Histochemical Demonstration of Transitory Esterase Activity in Venturia inaequalis

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ABSTRACT

Esterase activity was detected histochemically in spores and germlings of *Venturia inaequalis* during a 12-hr period after inoculation on host and nonhost surfaces. Esterase activity was present at the spore apex prior to germination, disappeared from the elongating germ tube, and reappeared in the appressorium. Morphological development and the pattern of esterase activity were similar for all isolates studied.

Activity at the apex of the ungerminated spore was suggested to facilitate germination by a plasticizing or rupturing of the spore wall components. Activity in the appressorium may represent the localization of hydrolytic enzymes necessary for host penetration.

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In an ultrastructure study of host-parasite interaction in the apple scab disease, Maeda (13) demonstrated that a membrane-bounded "infection sac" was present in appressoria of *Venturia inaequalis* (Cke.) Wint. and in contact with the host cuticle at the site of penetration. Furthermore, the sac appeared to be the first component of the fungal protoplast to contact the cuticle. She suggested that the sac might function as a reservoir of fungal products, including enzymes, necessary for penetration. Based on the discovery of the infection sac, studies have been initiated in this laboratory on components of the appressorium which may be significant in the apple scab interaction.

Localization of nonspecific esterase has been demonstrated histochemically in plant and animal tissue with indoxyl esters (1, 4, 11, 12, 17) which form crystals of indigo blue after hydrolysis. This study demonstrates, histochemically, transitory esterase activity in the development of *V. inaequalis* from the time of germination to appressorium formation.

MATERIALS AND METHODS.—*Fungus.*—Isolates of the five races of *V. inaequalis* (race 1, 1766-5; race 2, 1770-6; race 3, 1771-1; race 4, 1772-4, 1773-2; race 5, 1764-1, 1763-1) were grown in 8-oz prescription bottles on cheesecloth supports with 4% Difco malt medium at 19 C. Spores from 14-day cultures were washed by centrifugation (2,000 *g*) in distilled water and resuspended in distilled water, and concentrations were determined with a hemacytometer.

Host tissue.—Etiolated apple (*Malus sylvestris* Mill.) hypocotyls were used because symptoms of scab infection on these hypocotyls are similar to those on green leaves (16), and the tissue does not contain pigments which interfere in microscope studies. Open-pollinated McIntosh seeds (susceptible to all races of *V. inaequalis*) were planted in a mixture of 60% Vermiculite-40% soil and placed in the dark at 19 C for 10 days. Seedlings were washed with tap water and placed on supports in moisture chambers, and their roots were covered with deionized water. Seedlings were maintained in the dark at 19 C for 12 hr before inoculation. Spore suspensions were sprayed on hypocotyls and the plants were kept in the dark except when tissue samples were collected.

Nonhost surfaces.—Fungal morphology, development, and esterase activity were studied on nonhost surfaces for comparison with apple. Surfaces chosen were etiolated bean hypocotyls (*Phaseolus vulgaris* L. 'Perry Marrow'), glass (microscope slides), wettable cellophane, and tissue embedding medium.

Etiolated bean hypocotyls were grown according to Elliston et al. (3). Hypocotyls were inoculated and incubated as described for apple.

One-cm^2 surfaces were outlined with wax pencil on glass microscope slides (Micro Slides No. M6155, Scientific Products, Evanston, Ill.). Slides were sprayed with a spore suspension, placed in moisture chambers, and maintained in the dark at 19 C.

Pieces of wettable cellophane, 1 cm^2, were boiled in distilled water for 20 min, rinsed in sterile distilled water, and mounted on glass slides. Inoculation and incubation procedures were as described.

Paraplast tissue-embedding medium (Sherwood Medical Industries Inc., St. Louis, Mo.) was formed into blocks and cut with a microtome into sections 10 μ thick. Sections were placed on a film of water on clean glass slides, expanded on a warming plate, and dried. Inoculation and incubation procedures were as described.

Histochemical detection of esterase activity with indoxyl acetate.—Fresh solutions of indoxyl acetate (Nutritional Biochemicals Corp., Cleveland, Ohio) were prepared for each assay according to Barrnett & Seligman (1). Self-hydrolysis of indoxyl acetate occurs and substrate solutions must be prepared fresh for each assay. Epidermal strips were removed from etiolated hypocotyls at 2-hr intervals after inoculation, immersed directly in the substrate solution on glass slides, and examined after 5 min for the presence of crystals of indigo blue in spores and spore germlings (magnification X 800). Assays on nonhost surfaces were carried out by placement of a drop of substrate solution directly on the surface. Samples were taken at 2-hr intervals from the time of inoculation. Hydrolysis of indoxyl acetate was not observed in autoclaved spores.

Developmental stages of Venturia.—Four stages of fungal development preceding host penetration were defined. The number of spores or germlings in each stage that were esterase-positive and negative were counted at each 2-hr interval. Spores or germlings were considered esterase-positive when crystals of indigo blue were present at specific sites designated for each stage. Crystal deposition occurs regularly in lipid bodies and occasionally in the cytoplasm as elongate strands. Although these crystals represented hydrolysis of indoxyl acetate, they were not considered when designating a spore or germling as esterase-positive. Developmental stages and sites of activity were as follows:

1) *Pregermination stage* (PG).—Spores in this category had not germinated. Crystals of indigo blue at the spore apex represented an esterase-positive reaction. Spore germination nearly always occurs from the narrow end or apex of the spore.

2) *Just-germinating stage* (JG).—Germlings were placed in this category when the germ tube was just emerging through the spore wall. The length of the germ tube was limited to less than one-fourth the spore length. A positive esterase reaction was the presence of crystals at the tip of the germ tube.

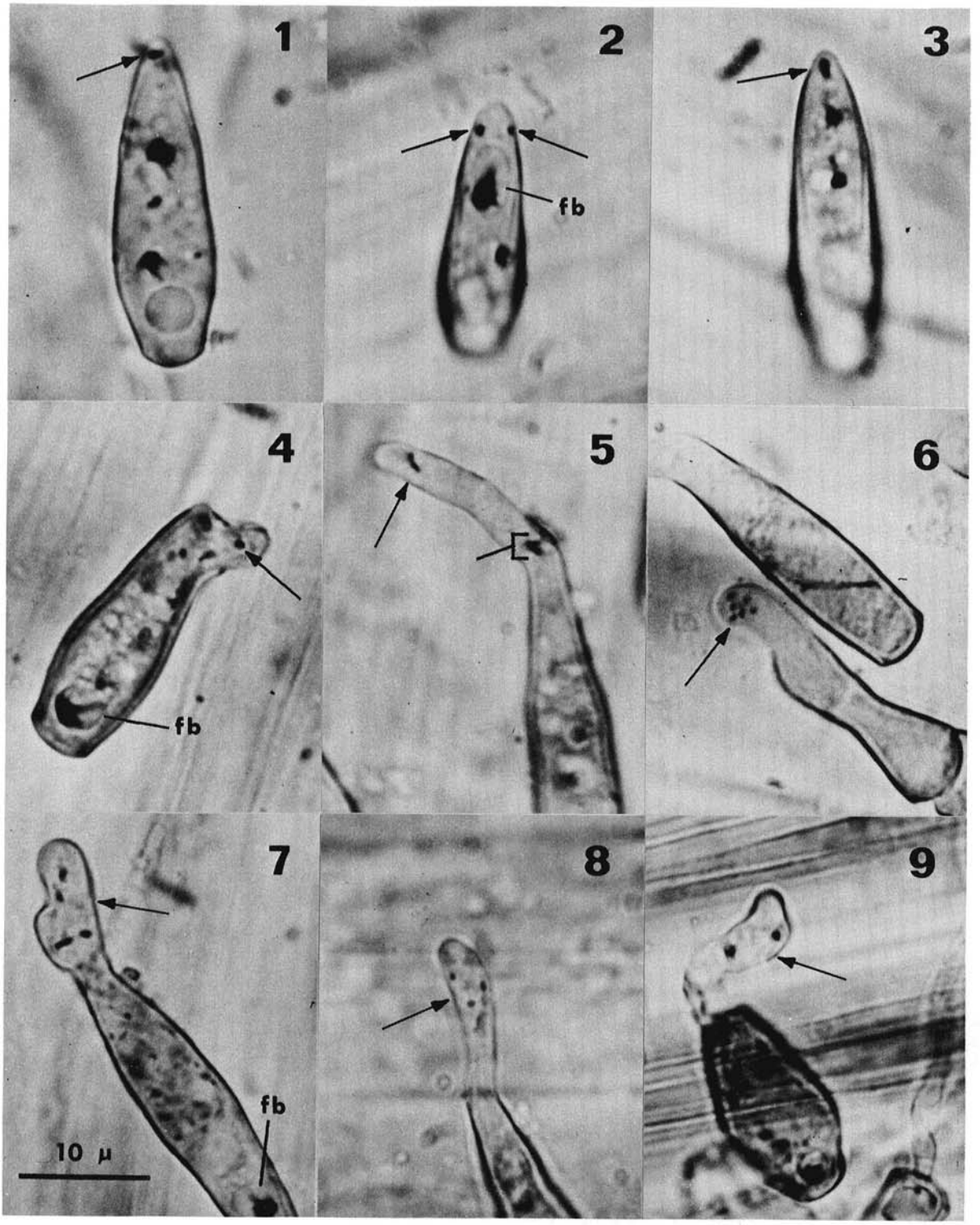
3) *Preappressorial stage* (PA).—Germlings were placed in this stage when the germ tube length was at least one-fourth the length of the spore. A positive esterase reaction was the presence of crystals at the tip of the developing germ tube.

4) *Appressorial stage* (AP).—Appressorium formation was recognized by enlargement of the tip of the germ tube. A positive esterase reaction was the presence of crystals of indigo blue in the appressorium.

RESULTS.—*Venturia inaequalis on etiolated apple hypocotyls.*—Positive esterase reactions in each developmental stage are shown in Fig. 1-9. In the PG stage, the percentage of ungerminated esterase-positive spores exhibited a downward trend with time (Fig. 10-B). This trend accompanied the decrease in the number of spores remaining in the PG stage and the increase in the number of spores that had progressed beyond pregermination (Fig. 10-A).

It is difficult to compare the JG and PA stages where different isolates of the fungus are used. Each isolate represents a population whose members develop morphologically at rates independent of other isolates. However, for each isolate the data show that the percentage of esterase-positive germlings in the JG stage consistently exceeds that of the PA stage. The AP stage precedes penetration for all isolates, and nearly all appressoria (90 to 100%) of each isolate studied were esterase-positive.

Venturia inaequalis on nonhost surfaces.—The developmental morphology of the fungus appeared the same on etiolated bean (Fig. 11-A) and Paraplast as on etiolated apple. Similarly, esterase activity on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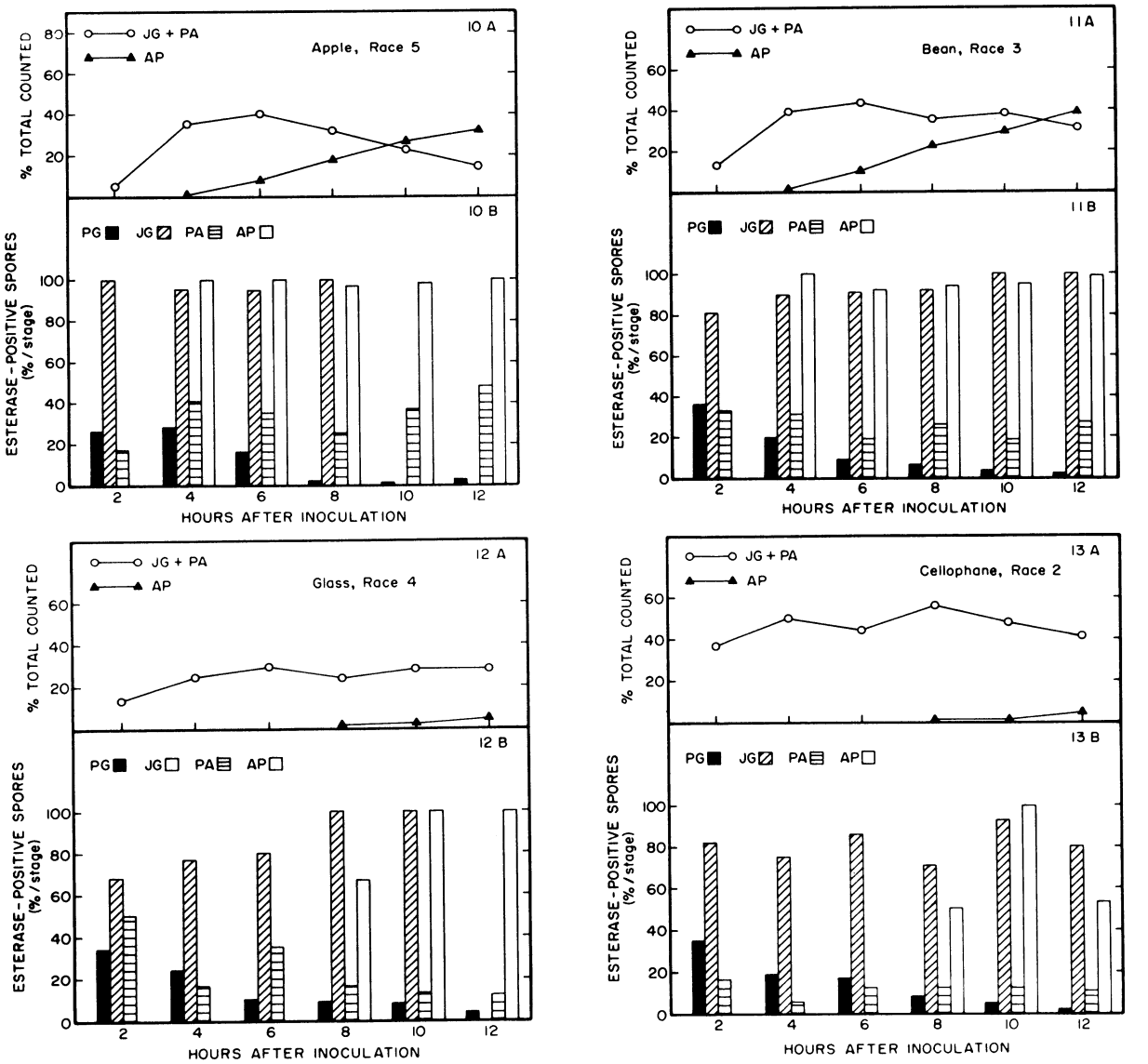

Fig. 10-13. Part A of each figure) Development of *Venturia inaequalis* after inoculation, expressed as percent of the total spores counted at each time interval (200 to 300 spores counted per time interval). The open circles equal spores in the just-germinating (JG) and preappressorial (PA) stages combined; the solid triangles equal spores in the appressorial (AP) stage. **Part B of each figure)** Esterase-positive spores in each developmental stage, expressed as percent of the total spores counted in each stage at each time interval. PG = pregermination stage; JG = just-germinating stage; PA = preappressorial stage; AP = appressorial stage. **10)** Host surface, etiolated open-pollinated McIntosh apple seedlings; fungus, race 5, isolate 1763-1. Germlings were not observed in the JG stage at 10 and 12 hr. **11)** Nonhost surface, etiolated Perry Marrow bean seedlings; fungus, race 3, isolate 1771-1. **12)** Nonhost surface, glass; fungus, race 4, isolate 1773-2. Germlings were not observed in the JG stage at 12 hr. **13)** Nonhost surface, cellophane; fungus, race 2, isolate 1770-6.

Fig. 1-9. Esterase-positive reactions in various stages of development of *Venturia inaequalis* on etiolated apple hypocotyls (bright field optics). Pregermination stage. **1)** Crystal of indigo blue (arrow) at the spore apex. **2)** Crystals of indigo blue (arrows) near the spore apex. Lipid body containing indigo blue crystals (fb). Just-germinating stage. **3)** Crystal of indigo blue (arrow) at the tip of the emerging germ tube. **4)** Crystals of indigo blue in the emerging germ tube (arrow). Note crystals in large lipid body (fb) at spore base. Preappressorial stage. **5)** Crystals of indigo blue (arrow) near the tip of the elongated germ tube. A crystal is also present where the germ tube bends away from the spore body (bracket). Appressorial stage. **6)** Crystals of indigo blue in the developing appressorium (arrow). **7)** Appressorium (arrow) formed directly upon spore germination. Crystals are present in the appressorium. **8)** Germ tube has started to enlarge (arrow). Crystals of indigo blue in the developing appressorium. **9)** Crystals present in the appressorium (arrow).

bean (Fig. 11-B) and Paraplast exhibited the same pattern as on apple.

When the fungus was placed on glass or cellophane (Fig. 12-A, 13-A), only 5% of the spores formed appressoria in the 12-hr incubation period. When the race-2 isolate (1770-6) assayed on cellophane was assayed on apple, ca. 35% of the spores formed appressoria within 12 hr, indicating that the surface on which spores germinate influences appressorium formation.

The pattern of esterase activity in the PG stage on glass and cellophane was the same as on apple (Fig. 12-B, 13-B). Activity in the JG stage was, as on apple, consistently greater than activity in the PA stage. The percentage of esterase-positive germlings in the PA stage was less relative to the PA stage on other surfaces.

DISCUSSION.—It was not possible to measure the esterase reaction of a single spore throughout its successive developmental stages. However, our findings, based on population studies, indicate that esterase activity appears prior to spore germination, disappears shortly after germination, and reappears with appressorium formation. This transitory pattern of activity was similar for all isolates studied.

Esterase activity was found at the spore apex (the normal site of germination) of ungerminated spores of *V. inaequalis* after 2-hr incubation on each surface. However, the percentage of ungerminated esterase-positive spores decreased as the number of germinated spores increased over a 12-hr period. These data suggest that spores which germinated had esterase activity at the site of germ tube emergence prior to germination.

The JG stage represents germlings which have recently advanced beyond pregermination. It is possible, therefore, that esterase activity at the tips of emerging germ tubes in the JG stage represents residual activity from the PG stage. The percentage of germlings in the JG stage showing esterase reactions was consistently greater than that of germlings in the PA stage. This suggests that as germ tube elongation occurs, esterase activity either disappears or is no longer detected by the method used. Nearly all appressoria were esterase-positive, suggesting the reappearance of activity in the appressorial stage.

Although the PA stage showed an over-all reduction in esterase activity as compared to the JG stage, the activity did not completely disappear. The population of germlings in the PA stage includes those at an interim of development between germination and appressorium formation. Thus, some are developmentally closer to appressorium formation than others. The population is also dynamic; i.e., spores are continually passing to more advanced stages of development. It is possible that activity in the PA stage represents the reappearance of esterase prior to appressorium formation. The following support this view: (i) When the fungus was assayed on cellophane and glass, both the extent of appressorium formation and the percentage of esterase-positive germlings in the PA stage were lower than for spores incubated on apple, bean, and Paraplast. This suggests an association between activity in the PA stage and appressorium formation. (ii) An appressorium was considered formed when enlargement of the germ tube tip could be detected. Nearly all appressoria observed were esterase-positive. Thus, esterase-positive appressoria were observed in different degrees of appressorium development, suggesting that activity was present from the time of appressorium initiation.

The function of esterase activity in the PG and AP stages is not known. However, suggestions may be made based on knowledge of spore germination and function of the appressorium. The protoplast of the ungerminated spore of many fungi, including *V. inaequalis*, is enclosed by a rigid wall (13) which must be ruptured in the germination process. Bracker (2) demonstrated vesicle accumulation at sites of germination in sporangiospores of *Gilbertella persicaria* and suggested that germination, like hyphal tip growth, occurs by vesicular additions at the site of cell expansion (5, 7). K. M. Maeda (*personal communication*) observed apical vesicles in germinating conidia of *V. inaequalis*. Localization of enzymes at the site of germination, whether free in the cytoplasm or associated with vesicles or other cytoplasmic components, could aid in plasticizing or rupturing the wall layers and thus facilitate emergence of the germ tube.

The primary role of the appressorium is in its association with penetration of the host. The only mechanical barrier to penetration by *V. inaequalis* is the cuticle, since development of the fungal stroma is subcuticular.

The trend in pathology has been to consider penetration of the cuticle strictly a mechanical event (6, 14). However, the inability to demonstrate cutinolytic enzymes may be because synthesis occurs only at the time of penetration or at a specific stage of fungal morphology. A probably substrate for such enzymes would be cutin, which is composed of intermolecular esters of polyhydroxy fatty acids (10, 15). One fungus, *Penicillium spinulosum*, produces a cutin esterase and carboxycutin peroxidase (8, 9).

Maeda (13) demonstrated the degradation of apple cuticle at the site of penetration by *V. inaequalis*. She suggested that enzymic dissolution of the cuticle had occurred. Thus, the appressorial "infection sac" of *V. inaequalis* could be a site of localization of cuticle-dissolving enzymes, possibly of the cutin esterase type, since it or its contents contact the cuticle throughout penetration (13).

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