Effect of Sodium Bisulfite on Peroxidase Activity and Electrolyte Leakage in Maize in Relation to Sporulation of *Bipolaris maydis* Race T[1]

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ABSTRACT. **In this study, we sought to determine whether the increases in peroxidase activity and electrolyte leakage induced in maize (*Zea mays* L.) leaves by sodium bisulfite were causally related to the sodium bisulfite-induced increases in sporulation of the pathogen *Bipolaris maydis* race T on infected maize leaves. Pretreatment of detached leaves of maize inbred W64 A with sodium bisulfite (500 µg/ml) for 24 h in the dark at 28°C increased peroxidase activity in the Tms cytoplasm (susceptible) isoline compared with the N cytoplasm (resistant) isoline. No such differences in peroxidase activity between the two isolines were observed when detached leaves were pretreated with double distilled water. The sodium bisulfite-induced increase in peroxidase activity persisted even when leaves pretreated with sodium bisulfite were inoculated with *B. maydis* race T and subsequently incubated for 48 h in the dark at 28° C. Similarly, pretreatment with sodium bisulfite caused a greater increase in electrolyte leakage as well as in sporulation on the leaves of the susceptible than on those of the resistant isoline when compared with leaves not treated with sodium bisulfite. Sodium bisulfite showed no effect on sporulation in vitro. Leachates from the susceptible isoline pretreated with sodium bisulfite also caused greater increase in sporulation than those from the resistant isoline pretreated with sodium bisulfite.**

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INTRODUCTION

Different kinds of stresses may induce a state of resistance or susceptibility of plants against pathogens depending upon the host-pathogen interactions (Byther and Steiner 1975, Chamberlain 1972, Daly et al. 1970, Stahmann et al. 1966). For example, chemicals such as ethylene have been shown to induce resistance of sweet potato to *Ceratocystis fimbriata* (Stahmann et al. 1966) and susceptibility of wheat to *Puccinia graminis* f. sp. *tritici* (Daly et al. 1970). In the above interactions, the possible involvement of peroxidase has been proposed, based on the role of peroxidase in several physiological processes (Gasper et al. 1982), including disease resistance (Hammerschmidt et al. 1982, Urs and Dunleavy 1974). Results, however, are not consistent. For example, ethylene-induced resistance of sweet potato to *C. fimbriata* as well as susceptibility of wheat to *P. graminis* f. sp. *tritici* were accompanied by an increase in peroxidase activity in either case. Thus, the role of increased peroxidase activity in either resistance or susceptibility is an open question.

Chemicals and other stresses such as high temperature could alter the resistance or susceptibility of plants to infection through their effects on membrane permeability. It is known that ethylene affects membrane permeability (Abrams and Pratt 1967, Goodman et al. 1986). Similarly, high temperature stress could induce susceptibility in maize through its effect on membrane permeability as measured by increased electrolyte leakage (Garraway et al. 1989). This might result in the loss of host cells' constituents which might be used by an invading pathogen as a source of nutrients. Thus, chemicals such as ethylene might function like other abiotic (Rist and Lorbeer 1984) or biotic (Stevens and Gudauskas 1982) stresses predisposing plants to disease.

In addition to the above literature, we have conducted preliminary studies which indicate that exposure of maize (*Zea mays* L.) leaves to chemicals such as the reducing agent sodium bisulfite prior to inoculation with a fungal pathogen *Bipolaris maydis* race T appears to increase the severity of infection and disease (Akhtar and Garraway 1988, 1989). We, therefore, sought in the present study to define the relationship, if any, of peroxidase activity and electrolyte leakage to the above phenomenon. Specific objectives were to determine: 1) the effect of sodium bisulfite on peroxidase activity and electrolyte leakage in two isolines of maize which differ in their degree of susceptibility to *B. maydis* race T; and 2) the relationship of sodium bisulfite-induced peroxidase activity and electrolyte leakage with sporulation of *B. maydis* race T on infected leaves of these isolines.

MATERIALS AND METHODS

HOST: Two isolines of the maize (*Zea mays* L.) inbred W64 A, i.e., normal (N) cytoplasm and Texas male sterile (Tms) cytoplasm were grown in the greenhouse as previously described (Birecka et al. 1975, Birecka and Garraway 1978). The fungal pathogen used in this study was *Bipolaris maydis* (Nisikado) Shoemaker (syn. *Helminthosporium maydis* Nisikado and Miyke, perfect stage *Cochliobolus heterostrophus* Drechlser) race T. Leaf samples of comparable age from 3-wk-old plants were detached from each isoline, washed with double distilled water, cut into pieces of about 5 x 2 cm in size, then placed on a sheet of Whatman No. 3 filter paper. Leaves thus prepared were floated either on 5 ml of double distilled water (control) or on 5 ml of an aqueous solution of sodium bisulfite (250, 500 and 1,000 µg/ml, J.A. Baker Chemical Company, Phillipsburg, NJ) for 24 h in the dark

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at 28° C in a water-vapor saturated incubator. Sodium bisulfite-treated and control leaves were then inoculated with a *B. maydis* race T spore suspension (10,000-15,000 conidia/ml) in double distilled water containing Tween-20 (50 µl/100 ml) as a surfactant. They were then incubated for 48 h in the dark at 28° C. Previous studies had indicated that this incubation time (48 h) was optimum for fungal colonization of the tissues. As the sensitivity of the pathotoxin produced by this fungus appeared to be higher in the dark than in the light, all incubations were carried out in the dark. The spore suspension used as inoculum was prepared from cultures grown on glucose-L-asparagine agar medium for 7 days at 28° C (Garraway 1973b).

Pathogen: A single spore isolate of *B. maydis* race T collected from a maize seed grown in Franklin County, OH, in 1970 (ATCC # 36180) was cultured on a glucose-L-asparagine agar medium in the dark at 28° C for 7 days as previously described (Garraway 1973b). Results obtained with this isolate in our previous preliminary studies were similar to those with other isolates of race T.

Determination of peroxidase activity: Procedures for extraction of peroxidase (donor: oxidoreductase: E.C. 1.11.1.7) were similar to those used previously (Birecka et al. 1975, Birecka and Garraway 1978, Garraway 1973a). The tissue was homogenized in 5 ml of 10 mM sodium phosphate buffer (pH 6) using a Brinkmann polytron homogenizer. The resulting homogenate was centrifuged at 20,000 g for 5 min at 4° C. The supernatant was assayed for the buffer-extractable, or soluble peroxidase activity. To recover salt-extractable, or ionically bound peroxidase activity, the washed pellet was resuspended in 5 ml of NaCl in 10 mM phosphate buffer (pH 6), stirred at 4° C for 1 h, and centrifuged at 20,000 g for 5 min at 4° C. The supernatant was then assayed. Our data are based solely on the activity of the soluble and ionically bound peroxidase fractions, as they constitute about 90% of the total peroxidase activity in maize and are recovered with ease and speed. Cadena-Gomez and Nicholson (1987), using the same method for peroxidase extraction in maize, have also recovered about 93% of the total peroxidase activity. The reaction mixture for the peroxidase assay included 200 µl of enzyme solution, 1300 µl of sodium phosphate buffer (10 mM pH 6), 500 µl of 50 mM guaiacol and 100 µl of 100 mM H_2O_2. One unit of the peroxidase activity has been defined as the change of 1.0 absorbance unit at 470 nm per minute per gram fresh weight of leaf tissues.

Determination of electrolyte leakage: Detached leaves were floated either on double distilled water or on an aqueous solution of sodium bisulfite (500 µg/ml) for 24 h in the dark at 28° C, inoculated with *B. maydis* race T as described above, then incubated in the dark for 24 h at 28° C. These leaves were cut into 3 cm sections, then washed with double distilled water. Each leaf section was immersed in 25 ml of sterile double distilled water in screw cap vials (40 ml-capacity). They were further incubated in the dark for 24 h at 28° C. To determine the quantity of electrolyte leakage, the change in conductance of the double distilled water with immersed leaf sections was measured at 0 and 24 h using a conductivity bridge (model 4959) and a conductivity cell (k=0.01, Leeds and Northrup Co., Philadelphia, PA). The amount of electrolyte leakage is expressed as micromhos (µ mhos) per milligram dry weight of leaf tissue.

Determination of sporulation on infected maize leaves: Detached leaves of both isolines were floated either on double distilled water or on an aqueous solution of sodium bisulfite (500 µg/ml) for 24 h in the dark at 28° C and inoculated with *B. maydis* race T as described above. They were then incubated in the dark for 48 h at 28° C in a water-vapor saturated incubator as reported previously (Birecka et al. 1975). At the end of the incubation period, infected leaf sections were placed in screw cap vials (15 ml-capacity) containing 3 ml of preservative solution (5% colorox, 20% ethanol and 2% NaOH) to inactivate the conidia. These vials were then agitated to dislodge conidia from the leaf surface. Conidium concentrations were determined with a hemacytometer as described previously (Garraway 1973b). Results were expressed as the number of *B. maydis* race T conidia produced per milligram dry weight of leaf tissues.

Determination of sporulation in vitro: To determine the effect of an aqueous solution of sodium bisulfite (500 µg/ml) on sporulation and mycelial dry weight of *B. maydis* race T in vitro, standard glucose-L-asparagine agar medium was prepared. Then this medium was either non-amended (double distilled water control) or amended with an aqueous solution of sodium bisulfite (500 µg/ml). Sporulation and mycelial dry weight were measured after 7 days of incubation in the dark at 28° C.

To study the effect of leachates on *B. maydis* race T sporulation, detached leaves of both isolines were floated either on double distilled water or on an aqueous solution of sodium bisulfite (500 µg/ml) for 24 h in the dark at 28° C, inoculated with B. *maydis* race T as described above and then incubated in the dark for 24 h at 28° C. The leaves were cut into 3 cm sections, rinsed and immersed in 50 ml of double distilled water in a beaker. After 12 h immersion, double distilled water containing leachates was autoclaved at 121° C for 20 min at 15 PSI and then used to constitute 20 ml of 2% agar media. This experimental media either non-amended (water agar) or amended with double distilled water containing leachates (leachates agar) was seeded with 0.5 ml of a sterile *B. maydis* race T spore suspension (30,000-40,000 conidia/ml). The conidia were uniformly distributed on the surface of each plate with a sterile spreader. Seeded plates were incubated in the dark at 28° C for 7 days. Conidia were collected from the culture by scraping and washing with 1 ml preservative solution and the number per ml was determined. Sporulation was expressed as conidia per milligram dry weight of fungus. Inoculation procedures, media preparation and sporulation, conidia and mycelial measurements have been described previously (Garraway 1973b).

All experiments involving peroxidase, electrolyte leakage, sporulation in vivo and in vitro were repeated three times with five replicates in each.

RESULTS

Effect of sodium bisulfite on peroxidase activity: When detached leaves of maize isolines were floated on an aqueous solution of sodium bisulfite for 24 h, sodium

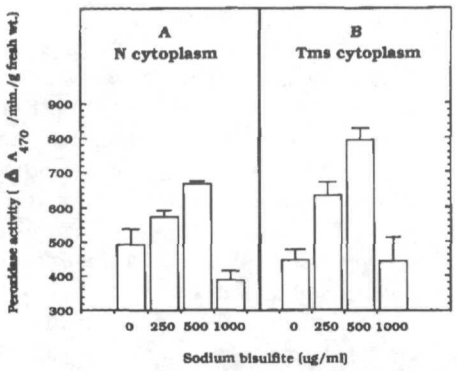

FIGURE 1. Peroxidase activity in detached leaves of normal (N) cytoplasm (A) and Texas male sterile (Tms) cytoplasm (B) maize isolines floated on different concentrations of sodium bisulfite for 24 h in the dark at 28° C. Peroxidase activity in leaves of the N and Tms cytoplasm isolines immediately after detachment (O h) was 230 ± 32 (SE) and 252 ± 47 (SE), respectively. The vertical lines represent standard error of the mean.

bisulfite at concentrations of 250 or 500 µg/ml significantly (P=0.05) enhanced the enzyme activity in both isolines compared with the double distilled water control. The 500 µg/ml concentration was found to be optimum (Fig. 1). A sodium bisulfite concentration of 500 µg/ml was therefore used in the follow-up time course experiments.

When detached leaves of both isolines were exposed to sodium bisulfite for 6, 12, or 24 h, peroxidase activity significantly (P=0.05) increased in both isolines only when detached leaves were floated on sodium bisulfite for a period of 24 h (Fig. 2). Interestingly, this increase in peroxidase activity was greater in the Tms cytoplasm isoline than in the N cytoplasm isoline, while control leaves of both isolines (leaves not floated on sodium bisulfite) showed no differences in peroxidase activity. When leaves with differential peroxidase activity were inoculated and then incubated for 48 h, the Tms cytoplasm isoline again showed a significant (P=0.05) increase in peroxidase activity compared with the N cytoplasm isoline in response to a prior exposure to sodium bisulfite (Fig. 3). Again, control leaves of both isolines showed similar levels of peroxidase activity (Fig. 3).

EFFECT OF SODIUM BISULFITE ON ELECTROLYTE LEAKAGE: Regardless of whether detached leaves were floated on

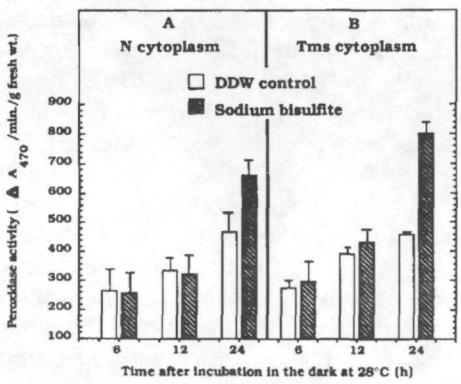

FIGURE 2. Time course changes in peroxidase activity in detached leaves of normal (N) cytoplasm (A) and Texas male sterile (Tms) cytoplasm (B) maize isolines floated either on double distilled water (control) or on sodium bisulfite solution (500 µg/ml) for 6, 12, or 24 h in the dark at 28° C. Peroxidase activity in leaves of the N and Tms cytoplasm isolines immediately after detachment (O h) was 230 ± 32 (SE) and 252 ± 47 (SE), respectively. The vertical lines represent standard error of the mean.

double distilled water (control) or on sodium bisulfite prior to inoculation, electrolyte leakage was significantly (P=0.05) greater from the leaves of the Tms cytoplasm isoline than those from the N cytoplasm isoline. The magnitude of electrolyte leakage was, however, much

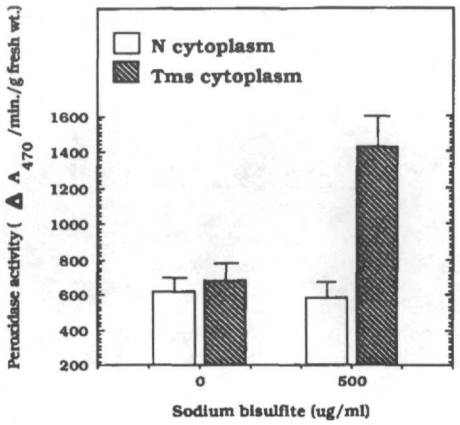

FIGURE 3. Differential changes in peroxidase activity between the detached leaves of normal (N) cytoplasm and Texas male sterile (Tms) cytoplasm maize isolines floated either on double distilled water (control) or on sodium bisulfite solution (500 µg/ml) for 24 h in the dark at 28° C, inoculated with 10,000-15,000 conidia/ml of *B. maydis* race T and then incubated for 48 h in the dark at 28° C. Peroxidase activity in leaves of the N cytoplasm and Tms cytoplasm isolines immediately after detachment (O h) was 230 ± 32 (SE) and 252 ± 47 (SE), respectively. The vertical lines represent standard error of the mean.

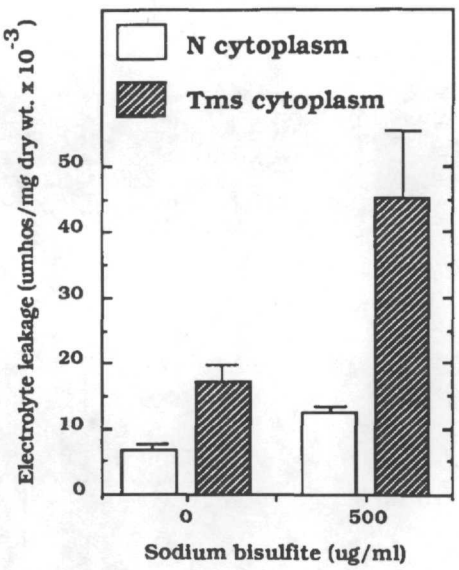

FIGURE 4. Electrolyte leakage from the detached leaves of normal (N) and Texas male sterile (Tms) cytoplasm maize isolines floated either on double distilled water (control) or on sodium bisulfite solution (500 µg/ml) for 24 h in the dark at 28° C, inoculated with 10,000-15,000 conidia/ml of *B. maydis* race T, then incubated for 24 h in the dark at 28° C. Such leaves were cut into 3 cm sections and then floated on 25 ml of double distilled water for 24 h in the dark at 28° C. The vertical lines represent standard error of the mean.

enhanced for both isolines by a prior exposure to sodium bisulfite (Fig. 4).

EFFECT OF SODIUM BISULFITE ON SPORULATION *B. MAYDIS* RACE T IN VIVO: Since prior exposure to sodium bisulfite significantly increased peroxidase activity in the Tms cytoplasm isoline compared with the N cytoplasm isoline in

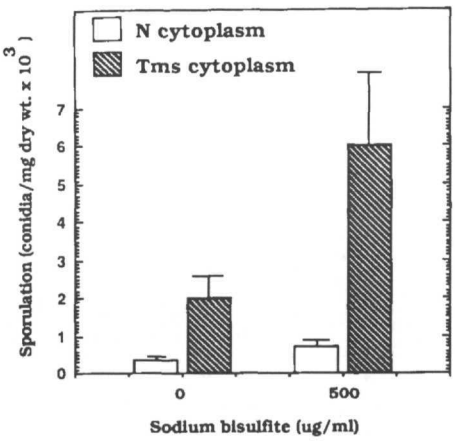

FIGURE 5. Sporulation of *B. maydis* race T on leaves of the normal (N) and Texas male sterile (Tms) cytoplasm maize isolines floated either on double distilled water (control) or on sodium bisulfite solution (500 µg/ml) for 24 h in the dark at 28° C, inoculated with 10,000-15,000 conidia/ml of *B. maydis* race T, then incubated for 48 h in the dark at 28° C. The vertical lines represent standard error of the mean.

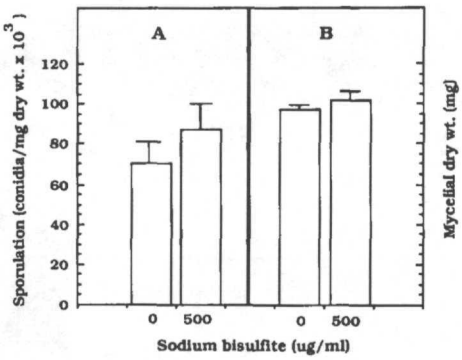

FIGURE 6. Sporulation (A) and mycelial dry wt. (B) of *B. maydis* race T after 7 days of incubation in the dark at 28° C on a standard glucose-L-asparagine agar medium either non-amended (double distilled water) or amended with sodium bisulfite solution (500 µg/ml). The vertical lines represent standard error of the mean.

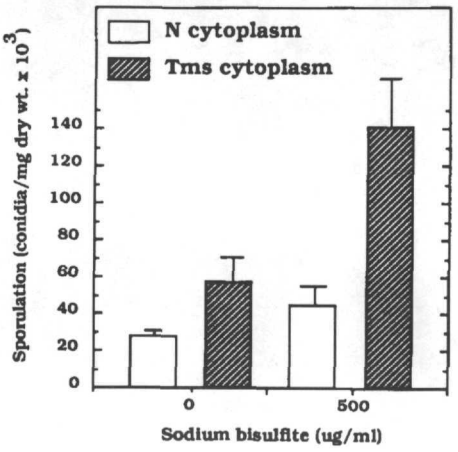

FIGURE 7. Sporulation of *B. maydis* race T after 7 days of incubation in the dark at 28° C on medium either non-amended (water agar) or amended with leachates from the normal (N) and from the Texas male sterile (Tms) cytoplasm maize isolines floated either on double distilled water (control) or on sodium bisulfite solution (500 µg/ml) for 24 h in the dark at 28° C, inoculated with 30,000-40,000 conidia/ml of *B. maydis* race T, then incubated for 24 h in the dark at 28° C. Sporulation on water agar medium was 18 ± 3 (SE) conidia/mg dry wt. x 10^3. Leachates were prepared as described in the Materials and Methods section. The vertical lines represent standard error of the mean.

the present study, a follow-up study was undertaken to compare the effect of a sodium bisulfite pretreatment on sporulation of *B. maydis* race T on leaves of both isolines. This study was motivated by the assumption that an increase in peroxidase activity in the Tms cytoplasm isoline (in response to sodium bisulfite) might alter the level of development of the pathogen in vivo. The Tms cytoplasm isoline showed greater sporulation over that seen on the N cytoplasm isoline when leaves were floated on either double distilled water or sodium bisulfite prior to inoculation. However, the sporulation was much enhanced by a prior exposure to sodium bisulfite (Fig. 5). This phenomenon prompted the following question: Is the stimulation in sporulation by sodium bisulfite in vivo a direct effect on the fungus or does sodium bisulfite induce changes in the peroxidase activity in the host?

EFFECT OF SODIUM BISULFITE ON SPORULATION AND MYCELIAL DRY WEIGHT OF *B. MAYDIS* RACE T IN VITRO: To address the first part of the above question, effect of sodium bisulfite on sporulation and mycelial dry weight was observed in vitro. When the fungus was grown on a glucose-L-asparagine agar medium which was either non-amended or amended with sodium bisulfite, after 7 days of incubation, sporulation and mycelial dry weight were comparable on either sodium bisulfite amended or non-amended medium (Fig. 6). These data suggest that the concentration of sodium bisulfite, which increased peroxidase activity differentially in the two isolines of maize, had no direct effect on sporulation and mycelial dry weight of the fungus.

EFFECT OF LEACHATES ON SPORULATION OF *B. MAYDIS* RACE T: The above results indicate a relationship of peroxidase activity and electrolyte leakage with the sporulation in vivo in response to a prior sodium bisulfite exposure. Since electrolytes contain constituents needed by *B. maydis* race T for its growth and development, we also examined their effect on sporulation in vitro. Sterilized leachates from the Tms cytoplasm isoline, floated on double distilled water (control) prior to inoculation, significantly (P=0.05) increased sporulation in vitro compared with the leachates from the N cytoplasm isoline. This increased sporulation in vitro was further enhanced when leachates from the leaves floated on sodium bisulfite were used (Fig. 7). The magnitude of increase in electrolyte leakage in response to prior exposure to sodium bisulfite appeared to be similar to the magnitude of increase in sporulation on media amended with leachates from sodium bisulfite-treated tissues.

DISCUSSION

The results of the present study suggested that peroxidase activity as well as sporulation in vivo concurrently increased in response to sodium bisulfite. Follow-up in vitro studies indicated that sodium bisulfite alone had no direct effect on sporulation. Previously, we demonstrated that both maize leaf extracts containing peroxidase activity and commercial peroxidase having enzyme activity equal to that present in maize leaf extracts increased in vitro sporulation to the same extent (Garraway et al. 1989). Thus, we concluded that sodium bisulfite-enhanced peroxidase activity may be involved in the in-

creased sporulation on infected maize leaves. These data also confirmed our previous findings where increased peroxidase activity caused by two other chemicals, i.e. 1-aminocyclopropane-1-carboxylic acid or methionine, appeared to be associated with increased sporulation (Akhtar and Garraway 1988). Daly et al. (1970) have shown that ethylene-induced peroxidase activity was also associated with increased sporulation of wheat rust. Thus, sodium bisulfite and other chemicals which increase peroxidase activity might also cause an increase in sporulation.

Electrolyte leakage from the two isolines used in this study indicated a difference in their degree of membrane sensitivity to the host-specific pathotoxin produced by *B. maydis* race T (Mertz and Arntzen 1977). Because of these differences, we also examined the effect of sodium bisulfite on electrolyte leakage. In the present study, exposure of maize leaves to sodium bisulfite prior to inoculation caused a significant amount of electrolyte leakage from both isolines; this leakage was even more acute from the susceptible isoline. Moreover, the magnitude of electrolyte leakage from the susceptible isoline appeared to be similar to the magnitude of sporulation in vivo. The observation that leachates from the susceptible isoline exposed to sodium bisulfite prior to inoculation produce similar increases in vitro to those seen in vivo supports the idea that the increased sporulation on sodium bisulfite-treated leaves may result from an increase in availability of nutrients to the pathogen. These findings are consistent with our previous studies (Garraway 1973a, Garraway and Evans 1977, Garraway and Evans 1984) and also with those of others who have established a relationship between electrolyte leakage and other parameters such as germ tube development and appresoria formation (Stevans and Gudauskas 1982) or rate of lesion development (Rist and Lorbeer 1984) on infected plants exposed to abiotic or biotic stresses. Moreover, our recent studies indicated that when sodium bisulfite was substituted for high temperature stress, similar results were obtained (Garraway et al. 1989). Thus, electrolyte leakage may be part of a more general response to stress, with the nutrients present in leachates playing a key role in the in vivo growth and development of the pathogen.

In the present study, the susceptible isoline (Tms cytoplasm) showed a greater increase in electrolyte leakage than the resistant isoline (N cytoplasm) when floated on double distilled water (Fig. 4). This is compatible with the observations of others that the membranes of the Tms cytoplasm isoline are more easily altered by the host-specific pathotoxin (HmT-toxin) produced by *B. maydis* race T (Mertz and Arntzen 1977, Miller and Koeppe 1971) as stated above. Interestingly, the difference in electrolyte leakage between the two isolines was much greater in response to a prior exposure to sodium bisulfite (Fig. 4). We think that sodium bisulfite increased electrolyte leakage either by its direct effect on membrane permeability or that sodium bisulfite-induced peroxidase might have altered the membrane permeability. There are reports that phenoloxidizing enzymes affect membrane permeability (Cory 1967), and in fact, our current unpublished data show that infiltration of maize leaves with purified horseradish peroxidase increases membrane permeability (as indicated by increased electrolyte leakage). A third possibility also exists — that sodium bisulfite pretreatment might have enhanced the synthesis or effectiveness of small molecular weight polypeptides (13 kD) recently reported in the Tms cytoplasm isoline of maize (Dewey et al. 1988). These peptides might alter membrane permeability, and thus facilitate greater electrolyte leakage from the leaves of the Tms cytoplasm isoline than those from the N cytoplasm isoline. Our current research goals are to test these hypotheses experimentally. These findings have great significance for the influence of sodium bisulfite on the establishment of *B. maydis* rate T on infected maize because this pathogen is a necrotroph (Heath 1987), with growth and development dependent upon metabolites released from the host cells. Previously, we demonstrated that high temperature stress affects the establishment of this pathogen on infected maize leaves through increased electrolyte leakage (Garraway et al. 1989). Thus, we believe that any kind of stress under field conditions that causes release of host cells' constituents would probably cause an increase in the severity of infection and disease.

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