An Improved Method for the Inoculation of Corn with Erwinia spp.

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Research supported by the College of Agricultural and Life Sciences and by the Graduate School, University of Wisconsin, Madison. Based on a portion of a Ph.D. thesis by the senior author.

The assistance of S. Vicen in preparation of figures is gratefully acknowledged.

Accepted for publication 8 December 1972.

ABSTRACT

Infection of corn plants using a whorl inoculation method with 0.7% Tween 40 and a corn pathotype of *Erwinia chrysanthemi* (ECZ) (5 X 10^7/ml) resulted in consistent rotting and collapsing of 3-week-old corn plants about 48 hr after inoculation. Most other inoculation techniques, including stem injections, were erratic or not successful under greenhouse or growth room conditions. In field tests, Tween 40 also enhanced infection and development of stalk rot of sweet corn and field corn following whorl inoculation with ECZ. Using this improved technique, ECZ isolates from North Carolina, Wisconsin, Egypt, and India were pathogenic to corn; whereas *E. carotovora*, *E. atroseptica*, and *E. aroideae* isolates were not. *Erwinia chrysanthemi* appeared to be weakly virulent to corn in certain tests.

An initial decrease (fivefold after 10 hr) in population of ECZ in inoculated plants was followed by a rapid increase in cells (100-fold after 36 hr) paralleling appearance of initial internal symptoms of stalk rot. In contrast, *E. carotovora* populations declined 20-fold during the entire 48-hr observation period.

Phytopathology 63:658-663

Additional key words: Tween 80, Tween 20, Eosin Y.

A destructive bacterial stalk rot of corn (*Zea mays* L.) plants mainly following overhead irrigation has been observed in a number of locations in the United States (7, 9). Disintegration of stalk tissue to the extent that the top breaks over and topples to the ground is the most typical symptom of the cornstalk rot disease. The causal agent was first described by Sabet (11) in Egypt as *Erwinia carotovora* f. sp. *zeae* mainly differing from *E. carotovora* in pathogenicity to corn. The question of the proper taxonomic position of this pathogen has been raised by a number of investigators (1, 2, 7, 13, 15).

The *Erwinia* isolates from corn were considered by Dye (1) to be synonomous with the *"chrysanthemi"* group and he included *E. carotovora* var. *zeae* Sabet under *E. carotovora* var. *chrysanthemi*. Hoppe & Kelman (7) also concluded that the cornstalk rot pathogen showed closer affinity to *E. chrysanthemi* than to *E. carotovora*. These limited studies on the taxonomic position of the cornstalk rot pathogen indicate that the bacterium should probably be classified as *E. chrysanthemi*. This corn pathotype of *E. chrysanthemi* will be referred to as ECZ in this paper.

In North Carolina, the disease has been controlled by adding chlorine to the irrigation water supplied in overhead irrigation systems (16). On the basis of this and observations on patterns of disease development (7), it has been assumed that natural infection is accomplished by entry of bacteria into the corn plant via the whorl.

Detailed studies of this disease required an artificial inoculation technique that would simulate field infection. In addition, the technique would have to provide reliable infections and uniform disease development. Development of such a technique would make it possible to evaluate pathogenicity of various soft rot bacterial isolates to corn and to determine relative resistance of corn cultivars to ECZ. Aspects of disease development and the physiology of the interaction between bacteria and its host plant could also be studied more precisely.

Corn plant inoculations by previous investigators have involved introduction of bacterial suspensions in wounds made in the stalks of 6- to 8-week-old plants (6, 7, 9, 10, 11, 12, 13, 14, 17, 18). Stem injections frequently gave variable results (3). Furthermore, stem wounding techniques were not desirable because they did not represent the probable means of entry of the bacteria into the corn plant under field conditions. However, artificial inoculations made by placing bacterial suspensions in the whorl to simulate natural field inoculations, were either erratic (7) or unsuccessful (6).

The primary objective of this investigation was to develop a uniform and reliable inoculation technique in order to study the factors influencing disease development with ECZ and related soft rot bacteria.

MATERIALS AND METHODS.—*Bacterial cultures.*—The two isolates mainly used, *Erwinia chrysanthemi*, corn pathotype (W-3-20) and *E. carotovora* (EC 208), will be referred to as ECZ W-20 and EC 208, respectively; the sources of these and other isolates are listed in Table 1.

Isolates were stored at 24 C in 5 ml of sterile distilled water in plastic capped 20-ml glass culture tubes. Suspensions consisted of ca. 1 X 10^6 cells per ml transferred by loop from 48-hr cultures grown on peptone (1.0%), casamino acids (0.1%), and glucose (1.0%) agar (PCG). Permanent stocks were maintained as bacterial suspensions in 1.5-ml vials

TABLE 1. Sources of cultures of soft rot *Erwinia* species used for pathogenicity tests

Isolate number	ICPB number[a]	Host	Location	Source
E. chrysanthemi, corn pathotype				
W-1-1		corn	Lincoln Co., Wis.	Kelman
W-3-20		corn	Spooner, Wis.	Kelman
EC 209	EC 209	corn	Egypt	Sabet
C-9		corn	North Carolina	Kelman
I-1, 5, 6, 9, 10[b]		corn	India	Payak
E. chrysanthemi				
18		chrysanthemum	New York	Dickey
176	EC 176	chrysanthemum	California	Starr
E. carotovora				
13	EC 13	carrot	California	Ark
105	EC 105	potato	California	Ark
153	EC 153	pepper	California	Kraght
169	EC 169	broccoli	England	Lacy
208	EC 208	carrot	Wisconsin	Jones
E. aroideae				
36	EA 144		England	Dowson
34		tobacco	New York	Burkholder
E. atroseptica				
54	EA 143	potato	England	Dowson

[a]Cultures kindly provided by Dr. M. P. Starr from the International Collection of Phytopathogenic Bacteria, Davis, California.

[b]Cultures from India (I-1, 5,6,9, and 10) were kindly sent by Dr. M. M. Payak.

containing 0.8% nutrient broth and immersed in liquid nitrogen.

Inoculation techniques.—Corn plants (inbred W-703) were grown from seed in soil in 15-cm pots in a growth chamber maintained at 28 C during a 12-hr light period (1,200 ft-c) and at 24 C during a 12-hr dark period. Inoculations were usually made on 21-day-old plants. Following inoculation, plants were incubated in a growth chamber continuously maintained at 28 C with a 12-hr light period.

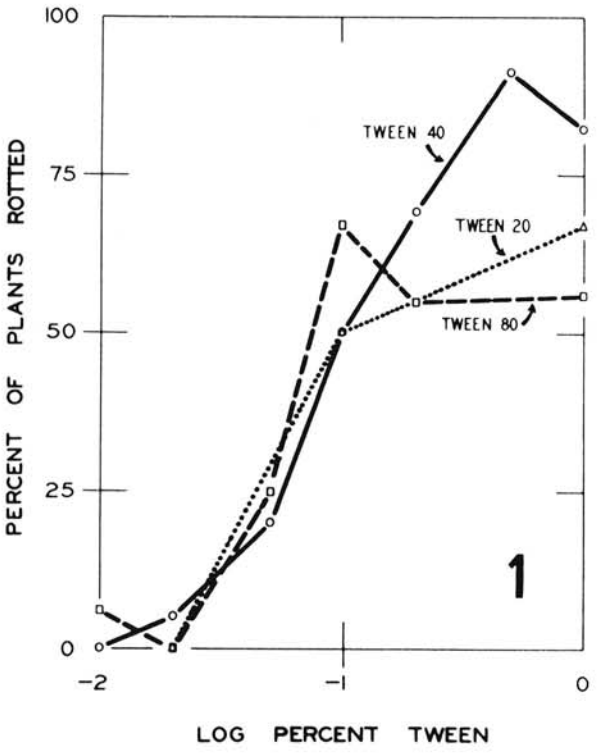
Bacterial cells removed from 48- to 72-hr cultures on PCG medium were suspended in distilled H_2O, centrifuged for 0.5 hr at 10,000 *g*, and resuspended in sterile distilled water to obtain the desired turbidity. Corn plants were inoculated by pipetting 1 ml of bacterial suspension into the whorl. Care was taken not to jostle the plant after inoculation so that all the liquid would remain in the whorl.

Disease ratings were based on percentage of plants showing stalk rot in relation to total inoculated plants 1 week after inoculation. Plants with stalk rot could be identified by collapse of the corn plants, and by easy removal of inner whorl leaves with the broken ends soft-rotted and browned. Occasionally, plants which had not collapsed and from which inner whorl leaves could not be removed easily did have small brown rotted areas within the stalks which could be detected when stalks were cut longitudinally with a scalpel. These stalks, however, were not included in the percentage of plants having stalk rot.

Infection and rotting of corn plants was determined as influenced by the type and concentration of Tween surfactants (Chemicals Division, Atlas Chemical Industries, Inc., Wilmington, Delaware, 19899) added to the inoculum. Suspensions of bacteria (ECZ W-20) (5×10^8 cells/ml) were prepared in solutions of Tween 20 [polyoxyethylene sorbitan monolaurate], Tween 40 [polyoxyethylene sorbitan monopalmitate], and Tween 80 [polyoxyethylene sorbitan monostearate]. Six concentrations of the Tween surfactants were used, ranging from 0.01% to 1.0% (v/v).

RESULTS.—*Effect of surfactants on infection.*—Use of Tween surfactants in the inoculum suspension noticeably enhanced infection and rotting of corn plants by ECZ W-20 inoculated into the whorl (Fig. 1). Tween 40 enhanced rotting more effectively at higher concentrations than did Tween 20 or 80; concentrations of Tween above 0.5% were necessary to obtain high levels of infection. By means of this method, severe stalk rot of 80 to 90% of inoculated plants was obtained routinely. A small percentage (about 10%) of plants had whorls that were so structured that the inoculum was not retained in the whorl. If these plants had been excluded from the inoculation tests, the data based on remaining plants would have indicated close to 100% infection in each instance.

Effect of concentration of cells in inoculum.—To determine the inoculum dose necessary for uniform



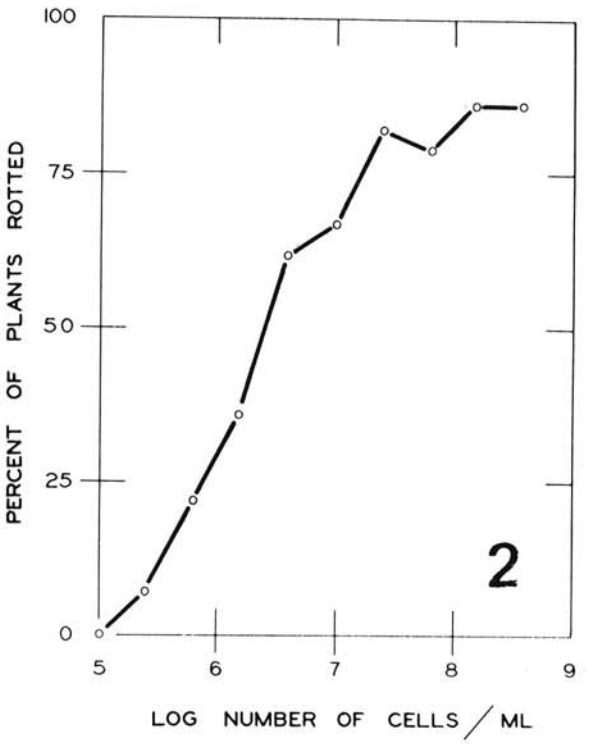


Fig. 1. (left). Relation of concentration and type of Tween surfactant to occurrence of stalk rot of corn plants (W-703) inoculated by placing 1 ml of a suspension of 5×10^8 cells/ml of *Erwinia chrysanthemi*, corn pathotype (W-20) into the whorl of 3-week-old plants.

Fig. 2. (right). Percent of corn plants (W-703, 3 weeks old) showing stalk rot in relation to number of *Erwinia chrysanthemi*, corn pathotype (W-20) cells suspended in 0.7% Tween 40. Plants inoculated by placing 1 ml of suspension into whorls.

infection and rotting of corn plants, 10 suspensions of ECZ W-20, ranging from 1×10^5 cells per ml to 4×10^8 cells per ml, were prepared in solutions of 0.7% Tween 40. One ml of each suspension was placed in the whorl of each of 16 to 24 corn plants.

Eighty to 90% infection and rotting was regularly obtained when 5×10^7 cells/ml (ECZ W-20) were suspended in a solution containing 0.7% Tween 40. No significant increase in infection rate occurred when the inoculum concentration was increased to more than 5×10^7 cells/plant (Fig. 2). Infection percentage declined when fewer than this number of cells was added in the inoculum. If less than 1×10^5 cells were used, neither infection nor rotting of plants occurred.

Development of symptoms.—Infection of corn plants using the whorl inoculation method with Tween 40 resulted in consistent rotting and collapsing of 3-week-old corn plants ca. 48 hr after inoculation (Fig. 3). Usually no external disease symptoms could be detected before 48 hr. After plants collapsed, browning and rotting of outer sheath leaves of the stalk could be seen and the remaining leaves of the plant soon became necrotic.

No visible internal symptoms were evident in cornstalks prior to 18 hr. Water soaking and decay of inner stalk tissues appeared 18 and 24 hr after inoculation. Soft rotting and browning increased in these tissues until the stalks weakened to the point where they toppled readily.

Inoculation of corn plants with EC 208 resulted in chlorotic areas on emerging leaves 1 week after inoculation. These symptoms were similar to those appearing 1 week after inoculation of ECZ W-20 in numbers too small to cause stalk rot.

Penetration of Eosin Y into the corn plant whorl using Tween 40.—To determine whether increased infection with Tween 40 was related to a more rapid and uniform spread of the bacteria into the leaf tissue of the inner whorl, eosin dye was added to Tween solutions and observations were made on movement of the dye in the whorl.

Eosin Y dye (0.5%) dissolved in a 0.7% solution of Tween 40 penetrated between inner whorl leaves in corn stalks within 1 hr, whereas a water solution of Eosin Y (0.5%) remained mainly in the whorl even after 5 hr. This striking difference between preparations with and without Tween is evident in the coloration of inner stalk leaves by Eosin Y. The dye solution with Tween penetrated between whorl leaves down to the growing point at the base of the stalk. Tween 40 apparently enhances the rate of

movement of the dye from the whorl down into the stalk by lowering of surface tension.

Effects of Tween on bacteria and plants.—Tween 40 at levels up to 20% aqueous solutions had no visible phytotoxic effects except that leaves exposed to concentrated solutions seemed to be smoother textured. The Tween 40 needed for this inoculation technique (less than 1.0%) had no noticeable effects on corn plants.

Neither ECZ W-20 nor EC 208 was capable of using Tween 40 as a sole source of carbon in a minimal salts medium. Furthermore, the same medium with added dextrose supported growth of ECZ and EC 208. No stimulation or inhibition of growth was observed for concentrations of Tween 40 as high as 6.3%. Thus, the enhancement of corn infection was not attributable to stimulation of bacterial growth by Tween after inoculations.

Pathogenicity tests using whorl inoculation.—The virulence of nine ECZ isolates to corn was compared with ten isolates of different soft-rot *Erwinia* species (Table 1). Suspensions of each of the 19 bacterial isolates were prepared in 0.7% Tween 40; populations ranged from 1.4×10^8 to 8.7×10^8 cells per ml. One ml of suspension of each isolate was placed in the whorls of each of six to eight corn plants (W-703).

All tested ECZ isolates were pathogenic to corn, whereas those designated as *E. carotovora, E. atroseptica, E. aroideae*, and *E. chrysanthemi* were not pathogenic to corn (Table 2). An exception was one isolate, *E. chrysanthemi* 18 which rotted a small percentage of plants in this experiment. There was no correlation between the small variations in inoculum concentrations (1.4 to 5.6×10^8 cells/ml) and amount of stalk rotting.

Changes in ECZ and E. carotovora populations following inoculations.—Changes in populations of ECZ W-20 and EC 208 were determined following whorl inoculations with suspensions containing 5.0×10^8 cells/ml. Bacterial populations per plant were determined from serial dilution plates of a puree made in a Waring Blendor of the entire aboveground portion of inoculated and infected corn plants. Samples were taken at 10 intervals from 0 to 54 hr with plants inoculated with ECZ W-20 and at five intervals from 0 to 48 hr for EC 208, each sample representing four replicates of three to four plants each.

An initial decrease (fivefold at 10 hr after inoculation) in population of ECZ W-20 was followed by a rapid increase (100-fold after 36 hr) in cells which paralleled the appearance of internal symptoms of stalk rot. In contrast, EC 208 populations declined 20-fold during the entire 48-hr observation period (Fig. 4).

In order to learn where in the corn plant these population changes were occurring, bacterial populations were determined from different portions of the plants. The proportion of bacteria recovered from the top of the corn plant (including the top of the whorl that was inoculated) compared to the bottom of the corn plant (the stalk portion that later

TABLE 2. Percent of corn plants (W-703) with stalk rot following whorl inoculation with suspensions of *Erwinia* isolates, in 0.7% Tween 40

Bacterial isolate	Percent plants with stalk rot[a]
E. chrysanthemi, corn pathotype	
W-1-1	100
EC 209	100
I-10	100
C-9	86
I-5	86
W-3-20	83
I-1	71
I-9	57
I-6	43
E. chrysanthemi	
18	29
176	0
E. aroideae	
36	0
34	0
E. atroseptica	
54	0
E. carotovora	
105	0
153	0
169	0
13	0
208	0

[a]Rotting 1 week after inoculation of six to eight plants.

Fig. 3. Typical symptoms produced at 30, 42, 48, and 54 hr after whorl inoculation of corn plants (W-703) with 5.0×10^8 cells/ml of *Erwinia chrysanthemi*, corn pathotype (W-20) suspended in 0.7% Tween 40.

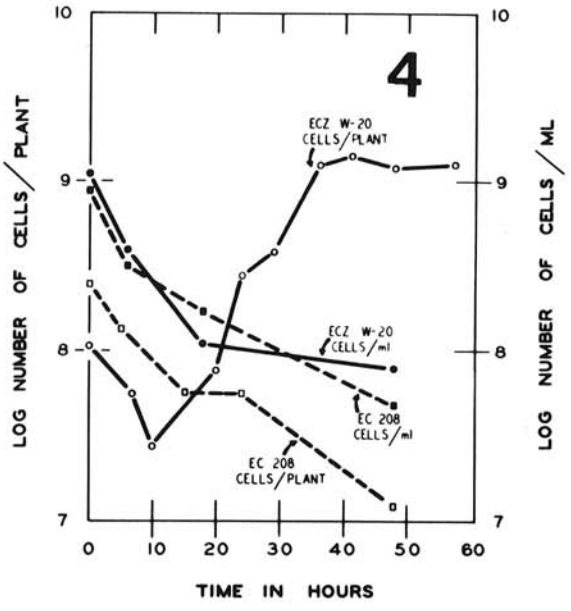


Fig. 4. Viable cells recovered per plant after whorl inoculation of corn plants (W-703, 3 weeks old) with *Erwinia chrysanthemi*, corn pathotype (ECZ W-20) and *E. carotovora* (EC 208) suspended in 0.7% Tween 40, and recovery of the same two species of *Erwinia* when held as suspensions in 0.7% Tween 40.

rots) after inoculation changed rapidly within 6 hr. Most of the bacteria inoculated (either ECZ W-20 or EC 208) moved downward between the leaves of the whorl and into the stalk during this time.

Changes in bacterial populations in an inoculum suspension (0.7% solution of Tween 40) held in capped culture tubes at 28 C were also determined by serial dilution plates at time intervals similar to those followed for plant bacterial population determinations. Viable populations of bacteria (both ECZ W-20 and EC 208) in control Tween 40 solutions decreased tenfold during 48 hr (Fig. 4).

Field inoculations.—Field-grown hybrid corn W-64A X W-117, age 7 weeks, and sweet corn hybrid Wisconsin Golden 900, age 6 weeks, were whorl-inoculated with 5 ml of ECZ W-20 (2×10^6 or 2×10^8 cells/ml), suspended in water or 0.7% Tween 40. Despite relatively cool weather (10 to 25 C) following inoculations, initial symptoms including wilting of inner whorl leaves, were evident on some plants within 4 days. Disease ratings were taken at 17 days after inoculation. The rating system used was 0 = no symptoms, 1 = small necrotic areas in tissue at the base of the whorl, 2 = 25% of the tissue rotted at the base of the whorl, 3 = 50%, 4 = 75%, and 5 = top broken over due to soft rot at the base of the whorl.

The disease ratings for hybrid corn inoculations (average of 30 plants/treatment) were as follows: No Tween, 2×10^6 cells/ml = 0.4, 2×10^8 cells/ml = 0.3; Tween 40, 2×10^6 cells/ml = 1.0, 2×10^8 cells/ml = 2.6. For sweet corn inoculations (average of 15 plants/treatment): No Tween, 2×10^6 cells/ml = 0, 2×10^8 cells/ml = 0; Tween 40, 2×10^6 cells/ml = 3.0, 2×10^8 cells/ml = 4.3. The hybrid corn tasselled during the first few days of the incubation period, whereas the sweet corn plants which were less mature, tasselled later or not at all resulting in retention of the bacteria in the whorl for a longer period of time. This may be the basis for the higher disease ratings on sweet vs. field corn.

DISCUSSION.—If overhead irrigation of corn fields with ECZ-infested water results in the development of bacterial stalk rot of corn (7, 9, 16), then inoculation of bacteria into the whorl of corn plants would most closely approximate natural inoculation. Previously, it was not possible to demonstrate that inoculation of bacteria into corn plant whorls would result in consistent infection (6). Use of Tween in the inoculum proved to be a useful additive for successful whorl inoculation, giving uniform and reproducible results compared to stem injections which were not as consistent (about 55% in one experiment). One unusual aspect of this method of inoculation was the demonstration that soft rot infection could occur in the absence of any mechanical injury to the plant.

With this inoculation technique the corn-ECZ host-pathogen system provides a number of advantages over other bacteria-plant interaction model systems since no infiltration or wounding of the tissues is needed. Studies on a differentially inhibitory fraction extracted from corn tissues have been facilitated by the use of this inoculation procedure (5).

At levels of Tween 40 needed for this inoculation technique (less than 1.0%) no adverse effects on corn plants have been observed. Previous studies with staphylococci (8) support the evidence obtained in our studies that Tween is not toxic to some bacteria at low concentrations.

It is apparent under these conditions that the effect of Tween 40 in this inoculation method is mainly physical. Penetration of Eosin Y into the stalk between inner whorl leaves, aided by presence of Tween 40, approximates penetration of inoculated bacteria into these between whorl leaf spaces in cornstalks. The primary effect of Tween in this inoculation technique appears to be to reduce surface tension and facilitate movement of inoculum downward between leaf blades in the whorl. In addition, exposure to Tween did not change cornstalk cell permeability to the extent that increased electrolyte leakage was evident (4).

Use of the whorl inoculation technique made it possible to study changes in bacterial populations in nonwounded corn plants following inoculation. Visible rotting symptoms followed increases in bacterial populations in cornstalks; production of stalk rotting enzymes may be associated with bacterial growth (3).

The artificial inoculation technique we developed does simulate natural infection in many respects and is an effective means of getting consistent infections of corn with ECZ. There appears to be some value in using artificial whorl inoculations to determine

whether or not given strains or isolates of this bacterium are virulent, and possibly to determine whether or not corn cultivars are resistant to cornstalk rotting bacteria.

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