Peroxidase Enzyme Markers for Ozone Sensitivity in Sweet Corn

Edward V. Podleckis, Charles R. Curtis, and Howard E. Heggestad

Former graduate research assistant, Department of Plant Science, University of Delaware, Newark 19711, now at Department of Botany, University of Maryland, College Park 20742; formerly professor of plant pathology, University of Delaware, Newark 19711, now at Department of Plant Pathology, Ohio State University, Columbus, OH 43210, and research plant pathologist, Plant Stress Lab, Plant Physiology Institute, USDA, Beltsville, MD 20705, respectively.

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ABSTRACT

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Fifteen inbreds and five hybrids of sweet corn (*Zea mays*) were evaluated for seedling sensitivity to a single acute ozone exposure. Seedlings at the three- to four-leaf stage were exposed to 492 $\mu g/m^3$ (25 pphm) ozone for 3 hr in a controlled environment chamber. Plants were visually rated for ozone sensitivity. Three genotypes, including the commercially important hybrid Silver Queen, were relatively sensitive. Four lines were ranked highly resistant and the remaining 13 were rated intermediate. Polyacrylamide gel isoelectric focusing was used to compare the protein banding patterns of sensitive, resistant, and intermediate lines. Peroxidase and general protein banding patterns were examined. Stepwise multiple regression analysis was used to construct models for predicting ozone sensitivity from the banding patterns. The model based on general protein banding patterns produced an unacceptably high error and variability. The peroxidase model accurately predicted the relative sensitivity of eight of 10 genotypes examined and shows promise as a screening technique for ozone sensitivity.

Foliar injury to sweet corn (*Zea mays* L.) caused by ozone and other ambient oxidants has been known for several years (3,10,17). Concentrations from 500 to 600 $\mu g/m^3$ (20–30 pphm) ozone at 25–35 C caused formation of water-soaked, interveinal streaks in field-grown sweet corn (23). Exposure to <200 $\mu g/m^3$ ozone for several hours daily for 3 wk caused substantial reductions in plant growth (10). Other symptoms included stunting, reduced leaf area, small ears, reduced seed set, and retarded flowering. Heagle et al (17) reported significant reductions in ear fresh weight, kernel number, and kernel dry weight at ozone levels from 100 to 200 $\mu g/m^3$. Heggestad (19) found that ambient ozone reduced yields of six sweet corn cultivars by about 9% in 1977 and 1978 at Beltsville, MD. Considering the evidence for ozone injury to sweet corn, plant breeders would benefit from having a reliable method for evaluating plant sensitivity to this stress.

In recent years, electrophoretically separated enzyme and protein markers have been used in some breeding programs (14,33). In this paper, isoenzymes are operationally defined as multiple molecular forms occurring in the same organism and having similar catalytic activity but separable by electrophoretic techniques (11). Isoenzyme markers may preclude the need for breeders to attach a unique morphological character to parental lines, or they may be used in instances in which no such character is available (26). If

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isoenzyme markers correlate well with ozone sensitivity or resistance, breeding pollutant-resistant lines may be greatly facilitated.

The objectives of this study were to determine the relative ozone sensitivities of 20 sweet corn genotypes, to perform background electrophoretic analyses on selected genotypes and relate these data to ozone sensitivity, and to develop a model to predict ozone sensitivity in selected genotypes.

MATERIALS AND METHODS

Fumigation studies. *Plant culture.* Twenty sweet corn genotypes of commercial importance or known ozone sensitivity were selected for fumigation studies. Fifteen inbred and five hybrid genotypes were chosen. The inbreds included NK6942, NK6604, 471-U6, and 81-1 obtained from James Cameron, University of California, Riverside; P51T, PF41, and P39 from Vern Gracen, Cornell University; and 453I, Cr853, 5125B, Cr825, 2132B, Fa32, 2256B, and Fa56A from Crookham Co., Caldwell, ID. The hybrids included Silver Queen, Butter and Sugar, and Iochief donated by Agway, Inc., Prospect, PA; NK51036 provided by Sun Seeds, Inc., Bloomington, MN; and Bonanza obtained from Ferry Morse Seed Co., Mountain View, CA.

Seedlings were grown in greenhouses with charcoal-filtered air at the U.S. Department of Agriculture, Beltsville, MD. Five to seven kernels of each line were sown per 10-cm-diameter plastic pot in a peat:perlite mixture (3:1, v/v), and later thinned to three plants per pot. All plants were watered daily and received a weekly feeding with a solution of 20-20-20 (NPK) fertilizer at 5 g/L. Plants were grown under conditions of natural sunlight and ambient temperatures ranging from 20 to 28 C.

Ozone fumigation. Three pots each of the 20 genotypes were exposed to ozone in a growth cabinet fumigation chamber (Controlled Environment, Inc., Pembina, ND) for a total of nine plants per genotype. Twenty pots, one per genotype, were arranged in each of three randomized complete blocks within the fumigation chamber. Control plants remained in the filtered-air greenhouse.

Plants were exposed at $492 \pm 2\ \mu g/m^3$ ozone for 3 hr when they reached stage I on Hanway's growth scale for corn (15). At this stage, the collar of the third leaf was fully visible on all plants. Exposure conditions were 26 ± 1 C, $86 \pm 5\%$ RH, and a mixture of fluorescent and incandescent lamps providing 200 $\mu E/cm^2/sec$ of photosynthetically active irradiation. Ozone was measured by a Mast ozone meter (Mast Development Co., Davenport, IA) calibrated with a Dasibi model 1003-PC ultraviolet ozone monitor (Dasibi Environmental Corp., Glendale, CA). Following ozone exposure, all plants were returned to the filtered-air greenhouse.

Injury rating. Forty-eight hours after fumigation, each plant was rated for visible foliar injury according to a nine-point modified McKinney index (28) defined as: 0 = healthy plant, no injury; 0.5 = minor flecking on some leaves; 1.0 = slight injury, $\leq$10% leaf area injured; 1.5 = some flecking, chlorosis of lower leaves; 2.0 = injury >10%, but $\leq$20% leaf area; 2.5 = some necrotic banding evident; 3.0 = moderate injury, 25–30% leaf area injured; 3.5 = extensive injury, lower leaves necrotic; 4.0 = severe injury, $\geq$40% leaf area injured. A total of 180 plants, nine per genotype, were evaluated. Controls were evaluated on the same scale.

Electrophoresis studies. *Plant culture.* Plant material for electrophoretic analyses was grown in the University of Delaware greenhouses, Newark. Ten genotypes comprised of five inbreds and five hybrids were selected for analysis. Three kernels were sown per 10-cm-diameter plastic pot in a mixture of peat, perlite, and vermiculite (3:4:2, v/v). All plants received a weekly feeding with a 5.0 g/L solution of 20-20-20 fertilizer plus a single 2.5 g/L application of iron sulfate.

Protein extraction. Plants grown for isoelectric focusing analyses were harvested after ~3 wk of growth when they reached Hanway's growth stage I. The second and third formed leaves were excised from each of three plants, chopped, shredded, and mixed together to form a 5-g sample. A 1-g subsample was drawn from the pooled tissue for electrophoretic analysis. Samples were ground in a chilled mortar with an equal weight of insoluble polyvinylpyrollidone (4×10^4 daltons), a small amount of ground glass and 5.0 ml of chilled extraction buffer. The pH 7.6 extraction buffer consisted of 0.01 g cysteine-HCl, 0.01 g of L-ascorbic acid, tris buffer, 1.0 g sucrose, and 6.1 ml 0.2 N HCl. The entire solution was diluted to 10 ml with distilled water. After it was filtered through cheesecloth, the filtrate was centrifuged 30 min at 29,000 *g* at 2–4 C. Protein concentration of the supernatant was determined by a modified Bradford colorimetric assay method (1). The supernatant was used for isoelectric focusing without further dilution.

Isoelectric focusing. Peroxidase (EC 1.11.1.7) isoenzymes and general proteins were separated by vertical slab isoelectric focusing by using a Bio-Rad model 220 dual slab electrophoresis unit (Bio-Rad Laboratories, Richmond, CA) and a 5.0% acrylamide, 2.0% pH 3–6 ampholyte gel. Samples of 75 μg of total protein per well were applied for peroxidase studies, while samples of 125 μg/well were applied for general protein determinations. A sample of the hybrid Iochief was included in every experiment as a standard to check for pattern variations. A discontinuous, 2.5% (v/v) phosphoric acid/monoethanolamine buffer system was employed. Focusing was performed at 100V constant voltage and 0.45 mA per well for 45 min, followed by 45 min at 300V and 1.5 mA and ending with 120 min at 500V. Following each run, the gels were removed from the unit, stained, mounted on cellulose film and photographed.

The pH gradient in the slab gel was determined by slicing representative gels into 5.0-mm sections, incubating the sections 24 hr in 1.0 ml of distilled water, then measuring pH.

Staining procedures. A Coomassie blue staining procedure was used for visualizing general proteins (36). Following fixation in an aqueous solution of 30% methanol (v/v):4% sulfosalicylic acid (w/v):11% trichloracetic acid (w/v) for about 60 min, the gels were soaked overnight in distilled water. The gels were then stained in an aqueous solution of 0.5% Coomassie blue R-250. Peroxidase banding patterns were visualized by the method of Hoyle (22). Relative mobilities and intensities of the bands were based on a minimum of six samples from at least three different gels.

Statistical analysis. Fumigation data were analyzed for differences between lines with respect to foliar injury rating by using the University of Delaware computer program NAOVAMAIN. Data were analyzed assuming a fixed effect, randomized complete block design with three blocks and three observations per treatment per block. Duncan's multiple range test and Fisher's least significant difference were used to separate genotypes into sensitive, intermediate, and resistant classes.

In recording electrophoretic results, each band was identified by its R_f value, pI, and intensity (1 = absent, 2 = light intensity, 3 = medium intensity, and 4 = heavy intensity) (25). Data were analyzed by a stepwise multiple regression using the University of Delaware computer library STPREG package. The predictors provided by stepwise regression were tested using the observed values for nine genotypes to develop a multiple regression model to predict the value for the 10th genotype. The process was repeated until each of the 10 genotypes was used as the unknown. A paired *t*-test was then utilized to test for significant differences between predicted and observed values for foliar injury.

RESULTS

Fumigation study. Plants reached Hanway's stage I maturity within 20–25 days of sowing. The ozone dose (3 hr at 492 $\mu g/m^3$) induced foliar symptoms without causing excessive injury. Symptoms included accelerated senescence of older leaves as evidenced by extensive chlorosis and necrosis. Younger leaves had a water-soaked appearance shortly after fumigation. The water-soaked areas developed into tan, bifacially necrotic zones within 48 hr of fumigation.

The mean injury ratings for visible foliar injury by ozone are presented in Table 1. There were no significant differences among replications. The grand mean was 1.51.

Analyses of these data according to Duncan's new multiple range test (27) and Fisher's least significant difference (27) enabled classification of the sweet corn lines into three distinct groups based

on mean injury ratings. The high-injury genotypes with mean injury ratings ≥2.94 were considered sensitive. Included in this group were the hybrid Silver Queen and the two inbreds NK6942 and 471-U6. Genotypes whose mean injury rating was ≤0.45 represented by four inbred genotypes: Fa32, 2132B, P51T, and 4531

TABLE 1. Mean ozone injury ratings of 20 sweet corn (*Zea mays* L.) lines from combined data of two experiments

Sensitivity Class	Line	Mean injury rating[y,z]
Sensitive	471-U6	3.44
	NK6942	3.11 a
	'Silver Queen'	2.94 a
Intermediate	'Bonanza'	2.50 b
	NK51036	2.39 bc
	'Butter and Sugar'	2.28 bc
	P-39	2.06 c
	CrR825	1.44 d
	NK6604	1.39 de
	81-1	1.35 de
	5125B	1.35 de
	CrR853	1.28 de
	Pf41	1.22 def
	'Iochief'	1.17 def
	2256	1.06 ef
	Fa56A	0.89 f
Resistant	Fa32	0.45 g
	2132 B	0.44 g
	P51T	0.39 g
	4531	0.38 g

[y]Mean of 18 observations per line.
[z]Means followed by a common letter not significantly different according to Duncan's new multiple range test, $P \leq 0.01$. Fisher's least significant difference = 0.33, $P \leq 0.01$.

were classified as resistant. Remaining genotypes with mean injury ratings ranging from 0.89 to 2.50 were designated intermediate and included four hybrid and nine inbred genotypes.

Electrophoresis studies. A total of 18 peroxidase bands were identified among the 10 genotypes that were analyzed. The isoelectric points of the bands ranged from pH 6.02 to 4.00. Fig. 1 is a diagrammatic interpretation of the gel patterns and represents a compilation of six observations per genotype. There were no significant differences among observations. No single gel stained for all 18 bands. Two genotypes (Iochief and 2132B) had 16 bands, the maximum number for a single genotype. The least number of bands was present in 471-U6, Silver Queen, Bonanza, and NK51036, each with 13 bands. The ozone-sensitive lines, 471-U6 and Silver Queen, were electrophoretically indistinguishable using peroxidase isoenzymes. These two genotypes had 13 common bands, all staining with identical intensities. Ten of the 18 total bands identified were common to all 10 genotypes examined. One band at 12 mm from the origin and pH (pI) of 5.78 was unique to the inbred line 2132B.

The Coomassie blue stained gels are represented in Fig. 2. A total of 22 bands was identified between pH 5.48 and 3.62. There were no significant differences among the six observations. No single genotype stained for all 22 bands, but Iochief and Bonanza each possessed 21 bands. NK6942, a sensitive genotype, had the fewest bands (only 14). There were no bands common to all 10 genotypes and no bands unique to a genotype.

Statistical analysis. Raw data for the banding patterns were entered into a stepwise multiple regression analysis. The analysis constructed a predictive model by adding predictors, in this case electrophoretic bands, to the model in order of their contribution to R^2. The analysis was effective in eliminating those bands with little or no predictive value by incorporating into the model only those variables that made a statistically significant contribution to the R^2 value of the model. Using the predictors supplied by stepwise

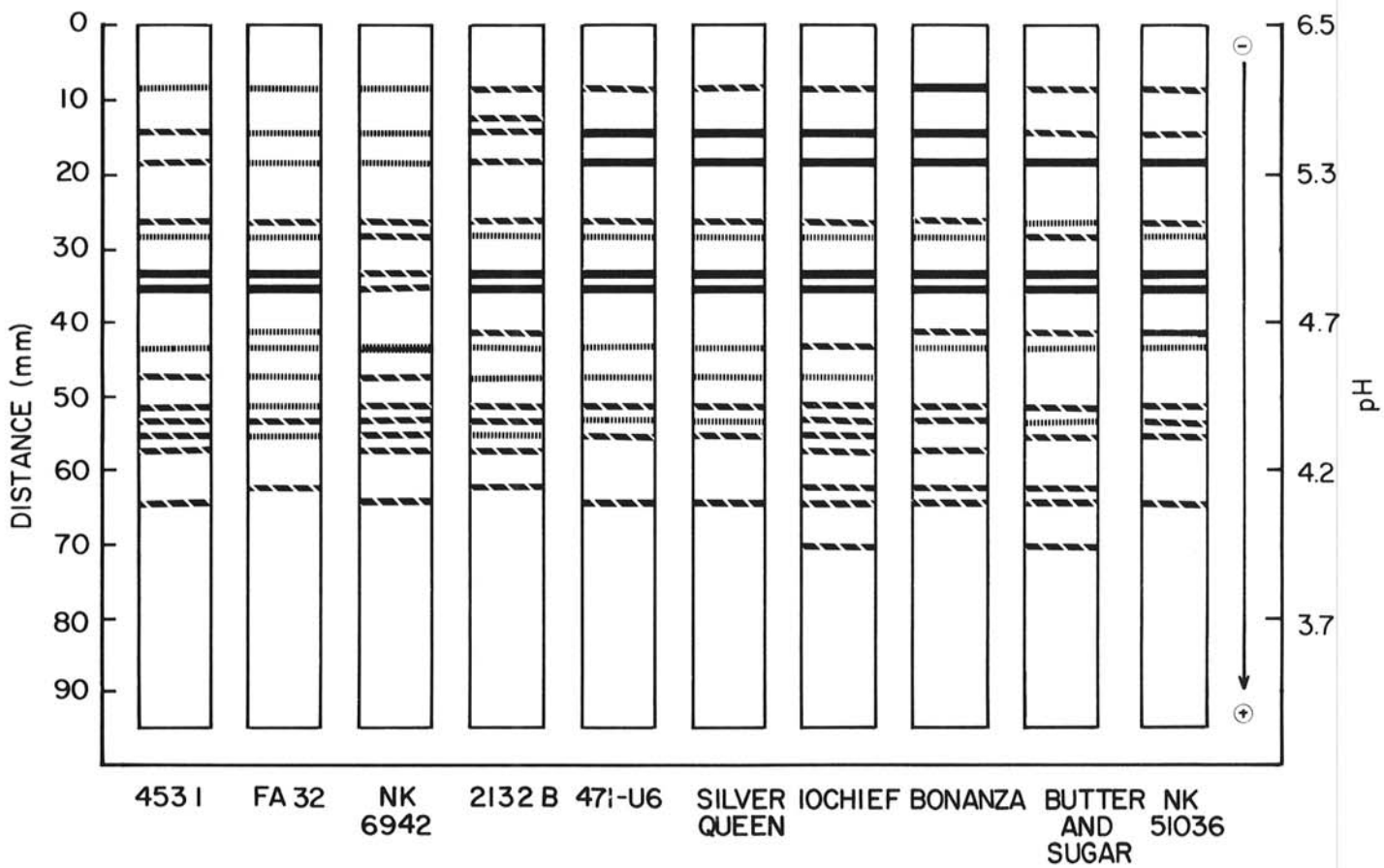

Fig. 1. Graphic respresentation of typical peroxidase gels (solid line indicates strong staining intensity, hatched line medium intensity, and shaded line light intensity for 10 sweet corn genotypes).

regression, a multiple regression equation was constructed for both staining systems to predict ozone sensitivity.

A seven-factor model was developed for peroxidase banding patterns. The model selected at the $P = 0.05$ level was:

$$Y = -1.95 + 1.30\ X_1 - 0.11\ X_2 - 1.62\ X_3 + 1.91\ X_4 - 0.58\ X_5 + 0.82\ X_6 + 0.11\ X_7$$

in which Y is the predicted mean injury rating, and the X values are staining intensities of the peroxidase bands used as predictors. The bands used as predictors in this model are given in Table 2. The variables are listed in order of insertion into the model on the basis of each band's contribution to R^2. The R^2 value for the model was 91.2% when adjusted for degrees of freedom. The predictive ability for the model is shown in Table 3. The model accurately predicted the ozone sensitivity class in eight of 10 genotypes. A paired t-test failed to demonstrate any significant differences between predicted and observed values at any level.

The predictive model adopted from stepwise regression analysis of Coomassie blue banding patterns contained seven factors. The model was as follows:

$$Y = 9.22 - 4.54\ X_1 - 1.11\ X_2 - 3.74\ X_3 + 1.86\ X_4 + 7.06\ X_5 - 6.81\ X_6 - 0.55\ X_7$$

in which Y is the predicted mean injury rating and X variables are the Coomassie blue-stained band predictors. The variables are listed in Table 4 according to their insertion into the model. The R^2 for the equation was 99.8% when adjusted for degrees of freedom. The model had only a six of 10 accuracy in predicting sensitivity class and an average difference from the observed value of 2.05 (Table 5).

TABLE 2. Coefficient of determination (R^2) and F values for peroxidase bands used as predictors of ozone injury to sweet corn by using the peroxidase banding pattern regression model

Variable[a]	Distance from cathode (mm)	pI	Contribution to R^2	F
X_1	18	5.42	0.481	7.405
X_2	62	4.16	0.231	5.592
X_3	53	4.38	0.112	3.776
X_4	43	4.64	0.074	3.577
X_5	41	4.68	0.071	8.671
X_6	12	5.78	0.019	4.447
X_7	14	5.66	0.003	0.666

[a] Variables listed in order of insertion into the model.

DISCUSSION

Reports vary regarding the relationships between visible injury and yield loss (17,20,31) and between seedling and mature plant responses to ozone (29,34). In our study, visible injury to seedling foliage was chosen as the basis for the sensitivity classification

TABLE 3. Ozone sensitivity of selected sweet corn (*Zea mays*) genotypes as predicted by a peroxidase banding pattern regression model

Line	Mean injury rating[a,b]		Ozone sensitivity class	
	Predicted	Observed	Predicted	Observed
471-U6	3.44	3.44	Sensitive	Sensitive
NK6942	1.90	3.11	Intermediate	Sensitive
Silver Queen	2.96	2.94	Sensitive	Sensitive
Bonanza	2.45	2.50	Intermediate	Intermediate
NK51036	2.35	2.39	Intermediate	Intermediate
Butter and Sugar	1.53	2.28	Intermediate	Intermediate
Iochief	3.10	1.17	Sensitive	Intermediate
Fa32	0.49	0.45	Resistant	Resistant
2132B	0.79	0.44	Resistant	Resistant
4531	0.83	0.38	Resistant	Resistant

[a] $t = 0.422$, NS at 9 df.
[b] Average difference = 0.49.

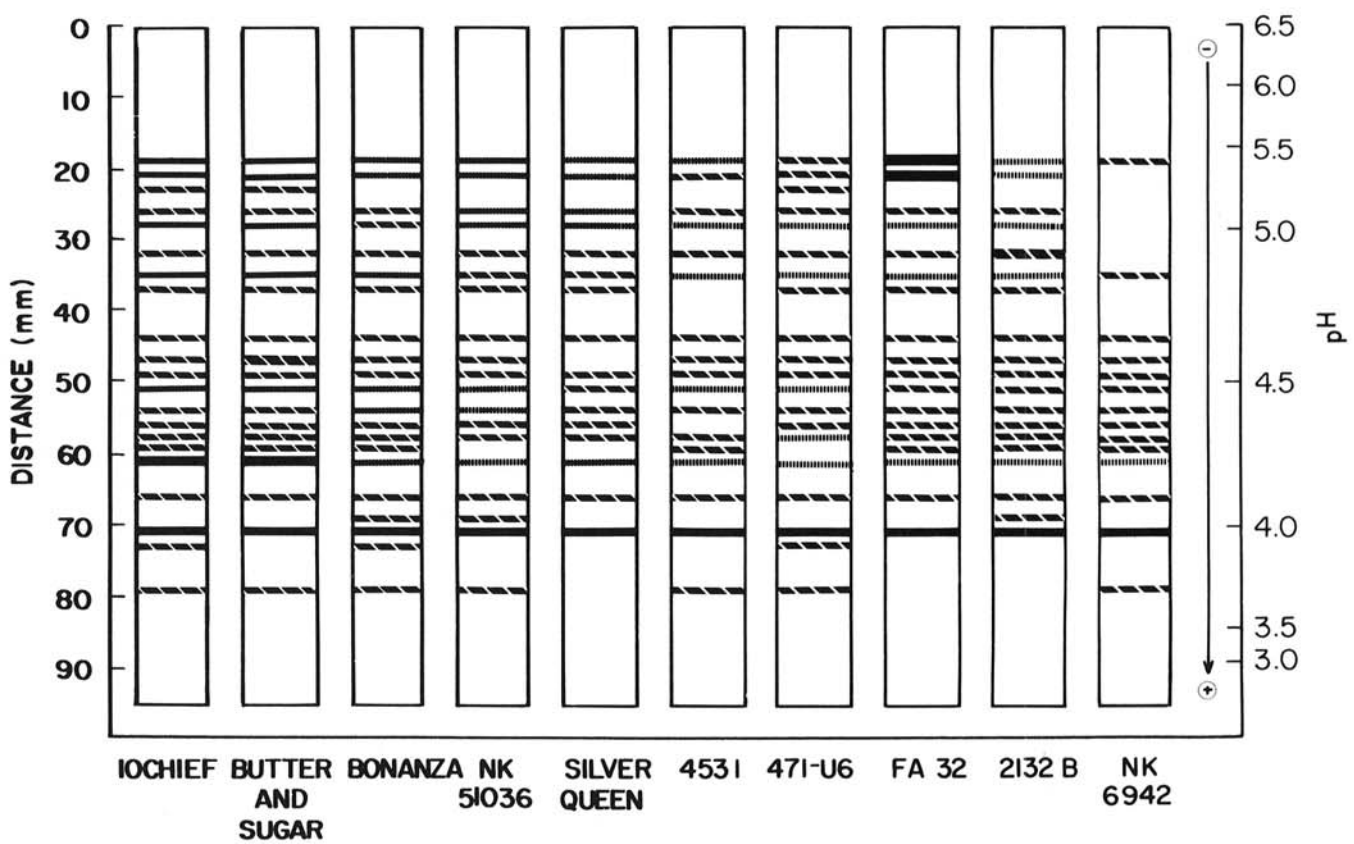

Fig. 2. Graphic representation of typical Coomassie blue-stained gels (solid line indicates strong staining intensity, hatched line indicates medium intensity, and shaded line indicates light intensity for 10 sweet corn genotypes).

because it is rapidly assessed. Sweet corn lines were chosen for this study because they were either commercially popular, of known ozone sensitivity, or both. NK6942, NK6604, 471-U6, and 81-1 were chosen because their relative sensitivities to ozone had been studied in California by Cameron (2). While Cameron rated 471-U6, NK6942 and 81-1 as ozone resistant, we found 471-U6 and NK6942 to be the two most sensitive genotypes, and 81-1 was ranked intermediate. Differences in ozone symptom expression under different environmental conditions have been reported (21). Cameron's estimates were made under field conditions of low humidity, high temperatures, and ambient air. There are reports of increased sensitivity to ozone under relatively cool, humid conditions (4). Lower temperatures and higher humidity conditions in our study may have altered plant response. Since filtered air, rather than ambient air, was used in our study, ozone-antagonistic elements of ambient air (18) may not have been present. Devos et al (8) observed that when high levels of peroxyacetylnitrate (PAN) were present, genes for sensitivity of petunia to PAN injury were almost completely dominant to those for resistance. A similar mechanism may have been operative in this study.

Five genotypes required earlier planting because of their slower growth rate. Three of these (2132B, Fa32, and 453I) were highly resistant to ozone injury while the remaining two (NK51036 and CrR825) were intermediate in response. Two of the most vigorous ones were Silver Queen and 471-U6, which were among the most sensitive to ozone. The literature reviewed by Harkov and Brennan (16) also suggests a positive relationship between slower growth rates and ozone resistance.

The differential response of the genotypes examined in this study indicates substantial genetic variability in sweet corn response to ozone. Since the most practical method for limiting ozone injury is the use of ozone-resistant cultivars (32), the use of the electrophoretic method in programs for breeding pollutant-resistant plants may be of some importance.

The utility of the electrophoretic data depends on the method for statistical analysis. Multiple regression was a logical choice for construction of a predictive model, but the complex nature of banding patterns warranted a method to eliminate bands with no predictive value. Draper and Smith (9) suggested that stepwise regression was the best variable selection procedure. Stepwise regression eliminates from the model any variable whose contribution to predictive ability is statistically insignificant. In our study, stepwise regression models proved effective in predicting injury ratings from banding patterns.

Generally satisfactory visualizations of banding patterns were obtained by using the Coomassie-blue staining system for general proteins, but the model they generated appeared to have only limited predictive ability. In this case, the model was neither acceptably accurate nor precise. Accuracy is defined here as the ability to correctly predict sensitivity class, and precision is the average difference between predicted and observed injury ratings. Successful predictions of sensitivity class were made in only six of 10 genotypes and the average difference of 2.05 on an injury scale of 0–4 was considered unacceptably high.

The model based on peroxidase banding patterns proved more accurate (eight of 10 correct predictions), as well as more precise (average difference, 0.49). This model predicted the exact observed mean injury rating for 471-U6 and was within 0.02 for Silver Queen. Both were sensitive and had identical banding patterns, suggesting that a characteristic profile may exist for these sensitive lines as well as others. Although NK6942 was ozone-sensitive, it was predicted to be intermediate in injury response to ozone. This apparent discrepancy may be due to this genotype's generally poor performance throughout all experiments. Despite efforts to reduce oxidation, leaf extracts from NK6942 occasionally exhibited phenolic browning. Although browned extracts were not used for analysis, in NK6942 there was apparently an undetermined problem in extraction and analysis procedures.

Many investigators have noted a correlation between disease resistance and peroxidase (13,24), and peroxidases have been postulated to play a role in air pollution tolerance (5,7,35). Increased peroxidase activity was implicated as a factor in the reduced response of a soybean cultivar resistant to ozone (6). Peroxidase may exert an influence on ozone tolerance through its role in univalent reduction of oxyradicals (12).

Concentrations of ambient oxidants ranging from 380 to 690 $\mu g/m^3$ (20–35 pphm) ozone caused injury to field-grown sweet corn in California (23). In North Carolina, visible injury and significant yield reduction in sensitive genotypes were reported at 100–200 $\mu g/m^3$ (5–10 pphm) ozone (17). At present, the Environmental Protection Agency maximum hourly concentration standard is 236 $\mu g/m^3$ (12 pphm) ozone (30). During the summer of 1980 this value was exceeded eight times at 15 Delaware monitoring stations operated by the State Division of Environmental Control. Symptoms resembling ozone injury were observed by the authors on several plant species, including sweet corn, on the University Farm at Newark, DE.

These data suggest the need for genotypes resistant to air pollutants. The most common technique for selection of pollution-resistant cultivars has been through ratings of visible foliar injury and yield loss assessments. The time and effort involved in these selection tests have limited plant breeders in selecting pollutant-resistant lines. Electrophoretic techniques such as those described here may provide a supplementary assay to distinguish between ozone resistant or sensitive cultivars.

TABLE 4. Coefficient of determination (R^2) and F values for Coomassie blue bands used as predictors of ozone injury to sweet corn in a Coomassie blue banding pattern regression model

Variable[a]	Distance from cathode (mm)	pI	Contribution to R^2	F
X_1	58	4.26	0.345	11.453
X_2	72	3.94	0.245	2.650
X_3	27	5.14	0.182	1.000
X_4	22	5.26	0.157	1.389
X_5	25	5.20	0.058	3.475
X_6	18	5.42	0.010	23.642
X_7	53	4.41	0.001	56.333

[a] Variables listed in order of insertion into the model.

TABLE 5. Ozone sensitivity of selected sweet corn (*Zea mays*) genotypes as predicted by a Coomassie blue general protein banding pattern regression model

Genotype	Mean injury rating[a,b]		Sensitivity class	
	Predicted	Observed	Predicted	Observed
471-U6	−1.20	3.44	Resistant	Sensitive
NK6942	7.69	3.11	Sensitive	Sensitive
Silver Queen	−1.59	2.94	Resistant	Sensitive
Bonanza	0.23	2.50	Resistant	Intermediate
NK51036	4.66	2.39	Sensitive	Intermediate
Butter and Sugar	1.16	2.28	Intermediate	Intermediate
Iochief	2.28	1.17	Intermediate	Intermediate
Fa32	0.41	0.41	Resistant	Resistant
2132B	0.45	0.44	Resistant	Resistant
453I	0.38	0.38	Resistant	Resistant

[a] $t = 0.50$, NS at 9 df.
[b] Average difference = 2.05.

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