Effects of *Pseudomonas fluorescens* F113 on ecological functions in the rhizosphere of pea is dependent on pH.

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Abstract

The aim of this microcosm study was to determine influence of the antibiotic 2,4 diacetylphloroglucinol (DAPG) on the effect of wild type and functionally modified *Pseudomonas fluorescens* F113 strains in a sandy loam soil of pH 5.4 planted with pea (*Pisum sativum* var Montana). The functional modification of strain F113 was a repressed production of DAPG, useful in plant disease control, creating the DAPG negative strain F113 G22 both were marked with a *lacZY* gene cassette. Lowering the soil pH to 4.4 significantly reduced the plant shoot and root weights and the root length, whereas, the bacterial inocula had no significant effect. However, both inocula significantly reduced the shoot/root ratio at pH 5.4 but this effect was not evident at the lowered or elevated (6.4) pH levels. The decrease in pH significantly increased the fungal and yeast colony forming units from the rhizosphere (root extract) but did not affect the total bacterial cfu's. Inoculation with strain F113 in the pH 4.4 soil resulted in a significantly greater total bacterial population The fungal and yeast cfu's were not significantly affected by the inocula under any of the pH's studied Increasing the pH significantly increased the indigenous *Pseudomonas* population in comparison to the reduced pH treatment and significantly increased both the introduced and total *Pseudomonas* populations. The antibiotic producing strain significantly reduced the total bacterial population and the NAGase activity (related to fungal activity) at pH 6.4 where the inocula population was the greatest. Alkaline phosphatase, phosphodiesterase, aryl sulphatase, β glucosidase, alkaline β galactosidase and NAGase activities significantly increased with increasing in pH. The F113 inocula reduced the acid phosphatase activity at pH 5.4 and increased the acid β galactosidase activity over all the pH treatments. The results presented illustrate the variation in impact with soil pH, with implications for variability in efficacy of *Pseudomonas fluorescens* biocontrol agents with soil pH.

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 [25]. Therefore a more comprehensive knowledge of the consequences of such releases on the rhizosphere must be provided before they can be utilised safely [34].

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 have been reported [6], however, differences between the two respective inoculants were not found. Other authors working on indigenous populations and ecosystem function in contained experimental systems also found such transient perturbations [30, 35, 38]. Perturbations have been recorded by several authors with the introduction of functionally modified GMMs, including displacement of indigenous populations [3]; suppression of fungal populations [33] reduced protozoa populations [1] and increased carbon turnover [37]. However, methods requiring microbial growth can be hampered by the non-culturability of many micro-organisms [5]. Molecular genetic methods are useful tools for assessing the ecology and population genetics of targeted microbial populations or communities [21, 23, 36], 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 to assess perturbations caused by the introduction of a *Flavobacterium* species [19], and with the inoculation of a *Pseudomonas fluorescens* strain

[26]. However, such perturbations in non-rhizosphere soil with the addition of an *Escherichia coli* strain were not found [7] and an effect was not found with addition of a GMM on either nitrogen transformations or populations of nitrogen transforming micro-organisms [11]. However both these works 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 a functionally modified *Pseudomonas fluorescens* strain in the rhizosphere of pea. The functional gene in the *P. fluorescens* strain F113 encoded the production of the antibiotic 2,4-diacetylphloroglucinol, useful in the control of damping off disease [9] and DAPG has isolated from soil [31]. 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 [32] and, indeed, it has shown that the wild type has an inhibitory effect *in vitro* on both bacteria and fungi. DAPG production has also been shown in the rhizosphere of microcosm grown plants [13, 18].

The wild type DAPG producing strain was previously released in field experiments [22] where natural field variation was found to be a more important factor influencing soil enzyme activities than the application of the biocontrol agent [29]. Siderophores as putative biocontrol metabolites for *Pseudomonas* spp. are strongly dependent on pH [16]. The effectiveness of a *Pseudomonas fluorescens* strain as a biological control agent of *Fusarium* wilt was found to be highly pH dependent [8], therefore, it is necessary to understand the dynamics and impact of such biocontrol inocula under varying pH's. Strain F113 was compared to its genetically modified non antibiotic-producing derivative in large numbers

in the pea rhizosphere to assess the impact of the inoculum and antibiotic production under varying soil pH conditions.

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. The pH of the soil was reduced one pH unit by the addition of 0.1 M HCl or increased one pH unit by the addition of 0.1M $NaCO_3$, until the desired pH, in 1:2 water, of samples of the soil was reached. The soil was allowed to equilibrate for 10 days prior to the experiment under the environmental conditions for the experiment described below. The pH of samples of the soil were checked every two days and amended accordingly. A total of 1.9 ml of 0.1M Na_2 CO3 and 4.4 ml 0.1M HCL were required, per 250g of soil, for the pH adjustments. Upon extraction from the soil, the availability of Na and Cl (water extract) did not significantly differ, and only the carbonate extractable Cl increased slightly with the addition of the HCl. Therefore, the direct effect of sodium and chloride was thought to be limited in this experiment.

Microcosm

Coarsely sieved (6 mm) loose soil (250 g) was placed in experimental microcosms [27], 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 F113 that 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 Tn*5* mutagenisis [32].

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

Nine treatments were applied consisting of pea seed inoculated with the two bacterial strains described above with a non inoculated control planted in soils of the three different pH's described above. 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 (including the water used in the pH adjustments) 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% and the light intensity was 10,000 lux at shelf level.

Sampling and analysis

After 17 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 (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1), phosphodiesterase (EC 3.1.4), aryl sulphatase (3.1.6.1), β glucosidase (3.2.1.21), β galactosidase (3.2.1.23) measured at acid and alkaline pH's and N-acetyl glucosaminidase (EC 3.2.1.52) by the methods of Naseby and Lynch [26].

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 [12] 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 were incubated for 7 days at 25^{o}C.

Statistical analysis

Data was analysed using the statistical software 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 with a p value of 0.05.

Results and discussion

Plant growth

Plant growth measurements (Table 1) were used to assess the potential impact of the different inocula on crop productivity. Lowering the soil pH significantly reduced the plant shoot and root weights and the mean root length with respect to the control and the increase in pH, whereas, the bacterial inocula did not significantly affect these plant measurements under any of the pH treatments. As neither shoot nor root weights showed significant differences between the inocula yet both the F113 (DAPG+) and G22 inocula resulted in smaller shoot weight and greater root weights at pH 5.4, the results were converted into shoot to root ratio (figure 1), to take both shoot and root weights into account together. The conversion into shoot/root ratio has been used extensively in the past [2,4] and has been used as an indicator of plant growth responses to *Azospirillium* inoculation [2]. Shoot/root ratios have also 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 [5]. 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. Both inocula significantly reduced the shoot/root ratio at pH 5.4 but this effect was not evident when the pH was reduced or increased. As the two bacterial inocula resulted in a significantly smaller shoot/root ratio than the control, it can be deduced that both these strains have attributes that cause stress in pea plants, at pH 5.4, such as causing increased C leakage from the root.

Microbial populations

The decrease in pH significantly increased the fungal and yeast colony forming units from the rhizosphere (root extract) but did not significantly affect the total bacterial cfu's (Table 2). Increasing the pH did not significantly affect any of these populations but significantly increased the populations of both the inocula. Inoculation with strain F113 in the pH 4.4 soil resulted in a significantly greater total bacterial population in comparison to the low pH control and the equivalent F113 G22 inocula however this effect was not evident under the two higher pH's. The fungal and yeast populations were not significantly affected by the either of the inocula under any of the pH's studied. Therefore the *in vitro* effects previously reported [32], whereby the F113, (DAPG+) inhibited the fungi and the non-producer did not, were not apparent *in vivo* in the fungal cfu's in this study. This is also contrary to previous work where it was found that fungal populations were suppressed by a strain of *Pseudomonas putida* inoculated into soil [33].

The microbial populations of the rhizosphere soil (Table 3) were affected by the changes in soil pH to a greater extent. The total bacterial and yeast populations were significantly greater pH 6.4 than the two lower pH's, whereas the fungal populations increased when the pH was both reduced to 4.4 or increased to 6.4. This is a logical progression as fungi prefer more acidic and bacteria less acidic conditions and reflects previous results where the number of culturable bacteria decreased sharply under acid cultivation conditions whilst the number of culturable fungi remained relatively constant over a pH range of 2.2-6.5 [17]. Increasing the pH significantly increased the indigenous *Pseudomonas* population in comparison to the reduced pH treatment and significantly increased both the introduced and total *Pseudomonas* populations in comparison to the control and the reduced pH treatments.

The bacterial inocula had varying effects at the different pH's, the antibiotic producing strain significantly reduced the total rhizosphere soil bacterial population at pH 6.4 in comparison to the control and the non-antibiotic producing inocula. Whereas, the same inocula significantly increased the total bacterial population at pH 5.4, but did not have a significant effect with the decreased pH. DAPG production by *Pseudomonas fluorescens* F113 was found not to be affected by pH [32]. However, the greater population of the F113 strain (by over a log unit) found on the root at pH 6.4 is thus likely to lead to a greater total production of DAPG in the rhizosphere and therefore, the effect upon the bacterial population would be greater. This then indicates that larger populations of F113 had a direct inhibitory effect upon the bacterial population related to the production of DAPG. Neither of the inocula had a significant effect upon the culturable fungal or yeast populations under any of the pH treatments and any of the *Pseudomonas* populations at pH 5.4 and 4.4. However, the F113 G22 inocula resulted in greater indigenous and total *Pseudomonas* populations than the control and F113 treatments at pH 6.4. The populations of the two *Pseudomonas* inocula were not significantly different from each other over the different pH's, which is in contrast to the results of Hartel *et al*. [10] who found that in soil of pH5 a wild type strain of *Pseudomonas putida* survival was 100 times greater than a genetically modified derivative after 28 days. The effect of a genetically modified derivative is highly dependent upon the nature of the modification [28] and the effect of the modification and site of insert upon the organism. Therefore, it is not surprising that the effects found in this experiment differ from those found by Hartel *et al*.[10].

Soil enzyme activities

The activities of all the P and S cycle enzymes (Figure 2) significantly increased with the increase in soil pH and the alkaline phosphatase, phosphodiesterase and the aryl sulphatase

activities significantly decreased with the reduction in soil pH. The only significant effect of the inocula was a reduction in the acid phosphatase activity with the F113 treatment at the control pH but this did not significantly differ from the G22 treatment. The C cycle enzyme activities (Figure 3) also varied with the changes in soil pH. β glucosidase, alkaline β galactosidase and NAGase activities significantly increased with the increase in pH, whereas, the acid β galactosidase activity did not. Only the β glucosidase activity significantly increased with the decrease in pH, whilst, the alkaline β galactosidase activity decreased. The acid β galactosidase activity significantly increased with the inoculation of strain F113 with all the pH treatments. This is not related to the β galactosidase activity introduced into the organism on a lacZY gene cassette, as it is *E coli* derived and therefore has an alkaline pH optimum. The β galactosidase activity is therefore derived from the plant and/or associated fungi, and as carbon leakage from the plant root with the F113 and other *Pseudomonas* inocula, has been shown previously [24, 28], it is therefore likely that the increased enzyme activity is of plant origin [14].

The NAGase activity was significantly greater in the control than with the two inocula at pH 5.4 bit not at the other pH treatments. As N-acetyl glucosaminidase (NAGase) activity has been shown correlate to fungal biomass [20] it follows that the fungal biomass increased with the increase in pH. It also follows that the fungal biomass was significantly reduced by both the inocula at pH 5.4 and was therefore independent of the antibiotic production. At pH 6.4 only the antibiotic producing strain significantly reduced the NAGase activity which is linked with an increased population of the inocula and is also dependent upon the production of DAPG. Similar effects upon NAGase activity with the F113 inocula have been found previously [28]. The reduction in the NAGase activity, which is an indicator of active fungal biomass [20], was not reflected in the fungal colony

forming units. However, this is not surprising, as fungal cfu's do not necessarily correspond with the active fungal biomass, indeed, in some cases they have been found to be inversely correlated [15]. This is primarily due to spore production by fungi, which do not contribute to the active fungal biomass but do form colonies on plates. Spore production increases with stressed conditions whilst the active fungal biomass decreases.

The results presented illustrate the variability of impact, with implications for possible variability in efficacy of *Pseudomonas fluorescens* biocontrol agents with soil physiochemical properties. Such biocontrol agents will be at their most effective at higher pH's and are more likely to be ineffective at low pH's at which fungal activity will be at its most predominant. It is clear that methods to measure fungal populations and activity other than colony forming units are required to establish the impact of biocontrol agents upon fungal populations. The measurement of NAGase activity is a promising alternative to the laboured conventional methods of measuring fungal biomass, like chitin measurement and ergosterol.

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Table 1. Pea growth as affected by pH with the inoculation of wild type and genetically modified *P. fluorescens*.

	pH 4.4			pH 5.4			pH 6.4		
Plant	Cont	G22	F113	Cont	G22	F113	Cont	G22	F113
Shoot wt (g)	0.75^{ab}	0.79^{abc}	0.72^{a}	0.87^{c}	0.81^{bc}	0.80^{bc}	0.84^{c}	0.85^{c}	0.78^{abc}
Root wt (g)	0.94^{ab}	1.01^{abc}	0.90^{a}	1.04^{bcd}	1.11^{cd}	1.12^{cd}	1.10^{cd}	1.14^{d}	1.11^{cd}
Root Length (cm)	14.6^{a}	14.13^{a}	14.30^{a}	17.67^{b}	18.48^{bc}	19.50^{c}	19.46^{c}	20.20^{cd}	21.80^{d}

Shoot wt, mean shoot weight; root wt, mean root weight
Cont, non inoculated control; G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.
Significant differences between treatments at p=0.05 level indicated by different letters.

Table 2. Log microbial populations from pea root extracts as affected by pH with the inoculation of wild type and genetically modified *P. fluorescens*.

	pH 4.4			pH 5.4			pH 6.4		
Microbe	Cont	G22	F113	Cont	G22	F113	Cont	G22	F113
Tot bact	7.45^{a}	7.43^{a}	7.61^{b}	7.41^{a}	7.45^{a}	7.45^{a}	7.37^{a}	7.28^{a}	7.38^{a}
Tot fungi	4.88^{ab}	4.58^{ab}	4.93^{b}	4.73^{ab}	4.53^{ab}	4.37^{ab}	4.38^{ab}	4.26^{a}	4.66^{ab}
Tot yeast	4.78^{ab}	4.83^{ab}	4.62^{ab}	4.26^{ab}	4.00^{a}	4.30^{ab}	4.56^{ab}	4.35^{ab}	4.92^{b}
Ind Pseu	6.41^{ab}	6.30^{a}	6.56^{ab}	6.62^{abc}	6.62^{abc}	6.77^{abc}	6.88^{bc}	6.94^{c}	6.88^{bc}
Int Pseu	N/A	6.00^{a}	6.30^{ab}	N/A	5.78^{a}	5.5^{a}	N/A	7.00^{b}	6.72^{b}
Tot Pseu	6.41^{a}	6.47^{ab}	6.72^{bc}	6.62^{ab}	6.68^{b}	6.79^{bc}	6.88^{cd}	7.27^{e}	7.08^{de}

Microbial populations expressed as c.f.u./g fresh root. N/A: not applicable as no genetically marked strains were introduced.

Tot bact, total bacteria; tot fungi, total fungi; tot yeast, total yeast; ind Pseu, indigenous *Pseudomonas*; int Pseu, introduced *Pseudomonas;* tot Pseu, *total Pseudomonas*
Cont, non inoculated control; G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.
Significant differences between treatments at p=0.05 level indicated by different letters.

Table 3. Log microbial populations from pea rhizosphere soil as affected by pH with the inoculation of wild type and genetically modified *P. fluorescens*.

	pH 4.4			pH 5.4			pH 6.4		
Microbe	Cont	G22	F113	Cont	G22	F113	Cont	G22	F113
Tot bact	7.54^{a}	7.58^{a}	7.64^{ab}	7.58^{a}	7.51^{a}	7.74^{bc}	7.82^{c}	7.78^{c}	7.64^{ab}
Tot fungi	5.95^{cd}	5.79^{abc}	6.03^{cd}	5.26^{ab}	4.30^{a}	4.78^{a}	5.95^{cd}	6.13^{d}	5.88^{bcd}
Tot yeast	5.34^{ab}	5.38^{ab}	5.72^{b}	5.3^{a}	5.24^{a}	5.41^{ab}	5.62^{b}	5.80^{b}	5.62^{b}
Ind Pseu	6.20^{a}	6.30^{ab}	6.08^{a}	6.68^{b}	6.41^{ab}	6.53^{ab}	6.62^{ab}	6.91^{c}	6.62^{ab}
Int Pseu	N/A	6.11^{a}	6.38^{ab}	N/A	5.60^{a}	6.38^{ab}	N/A	6.90^{b}	6.58^{ab}
Tot Pseu	6.20^{a}	6.52^{a}	6.56^{ab}	6.68^{ab}	6.48^{a}	6.76^{ab}	6.62^{ab}	7.21^{c}	6.90^{b}

Microbial populations expressed as c.f.u./g soil. N/A: not applicable as no genetically marked strains were introduced.

Tot bact, total bacteria; tot fungi, total fungi; tot yeast, total yeast; ind Pseu, indigenous *Pseudomonas*; int Pseu, introduced *Pseudomonas;* tot Pseu, *total Pseudomonas*
Cont, non inoculated control; G22, inoculated with *lac*ZY marked DAPG- (Tn*5* mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.
Significant differences between treatments at p=0.05 level indicated by different letters.

Figure 1: Ratio between plant shoot and root weights as affected by soil pH with the inoculation of wild type and genetically modified *Ps. fluorescens*.

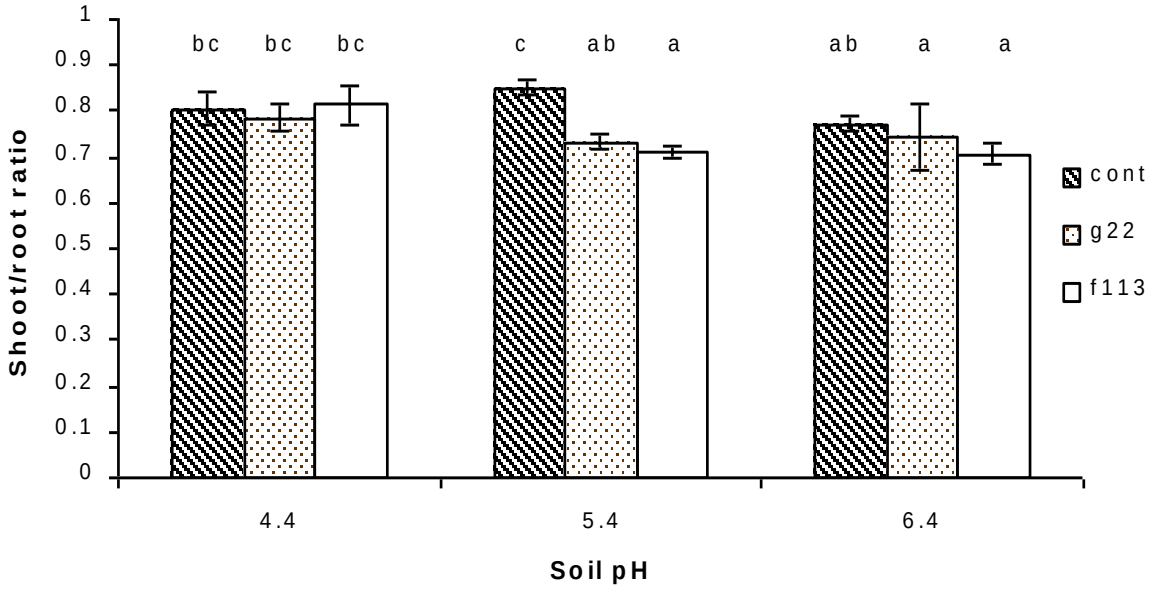
Cont, non inoculated control; G22, inoculated with *lac*ZY marked DAPG- (Tn5 mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.
Standard errors of means shown (n=5). Significant differences between treatments at p=0.05 level indicated by different letters.

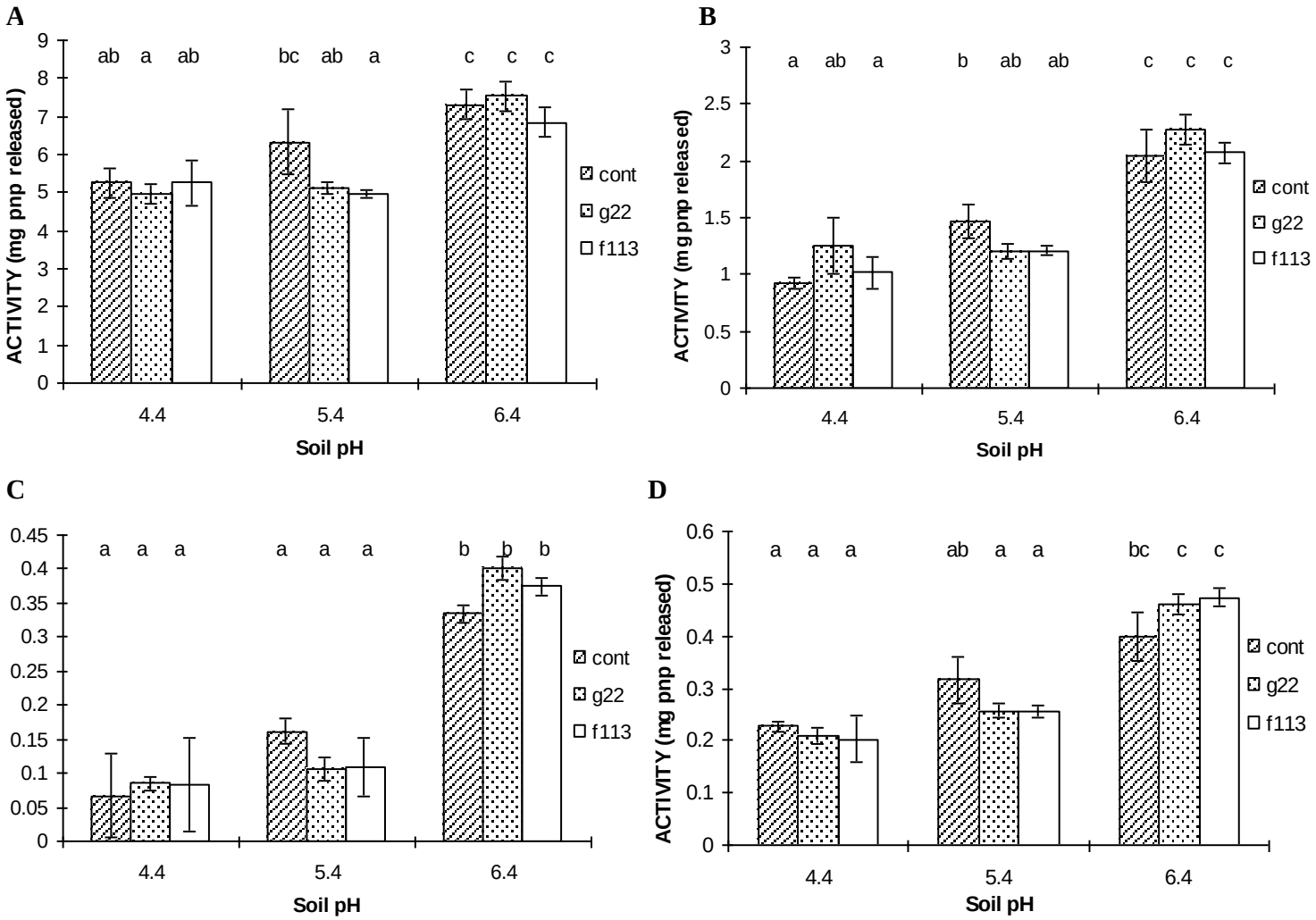
Figure 2: Phosphorus and sulphur cycle enzyme activities in the pea rhizosphere as affected by pH with the inoculation of wild type and genetically modified *P. fluorescens*.
A) acid phosphatase B) alkaline phosphatase, C) phosphodiesterase, D) aryl sulphatase

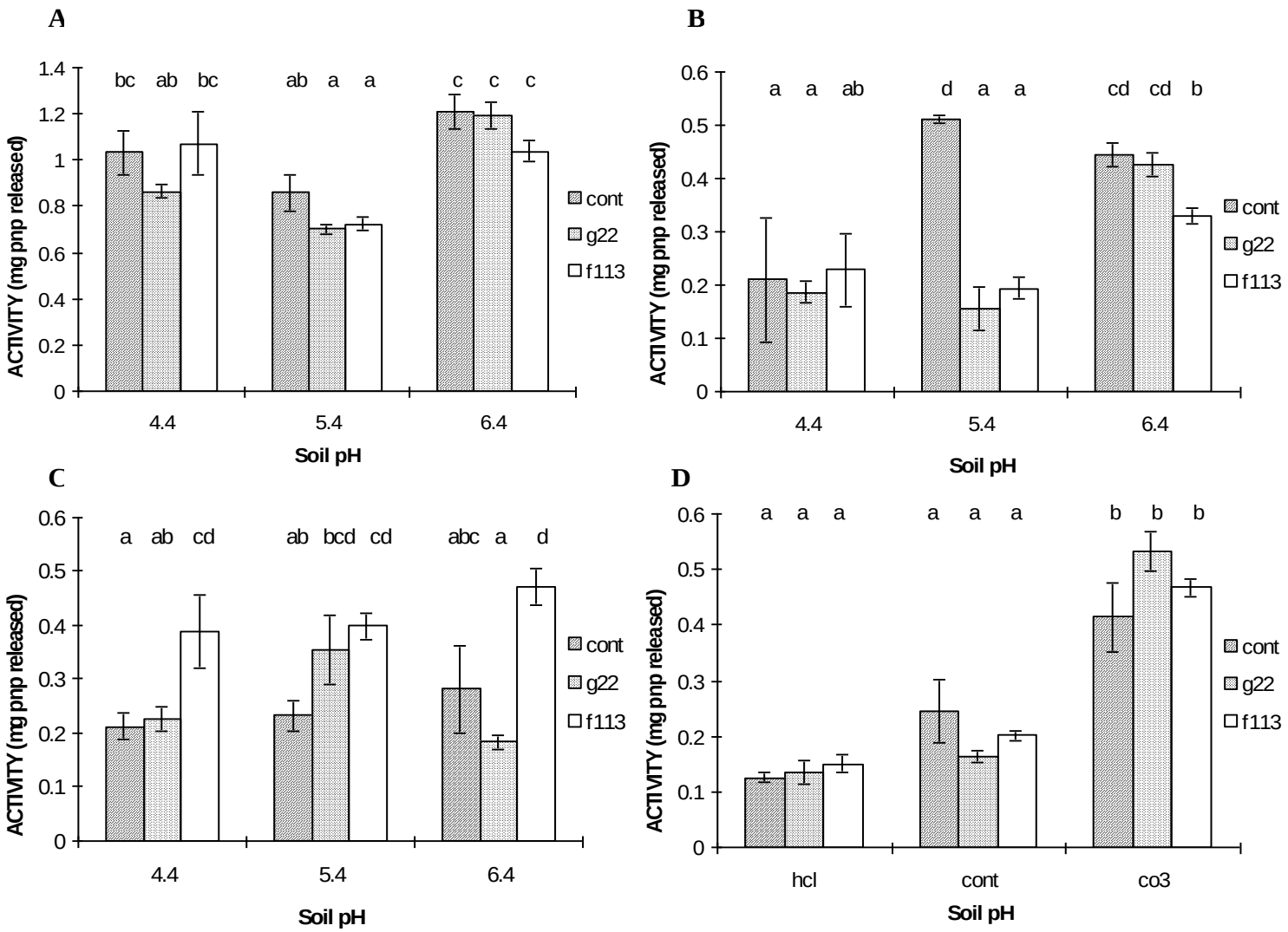
Cont, non inoculated control; G22, inoculated with *lac*ZY marked DAPG- (Tn5 mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.
Standard errors of means shown (n=5). Significant differences between treatments at p=0.05 level indicated by different letters.

Figure 3: Carbon cycle enzyme activities in the pea rhizosphere as affected by pH with the inoculation of wild type and genetically modified *P. fluorescens*.
A) β glucosidase B) N-acetyl glucosaminidase, C) acid β galactosidase, D) alkaline β galactosidase

Cont, non inoculated control; G22, inoculated with *lac*ZY marked DAPG- (Tn5 mutated) *P. fluorescens* F113 G22; F113, inoculated with *lac*ZY marked DAPG+ *P. fluorescens* F113.
Standard errors of means shown (n=5). Significant differences between treatments at p=0.05 level indicated by different letters.


Shoot/root ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
bc bc bc
c ab a
ab a a
4.4
5.4
6.4
Soil pH
cont
g22
f113


A
ab a ab bc ab a c c c
ACTIVITY (mg pnp released)
0 1 2 3 4 5 6 7 8 9
cont
g22
f113
4.4 5.4 6.4
Soil pH
B
a ab a b ab ab c c c
ACTIVITY (mg pnp released)
0 0.5 1 1.5 2 2.5 3
cont
g22
f113
4.4 5.4 6.4
Soil pH
C
a a a a a a b b b
ACTIVITY (mg pnp released)
0 0.05 0.1 0.15 0.2 0.25 0.3 0.35 0.4 0.45
cont
g22
f113
4.4 5.4 6.4
Soil pH
D
a a a ab a a bc c c
ACTIVITY (mg pnp released)
0 0.1 0.2 0.3 0.4 0.5 0.6
cont
g22
f113
4.4 5.4 6.4
Soil pH


A
bc ab bc ab a a c c c
ACTIVITY (mg pnp released)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
4.4
5.4
6.4
Soil pH
cont
g22
f113
B
a a ab d a a cd cd b
ACTIVITY (mg pnp released)
0.6
0.5
0.4
0.3
0.2
0.1
0
4.4
5.4
6.4
Soil pH
cont
g22
f113
C
a ab cd ab bcd cd abc a d
ACTIVITY (mg pnp released)
0.6
0.5
0.4
0.3
0.2
0.1
0
4.4
5.4
6.4
Soil pH
cont
g22
f113
D
a a a a a a b b b
ACTIVITY (mg pnp released)
0.6
0.5
0.4
0.3
0.2
0.1
0
hcl
cont
co3
Soil pH
cont
g22
f113