THE ROLE OF SPECIFIC GENES IN PRIMARY INFECTION OF WHEAT AND BARLEY BY ERYSIPHE GRAMINIS

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ABSTRACT

THE ROLE OF SPECIFIC GENES IN PRIMARY INFECTION OF WHEAT AND BARLEY BY ERYSIPHE GRAMINIS

By

Sheau-loh Yang

The process of primary infection of wheat and barley by Erysiphe graminis consists of a number of morphologically identifiable stages of development: spore germination, formation of appressoria, penetration into host cells, formation of haustoria in host cells, and formation of elongating seccondary hyphae (ESH) which are capable of initiating secondary and tertiary infections.

Conidia will germinate on a number of different natural and artificial surfaces. A high percentage of normal appearing, mature appressoria was observed on host leaves, the upper surface of epidermal strips, or on the upper surface of enzymatically isolated cuticles. No or few normal appearing, mature appressoria were formed on the lower surface of epidermal strips, the lower surface of isolated cuticles, on reconstructed wax layers, or on a number of different artificial surfaces. The presence or absnece of specific M*l* or Pm genes in the plants from which the epidermal strip or cuticles were isolated did not affect the formation of mature appressoria. Malformed appressoria

were observed on plants that possessed eceriferum (cer) mutations that affect the chemistry and physical structure of wax layers.

The *Ml* and Pm genes in barley and wheat, respectively, did not appear to interact with the corresponding *P* genes in the parasites to affect the morphological development of the parasites prior to penetration. The two effects of the incompatible parasite/host genotypes were: (1) the reduction in the percentage of parasite units that formed elongating secondary hyphae, and (2) the reduction of the infection type six days after inoculation.

In all four near-isogenic barley lines tested, the range of the percentages of ESH on the homozygous dominant (*Ml Ml*) and the homozygous recessive (*ml ml*) plants derived from selfing of heterozygous parents (*Ml ml*) was considerably larger than on homozygous dominant (*Ml Ml*) or recessive (*ml ml*) plants derived from homozygous parents. It appears that there is a carry-over effect from the genotype of the parent to the progenies.

No differences in the antigenicity of the protoplasts with different *Pm* genes were detected by homologous and heterologous agar gel double diffusion tests. The differences between rabbits of sensitivity to produce antibodies against isolated protoplasts were greater than the differences between tested protoplasts with different *Pm* genes.

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INTRODUCTION

The powdery mildew diseases of barley (Hordeum vulgare L.) and wheat (Triticum aestivum L.) are caused by Erysiphe graminis D.C. f. sp. hordei Em. Marchal and Erysiphe graminis D.C. f. sp. tritici Em. Marchal, respectively. These fungi are obligate ectoparasites. They are prevalent in most of the barley and wheat growing areas of the world. The diseases cause a very significant reduction in yield of grain in some areas of the world (46, 47, 83, 86).

The principal means of control of the mildew diseases have been through the selection and breeding of resistant cultivars. As a result, there is available a large array of host genotypes affecting mildew development, which give different degrees of resistance. The genes in the host interact with the genes in the parasite, a prediction of the gene-for-gene hypothesis, to affect parasite development and host response (29, 30, 31, 32, 52, 61, 79).

There are numerous reports on the comparative physiology and biochemistry of healthy and diseased tissues. Studies have also been made on the comparative physiology and biochemistry of "resistant" and "susceptible" infection types. The relationships of the differences observed to the

genes determining the various types of interactions are not clear. The confusion that surrounds the different interpretations of data by the various researchers is probably affected by several factors. Use of different cultures, environmental regimes, inocula, "resistant" and "susceptible" plants of different species, times of observations, etc., have probably contributed to the confusion in attempts by different workers to determine the critical processes in interactions between host and parasite, both for parasitism and pathogenesis.

A reasonably defined system for examining the early infection process of E. graminis on wheat and barley has been reported (52, 62, 64). The development of the fungus was shown to consist of a number of developmental stages which can be distinguished by changes in morphology and differential sensitivity to various environmental conditions. With the appropriate environmental conditions, the synchrony of morphogenesis can be increased and the percentage of successful infections can be maximized.

Conidia germinate under favorable environmental conditions and produce appressoria. Penetration pegs produced on appressoria provide the means for the fungi to get into the epidermal cells and produce haustoria. Further development of the fungus is by growth of the fungus structure on the surface of the host leaves and the production of additional penetration pegs and haustoria in the epidermal cell layer (Figure 1).

Figure 1. Diagram of Erysiphe graminis development during the stages of primary infection. (A) ungerminated spore, (B) germinated spore, (C) appressorial initial, (D) mature appressorium, (E) penetration peg, (F) haustorial body, (G) enlarged haustorial body and secondary hyphal initial, (H) elongating secondary hyphae and developing haustorial appendages, (I) elongating secondary hyphae and mature haustorium, (J) secondary appressorium. (From R. Slesinski's thesis, Michigan State University, 1969.)

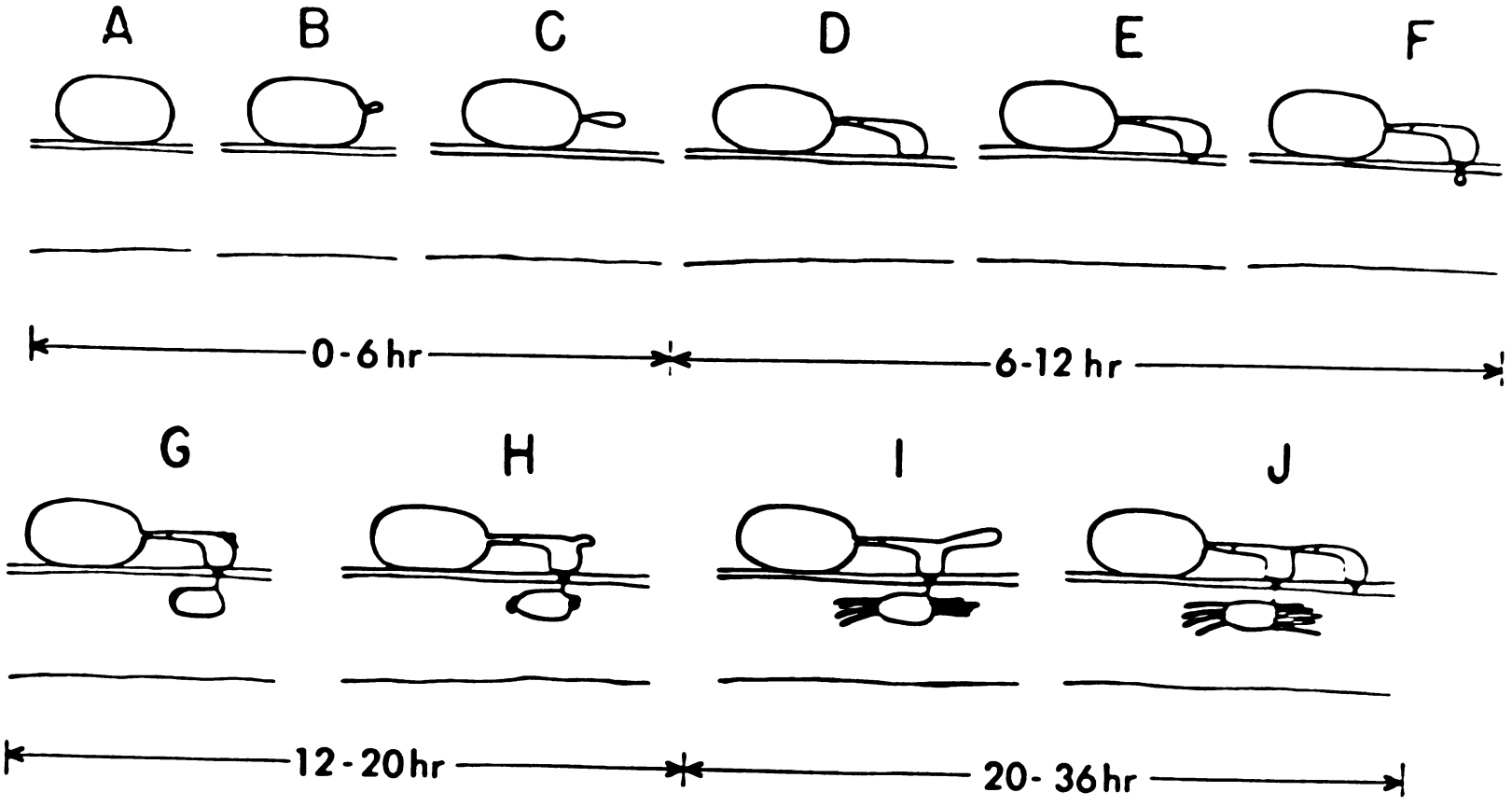

A
B
C
D
E
F
0-6 hr
6-12 hr
G
H
I
J
12-20 hr
20-36 hr
TIME AFTER INOCULATION

Figure 1

The objectives of this study were the following: (1) to determine the role of the host cuticle layer during the primary infection process, particularly on the formation of mature appressoria by the parasite population before penetration; (2) to examine the action of genes for incompatibility in the parasite and host on the sequential development of the parasite; (3) to determine the relationship between the percentage of elongating secondary hyphae to the segregation of genes for incompatibility in the host; and (4) to determine if antigenic differences on the cell protoplasts could be detected among near-isogenic host lines with and without genes for incompatibility.

LITERATURE REVIEW

Numerous articles have been published on the physiological, genetic and biochemical aspects of powdery mildew diseases. Many of the articles have contradictory conclusions. This review is to summarize the developments considered to be pertinent to the understanding of the diseases and to be related to the results reported herein.

Environmental conditions, i.e., light intensity, light period, relative humidity, temperature, etc., greatly affect powdery mildew development (12, 14, 33, 76, 94). Several different optimal conditions for mildew development have been reported (12, 36, 64, 76). Germination and subsequent development of conidia occur over a wide range of temperatures and relative humidities.

The development of E. graminis spores after deposition on the plant surface can be divided into several morphologically distinguishable stages: (1) germination, (2) production of appressorial initials, (3) formation of mature appressoria, (4) penetration of the cuticle and epidermal cells, (5) formation of haustoria, (6) development of elongating secondary hyphae (ESH), (7) initiation of secondary, tertiary, etc., infections, and (8) sporulation (12, 36, 51, 94). It has been shown that particular

temperature, relative humidity, and light conditions are required for synchronous development of each of the different stages of primary infection (51, 64). With optimum conditions for each stage, over 75% of the spores applied onto the host leaves proceed through the successive stages with a high degree of synchrony (51, 52, 53, 55). The production of elongating secondary hyphae (ESH) by the parasite indicates that the fungus is obtaining nutrients from the host which permit the continued growth of the parasite (52, 55). Furthermore, the number of ESH formed on the host surface corresponded to the number of haustoria in the host epidermal cells (52). The percentage of the applied conidia which develop ESH and, therefore, form a functional relationship with the host is defined as the infection efficiency (25).

Under natural conditions, spores of the fungus are conveyed to leaves by wind. There they germinate and form appressoria as the initial steps toward invasion of epidermal cells. The stimulus for forming appressoria was thought to be provided simply by a physical contact with the host surface (6, 16). Gold-leaf and collodion membranes were demonstrated to excite appressorium formation of Botrytis by physical contact (6). Physical contact in exciting rust spores to form appressoria was also emphasized (16). Chemical stimuli, too, may be important in the formation of appressoria (40, 41, 77). Spores of Puccinia coronata formed appressoria on gelatin if zinc ions were present (77).

A chemical stimulus is also apparent in the production of infection cushions by the soilborne pathogen Rhizoctonia solani (40, 41). The hyphae of the strain of R. solani specifically pathogenic to each host produced an appressorium-like structure on the surface of the cellophane placed over roots and indicated that the fungus responded to substances diffusing from the roots through the cellophane (40, 41, 27). Strains not pathogenic to a particular host showed no such response. The wax on living leaves of onion seemed to have no effect on the germination of spores of Alternaria porri, but the formation of appressoria was markedly influenced by the presence of wax on the leaves (1).

The host leaf surface penetrated is complicated both structurally and chemically. The pegs produced by the appressoria of a pathogen have to penetrate through several distinct layers, with indistinct interfaces, constructed of wax, cutin, pectin, cellulose, and protein (15, 54) to reach the phospholipid plasma membrane and to where the haustoria are formed.

The mechanism of wall penetration by obligately parasitic fungi has long been a subject of speculation. Many plant pathologists have believed that the mode of entry was mechanical (3, 7, 88). Some work on Puccinia graminis on barberries also supported the mechanical theory (58). However, indirect evidence for degradation of cuticle by powdery mildew comes from analyses of the surface layers of healthy and mildewed leaves of apple and turnip (93). The

cutin content of mildewed leaves is considerably lower than that of symptomless leaves. Rose leaves infected with Sphaerotheca pannosa have only one-fourth of the cutin content of healthy leaves (93). Penetration of the cell wall of the host plant by a pathogen may require the participation of cutinase, pectinases, pectin-methylesterase, cellulases, peptidases and proteases besides mechanical force (35). Polygalacturonase, cellulase and a hemicellulase had been reported in uredospores of Puccinia graminis var. tritici (81). Electron micrographs of some workers showed a crack across the center of the halo where penetration occurred (1) by Erysiphe graminis, while others did not find evidence for cracks in the cuticular layer (4). Cytochemical studies have also suggested that the cellulose wall in the zone around the infection peg is degraded by enzymes produced by the powdery mildew fungi (56). Only recently, observations of the penetration process of powdery mildew into the epidermal cells of barley by electron microscopy indicate the enzymatic digestion of the cuticle and cellulose portion of the epidermal wall by enzymes secreted from the developing mildew infection peg, and a mechanical pushing of the infection peg through a layer of material which has been deposited on the underside of the epidermal walls (22).

The electron microscope has been used for studies on the ultrastructure of the haustorial apparatus and the boundary region of the haustoria and host cytoplasm, the

so-called host-parasite interface (4, 23, 24, 57, 68, 78). Little attention has been given to the age of the haustoria examined and to the ultrastructural features of the developing haustoria.

Haustoria are thought to be specialized fungal structures for the uptake and transport of nutrients from the plant to the mycelium on the surface of the host (9, 12, 94). ESH never form if haustoria do not form (52). Fungus development on the leaf surface ceases after the destruction of the haustoria (9). Electron microscopy has shown that the haustorial wall of various pathogens is separated from the host cytoplasm by a sac-like membrane (4, 23, 24). The cytoplasm of the host and the cytoplasm of the parasite appear never to come into direct contact.

Biochemical studies on the nature of compatible or incompatible relationships between host and parasite (21, 37, 42, 92) are difficult, since two inseparable metabolic machineries are involved and both are changing continuously. Even if changes in an infected plant could be observed by various chemical or biochemical assays, the cause and effect relationships of these alterations as determinants of disease development is difficult to assess. The most convincing data of a known primary disease determinant comes from a nonobligate fungal parasite Helminthosporium victoriae. A host-specific toxin necessary for disease production was liberated by the fungus prior to the penetration (70, 75). The toxin affects only varieties of oats possessing the Vb

gene, which is thought to code for a receptor site for the toxin molecule (75, 87). No such determinants have been found in the physiological and biochemical studies of the powdery mildew diseases. Extracts of E. graminis conidia have been demonstrated to produce disease-like symptoms (8). No specificity of these extracts was found, however. Other substances, such as yeast extract, were shown to produce similar results (82). Many toxic compounds have been isolated from 'resistant' plants (82), but again they lack sufficient specificity and have not been shown to correlate with the specific genes conditioning incompatibility.

It is clear from many investigations (32, 65) that the interaction of host and parasite are controlled by complementary genetic information possessed by both host and parasite. The ability of Melampsora lini to grow and produce disease symptoms on flax lines containing genes for resistance was determined by specific corresponding genes in the pathogen (29). The existence of one gene in the pathogen for each gene in the host led to the development of the gene-for-gene hypothesis (31, 66). The gene-for-gene concept simply states that each R gene in the plant interacts with a specific corresponding P gene in the pathogen to determine mildew development or parasite/host incompatibility (26, 29, 66). With most diseases studied to date, resistance (R) and avirulence (P) are dominant and virulence (p) and susceptibility (r) are recessive (26, 48). This

concept has been found to apply to many other host-parasite systems (60, 67, 71) (Figure 2).

A biological test termed the 'quadratic check' has been proposed to study the physiological and biochemical effects of disease (72) (Figure 2). Incompatibility, or a low infection type, is specified only when the corresponding parasite/host genotype contains at least one P gene in the fungus and the corresponding R gene in the plant (P/R). Compatibility, or high infection type, is specified with the remaining P/r, p/R, and p/r, parasite/host genotypes (48, 72). No difference in the development of Puccinia sorghi on near-isogenic corn lines was observed with compatible parasite/host genotypes. With incompatible genotypes, however, reduced numbers of haustoria, encapsulation of haustoria in the cells, and fungus lysis were observed (38). The rapidity of cell collapse has also been suggested as a basis for resistance to disease development (70). Measurements of the rate of $^{35}SO_4$ transferred from a wheat leaf to the developing fungus during primary infection suggested that the rate of transfer was lowest with P/R, intermediate with p/R, and greatest with P/r and p/r (79, 80).

The concept that an immune response of a host may exert selective pressure on fitness for survival of many parasitic species has recently been supported by several lines of evidence in both animal and plant disease (17, 18, 19, 20, 39). The parasitic worm, Haemonchus contortus was found to display a greater antigenic disparity with rabbit

Figure 2. Diagram of the four possible parasite/host genotypes involving single loci governing compatibility in the plant and the pathogen. Rl and rl are the alternate alleles in the plant and Pl and pl are the alternate alleles in the pathogen corresponding to the Rl gene in the plant. Pl/Rl genotypes specify incompatibility. Pl/rl, pl/Rl, and pl/rl genotypes specify compatibility.

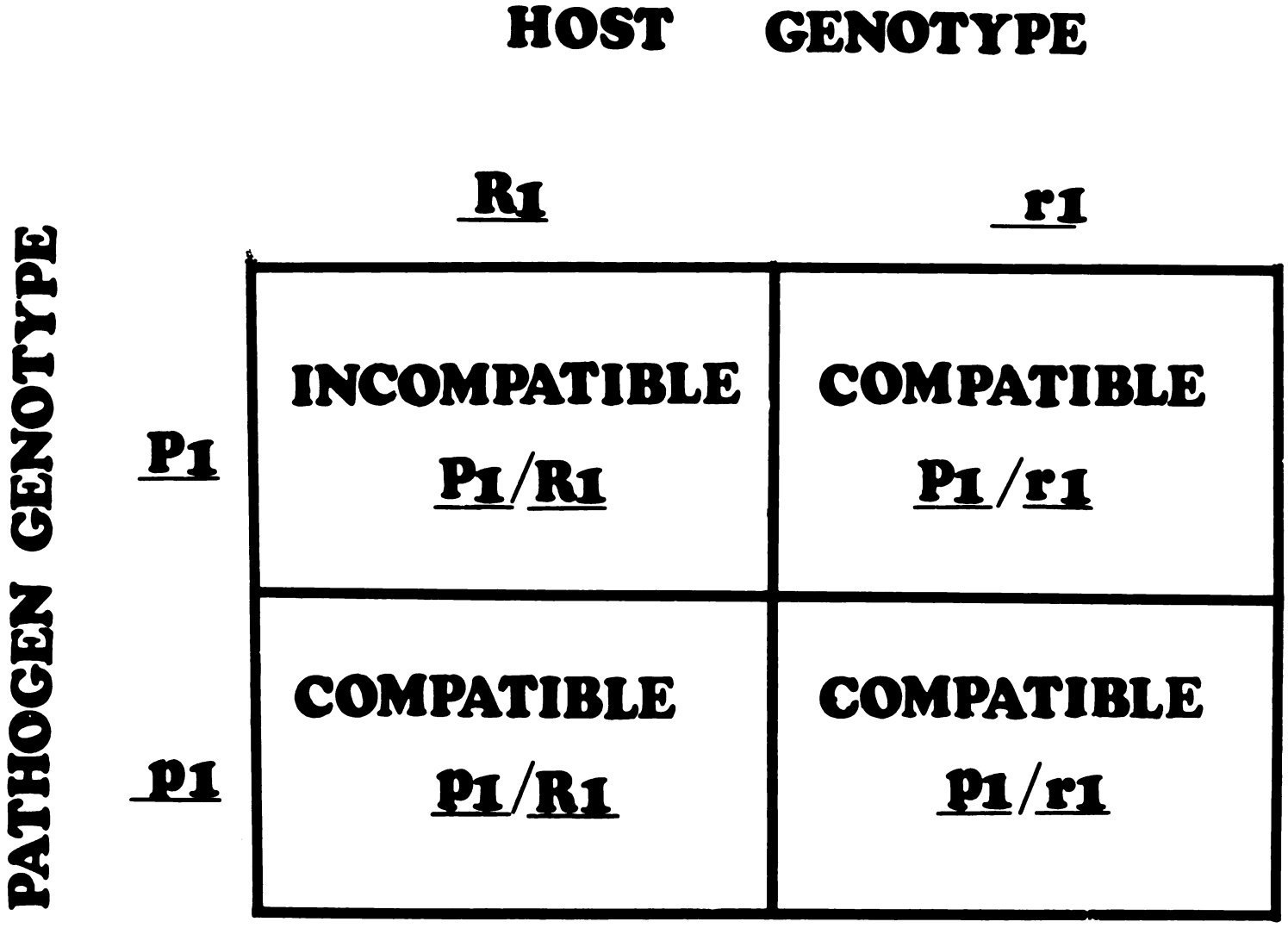

HOST GENOTYPE
R1
r1
PATHOGEN GENOTYPE
P1
p1
INCOMPATIBLE
P1/R1
COMPATIBLE
P1/r1
COMPATIBLE
p1/R1
COMPATIBLE
p1/r1

Figure 2

than with sheep, its natural host (18). The failure of sheep to produce antibodies to antigens common to both larval and adult worms was interpreted to indicate that these antigens were common to the host (sheep) and that such antigens constitute 'fitness characters.' At present there is no conclusive evidence that similar immune responses are produced in plants directed against pathogenic agents. Several authors, however, have alluded to the possible operation of an immune response in plants (10, 20, 73). Others (84, 85, 89, 90) have presented evidence to support the notion that new 'proteins' are formed by plant tissue following infection by pathogenic fungi and bacteria. Evidence was presented to suggest that a specific antigen in each of four races of the obligately parasitic rust fungus Melampsora lini was commonly shared by only those lines of flax that were susceptible to those races. A race of rust tested was avirulent to flax lines lacking the specific antigen (19, 20). The idea is plausible that common antigens between a host and a parasite might provide a less hostile environment for the parasite during the infection process and improve the chance for a successful parasitic relationship. However, more conclusive evidence is needed that the common antigens between host and parasite have some role in determining compatibility between host and parasite.

The research approaches to the problem of obligate parasitism have been varied. They range from studies of gross chemical differences in host varieties resistant and

susceptible to a given strain of pathogen to studies of the host-parasite interface by electron microscopy. Each approach has specific advantages, but none seem to be free from either conceptual or technical problems. It has become increasingly obvious that there will be no royal and direct road to an understanding of infections caused by obligate parasites. Much of the earlier work must be reevaluated as new techniques are available and more information is obtained. It is encouraging, however, that we are becoming aware of the limitations that many of our analytical procedures and experimental designs have imposed.

MATERIALS AND METHODS

Culturing of powdery mildew.--The strain CR-3 of Erysiphe graminis D.C. f. sp. hordei Em. Marchal was maintained on susceptible barley (Hordeum vulgare L. 'Manchuria'). The strain MS-1 of Erysiphe graminis D.C. f. Sp. tritici Em. Marchal was maintained on susceptible wheat (Triticum aestivum L. 'Little Club'). Wheat and barley plants were grown in 4-inch pots and were inoculated when they were 6-7 days old. Sets of wheat and barley plant were inoculated daily by dusting conidia produced 7 days after inoculation onto the leaves of the susceptible wheat and barley. Inoculated plants were maintained in a controlled environment chamber provided with adequate air circulation under the following conditions: Light intensity: 700 to 800 ft-c (650-705 ft-c from white VHO-fluorescent tubes and 50 ft-c from 25 watt incandescent bulbs). Light period: 15 hr/day. Temperature: $18\pm1^{\circ}C$ during the light period and $16\pm1^{\circ}C$ during darkness. The relative humidity (RH) was $65\pm5\%$ during the light period and $95\pm5\%$ during darkness. Mycelial growth of the culture was first macroscopically evident 3-4 days after inoculation. Conidia for experimental uses were abundant by 6 days after inoculation.

Lines of wheat and barley used.--Five lines of wheat were used. Chancellor, which contains no known major genes affecting mildew development, was used as the standard to which mildew development on other lines was compared. The four backcross-derived lines of wheat which contained Pm genes affecting mildew development were obtained from Dr. L. W. Briggle. They were designated as follows: Pm1 (Axminster x^8 Cc), Pm2 (Ulka x^8 Cc), Pm3 (Asosan x^8 Cc), Pm4 (Khapli x^8 Cc). The symbol, x^8 Cc, refers to the original cross and seven backcrosses to the cultivar Chancellor (2, 5). All barley lines were obtained from Dr. J. G. Moseman. Manchuria barley was used as the standard to which other lines were compared. Four other lines, each possessing a different gene affecting mildew development, were backcrossed to the variety Manchuria. The four near-isogenic lines and their derivations were as follows: M*la* (Algerian C.I. 1179 x_7^4 Manchuria C.I. 2330), M*lq* (Goldfoil C.I. 928 x_9^4 Manchuria C.I. 2330), M*lp* (Psaknon C.I. 6305 x_7^4 Manchuria C.I. 2330), M*lk* (Kwan C.I. 1016 x_8^4 Manchuria C.I. 2330). The symbol, x_7^4, refers to the original cross with Manchuria, three generations of backcrossing to Manchuria, and then the selfing of the heterozygous progeny in each of seven generations. After 7 to 9 generations of selfing, homozygous dominant and homozygous recessive lines were selected. These lines were highly isogenic to each other and are referred to as paired lines. Homozygous dominant, homozygous recessive and heterozygous plants were selected for

use in this study. Five lines of eceriferum mutants (eceriferum loci control the synthesis and/or excretion of the organic-specific wax components), cer-J[59], cer-J[71], cer-zd[67], cer-ze[81], and cer-zj[78], which were induced by either ionizing radiations or chemical mutagens, as well as wild type cultivar Bonus, were kindly supplied by Dr. P. von Wettstein-Knowles (49).

Designation of genotypes.--The barley variety Manchuria contains no known major genes affecting development of Erysiphe graminis. By definition, therefore, it contains the recessive alleles at the four loci that are known to determine mildew development. Rather than write out the complete genotype for each host line the following system of abbreviation is used:

Actual Genotype	Designation Used
Barley:	
m*la* m*la* m*lq* m*lq* m*lp* m*lp* m*lk* m*lk*	Manchuria
M*la* M*la* m*lq* m*lq* m*lp* m*lp* m*lk* m*lk*	M*la*
m*la* m*la* M*lq* M*lq* m*lp* m*lp* m*lk* m*lk*	M*lq*
m*la* m*la* m*lq* m*lq* M*lp* M*lp* m*lk* m*lk*	M*lp*
m*la* m*la* m*lq* m*lq* m*lp* m*lp* M*lk* M*lk*	M*lk*

Actual Genotype					Designation Used
Wheat:					
pm1 pm1	pm2 pm2	pm3 pm3	pm4 pm4		Chancellor
Pm1 Pm1	pm2 pm2	pm3 pm3	pm4 pm4		Pm1
pm1 pm1	Pm2 Pm2	pm3 pm3	pm4 pm4		Pm2
pm1 pm1	pm2 pm2	Pm3 Pm3	pm4 pm4		Pm3
pm1 pm1	pm2 pm2	pm3 pm3	Pm4 Pm4		Pm4

The genotype of the strain (MS-1) of E. graminis f. sp. tritici used in this study that is pertinent to this work is Pl P2 P3 P4. The genotype of the strain (CR-3) of E. graminis f. sp. hordei used in this study is Pa Pq Pp Pk.

Method of controlled inoculation.--Single 5 to 6 day-old plants grown in 2-inch pots were inoculated by the rolling method (63) in all experiments where the development of the powdery mildew fungus during primary infection was studied. Conidia were dusted onto a clean glass slide and transferred to a single wheat or barley plant with a cotton swab. Only the lower (abaxial) leaf surface of each plant was inoculated. The progress of the infection process is similar on either side of the leaf, but microscopic observations and the removal of the host epidermis are much easier on the lower side of the leaf (39). A uniform distribution of single conidia ranging from 80-120/cm^2 of leaf area was obtained by this method. Only single, well separated

parasite units were counted at each observation to eliminate the possibility of inhibition due to crowding (39).

Environmental conditions for experiments.--All experiments were carried out in Scherer-Gillett (Model CEL 512-37 and Model CEL 25-7) growth chambers. The conditions necessary for synchronous growth of each developmental stage of primary infection are as follows:

1. The first hr inoculated plants were kept in darkness at 18±1°C, and at approximately 100% relative humidity (RH).
2. The second through the sixth hr inoculated plants were kept in 260 ft-c of light (200 ft-c from white VHO fluorescent tubes and 60 ft-c from incandescent bulbs), 22±1°C, and RH of 65±5%.
3. The seventh through the twentieth hr the conditions were the same as (2.) above, except in darkness.
4. The twenty-first through the thirty-sixth hr the conditions were the same as (2.) above

Changes in temperature and relative humidity during experiments were monitored with wet and dry bulb thermometers and with a hygrothermograph calibrated with a sling psychrometer. Light intensity was measured at the distance of the plant from the lights with a Weston Model 756 light meter.

Examination of fungal development.--Observations of the percentage of the parasite population in each stage of development were performed by direct microscopic observations with a B & L microscope equipped with apochromatic objectives and at a magnification of 150X. At various times after inoculation, the development of the parasitic units on 1 cm leaf sections (excluding the 1 cm tip section) was determined. The leaf was discarded after the percentage of parasitic units in each stage of development was recorded. Observations at subsequent hours were made on other inoculated plants. Approximately 80-150 parasite units were counted at each observation.

Test of the segregation of Mℓ genes in barley.--Seeds derived from heterozygous parent barley plants with genotypes Mℓa mℓa, Mℓq mℓq, Mℓp mℓp, or Mℓk mℓk were planted individually in 2 inch pots. Barley seeds from homozygous recessive or homozygous dominant parents were planted as a control. The 5 to 6-day-old seedlings were inoculated by the rolling method (63). Inoculated plants were held under standard environmental conditions known to favor sequential parasite development. The percentage of conidia that produced elongating secondary hyphae (ESH) on a portion of the primary leaf on individual plants was recorded 28 hr after inoculation. The infection type was recorded on each plant 6 days after inoculation. Plants were then grown to maturity in the green house. The genotype of each tested plant

was determined by the segregation of infection types among progenies seven days after inoculation.

Enzyme solutions for the preparation of cuticle layers and protoplasts.--Cultures of Myrothecium verrucaria 460 obtained from the Quartermaster lab, Natick, Mass., were grown on potato dextrose agar slants until spores were produced, usually about 8 days. Sterile distilled water was added to the tubes. The mycelium was chopped into small pieces and the contents of the tubes were put into 500 ml of Whitaker's salt liquid medium containing 0.5 gm glucose and 5 gm ashless cellulose powder (product of W. & R. Balston Ltd., England). Vigorous reciprocal shaking at 22°C for 14 days resulted in nearly maximal amounts of 'cellulase' in the medium (the term cellulase is used to denote the material that digests the cell walls of wheat and barley). The cultural filtrate was evaporated to 35-40 ml in a vacuum evaporator at 43°C in approximately 2 hrs. This condensed crude solution was used as stock for the preparation of natural intact cuticle layers.

Further purification of the enzyme preparation was performed for the isolation of protoplasts from host coleoptiles. Crude extracts of 35-40 ml were fractionated with $(NH_4)_2SO_4$ at 2°C. The fraction precipitating at 35-70% of saturation was rediscovered in 2-3 ml of distilled water (the smallest amount needed to get complete solution) and desalted with a 1.2 x 20 cm column of Sephadex G-25 at 2°C.

One-tenth percent NaCl was used to maintain ionic strength and prevent binding to the Sephadex. The cellulase activity passed rapidly through the column in the front-running brown-pigmented band. Ions and certain pigments were retained. The first four 1 ml fractions were usually collected, mixed and frozen. Activity was retained for as long as 6 months, though frozen and thawed several times (74).

Preparation of natural intact cuticles.--Plants of 6-day-old seedlings were used. A section of about 3.5 cm long of the lower (abaxial) epidermis layer of the seedlings was peeled off and quickly transferred onto a 3-fold-diluted concentrated enzyme solution in a petri dish. The epidermal strips floated on the enzyme solution with their hydrophobic wax layer up until the epidermal cells were loosened from the cuticle and sedimented to the bottom of the solution. Approximately 12 hrs was needed to prepare cell-free cuticle layers. The isolated cuticles were then transferred to a 2% agar surface and subjected to various experiments.

Reconstruction of wax layer.--Chloroform was used to extract the waxy substances on either isolated intact cuticles or fresh leaves of Chancellor wheat. Extraction was performed by dipping cuticles or leaves 3 times, 10 seconds each time, into chloroform. To reconstruct a wax layer with approximately similar thickness as natural wax layers, the total surface area of extracted cuticles or leaves was calculated, and the chloroform solution was condensed so

that an equal reconstructed area could be made. Milky-white suspended wax particles were sometimes observed after condensation as a result of over saturation. These suspended particles were redissolved by elevating the temperature to 45°C. The chloroform solution was then spread over the surface of 2% agar. After the chloroform was completely evaporated, sterile water was gently poured into the petri dish. The reconstructed wax layers floated on the water surface. Reconstructed wax layers were then transferred to the surface of 2% agar and were used for various tests.

Preparation of protoplasts from coleoptiles.--Wheat seeds with and without Pm genes were soaked in water for 2 hr, surface sterilized with 15% (v/v) clorox, placed on wet paper towels under aseptical conditions, and exposed to red light for 24 hrs. They were then kept in the dark at 25°C until the coleoptiles were 35 mm long. To improve the penetration of cellulase, the first 1 cm from the tip was discarded. The remainder of each coleoptile was cut once longitudinally and then into 2 mm sections. The penetration of the enzyme was improved when the coleoptile was cut into small sections. For each experiment 20 coleoptiles were cut into pieces and placed in an enzyme-mannitol mixture consisting of 0.5 ml of the partially purified enzyme solution and 0.5 ml of 1 M Mannitol. Chloramphenicol (30 μg/ml) was used to inhibit the growth of bacteria. No buffer was used. Incubation was in the dark at 32°C. After 1 hr, the tube

was agitated gently to improve digestion. After 2 hr incubation, the cell suspension was filtered through 4 double layers of cheesecloth. The cells were washed 3 times with 0.5 M Mannitol under low speed centrifugation. Protoplasts were then resuspended in 2 ml 0.5 M Mannitol solution. The percent viable cells was estimated by observing the plasma streaming with a phase contrast microscope.

Serological techniques.--Experiments were designed to explore the antigenic differences of protoplasts from plants with and without a Pm gene. Protoplasts were prepared from four near isogenic lines of wheat containing genes Pm1a, Pm2a, Pm3a, or Pm4a and Chancellor which contains no known dominant Pm genes. Protoplasts were isolated from coleoptiles by the methods described above. Two separate experiments were performed approximately one year apart. In each experiment two pure-bred Checker rabbits were injected with protoplasts isolated from each of the different host lines. Intramuscular injection of 1 ml protoplast suspensions (4×10^4 cell/ml) mixed with equal amounts of adjuvent were performed at weekly intervals. The first antiserum was taken after three injections. The rabbits were subsequently injected biweekly, and antiserum was collected every other week up to the 7th bleeding. Homologous and heterologous reactions were carried out by agar-gel double diffusion tests.

Sample size and replication of experiments.--The experiments designed to characterize the development of either E. graminis f. sp. hordei or E. graminis f. sp. tritici on different near-isogenic lines of the respective hosts were repeated a minimum of five times on different weeks. Data presented in either figures or tables represent the mean of these observations. The number of parasite units counted at each observation varied from 80-150 depending upon the density of inoculation on each plant. The experiments were repeated on different days using different plants to eliminate small variations in either the plants or the inoculum.

When elongating secondary hyphae were used as a criterion for the identification of the segregation of the $M\ell$ genes, approximately 50 progeny from each heterozygous plant were tested on different days. The quality of the inoculum was tested by inoculating at least one plant of Manchuria each day. The results were not used unless the ESH produced on Manchuria was larger than 75%.

Statistical analyses.--Statistical analyses of the data were not made in most experiments either because the differences compared were very large and/or the variability around the mean was small and the populations compared did not overlap. Chi square analyses were performed on the data presented in Table 5 and Figure 8. Differences in the percentage of mature appressoria in the two treatments compared

against the control were not significant at the 5% level. Differences in length of ESH on the paired lines (with and without dominant *Ml* genes) for any one hour were nonsignificant, but the entire curves were significant at the 5% level for each set of paired lines.

RESULTS

CONFIRMATION OF PREVIOUS WORK.--The environmental conditions for germination, formation of mature appressoria, and the production of elongating secondary hyphae by E. graminis f. sp. tritici have been determined by other workers (53, 62, 64, 80). The first objective of this research was to reproduce earlier results on the process of primary infection. Of principal concern was the time course for the stages of primary infection, the synchrony of the parasite population in each stage, and the efficiency of infection. The results are presented in Figure 3, and are in close agreement with data already published (51).

Over 90% of the conidia applied to the host leaves germinated and developed matured appressoria by 8 hrs after inoculation (Figure 4A). Approximately 75-80% of the applied conidia produced elongating secondary hyphae (ESH) by 26 hrs after inoculation. The formation of ESH is possible only after the production of mature, lobed haustoria in the host cells (52). Therefore, the production of ESH, which, in turn, are capable of initiating secondary infections (80), was used in later experiments as the criterion for the formation of a functional relationship between host and parasite (52, 55).

Figure 3. Development of Erysiphe graminis f. sp. tritici during primary infection. (A) germination, (B) formation of appressorial initials, (C) formation of mature appressoria, (D) formation of elongating secondary hyphae (ESH).

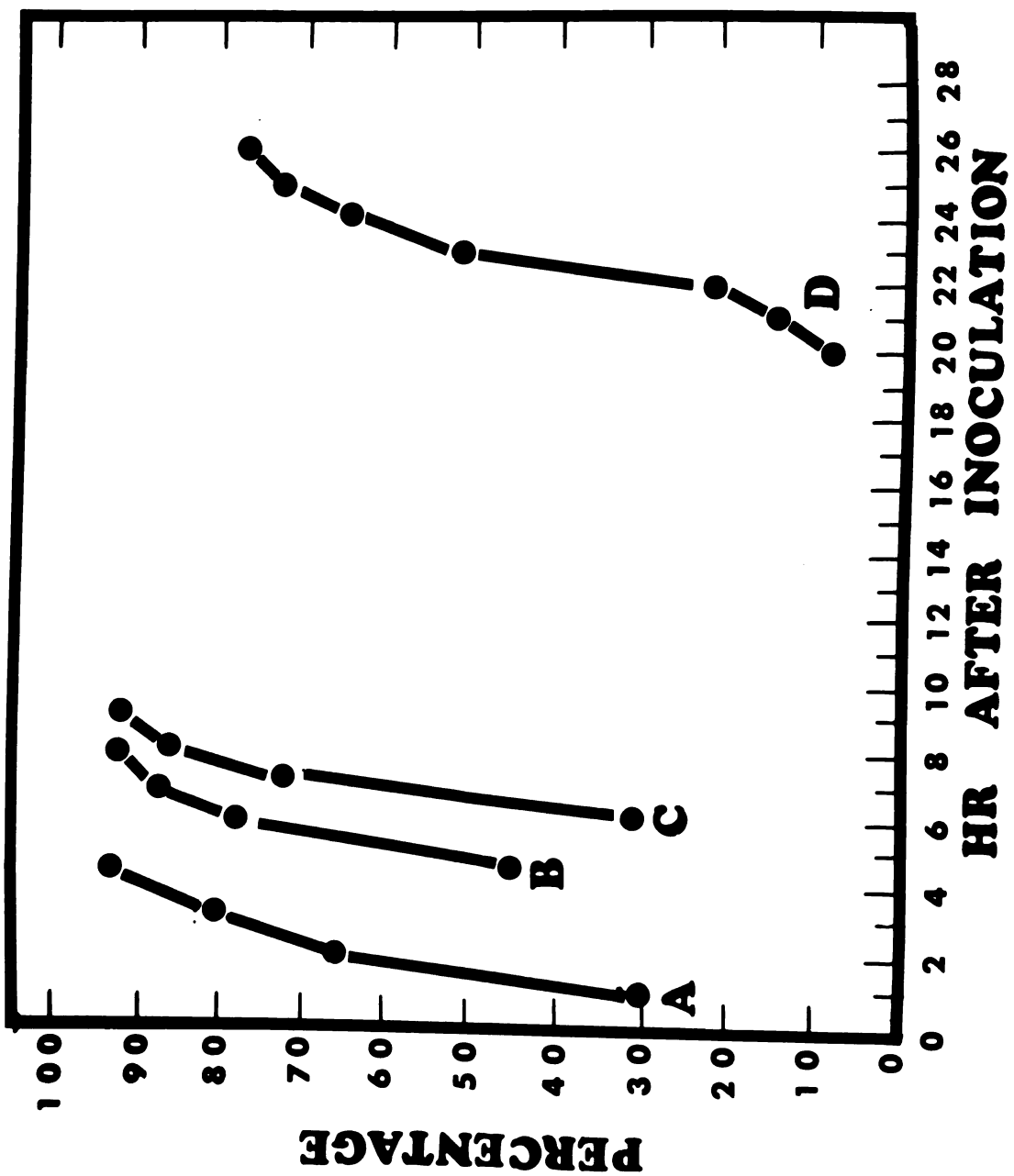

PERCENTAGE
100
90
80
70
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
HR AFTER INOCULATION
A
B
C
D

Figure 3

Figure 4. Formation of mature and malformed appressoria on different natural and synthetic surfaces. *E. graminis* f. sp. *tritici* on wheat epidermis (A), on isolated wheat cuticle (C), on the reverse surface of isolated wheat cuticle (D), on 2% water agar (E), on reversed surface of wheat epidermal strip (F), and on reconstructed wax layer (G). *E. graminis* f. sp. *hordei* on wheat epidermis (B) and on wax mutant *cer-Zj*^{78} (H).

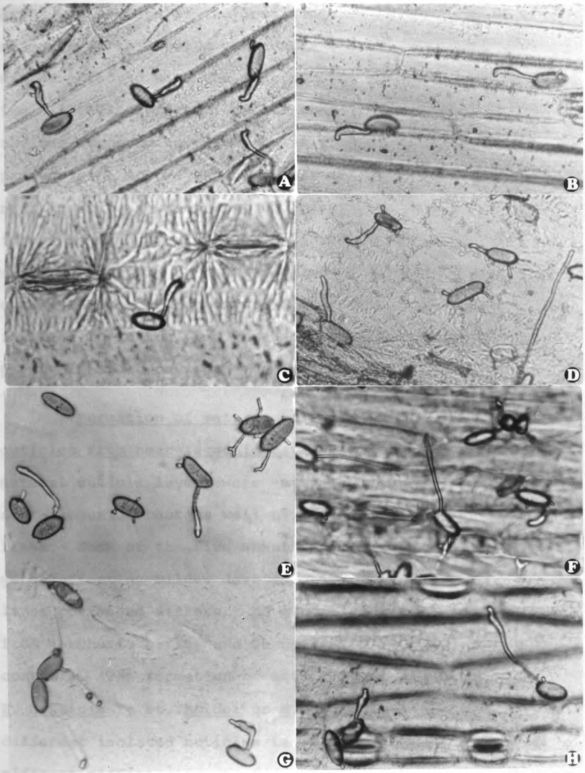


Figure 4

EFFECT OF THE CUTICLE ON THE FORMATION OF MATURE APPRESSORIA.--One of the long range objectives of this research is to develop procedures whereby all host cells can be infected with the fungus. The advantages of obtaining all cells infected, rather than less than 1%, is very obvious for studies on molecular changes associated with or prerequisite to the establishment of compatible, functional relationships between host and parasite. A series of experiments described below were designed to determine if it would be possible to get infection of different types of cell surfaces, to determine if the cuticle is necessary to initiate infection, and whether the chemical composition or physical structure is the determining factor.

Formation of mature appressoria on isolated natural cuticles from near-isogenic barley and wheat lines.--Intact natural cuticle layers were enzymatically isolated from five near-isogenic wheat as well as five near-isogenic barley lines. Each of the five wheat lines possessed different *Pm* genes affecting mildew development. Each of the five barley lines possessed different *Ml* genes. The cuticles isolated from Manchuria barley and Chancellor wheat were used as controls. The formation of mature appressoria of either *E. graminis* f. sp. *hordei* or *E. graminis* f. sp. *tritici* on different isolated cuticles is shown in Table 1. No significant differences were found in the formation of mature appressoria among cuticles isolated from plants with

Table 1. Formation of mature appressoria of Erysiphe graminis on isolated natural cuticles from near-isogenic barley and wheat lines

Near isogenic barley lines	Appressoria formed by Erysiphe graminis[a]	
	Barley mildew (f. sp. hordei)	Wheat mildew (f. sp. tritici)
	%	%
Manchuria (mℓ x)	88	85
Mℓa	92	--
Mℓq	89	--
Mℓk	86	--
Mℓp	90	--
Near-isogenic wheat lines:		
Chancellor (pm x)	83	86
Pm1a	--	94
Pm2a	--	90
Pm3a	--	91
Pm4a	--	86

[a]Eight hours after inoculation under standard condition.

different genes for reaction. This suggests the genes tested do not act to affect development of the parasite prior to penetration. These genes do not appear to impart a biological specificity to the cuticle layers. Moreover, the data also indicate that the formation of mature appressoria is not species specific, since E. graminis f. sp. hordei produced nearly the same per cent of mature appressoria on isolated wheat cuticle as on its natural host barley. Similar results were also observed with E. graminis f. sp. tritici inoculated onto barley. The results obtained with the isolated cuticles were consistent with results obtained with intact plants.

Formation of appressoria of Erysiphe graminis f. sp. tritici on various surfaces of isolated cuticles and epidermal strips.--The development of mature appressoria following inoculations of both sides of epidermal strips and both sides of isolated cuticle layers is presented in Table 2. About 85% mature appressoria were formed at the surfaces with wax layers on both isolated cuticles and epidermal strips 8 hrs after inoculation. Less than 5% mature appressoria were formed on the surface without the wax layer, and over 40% of the appressoria formed were malformed (Figure 4). The per cent mature appressoria on isolated cuticles grafted onto the lower surface of epidermal strips was essentially the same as on the upper surface of intact epidermal strips.

Table 2. Germination and formation of appressoria of E. graminis f. sp. tritici on various surfaces of isolated cuticles and epidermal strips

Surface	Germination[a]	Appressoria[a]	
		Malformed	Mature
	%	%	%
Isolated cuticle:			
upper face (with wax)	93	3	84
lower face (without wax)	91	42	1
Epidermal strip:			
upper face (with wax)	93	2	89
lower face (without wax)	90	45	2
Isolated cuticle grafted onto lower surface of the epidermal strip	89	5	82

[a]Eight hours after inoculation under standard conditions.

This result is also consistent with the interpretation that the wax layer is a major determining factor for the formation of mature appressoria.

Formation of mature appressoria of E. graminis f. sp. tritici on various artificial and reconstructed surfaces.--Urediospores of many species of rust fungi germinated on isolated host or nonhost cuticle and sequentially developed appressoria, infection pegs, vesicles, and infection hyphae, similar to those produced in nature during host infection. The effects of cuticle were duplicated by nitrocellulose membranes containing hydrocarbons isolated from the surface

wax of snapdragon leaves or containing mineral oil. The surface wax of the leaf, in addition, promoted the formation of haustorial mother cells and the branching of infection hyphae in bean and snapdragon rust fungi (50).

In the experiments described here seven different artificial surfaces were inoculated with conidia. Isolated cuticles were also inoculated and used as controls. The percentages of mature appressoria formed on these surfaces are shown in Table 3. The percentages of mature appressoria formed ranged from 0% to 17% on the artificial surfaces tested. Eleven per cent of the conidia produced mature appressoria on paraffin coated cellulose paper. Ninety per cent mature appressoria were formed on the isolated cuticles. Only 9 to 17% of the applied conidia formed mature appressoria on the reconstructed wax layers. Similar results were obtained whether the wax used for the reconstructed layers was extracted from fresh leaves or isolated cuticles. Since few parasite units produced mature appressoria on the reconstructed wax layers (without fractionation of the cuticular components), it appeared that the specific physical conformation of the wax layer may be one of the major factors affecting the formation of mature appressoria.

Formation of mature appressoria and elongating secondary hyphae by E. graminis f. sp. hordei on mutants affecting the wax layer of barley.--If the chemical composition and the physical structure of the wax layer is important

Table 3. Germination and formation of appressoria of *E. graminis* f. sp. *tritici* on various artificial and reconstructed surfaces

Surface	Germination[a]	Appressoria[a]	
		Malformed	Mature
	%	%	%
Isolated cuticle	98	2	90
Water agar (2%)	96	32	0
Glass slide:			
plain	30	0	0
paraffin coated	83	10	0
Cellulose paper			
plain	93	15	0
paraffin coated	96	42	11
Reconstructed wax layer:			
wax from fresh leaf blade	62	43	9
wax from isolated cuticle	90	54	17

[a]Eight hours after inoculation under standard conditions.

for the normal differentiation of appressoria of the parasite, mutations which affect the chemical composition and physical structure of the wax layer may differ in their effects upon the parasite. Several hundred independent mutations which give a glossy appearance to the leaves have been induced in barley by a variety of different mutagens (49). These mutations, called eceriferum mutants because they affect the wax layer on the leaves, map at several different loci. The loci are designated by the letter and the allele number by a superscript, e.g., cer-J^{59} is mutation number 59 at the J locus.

Five ecerferum (wax) mutations, cer-J^{59}, cer-J^{71}, cer-Zd^{67}, cer-Ze^{81}, cer-Zj^{78}, were available for use. These mutations have been partially characterized both by chemical analysis and with a scanning electron microscope. Primary leaves of barley with the mutations cer-J^{59} and cer-J^{71} produce per unit area 30% and 56% less wax, respectively, than the wild type Bonus barley. The wax mutations at the cer-J locus give a much smaller percentage of primary alcohols and a somewhat larger percentage of esters than that of wild type Bonus (49, 91). Approximately a 20% reduction in the amount of wax was found on the seedling leaves of barley with mutations cer-Zd^{67} and cer-Ze^{81} (91). Morphologically quite different wax coats were found on plants with either cer-Zd^{67} or cer-Ze^{81}, in addition to the wax bodies similar to those found on wild type Bonus. No obvious difference in the proportion of the lipid classes composing the wax could

be recognized with the latter two mutations, as compared to those of Bonus, the wild type barley (49, 91). Approximately 40% less wax was found on the primary leaves of plants with mutation cer-zj^{78} than on wild type Bonus. The wax bodies were smaller and irregularly distributed over the surface of the leaf (91). A higher percentage of aldehydes was found in plants with cer-zj^{78}, but the structural arrangements of the fibrils in the wax coating were less obvious.

Five to six-day-old seedlings with the different mutations were inoculated. Inoculations of Manchuria as well as wild type Bonus were also made as controls. The results are shown in Table 4. Eight hours after inoculation the percentage of malformed appressoria produced on barley plants with the various mutations ranged from 14% to 27%. The percentage of malformed appressoria depended on which mutation was involved. Less than 1% of malformed appressoria were produced on the controls. Reduction in the percentage of mature appressoria caused by the wax mutations ranged from approximately 15% to 35%. Reduction of the percentage of ESH 28 hrs after inoculation ranged from basically no differences to 40%. This result suggested the chemical components and the physical conformation, such as distribution of the wax bodies, are all important factors for the formation of mature appressoria.

Table 4. Germination, formation of appressoria and elongating secondary hyphae of Erysiphe graminis f. sp. hordei on barley seedlings with wax mutations

Host	Germination[a]	Appressoria[a]		ESH[b]
		Malformed	Mature	
	%	%	%	%
Manchuria	99	< 1	97	77
Bonus	97	< 1	96	71
Ecerferum mutations:				
cer-J^{59}	96	15	80	68
cer-J^{71}	92	19	72	62
cer-Zd^{67}	97	14	82	59
cer-Ze^{81}	97	14	83	55
cer-Zj^{78}	93	27	60	30

[a]Eight hours after inoculation.

[b]Twenty-eight hours after inoculation.

Formation of mature appressorium of E. graminis f. sp. tritici on isolated natural cuticles after washing with organic solvents.--The hypothesis that the wax layer is a determining factor for the formation of mature appressoria was also tested by intensive washing of isolated cuticle with organic solvents capable of desolving chloroform and ether which are waxy substances. Approximately 2 liters of an organic solvent were washed over a single 2.5 cm long isolated cuticle over a period of 6 hrs. Cuticles washed by the same method but with distilled water were used as control. The percentage of mature appressoria 8 hrs after inoculation was approximately 15% less on the cuticles washed with either chloroform or ether (Table 5). The material on or in the cuticle that stimulates the parasite to develop mature appressoria must not be entirely removed by the organic solvents used. This result also is in agreement with the hypothesis that some of the wax bodies are firmly impregnated into the cuticle structure (54).

EFFECT OF Mℓ GENES IN BARLEY AND Pm GENES IN WHEAT ON PRIMARY INFECTION.--Effect of Mℓ genes on the formation of elongating secondary hyphae of E. graminis f. sp. hordei.--Earlier work (52) has shown that ESH are formed only by parasite units that have formed haustoria. The formation of ESH has been used, therefore, as evidence that the parasite and host have established a compatible relationship. The effect of different Mℓ genes carried by four near-isogenic barley

Table 5. Spore germination and formation of mature appressoria on isolated natural cuticles after leaching with chloroform or ether

Organic solvent	Germination[a]	Mature appressoria[a]
	%	%
Chloroform	93	77
Ether	95	72
Control	98	89

[a]Eight hours after inoculation.

lines were tested by inoculation with CR-3 of E. graminis f. sp. hordei, a strain that possesses the complimentary genes Pa, Pq, Pk, and Pp for incompatibility with the four genes Mla, Mlq, Mlk, and Mlp, respectively. The formation of elongating secondary hyphae was used as a criterion of the establishment of compatible relations between host and parasite. The results of each gene pair, i.e., Pa/Mla (parasite/host genotype) on the formation of elongating secondary hyphae of the barley mildew fungus were recorded from 20 to 26 hours after inoculation (Figure 5). Approximately 8, 16, 32, and 37% ESH were observed 26 hrs after inoculation with the genotypes (parasite/host) Pa/Mla, Pq/Mlq, Pk/Mlk, and Pp/Mlp, respectively, specifying incompatibility. The possibility that genes other than the ones specified contributed to the results obtained was considered unlikely because essentially the same results were obtained on paired lines

Figure 5. Formation of elongating secondary hyphae (ESH) by Erysiphe graminis f. sp. hordei (CR-3) on near-isogenic barley lines that possessed the different Mℓ genes: mℓ (●-●), Mℓp (∗-∗), Mℓk (**0**-**0**), Mℓq (★-★), and Mℓa (✸-✸).

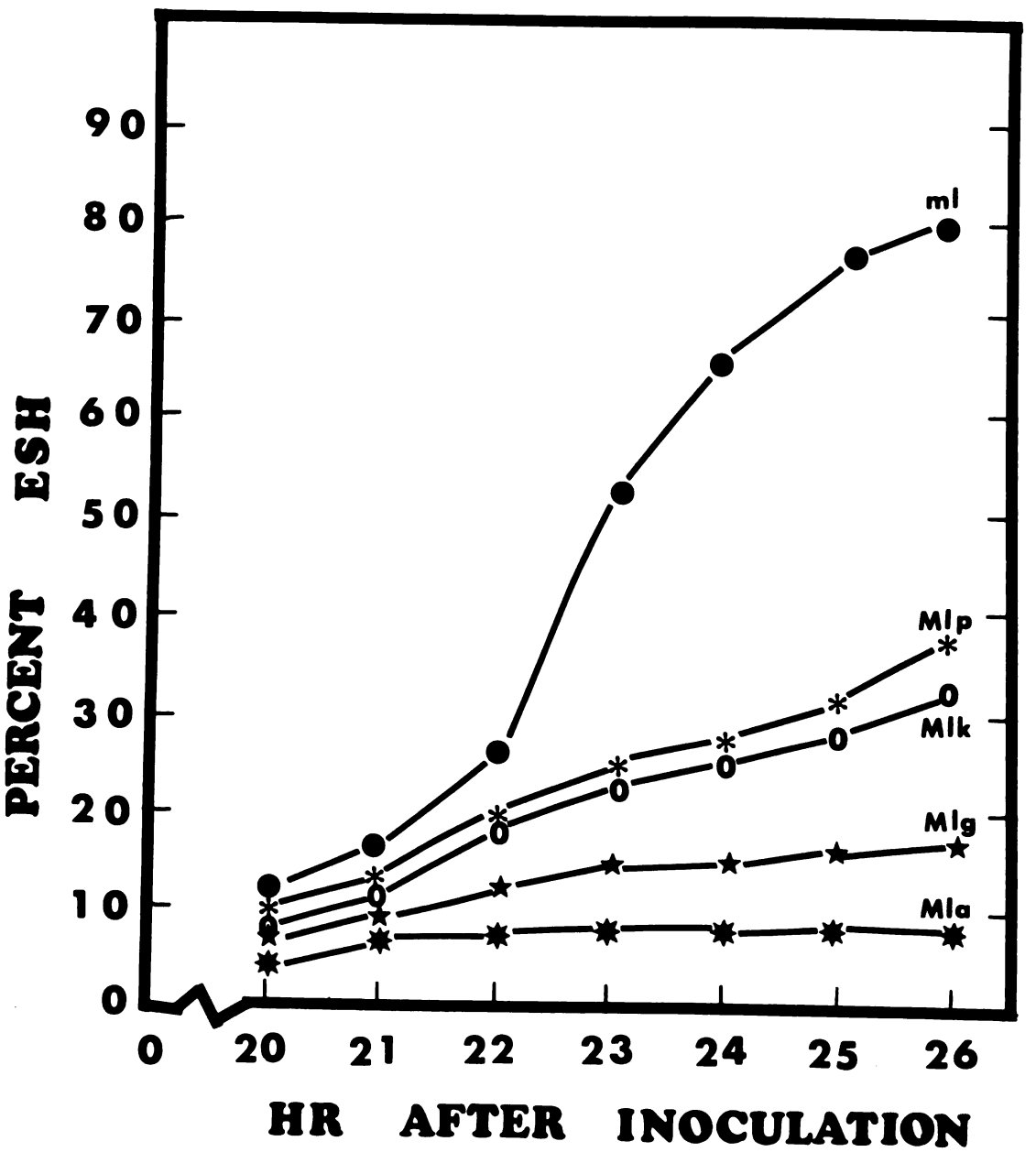

PERCENT ESH
90
80
70
60
50
40
30
20
10
0
0
20
21
22
23
24
25
26
HR AFTER INOCULATION
ml
Mlp
Mlk
Mlg
Mla

Figure 5

with no known dominant *Ml* genes, which were known to be very highly isogenic to the lines with *Ml* genes, as with Manchuria. The kinetics of formation of ESH on each near-isogenic homozygous recessive line was shown to closely parallel the results on Manchuria (Figure 6).

Effect of Pm genes in wheat on the formation of ESH of E. graminis f. sp. tritici.--The formation of ESH by E. graminis f. sp. tritici culture MS-1 on four near-isogenic wheat lines, each of which possessed a different Pm gene, was also tested. The culture MS-1 possesses the four complimentary genes P1, P2, P3, and P4 which specify incompatibility in the presence of Pm1, Pm2, Pm3, and Pm4, respectively.

The results of each gene affecting the formation of ESH were recorded from 20 to 26 hours after inoculation (Figure 7). Approximately 28, 84, 18, and 5% ESH were produced 26 hrs after inoculation in the presence of the parasite/host genotypes P1/Pm1, P2/Pm2, P3/Pm3, and P4/Pm4, respectively. The kinetics of ESH formation with P2/Pm2 genotype closely followed what was observed when the variety Chancellor was inoculated with strain MS-1. This indicated that the P2/Pm2 genotype does not alter the primary infection process, at least up to 26 hours after inoculation.

Figure 6. Formation of elongating secondary hyphae (ESH) by Erysiphe graminis f. sp. hordei (CR-3) on five near-isogenic barley lines all with recessive $m\ell$ genes. The line designated $m\ell a$, for example, was the homozygous recessive derived from the cross Algerian X_7^4 Manchuria.

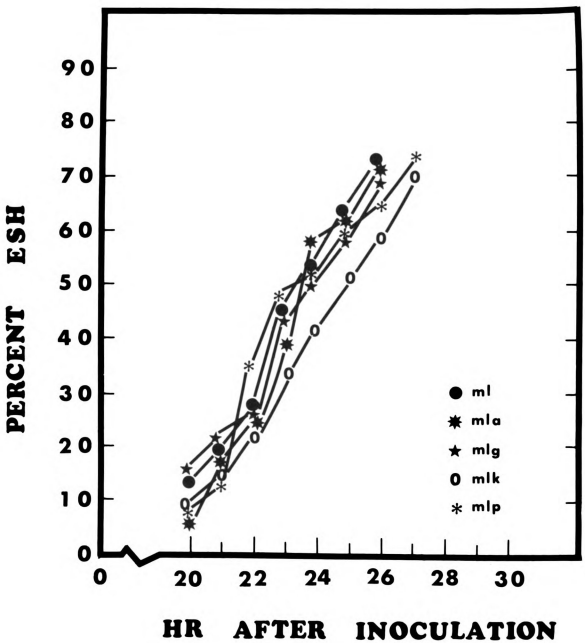

PERCENT ESH
90
80
70
60
50
40
30
20
10
0
0
20
22
24
26
28
30
HR AFTER INOCULATION
ml
mla
mlg
mlk
mlp

Figure 6

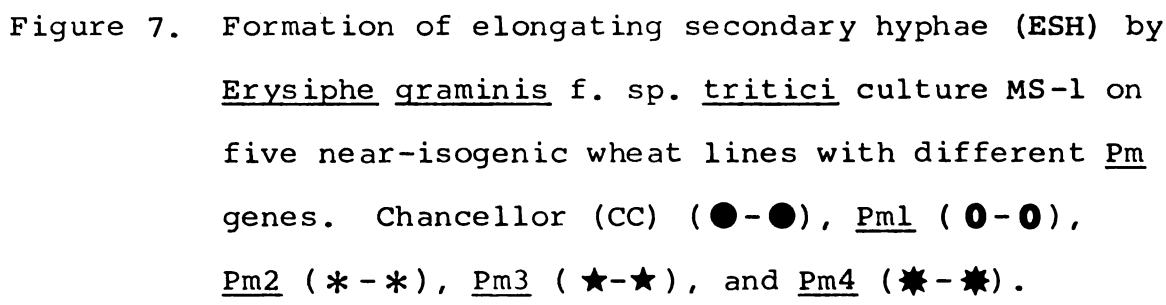


Figure 7. Formation of elongating secondary hyphae (ESH) by Erysiphe graminis f. sp. tritici culture MS-1 on five near-isogenic wheat lines with different Pm genes. Chancellor (CC) (●-●), Pm1 (**O**-**O**), Pm2 (✻-✻), Pm3 (★-★), and Pm4 (✸-✸).

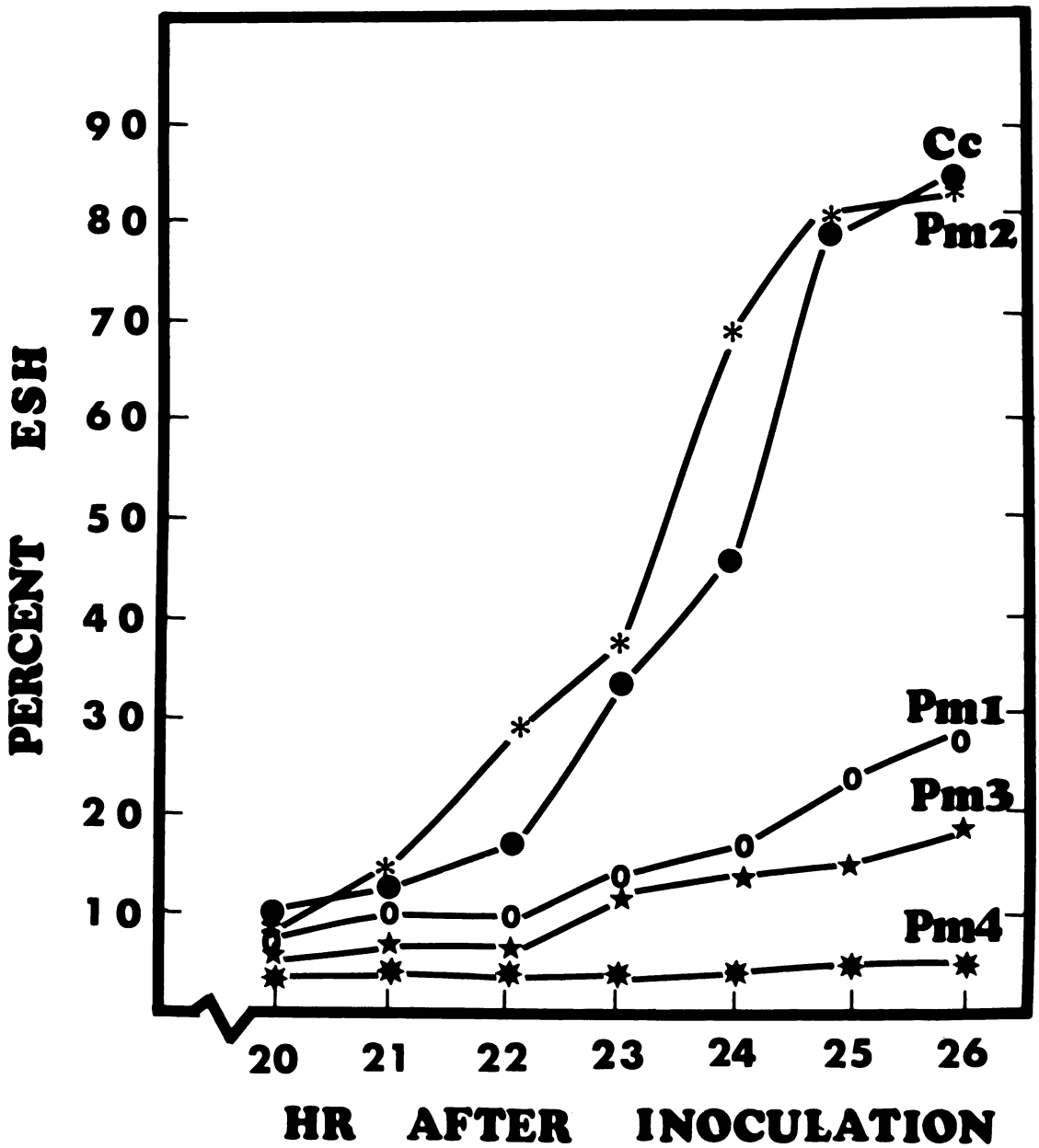

PERCENT ESH
90
80
70
60
50
40
30
20
10
Cc
Pm2
Pm1
Pm3
Pm4
20
21
22
23
24
25
26
HR AFTER INOCULATION

Figure 7

Effect of Pm and Mℓ genes on the infection type produced by the powdery mildew fungi on wheat and barley.--Near-isogenic lines of barley and wheat with and without Pm or Mℓ genes, respectively, were inoculated by dusting the conidia of the appropriate fungus onto 5 to 6-day-old seedlings. The inoculated plants were incubated under environmental conditions similar to that used for the maintenance of stock culture. The infection types were recorded 6 days after inoculation. Table 6 summarizes the effect of all the different genes on final infection type as well as the primary infection process. Incompatibility is expressed as the reduction of ESH formed during primary infection and the reduction of infection type observed 6 days after inoculation.

Effect of specific genotypes for incompatibility on the growth rate of elongating secondary hyphae produced by barley mildew.--One effect of 7 of the 8 different genotypes specifying incompatibility is to reduce the percentage of the conidia applied to the host surface that produce ESH by 26-28 hrs after inoculation. These results are consistent with published data (55). The fate of the approximately 17% of the parasite units that do form ESH with the Pq/Mℓq genotype, for example, is important in the determination of when the genes act to affect the interactions between host and parasite. Clearly the genes do more than reduce primary infection efficiency because the final infection type is reduced.

Table 6. Action of different genes in the hosts on the sequential development of barley and wheat powdery mildew, cultures CR-3 and MS-1 on barley and wheat, respectively

Sequential events	Hours after inoculation: 4 Germination	8 Mature appressoria	12 Penetration	22 Haustoria	26-28 Elongating secondary hyphae	29-120 Colonization sporulation	120 Infection type[b]
	%[a]	%[a]			%[a]		
Near-isogenic barley lines:							
Manchuria	> 95	> 90			75		4
M*la*	> 95	> 90			10		0
M*lq*	> 95	> 90			10-20		0,1
M*lk*	> 95	> 90			30-40		2
M*lp*	> 95	> 90			35-45		1,2
Near-isogenic wheat lines:							
Chancellor	> 95	> 90			75		4
Pm1a	> 95	> 90			20-30		0,1
Pm2a	> 95	> 90			75		2
Pm3a	> 95	> 90			15-25		3
Pm4a	> 95	> 90			10		0,1

[a]Percentage of total parasite population observed at at least that stage of development at the time of observation.

[b]Infection type: 0--no observable mildew development. 1--chlorotic flecking. 2--necrotic reaction. 3--significant reduction in mildew development from the rating of 4. 4--abundant mildew development (compatible).

The effect of different genotypes on the length of ESH 32 to 36 hrs after inoculation is presented in Figure 8. The data on paired, highly isogenic host lines which differ in the presence or absence of dominant Mℓ genes are also presented to aid in determining whether genes other than the Mℓ genes affect the growth of secondary hyphae.

The slope of the lines suggested no significant differences between growth rates of ESH formed on plants of different genotypes (Figure 8), at least up to 36 hrs after inoculation. The inhibition of mildew development, as indicated by the reduction of infection type, by the different genes for incompatibility must be at a stage of development of the parasite other than the elongation of secondary hyphae.

The genes for incompatibility must, therefore, function by two means. The formation of haustoria is an all or none development. Haustoria either form or they do not. No deficient or malformed haustoria were found during primary infection. The subsequent development of the parasite units that do form haustoria is more subtly affected by the different genotypes, at least as presently understood.

Formation of elongating secondary hyphae as a criterion for the identification of segregating Mℓ genes.--Since the percentage of the total applied parasite units which eventually form ESH is dependent upon different genes, it should be possible to identify the segregation of genes in

Figure 8. The length of elongating secondary hyphae (ESH) produced by Erysiphe graminis f. sp. hordei on near-isogenic paired barley lines which possessed different Mℓ genes. The line designated mℓa, for example, was the homozygous recessive derived from the cross Algerian X_7^4 Manchuria.

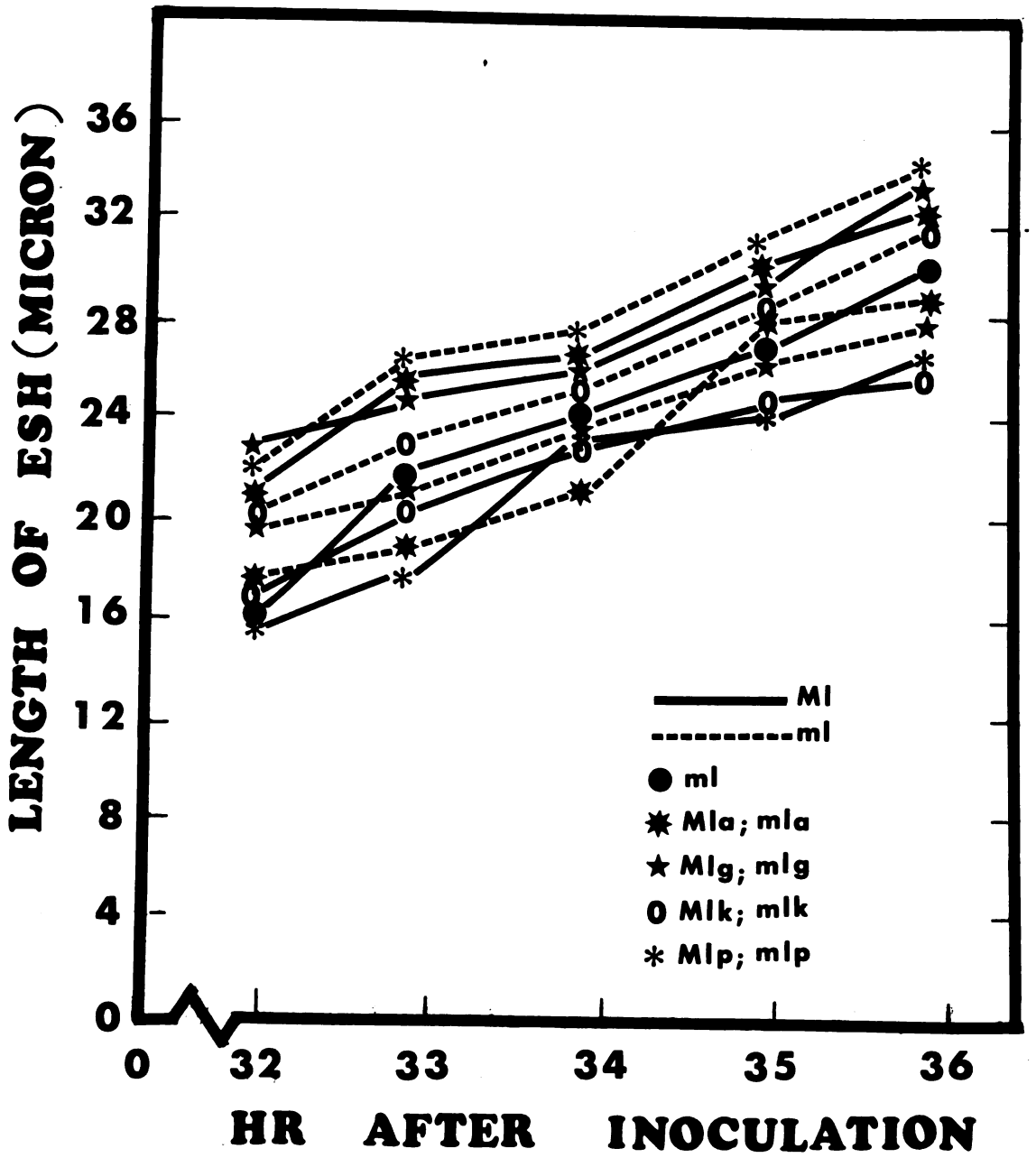

LENGTH OF ESH (MICRON)
36
32
28
24
20
16
12
8
4
0
0
32
33
34
35
36
HR AFTER INOCULATION
Ml
ml
ml
Mla; mla
Mlg; mlg
Mlk; mlk
Mlp; mlp

Figure 8

the host by the formation of ESH. The purpose of this experiment was to see whether, by use of the percentage ESH formed as a criterion, one can identify the three types of progenies expected, the homozygous dominant Mℓ, the homozygous recessive mℓ, and the heterozygote, from the selfing of heterozygous plants.

Seeds derived from the selfing of four near-isogenic heterozygous barley parents were planted individually in 2 inch pots. Approximately 50 seedlings of each line, 5 to 6-days-old, were inoculated with isolate CR-3. A one cm section of each seedling was removed, and the percentage of applied parasite units which formed ESH on that section 28 hr after inoculation was recorded. Infection types on the remaining portion of the inoculated leaves were observed 6 days after inoculation. The plants were grown to maturity and the genotype of each seedling was determined by the segregation of infection types among its progenies. The ranges of the percentages of ESH formed on seedlings were plotted against the number of seedlings which produced the same range of percentage of ESH. The percent ESH on each segregant derived by selfing of plants of the genotypes Mℓa mℓa, Mℓq mℓq, Mℓk mℓk, and Mℓp mℓp are shown in Figures 9, 10, 11, and 12, respectively. The percentages of ESH produced by control homozygous recessive plants derived from homozygous recessive parents of all four near-isogenic lines were always in the range of 75% to 90%. Per cent of ESH

Figure 9. The number of plants homozygous M *la* M *la* (■), heterozygous M *la* m *la* (◪), and homozygous m *la* m *la* (□) on which a particular range of percentage ESH was produced. All plants were derived from the selfing of heterozygous M *la* m *la* plants.

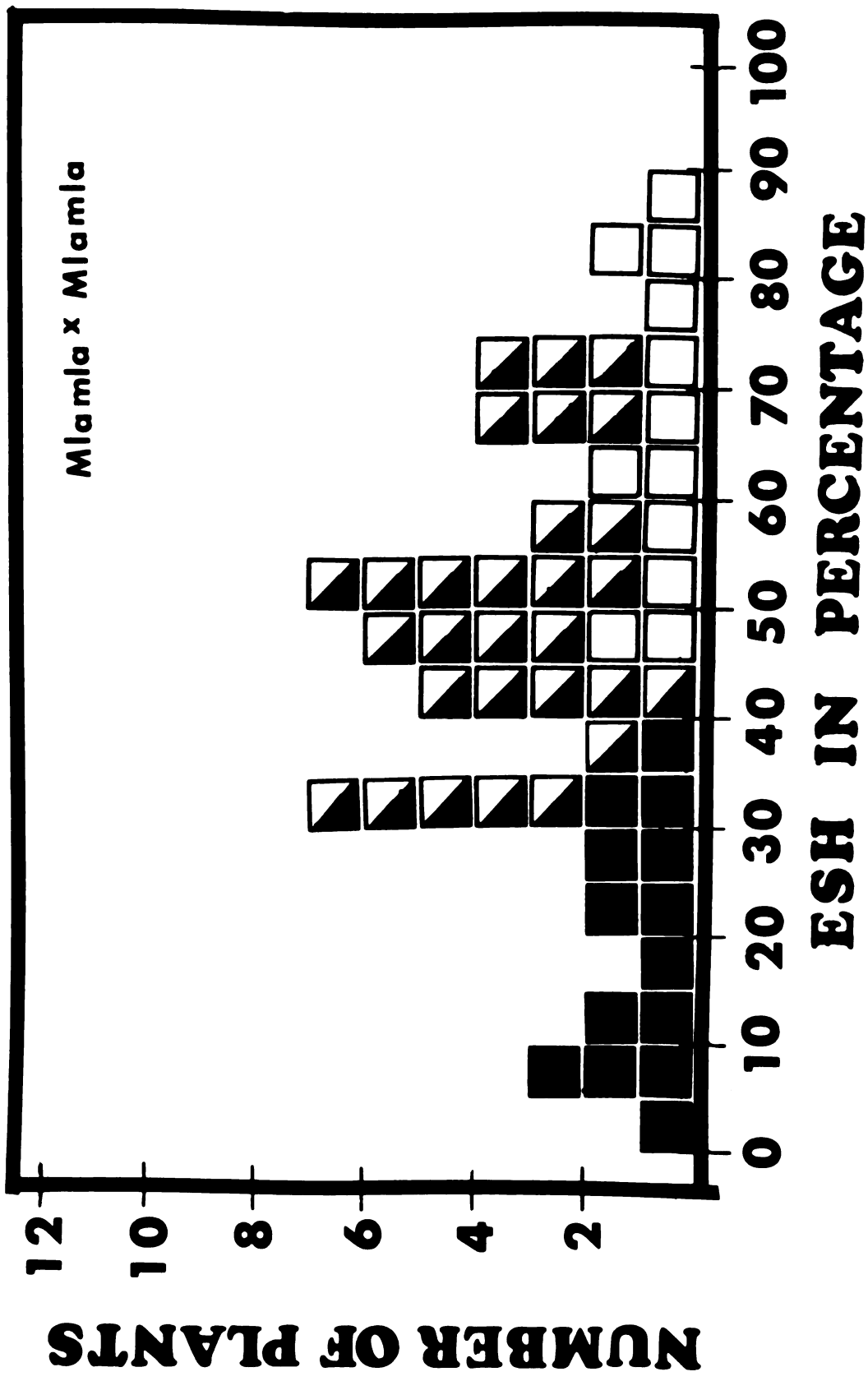
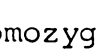

Mlamla x Mlamla
NUMBER OF PLANTS
12
10
8
6
4
2
0 10 20 30 40 50 60 70 80 90 100
ESH IN PERCENTAGE

Figure 9

Figure 10. The number of plants homozygous M *lg* M *lg* (), heterozygous M *lg* m *lg* (), and homozygous m *lg* m *lg* () on which a particular range of percentage ESH was produced. All plants were derived from the selfing of heterozygous M *lg* m *lg* plants.

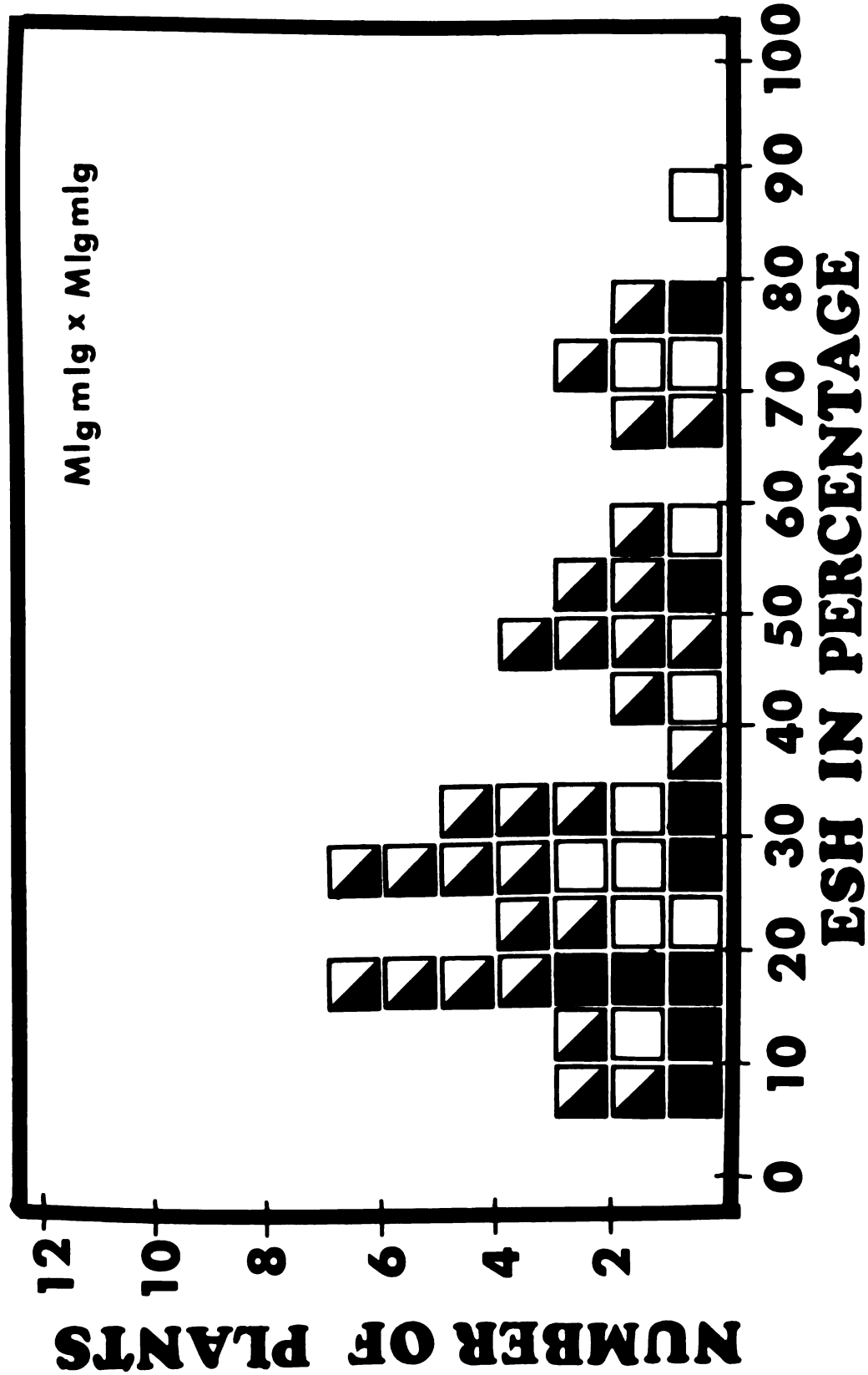

Mlg mlg x Mlgmlg
NUMBER OF PLANTS
12
10
8
6
4
2
0
10
20
30
40
50
60
70
80
90
100
ESH IN PERCENTAGE

Figure 10

Figure 11. The number of plants homozygous M/k M/k (■), heterozygous M/k m/k (◪), and homozygous m/k m/k (□) on which a particular range of percentage ESH was produced. All plants were derived from the selfing of heterozygous M/k m/k plants.

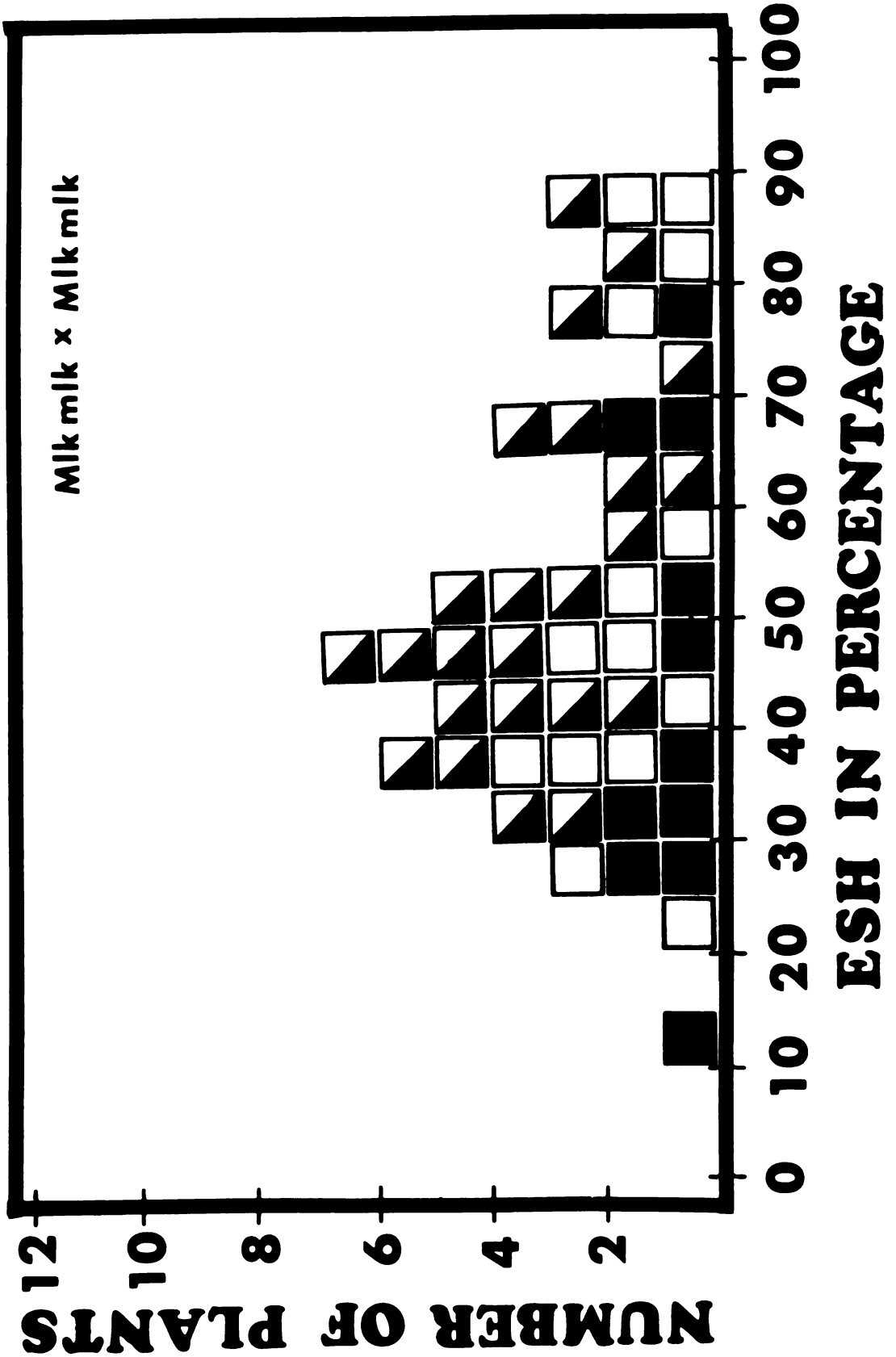

Mlk mlk x Mlk mlk
NUMBER OF PLANTS
12
10
8
6
4
2
0 10 20 30 40 50 60 70 80 90 100
ESH IN PERCENTAGE

Figure 11

Figure 12. The number of plants homozygous M*p* M*p* (), heterozygous M*p* m*p* (), and homozygous m*p* m*p* () on which a particular range of percentage ESH was produced. All plants were derived from the selfing of heterozygous M*p* m*p* plants.

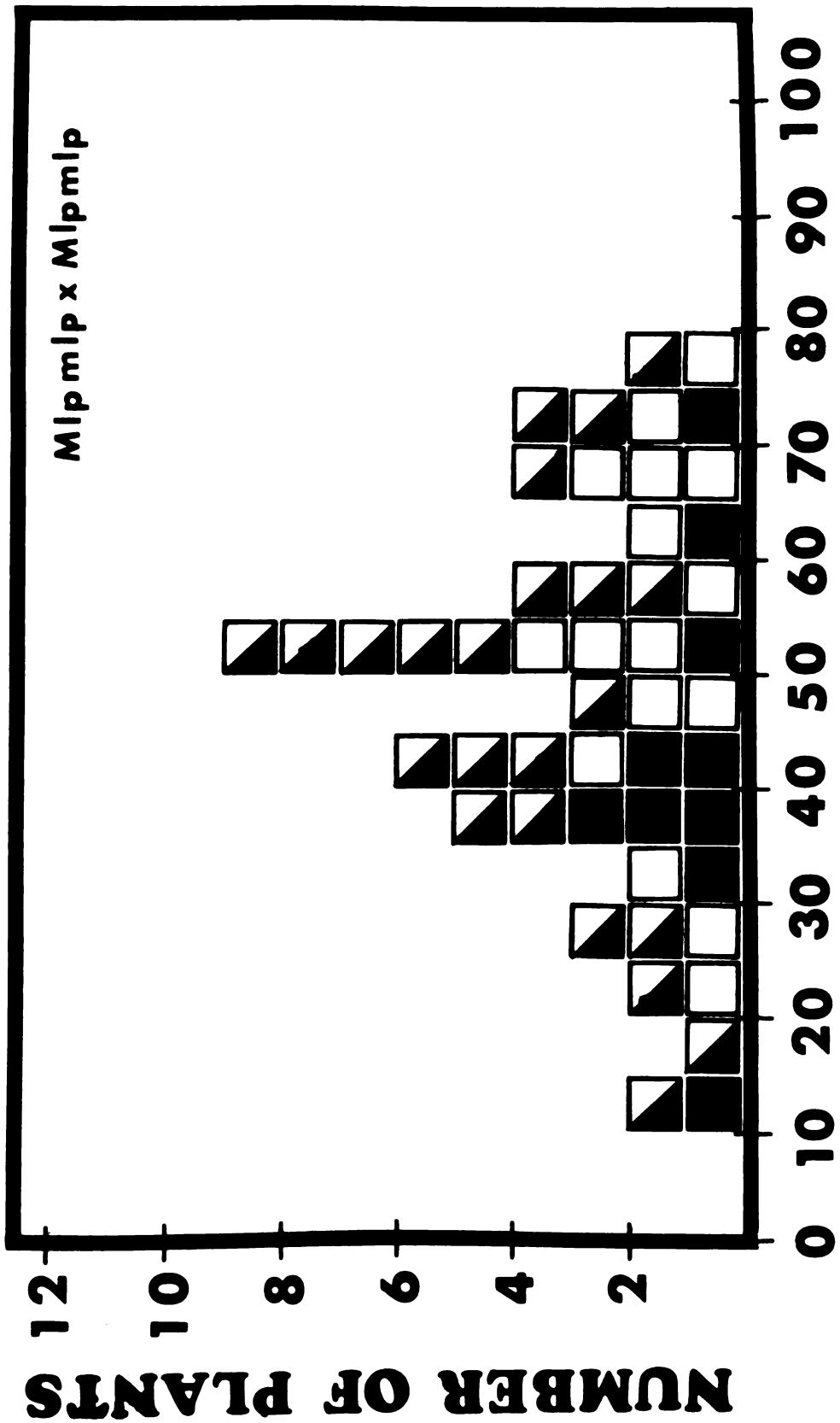

MlpmlpxMlpmlp
NUMBER OF PLANTS
2
4
6
8
10
12
0 10 20 30 40 50 60 70 80 90 100
ESH IN PERCENTAGE

Figure 12

produced by control homozygous dominant plants derived from homozygous parents of M*la* M*la*, M*lq* M*lq*, M*lk* M*lk*, and M*lp* M*lp* were observed to be 0-10%, 10-20%, 10-40%, and 25-45%, respectively. However, the range in the percentage of ESH produced on homozyghous plants of genotypes M*la* M*la*, M*lq* M*lq*, M*lk* M*lk*, and M*lp* M*lp* which were derived from heterozygous parents was 0-40%, 10-80%, 10-80%, and 10-75%, respectively. A greater range in the percentages of ESH was observed on homozygous recessive progenies derived from heterozygous parents than from homozygous parents.

The percentage of ESH could be safely used to distinguish homozygous progeny M*la* M*la* from m*la* m*la* derived from selfing M*la* m*la* plants. The percentage of ESH can not be safely used to distinguish the two types of homozygous progeny derived from selfing plants of the genotypes M*lq* m*lq*, M*lk* m*lk*, or M*lp* m*lp*. With all four genes, the range in the percentages of ESH produced by the parasite on either homozygous dominant or homozygous recessive plants derived from heterozygous parents is significantly larger than the ranges observed on homozygous plants derived from homozygous parents. These results suggest a carry-over effect of both alleles of the heterozygous parent to the homozygous progenies. These results suggest a function of recessive genes in heterozygous plants. The latter is also consistent with the observation that the M*lq*, and M*lp* genes were semi-dominant as evaluated by infection type 6 days after inoculation (55).

ANTIGENIC PROPERTIES ON THE SURFACE OF CELL PROTOPLASTS ISOLATED FROM WHEAT COLEOPTILES WITH DIFFERENT Pm GENES.--Observations in these studies, and other (55), suggest that the effect of various parasite/host genotypes that specify incompatibility is to reduce the percentage of the parasite units that produce haustoria. The various gene pairs do not appear to affect the parasite prior to penetration or in the penetration of the cuticle or cell wall. The development of haustoria in the epidermal cells with most genotypes for incompatibility is an all or none event. Either a parasite unit produces full sized, normal haustorial bodies by 18 hrs after inoculation or no haustoria at all are formed. The haustoria that do form with incompatible parasite/host genotypes appear to be similar in development at any hour, at least up to 20 hrs after inoculation, to haustoria produced with compatible genotypes. Small, partially developed haustoria have never been observed. The development of a certain portion of the parasite population appears to be stopped as the parasite reaches the host plasma membrane. These observations indicate that the "factors" conditioning incompatibility may be located on the host plasma membrane.

The idea that specificity of host-parasite relationships occur on the host plasma membrane has been suggested (75, 87). Helminthosporium victoriae produces a host-specific toxin. Protoplasts from oats with the Vb gene

burst very quickly in the presence of the toxin whereas protoplasts from oats with the vb gene do not (75).

If the host "factors" conditioning compatibility or incompatibility are located on the surface of protoplasts, the possibility exists that protoplasts isolated from near-isogenic host lines with different Pm genes might differ in their antigenicity.

Protoplasts were isolated from the coleoptiles of three near-isogenic wheat lines, Chancellor, Pm3, and Pm4. Intramuscular injections of protoplasts (4×10^4 cell/ml) into pure-bred Checker rabbits were made. The first antiserum from each rabbit was taken after three injections. The rabbits were subsequently injected bi-weekly and antiserum was collected every other week up to the 7th bleeding.

Homologous (antibody produced against protoplasts of one genotype diffused against protoplasts of the same genotype as antigen) as well as heterologous (antibody produced against protoplasts of one genotype diffused against protoplasts of a different genotype as antigen) reactions were carried out by agar-gel double diffusion tests. Three identical major bands appeared on either homologous or heterologous reactions when antisera produced from the 3rd to 7th bleedings of each rabbit were used (Figure 13). When antisera was diffused against soluble substances extracted from lypholized, ground coleoptiles by phosphate buffer (pH:7.2) no precipitating bands were observed (Figure 13). The antigenic substances extracted in buffer from

Figure 13. Agar gel double diffusion tests. In 1, 2, and 3, the antigens in the center wells were protoplasts of Chancellor, Pm3 and Pm4 host lines, respectively. In 4, 5, and 6, the antigens in the center wells were extracts from coleoptiles from Chancellor, Pm3, and Pm4 host lines, respectively. In 7 the antigen in the center well was an extract from E. graminis f. sp. tritici conidia. In 1 to 7, antibodies produced against protoplasts from Chancellor (C), Pm3, and Pm4 was in the outer wells.

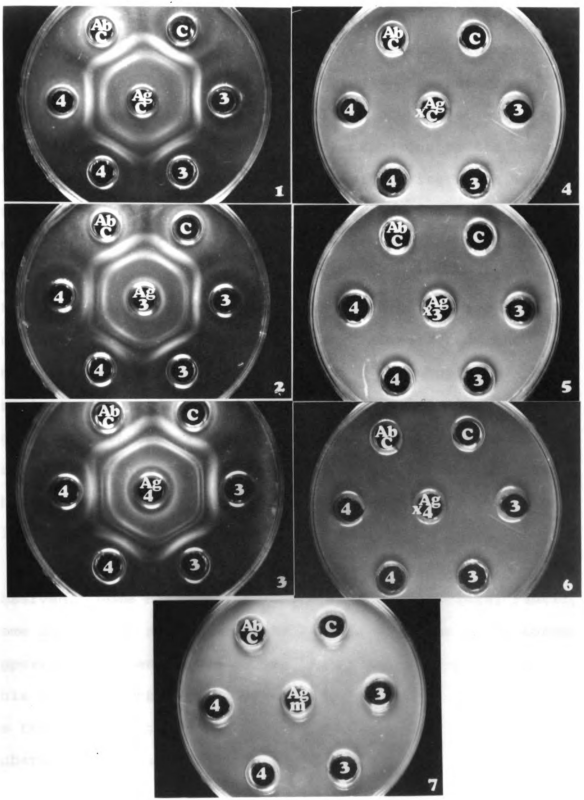

Ab C
C
4
Ag C
3
4
3
1
Ab C
C
4
Ag 3
3
4
3
2
Ab C
C
4
Ag 4
3
4
3
3
Ab C
C
4
Ag x
3
4
3
4
Ab C
C
4
Ag x
3
4
3
5
Ab C
C
4
Ag x
3
4
3
6
Ab C
C
4
Ag III
3
4
3
7

Figure 13

lypholized, ground coleoptiles, therefore, were not the same ones to which the rabbit responded when injected with protoplasts. Therefore, the antibodies produced in the serum were probably those corresponding to the antigenic substances associated with the isolated protoplast surface or released from the isolated cell surface due to the enzymatic treatment. Furthermore, no precipitating bands were formed by extracts of protoplasts made with 8 M urea or deoxycholate, or following ultrasonication of protoplasts when diffused against antiserum produced against protoplasts. This suggested the precipitating bands formed were due to antisera reacting with those antigens released from the cell surface after the preparation of the protoplasts. No precipitating bands were formed when antiserum was diffused against substances extracted in buffer from lypholized, ground conidia of the pathogen (Figure 13). This indicated that no common antigen was detected between the host protoplasts and the conidia of the pathogen.

One or two additional bands were occasionally observed. Some differences were due to an individual rabbit, some due to the number of bleedings used. These differences appeared in heterologous as well as homologous reactions. This suggested that the additional bands produced were due to the sensitivity of the individual rabbit to particular substances released by the host protoplast.

The data presented here do not show any differences in the antigenicity of protoplasts with different Pm genes. The differences between rabbits were greater than the differences between protoplasts for any one bleeding.

DISCUSSION

Infection of host tissues by many parasite fungi is often preceded by the formation of characteristic structures such as appressoria. It is generally assumed that these are a prerequisite for infection. Some fungi produce them readily when growing on a wide variety of surfaces, both of plants and nonliving structures (6). Others do so only, or much more frequently and characteristically, when they grow on plants that can function as hosts (50). In the first group, the stimulus which initiates the formation of characteristic structures by the parasite is apparently no more than contact of suitable hyphae with a surface having a certain degree of hardness. In the second group, the response may be conditioned by other properties of the surface as well as by its hardness. Moreover, factors not related to the surface may also operate, especially those substances produced by the host and accumulated in significant concentrations at its surface.

Appressoria are often regularly formed at some points on leaf surfaces, but not elsewhere. Positional effects of this nature are described for a number of parasites (28, 59). This suggested that there is some special stimulus in the particular zone of the host surface which

first stops linear growth of the hyphae, and then permits initiation and development of the appressoria.

In the primary infection process of E. graminis on wheat and barley, the formation of mature appressoria appears to depend upon properties of the host surface. The data presented indicated that when both upper and lower surfaces of epidermal strips were inoculated a high percentage of mature appressoria were formed on the upper surface of epidermal strips (with cuticle), and few mature appressoria were observed on the lower surface of the epidermal strips (without cuticle). This indicates that the cuticle layer was the major factor that stimulated the formation of mature appressoria. The possibility remains that chemical substances are diffusing from epidermal cells and are responsible for the stimulation of the formation of mature appressoria. However, since chemicals could diffuse out from both sides of the epidermal cell layer and stimulate the formation of mature appressoria, mature appressoria should form on both sides of an epidermal strip. Mature appressoria were observed to form on the upper surface of the enzymatically isolated cuticle but few mature appressoria formed when the lower surface was inoculated. This suggested that the wax layer of the cuticle surface is the major factor responsible for the stimulation of maturation of appressoria. A large percentage of malformed appressoria was observed on the under surface (without wax) of isolated cuticles and epidermal strips. Malformed appressoria were

prevalent on artificial surfaces which are believed to closely simulate the chemical and/or physical nature of the cuticle surface, including reconstruction of the wax layer with wax extracted from either fresh seedlings or the isolated cuticles. The appressoria were termed malformed by the fact that their irregular shapes were easily distinguishable from those of regular "normal" appressoria by microscopic observation. The malformed appressoria also gave no evidence of an attempt to penetrate the leaf surface.

Under favorable conditions, over 90% of the inoculated conidia of either E. graminis f. sp. tritici or E. graminis f. sp. hordei produced mature appressoria on the leaf surface of wheat and barley, respectively, 8 hr after inoculation. Essentially the same percentages of mature appressoria were observed on the isolated intact cuticles from near-isogenic host lines with different dominant Ml or Pm genes. These results suggest that all the various parasite/host genotypes for incompatibility do not act to affect the development of the parasite before penetration. The host genes tested must not have affected the cuticle structure, and no chemical substances must have diffused out from the host epidermal cells to inhibit the formation of mature appressoria. Moreover, conidia of either fungus produced approximately the same percentages of mature appressoria on cuticles isolated from either wheat or barley

lines. The formation of mature appressoria is not host specific, therefore, at least in two species tested

The hypothesis that the wax layer is a determining factor for the formation of mature appressoria was checked by inoculating plants with eceriferum (cer) mutations. The cer loci control the synthesis and/or excretion of the organ specific wax components (49). Five eceriferum mutants (cer-J^{59}, cer-J^{71}, cer-Zd^{67}, cer-Ze^{81}, and cer-Zj^{78}), which were identified by glossy leaves, were inoculated. Malformed appressoria were formed on the primary leaf surface of all five mutants. Much lower percentages of ESH were observed on plants with mutations cer-Zd^{67}, cer-Ze^{81}, and cer-Zj^{78} than expected based on the percentages of mature appressoria formed. This suggested that though some appressoria appeared normal, they did not function to give haustoria and ESH. Slightly malformed appressoria may be indistinguishable from mature appressoria by microscopic observation. Malformed appressoria were found on plants with mutation cer-Zj^{78}. This mutation gives wax bodies that are smaller, less densely scattered, and irregularly distributed over the leaf surface (91). This suggests the distribution of wax bodies is an important factor for the formation of mature appressoria. The suggestion is that the more homogeneous the distribution of the wax bodies, the better is the chance for each parasite unit to be stimulated to differentiate.

Though the eceriferum mutations cause a reduction in the percentage of mature appressoria, the final infection types were the same as with Manchuria and wild type Bonus. This suggested that, even though part of the population of applied parasite units was stopped at the stage of malformed appressoria, some of the remaining parasite units did establish a compatible host-parasite relationship and produce pustules 6 days after inoculation. Since the formation and excretion of wax bodies were affected tremendously by the environmental factors (54), the wax layer could possibly be one of the factors that contributed to the so-called field resistance.

The large percentage of mature appressoria formed on the isolated cuticles leached by the organic solvents is possibly due to the wax molecules that are considered to be firmly impregnated into the cuticle structure (54).

Although no attempt was made to purify the wax substances of the isolated cuticles and the extracted wax used for reconstruction of wax layers, the genetic analysis as well as the leaching experiments make it appear that the stimulus for the formation of mature appressoria is by contaminating chemicals on the cuticle is very unlikely.

Elongating secondary hyphae have been used as an indication of the establishment of compatible, functional host-parasite relationships, since the formation of elongating secondary hyphae is completely dependent on the

formation of haustoria in the host epidermal cells (52). The effect of different near-isogenic barley and wheat lines with different Mℓ or Pm genes, respectively, on the formation of ESH was examined. A significant reduction of the formation of ESH was found in seven out of eight genotypes for incompatibility tested. The kinetics of ESH formation with P2/Pm2 followed closely the results obtained with inoculations of Chancellor.

Though the percentage of ESH-formed was reduced by seven different genotypes for incompatibility, the growth rate of the ESH with 4 genotypes was not affected, at least up to 36 hr after inoculation. This result is consistent with the observation that no malformed haustoria were found during primary infection with or without genes for incompatibility. If haustoria were formed, they always attained full size. The simplest explanation of this result is that either the products of the Mℓ or Pm genes were located at the surface (membrane or wall) of the epidermal cells, or the product of the gene is synthesized very rapidly in the epidermal cells after contact by the parasite. The product of the P gene in the parasite may also be constitutive or synthesized after contact with the host. The initiation of the formation of haustoria was supposedly stopped by the interaction between the product of the Mℓ or Pm genes in the host and the product of the P genes produced by the parasite as soon as penetration by the parasite reached the host cell plasma membrane. The different percentages of the

total applied parasite units which formed haustoria or ESH with the different genotypes for incompatibility can be rationalized if a nonrandom distribution of the products of the Mℓ or Pm genes on the plasma surface is considered a possibility. The presence and/or concentration of the products of host and parasite genes at the point of penetration may determine whether or not a haustorium will form.

In summary, the action of Mℓ or Pm genes with their corresponding P genes in the parasite was not detected before the penetration process by the pathogen. Two effects of genes for incompatibility were observed: (1) the reduction in the percentage of ESH formed in the primary infection process, and (2) the reduction of the infection type at 6 days after inoculation.

The use of the criterion of the percentage of ESH formed for the identification of genes for incompatibility gave surprising results when applied to the identification of segregating Mℓ genes in barley. The identification of three different genotypes, the homozygous recessive (mℓ mℓ), the homozygous dominant (Mℓ Mℓ), and the heterozygote (Mℓ mℓ) was attempted by the inoculation of progeny obtained from the selfing of heterozygous plants (Mℓ mℓ). In all four near-isogenic barley lines tested, the range of the percentages of ESH on the homozygous dominant (Mℓ Mℓ) and the homozygous recessive (mℓ mℓ) plants derived from selfing of heterozygous parents (Mℓ mℓ) was considerably larger than on homozygous

dominant (Mℓ Mℓ) plants derived from homozygous dominant parents (Mℓ Mℓ) and homozygous recessive (mℓ mℓ) plants derived from homozygous recessive (mℓ mℓ) parents. It appears that there is a carry-over effect from the genotype of the parent to the progenies. The functioning of both dominant and recessive genes in the parent is suggested. No attempt was made to resolve the mechanism of this carry-over effect. The carry-over effect of the parent on the homozygous progeny can not be from the genotype of the endosperm, unless double fertilization occurred, because the endosperm would be homozygous. Both Mℓq and mℓp were semidominant as evaluated by infection type 6 days after inoculation (55). It was also observed that an intermediate infection type, type 2, was found on some plants derived from the selfing of Mℓa mℓa. The progenies of plants with infection type 2 were inoculated and segregation patterns were observed. Two types of segregation were observed. Progeny from some plants gave all infection type 0. Progeny from some plants had progeny with infection types 0, 2, or 4 but not in the ratio of 1:2:1 expected with partial dominance. These results strongly suggest a function of both Mℓa and mℓa genes and a carry-over effect from heterozygous parents to the progeny that affects the expression of the genotype of the progeny.

The observations that either a full sized haustorium is formed or no haustorium at all is formed suggest that a factor located on the epidermal cell surface determines

compatibility between host and parasite. Electron micrographs show that there is probably no direct mixing of the cytoplasm of the haustoria and the cytoplasm of the host cell (4). The investigations of the host cell surfaces were obviously important.

Experiments were performed to examine the antigenicity of the protoplasts isolated from three near-isogenic host lines, each of which had different Pm genes. Three major bands appeared on either homologous or heterologous reactions by agar-gel double diffusion. No bands were observed when antisera was diffused against soluble substances extracted from lypholized, ground coleoptiles by phosphate buffer. The antigenic substances extracted in buffer from lypholyzed ground coleoptiles, therefore, were not the same ones to which the rabbit responded when injected with protoplasts. Therefore, the antibodies produced in the serum were probably those corresponding to the antigenic substances associated with the isolated protoplast surface or released from the protoplasts. Furthermore, no precipitating bands were formed by extracts of protoplasts made with 8 M urea, or deoxycholate, or following ultrasonication of protoplasts, when diffused against antiserum produced against protoplasts.

Two kinds of cells were found in the protoplast preparation. Approximately 40-60% of the cells were spherical protoplast. The remaining cells were irregularly shaped

single cells due to incomplete digestion of the cell wall. Additional incubation with the cellulose enzyme preparation did not increase the percentage of spherical protoplasts. This was possibly due to either different aging or different kinds of cells whose cell walls were undigestible by the enzyme solution used. Therefore, whether the antigenic substances were located on cell walls or cell membranes remains to be answered.

Primary cell wall has been considered a cell particle or organelle (43) with its own complement of proteins and enzymes (11, 34, 44, 45), and perhaps even ribonucleic acid (69). Cell wall enzymes could be supposed to bring about the following: (a) synthesis of cell wall macromolecules, (b) transfer and hydrolysis of cell wall macromolecules, (c) modification of extracellular metabolites to facilitate their transport into the cell, and (d) as a defense mechanism. All these functions may be involved directly or indirectly in the mechanism of early host and parasite interactions during the primary infection process.

No precipitating bands were formed when the prepared serum was diffused against substances extracted in buffer from lypholized, ground conidia of the pathogen. This indicated that no common antigen existed between the host protoplasts and the conidia of the pathogen.

One to two additional bands were occasionally observed. Some differences were due to individual rabbits.

The differences appeared in heterologous as well as homologous reactions. This suggested that the additional bands produced were really due to the sensitivity of the individual rabbits rather than particular antigenic substances released by the host protoplasts.

The data presented do not show any differences in the antigenicity of protoplasts with different Pm genes. The differences of sensitivity to produce antibodies against isolated protoplasts between rabbits were greater than the differences between tested protoplasts carrying different Pm genes.

SUMMARY

The process of primary infection of wheat and barley by Erysiphe graminis consists of a number of morphologically identifiable stages of development: spore germination, formation of appressoria, penetration into host cells, formation of haustoria in host cells, and formation of elongating secondary hyphae which are capable of initiating secondary and tertiary infections.

The objectives of this study were the following: (1) to determine the role of the host cuticle layer during the primary infection process, particularly on the formation of mature appressoria by the parasite population before penetration; (2) to examine the action of genes for incompatibility in the parasite and host on the sequential development of the parasite; (3) to determine the relationship between the percentage of elongating secondary hyphae to the segregation of genes for incompatibility in the host; and (4) to determine if antigenic differences on the cell protoplasts could be detected among near-isogenic host lines with and without genes for incompatibility.

Conidia will germinate on a number of different natural and artificial surfaces. A high percentage of normal appearing, mature appressoria was observed on host

leaves, on the upper surface of epidermal strips, and on the upper surface of enzymatically isolated cuticles. No or few normal appearing, mature appressoria were formed on the lower surface of epidermal strips, the lower surface of isolated cuticles, on reconstructed wax layers, or on a number of different artificial surfaces. The presence or absence of specific Mℓ or Pm genes in the plants from which the epidermal strip or cuticles were isolated did not affect the formation of mature appressoria. Malformed appressoria were observed on plants that possessed eceriferum (cer) mutation that affect the chemistry and physical structure of wax layers.

The Mℓ and Pm genes in barley and wheat, respectively, did not appear to interact with the corresponding P genes in the parasites to affect the morphological development of the parasites prior to penetration. The two effects of the incompatible parasite/host genotypes were: (1) the reduction in the percentage of parasite units that formed elongating secondary hyphae, and (2) the reduction of the infection type six days after inoculation.

In all four near-isogenic barley lines tested, the range of the percentages of ESH that developed on the homozygous dominant (Mℓ Mℓ) and the homozygous recessive (mℓ mℓ) plants derived from selfing of heterozygous parents (Mℓ mℓ) was considerably larger than on homozygous dominant (Mℓ Mℓ) or recessive (mℓ mℓ) plants derived from homozygous

parents. There appears to be a carry-over affect from the genotype of the parent to the progenies.

No differences in the antigenicity of the protoplasts with different Pm genes were detected by homologous and heterologous agar gel double diffusion tests. The differences between rabbits were greater than the differences between protoplasts for any one bleeding.

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LITERATURE CITED

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