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Colonization of the developing rhizosphere of sugar beet seedlings by potential biocontrol agents applied as seed treatments

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Aims: Poor colonization of the rhizosphere is a major constraint of seed treatment biological control. The objectives of this study were to; examine the colonization of the rhizosphere of sugar beet seedlings by selected rhizobacteria; determine the influence of the host rhizosphere and percolating water on the distribution of the bacteria; and deliver two biological control agents (BCAs) by co-inoculation.
Methods and Results: Rifampicin-resistant bacterial strains (*Rif*$^{+}$) applied as single treatments to seed sown in columns of field soil produced persistent populations of 5–9 $\log_{10}$ cfu g^{-1} in the infection court of the damping-off pathogen *Aphanomyces cochlioides* in a controlled environment. However, isolates varied in their ability to colonize the lower rhizosphere. Percolating water significantly increased the colonization of the upper rhizosphere. Bacterial populations in the soil profiles of ''non-rhizosphere'' controls declined markedly with time. There was no interaction between the two selected BCAs applied as a seed treatment mixture.
Conclusions: The distribution of the bacteria resulted primarily from root colonization although percolating water may modify the colonization profiles. Co-inoculation of the sugar-beet rhizosphere is a viable proposition.
Significance and Impact of Study: Potential BCAs were successfully delivered to the known infection court of *A. cochloides* and persisted for the infection period. This bioassay can be used as a tool for the selection of BCAs for field trials.

INTRODUCTION

One of the major constraints of seed treatment biological control is poor colonization of the rhizoplane and rhizosphere by the introduced biocontrol agent (BCA) (Deacon 1994). This has been variously attributed to; predation, competition and nutrient limitation (Thompson *et al.* 1990); the variability of physical, chemical, microbiological and environmental factors (Kim and Misaghi 1996); and poor root surface colonization or an inability to be transported through the soil profile via the root (Chao *et al.* 1986).

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This study was undertaken to examine the colonization of the developing seedling rhizosphere of sugar beet by selected rhizobacteria and to determine the respective roles of the host and the movement of percolating water on this pattern of colonization. The effect of co-inoculation of two selected rhizobacteria on their colonization profiles in the seedling rhizosphere was also determined.

Five bacterial isolates have previously been selected from biocontrol studies which, when applied to pelleted sugar-beet seed, showed consistent activity against *Aphanomyces cochlioides* blackleg in controlled environment tests (Williams and Asher 1996). This approach is being developed as a possible alternative to Tachigaren® seed treatment (active ingredient: hymexazol) which is currently used on over 2 million hectares per annum in Europe (Asher and Dewar 1994). This constitutes an enormous selective pressure for

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the development of hymexazol-resistant strains of the pathogen and no alternative form of *Aphanomyces* control is currently available.

A. cochlioides is a soil-borne pathogen that causes damping-off of sugar-beet for up to four weeks postemergence in the field. The first symptoms appear as a shrunken black lesion on the hypocotyl spreading to the cotyledons and tap root. As the first symptoms are observed on the upper root and hypocotyl at the soil surface (Papavizas and Ayers 1974), for successful control it is imperative that this infection court is targeted and well colonized by the introduced BCAs. However, observations indicate that the pathogen may also infect the lower root system, particularly when plants survive an earlier attack. Colonization of the entire rhizoplane by a BCA could therefore offer a distinct advantage in terms of disease control.

To test the comparative rhizosphere competence of the BCAs, a rifampicin-resistant-marked strain of each isolate was introduced as a pelleted seed treatment into a field soil bioassay under controlled environmental conditions. During four weeks of seedling development, the bacterial numbers present on sections of the rhizosphere plus the seed pellet, raw seed and hypocotyl were determined for each isolate. These data were used to construct a rhizosphere colonization profile for each BCA. Non-viable seed treated with each BCA was used as 'non-rhizosphere' control to determine the influence of the host rhizosphere. The effect of percolating water on the location and distribution of *B. megaterium* in the soil profile was examined. Co-inoculation of the two most promising rhizosphere colonizers was also carried out to investigate the feasibility of delivering two bacteria into the infection court to act in concert.

MATERIALS AND METHODS

Chemicals and microbiological media

Unless otherwise stated all chemicals were obtained from Sigma (Dorset, UK) and microbiological media from Oxoid (Basingstoke, UK).

Bacterial wild-type and antibiotic-resistant marked strains

The five strains of bacteria used throughout this study were previously isolated from roots of sugar-beet seedlings and selected on the basis of their antagonism *in planta* to *Aphanomyces cochlioides* (Williams and Asher 1996). The isolates were identified by fatty acid methyl ester (FAME) profiling as strains of *Arthrobacter histidinolovorans*, *Bacillus megaterium*, *Cytophaga johnsonae*, *Pseudomonas fluorescens* and *Pseudomonas syringae* (Williams and Asher 1996).

A rifampicin-resistant strain of each of the five wild-types was generated by spread plating bacterial liquid cultures in Tryptone Soya Broth (TSB), that had been incubated for 16 h at 20°C, onto Nutrient Agar (NA) containing 25 $\mu g\ ml^{-1}$ rifampicin. The resulting rifampicin-resistant colonies were then streak plated onto NA containing 250 $\mu g\ ml^{-1}$ rifampicin (NAR) and Rif^{+} strains were subsequently maintained on this medium. A strain of *B. megaterium* with dual antibiotic-resistance to streptomycin and kanamycin was also produced to facilitate selective re-isolation of the two species from a dual mixture in the co-inoculation study. The $Strep^{+}$ strain was selected on NA containing 25 $\mu g\ ml^{-1}$ streptomycin and maintained on NAS (streptomycin @ 250 $\mu g\ ml^{-1}$). Resistance to the second antibiotic was then generated on NA containing 10 $\mu g\ ml^{-1}$ kanamycin and 250 $\mu g\ ml^{-1}$ streptomycin. The resulting $Strep^{+}/Kan^{+}$ strain was subsequently maintained on NA containing 100 $\mu g\ ml^{-1}$ kanamycin and 250 $\mu g\ ml^{-1}$ streptomycin (NASK).

Application of bacterial strains to pelleted seed

All liquid cultures were incubated at 20°C and 100 rev min^{-1} in rotary culture (Gallenkamp, UK). Conical flasks containing 100 ml TSB were inoculated with 100 μl bacterial suspension from a 24-h culture in Nutrient Broth (NB; Oxoid, CM1). Flasks were incubated until each isolate/strain had reached the mid-log phase of growth (determined previously by growth curve studies). Resulting bacterial cells were spun down at 8000 *g* and 22°C for 30 min (4K10 Centrifuge, Sigma, Poole, Dorset, UK), the supernatant was discarded and the pellet re-suspended in 100 ml sterile distilled water (SDW). This washing procedure was repeated twice. The concentration of each bacterial isolate was determined by total cell counts in an Improved Neubauer haemocytometer (Weber, Lancing, Sussex, UK) and was adjusted to 9 log_{10} cells ml^{-1}.

Bacterial strains were applied to seed as described by Williams and Asher (1996). Standardization was achieved by applying 500 μl aliquots of the cell suspensions to 10 pelleted seeds in a single compartment (20 mm × 20 mm) of a 25 well repli dish (Bibby Sterilin, Stone, Staffordshire, UK) to give a final application rate of 7·5 log_{10} cells $seed^{-1}$. The treatments were allowed to soak into the seed pellet for 6 h and then dried for 16 h in a laminar flow cabinet at *ca.* 20°C.

Rhizosphere competence bioassay

The location and distribution of the isolates in the rhizosphere was determined using a split-tube bioassay modified from the methods of Williams *et al.* (1997). Tubes 30 cm in height were prepared from plastic tubing with an internal diameter of 6·5 cm. The tubes were cut in two

lengthways and the two halves re-joined with PVC tape. A strip of mesh was secured to the bottom of each tube with an elastic band to retain the soil whilst allowing for drainage. The tubes were filled with field soil obtained commercially (Hewitt Toptex Sportsturf, Petersfield Products, Cosby, Leicestershire, UK). Soil was firmed and levelled until 3·5 cm from the top of the column. Five seeds treated with a single bacterial suspension were then sown in each tube, covered with soil to 0·5 cm from the top of the column and watered immediately with 100 ml water. Columns were covered with polythene and newspaper secured with elastic bands to prevent drying out. They were arranged in randomised blocks according to sampling date in a controlled environment at 20°C constant temperature with a 16 h photoperiod of 250 μmol/m^2 s^{-1}. Covers were removed after first seedling emergence and tubes watered daily from above thereafter.

At four sample times after sowing, the tubes were assessed for bacterial survival. The shoots of the seedlings were severed just above soil level and the leaves removed from the upper hypocotyl sections. Columns were opened and the root systems carefully removed without damaging the tap roots. Loosely adhering soil was removed by gently rubbing along the tap root length. Seedlings were then divided into sections consisting of the lower part of the hypocotyl (below the soil surface) and five root sections @ 5 cm in length, numbered from 1 to 5 starting with the uppermost section. The pelleted seed was also recovered and the seed coat and pellet assessed separately for survival of bacteria. For each section type, the tissue samples from the five seedlings in each replicate column were bulked, weighed and homogenized in Maximum Recovery Diluent (MRD) 1 g tissue in 9 ml MRD for 15–30 s at *ca*. 10 000 *g*, using an Ultra-Turrax T25 (IKA®-Labortechnik, Janke & Kunkel, GmbH & Co., KG, Staufen, Germany). Homogenized samples were serially diluted in MRD and selected dilutions were spiral-plated (Model D Spiral Plater, Don Whitley Scientific Limited, Shipley, West Yorkshire, UK) onto NAR amended with the antifungal agent cycloheximide (NARC). Plates were incubated at 30°C and the numbers of re-isolated colony forming units (cfu) calculated.

Comparison of rhizosphere colonization profiles of the *Rif*-marked isolates

Each of the *Rif*$^+$ isolates (excluding *B. megaterium*) was cultured, applied to seed and sown in a separate rhizosphere competence bioassay, as described above. At sample times of 7, 14, 21 and 28 d after sowing, triplicate were assessed for bacterial survival on each of the plant sections described. The mean data were used to construct a rhizosphere colonization profile for each of the *Rif*-marked isolates which were then compared statistically.

The influence of the host rhizosphere on the distribution of the isolates in the soil profile

Additional treatments of each of these four isolates were prepared as 'non-rhizosphere' controls to determine the influence of the host plant and the movement of percolating water on the location and distribution of each of the isolates in the soil profile. The controls consisted of tubes sown with seed killed by heat treatment at 80°C for 24 h. The nonviable seed was then treated with bacterial suspensions and sown as described previously. One 'non-rhizosphere' control was assessed per isolate at each sample time. Statistical comparisons were made between the rhizosphere colonization profile and the corresponding control for each isolate tested.

The influence of percolating water on the distribution of *Bacillus megaterium* in the soil profile

Additional columns prepared with seed treated with the *Rif*$^+$ isolate of *B. megaterium* were watered from reservoirs at the column base. Duplicate columns were assessed at each sample time concurrently with the columns of this isolate watered from above referred to previously. Statistical comparisons were made between these treatments to determine the effect of the movement of percolating water in the presence of the host on the rhizosphere colonization profile of *B. megaterium*.

Effect of co-inoculation of *Ps. syringae* and *B. megaterium* on rhizosphere colonization

A strain of *B. megaterium* with dual antibiotic-resistance to streptomycin and kanamycin was used to facilitate selective re-isolation when co-inoculated with *Rif*-marked *Ps. syringae*. Split-columns were prepared containing seed treated with these two antibiotic-marked isolates applied as single strains or as a mixture. Mixtures were applied by combining 250 μl aliquots of a suspension (9 $\log_{10}$ cells ml^{-1}) of each isolate and adding this to 10 seeds, using the method described previously. At the sample time described previously, columns were assessed in triplicate for each of the three treatments.

Statistical analysis

Bacterial numbers were expressed as cfu g^{-1} sample following logarithmic transformation. Significant differences between sample means (Fisher's protected LSD ($P = 0{\cdot}05$)) were determined by ANOVA analysis of variance performed with Genstat V.

RESULTS

Rhizosphere competence bioassay

Although the numbers of bacterial cells declined below the initial application rate during seed drying, once the seed treatments had been re-hydrated in the bioassay system the cell numbers rapidly increased in the infection court (Fig. 1). High cell numbers (6–9 $\log_{10}$ cfu) of all five bacterial isolates were consistently recovered from the pellet and seed over the 28 d test period. All isolates were also present on the lower hypocotyl and first root section at all sample times. However, the bacteria varied in their ability to colonize the middle and lower root sections. In general, bacterial numbers were highest on the seed and pellet and declined down the tubes away from the point of inoculation.

Comparison of rhizosphere colonization by the five *Rif*$^{+}$-marked isolates

The rhizosphere colonization profiles of the isolates at 7 d and 28 d are shown in Fig. 1a and 1b, respectively. *Ps. syringae* was the most rhizosphere competent isolate of the five bacteria tested, displaying consistent colonization with high cell numbers (5–9 $\log_{10}$ cfu g^{-1}) on all rhizosphere sections at all sample times. Even the upper hypocotyl, above soil level, supported a high population of cells. *B. megaterium* displayed promising rhizosphere activity on all samples taken from the infection court and also colonized the rest of the developing rhizosphere. All these bacterial populations persisted for the duration of the experiment. *A. histidinolovorans* was also observed in high numbers on all samples taken from the infection court. Smaller populations were detected on the upper hypocotyl and almost all of the lower root samples.

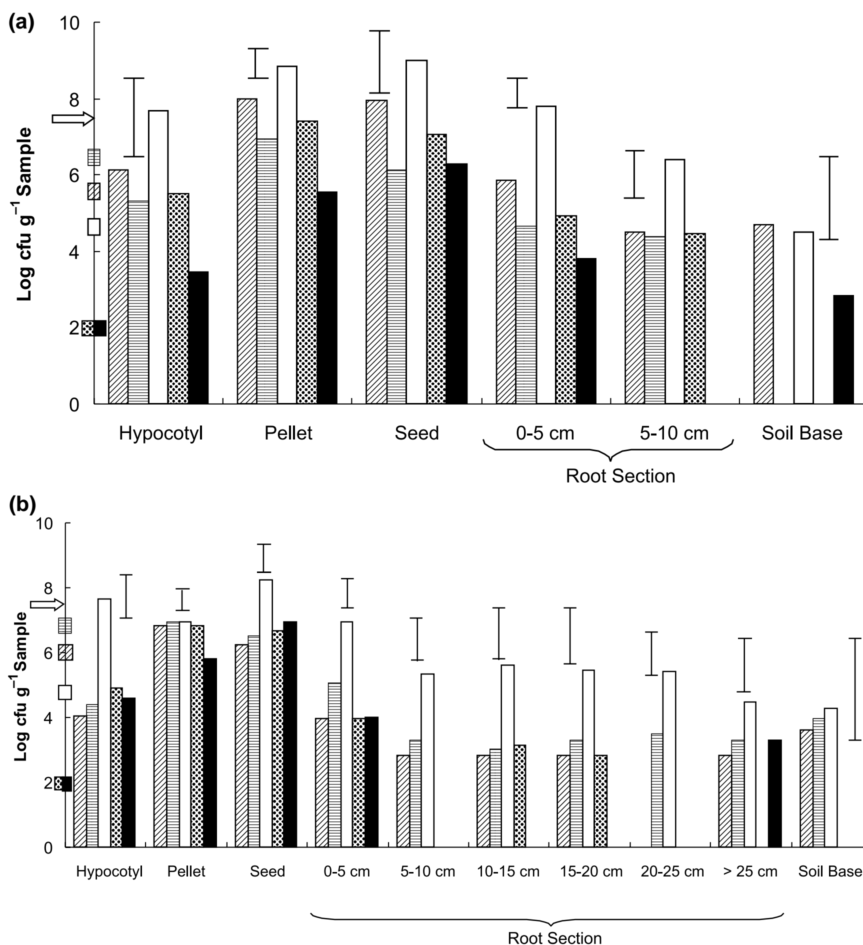

Fig. 1 Comparison of the colonization profiles of five potential biocontrol agents on sugar beet seedlings (a) 7 d and (b) 28 d after sowing. → Application rate to seed. Blocks on *y*-axis denote population of bacteria surviving seed drying. ▨, *Arthrobacter histidinolovorans*; ▤, *Bacillus megaterium*; □, *Pseudomonas syringae*; ⚄, *Pseudomonas fluorescens*; ■, *Cytophaga johnsonae*. Bars represent Least Significant Differences between sample means (Fisher's protected LSD ($P = 0{\cdot}05$)), determined by ANOVA analysis of variance

Therefore, whilst *A. histidinolovorans* colonized almost the entire rhizosphere, its activity was not as prolific as that of *Ps. syringae* or *B. megaterium*. In contrast, although *Ps. fluorescens* and *Cyt. johnsonae* displayed good survival in the infection court, these isolates showed comparatively poor colonization of the middle and lower root sections. The overall rhizosphere competence of the isolates has been determined statistically as; *Ps. syringae*^{a} > *B. megaterium*^{b} > *A. histdinolovorans*^{b} > *Ps. fluorescens*^{c} > *Cyt. johnsonae*^{c} (where significant differences between isolates are denoted by 'a–c').

The influence of the host rhizosphere on the distribution of the isolates in the soil profile

The rhizosphere colonization profiles of the five *Rif* -marked isolates and their behaviour in the corresponding 'non-rhizosphere' controls are compared in Table 1. In contrast to the high degree of colonization in the rhizosphere profiles (A) only the spermosphere samples consistently supported large bacterial populations of the isolates in the 'nonrhizosphere' controls (B). The exception to this was *Cyt. johnsonae* which displayed poor colonization throughout the column. In general, bacterial populations were also present at the soil base and at the soil surface. Population densities equivalent to those in the corresponding colonization profiles were found in these samples and in the spermosphere. Apart from the soil samples directly below the spermosphere (Soil 1) the remaining soil samples were largely devoid of bacteria. A typical comparison of the vertical distribution of bacteria in the presence and absence of the developing host over the duration of the experiment is presented in Fig. 2, using *Ps. syringae* as an example. This isolate showed consistent colonization of all rhizosphere sections at all sample times

Table 1 Comparison of (A) the rhizosphere colonization profiles with (B) the distribution in the 'non-rhizosphere' controls of the five *Rif*-marked isolates

A Colonization (log cfu g^{-1}) of rhizosphere sections*

Rhizosphere section‡	*Arthrobacter histidinolovorans*	*Cytophaga johnsonae*	*Pseudomonas fluorescens*	*Pseudomonas syringae*	LSD†
Upper Hypocotyl	2·05^{b}	0·93^{b}	1·80^{b}	5·38 ^{a}	1·56
Lower Hypocotyl	4·87^{b}	3·33^{c}	4·16	7·05^{a}	1·18
Pellet	7·10^{b}	5·83^{c}	6·73	7·64^{a}	0·40
Seed	6·79	7·05^{b}	6·22^{c}	7·93^{a}	0·70
Root 1 (0–5 cm)	5·02	3·49^{c}	3·43^{c}	7·01^{a}	0·77
Root 2 (5–10 cm)	2·90^{b}	0·36^{c}	1·40^{c}	5·34^{a}	1·06
Root 3 (10–15 cm)	1·27	0·00	0·71^{c}	4·38^{a}	1·30
Root 4 (15–20 cm)	1·28^{b}	0·36^{b}	0·37^{b}	3·65^{a}	1·57
Root 5 (20–25 cm)	0.00	0·00	0·00	2·62	1·36
Root Base (> 25 cm)	0·86^{b}	0·65	0·00	2·26	1·63

B Colonization (log cfu g^{-1}) of soil sections in 'non-rhizosphere' controls*

Soil column section	*Arthrobacter histidinolovorans*		*Cytophaga johnsonae*		*Pseudomonas fluorescens*		*Pseudomonas syringae*	
Soil surface	1·34	(0·76)†	0·00	(3·00)	**4·16**§	(1·53)	1·20	(0·60)
Pellet	6·42	(0·37)	1·40	(0·86)	**7·64**	(0·41)	**7·58**	(0·90)
Seed	6·40	(0·23)	2·55	(1·63)	**7·63**	(0·30)	7·39	(0·71)
Soil 1 (0–5 cm)	0·00	–	0·90	(1·61)	1·65	(1·52)	1·02	(0·92)
Soil 2 (5–10 cm)	0·00	–	0·00	–	0·00	–	3·27	–
Soil 3 (10–15 cm)	0·00	–	0·00	–	0·00	–	0·83	–
Soil 4 (15–20 cm)	0·00	–	0·00	–	0·00	–	0·00	–
Soil 5 (20–25 cm)	0·00	–	0·00	–	0·00	–	0·00	–
Soil Base (> 25 cm)	**1·04**	(2·38)	**1·33**	(1·27)	**3·05**	(1·27)	**2·17**	(2·55)

*Data shown are means from triplicate columns over four sampling times pooled for each isolate.
†Significance tested using Fisher's protected LSD ($P = 0{\cdot}05$) shown in parentheses.
‡Significant differences between treatments within a rhizosphere section type denoted by 'a–c'.
§Values in bold type are not significantly lower than corresponding values in presence of host rhizosphere (Table 1B).

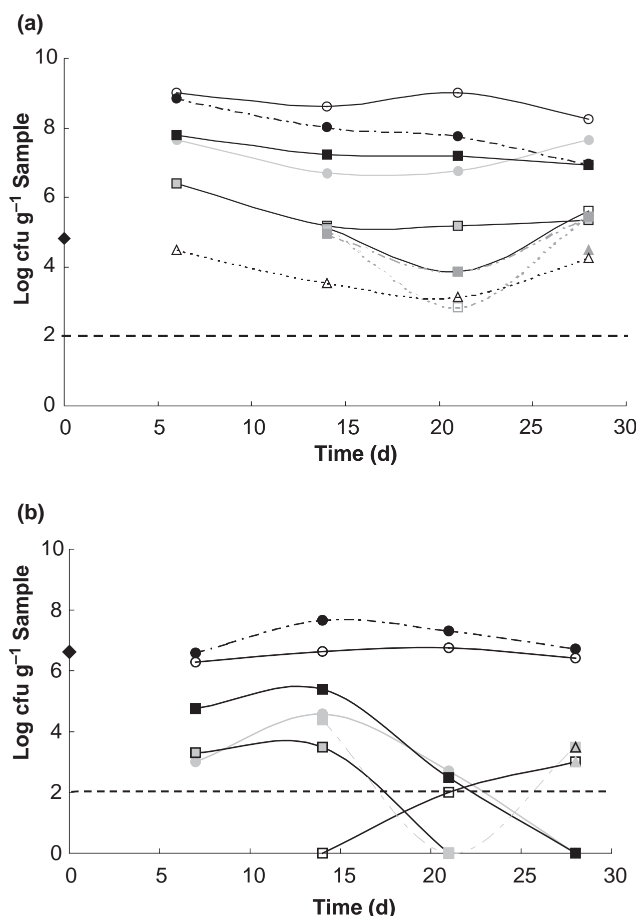

Fig. 2 Comparison of (a) the rhizosphere colonization profile of *Pseudomonas syringae* (P22P104) with (b) the nonrhizosphere control over 28 d from sowing. ●, Pellet; ○, Seed; ● Hypocotyl; ■, Root 1 (0–5 cm); ■, Root 2 (5–10 cm); □, Root 3 (10–15 cm); ■ Root 4 (15–20 cm); □, Root 5 (20–25 cm); ▲ Root Base (> 25 cm); △, Soil Base; ◆, Air-Dried Seed; – – – – –, Detection Limit

in high cell numbers (5–8 $\log_{10}$ cfu) (Fig. 2a). Despite the prolific colonization in the spermosphere, the cell numbers in the soil samples of the 'nonrhizosphere' control varied between 3 and 5 $\log_{10}$ cfu at 7 d and then declined with time to nondetectable levels by 28 d (Fig. 2b). Stable populations in the spermosphere and declining populations in the soil profiles were also observed in the 'non-rhizosphere' controls of the remaining isolates; in *Cyt. johnsonae* no viable cells were detected in the rhizosphere after 14 d (data not shown).

The influence of percolating water on the distribution of *B. megaterium* in the soil profile

The movement of percolating water had a significant effect on the rhizosphere colonization profile of *B. megaterium* (Table 2). In the upper rhizosphere, despite large bacterial populations surviving in the spermosphere, colonization was significantly lower in the columns that were watered from below when pooled data from all sample times were analysed. Although there were no significant differences between the two watering treatments in the lower rhizosphere, the columns watered from below again supported smaller bacterial populations on all sections except Root 5. When comparing individual sampling times it is clear that, with the exception of the spermosphere samples, none of the rhizosphere samples watered from below could support a stable bacterial population. Populations fluctuated markedly, falling below the detection limit on most of the root samples after 14 d. This contrasts with the stable bacterial populations in the columns watered from above. Despite this the isolate could still produce peak populations in the infection court of > 7 $\log_{10}$ cfu g^{-1} in the absence of percolating water.

Table 2 Comparison of rhizosphere colonization profiles of *Bacillus megaterium* when watered from above and below

Colonization (log cfu g^{-1})*	Watered from above					Watered from below					
Rhizosphere sections	7 d	14 d	21 d	28 d	Total	7 d	14 d	21 d	28 d	Total	LSD†
Hypocotyl	4·57	4·92	4·13	2·20	3·96^a	1·65	3·66	1·35	0·00	1·67^b	2·05
Pellet	6·98	7·72	8·02	6·89	7·40^a	6·59	7·65	7·14	6·64	7·01^b	0·37
Seed	6·16	7·07	6·69	6·52	6·60	6·11	6·59	6·74	6·37	6·45	0·67
Root 1 (0–5 cm)	4·41	5·19	4·88	4·17	4·66^a	4·18	5·26	1·89	0·00	2·83^b	1·37
Root 2 (5–10 cm)	3·58	4·33	1·65	3·31	3·22^a	1·80	1·89	0·00	0·00	0·92^b	1·96
Root 3 (10–15 cm)	2·67	3·55	1·89	1·74	2·46	0·83	0·00	1·65	1·65	1·03	2·46
Root 4 (15–20 cm)	1·68	1·02	1·65	1·80	1·54	0·00	0·00	0·00	0·00	0·00	–
Root 5 (20–25 cm)	1·66	2·16	1·65	1·65	1·78	1·98	2·34	0·00	3·46	1·95	2·67
Root Base (> 25 cm)	2·54	2·54	1·80	3·31	2·55	1·75	1·75	0·00	3·46	1·74	1·78

*Mean data from duplicate columns presented at each sampling time.
†Significant differences between totals tested using Fisher's protected LSD ($P = 0{\cdot}05$) and denoted by 'a-b'.

Table 3 Comparison of the rhizosphere colonization profiles of *Bacillus megaterium* and *Pseudomonas syringae* applied as single strains and as a mixture

Colonization (log cfu g^{-1}) of rhizosphere sections*						
	Bacillus megaterium			*Pseudomonas syringae*		
Rhizosphere section	Single strain	In mixture	LSD†	Single strain	In mixture	LSD†
Upper Hypocotyl	2·78	2·78	1·34	4·99	4·94	0·79
Lower Hypocotyl	5·11[b]‡	5·64[a]	0·24	6·83	6·62	0·38
Pellet	6·48	6·81	0·55	8·02	7·96	0·47
Seed	7·23	7·76	0·95	8·59	8·23	0·56
Root 1 (0–5 cm)	5·03	5·30	0·27	6·03	5·72	0·56
Root 2 (5–10 cm)	3·94	4·49	1·52	3·70	4·67	1·28
Root 3 (10–15 cm)	4·58	5·18	1·57	2·16[b]	4·26[a]	1·70
Root 4 (15–20 cm)	4·87	4·37	1·43	2·03[b]	3·57[a]	1·42
Root 5 (20–25 cm)	4·20	4·63	1·85	2·12	2·71	1·28
Root Base (> 25 cm)	5·16	5·61	0·86	4·99	4·20	0·83

*Data shown are means from triplicate columns over four sampling times pooled for each isolate.
†Significance tested using Fisher's protected LSD ($P = 0{\cdot}05$).
‡Significant differences between single strains and mixture within each isolate denoted by 'a–b'.

Effect of co-inoculation of *Ps. syringae* and *B. megaterium* on rhizosphere colonization

The colonization by *Ps. syringae* or *B. megaterium* was not significantly reduced on any of the rhizosphere sections tested when the bacteria were coinoculated compared with their application as single strains (Table 3). The populations of *B. megaterium* and *Ps. syringae*, when coinoculated, were actually significantly greater on the lower hypocotyl and the middle root sections, respectively, compared with the populations produced from single strains.

DISCUSSION

The five bacterial isolates displayed different degrees of population decline on the seed during drying. Despite this, the isolates recovered *in situ* following sowing to proliferate in the sugar-beet rhizosphere. Bacterial numbers in excess of the original application rate to seed were re-isolated consistently from the pellet and the seed for all the bacterial strains over the 28 d test period. Studies made by other workers have also observed a population revival when seed bearing apparently declining bacterial numbers are introduced into the soil environment. Geels and Schippers (1983) demonstrated that even after large decreases in viable cell numbers during drying of treated potato seed tubers, bacterial numbers subsequently increased substantially during the first three d after sowing. The general trend in rhizosphere population dynamics with bacteria introduced as seed treatments is typified by a rapid increase in numbers (especially in sterile soil) followed by a period of stabilization and slow decline to non-detectable levels (Geels and Schippers 1983; Kloepper *et al*. 1992; Kluepfel and Tonkyn 1992; Kluepfel 1993). Kluepfel (1993) reported that for a fluorescent pseudomonad seed treatment, regardless of the initial concentration of inoculum applied (within a range of 10^4–10^9 cells ml^{-1}), an optimum rhizosphere population potential was reached which represented the carrying capacity of the root.

All isolates were present on the hypocotyl and first root section at all sample times. Therefore the isolates could proliferate in the known infection court of *A. cochlioides* and formed stable populations for the duration of the infection period. High bacterial numbers in the infection court denote good biocontrol potential as the first symptoms of pathogen infection occur at these sites. However, the isolates varied in their ability to colonize the middle and lower root sections. In general, bacterial numbers were highest on the seed and the pellet and declined down the tubes away from the point of inoculation. This gradient of colonization has been observed in other studies using bacteria as seed treatments in soil columns (Misaghi *et al*. 1992; Dandurand *et al*. 1997) and has been attributed to adsorption of bacteria to soil, forced removal from growing roots or the inability to keep pace with root growth.

The ability to colonize distal parts of the rhizosphere from an initial point of inoculum, especially in the absence of percolating water flow, has been termed 'mobility' (Misaghi *et al*. 1992) and has been implicated as an important trait for biocontrol in the rhizosphere. Percolating water flow was shown to have a significant effect on the colonization of the upper rhizosphere sections by one of the candidate bacteria. However, the *B. megaterium* isolate could still produce peak populations of > 7 $\log_{10}$ cfu g^{-1} plant material in the

infection court and therefore displays mobility in the absence of percolating water. The rhizosphere competent nature of this *Bacillus* isolate in the presence of percolating water is also encouraging as, typically, fluorescent pseudomonads are the dominant rhizosphere colonizers due to their high affinity for amino-acid exudates and short generation time on roots (Suslow and Schroth 1982). However, the use of a rhizosphere competent spore-forming *Bacillus* isolate would facilitate the long-term storage of a commercial seed treatment product.

A comparison of the rhizosphere colonization profiles with the data from the 'nonrhizosphere' controls show that the patterns of distribution of the isolates in the presence of the host result primarily from the development of the host rhizosphere. Studies by other workers have also reported that typical rhizobacteria show only sparse migration to non-rooted soils (Wiehe and Höflich 1995a,b) and survive in only low numbers in root-free soil (Chao *et al.* 1986; Wiehe and Höflich 1995a; De Leij *et al.* 1998). In the absence of the developing host rhizosphere, only the spermosphere samples supported bacterial populations for the duration of the experiment. None of the isolates produced stable populations in the soil profiles, apart from those adjacent to the seed and at the column base. This suggests that the cells were being flushed directly from the spermosphere to the base of the column by percolating water. However, the movement of percolating water was shown to significantly increase the colonization of the upper sugar beet rhizosphere by *B. megaterium*. Therefore the colonization profiles established in the host rhizosphere are modified by the movement of percolating water. This is supported by the observations of other workers. Preferential water flow has been shown to move along the rhizoplane surface in soil systems (Natsch *et al.* 1996) and bacteria can be carried in large numbers along root surfaces by this mechanism (Parke *et al.* 1986; Kluepfel 1993). Chao *et al.* (1986) demonstrated that the depth of rhizosphere colonization by bacteria was influenced by the volume of percolating water added to a root system. In a field situation, seed treatment bacteria would also be distributed along roots by preferential flow from rainfall (Natsch *et al.* 1996) although this movement would also be influenced by other environmental factors.

There was no suppression of population levels of *Ps. syringae* or *B. megaterium* when the bacteria were applied as a mixture compared with their application as single strains. Previous investigations by other workers have shown that co-inoculation with two BCAs can either suppress the rhizosphere activity of one of the isolates or that the activity of both isolates remains unaffected. For example, Dunne *et al.* (1998) demonstrated that the rhizosphere competence of *Pseudomonas fluorescens* F113 and *Stenotrophomonas maltophilia* W81 were essentially similar when the two strains were applied to sugar beet seed as single seed treatments or co-inoculated. In contrast, Chiarini *et al.* (1998) reported that rhizosphere populations of a strain of *Burkholderia cepacia* on sorghum were suppressed by a species of *Enterobacter* but not by *Ps. fluorescens*.

Competition for space and nutrients may each play a role in the outcome of co-inoculation. Using geostatistical analysis of photomicrographs, Dandurand *et al.* (1997) demonstrated that a high degree of bacterial aggregation occurred on the pea rhizoplane when a *Pseudomonas fluorescens* isolate was introduced as a seed treatment. Roots were typically colonized heavily in a few regions with the remaining area colonized lightly or not at all. This bacterial aggregation could not be correlated with root exudation, suggesting spatial competition may not be the most important determining factor. Strain compatibility has been partly attributed to the utilization of differing carbon substrates (Wilson and Lindow 1994) although the outcome of such interactions remains difficult to predict (Schisler *et al.* 1997). The rate of application of bacterial strains has also been shown to be an important parameter in determining the outcome of co-inoculation. Fukui *et al.* (1994) showed that antagonism between supposedly compatible strains of *Pseudomonas* species on sugar beet seed often occurred if the strains were co-inoculated at different application rates.

In this study there was no interaction in the mixture between the two isolates and therefore co-inoculation of the sugar-beet rhizosphere with these two potential BCAs is a viable proposition. This has implications for the delivery of a mixture of BCAs with different modes of action or environmental adaptations.

Resistance to rifampicin is unusual in soil bacteria, being mediated by a chromosomal mutation in the β subunit of RNA polymerase (Sippel and Hartmann 1968). This renders the resistance more stable and less easily transferable than plasmid-borne markers (Compeau *et al.* 1988) and makes rifampicin resistance a useful selective agent for rhizosphere colonization studies. However, antibiotic resistance can have deleterious effects on the growth and competitiveness of the marked strains (Paulitz 2000). The Rif^+ strain of *B. megaterium* used in this study, in comparison to the parental wild-type isolate from which it was derived, is more slow growing in pure culture and enters the decline phase earlier (data not shown). Despite this, the *Rif*-marked *B. megaterium*, and the Rif^+ strains of the other isolates tested, could effectively colonize the known infection court of *Aphanomyces*.

This study has demonstrated that effective colonization of the seedling rhizosphere of sugar beet can be achieved in nonsterile field soil by isolates of rhizobacteria applied to pelleted seed. The known infection court for *A. cochlioides* was successfully targeted by the seed treatments and the introduced bacteria formed stable

populations for the duration of the pathogen infection window. Suslow and Schroth (1982) described bacterization of sugar beet seed with fluorescent pseudomonads resulting in rhizosphere colonization of 10^5–10^7 cfu cm^{-1} root on 15-day-old seedlings, which is consistent with this study. This bioassay is therefore considered an effective method for ranking candidate rhizobacteria for rhizosphere competence as an aid to the selection of potential BCAs for future field trials.

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