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Influence of soil type and pH on the colonisation of sugar beet seedlings by antagonistic *Pseudomonas* and *Bacillus* strains, and on their control of Pythium damping-off

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Abstract

In five different soils originating from Scotland (Craibstone and Cruden Bay), Germany (Magdeburg and Uelzen) and Greece (Tymbaki), *Pseudomonas fluorescens* B5 reached higher population sizes (4.7–5.7 log CFU/plant) on 12-day-old sugar beet seedlings than *Bacillus subtilis* MBI 600 (4.1–4.8 log CFU/plant). Total population size per plant was not affected by soil type. In all five soils, the antagonists reached highest population densities in the hypocotyl and the upper 2 cm root section (*P. fluorescens* B5: 5.2–6.8 $\log_{10}$ CFU/g plant fresh weight, *Bacillus subtilis* MBI 600: 5.2–6.1 $\log_{10}$ CFU/g plant fresh weight) and declined to 0–3 $\log_{10}$ CFU below 4 cm root depth. Colonisation by *P. fluorescens* B5 down the root was slightly increased in the soils from Craibstone, Magdeburg, and Uelzen compared to the sandy clay loam from Tymbaki. In *lux*-marked *P. fluorescens* B5, population density was positively correlated with light emission in all soils; the light emission indicated physiological activity of the strains. However, *P. fluorescens* B5 reduced Pythium damping-off (measurement after 14 days plant growth) only in three of the five soils (Craibstone, Cruden Bay and Magdeburg). Co-inoculation of *B. subtilis* MBI 600 increased downward colonisation of the root by *P. fluorescens* B5, but not the total population of *P. fluorescens* B5 per plant. *Bacillus subtilis* MBI 600 did not reduce Pythium damping-off in any of the soils nor did it influence the efficiency of co-inoculated *P. fluorescens* B5; its population consisted mainly of physiologically inactive spores. In Craibstone soil, pH did not affect population density, distribution along the root or biocontrol activity against *P. ultimum* of *P. fluorescens* B5 or *B. subtilis* MBI 600.

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

Introduction

Damping-off of sugar beet (caused by *Pythium* spp.) is a soil-borne disease of major economic significance (Martin and Loper, 1999). *Pythium ultimum* and other soil-borne seedling pathogens (e.g. *Aphanomyces euteiches*, *Rhizoctonia solani*) are currently controlled by the addition of fungicides to the seed coat. Biocontrol agents, especially bacteria of the genera *Pseudomonas* and *Bacillus*, have been investigated as an alternative to chemical seed treatment (Suslow and Schroth, 1982;

Martin and Loper, 1999). As sugar beet seeds are pelleted to permit use of a seed drill, biocontrol agents could easily be added in substantial numbers to the pelleting substances. In soil, microorganisms encounter a more protected and less hostile environment than on aerial plant surfaces (Elad, 1990); however, there is a lot of competition by the indigenous microflora, which occupies the favourable niches. Reliable control of soil-borne pathogens has rarely been achieved under field conditions (Suslow and Schroth, 1982; Schippers et al., 1987). Therefore, few biocontrol agents have been commercialised, despite several decades of research.

The main reasons for the variability of biocontrol performance in the field are variable environmental conditions (Weller, 1988), which affect survival, activity and antibiotic production of biocontrol inoculants. Soil type and composition affect the composition of the indigenous flora of fluorescent pseudomonads, the survival of introduced inocula (Bahme and Schroth, 1987; Hartel et al., 1994; Latour et al., 1999) and also their biocontrol performance (Stutz et al., 1989; Ownley et al., 1990/1991). Ownley et al. (1991) established correlations between biocontrol of *Gaeumannomyces graminis* by *P. fluorescens* 2-79 and sand, silt and clay content, organic matter, quantity of a range of minerals and soil pH. Soil pH is an important environmental factor, which can vary widely between soils. Growth and antagonistic activity *in vitro* is influenced by pH (Ownley et al., 1992; Slininger and Shea Wilbur, 1995). Soil pH has also been shown to influence soil suppressiveness (Scher and Baker, 1980), biocontrol performance (Duijff et al., 1995; Naseby and Lynch, 1999) and root colonisation by biocontrol organisms (Naseby and Lynch, 1999). Soil pH can be manipulated and this has facilitated studies on its influence. In addition, knowledge about pH optima of antagonists could lead to guidelines for soil treatment, in order to optimise disease control.

Most studies on the effect of soil type and pH have dealt with true fungi such as take-all of wheat (*Gaeumannomyces graminis*) and *Fusarium*. These fungi are not taxonomically related to the oomycetes *Pythium* and *Aphanomyces*. Although some antifungal compounds produced by biocontrol organisms are broad-spectrum antibiotics, others may be more specific (Dowling and O'Gara, 1994; Leifert et al., 1995). The production of different compounds may be affected differently by environmental conditions. Thus, it is likely that soil type and soil pH affect biocontrol of Pythium damping-off in a different way than biocontrol of take all and other diseases caused by true fungi. In addition, only the study of Ownley et al. (1990, 1991) involved a wide range of soils. Most other investigations on the effect of soil have concentrated on only two or three soils.

Root colonisation and physiological activity *in situ* are considered necessary predictors for successful biocontrol (Bull et al., 1991; de Weger et al., 1995; White et al., 1996; Sørensen et al., 2001). The introduction of *lux*-genes and measurement of resulting bioluminescence has proved to be a sensitive marker system for the estimation of physiological activity (White et al., 1996; Killham and Yeomans, 2001). Most studies on the influence of soil type and soil pH have focussed on either root colonisation and activity of the biocontrol strains or on the control of a certain pathogen. A combined investigation of all three traits, however, is crucial to assess whether *in-situ* colonisation and activity are truly reliable and sufficient predictors for biocontrol efficacy. This is particularly important in investigations on damping-off where, apart from *Pythium* species, other pathogens (e.g. *Aphanomyces euteiches*, *Rhizoctonia solani*) might cause the disease.

In combinations of biocontrol strains, strains with different ecological optima may complement each other. This is thought to be the main reason for their greater reliability in a number of cases (Duffy and Weller, 1995; Pierson and Weller, 1994; Dunne et al., 1998). However, this assumption has not yet been systematically tested under laboratory conditions.

The objective of the following study was to investigate whether the colonisation of infection sites (roots and hypocotyl of sugar beet seedlings), physiological activity *in situ* (measured as expression of the inserted *lux*-genes) and antagonistic activity of biocontrol bacteria (*Pseudomonas* spp., *B. subtilis*) differs in soil types from various regions in Europe and is affected by soil pH. Furthermore, the effects of the application of combined inocula on root colonisation by the antagonists and on biocontrol of Pythium damping-off disease were also investigated.

Materials and methods

Strains and seeds used

The *Pythium ultimum* isolate originated from the culture collection of the Department for Plant and Soil Science, University of Aberdeen. *Pseudomonas fluorescens* B5 (Heupel, 1992) was obtained from Professor G.A. Wolf, Institute of Plant Pathology and Plant Protection, University of Göttingen, Germany. *Bacillus subtilis* MBI 600 (Fiddaman and Rossal, 1994) was obtained from Microbio Ltd., Hemel Hempstead, UK (now Beckerunderwood Inc.®, Littlehampton, West Sussex, UK). For root colonisation studies, *lux*-genes from *Vibrio fischerii* were introduced into the bacterial strains. The *Pseudomonas* strains were chromosomally marked using a mini-Tn5 transposon with a *lux*AB gene cassette and a tetracycline resistance gene (strains B5L9) according to the protocol described by de Lorenzo et al. (1990). *B. subtilis* MBI 600 was transformed with plasmid pSB340 containing a *lux*AB gene fusion cassette and chloramphenicol and erythromycin resistance genes (strain MBI 600(33)) as described by Knox et al. (2002). 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 (Russell, 1996; Knox, 2000; Knox et al., 2002). Thus, bioluminescence was a true indicator of their physiological activity (White et al., 1996; Killham and Yeomans, 2001). However, *lux*-marking negatively affected biocontrol activity of *B. subtilis* MBI 600 (Knox et al., 2002). Therefore, the wild type strains of both antagonists were used in assays on biocontrol activity. Sugar beet seeds (*Beta vulgaris*) of the cultivar Samantha were obtained unpelleted and pelleted (grey pellets; without final colour layer and fungicide) from KWS SAAT AG, Einbeck, Germany: pelleting was carried out as described below.

Soils

Five arable topsoils (originating from Craibstone and Cruden Bay, Scotland: Magdeburg and Uelzen, Germany: Tymbaki from Crete, Greece) were used; their properties are shown in Table 1. Soil was sieved to less than 3.25 mm before use. Gravimetric water content of the soils was adjusted to water retention capacity (–10 kPa; Table 1) previously determined using a tension table (Townend et al., 2001). In the experiments investigating the effect of soil pH, Craibstone soil was used; the pH was adjusted with $Al_3(SO_4)_2$ and $CaCO_3$. Soil pH was determined in water and in 0.01 M $CaCl_2$. The adjusted pH values were pH 5.0–5.2 (5.0), pH 6 (5.4–5.6; no amendments), pH 6.8–7.0 (6.3–6.5), pH 7.3–7.8 (6.9–7.2); pH values measured in $CaCl_2$ are presented in brackets.

Microcosm set-up

Microcosms consisted of rectangular PVC columns (made from electrical cable conduit) with a Perspex plate fitted to their base. The front panel was removable for the recovery of roots. They were 29.8 cm high and 7×7 cm wide. The soil column height was 25 cm. A 1 cm thick layer of gravel was packed at the bottom of each microcosm. To obtain a uniform penetration resistance below 0.7 MPa, soils were packed at individual bulk densities (Table 1). A uniform bulk density was achieved by packing the required quantity of soil in 4 cm thick layers (3 and 2 cm at the top). In assays on antagonistic activity against *P. ultimum*, but not in assays on bacterial root colonisation, *P. ultimum* inoculum was added to the top 9 cm of soil as described below. It was necessary to omit *Pythium* inoculation in colonisation assays in order to have sufficient plant material for analysis in all treatments at the end of the experimental run. Sixteen sugar beet seeds were sown at a depth of 2 cm in each microcosm. 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. The microcosms were incubated in a growth chamber (Fitotron SGC066.PPX.F, Sanyo Gallenkamp plc, Loughborough, UK) for 12 days until the plants had developed the first true leaves (2 mm length). In biocontrol assays, the growth period was prolonged to 14 days to allow more time for disease development. The study focussed on the control of damping-off during the very early, most susceptible stages; pre-emergence damping-off prevailed under the chosen conditions. Relative humidity was 85%, light irradiation intensity (measured at bead layer surface level) 309–510 μmol m^{-2} s^{-1} of

Table 1. Origin and properties of the soils

Soil origin	Soil classification according to FAO (1998)	Mass fraction in particle size analysis [µm]				Soil texture	pH (H_2O)	pH ($CaCl_2$)	Organic carbon [g/100 g]	Organic matter [g/100 g]	Volumetric water content ($cm^3\ cm^{-3}$) at −10 kPa	Bulk density ($g\ cm^{-3}$)	Penetration resistance (MPa)[a]
		2000–60 (sand)	60–20 (coarse silt)	20–2 (fine silt)	>2 (clay)								
Craibstone (Sc)[b]	Humic podzol	0.68	0.13	0.12	0.07	SL[c]	6.0	5.4	2.91 ± 0.04	5.00 ± 0.07	0.31	1.0	0.22
Cruden Bay (Sc)	Dystric podzol	0.18	0.04	0.4	0.38	ZC	6.3	5.9	1.64 ± 0.10	2.82 ± 0.17	0.31	1.0	0.071
Magde-burg (G)	Luvic chernozem	0.28	0.28	0.23	0.21	CL	7.5	7.2	1.25 ± 0.01	2.15 ± 0.02	0.28	1.0	0.11
Uelzen (G)	Haplic cambisol	0.82	0.09	0.04	0.05	LS/SL	6.8	6.6	0.26 ± 0.04	0.45 ± 0.07	0.14	1.4	0.19
Tymbaki (Cr)	Rhodic luvisol	0.73	0.12	0.04	0.11	SL	7.8	7.7	0.26 ± 004	0.45 ± 0.07	0.16	1.4	0.25

Topsoil was used. Calculation of organic matter and organic carbon is based on loss on ignition (375 °C, 24 h) using the formula of Ball (1964). Soil was adjusted to the VWC (volumetric water content) as shown below to achieve a target matric potential of −10 kPa.

[a] Penetration resistance was measured throughout the soil profile with a penetrometer; values show the mean of values measured between 2 and 10 cm depth.

[b] Abbreviations for Countries: Sc = Scotland, G = Germany, Cr = Crete, Greece.

[c] Abbreviations for soil textures: SL = sandy loam, LS = loamy sand, CL = clay loam, ZC = silty clay.

white fluorescent light, day night cycle 12 h/12 h. The air temperature during the dark phase was set to 14.5 °C. During the light phase, it was lowered to 11.5 °C to counteract warming of the soil through light irradiation. This way, diurnal temperature fluctuation in soil was minimised and a soil temperature between 14 °C (night) and 18 °C (day) was achieved. Soil volumetric water content, was monitored at 3.2, 11.5 and 19.5 cm soil depth using the datalogger DL3000 with theta probes type ML1 (Delta T-Devices, Burwell Cambridge, UK). Theta probes were calibrated specifically for each soil. Matric potential and temperature were monitored at 2 cm soil depth (= seed depth) and 10 cm soil depth using the datalogger Skye DataHog 1 and 2 with the pressure sensor SKT 600S/I and temperature sensor SKT 200U/I (Skye Instruments, Powys, UK), respectively. The probes were placed in separate control columns, which had not received antagonist treatment, and a similar behaviour of the measured parameters was assumed for all other microcosms within one experimental run and containing the same soil type. To maintain a stable matric potential (−5 to −20 kPa) during the incubation period, columns were watered regularly by injection of water through rubber suba-seals (Fisher Scientific Ltd., Loughborough, UK) at five different soil depths (2.3, 6.2, 10.5, 14.2, and 18.2 cm) at two opposite sides of the soil column with a syringe. The necessary amounts of water were calculated using the theta probe readings. Microcosms were re-arranged at least two times during the run to counteract possible positional effects caused by microclimatic variation in the growth chamber. One microcosm per treatment was set up in assays on root colonisation and *in situ* activity, where the replicates consisted in groups of two individual plants; three replicate microcosms per treatment were set up in assays on biological control of Pythium damping-off.

Seed treatment

Pseudomonas fluorescens B5 was applied by soaking pelleted seeds in bacterial suspensions from 24 day old cultures before sowing. In the experiments on the effects on soil pH, *B. subtilis* MBI 600 was applied in the same way; 100% of cells applied and recovered were vegetative cells. In the experiments on the effect of soil type, a spore suspension from *B. subtilis* MBI 660 was incorporated into the seed pellet during the pelletting process. In detail, the seed treatment was done as following: *Pseudomonas fluorescens* B5 was cultured overnight (150 ml, 24 °C, swirled at 200 rpm) in Luria-Bertani medium (LB: 10 g l^{-1} Yeast extract, 10 g l^{-1} tryptone, both from Oxoid Limited, Basingstoke, Hampshire, UK, 5 g l^{-1} NaCl). *B. subtilis* MBI 600 was cultured under the same conditions in baffled flasks (500 ml, 30 °C, LB-Medium + 0.267 *g* $MnSO_4 \times 4H_2O$) When *lux*-marked strains were cultured, the appropriate antibiotics were added to the medium. This was 50 ppm tetracycline (Tetracycline hydrochloride, Merck, Darmstadt, Germany) for *P. fluorescens* B5 and 50 ppm erythromycin and 25 ppm chloramphenicol (both Sigma Chemical Company, St Louis, USA) for *B. subtilis* MBI 600. The cultures were centrifuged (*P. fluorescens* $2710 \times g$, *B. subtilis* $2556 \times g$, 20 min, 4 °C) and cells were suspended in 1/4 strength Ringer solution (Oxoid Limited, Basingstoke, Hampshire, UK). The optical density of the suspensions was adjusted to $OD_{550\ nm} = 5$ using the photometer LKB Ultrospec 4050. Pelleted seeds were soaked for 15 min in the suspension and sown after 1 h drying period under the laminar flow. The treatment resulted in a bacterial inoculum of 10^7–10^8 CFU/seed pellet (*P. fluorescens* B5), 2×10^5 CFU/seed pellet (*B. subtilis* MBI 600); this was determined by suspending 10 seeds in 5 ml $1/4 \times$ Ringer solution and plating of serial dilutions of this suspension as described below. Pelleting of seeds and incorporation of spores of *Bacillus subtilis* MBI 600 was carried out in a rotating vessel (Trybuhl DS 20.FU; Trybuhl Dragiertechnik GmBH, Einbeck, Germany; 45 rpm). Pelleting mass (KWS recipe 13) and adhesive (KWS recipe 4) were obtained from KWS SAAT AG, Einbeck, Germany. To 6000 seeds, 68 *g* of pelleting mass was added and 140 ml of a 2% adhesive solution (in tap water) were sprayed using the pneumatic pistol SATA LM-92 (SATA Farbspritztechnik GmbH Kornwestheim, Germany). A sporulating culture of *B. subtilis* MBI 600 (4 day old, conditions see above) was centrifuged ($2556 \times g$) and the cells suspended in the 140 ml adhesive solution used for pelleting. Pelleted seeds were blow dried to a moisture content of 7–8%. The treatment resulted in a bacterial inoculum size of 10^6–10^7 CFU/seed pellet (*B. subtilis* MBI 600), consisting mainly (60–100%) of spores. Dried

pellets were soaked in 1/4 × Ringer solutions at the start of the experiment to create conditions similar to the treatments containing *P. fluorescens*. To obtain combined inocula, pellets containing *B. subtilis* MBI 600 were soaked in cell suspensions of *P. fluorescens* B5.

Pythium *inoculation in antagonist assays and assessment of* Pythium *damping-off disease*

Pea seeds (marrowfat peas, Great Scot, Scotia Haven Foods Ltd., Warrington, UK) were pregerminated in water for 24 h and then homogenised 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 glass Petri dishes were then autoclaved three times for 20 min at 121 °C. They were inoculated using three agar plugs of 3 day old corn meal agar cultures of *P. ultimum* and incubated at 25 °C for 4 days. A specific amount of inoculated pea mash was crumbled and mixed gently into the soil after the water content had been brought to water retention capacity. The inoculated soil was incubated in plastic bags for 3 days at room temperature before the start of the experiment. Only the upper part of the soil column (top 9 cm) consisted of inoculated soil. Per microcosm, 14.3 g inoculated pea mash were added. This treatment significantly increased incidence of Pythium damping-off (pre-emergence damping-off prevailed) whereas sterile non-inoculated pea mash had no effect (unpublished results). In assays investigating bacterial root colonisation, the soil was not inoculated with *P. ultimum*. Non-inoculated soil contained some natural inoculum (up to 30% of recovered plants in assays on root colonisation showed symptoms). Therefore, no non-inoculated controls were set up in antagonist assays, but emergence in non-inoculated soils was assessed in the assays on root colonisation. At the end of the experiments, shoots of all healthy (symptomless) plants in three replicate microcosms (each sown with 16 seeds) per treatment were recovered, counted and collected for fresh weight determination at the end of the growth period (14 days). Shoots of plants, which showed typical symptoms of Pythium damping-off, such as 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

Three replicates, each consisting of two plants were sampled within one microcosm. Pre-experiments (unpublished data) revealed, that there was no substantial root-to-root travel of the antagonists (except for the vegetative cells of *B. subtilis* MBI 600) and thus plants taken within one microcosm can be considered as truly independent replicates. Seed pellets and seed coats were removed. The plants were then dissected as hypocotyl (0–2 cm above seed level), 0–2 cm root section, 2–4 cm root section, >4 cm root depth (corresponding to 0–2 cm soil depth, 2–4 cm soil depth, 4–6 cm soil depth, >6 cm soil depth). Two millilitres of 1/4 strength Ringer solution (Oxoid Limited, Basingstoke, Hampshire, UK) were added to the root and hypocotyl samples. Plant tissues were then vortexed and sonicated (Ultrasonic bath DECON, Ultrasonics Ltd., Hove, Sussex, UK) for 3 min (*Pseudomonas*) or 5 min (*B. subtilis*). In mixed treatments, dilution series for detection of *P. fluorescens* B5 were plated after 3 min sonication, and then the sample was sonicated for further 2 min, before the dilution series for detection of *B. subtilis* MBI 600 were performed. From each dilution step, three 10 µl drops were placed on to LB plates with appropriate antibiotics (see above). For spore counts, samples containing *B. subtilis* MBI 600 were then subjected to heat treatment (80 °C, 10 min) and plated again. No tetracycline resistant indigenous flora was found in samples of untreated control plants whereas chloramphenicol and erythromycin resistant soil bacteria grew on isolation plates in some experiments. However, *B. subtilis* MBI 600 could be distinguished by the distinct morphology of its colonies. Total populations per plant where calculated by adding the population sizes recovered in each plant section together and dividing that sum by 2 (sections from two plants per replicate sample).

Measurement of luminescence

Bioluminescence was measured as Relative Luminescence Units (RLU) according to Rattray et al. (1990). In the *Pseudomonas* strains, luminescence was recorded 3 min after the addition of 10 µl 5% *n*-decyl aldehyde (dissolved in 96% ethanol) to 1 ml of the sonicated sample using the luminom-

eter Biorbit LKB 1251 (Labtech International, Uckfield, UK). The measuring times were 3×2 after 4 s shaking. In *B. subtilis*, bioluminescent activity was immediately measured after addition of 10 µl 0.05% *n*-decyl aldehyde to 1 ml sonicated sample using the Jade portable luminometer (Labtech Inc. Andover, MA, USA). Values measured in samples from control plants, which grew in the same soil type but did not receive antagonist treatment, were subtracted as blank values. No luminometry was performed in the combined inoculum treatments.

Statistical analysis

Plant fresh weight data were square root transformed to obtain normally distributed residuals. Population sizes and densities (Colony forming units CFU) and RLU-measurements were log-transformed before analysis of variance was carried out because they show a lognormal distribution (Loper et al., 1984). In antagonist assays Tukey's Honestly Significant Difference test was used to determine (a) the effect of antagonist treatment within each soil factor and (b) the effect of soil factor within each antagonist treatment.

Multiple regression was used to test the extent to which biocontrol efficacy of *P. fluorescens* B5 could be explained by the soil properties measured in this study (Table 1). Biocontrol efficacy was calculated as the increase in plant number in the treatment containing *P. fluorescens* compared to the corresponding treatment which did not contain *P. fluorescens* (i.e. the *P. fluorescens* treatment was compared to the control, the combined treatment was compared to the treatment with single *B. subtilis* MBI 600, respectively). Each *P. fluorescens* B5 treated microcosm was paired with a corresponding control microcosm without *P. fluorescens* B5. Increase in plant number (y) was transformed using the formula $y' = \sqrt{(y + 5)}$ for the multiple regression analysis. Five was added in each case to remove negative values from the data set. The correlation of single soil properties (pH, organic matter content, sand, silt, and clay content) with biocontrol efficacy of *P. fluorescens* B5, was investigated with curve fits.

In root colonisation assays, a mixed (split plot) ANOVA was carried out. Root depth was treated as a repeated measure within one subject and all other factors as independent (between subject) factors. If Mauchley's test of sphericity was significant, degrees of freedom were adjusted according Greenhouse-Geiser (Howell, 1997). The effect of pH was also investigated at each single depth using linear and quadratic regression. All statistical calculations were performed using SPSS release 9.0.0 (SPSS Inc., Chicago, IL, USA); the multiple linear regression was done using Minitab statistical software (Minitab™, 2000).

Results

Sugar beet seedling colonisation in different soils

P. fluorescens B5 and *B. subtilis* MBI 600 colonised sugar beet seedlings in all five soil types (Table 2 shows the total population size, Figures 1 and 2 show the distribution along the root). *In situ* bioluminescence was measurable in all samples containing *lux*-marked *P. fluorescens* B5 (Figure 1A, insert). However, bioluminescence was not measurable in samples containing *lux*-marked *B. subtilis* MBI 600 alone. The population densities of *B. subtilis* spores (determined after heat treatment of the samples) were mostly as large or even larger (1.2 to 35-fold) than the population density determined before heat treatment. This indicated that the population of *B. subtilis* MBI 600 consisted largely of spores, which were activated by heat treatment, whereas there was a negligible or only a minor proportion of physiologically active vegetative cells. Thus, only the spore counts are presented here.

The total population per seedling of both antagonists was not significantly affected by soil type or by the application of combined inocula (ANOVA). The total population sizes per plant of *P. fluorescens* (4.7–5.7 $\log_{10}$ CFU) were greater than those of *B. subtilis* (4.1–4.8 $\log_{10}$ CFU; Table 2).

In all five soils, both antagonists reached greatest population densities on the hypocotyl and the upper root region, 0–2 cm below the seed (3×10^5–1×10^7 CFU/g FW for *P. fluorescens* B5, 10^5–10^6 CFU/g FW for *B. subtilis* MBI 600). Population densities decreased significantly with depth ($P < 0.001$; Figures 1 and 2).

Root colonisation and distribution along the root (i.e. interaction between root depth and soil type) of *P. fluorescens* B5 was significantly affected

Table 2. Total population per sugar beet seedling of *Pseudomonas fluorescens* B5 and *Bacillus subtilis* MBI 600 (spores) in different soils, in the single antagonist and combined treatments (*Pseudomonas fluorescens* B5 combined with *Bacillus subtilis* MBI 600)

	$\log_{10}$ (total CFU/plant)		
	P. fluorescens B5		*B. subtilis* MBI 600
	Single	Combined	Single
Craibstone	5.2 ± 0.09 a	5.2 ± 0.27 ab	4.4 ± 0.12 ab
Cruden Bay	5.5 ± 0.22 a	5.7 ± 0.27 a	4.8 ± 0.09 a
Magdeburg	4.8 ± 0.15 a	4.7 ± 0.22 b	4.3 ± 0.24 ab
Uelzen	5.2 ± 0.14 a	5.2 ± 0.13 ab	4.6 ± 0.05 ab
Tymbaki	5.0 ± 0.07 a	4.9 ± 0.63 b	4.1 ± 0.18 b
LSD	0.79		0.48

Averages ± standard error were based on three replicates (2 plants per replicate), recovered after 12 days growth. Seed and seed pellet were not recovered. The total amount of recovered bacteria per plant was calculated as the sum of the CFU recovered from each section/2 (2 = number of recovered plants per replicate). CFU = colony forming units. The initial inoculum was 7.6–8.0 $\log_{10}$ (CFU/seed pellet) for *P. fluorescens* B5); 6.5 $\log_{10}$ (CFU/seed pellet) for *B. subtilis* MBI 600 (applied as spores). ANOVA revealed no significant effect of soil type and combination on total populations per plant. LSD at $P < 0.05$.

by soil type and combination with *B. subtilis* MBI 600 (Figure 1). Colonisation of deeper root regions >2 cm below the seed was greater in the clay soils from Cruden Bay and Magdeburg compared to the sandy clay loam from Tymbaki for both the single antagonist treatments and the combined treatment (Figure 1). This was confirmed in a second experiment (data not shown). In Tymbaki soil, *P. fluorescens* B5 could not be re-isolated from deeper sections of the root (Figure 1). However, bioluminescence was detected indicating that the organisms were present at low numbers (Figure 1A, insert). Combined treatment with *B. subtilis* MBI 600 had no effect on the total population of *P. fluorescens* B5 per plant (Table 2). However, it affected significantly the population density of *P. fluorescens B5* measured in single plant sections ($P = 0.0005$; Figure 1). Amendment with *B. subtilis* MBI 600 significantly increased colonisation down the root by *P. fluorescens* B5 except for the sandy soil from Uelzen (Figure 1). For the Uelzen soil, downward colonisation was relatively high ($\sim 10^3$ CFU/g FW) in the single antagonist treatment. In the other four soils, *P. fluorescens* B5 cells could be recovered from root regions below 4 cm depth only in the combined treatment (up to 10^3–10^5 CFU/g FW; Figure 1). Downward colonisation of *P. fluorescens* B5 was more pronounced and the effect of *B. subtilis* MBI 600 was not significant in a second experimental run involving only the soils from Cruden Bay, Magdeburg and Tymbaki (data not shown). However, when the data from both experiments for these three soils were analysed together, the amendment with *B. subtilis* MBI 600 still increased population density of *P. fluorescens* B5 in deeper root sections significantly ($P = 0.0005$, interaction with root depth $P = 0.0004$).

In situ bioluminescence was measured only in the single antagonist treatment of *P. fluorescens* B5, as interference between the co-inoculated luminescent *B. subtilis* strain could not be excluded in the combined treatment. There was no significant difference in bioluminescence between the different soils (Figure 1A, insert), indicating that the introduced *P. fluorescens* B5 was equally active in all tested soils. As with population density, bioluminescence decreased significantly with depth (Figure 1A, insert), and there was a significant linear relationship between bioluminescence and population density ($\log_{10}$ [RLU/(g FW + 1)] = 0.234 $\log_{10}$ [CFU/(g FW + 1)] + 2.4295, $R^2 = 0.45$, $P < 0.001$). However, bioluminescence was also measurable in the samples from deeper root regions (≥4 cm below seed level), where no cells could be recovered (Figure 1A).

Soil type had no significant effect on population density and distribution of *B. subtilis* MBI 600 along the roots and hypocotyl of sugar beet seedlings (Figure 2). Also the combination with *P. fluorescens* B5 had no significant effect on rhizosphere populations of *B. subtilis* MBI 600 (data not shown).

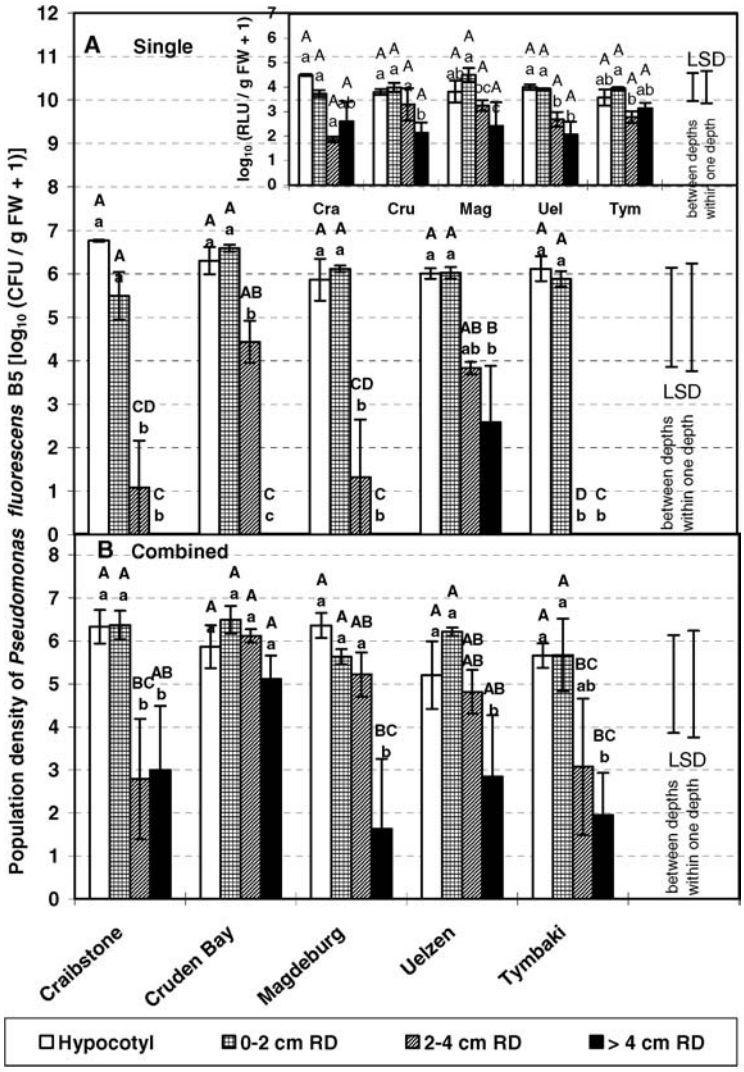

Figure 1. Population density and bioluminescence (insert) of *Pseudomonas fluorescens* B5 (*lux*-marked strain L9) in different soils on sugar beet seedlings at different root depths (RD): Averages and standard error. (A) Single antagonist treatment with *P. fluorescens* solely. (B) Combined inoculum with *P. fluorescens* B5 and *Bacillus subtilis* MBI 600 (spores); only *P. fluorescens* B5 was counted. Bioluminescence was measured in the single strain inoculum only. CFU = colony forming units. RLU = relative Luminescence Units. FW = fresh weight**.** Three replicates, each consisting of two plants were recovered without seed pellet after 12 days growth. ANOVA with root depth as repeated measure within subjects and all other factors as independent between subjects factors was done; degrees of freedom where adjusted according Greenhouse–Geiser for the within subjects analysis. LSD at $P < 0.05$. Large letters are for the comparison between treatments within one root depth, small letters are for the comparison of different root depths within one treatment. Significant effects were found as following: Soil $P = 0.0097$**, combination with *B. subtilis* $P = 0.0005$***, root depth $P < 0.001$***, root depth × soil $P = 0.0133$*, root depth × combination with *B. subtilis* $P < 0.001$***. Bioluminescence was significantly affected by root depth ($P < 0.001$***). Regression between population density [$\log_{10}$ (CFU/g FW + 1) = x] and bioluminescence: [$\log_{10}$ (RLU/g FW + 1) = y]: $y = 0.234\ x + 2.4295$, $R^2 = 044985$, $P < 0.001$***.

Biological control of Pythium *damping-off in different soils*

In the assays on root colonisation, where no artificial *P. ultimum* inoculum was applied, plant emergence was generally higher in the soils from Craibstone and Cruden Bay with a high organic matter content (average 80–90% emergence) compared to the soils from Uelzen and Tymbaki with a low organic matter content (average 70%

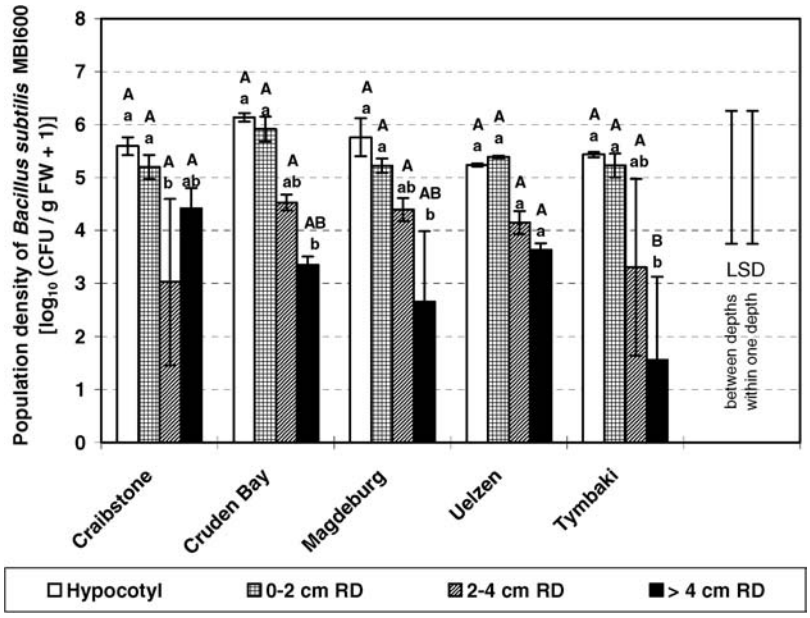

Figure 2. Population density of *Bacillus subtilis* MBI 600 (*lux*-marked strain 33, spores) in different soils on sugar beet seedlings at different root depths (RD). Averages ± standard error. CFU = colony forming units arising from spores. Initial inoculum: 6.5 $\log_{10}$ (spores/seed pellet). Three replicates, each consisting of two plants were recovered without seed pellet after 12 days growth. ANOVA with root depth as repeated measure within subjects and all other factors as independent between subjects factors was done; degrees of freedom where adjusted according Greenhouse-Geiser. LSD at $P < 0.05$. Large letters are for the comparison between treatments within one root depth, small letters are for the comparison of different root depths within one treatment. No significant effects of soil type but a significant effect of root depth ($P = 0.0001$***) were found. Combination with *P. fluorescens* had no consistent significant effect (data not shown).

emergence). Plant development was also more vigorous in these two soils, and average root length was significantly greater (14–19 cm compared to 9–10 cm in all other soils). Emergence started after 10 days in the Tymbaki soil and 5–6 days in all other soils. Maximum emergence was reached after 10–12 days. Average emergence in the root colonisation assays was significantly greater (88%) when seeds received a combined treatment with *P. fluorescens* and *B. subtilis* compared to untreated controls (80%) and seeds treated with only one of the two antagonists (63–73%). Up to 30% of emerged plants showed damping-off symptoms (brown discoloration or dry thin sections of the hypocotyl) in non-inoculated soil. This indicates the presence of natural *Pythium* inoculum in the soils.

In soils inoculated with *P. ultimum*, plant survival in the controls without antagonist treatment was low (Figure 3). This confirms that the disease pressure was very high. Disease pressure was significantly lower in the soil from Tymbaki with 37.5% survival (average of six plants/microcosm) than in the soil from Uelzen, which showed the highest disease pressure with only 0–6% survival (0–1 plant per microcosm) in the untreated control (Figure 3). The controls of all other soils were not significantly different from each other and from Uelzen and Tymbaki soil (Figure 3). Depending on soil and antagonist treatment, 20–100% of the seeds did not emerge (pre-emergence damping-off), and a smaller proportion (0–40%) was lost due to post-emergence damping-off.

Plant survival (number of plants at the end of the experiment) and the shoot fresh weight of all healthy plants were significantly influenced by soil type ($P < 0.001$) and treatment with *P. fluorescens* B5 ($P < 0.001$), and there was a significant interaction between soil type and *P. fluorescens* B5 treatment for plant survival ($P = 0.006$) and fresh weight ($P < 0.001$). This demonstrates that soil type affects biocontrol activity by *P. fluorescens* B5 (Figure 3). Treatment with *P. fluorescens* B5 increased the number of apparently healthy plants and their fresh weight significantly in Craibstone, Cruden Bay and Magdeburg soils, but not in the

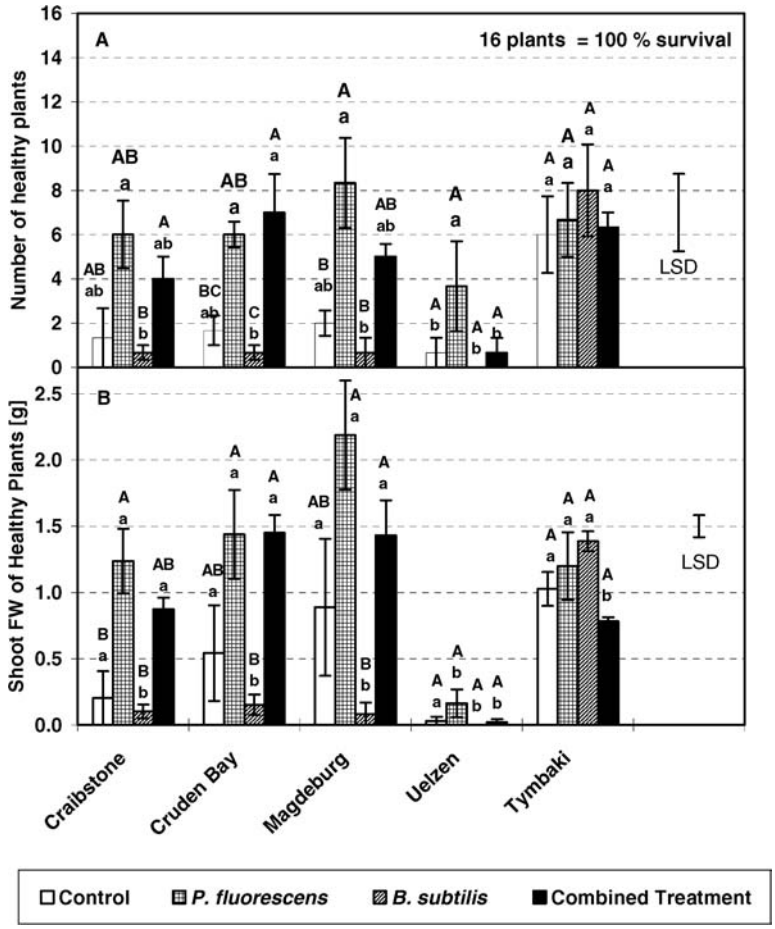

Figure 3. Effect of soil type on antagonistic activity of *Pseudomonas fluorescens* B5 and *Bacillus subtilis* MBI 600 against Pythium damping-off. Number and fresh weight (FW) of healthy sugar beet after 14 days growth. There were three replicate microcosms per treatment with 16 seeds per replicate. Initial antagonist inoculum: *P. fluorescens* B5 8 $\log_{10}$ (CFU/seed pellet); *B. subtilis* MBI 600 7 $\log_{10}$ (spores/seed pellet). Fresh Weight (FW) was square root transformed before analysis of variance and Tukey's Honestly Significant Difference tests were done. Large letters above bars are for comparison of antagonist treatments within each soil type; small letters for comparison of soil types within each antagonist treatment. LSD at $P < 0.05$ calculated from analysis of variance, not from Tukey's HSD tests. Significant effects of *P. fluorescens* ($P < 0.001$***), soil type ($P < 0.001$***) and a significant interaction between the factors soil type × *P. fluorescens* (number of healthy plants: $P = 0.006$**, square root FW: $P < 0.001$***) were found. *Bacillus subtilis* had a significant influence on square root FW ($P = 0.0064$**) but not on the number of healthy plants. There was no significant interaction between *P. fluorescens* B5 and *B. subtilis* MBI 600.

Uelzen and Tymbaki soils (Figure 3). The effect of *P. fluorescens* B5 in the three former soils was significant when *P. fluorescens* B5 as single antagonist treatment was compared to the corresponding untreated control, or when the combined treatment (*P. fluorescens* + *B. subtilis*) was compared to the corresponding treatment with *B. subtilis* MBI 600 alone (Figure 3). Disease pressure was significantly lower in Tymbaki soil (where *P. fluorescens* B5 had no significant effect) and was not significantly different between all the other soils (see above). Thus the observed effects are due to differences in soil type and not to differences in disease pressure in different soils.

B. subtilis MBI 600 had no significant antagonistic effect and also did not increase the performance of *P. fluorescens* B5 significantly (Figure 3). There was no significant interaction between the two antagonists when combined antagonist treatments were assessed.

The effect of single soil properties on biocontrol efficacy of *P. fluorescens* B5 (i.e. the increase in

plant number compared to the correspondent treatment without *P. fluorescens* B5) was investigated in more detail. Using multiple regression we found that overall 38% of the variation in biocontrol efficacy (transformed values) could be explained by the fractions of coarse silt (particle size 20–60 μm), fine silt + clay (particle size <20 μm) and pH ($P = 0.005$), demonstrating the influence of these soil properties over biocontrol efficacy (Table 3). The coefficient for organic carbon was not significant when this was included in the regression but this is probably because it is correlated with some of the other variables. When tested on its own there was a highly significant correlation with biocontrol (Table 3). Sand content was included implicitly in the regression model as this equals the remainder not included in coarse silt, fine silt and clay. When single parameters were tested, sand (particle size 60–2000 μm), fine silt (particle size 2–20 μm) and organic carbon formed highly significant correlations with biocontrol efficacy ($P < 0.006$, Table 3); fine silt and organic carbon had a positive effect, sand had a negative effect on biocontrol efficacy. Significant relationships with biocontrol efficacy could also be established for all other investigated soil properties, except soil pH, but correlation coefficients were rather low (R^2 0.15–0.21, Table 3).

Effect of soil pH on sugar beet seedling colonisation

Pseudomonas fluorescens B5 and *Bacillus subtilis* MBI 600 colonised sugar beet seedlings over the whole range of soil pH values included in this study (pH 5.0–7.8 measured in water, pH 5.0–7.2 measured in $CaCl_2$). In contrast to the experiments on the effect of soil type described above, *B. subtilis* MBI 600 was applied to the seeds as vegetative cells here. Only vegetative cells (i.e. no spores) could be recovered. Total rhizosphere populations of both *Pseudomonas* strains were greater (5.4–5.9 $\log_{10}$ CFU/plant) than those of *B. subtilis* MBI 600 (3.5–5.2 $\log_{10}$ CFU/plant; Table 4). The total population per plant increased significantly with increasing pH for *P. fluorescens* B5 and *B. subtilis* MBI 600 ($R^2 = 0.55$, $P = 0.0055$; Table 4).

Neither analysis of variance nor regression analysis (linear and polynomial, performed in each depth separately; data not shown) revealed any significant influence of soil pH on population density and distribution along the root of both *P. fluorescens* B5 and *B. subtilis* MBI 600 (Figures 4 and 5). This could be reproduced for *P. fluorescens* B5 in a repeated experimental run (data not shown). The population size of *P. fluorescens* B5 was greatest ($1–4 \times 10^6$ CFU/g FW) in the hypocotyl region and the upper root region (0–2 cm depth) and decreased significantly ($P < 0.001$) with increasing depth at all pH-values tested (Figure 4). Population densities of *B. subtilis* MBI 600 (vegetative cells) were 1–2 orders of magnitude less (log 4–log 5; Figure 5) than those of *P. fluorescens* B5 (log 6–log 6.5; Figure 5), especially in the upper regions (hypocotyls and 0–2 cm root depth) and did not decrease with root depth in the pH experiment. In contrast, a decrease of the population with depth was found in the experiment on the influence on different soil types, where cells had been applied and recovered as spores (Figure 2).

A highly significant relationship between population density and *in situ* bioluminescence was observed in *P. fluorescens* B5 ($R^2 = 0.465$, $P < 0.001$). As with population density, bioluminescence decreased significantly with depth ($P < 0.001$) for *P. fluorescens* B5. Soil pH had no effect on bioluminescence of *P. fluorescens* B5 (Figure 4, insert). Bioluminescence in deeper root regions was increased at higher soil pH (Figure 4, insert). It is notable that *in situ* bioluminescence was up to two orders of magnitude less at 0–2 cm root depth than in the hypocotyl section in the pH experiments, although population densities were similar in both sections (Figure 4).

In situ bioluminescence of vegetative populations of *B. subtilis* MBI 600 could be demonstrated in only one experimental run. Unlike root colonisation of *B. subtilis* MBI 600, *in situ* bioluminescence followed a pH optimum curve (Figure 5, insert). Around 10^5 relative luminescence units per gram root fresh could be measured at pH 6.0–6.7 whereas bioluminescence dropped 2–3 orders of magnitude at pH 5.0 and pH 7.5 (Figure 5, insert). However, in subsequent experimental runs, no *in situ* bioluminescence of vegetative (and spore) populations of *Bacillus subtilis* MBI 600 could be detected.

Effect of soil pH on biological control of Pythium *damping-off*

In the presence of *P. ultimum*, antagonist treatment had a highly significant effect on the number and shoot fresh weight of healthy plants ($P < 0.001$),

Table 3. Effect of soil properties (see Table 1) on biocontrol efficacy of *Pseudomonas fluorescens* B5

Soil properties	Multiple linear regression (R^2 = 0.38, P = 0.005)		Curve fit models for single properties					
	Coefficient	Significance of coefficient (P)		R^2	P	Constant	$b0$	$b1$
Constant	4.855 ± 1.08	<0.001***	–	–	–	–	–	–
soil pH	−0.45 ± 0.17	0.013*	Linear	0.120	0.060	13.050	–1.502	
Sand (60–2000 µm)	–	–	Linear	0.243	0.006**	6.929	–6.932	
Coarse silt (20–60 µm)	3.68 ± 1.68	0.038*	Quadratic	0.210	0.041*	7.875	–84.056	268.855
Fine silt (2–20 µm)	1.06 ± 0.5[a]	0.043*	Inverse	0.343	0.001***	5.976	−0.213	
Clay	–	–	Linear	0.146	0.037*	1.328	11.416	
Organic carbon	–	–	Inverse	0.312	0.001***	5.578	−1.259	
			Quadratic	0.341	0.004**	−1.033	7.141	−1.865

Multiple linear regression and curve fit. Data from Figure 3: Biocontrol efficacy of *P. fluorescens* B5 was calculated as the increase of plant number in the treatment containing *P. fluorescens* compared to the corresponding treatment which did not contain *P. fluorescens* (i.e. the *P. fluorescens* treatment was compared to the control, the combined treatment was compared to the treatment with single *B. subtilis* inoculum, respectively). Curve fit analysis was done with single properties as the independent value and biocontrol efficacy of *P. fluorescens* B5 as the dependent value. Linear, inverse and quadratic relationships were checked; only the most significant relationships are displayed. Coefficients $b0$ and $b1$ were always significant ($P < 0.03$) except for soil pH. The multiple linear regression equation was √(Increase in Plant No. +5) = 4.86 + 3.68 (coarse silt content) + 1.06 (fine silt + clay content) − 0.447 (soil pH in $CaCl_2$) (R^2 = 0.38, P = 0.005). Sand content was excluded from the multiple regression model because it was highly correlated with silt and clay content. Organic carbon was significant in curve fit analysis, but not in the multiple regression model.

[a] In the multiple linear regression model clay and fine silt were pooled (particle size <20 µm).

Table 4. Total population per sugar beet seedling of *Pseudomonas fluorescens* B5 and *Bacillus subtilis* MBI 600 (vegetative cells) at different soil pH valus (± standard error)

Soil pH ($CaCl_2$)	log_{10} (total CFU/plant)	
	Pseudomonas fluorescens B5	*Bacillus subtilis* MBI 600
5.0	5.4 ± 0.15 b	3.5 ± 0.41 b
5.4	5.7 ± 0.02 ab	4.1 ± 0.40 b
6.3	5.8 ± 0.13 a	4.2 ± 0.23 ab
7.2 (6.9)[a]	5.9 ± 0.07 a	5.2 ± 0.24 a
LSD	0.35	1.08
Increase with pH (LR)	$R^2 = 0.553$, $P = 0.0055$**	$R^2 = 0.552$, $P = 0.0056$**

Averages ± standard error were based on three replicates (2 plants per replicate), recovered after 12 days growth. Seed and seed pellet were not recovered. The total amount of recovered bacteria per plant was calculated as the sum of the CFU recovered from each section/2 (2 = number of recovered plants per replicate). Natural pH of Craibstone soil was pH 5.4 ($CaCl_2$); pH adjustment was done with $Al_3(SO_4)_2$ or $CaCO_3$, respectively. Soil pHs were measured in $CaCl_2$. Initial inoculum: *P. fluorescens* B5: 7.7 log_{10} (CFU/seed pellet), *B. subtilis* MBI 600: 5.3 log_{10} (CFU/seed pellet); *B. subtilis* MBI 600 was applied and recovered as vegetative cells. LSD at $P < 0.05$; linear regression (LR) revealed a significant increase of the total population per plant with soil pH.
[a] pH = 7.2 in Experiment with *P. fluorescens* B5, pH = 6.9 in experiments with *B. subtilis* MBI 600.

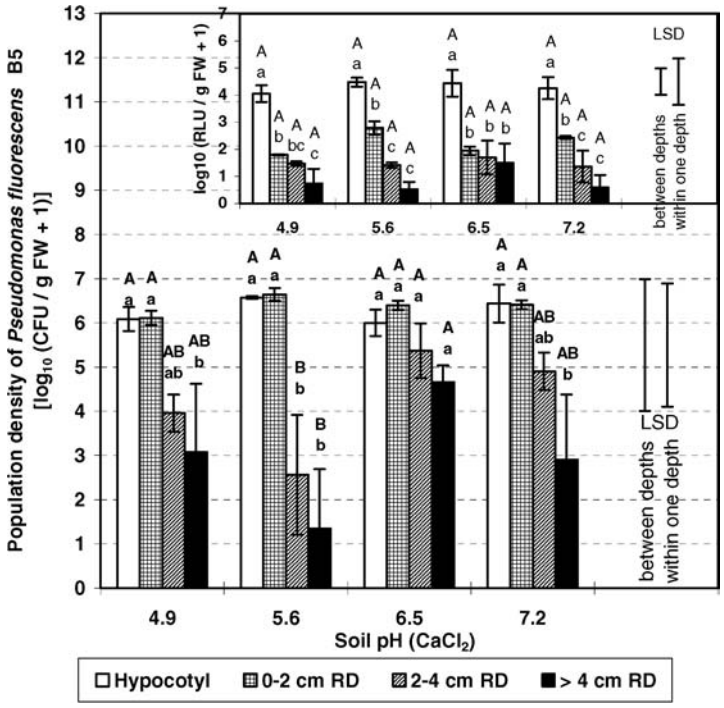

Figure 4. Population density and Bioluminescence (insert) of *Pseudomonas fluorescens* B5 (*lux*-marked strain) at different soil pH values on sugar beet seedlings at different root depths (RD). Averages + standard error (SE). CFU = colony forming units; RLU = relative luminescence units; FW = fresh weight. Natural pH of Craibstone soil was 5.4 ($CaCl_2$), pH 6 (H_2O); pH adjustment with $Al_3(SO_4)_2$ or $CaCO_3$, respectively. Soil pH was measured in $CaCl_2$. Initial inoculum: Three replicates, each consisting of two plants were recovered without seed pellet after 12 days growth. LSD at $P < 0.05$. Large letters are for the comparison between treatments within one root depth, small letters are for the comparison of different root depths within one treatment. Bioluminescence was significantly correlated with population density for *P. fluorescens* B5 ($y = 0.5002\ x - 0.266$, $R^2 = 0.4653$, $P < 0.001$***). Linear and polynomial regression and ANOVA with root depth as repeated measure within subjects and all other factors as independent factors revealed no significant influence of soil pH on the population density of *P. fluorescens*. Root depth had a significant effect on the population density and bioluminescence of *P. fluorescens* ($P < 0.001$***).

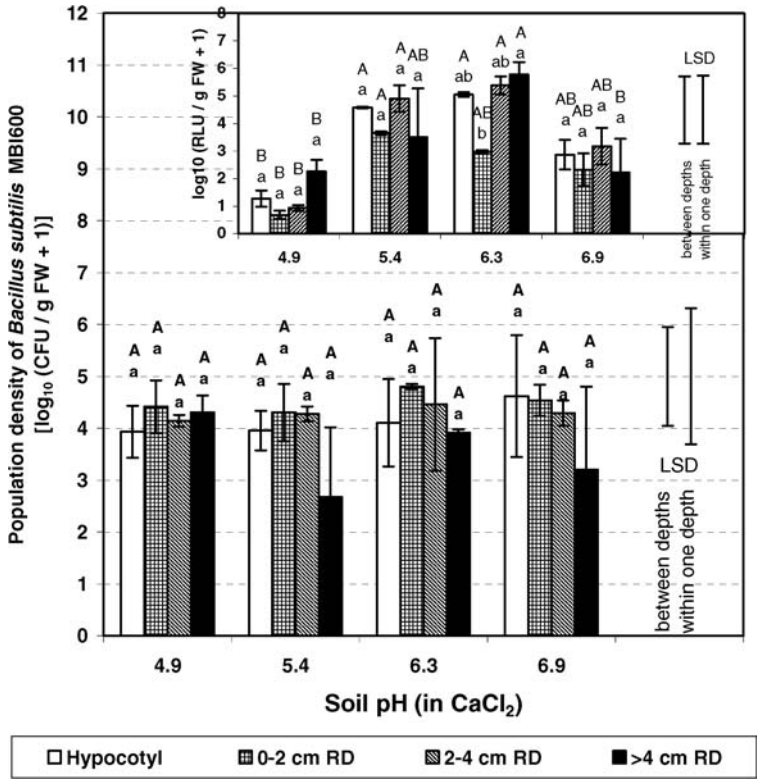

Figure 5. Population density and bioluminescence (insert) of *Bacillus subtilis* MBI 600 (*lux*-marked strain 33, vegetative cells) at different soil pH values on sugar beet seedlings at the hypocotyl and at different root depths (RD): Averages ± standard error (SE). CFU = colony forming units; RLU = relative luminescence units; FW = fresh weight; Craibstone soil, natural pH 5.4 ($CaCl_2$), pH 6 (H_2O); pH adjustment with $Al_3(SO_4)_2$ or $CaCO_3$, respectively. Soil pH was measured in $CaCl_2$. Three replicates, each consisting of two plants were harvested without seed pellet after 12 days growth. LSD at $P < 0.05$. Large letters are for the comparison between treatments within one root depth, small letters are for the comparison of different root depths within one treatment. Linear and polynomial regression and ANOVA with root depth as repeated measure within subjects and all other factors as independent factors revealed no significant influence of pH and root depth on the population density of *B. subtilis* MBI 600. Bioluminescence was significantly affected by soil pH ($P < 0.001$***).

but there was no significant effect of soil pH and also no significant interaction between soil pH and antagonist treatment (Figure 6). At all soil pH values, treatment with *P. fluorescens* B5 increased the survival of plants from 0 to 12.5% (average 0–2 plants per microcosm; 0–0.2 g shoot FW) observed in the untreated controls to 31–50% (average 5–8 plants per microcosm, 0.7–1.2 g shoot FW) in treated plants. Also, comparison of the number and shoot fresh weight at different pH values within each treatment using Tukey's Honestly Significant Difference test revealed no significant difference between the four soil pH's except in *B. subtilis* (Figure 6). However, when the treatments were compared within each pH, the increase of plant number and fresh weight in treatments with *Pseudomonas fluorescens* B5 was only significant at pH values above 6 (Figure 6).

The number and fresh weight of healthy plants in treatments with *B. subtilis* MBI 600 was significantly smaller than in treatments with the *Pseudomonas* strains in most cases. *B. subtilis* treatment resulted in a (small) increase in the number and fresh weight of healthy plants compared to the untreated control, but this effect was only significant at pH 6.9 (Figure 6).

Discussion

The plant survival rate in *Pythium* inoculated soil was uniformly low (<10%) compared to

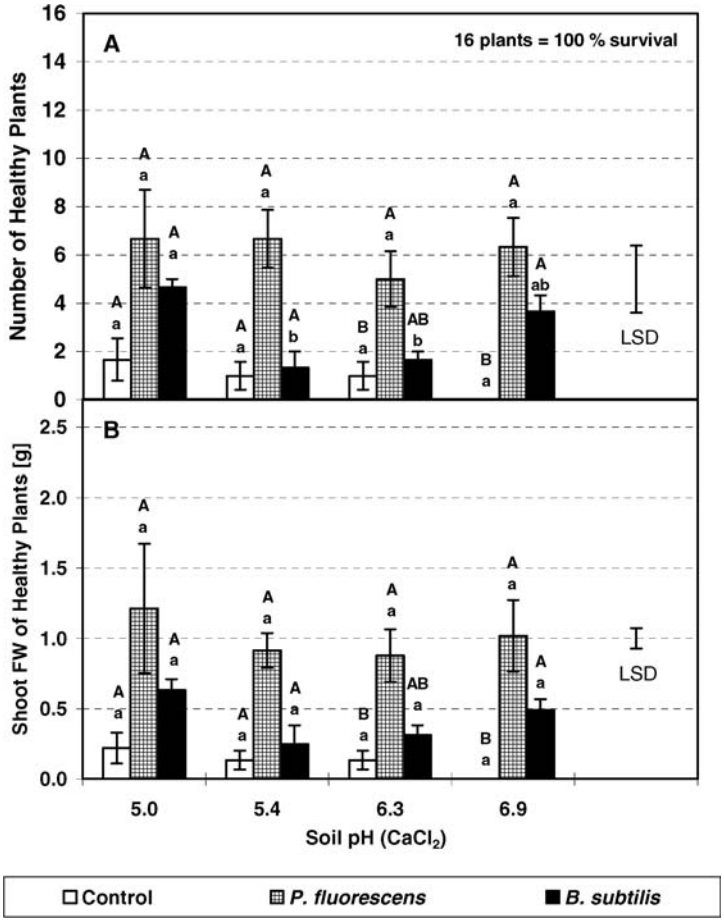

Figure 6. Effect of soil pH on antagonistic activity of *Pseudomonas fluorescens* B5 and *Bacillus subtilis* MBI 600 against Pythium damping-off. Number and fresh weight (FW) of healthy sugar beet plants after 14 days growth. There were three replicate microcosms with 16 seeds per microcosm. Initial inoculum: *P. fluorescens* B5: 7.8 $\log_{10}$ (CFU/seed pellet, *B. subtilis* 5.6 $\log_{10}$ (CFU/seed pellet); *B. subtilis* MBI 600 was applied as vegetative cells. Soil pH was measured in $CaCl_2$. Fresh weight (FW) was square root transformed test before analysis of variance and Tukey's Honestly Significant Difference tests were done. Large letters above bars are for comparison of antagonist treatments within each soil type; small letters for comparison of soil types within each antagonist treatment. LSD was calculated from analysis of variance, not from Tukey's HSD tests. A highly significant effect of antagonist treatment on number and fresh weight of healthy plants ($P < 0.001$***), but no effect of soil pH and no significant interaction between the factors soil pH and antagonist treatment was found.

non-inoculated soil (70–90%). This indicates a very high disease pressure. Thus, this is a worst case for antagonistic effects, since it has been shown that biocontrol is less effective at high disease pressures. For example, biocontrol efficiency of *Pseudomonas* against Fusarium wilt was only 0–20% under high disease pressure (80% disease incidence), whereas it rose to 100% at low disease pressure (20% disease incidence, Raaijmakers et al., 1995). The high disease pressure is likely to be the reason for the limited biocontrol efficacy of *P. fluorescens* B5 (~40% of plant survival even under favourable conditions). Disease pressure in the field is likely to be lower. The disease incidence was remarkably reproducible. Considerable variation of disease pressure has been observed in other studies (Fiddaman and Rossal, 1995) where similar semi-solid state inocula of soil-borne fungi had been applied.

Differences in disease pressure are not likely to be a significant reason for the observed differences in biocontrol efficacy in different soils. Low antagonistic activity was observed in the soil with the lowest disease pressure (Tymbaki) as well as in

the soil with the highest disease pressure (Uelzen), whereas significant biocontrol occurred in soils with intermediate disease pressure. Also, disease pressure was uniformly high at different soil pH values, although an effect of soil pH on *Pythium* species has been found in other studies (Dick and Ali-Shtayeh, 1986; Fukui et al., 1994a). Schmidt et al. (2004a) showed that matric potential and soil temperature had a pronounced influence on disease incidence.

A short growth period (12 days) was chosen in our root colonisation assays because this is the stage where sugar beet seedlings are most susceptible to Pythium damping-off (Martin and Loper 1999). Short-term survival and redistribution of the inoculum rather than active root colonisation may occur during this short time period (Russo et al., 1996). However, the population of *Pseudomonas* isolates can increase even during growth periods of 1–12 days (Ramos et al., 2000, Schmidt et al., 2004b) indicating active root colonisation (rhizosphere competence) by these isolates.

The small differences observed in root colonisation and activity in different soils are not likely to explain the large differences observed in biological control of Pythium damping-off by *P. fluorescens* B5. Root colonisation and general physiological activity alone appear to be insufficient predictors of antifungal activity. Soil composition may specifically affect the production of active compounds rather than the growth of the biocontrol strains in general (Bonsall et al., 1997). Antibiotics and siderophores have been shown to be the active compounds for antagonistic activity in *P. fluorescens* B5 (Schulz and Wolf, 2002). Antibiotics from biocontrol organisms have been isolated *in situ* only from nutrient rich microsites such as: the rhizosphere, spermosphere, plant debris, wounds and lesions (Weller and Thomashow, 1990). There was a weak but significant link between organic matter content and suppressiveness of soils (Persson and Olson, 2002) and compost had a general beneficial effect on the biological control of soil-borne diseases like Pythium damping-off and Rhizoctonia root rot (Hoitink and Boehm, 1999). In this study, the antagonist was efficient against Pythium damping-off only in the three soils richer in organic carbon, but not in two soils with low organic carbon content; the relationship between organic carbon content and biocontrol efficacy of *P. fluorescens* B5 was significant. This also points to a possible role of nutrient and carbon supply for effective biocontrol.

Contrary to these observations, organic matter and carbon content were negatively correlated with the biocontrol of *Gaeumannomyces graminis* by *P. fluorescens* 2-79 (Ownley et al., 1990, 1991). Differences between both biocontrol systems appear to exist also with respect to sand content. Ownley et al. (1990) found a positive correlation with sand content and biocontrol activity whereas biocontrol activity of *P. fluorescens* B5 against Pythium damping-off was very poor in the sandiest soil (Uelzen). Sandy soils were found to be less favourable to antagonistic microflora and more conductive to *Fusarium* wilt (Lindemann et al., 1983). Differences between strains and chosen target pathogens may account for the difference in outcome as strains of *Pseudomonas* differ in their environmental optima for biocontrol in relation to soil type (Ross et al., 2000).

Biocontrol efficacy of *P. fluorescens* B5 correlated positively with fine silt content (2–20 μm particle size). This fraction may reduce the accessibility of the bacteria to protozoan predators (Thirup et al., 2000) by providing pore sizes which can be colonised solely by the bacteria, but not by their predators.

Apart from soil components, the generally more vigorous growth of the sugar beet seedlings may be another reason for the improved biocontrol performance of *P. fluorescens* B5 in Craibstone, Cruden Bay and Magdeburg soils. This may lead to increased root exudation, which provides the biocontrol strain with the necessary nutrients. Root exudates and sloughed off cells from the root cap can contain up to 18% of assimilated carbon in plants (Barber and Martin, 1976) and are likely to contribute significantly to the carbon supply of the antagonists.

In this study, total rhizosphere population of *P. fluorescens* increased slightly with pH, but the effect was too small to be apparent in the population density of the different root sections. We detected no effect of soil pH on biocontrol efficiency of *Pseudomonas fluorescens* B5 and a concomitantly tested *P. corrugata* strain (strain 2140, isolated from Ryder and Rovira (1993), data not shown). Both pseudomonads exerted a similar degree of control in the range of pH 4.5–7. This was further revealed by the investigation of different soil types, as colonisation, activity and

biocontrol efficiency were equally high in Cruden Bay, Craibstone and Magdeburg soils, which varied in their pH from 6.0 to 7.2. Similar observations were made with *P. fluorescens* strains active against *Gaeumannomyces graminis* (Howie et al., 1987; Thomashow et al., 1990; Ownley et al., 1992). On the contrary, other authors (Duijff et al., 1995; Naseby and Lynch 1999) have found an increase of biocontrol performance of pseudomonads with soil pH in this range. Different bacterial strains, plant hosts and different target fungi and methodology (quantification of antifungal activity by measurement of fungal enzymes in soil) might account for these differences. In very alkaline soils (pH 8), Ross et al. (2000) found poor expression of biocontrol related genes. This may be one explanation for the observed negative correlation between soil pH and biocontrol activity in our experiment involving soils with higher pH (7.7). However, this conclusion remains speculative because there was considerable variation in other factors in the different soils, and the outcome also depended on the statistical method used.

There was no marked effect of soil type on the population densities of *P. fluorescens* B5 (i.e. treatment differences were mostly smaller than 1–2 orders of magnitude) in this study, which concentrated on the rhizosphere. On the contrary, studies performed in bulk soil showed a very pronounced effect of soil type on the survival of introduced *Pseudomonas* inocula (3–5 orders of magnitude; Hartel et al., 1994). Clay minerals increased the population density of introduced *P. fluorescens* inocula by three orders of magnitude in bulk soil, but only by one order of magnitude in rhizosphere soil, where it was generally higher (Van Elsas et al., 1986; Heijnen et al., 1993). Thus, the rhizosphere appears to be a more protected environment than bulk soil and buffers against extreme environmental conditions. This may also be true for adverse pH conditions, as the pH in rhizosphere might be different from the pH in soil. In the rhizosphere of sugar beet, survival of *P. fluorescens* B5 (and *Pseudomonas corrugata* 2140) was not diminished even at a soil pH of 4.5–5 whereas in bulk soil studies, populations of *P. corrugata* decreased markedly from 10^9 CFU/g soil to zero within a similar incubation period of 15 days at pH 4.0 (White et al., 1996).

At an extreme soil pH of 4.5, sugar beet seedlings showed stunted root growth and root length did not exceed 2 cm, probably due to aluminium toxicity. The aluminium sulphate used to lower the soil pH can increase availability of phytotoxic Al^{3+} (White and Cresser, 1998). No negative effects on plant growth were observed at a pH of 5.0 and above.

Increased colonisation down the root by rhizosphere pseudomonads in sandy soils was found in this as well as other studies (e.g. Bahme and Schroth, 1987; Trevors et al., 1990). The maximal population size in the upper root reached $\sim 10^5$ CFU/cm root in this and most other studies (e.g. Howie et al., 1987; Liddell and Parke, 1989; Heupel, 1992) irrespective of soil type, soil pH and plant host. This appears to be the carrying capacity of the root. The observed decrease in the population size with root depth of the seed inoculants is in accordance with the findings of numerous authors (e.g. Bahme and Schroth, 1987; Trevors et al., 1990; Ramos et al., 2000). Liddell and Parke (1989) found that movement of *Pseudomonas* inocula down the root was proportional to downward water percolation (i.e. the amount of water added the top of the soil column). We minimised water percolation by compensating for water losses by the addition of water at several depths in order to create conditions least favourable for the spread of the biocontrol inoculum down the root and to simulate a worst-case scenario. However, as water had to spread a distance of up 2 cm from the points of injection, localised microbial movement after rewatering could not be completely excluded. The spread of *P. fluorescens* B5 down the root was decreased compared to Heupel's (1992) study, where plants were not rewatered and thus water percolation was minimised. Our results were also similar to the distribution observed by Liddell and Parke (1989) in another strain of *Pseudomonas* when they added small amounts (8.8 mm) of water from the top. These comparisons suggest that water movement played only a minimal role in distribution of the microbial inoculum in the present study.

Bioluminescence of the *Pseudomonas* strains used in this study was strongly correlated with dehydrogenase activity in *in vitro* cultures and is thus a good indicator for physiological activity (Russell, 1996; Knox, 2000; Knox et al., 2002). In addition, bioluminescence responds even more readily to changes in the physiological status (Meikle et al., 1994) than dehydrogenase activity

and thus represents physiological activity more accurately. Particularly at greater root depth, bioluminescence could be measured, although no colonies could be recovered by plating methods. This indicates the presence of a larger proportion of unculturable cells, because plate counts are more sensitive than bioluminescence measurements (i.e. the detection limit is 10^2 cells per cm root compared to 10^4–10^5 cells per cm root; Meikle et al., 1992; Ramos et al., 2000; Sørensen et al., 2001). Unculturable cells can form a significant proportion of the population of pseudomonads in rhizosphere and soil (Sørensen et al., 2001; Chapon et al., 2003). Thus the measured increase in downward colonisation of *P. fluorescens* B5 in the presence of *B. subtilis* MBI 600 might be either due to a real increase in the number of cells or through an increase in the culturability of the cells.

Pseudomonas strains belonging to the species *P. putida* and *P. fluorescens* had been shown to prevent sugar beet pericarp seed colonisation of co-inoculated *B. subtilis* at temperatures lower than 36 °C (Fukui et al., 1994b). We found no negative interaction; on the contrary, colonisation down the root by *P. fluorescens* B5 was enhanced in different soils when supplied as a mixed inoculum with *B. subtilis* MBI 600. A positive interaction between these two strains was also observed *in vitro* (Georgakopoulos et al., 2002). Differences in inoculum preparation and physiological state of *Bacillus* (presence as vegetative inoculum or as spores) could lead to differences in root colonisation behaviour and antagonistic properties as shown in this study. Furthermore it must be noted, that Fukui et al. (1994b) studied root colonisation on naked sugar beet seeds, whereas pelleted seeds were used in this study.

The rhizosphere population of *B. subtilis* MBI 600 consisted mainly of dormant spores if it was applied as spore inoculum. This is the likely explanation for its observed lack of biocontrol activity against *P. ultimum* (Georgakopoulos et al., 2002; this study). *Bacillus subtilis* MBI 600 is registered as an antagonist effective against *Fusarium*, *Rhizoctonia* and *Aspergillus* (Becker Underwood Inc., Ames, Iowa, USA). The vegetative inoculum applied in the experiment on soil pH showed some, albeit very weak biocontrol activity. The dormant state (as spores) also explains why rhizosphere populations of *B. subtilis* MBI 600 were generally less affected by soil type compared to *P. fluorescens* B5. No large turnover from vegetative cells to spores and from spores to vegetative cells was observed, contrary to other investigations (Mahaffee and Backman, 1993, Berger et al., 1996; Lee and Stotzky, 1999); although short cycles of spore germination and re-sporulation as observed by Van Elsas et al. (1986) cannot completely be excluded.

Soil pH between 4.5 and 7.2 is not a limiting factor for the biocontrol of Pythium damping-off by *P. fluorescens* B5 in sugar beet, but the biocontrol performance is clearly dependent on soil type. If insufficient nutrient supply is the reason for the failure of *P. fluorescens* B5 in some soils, appropriate formulation additives might solve the problem.

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