Preservation of Corynebacterium insidiosum in a Sterile Soil Mix Without Loss of Virulence

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ABSTRACT

Forty-five single-cell isolates of *Corynebacterium insidiosum* were maintained for 1 year at 4 and 21 C in sterile, distilled water, on beef-lactose agar (BLA), in a sterile 3:1:1 soil:peat:perlite mix. Forty-two of the isolates kept at each temperature in soil persisted over this interval and produced typical colonies when cultured on BLA. No variant colonies were detected. Parent isolates of the single-cell isolates persisted for 20 months in a sterile soil mix with no apparent change. In contrast, all isolates maintained on BLA persisted but grew poorly, produced little of the typical blue pigment, and produced variant colonies. Only 50% of the isolates could be recovered from sterile water. These grew poorly on BLA and produced only a small amount of the pigment. Thirty-two of the isolates held at 21 C on BLA and in sterile soil were tested for virulence. Results showed a loss of virulence for all isolates maintained on agar in contrast to those in soil. No change in virulence due to soil-storage temperature was detected when eight virulent isolates were tested on seedlings grown under sterile conditions. This method is more convenient and reliable than previously described methods, and should be useful for maintaining a large number of isolates for long periods without loss of virulence. Phytopathology 61:688-690.

Additional key words: bacterial wilt, alfalfa.

Maintenance of virulent inoculum of *Corynebacterium insidiosum* (McCull.) H. L. Jens. is difficult because of the rapid loss of virulence in culture. In several studies, the effect of storage methods on persistence and virulence of the organism was determined. These included storage in culture (1, 7), in frozen host tissue (9), in dried host tissue (3, 4), in sterilized and natural soil (11), and in sterile glass-distilled water (6). In our study, we compared three methods of storage of *C. insidiosum* cultures and have described a method for the maintenance of virulence of isolates of the bacterium in a sterile soil mix. A preliminary report of the study was previously given (2).

MATERIALS AND METHODS.—The *C. insidiosum* employed in this study was isolated initially from infected alfalfa plants (cultivars Glacier and Stride) by the method of Kreitlow (10).

Forty-five single-cell isolates were obtained from eight parent isolates by the method of DeVay & Schnathorst (6). After 9 to 12 days' growth on beef-lactose agar (BLA), the single-cell isolates and their parent isolates were transferred to 5 g of a sterile 3:1:1 soil:peat:perlite mix moistened with 1 ml water and contained in a 4-dr screw-cap vial. The mix was sterilized by autoclaving at 121 C for 1 hr on 2 consecutive days (final pH 6.0). Two loopfuls (2-mm diam) of the bacterial cells were transferred to each vial and stirred thoroughly into the soil. One loopful of each isolate was also transferred to 5 ml of sterile, glass-distilled water contained in a screw-cap tube and mixed thoroughly. In addition, each isolate was maintained on BLA slants sealed with Parafilm, by periodic transfer at 14- to 20-day intervals.

Single-cell isolates were kept in the dark at 21 C and 4 C for 1 year. At the end of this period, they were tested for persistence by streaking from the water and agar onto freshly prepared BLA plates which were incubated at 21 C.

All parent and single-cell isolates were tested for virulence in the greenhouse, prior to storage, by the wounded-cotyledon method of Kreitlow (10). Thirty-two isolates maintained in soil and on agar at 21 C, for 1 year, were also tested in the same manner. Seven-day-old DuPuits alfalfa seedlings were inoculated with each isolate by clipping the cotyledons and immediately atomizing with a water suspension of the bacteria. Inoculated seedlings were covered with a plastic bag for 24 hr. Controls were treated in the same manner, except that they were atomized with sterile distilled water. Thirty seedlings were inoculated with each isolate, and 100 seedlings served as controls. In addition, 15 resistant Vernal alfalfa seedlings were inoculated with each isolate. Plants were evaluated for disease development after 6 weeks utilizing the scale of Cormack et al. (4). Following evaluation, isolations were made from the seedlings to check for the presence of *C. insidiosum*.

The effect of storage temperature on virulence was determined. A random selection of eight virulent isolates, maintained in sterile soil at 4 C and 21 C for 1 year, was tested on 6-week-old DuPuits alfalfa seedlings grown under sterile conditions in 20-cm test tubes at 19-23 C. Sterile culture of the seedlings was accomplished by surface-sterilizing the seeds for 20 min in 0.2% (w/v) aqueous mercuric chloride solution and rinsing in sterile distilled water according to the method of Dadd & Jacobs (5). Tubes contained 7 cm of washed, sterile sand moistened with Hoagland's solution No. 1 (8). Inoculation was accomplished with the use of a fine, sterile needle to injure the roots of seedlings. Two ml of a bacterial suspension from a 10-day-old culture on BLA were then dispensed into the

sand. Five tubes containing four seedlings each were inoculated with each isolate. The controls were treated in the same manner, except that sterile water was used. Twenty seedlings served as controls, and twelve Vernal seedlings were also inoculated with each isolate. Disease evaluations and isolations for *C. insidiosum* were made 4 weeks after inoculation. The same scale employed in the greenhouse tests was used for the ratings.

RESULTS.—Forty-two of the 45 single-cell isolates kept at each temperature in soil persisted over the 1-year interval and produced typical colonies, with no variants, when subsequently cultured on BLA. Parent isolates persisted for 20 months in sterile soil held at 4 and 21 C with no apparent cultural change. In contrast, only 50% of the isolates could be recovered at the end of 1 year from sterile distilled water kept at 4 and 21 C. These grew very poorly on BLA, had several cultural variants, and produced only a small amount of the typical blue pigment. All the isolates maintained on agar persisted at both temperatures, but also grew poorly. These all had cultural variants and produced very little of the blue pigment.

In greenhouse tests (Table 1), the isolates maintained in soil showed a high degree of virulence when compared to the same isolates on agar. Table 1 also illustrates that there were only slight differences in virulence between the single-cell isolates and their parent isolates both prior to and after the period of storage. Of greater importance is the lack of any significant changes of virulence during storage in the sterile soil. In all instances, there was a loss of virulence in isolates maintained on agar. The isolates stored in sterile soil showed there were some small differences in virulence due to storage temperature, but no consistent pattern. Some isolates were slightly more virulent when stored at 21 C than at 4 C, while the reverse was true for other isolates. For example, isolate S-2C showed an average rating of 4.1 at 21 C and 3.6 at 4 C, while isolate G-2E gave an average rating of 3.8 and 4.2, respectively, at the same temperatures.

TABLE 1. Virulence of parent and single-cell isolates of *Corynebacterium insidiosum* before and following 1 year of storage at 21 C

Isolate no.[a]	Before storage, rate[b]	Method of storage: Beef-lactose agar, rate	Method of storage: Sterile soil mix, rate
G-1 Parent	3.2[c]	0.4	3.2
G-1 F	3.8	0.4	3.6
G-2 Parent	3.6	0.6	3.2
G-2 C	3.8	0.5	3.5
G-3 Parent	1.8	0.4	2.0
G-3 A	2.2	0.0	1.8
S-1 Parent	2.4	0.0	2.6
S-1 A	2.8	0.1	2.9
S-2 Parent	3.2	0.4	2.8
S-2 C	3.0	0.0	2.6
S-3 Parent	3.2	0.4	2.9
S-3 A	3.0	0.5	2.7
S-4 Parent	2.4	0.4	2.0
S-4 A	2.6	0.0	2.8
S-5 Parent	3.7	0.2	3.2
S-5 D	3.0	0.0	2.9

[a] Only one single-cell isolate/parent isolate is shown as representative of the total number of isolates tested.
[b] Average rating of 30 seedlings/isolate.
[c] 0. = no infection; . . . . 5. = dead or dying.

The disease ratings for the isolates tested on plants grown under sterile conditions were as high or higher in every case than those obtained under greenhouse conditions. In some the rating was twice as high when seedlings were tested under sterile conditions. *Corynebacterium insidiosum* was isolated from infected seedlings grown in both ways.

No contaminants were isolated from seedlings grown under sterile conditions. Several other bacteria, however, appeared in isolations from seedlings grown in the greenhouse. Controls remained healthy in all cases, as did the inoculated Vernal plants, and *C. insidiosum* could not be isolated from either.

DISCUSSION.—Maintenance of *C. insidiosum* on BLA for a long time interval was unsatisfactory, as variants appeared in culture and virulence was lost. This is in partial agreement with the findings of Fulkerson (7), who demonstrated the lack of stability of isolates under prolonged culture on BLA at both 21 and 2-4 C. He detected variants in nearly all cultures. However, he did not detect any change of virulence for two isolates after 365 days of selective transfer at 21 C. It is not probable that the differences of results reported herein and those reported by Fulkerson are due to the slight difference in interval between transfers.

Storage in sterile water was also unsatisfactory, as 50% of the isolates did not persist for 1 year and variants appeared in subsequent culture of those isolates that did persist. This is in contrast to previously reported results (6).

Maintenance of single-cell isolates of *C. insidiosum* in the sterile soil mix appears to be a very satisfactory method, as the bacterium persisted in a virulent state without the production of variants. The differences in results reported here and in a previous study (11) may be partially due to the use of the soil mix rather than field soil. The temperature of storage in sterile soil appeared to have little or no effect on persistence or virulence of the bacterium. Subsequent growth on BLA was good, much of the typical blue pigment was produced, and no variant colonies were detected after 1 year. Similarly, Cormack et al. (4) found storage temperature had no effect on infective capacity of the bacterium maintained for a long interval in dried roots and tops of diseased plants.

When highly standardized inoculum is required or desirable, cultures of known virulence are needed. Methods for maintaining virulent inoculum in host plant material are then unsatisfactory, as the bacterium must be isolated, checked for purity, and tested each time inoculum is needed. Maintenance in sterile soil is more convenient and reliable than previously described methods, and should be useful for maintaining a large number of isolates for a long interval without loss of virulence.

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