Maintenance of Viability and Virulence of Corynebacterium nebraskense

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Published with the approval of the Director as Journal Series Paper No. 5263, Nebraska Agricultural Experiment Station. The work reported was conducted under Nebraska Agricultural Experiment Station Project No. 21-21.

I thank Randall Carlson, Susan Reimers, and Delbert Siefkin for technical assistance.

Accepted for publication 29 December 1976.

ABSTRACT

VIDAVER, A. K. 1977. Maintenance of viability and virulence of Corynebacterium nebraskense. Phytopathology 67:825-827.

Maintenance of viability and virulence of *Corynebacterium nebraskense*, causal agent of Goss's bacterial wilt and blight of corn (leaf freckles and wilt), was optimal either on solid complex media maintained at 6 C for 2 yr or as lyophilized cultures maintained at −20 C for 5 yr. Qualitative and quantitative measurements indicated that storage in sterile distilled or deionized water or phosphate buffer at 6 C or room temperature (24 ± 3 C) was unsatisfactory for maintenance of viability and virulence; such treatments also gave rise to colony type variants. Bacterial strains maintained in dried leaves of greenhouse grown plants were viable and virulent up to three months.

Additional key words: *Zea mays*, maize.

Corynebacterium nebraskense, causal agent of Goss's bacterial wilt and blight (leaf freckles and wilt) of corn, was first isolated in 1969 from diseased plants in central Nebraska. Subsequently, taxonomy of the organism (12); symptomatology, host range, and field survival (5, 8); distribution and occurrence (13); and variety resistance studies (1, 6) were reported. During early studies it became clear that the bacterium readily loses virulence in culture. Consequently, experiments were undertaken to maintain virulent reference cultures.

Schuster (5, 7) claimed that loss of virulence could be prevented by storage of laboratory cultures in mineral oil or distilled water and by storage of field isolates in plant parts obtained from the field. However, no quantitative data or disease ratings were presented along with the qualitative pathogenicity data on survival.

The present study shows that strain differences in loss of virulence occur, that the maintenance medium and temperature affect qualitative and quantitative survival, and that lyophilization is the best means for both long-term survival and maintenance of virulent reference cultures.

MATERIALS AND METHODS

Bacterial strains.—Twelve strains of *C. nebraskense* were used in this study. The strains were isolated during the summers of 1970 and 1971 from different geographic locations within Nebraska and Iowa. The strains were evaluated for pathogenicity on both sweet corn (cultivar Golden Cross Bantam) and field corn (inbred line A619), and were rated for virulence at the beginning of the tests reported here. Three strains of low-, four of moderate-, and five of high virulence were chosen as representing the spectrum of virulence observed in newly isolated strains.

Inoculation procedure and disease rating.—To determine pathogenicity of bacterial strains, sweet corn (cultivar Golden Cross Bantam) was grown to the two- to three-leaf stage (about 10 days) in the greenhouse (21-27 C). Each of at least three plants was inoculated with a No. 8 sewing needle (calibrated to deliver 1.5×10^{-4} ml upon penetration) thrust needle-eye first into the stem. Each plant was inoculated at approximately 3.0, 3.5, and 4.0 cm from the soil line, in a 90° horizontal plane relative to each puncture. The inoculum was adjusted to a concentration between 0.5 to 2.5×10^{9} CFU/ml (A_{420nm} = 0.2 to 0.4) in 0.01 M potassium phosphate buffer, pH 7.2. Cell suspensions were kept on ice until inoculated. Control plants were inoculated with sterile phosphate buffer by the same procedure.

A rating scale of 0 (no infection) to 5 (dead plants) was used to evaluate disease symptoms after 10 days. Disease ratings were based on the extent of discontinuous water-soaking, and severity of stunting, wilting and necrosis. Generally, evaluation was performed by two observers.

Survival determinations.—For survival determinations on solid media, the 12 strains were transferred to duplicate slants of NBY agar [a nutrient broth, yeast-extract medium (10)], GYC agar [a glucose-yeast-calcium carbonate medium (3)], and SSM agar [a semi-synthetic medium (11) with added yeast extract (0.05% w/v)]. After incubation for four days at 24 to 26 C, half of the cultures were placed at room temperature (24 ± 3 C) and the remainder at 6 C. At 60-day intervals, the cultures were transferred from NBY, GYC, or SSM agar to slants of the same respective media, and also to NBY plates. Colony types were observed on NBY and pathogenicity tests were performed on 4-day-old NBY cultures, suspended in phosphate buffer as described above.

Survival in liquid was determined by transferring a loopful of cells from 5-day-old NBY slants into 10 ml of sterile 0.01 M potassium phosphate buffer, pH 7.2 containing 10^{-3} M $MgSO_4 \cdot 7\ H_2O$ or 10 ml of glass-distilled or deionized water in screw-cap tubes. The initial $A_{420nm} = 0.5$ to 0.8. Appropriate dilutions were made at intervals for viable counts on NBY agar plates. After incubation for 4 to 5 days at 24 to 26 C, cell suspensions from the plates were tested for pathogenicity as above.

For lyophilized cultures, the strains were grown for 4 days at room temperature on NBY agar slants, suspended in sterile skimmed milk, and dispensed in 0.5-ml quantities into VirTis 5-ml screw-cap vials. After freezing the cultures in a dry ice-acetone bath, the cultures were dried under vacuum with a VirTis manifold freeze-dryer equipped with vented adapters and Quickseal®(The VirTis Co., Gardiner, NY 12525) valves. The freeze-dried cultures were stored in a −20 C freezer. Survival was determined by resuspending bacteria in 0.5 ml NBY broth; after a 15- to 30-min interval at room temperature, the vials were shaken gently and sampled for viable counts on NBY agar plates. Subsequently, pathogenicity was determined as described above.

Survival of reference strains in greenhouse-infected plants was determined by air-drying infected leaves from plants inoculated at the two- to three-leaf stage. Leaves were harvested 10 days after inoculation, dried for 2 days, and placed in separate glassine bags at 6 C. At intervals, about 2.5-cm^2 pieces of infected plant material were cut with sterile scalpels and placed in 3 ml of phosphate buffer, pH 7.2. After 30 min at room temperature, a loopful of liquid was streaked onto NBY agar plates. Pathogenicity of the cultures was determined as above.

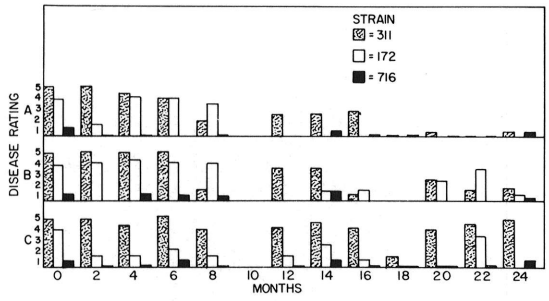

Fig. 1-(A to C). Disease ratings for *Corynebacterium nebraskense* grown on three solid media and transferred at 60-day intervals. Cultures were stored at 6 C after transfer. **A)** = SSM; **B)** = NBY; **C)** = $CaCO_3$ medium. Rating scale. 0 = no infection; 1 = water-soaked lesions on one to two leaves, with or without stunting; 2 = less than 25% leaf area affected on three or more leaves: 3 = less than 50% of leaf area affected; 4 = extensive water-soaking, wilting, and lesion development over 75% of leaf area affected; 5 = dead plants.

RESULTS

On solid media, both viability (measured qualitatively) and virulence were retained well at 6 C but not at room temperature (24 ± 3 C). Media NBY or GYC were superior to SSM for maintaining virulence of all strains, whether the strains were of high (strain 311), moderate (strain 172), or low (strain 716) virulence (Fig. 1). Room temperature cultures lost virulence more rapidly than viability, regardless of the medium. No detectable loss of viability of refrigerated cultures occurred for 2 yr, in contrast to cultures kept at room temperature.

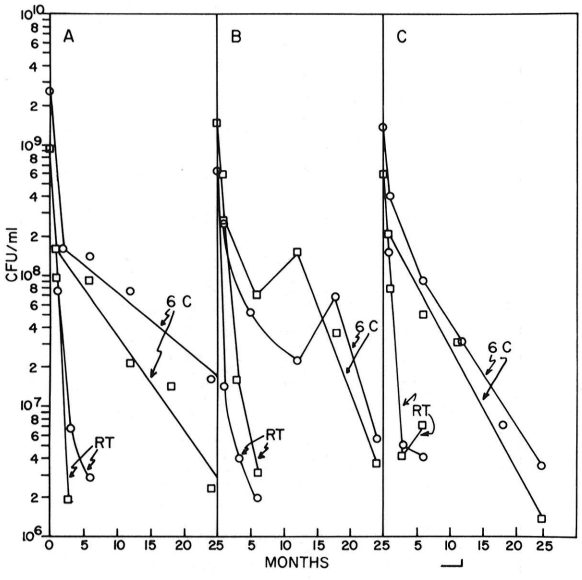

Fig. 2-(A to C). Survival of strains 311 **A)**, 172 **B)**, and 176 **C)** in sterile distilled water □ or phosphate buffer● Temperatures were room temperature (RT) or 6 C.

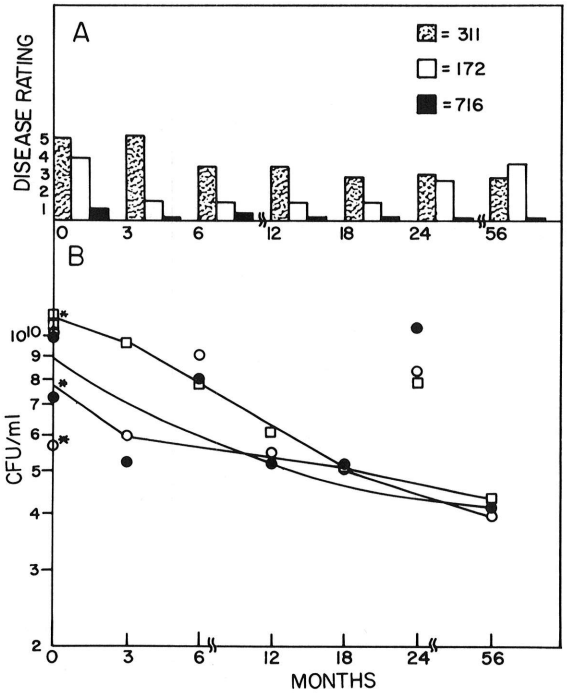

Fig. 3-(A,B). Disease rating **A)** and survival **B)** of lyophilized strains 311 □, 172 ● and 716○. Asterisk indicates initial reconstituted viable count.

In liquid, all 12 strains survived more satisfactorily at 6 C than at room temperature. In repeated tests, deionized water gave essentially the same results as distilled water (Fig. 2). Two or three strains showed a slight increase in population after a few months (e.g., strain 172, Fig. 2) before continuing to decline. The majority of strains survived at higher population levels in phosphate buffer than in water, whether at 6 C or at room temperature. Cultures maintained at room temperature not only lost viability quickly (Fig. 2), but also decreased in virulence (not shown) with the degree of loss varying with the strain. In addition, both the room temperature cultures and, to a lesser degree, the 6 C cultures gave rise to colony-type variants. Wild-type colonies from nearly 200 strains are of three mucoid types (12); colonies arising from liquid storage conditions were more variable in size and commonly very wrinkled. With only one exception (strain 39 kept at 6 C), virulence was lower with cultures maintained for 24 mo in the storage liquids than on solid media.

Lyophilized cultures retained virulence reasonably well for about 5 yr: four showed no loss in virulence; six showed slight loss (about one disease rating unit); one of low virulence showed complete loss, and one showed a slight gain in virulence (one disease rating unit) (Fig. 3-A). There was a modest decline (less than one log unit) of viability for the lyophilized cultures (Fig. 3-B).

Survival of cultures in dried leaves of greenhouse-grown plants was unsatisfactory, being less than 3 mo; virulence, however, was unchanged. In contrast, isolations from naturally infected field material could be made after 1 yr.

DISCUSSION

This study indicates that virulence of *C. nebraskense* can be maintained satisfactorily either on solid complex media maintained at 6 C or by lyophilization. Liquid storage at any temperature is unsatisfactory because of loss of virulence and emergence of variants. Schuster et al. (5, 7) also observed such colony-type variants under similar conditions with *C. nebraskense*. With the taxonomically related *C. insidiosum*, storage in liquid (distilled water) also gave rise to different colony-type variants; such variants were associated with the loss of virulence (2). In a separate study, cultures of *C. insidiosum* transferred on solid media at 14-day intervals showed colony morphology and pigment variation, but no detectable change in virulence after 1 yr (4).

The poor survival of *C. nebraskense* in artificially inoculated corn plants may be due to a variety of reasons, ranging from environmental conditions and choice of cultivar to the age of leaves collected. These results contrast with the fact that the bacterium can be obtained readily from naturally infected leaves and stalks, whether stored (at 6 C) or maintained in the field.

Variability in disease ratings was frequently seen from one test period to the next, but could not be correlated with the time of year or the individual strain tested. Duplicate tests begun 6 mo later than those reported here showed the same trends in viability and virulence. In each test period, however, the trends generally were observed so that the disease ratings were higher or lower as a group. Plants grown in more restrictive environments, such as growth chambers, may be more satisfactory for critical virulence tests.

The apparent slight loss in virulence of lyophilized cultures of *C. nebraskense* may be caused by either a direct effect on genetic material or a selection phenomenon. Despite routine maintenance of phytopathogenic bacteria by lyophilization, documented evidence is lacking on the effects of freeze-drying on virulence. For human and animal pathogens, virulence may increase, decrease, or generally remain unchanged after lyophilization (9).

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