Streptomycin Resistance and Copper Tolerance Among Strains of *Pseudomonas cichorii* in Celery Seedbeds

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ABSTRACT

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Strains of *Pseudomonas cichorii* were isolated from celery plants exhibiting symptoms of bacterial blight at four seedbed sites in Florida. In 1991, 70% of 87 isolated strains were resistant to 200 μg/ml of streptomycin in vitro. In 1992, 32% of 106 strains were streptomycin-resistant. Most of the reduction in streptomycin resistance in 1992 was accounted for by a reversal from 100% to 0% resistance among strains collected from one seedbed site. Strains were also assayed for tolerance to 0.64 mM $CuSO_4$. In 1991, 32% of the strains were classified as sensitive (growth 0–30% of that on controls), 44% as moderately tolerant (growth >30 to <60% of that on controls), and 25% as highly tolerant (growth ≥60% of that on controls). In 1992, 36% were sensitive, 27% were moderately tolerant, and 37% were highly tolerant. When exposed to 1.2 g/L of a commercial copper hydroxide bactericide for 4 hr in the laboratory, populations of tolerant strains were reduced from 10^8 to only 10^6 cfu/ml. In contrast, populations of sensitive strains were typically less than 100 cfu/ml. Strains identified as copper-tolerant became more sensitive as cryogenic storage time increased. After 10 mo of storage, 10^3–10^4 cells survived exposure to copper hydroxide. Phenotypic expression of copper tolerance (10^7 cfu/ml) could be retrieved by growing cultures from cryogenic storage on media containing a sublethal dose of copper (0.32 mM $CuSO_4$).

Bacterial blight, caused by *Pseudomonas cichorii* (Swingle) Stapp, can cause widespread damage to the celery crop (*Apium graveolens* L. var. *dulce* (Mill.) Pers.) in Florida. It is especially destructive in those seedbed operations where seedlings are produced for the fall and early winter crops. Most celery seedlings for the transplant industry are produced outdoors in raised beds under shadecloth. Hot and rainy weather, combined with extremely high plant populations, results in an ideal environment for development and spread of bacterial blight.

Growers routinely apply copper bactericides for control of bacterial blight, especially during transplant production, but control has been less than satisfactory in many cases. Other workers have reported tolerance to copper for several bacterial phytopathogens (1-3,9,14,17). Therefore, one objective of this study was to determine the presence and prevalence of copper tolerance among strains of *P. cichorii* in celery seedbeds.

When first introduced, streptomycin provided promising control of bacterial blight (4). However, resistance in *P. cichorii* populations developed soon thereafter (18). As control diminished, use of streptomycin steadily declined. Very little of the antibiotic has been used commercially in the Everglades in the last 20 yr. A second objective of this study was to determine if streptomycin resistance is still widespread despite a long hiatus in routine use of the antibiotic. A preliminary report of a portion of this work has been published (13).

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MATERIALS AND METHODS

Sample collection and pathogen isolation. Samples of celery leaves with bacterial blight symptoms were collected from three transplant production sites in Belle Glade and one in Zellwood, Florida, between 27 August and 23 September 1991 and 8 September and 12 October 1992. These sites represent about 95% of the state's commercial production of celery transplants. Transplant production begins in June, with the first plants set in production fields in August. Commercial production is curtailed in May. Therefore, celery is in some stage of production in southern Florida yearround.

At all locations, celery plants were produced on raised, fumigated beds at a density of 7.7 million plants per hectare. Bedding areas were covered with polypropylene shading (55% light attenuation) stretched over support poles approximately 2.5 m above the top of the plants. Transplants were mowed with a mechanical clipping device about once a week in order to maintain uniformity in transplant size.

Beds from which plant material was taken were selected by means of a random numbers table. Workers began searches for bacterial blight at either end of rows of transplants. Approximately 25 samples, consisting of three or four diseased leaves, were taken from each field. In the laboratory two leaves were used to select water-soaked, young lesions for isolation. One colony from one of the two plates was chosen for culture increase and storage. An average of 22 strains of *P. cichorii* was successfully recovered per field survey.

The isolation technique was modified from Goth (6) as previously described (14). Bacteria were recovered from each sample on duplicate plates of King's medium B (7). Autoclaved isolation medium cooled to 55 C was supplemented with 50 μg/ml of cycloheximide added from a filter-sterilized 1% solution in 50% ethanol. Plates were incubated at 27 C for 48 hr. Fluorescent colonies characteristic of *P. cichorii* were purified by restreaking on King's medium B. Cultures identified as *P. cichorii* were maintained in sterile 15% aqueous glycerol at −70 C (16). Working cultures were kept up to 1 mo on King's medium B slants at 4 C.

Pathogen identification. Presumptive cultures of *P. cichorii* were tested for oxidase (8) and arginine dihydrolase (19) reaction. Those that were oxidase-positive and arginine-dihydrolase-negative were retained and tested for virulence on celery.

Seedlings of celery cultivar Florida M-68 were transplanted into a growth medium (Metro-Mix 300, W. R. Grace & Co., Cambridge, MA) in 250-ml Styrofoam cups. At the three-true-leaf stage, plants were inoculated by a cotton swab abrasion technique (11). Sterile cotton swabs were dipped in 10^6 cfu/ml of each test strain suspended in sterile phosphate-buffered saline to which a small amount of Carborundum had been added. Bacterial suspensions were rubbed over adaxial surfaces of all leaves of test plants. Control plants were treated with saline plus Carborundum. Plants were kept in a mist bed (30 C) and examined for symptom development over the next 7 days.

Sensitivity to streptomycin and copper. All strains were assayed for sensitivity to streptomycin by comparing growth on nutrient-glucose agar only or amended with 100 or 200 μg/ml of streptomycin sulfate in 1991 and 200 μg/ml

in 1992. Data were recorded after 48 hr of incubation at 27 C.

All strains were tested for sensitivity to copper in an agar plate assay. Cultures were taken from −70 C storage, streaked on King's medium B, and incubated at 27 C for 48 hr. Small amounts of bacteria were removed from the plates and streaked on duplicate plates of casitone-yeast extract (CYE) agar (2), a low-metal-complexing medium with or without copper sulfate supplement. Two concentrations of copper sulfate tested, 0.16 and 0.64 mM, were obtained by incorporating appropriate volumes of a filter-sterilized solution of 0.25 M $CuSO_4$ into batches of autoclaved media cooled to 55 C. Estimates of the percentage growth of strains were made on the copper-amended medium compared to unamended control plates after 5 days of incubation at 25 C.

The conclusions concerning the relative tolerance of strains to copper were confirmed by an additional quantitative test. Strains labeled as sensitive, moderately tolerant, or highly tolerant in the agar plate assay were selected at random from our culture collection. Strains were increased on King's medium B for 48 hr. Suspensions of each strain were made in sterile, buffered saline and adjusted turbidimetrically to approximately 2.0×10^8 cfu/ml. Then, 5 ml of suspension of each test bacterium was mixed in culture tubes with 5 ml of a 2.4 g/L suspension of a commercial copper hydroxide bactericide (Kocide 101). The dosage of copper hydroxide was based on recommendations of the manufacturer for field control of bacterial blight. Tubes were placed on an orbital shaker at room temperature and 220 rpm for 4 hr. Aliquots (0.1-ml) of appropriate dilutions then were plated on triplicate plates of King's medium B, evenly spread with a sterile, L-shaped glass rod, and incubated at 27 C for 48 hr. Treatments were replicated three times. Mixtures of bacterial suspensions and buffered saline served as controls.

After storage at −70 C for about 6 mo, populations of several tolerant strains were severely depressed upon exposure to copper. A set of experiments was designed to determine if copper tolerance is inducible in those cultures maintained for some time in the laboratory. Cultures were grown for 48 hr on King's medium B and transferred to CYE medium with or without addition of a sublethal dose of copper sulfate at a final concentration of 0.32 mM. After 48 hr on CYE, cultures were assayed for sensitivity to copper hydroxide as described above.

All experiments were carried out two to four times, with similar results. Data from representative trials are shown.

RESULTS

In 1991, resistance to streptomycin was widespread among strains of *P. cichorii* isolated in our study. Sixty-one (70%) of the strains tested were highly resistant to streptomycin in vitro (Fig. 1). No differences were noted in the reaction of strains to 100 or 200 μg/ml of streptomycin sulfate. Over one-half of the sensitive strains were from one seedbed site (Farm B in Fig. 1). Streptomycin resistance profiles were similar in 1992 for three of the four sites (Fig. 1). The 1992 strains for Farm A were all sensitive, whereas in 1991, all strains from this farm were resistant.

Sensitivity of strains to copper varied. We divided strains into three groups based on their relative growth on the low copper-complexing medium: sensitive, growth 0–30% of that on controls; moderately tolerant, growth >30 to <60% of that on controls; and highly tolerant, growth ≥60% of that on controls. In 1991, about two-thirds of the strains showed some tolerance to copper (Fig. 2). Of these strains, one-third were highly tolerant. Classification of data by production site revealed that most of the sen-

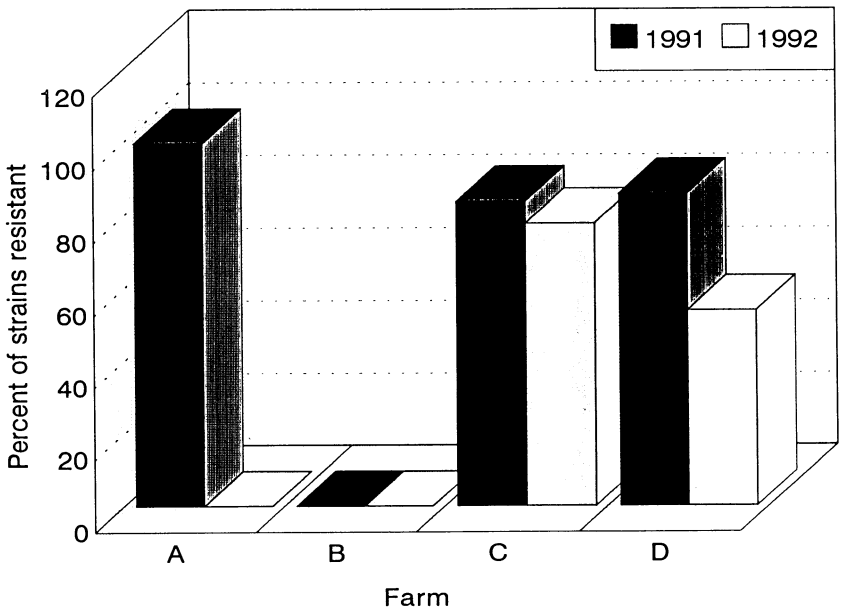

Fig. 1. Percentages of strains of *Pseudomonas cichorii* resistant to 200 μg/ml of streptomycin sulfate incorporated into King's medium B plates. Data are for four seedbed farms sampled in early fall of 1991 and again in 1992.

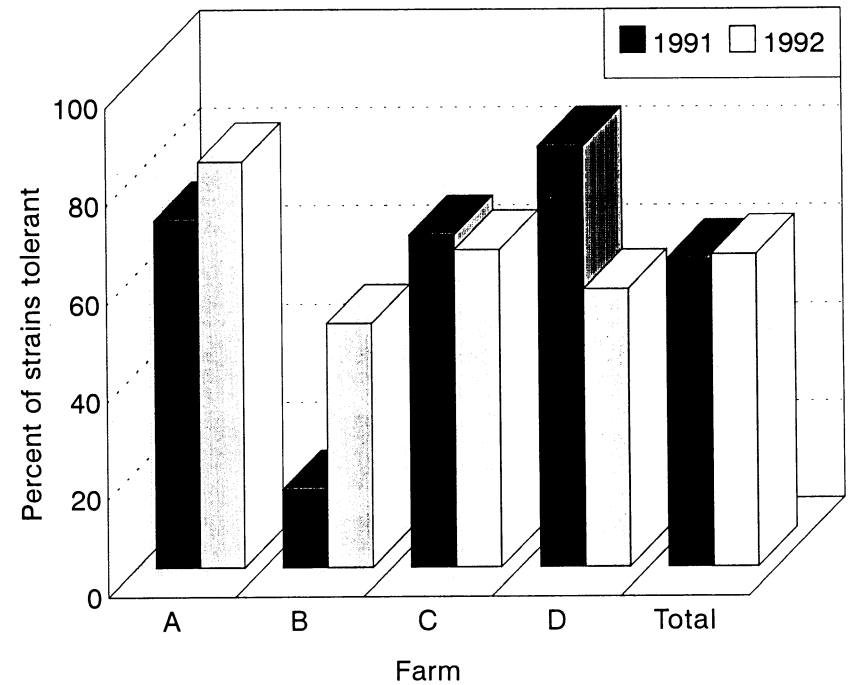

Fig. 2. Percentages of strains showing some degree of tolerance to 0.64 mM $CuSO_4$ incorporated into CYE medium. Strains characterized as moderately tolerant (growth >30 to <60% of that on controls) and highly tolerant (growth ≥60% of that on controls) were combined to produce this graph.

sitive strains were collected from one of the four farms surveyed. In three of the four seedbeds, approximately 80% of the strains were at least moderately tolerant.

In 1992, 39 of the 104 strains collected were highly tolerant, 28 were moderately tolerant, and 37 were sensitive. Incidence of copper tolerance was similar to that in 1991 for three of the four farms surveyed.

The 4-hr exposure to labeled rates of copper hydroxide confirmed the results of the agar plate assay (Table 1). Representative sensitive strains were completely killed or reduced to very low levels (<30 cfu per plate from undiluted preparations). On the other hand, more than 1 million cfu/ml of highly tolerant strains usually remained in culture tubes containing copper. Results for strains classified as moderately tolerant in the agar plate assay tended to closely approximate those of tolerant strains. Final populations of moderately tolerant strains were generally 10^5 cfu/ml, only one log unit lower than highly tolerant strains but four log units higher than sensitive strains.

Tolerance to copper bactericide was less evident as cryogenic storage time increased. Only 10^4 cfu/ml of tolerant strains survived when cultures were assayed 8 mo after storage (Table 2). At 10 mo, recoverable populations were reduced to 10^3 cfu/ml. However, phenotypic expression of copper tolerance was dramatically recovered by exposing cells of tolerant strains to sublethal doses of copper ion. When grown on media containing 0.32 mM $CuSO_4$, 10^7 cfu/ml were recoverable after the standard exposure to copper hydroxide.

In 1991, 57 of the 87 strains were both streptomycin-resistant and copper-tolerant, 21 strains were sensitive to streptomycin and copper (16 of these from Farm B), and only 9 strains were a mixed phenotype, i.e., 2 were streptomycin-sensitive and copper-tolerant and 7 were streptomycin-resistant and copper-sensitive. In 1992, 30 of 104 strains were sensitive to both streptomycin and copper and 27 were streptomycin-resistant and copper-tolerant; mixed phenotypes were 40 streptomycin-sensitive and copper-tolerant and 7 streptomycin-resistant and copper-sensitive.

Table 1. Quantitative assay of effect of exposure to copper hydroxide in vitro on populations of representative strains of *Pseudomonas cichorii* varying in sensitivity to copper[a]

Strain	Sensitivity classification[b]	Population after 4 hr (cfu/ml)
PC40	Sensitive	0
PC46	Sensitive	0
PC51	Sensitive	8.7
PC108	Sensitive	1.3×10^1
PC36	Sensitive	3.5×10^1
PC57	Moderately tolerant	5.4×10^5
PC92	Moderately tolerant	6.1×10^5
PC12	Moderately tolerant	1.8×10^4
PC25	Moderately tolerant	2.5×10^5
PC83	Tolerant	1.4×10^6
PC94	Tolerant	1.6×10^6
PC62	Tolerant	3.8×10^6
PC72	Tolerant	2.8×10^6

[a] Suspensions (5 ml) of bacteria in sterile, buffered saline at initial populations of 2.0×10^8 cfu/ml exposed to 5 ml of a 2.4-g/L suspension of commercial copper hydroxide bactericide on an orbital shaker for 4 hr. Data are averages of three replications.
[b] Based on qualitative assay.

DISCUSSION

Data over two seasons support the conclusion that resistance to streptomycin is still widespread among strains of *P. cichorii* causing blight of celery. This is the first time since the early 1960s that a comprehensive survey has been made of streptomycin sensitivity in the Everglades Agricultural Area. Even though little streptomycin has been used over the past 25 yr, resistant strains are still readily isolated. Bacterial blight caused by *P. cichorii* does not occur in the areas of California where the seed for Florida is produced. Therefore, variation in copper sensitivity among Florida strains is not readily explained by differences in seedborne strains. It is more likely that resistant strains have survived year-round in the Everglades Agricultural Area even in the absence of continued selection pressure (15).

The profile in a given seedbed may vary dramatically from one year to the next. On one farm, the percentage of streptomycin-resistant strains changed from 100 to 0 between the 1991 and 1992 seasons. This reversal in the streptomycin resistance profile occurred despite any known change in the grower's selection of spray materials. The celery seedbed is a very different agroecosystem from that encountered in many vegetable production fields. Millions of plants are crowded into areas as small as 1.5 ha, so that considerable plant-to-plant contact occurs. An ideal situation exists for rapid, efficient pathogen spread during passes of the mechanical clipping device (5). Under these circumstances, a relatively small number of introduced pathogen strains may quickly infect large portions of the transplant range. Strains that might otherwise become established are excluded, because suitable ecological niches are already occupied. In this way, a resistant or susceptible strain may be the predominant phenotype when a seedbed is sampled.

To the authors' knowledge, this is the first report of copper tolerance among populations of *P. cichorii*. Celery seedbeds are intensively managed, receiving frequent sprays of copper bactericides throughout the period of transplant production. It is not surprising that often 40% or more of the strains assayed in transplant ranges are tolerant of copper. Tank mix combinations of copper and EBDC fungicides can help overcome copper tolerance (9). However, growers cannot use this option, because maneb and other EBDCs are no longer registered for celery.

We have found evidence that tolerance to copper among strains of *P. cichorii* diminishes over time during storage in the laboratory. The number of cells surviving exposure to copper hydroxide can be restored by "inducing" cultures. We did this by growing recovered bacteria on a medium with a low binding capacity for transition metal ions (2) amended with a sublethal dose of copper. Copper tolerance can be induced in strains of other bacterial pathogens (2,10). It is interesting to consider whether copper tolerance is induced in the field. It is possible that, at least to some degree, the expression of copper tolerance is being induced by exposure of *P. cichorii* to frequent copper sprays. If so, more judicious use of copper pesticides might maximize the efficacy of those sprays that are applied. Higher frequencies of copper resistance than we anticipated may also occur in Florida.

Because the profile of bactericide resistance can shift appreciably from one season to the next, it is difficult to advise growers as to probable bactericide efficacy. Frequent tests of sensitivity of strains might be of value to growers implementing an integrated pest management program in celery seedbeds (12).

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Table 2. Reestablishment of copper tolerance in stored cultures of *Pseudomonas cichorii* by exposure to sublethal concentrations of copper[a]

	Number of months in storage			
			10	
Strain	4	8	No Cu induction	Cu induction
PC72	2.8×10^6	4.7×10^4	5.6×10^3	5.0×10^7
PC94	1.6×10^6	5.1×10^4	8.8×10^3	3.6×10^7

[a] Culture removed from cryogenic storage, cultured on King's medium B for 48 hr, and then transferred and grown for 48 hr on CYE medium amended with a sublethal dose of copper (0.32 mM $CuSO_4$). Initial populations of approximately 2.0×10^8 cfu/ml exposed to a 2.4-g/L suspension of commercial copper hydroxide bactericide on an orbital shaker for 4 hr. Data are averages of three replications.

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