The Recovery of *Corynebacterium sepedonicum* from Sugar Beet Seed

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ABSTRACT

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Pathogenic strains of *Corynebacterium sepedonicum*, the cause of bacterial ring rot of potato, were recovered from sugar beet seeds that were produced in the Willamette Valley of Oregon in 1984. The bacterium was recovered directly from culture plates of diluted sugar beet seed extracts and from eggplants that had been inoculated with sugar beet seed strains of the bacterium. All strains of *C. sepedonicum* recovered from sugar beet seed were pathogenic to potato and eggplant. Indirect immunofluorescent antibody staining (IFAS) using highly specific monoclonal antibodies detected the bacterium in seed extracts. Seed extracts from one of seven cultivars produced in Oregon in 1985 and two of 26 foreign seed lots were IFAS positive, but the bacterium could not be recovered from dilution plates or eggplants that were inoculated with centrifuged seed extracts.

Additional key words: bacterial diseases, bacterial ring rot, *Clavibacter michiganense* subsp. *sepedonicum*, endophytes, latent infections.

Corynebacterium sepedonicum (Spiek. & Kotth.) Skapt. & Burkh. (syn. *Clavibacter michiganense* subsp. *sepedonicum* (Spieck. & Kotth.) Davis et al), the bacterium that causes ring rot of potato, has been isolated from symptomless sugar beet roots (1). The bacterium was first recovered from a 6-wk-old sugar beet seedling that had grown in nonsterile soil in the greenhouse. Further attempts to recover the bacterium from field-grown sugar beet roots by direct dilution plating of root extract failed. Subsequent recoveries were made by enrichment of the *C. sepedonicum* population in eggplant (1). It was theorized that the bacterium must have been seedborne in our initial recovery because 6 wk would not have been long enough for the bacterium to infect and colonize the sugar beet seedling in the greenhouse from the soil considering the slow growth of this pathogen. Further, the bacterium was isolated from sugar beet roots taken from fields not previously planted to potatoes (1).

All of the hybrid sugar beet seed for domestic use is produced in Oregon. There are international imports or exports of parental germ plasm and hybrid seed. It might be difficult to eradicate potato ring rot from certain regions of the United States if the bacterium is present in sugar beet seeds that are planted in potato-growing regions; therefore, our objective was to determine if *C. sepedonicum* is carried in sugar beet seed.

MATERIALS AND METHODS

Fifty-gram quantities of each seed sample were assayed for *C. sepedonicum*. The seeds were from six cultivars produced in 1984 and seven cultivars produced in 1985. These seeds were produced in the Willamette Valley of Oregon. In addition, 26 seed lots from Europe were assayed twice. The procedure that was used to recover *C. sepedonicum* from sugar beet seed was similar to that developed for the detection of latent ring rot infections in potato tubers (10,13). Seed lots that had been treated with fungicides were washed 1 hr in running tap water to remove the fungicide. The seeds were surface disinfected in 0.5% sodium hypochlorite (NaOCl) for 2 min followed by three rinses in 200 ml of sterile distilled water (SDW). The seeds then were steeped 1 hr in 100 ml of sterile 0.02 M potassium phosphate buffer (KPB), pH 7.2, at room temperature to soften the seed. The seeds were homogenized in the same buffer in a sterile food blender jar then shaken in a rotary shaker at 125 rpm for 30 min. The homogenate was centrifuged for 5 min at 300 *g* to remove large particles. The supernatant was centrifuged for 30 min at 10,000 *g* to concentrate the remaining bacteria and plant particles into a pellet. A 1-ml sample of the pellet was diluted in sterile distilled water in a 10-fold series and plated on nutrient broth yeast extract (NYS) agar containing: nutrient broth (Difco) (8 g), yeast extract (Difco) (2 g), K_2HPO_4 (0.5 g), sucrose (5 g), agar (15 g), and added after autoclaving 1 ml of 1 M $MgSO_4 \cdot 7H_2O$. Pellet material also was examined by indirect immunofluorescent antibody staining (IFAS) (1). The remainder of the pellet (3–5 ml) was used immediately to inoculate eggplant (*Solanum melongena* L. 'Black Beauty') or was stored at −22 C to be used later for inoculation. The method of disinfecting seed surfaces was tested on seed that had been inoculated with 10^6 colony-forming units (cfu)/ml of *C. sepedonicum*. The method was successful because the bacterium was not detected in dilution culture plates of seed extract.

After 9–11 days of incubation, small bacterial colonies on dilution plates were transferred to new NYS plates and tentatively identified as *C. sepedonicum* according to colony characteristics, Gram stain reaction, and cellular morphology. Final identification was based on pathogenicity testing (11). The methods for increase of inoculum and inoculation of potato (*Solanum tuberosum* L. 'Red Norland') and eggplant for pathogenicity testing were reported previously (1). Potato sprouts about 7 mm long were removed from tubers with a household melon scoop. A disposable 27-gauge syringe needle containing 0.1 ml of inoculum was inserted into the base of the sprout. The sprouts then were planted in a commercial potting mix (Sunshine Mix #1, Fisons Western Corp., Vancouver, B.C., Canada) in 15-cm clay pots in the greenhouse. Eggplants were inoculated by loading 0.1 ml of pellet material into 26- or 27-gauge syringe needles and hub and inserting two needles on opposite sides of the stem base. A third inoculation was done on each plant at the first leaf axil. The needles remained in the plants overnight. Control plants were inoculated with two potato strains

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(NDCs OFF or NDCs N-1-1) of the bacterium or sterile 20 mM KPB, pH 7. The onset and prevalence of ring rot symptoms were recorded for 12–14 wk for potato and 4–6 wk for eggplant. Stems were harvested and extracted to recover the bacterium. The excised stems were surface disinfested by flaming-off 95% ethanol, then homogenized in 30 ml of sterile KPB in a sterile tissue homogenizer. The homogenate was centrifuged as described previously and samples of the pellet were plated on NYS and examined with IFAS.

Confirmation of the identity of sugar beet seed strains of the bacterium was based on physiological and biochemical tests reported earlier (1). Seed strains of *C. sepedonicum* were compared with three strains of *C. sepedonicum* from the American Type Culture Collection (ATCC), 10 potato strains, and four other species of *Corynebacterium* from ATCC (*C. michiganense* (Smith) Jensen ATCC 14456, *C. betae* Keyworth et al ATCC 13437, *C. insidiosum* (McCulloch) Jensen ATCC 10253, and *C. nebraskense* Shuster et al ATTC 27822).

To determine the percentage of seed infected, 60 seeds of cultivar Dippe 2 that were produced in 1984 were assayed individually. The seeds were surface disinfected as described previously then steeped in sterile KPB in a sterile petri dish for 5 days at room temperature to soften the seed. Each seed was ground individually with a sterile mortar and pestle in 0.9 ml of sterile KPB. Samples from each of three 10-fold dilutions were placed in wells of toxoplasmosis slides and examined for *C. sepedonicum* using the IFAS assay.

RESULTS

Five of six commercial sources of sugar beet seed that were produced in 1984 in Oregon were positive for *C. sepedonicum* using the IFAS assay, and the bacterium was isolated from dilution plates for all five seed sources. One of seven commercial seed lots that were produced in 1985 was IFAS positive, but the bacterium was not detected on dilution plates of sugar beet seed extracts or in extracts from eggplants that were inoculated with the centrifuged seed extracts. The five seed strains of *C. sepedonicum* that were recovered from sugar beet seed produced in 1984 were pathogenic on potato and produced symptoms of chlorosis and wilt identical to those symptoms that were produced by the known potato strains. When four eggplants in each of two tests were inoculated with the five seed strains, one to four plants developed ring rot symptoms that were identical to those that were produced by the known potato strains (Fig. 1, Table 1). Eggplants injected with buffer produced no symptoms. Centrifuged extracts of infected eggplant stems contained bacteria that reacted with IFAS and *C. sepedonicum* was reisolated from the dilution plates. Extracts from uninoculated eggplants had no IFAS positive reactions.

Fig. 1. Ring rot symptoms of interveinal chlorosis and wilting on eggplants cv. Black Beauty that were inoculated by a known potato strain or a sugar beet seed strain of *C. sepedonicum* from cultivar Dippe 2. Plants inoculated with sterile broth diluent remained healthy.

The results of physiological and biochemical comparisons of five seed strains with three ATCC and 10 potato strains of *C. sepedonicum* and four other species of *Corynebacterium* showed the seed strains were identical to the potato strains in biochemical and physiological comparison tests but distinct from *C. michiganese*, *betae*, *insidiosum*, and *nebraskense* (Table 2). The four strains were phenotypically identical to sugar beet root strains reported earlier (1).

Two of 26 foreign seed lots were positive in the IFAS assay in the first trial. One infected seed lot was produced in England and one was produced in France. No positive IFAS seed lots were found in the second trial. The bacterium was not detected on dilution plates or recovered from the extract of eggplants that were inoculated with IFAS-positive centrifuge pellets from the first trial.

C. sepedonicum was more prevalent at the higher dilutions on dilution plates of seed extracts from cultivar Dippe 2. No *C. sepedonicum* was present on culture plates at dilutions of 10^{-3} or 10^{-4} but did appear at a dilution of 10^{-5}. At these higher dilutions, other bacteria were at a low concentration or absent. Therefore, antagonism resulting from the presence of bacterial species other than *C. sepedonicum* may explain our inability to recover the ring rot bacterium at the lower dilutions.

Cultivar Dippe 2 had the highest concentration of *C. sepedonicum* of all the seed lots that were assayed. The population estimate for this seed lot was 1.7×10^5 cfu gm^{-1} of seed. The results from the assay of 60 seeds of cultivar Dippe 2 1984, using IFAS, showed that 80% were positive for *C. sepedonicum*.

DISCUSSION

The evidence reported here shows that the potato ring rot bacterium can be seedborne in sugar beet. The pathological, biochemical, physiological, and serological tests showed that strains of *C. sepedonicum* isolated from sugar beet seed were identical to strains isolated from potato. We were able to recover the bacterium directly from dilution plates of five of eight seed extracts that were IFAS positive, whereas sugar beet root extracts needed to be enriched by inoculation into eggplants before the bacterium could be recovered (1). The success with direct plating may have been due to a larger bacterial population per unit volume of seed compared with root tissue.

TABLE 1. The detection of *Corynebacterium sepedonicum* in sugar beet seed extract by immunofluorescent antibody staining (IFAS) and frequency of symptoms on eggplant after inoculation with the recovered seed strains

Sugar beet cultivar	Strain designation	IFAS reaction 1984 Seed	No. eggplants with symptoms/ No. inoculated eggplants	
			Test 1	Test 2
Dippe 2	1	+[a]	1/4	4/4
Ultramono	2	+	2/4	4/4
Monoricca	3	+	3/4	3/4
BJ-19	4	+	1/4	4/4
Beta 1230	5	+	0/4	1/4
Mono-Hy R103	–	–[b]	...	...
Inoculated with sterile buffer			0/4	0/4
1985 Seed				
BJ-19	–	+[c]	0/4	0/4
KW-3394	–	–	0/4	0/4
Mono-Hy R103	–	–	0/4	0/4
Monoricca	–	–	0/4	0/4
Monohikari	–	–	0/4	0/4
Ultramono	–	–	0/4	0/4
ACH 124	–	–	0/4	0/4

[a] + = A positive IFAS reaction.
[b] – = A negative IFAS reaction.
[c] *C. sepedonicum* was not recovered from seed extract.

TABLE 2. Biochemical comparison of five sugar beet seed strains of *Corynebacterium sepedonicum* with known strains of plant pathogenic corynebacteria

	C. sepedonicum			*Corynebacterium*			
Test	Potato[a]	Sugar beet	ATCC[b]	*michiganense*	*betae*	*nebraskense*	*insidiosum*
Color	Pale yellow 3A3[c]	Pale yellow 3A3	Pale yellow 3A3	Wax yellow 3B5	Naples yellow 3B7	Golden yellow 5B7	Curry yellow 4C8
Utilization							
Acetate	+[d]	+	+	+	+	+	+
Fumarate	+	+	+	+	−	+	+
Acid production from							
Lactose	−	−	−	+	+	+	+
Maltose	v[e]	v	v	+	+	+	+
Mannitol	+	+	+	+	+	−	+
Glycerol	v	v	v	+	+	+	+
Melezitose	−	−	−	−	+	−	−
Trehalose	−	−	−	+	+	w	w
Rhamnose	−	−	−	−	+	−	−
Melibiose	−	−	−	+	+	w	w
Cellobiose	v	v	v	+	+	+	+
Growth on							
CNS[f]	−	−	−	+	+	+	+
TTC[g]	−	−	−	+	+	−	+
H_2S production	v	−	v	+	+	+	+
Indigoidine produced	−	−	−	−	−	−	+
Gelatinase	−	−	−	+	−	−	−
Lipase							
Tween 80	−	−	−	−	+	−	−

[a] Ten potato strains were tested.
[b] Three strains were tested.
[c] Color code from Methuen Handbook of Color (Kornerup & Wanscher 1967) (9).
[d] + = Positive; − = negative.
[e] w = Weak; v = variable among strains.
[f] CNS = Growth medium containing cycloheximide, nalidixic acid, and polymixin B sulfate (3).
[g] TTC = Growth medium containing tetrazolium chloride (6).

The source of the seedborne *C. sepedonicum* is not known. The domestic seed that were assayed were produced in Oregon as is most of the U.S.-grown sugar beet seed. Although potato ring rot is present in Oregon, the amount of potato acreage is small and not located near the sugar beet seed production areas. The bacterium may be systemic because it was recovered from surface disinfested seed, which indicates deep infection. The prevalence of the bacterium in sugar beet seed, as shown with cultivar Dippe 2, indicates that high percentage infection is possible. The bacterium could have moved into seed from infected roots; systemic movement of plant pathogens in sugar beet is known. *C. betae*, which systemically infects red beets causing leaf silvering symptoms followed by wilting and death, also may be seed contaminants (7). *Phoma betae*, a fungal pathogen of sugar beet, moved into flowers and seed from infected roots (8). Healthy sugar beet roots contain populations of bacteria including various species of Corynebacteria (1,5). The roots of parental germ plasm could become infected at the site of production anywhere in the world and hybrid seed produced from these roots could be infected with *C. sepedonicum* when sent to Oregon for local production.

The IFAS method used a specific monoclonal antibody (2) that enhanced the sensitivity of the assay. However, a positive IFAS reaction did not always result in recovery of the bacterium from dilution plates or the inoculated eggplants. Low virulence (4) or population (12) level of the bacterium in the extracts and the apparent antagonism at low dilutions reported here might account for the failure.

Our inability to recover *C. sepedonicum* using dilution plates from the European sugar beet seed lots, where bacterial ring rot does not commonly occur, was extremely interesting. This is especially so considering the relative ease with which we isolated this bacterium from domestic seed lots. This disparity may be due to the difference in the incidence of bacterial ring rot of potato in the respective geographic regions. Ring rot is not commonly reported in most potato-growing areas of Europe.

It remains to be proven that infected sugar beets are a source of inoculum for infecting potatoes in the field. However, the importation of infected sugar beet seed or roots into potato-growing regions might prevent the successful and permanent eradication of the potato bacterial ring rot disease.

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