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Evaluation of antagonistic bacteria for suppression of bacterial ring rot of potato

P. Gamard[1] & S. H. De Boer*

**Author for correspondence: Agriculture Canada, Pacific Agriculture Research Centre, 6660 N. W. Marine Drive, Vancouver, British Columbia, Canada, V6T 1X2 [1]Present address: McGill University, Macdonald Campus, Department of Plant Science, Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9*

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Abstract

Bacterial strains with potential for biological control of bacterial ring rot of potato caused by *Clavibacter michiganensis* subsp. *sepedonicus* were isolated from the surface of potato tubers. Eighty-eight potential biocontrol candidates, selected on the basis of *in vitro* antibiosis to *C. m. sepedonicus*, produced inhibition zones with radii ranging from 0.5 to 16 mm on test plates. All antagonistic isolates were screened in the greenhouse for biocontrol activity on micropropagated potato plantlets root-inoculated with *C. m. sepedonicus*. Eight strains consistently prevented infection of plantlets but there was no significant correlation between the width of the inhibition zone in the *in vitro* assay and ring rot suppression in the plant bioassay. Three strains that showed a high level of biological control potential were identified as a saprophytic enteric bacterium (strain 7G), an *Arthrobacter* sp. (strain 16C), and a soil coryneform bacterium (strain 18A). These were tested in a field plot by co-inoculating cut seed potato tubers with *C. m. sepedonicus* and antagonists. Strains 7G and 18A significantly increased plant stand whereas 16C decreased disease incidence. The relative number of ostensibly ring rot-free progeny tubers was generally greater when antagonists were present.

Introduction

Bacterial ring rot of potato, caused by *Clavibacter michiganensis* subsp. *sepedonicus* (Spieck. and Kotth.) Davis *et al.*, remains a concern of the potato industry in North America. Although the disease has been significantly reduced by a zero certification tolerance [De Boer and Slack, 1984], it continues to persist in the potato industry, in part, due to asymptomatic infections which serve to spread the bacterium to contact seed lots.

It may be possible to reduce the incidence of ring rot in seed lots that contacted a *C. m. sepedonicus* inoculum source by a biological control strategy. Previously we noted that ring rot infected tubers sometimes give rise to ostensibly disease-free plants [De Boer and McNaughton, 1986], and preliminary observations indicated that this may occur when the seed piece decays prior to translocation of the bacteria from the seed piece into the stem [S.H. De Boer, unpubl.]. This suggested that by increasing the population of microorganisms which are antagonistic to *C. m. sepedonicus* on seed tubers, it may be possible to inhibit multiplication and spread of the ring rot pathogen to stem tissue, and thereby prevent disease from developing. Recently De La Cruz *et al.* [1992] showed that ring rot was suppressed by antagonistic fluorescent pseudomonads in greenhouse experiments with stem-cultured potato seedlings. Biological control of the brown rot disease of potato, caused by *Pseudomonas solanacearum*, has also been reported to be successful with an antagonistic microorganism in experimental trials [Ciampi-Panno *et al.*, 1989].

In this study we selected potential biocontrol microorganisms from among naturally occurring bacteria associated with potato. Bacteria which showed *in vitro* antibiosis of *C. m. sepedonicus*, and reduced the incidence of ring rot in greenhouse trials, were tested

for suppression of disease development in the field. A preliminary report on a part of this study has been presented [Gamard and De Boer, 1994].

Materials and methods

Bacteria

C. m. sepedonicus strain R8, isolated in 1984 from cv. Russet Burbank was used in all experiments. It was stored in 10% glycerol at −80 °C and grown at 23 °C on yeast extract, glucose, mineral salts medium (YGM) [De Boer and Copeman, 1980].

Isolation and selection of antagonists

Potato tubers of six cultivars were sampled from New Brunswick and British Columbia. Bacteria inhibitory to *C. m. sepedonicus* were isolated mainly from the surface of potato tubers, but also from tuber sap and potato rhizosphere soil. Suspensions of tuber surface and rhizosphere microflora were prepared simply by rinsing tuber surfaces or roots with 100 ml of sterile distilled water and collecting the wash water. Tuber sap samples were extracted from tissue at the stolon end of non-surface sterilized tubers in the same manner as samples for ring rot tests are prepared [Anon., 1987]. Three 10-fold dilutions of each suspension were prepared in sterile one quarter strength Ringer solution (QSRS) and 100 μl of each dilution were added to 3 ml of molten soft (0.6% agar) YGM medium. The molten agar was poured over a 15 ml layer of standard (1.5% agar) YGM medium in Petri dishes. A third layer of 3 ml sterile soft YGM medium was poured over the bacteria-containing layer after it solidified. Plates were incubated for 24 h at 23 °C to initiate bacterial growth in the sandwiched layer of agar, after which a final layer containing about 10^9 colony forming units (cfu)/ml of *C. m. sepedonicus* R8 in soft YGM medium was added. Plates were further incubated at 23 °C for 3 to 5 days. The ring rot bacteria developed a confluent lawn of microcolonies in the top layer, except in zones of inhibition immediately above antagonistic colonies. Antagonistic bacteria were isolated by stabbing the colonies through the zone of inhibition, restreaking, and subsequent single colony selection. Antagonistic isolates were stored at −80 °C in 10% glycerol. Inhibition by each isolate was retested by point inoculation of plates containing a layer of *C. m. sepedonicus* in soft YGM poured over standard YGM plates. After 3 days at 23 °C, radii of inhibition zones were measured. Inhibition tests were repeated at least twice.

Bioassay for biocontrol potential

Micropropagated plantlets of the ring rot susceptible cultivar, Red Pontiac, were obtained from nodal cuttings rooted in glass jars, on Murashige and Skoog medium (Murashige minimal organic medium, Gibco Canada Ltd., Burlington, ON, supplemented with 30 g/L sucrose, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 2 mg/L glycine, 4 μg/L kinetin, 10 g/L agar). Three-week-old plantlets were removed from the agar medium and the roots were cut back by one-third. The remaining root systems were first immersed for 30 min in a suspension of an antagonistic bacterium at 10^8 cfu/ml in QSRS, then for 30 min in a suspension of strain R8 at 5×10^6 cfu/ml in QSRS. Each treatment was replicated on 6 plantlets. For each experiment in which 8–10 antagonists were tested, six positive control plantlets were treated with sterile QSRS prior to the R8 suspension and six negative control plantlets were treated with sterile QSRS only. Treated plantlets were planted into a soil mix (Metro-mix, Grace Hort. Products, Vancouver, BC) in individual containers and grown in the greenhouse at 21 ± 2 °C with a 16 h daylight photoperiod supplemented by sodium vapour lamps. After 2–3 weeks a 1- cm basal stem section was tested in ELISA to detect *C. m. sepedonicus* infections as described previously [De Boer *et al.*, 1988]. Plantlets were considered positive when absorbance readings were at least twice the mean of readings for the six uninoculated controls. The bioassays were repeated at least twice for antagonistic strains which reduced disease incidence in the first trial.

Preliminary identification of antagonists

Eight antagonistic isolates were tentatively identified on the basis of Gram stain and carbohydrate utilization patterns (Biolog Inc., Hayward, Calif.). Four of these strains were submitted to MIDI (Microbial ID Inc., Newark, Delaware) for identification by fatty acid analysis.

Field trial

Three antagonistic isolates, 7G, 16C, and 18A which differed in colony morphology, identifications, and the size of inhibition zones were selected for testing in field plots for potential biocontrol activity. Seed potato tubers (cv. Red Pontiac) were inoculated with *C. m. sepedonicus* strain R8 by one of two ways. One proce-

dure was vacuum infiltration of cut tuber seed pieces with a bacterial suspension containing 10^6 cfu/ml as described previously [De Boer and McCann, 1990]. The other procedure involved cutting tubers into seed pieces with a knife dipped into an agar (0.4%) slurry of bacteria at 10^9 cfu /ml. Vacuum-infiltrated seed pieces were dried overnight at room temperature before treatment with antagonists, whereas knife-inoculated seed pieces were treated with antagonists one day prior to inoculation with the pathogen. Treatment with antagonistic bacteria was done by dipping tubers or seed pieces into aqueous suspensions of 24-h-old washed cells at 10^8 cfu/ml for 30 min. Positive controls were inoculated only with *C. m. sepedonicus*. Negative controls were inoculated and treated with sterile QSRS in place of bacterial suspensions.

Plots of 24 seed pieces for each of ten treatments were planted in a randomized block design with four replications. Plots within rows were separated with five plants of a white-skinned cultivar (cv. Superior) and two guard rows were planted along the perimeter of the field. Spacing of seed pieces was 15 cm within rows and 1 m between rows. Standard agronomic practices were used for cultivation, fertilization, pest control, and vine-killing.

During the growing season, the number of plants in each plot was recorded as well as the number of plants with ring rot symptoms. Petioles from 12 randomly selected stems were sampled from each plot and tested for the presence of *C. m. sepedonicus* by ELISA. The proportion of plants with latent ring rot infections was estimated from the number of asymptomatic ELISA-positive plants.

At harvest, the total number of tubers and the number with external ring rot symptoms were counted. Sixteen symptomless tubers from each plot were tested by an indirect sandwich ELISA [De Boer *et al.*, 1988] to determine the proportion of latently infected tubers. Statgraphics software (Statistical Graphics Corporation, Rockville, MD) was used for analyses of variance and multiple range tests. Treatments were compared using the 95% least significant difference method.

Results

Isolation and selection of antagonists

Eighty-three bacterial strains antagonistic to *C. m. sepedonicus* were isolated from tuber wash samples, one from rhizosphere soil, and four from tuber sap. The radii of inhibition zones ranged from 0.5 to 16 mm in the plate assay and were consistent in repeated assays with the same isolate. Almost 49% of the 83 isolates, including the isolate from the rhizosphere and two isolates from tuber sap, produced narrow ($\leq$4 mm) zones of inhibition.

Preliminary greenhouse experiments indicated that although ring rot symptoms did not develop in root inoculated plantlets until at least eight weeks after inoculation, infections could be detected in stems in as little as two weeks. Only eight of the 81 antagonistic strains tested prevented infection in all of the six inoculated plantlets in each of the 2–4 replications performed with these strains. Other strains inhibited development of *C. m. sepedonicus* in 1–5 of the test plants. There was no significant correlation between the width of the inhibition zone in the plate assay and the biocontrol potential in the plant bioassay. The eight isolates with the greatest apparent biocontrol potential gave inhibition zones from 0.5–16 mm in the *in vitro* assay.

Preliminary identification of antagonists

Six of the eight strains that inhibited *C. m. sepedonicus* in potato plantlets were tentatively identified with the Biolog system (Table 1). Fatty acid profile analysis by MIDI of four of these strains suggested different identifications from the Biolog results. Subsequent comparison of the antagonistic strains with type strains, obtained from LMG (Gent, Belgium) and ATCC (Rockville, MD) collections, of each species which gave the best match by Biolog and MIDI, indicated that the antagonistic strains were similar to but differed in carbohydrate utilization pattern from the type strains of these species.

Field trial

Severity and incidence of the ring rot disease was generally greater in plots which were vacuum infiltrated with *C. m. sepedonicus* compared to plots inoculated by the cutting knife (cf. Figs. 1A & C with Figs. 1B & D). In plots inoculated by vacuum infiltration, the number of surviving plants by mid-season (plant stand) was significantly greater if they had received treatment with antagonistic bacteria, although numbers were still lower than for the uninoculated control (Fig. 1A). In plots inoculated by cutting knife, antagonist strains 7G and 18A significantly increased plant stand, whereas strain 16C had the greatest significant effect on decreasing disease incidence (Fig. 1B). The method

Table 1. Isolates of bacteria inhibitory to *Clavibacter michiganensis* subsp. *sepedonicus*, and the species identifications giving the best match on the basis of carbohydrate utilization pattern (Biolog) and fatty acid profile (MIDI)

Strain	Width of inhibition zone (mm)	Biolog best match	MIDI best match
6A	8	*Serratia plymuthica*	ND[a]
7G	8	*Serratia plymuthica*	*Erwinia herbicola*
16C	16	*Arthrobacter histidinolovorans*	*Arthrobacter protophormiae*
17C	6	*Curtobacterium flaccumfaciens*	ND
18A	0.5	no identification	*Curtobacterium citreum*
20A	4	*Pseudomonas marginalis*	ND
26D	0.5	no identification	ND
45A	11	*Pseudomonas fluorescens* B	*Pseudomonas aureofaciens*

[a]Not done.

Table 2. Percentage of ostensibly healthy plants and tubers produced from seed co-inoculated with *Clavibacter michiganensis* subsp. *sepedonicus* and one of three antagonistic bacteria, relative to uninoculated controls

Treatment	Plants		Tubers	
	Knife[a]	Vacuum[b]	Knife	Vacuum
Uninoculated	100.0a[c]	100.0a	100.0a	100.0a
Cms[d] alone	29.3b	30.1b	18.3c	7.6b
Cms + 16C	79.1a	32.3b	48.1b	12.0b
Cms + 18A	28.8b	43.1b	26.3bc	14.7b
Cms + 7G	32.7b	41.8b	28.8bc	15.2b

[a] *C. m. sepedonicus* inoculated into seed potatoes with a contaminated cutting knife (see text for details).
[b] *C. m. sepedonicus* inoculated into seed potatoes by vacuum infiltration (see text for details).
[c] Values in each column followed by the same letter are not significantly different at p=0.05.
[d] *C. m. sepedonicus.*

of inoculation and treatment with antagonistic bacteria had a very similar effect on tuber yield and disease incidence as on the plants in the field. Strains 7G and 18A had the greatest effect on the number of tubers produced while strain 16C had the greatest impact on reducing disease incidence (Figs. 1C and D). Strain 16C significantly increased the relative percentage of healthy plants and tubers produced by the knife-inoculated seed tubers (Table 2). The proportion of asymptomatic infections was generally greater in knife-inoculated compared to vacuum-inoculated plants and tubers and was not consistently affected by the antagonists (Fig. 1).

Discussion

When *C. m. sepedonicus* is isolated from diseased potato stems and tubers, zones of inhibition are often observed around some colonies of contaminating saprophytic bacteria. The procedure we used to detect bacteria inhibitory to *C. m. sepedonicus* confirmed that almost all samples with tuber-associated bacteria contained strains that were antagonistic to the ring rot bacterium. We isolated bacteria primarily from tuber surfaces because they would be the ones most likely to survive well on potato seed pieces for biocontrol.

Although only some bacteria which show antibiosis *in vitro* will be useful as biological control agents, antibiosis is a convenient criterion for an initial screen to distinguish potential biocontrol candidates from the total soil microflora. The colony morphologies of many of the antagonistic bacteria differed from one another which suggested that many different species produced substances inhibitory to *C. m. sepedonicus*. The variation in diameter of the inhibition zones, which presumably is a function of the molecular weight and/or concentration of the compounds, also suggested that different inhibitory compounds were being produced.

Our second phase of testing on micropropagated potato plantlets, identified strains that were most adept in suppressing development of *C. m. sepedonicus* in potato plants. Several strains entirely prevented infection whereas all control plants not treated with antagonistic bacteria developed detectable infections. The procedure tested the ability of antagonistic isolates to suppress bacterial ring rot *in planta* but did not test other characteristics that are essential for successful

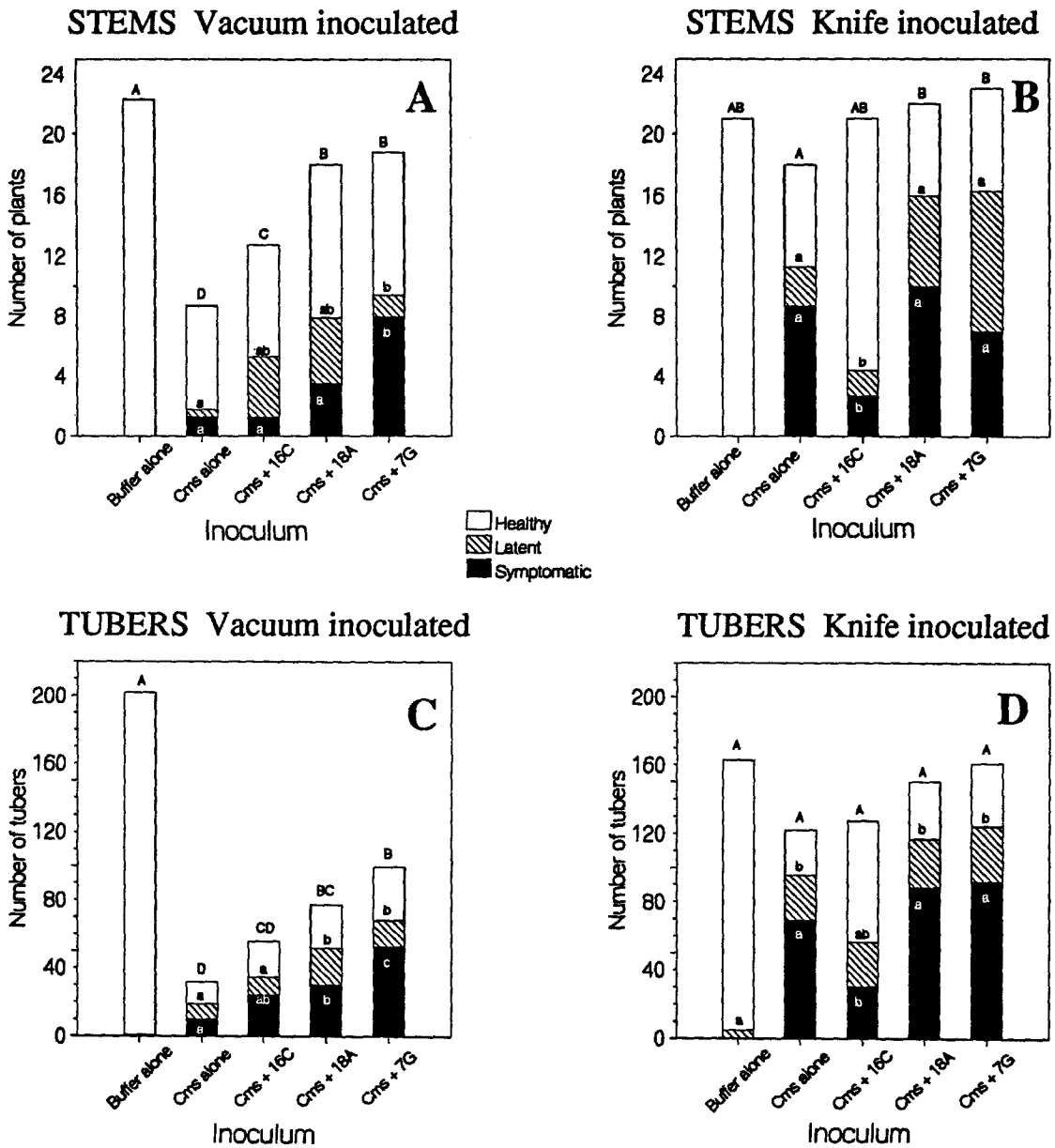

Fig. 1. Number of plants at midseason and tubers at harvest that were ostensibly healthy and that had latent or symptomatic bacterial ring rot infections in field plots planted with seed pieces inoculated with *Clavibacter michiganensis* subsp. *sepedonicus* and one of three antagonistic bacteria. Seed tubers were either inoculated with *C. m. sepedonicus* by vacuum infiltration (A and C) or with a contaiminated knife (B and D). Different uppercase letters above bars signify significant differences in total number of plants and tubers within each graph. Different lowercase letters above cross-hatched bars and on solid bars indicate significant difference, respectively, in the number of latently infected and symptomatic plants and tubers within each graph.

biocontrol agents. Our test differed from the greenhouse test by which De La Cruz *et al.* [1992] tested efficacy of antagonistic pseudomonads for controlling ring rot in that they did not directly inoculate test plantlets with the pathogen but rather planted them in *C. m. sepedonicus* infested soil. We used the more stringent test to discriminate strains with the greatest capability of preventing ring rot from developing.

However, field testing is essential to determine whether suppression of the ring rot disease with antagonistic bacteria will be efficacious for growers.

Only tentative identification was made of antagonistic strains because complete identification of saprophytic bacteria from soil is tedious and the taxonomy of many soil bacteria is incomplete. On the basis of our tests, strains 6A and 7G appeared to be saprophytic enterobacteria because they typed variously as *Serratia plymuthica* or *Erwinia herbicola*. Both of these species have been isolated from the surface of plants and some *E. herbicola* strains produce antibiotics [Greiner and Winkelmann, 1991] and have been studied as possible biocontrol agents for the fireblight disease incited by *Erwinia amylovora* [Chatterjee *et al.*, 1969]. Strains 20A and 45A are fluorescent pseudomonads, which as a group have also been widely studied for the control of soilborne plant diseases [Weller, 1988]. Strains 16C, 17C and 18A are members of the Gram positive soil corynebacteria which form an important component of the indigenous soil microflora [Veldkamp, 1970]. Saprophytic soil corynebacteria generally have properties that are attractive for biological control such as the ability to withstand prolonged periods of drought and nutrient starvation [Boylen and Mulks, 1978]. Strain 16C, particularly, showed a suppressive effect on bacterial ring rot and appears to be an *Arthrobacter* sp. similar to *A. protophormiae* and *A. histidinolovorans*. Accurate identification of arthrobacters by Biolog and MIDI is limited by the number of strains that have been entered into the respective computer libraries. However, both *A. protophormiae* and *A. histidinolovorans* were classified as typical arthrobacters in a large numerical study [Seiler, 1983] and strain 16C had a fatty acid profile and carbohydrate utilization pattern similar to this group. Production of anti-bacterial compounds by arthrobacters have not been described but some species do release bacterial growth factors [Veldkamp *et al.*, 1966] and auxins [Katznelson and Sirois, 1961] into the soil. Other *Arthrobacter* spp. have potential as biological control agents for plant diseases caused by soil-borne fungi [Mitchell and Hurwitz, 1965; Sneh, 1981].

Biological control of bacterial ring rot is most logically directed toward suppressing the development of the disease when a seed lot has become contaminated with a low level of inoculum through inadvertent contact with contaminated equipment or a field source. Seed lots with high levels of bacterial ring rot contamination would not be considered for planting under any circumstances. Nevertheless, evidence for biocontrol activity is easier to ascertain in small field experiments when a high incidence of disease occurs in positive control treatments than when disease incidence is low. The highly susceptible cultivar, Red Pontiac, was, therefore, used with a high inoculum dosage in the field experiment. The vacuum infiltration treatment was meant to simulate natural primary infection, while the cutting knife inoculation treatment was meant to simulate secondary infection from contaminated equipment.

The three antagonistic bacteria tested in the field experiment suppressed the ring rot disease. Strains 7G and 18A had a greater effect on plant stand than 16C, but 16C treated plots had the lowest disease incidence. Perhaps strains 7G and 18A inhibited *C. m. sepedonicus*-induced breakdown of the seed pieces so the reduction in plant stand observed with *C. m. sepedonicus* alone was attenuated by these antagonists, whereas 16C impeded movement of *C. m. sepedonicus* from the seed pieces into the plants. An increase in latent infections was observed in some of the vacuum infiltrated plots treated with antagonists. Although this would be a problem for seed potato production, increased latent infections probably would not occur at lower disease pressure as observed with the knife inoculation treatments.

The suppression of bacterial ring rot with antagonistic bacteria in field experiments with high levels of ring rot inoculum is encouraging. Biological suppression of the disease at low levels of inoculum is expected to be even more effective but large scale field experiments are required to test whether low incidence of ring rot infections can be controlled entirely by inoculation of seed pieces with an antagonistic bacterium.

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