Effects of Helminthosporium maydis T-toxin on the Uptake of Uranyl Salts in Corn Roots

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ABSTRACT

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Bioassays of *Helminthosporium maydis* T-toxin based on root growth inhibition, electrolyte loss, and inhibition of transpiration were compared. With four corn inbred lines, those carrying Texas male sterile (Tms) cytoplasm could be separated readily from those with normal (N) cytoplasm by each of these bioassays. The root growth test, however, was more sensitive by at least one order of magnitude than the other two. More than 50 chemical and physical agents were screened but, other than HmT-toxin, none had clear-cut selective effects on Tms inbred lines. As a post-treatment, HmT-toxin at a concentration which inhibited root growth by 90% did not remove uranyl crystals bound to cell walls or release uranyl sequestered in vacuoles of corn root cells. Pretreatment with HmT-toxin did not interfere with binding of uranyl to cell walls but did prevent uranyl uptake into vacuoles and this effect was selective for Tms roots. Inhibition of uptake of uranyl into vacuoles was detectable in Tms roots exposed to HmT-toxin for 30 min and the process was completely blocked in thos pretreated with toxin for 1 hr. In contrast, exposures of 2 hr to the same concentration of HmT-toxin were required to induce significant increases in electrolyte loss. These results support previous suggestions that HmT-toxin alters cell permeability through effects on a specific component of the transport system rather than by general disruption of membranes.

Additional key words: southern corn leaf blight, pathotoxin, ultrastructure.

Since the outbreak of southern corn leaf blight in 1970, the cause of this disease, *Helminthosporium maydis* race T Nisikado and Miyake, and the pathotoxin that this pathogen produces have been subjected to investigation in many laboratories. Extensive and, in some cases, conflicting data have accumulated (4, 5, 6, 9, 13). Previously published results have led to two main hypotheses which were constructed to account for the mode of action and role of HmT-toxin in this disease.

Initial physiological studies revealed that HmT-toxin produced striking selective effects (swelling and loss of respiratory control) in mitochondrial preparations from Tms plants (8). These findings, which have been confirmed by several different investigators, led to the hypothesis that mitochondria were the sites of toxin action. This hypothesis was attractive because an effect on a cytoplasmic organelle would account for the cytoplasmic inheritance of disease reactions (6, 8). A recent report that mitochondria from resistant N plants can be rendered sensitive to HmT-toxin by removal of their outer membranes (11) suggests that this membrane may play a key role in selective toxin activity.

The second hypothesis assumes that the initial effect of HmT-toxin is on cell permeability. This hypothesis was based on reports that effects of HmT-toxin on root growth (3), K^+ fluxes (2, 3), and cellular electrochemical potentials (2,3) occur very rapidly (in 30 min or less), whereas changes in tissue respiration and ultrastructure, if detected at all, occur much later. A report of selective inhibition of a K^+-stimulated ATPase in microsomal fractions from Tms corn led to the more specific suggestion that the initial effect of HmT-toxin was exerted on this component of the membrane transport system (10). It should be noted that this reported inhibition of ATPase was not confirmed by work in another laboratory (7). Other investigators (4) have pointed out that reported rapid effects have been obtained only with very high concentrations of toxin, several orders of magnitude greater than those which cause well-defined inhibitions of dark CO_2 fixation. These results raise the question of why presumed initial events should require much higher toxin concentrations than those thought to be secondary.

One objective of this investigation was to test the hypothesis that selective effects of HmT-toxin might be a reflection of a general inability of Tms corn tissues to withstand stress. A prerequisite for such a test was the development of suitable bioassay systems. A second objective was to investigate the nature of permeability

changes induced by HmT-toxin through the use of uranyl salts to provide electron-dense markers in the electron microscope. Some of the results obtained have been published in abstracts (1, 14).

MATERIALS AND METHODS

Methods of production and root growth bioassay of HmT-toxin were those previously described (18). Most work was done with a single batch of toxin which gave 50% inhibition of root growth of Tms seedlings at a dilution of 1/1,000. Both crude culture filtrates (8.6 mg total solids per ml) and preparations partly refined by freezedrying and extraction with methanol (2.0 mg total solids per ml) were used in all tests. Unless otherwise noted, toxin was used at a concentration which gave 90% inhibition in the root growth assay. For the batch described above, this was a 1/50 dilution. Toxin preparations used in transpiration tests were those that had passed through dialysis tubing to remove high-molecular-weight substances. Control values represent both results with toxin solutions deactivated with alkali (18) and those obtained with distilled water, since the two never differed significantly.

Four pairs of corn inbred lines (W64A, B37, MO17, and 33-16) with one member of each pair carrying Tms and the other N cytoplasm were grown in a controlled environment chamber at 26 C under a 16-hr photoperiod of 8,800 lux. Transpiration was measured as previously described (16) with first and second leaves of plants 10 to 15 days olds, the cut ends of which were placed in test solutions at time zero. To measure electrolyte loss, 25-mm sections of similar leaves were placed in test solutions at time zero and the conductance of the bathing solutions was recorded at 1-hr intervals (16).

Corn seedlings with primary roots 10-mm long were used to study the effects of HmT-toxin on the binding and uptake of uranyl salts. Control roots were exposed either to 1mM uranyl acetate for 1 hr to label cell walls or for 20 hr to 0.1mM uranyl acetate to label vacuoles. Experimental roots were treated with toxin either before (pretreatment) or after (post-treatment) they had been exposed to uranyl solutions. Root tips were then excised and processed for electron microscopy as in previous work (17).

RESULTS

Responses of Tms and N inbred to stress.—In a preliminary test, more than 50 agents were screened for selective inhibition of root growth on Tms and N seedlings from a single lot of W64A seed. Among these were mineral salts, metal chelators, plant growth regulators, metabolic inhibitors, antibiotics, and temperature stresses. Results indicated that a number of these agents were more inhibitory to Tms than to N seedlings. However, when the test was repeated with a different lot of W64A seed and with seedlings of B37, MO17, and 33-16, results were consistent. Agents which were selectively inhibitory to Tms seedlings of one inbred failed to show such effects on the other three, or in several cases, were more inhibitory to N than to Tms seedlings. Analysis of these results and those obtained when Tms and N seedlings of the four inbreds were grown in distilled water, indicated that lack of uniformity in seedling growth and vigor, rather than inherent differences in the two cytoplasms, were responsible for the apparent selective effects observed in the preliminary screening test. This problem, which has been encountered by others (4), led to a search for more uniform lots of Tms and N seed, and in the meantime, an attempt to develop a more satisfactory bioassay.

Detached leaves were used in tests for effects of HmT-toxin on electrolyte loss and transpiration. Results with W64A tissues (Fig. 1) exposed to T-toxin at a concentration which inhibited root growth of Tms

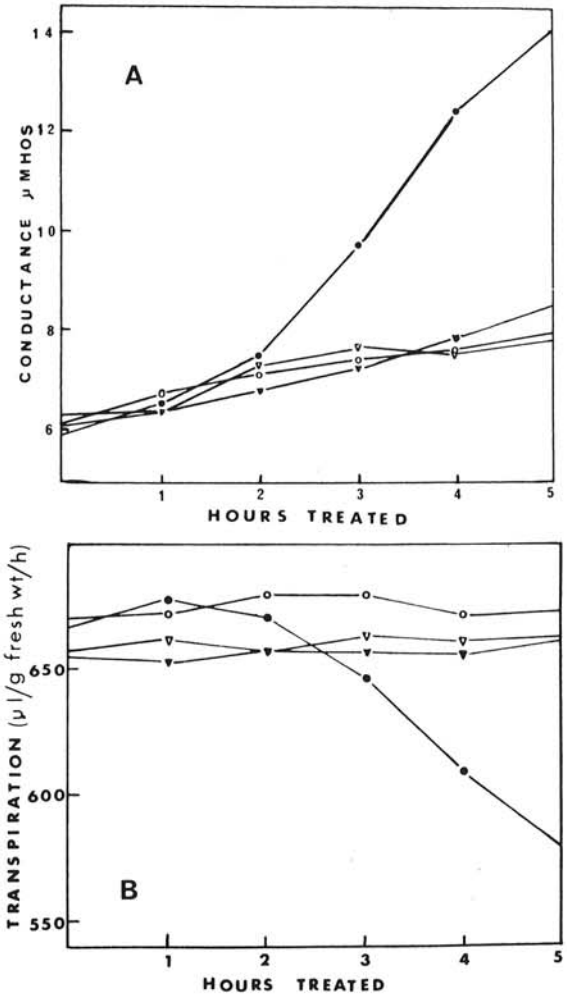

Fig. 1-(A, B). Effects of *Helminthosporium maydis* T-toxin on loss of electrolytes **A)** and transpiration **B)** of W64A corn leaves. Solid symbols represent results with tissues treated with T-toxin at a concentration which inhibited root growth of Tms seedlings 90%. Open symbols are results with controls exposed to deactivated toxin. Circles are results with Tms tissues; triangles represent those with N tissues. In (A) results with toxin-treated Tms tissues differed significantly ($P<0.01$) from all other treatments at 3 hr and thereafter. In (B) toxin-treated Tms tissues differed significantly ($P<0.01$) from the Tms control at 3 hr and from all other treatments thereafter. Other differences in (A) and (B) were not significant.

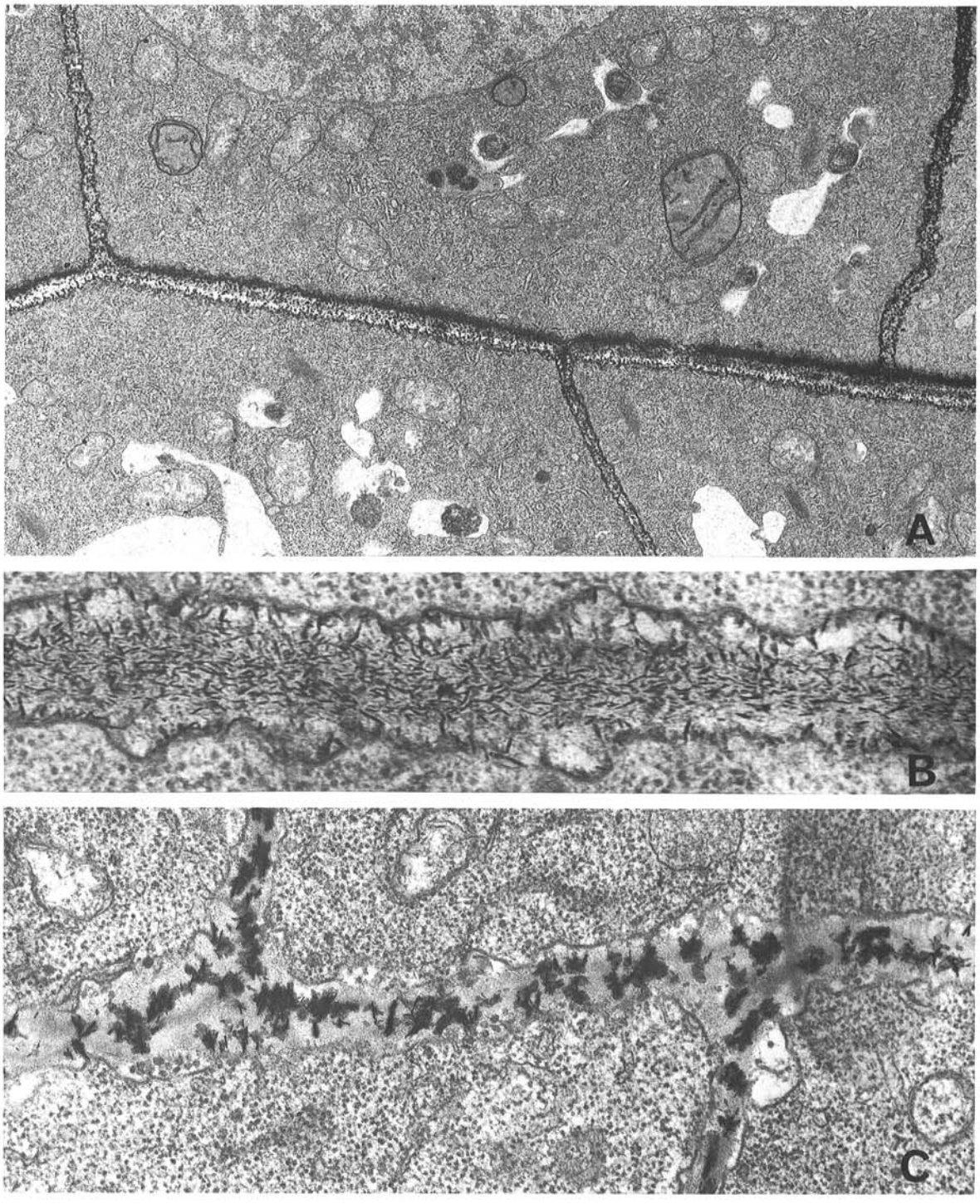

Fig. 2-(A to C). Effects of *H. maydis* T-toxin on the labeling of cell walls of corn roots exposed to 1 mM uranyl acetate for 1 hour. **A)** Typical pattern of labeling of control roots exposed only to uranyl with uranyl crystals confined to cell walls (× 16,000). **B)** Enlarged from (A) to show scattered distribution of uranyl crystals. This arrangement was not altered in Tms or N roots post-treated with toxin for 1 hr or pretreated for 2 hr (× 60,000). **C)** Clustered arrangement of uranyl crystals commonly seen in Tms but not in N roots pretreated with toxin for 4 hr or more (× 29,900).

seedlings by 90%, are typical of those obtained with the other three inbred lines. These data show that exposures to T-toxin of more than 2 hr were required to produce significant selective effects on Tms tissues. Even when the toxin concentration was increased 10-fold, changes in transpiration or electrolyte loss could not be detected in

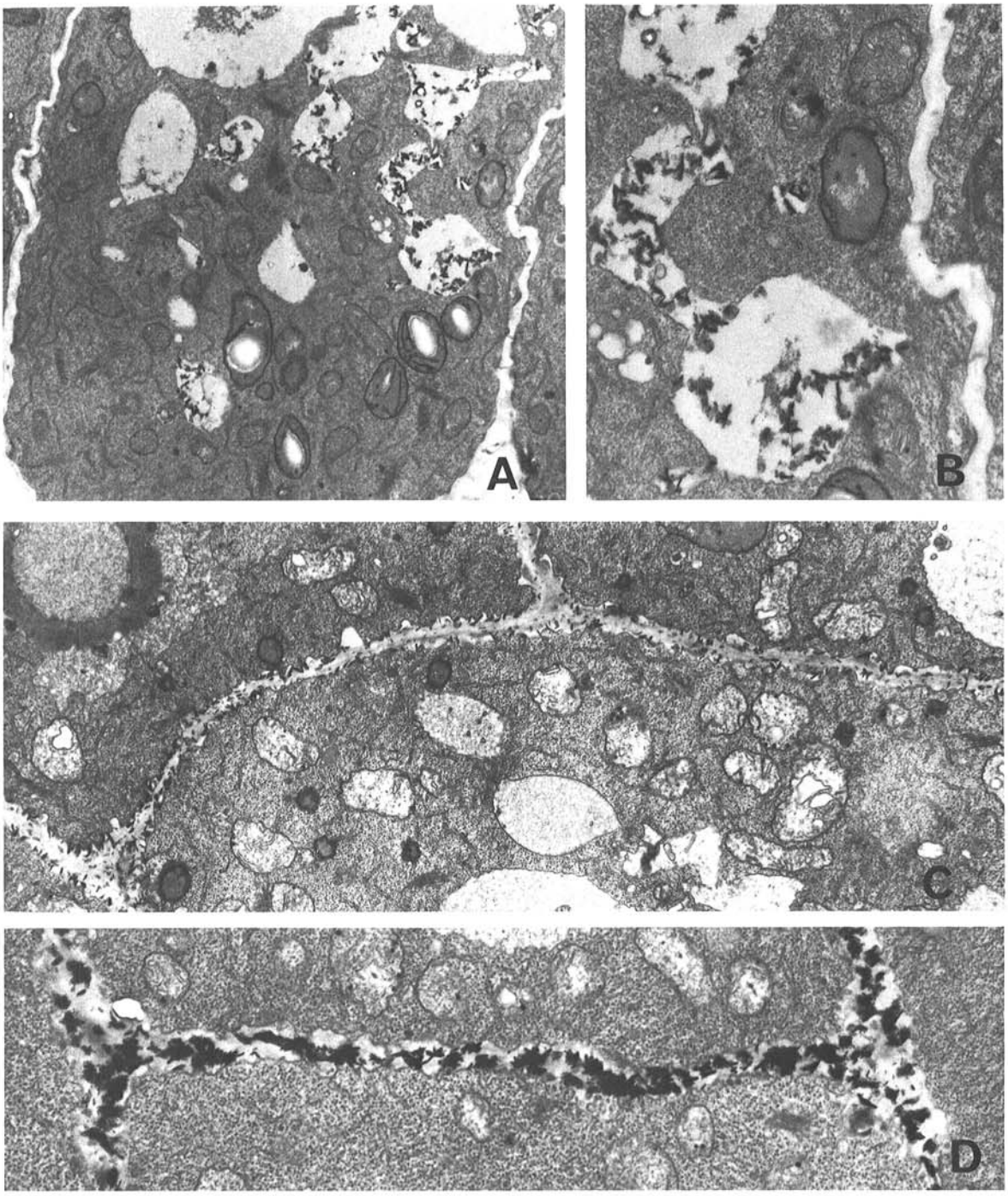

Fig. 3-(A toD). Effects of *Helminthosporium maydis* T-toxin on uptake of uranyl into vacuoles in corn roots exposed to 0.1 mM uranyl acetate for 20 hr. **A)** Localization of uranyl crystals typical of results with Tms and N controls exposed only to uranyl (× 8,000). **B)** Enlarged from (A). This distribution of uranyl crystals was not altered in Tms or N roots post-treated with toxin for periods up to 24 hours (× 24,000). **C)** Tms root pretreated with toxin for 1 hr. Uranyl crystals confined to cell walls and absent from vacuoles. Pretreatments of 24-30 hr were required to obtain similar results with N roots (× 18,000). **D)** Clustered arrangement of uranyl crystals in Tms roots pretreated with toxin for 4 hr or more (× 23,000).

tissues exposed to toxin for less than 1 hr. In contrast, as others have reported (3), inhibition of root growth could be detected within 30 min with toxin at the concentration used (Fig. 1).

Despite its insensitivity, electrolyte loss was used to screen all agents which in previous tests had given some indication of selective effects on Tms seedlings. Results were entirely negative for all four inbred lines tested.

Eventually, two lots of Tms and N seed of W64A were obtained which were uniform enough for further root growth tests. The same agents previously screened for selective effects were retested three times with triplicate treatments in each test. Statistically significant differences between Tms and N seedlings were not obtained with any of the agents tested.

Effects of Helminthosporium maydis T-toxin on corn root ultrastructure.—In these experiments, at lease two, and in most cases three, Tms and N roots of all four inbred lines were examined for each treatment. Where effects of toxin treatment were observed, results were checked with two additional seed lots of W64A and one of 33-16.

Prior to tests with uranyl-labeled tissues, roots held in distilled water were compared to those treated with toxin alone for 1, 2, 4, and 8 hr. Among controls in distilled water, no differences in ultrastructure were found either between Tms and N lines or among the four inbreds examined. No effects of toxin treatment were detected in Tms roots treated for up to 2 hr or in N roots treated for up to 8 hr. With exposures to toxin of 4 and 8 hr, Tms roots showed the same general and gradual disorganization of cellular ultrastructure reported by others (3).

Effects on uranyl labeling of cell walls.—Exposure of corn roots to 1mM uranyl acetate for 1 hr followed by desorption in distilled water for 30 min or 1 hr, resulted in the same labeling of cell walls reported for roots of other cereals (15, 17). In both Tms and N roots of all four inbreds, uranyl crystals were confined to cell walls [Fig. 2-(A, B)] with the heaviest depositions in the outer three or four layers of cells. Post-treatment with toxin for 1 hr failed to remove the crystals or alter their arrangement. Longer post-treatments were not attempted because exposure to 1mM uranyl for 1 hr results in extensive cellular disruption if roots are held in distilled water more than 1 hr before fixation (17). Pretreatment with toxin for up to 2 hr also had no effect on the binding or distribution of uranyl crystals in cell walls of Tms or N roots. However, in Tms roots pretreated for 4 hours, and especially in those pretreated for 8 hours, uranyl crystals usually were found in clusters (Fig. 2-C) rather than randomly scattered throughout the wall (Fig. 2-B). Since this did not occur with N roots, these results indicate that the toxin is capable of selective effects on the properties of Tms cell walls. This effect was not entirely consistent and, on rare occasions, similar clustered arrangements of uranyl crystals were found in both Tms and N roots that had not been treated with toxin.

Effects on uranyl labeling of vacuoles—In agreement with previous results (15, 17), uranyl crystals in subepidermal cells of corn roots exposed to 0.1 mM uranyl acetate for 20 hr were localized in vacuoles [Fig. 3-(A, B)]. Post-treatments with T-toxin for periods as long as 24 hr failed to remove or change the localization of these crystals in either Tms or N roots. Pretreatment with T-toxin however, had striking effects on the localization of uranyl in Tms roots. In those pretreated with toxin for 30 min, crystals rarely were found in vacuoles but were abundant in cell walls. Pretreatment of Tms roots with toxin for 1 hr completely blocked uptake into vacuoles and all uranyl crystals were confined to cell walls (Fig. 3-C). Pretreatments of Tms roots for 4 or 8 hr resulted in clustered arrangements of crystals in cell walls (Fig. 3-D) similar to those seen with the same pretreatments of roots exposed to 1mM uranyl for 1 hr (Fig. 2-C). With N roots, pretreatments with T-toxin for 24 to 30 hr were required to prevent uptake of uranyl into vacuoles. These results were consistent with those of root growth tests which indicate that Tms roots are approximately 25 times more sensitive than N roots to HmT-toxin (18).

DISCUSSION

Failure to demonstrate selective toxic effects with any agent other than T-toxin does not support the hypothesis that general inability to withstand stress is responsible for toxin sensitivity and disease susceptibility of Tms plants. Instead, these results suggest that some specific character associated with Tms cytoplasm is responsible for the selective effects of this pathotoxin.

Although Tms and N plants could be clearly separated by all of the bioassays for T-toxin used in this study, none of these assays was capable of discriminating among Tms plants of the four inbred lines studied. These four lines have been reported to differ markedly in field reactions to southern corn leaf blight (4, 12). Initially these results were thought to indicate that factors other than sensitivity to T-toxin must be responsbile for differences among Tms inbreds observed in the field. Recently, however, a highly sensitive bioassay based on inhibition of dark CO_2 fixation has been reported to be capable of distinguishing among different Tms inbreds (4). It is possible, therefore, that our bioassays were not sufficiently sensitive to detect small differences in responses of Tms inbred lines to the toxin.

Others have reported that toxin-induced inhibition of root growth can be detected within 30 min, whereas increased losses of electrolytes occur at least 1 hr later (2). On the basis of these results and on selective effects of toxin treatment on ion fluxes (3), they concluded that an effect on some specific component of the transport system rather than a general disruption of membranes was responsible for alterations in cell permeability caused by T-toxin. We have confirmed their findings in regard to effects on root growth and electrolyte loss and, in addition, have found that toxin-induced inhibition of uranyl uptake into vacuoles occurs at about the same time as root growth inhibition.

In view of evidence that most, if not all, uptake of uranyl takes place in endocytotic vesicles (15, 17), our results suggest that this may be the specific component of the transport system first disrupted by T-toxin.

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