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Influence of inoculum density of the antagonistic bacteria *Pseudomonas fluorescens* and *Pseudomonas corrugata* on sugar beet seedling colonisation and suppression of Pythium damping off

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Abstract

The effect of initial inoculum density of the antagonistic bacterial strains *Pseudomonas fluorescens* B5 and *Pseudomonas corrugata* 2140 (10^3 to 10^8 CFU per seed pellet) on sugar beet seedling colonisation, *in situ* bioluminescence and antagonistic activity towards *Pythium ultimum* was investigated. Populations of the bacteria colonising sugar beet root systems approached an apparent carrying capacity of 10^5 to 10^6 CFU per plant after 12 d growth, irrespective of inoculum density. This meant an up to 320-fold population increase at low inoculum densities and a decrease at high densities. Population densities of both bacteria and their corresponding *in situ* bioluminescence (resulting from luciferase enzyme expression from the inserted *lux*AB genes) reached highest levels in the hypocotyl region and in the upper root region 0–20 mm below seed level (10^4–10^6 CFU/cm section, 10^1–10^3 RLU/cm section) and decreased with root depth. *In situ* bioluminescence, which indicates physiological activity, was measurable at lowest antagonist initial inoculum density (10^3 CFU per seed pellet) and did not increase significantly with increasing inoculum density. Bioluminescence was also significantly correlated with population density. For *Pseudomonas fluorescens* B5, the total population size per plant and downward colonisation of the root (below 40 mm depth) increased significantly with antagonist inoculum density applied to the seeds. For *Pseudomonas corrugata* 2140, no significant influence of initial inoculum density on root colonisation was observable. Survival and dry weight of sugar beet seedlings in *Pythium* infested soil increased significantly with increasing inoculum density of *Pseudomonas fluorescens* B5, whereas for *Pseudomonas corrugata* 2140, initial densities of 10^4 to 10^6 CFU per seed resulted in maximal survival of plants.

Abbreviations: CFU – colony forming units; RLU – relative luminescence units.

Introduction

Damping-off of sugar beet by *Pythium spp.* is a soil-borne disease of major economic significance (Martin and Loper, 1999). Although damping-off is controlled currently by the addition of fungicides to the seed coat, biocontrol agents, especially bacteria of the genera *Pseudomonas* and *Bacillus*, may be an ecologically sound alternative to chemical seed treatment (Suslow and Schroth, 1982; Martin and Loper, 1999). Seed treatment with biocontrol agents is an economically viable option of disease control on high value crops like sugar beet. Seed treatment ensures that the biocontrol agent is in close proximity to the sites of pathogen entry, in the seed and the emerging seedling. In such a targeted application, much less antagonist

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inoculum is needed than for soil treatment, which will reduce crop production costs. Furthermore, sugar beet seed is contained within a wrinkled pericarp that has to be pelleted to permit use of a seed drill. Consequently, biocontrol agents can easily be added in substantial numbers in the pelleting process.

As with chemical control, the correct dose (inoculum density) of a biocontrol agent is of crucial importance for disease control. An insufficient dose may result in biocontrol activity that is too low, especially at high pathogen density (Berger et al., 1996). On the other hand, antagonist inoculum density should be kept to a minimum for economic reasons; too high doses might also have phytotoxic effects (Maurhofer et al. 1992; Raaijmakers et al., 1999). Consequently, studies of dose-response relationships have been carried out in a number of biocontrol systems (Montesinos and Bonaterra, 1996; Frey-Klett et al., 1999; Larkin and Fravel, 1999; Bull et al., 1991) and mathematical models have been developed to decribe dose-response-relationships for biological control agents (Johnson, 1994; Montesinos and Bonaterra, 1996). In addition, studies focused on rhizosphere colonisation by the biocontrol agent as a function of initial dose (Loper et al., 1985; Bull et al., 1991; Ramos et al., 2000), as subsequent colonisation of the infection sites (i.e., the roots and hypocotyl of the emerging seedling) is a necessary prerequisite for biocontrol. Raaijmakers et al. (1999) found a linear correlation between the density of the rhizosphere population of *Pseudomonas fluorescens* Q2-87 and *in situ*-concentration of 2,4-diacetylphloroglucinol, an antibiotic produced by the bacterium that is active against *Pythium ultimum*. In sugar beet, Fukui et al. (1994a, b) demonstrated the importance of high initial pericarp colonisation by *Pseudomonas fluorescens* for antagonism against *Pythium ultimum*; however, comparisons between strains, rather than dose response relationships, were the main focus of their studies.

The metabolic activity of an antagonist and not its mere presence at the infection sites is considered an essential prerequisite for biological control (Sørensen et al., 1994; White et al., 1996). However, the relationship between metabolic activity, soil population density and the efficacy of biological control has rarely been studied. The introduction of *lux* genes coupled to measurement of resulting bioluminescence has represented a particular useful and sensitive marker system for the determination of metabolic activity in soil (Meikle et al., 1992; White et al., 1996; Killham and Yeomans, 2001), but to our knowledge, there is only one study (Ramos et al., 2000) where this technique was employed to investigate the effects of initial antagonist dose (as seed inoculum on barley) on rhizosphere colonisation.

In the following study, different inoculum densities of antagonists were incorporated into a commercial pellet formulation of sugar beet seeds (KWS SAAT AG, Einbeck, Germany). This enabled us to investigate the effect of inoculum density of the antagonistic bacteria *Pseudomonas fluorescens* B5 and *Pseudomonas corrugata* 2140 on root colonisation and distribution of these antagonists along the root. It also enabled us to study the physiological activity *in situ* (light emission resulting from luciferase enzyme expression from the inserted *lux*AB genes) and assessment of antagonistic activity against *Pythium ultimum* at different inoculum densities.

Materials and methods

Strains and seeds used

Pseudomonas fluorescens B5 (Heupel, 1992) was provided from the research group of Prof. G.A. Wolf, Institute of Plant Pathology and Plant Protection at the University of Göttingen, Germany. *Pseudomonas corrugata* 2140 was isolated by Ryder and Rovira (1993) and was obtained from CSIRO Land and Water Division, Australia. Antagonistic properties of *P. fluorescens* B5 against *Pythium ultimum* had been demonstrated in glasshouse and field experiments (Heupel 1992). *Pseudomonas corrugata* 2140 has proved to be effective against take all disease (*Gaeumannomyces graminis*) in greenhouse trials and against *Rhizoctonia solani in vitro* (Ryder and Rovira, 1993). The *Pythium ultimum* isolate was obtained from the culture collection of the Department for Plant and Soil Science, University of Aberdeen. For root colonisation studies, the *Pseudomonas* strains were chromosomally marked using a mini-Tn5 transposon with a *lux*AB gene cassette from *Vibrio fischerii* and a tetracycline resistance gene (strains B5L9, 2140*lux*) according to the protocol described by de Lorenzo et al. (1990). The *lux*-marked derivatives did not differ in their *in vitro* growth characteristics from their wild type parent strains and bioluminescence was significantly correlated with dehydrogenase activity in *in-vitro* cultures (Knox, 2000; Russell, 1996).

Pelleted sugar beet seeds (*Beta vulgaris* L.) of the cultivar Samantha (grey pill; without addition of the fi-

nal colour layer and without fungicides) were provided by KWS SAAT AG (Einbeck, Germany).

Microcosm set-up

Microcosms consisted of rectangular PVC columns (made from electric cable conduit) with a perspex plate fitted to their base. The front panel was removable for recovery of roots. They were 29.8 cm high and 7×7 cm wide. The soil column height was 25 cm. A one-cm thick layer of gravel was packed at the bottom of each microcosm to facilitate drainage and soil aeration. Sieved (< 3.25 mm) sandy loam topsoil from Craibstone (Aberdeenshire, Scotland) was wetted to a gravimetric water content of 0.32 g g^{-1} which corresponded to a matric potential of -10 kPa and packed to a bulk density of 1.0 g cm^{-3}. A uniform bulk density was achieved by packing the required quantity of soil in five 4 cm thick layers followed by 3 and 2 cm at the top. In assays on antagonistic activity against *Pythium ultimum*, but not in assays on bacterial root colonisation, *Pythium ultimum* inoculum was added to the top 9 cm of soil as described below. In each microcosm, 16 sugar beet seeds were sown at a depth of 2 cm. The soil surface was covered with a 2 cm thick layer of white plastic beads (50 g) to minimise warming of the soil through light irradiation. Thermistors and ceramic tensiometer tubes (5 mm diameter) were inserted horizontally during packing. One microcosm per treatment (dose) was set up in assays on root colonisation and *in situ* activity; two replicate microcosms per treatment were set up in assays on biological control of *Pythium ultimum*.

The microcosms were incubated in a growth chamber (Fitotron SGC066.PPX.F, Sanyo Gallenkamp plc, Loughborough, UK) for 12 days in assays on bacterial root colonization. At this stage, the cotyledons were fully developed and the first true leaves reached 1–3 mm length. In biocontrol assays, the growth period was prolonged to 14 days to allow more time for disease development. Relative humidity was 85%, irradiance (measured as photosynthetic active radiation at the surface of the bead layer) was 309–510 μmol m^{-2} s^{-1}, and the day/night cycle was 12 h/12 h. Air temperature at night was set at 14.5 °C, and reduced to 11.5 °C during the daytime to counteract warming of the soil through radiation. The soil temperatures measured were 16 °C (night) to 20 °C (day). The volumetric water content of the soil was monitored at 3.2, 11.5 and 19.5 cm soil depth with thetaprobes type ML1 and a DL3000 data logger (Delta T-Devices, Burwell Cambridge, UK). Matric potential and temperature were monitored at 2.0 cm soil depth (=seed depth) and 6.2 cm soil depth using SKT 600S/I tensiometers and SKT 200U/I temperature sensors attached to Skye DataHog 1 and 2 data loggers, respectively (Skye Instruments, Powys, UK). Columns were watered regularly by injection of water with a syringe through rubber stoppers (Suba-Seal, Fisher Scientific Ltd., Loughborough, UK) at 5 different soil depths (2.3, 6.2, 10.5, 14.2, and 18.2 cm) through either side of the soil column. The amounts of water required to maintain a constant water content were calculated using the theta probe readings. With this method, a matric potential of -5 to -19 kPa was maintained during the incubation period.

Seed treatment

Pseudomonas fluorescens B5 and *Pseudomonas corrugata* 2140 were cultured overnight (150 mL, 24 °C, 200 rpm) in Luria Bertani medium (10 g L^{-1} Yeast extract, 10 g L^{-1} tryptone, both from Oxoid Limited, Basingstoke, Hampshire, UK, 5 g L^{-1} NaCl). When *lux*-marked strains were cultured, the appropriate antibiotics were added to the medium (50 ppm tetracycline for *Pseudomonas fluorescens* B5, 12.5 ppm tetracycline for *Pseudomonas corrugata* 2140). The cultures were centrifuged (2710 g, 20 min, 4 °C). Cells were resuspended in 30 mL of sterile $\frac{1}{4}$ Ringers solution (Oxoid Limited, Basingstoke, Hampshire, UK). Pelleted sugar beet seeds were soaked in this concentrated suspension or serial dilutions of it for 15 min. To determine the resulting inoculum density per seed 10 pellets of each treatment were suspended in 5 mL $\frac{1}{4}\times$ Ringers solution and serial dilutions of this suspension were plated as described below. The seed treatment resulted in bacterial inoculum densities in the range of 10^3 to 10^8 CFU/seed pellet.

Pythium *inoculation in antagonist assays*

Pea seeds (marrowfat peas, Great Scot, Scotia Haven Foods Ltd., Warrington, UK) were pre-germinated in water for 24 h and then homogenized for 30–60 s in a blender. Thirty-five grams of homogenised peas were then spread into a 9-cm-diameter glass Petri dish. The Petri dishes were autoclaved 3 times for 20 min at 121 °C. They were inoculated using three agar plugs of 3 d old corn meal agar cultures of *Pythium ultimum* and incubated at 25 °C for 4 d. Inoculated pea mash was crumbled and mixed gently but thoroughly

into the soil which was adjusted to a gravimetric water content of 0.32 g g^{-1}. The end concentration of inoculated pea mash was 2.5% related to wet soil weight. The inoculated soil was incubated in plastic bags for 3 d at room temperature. Only the upper 9 cm of the soil column consisted of inoculated soil; each microcosm (1610 g wet soil mass) contained 14.3 g inoculated pea mash in the upper 9 cm. In assays on bacterial root colonisation, the soil was not inoculated with *Pythium ultimum*. Non-inoculated soil contained natural inoculum (60–90% sugar beet emergence in non-inoculated soil). Therefore, no non-inoculated controls were set up in antagonist assays.

Assessment of disease

Shoots of all symptomless plants (termed 'healthy') in two replicate microcosms for each antagonist dose were recovered, counted and collected for dry weight determination at the end of the growth period (14 d). Shoots of plants, which showed typical symptoms of *Pythium* damping-off like brown discoloration of the hypocotyl and dry thin sections of the hypocotyl (in a later stage of disease development), were discarded. The term shoot was applied to all plant parts above seed level.

Plant recovery in colonisation experiments

The microcosms were opened and the plants were carefully removed with a dissecting needle. Six plants were taken from each microcosm. The plants were dissected into: hypocotyl (0 to 2 cm above seed level), 0 to 2 cm root section, 2 to 4 cm root section and the section below 4 cm root depth (corresponding to 0–2, 2–4, 4–6, and $>$ 6 cm soil depth). Three mL of $\frac{1}{4}$ strength Ringers solution were added to the root and hypocotyl samples. They were vortexed and sonicated (Ultrasonic bath DECON, Ultrasonics Ltd., Hove, Sussex, UK) for 3 min before dilution series and bioluminescence measurements were performed. From each dilution step three 10 μL drops were placed on plates with Luria Bertani medium containing appropriate antibiotics (50 ppm tetracycline for *Pseudomonas fluorescens* B5, 12.5 ppm tetracycline for *Pseudomonas corrugata* 2140). No tetracycline resistant indigenous flora were found in samples of untreated control plants.

Measurement of luminescence

Bioluminescence was measured as Relative Luminescence Units (RLU) according to the methods described by Rattray et al. (1995). Luminescence was recorded 3 min after the addition of 10 μL 5% n-decyl aldehyde (dissolved in 96% ethanol) to 1 mL of the undiluted sonicated sample using the Luminometer Biorbit LKB 1251 (Biorad Inc., Hemel Hempstead, UK). The measuring times were 3 $\times$ 2 s after 4 s shaking. Values measured in root samples from control plants, which did not receive antagonist treatment, were subtracted as blank.

Statistical analysis

Linear and polynomial regression was used to investigate the effect of initial dose on colonisation, *in situ* bioluminescence and antagonistic activity and to establish a relationship between root colonisation (log CFU/cm section $+1$) and bioluminescence (log RLU/cm section $+1$). The calculations were carried out using the analytical tools of the following software packages: Microsoft Excel Version 5.0 (Microsoft Corporation), Sigma Plot 4.0 and SPSS release 9.0.0 (SPSS Inc. Chicago, IL, U.S.A.).

Results

Effect of initial antagonist dose in sugar beet seedling colonisation

Even when low inoculum densities of both *Pseudomonas* strains were applied to the seed (10^3 CFU/seed pellet) high populations ($\geq$ 10^5 CFU per plant) and *in situ* bioluminescence were detectable on 12 d old seedlings (Table 1, Figures 1 and 2). The effects of initial dose on the population size were small and, in most cases, not significant (Table 1 and 2, Figures 1 and 2). The ratio between applied bacteria (CFU/Seed) and recovered bacteria per plant (Table 1) clearly showed that the *Pseudomonas* inocula not only persisted, but also propagated on the seedling surfaces. At low doses, up to 330-fold more cells than initially applied could be recovered (Table 1). However, the ratio between applied and recovered cells fell rapidly with increasing dose (Table 1). At doses exceeding 10^5–10^6 CFU/seed, the number of cells recovered was less than the initial dose applied. Thus, a saturation point appeared to be reached with 10^5 to 10^6 CFU per plant in both *Pseudomonas* strains (Table 1).

Table 1. Pseudomonas fluorescens B5 and *Pseudomonas corrugata* 2140: Relationship between the total antagonist population recovered per plant (CFU/plant) and initial inoculum density (CFU/seed pellet). Total amount of recovered bacteria per plant was calculated as the sum of the CFU recovered from each section/6 (6 = number of recovered plants). The seed pellet itself was not recovered, only plants. For the determination of initial antagonist inoculum, 10 freshly treated seed pellets were suspended in $\frac{1}{4}\times$ Ringers solution and the suspension was plated out at the start of the experiment. For *Pseudomonas fluorescens* B5, the relationship between applied dose (x) and recovered population size (y) could be described by the equation $\log_{10} y = 0.185 \log_{10} \times +4.2231$ ($r^2 = 0.6308$, $P = 0.033^*$), whereas no significant relationship existed for *Pseudomonas corrugata* 2140

Pseudomonas fluorescens B5			*Pseudomonas corrugata* 2140		
Applied (CFU/seed) =x	Recovered (CFU/plant) =y	Ratio Recovered/ Applied	Applied (CFU/seed) =x	Recovered (CFU/plant) =y	Ratio Recovered/ Applied
1.42×10^3	1.31×10^5	92.72	9.83×10^2	3.23×10^5	328.67
6.26×10^3	3.62×10^4	5.79	6.12×10^3	8.50×10^4	13.89
5.29×10^4	1.11×10^5	2.09	5.45×10^4	6.48×10^4	1.19
6.15×10^5	1.52×10^5	0.25	5.14×10^5	2.54×10^5	0.49
3.09×10^6	5.73×10^5	0.19	4.21×10^6	1.37×10^6	0.33
1.71×10^7	3.21×10^5	0.02	4.12×10^7	2.75×10^5	0.01
6.08×10^7	4.02×10^5	0.01	3.29×10^8	1.85×10^5	0.001

Total population size per seedling ($\log_{10}$ CFU/plant) increased slightly with dose for *Pseudomonas fluorescens*, but not for *Pseudomonas corrugata* (Table 1). Also quadratic and cubic equations revealed no significant relationship between the initial dose and the total recovered population in *P. corrugata* 2140.

Population densities and measured *in situ* bioluminescence of both *Pseudomonas* strains reached highest levels in the hypocotyl and the root regions nearest to the seed (10^4 to 10^5 CFU per cm at 0–2 cm depth; Figures 1 and 2). They decreased to $10^1 - -10^3$ CFU/cm section in the deeper root regions (Figures 1 and 2). The high densities in the hypocotyl region and in the root regions nearer to the seed (0–2 and 2–4 cm depth), were already reached at the lowest applied dose (10^3 CFU/seed) and did not increase significantly with inoculum density, indicating that a saturation point was reached in these regions (Figures 1 and 2). Only in deeper root regions (4 cm below seed level) did the population size of *Pseudomonas fluorescens* B5 increase with dose (Figure 1). *In-situ* bioluminescence of *Pseudomonas corrugata* 2140 in the root regions more than 2 cm below the seed appeared to reach a maximum at 6.67 log ((CFU/seed)+1) (Figure 2). At 2–4 cm root depth, regression with a cubic equation ($\log \text{RLU} = -0.0557\ [\log((\text{CFU/seed})+1)]^3 + 0.875\ [\log((\text{CFU/seed})+1)]^2 - 4.138\ [\log((\text{CFU/seed})+1)] + 6.526$) was significant ($R^2 = 0.910$; $P = 0.043$). Apart from these two observations, no significant effect of initial antagonist inoculum density on population density as well as *in-situ* bioluminescence in plant sections was detected (Figures 1 and 2).

In both strains, *in situ* bioluminescence (log (RLU/cm +1)) decreased with depth (Figures 1 and 2) and was significantly correlated with population density (Table 2). However, bioluminescence was highest in the hypocotyl region (Figures 1 and 2) although maximum population densities occurred on the upper root in 0–2 cm depths. Consequently, linear regression with population density as a predictor and bioluminescence as a dependent value showed a greater increase of bioluminescence with population density in the hypocotyl sections than in the root sections (Table 2). Furthermore, higher correlation coefficients indicate that the strength of the relationship was greater in hypocotyl samples than in root samples (Table 2). In hypocotyl as well as root sections, *Pseudomonas corrugata* 2140 showed a slightly greater increase of bioluminescence with population density than did *Pseudomonas fluorescens* B5 (Table 2), indicating a greater level of *lux* gene expression in the former. Despite the differences between the strains and between root and hypocotyl sections, the relationship between population density and bioluminescence remained highly significant ($P < 0.001$) when the data were pooled (Table 2).

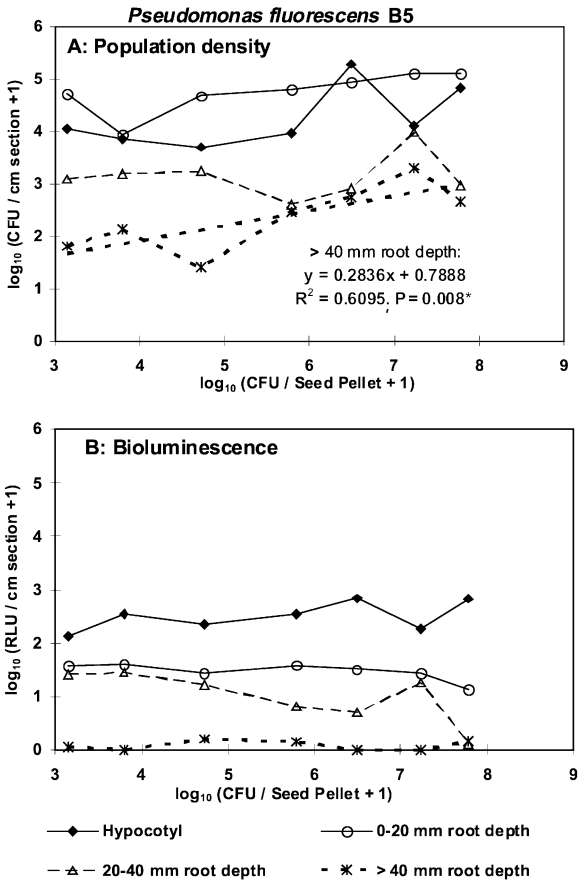

Figure 1. Sugar beet seedling colonisation by *Pseudomonas fluorescens* B5 after different initial concentrations were applied to the seed: Resulting population densities (A) and physiological activity measured as bioluminescence (B) on sugar beet seedlings at the hypocotyl and at different root depths. Growth time was 12 days. Seeds were soaked in serial dilutions of the *lux*-marked antagonist. CFU = colony forming units, RLU = Relative Luminescence units. Significant relationships (Population density at > 40 mm root depth) are indicated with a trend line and inserted equation.

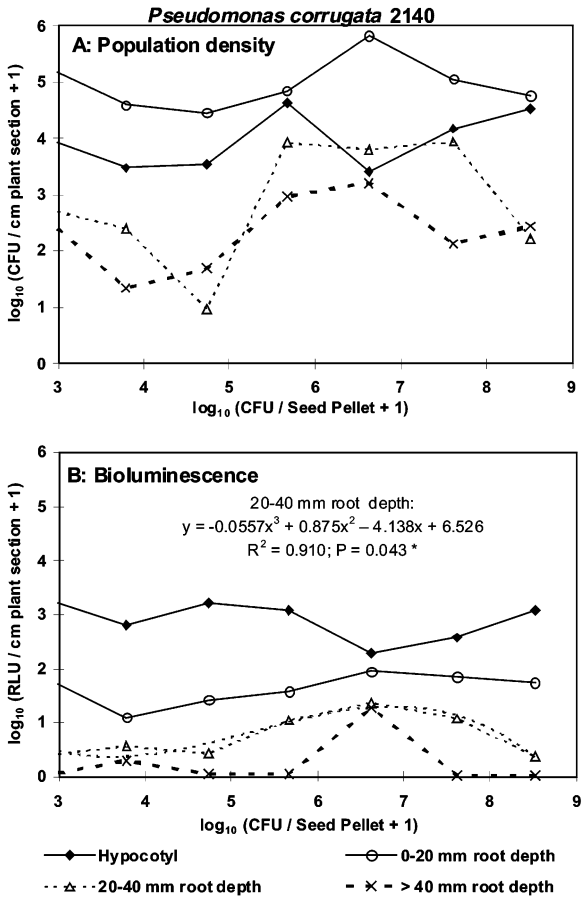

Figure 2. Sugar beet seedling colonisation by *Pseudomonas corrugata* 2140 after different initial concentrations were applied to the seed: Resulting population sizes (A) and physiological activity measured as bioluminescence (B) on sugar beet seedlings at the hypocotyl and at different root depths (RD). Growth time was 12 days. Seeds were soaked in serial dilutions of the *lux*-marked antagonist. CFU = colony forming units, RLU = Relative Luminescence units; CFU = colony forming units, RLU = Relative Luminescence units. Significant relationships (bioluminescence at 20–40 mm root depth) are indicated with a trend line and inserted equation.

Effect of initial antagonist inoculum density on antagonistic activity towards Pythium ultimum

The two *Pseudomonas* isolates showed different dose response curves. For *Pseudomonas fluorescens* B5, the percentage of surviving, symptomless ('healthy') plants increased significantly ($P < 0.01$) with antagonist dose from an average of 1–7 plants / microcosm (6–43% survival) in untreated controls to 10 plants/microcosm (62% survival) at a dose of 10^7–10^8 cells/seed (Figure 3). This increase was also reflected in their respective dry weights (Figure 3). When the untreated control was omitted from the calculation (comparison in the range of 10^3 to 10^8 CFU per seed pellet) the increase of fresh and dry weight of healthy plants was still significant ($P > 0.05$, data not shown). Although the variation was considerable (R^2 values in the range of 0.4–0.5; Figure 3) it can be concluded that the antagonistic activity of *Pseudomonas fluorescens* B5 increases with dose and that high initial doses ($\geq 10^7$–10^8 cells per seed) provide the best protection from damping off disease.

The relationship between inoculum density and antagonistic effect of *Pseudomonas corrugata* 2140 was not linear, but followed an optimum curve (Figure 4). Doses of 10^4 to 10^6 CFU per seed pellet resulted in the highest number (percentage) and dry weight of healthy plants in *Pythium* infested soil, whereas at

Table 2. Linear regression analysis with population density as predictor ($\log_{10}$ (CFU/cm plant section) $= x$) and measured activity of the inserted *lux* gene (bioluminescence) as dependent value ($\log_{10}$ (RLU/cm plant section) $= y$): Equations, correlation coefficients (R^2) and significance (P) of the relationship. CFU = colony forming units; RLU = relative luminescence units. Calculations are based on the data shown in Figures 1 and 2. The relationship between population density and bioluminescence was still highly significant ($P < 0.001^{***}$) when all data where pooled

	Equation y = log (RLU/cm section+1) x = log (CFU/cm section+1)	R^2	P
P. fluorescens B5			
Hypocotyl	$y = 0.5599x + 0.0974$	0.951	$3.80 \times 10^{5***}$
Roots	$y = 0.3378x - 0.2713$	0.614	$5.99 \times 10^{6***}$
P. corrugata 2140			
Hypocotyl	$y = 0.6842x + 0.1687$	0.879	$5.87 \times 10^{4***}$
Roots	$y = 0.3624x - 0.3047$	0.784	$8.79 \times 10^{9***}$
All			
Hypocotyl	$y = 0.6004x + 0.2026$	0.830	$9.18 \times 10^{7***}$
Roots	$y = 0.3511x - 0.2914$	0.702	$1.16 \times 10^{13***}$
Hypocotyl + roots	$y = 0.4661x - 0.3036$	0.524	$1.40 \times 10^{11***}$

doses exceeding these values, number and dry weight of survived seedlings decreased with antagonist dose (Figure 4). This curve was even more significant, when the values from the untreated controls were omitted from the calculation ($P < 0.001$).

Discussion

The present study confirms the capability of *Pseudomonas* strains used for biological control to propagate in the rhizosphere. The total population size of the strains after 12 d plant growth was probably even higher than the estimated population size on the plants, since the recovered plants did not include the seed pellet, which contained the original inoculum. Irrespective of initial inoculum density, the pseudomonad populations appear to adjust to an equilibrium determined by the carrying capacity of the host plant. No correlation between initial dose and final population size was found in *Pseudomonas corrugata* 2140 and the increase of the rhizosphere population of *Pseudomonas fluorescens* B5 was only slight (one order of magnitude). Similar observations were made with *Pseudomonas putida* KT2440 on barley (Ramos et al., 2000), which reached a final population density of 10^8 CFU/root irrespective of initial doses (3.3–6.7 log (CFU/seed)). In the spermosphere of naked seeds of sugar beet, the populations of several *Pseudomonas* strains rose from 10^3 to 10^7 CFU/seed within one day (Fukui et al., 1994b, c). Our study demonstrated that populations of 10^5–10^6 CFU per plant were able to persist on the developing seedling for at least 12 days. This appears to be the carrying capacity of sugar beet seedlings, as similar population sizes were found on 6 day old seedlings of sugar beet by Osburn and Schroth (1984), as well as on seedlings of other plant species (Beauchamp et al., 1993).

In contrast to this study, other investigators (e.g. Loper et al., 1985; Bull et al., 1991; Raaijmakers et al., 1995) have found a marked impact of initial inoculum density on resulting rhizosphere populations of *Pseudomonas* strains. A difference of several orders of magnitude was observed in the rhizosphere of wheat (Bull et al., 1991). Differences between the strains of *Pseudomonas fluorescens* used and different plant hosts probably account for these differences in the outcome.

Numerous authors have reported a decrease of the population size of seed inoculants with root depth (e.g. Howie et al., 1987; Liddell and Parke, 1989; Bahme and Schroth, 1987; Trevors et al., 1990; Beauchamp et al., 1993; Ramos et al., 2000); this was also shown for the *Pseudomonas* strains used here (Heupel, 1992; Ryder and Borrett, 1991). However, to our knowledge, the present study is the first one where the influence

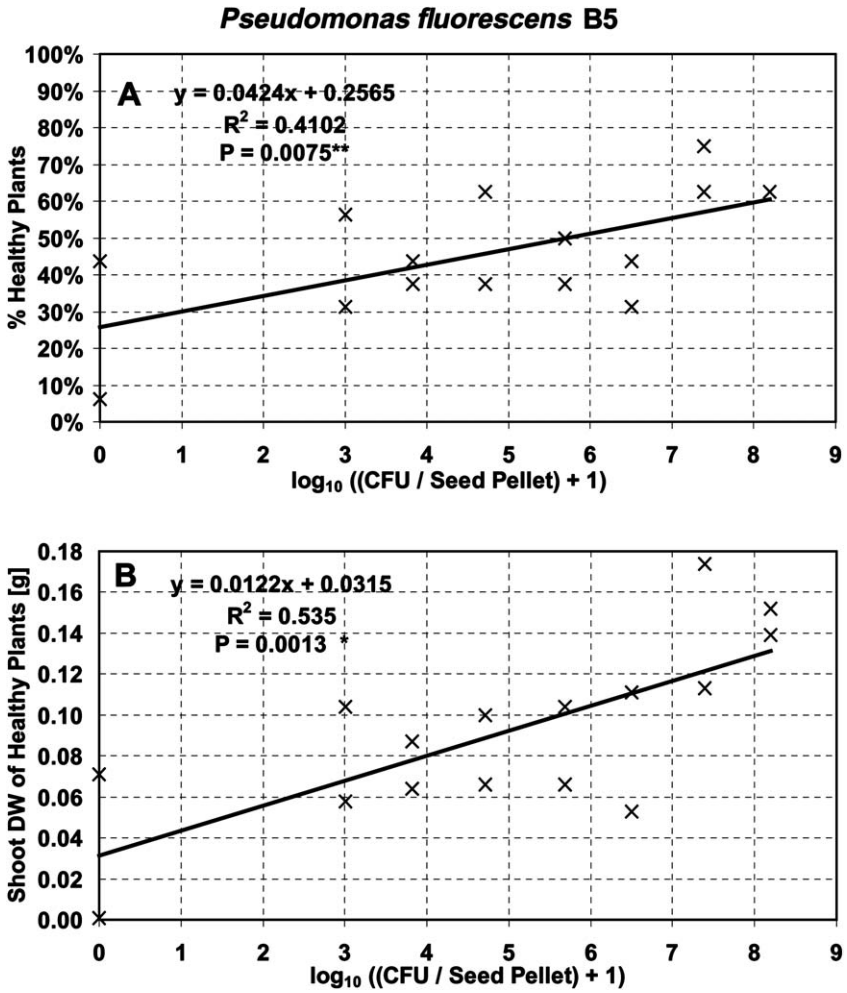

Figure 3. Dose response curve of *Pseudomonas fluorescens* B5 showing the antagonistic activity against damping-off of sugar beet caused by *Pythium ultimum*. A: Percentage (16 plants = 100%). B: dry weight (DW) of sugar beet seedlings without damping-off symptoms. 16 sugarbeet seeds (= 100%) were sown per microcosm with two replicate microcosms set up per treatment. Growth period was 14 days.

of initial antagonist dose on the population size and activity at different root depths was a main interest of investigation. The increase of the population of *Pseudomonas fluorescens* B5 with inoculum density was only apparent in the root sections below 4 cm depth, indicating slightly enhanced downward colonisation at higher doses. Downward movement on the root is substantially increased by percolating water (Parke et al., 1986; Liddell and Parke, 1989). Our intention was to create a worst case scenario for the spread of the bacterial inocula along the root. Surface applications of water were avoided in order to minimise percolation in our microcosms. Thus, percolating water plays no or a limited role in downward movement of the bacterial inocula in our study. Alternatively, the bacteria may have been transported downwards by vectors like nematodes, fungal hyphae or the root itself (Bowen and Rovira, 1976; Howie et al., 1987). Rhizosphere-colonising bacteria may also move in the water film at the root surface (Huck et al., 1970; Bandoni and Koske, 1974; Bowen and Rovira, 1976). Movement of this water film at the root-soil interface, caused by diurnal shrinking and swelling of roots, may facilitate passive dispersal of rhizobacteria along longer distances (Huck et al., 1970; Faiz and Weatherley, 1982).

In accordance with previous studies (Rattray et al., 1995; Shaw et al., 1992), we found a linear correlation between the logarithm of bioluminescence and the logarithm of the rhizosphere population size. *Pseudo-*

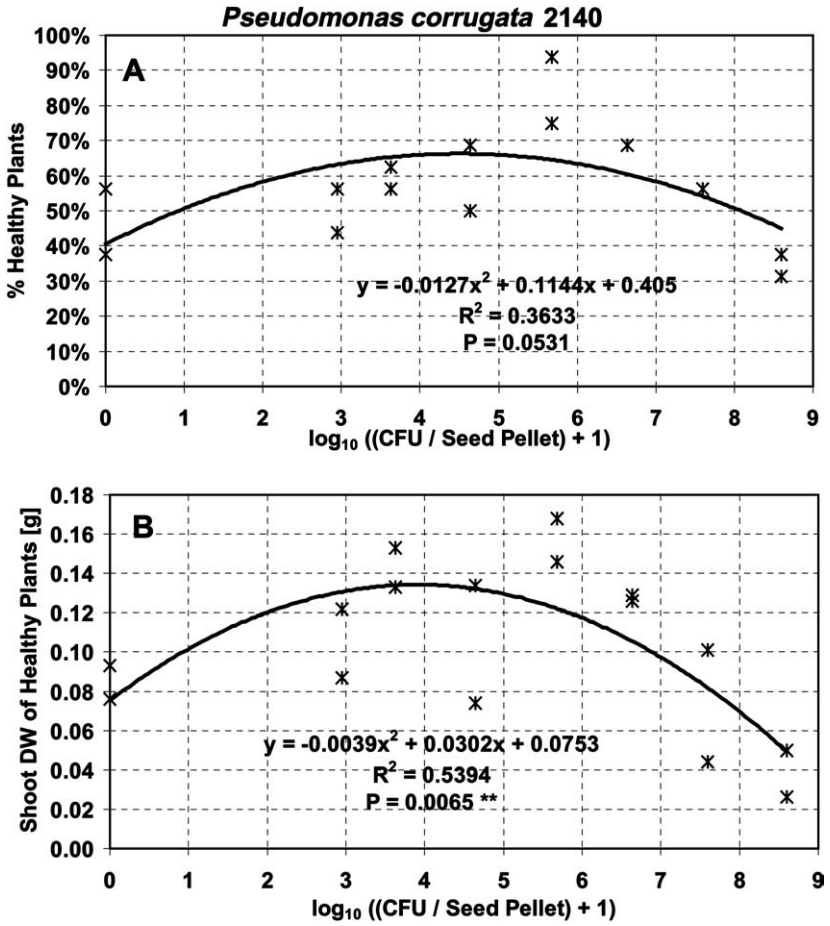

Figure 4. Dose response curve of *Pseudomonas corrugata* 2140 showing the antagonistic activity against damping-off of sugar beet caused by *Pythium ultimum*. A: Percentage (16 plants = 100%). B: dry weight (DW) of sugar beet seedlings without damping-off symptoms. 16 sugarbeet seeds (= 100%) were sown per microcosm, with two replicate microcosms set up per treatment. Growth period was 14 days.

monas corrugata 2140 showed a steeper increase of *in situ* bioluminescence with increasing population size than *Pseudomonas fluorescens* B5. This might be due to a slightly higher metabolic activity per cell or a higher expression rate of the inserted *lux* gene in *Pseudomonas corrugata* 2140. Similar differences in the bioluminescence between different strains of *lux*-marked pseudomonads were also observed by Beauchamp et al. (1993). However, the most significant difference in the increase of bioluminescence with population density was observed between hypocotyl sections and rhizosphere sections. Samples of the upper root region (0–2 cm) showed on average 10 to 100-fold lower bioluminescence than hypocotyl samples, although the rhizosphere population was similar or even greater in the root samples. A probable explanation is the quenching effect of suspended soil particles from adhering soil, which were present in root samples, but not in hypocotyl samples. The addition of 0.2–1 g of soil to suspensions of *lux*-marked bacteria reduced measured bioluminescence by 0.5–2 orders of magnitude (Rattray et al., 1995; Grant et al., 1992). Therefore, the comparison between soil-less hypocotyl samples and soil-containing rhizosphere samples has to be made with caution. We did not add defined amounts of soil to the hypocotyl samples in order to make them more comparable because this would have reduced the sensitivity of the assay further. Also, we did not try to remove the soil particles from rhizosphere samples because we wanted to avoid the removal of adhering inoculum. The effect of suspended soil particles might also explain lower correlation and greater variability between population size and bioluminescence in root samples compared to hypocotyl samples.

As in numerous other studies (e.g. Sørensen et al., 2001; de Weger et al., 1991; Shaw et al., 1992; Meikle et al., 1992; Ramos et al., 2000), high population sizes of at least 10^3–10^5 CFU/cm root were necessary to detect *in situ* bioluminescence, whereas the detection limit of plate counts was at least tenfold lower (10^2 CFU/cm root). Ramos et al. (2000) were able to detect *in situ* bioluminescence only in the upper region of the root, similar to this investigation. Bioluminescence was correlated with dehydrogenase activity in the used strains (Knox, 2000; Russell, 1996). Thus bioluminescence is a valuable indicator of physiological activity under the given conditions (Killham and Yeomans, 2001; White et al., 1996), whereas CFU counts allow no conclusions to be drawn about the metabolic activity of the strains. Contrary to the study of Ramos et al. (2000) we found no increase in activity (bioluminescence) of the applied *Pseudomonas* strains at lower initial doses. The difference in growth time might be the reason for this difference. Ramos et al. (2000) measured *in-situ* bioluminescence after one day plant growth when the population at low doses increased rapidly whereas it was already static at higher doses; an increasing population should be physiologically more active than a static one. The population had reached its final size after just 1–3 d plant development. Contrary to this, measurements were made in a much later stage of plant development (12 d) here; by then, the final population sizes have probably been established for some time and populations may already have reached a static state even at lower initial doses.

Increased population sizes of *Pseudomonas fluorescens* in the lower root zone might be one reason for the increased biocontrol effect against *Pythium* at higher initial antagonist densities. The size of the rhizosphere population of *Pseudomonas fluorescens* Q2-87 was positively correlated with the *in situ*-concentration of 2,4-diacetyl-phloroglucinol (Raaijmakers et al., 1999), an active antibiotic against *Pythium ultimum* (Dowling and O'Gara, 1994), and population size of another strain (2-79) was negatively correlated with the number of root lesions caused by *Gaeumannomyces graminis* (Bull et al., 1991).

The assessment of antagonist population size and metabolic activity after emergence may, however, offer insufficient explanation for the marked effect of initial dose on biocontrol activity against *Pythium ultimum*. In different strains of pseudomonads, Fukui et al. (1994b) found that the length of the lag phase rather than the final population size reached after 24 h was crucial for the prevention of sugar beet pericarp colonisation by *Pythium ultimum*. The same authors also found a strong linear correlation between the logarithm of population density and prevention of pericarp colonisation by *Pythium ultimum*. *Pythium* infection of seed coats can commence 4 h after sowing and can be nearly complete after 24 h (Osburn and Schroth, 1984). Thus, sufficient protection is needed in a very early stage. In the present study, pre-emergence damping off prevailed and only a few plants fell prey to post emergence damping off, indicating that infection had taken place in an early stage of germination. These data suggest that insufficient population increase in the spermosphere or during early seedling development might explain for the observed decrease of biocontrol activity at lower initial doses. Nevertheless, it was demonstrated, that high populations were achieved at all doses at least in later stages and that the population persisted during the susceptible phase of seedling development. If infection occurs later in plant development (post-emergence damping off), these high population levels may ensure successful biocontrol.

In-vitro germination assays in the absence of *Pythium* hinted towards a reduction of the germination rate of sugar beet at high doses of *Pseudomonas corrugata* 2140 (unpublished results). This suggests that negative effects of high initial densities of *Pseudomonas corrugata* 2140 on plant numbers and fresh weight were probably due to phytotoxic effects and not due to a reduction in antagonistic activity against *Pythium*. High doses of the strain were phytotoxic to wheat (Ryder, unpublished data). The antibiotics produced by the strains used in the present study were not characterised. However, Katarýan and Torgashova (1976) and Raaijmakers et al. (1999) reported herbicidal properties of 2,4-diacetylphloroglucinol, a main active component against *Pythium ultimum* produced by many antagonistic *Pseudomonas* strains. Unfortunately it was not possible to run controls without *Pythium in situ* because the soil contained an indigenous inoculum (disease incidence in non-inoculated soil 12–40%) and we did not want to sterilise the soil in order to maintain conditions as close to the field as possible.

Due to the presence of non-characterised indigenous inoculum the introduced *Pythium* inoculum may have not accounted for all infections of the plants. Furthermore, the introduced pea mash may have encouraged the growth of the indigenous inoculum, and there may have been interactions between the native and the introduced inoculum of soilborne pathogens. How-

ever, amendment of pea mash inoculated with *Pythium ultimum* significantly increased damping off disease incidence compared to non-inoculated controls in optimisation experiments; the addition of pea mash alone had no significant effect (unpublished results). Thus it can be assumed that the introduced *Pythium* inoculum was the limiting factor for emergence in our antagonist assays and the observed increase in emergence was due to the antagonistic effect of the isolates.

Contrary to most previous studies on biocontrol of sugar beet diseases, where methylcellulose formulations were applied on naked seeds, seeds with a commercial pellet formulation were used here. This gives the present study a particular practical relevance. However, the necessary high inoculum densities of pseudomonads ($> 10^6$ CFU/seed pellet) could only be achieved by soaking pelleted sugar beet seeds in suspensions of the antagonist and immediate sowing of the moist pellets. This method cannot be applied to field production systems. In commercial dry pellet formulations, a large proportion of cells is killed in the drying process and only 10^3 to 10^4 CFU per seed can so far be realised (Tilcher, KWS SAAT AG Einbeck, Germany, unpublished information), a dose which was shown to be ineffective here. Formulation methods and drying procedures still have to be found which either achieve and maintain the necessary high doses or, alternatively, ensure a sufficiently rapid population build-up and antagonistic activity from lower initial doses.

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References

Bahme J B and Schroth M N 1987 Spatial temporal colonization pattern of a rhizobacterium on underground organs of potato. Phytopathology 77, 1093–1100.

Bandoni R J and Koske R E 1974 Monolayers and microbial dispersal. Science 183, 1079–1080.

Beauchamp C J, Kloepper J W and Lemke P A 1993 Luminometric analysis of plant root colonization by bioluminescent pseudomonads. Can. J. Microbiol. 39, 434–441.

Berger F, Hong Li, White D, Fraser R and Leifert C 1996 Effect of pathogen inoculum, antagonist density and plant species on biological control of *Phytophtora* and *Pythium* damping-off in high-humidity fogging glasshouses. Phytopathology 86, 428–433.

Bowen G D and Rovira A D 1976 Microbial colonization of plant roots. Ann. Rev. Phytopathol. 14, 121–144.

Bull C T, Weller D M and Thomashow L S 1991 Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *triticii* by *Pseudomonas fluorescens* strain 2-79. Phytopathology 81, 954–958.

Dowling D N and O'Gara F 1994 Metabolites of *Pseudomonas* involved in biocontrol of plant disease. Trends Biotechnol. 12, 133–141.

Faiz S M A and Weatherly P E 1982 Root contraction in transpiring plants. New Phytol. 92, 333–343.

Frey-Klett P, Churin J-L, Pierrat J-C, Garbaye J 1999 Dose effect in the dual inoculum of an ectomycorrhizal fungus and a mycorrhiza helper bacterium in two forest nurseries. Soil Biol. Biochem. 31, 1555–1562.

Fukui R, Schroth M N, Hendson M and Hancock J G 1994a Interaction between strains in sugar beet spermospheres and their relationship to pericarp colonization by *Pythium ultimum* in soil. Phytopathology 84, 1322–1330.

Fukui R, Schroth M N, Hendson M, Hancock J G and Firestone M K 1994b Growth Patterns and metabolic activity of pseudomonads in sugar beet spermospheres: relationship to pericarp colonization by *Pythium ultimum*. Phytopathology 84, 1331–1338.

Fukui R, Poinar E I, Bauer P H, Schroth M N, Hendson M, Wang X-L and Hancock J G 1994c Spatial colonization patterns and interactions of bacteria on inoculated sugar beet seed. Phytopathology 84, 1338–1345.

Grant F A, Prosser J I, Killham K and Glover L A 1992 Luminescence based detection of *Erwinia carotovora* subsp. *carotovora* in soil. Soil Biol. Biochem. 24, 961–967.

Heupel M 1992 Biologische Bekämpfung bodenbürtiger Wurzelbranderreger der Zuckerrübe (*Beta vulgaris* L. ssp. *vulgaris* var. *altissima* DOELL) durch den Einsatz mikrobieller Antagonisten. Ph.D. thesis. University of Göttingen.

Howie W J, Cook R J and Weller D M 1987 Effects of soil matric potential and cell motility on wheat root colonization by fluorescent *Pseudomonas* suppressive on take all. Phytopathology 77, 286–292.

Huck M G, Klepper B and Taylor, H M 1970 Diurnal variations in root diameter. Plant Physiol. 45, 529–530.

Johnson K B 1994 Dose response relationships and inundative biological control. Phytopathology 84, 780–784.

Katarýan B T and Torgashova G G 1976 Spectrum and activity of the herbicidal effect of 2,4-Diacetylphloroglucinol. Dokl. Acad. Nauk. Arm. SSR 63, 109–112.

Killham K S and Yeomans C V 2001 Rhizosphere carbon flow measurement and implications: from isotopes to reporter genes. Plant Soil 232, 91–96.

Knox, O G G 2000 Exploiting nitrate respiration to optimise antagonistic control of root disease in soil. Thesis submitted for the degree of Doctor of Philosophy, University of Aberdeen.

Larkin R P and Fravel D R 1999 Mechanisms of action and dose response relationships governing biological control of *Fusarium*

wilt of tomato by nonpathogenic *Fusarium spp*. Phytopathology 89, 1152–1161.

Liddell C M and Parke J L 1989 Enhanced colonization of pea taproots by a fluorescent pseudomonad biocontrol agent by water infiltration into soil. Phytopathology 79, 1327–1332.

Loper J E, Haack C A and Schroth M N 1985 Population dynamics of soil pseudomonads in the Rhizosphere of potato (*Solanum tuberosum* L.) Appl. Env. Microbiol. 49, 416–422.

De Lorenzo V, Herrero M, Jakubzik U and Timmis K N 1990 Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. 172, 6568–6572.

Martin F N and Loper J E 1999 Soilborne diseases caused by *Pythium* spp. Ecology, epidemiology and prospects for biological control. Crit. Rev. Plant Sci. 18, 111–181.

Maurhofer M, Kell C, Schnider U, Volsard C, Haas D and Defago G 1992 Influence of enhanced antibiotic production in *Pseudomonas fluorescens* strain CHA0 on its disease suppressive capacity. Phytopathology 82, 190–195.

Meikle A, Killham K, Prosser J I and Glover L A 1992 Luminescence measurement of population activity of genetically modified *Pseudomonas fluorescens* in the soil. FEMS Microbiol. Lett. 99, 217–220.

Montesinos E and Bonaterra A 1996 Dose-Response Models in biological control of plant pathogens: an empirical verification. Phytopathology 86, 464–472.

Osburn R M and Schroth M N 1984 Dynamics of *Pythium ultimum* infection and damping-off of sugar beets and biocontrol by a strain of *Pseudomonas putida*. (abstract) Phytopathology 74, 835.

Parke J l, Moen R, Rovira A D and Bowen G D 1986 Soil water flow affects the rhizoshere distribution of a seed borne biocontrol agent, *Pseudomonas fluorescens*. Soil Biol. Biochem. 18, 583–588.

Raaijmakers J M, Bonsall R F and Weller D M 1999 Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. Phytopathology 89, 470–475.

Raaijmakers J M, Leeman M, van Oerschot M M P, van der Sluis I, Schippers B and Bakker, P A H M 1995 Dose response relationships in biological control of *Fusarium* wilt of radish by *Pseudomonas ssp*. Phytopathology 85, 1075–1081.

Rattray E A S, Prosser J I, Glover L A and Killham K 1995 Characterization of rhizosphere colonization by luminescent *Enterobacter cloacae* at the population and single cell levels. Appl. Env. Microbiol. 61, 2950–2957.

Ramos C, Molina L, Mølbak L, Ramos J L and Molin S 2000 A bioluminescent derivative of *Pseudomonas putida* KT2440 for deliberate release into the environment. FEMS Microbiol. Ecol. 34, 91–102.

Russell C N 1996 Lux gene reporting of the interaction between *Gaeumannomyces graminis* and an antagonistic pseudomonad in the wheat rhizosphere. Thesis submitted for the degree of Doctor of Philosophy, University of Aberdeen.

Ryder M H and Borrett M A 1991 Root colonization by non-fluorescent pseudomonads used for the control of wheat take-all. *In* Plant Growth-Promoting Rhizobacteria–Progress and Prospects. Eds. C Keel, B Koller and G Défago. pp. 302–307. International Organization for Biological and Integrated Control of Noxious Animals and Plants IOBC/WPRS Bulletin XIV/8.

Ryder, M H and Rovira A D 1993 Biological control of take-all of glasshouse-grown wheat using strains of *Pseudomonas corrugata* isolated from wheat field soil. Soil Biol. Biochem. 25, 311–320.

Shaw J J, Dane F, Geiger D and Kloepper J W 1992 Use of bioluminescence for the detection of genetically engineered microorganisms released into the environment. Appl. Env. Microbiol. 58, 267–273.

Sørensen J, Jensen L E and Nybroe O 2001 Soil and rhizosphere as habitats for *Pseudomonas* inoculants: new knowledge on distribution, activity and physiological state derived from micro-scale and single-cell studies. Plant Soil 232, 97–108.

Suslow T V and Schroth M N 1982 Rhizobacteria on sugar beets: Effects of seed application and root colonization on yield. Phytopathology 72, 199–206.

Trevors J T, van Elsas J D, van Overbeek, L S and Starodub M E 1990 Transport of a genetically engineered *Pseudomonas fluorescens* strain through a soil microcosm. Appl. Env. Microbiol. 56, 401–408.

White D, Leifert C, Ryder M H and Killham K 1996 *Lux* gene technology – A strategy to optimize biological control of soil borne diseases. New Phytol. 133, 173–181.

de Weger L A, Dunbar P, Mahaffee W F, Lugtenberg B J J and Sayler G S 1991 Use of bioluminescence markers to detect *Pseudomonas spp*. in the rhizosphere. Appl. Env. Microbiol. 57, 3641–3644.

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