseby and Lynch, 1997b) a similar effect was found, where the inoculation of SBW25 into the rhizosphere of wheat caused a reduction in the alkaline phosphatase and phosphodiesterase activities. However, the addition of enzyme substrates to the soil caused a reversal of this trend, with the inocula causing higher activities than the control. Increased available inorganic soluble phosphate is known to decrease soil phosphatase activity (Tabatabai, 1982 and Tadano *et al*, 1993). Therefore, the F113 (DAPG+) strain must have caused a decrease in the available phosphate, thus causing an overall increase in activity. The decrease in available P may have taken the form of an increase in the available carbon in the rhizosphere (by stimulation of root exudation or leakage, as there was a decrease in the shoot/root ratio). Other studies have highlighted changes in root exudation caused by biocontrol *Pseudomonas fluorescens* strains (Mozafar *et al* 1992). Increasing the ratio of C to P available would therefore increase the microbial P demand.

The trend found in the alkaline phosphatase activity was not repeated with the acid phosphatase activity (Table 4) which was lowest with the inoculation of the F113 strain, but only significantly so to the SBW25 WT treatment ($p<0.05$). However, the majority of the acid phosphatase activity may be of a different origin to the alkaline phosphatase. Acid phosphatase is mostly of plant and associated fungal origin

(Tarafdar and Marschner, 1994), whereas the alkaline phosphatase is more likely to be of microbial origin. If this is the case, then the effects of the inocula upon acid and alkaline phosphatase, in some circumstances, can be independent. This is supported by the work described earlier (Naseby and Lynch 1997b) where rhizosphere acid phosphatase did not show significant differences with the inoculation of bacteria, addition of substrates and did not show a trend with soil depth. The acid phosphatase activity would be more dependent upon the nutritional status of the plant, which had a lower shoot/root ratio with the F113 treatment and would lose comparatively larger amounts of C, resulting in a reduced P demand.

The aryl sulphatase activity (Table 4) showed a similar trend to the alkaline phosphatase and the phosphodiesterase activities, with the F113 (DAPG+) inoculum causing the greatest activities, and the other treatments resulting in similar lower activities. Therefore the same mechanisms are involved with the aryl sulphatase activity as those that influenced the alkaline phosphatase and phosphodiesterase activities, i.e. aryl sulphatase activity is inversely linked to the available inorganic sulphate in the rhizosphere.

Inverse trends were found with the C and N cycle enzymes (Table 5) in comparison to the general trend found in the P and S cycle enzymes (as was found in Naseby and Lynch, 1997b). The F113 (DAPG+) strain was associated with the lowest acid β galactosidase activity which was significantly lower than the SBW25 WT treatment. The F113 (DAPG+) strain produced the lowest β glucosidase activity (Table 5) which was significantly smaller than the control, the SBW25 WT and the F113 G22

treatments, which all had similar activities. As with the β galactosidase and β glucosidase activities the F113 (DAPG+) strain produced the lowest N-acetyl glucosaminidase activity (Table 5). This was a significantly lower activity than the control, the SBW25 WT and the F113 G22 treatments, which all had comparable activities. All three carbon cycle enzyme activities, therefore, also indicate an increase in carbon availability.

The results presented evaluated a range of methods for assessing the impact of introducing a range of GM *Pseudomonas* into soil and showed that the nature of the genetic modification is important. The soil enzyme measurements proved to be a sensitive indicator of perturbations caused by the inocula and provided information as to the nature of the perturbations. Total microbial populations were not a sensitive indicator of perturbation. However, r and K strategy measurement was a more reliable measure of perturbation than total populations, but this too is non-functional and unlike soil enzyme measurements does not give an indication of the effects of the inocula upon ecosystem function. The *Pseudomonas* populations were very sensitive as expected of populations related to the inocula. Significant effects were not found with the addition of marker genes (*lacZY, xylE* and Kan^r) where as a number of significant effects were found with the wild type functional gene (DAPG). The results therefore indicate not surprisingly that such functional genes in the genome of *Pseudomonas* strains would have an impact on rhizosphere populations and function. However this is a wild type and the genetic modification (deletion) reduced these effects, but this still gives an indication of the effects of introducing such antibiotic genes into other strains. The functional wild type antibiosis gene used here targeted at

plant disease control is likely to be far more beneficial than any small effects found in this study.

Acknowledgement

This work was supported by the EU Biotechnology Programme contract no. BI02 CT93-0053, the IMPACT project which incorporates 17 European partners.

We thank University College Cork for the provision of the F113 constructs and IVEM, Oxford for the provision of the SBW25 construct.

References

Andrews JH, Harris RF (1986) r- and K-selection and microbial ecology. *Advances in Microbial Ecology* **9**, 99-147.

Austin HK, Hartel PG, Coleman DC (1990) Effect of genetically altered *Pseudomonas solanacearum* on predatory protozoa. *Soil Biology and Biochemistry* **22**, 115-117.

Bailey MJ, Thompson IP, Rainey PB, Ellis RJ (1995) Site directed chromosomal marking of a fluorescent pseudomonad isolated from the phytosphere of sugar beet: stability and potential for marker gene transfer. *Molecular Ecology* **4,** 755-762.

Bolton H Jr, Fredrickson JK, Bentjen SA, Workman DJ, Scu-mei WLi, Thomas JM (1991) Field calibration of soil core microcosms: fate of genetically altered rhizobacterium. *Microbial Ecology* **21,** 163-173.

Clark RB, Reinhard N (1991) Effects of soil temperature on root and shoot growth traits and iron deficiency chlorosis in sorghum genotypes grown on low iron calcarious soil. *Plant and Soil* **130,** 97-103.

Colwell RR, Brayton PR, Grimes DJ, Rosak DB, Huq SA, Palmer, LM (1985) Viable but unculturable *Vibrio cholerae* and related pathogens in the environment:

implications for the release of genetically engineered micro-organisms. *Biotechnology* **3,** 817-820.

De Leij FAAM, Whipps JM, Lynch JM (1993a) The use of colony development for the characterisation of bacterial communities in soil and on roots. *Microbial Ecology* **27,** 81-97.

De Leij FAAM, Bailey MJ, Whipps JM, and Lynch JM (1993b) A simple most probable number technique for the sensitive recovery of a genetically-modified *Pseudomonas-aureofaciens* from soil. *Letters In Applied Microbiology* **16,** 307-310.

De Leij FAAM, Sutton E J, Whipps J. M., Fenlon JS, Lynch J M (1995a) Field release of a genetically modified *Pseudomonas fluorescens* on wheat: establishment, survival and dissemination. *Applied & Environmental Microbiology* **61**, 3443-3453.

De Leij FAAM, Sutton EJ, Whipps JM, Lynch JM (1995b) Impact of genetically modified *Pseudomonas fluorescens* on indigenous populations of wheat. *FEMS Microbiology Ecology* **13,** 249-258.

Doyle JD, Stotzky G (1993) Methods for the detection of changes in the microbial ecology of soil caused by the introduction of micro-organisms. *Microbial Releases* **2,** 63-72.

Drahos DJ, Hemming BC, Mcpherson S (1986) Tracking recombinant organisms in the environment; B-galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. *Biotechnology* **4**, 439-444.

Fenton AM, Stephens PM, Crowley J, O'Callaghan M, O'Gara F (1992) Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capacity to *Pseudomonas* strain. *Applied and Environmental Microbiology* **58**, 3873-3878.

Hattori T (1982) Analysis of plate count data of bacteria in natural environments. *Journal of General and Applied Microbiology* **28,** 13-22.

Hattori T (1983) Further analysis of plate count data of bacteria. *Journal of General and Applied Microbiology* **29,** 13-22.

Jones RA, Broder MW, Stotzky G (1991) Effects of genetically engineered micro-organisms on nitrogen transformations and nitrogen-transforming microbial populations in soil. *Applied and Environmental Microbiology* **57**, 3212-3219.

Katoh K, Itoh K (1983) New selective media for *Pseudomonas* strains producing fluorescent pigment. *Soil Science and Plant Nutrition.* **29,** 525-532.

Keel C, Schnider U, Maurhofer M, Voisard C, Laville J, Burger U, Wirthner P, Hass D, Defago G (1992) Suppression of root diseases by Pseudomonas

fluorescens CHAO: importance of the bacterial metabolite 2,4-diacetylphloroglucinol. Molecular Plant –Microbe Interactions **5,** 4-13.

Maurhofer M, Keel C, Haas D, Defago G (1995) Influence of plant species on disease suppression by Pseudomonas fluorescens strain CHAO with enhanced antibiotic production. Plant pathol. 44, 40-50.

Mawdsley JL, Burns RG (1994) Inoculation of plants with *Flavobacterium* P25 results in altered rhizosphere enzyme activities. *Soil Biology and Biochemistry* **26,** 871-882.

Mills PR (1994) DNA based methods for identification and characterisation. In: *The identification and characterisation of pest organisms*, pp 427-435. Edited by DL Hawksworth. CAB International, Wallingford.

Moenne-Loccoz Y, Powell J, Higgins P, McCarthy J, O'Gara F (1997) An investigation of the impact of biocontrol *Pseudomonas fluorescens* F113 on the growth of sugar Beet and the performance of subsequent clover-*Rhizobium* symbiosis. Applied Soil Ecology In press.

Morgan JAW (1991) Molecular biology: new tools for studying microbial ecology. *Scientific Progress Edinburgh* **75,** 265-278.

Mozafar A, Duss F, Oertli JJ (1992) Effect of *Pseudomonas-fluorescens* on the root exudates of 2 tomato mutants differently sensitive to Fe chlorosis. *Plant And Soil* 144:167-176.

Naseby DC, Lynch JM (1997a) Functional impact of genetically modified micro-organisms on the soil ecosystem. In *Ecotoxicology: Responses, Biomarkers and Risk Assessment.* Edited by JT Zelikoff, J Schepers, JM Lynch. SOS Publications, Fair Haven, NJ, USA.

Naseby DC, Lynch JM (1997b) Rhizosphere soil enzymes as indicators of perturbation caused by a genetically modified strain of *Pseudomonas fluorescens* on wheat seed. *Soil Biology and Biochemistry* **29** 1353-1362.

Panikov N (1994) Population dynamics of micro-organisms with different life strategies, p 47-65. In: *Environmental gene release: models, experiments and risk assessment*. Edited by MJ Bazin, JM Lynch. Chapman and Hall, London.

Seidler RJ (1992). Evaluation of methods for detecting ecological effects from genetically engineered micro-organisms and microbial pest control agents in terrestrial environments. *Biotechnology Advances* **10**, 149-178.

Shanahan P, Borro A, Ogara F, Glennon JD (1992a) Isolation, trace enrichment and liquid-chromatographic analysis of diacetylphloroglucinol in culture and soil

samples using uv and amperometric detection. *Journal of Chromatography* **606,**171-177

Shanahan P, O'Sullivan DJ, Simpson P, Glennon JD, O'Gara F (1992b) Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Applied and Environmental Microbiology* **58**, 353-358.

Short KA, Seidler RJ, Olson RH (1990) Survival and degrative capacity of *Pseudomonas putida* induced or constitutively expressed plasmid mediated degradation of 2,4-dichlorophenoxyacetate (TFD) in soil. *Canadian Journal of Microbiology* **36**, 821-826.

Smit E, Van Elsas JD, Van Veen JN (1992) Risks associated with the application of genetically modified micro-organisms in terrestrial environments. *FEMS Microbiology Reviews* **88,** 263-278.

Stotzky G, Broder MW, Doyle GD, Jones RA (1993) Selected methods for the detection and assessment of ecological effects resulting from the release of genetically engineered micro-organisms to the terrestrial environment. *Advances in Applied Microbiology* **38**, 1-98.

Tabatabai MA (1982) Soil Enzymes. In *Methods in Soil Analysis, part 2, Chemical and Microbiological Properties.* 2nd Edn pp. 903-948. Edited by A.L. Page, R.H. Miller, D.R. Keene. American Society of Agronomy, Madison.

Tadano T, Ozowa K, Satai M, Osaki M, Matsui H (1993) Secretion of acid phosphatase by the roots of crop plants under phosphorus-deficient conditions and some properties of the enzyme secreted by lupin roots. *Plant and Soil* **155-156,** 95-98.

Tarafdar JC, Marschner H (1994) Phosphatase activity in the rhizosphere and hyphosphere of VA mycorrhizal wheat supplied with inorganic and organic phosphorus. *Soil Biology and Biochemistry* **26,** 387-395.

Van Elsas JD, Waalwijk C (1991) Methods for the detection of specific bacteria and their genes in soil. *Agriculture, Ecosystem and Environment,* **34,** 97-105.

Wang Z, Crawford DL, Magnunson TS, Bleakley BH, Hertel G (1991) Effects of bacterial lignin peroxidase on organic carbon mineralisation in soil using recombinant *Streptomycin* strains. *Canadian Journal of Microbiology* **37**, 287-294.

Whipps JM, De Leij FAAM, Lynch JM, Bailey MJ (1996) Impact of genetically modified micro-organisms on the terrestrial microbiota including fungi. In *Fungi and Environmental Change* pp219-316. Edited by J Frankland N Magan, G Gadd. Cambridge University Press, Cambridge.

Figure Legend

Figure 1: Total bacterial community structure (colony formation over time - r and K strategy) in the rhizosphere of pea inoculated with wild type and genetically modified *P. fluorescens* strains.

*Treatments; control, not inoculated; SBW25 WT, inoculated with *P. fluorescens* SBW25 wild type; SBW25 GMM, inoculated with recombinant *P. fluorescens* SBW25; F113 G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.

Standard errors of means (n=7) indicated. Significant differences, 36 hrs F113 lower than all other treatments (p=0.05); 60 hrs F113 higher than other treatments (p=0.05).

Table 1 Mean pea plant shoot and root weights and the ratio between the two, as affected by wild type and GMM *P. fluorescens* inoculation.

Treatment	Shoot weight	Root weight	Shoot/root ratio
Control	1.286 ±0.032	1.092 ±0.033	1.182 ±0.037[a]
SBW25 WT	1.286 ±0.028	1.094 ±0.024	1.179 ±0.038[a]
SBW25 GMM	1.257 ±0.038	1.076 ±0.027	1.172 ±0.04[a,b]
F113 G22	1.259 ±0.04	1.076 ±0.048	1.176 ±0.038[a]
F113	1.218 ±0.024	1.133 ±0.021	1.075 ±0.002[b]

*Treatments; control, no inocula; SBW25 WT, inoculated with *P. fluorescens* SBW25 wild type; SBW25 GMM, inoculated with recombinant *P. fluorescens* SBW25; F113 G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.

Standard errors for means (n=7) indicated. Significant differences at p=0.05 level indicated by letters.

Table 2: Log fluorescent *Pseudomonas* and lactose utilising populations in the rhizosphere of pea plants inoculated with wild type and genetically modified *P. fluorescens* strains.

Treatment*	Indigenous *Pseudomonas*#	Introduced *Pseudomonas*#	Total *Pseudomonas*#	Other lactose utilisers#
Control	6.3 ±0.015^{ab}	N/A	6.3 ±0.015^{a}	6.42 ±0.048^{b,c}
SBW25 WT	N/A	N/A	6.771 ±0.018^{b}	6.104 ±0.046^{a,b}
SBW25 GMM	6.146 ±0.066^{b}	6.555 ±0.025^{a}	6.698 ±0.025^{b}	5.854 ±0.081^{a}
F113 G22	6.594 ±0.044^{a}	6.151 ±0.058^{b}	6.728 ±0.037^{b}	6.471 ±0.036^{c}
F113	6.136 ±0.056^{b}	6.168 ±0.025^{b}	6.422 ±0.029^{a}	6.542 ±0.039^{c}

*Treatments; control, no inocula; SBW25 WT, inoculated with *P. fluorescens* SBW25 wild type; SBW25 GMM, inoculated with recombinant *P. fluorescens* SBW25; F113 G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.

c.f.u./g fresh root. Standard errors for means (n=7) indicated. Significant differences between treatments at p=0.05 level indicated by different letters.

N/A: not applicable as no genetically marked strains were introduced.

Table 3: Log total fungal, yeast and bacterial populations in the rhizosphere of pea plants inoculated with wild type and genetically modified *P. fluorescens* strains.

Treatment*	total fungi#	total yeast#	total bacteria#
Control	4.354 ±0.08	4.316 ±0.088	7.639 ±0.034
SBW25 WT	4.371 ±0.069	4.245 ±0.095	7.603 ±0.048
SBW25 GMM	4.383 ±0.063	4.212 ±0.088	7.556 ±0.041
F113 G22	4.540 ±0.051	4.119 ±0.055	7.565 ±0.042
F113	4.490 ±0.063	4.251 ±0.096	7.524 ±0.073

*Treatments; control, no inocula; SBW25 WT, inoculated with *P. fluorescens* SBW25 wild type; SBW25 GMM, inoculated with recombinant *P. fluorescens* SBW25; F113 G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.

c.f.u./g fresh root. Standard errors for means (n=7) indicated.

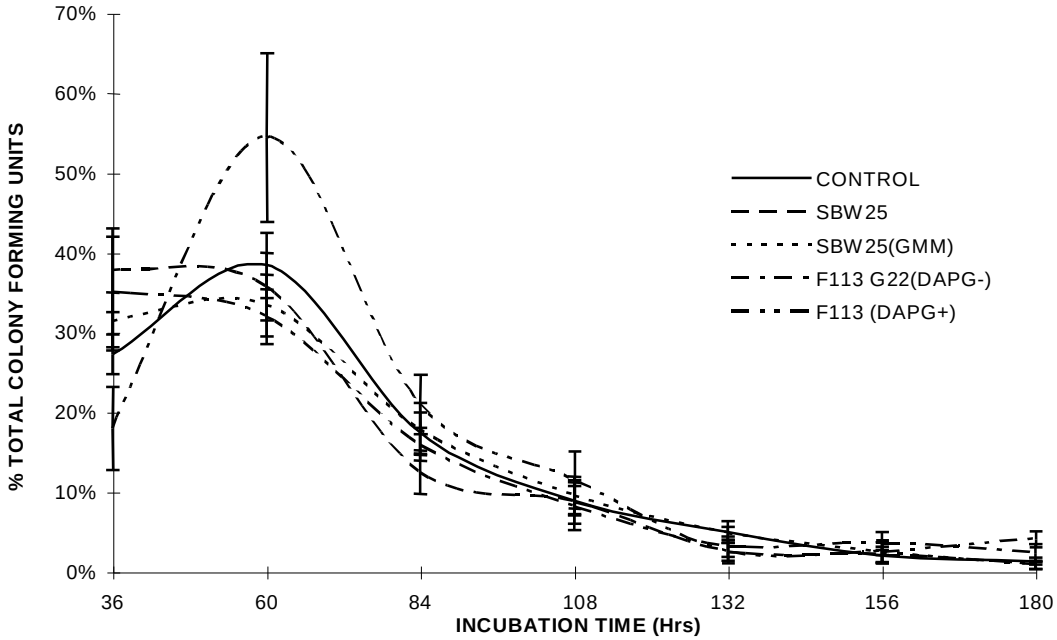
70%
60%
50%
40%
30%
20%
10%
0%
% TOTAL COLONY FORMING UNITS
36
60
84
108
132
156
180
INCUBATION TIME (Hrs)
CONTROL
SBW25
SBW25(GMM)
F113 G22(DAPG-)
F113 (DAPG+)

Table 4: Phosphorus and sulphur cycle enzyme activities in the rhizosphere of pea plants inoculated with wild type and genetically modified *P. fluorescens* strains.

Treatment*	acid phosphatase#	alkaline phosphatase#	phospho-diesterase#	aryl sulphatase#
Control	7.911 ±0.407^{a,b}	1.235 ±0.153^{b,c}	0.111 ±0.018^{a}	0.091 ±0.012^{a}
SBW25 WT	9.282 ±0.414^{b}	0.757 ±0.172^{a,b}	0.086 ±0.016^{a}	0.094 ±0.014^{a}
SBW25 GMM	7.857 ±0.45^{a,b}	0.934 ±0.147^{a,b}	0.119 ±0.017^{a,b}	0.117 ±0.009^{a,b}
F113 G22	8.241 ±0.692^{a,b}	0.704 ±0.159^{a}	0.087 ±0.025^{a}	0.076 ±0.023^{a}
F113	7.058 ±0.398^{a}	1.523 ±0.128^{c}	0.168 ±0.014^{b}	0.161 ±0.023^{b}

*Treatments; control, no inocula; SBW25 WT, inoculated with *P. fluorescens* SBW25 wild type; SBW25 GMM, inoculated with recombinant *P. fluorescens* SBW25; F113 G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.

Expressed as mg pNP released/g dry soil. Standard errors for means (n=7) indicated. Significant differences between treatments at p=0.05 level indicated by different letters.

Table 5: Carbon and nitrogen cycle enzymes activities in the rhizosphere of pea plants inoculated with wild type and genetically modified *P. fluorescens* strains.

Treatment*	acid β-galactosidase#	β-glucosidase#	NAGase#
Control	0.515 ±0.042^{a,b}	1.019 ±0.098^{b}	0.321 ±0.03^{b}
SBW25 WT	0.623 ±0.047^{b}	1.026 ±0.072^{b}	0.334 ±0.017^{b}
SBW25 GMM	0.501 ±0.032^{a,b}	0.765 ±0.088^{a,b}	0.267 ±0.044^{a,b}
F113 G22	0.505 ±0.047^{a,b}	1.022 ±0.138^{b}	0.295 ±0.029^{b}
F113	0.452 ±0.04^{a}	0.624 ±0.068^{a}	0.184 ±0.019^{a}

*Treatments; control, no inocula; SBW25 WT, inoculated with *P. fluorescens* SBW25 wild type; SBW25 GMM, inoculated with recombinant *P. fluorescens* SBW25; F113 G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.

Expressed as mg pNP released/g dry soil. Standard errors for means (n=7) indicated. Significant differences between treatments at p=0.05 level indicated by different letters.