Effect of 2,4 diacetylphloroglucinol producing, over producing and non-producing *Pseudomonas fluorescens* F113 in the rhizosphere of pea.

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Abstract

Pseudomonas fluorescens F113*lacZY* and modified strains carrying different functions modifications were assessed for their impact in the rhizosphere of pea. Strain F113*lacZY* naturally produces the anti-fungal metabolite 2,4 diacetylphloroglucinol (Phl) useful in plant disease control. The first modified strain of F113 was repressed in production of Phl, creating the Phl negative strain F113G22. The second was a plasmid based over-producer of Phl (F113Rif (pCUGP)). Both the F113*lacZY* and the F113Rif (pCUGP) strains increased the rhizoplane fungal populations whereas the same strains reduced the rhizosphere soil fungal populations with respect to the control. Similar results were found with the rhizoplane and rhizosphere soil bacterial populations. The F113G22 treatment resulted in a significantly greater indigenous fluorescent *Pseudomonas* population than the F113*lacZY* and F113Rif (pCUGP) treatments and a greater total *Pseudomonas* population than the control, F113*lacZY* and F113Rif (pCUGP) treatments. Over-production of Phl did not affect the establishment of the introduced *Pseudomonas* population. None of the inocula displaced the indigenous populations, but the F113G22 inocula had an additive effect on the total *Pseudomonas* population. P (phosphatase), S (sulphatase) and N (urease) cycle enzyme activities were increased whilst C (glucosidase, NAGase) cycle activities were decreased by the F113*lacZY* and F113Rif (pCUGP) treatments suggesting C leakage from the roots. Overall, most effects of inoculation compared to the wild type were found with the non-Phl producing strain. Over-production of Phl had little environmental effect in relation to wild type inocula.

Introduction

The effect of genetically modified micro-organisms (GMMs), released as biocontrol agents, on the environment has increasingly become of public concern. Therefore 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. 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). 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; Van Elsas and Waalwijk, 1991), whilst Natsch et al (1997, 1998) found transient effects of an antibiotic overproducing *Pseudomonas* strain on pseudomonad diversity and the resident bacterial community. However 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 *P. fluorescens* strain. Naseby and Lynch (1998b) and Naseby et al. (1999) found evidence, using soil enzyme activities, for changes in carbon availability caused by increased C leakage from the roots with the inoculation of the same *Pseudomonas* strain used in this study. However these two studies only assessed the deletion of anti-fungal metabolite production in a biocontrol strain and did not assess the effect of enhanced biocontrol properties. It should also be noted that the type and amount of available C source can influence Phl production (Yuan et al 1998).

The deletion of 2,4-diacetylphloroglucinol (Phl) production in the *P. fluorescens* F113G22 strain and over production of the same anti-fungal metabolite in F113Rif (pCUGP) are functional modifications, and allow the assessment of the impact of an antimicrobial-producing inoculum in comparison with a non-producer and a strain enhanced in production. All three strains have been used before in an experiment to determine the effect on arbuscular mycorrhiza formation (Barea et al 1998), no adverse effects on mycorrhizal performance were found. 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 Phl from soil (Shanahan et al. 1992a). Phl production has also been shown in the rhizosphere of microcosm grown plants (Keel et al. 1992; Maurhofer et al., 1995) and is useful in the control of damping off diseases (Fenton et al. 1992).

The aim of the experiments reported here was to investigate the effect of inoculation of the pea rhizosphere with a strain F113 carrying functional modifications on early growth of pea where *P fluorescens* has been investigated for use as a seed inocula to prevent seedling diseases such as *Pythium* (Dunne et al. 1998). A *lacZY* marked strain was compared to its *Tn5* mutated non-anti-fungal metabolite producing derivative and a Phl overproducing

derivative in the pea rhizosphere to assess the impact of the inoculum and anti-fungal metabolite production on rhizosphere populations and enzyme activities.

Materials and Methods

Soil description

The soil used was a sandy loam of the Holiday Hills series, Merrist Wood Agricultural College (Surrey), and had been under permanent pasture for at least 15 years. The soil was taken from the top 30 cm under the turf and was stored for 2 weeks at 4^{o}C before use. 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. The total NPK, measured by the standard methods of MAFF/ADAS (1986), contents by weight were 0.124%, 0.033% and 0.861% respectively.

Microcosm

Coarsely sieved (6 mm) loose soil (250 g) was placed in experimental microcosms, as used by Naseby and Lynch (1998a), consisting of 210 mm high acetate cylinders with a 90 mm diameter, slotted between the top and base of plastic 90 mm diameter Petri dishes creating semi-enclosed microcosms.

Bacterial strains and treatments

Three strains of *Pseudomonas fluorescens* were used with different modifications. Strain F113*lacZY* that produces the anti-fungal metabolite 2,4 diacetylphloroglucinol (Phl), and was marked with a *lac*ZY gene cassette, a Phl negative derivative (strain F113G22) produced by Tn*5* mutagenisis (Shanahan et al. 1992b) and a constitutive plasmid based Phl over-producer (F113Rif (pCUGP)) marked with a *gusA* gene cassette. The over producer

produced approximately ten times more Phl (measured by the method of Shanahan et al 1992a) than the wild type in liquid media and a three fold increase in the amount of Phl extracted from rhizosphere soil was found with the inoculation of the overproducer in comparison to the wild type

The bacteria were grown on full strength tryptone soya agar (Oxoid) for 3 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 8 x 10^{8} c.f.u./ml were subsequently used to imbibe pea seeds (*Pisum sativum* var. Montana), at a ratio of one seed per ml, for 6 hours (stirred every 30 minutes) resulting in between 6 and 8 x 10^{7} c.f.u. per pea seed.

Experimental design

Each microcosm consisted of eight imbibed seeds, planted at a depth of approximately 1 cm below the soil surface. Each treatment was replicated seven times (7 microcosms consisting of a pool of 8 plants). Twenty five 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 at 21^{o}C, no further water was added. The relative humidity was maintained at 70% and the light intensity was 10,000 lux at shelf level.

Sampling and analysis

After 17 days growth the microcosms were harvested in random order, 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, N-acetyl glucosaminidase (NAGase) and urease by the methods of Naseby and Lynch (1997b).

Shoot and root fresh weights (after the remains of the seeds were discarded) were measured and a 1g root sample from each replicate (containing a whole root system) was macerated in 9 ml of sterile quarter strength Ringers solution using a pestle and mortar. Rhizosphere soil (1g) and rhizoplane (including endorhizosphere) filamentous fungal populations were quantified by plating a ten fold dilution series of each root macerate or soil sample 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 rhizoplane, fluorescent *Pseudomonas*. To enable quantification of introduced *P. fluorescens* strains F113*lacZY* and F113G22, this medium was amended with 50 ppm X-Gal upon which recovered *lac*ZY modified *Pseudomonas* could be identified as blue colonies. P1 media was amended with X-Gluc for the identification of the *gusA* marked F113Rif (pCUGP). 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; plates were incubated at 25^{o}C and enumerated after 7 days growth.

Statistical analysis

Data were 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 (LSD) as the post-hoc test.

Results and Discussion

Plant growth

Plant growth measurements (Table 1) were used to assess the potential impact of the different inocula on crop productivity. The shoot weights were not significantly affected by any of the treatments. However, the root weights were significantly greater with the F113*lacZY* and F113Rif (pCUGP) treatments, which resulted in both these treatments having significantly smaller shoot to root ratios and suggesting a greater root density per unit root length. The conversion into shoot/root ratio has been used extensively in the past (Clark and Reinhard 1991, Andrews et al 1999) 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*lacZY* and F113Rif (pCUGP) strains resulted in significantly smaller shoot/root ratios than the control and the F113G22, it can be deduced that these strains have attributes that have an impact on pea plants. These attributes have been deleted in the F113G22 strain, and thus the plant stress is the result of Phl production. Either the anti-fungal metabolite 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). A similar decrease in the shoot/root ratio was found by Naseby et al. (1999) and was related to an increase in C leakage from the roots.

Microbial populations

Both the F113*lacZY* and the F113Rif (pCUGP) strains increased the rhizoplane fungal populations whereas the same strains reduced the rhizosphere soil fungal populations with respect to the control (Table 2). The F133 OP treatment also resulted in a lower rhizosphere fungal population than the F113G22 treatment. Similar results were found with the rhizoplane and rhizosphere soil bacterial populations with the F113*lacZY* treatment significantly increasing rhizoplane bacteria and the F113Rif (pCUGP) significantly reducing rhizosphere soil bacteria (Table 2). These results are therefore contradictory in nature, however the explanation is linked to an increase in root exudate/leakage with the inoculation of these strains (Naseby et al. 1999), which would support a greater rhizoplane population. The soil populations were not affected in the same way by the increased root exudate/leakage and therefore may be more strongly influenced by the anti-fungal metabolite production of strains F113*lacZY* and F113Rif (pCUGP).

The possibility that the reduced populations in the rhizosphere soil may be linked to the anti-fungal metabolite production by the Phl producing strains is supported by Short et al. (1990), who found that soil fungal populations were suppressed by a strain of *Pseudomonas putida* inoculated into soil. Mackie and Wheatley (1999) found that volatile organic compounds produced by various soil bacteria mediated effects in fungi, which appeared to be species-specific, with each fungus responding uniquely to the products of each of the bacterial cultures.

Other studies investigating the effect of Phl producers on soil or root microbial activities have found similar effects. Natsch et al. (1998) found that *P fluorescens* CHA0 and a Phl and Pyoluteorin over producing derivative had a similar but transient increase in the

metabolic activity of resident rhizoplane bacterial community. Furthermore, Brimecombe et al. (1998) found that the F113*lacZY* strain significantly reduced the soil microbial activity in the rhizosphere of pea. However, it must be noted that none of these studies have investigated the effect of Phl producers on rhizoplane and soil microbial populations concurrently.

Pseudomonas populations

It is conceivable that the inoculation of a micro-organism into the environment will cause the largest effects upon populations of a similar nature to the released strain, i.e. the indigenous *Pseudomonas* populations. Therefore, it was necessary to enumerate not only the introduced strains but also the indigenous population.

The F113G22 treatment resulted in a significantly greater indigenous fluorescent *Pseudomonas* population (Table 3) than the F113*lacZY* and F113Rif (pCUGP) treatments, whilst the control had an intermediate indigenous *Pseudomonas* population. This suggests that the F113G22 strain promoted the *Pseudomonas* indigenous population. However as the F113*lacZY* inocula resulted in a smaller indigenous *Pseudomonas* population than its Phl mutated derivative (F113G22) it is therefore directly attributable to the Phl production and caused by repression of the indigenous population by the anti-fungal metabolite.

From the total *Pseudomonas* populations (Table 3), which is the sum of the introduced and indigenous *Pseudomonas* populations, it is evident that none of the inocula displaced the indigenous populations. It is, therefore, plausible that the F113G22 inocula 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 smaller

indigenous *Pseudomonas* population with introduction of F113*lacZY* and F113Rif (pCUGP), 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* populations, which were similar to the control with the F113*lacZY* and F113Rif (pCUGP) treatments, and significantly ($p<0.05$) smaller than the F113G22 treatment. These results are similar to those of Natsch *et al.* (1997), who found that *P fluorescens* CHA0 and a Phl and pyoluteorin over-producing derivative had a similar but transient effect on the indigenous *Pseudomonas* population structure.

Soil enzyme activities

Soil enzyme activities have been used previously to assess the general functioning of biogeochemical cycling in the rhizosphere of inoculated plants (Naseby and Lynch, 1998b). The alkaline phosphatase activity (Table 4) was significantly greater with the inoculation of the F113*lacZY* and the Phl overproducing strain than the control, whilst the F113G22 treatment was intermediate in activity. The F113Rif (pCUGP) strain resulted in a significantly greater phosphodiesterase activity (Table 4) than the F113*lacZY* strain and both resulted in significantly greater activities than the control and F113G22 treatments. Therefore, the alkaline phosphatase and phosphodiesterase activities were increased by the presence of the Phl-producing strains whilst the non-producing strain did not significantly affect the activities with respect to the control.

Similar increased activities were found with sulphatase (Table 4) and urease (Table 5) activities with the inoculation of the two Phl producing strains. Therefore, the same mechanisms are involved with the aryl sulphatase and urease activities as those that influenced the alkaline phosphatase and phosphodiesterase activities.

In previous work (Naseby et al. 1999) a similar effect was found, where the inoculation of strain F113*lacZY* into the rhizosphere of pea caused an increase in P and S cycle enzyme activities associated with an increase in organic acid leakage from the roots. Increased available inorganic soluble phosphate is known to decrease soil phosphatase activity (Tabatabai, 1982 and Tadano et al., 1993). Therefore, Phl producing strains caused a decrease in the available N, P and S, as these nutrients became limiting due to an increase in available C, thus causing an overall increase in enzyme activity. Other studies have highlighted changes in root exudation caused by *Pseudomonas fluorescens* strains (Mozafar et al. 1992).

The trend found in the alkaline phosphatase activity was not repeated with the acid phosphatase activity (Table 4) which was not significantly affected by any of the inocula. 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, are 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 Phl producing treatments and would lose comparatively larger amounts of C, resulting in a reduced P demand.

Inverse trends were found with the C cycle enzymes (Table 5) in comparison to the general trend found in the N, P and S cycle enzymes (as were found by Naseby and Lynch 1998b). The two Phl producing strains resulted in significantly lower β-glucosidase and NAGase activities than the control whilst the F113G22 treatment was intermediate in activity. These carbon cycle enzyme activities, therefore, also indicate an increase in easily available carbon sources (Naseby et al. 1999), which would reduce the carbon demand and the production of enzymes for the release of carbon from more recalcitrant compounds.

The results therefore indicate not surprisingly that such functional genes in the genome of *Pseudomonas* strains have an impact on rhizosphere populations and function. However, most of differences in effect of the inocula, in comparison to the non-functionally modified F113*lacZY* strain, were found with the non-Phl producing F113G22 strain. This indicates that there would be an effect of introducing such anti-fungal metabolite genes into other non-producing strains. However, few differences in effect were found between the F113*lacZY* and the Phl overproducing strain. Therefore the actual functional modification for biocontrol purposes, in this experiment, had very little environmental effect in relation to the non-functionally modified strain.

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Table 1: Mean dry pea plant weights, the ratio between the two and root length as affected by Phl producing over producing and non producing *P. fluorescens* inoculation.

Plant#	Control*	F113G22*	F113*	F113Rif (pCUGP)*
Shoot wt (g)	0.069±0.002	0.071±0.001	0.070±0.002	0.073±0.002
Root wt (g)	0.086±0.004[a]	0.088±0.003[a]	0.102±0.006[b]	0.098±0.001[b]
S/R Ratio	0.813±0.046[b]	0.806±0.034[b]	0.699±0.039[a]	0.742±0.023[a]
Root length (cm)	20.50±1.82	20.70±0.38	20.02±1.24	21.50±2.08

*Treatments; control, no inocula; F113G22, inoculated with *lac*ZY marked Phl- (Tn*5* mutated) *P. fluorescens* F113G22; F113, inoculated with *lac*ZY marked Phl+ *P. fluorescens* F113; F113Rif (pCUGP), inoculated with *gusA* marked *P. fluorescens* F113R:pCUGP (Phl over producer).

S/R ratio: ratio of shoot weight to root weight.
Significant differences between treatments (within a row) at p=0.05 level indicated by different letters. Standard errors for means (n=7) indicated.

Table 2: Mean log soil and rhizoplane microbial populations as affected by Phl producing, over producing and non producing *P. fluorescens* inoculation.

Population#	Control*	F113G22*	F113*	F113Rif (pCUGP)*
Rhizoplane fungi	4.669±0.036[a]	4.641±0.030[a]	4.842±0.023[b]	4.812±0.047[b]
Rhizopshere fungi	4.632±0.060[c]	4.573±0.056[bc]	4.413±0.034[ab]	4.241±0.043[a]
Rhizoplane bacteria	7.762±0.047[a]	7.814±0.060[a]	7.942±0.040[b]	7.868±0.053[ab]
Rhizosphere bacteria	7.227±0.055[b]	7.227±0.073[b]	7.151±0.068[ab]	7.091±0.067[a]

*Treatments; control, no inocula; F113G22, inoculated with *lac*ZY marked Phl- (Tn*5* mutated) *P. fluorescens* F113G22; F113, inoculated with *lac*ZY marked Phl+ *P. fluorescens* F113; F113Rif (pCUGP), inoculated with *gusA* marked *P. fluorescens* F113R:pCUGP (Phl over producer).

Expressed as mean $\log^{10}$ c.f.u./g fresh root or soil.

Significant differences between treatments (within a row) at p=0.05 level indicated by different letters. Standard errors for means (n=7) indicated.

Table 3: Log fluorescent *Pseudomonas* populations in the rhizoplane of pea plants inoculated with Phl producing, over producing and non producing *P. fluorescens* strains.

Population#	Control*	F113G22*	F113*	F113Rif (pCUGP)*
Ind Pseu	6.38 ±0.024[ab]	6.59 ±0.051[b]	6.19 ±0.043[a]	6.14 ± 0.038[a]
Int Pseu	N/A	6.19 ±0.043	6.18 ±0.032	6.15 ± 0.028
Tot Pseu	6.38 ±0.024[a]	6.74 ±0.031[b]	6.49 ±0.028[a]	6.45 ± 0.026[a]

*Treatments; control, no inocula; F113G22, inoculated with *lac*ZY marked Phl- (Tn*5* mutated) *P. fluorescens* F113G22; F113, inoculated with *lac*ZY marked Phl+ *P. fluorescens* F113; F113Rif (pCUGP), inoculated with *gusA* marked *P. fluorescens* F113R:pCUGP (Phl over producer).

Expressed as mean $\log^{10}$ c.f.u./g fresh root. Ind pseu, indigenous *Pseudomonas;* int pseu, introduced *Pseudomonas;* tot pseu, total *Pseudomonas.*
N/A: not applicable as no genetically marked strains were introduced.

Significant differences between treatments (within a row) at p=0.05 level indicated by different letters. Standard errors for means (n=7) indicated.

Table 4: Soil enzyme activities in the rhizosphere of pea plants inoculated with Phl producing, overproducing and non-producing *Pseudomonas fluorescens* strain F113.

Enzyme#	Control*	F113G22*	F113*	F113Rif (pCUGP)*
Acid phosphatase	13.02±0.74	12.74±0.60	12.58±0.20	12.07±0.37
Alkaline phosphatase	7.56±0.56[a]	8.26±0.59[ab]	8.85±0.41[b]	8.86±0.26[b]
Sulphatase	0.35±0.03[a]	0.40±0.02[a]	0.47±0.03[b]	0.49±0.03[b]
Phosphodiesterase	1.09±0.11[a]	0.98±0.06[a]	1.29±0.11[b]	1.60±0.27[c]

Expressed as mg pNP released/g dry soil.

*Treatments; control, no inocula; F113G22, inoculated with *lac*ZY marked Phl- (Tn*5* mutated) *P. fluorescens* F113G22; F113, inoculated with *lac*ZY marked Phl+ *P. fluorescens* F113; F113Rif (pCUGP), inoculated with *gusA* marked *P. fluorescens* F113R:pCUGP (Phl over producer).

Significant differences between treatments (within a row) at p=0.05 level indicated by different letters. Standard errors for means (n=7) indicated.

Table 5: C and N cycle soil enzyme activities in the rhizosphere of pea plants inoculated with Phl producing, overproducing and non-producing *Pseudomonas fluorescens* strain F113.

Enzyme#	Control*	F113G22*	F113*	F113Rif (pCUGP)*
β-glucosidase	4.63 ± 0.39^{b}	4.00 ± 0.32^{ab}	3.86 ± 0.20^{a}	3.79 ± 0.17^{a}
N-acetyl glucosaminidase	2.12 ± 0.31^{b}	1.80 ± 0.20^{ab}	1.51 ± 0.07^{a}	1.55 ± 0.08^{a}
Acid β galactosidase	1.80 ± 0.18	1.55 ± 0.10	1.49 ± 0.11	1.59 ± 0.09
Urease	37.78 ± 1.65^{a}	40.34 ± 2.32^{ab}	44.16 ± 2.54^{b}	45.21 ± 2.91^{b}

Expressed as mg pNP released/g dry soil.

*Treatments; control, no inocula; F113G22, inoculated with *lac*ZY marked Phl- (Tn*5* mutated) *P. fluorescens* F113G22; F113, inoculated with *lac*ZY marked Phl+ *P. fluorescens* F113; F113Rif (pCUGP), inoculated with *gusA* marked *P. fluorescens* F113R:pCUGP (Phl over producer).

Significant differences between treatments (within a row) at p=0.05 level indicated by different letters. Standard errors for means (n=7) indicated.