*Un*8 Allele for Loose Smut Resistance Associated with Necrosis in Embryos of Infected Barley

B. K. Gabor and P. L. Thomas

Agriculture Canada Research Station, 195 Dafoe Road, Winnipeg, Manitoba, Canada R3T 2M9.
Present address of the first author: Department of Plant Pathology, University of California, Riverside 92521.
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ABSTRACT

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The barley line CI 13662 carries the *Un*8 allele for resistance to loose smut. Inoculated florets of this line produce seeds from which adult plants are free from sporulation. Extensive necrosis was histologically observed in the embryos of inoculated seeds, but not in seed of cultivars in which resistance is conditioned by dominant alleles at three other loci. The stage of plant growth at which resistance was expressed was also different. The alleles *Un*3 and *Un*6, in the cultivars Jet and Conquest, appeared to confer resistance in the seedling stage. The allele *Un*, in the cultivars Trebi and Warrior, appeared to confer resistance in which penetration of the embryo was inhibited. Electron microscopy showed that the necrosis of cells of embryos of CI 13662 was accompanied by secondary wall thickening and vacuolization in the host and by necrosis of hyphae in the fungus.

Additional key word: *Ustilago nuda*.

Studies of the embryo infecting loose smut of barley (*Ustilago nuda* (Jens.) Rostr.) and of the closely related loose smut of wheat (*U. tritici* (Pers.) Rostr.) revealed that the time and site of the resistance reaction is dependent on the particular cultivar and pathogen strain involved. In susceptible hosts the number of infected embryos was usually found to be closely correlated to the number of adult plants with sporulation (2,6,10,16). In resistant hosts, fungal growth was found to be arrested at specific sites, e.g., the pericarp, the embryo, in the seedling stage, or at different stages of subsequent development (8,14). In some interactions deemed to be resistant, sporulation occurred in a small percentage of the adult plants (5). The effect of the fungus on the health of the plant ranged from little to severe, at any stage from the seedling to the adult (8,10).

Rasmusson and Mumford (17) examined embryos from inoculated florets of cultivars with the resistance genes *Un, Un*3, and *Un*6. They did not observe sporulation in the barley cultivar Jet (*Un*3 and *Un*6) grown from seed in which 36% of the embryos contained hyphae. In contrast, inoculated florets of the cultivar Trebi (*Un*) produced only 6% infected embryos, none of which produced adult plants with sporulation. A microscopic study of young seedlings of the cultivar Jet revealed that infected shoots failed to develop, while some plants recovered by producing noninfected tillers (12). The authors suggested that resistance was conferred by two mechanisms: a general resistance expressed before embryo infection (i.e., 50% embryo infection in the resistant cultivar Jet compared with 90% in the susceptible cultivar Newal) and a second mechanism expressed after embryo infection, approximately 3 wk after germination. Light microscopy of embryos of the cultivar Trebi revealed resistance occurring at the pericarp (19).

The object of this study was to compare histologically the resistance conferred by *Un*8 with that of *Un, Un*3, and *Un*6.

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MATERIALS AND METHODS

Two cultivars and one line of barley, each with known genes for resistance, were used as hosts: Warrior (CI 6991) probably is homozygous for the allele *Un* from Trebi (22), Conquest (CI 11638) possesses *Un*3 and *Un*6 from Jet (11), and CI 13662 has *Un*8 from Milton (25). The cultures used were 72-66, which is virulent (sporulates) on Conquest but not Warrior, and 72-146, which is virulent on Warrior but not Conquest (21,24). Neither culture is virulent on CI 13662; no known cultures are virulent on barley containing *Un*8 (25).

Barley was inoculated with teliospores at anthesis as previously described (23). At least 100 florets of each cultivar were inoculated with each race for each test. All plants were grown in growth cabinets and/or greenhouses.

Embryos from inoculated plants were extracted and examined using the "Scottish Method" described by Khanzada et al (7), with the following modifications. Complete separation of the embryo from the rest of the seed was facilitated by agitation in a 1-L beaker in water at approximately 50 C. The separated embryos were then flushed through a 10-mesh sieve with water at 5 C and caught on a 50-mesh sieve before dehydration in ethanol. After clearing, the embryos were quickly rinsed in distilled water to remove the lactophenol, transferred into glycerin for 2 hr, and mounted in glycerin for viewing. The blue stained mycelium was readily visible in the embryos at ×6 to ×40 magnification, allowing their unambiguous classification as infected or noninfected.

The outer epidermis of developing barley kernels was also examined at approximately 48-hr intervals for 12 days after inoculation. The developing kernels, freed of lemma and palea, were stained in a 0.01% solution of Calcofluor White M2R (Polysciences, Inc.) in distilled water for 3 min, rinsed in distilled water, mounted in distilled water on glass slides, and examined with a Zeiss incident light fluorescence microscope equipped with the HBO 50 light source, exciter filter BP 405/6, dichromatic beam splitter FT 425, and barrier filter LP 435.

Further examination of necrotic areas in the pericarp of inoculated CI 13662 required an alternate method of preparation because nonspecific staining occurred in sections prepared by the above method. Sections approximately 0.5 mm thick were cut by

hand with a razor blade through the necrotic areas of the kernels and then prepared by the method of Rohringer et al (18) (omitting boiling before fixing).

Mature seeds of Conquest and CI 13662 from florets inoculated with cultures 72-66 and 72-146, as well as seeds from uninoculated florets, were imbibed in distilled water for approximately 15 hr at room temperature. A 1.0-mm-thick longitudinal sample was cut from the growing point region of the embryos using a razor blade. Nonembryo material was trimmed away and the samples were fixed in 3% glutaraldehyde in 0.025 M phosphate buffer, pH 6.8, for 16 hr at 3 C, after vacuum infiltration for approximately 15 min to sink the samples to the bottom of the vials. Samples were washed in the phosphate buffer six times over the next 24 hr, then run through an ethanol dehydration series, and embedded in glycol methacrylate (13). Sections were cut with a glass knife, stained with 1% toluidine blue in 1% borax for 5 sec, and viewed with the light microscope.

Material for electron microscopy was fixed in glutaraldehyde and washed in phosphate buffer as first described. Samples were then postfixed for 5 hr in 2% osmium tetroxide, washed in distilled-deionized water for 2 hr before running through the ethanol dehydration series, followed by a propylene oxide:ethanol series to 100% propylene oxide, before embedding with Spurr resin mixture (20). Ultrathin sections were cut from the growing point region with glass knives. The sections were mounted on formvar and carbon-coated 100-mesh copper grids, stained with 5% uranyl acetate in 50% ethanol aqueous solution for 10 min, stained in lead citrate for 6 min, and then examined using a Phillips 420 electron microscope.

RESULTS

Figure 1 shows a whole embryo extracted from an uninoculated floret. An example of the distribution of hyphae in infected embryos of Conquest (*Un*3,6) is shown in Figure 2. There was no visible difference in the amount or location of hyphae in embryos of this cultivar infected with either culture 72-66 or 72-146.

Embryos of Warrior (*Un*) infected with 72-146 appeared the same as infected Conquest embryos. In contrast, only 3% of the embryos of Warrior inoculated with 72-66 contained hyphae and these embryos were usually only partially invaded by hyphae (Fig. 3).

With the exception of the partially infected embryos of Warrior, embryos containing hyphae appeared similar for all three hosts, until after the hyphae penetrated the scutellum. Hyphae growing beyond this point in CI 13662 (*Un*8) were associated with heavily stained cells in the vicinity of the embryo axis, the growing point, and sometimes in the primordial foliage leaves (Fig. 4). No instances of such heavy staining were observed in infected embryos of Conquest and Warrior (Figs. 2 and 3).

The observed relationship between embryo infection and sporulation in adult plants is recorded in Table 1. Sporulation was observed in adult plants of Conquest grown from seed infected with 72-66, and in adult plants of Warrior grown from seed infected with 72-146 but not in adult plants of CI 13662.

Yellow autofluorescence (AF) was observed in the pericarp under most appressoria. The AF regions varied in size from the intercellular spaces around a few cells to the entire contents of many cells. Varying numbers of necrotic cells were often associated with the AF regions. The AF and necrotic regions were detected as early as 5 days after inoculation. Cross sections through the necrotic regions revealed that AF and necrosis extended from the outer epidermis deep into the pericarp (Fig. 5). A comparison of the amount of AF and necrosis occurring in Conquest, Warrior, and CI 13662, inoculated with both smut cultures, revealed no quantifiable differences between any of the combinations. None of the uninoculated controls had either AF or necrosis.

TABLE 1. Relationship between embryo infection and sporulation, observed after inoculation of florets of two cultivars and one line of barley with teliospores of two cultures of *Ustilago nuda*

U. nuda culture	Percentage of embryos containing hyphae (E) and adult plants with sporulation (A)					
	Warrior		Conquest		CI 13662	
	E	A	E	A	E	A
72-66	3	0	51	49	81	0
72-146	84	90	50	0	58	0

Light microscopy of glycol methacrylate embedded and sectioned embryos of Conquest showed that the pathway of infection appeared to be the same for hyphae of both 72-66 and 72-146, but it was difficult to distinguish whether their growth was intercellular or intracellular. Hyphae were apparently able to penetrate from anywhere on the scutellum and invade the growing point region through the embryo axis. Hyphae were always found in the embryo axis region of infected embryos, but not always in the growing point. Neither necrosis nor any other adverse reaction was detected in host cells past the pericarp (Fig. 6).

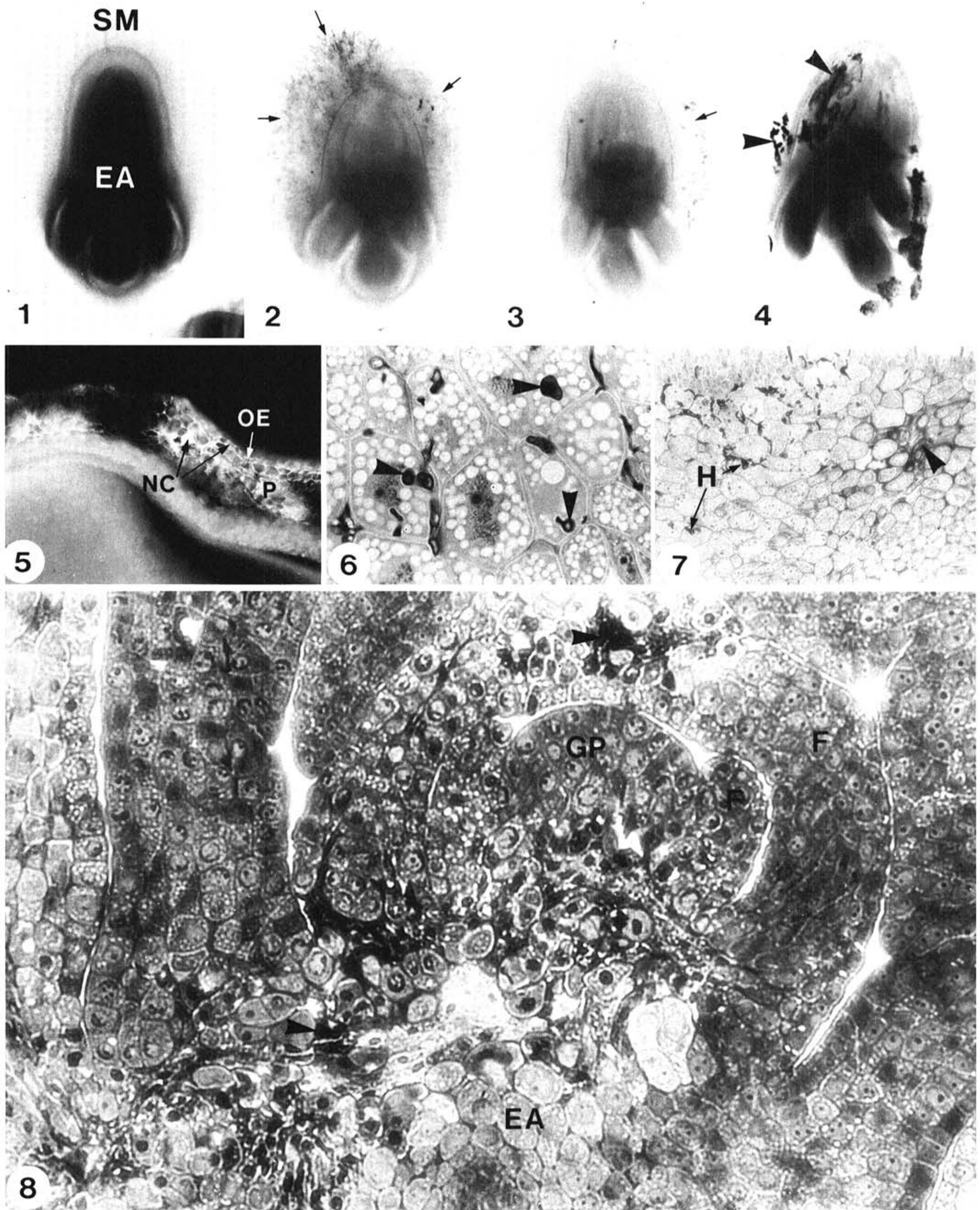
The embryo tissues in glycol methacrylate sections of CI 13662 infected with 72-66 had the same appearance as those infected with 72-146. Hyphae were observed to penetrate from anywhere on the scutellum as in Conquest. Again it was not obvious whether hyphal growth was intercellular or intracellular. However, unlike Conquest and Warrior infected with avirulent cultures, embryos of CI 13662 contained necrotic cells in the area where the embryo axis is attached to the scutellum (Fig. 7). Similarly reacting cells were also observed in the growing point and in the foliage leaves, sometimes appearing to stunt the development of these tissues (Fig. 8). The location of these necrotic cells corresponds to the intensely stained regions observed in whole embryos (Fig. 4). Hyphae could not be identified in or between the necrotic cells but were observed in apparently healthy cells near the sites of the reaction (Fig. 7). The number of affected cells observed in each necrotic area varied from a few to over 50. The contents of the distorted and collapsed necrotic cells were obscured by intense staining (Figs. 7 and 8).

In electron micrographs of infected embryos of Conquest the hyphae were found to be mostly intracellular, and they were surrounded by a "sheath" of material similar in appearance to and continuous with the cell wall (Fig. 9). An electron dense layer (EDL) was observed between the sheath and the fungal cell wall. Up to eight distinct layers, counting the EDL, were visible between the host and hyphal plasmalemmas, but distinguishing between host and hyphal cell wall material in these layers was not possible (Fig. 10A and B). Hyphae appeared viable because lipids, nuclei, and organelles could be identified (Fig. 9). There was no apparent degradation of contents in host cells containing the fungus.

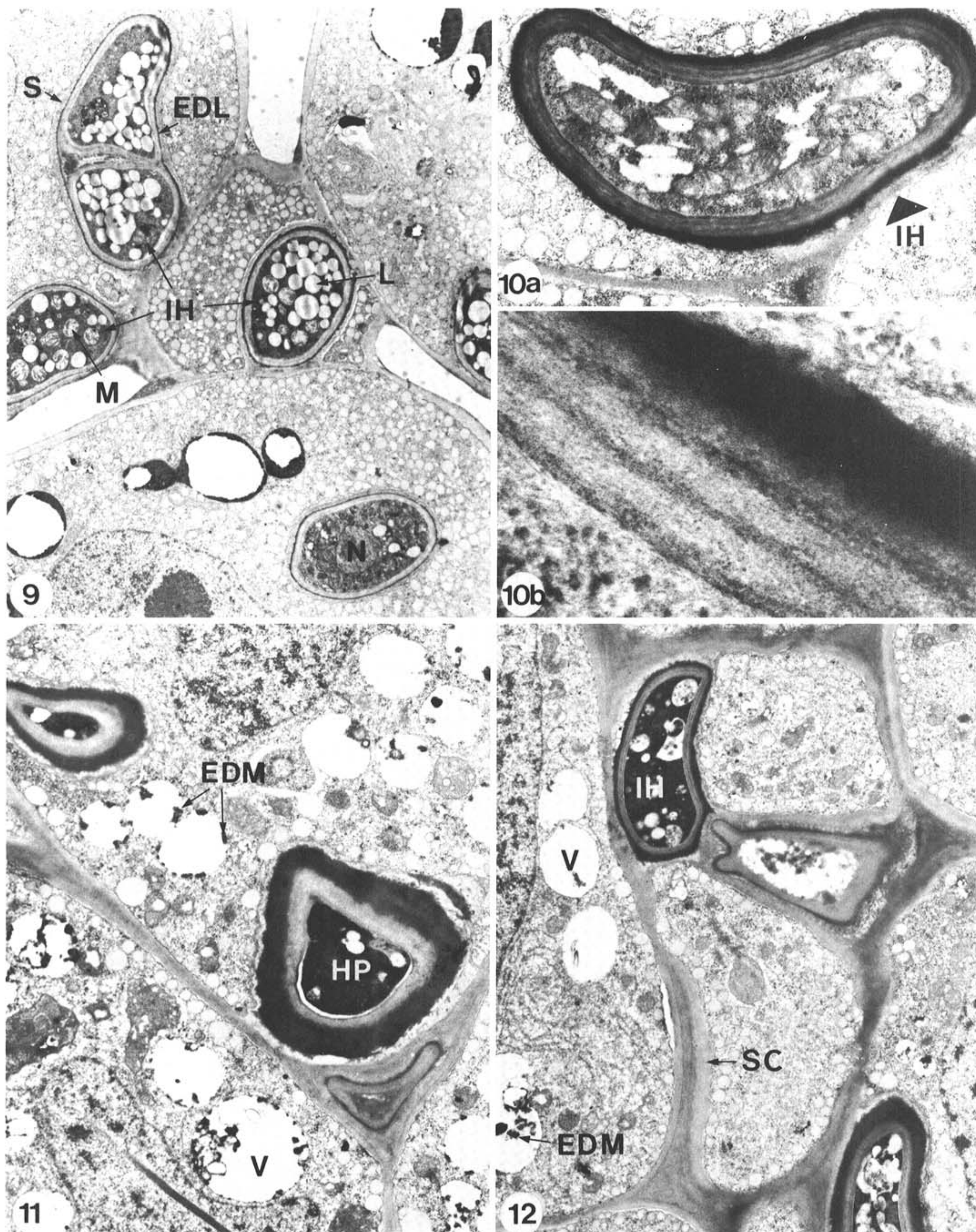
Hyphal growth was also mainly intracellular in embryos of CI 13662, and again the hyphae were surrounded with the characteristic sheath mentioned earlier (Figs. 11 and 12), but in CI 13662 the EDL was much thicker than in Conquest (Fig. 11). Some hyphal cells in embryos of CI 13662 appeared necrotic because organelles were difficult to identify and the protoplasm was electron dense (Fig. 11). Hyphae in the vicinity of the necrotic host cells appeared to have internal degeneration (Fig. 12) compared with hyphae in Conquest (Fig. 9). Hyphae were not always apparent within the areas of necrotic cells in CI 13662 (Fig. 14). Large vacuoles and electron-dense particles were observed in cells of CI 13662 in the affected regions (Figs. 11–14). Secondary thickening of host cell walls occurred in cells next to necrotic cells as well as in and beside heavily vacuolated cells (Figs. 12–14). Increased vacuolization of host cells and extensive secondary thickening of cell walls was not apparent in noninfected embryos of CI 13662 or in infected embryos of Conquest.

DISCUSSION

Resistance in each of the two cultivars and the barley line appeared to take place at different stages of plant growth and, therefore, may have been the result of different mechanisms. First, Warrior inoculated with the avirulent culture (72-66) had a low



Figs. 1–8. 1–4. Whole extracted barley embryos. **1,** Uninoculated Conquest. No hyphae present in the scutellum (SM) or embryo axis (EA). **2,** Conquest infected with 72-66. Hyphal penetration (arrows) from all sides of the scutellum with growth into the embryo axis. **3,** Warrior infected with 72-66. Hyphal penetration (arrow) into the embryo is slight and the mycelium is limited to a portion of the scutellum. **4,** CI 13662 infected with 72-146. The incompatible reaction (arrowheads) extends into the foliage leaves. Magnification: All ×20. **5,** Freehand section of kernel of Conquest inoculated with 72-66. Autofluorescence and necrotic cells (NC) occur in the outer epidermis (OE) and pericarp (P). Magnification: ×50. **6–8,** Light micrographs of glycol methacrylate embedded embryos. **6,** Conquest, infected with 72-146. Hyphae in the scutellum appear to be mainly intracellular (arrowheads), with no adverse reaction visible in host cells. (×500) **7,** Scutellum of CI 13662 infected with 72-146. Some hyphae (H) are outside of the zone of incompatibility (arrowhead). (×125) **8,** CI 13662 infected with 72-66. The incompatible reaction (arrows) is in the embryo axis (EA), the growing point (GP), and the young foliage leaves (F). (×350)



Figs. 9–12. Electron micrographs of Spurr embedded embryos. **9,** Conquest infected with 72-66. Viable intracellular hyphae (IH) containing nuclei (N), mitochondria (M), and lipid bodies (L). Hyphae are surrounded by a sheath (S) and an electron dense layer (EDL). (×5,300) **10A,** Conquest infected with 72-146. Apparently viable hyphae (IH) in an apparently viable host cell. At least eight distinctive layers are present between the host and fungal plasmalemmas. (×17,000) **10B,** Greater magnification of fungal wall layers (72-146 in Conquest). (×122,000) **11,** CI 13662 infected with 72-146. Thick EDL surrounds the hyphae. Hyphal protoplasm (HP) is electron dense, indicating necrosis. Extensive vacuolization (V) has occurred in host cells, with electron dense material (EDM) present within the vacuoles. (×12,000) **12,** CI 13662 infected with 72-146. Intracellular hyphae (IH), secondary thickening of host cell walls (SC), and large vacuoles (V) are present. (×8,000)

level of embryo infection (3%) compared with the high level (84%) obtained with the virulent culture (72-146). This indicates that resistance was expressed before embryo infection. The presence of some hyphae in 3% of the embryos indicated that resistance in the pericarp was not complete, but because no sporulation was observed in the adult plants, resistance must also have been expressed after embryo infection.

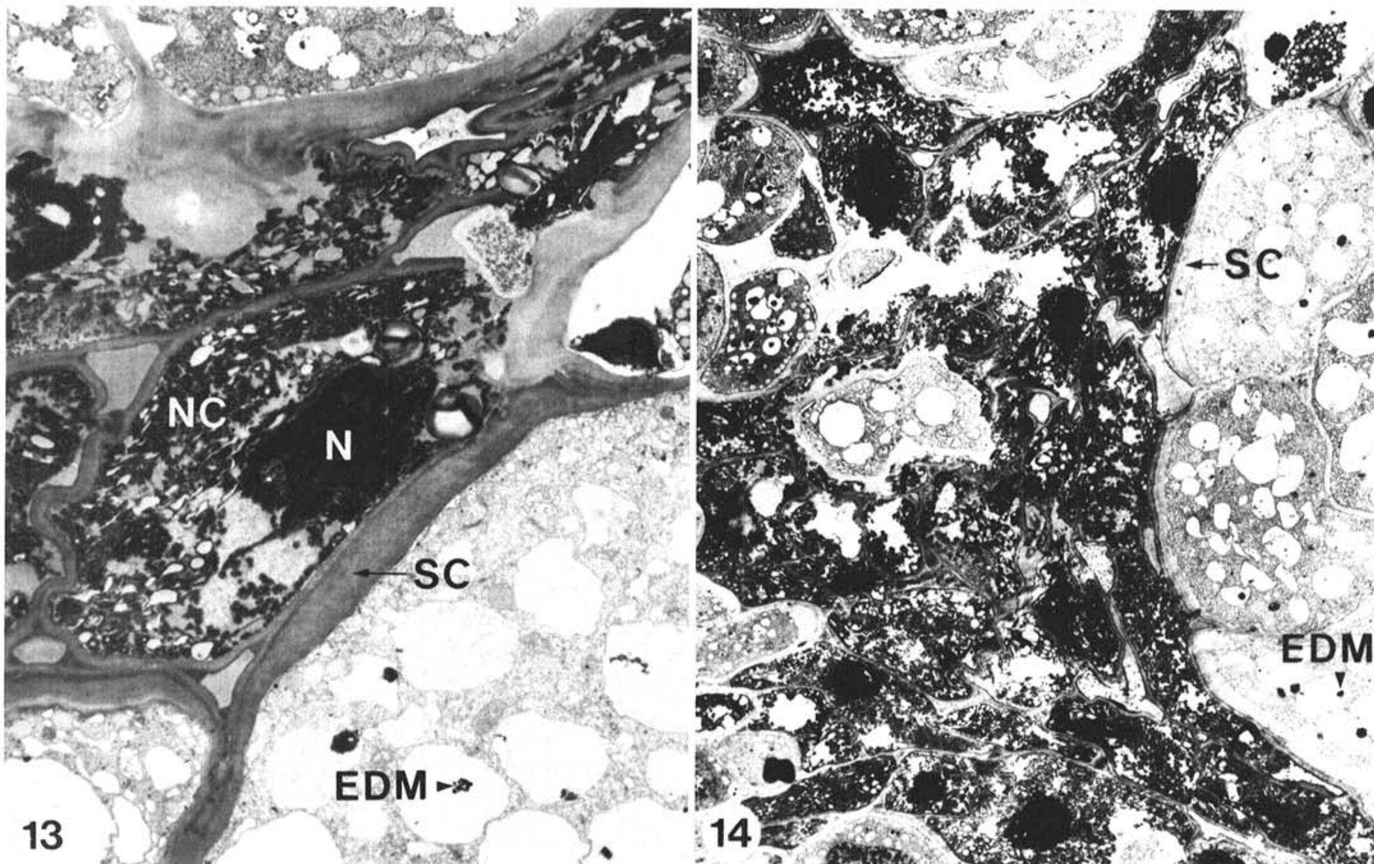
It was previously thought that the resistance of Trebi, which carries the same resistance gene (*Un*) as Warrior, was related to a necrotic reaction of the cells of the epidermis and pericarp in response to hyphal penetration (19). However, this necrotic reaction was found in Warrior inoculated with both the avirulent and virulent cultures as well as in Conquest and CI 13662 inoculated with both smut cultures. This suggests that necrosis at pericarp penetration may not play a role in resistance to loose smut in these cultivars, although necrosis in reaction to avirulent cultures may have been related to the unnaturally high concentration of inoculum used.

A second type of resistance was expressed after embryo infection in Conquest (*Un*3,6) inoculated with 72-146. This combination produced 50% of embryos with hyphae but gave no adult plants with sporulation (Table 1). Embryo infection developed the same in Conquest as in the parent (Jet) from which it derived its resistance. In Jet, the infected main shoot fails to develop and later tillers carry healthy spikes (12).

A possible third type of resistance was revealed in a comparison of the reaction of the extracted embryos of the two cultivars and one line (Figs. 1–4). Hyphae in Conquest and Warrior appeared compatible with the host tissue as was described in previous studies on susceptible cultivars inoculated with virulent races of loose smut (1,15). Yet in CI 13662 (*Un*8), after the hyphae passed the scutellum, the reaction became incompatible (Fig. 4). Very little is known about this type of incompatible reaction. Batts and Jeater (2) observed one embryo of a scutellum-susceptible, but field-resistant wheat cultivar in which cells in the region between the scutellum and embryo axis appeared swollen and brown in color. Hyphae were not found in the growing point region of these embryos. Wells and Platt (27) reported that susceptible barley cultivars had embryos that were partially or completely destroyed by the fungus, but details of the type of destruction were not given. Therefore, this study provides the first detailed report of the third type of reaction in a resistant barley line (CI 13662).

The incompatible reaction observed in CI 13662 appeared to be hypersensitive in nature. The presence of the fungus was always associated with necrosis of host cells in and around the growing point. Some hyphae in this region were surrounded by a thickened EDL and also appeared to be necrotic (Fig. 11), but whether this was due to normal senescence or related to the presence of the thick EDL is not known. All hyphae in the growing point region of CI 13662 appeared to be degenerating (Figs. 11 and 12), when compared with hyphae in Conquest (Fig. 9). Whether this was indicative of eventual death of all hyphae or whether they were capable of growing past this necrotic reaction, with resistance being conferred in the seedling stage, was not examined. If hyphae can grow past the necrotic areas, the reaction would not conform to the definition of hypersensitivity. Therefore, classification of this reaction should be held in abeyance until a thorough examination of developing seedlings verifies the exact site where hyphal growth ceases. Not all cells containing hyphae were necrotic (Figs. 11 and 12), and necrotic cells without hyphae were observed (Fig. 14), suggesting that direct contact with hyphae may not be necessary for necrosis.

Extensive secondary thickening of host cell walls was observed adjacent to necrotic cells and to heavily vacuolated cells of CI 13662 (Figs. 12–14). Secondary thickening of host cell walls can occur as a defense mechanism in response to fungal penetration (26). Penetration of hyphae through the secondary thickening of these walls probably would be difficult if not impossible.



Figs. 13 and 14. 13, CI 13662 infected with 72-146. Necrotic host cells (NC) lacking membrane bound organelles. The host nucleus (N) is very electron dense. Extensive secondary thickening (SC) has occurred in the walls of apparently viable, extensively vacuolated, adjacent cells. (×5,000) **14,** CI 13662 infected with 72-146. Large necrotic areas are present in the growing point. Secondary thickening of cell walls is obvious in heavily vacuolated cells. (×1,500)

Previous studies have suggested that hyphal growth is mainly intercellular in the embryo (9,15). However, growth was mainly intracellular in the scutellum of Conquest (Fig. 6) as well as in the growing point region of Conquest and CI 13662 (Figs. 9 and 12).

Electron microscopy showed that intracellular hyphae of other smut species were surrounded by a sheath (4). In this study, hyphae in Conquest and CI 13662 embryos were also always surrounded by a sheath. A distinct EDL, not reported by Fullerton (4), was observed adjacent to the sheath (Figs. 9 and 11). The thickness of the EDL varied from approximately 60 nm in Conquest inoculated with 72-66 (Fig. 9), to an extreme thickness of approximately 500 nm in CI 13662 inoculated with culture 72-146 (Fig. 11). Fullerton (4) suggested that sheath formation may cause hyphal degeneration. This study indicates that the thickness of the EDL may also be associated with hyphal degeneration.

Cytochemical studies of the wheat stem rust fungus (*Puccinia graminis* f. sp. *tritici* Erikss. & E. Henn.) in a compatible host revealed up to six possible layers in the haustorium mother cell walls and four layers in the hyphal walls (3). At least eight distinct layers were visible between the host and hyphal plasmalemmas in both Conquest and CI 13662 infected with culture 72-146 (Fig. 10A and B). The layer constituting the sheath appears to be a continuation of the host cell wall, but distinction between host and fungus composition would require cytochemical tests.

Approximately one-third fewer adult plants were produced from the inoculated seeds of CI 13662 than were expected from the proportion of noninfected seeds detected in the embryo test. This may be indicative of death of infected embryos before or soon after germination. However, the number of embryos examined and the number of seeds germinated were too small to allow a correlation of death with hyphal presence. The death of embryos from loose smut infection has been referred to as a false type of resistance (14). Nevertheless, cultivars with this type of resistance are of practical value because the crop remains free of smut in subsequent generations.

The *Un*8 gene in CI 13662 appears to confer resistance at the embryo stage. Resistance is probably associated with the necrotic host cell reaction observed in the presence of the fungus in the region around the attachment of the scutellum to the embryo axis, and in the embryo axis, the growing point, and the foliage leaves. Whether this necrotic reaction is indicative of death of all hyphae, or whether further resistance is conferred at a later stage, is not known.

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