

## Pathological and Physiological Identification of Race C of *Bipolaris maydis* in China

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### ABSTRACT

Wei, J.-K., Lui, K.-M., Chen, J.-P., Luo, P.-C., and Lee-Stadelmann, O. Y. 1988. Pathological and physiological identification of race C of *Bipolaris maydis* in China. *Phytopathology* 78:550-554.

Five of 116 isolates of *Bipolaris maydis* collected from diseased corn (*Zea mays*) leaves in 12 provinces in China induced significantly larger lesions on leaves of inbred lines of corn with cms-C than on leaves of the same lines with cms-T, cms-S, or normal (N) cytoplasm. The greater virulence to plants with cms-C was observed both in seedlings in the greenhouse and in adult plants inoculated in the field. The isolates with virulence specific to cms-C *Z. mays* are designated race C, a new race of *B. maydis*. Crude preparations of race C toxin were obtained by chloroform extraction from filtrates of 2-wk-old cultures of race C in Czapek's solution. Treatment of leaf sections with C-toxin at 1,000 µg/ml greatly increased the rate and total amount of electrolyte leakage from leaves of cms-C but not cms-T,

cms-S, or N cytoplasm. C toxin also significantly enhanced activity of phenylalanine ammonia-lyase in treated tissue of cms-C but not cms-T, whereas T toxin from race T enhanced activity in material of cms-T but not cms-C. Viscosity of cytoplasm as indicated by time of centrifugation required for displacement of chloroplasts in subepidermal cells of *Z. mays* coleoptiles was reduced in plants of cms-C by C-toxin and in plants of cms-T by T-toxin, but not vice versa. Thus, the evidence from several physiological and pathogenicity tests supports the designation of race C as a new race of *B. maydis*, which produces a toxin specifically active against *Zea mays* lines with cms-C.

*Additional keywords:* cytoplasmic male sterile inbred lines, cytoplasmic streaming, cytoplasmic viscosity.

### MATERIALS AND METHODS

Enhanced susceptibility of *Zea mays* lines with cms-T to isolates of *Bipolaris maydis* (Nisik.) Shoemaker (*Helminthosporium maydis*) was first observed in the Philippines in 1960 (4). Subsequently, it became apparent that the Philippine isolates of *B. maydis* were of a new race, designated race T (5). Later *B. maydis* race T was also observed in China (H8) and in the United States (1,5). Race T isolates produce a toxin specifically active only against corn with cms-T. No further cases of race specificity of *B. maydis* have been reported outside China since the discovery of race T.

Recently, while a large number of *B. maydis* samples from 12 provinces of China were being screened, isolates were found to which *Z. mays* with C cytoplasm was highly sensitive (3). This paper provides evidence that these isolates (represented by isolate 523) belong to a new pathotype both pathologically and physiologically, which should be defined as new race C.

**Plant material.** Seedlings of inbred line C103 in cms-T, cms-C, cms-S, and normal (N) cytoplasm were used in laboratory and greenhouse tests. Seedlings of inbred VA35 in cms-C and N cytoplasm were also used in the greenhouse test. Seeds were presoaked in tap water at room temperature for 48 hr, then sown in germination trays containing sterile soil and placed in a growth chamber at 25 C. Two-week-old plants were used for tests of cytoplasmic viscosity, toxin-induced electrolyte leakage, and virulence tests in the greenhouse. For determinations of the effects of toxin on phenylalanine ammonia-lyase, the soaked seeds were kept in rolls of moist paper in the dark, and etiolated, 1-wk-old seedlings were used. For experiments on root growth, inbred line C107 in cms-C and normal (N) cytoplasm were used. The soaked seeds were allowed to germinate at 25 C. The germinated seedlings with 0.5 mm primary roots were tested.

For field evaluation, seeds of inbred lines C103, B37, B73, and WF9 in cms-T, cms-C, cms-S and N cytoplasm were planted at Shijiazhuang, Hebei Province, China, on 14 May 1986. The plants

were sprayed with spore suspensions of race T and isolate 523 on 10 July 1986 when they were in the 9- to 10-leaf stage. Disease reactions were evaluated 3 wk after inoculation.

**Preparation of inoculum and inoculation procedure.** For preparation of the inoculum, slanted test tubes were one third filled with potato-dextrose agar (PDA) medium and inoculated from a stock culture of race T or isolate 523. After 1 wk, spores were washed from the cultures with 10 ml of sterile water per tube, and the spore suspension was filtered twice through two layers of cheesecloth. A drop of the filtered spore suspension was placed on a slide, and 10 microscopic fields (objective 10 $\times$ , eyepiece 10 $\times$ ) were examined for spore numbers. A suspension with a spore concentration corresponding to five to 10 spores per microscopic field was used for inoculation in the greenhouse. The inoculated seedlings were kept in a moisture chamber for 24 hr and then put on greenhouse bench at 25 C. The disease response was recorded 1 wk after inoculation by measuring the length and width (mm) of 10 lesions. Average values were calculated.

Plants in the field were inoculated by spraying with a spore concentration corresponding to five to 10 spores per microscopic field. Three weeks after inoculation, the lengths of 50 lesions were measured and the average value determined.

**Preparation of toxin.** Culture of *B. maydis* and extraction and preparation of toxin followed the procedure described by Wang and Xue (7). Race T and isolate 523 (virulent on plants with cms-C) were grown in still culture in Czapek's solution at 25 C. After 2 wk, cultures were filtered through nylon and then through four layers of filter papers. The culture filtrate was extracted in a separatory funnel with chloroform three times. The chloroform was removed by evaporating the extract over a water bath at 60–70 C. The brownish residue was weighed, dissolved in distilled water, and used as toxin.

**Activity of phenylalanine ammonia-lyase (PAL).** The method described by Wang and Xue (6) was used for testing the activity of PAL. Plants were treated with 200  $\mu\text{g/ml}$  of toxin of race T or isolate 523. Controls were treated with distilled water. Roots and leaf blades were cut off before toxin application, which lasted for 24 hr.

**Toxin-induced electrolyte leakage.** Second leaves of maize at the three-leaf stage were collected and 0.5 g of leaf tissue was washed three times with double distilled water and transversally cut into pieces of 0.5 cm width, which were placed into test tubes. To these, 10 ml of 1,000  $\mu\text{g/ml}$  of toxin (either race T or isolate 523) was added; double distilled water served as control. The tissues were infiltrated twice for 10 min each time. The conductivity of the solution was measured with a Wescon conductivity meter after 30 min in the test solution and subsequently at intervals of 1 hr for up to 21 hr. Finally, the tubes were placed in a water bath at 90 C for 30 min to kill the cells, after which the tubes were cooled and the final conductivity was determined. The time needed to reach 50% of final conductivity ( $t_{50}$ ) was determined for the graph (Fig. 1).

**Cytoplasmic viscosity of coleoptile subepidermal cells.** Cytoplasmic viscosity indicates the resistance of the protoplasm (ground plasm) against flow. Chloroplast displacement (Fig. 2A and B) by centrifugation can serve as relative measure for it (for references see 2). Seedlings at the two- to three-leaf stage (Fig. 2A and B) (root removed and shoot cut to 10 cm in length) were placed for 4 hr in 1,000  $\mu\text{g/ml}$  of toxin solutions in distilled water and subsequently centrifuged in "equilibrated water" (a mixture of equal volume of 3.7 mM  $\text{CaCl}_2$  + 25 mM KCl) at 2,500 rpm (swinging bucket rotor; radius = 18 cm) at 18 C. For each experiment, a series of individual runs was needed. Each run used a fresh seedling, and the centrifugation time was increased in subsequent runs in increments of 2 min (i.e., 2 min for the first run, 4 min for the second, 6 min for the third run, and so on). The experiments were repeated three times. The time of centrifugation needed for about 95% of chloroplasts to be accumulated at the centrifugal transversal cell wall was used as an indicator of the relative cytoplasmic viscosity in plant cells (Wei et al, unpublished).

**Root elongation.** Seeds with 0.5-cm long primary roots were placed on dishes and 10 ml of 523-toxin solution (treatment) or 10

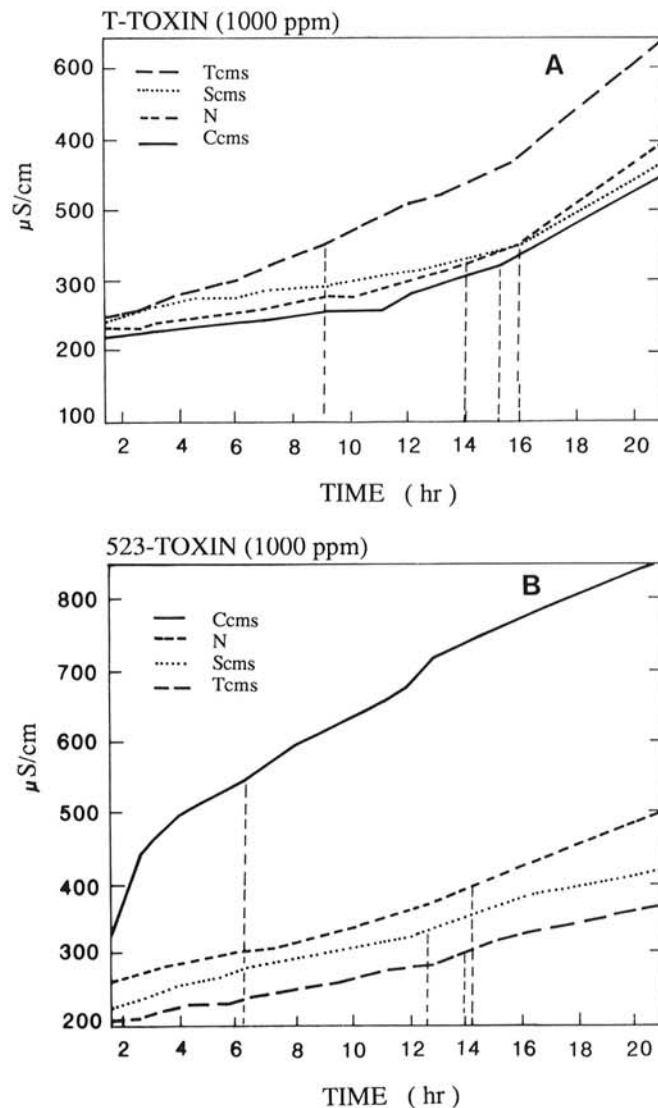
ml of distilled water (control) added. The dishes were placed into an incubator (25 C) for 90 hr. In each experiment, the length of the primary root was determined for 15 seedlings.

## RESULTS AND DISCUSSION

**Reaction of cms-C lines to *B. maydis*.** Liu et al (5) reported the existence of *B. maydis* that was pathogenic to the cms-C lines of maize from field observation in the principal maize-growing regions in China. Among 116 isolates of the pathogen from 12 provinces that were tested in their study, five had high virulence to lines with C but not high virulence on T, S, or N cytoplasm.

In our greenhouse experiments with these five isolates, the leaf lesions of C103 and VA35 plants with C cytoplasm were significantly longer than those on C103 and VA35 with N cytoplasm. Average lesion length was 5.0 mm on cms-C plants and 2.0 mm on N plants (Table 1). Both, cms-T lines infected by race T and cms-C lines infected by isolate 523, developed a wilting type of spot (Fig. 3A and B), while N-type lines when infected by any isolate of the pathogen developed necrotic lesions (Fig. 3C).

Race T induced significantly larger lesions on cms-T plants of C103, B37, B73, and WF9 inoculated in the field than on cms-C,



**Fig. 1. A,** Effect of T-toxin on electrolyte leakage from leaf tissues with different cytoplasm of *Zea mays*, genotype C103. Ordinate: conductivity. Vertical broken line indicates time ( $t_{50}$ ) for 50% of final conductivity. **B,** Effect of 523-toxin on electrolyte leakage from leaf tissues with different cytoplasm of *Z. mays*, genotype C103. Ordinate: conductivity. Vertical broken line indicates time ( $t_{50}$ ) for 50% of final conductivity.

cms-S, or N cytoplasm plants of these lines (Table 2). Race T lesions on cms-T lines were of the wilt type typical of a toxin-sensitive reaction. Isolate 523 induced wilt type lesions on cms-C plants of C103, B37, B73, and WF9 but not on cms-T, cms-S, or normal cytoplasm plants of those lines. The lesions of isolate 523 on all cms-C lines were significantly larger than those on lines of the other cytoplasm types (Table 2).

These observations indicate that race T and isolate 523 produced toxins specifically active on cms-T and cms-C lines, respectively, both in the adult stage in the field and in the seedling stage in the greenhouse. Therefore, we consider the pathotype that is specifically pathogenic to the cms-C line as a new type and designate it as race C. Additional evidence that race C is physiologically distinct is shown in the following tests.

**Activity of phenylalanine ammonia-lyase.** Wang and Xue (9)

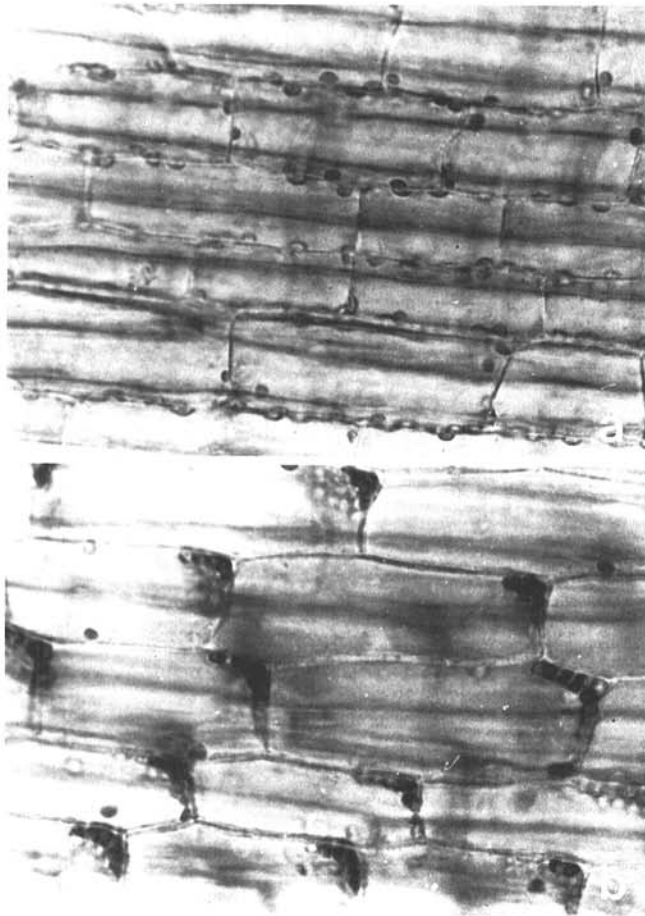


Fig. 2. A, Chloroplast distribution in subepidermal parenchyma cells of the leaf sheath of the first leaf of a *Zea mays* plant, 2 wk old. B, Same cells after centrifugation. Chloroplasts accumulated at the centrifugal transversal cell wall.

showed that T-toxin strongly stimulated activity of PAL of cms-T plants but had no evident effect on plants with other cytoplasm. In the present investigation, we also found that T-toxin stimulated the activity of PAL for plants with cms-T only but had no effect on the activity of PAL in the other three cytoplasm (Table 3). The difference for the T cytoplasm between the control and the treatment was highly significant. Furthermore, the toxin produced by isolate 523 stimulated PAL of C cytoplasm only (Table 3). The difference between the control and toxin treatment was highly significant.

**Electrolyte leakage.** The effect of toxin on the efflux of electrolyte from leaf tissues of maize is shown in Figure 1A and B.

The time required for C103 cms-T after treatment with 1,000  $\mu\text{g/ml}$  of T-toxin to reach 50% of the final conductivity ( $t_{50}$ ) was 9 hr, while for the C103 cms-C, C103 cms-S, and C103 this time was 14–16 hr (Fig. 1A).

After 9 hr of treatment, the conductivity of the C103 cms-T was 350  $\mu\text{S/cm}$ , but conductivity was below 292  $\mu\text{S/cm}$  for the other three cytoplasm. After 21 hr of treatment, the conductivity of C103 cms-T increased to 643  $\mu\text{S/cm}$ , while it remained below 491  $\mu\text{S/cm}$  for the other cytoplasm lines. The difference was highly significant, indicating the specificity of T-toxin in inducing electrolyte efflux from leaves of cms-T.

The time  $t_{50}$  for C103 cms-C treated with 1,000  $\mu\text{g/ml}$  of toxin from isolate 523 was only slightly more than 6 hr, while it was between 12.5–14.4 hr for C103 cms-T, C103 cms-S, and C103 (Fig. 1B). After 6 hr of treatment with the toxin of isolate 523, the conductivity of C103 cms-C increased to 537  $\mu\text{S/cm}$ , while those of

TABLE 1. The reaction of *Zea mays* with cms-C or normal (N) cytoplasm to six isolates of *Bipolaris maydis* in China

Source of pathogen	Isolate no.	Host genotype	Cytoplasm	Reactions	Longest length of spot (mm)
Yan shan, Hebei	356	C103	C	S <sup>a</sup>	3.4* <sup>b</sup>
			N	R	1.3
Tianjin	627	C103	C	S	2.2*
			N	R	0.5
Zun Hua Hebei	156	VA35	C	S	5.5*
			N	R	2.0
Yi County Hebei	353	VA35	C	S	4.7*
			N	R	1.7
Ya An, Sichuan	523	VA35	C	S	9.0*
			N	R	1.4
Average			C	S	5.0*
			N	R	2.0

<sup>a</sup>S susceptible, R resistant.

<sup>b</sup>Significantly different at  $P = 0.01$  from spot length with N cytoplasm.

TABLE 2. The reactions of inbred lines of *Zea mays* with different male-sterile cytoplasm to inoculation with *Bipolaris maydis* race C and T in the field

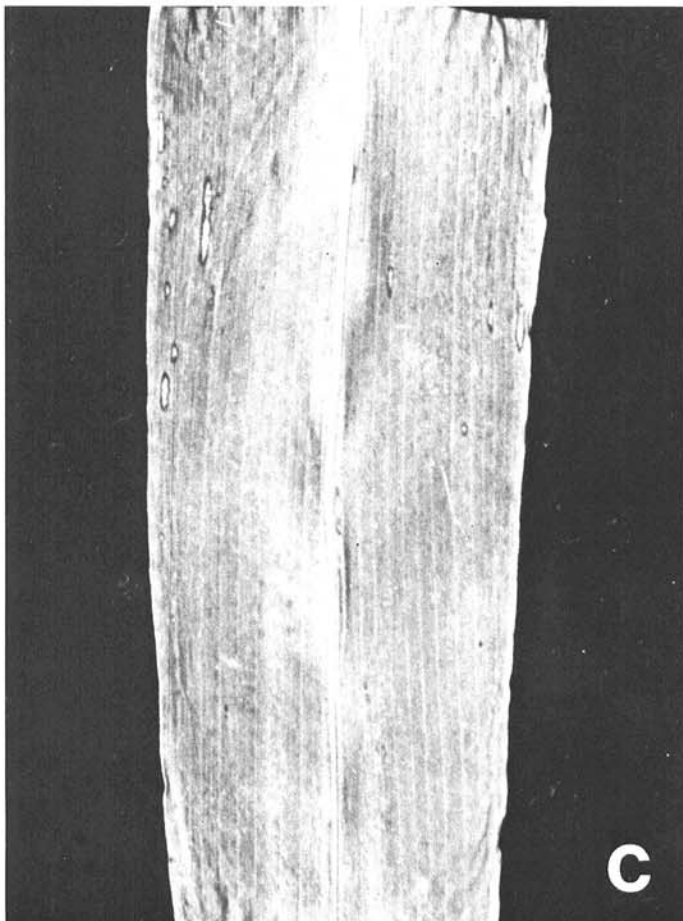
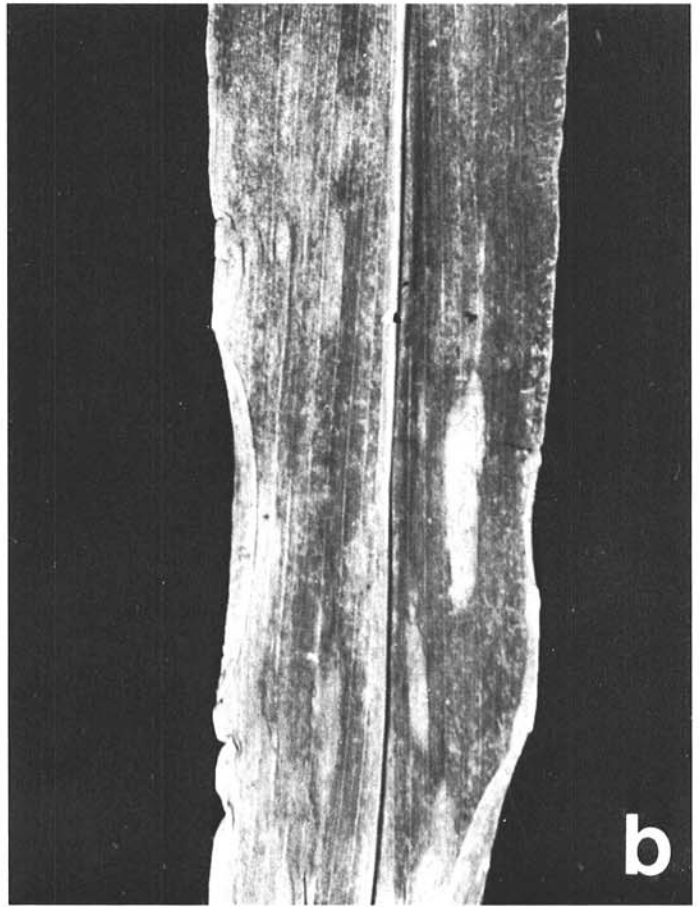
Genotypes	Lesion length (mm)							
	Race C				Race T			
	T <sup>a</sup>	C <sup>b</sup>	S <sup>a</sup>	N <sup>a</sup>	T <sup>c</sup>	C	S	N
C103	13.2 ± 4.9 <sup>d</sup>	6.2 ± 2.3	4.8 ± 1.7	2.9 ± 2.0	6.2 ± 2.7	9.8 ± 4.4 <sup>d</sup>	4.8 ± 1.7	4.0 ± 1.6
B73	19.5 ± 7.4 <sup>d</sup>	7.5 ± 2.1	8.6 ± 2.7	5.8 ± 2.4	5.7 ± 2.1	13.5 ± 3.5 <sup>d</sup>	6.0 ± 2.7	7.3 ± 2.4
B37	12.1 ± 4.6 <sup>d</sup>	6.5 ± 2.3	8.7 ± 2.6	7.5 ± 2.8	8.4 ± 3.2	11.8 ± 5.7 <sup>d</sup>	5.7 ± 1.7	6.7 ± 2.1
WF9	14.2 ± 3.1 <sup>d</sup>	8.1 ± 3.3	7.6 ± 1.9	6.9 ± 2.3	6.9 ± 3.2	13.2 ± 3.4 <sup>d</sup>	7.4 ± 2.3	7.0 ± 2.1

<sup>a</sup>Lesions induced by race C on *Z. may* with T, S, and N cytoplasm were necrotic with no wilting.

<sup>b</sup>Lesions on lines with C cytoplasm display the wilt reaction. On C103 the wilted area extended beyond the necrotic lesion to a distance greater than 1 times the lesion diameter.

<sup>c</sup>Lesions on T cytoplasm displayed a wilting reaction. The wilted area extended from 1/2 to 1 times the lesion diameter.

<sup>d</sup>Significantly greater than lesions on normal cytoplasm ( $P = 0.01$ ).



**Fig. 3.** **A,** Leaf of a plant with cms-T cytoplasm, infected by race T of *Bipolaris maydis*. **B,** Leaf of a plant with cms-C cytoplasm, infected by isolate 523 of *B. maydis*. **C,** Leaf of a plant with normal cytoplasm, infected by *B. maydis* race T or isolate 523.

the other three cytoplasm lines were all below  $304 \mu\text{S}/\text{cm}$ . After 21 hr of treatment, the value of conductivity of C103 cms-C was  $851 \mu\text{S}/\text{cm}$ , compared with less than  $495 \mu\text{S}/\text{cm}$  of the other three lines. The difference was highly significant. These results clearly indicate the specificity of the toxin of isolate 523 in inducing the efflux of electrolytes from leaves of cms-C. It seems, therefore, reasonable to consider the toxin produced by isolate 523 as C-toxin.

**Cytoplasmic viscosity.** Our earlier experimental results (Wei et al, *unpublished*) showed that T and C cytoplasm have distinctly lower cytoplasmic viscosity than N and S cytoplasm. The centrifugation times for 95% accumulation of chloroplasts were 13, 15, 21, and 30 min in C103 cms-T, C103 cms-C, C103 cms-S, and C103, respectively. This agrees with the results in earlier studies (Wei et al, *unpublished*). If the cms-T coleoptiles were presoaked 4 hr in T-toxin or cms-C coleoptiles in toxin of isolate 523 (each  $1,000 \mu\text{g}/\text{ml}$ ) in "equilibrated water," the centrifugation time for 95% accumulation of chloroplasts (Fig. 2B) was shortened by 2–4 min (Fig. 4). No appreciable difference in the degree of cytoplasmic hydration can be expected between these CMS races. The viscosity differences, therefore, seem to indicate differences in the concentration and/or structure of macromolecules and other highly hydrated molecules (cf. 2).

**Root elongation.** The 523-toxin specifically inhibited the elongation of the primary root of cms-C plants only; while root length of cms-N and cms-C seedling in water was 7.5 and 7.9 cm, respectively, in C-toxin ( $1,000 \mu\text{g}/\text{ml}$ , 90 hr of treatment) the cms-C root was only 0.5 cm long compared with 4.3 cm for the cms-N seedling.

TABLE 3. The stimulating effect on the activity of phenylalanine ammonia-lyase (PAL) by the toxin produced by race T and the isolate 523 of *Bipolaris maydis*

	PAL activity <sup>a</sup> (Genotype C103)							
	T		C		S		N	
	Control	Toxin	Control	Toxin	Control	Toxin	Control	Toxin
Toxin <sup>b</sup> of isolate 523	1.395	1.480	0.901	3.350 <sup>c</sup>	0.895	0.905	0.355	0.530
Toxin <sup>b</sup> of race T	1.395	3.565 <sup>c</sup>	0.901	1.080	0.895	0.940	0.355	0.380

<sup>a</sup>Numbers indicate absorbance at 290 nm for a 1-cm cuvette. The data were normalized to 1 g fresh weight and 1-hr exposure to the toxin.

<sup>b</sup>Treated 24 hr, 200 µg/ml.

<sup>c</sup>Highly significant at  $P = 0.01$  between control and toxin treatment.

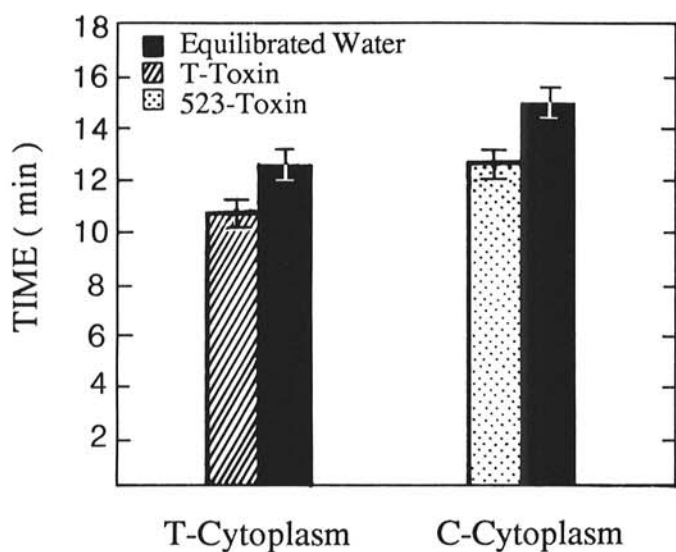


Fig. 4. Viscosity of the cytoplasm expressed as time required for 95–100% chloroplast displacement after 4 hr of treatment with T-toxin and 523-toxin. Bar indicates standard deviation.

### CONCLUSION

The evidence from all of the pathological and physiological tests in this study supports the proposal that isolate 523 is a new pathotype that it be designated as race C of *B.maydis* and the toxin be called C-toxin.

In earlier experiments (Wei et al, unpublished), specific protoplasmic parameters (water permeability, cytoplasmic viscosity, and cytoplasmic streaming) were tested. Those of C-cytoplasm were always found to be closer to those of T-cytoplasm than to other CMS types. The catastrophe of 1970 in production of *Z. mays* in the United States occurred because most hybrids carried the T-cytoplasm. Therefore, it became imperative to examine the

possibility that there exists a *B. maydis* race for which the C-cytoplasm of *Z. mays* have a similarly high degree of susceptibility.

The above results indicate that such a race exists in China. The catastrophic situation of 1970, therefore, may repeat itself if production of *Z. mays* were to be based on C-cytoplasm. In making use of the different cytoplasm of male-sterile corn, it is, however, possible to select new resistant variants and "multiplasms," which can be used for optional breeding.

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