Histochemistry of Beta-Glucosidase in Isolines of Zea mays Susceptible or Resistant to Northern Corn Leaf Blight

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ABSTRACT

Localization of β-glucosidase activity was determined histochemically in the leaves of noninoculated and inoculated isolines of inbred corn susceptible or resistant to northern corn leaf blight incited by *Helminthosporium turcicum*. The β-glucosidase activity in susceptible and resistant corn lines generally was restricted to the phloem of the small vascular bundles. A trace of enzyme activity occasionally was observed in the phloem of the large vascular bundles after prolonged incubation. Enzyme activity was detected at particulate sites in the cytoplasm. Localization of the host β-glucosidase activity in the inoculated leaves of the isolines was identical to that in noninoculated control leaves. The β-glucosidase of *H. turcicum* also occurred at particulate sites in the cytoplasm of germ tubes, appressoria, and mycelium. The growth rate of *H. turcicum* was comparable in susceptible and resistant leaves during the 2-3 days required for the mycelium to reach the xylem of the vascular bundles. This observation, along with the histochemical data, suggests that any release of the fungitoxic aglycone from the 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one glucoside present in corn leaves occurs in the vascular bundle after hydrolysis of the glucoside by the β-glucosidase of the host or pathogen.

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The chlorotic-lesion resistance to northern corn leaf blight is controlled by a single dominant gene, Ht (7). The Bx gene appears to influence the expression of the Ht gene by regulating the production of the cyclic hydroxamic acid, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (4). Resistance is expressed as reduced sporulation of the fungus and subsequent retardation of secondary spread of the disease. Hilu & Hooker (8) have documented the pathological histology of chlorotic-lesion resistance in inbred *Zea mays*.

Virtanen (13) and Wahlroos & Virtanen (14) initially reported the occurrence of the monoglucoside of 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one in corn leaves and the cleavage of the glucosidic bond by corn glucosidases. Klun et al. (9) established that the aglycone, 2,4-dihydroxy-7-methoxy-2H-1,4- benzoxazin-3-one (DIMBOA), as determined by the levels of its decomposition product, 6-methoxy-2(3)-benzoxazolinone, constitutes the major cyclic hydroxamic acid in several inbred corn lines.

The aglycone, DIMBOA, has many fungitoxic effects against such disease fungi as *Fusarium nivale* (14), *Puccinia graminis* var. *tritici* (5), and *Helminthosporium turcicum* (4); however, the DIMBOA-glucoside has little or no fungitoxic effect on these fungi. Nearly complete inhibition of the germination of *H. turcicum* spores was achieved at 2.84×10^{-5} M DIMBOA (4).

Molot & Anglade (12) reported a significant correlation (-0.95) between levels of DIMBOA, as determined by the levels of its decomposition product, 6-methoxy-2(3)-benzoxazolinone, and the resistance of several corn inbreds to *H. turcicum*. Couture et al. (4) recently suggested that the resistance of inbred corn to *H. turcicum* is associated with the enzymatic hydrolysis of the DIMBOA glucoside by host β-glucosidases after infection. The objectives of this study were to demonstrate histochemically the distribution of β-glucosidase in healthy and infected corn inbreds susceptible or resistant to northern corn leaf blight, and to determine whether the distribution of this enzyme was of possible significance to the DIMBOA fungal toxicity hypothesis.

MATERIALS AND METHODS.—Two pairs of nearly isogenic inbred lines of corn (*Zea mays* L.) were selected for study. These nearly isogenic pairs were (i) susceptible B37 and resistant RB37Ht; and (ii) susceptible Oh51A and resistant Oh51AHt. The resistant inbreds, RB37Ht and Oh51AHt, were developed by introducing, from resistant breeding lines, the dominant gene (Ht) for chlorotic lesion resistance to *H. turcicum* into B37 and Oh51A, previously established as susceptible inbreds. Subsequent backcrosses for five generations to the susceptible inbred parents and selection of the progeny for resistance to *H. turcicum* resulted in the production of the two nearly isogenic lines. All four inbreds carry the *Bx* (normal) gene. Plants were grown in the greenhouse in a 2:1 soil-vermiculite mixture, and soluble 20:20:20 fertilizer was applied as part of the watering regime.

The pathogen, *Helminthosporium turcicum* Pass., was obtained from the American Type Culture Collection, Rockville, Md. (ATCC No. 11536). The genetic stability of the pathogen from one experiment to the next was insured by using lyophilized stock cultures for each experiment. *H. turcicum* spores harvested from 10-day-old V-8 agar cultures were lyophilized essentially as previously described for *Fusarium* spores (11); however, it was necessary to pretreat the *H. turcicum* spores at 4 C for 4 hr followed by −24 C for 1 hr before placing them in liquid nitrogen.

Spores for inoculations were harvested with distilled water from 10-day-old V-8 agar cultures seeded with lyophilized spores, collected on a 1.2 μ pore

filter, washed with distilled water, and suspended in distilled water at 50,000 spores/ml. Plants at the five- to six-leaf stage (ca. 1 month old) were inoculated by atomizing the spore suspension onto the leaves and placing the plants in a moist chamber at 25-27 C for 18 hr. Similar plants treated with atomized distilled water served as controls. All plants were removed from the moist chamber after 18 hr and held in the greenhouse for 1 week at 25-27 C.

The youngest fully expanded leaves of control and inoculated plants were harvested 16-20 hr after inoculation and at daily intervals thereafter. Fresh sections ca. 100 μ thick were cut with a sliding microtome and used routinely for β-glucosidase local- ization. The sections were washed in distilled water and immediately placed in the histochemical reagents. Fixation of sections in 5% Formalin saturated with $CaCO_3$ at 4 C for 24 hr did not enhance enzyme localization and, therefore, was discontinued.

The simultaneous azo-coupling technique of Ashford (1) was used for histochemical localization of β-glucosidase. The enzyme substrate (6-bromo-2- naphthyl-β-D-glucopyranoside) solution was that developed by Cohen et al. (3). The diazonium salt, Fast Garnet GBC, was used to couple with the aglycone, 6-bromo-2-naphthol, enzymatically released from the substrate. All reactions were conducted in a phosphate-citrate buffer at pH 6.0 for 15 min at 37 C. The β-glucosidase activity in host and pathogen can be detected histochemically at 22-23 C; however, the prolonged incubation period required does not permit good enzyme localization. Controls consisted of enzymatically active sections incubated in the test solutions without the substrate and sections heat inactivated in distilled water at 85-90 C for 10 min and then incubated in a test solution with substrate. Control and test sections were removed from the assay solutions, washed in distilled water for 2 min, and mounted in distilled water for color photography.

RESULTS.—Epidermal cells of corn root tips have been shown by Ashford (1) to possess very high levels of β-glucosidase activity. As a check on the test conditions used in my study, root tips were placed in the complete assay solution; after a 5-min incubation period, intense β-glucosidase activity was detected in the epidermal cells. This confirmed that the test conditions were optimal or nearly so for the histo- chemical demonstration of β-glucosidase activity.

Most β-glucosidase activity in corn leaf tissue was restricted to the small vascular bundles in the noninoculated leaves of the susceptible and resistant inbred lines used in this study (Fig. 1-A,C). This activity was generally restricted to the phloem paren- chyma area near the center of the bundle, and it occurred at particulate cytoplasmic sites. In a few instances, a trace of activity was observed in the bundle sheath cells. In most instances, no activity was detectable histochemically in the large vascular bundles (Fig. 1-B); however, after 30-min incubation, a faint trace of activity was noted occasionally in the phloem parenchyma area. No β-glucosidase activity was detected in the epidermal or mesophyll cells. In both control assays (no substrate present and heat inactivated sections of inoculated and noninoculated leaves of all inbred lines), β-glucosidase activity was absent; however, an intense red stain noted in Fig. 1-B also was observed in the fibrous sheath cells associated with the large vascular bundles and in metaxylem vessel walls. This nonenzymatic staining probably was caused by the coupling of phenolic compounds in the secondary cell walls with Fast Garnet GBC to form the the red azo dye. The absence of β-glucosidase activity in both host and pathogen after heat inactivation is illustrated in Fig. 1-D.

As in corn leaf tissue, intense β-glucosidase activity was detected as particulate azo dye deposits in the cytoplasm of the germ tubes and appressoria of *H. turcicum* by 16 hr after inoculation of the upper leaf surfaces of the four corn inbreds used in this study (Fig. 1-E). As the cytoplasm moved out of the spore case and into the germ tube, the particulate sites of enzyme activity migrated with the cytoplasm, leaving no evidence of enzyme activity in the spore wall. Similar enzyme activity could also be observed in ungerminated spores at the time of inoculation.

During the 2-3 days required for *H. turcicum* to colonize the leaf mesophyll and reach the vascular bundle, all β-glucosidase activity outside of the vascular bundle appeared to be in the fungal my- celium (Fig. 1-F,G,H). As was true for the germ tube and appressorium on the leaf surface, the enzyme activity was associated with particulate cytoplasmic

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Fig. 1. The localization of β-glucosidase in fresh, unfixed sections of leaves of *Zea mays* noninoculated or inoculated with *Helminthosporium turcicum*. Particulate deposits of cherry red azo dye (single arrows) occur within the cytoplasm of host and pathogen at sites of β-glucosidase activity. With the exception of E, G, and H, all figures are cross-sections. A) A noninoculated resistant RB37Ht leaf illustrating the restriction of β-glucosidase activity (white arrow) to the phloem of the small bundle (X 600). B) A large bundle of a noninoculated susceptible B37 leaf illustrating the absence of β-glucosidase activity in the phloem (black arrow) and the nonenzymatic staining (double white arrows) of the fibers and some vessel walls (X 1,200). C) A noninoculated susceptible Oh51A leaf illustrating the restriction of β-glucosidase activity (white arrow) to the phloem of the small bundle. Nonenzymatic staining of chloroplasts is evident in the bundle sheath cells (X 2,700). D) A heat-inactivated control (+ substrate) of a susceptible B37 leaf 24 hr after inoculation (X 600). E) β-glucosidase localization (white arrows) in germ tubes and appressoria of *H. turcicum* on the upper surface of a whole mount of a susceptible Oh51A leaf 16 hr after inoculation (X 600). F) A susceptible B37 leaf 24 hr after inoculation. Note the granular nature of the red azo dye in the mycelium (white arrows) and the slightly diffuse nature of the dye in the epidermal layer (black arrow). In the subepidermal layers, the azo dye (white arrows) is confined to the mycelium (X 600). G) A longitudinal section of a resistant RB37Ht leaf 24 hr after inoculation (X 600). H) A longitudinal section of a resistant RB37Ht leaf 72 hr after inoculation. The β-glucosidase is localized (white arrows) within the mycelium. Note the nonenzymatic staining (double arrows) of the cuticle (X 600).
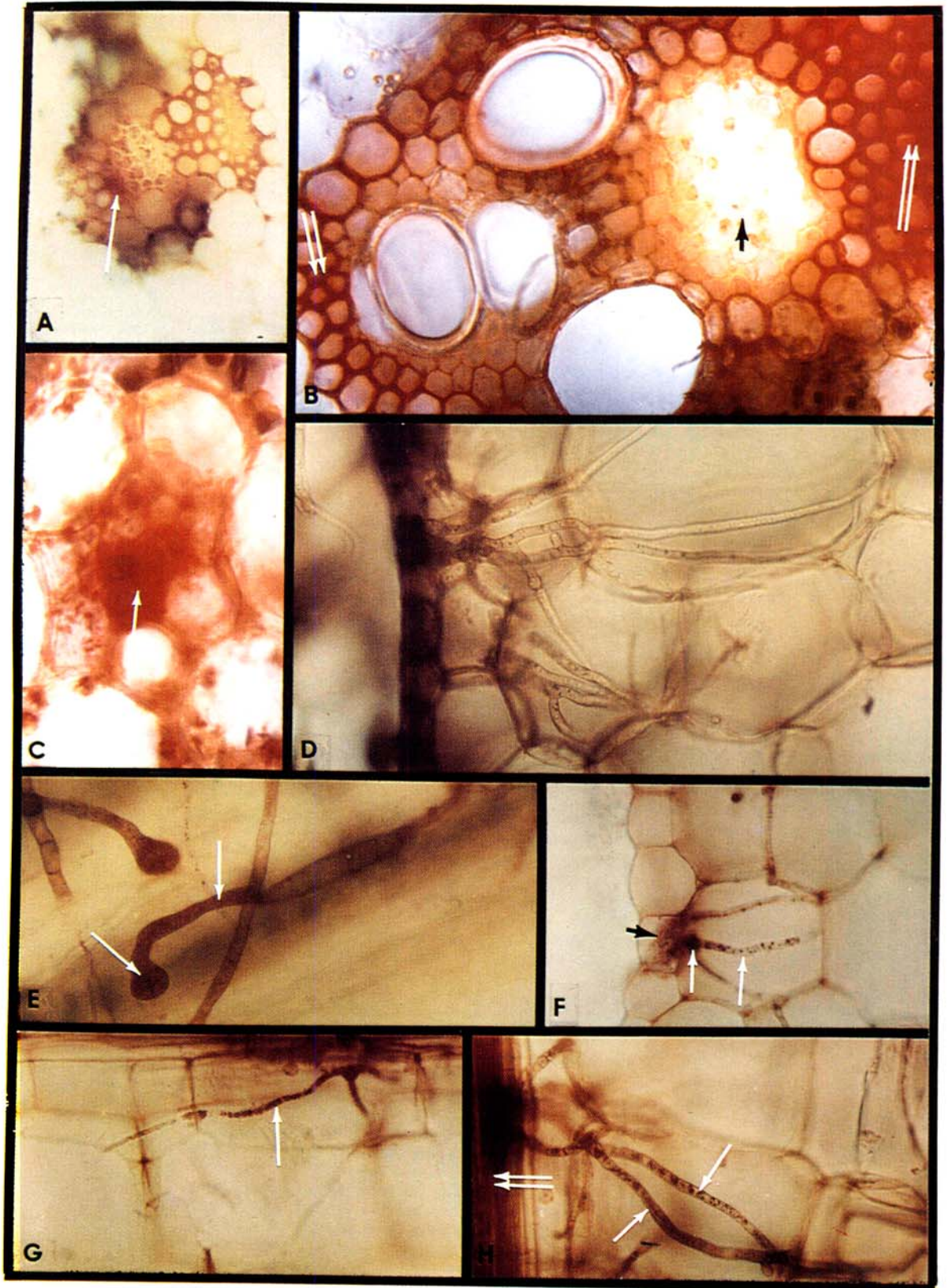
sites. At sites where the mycelium was ruptured during sectioning, the fungal cytoplasm flowed out into the host tissue, and a halo of β-glucosidase activity was observed around the site of rupture. No obvious changes in the histochemical pattern of localization of host β-glucosidase in the vascular bundles were noted in the infected leaves of any of the inbred corn lines, nor were new or additional sites of localization of the host enzyme observed as a result of infection. The time (2-3 days) required for *H. turcicum* mycelium to penetrate the leaf and enter the vascular bundles was comparable in both susceptible and resistant varieties.

DISCUSSION.—The histochemical data indicate that the β-glucosidase activity in *H. turcicum* mycelium and in the leaves of the four inbred corn lines used in this study is localized at particulate sites in the cytoplasm. Ashford (1) has made similar histochemical observations for corn roots. Chkanikov et al. (2) reported that most, if not all, β-glucosidase activity in homogenates of corn coleoptiles and oat, bean, and potato leaves was associated with the cell wall fraction. Our histochemical data for corn leaves suggest that the β-glucosidase detected by Chkanikov in corn coleoptiles was adsorbed to the cell wall fraction after homogenization.

Couture et al. (4) suggest that the toxic DIMBOA is released from the DIMBOA-glucoside by the corn leaf β-glucosidase after the penetration by *H. turcicum* of the first one or two cell layers of the upper leaf surface. DIMBOA-glucoside is known to be hydrolyzed by corn glucosidase after incubation in juice pressed from corn seedlings (14). The low specificity of β-glucosidases (6, 10) strongly suggests that the *H. turcicum* β-glucosidase also would hydrolyze the DIMBOA-glucoside. The present study and that of Hilu & Hooker (8) establish that 2-3 days are required for *H. turcicum* mycelium to penetrate the epidermis and mesophyll and reach the xylem vessels of the vascular bundles of the chlorotic-lesion resistant or susceptible inbred lines. It is apparent from the histochemical data that no host β-glucosidase is present at the epidermis or in the mesophyll cells encountered by *H. turcicum* during the first 2-3 days after mycelial penetration; however, profuse β-glucosidase activity is present in the *H. turcicum* mycelium before and after host penetration. This suggests that any release of toxic DIMBOA from the glucoside which might occur during the first 2-3 days after penetration must be effected by the β-glucosidase of *H. turcicum*. After entry into the vascular bundles at 2-3 days, the β-glucosidases of both host and pathogen may hydrolyze any DIMBOA-glucoside present in the vascular bundle.

Observations of this study and those of Hilu & Hooker (8) that no apparent inhibition of *H. turcicum* growth occurs in the susceptible or chlorotic-lesion resistant inbred corn lines before entry into the xylem appear to preclude any involvement of DIMBOA in the susceptible or resistant interactions during the first 2-3 days after penetration. Both host and pathogen β-glucosidases might act to hydrolyze DIMBOA-glucoside and release the toxic

DIMBOA at the time of vascular bundle penetration; however, the localization of the glucoside must be determined before this hypothesis can be evaluated.

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