Differential Inhibitory Activity of a Corn Extract to Erwinia spp. Causing Soft Rot

John R. Hartman, Arthur Kelman, and C. D. Upper

Assistant Professor, Department of Plant Pathology, University of Kentucky, Lexington 40506; Professor, Department of Plant Pathology; and Research Biochemist, Agricultural Research Service, U.S. Department of Agriculture, Department of Plant Pathology, University of Wisconsin, Madison 53706, respectively.

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ABSTRACT

A water-soluble fraction extracted from corn leaves and stalks was more inhibitory to soft rot bacteria (*Erwinia* spp.) that are nonpathogenic to corn than to the bacterial corn stalk rot pathogen (ECZ), which is a pathotype of *E. chrysanthemi*. Concentrations of the differentially inhibitory fraction (DIF) greater than 0.75 grams equivalent fresh weight of plant tissue extracted per milliliter of culture medium killed *E. carotovora* (EC 208) in broth within 20 hours, whereas ECZ isolates were not killed at this concentration. Other corn pathogens such as *P. syringae*, and *Erwinia stewartii* were less susceptible to the inhibitory fraction than were various *Erwinia* isolates and several other phytopathogenic bacteria nonpathogenic to corn. DIF prepared by thawing of frozen crushed corn tissue in boiling water prior to extraction had low inhibitory activity. DIF preparations from several different corn lines were each active. Activity was higher in DIF extracts from whorl tissues than from an equal weight of leaf or stalk sheath tissue.

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Additional key words: blackleg of potatoes, bacterial soft rot, Stewart's wilt, bacterial leaf blight, holcus or bacterial leaf spot, cyclic hydroxamates, benzoxazolinone.

The causal agent of bacterial stalk rot of corn (*Zea mays* L.) is the only member of the *Erwinia* group of soft rot bacteria that is known to be pathogenic to corn (10, 13, 15, 20, 22, 23, 25). The bacterium, a corn pathotype of *Erwinia chrysanthemi* (ECZ), is closely related to *E. chrysanthemi* Burkholder et al. and also has many physiological characteristics in common with the *E. carotovora* (L. R. Jones) Holland group of soft rot bacteria (5, 20). Although host specificity is characteristic of the *E. chrysanthemi* group (8, 26), all soft rot *Erwinia* spp. have some common hosts such as potato (*Solanum tuberosum* L.) tubers and carrot (*Daucus carota* L.) roots.

Pathogenesis by soft rot bacteria in the Erwinia group has been attributed mainly to the production of hydrolytic enzymes by these bacteria (1, 4); similarly, tissue disintegration of corn stalk and leaf tissue by ECZ has been attributed to extracellular cell-wall degrading enzymes formed by this pathogen (9). Soft rot *Erwinia* did not differ significantly from ECZ with respect to production of pectin lyase, cellulase (C_x), protease, and phosphatase (9), and both produced high levels of polygalacturonases. When corn plants (W-703) susceptible to ECZ were inoculated with a wide range of soft rot *Erwinia* spp. isolates, only ECZ isolates were pathogenic to corn (10). *Erwinia* species included in these studies were *E. chrysanthemi, E. carotovora, E. atroseptica* (van Hall) Jennison, and *E. aroideae* (Town.) Holland. All of these pathogens including ECZ caused decay of potato tubers and other fleshy vegetables and also of autoclaved corn tissues in culture (9). Thus, inability of soft rot *Erwinia* (other than ECZ) to infect corn could not be attributed to absence of enzymes capable of degrading corn. This conclusion, supported by data presented by Turner and Bateman (28), indicates that differences in pathogenicity cannot be attributed to differences in cell-wall macerating enzymes or differences between hosts with respect to pectic substrates.

The development of a standardized inoculation procedure made it possible to compare the population changes of ECZ and *E. carotovora* after adding known numbers of cells to corn whorls (10). After an initial decrease, numbers of ECZ cells increased rapidly, whereas numbers of *E. carotovora* decreased about 100-fold in 48 hours. Plants inoculated with ECZ showed symptoms of stalk rot within 48 hours, whereas, no soft rot symptoms appeared in *E. carotovora*-inoculated plants. Growth of *E. carotovora* was apparently inhibited within the inner whorl after inoculation.

The objectives of this investigation were to determine whether corn leaf and stalk tissues contain factor(s) more inhibitory to the soft rot *Erwinia* isolates that cannot infect corn than the ECZ isolates that are pathogenic on corn and to determine susceptibility to the inhibitor(s) of other bacteria pathogenic and nonpathogenic to corn. Preliminary reports on these studies have been presented (11, 16).

MATERIALS AND METHODS.—*Bacterial cultures.*—Isolates of soft rot bacteria were stored at 24 C in 5 ml of sterile distilled water in plastic-capped 20-ml glass culture tubes. Suspensions consisted of approximately 1×10^6 cells per ml transferred by loop from 48-hour cultures grown on peptone (1.0%), casamino acids (0.1%), glucose (1.0%), and agar. Other bacterial isolates were stored on potato-dextrose-agar slants at 5 C. Permanent stocks were maintained as

bacterial suspensions in 1.5-ml sealed vials containing 0.8% nutrient broth; vials were then immersed in liquid nitrogen in a Linde container. The source of each bacterial isolate is listed in Table 1. The two most commonly used isolates were *E. carotovora* (EC 208), and a corn pathotype of *E. chrysanthemi* (W-3-20). These are designated as EC 208 and ECZ W-20, respectively, in the text.

Extraction of inhibitory fraction from corn.—Corn plants (most commonly inbred line W-703) were grown from seed in soil in 15-cm pots in a growth chamber maintained at 28 C during 12-hour light periods 12,912 lux (1,200 ft-c) and at 24 C during 12-hour dark periods. The aboveground portions were usually harvested when the corn plants were 21 days old.

Extracts were obtained from corn tissue using the following procedure. Fresh corn leaf and stalk tissue (100 grams) was cut into segments (approximately 5 cm each), frozen in liquid nitrogen, and crushed while frozen with mortar and pestle. The crushed tissue was suspended in 500 ml H_2O and allowed to stand for 1 hour at ambient temperatures (24 ± 2 C), filtered through cheesecloth, and the filtrate was centrifuged at 10,000 *g* for 1 hour. The pellet was discarded and the supernatant was lyophilized yielding approximately 3.3 grams of a fine powder which was used in the bioassays. This extract powder could be stored for several months at 5 C without appreciable loss in activity. Extracts were also obtained by chopping 100 grams of corn tissue in a Waring Blendor with 500 ml H_2O for 3 minutes. The filtrate obtained from this material was processed as outlined above and the lyophilized powder obtained had inhibitory activity equivalent to that obtained by the first method. About one-third of the dry weight of the aerial portion of a 3-week-old corn plant (W-703) constituted the water-soluble fraction. In all experiments, the amount of extract added to the bioassay medium was expressed as grams of fresh weight of corn tissue extracted/ml of bioassay medium.

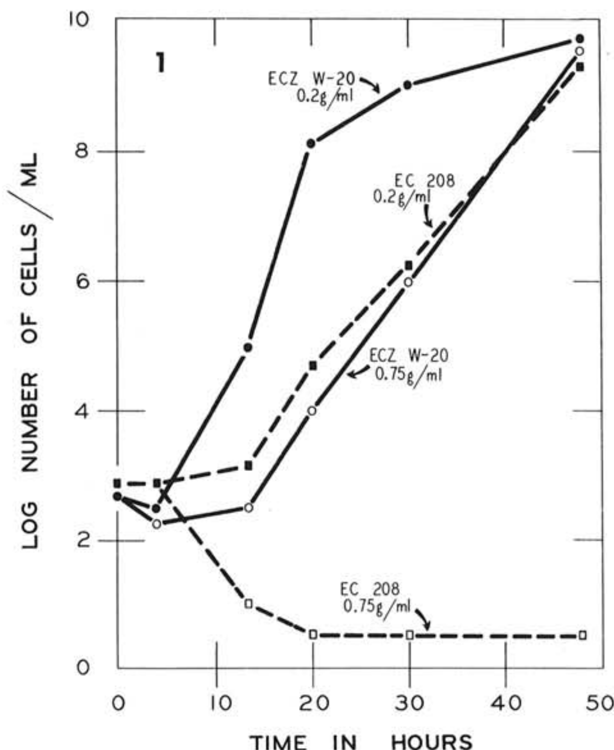

Fig. 1. Growth of *Erwinia chrysanthemi* corn pathotype (ECZ W-20) and *E. carotovora* (208) on peptone-casamino acids-glucose medium at two concentrations of lyophilized inhibitory fraction (DIF) from corn (W-703). Inhibitory fraction concentration is based on grams fresh weight of corn tissue extracted per milliliter of growth medium. Shake cultures were incubated at 28 C. Values < 1.0 for $\log_{10}$ cells per milliliter indicate that bacterial cells probably were killed; the dilution plate method which was used did not detect fewer than 10 cells/ml.

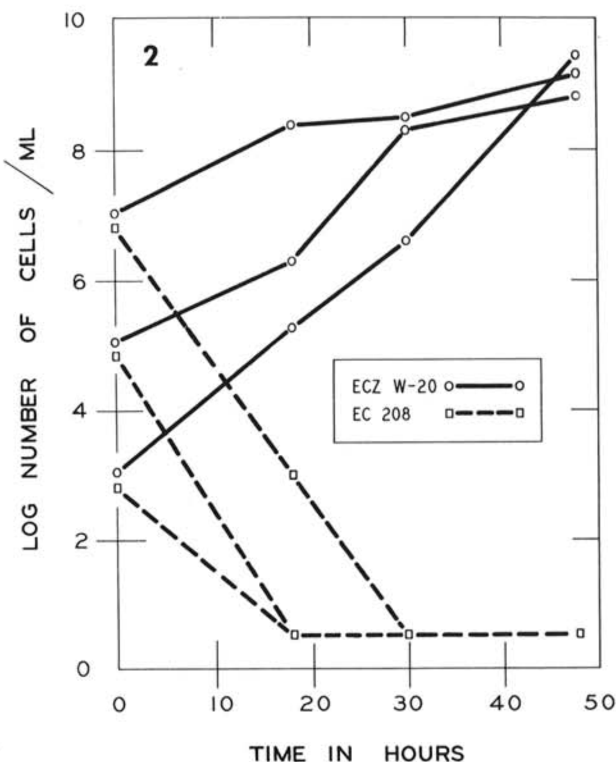

Fig. 2. Relationship of cell number in inoculum to growth of *Erwinia chrysanthemi* corn pathotype (ECZ W-20) and *E. carotovora* (EC 208) in peptone-casamino acids-dextrose liquid medium containing the amount of lyophilized inhibitory fraction extracted from 0.75 g fresh weight of corn (W-703) tissue per milliliter of growth medium.

Bioassay techniques.—Two liquid culture media were used: (i) Peptone-casamino acids-glucose broth containing 1.0% peptone (Difco), 0.1% casamino acids (Difco), and 1.0% glucose in distilled water, and (ii) modified Husain-Kelman medium (14) with 1% glucose, 0.1 M sodium phosphate buffer (pH 6.9) and 0.1% yeast extract. After extracts to be bioassayed were added, media were filter-sterilized. To each 25-ml Erlenmeyer flask containing 3 ml of liquid medium, 0.1 ml of bacterial suspension was added. Inoculum was prepared in sterile distilled water by suspending cells from cultures grown on peptone-casamino acids-glucose agar plates for 48 hours.

Flasks were incubated at 28 C in a water bath on a reciprocating shaker.

The two criteria for determining bacterial growth were: (i) viable cell counts by dilution platings and (ii) turbidimetric determinations at 600 nm with a 1-cm light path using a Beckman model DB spectrophotometer. In (ii), numbers of cells in the initial inoculum were increased so that "0" time determinations would give O.D. values near 0.1 (about 1×10^8 cells/ml determined from a standard curve with conversion factor of 1.0 O.D. = 1.0×10^9 cells/ml).

RESULTS.—The effect of different concentrations of corn tissue extract on growth of ECZ W-20 and EC 208 was determined (Fig. 1). Concentrations of corn extract greater than 0.75 grams equivalent/ml killed EC 208 within 20 hours, whereas ECZ W-20 was not killed unless 2.0 grams equivalent/ml was present.

At nonbactericidal concentrations of the extract the lag growth phase was extended. This lag was more evident in turbidimetric assays than in viable cell count assays because more points were recorded per growth curve. The initial inoculum in these assays was greater than in viable

TABLE 1. Bacterial isolates tested for susceptibility to inhibitory fraction from corn

Isolate	Host and source location	Source
Soft rot bacteria:		
Erwinia aroideae		
36 (EA 144-ICPB)[a]	Britain	Starr, Dowson
E. atroseptica		
54 (EA 143-ICPB)	potato, Britain	Starr, Dowson
E. carotovora		
EC 105 (ICPB)	potato, California	Starr, Ark
EC 169 (ICPB)	broccoli, Britain	Starr, Lacy
EC 208 (ICPB)	carrot	Starr, Jones
E. carnegiana	cactus	Goodman
E. chrysanthemi		
18	chrysanthemum, New York	Dickey
EC 176 (ICPB)	chrysanthemum, New York	Starr, Dimock
E. chrysanthemi		
corn pathotype (ECZ)		
W-1-1	corn, Wisconsin	Kelman
W-3-20	corn, Wisconsin	Kelman
C-9	corn, North Carolina	Kelman
EC 209 (ICPB)	corn, Egypt	Starr, Sabet
I-1,6	corn, India	Payak
E. chrysanthemi var. *graminarum*	sugar cane, Australia	Lelliott, Hayward
Other plant pathogens:		
E. amylovora	apple	Goodman
E. stewartii	corn	Vidaver
Pseudomonas syringae		
C3A	corn	Vidaver
A	corn	Vidaver
C1A	corn	Vidaver
11	corn	Vidaver
59 A	bean, Wisconsin	Kelman
Y-12	cherry, Wisconsin	Kelman
Xanthomonas campestris	cabbage, Wisconsin	Williams
Bacteria nonpathogenic to plants:		
Bacillus cereus WDA 201		McCoy
Enterobacter aerogenes NRRL 199		McCoy
Enterobacter cloaceae ATCC 961		McCoy
Escherichia coli WBS H-52		McCoy
Pseudomonas fluorescens		McCoy
Staphylococcus aureus FDA 209		McCoy

[a]ICPB = International Collection of Phytopathogenic Bacteria. Cultures were kindly provided by M. P. Starr, University of California, Davis. Other cultures were provided by the individuals listed. Second name in series refers to individual who originally obtained the isolate.

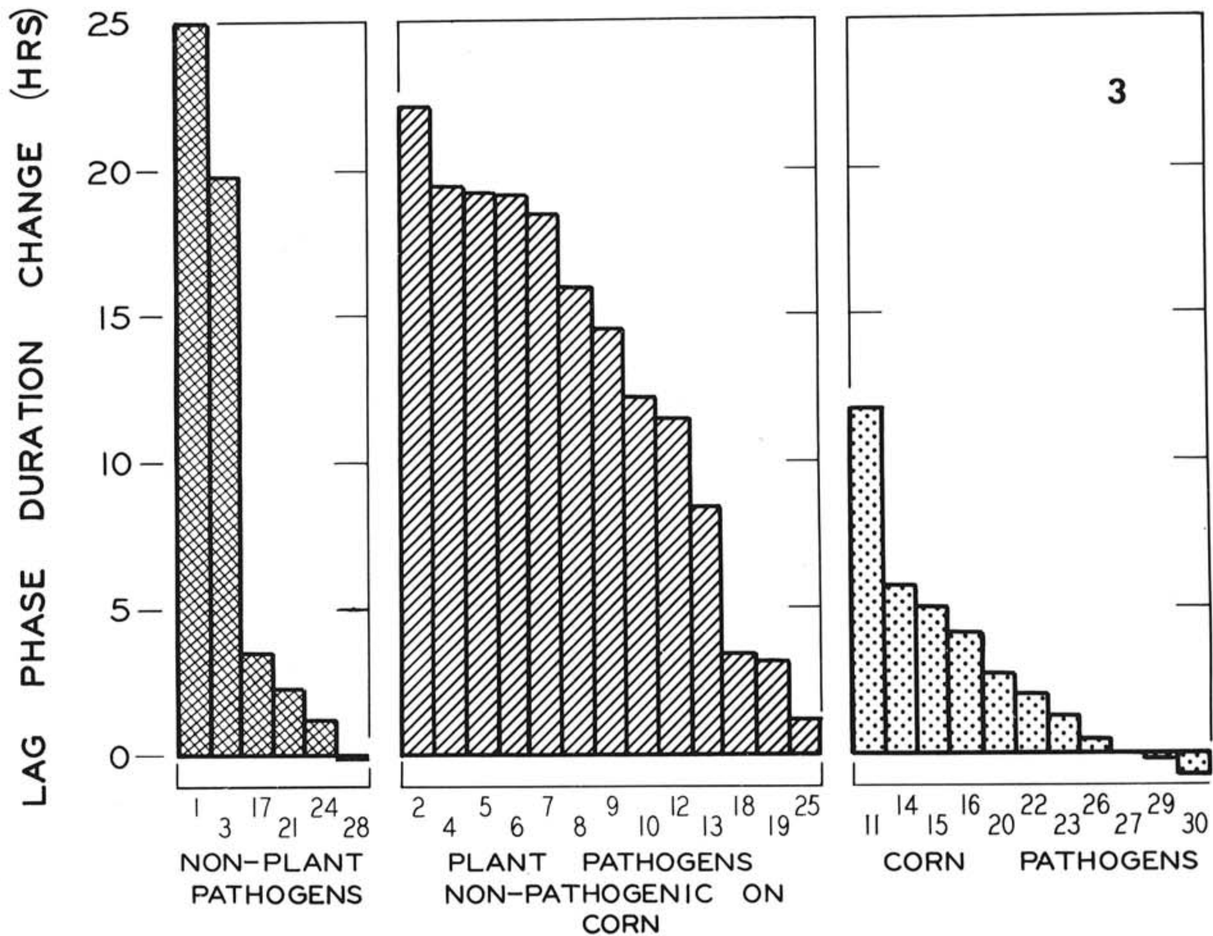

Fig. 3. Effect of differentially inhibitory fraction from W-703 corn plants (0.5 grams fresh weight of corn tissue extracted/ml culture medium) on change in duration of lag phase (hours required for culture containing DIF to reach level of O.D. 0.5 at 600 nm minus hours for culture without extract to reach same turbidity for selected bacteria. Non-plant pathogens were: 1) *Bacillus cereus* WDA 201, 3) *Staphylococcus aureus* FDA 209, 17) *Escherichia coli* WBS H-52, 21) *Pseudomonas fluorescens*, 24) *Enterobacter cloaceae* ATCC 961, and 28) *E. aerogenes* NRRL 199. Plant pathogens nonpathogenic on corn were: 2) *Erwinia chrysanthemi* EC 176, 4) *E. amylovora*, 5) *E. chrysanthemi* 8, 6) *E. carotovora* EC 169, 7) *E. chrysanthemi graminarum*, 8) *E. carotovora* EC 208, 9) *E. carotovora* EC 105, 10) *E. atroseptica* EA 143, 12) *Xanthomonas campestris*, 13) *E. carnegiana*, 18) *E. aroideae* EA 144, 19) *Pseudomonas syringae* Y-12, and 25) *P. syringae* 59A. Corn pathogens were: 11) *Erwinia chrysanthemi* ECZ C-9, 14) *E. stewartii*, 15) *E. chrysanthemi* ECZ W-20, 16) *E. chrysanthemi* ECZ W-1, 20) *E. chrysanthemi* ECZ EC 209, 22) *Pseudomonas syringae* C1A, 23) *E. chrysanthemi* ECZ I-6, 26) *E. chrysanthemi* ECZ I-1, 27) *P. syringae* 11, 29) *P. syringae* C3A, and 30) *P. syringae* A.

cell count assays and, as a result, cells reached the maximum stationary phase sooner. Decreases in turbidity representing lysis of cells were not detected. In order to obtain the same degree of inhibition of ECZ W-20 as EC 208, a concentration of the extract approximately threefold was required. This fraction which had greater inhibition of EC 208 than ECZ W-20 was designated the *differentially inhibitory fraction* (DIF).

When the time interval between chopping of tissues in the Waring Blendor and freezing of supernatant for lyophilization was varied from 1 hour at 4 C to 0.5 to 3 hours at ambient temperature, the activity of DIF did not vary significantly.

To determine the relation of cell number in inoculum to growth of ECZ W-20 and EC 208 in a peptone-casamino acids-glucose medium containing DIF (0.75 grams equivalent/ml) bacterial suspensions were added to obtain 10^3, 10^5, or 10^7 cells/ml and viable cell counts were made periodically. Cultures containing levels of DIF bactericidal to EC 208 and bacteriostatic to ECZ W-20 showed little difference in pattern of growth with the three different inoculum levels (Fig. 2). When the number of cells in the inoculum was increased, there was an expected decrease in time needed to reach maximum stationary growth phase by ECZ W-20 and an increase in time needed for killing of cells of EC 208.

Effect of DIF on various bacterial isolates.—The effect of DIF on growth of 30 different bacterial isolates was determined. Of the 30, six were nonpathogenic to plants (2 Gram +); 11 were pathogens of corn, and the others were pathogens of other plants. Of the 15 soft rot bacteria used, six were isolates of ECZ from corn.

The susceptibility of various bacteria to DIF in the

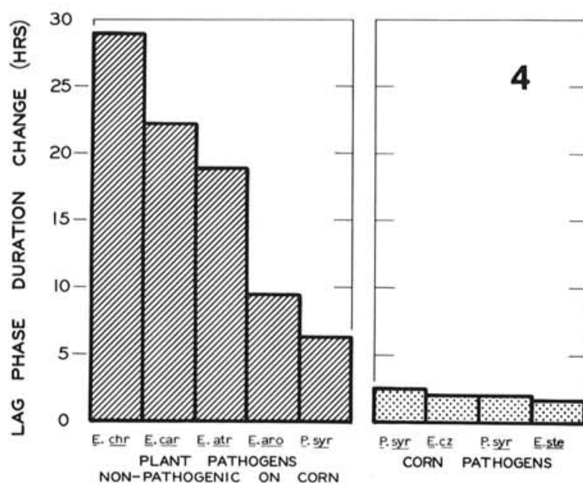

Fig. 4. Effect of differentially inhibitory fraction from W-703 corn plants (0.5 g fresh weight of corn tissue extracted/ml culture medium) on change in duration of lag phase (hours required for culture containing DIF to reach level of 1×10^7 cells/ml minus hours for culture without extract to reach same cell number) for selected phytopathogenic bacteria as determined by viable cell counts. Plant pathogens not pathogenic on corn include *Erwinia chrysanthemi* 18, *E. carotovora* EC 208, *E. atroseptica* EA 143, *E. aroidea* EA 144, and *Pseudomonas syringae* 59A. Corn pathogens include *P. syringae* A, *E. chrysanthemi* ECZ W-20, *P. syringae* C1A, and *E. stewartii.*

growth medium ranged from strong inhibition to slight stimulation (at 0.5 grams equivalent/ml) (Fig. 3). Change in duration of lag growth phase ranged from +25 hours to −0.6 hours. When the 30 isolates used were ranked in order of increasing susceptibility to DIF at 0.5 grams equivalent/ml, the 11 corn pathogens were among the 20 least sensitive organisms. Of the 24 plant pathogens used, the corn pathogens ranked among the 16 least sensitive organisms. Of the 15 soft rot isolates used, the six corn stalk rotting bacteria ranked among the eight least sensitive organisms.

The effect of DIF on lag phase duration for nine selected phytopathogenic bacterial isolates was also determined using viable cell count procedures. Of the nine, four were corn pathogens. Each isolate was grown in modified Husain-Kelman medium containing 0, 0.25, and 0.5 g/ml DIF. The lag phase duration change ranged from +29.0 to −3.3 hours (Fig. 4, Table 2). Ranking the isolates in increasing order of susceptibility to DIF at 0.5 g equivalent/ml indicates that of the nine isolates used, the four corn pathogens were least susceptible to inhibitory activity of DIF. Ranking the same bacteria at 0.25 grams equivalent/ml did not result in an identical order of sensitivity to DIF; there was a reversal of position only for two nonpathogens of corn. The lag phase duration change at 0.25 grams equivalent/ml shown by these isolates was not always proportional to the duration change at 0.5 grams equivalent/ml. Negative values indicate a decrease in lag phase of growth relative to cultures without DIF.

General characteristics of the fraction.—Inhibitory properties of DIF extracted from crushed, boiled corn tissue were compared to DIF extracted in the usual manner without boiling. Boiled DIF was prepared by the same procedure except that the frozen powder was suspended directly in boiling water and the suspension boiled for 30 minutes before filtration and centrifugation.

Boiled DIF was added to peptone-casamino acids-glucose medium (0.675 grams equivalent per milliliter). To this medium was added 0.075 grams equivalent/ml of active DIF prepared by the normal procedure, i.e., without boiling. Flasks containing 0.075 grams equivalent/ml of nonboiled extract or 0.75 grams/ml boiled DIF in peptone-casamino acids-glucose medium were used as controls. The series of preparations was shaken at 28 C for 8 hours before suspensions of ECZ W-20 or EC 208 were added; growth was determined by viable cell counts. Boiled DIF had low inhibitory activity. Addition of a small amount (10% of the total) of active DIF to the inactive DIF prepared by boiling resulted in a gain of differential inhibitory activity which was not proportional to the small amount of active DIF added. Not all of the DIF activity was restored, however, since 0.75 g total of nonboiled DIF was usually bactericidal to EC 208, whereas in this instance the combination of boiled and nonboiled did not completely kill the cells (Table 3).

TABLE 2. Effect of differentially inhibitory fraction from corn (W-703) on change in duration of lag phase for pathogens from corn and other hosts as determined by viable cell counts

Bacterial isolates	Host	Change of duration of lag phase[a] at indicated inhibitory extract conc[b]	
		0.5 g/ml	0.25 g/ml
Erwinia chrysanthemi (18)	chrysanthemum	+29.0	+3.5
E. carotovora (EC 208)	carrot	+22.3	+6.7
E. atroseptica (EA 143)	potato	+19.0	+3.8
E. aroideae (EA 144)	tobacco	+ 9.5	+0.3
Pseudomonas syringae (59A)	bean	+ 6.4	−0.7
P. syringae	corn	+ 2.5	−0.7
E. chrysanthemi (ECZ W-20)	corn	+ 2.0	−1.0
P. syringae (C1A)	corn	+ 2.0	−1.8
E. stewartii	corn	+ 1.6	−3.3

[a]Change in duration of lag phase = hours required for culture containing inhibitory fraction to reach level of 1×10^7 cells/ml minus hours for culture without extract to reach same cell number.

[b]Grams fresh weight equivalent to amount of corn (W-703) tissue extracted per milliliter.

TABLE 3. Growth of *Erwinia chrysanthemi*, corn pathotype (ECZ W-20) and *E. carotovora* (EC 208) in medium[a] containing inhibitory fractions from lyophilized corn tissues prepared either by thawing frozen crushed corn tissue in boiling water for 30 minutes or at ambient temperatures (approximately 24 C)

Source and amount of extract[b]		Log_{10} number of viable cells/ml					
		EC 208 Incubation period (hr)			ECZ W-20 Incubation period (hr)		
Boiled tissue	Nonboiled tissue	0	24	48	0	24	48
...	0.75	3.0[c]	1.0	1.0	2.9	8.1	9.7
0.75	...	3.0	7.0	9.7	2.9	9.0	9.5
0.675	0.075	3.0	3.1	2.4	2.9	8.7	9.3
...	0.075	3.0	8.5	9.5	2.9	8.8	9.1

[a]Cultures were grown in basal peptone-casamino acids-glucose liquid medium.
[b]Fresh weight equivalent of corn (W-703) tissue extracted per milliliter.
[c]Average of two experiments.

In addition to W-703, active DIF extracts were obtained from a number of different corn lines including: WF 9, 22 R, 64 A × 117 N and 64 A × 117 (Texas male sterile) obtained from J. Lonquist, Department of Agronomy, University of Wisconsin, Madison; and 8HL6, 8HL8, 8HL6 × 8HL8, NC 601, 73 HB10, and 7HB10 obtained from D. L. Thompson, North Carolina State University, Raleigh.

North Carolina inbreds 8HL8 (field-tolerant) and NC 601 (field-susceptible) (D. L. Thompson, *personal communication*) showed little difference in disease reaction in growth room inoculation tests, nor were they different in terms of activity of DIF extracted from tissues of 3-week-old plants.

Extracts of DIF from whorl (inner achlorophyllous stalk leaf) tissue had higher levels of activity than did extracts of equivalent weight of leaf (all nonstalk tissue) or stalk sheath (outer stalk leaf) tissue. Differential growth inhibition of ECZ W-20 and EC 208 was detected in preparations derived from whorl tissue weighing as little as 0.1 g/ml, whereas higher levels of leaf tissue (0.25 g/ml) were required to get equivalent activity.

DISCUSSION.—In general, bacterial pathogens of corn were less sensitive to the effects of DIF than bacterial pathogens of other crops. This was evident both in turbidimetric and viable cell-count assays. By means of growth-curve bioassays, the effect of DIF was mainly manifested as an increase in duration of the lag phase of growth. The increase in lag phase duration with susceptible bacterial isolates provided a semi-quantitative estimate of the relative activity of DIF in various preparations. Growth-curve bioassays were a repeatable and reliable means of determining differences in bacterial response to various extracts of DIF.

It may be expected that the corn pathogens would be less susceptible to the inhibitory fraction since they may have evolved in the presence of the material. Certain nonphytopathogenic bacteria are also not susceptible to DIF; lack of pathogenicity to corn of a given bacterium would not be completely attributable to susceptibility to DIF, however.

Studies have been made of the role of several cyclic hydroxamates and their derivatives with respect to resistance to insects (2, 17, 18) and various diseases of corn (3, 7, 12, 21, 29, 30, 31, 32) and stem rust of wheat (19). Preliminary data of Corcuera et al. (6) indicate that 2,4-dihydroxy-7-methoxy-1,4-(2H)-benzoxazin-3-one (DIMBOA), one of the cyclic hydroxamates present in most corn varieties, causes a greater increase in lag phase of EC 208 than of ECZ W-20. If the activity in DIF is attributable to DIMBOA or a similar compound which is released enzymically from a relatively inactive glycoside, boiling of tissues before extraction of DIF should result in a preparation with lower activity than that obtained by the normal extraction method; this result was observed.

Differences in field resistance to bacterial stalk rot in certain corn lines have been observed (24, 27). Some of these lines differed neither in susceptibility when challenged by means of an artificial inoculation procedure (J. R. Hartman, *unpublished*), nor in the activity of DIF preparation derived from them. Thus no clear relationship between field resistance to ECZ and DIF content can be drawn on the basis of our data at present.

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