THE DEVELOPMENT OF APPRESSORIA, HAUSTORIA AND SECONDARY HYPHAE DURING THE PRIMARY INFECTION OF WHEAT AND BARLEY BY ERYSIPHE GRAMINIS

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ABSTRACT

THE DEVELOPMENT OF APPRESSORIA, HAUSTORIA AND SECONDARY HYPHAE DURING THE PRIMARY INFECTION OF WHEAT AND BARLEY BY ERYSIPHE GRAMINIS

By Shawki S. Masri

The differential response of the various stages of primary infection of powdery mildew (E. graminis) to different environmental conditions suggests that the primary infection is made up of several stages. These are: 1) germination, 2) formation of appressorial initials, 3) maturation of appressoria, 4) development of the haustorial body, 5) the formation of secondary hyphal initials, and 6) the formation of functional secondary hyphae. At the optimum conditions of every phase, the parasite population was synchronized; that is, most units of the population reached each phase within a 4 hr period. The host was penetrated around the 10th hour (hr) after inoculation. The haustorial bodies appeared 16 hr after inoculation and full-sized haustoria were detected 34-36 hr after inoculation.

Prior to infection, succinate dehydrogenase activity was detected only in the parasite. Two hr after penetration, succinate dehydrogenase activity was detected on the surface of the young developing haustoria. This indicated that either the enzyme moved from the parasite to the host or its production was stimulated, by the parasite, in the host.

Through the use of various strains of barley with major genes for resistance, it was demonstrated conclusively that only functional secondary hyphae should be taken as a criterion for the establishment of a successful host-parasite

relationship. In susceptible, moderately resistant and highly-resistant hosts studied, the data showed that the number of functional secondary hyphae on the surface of the host equaled the number of haustoria formed inside the host. Hence, either the functional secondary hypha or the haustorium could be taken as a criterion for the establishment of primary infection.

All major genes for disease resistance exhibited more than one effect in their mode of action and, therefore, these genes can be considered pleiotropic. The various ways and means by which these genes conferred resistance to their respective hosts were: 1) exclusion of the pathogen from the host; 2) delay in early haustorial development; 3) distortion of some haustoria; 4) destruction and distortion of the majority of haustoria 5 days after inoculation; and 5) suppression of fungus sporulation after the establishment of primary infection thereby preventing the occurrence of repeating disease cycles.

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By

Shawki S. Masri

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Dedicated To My

Father and Mother

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INTRODUCTION.--Powdery mildew of wheat (caused by Erysiphe graminis f. sp. tritici) has been reported from nearly all wheatgrowing countries of the world (8,20,21). Although almost ubiquitous in distribution, powdery mildews cause less spectacular losses than those caused by several other important groups of plant pathogens (53). Nevertheless powdery mildew is ranked second to rust in its economical importance (20,24,25).

The purpose of this investigation was to: 1) define the various component phases that make up the process of primary infection; 2) synchronize the parasite population in its advance through the various stages of primary infection; 3) study the formation and development of haustoria in susceptible, moderately resistent and resistant hosts in an attempt to gain an insight to host-parasite interaction; 4) examine the histochemical changes that accompany the early stages in the development of haustoria in order to determine whether or not there is a detectable interaction between the host and the parasite in the initiation of infection; and 4) determine useful criteria for the establishment of a compatible host-parasite relationship.

The ectoparasitic nature of most powdery mildews and the availability of different strains of barley with various levels of resistance made this host-parasite combination a very useful system for investigating the several aspects of host-parasite interaction.

LITERATURE REVIEW.--A rather voluminous literature is available on the various powdery mildews. A great deal of emphasis has been placed on the different environmental factors such as temperature, relative humidity (RH) and light as they affect conidial germination and the overall development of the disease. It was not until recently, however, that the infection process was considered as being made up of several distinct phases, each subject to experimentation, rather than a single process (27,30). For experimental purposes, these stages were considered to be: 1) germination, 2) formation of appressorial initials, 3) maturation of appressoria, 4) formation of haustoria, and 5) development of secondary hyphae (30).

Yarwood et al. (52) reviewed temperature relations of powdery mildews in general and concluded that the optimum ranges from about 11-28 C and averages about 22 C, while others (8, 39) reported maximum development at 17-19 C and 15-20 C. Dale (12) and Cormack et al. (10) noticed absence or scarcity of powdery mildew near the ground and it was proposed (53) that this was due to high temperatures. Delp (14) investigated rather extensively the effect of temperature on the grape powdery mildew fungus and reported that the minimum leaf temperature for conidial germination was below 6 C, but infection did not take place below 7 C. Growth was very slow at 7 C with more than 32 days from inoculation to sporulation. The growth rate increased with corresponding rise in temperature to a maximum of about 26 C. It was

concluded that temperature is the primary factor which limits the growth of powdery mildew of grapes.

Nair and Ellingboe (33) reported that: "Germination of conidia was insensitive to light, but was dependent on temperature and relative humidity. Maximum germination was obtained by an amphidirectional change in relative humidity accompanied by a unidirectional change in temperature from 17-22 C." Although germination occurred at 17 or 22 C and 65 or 100% RH, the most favorable temperature for the maturation of appressoria was 22 C (30). These results were verified in a later study (27).

The relation of RH to disease has been one of the most controversial aspects of our knowledge of powdery mildews (53). Powdery mildews have been classified as dry-weather fungi (50). The germination of Erysiphe polygoni (5, 50) and Erysiphe graminis (8) occurred in the absence of water or at RH approaching zero. Other workers (9, 21) failed to get any germination of Erysiphe graminis below 85% RH. Free water may be necessary for grape mildew (Uncinula necator) development (18) and development of other mildews is limited by low RH (18). Yarwood (53) correlated the importance of powdery mildews on the same host species in different regions with the rainfall distribution. The powdery mildews of peach, apple, rose, cucurbits, beans, peas and grapes are commonly severe with rainfree summers. In fact, it was suggested (8, 51) that mildew development is checked by sprinkling infected plants with water, and Yarwood (51) even

proposed control of powdery mildew with a water spray. Relative humidity close to 100% gave better germination of E. graminis f. sp. tritici than did 65% RH (32).

A stimulatory effect of light on the germination and development of mildew has been reported (8, 38, 40, 48). Yarwood (48) observed that several powdery mildews gave higher germination percentages in light than in darkness. Light is essential for normal mildew development (46). With increase in day-length, vigorous host plants were produced and better development of mildew resulted (38).

Work with the rusts also emphasized the importance of light on germination and overall development of the disease. Sharp et al. (40) found that germination and appressorial development of urediospores of wheat stem rust (Puccinia graminis f. sp. tritici) occurred equally well over a range of 15.5-24 C and at light intensities below 300 foot-candles (ft-c). Post-appressorial development was favored by a higher temperature and light intensity than that which favored germination and appressorial formation. Generally, a reduction in light intensity or total darkness after inoculation reduced the prevalence of rust infection (17, 22, 44). Post-appressorial development was greatest at 29.5 C and at light intensities greater than 500 ft-c (40). When appressoria were exposed to natural light of 1500 ft-c or more at about 29.5 C, they produced the substomatal vesicle and completed the infection process (40). Whereas the effect of light was emphasized by several

workers, Rowell *et al.* (37) also working with *P. graminis tritici* found that under conditions of dew at 21 C and little or no light, urediospores germinated during the first 4 hr and a majority of the appressoria developed in the next 4 hr.

The effect of darkness and low light intensities on mildew development was studied by many workers. While Cherewick (8) reported slight or no effect on development of *E. graminis* f. sp. *tritici* when plants were put in darkness after inoculation, Yarwood (49) observed a definite reduction in development under the same conditions with *Erysiphe polygoni*. A more luxurious development of strawberry mildew in shade than in full natural light has been observed (13). Part of the favorable effect of shade could be due to reduced temperature and increased RH and of the 2, temperature is considered more important (53). Yarwood (53) believed that mildew severity on plants increases with duration of light up to a point (which is not defined) and then decreases, and that light may affect powdery mildew through its effect on the host. The effect of light on wheat powdery mildew (*E. graminis* f. sp. *tritici*) varied with the stages of primary infection (30). Whereas germination and formation of appressorial initials were light independent, the maturation of appressoria was light sensitive. These findings were verified by a later study (27) where it was found that the maturation of appressoria was light sensitive

and inhibited by darkness and high light. The optimum light treatment for this stage of infection was 240 ft-c applied 2 hr after inoculation.

Powdery mildews, like rusts and many other pathogens, penetrate plants on which they will make no further growth (11, 47). A fine penetration tube emerges from the center of the appressorium and grows into the lumen of the cell (11, 40, 41). It has been repeatedly reported (1, 43, 47) that certain fungi penetrated resistant varieties as easily as susceptible varieties.

The occurrence of special stimuli for penetration in susceptible hosts can be questioned (1, 43). According to Corner (11), the mature appressoria of powdery mildews penetrate the host and produce haustoria in the host epidermal cell. Penetration begins 24 hr after inoculation at about 20 C and 100% RH. The penetration through the cell wall by a fine infection peg below an appressorium has been regarded as an entirely mechanical process (6). Failure to penetrate could have been due to: 1) the lack of infection pegs, 2) the mechanical toughness of the cuticle and epidermal cell wall, and 3) the poor attachment of the appressoria and consequent failure to take the back pressure of penetration (16). The resistance of older barley plants to E. graminis f. sp. hordei has been attributed to the abortion of the infection peg due to the hardness or thickness of the cuticle and epidermis (20). After penetration, a

haustorium is formed which is restricted to the epidermal cells in most powdery mildews. A sheath has been shown to be present around the haustorium of E. graminis (4, 15, 42). Smith (42) believed it represented cell-wall debris which accumulated around the haustorium at the time of penetration. According to Ehrlich and Ehrlich (15), Hirata and Kojima in 1962 proposed that the body of the sheath consisted of metabolic products of the host which accumulate around the haustorium via osmosis, and suggested that the sheath is important in establishing parasitic relationships and in preventing death of the host cell, but the mechanism of its action is uncertain. It was also observed (15) that the host plasma membrane invaginates during penetration, but the sheath boundary is not simply the host plasma membrane but is a membrane partly of fungal origin, or a special membrane produced by the host in response to the presence of the pathogen. A recent study (4) indicated that the sheath membrane is continuous with the host ectoplast and the organization of the haustorial protoplast is similar to that of many plant and fungal cells.

The formation of a functional haustorium is the crucial step in the development of a compatible host-parasite relationship. Corner(11), working with resistant and immune hosts, observed that the development of powdery mildew is arrested at the papillae stage before the haustoria are formed. Physiological resistance was attributed to death of mesophyll

cells adjacent to the epidermal cell in which the haustoria are formed (8, 11). White and Baker (47) used host plants with varying degrees of resistance. When susceptible varieties of barley were used, abundant development of mycelium on the leaf surface took place. There were 20-30 haustoria in the epidermal cells beneath each colony. With semi-resistant varieties, the infection site was surrounded by a zone of dead host tissue. The mycelium was well developed in each colony, but was less dense than in the susceptible host. There were 10-15 haustoria in the epidermal cells beneath each colony. In the resistant variety Goldfoil, the mycelium was restricted in development and a few conidial branches were produced by each colony. There were 5-12 haustoria in the epidermal cells beneath each colony. With highly-resistant varieties, mycelial development was very limited. Occasionally, 1 conidial branch was formed by a colony. Each infection court contained 1 or 2 haustoria in the epidermal cells. When rye, oats and certain wheat varieties were inoculated with E. graminis f. sp. hordei, germ tubes, appressoria and short infection papillae were formed, but there were no haustoria.

With almost every aspect of the powdery mildews, there is a great deal of controversy. Early work (8) with E. graminis indicated that the organism is homothallic, while later reports (35, 36) asserted heterothallism. Hiura (23) demonstrated hybridization between E. graminis f. sp. hordei

and E. graminis f. sp. tritici on wheat, but ascopsores from such crosses failed to germinate.

Plants infected with powdery mildews displayed pronounced increases in respiration, but these respiratory changes were not detected until 6 days after inoculation (2, 7). Infection with E. graminis stimulated respiration of areas of barley leaves adjacent to the cells invaded by the fungus (2, 7).

A high concentration of acid phosphatase was shown to be associated with the haustoria of powdery mildew on barley (3). It was suggested that these enzymes play a role in the utilization of phosphorylated intermediates by obligate parasites. An increased number of isozymes of acid and alkaline phosphatase, succinate dehydrogenase and malate dehydrogenase was reported in a recent study of rust-infected bean plants (45). The activity of acid phosphatase decreased after infection whereas a small increase in peroxidase activity was detected. Malca et al. (26) observed that the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase increased in mildewed barley leaves at the time of or shortly after the appearance of symptoms, and continued to increase as the disease developed. However, part of the increased activity was due to enzymes contributed by the fungus.

MATERIALS AND METHODS.--Maintenance of stock culture.--Two strains of mildew were used. The strain of E. graminis f. sp. tritici (cause of powdery mildew on wheat) was collected in Michigan. The strain of E. graminis f. sp. hordei (cause of powdery mildew on barley) was CR-3 (59.1), obtained from Dr. J.G. Moseman.

Little Club wheat (Triticum compactum Host) and Manchuria barley (Hordeum vulgare L.), on which the mildew cultures were maintained, were grown in 4-in. pots and inoculated when 7-8 days old. Inoculations were made daily toward the end of the light period by shaking conidia onto the leaves of the host plants. Inoculated plants were kept under incandescent bulbs (50 ft-c) and cool, white fluorescent tubes (400 ft-c) with a total light intensity of 450 ft-c (at the leaf surface level) over a 15-hr day. The temperature was maintained at 18 $\pm$ 2 C and the RH fluctuated between 60-70% during the day and near 100% at night. Mildew pustules were visible in 3 days, and an abundant supply of good quality conidia was available 6 days after inoculation (Fig. 1A).

Inoculations of host plants.--The "rolling method" (31) of inoculation, whereby conidia were applied by cotton swabs onto the host plants, provided a rather uniform distribution of single conidia on a leaf, and the means for the study of primary infection on a quantitative basis. All observations of conidial germination, appressorial initials, and

mature appressoria were made on inoculated 5 or 6-day-old plants of wheat or barley, grown singly in 2-in. pots. About 2.5 cm of leaf surface was inoculated on each single plant with 40-130 conidia per cm. Heavier inoculations with as many as 500 conidia on 1-cm-leaf surface were needed in some experiments. Inoculations were made on either the upper or lower side of the leaf. The lower side was always inoculated for experiments in which the epidermal layer was peeled off for closer examinations, because of the ease with which the lower epidermis can be peeled. Mildew susceptible Little Club wheat and susceptible Manchuria barley were used. Also used were 4 additional barley strains each of which possessed a major gene for resistance backcrossed into the variety Manchuria. The pedigrees of the 4 strains were as follows: 1) Algerian C.I. 1179 ($\underline{Ml}_a$) X_7^4Manchuria C.I. 2330 (crossed to Manchuria 4 times and then selfed 7 times); 2) Goldfoil C.I. 928 ($\underline{Ml}_g$) X_9^4 Manchuria C.I. 2330; 3) Kwan C.I. 1016 ($\underline{Ml}_k$) X_8^4 Manchuria C.I. 2330; and 4) Psaknon C.I. 6305 ($\underline{Ml}_p$) X_7^4 Manchuria C.I. 2330. The genes conditioning resistance in these varieties have been designated $\underline{Ml}_a$, $\underline{Ml}_g$, $\underline{Ml}_k$, and $\underline{Ml}_p$, respectively (28).

Control of environmental conditions.--The various environmental conditions of temperature, RH and light were obtained with Sherer Gillett, Model No. CEL 512-37 growth chambers.

In all experiments, inoculated plants were incubated

for 1 hr at 18 C, 100% RH with or without light depending on the experiment. An accuracy of ± 1 C temperature and ± 3% RH control was obtained in these chambers. Light was supplied by 60-watt incandescent bulbs and cool, white fluorescent tubes. The light treatments used and their respective intensities were:

1) darkness(0 ft-c)
2) three 60-watt incandescent bulbs (25 ft-c)
3) six 60-watt incandescent bulbs (50 ft-c)
4) three 60-watt incandescent bulbs (25 ft-c) and 2 fluorescent tubes (215 ft-c) - a total of 240 ft-c (designated as the low light treatment)
5) six 60-watt incandescent bulbs (50 ft-c) and 22 fluorescent tubes (2750 ft-c) a total of 2800 ft-c (designated as the high light treatment)
6) six 60-watt incandescent bulbs (50 ft-c) and 11 fluorescent tubes (1375 ft-c) - a total of 1425 ft-c.
7) three 60-watt incandescent bulbs (25 ft-c) and 11 fluorescent tubes (1375 ft-c) - a total of 1400 ft-c.

Temperature and RH treatments ranged from 60-90 C and 55-100% RH respectively depending on the kind of experiment and the effect investigated. However, the most frequently used temperatures and relative humidities were 65 or 72 C, and 65 or 100% RH.

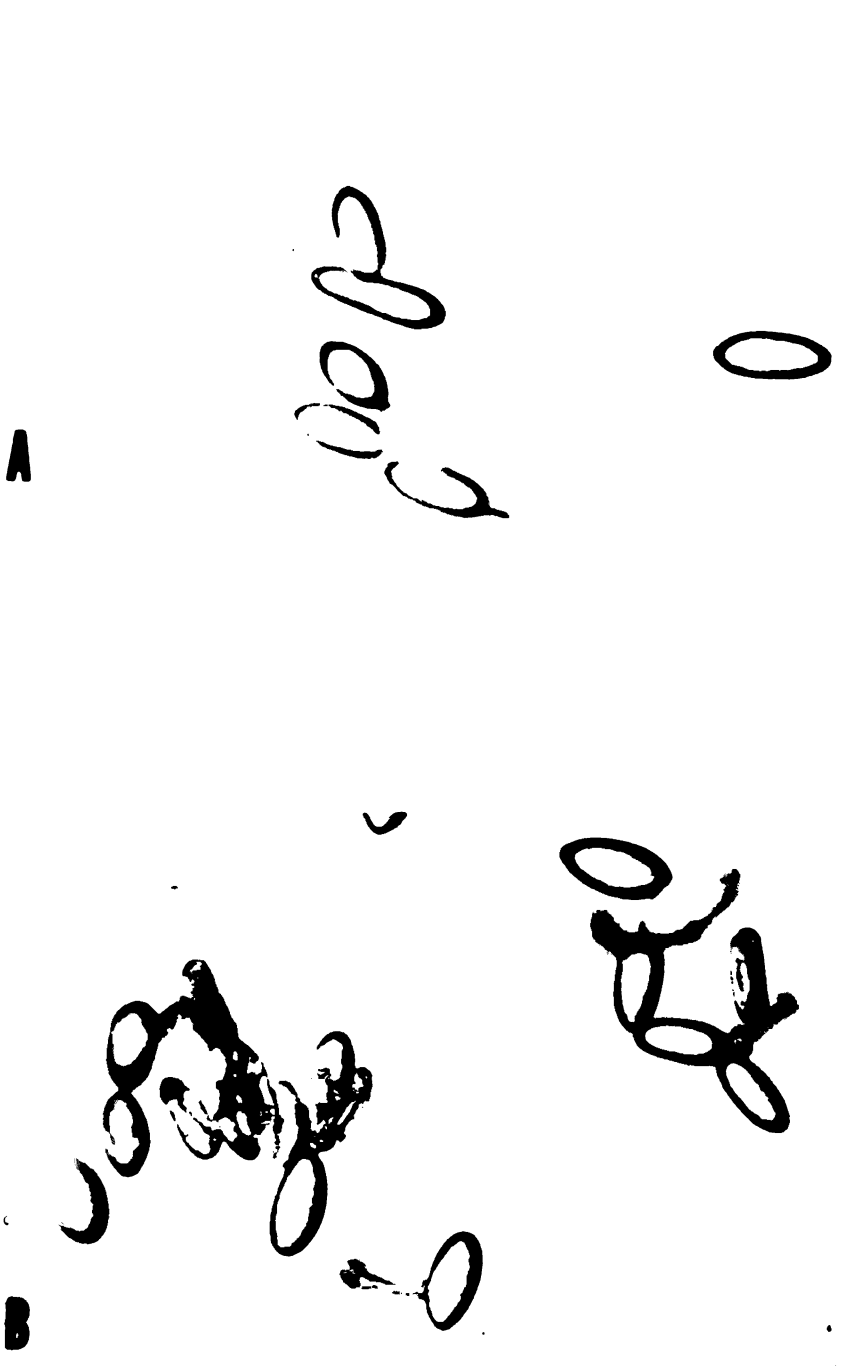
One hygrothermograph, calibrated with a sling psychrometer

every 2 weeks, was installed inside each chamber.

Examination of external structures.--Plants were removed from the growth chambers after receiving the desired treatment and at regular intervals, 1 plant at a time. The inoculated portion of the leaf was cut into sections approximately 1 cm long and examined under a light microscope at 125x magnification. The various fungal structures were examined, with as little disturbance as possible, on the surface of the host plant. All conidia at the various stages of development were counted and recorded. Individual plants were then discarded. The external structures observed were: 1) germ tubes, 2) appressorial initials, 3) mature appressoria, 4) secondary hyphal initials, and 5) functional secondary hyphae.

Examination of internal structures.--In most ectoparasitic powdery mildews, the only internal structure is in the epidermis. This structure, the haustorium, serves to absorb nutrients from the host. Since it was hard to examine this relatively transparent structure in epidermal strips, the haustoria were stained to aid in distinguishing them from the rest of the cell cytoplasm. The stain used was acid fuchsin in lactophenol. Epidermal strips (lower epidermis) were removed directly into a drop of acid fuchsin on a glass slide, covered with a cover slip, and examined under the microscope. Low power (125x), high power (594x), bright field, and phase-contrast microscopes were used. Haustoria stained a blue color. The time needed for examining a 1-cm-section of the

Fig. 1. Conidia of *E. graminis* f. sp. *tritici* harvested A) 5 and B) 7 days after inoculation. (575x)


A
B

lower epidermis varied from 8-20 minutes depending on the stage of development of the haustoria. For some experiments, it was necessary to examine secondary hyphae on the intact leaf and haustoria in the same leaf section after stripping the epidermis. This was more time-consuming and required 30-45 minutes.

Histochemical tests.--The activities of acid phosphatase, succinate dehydrogenase, and glucose-6-phosphate dehydrogenase were tested. Succinate dehydrogenase was chosen because it is involved in the Krebs cycle; glucose-6-phosphate dehydrogenase was chosen because it is a part of the hexosemonophosphate shunt; and acid phosphatase was chosen because it is produced in the host in response to injury. Increased acid phosphatase activity was also reported (3) in and around the haustoria.

The histochemical methods used were:

1) Takeuchi and Tanoue modified lead nitrate method for acid phosphatase (34).
2) Gomori improved histochemical technique for acid phosphatase (19). In this method, the enzyme reacts with its substrate and inorganic phosphate is released. Lead combines with the phosphate to form a precipitate which is subsequently converted into the brown sulfide of lead.
3) Nachals et al. method for determining succinate dehydrogenase (34). The tetrazolium salt (Nitro-BT),

which is a hydrogen acceptor, is reduced in the reaction mixture, and turns blue.

4) Hess, Scarpelli and Pearse methods for testing glucose-6-phosphate dehydrogenase (34). In this case, the tetrazolium salt (MTT) was used which, upon reduction in the reaction, turns grey-black in color.

All of these methods were designed to locate and qualitatively determine enzymatic activity as reflected by a color reaction. They were originally used for work with animal tissues and needed modification in various ways when used on plant material. The modification of these methods was difficult because of basic differences between plant and animal tissues. In general, the time of exposure was prolonged sufficiently (more than doubled sometimes) to permit the various chemicals to penetrate the thick cell walls. It was also necessary to determine whether or not to fix, to prefix or postfix the tissue, and the kind of fixative to use. Most of these adjustments were made by trial and error. The lower epidermis of inoculated host plants was used in all these tests and special attention was focused on enzymatic activities in and around the haustoria as revealed by a color reaction for a specific procedure. The epidermal strips were mounted in glycerine jelly or other synthetic media and examined microscopically. Phase-contrast microscopy was very useful in locating young haustoria, especially when they

retained their relatively transparent appearance.

The shortcomings and limitations of histochemical techniques in this study were numerous. These will be discussed later.

Sample size and statistical analysis.--When external structures were examined, the number of conidia observed varied from 40-130 with an average of around 60 on a 1-cm-leaf section. When haustoria were examined, the sample size varied appreciably depending on the stage of development, the kind of experiment, and the type of host used. All experiments were replicated 3-12 times on successive days. Replication in days, although time-consuming, has the advantage of compensating for variability in the quality of conidia.

Two-way analysis of variance was used and a level of 5 per cent was considered statistically significant, whereas 1 per cent level was considered highly significant.

RESULTS.--Germination of conidia and formation of appressoria.--Several experiments were conducted on the germination of conidia under the best environmental conditions found in a previous study (32). Inoculated wheat plants of the Little Club variety were incubated for 1 hr at 100% RH and 17 C followed by 3 hr at 22 C and 65% RH. Under these conditions, 94% of the applied conidia germinated in 4 hr (Fig. 2). Essentially identical results were obtained in polyethylene-covered wooden boxes in which RH was controlled with sulfuric acid solutions and in Sherer Gillett, Model No. CEL 512-37 growth chambers in which RH was controlled by mechanical means.

The enlargement and partial elongation of germ tubes resulted in the formation of club-shaped appressorial initials beginning 4 hr after inoculation. There was usually 1 appressorial initial per germinating conidium regardless of the number of germ tubes formed. The formation of these structures was observed at 22 C, 65% RH in darkness or in low light (50 ft-c) applied the 2nd hr after inoculation (30). There was no detectable difference between the 2 treatments; 91 and 96% of the applied conidia produced appressorial initials 7 and 8 hr, respectively, after inoculation. The curve representing the per cent of units forming appressorial initials was almost identical to the curve of per cent conidial germination (Fig. 2).

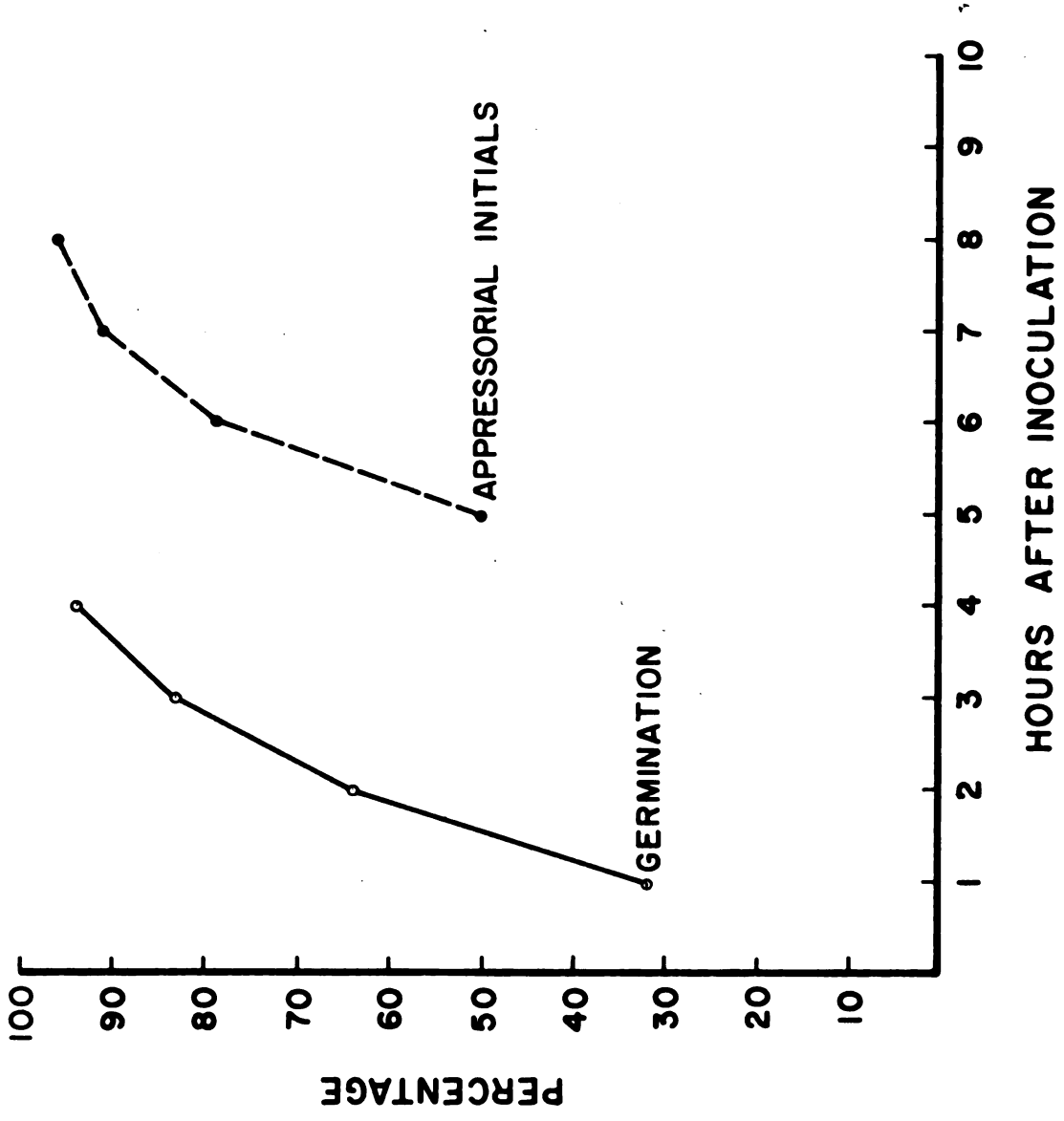
In a previous study (30) using the same environmental

conditions (except that wooden boxes were used as growth chambers) only 60% of the applied conidia produced initials 8 hr after inoculation. One basic difference between the wooden boxes and the Sherer Gillett chambers was the air circulation provided by the latter. This factor could account, at least in part, for the different results obtained in the 2 types of chambers.

The enlargement, elongation and bending of an appressorial initial resulted in a conspicuous structure which was cut off from the rest of the germ tube by a septum; this structure was considered an indication of maturity of appressoria. Both length and bending (to form a hook-like structure) were taken as criteria for a mature appressorium (30). A fully-formed appressorium was approximately 30-35 u long as compared to 30 u for an average length of conidia. Mature appressoria were detected 5 hr after inoculation and over 90% of the applied conidia produced mature appressoria, in most experiments, 10 hr after inoculation.

Effect of light on maturation of appressoria.--The effect of light treatments on this phase of primary infection was investigated next. With all light treatments, 22 C and 65% RH were used (30) beginning the 2nd hr after inoculation; only treatments of light were varied. High light (2800 ft-c) when applied 4 hr after inoculation delayed maturation of appressoria (Fig. 3). Although 89% of the appressoria were mature in 10 hr, yet the percentage was lower than

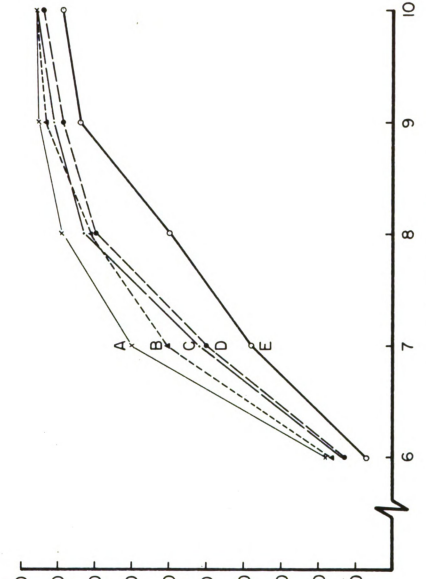
Fig. 2. Germination of conidia and formation of appressorial initials of E. graminis f. sp. tritici, expressed as a percentage of the total population reaching these stages.


APPRESSORIAL INITIALS
GERMINATION
PERCENTAGE
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
HOURS AFTER INOCULATION

other treatments during earlier hours (Fig. 3). On the other hand, darkness (0 ft-c) throughout the 10-hr-period gave a slightly higher percentage of mature appressoria at each hr. Incandescent light (50 ft-c), when applied 4 hr after inoculation, results did not differ statistically from complete darkness. However, a combination of incandescent (25 ft-c) and fluorescent light (215 ft-c) applied for the same period of time resulted in a still higher percentage of mature appressoria (Fig. 3). An appreciable increase in maturation of appressoria was detected when the last treatment was started 2 hr rather than 4 hr after inoculation.

These results showed that about 90% of the applied conidia were capable of forming appressoria when given enough time regardless of the treatment. However, it was also observed that certain light treatments reduced maturation of appressoria particularly between the 7-9th hr after inoculation (Fig. 3). Whereas 70% of the conidia formed mature appressoria under the best light treatment 7 hr after inoculation, only 39% formed appressoria during the same period with very high light (2800 ft-c) (Fig. 3). Although not to the same extent, there was a delay in maturation of appressoria with no light during the entire 10-hr-period. This definite lag in maturation with both high light (2800 ft-c) and darkness (o ft-c) as compared to low light (240 ft-c) indicated that this phase of the

Fig. 3. Effect of light treatments and time of application on maturation of appressoria of E. graminis f. sp. tritici on wheat leaves. A) Incandescent and fluorescent light (240 ft-c) beginning 2 hr after inoculation. B) Incandescent and fluorescent light (240 ft-c) beginning 4 hr after inoculation. C) Incandescent light (50 ft-c) beginning 4 hr after inoculation. D) Darkness (0 ft-c) throughout 10-hr period. E) Incandescent and fluorescent light (2800 ft-c) beginning 4 hr after inoculation. Treatment A differed statistically from C, D and E but not from B. Treatment B differed statistically from E but not from C and D.


PERCENTAGE OF MATURE APPRESSORIA
100
90
80
70
60
50
40
30
20
10
A
B
C
D
E
6
7
8
9
10
HOURS AFTER INOCULATION

primary infection was light-sensitive. It was also concluded that the time of starting a light treatment was an important factor affecting appressorial maturation.

Under the best light treatment, the percentage of appressoria which matured in each hr, respectively, were: 6th hr, 15%; 7th hr, 53%; 8th hr, 18%; 9th hr, 7%; and 10th hr, 1%. From this, it was obvious that 86% of the appressoria matured within a 3 hr period (6-9th hr after inoculation) and 53% of these matured during the 7th hr (Fig. 4). The curve representing per cent maturation of appressoria which was obtained with this treatment was very similar to the previous curves representing the first 2 phases of infection, namely germination and formation of appressorial initials (Fig. 5). This indicated that the conidial population was synchronized in the first 3 stages of primary infection; that is, most units of the population reached each phase within a 4 hr period.

Formation of secondary hyphal initials.--The secondary hyphal initials began as bud-like structures that emerged from almost any part on the primary appressorium. However, most often they arose from around the hook at the end of the primary appressorium. The secondary hyphal initials, which in previous work (30) were considered to indicate the establishment of a successful host-parasite relationship usually began to appear Ca. 16 hr after inoculation. Usually more than 1 hyphal initial formed on an appressorium,

but in most cases only 1 was observed to form a functional secondary hypha.

The stages preceding secondary hyphal initials were synchronized; that is, most units of the population reached each phase within a 4 hr period. Therefore, the formation of secondary hyphal initials was expected to follow the same pattern. In preliminary experiments, formation of secondary hyphal initials spread over a 14 hr time period. Thus the population was not synchronized for this stage, as it was for the preceding stages. An effort was made, therefore, to synchronize the parasite units in this stage of primary infection. To do this, the effects of temperature, RH and light on the formation of secondary hyphal initials were investigated.

For all studies on the formation of secondary hyphal initials, the previously determined optimum environmental conditions for conidial germination, formation and maturation of appressoria were used for the first 8 hr after inoculation. After the 8th hr, 1 of the 3 environmental factors (light, temperature and RH) was varied while the other 2 were kept constant.)

Effect of temperature on development of secondary hyphal initials.--The following temperature regimes were tested for effects on development of secondary hyphal initials: 1) 30 C during 8-30th hr after inoculation; 2) 30 C during 8-12th hr and 22 C during 12-30th hr after inoculation; 3) 22 C during 2-30th hr after inoculation; 4) 18 C during 8-30th hr

Fig. 4. Structure of the population of parasite units in respect to time in the 3rd stage (Maturation of appressoria) of primary infection of E. graminis f. sp. tritici on wheat leaves.

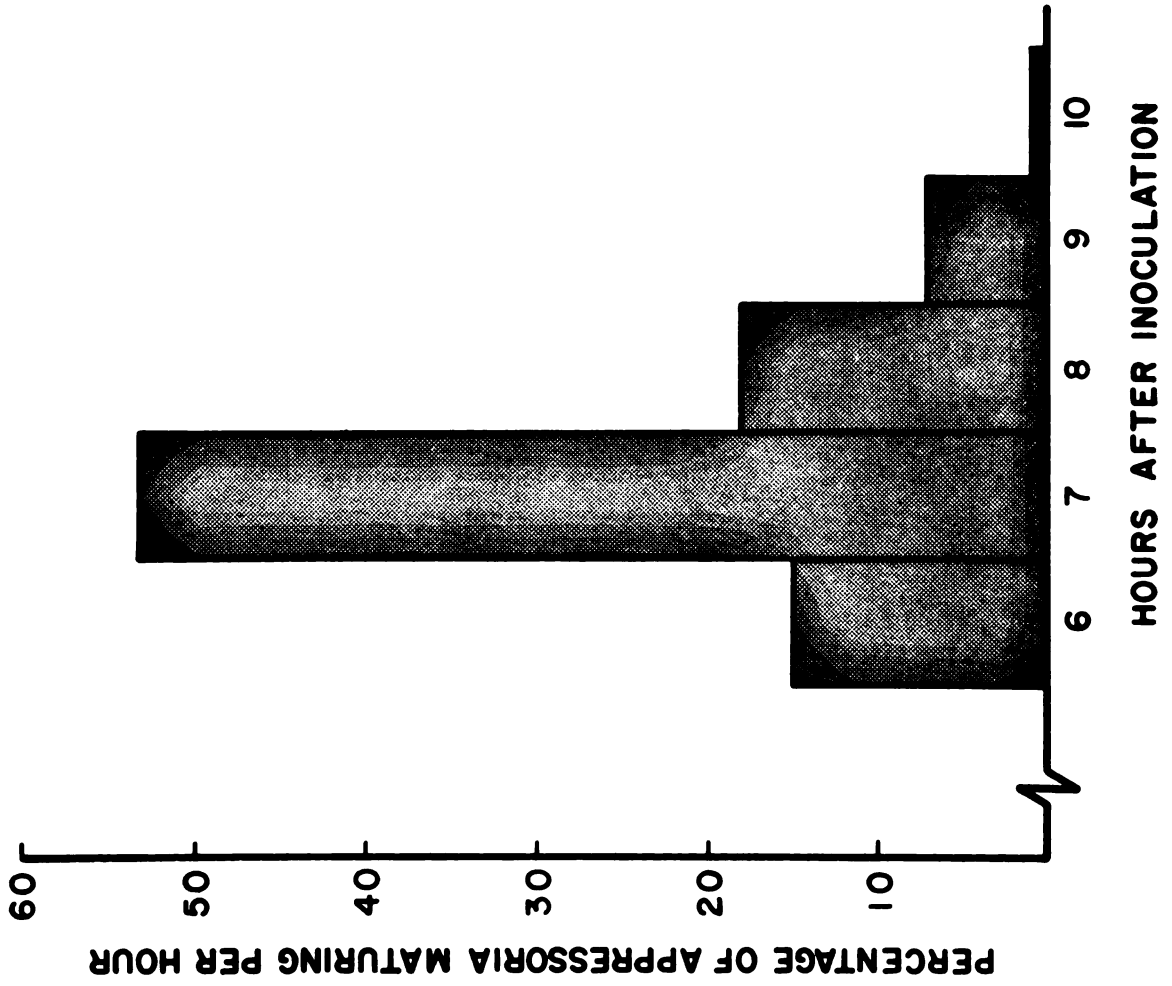
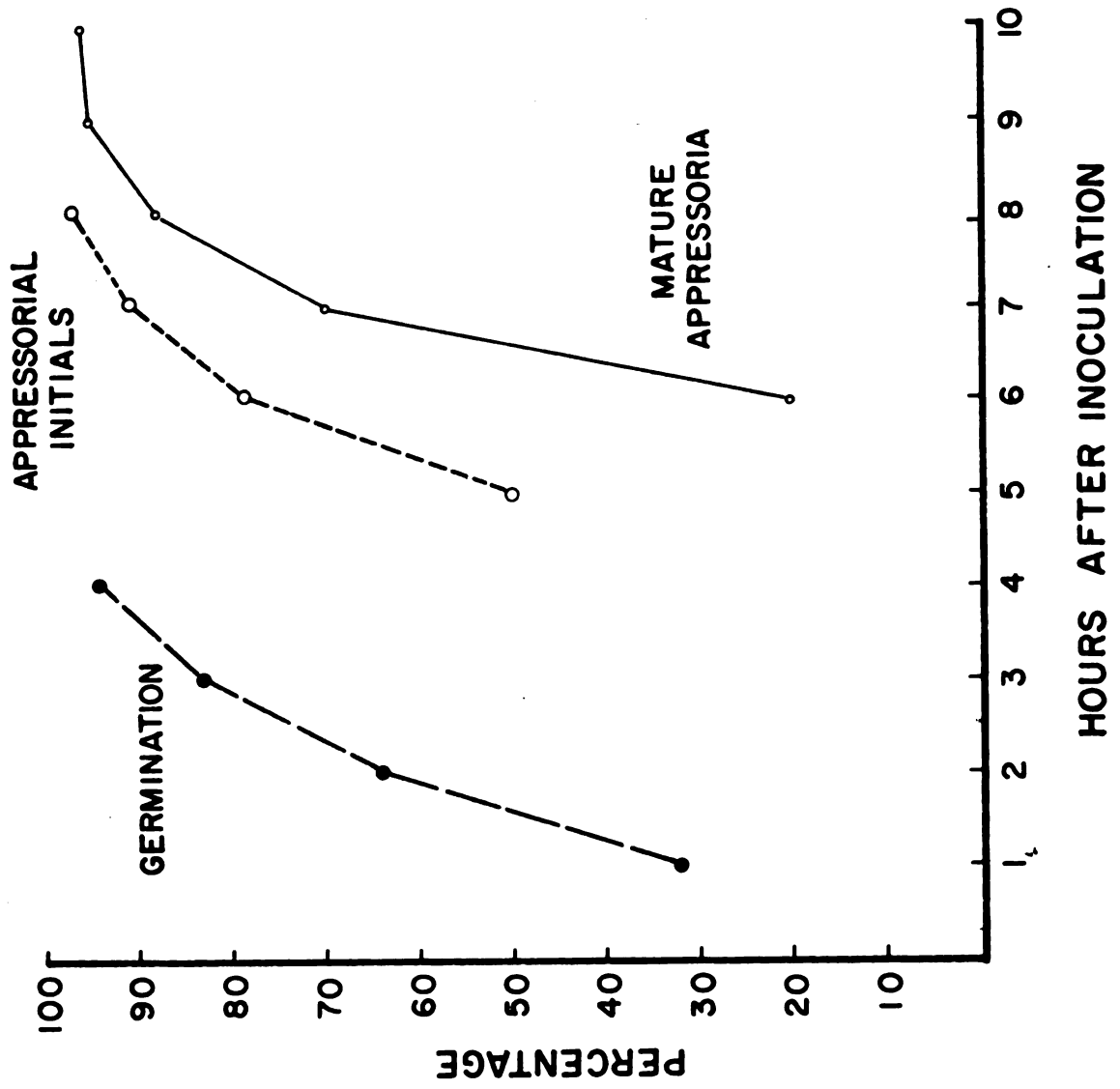

PERCENTAGE OF APPRESSORIA MATURING PER HOUR
60
50
40
30
20
10
6
7
8
9
10
HOURS AFTER INOCULATION

Fig. 5. Conidial germination, formation of appressorial initials, and maturation of appressoria in a population of *E. graminis* f. sp. *tritici* on wheat leaves. Note that most of the units reach each stage within a limited time period, indicating a synchronization of units within the population.


APPRESSORIAL INITIALS
MATURE APPRESSORIA
GERMINATION
PERCENTAGE
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
10
HOURS AFTER INOCULATION

after inoculation; and 5) 18 C during 8-16th hr and 22 C during 16-30th hr after inoculation. Results showed that the latter represented the best temperature treatment because 94% of the parasite units formed secondary hyphal initials 30 hr after inoculation (Fig. 6). Although this represented a significant improvement over other treatments, the parasite units were not as well synchronized as in the preceding stages (Fig. 5).

These experiments indicated that development of secondary hyphal initials was temperature sensitive and that temperatures of 18 to 22 C are more favorable than 30 C.

Effect of relative humidity on development of secondary hyphal initials.--The effect of RH on development of secondary hyphal initials was not emphasized mainly because the parasite should not respond to such treatments since it was already in the host. The only treatment used was 100% RH between the 8-16th hr after inoculation. A temperature of 22 C and 240 ft-c was maintained starting the 2nd hr after inoculation and continued until 30 hr after inoculation. The RH before and after applying the treatment was kept at 65%. High RH had an inhibitory effect on development of secondary hyphal initials. With this treatment, 85% of the secondary hyphal initials were formed 30 hr after inoculation (Fig. 7).

Effect of light on development of secondary hyphal initials.--With the optimum temperature treatment, ca. 90%

of the parasite units formed secondary hyphal initials between the 16-30th hr after inoculation. Formation of secondary hyphal initials took a longer time than the other preceding stages of primary infection, possibly because the optimum conditions for the formation of secondary hyphal initials were not yet reached. Therefore, light conditions were varied to see if this stage could be shortened.

In all experiments, a temperature of 22 C and 65% RH was maintained starting the 2nd hr after inoculation. Before and after the light treatments were applied, a 240 ft-c intensity was maintained.

High light treatments of 2800 ft-c applied between the 8-16th hr after inoculation seemed to have an inhibitory effect on the formation of secondary hyphal initials (Fig. 8). On the other hand, darkness (0 ft-c) during the same period gave significantly more secondary hyphal initials than did high light (2800 ft-c). Darkness, although superior to high light, had an inhibitory effect on a portion of the conidial population as was evidenced by the levelling of the curve between the 20-22nd hr after inoculation (Fig. 8). Identical results were obtained with very low light (25 ft-c) when applied the 8-16th hr after inoculation. When darkness was started at 6 hr, rather than at 8 hr after inoculation and continued until the 16th hr, a remarkable increase in the percentage of secondary hyphal initials

Fig. 6. Effect of temperature on development of secondary hyphal initials of E. graminis f. sp. tritici on wheat leaves. A) 18 C 8-16th hr after inoculation followed by 22 C after the 16th hr. B) 18 C 8-30th hr after inoculation. C) 22 C 2-30th hr after inoculation. D) 30 C 8-12th hr after inoculation followed by 22 C after the 12th hr. E) 30 C 8-30th hr after inoculation. Treatment A did not differ statistically from B and C, but differed from D and E. Both B and C differed statistically from D and E.

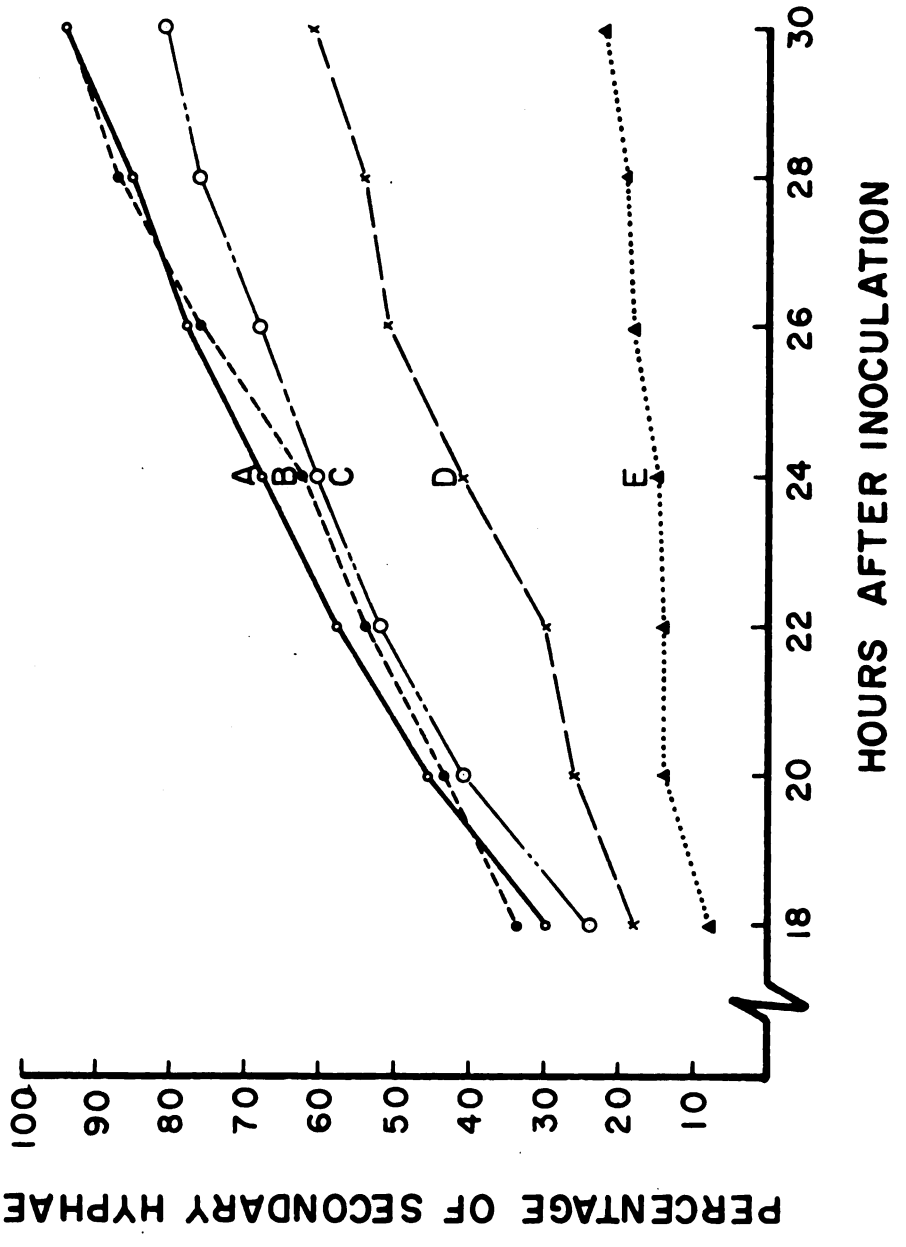
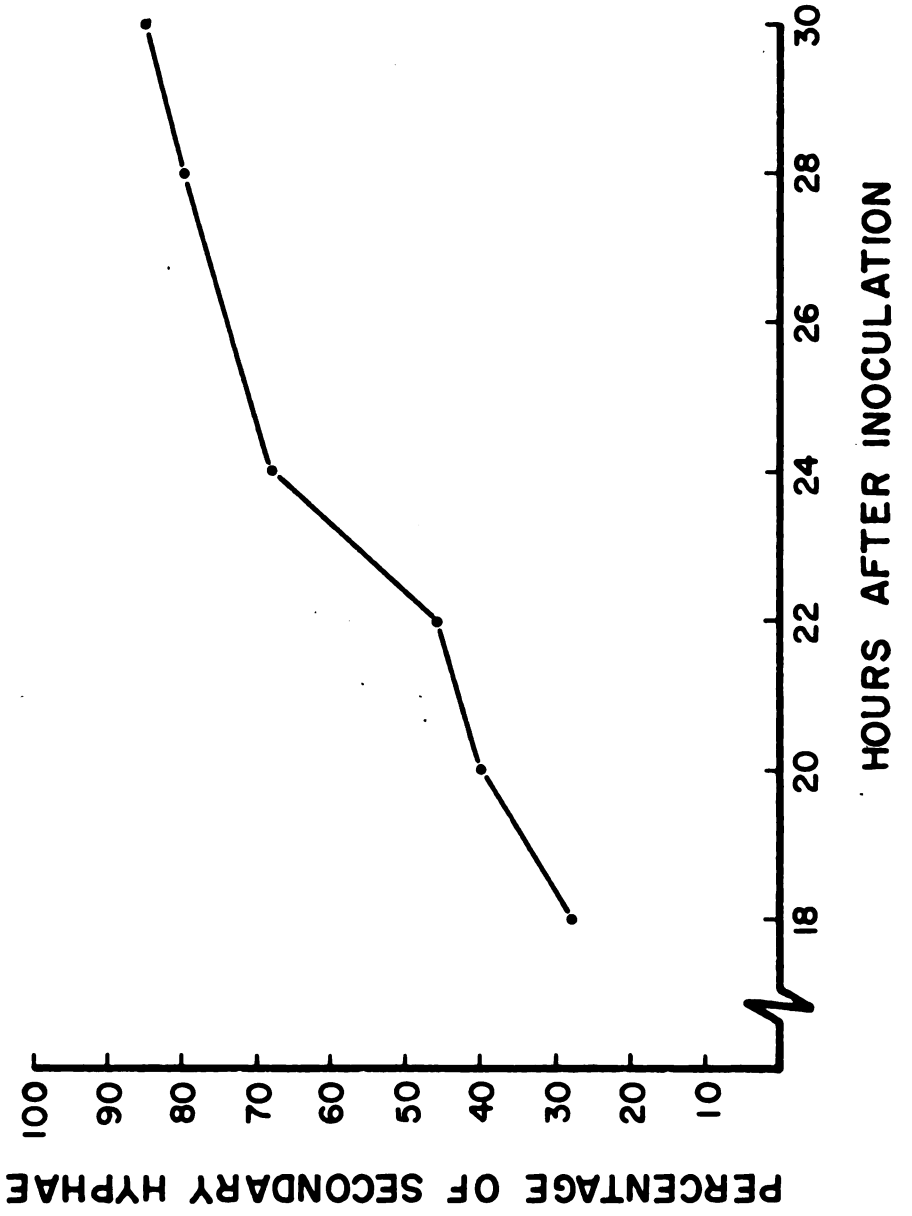

PERCENTAGE OF SECONDARY HYPHAE
100
90
80
70
60
50
40
30
20
10
18
20
22
24
26
28
30
HOURS AFTER INOCULATION
A
B
C
D
E

Fig. 7. Per cent of appressoria of E. graminis f. sp. tritici developing secondary hyphal initials on wheat leaves at 100% RH during the 8-16th hr after inoculation.


PERCENTAGE OF SECONDARY HYPHAE
100
90
80
70
60
50
40
30
20
10
18
20
22
24
26
28
30
HOURS AFTER INOCULATION

formed was detected, and a steep smooth curve was obtained (Fig. 8). This seemed to be the optimum light condition for the formation of secondary hyphal initials since 90% secondary hyphal initials were formed in 6 hr (16-22nd hr). This was a significant improvement over all previous treatments because it shortened the period for the formation of secondary hyphal initials from 14 to 6 hr.

It was rather hard to conceive how a 2-hr increase in the dark period (as early as 6 hr after inoculation) could affect formation of secondary hyphal initials 10-16 hr later. One plausible explanation was that a portion of the conidia was inhibited by light between the 6-8th hr after inoculation and that this portion of the population was "released", so to speak, when light (240 ft-c) was replaced by darkness (0 ft-c).

These data suggested that the parasite population was progressing through the various stages of primary infection, namely germination, formation of appressorial initials, maturation of appressoria, and development of secondary hyphal initials in a synchronized manner (Fig. 9).

Development of haustoria.--Prior to the appearance of secondary hyphal initials on the surface of inoculated leaves, a series of host-parasite interactions took place during a seemingly quiescent period (between the 10-16th hr after inoculation). It was during this period that infection pegs were formed on the lower side of the appressoria (Fig. 10). Infection pegs penetrated the epidermis and developed

Fig. 8. Effect of light on development of secondary hyphal initials of E. graminis f. sp. tritici on wheat leaves. A) Darkness (0 ft-c) 6-16th hr after inoculation. B) Darkness (0 ft-c) 8-16th hr after inoculation. C) Incandescent light (25 ft-c) 8-16th hr after inoculation. D) Incandescent and fluorescent light (240 ft-c) 8-16th hr after inoculation. E) Incandescent and fluorescent light (2800 ft-c) 8-16th hr after inoculation.

Treatment A differed statistically from B, C, D and E. B and C differed statistically from D and E. D differed statistically from E.

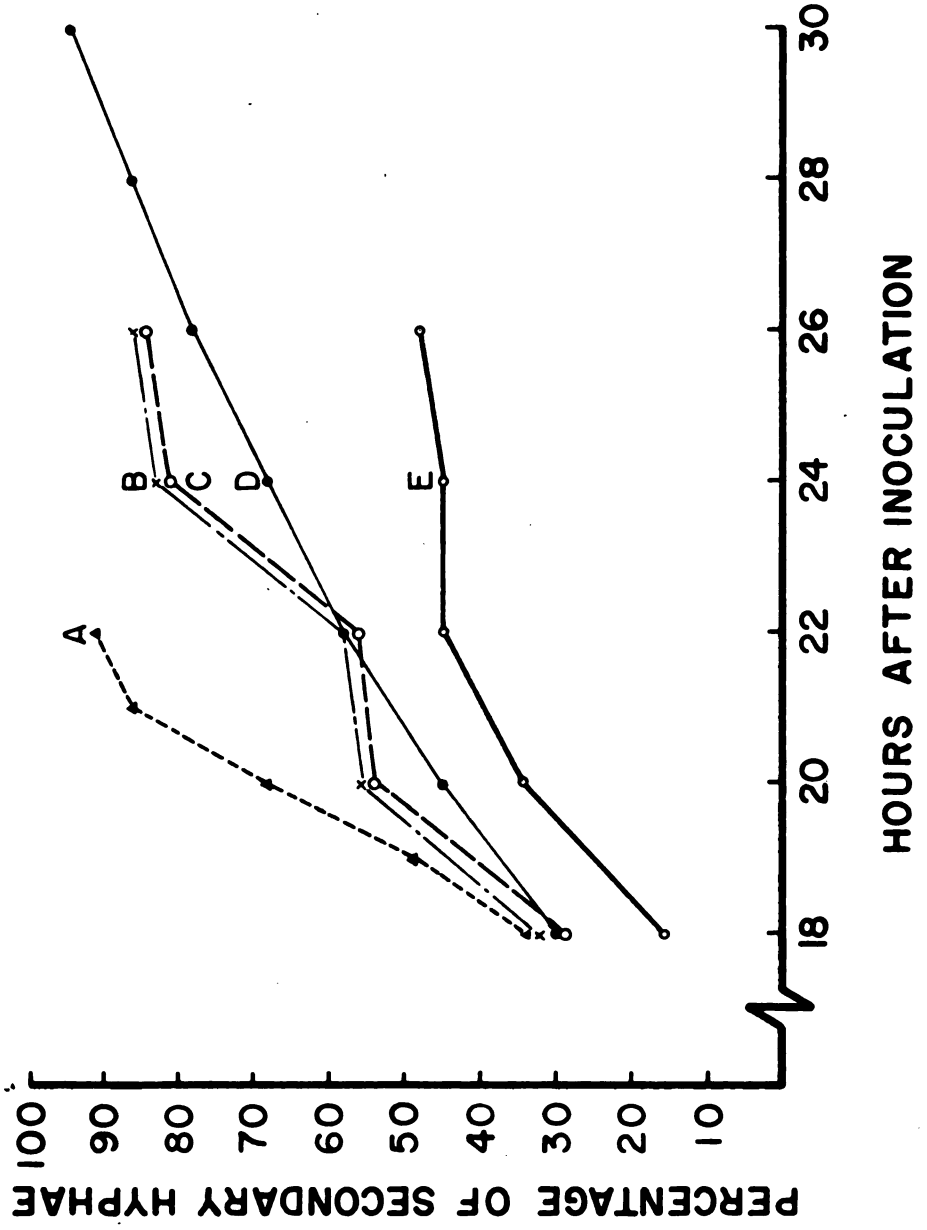
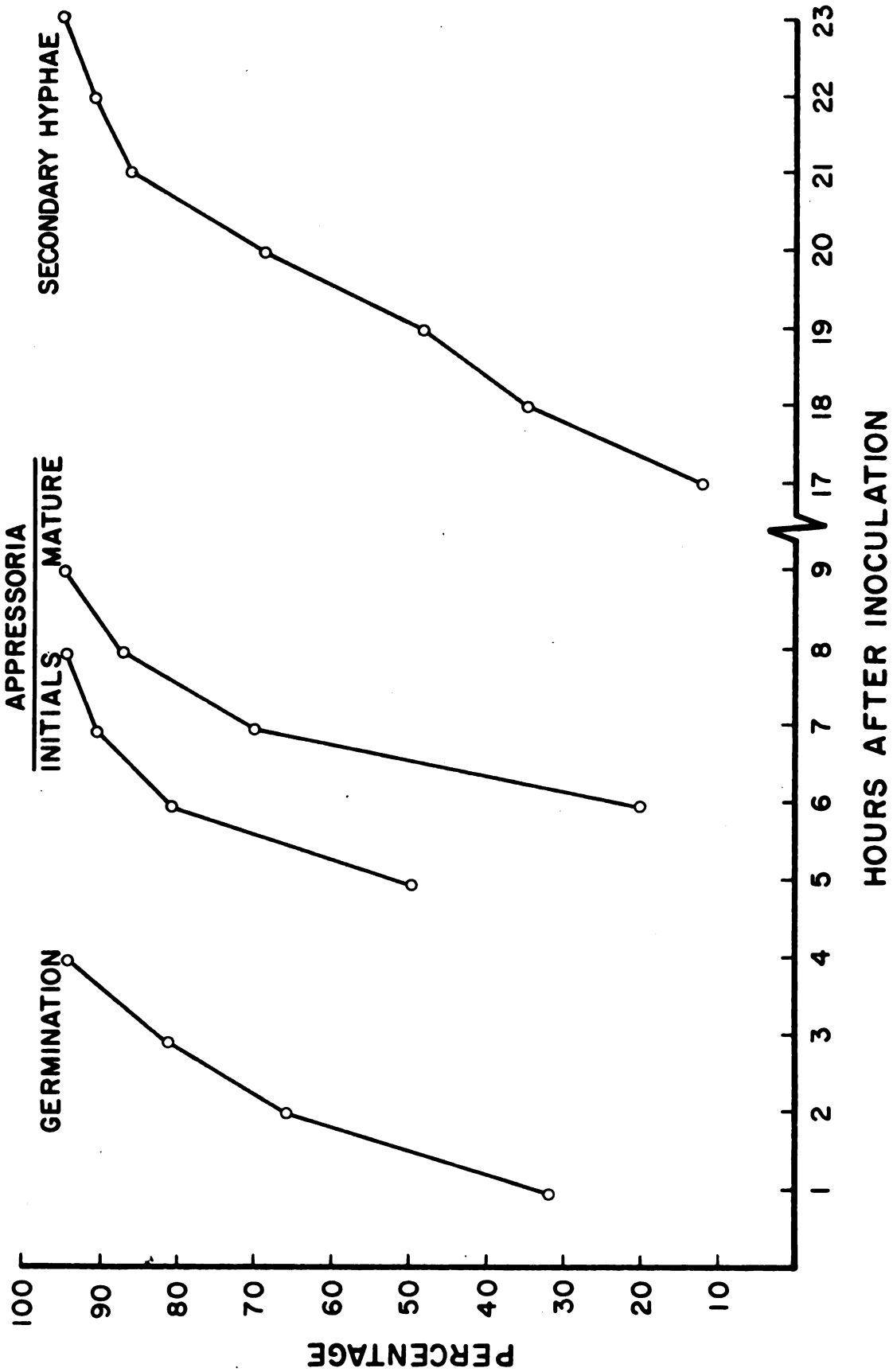

PERCENTAGE OF SECONDARY HYPHAE
100
90
80
70
60
50
40
30
20
10
18
20
22
24
26
28
30
HOURS AFTER INOCULATION
A
B
C
D
E

Fig. 9. Comparison of 4 curves representing percentage of the population in 4 stages of primary infection of *E. graminis* f. sp. *tritici* on wheat leaves. The stages are A) Germination B) Formation of appressorial initials C) Maturation of appressoria and D) Formation of secondary hyphal initials.


GERMINATION
APPRESSORIA
INITIALS
MATURE
SECONDARY HYPHAE
PERCENTAGE
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
17
18
19
20
21
22
23
HOURS AFTER INOCULATION

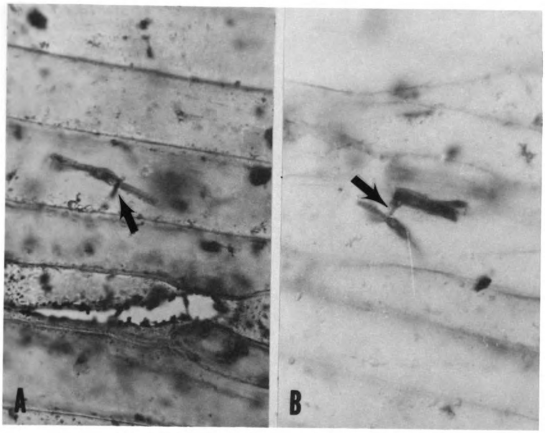
into the only internal structure of the fungus, the haustorium. Preliminary experiments showed that 36 hr (after inoculation) were required for full-sized haustoria (65-70 u) to develop. An effort was made to hasten maturation of haustoria by manipulation of the environment.

Effect of light on development of haustoria.--Light, by increasing photosynthetic activity in the host, might affect maturation of haustoria. In all experiments, temperature and RH were kept at 22 C and 65% respectively starting the 2nd hr after inoculation. Light was 240 ft-c from the 2-6th hr after inoculation, 0 ft-c 6-16th hr, and 240 ft-c the 16-36th hr. Four different treatments of 2800 ft-c, 1400 ft-c, 240 ft-c and darkness (0 ft-c) when applied 16-36th hr after inoculation did not show statistically significant differences in their effect on haustorial development (Fig. 11).

This lack of response to all light treatments indicated that this stage of primary infection was light sensitive and that development of haustoria proceded unaffected by light or darkness.

Effect of temperature on development of haustoria.--When a temperature of 35 C was applied the 16-36th hr after inoculation, complete inhibition of haustorial development was observed. Examination of secondary hyphal initials on the same leaf sections revealed that none reached the functional stage. When the temperature was 30 C, very few

Fig. 10. Infection pegs of E. graminis f. sp. tritici. A) Infection peg located between the "haustorial body" and the appendages. B & C) Relative position of infection peg between the appressorium and the haustorium. (575x)


A
B

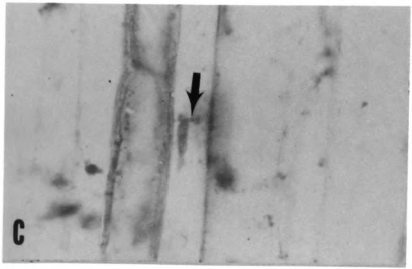
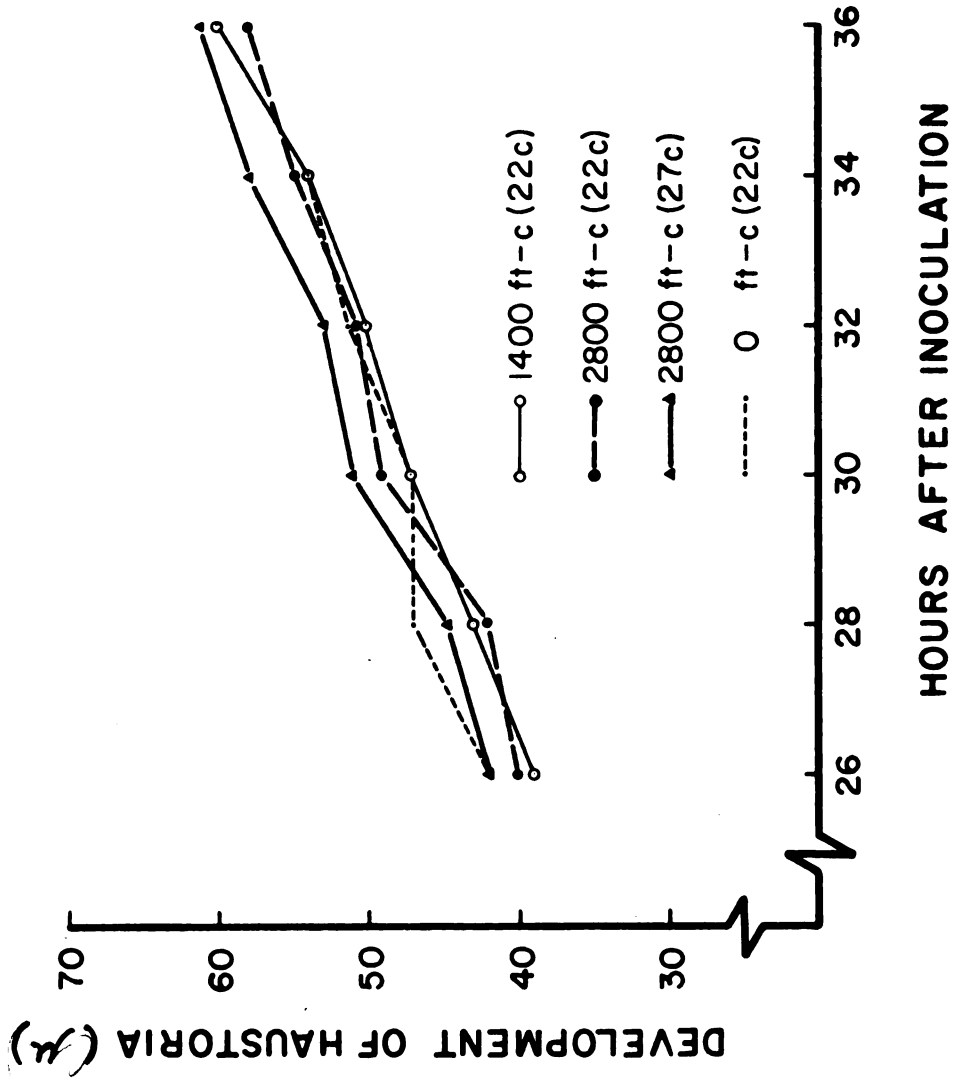

C

Fig. 11. Effect of light on development of haustoria of E. graminis f. sp. tritici. All treatments were applied the 16-36th hr after inoculation. Treatments did not differ statistically.


DEVELOPMENT OF HAUSTORIA (%)
70
60
50
40
30
26
28
30
32
34
36
HOURS AFTER INOCULATION
1400 ft-c (22c)
2800 ft-c (22c)
2800 ft-c (27c)
0 ft-c (22c)

undersized haustoria formed; they attained a length of 30 u in 36 hr. High temperature restricted elongation of the appendages on both sides of the haustoria. At 22 C the 2-36th, haustoria were 65-70 u. At 17 C from the 16th hr onward, haustoria development was slower than at 22 C, but the inhibition was less than at high temperatures. The haustoria attained a length of 53 u in 36 hr.

It was thus inferred that although development of haustoria was light independent, it was temperature sensitive. The most favorable temperature for haustorial development was 22 C during the 2-36th hr after inoculation.

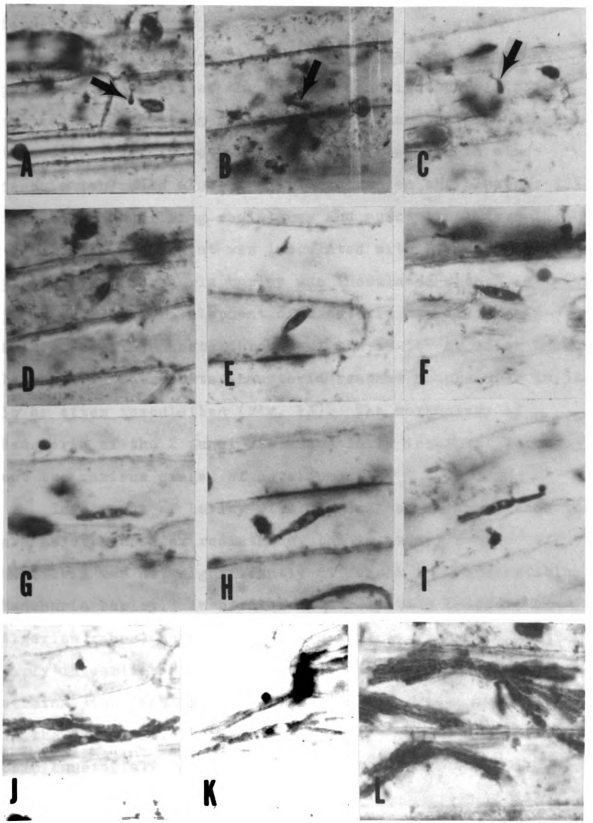
Since attempts to hasten the rate of growth of haustoria were unsuccessful, subsequent studies of the haustoria were all made under the following conditions: 1) 22 C starting the 2nd hr after inoculation; 2) 240 ft-c during the 2-6th hr, 0 ft-c during the 7-16th hr, 240 ft-c after the 16th hr; and 3) 65% RH beginning the 2nd hr after inoculation.

Morphological development of the haustorium.--The development of haustoria was observed under optimum conditions of primary infection as reported earlier. With staining and use of high magnification (594x), it was possible to follow the various morphological and cytological changes that accompanied development of haustoria in the 11-34th hr after inoculation. Little Club wheat was used for this purpose in most experiments; the barley

variety Manchuria was also used with similar results. As early as 11-12 hr after inoculation, few infection pegs were formed in epidermal cells. A slight swelling was detectable towards the tip of each peg (Fig. 12 A). Two hr. later, after further elongation and enlargement (Fig 12 B), the resulting structure started turning towards a position parallel with the leaf surface (Fig. 12 C). It was also at this time that the haustorial sheath was first detected. Two hr later, i.e. 16 hr after inoculation, a pear-like structure became conspicuous, and this structure also assumed a horizontal position (Fig. 12 D). The haustorial sheath was very prominent at this stage. Eighteen hr after inoculation, the haustorial body was fully formed with a distinct sheath, a nucleus, and a vacuole at each side of the nucleus (Fig. 12 E). The haustorial body measured 18-20 u long and 3-4 u wide. The only detectable development, after the body was formed, was elongation of finger-like structures on both ends of the haustorial body (Fig. 12 F-K). The elongation of these "fingers" proceded at about 2.5 u/hr starting 18 hr after inoculation. By the 34-36th hr, the haustoria reached their mature size of 65-70 u. An occasional extremely large haustorium was noted (Fig. 12 L).

The earliest that infection pegs were detectable was between the 11th and 12th hr after inoculation. This meant that penetration had started around the 10th hr after

Fig. 12. Morphological development of haustoria of E. graminis. A) Infection peg 11 hr after inoculation. B) Infection peg 13 hr after inoculation. C) Infection peg swelling to form haustorial body initial (14 hr). D) Haustorial body 16 hr after inoculation. E) Full-sized haustorial body 18 hr after inoculation. F) Haustorial appendages start forming on both sides of haustorium 20 hr after inoculation. G-K) Haustoria at 24, 26, 28, 32, 34 hr after inoculation. K) Mature haustorium. L) "Giant" haustoria. (575x)


A
B
C
D
E
F
G
H
I
J
K
L

inoculation. Over 90% of the mature appressoria had been formed by the 8th hr.

Comparative development of haustoria in 6 different hosts.-- The effect of the genetic constitution of different hosts on development of haustoria was studied. Examination of changes in both host and parasite when in close association could also give information on the possible mechanisms underlying resistance and susceptibility.

Little Club wheat was inoculated with E. graminis f. sp. tritici, and Manchuria barley was inoculated with E. graminis f. sp. hordei. Development of haustoria was similar in both susceptible hosts throughout the 18-36th hr after inoculation. In both hosts, haustoria reached mature size in 34-36 hr after inoculation (Fig. 13). The morphology of haustoria of the 2 fungi was essentially identical throughout the various stages of development.

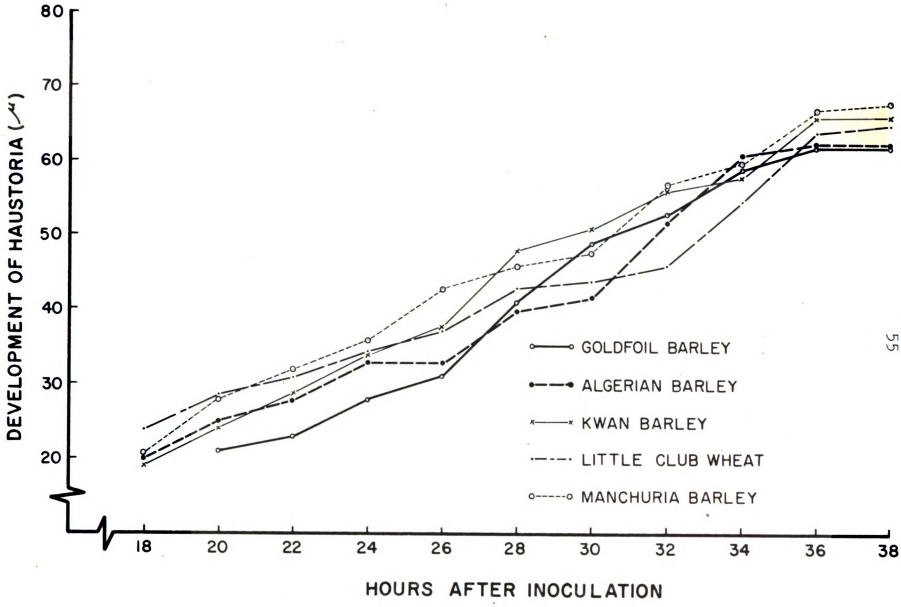
In strains of barley with the Psaknon or kwan genes that are major genes for resistance, the increase in size of haustoria was not significantly different from susceptible Manchuria barley. In strains of barley with Goldfoil or Algerian genes, haustorial development was retarded particularly in earlier stages (Fig. 13). In the latter two strains, the final length of haustoria was less than those in the susceptible barley variety, Manchuria (Fig. 13). Some haustoria were malformed. The kwan gene gave a hypersensitive reaction 5 days after inoculation. This

delayed hypersensitivity resulted in disintegration or distortion of the great majority of haustoria (Fig. 14). The hypersensitive reaction was not exhibited with the other genes.

It was observed repeatedly that fewer haustoria developed in a host with any major gene for resistance than in susceptible hosts. Experiments were designed to determine the percentage of conidia that could form haustoria in plants carrying the various genes for resistance.

The various host plants were inoculated in the usual manner and incubated under the optimum conditions for conidial germination, formation of appressoria, and development of secondary hyphal initials. The plants were examined 30 hr after inoculation when a successful host-parasite relationship was established. The haustoria could then be detected with relative ease. A 1 cm section of the inoculated leaf was examined, and the number of parasite units with secondary hyphal initials and those with functional secondary hyphae was determined. The epidermis of the same 1-cm-leaf section was removed into a drop of stain on a glass slide and the number of haustoria formed was counted. The number of haustoria formed in each case was expressed as a percentage of the total number of conidia with secondary hyphal initials. The number of conidia per cm leaf section was not counted due to the limitation of time, but it was known from other experiments that over 90% of the conidia produce

Fig. 13. Development of haustoria in 5 different hosts. Development in Manchuria differed statistically from both Goldfoil and Algerian, but not from Kwan. Development of Kwan differed statistically from both Goldfoil and Algerian. Algerian and Goldfoil did not differ statistically.


DEVELOPMENT OF HAUSTORIA (μ)
80
70
60
50
40
30
20
18
20
22
24
26
28
30
32
34
36
38
HOURS AFTER INOCULATION
GOLDFOIL BARLEY
ALGERIAN BARLEY
KWAN BARLEY
LITTLE CLUB WHEAT
MANCHURIA BARLEY

secondary hyphal initials by the 30th hr on all hosts used.

With hosts susceptible to either fungus form, about 88% of the units, with secondary hyphal initials also produced haustoria (Table 1). When the kwan and Psaknon genes were present, ca. 30% of the units with secondary hyphal initials formed haustoria. With the genes Algerian and Goldfoil, 5 and 15%, respectively, of the units with secondary hyphal initials formed haustoria (Table 1).

It was thus very evident that all 4 major genes for resistance affected the percentage of applied conidia that formed haustoria and established a successful host-parasite relationship.

Relationship between haustoria and functional secondary hyphae.--An attempt was made to correlate the number of haustoria and the number of functional secondary hyphae formed on the same leaf section. This was done with the 6 available hosts and under the same environmental conditions used in the study of the development of haustoria. These experiments were started 30 hr after inoculation to allow the functional secondary hyphae and haustoria to develop and be seen with relative ease. In each case, the number of functional secondary hyphae per cm leaf section was recorded, the epidermis of the same section removed, and the number of haustoria was counted. The number of haustoria formed in the epidermal cells approximated the number of functional secondary hyphae on the surface of the leaf (Table 1). The

Fig. 14. Hypersensitive reaction induced by the fungus in the presence of the Kwan gene 5 days after inoculation. A) Disorganized cytoplasm and loss of appendages on both sides of haustorium. B) Disorganized host cytoplasm and oozing of cytoplasm from distorted haustorium. C) Collapsed host cytoplasm and abnormal haustoria. D) Disorganized host cytoplasm. E) Normal haustoria and host cytoplasm 5 days after inoculation, of the susceptible Manchuria variety. (575x)

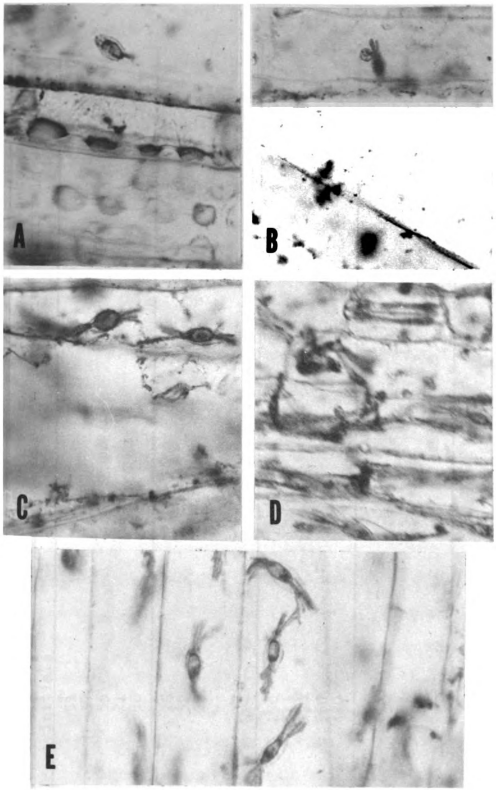

A
B
C
D
E

TABLE 1. The number of secondary hyphal initials, functional secondary hyphae and haustoria on the same inoculated leaf section

Hosts	Total secondary hyphal initials	Functional secondary hyphae	Haustoria	%of secondary hyphal initials with haustoria	
Little Club	51	48	44	86	
	37	36	34	92	
	35	34	28	80	
	21	16	19	91	
	58	46	51	89	Avg 88
Manchuria	26	24	22	85	
	32	26	28	87	
	22	19	19	87	
	--	--	--	--	
	--	--	--	--	Avg 87
Algerian	150	10	10	7	
	341	10	12	4	
	280	11	13	5	
	560	15	19	3	
	420	19	23	6	Avg 5
Goldfoil	65	3	4	6	
	130	14	17	13	
	25	5	6	24	
	60	13	11	18	
	--	--	--	--	Avg 15
Kwan	44	14	12	27	
	76	29	24	32	
	70	24	22	31	
	54	18	16	30	
	23	7	7	31	Avg 30
Psaknon	54	23	20	37	
	84	43	40	48	
	60	9	11	18	
	--	--	--	--	Avg 33

correlation occurred in all susceptible and resistant host combinations. Consequently, a safe conclusion is that for every functional secondary hyphae on the surface of the host, there is a corresponding haustorium in the epidermis.

The formation of functional secondary hyphae clearly indicated a functional host-parasite relationship. Since the functional secondary hyphae were formed on the surface of the host and were easily detectable by microscopic examination, they were considered a useful criterion in studying host-parasite relationships.

Macroscopic assay of relative resistance in the barley varieties.--To further test the relative resistance in the 5 strains of barley and correlate microscopic evidence of resistance with actual numbers of lesions formed on inoculated hosts, the following experiment was performed. Three 6-day-old plants of each variety were inoculated in the usual manner and kept under optimum conditions for germination, appressorial maturation, and development of secondary hyphae. Four days after inoculation, a lesion count on 1-cm-leaf sections were made on all plants. Each plant was considered as 1 replicate, and the 3 replicates of each variety averaged as shown in Table 2. Seventy-two lesions were formed on the susceptible variety Manchuria as compared to 0, 4, 36, and 39 lesions on the strains of barley with genes Algerian, Goldfoil, Kwan, and Psaknon,

respectively. These results were in agreement with the microscopic evidence of relative resistance of the various hosts. The percent of parasite units producing haustoria and functional secondary hyphae was 5, 15, 30, and 33 in the presence of the 4 genes (Algerian, Goldfoil, Kwan, and Psaknon) respectively (Table 1).

The count of the number of colonies on the leaf surface was done macroscopically in all cases. Microscopic examination of the strain with the Algerian gene revealed, however, some mycelial growth in a few places on the inoculated leaf surface. Microscopic examination of the epidermis of these same leaf sections revealed the formation of a few haustoria (4-5%). From this, it was inferred that the major gene for resistance in this variety suppressed the reproductive activity even though 4-5% of the parasite units had succeeded in establishing a successful, though short-lived, host-parasite relationship.

The various mechanisms by which the Algerian gene conferred resistance to the host could be identified as follows:

1) Exclusion of 95% of the applied conidia from entering the host
2) Delay in early haustorial development
3) Reduction of final size of haustoria
4) Distortion of a small portion of the haustoria
5) Suppression of fungus sporulation even after a successful host-parasite relationship was established.

Of all these, the first and the last mechanisms appeared to be of most importance in insuring a high level of macroscopically observable resistance in the host.

Brief review of sequential events of primary infection.--

1) Germination of conidia--over 90% of the applied conidia germinated in 4 hr.

2) Formation of appressorial initials-over 90% of the applied conidia produced appressorial initials in 4 hr (5-8th hr after inoculation).

3) Maturation of appressoria--over 90% of the applied conidia produced mature appressoria in 4 hr, (6-9th hr after inoculation).

4) Penetration--this most probably occurred around the 10th hr after inoculation.

5) Formation of haustoria--infection pegs were detected in epidermal cells 11 hr after inoculation. The haustorial body reached full size 18 hr after inoculation. Finger-like projections on the haustorium elongated between the 18-36th hr.

6) Appearance of secondary hyphal initials--over 90% of the applied conidia formed secondary hyphal initials in 6 hr (17-23rd hr after inoculation).

7) Development of functional secondary hyphae--ca. 90% of the applied conidia produced functional secondary hyphae by the 30th hr (in susceptible host-parastie combinations).

When a successful host-parasite relationship was

established, functional secondary hyphae were formed which gave rise to secondary and tertiary appressoria, and subsequently conspicuous pustules were formed on the inoculated leaf.

Histochemical studies.--Few, if any histochemical studies reported in the literature were on the very early interactions. Comparisons of healthy and diseased tissues are usually studied several days after inoculation. By this time, host-parasite relationships are established and external symptoms are apparent. There seemed to be a general agreement that histochemical changes in the host occurred simultaneously with macroscopic symptom development and sporulation of the fungus.

An attempt was made to detect early interactions between the host and the parasite before the onset of external visible symptoms. These early interactions are of prime importance in establishing parasitism. Since penetration of the host began around the 10th hr after inoculation and secondary hyphal initials began to form at the 16th hr, it was this 6-hr-period that was mainly investigated. However, histochemical changes beyond this period were also studied.

As evaluated by the procedures used, there was an increased succinate dehydrogenase activity around the haustorium as early as 12 hr after inoculation, i.e. shortly after penetration. Tests made 12, 14, 16, 18, 24, 48, 72, and 144 hr after inoculation showed a marked increase of succinate

TABLE 2. Effect of genes for resistance on lesion formation on leaves of barley inoculated with *E. graminis* f. sp. *hordei*. Data are expressed as lesions per linear cm of inoculated leaf

Manchuria	Algerian	Goldfoil	Kwan	Psaknon
110	0	3	33	56
55	0	0	29	49
51	0	6	46	42
Avg 72	Avg 0	Avg 4.5	Avg 36	Avg 49

Percent[a] functional secondary hyphae formed on the various varieties are:

Manchuria	Algerian	Goldfoil	Kwan	Psaknon
87	5	15	30	33

a) Values obtained from another experiment.

dehydrogenase activity around the haustorium (Fig. 15). A positive test was characterized by numerous blue crystals of diformazan formed around the haustorium, while the lack of such crystals constituted a negative test. Three types of controls were run with every experiment. In one control, the tetrazolium salt (Nitro-BT) was not added to the reaction mixture. In another, no succinate was added. In the third, the succinate was replaced by substrates of other dehydrogenase systems, i.e. glucose-6-phosphate, lactate, malate, citrate, and acetaldehyde. In all controls where the tetrazolium salt was omitted, there was a complete absence of blue crystals, whereas when no substrate was added, very few and sporadic blue crystals formed most likely because of the occurrence of succinate in the host cells (Fig. 15). The increase in enzymatic activity was limited to the inside and around the haustoria. The only other places where such activity was detected were the chloroplasts and both ends of the closed stomata (Fig. 15).

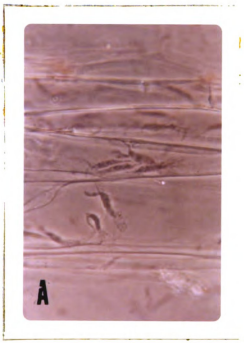
These findings indicated that even 2 hr after penetration some kind of interaction between the host and the parasite occurred. Whether this increased enzymatic activity was stimulated in the host by the parasite or originated in the parasite was not determined. In either case, the results indicate an interaction between the host and the parasite at an early stage in primary infection.

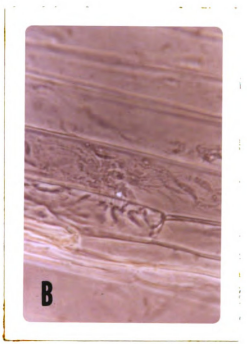
The activity of acid phosphatase was difficult to

determine. Results were not conclusive although 2 general methods for testing this enzyme and several other modifications were tried. A major modification in fixing the tissue was tried. This consisted of fixing the intact inoculated leaf for 16-24 hr in Baker's solution (1.3% calcium chloride in formaldehyde) prior to peeling the epidermis and running the test. This eliminated the massive production of acid phosphatase in the host cells caused by stripping the epidermis, and permitted the examination of any enzymatic changes around and in the haustoria. Controls consisted of omitting either the substrate or the eosin red. In some experiments, haustoria stained red-brown color (Fig. 16), but these results were erratic and hard to reproduce. What was more important was the continuous erratic results in critical controls for these tests. Although Atkinson and Shaw (3) reported increased acid phosphatase on and in the haustoria, all attempts to reproduce their work, using identical material and techniques, failed. Tests were run as early as 11 hr after inoculation to as late as 6 days after inoculation.

No modifications of the original histochemical method for glucose-6-phosphate dehydrogenase (34) were attempted other than increasing the time of exposure of the tissue to the various chemicals used. Controls consisted mainly of omitting the substrate, the tetrazolium salt (MTT), or the cobalt chloride. In some experiments, black-gray deposits

Fig. 15. Succinate dehydrogenase activity on and in the haustoria of E. graminis f. sp. tritici. A) Blue diformazan crystals on haustoria. B) Control (omitting the succinate from reaction mixture). C) Control (omitting tetrazolium salt from reaction mixture.) D) Enzymatic activity in the chloroplasts. (370x)


A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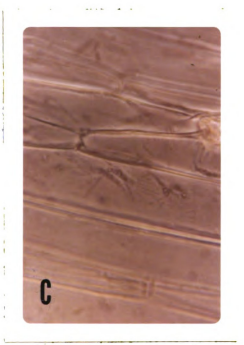
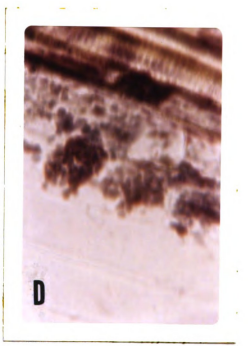

C

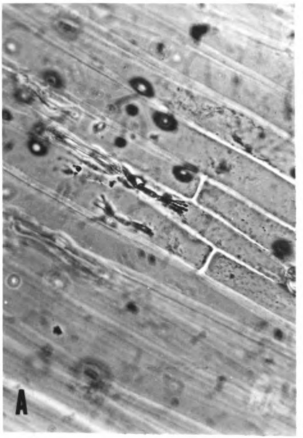

D

were formed on and around the haustoria, but these results were not reproducible and similar deposits were observed in most control experiments.

Cross-inoculation experiments.--The purpose of these experiments was to determine if infection occurs in a supposedly incompatible system, and to further test the formation of functional secondary hyphae as a criterion for the establishment of a successful host-parasite relationship.

Manchuria barley was inoculated with E. graminis f. sp. tritici and Little Club wheat was inoculated with E. graminis f. sp. hordei. Inoculated plants were kept under optimum conditions for infection and the percentage of secondary hyphal initials formed was determined 18 hr after inoculation, and at 2-hr intervals thereafter. Even with these supposedly incompatible systems, secondary hyphal initials were formed. When wheat mildew was inoculated onto barley, 69% of the applied conidia formed secondary hyphal initials 23 hr after inoculation (Fig. 17). Of the 69%, only 4% formed haustoria and functional secondary hyphae. When wheat plants were inoculated with E. graminis f. sp. hordei, 24% of the applied conidia formed secondary hyphal initials and of these only 0.75% were capable of producing haustoria and functional secondary hyphae (Fig. 17). These results once again emphasized the fact that only the formation of functional secondary hyphae (or haustoria) should be taken as a

Fig. 16. Acid phosphatase activity on and in the haustoria of E. graminis f. sp. tritici. A) Enzymatic activity 48 hr after inoculation. B) Enzymatic activity 72 hr after inoculation. C) Control. (575x)


A


B

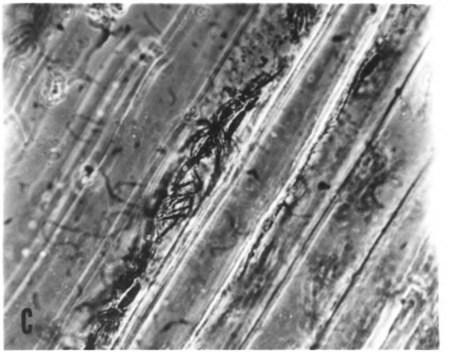
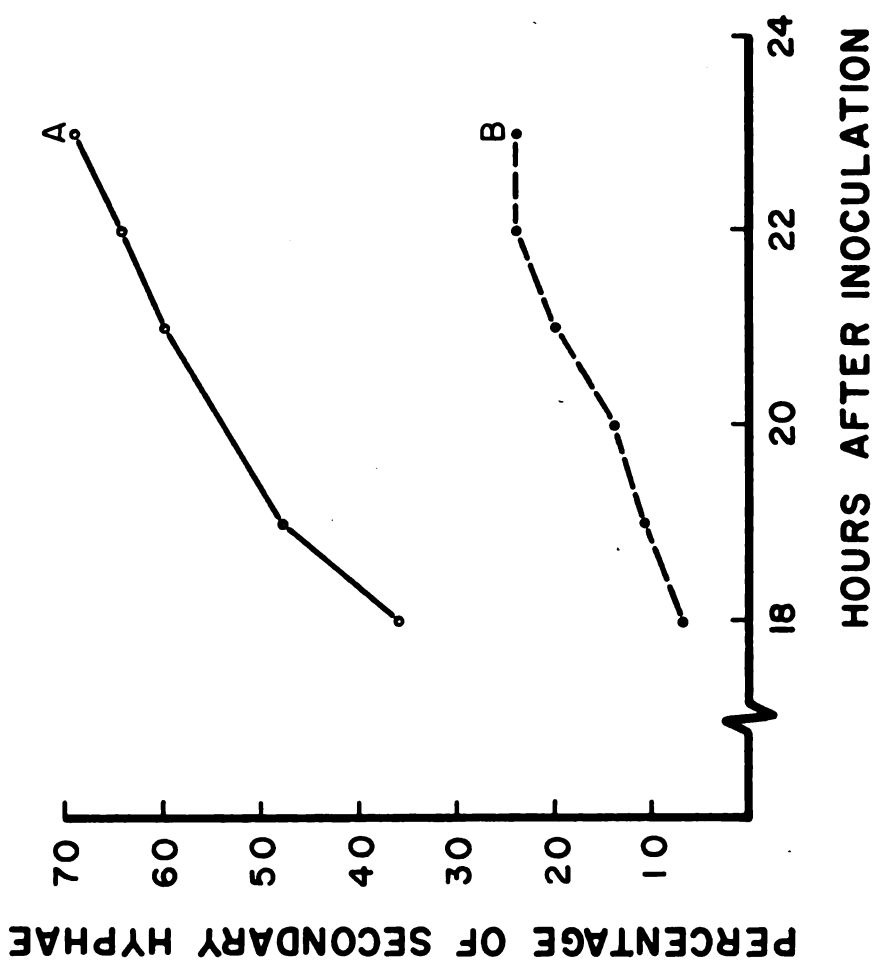

C

Fig. 17. Formation of secondary hyphal initials on nonsusceptible hosts. A) Wheat mildew (E. graminis f. sp. tritici) on Manchuria barley. B) Barley mildew (E. graminis f. sp. hordei) on Little Club wheat.


A
B
70
60
50
40
30
20
10
18
20
22
24
HOURS AFTER INOCULATION
PERCENTAGE OF SECONDARY HYPHAE

criterion for the establishment of a successful host-parasite relationship.

DISCUSSION.--Primary infection in powdery mildew is made up of several component phases (30). These phases are distinguished on a morphological basis and by a difference in response of each phase to various environmental conditions. When optimum conditions for every phase were reached, the parasite population was synchronized; that is, most units of the population reached each phase within a 4 hr period. Also, the primary infection process was shortened appreciably and the infection efficiency was greatly increased. About 90% infection was obtained in susceptible host-parasite combinations of wheat and barley; this is far higher than any other known host-parasite combination. This host-parasite combination, with a fairly well-defined kinetics, lends itself to quantitative studies of host-parasite interaction. It is possible to predict what portion of the parasite population is in a particular stage of development at a particular time.

After maturation of appressoria, a fine penetration peg emerges from the center of the appressorium into the lumen of the cell (11, 40, 41). Under optimum conditions, penetration starts as early as the 10th hr after inoculation. Corner (11) reported that penetration by powdery mildew fungi begins 24 hr after inoculation at 20 C and 100% RH. There seemed to be a considerable lag in the progress of primary infection as reported by Corner (11). Slow

progress of infection reported by Corner may be due to sub-optimal environmental conditions for each component phase.

The infection pegs undergo a series of morphological and cytological changes between the 11-18th hr after inoculation. The changes result in formation of haustorial bodies which reach their full size 18 hr after inoculation. At this time (18 hr after inoculation), secondary hyphal initials begin to appear on the sruface of the inoculated leaf. There was a correlation between the formation of haustorial bodies inside the epidermal cells and the simultaneous appearance of secondary hyphal initials on the leaf surface. It is reasonable to assume that secondary hyphal initials are not formed until the haustoria reach a certain stage of development. In all hosts studied, susceptible as well as resistant, there was a uniform pattern of morphological and cytological development of haustoria except for minor modifications brought about by the major genes for resistance.

Microscopic observations of the development of haustoria in susceptible and resistant hosts gave an insight to some mechanisms underlying resistance and susceptibility. The action of these major genes for resistance was during the early stages of the initiation of primary infection. The effect of all genes was to exclude a portion of the parasite population from infecting plants carrying the genes. It was shown e.g. that whereas about

90% of the parasite units could infect susceptible Manchuria barley, only 5, 30, and 33% of the parasite units could infect Algerian, Kwan, and Psaknon barley respectively. It is very unlikely that these genes act during pre-penetration stages.

Conclusions regarding infection by mildew do not agree entirely with conclusions of other workers, using other models. Flentje (16) ascribed failure in host penetration to: 1) lack of infection pegs; 2) mechanical toughness of the cuticle; and 3) poor attachment of the appressoria and consequent failure to take the back pressure of penetration. Graf-Martin (20) attributed resistance of older barley plants to E. graminis f. sp. hordei to the abortion of the infection peg due to the hardness or thickness of the cuticle and epidermis. It is conceivable that one or more of these mechanisms could be involved in mildew resistance, but other possibilities should not be overlooked. It is quite possible that even after infection takes place, the progress of the pathogen could be halted thus constituting a later line of defense in the host. It was demonstrated that even after a fully compatible host-parasite relationship was established, the Algerian gene suppressed completely the sporulation of the fungus and, consequently, repeating disease cycles were avoided. The Kwan gene induced a hypersensitive reaction 5 days after inoculation. This

resulted in the collapse of host cytoplasm and eventual disintegration of most haustoria.

Several of the aforementioned mechanisms might be operative in resistant hosts. It is plausible to assume that highly resistant or immune hosts are very well "buffered" by having several of these "protective" mechanisms as compared to less resistant hosts. It would be conceivable, therefore, that the Algerian gene confers most of these mechanisms of resistance, whereas the Psaknon gene confers a more limited number of these mechanisms. Consequently, whereas the Algerian strain of barley is almost immune to barley mildew, the Psaknon strain is relatively susceptible.

In compatible host-parasite combinations, secondary hyphal initials elongate and branch giving rise to secondary and tertiary appressoria which eventually develop into macroscopically visible pustules. A portion of the secondary hyphal initials never advanced beyond 2 u in length (i.e. never reached the functional form). On all hosts studied (resistant and susceptible), over 90% of the applied conidia produced secondary hyphal initials under optimum conditions. Even in non-compatible host-parasite combinations (barley mildew on wheat and wheat mildew on barley), secondary hyphal initials were formed. Since secondary hyphal initials can form in the absence of developed haustoria, the initiation of secondary hyphal

initials might be the result of a mechanical stimulus. Although this might be true, other factors could be involved. There seemed to be some specificity even in the formation of secondary hyphal initials. This was substantiated by the fact that whereas over 90% of the parasite units produced secondary hyphal initials on susceptible hosts, only 69 and 24% of the parasite units produced secondary hyphal initials on Manchuria barley and Little Club wheat when inoculated with wheat and barley mildew respectively.

In all hosts studied, there was a good correlation between the number of functional secondary hyphae on the surface of the leaf and the number of haustoria in the epidermis of the same leaf. The same relationship did not hold for secondary hyphal initials. This meant that secondary hyphal initials could form regardless of whether or not haustoria were formed. In fact, it was observed that while 95% of the applied conidia produced secondary hyphal initials, only 5% of those same conidia produced functional secondary hyphae and haustoria when the host plant possessed the Algerian gene. Therefore only functional secondary hyphae or haustoria should be the criterion for the establishment of a successful host-parasite relationship.

All attempts by other workers to study biochemical changes in the hosts, in response to infection, have shown

differences at the time of flecking or sporulation of the fungus (2, 3, 7, 26, 45). Allen (2) and Bushnell and Allen (7) reported that plants infected with powdery mildew had increased respiration, but these respiratory changes were not detected until 6 days after inoculation. Several isozymes of succinate dehydrogenase and malate dehydrogenase were reported in rust-infected bean plant 12 days after inoculation (45). In my study, histochemical tests revealed certain enzymatic changes in the host 2 hr after penetration. This was rather well established with succinate dehydrogenase activity at 12, 14, 16, 18, 24, 48, 72 and 144 hr after inoculation. The detection of succinate dehydrogenase in the appressoria before penetration, and around the haustoria (in the host) 2 hr after penetration indicate that either the enzyme moved from the parasite to the host or its synthesis was stimulated in the host by the parasite. Whichever the case may be, this is an indication of a host-parasite interaction early in the establishment of parasitism. Whether this is necessary to parasitism or is merely a side effect was not determined.

The optimum environmental conditions for germination, formation of appressorial initials, and maturation of appressoria were determined with relative ease. This was not the case with optimum conditions for the formation of functional secondary hyphae. This is not hard to conceive

since all processes conducive to the establishment of infection take place prior to the formation of functional secondary hyphae. It is the formation of infection pegs at the lower side of the appressoria, the penetration of host cuticle and epidermis, and the development of haustoria that precede the appearance of functional secondary hyphae on the surface of the host. The progress of the parasite could, theoretically, be halted at any one of these steps, and finding the overall optimum conditions for this phase of infection might require finding the optimum conditions for each individual step.

It was generally observed that the external structures of the fungus were sensitive to various environmental conditions. This was not the case with the haustoria, the only internal structures of the fungus. The development of haustoria was insensitive to light and RH. Insensitivity to light could be partly due to limited photosynthetic activity in epidermal cells. Lack of response to RH could mean that the fungus, being inside the host, has plenty of water. High temperature of 35 C blocked haustorial development. This temperature is well above the optimum for growth of many fungi.

This host-parasite combination is a very useful system for the study of host-parasite interaction. Because of the ectoparasitic nature of the fungus, changes in both the host

and the parasite can be studied when in close association. The fungus population can be synchronized, allowing for kinetic studies.

SUMMARY

The differential response of the various stages of primary infection of powdery mildew (E. graminis) to different environmental conditions suggests that the primary infection is made up of several stages. These are: 1) germination, 2) formation of appressorial initials, 3) maturation of appressoria, 4) development of the haustorial body, 5) the formation of secondary hyphal initials, and 6) the formation of functional secondary hyphae.

Optimum environmental conditions were established for development of each phase. Under optimum conditions, the parasite population was synchronized and most units of the population reached each phase within a 4 hr period. The host was penetrated around the 10-12th hr after inoculation. The haustorial bodies appeared 16 hr after inoculation and full-sized haustoria were detected 34-36 hr after inoculation.

Prior to penetration, succinate dehydrogenase activity was detected only in the parasite. Two hr after penetration, succinate dehydrogenase activity was detected on the surface of the young, developing haustoria. This indicated that either the enzyme moved from the parasite to the host or its synthesis was stimulated by the parasite in the host.

In susceptible hosts and in strains of barley with major genes for resistance, the number of functional

secondary hyphae on the surface of the host equalled the number of haustoria formed inside the host. Hence, either the functional secondary hypha or the haustorium could be taken as a criterion for the establishment of primary infection.

All major genes exhibited more than one effect in their mode of action, and therefore, these genes are pleiotropic. The various ways and means by which these genes conferred resistance on host plants were: 1) exclusion of the pathogen from the host; 2) delay in early haustorial development; 3) distortion of some haustoria; 4) destruction and distortion of the majority of haustoria 5 days after inoculation; and 5) suppression of fungus sporulation after the establishment of primary infection thereby preventing the occurrence of repeating disease cycles.

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