Journal of Applied Microbiology 1998, **84**, 791–801

Bacillus isolates from the spermosphere of peas and dwarf French beans with antifungal activity against *Botrytis cinerea* and *Pythium* species

R. Walker*, A.A. Powell and B. Seddon

Department of Agriculture, MacRobert Building, University of Aberdeen, Aberdeen, Scotland, UK

6266/06/97: received 9 June 1997, revised 12 September 1997 and accepted 16 September 1997

R. WALKER, A.A. POWELL AND B. SEDDON. 1998. A range of isolation procedures including washing, sonication and incubation in nutrient broth were used separately and in combination to obtain potential bacterial antagonists to *Botrytis cinerea* and *Pythium mamillatum* from the testae and cotyledons of peas and dwarf French beans. Heat treatment was also used to bias this selection towards spore-forming bacteria. Ninety-two bacterial isolates were obtained, 72 of which were provisionally characterized as species of *Bacillus*. Four of these *Bacillus* isolates (B3, C1, D4 and J7) displayed distinct antagonism *in vitro* against *Botrytis cinerea* and *P. mamillatum* when screened using dual culture analysis. Further characterization of these antagonists using API 50CHB biochemical profiling identified isolate D4 as *Bacillus polymyxa* and isolates B3, C1 and J7 as strains of *B. subtilis*. *In vitro* screening techniques, using cell-free and heat-killed extracts of liquid cultures against *Botrytis cinerea*, demonstrated the production of antifungal compounds by these four *Bacillus* antagonists. With each isolate the antifungal activity was found not to be either exclusively spore-bound nor released entirely into the medium but present in both fractions. The antifungal compounds produced by these isolates were shown to be heat-stable. Their identification, production and release require further study for exploitation as biocontrol systems.

INTRODUCTION

Damping-off diseases are responsible for considerable yield losses to a wide range of agricultural crops throughout the world (Cline *et al.* 1988), including pea (*Pisum sativum*) and dwarf French bean (*Phaseolus vulgaris* L.). A variety of soil-borne plant pathogens are responsible for damping-off, primarily species of *Pythium*, *Rhizoctonia*, *Fusarium*, *Phytophthora* and *Aphanomyces* (Garrett 1970). The major pathogens associated with the damping-off of seeds and seedlings are *Pythium* species which are widely distributed in soil and decomposing leaf litter where they infect mainly juvenile or succulent tissues (Garrett 1970). *Botrytis cinerea*, the causal agent of grey mould, is an unspecialized plant pathogen with a vast host range. Although more commonly associated with the phylloplane the pathogen infects young succulent tissue and has also been implicated in the damping-off of seeds (Duffy and Gardener 1994) and seedlings (Sutherland 1991).

Elimination of these pathogens from soil is unrealistic and the use of single disease control measures has met with limited success (Martin *et al.* 1985). The integration of various disease control strategies, including biological control, should be considered to improve efficacy and reduce fungicide levels in the environment (Nelson 1989).

Bacillus species are characterized by their ability to form highly resistant endospores (Sadoff 1972) and to produce a wide range of antibiotics (Katz and Demain 1977). Many of these *Bacillus* species and their antibiotics have antifungal activity against phytopathogenic fungi (Edwards *et al.* 1994) and are generally soil-inhabiting micro-organisms or exist as epiphytes and endophytes in the spermosphere (Mundt and Hinckle 1976) and rhizosphere (Zhang *et al.* 1993). *Bacillus* species are also found in many other environments (Sneath 1986) as their survival is aided by this ability to form endospores. These dormant structures are resistant to desiccation, heat, u.v. irradiation and organic solvents; characteristics

*Correspondence to Richard Walker, *present address: IACR-Broom's Barn, Higham, Bury St Edmunds, Suffolk IP28 6NP, UK (e-mail: walker@mserv.res.bbsrc.ac.uk).*

which allow for further formulation and commercialization procedures (Rhodes 1990). *Bacillus* species are therefore ideal candidates for use as biocontrol agents (BCAs) in seed treatment programs against soil-borne pathogens.

The aims of this study were to isolate *Bacillus* species from the spermosphere of peas and dwarf French beans, screen these isolates *in vitro* for antagonism against *Botrytis cinerea* and pathogenic species of *Pythium*, select and identify isolates showing promising antagonism and determine the mode of action of this activity with a view towards the development of biocontrol systems against the damping-off of seeds of pea and dwarf French bean. The detailed methodology and experimental observations of this work are presented here with preliminary observations having been reported elsewhere (Walker *et al.* 1994).

MATERIALS AND METHODS

Micro-organisms

The strain of *Botrytis cinerea* used in this study was isolated from leaves of Chinese cabbage var. Kasumi in 1989 from a field crop at Allerthorpe (Humberside, UK) and has been shown in this laboratory to be an effective damping-off agent of seeds (Edwards 1993). The procedure for harvesting conidia was based on that described by Wale (1980) and detailed further by Walker *et al.* (1996). The pathogen was maintained by harvesting conidia in suspension and storage at −20 °C. The strain of *Pythium mamillatum* used was isolated from pea seeds sown in contaminated soil obtained from a walled garden at Craibstone Farm 4 miles north-west of Aberdeen (Scotland, UK) and was identified at the International Mycological Institute (Egham, Surrey, UK). The strains of *P. aphanidermatum* and *P. ultimum* were supplied by Dr Eirian E. Jones (Bacteriology Division, Institute of Cell and Molecular Biology, Edinburgh University, Edinburgh, UK). *Pythium aphanidermatum* CBS strain 634.70 was isolated from *Lycopersicon esculentum* (common tomato) in Israel and *P. ultimum* strain 39 was isolated from soil in Greece. The fungi were maintained by cultivation on plates of Potato Dextrose Agar (PDA; Oxoid, Basingstoke, UK, CM139) for 3 d at room temperature. The plates were then sealed with parafilm and refrigerated at 4 °C. The pathogens were subcultured onto fresh PDA plates within a period of 3 months.

Bacillus brevis strain Nagano and *B. licheniformis* were included in this study because they have activity against the damping-off fungi *Phoma lingam*, *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Edwards *et al.* 1991; McKay *et al.* 1991; Edwards 1993). The strain of *B. brevis* used produces a single cyclic decapeptide antibiotic gramicidin S (Saito *et al.* 1970). The isolate of *B. licheniformis* used was isolated in this laboratory from seeds of Chinese cabbage (Edwards *et al.* 1991).

Plant material

Two pea seed lots of the cultivar Puget and two cultivars of dwarf French bean (black seeded cultivar Sunray and white seeded cultivar Processor) were used in this work. The two pea seed lots consisted of a high vigour lot (lot reference 743) and a low vigour lot (lot reference PWB/095/B/8353). This plant material was obtained from Sharpe's (Sleaford, Lincolnshire, UK).

All chemicals were obtained from Sigma (Poole, UK) and microbiological media from Oxoid.

Isolation and characterization of potential bacterial antagonists

Isolation. A combination of techniques was used to obtain bacterial isolates in an attempt to maximize the variety and number of bacterial species obtained. The isolations involved washing and sonication of seeds of pea and dwarf French bean, with and without the testa separated from the remainder of the seed, in Ringer's solution or Nutrient Broth (NB; CM1). The washing/sonication in NB was followed by incubation at 10, 25 or 37 °C for 48 h or 5 d (to allow mature spores to develop). Isolations were also performed with heat treatment to further bias this selection towards spores of *Bacillus* species. Twelve replicate seeds of each of the four seed lots were exposed to the following isolation procedures.

(i) Isolation in Ringer's solution. The seeds were washed by agitating seeds individually in Ringer's solution for 1 min using a whirlimixer. Single seeds were sonicated individually in Ringer's solution for 10 s at the lowest setting using an MSE 10/75 Mk2 (MSE, Crawley, UK) bench sonicator. Sonication was followed by 20 s cooling in ice. This process was repeated twice. Serial dilutions of the washings/sonications in maximum recovery diluent (CM733) were used to prepare spread plates on Nutrient Agar (NA; CM3) supplemented with 0·005% cycloheximide (C-6255) (NAc). The plates were incubated at 30 °C for 48 h. The numbers of colony-forming units were then counted and distinct single colonies were subcultured onto NA for characterization.

(ii) Incubation in nutrient broth. Seeds were subjected to the same treatment as described above using NB as the recovery medium instead of Ringer's solution. The seeds were incubated for 48 h at either 10, 25 or 37 °C in 5 ml broth. Each replicate group of seeds of each seed type was incubated at a different temperature in order to maximize the number of isolates obtained. Spread plates, colony counts and subculturing of distinct isolates were performed as described above.

(iii) Heat treatment. This procedure was carried out as for the incubation in NB method previously described except that the seeds were incubated at 10, 25 or 37 °C

for 5 d to encourage any *Bacillus* species present to produce spores. One-ml samples of these treatments were placed in a water-bath in thin-walled test-tubes at 80 °C for 10 min to select for spore-producing isolates by heat-killing the vegetative cells present (Walker *et al.* 1994). A dilution series was prepared and the selected dilutions were plated out onto NAc plates. The preparation of spread plates, colony counts and subculturing of distinct isolates were then performed as described previously.

Preliminary characterization. The bacterial isolates obtained by these isolation techniques were characterized by simple classical methods as outlined by Bradbury (1988). The tests carried out in duplicate were Gram staining, nitrate reductase, oxidation and fermentation of glucose, fluorescent pigment formation, Kovacs oxidase, arginine dihydrolase, levan and starch hydrolysis.

Screening of isolates for antagonism *in vitro*

The characterized bacterial isolates were screened for antagonism *in vitro* against *Botrytis cinerea* and *P. mamillatum* using the technique of dual culture analysis (Whipps 1987). A single colony of the bacterial isolate was used to inoculate dual culture plates of NA, Malt Extract Agar (CM59) and PDA by producing a single streak of inoculum at one side of the plate. A loopful of conidial suspension ($2{\cdot}6 \times 10^7$ ml^{-1}) was used to produce a streak of *Botrytis cinerea* inoculum in parallel and at a distance of 5 cm from the first streak. Plugs of mycelium from 3-d old plates of *P. mamillatum* were used to inoculate the dual culture plates of this pathogen. A template was used to achieve uniformity of inoculation between plates and two negative controls were used in this experiment. Firstly, a *B. brevis* antibiotic-negative mutant E-1 was used (Iwaki *et al.* 1972) which lacks antifungal activity against *Botrytis cinerea* and has been used as a negative control in previous biocontrol studies with *Botrytis cinerea* (Edwards and Seddon 1992; Edwards 1993). Secondly, control plates which were not inoculated with bacterial isolates were prepared. *Bacillus brevis* Nagano wild-type, which produces the antifungal compound gramicidin S, was used as a positive control (Edwards and Seddon 1992). The plates were incubated at 25 °C and the microbial interactions were analysed by measuring the radius of zones of inhibition produced when the control plates were fully developed.

Selection and further identification of antagonists

Those isolates identified as *Bacillus* species and showing distinct antagonism *in vitro* to both *Botrytis cinerea* and *P. mamillatum* were selected for further characterization using API 20E and 50CHB test strips (bioMérieux, Marcy l'Etoile, France). When used in conjunction with an Analytical Profile Index and APILAB software package this system can positively identify bacilli down to species level. The identification is measured as a percentage probability and is presented with any anomalous test results and comments for further clarification if required.

Mechanism of antagonism of *Bacillus* isolates

Antagonistic isolates along with *B. brevis* strain Nagano and *B. licheniformis* were then screened for production of antifungal metabolites *in vitro* against *Botrytis cinerea* using membrane filters. Both *B. brevis* Nagano (Edwards and Seddon 1992) and *B. licheniformis* (Edwards *et al.* 1991; McKay *et al.* 1991) have previously been shown to inhibit the growth of *Botrytis cinerea*. Nutrient agar was dispensed into Petri dishes in 20-ml aliquots. A sterile 9-cm diameter membrane filter with a pore size of 0·22 μm (Millipore, Bedford, UK) was smoothed onto the surface of the medium on each plate so that there was good contact between the filter and the medium. The isolates were cultivated for 48 h on NA and then harvested into 1-ml samples of sterile distilled water (SDW) until the turbidity of each of the seven solutions was equivalent to Mcfarland's Standard no. 2 (approximately 6×10^8 cells ml^{-1}) (Blair *et al.* 1970). One drop (approximately 30 μl) of this suspension for each isolate was placed onto the centre of the membrane filter in triplicate plates of NA using a sterile pipette. After incubation (30 °C for 24 h) the filters were removed aseptically and the plates were overlaid with 20-ml aliquots of PDA containing a conidial suspension of *Botrytis cinerea* (approximately 5×10^5 ml^{-1}). The plates were then placed in a refrigerator for 24 h to delay conidial germination whilst allowing diffusion of the metabolites. The plates were then incubated at 20 °C for 48 h before being assessed for the production of antifungal metabolite by measuring the clearing zones of inhibition produced in the developing lawn of the pathogen.

Assay of supernatant filtrates and autoclaved cultures of antagonists against conidial germination of *Botrytis cinerea*

All antagonists were cultured in 100-ml volumes of both NB and Tryptone Soya Broth (TSB; CM129) in baffled 250-ml conical flasks on an orbital shaker for 7 d at 100 rev min^{-1} and 30 °C. A 20-ml sample of each of the spore suspensions produced was spun down at 2500 *g* for 15 min (MSE Chilspin centrifuge) and the supernatant fluids were removed. A 2-ml aliquot of each supernatant fluid was then filtered through a sterile acrodisc filter of pore diameter 0·2 μm (Millex®-FG, Millipore). The remaining whole cultures were mixed to resuspend the spores and autoclaved at 121 °C for 30 min.

The autoclaved spore suspensions and supernatant filtrates were then screened for antagonism to conidia of *Botrytis cinerea* using the method developed by Edwards (1993) and described by Walker *et al.* (1996). Sterile 1·5-ml vials were taken and the following components pipetted into them sequentially: 500 μl of double-strength glucose peptone broth (GPB), 250 μl of the test solution and 250 μl of 4×10^5 conidia ml^{-1} of *Botrytis cinerea*. Glucose peptone broth was prepared by adding 4·0 g of Bacteriological Peptone (Oxoid, L37) and 20·0 g glucose to 1 l distilled water, adjusting the pH to 6·0 with 1 mol l^{-1} NaOH and autoclaving for 121 °C for 15 min. Control samples replaced the test solution with the same volume of sterile medium (either NB or TSB). The vials were mixed for 10 s and three drops (each drop approximately 30 μl) from each were then pipetted onto sterile glass microscope slides using a 100 μl Gilson Pipetman (Villiers-le-Bel, France). The slides were then incubated for 6 h at 25 °C and 100% r.h. After incubation, one drop of concentrated sterile Viablue 2 fluorescent stain (Seddon *et al.* 1993) was added to each drop of conidial suspension to help visualize the conidia. The conidia were assessed microscopically under a glass cover-slip at $\times 100$ magnification (Polyvar Microscope, Reichert, Austria). A conidium was deemed to have germinated if a germ tube greater in length than the radius of the spore was visible (Walker *et al.* 1996). The percentage germination of conidia was determined by counting the numbers of germinated conidia from a total of 500 counts.

Comparative quantification of antifungal metabolite production

The aim of these experiments was to correlate by comparison the antifungal activity of the isolates to corresponding concentrations of gramicidin S (GS) standards and to determine the nature of production of these antifungal metabolites by the different *Bacillus* isolates. Gramicidin S is the cyclic decapeptide antibiotic produced by the BCA *B. brevis* Nagano which has strong antifungal activity against both conidia and mycelia of *Botrytis cinerea* (Edwards *et al.* 1994). Purified GS is available which enables direct comparative studies of *B. brevis* cultures against pure antibiotic and therefore provides a standard by which antifungal activity of other *Bacillus* species antagonists can be compared and quantified on a relative basis. Inhibition levels can therefore be quoted as 'equivalent concentrations of GS' and a relative comparison made.

Assay of ethanolic extracts of antibiotics. The seven antagonists were cultivated in NB and TSB at 30 °C for 5 d. Ethanolic extraction was then performed on these cultures (Walker 1995). A 20-ml sample of bacterial culture was spun down at 2500 *g* for 15 min (MSE Chilspin centrifuge). The supernatant fluid was removed and the pellet was resuspended in 1·5 ml of ethanol. The suspension was mixed for 1 h on an orbital shaker at 30 °C and 150 rev min^{-1} and then spun down at 10 000 *g* for 3 min (MSE Microcentaur centrifuge). The supernatant fluid was then removed and assayed for antifungal activity against *Botrytis cinerea* as follows. Molten PDA (150 ml) was seeded with conidia of *Botrytis cinerea* to give a final concentration of 10^5 ml^{-1}. The seeded agar was then poured immediately into a sterile Petri dish, 243 × 243 × 18 mm, especially designed for agar diffusion assays (Nunc, Rofkilde, Denmark) and balanced using a three-way spirit level technique (Strang 1992) to produce a uniform thickness of medium throughout the plate. Wells were made in the medium using a sterile 7-mm diameter cork borer. Treatments were applied to the plates randomly and in triplicate. The treatments were: standards of GS over the range 0–1000 μmol l^{-1}, supernatant filtrates of the seven bacterial isolates which had been grown in NB and TSB shake culture for 7 d at 20 °C and autoclaved spore suspensions of the same cultures. The wells were filled with the samples using a Gilson Pipetman. Control wells of ethanol alone were tested. The plates were incubated for 48 h at 25 °C to provide optimum growth conditions for the pathogen. Plates were then assessed for antifungal activity by measuring the radius of the zones of inhibition produced.

The theory of antibiotic inhibition zones describes a correlation between antibiotic concentration and the size of the zone of inhibition produced (Cooper and Woodman 1946). This states that Log_{10} of the antibiotic concentration is linearly related to the square of the inhibition zone (Cooper 1963; Linton 1983). Inhibition by GS is well defined (Edwards and Seddon 1992; Edwards *et al.* 1994), whereas the antifungal metabolites produced by the isolates studied here are not yet fully characterized. In order to make comparisons between the relative activity of the antagonists, the data were analysed by linear regression and expressed as 'equivalents of GS' (Walker 1995).

Assay of filtered ethanolic extracts of antibiotics. The method outlined above was repeated with the following amendment. Each of the ethanolic extracts was passed through a sterile 0·2 μm acrodisc filter (Millex®-FG, Millipore) before screening for antibiotic activity.

Assay of ethanolic extracts of autoclaved cultures. After incubation, 10-ml samples of the bacterial cultures were autoclaved at 121 °C for 30 min. The procedure of ethanolic extraction and screening was then carried out as described previously.

Assay of autoclaved ethanolic extracts. In this experiment

ethanolic extraction was carried out as described previously. The extracts were then autoclaved at 121 °C for 30 min. After autoclaving, the slurries produced were brought back to the original volume with ethanol before being assayed.

Assay of supernatant filtrates of cultures. After incubation, a 20 ml sample of each culture was spun down at 2500 *g* for 15 min (MSE Chilspin centrifuge). A 2 ml aliquot of each sample was then passed through a sterile 0·2 μm acrodisc filter (Millex®-FG from Millipore). These supernatant filtrates were then assayed.

RESULTS

Isolation and characterization of potential bacterial antagonists

Using the various isolation procedures described, 92 different bacterial isolates were selected on the basis of colony colour, size and morphology. Seventy-two of these isolates were identified as members of the genus *Bacillus*. The remaining isolates belonged to the genera *Erwinia*, *Corynebacterium*, *Staphylococcus*, *Pseudomonas* and *Rhodococcus* although some isolates could not be assigned to any single genus at this stage of the characterization (Table 1).

Screening of isolates for antagonism *in vitro*

Of the 92 isolates screened for antagonism, five *Bacillus* isolates produced zones of inhibition against both *Botrytis cinerea* and *P. mamillatum* on all three types of media tested. These isolates were subsequently screened against *P. aphanidermatum* and *P. ultimum*. These data, together with the results of the screenings of *B. brevis* and *B. licheniformis* against the four pathogens, are presented in Table 2.

The isolates B3, C1, D4 and J7 showed distinct antagonism against both *Botrytis cinerea* and *P. mamillatum* on all three types of media tested. The zones of inhibition against *Botrytis cinerea* were particularly well developed. Isolate D4 showed good antagonism against all four pathogens. *Bacillus brevis* Nagano wild-type and *B. licheniformis* produced zones of inhibition in certain combinations whereas the antibiotic-negative mutant E-1, as expected, did not show inhibition.

Mechanism of antagonism of *Bacillus* isolates

The results demonstrating antifungal activity using membrane filters for the four isolates which showed strong antagonism, together with observations on *B. brevis* and *B. licheniformis*, are shown in Table 3. All of the isolates screened produced zones of inhibition indicating the production of antifungal metabolites active against *Botrytis cinerea*.

Table 1 Characterization of bacterial isolates to genus level

Isolate	Source	Genus
A1	DFW	*Bacillus*
A2	DFW	*Bacillus*
A3	DFW	*Bacillus*
A4	DFW	*Bacillus*
A5	DFW	*Bacillus*
A6	DFW	*Bacillus*
B1	DFB	*Bacillus*
B2	DFB	*Bacillus*
B3	DFB	*Bacillus*
B4	DFB	*Bacillus*
B5	DFB	*Bacillus*
B6	DFB	*Bacillus*
B7	DFB	*Bacillus*
B8	DFB	*Bacillus*
B9	DFB	*Staphylococcus*
B10	DFB	*Bacillus*
C1	Pea	*Bacillus*
C2	Pea	*Bacillus*
C3	Pea	*Bacillus*
C4	Pea	*Bacillus*
C5	Pea	*Bacillus*
C6	Pea	*Bacillus*
C7	Pea	*Bacillus*
D1	Pea	*Bacillus*
D2	Pea	*Bacillus*
D3	Pea	*Bacillus*
D4	Pea	*Bacillus*
D5	Pea	*Bacillus*
D6	Pea	*Bacillus*
E1	DFW	*Bacillus*
E2	DFW	*Staphylococcus*
E3	DFW	*Rhodococcus*
E4	DFW	*Corynebacterium*
F1	DFB	*Bacillus*
F2	DFB	*Bacillus*
F3	DFB	*Bacillus*
F4	DFB	*Bacillus*
F5	DFB	*Bacillus*
F6	DFB	*Staphylococcus*
F7	DFB	*Bacillus*
F8	DFB	*Staphylococcus*
F9	DFB	*Bacillus*
F10	DFB	*Bacillus*
F11	DFB	*Bacillus*
G1	Pea	*Bacillus*
G2	Pea	*Bacillus*
G3	Pea	*Bacillus*
G4	Pea	*Bacillus*
G5	Pea	*Bacillus*
H1	Pea	*Staphylococcus*
H2	Pea	*Staphylococcus*
H3	Pea	*Corynebacterium*
H4	Pea	*Corynebacterium*

cont.

Table 1 —*continued.*

Isolate	Source	Genus
H5	Pea	*Corynebacterium*
H6	Pea	*Corynebacterium*
H7	Pea	*Bacillus*
J1	DFW	*Bacillus*
J2	DFW	*Bacillus*
J3	DFW	*Bacillus*
J4	DFW	*Bacillus*
J5	DFW	*Pseudomonas/Agrobacterium*
J6	DFW	*Bacillus*
J7	DFW	*Bacillus*
J8	DFW	*Erwinia*
J9	DFW	*Erwinia*
J10	DFW	*Bacillus*
K1	DFB	*Bacillus*
K2	DFB	*Erwinia*
K3	DFB	*Bacillus*
K4	DFB	*Pseudomonas*
K5	DFB	*Bacillus*
L1	Pea	*Bacillus*
L2	Pea	*Erwinia*
L3	Pea	*Bacillus*
L4	Pea	*Bacillus*
L5	Pea	*Bacillus*
L6	Pea	*Bacillus*
L7	Pea	*Bacillus*
L8	Pea	*Staphylococcus*
L9	Pea	*Bacillus*
L10	Pea	*Bacillus*
M1	Pea	*Bacillus*
M2	Pea	—
M3	Pea	*Bacillus*
M4	Pea	*Bacillus*
M5	Pea	*Aeromonas/Azotomonas*
M6	Pea	*Corynebacterium*
M7	Pea	*Bacillus*
M8a	Pea	*Bacillus*
M8b	Pea	*Bacillus*
M9	Pea	*Bacillus*
M10	Pea	*Bacillus*

DFW, White dwarf French bean; DFB, black dwarf French bean.

Selection and further identification of antagonists

Profiles obtained from API tests for each isolate were analysed using a bioMérieux *Bacillus* software package as described in Materials and Methods. The identification of each isolate down to species level is represented as a percentage probability in accordance with the software analysis (Table 4). Positive identification was obtained for the four isolates which were identified as *B. polymyxa* and three strains of *B. subtilis*.

Assay of supernatant filtrates and autoclaved cultures of antagonists against conidial germination of *Botrytis cinerea*

The results of the screenings of the supernatant filtrates and autoclaved samples of the antagonists cultivated in NB and TSB and tested against germination of conidia of *Botrytis cinerea* are shown in Table 5. All supernatant filtrates and autoclaved samples from both broth cultures significantly reduced the levels of conidial germination compared with controls when analysed by the Chi-squared test. Autoclaved samples from both NB and TSB broth cultures of *B. brevis* wild-type, which produces GS, showed the strongest activity, with complete inhibition of conidial germination.

Comparative quantification of antifungal metabolite production

The results for all types of ethanolic extracts and the supernatant filtrates are presented in Table 6. All isolates showed antifungal activity against *Botrytis cinerea*. The most active antagonists appeared to be *B. brevis* and isolate J7. *Bacillus brevis* showed some antifungal activity against *Botrytis cinerea* in all screenings. Isolate J7 showed activity against *Botrytis cinerea* in over half of the screenings.

Antifungal activity was present in the supernatant filtrates of the cultures of all isolates. However, in all but two cases the activity of the supernatant filtrate of *B. brevis* was minimal compared with the ethanolic extractions.

The supernatant filtrates of isolates B3, C1, D4 and *B. licheniformis* appeared to be more active against *Botrytis cinerea* than the ethanolic extractions. There was antifungal activity in both the supernatant filtrate and the ethanolic extractions of isolate J7.

DISCUSSION

Seventy-two of the 92 isolates obtained were found to be species of *Bacillus*. This high isolation rate of *Bacillus* species may be due, in part, to the use of heat treatment and an optimum incubation temperature of 37 °C for the growth of organisms of this genus. However, almost half of these *Bacillus* isolates were obtained non-selectively via Ringer's washings, or incubation in NB at lower temperatures, suggesting that *Bacillus* species are still the predominant bacteria in this spermosphere environment. There is some evidence in the literature of *Bacillus* species surviving as endophytes in this environment. Mundt and Hinckle (1976) isolated endophytic bacteria from within surface-sterilized ovules and seeds of 27 plant species including *Phaseolus* sp. (bean) and *Pisum* sp.

Table 2 *In vitro* antagonism against damping-off pathogens

Isolate	Media	*Pythium aphanidermatum*	*Pythium mamillatum*	*Pythium ultimum*	*Botrytis cinerea*
B3	Nutrient agar	+ +	+ +	+ +	ZI
	Malt extract agar	+	+ + +	+ +	+ + +
	Potato dextrose agar	−	+ +	−	+ + +
C1	Nutrient agar	+ +	+ + +	+ +	ZI
	Malt extract agar	+	+ +	+ +	+ + +
	Potato dextrose agar	+	+ +	−	+ + +
D4	Nutrient agar	+ + +	+ + +	+ + +	ZI
	Malt extract agar	+ +	+ + +	+ + +	+ +
	Potato dextrose agar	+	+ + +	+ +	+ + +
J7	Nutrient agar	+ + +	+ + +	+ + +	ZI
	Malt extract agar	+ +	+ +	+ +	+ + +
	Potato dextrose agar	+	+ +	−	+ + +
Bacillus brevis	Nutrient agar	+ + +	ZI	+ + +	ZI
	Malt extract agar	−	−	+	−
	Potato dextrose agar	−	−	+	+ +
Bacillus licheniformis	Nutrient agar	+ + +	ZI	+ + +	ZI
	Malt extract agar	+ +	+	+ +	+ +
	Potato dextrose agar	+	−	+	+ +
Bacillus brevis E-1	Nutrient agar	−	−	−	−
	Malt extract agar	−	−	−	−
	Potato dextrose agar	−	−	−	−

+ + +, Strong inhibition of fungal growth; + +, good inhibition of fungal growth; +, some inhibition of fungal growth; −, no zone of inhibition; ZI, large zone of inhibition associated with poor growth of pathogen on nutrient agar.

(English pea). *Bacillus* species occurred more frequently than the species of any other single bacterial genus, accounting for almost one-third of all bacteria isolated.

The analysis of API 50CHB biochemical profiles of the isolates by the bioMérieux APILAB *Bacillus* software package provided a rapid method of identification to species level. Identification of the antagonists is also necessary to prevent the environmental release of undesirable micro-organisms in the guise of BCAs (Markellou *et al.* 1995). Such analysis is also vital in the formulation of a good biocontrol system as a knowledge of a potential BCA's mode of antagonism, nutrient requirements, environmental adaptability and chemical compatibility will facilitate enhancement of this system (Baker 1986; Pusey 1990; Edwards *et al.* 1994).

Results from the membrane filter experiment showed that all four selected *Bacillus* isolates produced a compound or

Table 3 Screening for antifungal activity against *Botrytis cinerea* using membrane filters

	Zone of Inhibition (mm)			
Isolate	Replicate 1	Replicate 2	Replicate 3	Mean
Control	—	—	—	—
B3	15*	16*	—	10·3
C1	48	48	47	47·7
D4	22	26*	21	23·0
J7	22	22	18	20·7
Bacillus brevis	32	27	28	29·0
Bacillus licheniformis	28	29	28	28·3

* Limited fungal growth in zone of inhibition; —, no inhibition.

Table 4 Identification of isolates to species level

Isolate	Species name	Source	% Identification	Tests against	Comments
B3	*Bacillus subtilis*	DFB	99·8	L-sorbose dulcitol	Very good identification
C1	*Bacillus subtilis*	Pea	94·7	Lactose inulin	Good identification
D4	*Bacillus polymyxa*	Pea	99·5	L-rhamnose methyl-D-mannoside	Very good identification
J7	*Bacillus subtilis*	DFW	95·5	—	Excellent identification to the genus

DFW, White dwarf French bean; DFB, black dwarf French bean.

Table 5 Screening of isolates for inhibition of germination of conidia of *Botrytis cinerea*

NB cultures		TSB cultures	
Treatment	% Germination	Treatment	% Germination
Control	80·0 ± 1·53	Control	74·7 ± 0·33
SF B3	55·0 ± 1·33	SF B3	55·7 ± 1·45
SF C1	50·3 ± 1·15	SF C1	57·3 ± 0·33
SF D4	52·3 ± 1·33	SF D4	63·3 ± 1·86
SF J7	65·7 ± 0·88	SF J7	49·0 ± 0·85
SF *Bacillus brevis*	37·0 ± 1·53	SF *Bacillus brevis*	33·0 ± 0·00
SF *Bacillus licheniformis*	43·3 ± 0·88	SF *Bacillus licheniformis*	59·0 ± 1·32
Control	78·7 ± 1·45	Control	83·7 ± 1·86
A B3	23·0 ± 0·00	A B3	21·3 ± 1·53
A C1	68·0 ± 1·00	A C1	48·0 ± 1·15
A D4	51·7 ± 1·53	A D4	44·7 ± 1·00
A J7	59·0 ± 1·00	A J7	42·7 ± 0·88
A *Bacillus brevis*	0·0 ± 0·00	A *Bacillus brevis*	0·0 ± 0·00
A *Bacillus licheniformis*	58·3 ± 1·45	A *Bacillus licheniformis*	26·0 ± 1·33

SF, supernatant filtrate samples; A, autoclaved whole culture samples; NB, nutrient broth; TSB, tryptone soya broth.

compounds which had antifungal activity against the growth of *Botrytis cinerea*. Although these antifungal metabolites were not further characterized in this study it is known that four different antibiotics (polymyxin A, B, D and E) are produced by certain strains of the species *B. polymyxa* (Ramachandran *et al.* 1982) and that isolates of *B. subtilis* produce a range of antibiotics, including subtilin (Kurahashi *et al.* 1982) and iturins (Gueldner *et al.* 1988). Certain strains of *B. licheniformis* produce bacitracins of which there are six different types (A–F) (Ishihara *et al.* 1982).The results of the membrane filter screening were supported by the results from the bioassay screenings against *Botrytis cinerea*.

A GS standard plot was made for comparison with all of the bioassay screenings described to determine the concentration of antifungal metabolites in the samples as 'equivalents of GS'. As *B. polymyxa*, *B. licheniformis* and the three strains of *B. subtilis* isolated produce antifungal metabolites which have not yet been characterized and which may differ from GS in their modes of action, their activities cannot be directly comparable with GS standards. Nevertheless, it was thought that such a comparison could give some measure of the respective antagonistic abilities of the isolates and is therefore useful in rating the antifungal activity of these prospective BCAs. For all the bioassay screenings described in this paper, a linear relationship was only true within the range 1–100 μmol l^{-1} GS for each GS standard plot. This supports earlier work by Edwards (1993) which described linearity within the same range for this assay. 'Equivalents of GS' lying outside this linearity have been expressed as activity >100 μmol l^{-1} GS and, although no longer linear, indicate high levels of antifungal activity present in these samples.

Analysis of the ethanolic extracts and supernatant filtrates from the cultures also provided information on the nature of each antifungal metabolite and its production by the spo-

Table 6 Antifungal effects of samples against *Botrytis cinerea* (data expressed as the mean radius of the inhibition zones from triplicate wells)

Medium	Isolate	Ethanolic extracts	Filtered ethanolic extracts	Ethanolic extracts of autoclaved cultures	Autoclaved ethanolic extracts	Supernatant filtrates
Nutrient broth	B3	0·0	0·0	0·0	0·0	3·7 (>100 μmol l^{-1})
	C1	0·0	0·0	0·0	0·0	3·5 (>100 μmol l^{-1})
	D4	0·0	0·0	0·0	0·0	3·0 (>100 μmol l^{-1})
	J7	2·2 (29·8 μmol l^{-1})	0·0	0·0	1·7 (21.1 μmol l^{-1})	3·5 (>100 μmol l^{-1})
	Bacillus brevis	4·3 (>100 μmol l^{-1})	4·0 (>100 μmol l^{-1})	4·0 (>100 μmol l^{-1})	4·7 (>100 μmol l^{-1})	2·0 (34·2 μmol l^{-1})
	Bacillus licheniformis	2·0 (20·3 μmol l^{-1})	0·0	0·0	0·0	6·3 (>100 μmol l^{-1})
Tryptone soya broth	B3	3·7 (>100 μmol l^{-1})	0·0	0·0	0·0	4·2 (>100 μmol l^{-1})
	C1	3·3 (>100 μmol l^{-1})	0·0	0·0	0·0	6·5 (>100 μmol l^{-1})
	D4	5·7 (>100 μmol l^{-1})	0·0	0·0	0·0	0·0
	J7	5·0 (>100 μmol l^{-1})	3·7 (>100 μmol l^{-1})	0·0	2·7 (>100 μmol l^{-1})	7·0 (>100 μmol l^{-1})
	Bacillus brevis	4·7 (>100 μmol l^{-1})	1·2 (12·4 μmol l^{-1})	1·7 (32·1 μmol l^{-1})	5·0 (>100 μmol l^{-1})	2·2 (50·7 μmol l^{-1})
	Bacillus licheniformis	2·0 (20·3 μmol l^{-1})	0·0	0·0	0·0	7·0 (>100 μmol l^{-1})

Figures represent radius of zones of inhibition in mm. Equivalent concentrations of gramicidin S in brackets.

res/cells of the antagonist. The antifungal activity present in the supernatant filtrates was due to the metabolite(s) being released from the bacteria into the culture medium. If antifungal activity was found to be present in the ethanolic extracts of the culture pellet this denoted that the metabolite(s) was cell-bound. Antifungal activity was present in the supernatant filtrates of the cultures of all isolates suggesting that the antifungal metabolites were released into the medium. However, in all but two cases, the activity of the supernatant filtrate of *B. brevis* was minimal compared with the extracts from the spore component of the culture and this supports earlier work which established that the antibiotic activity of *B. brevis* is mainly cell-bound (Edwards 1993).

The supernatant filtrates of isolates B3, C1, D4 and *B. licheniformis* appeared to be more active against *Botrytis cinerea* than the extracts from the spore component of their cultures. This indicates that the metabolites produced by these isolates are not tightly spore-bound but released into the surrounding medium. There was antifungal activity in both the supernatant filtrate and the spore component of the cultures of isolate J7 showing that this metabolite, although spore-bound, can be released into the medium. From a range of studies on antibiotics produced by *Bacillus* species the relationship between bound and released levels of these antifungal metabolites appears to relate to the physiological and developmental stages of the *Bacillus* species (vegetative growth, sporulation, dormant spore or germinating spore) and provides another aspect on which manipulation of biological control might be focused. It is possible that the same *Bacillus* species is capable of producing an array of peptide antibiotics (Sadoff 1972) and that antibiotic production may vary between different strains of the same species. A full identification of these antifungal metabolites will be useful to enhance the biocontrol potential of these *Bacillus* isolates as BCAs against both *Botrytis cinerea* and pathogenic *Pythium* species.

When the supernatant filtrates and autoclaved NB and TSB cultures of the antagonists were screened for antifungal activity against germinating conidia of *Botrytis cinerea*, the percentage conidial germination was significantly reduced. In the case of *B. brevis* it is clear that this activity is associated with the spores. The antifungal activity of the remaining isolates also appeared to be present in both the supernatant filtrates and the whole autoclaved culture. The characteristic production and release of antifungal metabolites by these *Bacillus* isolates require further study. Once a full understanding of these phenomena has been achieved then it may be possible to manipulate the isolates for effective production of antifungal metabolites and possibly produce the metabolites in the infection court.

The results also showed that the metabolites produced inhibited conidial germination of *Botrytis cinerea* as well as showing activity against the growth of hyphae. This is important as inhibition of the pathogen at more than one stage in its life-cycle could lead to improved biocontrol efficiency (Seddon *et al.* 1997). It is also clear that growth of the pathogens initiated from mycelial plugs is more resistant to antifungal activity than the initiation of germ tubes from conidia. In this study conidial germination was inhibited by 20 μmol l^{-1} GS whereas the growth of mycelia from plugs was

inhibited by 50 μmol l^{-1}. Similar results have been presented by other workers (Edwards and Seddon 1992; Edwards 1993) from studies involving the inhibition of growth of *Botrytis cinerea* in liquid culture (GPB) inoculated with either mycelial plugs or conidia of the pathogen. In these studies GS was found to be more active in inhibiting conidial germination (5 μmol l^{-1}) than mycelial growth (50 μmol l^{-1}).

Each potential BCA produced different biocontrol profiles depending on the screening procedures used. *Bacillus subtilis* isolate C1 showed promising activity against the mycelial growth of *Botrytis cinerea* on PDA in the membrane filter bioassay. Isolates B3 and J7 (both strains of *B. subtilis*) showed promising antagonism against germinating conidia of *Botrytis cinerea* on glass slides and agar, respectively. These differing biocontrol profiles may have implications in the targeting of BCAs either singly or in concert against the pathogen in different stages of its life-cycle. These *Bacillus* species antagonists will now be investigated in more detail to develop biocontrol systems.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Eirian E. Jones of the Bacteriology Division, Institute of Cell and Molecular Biology, Edinburgh University for supplying some of the fungal material, Dr Niall Logan of Glasgow Caledonian University for his help with identification of the *Bacillus* isolates and Dr E. J. Allan for useful discussion and comments on the manuscript. This work was supported by a studentship from the University of Aberdeen to R.W.

REFERENCES

Baker, R. (1986) Biological control: an overview. *Canadian Journal of Plant Pathology* **8,** 218–221.

Blair, J.E., Lennette, E.H. and Truant, J.P. (1970) *Manual of Clinical Microbiology*, 1st edn. pp. 677–678. Bethesda: American Society for Microbiology.

Bradbury, J.F. (1988) Identification of cultivable bacteria from plants and plant tissue cultures using simple classical methods. *Acta Horticulturae* **225,** 27–37.

Cline, M.N., Chastagner, G.A., Aragaki, M., Baker, R., Daughtrey, M.L., Lawson, R.H., MacDonald, J.D., Tammen, J.F. and Worf, G.L. (1988) Current and future research directions of ornamental pathology. *Plant Disease* **72,** 926–934.

Cooper, K.E. (1963) The theory of antibiotic inhibition zones. In *Analytical Microbiology* ed. Kavanagh, F. pp. 1–86. New York: Academic Press.

Cooper, K.E. and Woodman, D. (1946) The diffusion of antiseptics through agar gels with the specific reference to the agar cup assay method of estimating the activity of penicillin. *Journal of Pathogenic Bacteria* **58,** 75–84.

Duffy, B.K. and Gardner, D.E. (1994) Locally established *Botrytis* fruit rot of *Myrica faya*, a noxious weed in Hawaii. *Plant Disease* **78 (9),** 919–923.

Edwards, S.G. (1993) *Biological control of* Botrytis cinerea *by* Bacillus brevis *on protected Chinese cabbage*. PhD thesis, University of Aberdeen, UK.

Edwards, S.G., Watson, D.G., McKay, T. and Seddon, B. (1991) Isolation and identification of a biological control agent from Chinese cabbage seeds as a *Bacillus licheniformis*. *Journal of Applied Bacteriology* **71,** xxix (Abstr.).

Edwards, S.G. and Seddon, B. (1992) *Bacillus brevis* as a biocontrol agent against *Botrytis cinerea* on protected Chinese cabbage. In *Recent Advances in Botrytis Research* ed. Verhoeff, K., Malathrakis, N.E. and Williamson, B. pp. 272–276. Wageningen: Pudoc Scientific Publishers.

Edwards, S.G., McKay, T. and Seddon, B. (1994) Interaction of *Bacillus* species with phytopathogenic fungi – Methods of analysis and manipulation for biocontrol purposes. In *Ecology of Plant Pathogens* ed. Blakeman, J.P. and Williamson, B. pp. 101–118. Wallingford: CAB International.

Garrett, S.D. (1970) Unspecialised parasites. In *Pathogenic Root-Infecting Fungi*, p. 31. London: Cambridge University Press.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrandale, R.F., Cox, R.H., Himmelsbach, D.S., Crumley, F.G. and Cutler, H.G. (1988) Isolation and identification of iturins as antifungal peptides in the biological control of peach brown rot with *Bacillus subtilis*. *Journal of Agricultural Food Chemistry* **36,** 366–370.

Ishihara, H., Ogawa, I. and Shimura, K. (1982) Component I protein of bacitracin synthetase: A multifunctional protein. In *Peptide Antibiotics*: *Biosynthesis and Functions* ed. Kleinkauf, H. and von Döhren, H. pp. 289–296. Berlin: Walter de Gruyter.

Iwaki, M., Shimura, K., Kanda, M., Kaji, E. and Saito, Y. (1972) Some mutants of *Bacillus subtilis* deficient in gramicidin S formation. *Biochemical and Biophysics Research Communication* **48,** 113–118.

Katz, E. and Demain, A.L. (1977) The peptide antibiotics of *Bacillus*: Chemistry biogenesis and possible functions. *Bacteriological Review* **41,** 449–474.

Kurahashi, K., Komura, S., Akashi, K. and Nishio, C. (1982) Biosynthesis of antibiotic peptides polymyxin E and gramicidin A. In *Peptide Antibiotics: Biosynthesis and Functions* ed. Kleinkauf, H. and von Döhren, H. pp. 275–288. Berlin: Walter de Gruyter.

Linton, A.H. (1983) Theory of antibiotic inhibition zone formation, disc sensitivity methods and MIC determinations. In *Antibiotics*, pp. 19–30. Society for Applied Bacteriology. Oxford: Blackwell Scientific Publications.

Markellou, E., Malathrakis, N.E., Walker, R., Edwards, S.G., Powell, A.A. and Seddon, B. (1995) Characterisation of bacterial isolates to *Botrytis cinerea* from the biotic environment and its importance with respect to *Bacillus* species and biocontrol considerations. In *Environmental and Biotic Factors in Integrated Plant Disease Control* ed. Mañka, M. 3rd Conference of European Foundation for Plant Pathology, pp. 385–389. Poznañ: The Polish Phytopathological Society.

Martin, S.B., Abawi, G.S. and Hoch, H.C. (1985) Biological control of soilborne pathogens with antagonists. In *Biological Control in Agricultural IPM Systems* ed. Hoy, M.A. and Herzog, D.A. pp. 433–434. London: Academic Press.

McKay, T., Edwards.G. and Seddon, B. (1991) Biocontrol of damp-

ing-off disease using *Bacillus* species. *Journal of Applied Bacteriology* **71**, xxix (Abstr.).

Mundt, J.O. and Hinckle, N.F. (1976) Bacteria within ovules and seeds. *Applied and Environmental Microbiology* **32,** 694–698.

Nelson, M.R. (1989) Biological control; the second century. *Plant Disease* **73,** 616.

Pusey, P.L. (1990) Control of pathogens on aerial plant surfaces with antagonistic microorganisms. In *Biological and Cultural Tests for Control of Plant Diseases*, Vol. 5. ed. Wilcox, W.F. pp. v–vii. APS: St Paul.

Ramachandran, L.K., Srinivasa, B.R. and Radhakrishna, G. (1982) Structural requirements for the biological activity of Polymyxin B. In *Peptide Antibiotics: Biosynthesis and Functions* ed. Kleinkauf, H. and von Döhren, H. pp. 427–443. Berlin: Walter de Gruyter.

Rhodes, D.J. (1990) Bacterial antagonist-fungal interactions on the plant aerial surface. In *The Exploitation of Microorganisms in Applied Biology. Aspects of Applied Biology*, No. 24, pp. 145–153. Warwick: Association of Applied Biologists.

Sadoff, H.L. (1972) Sporulation antibiotics of *Bacillus* species. In *Spores V* ed. Halvorson, H.O., Hanson, R. and Campbell, L.L. pp. 157–166. Bethesda: American Society for Microbiology.

Saito, Y., Otani, S. and Otani, S. (1970) Biosynthesis of gramicidin S. *Advances in Enzymology* **33,** 337–380.

Seddon, B., McKay, T., MacKenzie, L., Kemp, J.S., Fenlon, D.R. and Farr, L. (1993) The use of Viablue and DEFT for the detection of yeasts, moulds and bacteria. *International Biodeterioration and Biodegradation* **32,** 3–18.

Seddon, B., Edwards, S.G., Markellou, E. and Malathrakis, N.E. (1997) Formulation requirements for biological control agents. In *Multitrophic Interactions in Terrestrial Systems*. The 36th Symposium of The British Ecological Society ed. Gange, A.C. and Brown, V.K. pp. 3–25. London: Blackwell Science.

Sneath, P.H.A. (1986) Endospore-forming Gram-positive rods and cocci. In *Bergey's Manual of Systematic Bacteriology,* Vol. 2. ed. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. pp. 1104–1207. Baltimore: Williams & Wilkins.

Strang, J. (1992) Bacillus brevis *Nagano L-forms: Induction, Cultivation and Potential as Biocontrol Agents*. MSc thesis, University of Aberdeen, Scotland, UK.

Sutherland, J.R. (1991) Management of pathogens in seed orchards and nurseries. *Canadian Institute of Forestry* **67,** 481–485.

Wale, S.J. (1980) *The Post-harvest Pathology of Dutch White Cabbage in Refrigerated Storage*. PhD thesis, Manchester University, UK.

Walker, R. (1995) *Identification of Potential Biocontrol Agents of the Genus* Bacillus *for the Control of Damping-off of Seeds caused by* Botrytis cinerea *and* Pythium *Species*. M.Phil. thesis, University of Aberdeen, Scotland, UK.

Walker, R., Powell, A.A. and Seddon, B. (1994) Tests for biological control of seed and seedling damping-off diseases of peas and beans using *Bacillus* species. In *BCPC Monograph No. 57. Seed Treatments: Progress and Prospects* ed. Martin, T. pp. 333–338. Farnham: British Crop Protection Council.

Walker, R., Emslie, K.A. and Allan, E.J. (1996) Bioassay methods for the detection of antifungal activity by *Pseudomonas antimicrobica* against the grey mould pathogen *Botrytis cinerea*. *Journal of Applied Bacteriology* **81,** 531–537.

Whipps, J.M. (1987) Effect of media on growth and interactions between a range of soil-borne glasshouse pathogens and antagonistic fungi. *New Phytologist* **107,** 127–142.

Zhang, X.J., Miao, W.G., Zhu, G.N. and Wang, J.S. (1993) Screening of bacterial antagonists against several important crop disease pathogens. *Chinese Journal of Biological Control* **9,** 126–129.