GENETIC AND HOST-PARASITE INTERACTION STUDIES OF THE FUNGAL PATHOGEN PHYLLOSTICTA MAYDIS USING TEMPERATURE-SENSITIVE AND AUXOTROPHIC MUTATIONS

Thesis for the Degree of M. S.
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ABSTRACT

GENETIC AND HOST-PARASITE INTERACTION
STUDIES OF THE FUNGAL PATHOGEN PHYLLOSTICTA MAYDIS
USING TEMPERATURE SENSITIVE AND AUXOTROPHIC MUTATIONS

By

Dean William Gabriel

The mutagenic agent nitrosoguanidine was used to produce thirty temperature-sensitive, ten auxotrophic, and five morphological mutants of Phyllosticta maydis Arny and Nelson. The auxotrophic mutants have been characterized as to nutritional requirements, and complementation groups have been determined. The temperature-sensitive mutants fell into three categories: those temperature-sensitive on artificial agar media, those temperature-sensitive on host plants, and those temperature-sensitive on both. These temperature sensitive-mutants provide evidence that P. maydis follows the gene-for-gene pattern of interaction on Normal (N) cytoplasm corn. Mutations to increased virulence were also obtained, and together with the temperature-sensitive mutants, provide support for the hypothesis that the avirulence gene(s) of the parasite is (are) active in promoting incompatibility, and that the virulence allele(s) arise(s) through loss of function.

The apparently multinucleated condition of the conidia, and restrictions on the homothallic capability of the perfect stage, are

discovered and discussed in terms of the variability they afford the fungus.

GENETIC AND HOST-PARASITE INTERACTION STUDIES OF THE FUNGAL PATHOGEN *PHYLLOSTICTA MAYDIS* USING TEMPERATURE-SENSITIVE AND AUXOTROPHIC MUTATIONS

By

Dean William Gabriel

A THESIS

Submitted to
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To my parents, Evelyn and Bill,

my wife, Meri Anne,

and my son, Ryan

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INTRODUCTION

Yellow leaf blight (Phyllosticta maydis Arny and Nelson) of corn (Zea mays L.) is known to occur across the northern part of the corn belt of the U.S. and in Ontario, and in 1968 appeared in near epidemic proportions in Michigan. A Phyllosticta leaf blight caused by Phyllosticta zeae was first described in 1930 (45). Isolates of Phyllosticta with larger spores were found to be widespread in the northern corn belt regions in the 1960's, and these larger spored isolates were considered to be a new species (2). The economic importance of the species was correlated with the use of Texas male sterile cytoplasm (Tms) in corn.

The immediate cause of the P. maydis epidemic is considered to be related to its ability to produce a toxin specific for plants with the Tms cytoplasm (6,7,54). This toxin is not mandatory for parasitism, however, as P. maydis is capable of infecting normal (N) cytoplasm corn in some cases as severely as Tms lines. Studies on resistance and susceptibility of N cytoplasm corn indicate that resistance is dominant (3,4,29,32). P. maydis may be a valuable link between pathogens in which toxin production is essential for pathogenicity, and those which produce no toxin.

One of the purposes of this study was to obtain temperature sensitive mutants and use them to help analyze the genetics and physiology of the host-parasite interactions. Mutants which are temperature-sensitive both on agar and on host plants should affect

spore germination, differentiation of germ tubes, appressoria, and basic life processes. Mutants which are temperature-sensitive on agar media but not on the host should be useful to study transfer of materials from the host to the parasite. Most desireable of all are mutants which are not temperature-sensitive on agar media, but which are temperature-sensitive on the host, for these mutants affect the parasitic ability of the fungus. This last type should exhibit at least two types of reaction: 1) At the restrictive temperature, the fungus would not be parasitic, possibly because of a failure of a developmental step or sequence necessary to an active parasitism, such as formation of appressoria, or penetration through host cuticle, or production of a toxin. Temperature-sensitive mutants of this kind could conceivably be used to determine the developmental gene sequences necessary to the life cycle of P. maydis, and especially to resolve whether or not parasitism is an active process, the latter an hypothesis which has wide acceptance but with little supporting evidence. 2) At the restrictive temperature, the fungus would be parasitic, because of a loss of the specific gene-for-gene interaction(s) between resistance gene(s) of the host and avirulent gene(s) of the parasite. The P6/Sr6 interaction of wheat stem rust is one example of a temperature-sensitive interaction following this pattern (15).

P. maydis is an ascomycete, and therefore haploid. Its sexual stage, recently described, is Mycosphaerella zeae-maydis Mukunya and Boothroyd (31). With the discovery of the sexual stage, coupled to the fact that P. maydis is saprophytic and easily cultured in the laboratory, comes

the possibility of genetic analyses of induced mutations. Another purpose of this study was to obtain auxotrophic mutants, and to obtain linkage data not only for the auxotrophic markers, but also to attempt to determine linkage relationships between auxotrophic markers and temperature sensitive mutations, since these mutations are indistinguishable unless they are developmentally (temporally) or biochemically distinct.

A final purpose of the study was to determine whether or not P. maydis would form a heterokaryon, and if so, undergo subsequent parasexual recombination. Heterokaryosis is regarded as a prominent feature of the fungi and of great importance to plant pathogens in particular, since the proportion of different nuclei in a heterokaryon may change in response to selection, and since segregation of dissimilar nuclei in conidia can generate variation (33). Occasionally unlike nuclei may fuse to form a diploid, which then apparently undergo mitotic non-disjunction back to the haploid state through stages of aneuploidy, in a process termed parasexuality (38). Since P. maydis under laboratory conditions produces ascospores (sexual cycle) only sparsely, demonstration of parasexuality or heterokaryosis in this fungus would indicate an important alternate source of variability in this pathogen.

LITERATURE REVIEW

Specific recognition: the gene-for-gene hypothesis.---One of the essential features of man's success in supporting such a large population of his own species has been the large-scale planting and cultivation of crops in monoculture. With our basic philosophy of discarding disease-prone or low yielding plant lines and substituting disease-resistant ones (15), together with our basic economic philosophy of uncontrolled use of certain preferred lines, we have replaced in each major crop older, lower yielding varieties by a few, closely related, "superior" forms (9a). Selection for uniformity in crops---where once there was diversity---has encouraged concommitant selection for, and rapid multiplication of, particular parasite races.

Plant breeders tend to introduce and thus rely upon relatively few genes for resistance to any one disease at any one time. The effect is to expose the host genes one at a time. Few genetic changes in the parasite are necessary to render the host genes so exposed ineffective. One can follow the appearance and disappearance of physiological races of fungal pathogens according to the use and disuse of various genes for resistance (34). Johnson has called the phenomenon "man-guided" evolution (25).

Careful genetic studies of host and parasite variability has revealed a particular pattern, first articulated by Flor as the gene-for-gene hypothesis (18,19,20). A host plant with a given gene will be resistant to its parasite only in the presence of a specifically

recognized parasite gene, called then, by definition, a gene for avirulence. The given host gene is then, by definition, a gene for resistance. This interaction has been demonstrated or suggested with at least some of the following parasites: rusts, smuts, bunts, mildews, nematodes, insects, bacteria, and viruses (9b).

A simple heuristic interpretation of this gene-for-gene interaction (see Figure 1) is that a host "Resistance" (R) gene "recognizes" a parasite "Avirulence" (A) gene. With the use of a host line r1r1, the segregation of A1 from a1 is undetectable in crosses of the pathogen. Since an A gene, until the appropriate R gene is found, is initially associated with virulence, it is probable that the "primary role of the A gene is to function in support of the parasite's success as an intrinsically viable organism" (36).

The converse is only partially true; that is, Flor demonstrated the importance of using a wide variety of parasite isolates because the use of parasite strain a1a1 does not allow detection of segregation of R1 from r1. Unlike A genes, however, R genes are thought to increase in frequency in response to pathogenic selection, and as such, more accord with the general expectation that dominant genes afford a swifter selective advantage than recessive ones. (Note that the gene for virulence, a, behaves as a recessive, and is not in accordance with this expectation.) The initial situation of pathogenic encounter is hypothesized to be the compatible A1A2A .../r1r2r ... . The host responds by selection of mutations for a recognized R gene, giving the incompatible interaction A1A2A .../R1r2r ... . The primary role of

Figure 1a.---The 9 possible parasite/host genotypes involving a single gene pair governing compatibility in diploid hosts and parasites.

The terms compatible and incompatible are used quite arbitrarily to grade a disease reaction into two categories: "+" denoting compatibility, and "-" incompatibility. These terms are useful in calling attention to the parasite/host interaction, alleviating the confusion involved in describing a host as resistant or susceptible, or a parasite as avirulent or virulent. The nine possible combinations involving a single gene pair in both host and parasite are shown above right. This figure is often shown in abbreviated form, and called the "Quadratic Check", as below right.

Figure 1b.---The Quadratic Check.

		Host Genes		
		RR	Rr	rr
Parasite Genes	AA	-	-	+
	Aa	-	-	+
	aa	+	+	+

a

R = gene specifying resistance in the presence of specifically recognized A gene.

r = alternate allele of R.

A = gene specifying avirulence in the presence of specifically recognized R gene.

a = alternate allele of A.

		Host Genes	
		R-	rr
Parasite Genes	A-	-	+
	aa	+	+

b

Figure 1

the R gene, then, is probably not in support of the host's success as an intrinsically viable organism, as argued for the A gene in the pathogen.

Because resistance is often phenotypically expressed several days after intimate contact between host and parasite is established, R genes are often thought of as inducible genes which are activated by and respond to the presence of the pathogen. At least one gene, however, which specifies resistance has been demonstrated to be constitutive: the Vb gene in oats. The dominant Vb gene conditions susceptibility to Helminthosporium victoriae toxin and resistance to Puccinia coronata (49). Reaction of oat protoplasts with the Vb gene to H. victoriae toxin is immediate (40,51), and indicates that the Vb gene is constitutive in function. The site of action of the toxin appears to be in the plasma membrane; resistant (vb) cell membranes appear to lack the receptor site (40). Since this same Vb gene confers resistance to P. coronata, then it is by definition an R gene, and has been demonstrated to be constitutive. Other R genes may be constitutive; it is also possible that R genes may function in a partially inducible manner, responding to parasitic invasion with increased levels of activity (15).

There may be many host genes for resistance, each specific for a corresponding parasite avirulent gene, and any combination specifying incompatibility is epistatic to all others which would allow parasitism to proceed. For a parasite population to overcome a given resistance factor, it needs to lose or alter its gene for avirulence, which is recognized by the host, and if it was a necessary gene, replace it with a gene of identical function, but one which is now unrecognized. Hence

the disappearance of certain physiological races, and the appearance of a few, "select" others.

The significance of exposing resistance genes one at a time now becomes apparent. The use of plant stocks which are genetically homogenous except for a few discovered resistance genes facilitates the parasite's change to negate the recognition of the host R genes. Reliance on horizontal, or general resistance is not likely to be of much value either, since all non-toxin mediated host-parasite interactions appear to follow the gene-for-gene pattern (14).

Specific recognition: toxin mediated parasitism.---P. maydis gained its prominance as a pathogen, not because of selection to avoid a specific dominant resistance factor, but rather because of its ability to produce a host-specific toxin.

In commercial seed production, a great yield advantage may be realized through the use of hybrid seed, which seedsmen successfully promoted in the 1930's (9c). Self fertilization may be prevented either by hand detasseling the anthers, or by the use of a cytoplasmic male sterility factor, transmitted by the female parent to all the progeny. While there are many sources of male sterile cytoplasms, Texas male sterile (Tms) had unusual favor because it caused full sterility (no anthers exerted) in a majority of corn inbred backgrounds. Most other sterility factors cause only partial sterility or full sterility in only a few inbred backgrounds.

The widespread use of Tms hybrids provided a man-made, genetically uniform background of another kind, allowing epidemic sized parasitic

infection by any organism able to exploit it. In this case, by genetically uniform is not meant that each host plant was genetically identical---indeed, the Tms cytoplasm was used with a great many different inbred backgrounds---but rather, that a single recognizable genetic factor was present. This factor appeared to be the Tms factor, for all Tms lines and Tms lines with nuclear fertility restorer (Tms-Rf) genes tested were reported uniformly susceptible, while almost all inbreds without the sterility factor (Normal, or N cytoplasm) were reported uniformly resistant (43).

The ability of a fungal pathogen to attack a host containing a single factor (susceptibility dominant) as opposed to inability to attack a host containing a single factor (resistance dominant) is characteristic of toxin mediated parasitism (See Figure 2),which is quite distinct from gene-for-gene interactions (Compare Figure 1). The former interactions are thought to be relatively early host-parasite encounters in an evolutionary time scale, and the latter are thought to be the more highly evolved and hence more stable or equilibrated interactions (15).

For example, H. victoriae, mentioned previously, produces a toxin which renders all oat varieties containing the single recognizable genetic factor, the nuclear Vb gene, susceptible. Specificity is, therefore, for a nuclear gene, the site of action appearing to be the plasma membrane (40). Toxin specificity of P. maydis and Helminthosporium maydis, on the other hand, is not for a nuclear gene as such, but rather for the cytoplasmically inherited sterility factors, such as

Parasite	Host: Oats Vb	Oats vb	Corn Tms	Corn N
H. victoriae Race T	+			-
H. maydis Toxin		+		-
P. maydis Toxin			+	-
H. victoriae Race O	-			-
H. maydis No toxin		-		-
P. maydis No toxin			-	-

Figure 2.---Toxin mediated parasitism in three systems. "+" = compatible reaction, "-" = incompatible reaction. Vb and vb are alternate alleles of a nuclear gene of oats. Tms and N refer to Texas male sterile and Normal cytoplasms of corn, respectively. Note that this pattern is the exact reverse of the "Quadratic Check" gene-for-gene pattern of Figure 1.

as Tms (6,7,8,41,54).

Evidence has been presented which suggests that the specificity in reaction to the toxin produced by H. maydis and P. maydis is for Tms mitochondria (6,7,50). Toxin in low concentrations is highly specific, causing uncoupling of oxidative phosphorylation from the electron transport chain and immediate and irreversible swelling of isolated Tms mitochondria, while having no effect on isolated N cytoplasm mitochondria, even when concentrated a thousand fold (6,7). Nuclear genes which restore fertility (Rf) to plants with Tms cytoplasm give a toxin reaction intermediate between that observed with N and Tms plants and mitochondria. In one case toxin had a greater effect on Tms-Rf mitochondria than on Tms without Rf (50). Several possibilities are proposed to account for the observations: 1)Rf genes may be closely linked to other genes conditioning toxin sensitivity; 2) Rf genes may modify the toxin itself; and 3) Rf genes may alter mitochondrial or plasma membranes, or both (50).

As mentioned previously, without the Tms or other dominant susceptibility factors present, P. maydis exhibits the more usual pattern of resistance being dominant. That is, when one considers N cytoplasm corn only, then F1 progeny of crosses of these corn lines always show resistance approximately equal to that of the most resistant parental line. This pattern follows the gene-for-gene interaction (Figure 1), and argues strongly against toxin mediated parasitism (Figure 2) in N cytoplasm corn, where susceptibility in the F1 would at least be equal to that of the most susceptible parent.

If further studies bear out the preliminary data, then it becomes apparent that toxin production is useless to P. maydis in promoting parasitism of N cytoplasm corn. This conclusion is in conflict with an abstract published in 1972 by Yoder and Mukunya (55), in which they report that culture filtrates of P. maydis contain host-specific toxic metabolite(s) having the same specificity as the pathogen in N cytoplasm corn. If this is the case, then they have discovered a metabolite with different specificities than the Tms specific toxin (7). They have published nothing further about it.

Since Tms cytoplasm rendered host lines which carried it susceptible to P. maydis, hybrid seed producers have resorted to N cytoplasm corn (with hand detasseling). An advantage is that N hybrids appear to perform better than Tms hybrids even when disease is not a problem (16,39). Of course, conversion to N cytoplasm only obviates the toxin specificity of P. maydis, and incorporation of genetic sources of resistance in corn hybrids are needed for protection and control.

Genetics of pathogenicity of P. maydis.--- Yellow leaf blight was first observed on corn in Ohio in 1965 (29), and by 1967 in Wisconsin (3) and in Ontario (24). It was first described by Scheifele and Nelson (42) in 1969 as a Phyllosticta leaf spot, and the causal organism was assumed to be P. zeae Stout (45), but nine months later Gates and Mortimer (24) determined that it was not. During 1970 it was referred to as Phyllosticta sp. until described as a new species by Arny and Nelson in 1971 (2).

In terms of pathogenicity, P. maydis is considered weak, due largely to its predilection for young seedlings, older leaves of larger plants, physiologically weakened or stressed plants, or those already parasitized (1,24,46). Several studies have been made of the reactions of various inbreds (3,4,29,32,42,43) and these studies all indicate that even though a considerable amount of resistance may be present in inbred lines, the resistance will be partially or totally lost upon conversion to Tms or Tms-Rf cytoplasm. The results of these studies on N cytoplasm corn indicate that resistance in the F1 is as good as the most resistant parent, or is at least intermediate between the two parental lines. These intermediate reactions are not surprising in view of the apparent lack of appreciation of Flor's work demonstrating the necessity of using single isolates to detect different resistance factors. In only two studies are single isolates used to obtain data on pathogenicity. To cite one example, the rating of W64A in the data of Arny et al (3) varied from 2.5 to 5.0 on a 12 point scale. They used "several isolates" of P. maydis.

Formal genetics.--- The sexual stage of P. maydis was first described in 1973 as Mycosphaerella zeae-maydis by Mukunya and Boothroyd (31). Mycosphaerella is in the Order Dothideales, of the Subclass Loculoascomycetidae. Diagnostic characteristics are a bitunicate ascus, with the asci borne in locules, the ascocarps of which are stroma. The locules are thus not perithecia, which

they resemble, but pseudoperithecia, or pseudothecia.

As with many fungi, sexual reproduction in P. maydis is favored by relatively low, and inhibited by relatively high, temperatures. In Pleospora herbarium, sexual reproduction may be triggered by either U.V. light or low temperatures, and subsequent maturation requires light (27). In Venturia inaequalis, initiation of perithecia requires a temperature of 13° C, while the maturation of the ascospores requires a temperature of 20° C (53). P. maydis develops pseudothecial initials in corn debris over winter, and maturation occurs in the spring; the optimum temperature is about 20° C, with neither light nor cold treatments necessary. Mukunya and Boothroyd report that all of 72 single ascosproe cultures developed mature pseudothecia; they thus considered P. maydis to be homothallic (31). Whether the homothallic mechanism is primary or secondary is unknown.

Homothallism is thought to be favored by natural selection due to its lack of dependence on spores or hyphae of compatible mating type to ensure sexual reproduction (34). This advantage may be gained by essentially heterothallic fungi as well, such as those capable of secondary homothallism [Neurospora tetrasperma (52)], those which are weakly heterothallic [Glomerella cingulata (52)], and those which mutate from one mating type to the other [Saccharomyces lactis (23), Chromocrea spinulosa (28)]. Indeed, primary homothallism, in which single spore cultures are never self sterile, and in which mating type is stable, appears to be relatively rare and not well understood (48).

The most important aspect of heterothallism to a fungus is the requirement of heterokaryon formation [the mixing of unlike nuclei in a common cytoplasm (33)] and the possibilities of recombination afforded it by meiosis or parasexuality. In those fungi in which meiosis is restricted, rare, or unknown (as with the Imperfect Fungi, most of which are considered to be related to the Ascomycetes), parasexual recombination is assumed to be important in generating variation. Mitotic crossing over occurs in A. nidulans according to the following scheme (17): 1) Plasmogamy (heterokaryon formation); 2) Karyogamy at a frequency of about 10^{-6} to form diploid heterozygous nuclei with sectoring of diploid and haploid nuclei; 3) Mitotic crossing over (at a frequency of about 10^{-2} per nuclear division, compared to 10 per nuclear division for meiotic crossing over); and 4) Haploidization at the rate of about 10^{-3} per nuclear division. Mitotic, like meiotic, crossing over is reciprocal, occurs at the four strand stage, and only two of the four strands participate in any one exchange (26).

Parasexuality in the Ascomycetes has been demonstrated in Helminthosporium sativum and Glomerella cingulata and indicated in Leptosphaeria maculans (47). In all of the above, heterokaryons were forced using auxotrophic or drug resistant mutations, but linkage relationships of markers were not established. In plant pathogens generally, well marked chromosome arms are lacking (33). In part this is due to the use of multiple isolates presenting many heterogenous genetic backgrounds, unlike the cases of N. crassa, and A. nidulans,

where genetic analysis is based entirely on two and on one wild type stocks, respectively. In part this is also due to the lack of development of the shortcuts needed to expedite genetic manipulations with a particular (and almost by definition, peculiar) fungus.

Perhaps the most extensive genetic work on a single pathogen in terms of identification of avirulent ("pathogenic") genes, auxotrophic and chemical resistant markers, and their linkage has been done on Venturia inaequalis (5). Nitrogen mustard methyl-bis (P chloroethyl) amine and U.V. radiation were used to obtain 30 amino acid, 19 vitamin, and 39 nucleic acid auxotrophs, representing 11, 8, and 12 loci, respectively. Only the biotin, inositol, nicotinic acid, and pantothenic acid mutants were pathogenic, and the specificity to different host lines was unaffected by the mutations. The rest were all generally nonpathogenic. Although nutritional mutants were able to grow together in complementary fashion, or syntropically, on minimal media, and even though hyphal anastamoses occurred regularly, heterokaryosis was not demonstrated.

V. inaequalis proved to be a more difficult fungus to work with than Neurospora, despite their resemblance in many respects to each other. Linkage relationships, for example, demonstrated more centromeres than the observed number of chromosomes. Of particular interest are their mapping studies of the 19 avirulence genes, none of which were found to be closely linked or allelic. This finding is in accordance with the general observation that parasite genes recognized as avirulent are numerous and scattered, while host genes

recognized as resistant are few, and in general, form multiple allelic series, or are closely linked (22).

The significance of many unlinked A genes in parasites and of the long allelic series for R genes in the host may be appreciated in light of continuing interpretations of the term "virulence" as necessarily indicating an active process on the part of the pathogen gene so labeled in support of the pathogen. For example, Person (1975) writes, "it appears that the primary function of the virulence gene is to negate the primary function of R" (35). And Boone (1971) writes, "the allele for virulence might somehow be active in repressing the expression of the resistance gene of the host" (5). The problem with these interpretations is that alleles are alternative varieties of the same gene at a given locus. Of those alleles which are known to be functional (due to their specific reaction to A or R), they might be expected to differ from one another by only a few base pairs, perhaps arising via a missense mutation. (A mutation on the order of a nonsense mutation would be expected to eliminate most of the function of the cistron.) Yet the specificity of those R alleles is for A genes which map all over the parasite genome. If a gene for virulence repressed the expression of one R allele, why would it not also repress an alternate allele of the same locus in a diploid, that allele presumably almost identical to the one supposedly repressed?

Similarly, if the a gene were providing something the parasite needed for growth which the host r gene normally supplies and the R gene does not, it would require an incredible flexibility of the R

cistron to provide so many different gene products from a set of alleles. A far simpler explanation is that a gain in virulence represents either a loss in function, or a shift to a non-identifiable function. If this is so, then mutations to virulence should be easily obtained, but if virulence is an active allele, then mutations to virulence should be difficult. With M. lini, at least, Flor found the former to be the case (21). Boone's group apparently did not attempt similar studies (5).

Further support for the idea that A and R genes actively interact to produce incompatibility is provided by a temperature sensitive interaction. In stem rust of wheat, the P6 gene of Puccinia graminis conditions incompatibility with the wheat gene Sr6. This interaction is temperature-sensitive; that is, at the permissive temperature (20° C), the P6/Sr6 interaction specifies an incompatible reaction, while at the restrictive temperature (25° C), the P6/Sr6 interaction gives a compatible reaction (See Figure 3).

This is not to say that gene-for-gene interactions are the only interactions possible. Some host-parasite combinations exist where no gene-for-gene interaction is demonstrable (15). Since the active, specific interaction appears to be for incompatibility, and since for parasitism to occur, this interaction cannot be present, then the gene-for-gene interaction must develop later in an evolutionary time scale than basic compatibility. The gene-for-gene interaction is thus superimposed on a basic parasitic capability (Figure 4). If this is so, then mutations which affect this basic compatibility ought to be

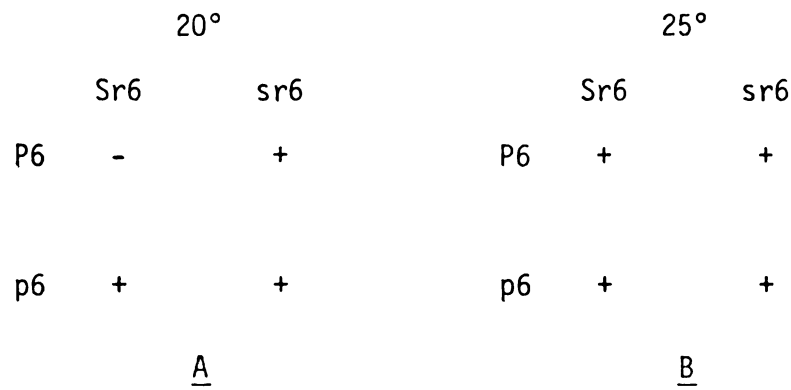

Figure 3.---A temperature-sensitive gene-for-gene interaction.

A. The Sr6 gene in the wheat variety Red Egyptian interacts with gene P6 of *P. graminis* to produce the standard gene-for-gene pattern of interaction at 20° C.

B. The same interaction as in A, except that the temperature has been raised to 25° C.

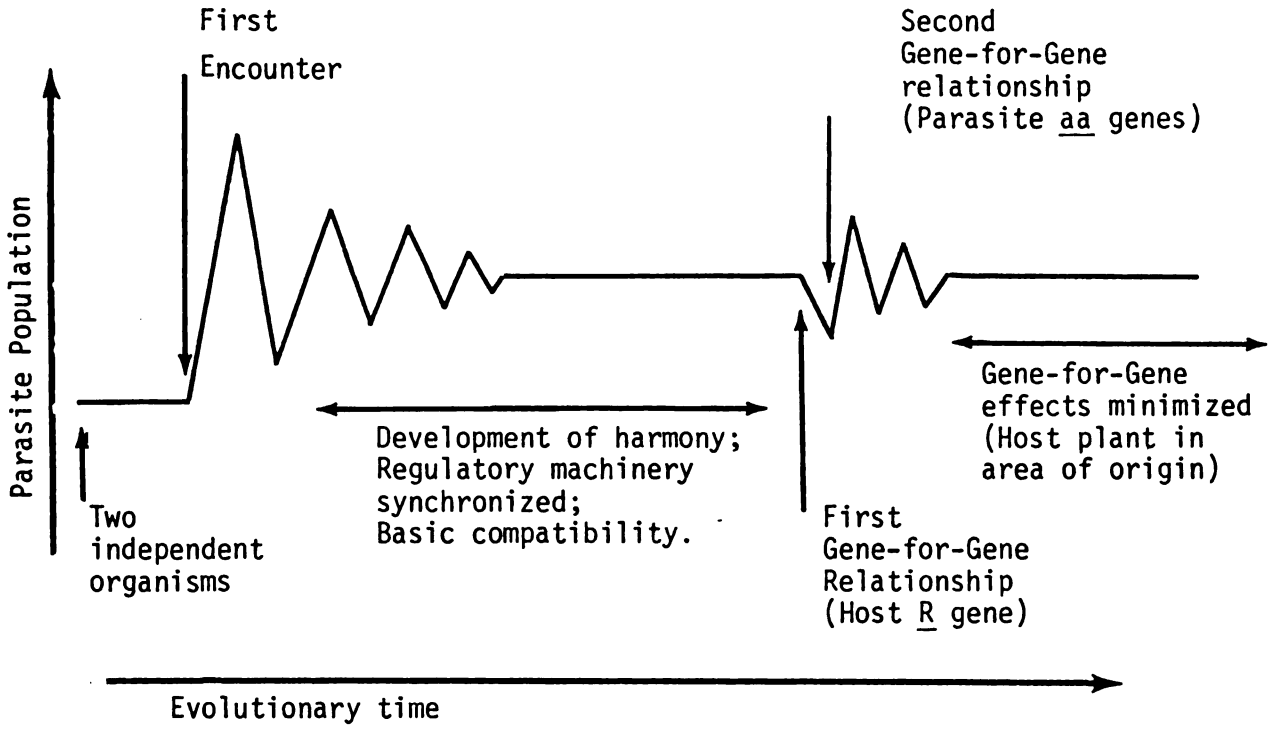

Figure 4.---Expected variations in parasite populations over time during the evolution of a gene-for-gene relationship and the establishment of basic facultative parasitic capability. According to this scheme, the gene-for-gene interaction is superimposed on a basic ability to overcome constitutive physical barriers to parasitism. [After Ellingboe (15)].

obtainable. In particular, temperature-sensitive mutants should be obtainable that disallow this basic compatibility at the restrictive temperature. If P. maydis follows the gene-for-gene interaction, then temperature-sensitive mutants which disallow incompatibility at the restrictive temperature (like P6/Sr6) should be easily obtained also.

MATERIALS AND METHODS

Phyllosticta maydis cultures.---All cultures of P. maydis used in this study, unless otherwise noted, are descended from a single ascospore isolate obtained from a culture isolated from the field in 1969. This culture grows and sporulates well on minimal media (below), and both ascospores and conidia germinate on water agar, but mycelial growth is light and no sporulation of any type is observed on the latter.

Conidial spores measure 12-16 X 4-6 µ, and ascospores are 13-19 X 5-6 µ, closely fitting the descriptions of P. maydis Arny and Nelson, and M. zeae-maydis Mukunya and Boothroyd.

All stock cultures were maintained by serial transfer on complete or minimal media. All sectors which arose in the stock cultures were seperately transferred and appropriately labeled.

Media.---Minimal, Complete, and Corn Leaf Decoction agar media were prepared according to the following recipes:

Minimal Media	
Glucose	10 g
Salt solution	62 ml
KNO_3	3 g
KH_2PO_4	0.5 g
K_2HPO_4	1 g
Noble Special Agar	20 g
Water to 1 liter	

Complete Media	
Glucose	10 g
Salt solution	62 ml
KNO_3	3 g
KH_2PO_4	0.5 g
K_2HPO_4	1 g
Yeast extract	2 g
Bacto-Peptone	2 g
Agar	20 g
Water to 1 liter	

Salt Solution

KH_2PO_4	16 g
Na_2SO_4	4 g
KCl	8 g
$MgSO_4 \cdot 7 H_2O$	2 g
$CaCl_2$	1 g
Water to 1 liter	

Corn Leaf Decoction

Corn leaves	50 g
Water to 1 liter, combine and autoclave 20 minutes, then filter.	
Infusion filtrate	1 L
K_2HPO_4	2 g
Agar	20 g
Adjust pH to 6.2	

The Minimal Media is patterned after Ustilago Genetics Media; the Complete Media is patterned after Schizophillum Complete Media; and the Corn Leaf Decoction Media is patterned after Hay Infusion Media (44).

When compacted colony growth was desired, 5 g of sorbose was substituted for 5 g of the glucose in the medias.

For the screening of auxotrophic mutations, variously supplemented media were used, based upon the Minimal Media recipe, to which the supplements were added. All supplements save casein (used at 2g / liter) were kept in stock solutions. All amino acids and nucleic acids were used at a final concentration of 10^{-4} M. Vitamins were used at the following concentrations: inositol, 0.4%; ascorbic acid, choline, niacin, pantothenate, 0.2%; thiamine, 0.1%; para-amino benzoic acid (PABA), pyridoxine, riboflavin, 0.05%; and biotin, 0.025%.

Protocol for ultraviolet light (UV) mutagenesis.---Conidia were obtained from one to two week old minimal media plates. Plates were flooded with distilled water, the mycelial mat was rubbed lightly with a bent glass rod dipped into Tween-80, and the resulting spore suspension was passed through sterile triple layered cheesecloth to

filter out germinated spores, mycelial fragments and whole pycnidia. Spore concentration was adjusted to 10^2 spores per ml.

All U.V. treatments were performed in a Microvoid laminar flow hood. The flourescent light in the hood was turned off and plates subsequently kept as dark as possible to avoid photoreactivation of the treated conidia. Seventy-five ml of spore suspension was poured into a glass petri dish, and a magnetic stirrer provided constant swirling throughout the treatment. A low pressure (90% of output @ 2,537 A) .25 amp. Model V-41 Mineralight (U.V. Products, San Gabriel, Ca.) placed 5 inches from the plate was used as the U.V. source. One ml. alliquots of liquid were withdrawn every 30 seconds for 15 minutes after the start of the treatment, and plated on complete or minimal media. Depth of spore suspension varied from an initial 3-4 mm to a final 2-3 mm. of liquid.

Protocol for N-Methyl-N'-Nitro-N-Nitrosoguanidine (NG) Mutagenesis.--- Conidia were obtained as above, except that liquid complete media was used in place of water to wash off the spores. Spore concentration was adjusted to 1.0 - 2.5 X 10^5 spores / ml. NG supplied by Aldrich Chemical Co. of Milwaukee was kept for up to a month in a stock solution and used at a final concentration of 100 µg / ml. The spore suspension was constantly agitated during NG treatment. Spores were not washed following treatments; rather, the suspension was diluted by 5 X 10^3 prior to plating. Treated spores were plated on either minimal or complete media (see selection procedure).

Selection procedure: auxotrophic mutants.---Spores treated with either NG or UV were screened by either of two procedures. In the first, the layer plate method, spores were evenly dispersed over petri plates of minimal media at about 100 spores per plate, allowed to grow for two days, and those which germinated were marked as prototrophs. A layer of 45° C complete media was then poured over the top of the minimal plates. The colonies which grew after two days were isolated as presumptive auxotrophs for further testing. In the second procedure, the random isolate method, spores were plated at about 100 spores per plate directly onto complete plus sorbose media, allowed to germinate, and then 16 hyphal tips were each transferred onto one plate of complete media. Replica plates of these were then made onto minimal media, and those which grew well on complete and not at all on the minimal were isolated as presumptive auxotrophs.

Presumptive auxotrophs from either method were then plated onto minimal plus casein, minimal plus vitamins, and minimal plus nucleic acids. Auxotrophs which grew on one of these three and not on the other two were kept for further analysis. Auxotrophs which responded to casein were presumably amino acid requirers, and were plated on amino acid "pool plates"---minimal media supplemented as follows:

	#1	#2	#3	#4	#5
#6	alanine	aspartic acid	leucine	serine	tryptophan
#7	isoleucine	cystine	phenylalanine	glycine	threonine
#8	cysteine	lysine	glutamic acid	tyrosine	arginine
#9	histidine	methionine	proline	glutamine	valine

An auxotroph requiring, for example, arginine, would grow on plates #5 and #8, but on no others.

Auxotrophs which grew only on the vitamin supplemented plates were plated on vitamin pool plates---minimal supplemented as follows:

	#1	#2	#3
#4	riboflavin	pyridoxine	inositol
#5	niacin	pantothenate	ascorbic acid
#6	PABA	choline	biotin

No auxotroph isolated responded to nucleic acid-supplemented plates, so further testing of this expected type was unnecessary.

Selection procedure: temperature-sensitive mutants.---Conidia treated with NG were allowed to germinate, and hyphal tips were transferred 16 per plate onto complete plus sorbose media. After two days the colonies were replica plated onto complete media to be held at 30° C, onto minimal to be held at 22° C, and onto minimal to be held at 30° C. This method thus served to screen for auxotrophs as well as temperature-sensitive (ts) mutations. Growth on complete media at 22° was compared to growth on complete at 30°, and little or no growth at high temperature, with good growth at low, indicated temperature-sensitivity. Temperature-sensitive auxotrophs were tested for auxotrophic requirement.

Another, simplified method that has the advantage of considerable savings in time and in petri dishes and agar was also used. In this method, a temperature-upshift experiment, hyphal tips were transferred 16 per plate onto complete media, and allowed to grow at 22° for five days. The extent of the mycelial growth was indicated by circling the perimeter of growth with a marking pen on the bottom of the plate.

This complete media plate was replicated once onto minimal media, and then transferred into an incubator to be held for another five days at 30°. Much growth beyond the circled area indicated insensitivity to temperature, while ts mutants would not grow at all, or only very little. After marking the ts mutants, these plates were then placed at room temperature (22°), and checked for lethality. An added advantage of this method is that ts auxotrophs are automatically eliminated, since they continue to grow beyond the circled boundary. Each mutant was tested at least three, and sometimes four times; most were tested by both methods.

Screening for temperature-sensitivity and for changes in virulence.--- Corn lines W117, W64A, and MS500 in both N and Tms cytoplasms were innoculated with wild type (wt) P. maydis to obtain normal reaction variability; all mutants were innoculated onto W64A in N cytoplasm. Conidial suspensions of each mutant tested were obtained by rubbing a two-week-old mycelial mat with a bent glass rod in about 10 ml of distilled water to which a drop of Tween 80 was added. Each suspension was atomized onto two sets of corn plants (one set = 4-6 plants in one pot) at the 3-4 leaf stage. Fertilizer was added to the pots to help prevent yellowing of the lower leaves. The innoculated plants were placed into a mist chamber in the greenhouse, which provided a water-saturated atmosphere. Temperature in the mist chamber normally varied from 65° -75° F, except where indicated. Innoculated sets of plants were held in the mist chamber for 48 hours, and were then divided and transferred into two Sherer-Gillette Controlled Environment chambers,

one set at 22° C, and the other at 30° C. The chambers varied by ± 1° - 2° in temperature, and from 35% - 85% in relative humidity. The chambers were set for a photoperiod of 16 hours on a 24 hour cycle.

Notes were taken daily beginning with the first day of transfer and continued for about two weeks. Reaction type was scored on a scale of 0 for no reaction, F for fleck reaction, 1 for first leaves yellowing, 2 for second leaves yellowing and first leaves browning, and 3 for second leaves almost brown, first leaves completely brown. Controls of uninoculated plants were always present.

Scoring of the temperature-sensitive mutants on the host plant.--- Factors such as variations in moist chamber temperature, watering frequency, height of the plants, density of the plants, fertilizer concentration, and variation within the environmental chambers made raw data comparisons between sets innoculated on different dates difficult. An effort to control such factors was made by comparing the experimental group scores to wt and control scores obtained on the same date. The raw data was then reduced to a simplified numerical rating system to make inter-group comparisons more meaningful.

A score of 3 by the wt on a particular day was taken as the norm for the + reaction, by which all other mutants were scored, according to their deviation from this norm. Plants which reached a reaction of 3 at the same time or before the wt were scored "+", or "+(the number of days the score of 3 preceeded the wt)", respectively. Plants which scored 0 at the beginning or which never reached a 3 score ("-?")

were considered as reacting much like an uninoculated control. A flecking reaction which later turned to 0 ("F/0") was considered one of the most diagnostic of scores, for control plants never show it, and it is characteristic of a ts mutant. Finally, plants which reached a reaction of 3 later than wt were scored "-(the number of days the score of 3 succeeded the wt)".

Inducement of the perfect stage.---Several methods were used in an attempt to obtain easily manageable pseudothecia. In the first, sterile, senescent corn leaves were placed on water agar, a block of mycelium was transferred to the center of the leaf, and incubated for three weeks at 21° C in darkness. This method gives the best growth, and the most consistent looking pseudothecia, but since these structures lie buried in the leaf, their clean removal is difficult.

In the second, corn extract agar was used in place of water agar and corn leaves. This method was poor due to the tendency of the mycelium to grow through, rather than on top of the agar, a phenomenon not observed with minimal or complete media.

In the third, and perhaps best method, corn extract agar was covered with a piece of cellulose. Mycelial growth usually remained on top, so pycnidia and pseudothecia were easily and cleanly peeled off the cellulose mat. Growth of mycelium and numbers of fruiting structures were reduced compared to either of the two previous methods, however.

With all three methods, plates were held at a constant 21 ± 1° C in the dark.

Complementation analysis.---Six of the best auxotrophs were gorwn together in all possible pairwise combinations in liquid media on a shaker for one week. The mycelial spheres were blended in a sterile blender cup and centrifuged. The mycelial pellet was resuspended in distilled water, centrifuged again, and the washed pellet divided and spread out on three minimal media plates. The plates were inspected after a week's time to determine if new mycelial growth was observed, and if so, a small piece was transferred to a minimal plate. In those plates where mycelial growth appeared limited to the edge of the innoculum, a 1 cm square block of agar was transferred to minimal media. Control plates were made of all six auxotrophs grown in liquid media on the shaker alone and treated similarly to the pairwise mixtures.

Complementation was scored as positive if two auxotrophs were able to grow together on minimal, either syntropically, or by heterokaryon formation. Lack of complementation was indicated by failure to grow even from an agar block transfer.

To determine whether or not heterokaryons were formed in the complementing groups, colonies grown on minimal plates were allowed to grow for several days until spores were produced. Conidia were harvested and plated on minimal media at about 50 spores per plate. Sixteen hyphal tip isolations were made of each complemting group as well, and these were plated on minimal media.

Crosses.---Since only limited mycelial growth of auxotrophs occurred on the corn leaves, crosses of complementing auxotrophs were made on sterile corn leaves. A small chunk of minimal media from the

growing edge of two complementing auxotrophs was placed face down on a sterile corn leaf, the corn extract agar, and the corn extract agar covered with cellulose.

Cytological studies.---Since no report exists in the literature of the number of nuclei per conidial spore, an attempt was made to stain the nuclei with Feulgen reagent. Glass slides thinly smeared with a small amount of albumen were pressed onto the surfaces of mycelial mats of two-week-old P. maydis cultures. The slides were lifted, the albumen allowed to dry, and the adhering spores were then fixed for 30 minutes in Carnoy's solution. The slides were then transferred to 1 N HCl for another 30 minutes, after which they were immersed in freshly prepared leuco basic fuchsin for 30 minutes. As a final step, the slides were rinsed briefly in 45% acetic acid, blotted dry, and covered with Permount.

RESULTS

U.V. Mutagenesis.---Ultraviolet light proved to be very effective in producing lethal mutants and presumptive auxotrophs. Of the approximately 7,000 spores treated in four experiments, 56 presumptive auxotrophs (0.8%) were isolated. Only one of these remained stable, unleaky, and characterizable. (See Table 1).

A plot of survival on both minimal and complete media indicated the optimum treatment range for production of auxotrophic mutants, since the survivors on complete media would be comprised of both auxotrophs and prototrophs, whereas survivors on minimal media would be prototrophic only. The curves (Figure 5) diverge the most at the 50-80% survival levels, indicating an optimum treatment time of 4-6 minutes of U.V. exposure.

The survival curves unexpectedly demonstrated multiple hit kinetics, suggesting that more than one nucleus is present in each conidia.

N.G. Mutagenesis.---Nitrosoguanidine proved to be an effective mutagenic agent, being more efficacious than U.V. light in producing mutants. Of some 2,160 isolates removed by the random isolation method, 76 were presumptive auxotrophs and 92 were presumptive ts mutations; thus mutation rates were 3.5% and 4.2%, respectively. (See Table 1). Nine stable, Nine stable, unleaky, characterizable auxotrophs, and 30 stable ts mutations were obtained.

Table 1.---Numbers of mutations obtained using U.V. and NG mutagenic treatments.

Treated With	Experiment Number	Number of Spores Tested	Number of Presumptive Auxotrophic Mutations	Number of Presumptive Agar Media TS Mutations	Number of Usable Auxotrophs	Number of Usable TS Mutants
UV	1	1,000	10	--	0	--
UV	2	2,000	13	--	0	--
UV	3	2,000	16	--	0	--
UV	4	2,000	17	--	1	--
		7,000	56	--	1	--
NG	5	80	0	0	0	0
NG	6	160	7	12	2	6
NG	7	160	12	11	0	5
NG	8	320	9	20	0	4
NG	9	160	6	2	3	2
NG	10	160	3	12	2	3
NG	11	160	4	12	1	4
NG	12	160	5	1	0	0
NG	13	0	0	0	0	0
NG	14	160	3	6	0	3
NG	15	160	8	4	1	1
NG	16	160	12	3	1	0
NG	17	160	0	0	0	0
NG	18	160	4	4	0	2
		2,160	76	92	10	30

Figure 5.--- Semi-log survival curves of P. maydis conidia on both minimal and complete media following U.V. light treatments, expressed as percentage survival plotted against the duration of U.V. exposure.

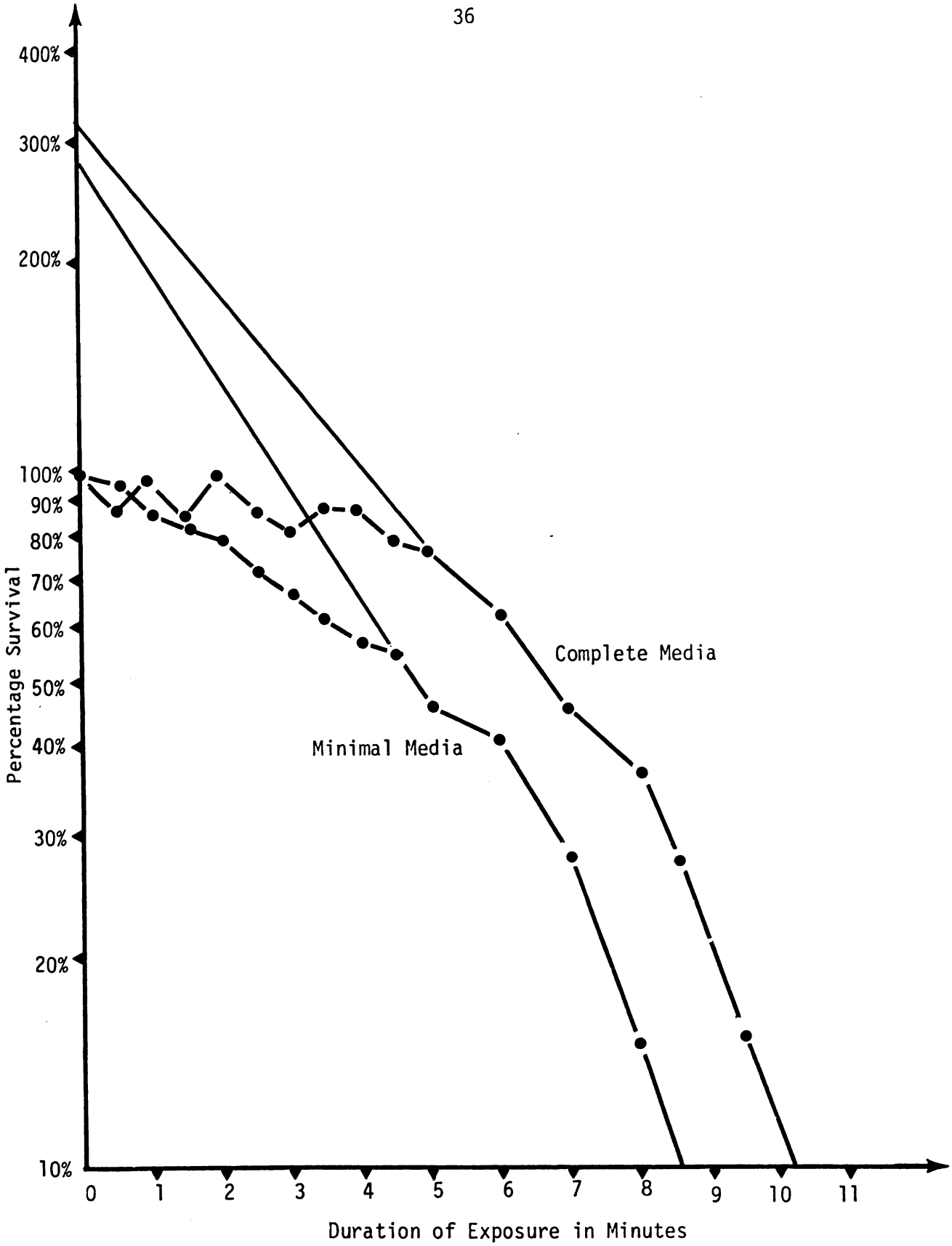
400%
300%
200%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
Percentage Survival
Complete Media
Minimal Media
0
1
2
3
4
5
6
7
8
9
10
11
Duration of Exposure in Minutes

Figure 5

Characterization of auxotrophic mutants, complementation and crossing data.--- In Table 2 are listed ten auxotrophic and five morphological mutants obtained, their nutritional requirements, and descriptions. Four arinine, two glutamic acid, one cystine, one lysine, one leucine, and one isoleucine requiring mutants were obtained. Although they were expected, no vitamin or nucleic acid requiring mutants were found.

Six of the auxotrophic mutants were tested for complementation, and these results are summarized in Figure 6. Four complementation groups were obtained, the first containing NG-39, a glutamic acid requirer (glu^-), the second containing NG-38, an arginine requirer (arg^-), the third containing NG-30 and NG-35, lysine (lys^-) and leucine (leu^-) requirers, respectively, and the fourth containing NG-36 and UV-53, both arg^-. The complementing groups were scored unambiguously except where indicated. Individual auxotrophic controls scored completely negative.

No crosses have been obtainable after repeated attempts to find a single pseudothecia amongst all possible combinations of mutants; the wt under the same conditions produced few pseudothecia. A single ascospore was isolated from one of these few pseudothecia, and all attempts to pass this isolate through the perfect stage have failed. This indicates a restriction or limitation on homothallism for this fungus which is not apparent until consecutive passes of single ascospore isolates through the perfect stage are made.

Table 2.---The ten auxotrophic and five morphological mutants obtained, their nutritional requirements, and descriptions.

a. Auxotrophic mutants

Name	Requirement	Description
UV-53	Arginine	wt in appearance
NG-3	Cystine	wt in appearance
NG-30	Lysine	Darker than wt, dense growth
NG-35	Leucine	Lighter brown than wt.
NG-36	Arginine	wt in appearance
NG-38	Arginine	Frequent sectoring
NG-39	Glutamic acid	Fuzzy surface
NG-72	Glutamic acid	wt in appearance
TS-9	Isoleucine	wt in appearance
TS-74	Arginine	wt in appearance

b. Morphological mutants

Name	Description
Sandy	Very sick, flat appearance. White border, with overall tan color. Spores misshapen, guttules irregular or lacking. Also known as TS-10.
Cotton	Surface of mycelia when fresh grows a fluffy white layer which begins to disintegrate to a moldy appearance when old.
Rust	Orange in color when fresh, ages to a rich dull orange.
Yellow	A bright yellow color.
Band	Produces a very distinct banding pattern. Also known as TS-22.

Figure 6.---Complementation of six auxotrophic mutants on minimal minimal media.

	UV-53	NG-30	NG-35	NG-36	NG-38	NG-39
UV-53	-	+	+	-	+	+
NG-30		-	-	+-	+-	+
NG-35			-	+-	+	+
NG-36				-	+	+
NG-38					-	+
NG-39						-

The four complementation groups determined by the above:

NG-39, glu^-	NG-38, arg^-	NG-30, lys^-	NG-36, arg^-
		NG-35, leu^-	UV-53, arg^-
1	2	3	4

"+" = growth, "-" = no growth, "+-" = sparse growth, but more than each of the two cultures growing seperately

Attempts to force syntropic growth of two auxotrophs on minimal media have been successful, but repeated attempts to demonstrate heterokaryosis have failed.

Cytological studies.---Slides prepared as described in Materials and Methods revealed four to six intensely stained objects per conidia. Since the Feulgen reaction is specific for DNA, this observation was taken as evidence that the objects were in fact nuclei. Photographs of two of the slides are presented in Figure 7.

Characterization of temperature-sensitive mutants.---Ninety-two temperature-sensitive mutants were selected initially on the basis of their indicating a temperature sensitivity on complete media. Of these, a total of 26 remained stable. Tests were repeated at least three and in some cases four times; this data and a summary is given in Table 3. None of the mutants were lethal after one week at the restrictive (high) temperature.

Of the 26 stable mutants which are ts on agar, 9 also proved to be temperature-sensitive on the host plant. Four isolates of the 92 originally selected as agar ts mutants subsequently lost their temperature sensitivity on the agar media, but tested temperature-sensitive on the host plants. A summary of the 30 ts mutants is given in Table 4. Only mutants tested for pathogenicity at least twice are included in the results.

Original data on all 13 ts mutants are included in Table 5. Also included are the original data on several controls (fertilized,

Figure 7.---Photographs of feulgen stained spores of P. maydis, 2,000 X magnification. The intensely stained bodies which appear in these conidia are taken to be nuclei; in these pictures, there are four nuclei per spore.

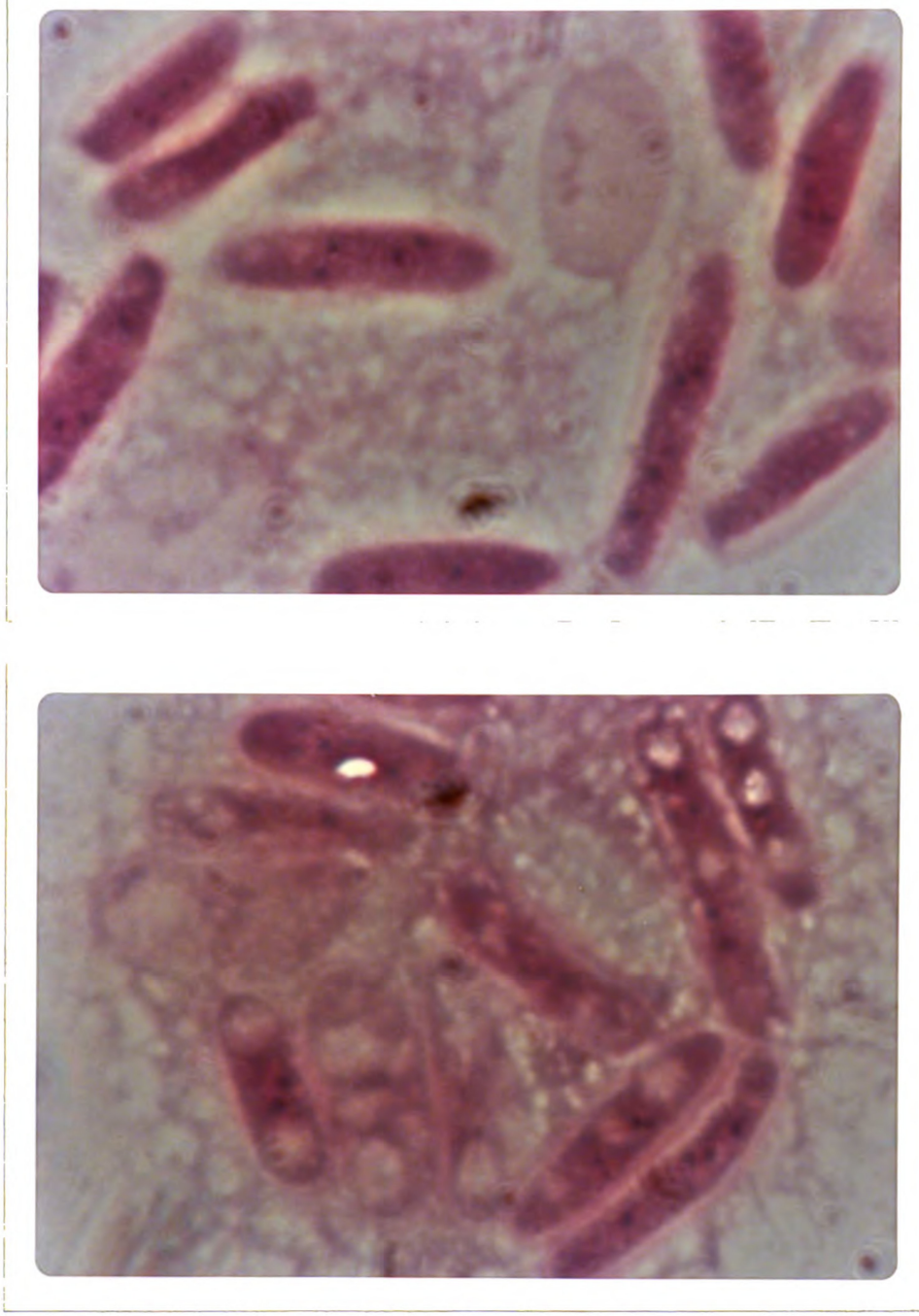

Figure 7

Table 3.--Growth of presumptive temperature-sensitive mutants at 30° C on complete media. "+" = growth, "-" = no growth.

Table 3

Name	5-1	5-6	6-1	6-1	Summary	Name	5-1	5-6	6-1	6-1	Summary
TS-1	+		+	+	+	50	+		+	+	+
2	+		+	+	+	51	+-		+	+	+
3	-		-	+	-	52	+		+	+	+
4	+		+	+	+	53	-		-	-	-
5	+		+	+	+	54	-		-	+	-
6	-		-	+-	-	55	+-		+	+	+
7	+		+	+	+	56	+		+	+	+
8	-		-	+	-	57	+		+	+	+
9	-		-	+	-	58	+		+	+	+
10	-		-	-	-	59	+		+	+	+
11	+		+	+	+	60	+		+	+	+
12	+		+	+	+	61	-		-	+	-
13	+		+	+	+	62	+-		+	+	+
14	+		+	+	+	63	+-		+	+	+
15	+		+	+	+	64	+-		+	+	+
16	+		+	+	+	65	-		+	+	+
17	+		+	+	+	66	-		-	+	-
18	-	+	+	+	+	67	-		-	+	-
19	-	-	-	-	-	68	+-		+	+	+
20	-	-	-	-	-	69	+-		+	+	+
21	-	+-	-	+-	-	70	+-		+	+	+
22	+-	+	-	+-	-	71	-		-	+	-
23	+-	+	+	+	+	72	+-		+	+	+
24	+		+	+	+	73	+		+	+	+
25	+		+	+	+	74	-		-	+	-
26	+		+	+	+	75	+		+	+	+
27	+		+	+	+	76			-	-	-
28	+		+	+	+	77			-	+	-
29	+		+	+	+	78			-	-	-
30	+		+	+	+	79			+	+	+
31	+		+	+	+	80			+	+	+
32	+		+	+	+	81			+	+	+
33	-		-	+	-	82			-	-	-
34	+		+	+	+	83			+	+	+
35	+		+	+	+	84			+	+	+
36	+		+	+	+	85			+	+	+
37	+		+	+	+	86			+	+	+
38	+		+	+	+	87			+	+	+
39	+		+	+	+	88			+	+	+
40	-	+	-	+	-	89			+	+	+
41	+		+	+	+	90			-	-	-
42	+		+	+	+	91			-	-	-
43	-		-	-	-	92			+	+	+
44	+		+	+	+						
45	+		+	+	+						
46	+		+	+	+						
47	+		+	+	+						
48	+		+	+	+						
49	-		-	-	-						

Table 4.---Summary list of temperature-sensitive mutants.

On agar	On the host	On both	
TS-3			
	TS-4		
6	6	TS-6	
8*			
9*			
10*			
	17		
19			
20*			
21			
22*			
33	33	33	
	34		
40	40	40	
43	43	43	
49	49	49	
	50		
53*			
54			
61	61	61	
66	66	66	
67*			
71	71	71	
74*			
76	76	76	
77			
78			
82			
90			
91			
26	13	9	Totals

*Indicates discovery of auxotrophy, after initially scoring prototrophic. Except for TS-9 and TS-74, however, the nutritional requirements of these ts mutants could not be determined.

Table 5.---Selected examples of raw data scores of control plants, wild type innoculated, and temperature-sensitive mutant innoculated plants. "0" = no fleck observed; "F" = flecking observed; "1" = first leaves turning yellow; "2" = second leaves yellowing, first leaves brown; "3" = third leaves yellowing, first and second leaves brown.

Table 5

Innoculated with	Date	Daily scores 48 hrs. after innoculation 22° 1 5 10 15	30° 1 5 10 15
Control--No fertilizer	3-6	001122222222222	00011223
	3-15	001111222222222	0111111223
Control-- fertilizer	3-6	000000001111111	0000011111
	3-15	000001122222222	01111112223
Acetone	5-13	000001111222222	0111223
Tween-80	5-13	000000011112222	0122223
	5-13	000000011112222	0122223
Wild type	3-6	122222223	12223
	3-15	FFF111223	1122223
	4-8	F1112222223	F112223
	4-29	11223	123
	5-13	112223	F1223
	5-22	1222223	122223
	5-26	FF112222223	F11122223
	6-4	FF11112223	FF1112223
TS-4	3-6	12222223	1111112223
	5-22	11222222222222	123
	5-26	FFF111222222222	FFF122223
TS-6	3-6	1112222223	122223
	4-8	FFFFFFF1111223	FF11223
	5-26	FFF111222222222	FF1223
	6-4	F001111111111	FF111123
TS-17	4-8	FFFF11122222223	F111223
	5-22	111222222222	112223
TS-33	3-15	FFFFFF0	F1122223
	4-8	FFFFFFFF112223	F1123
	5-22	122222223	13
TS-34	3-15	FF111123	12222223
	5-22	12223	1222223

Table 5.---(Cont.)

Innoculated with	Date	Daily scores 48 hrs. after innoculation 22° 1 5 10 15	30° 1 5 10 15
TS-40	3-15	FFFFF0	F112223
	4-8	FFFFFFF112223	1111223
	5-26	0000001122223	000011223
	6-4	01111222	000123
TS-43	3-15	FFFFF00	FF1111223
	4-8	FF00111122223	FF11223
	5-22	0111111222222	122223
TS-49	4-8	F000000000011112	FF11223
	4-29	F11122222222223	F11122223
	6-4	F1222223	FFF22223
TS-50	4-29	11222223	123
	6-4	F1222223	F1223
TS-61	4-29	F111111111111223	FF11223
	5-13	1111111223	F112223
	5-26	FFFF000000000000	FFF1123
	6-4	F1112222223	F11223
TS-66	5-13	1111111123	0112223
	5-26	FFFF011112222222	FFF122223
TS-71	5-13	1111111122223	F12223
	5-26	FF11222222223	F12222223
TS-76	5-26	FFFFF11111222	FFF1222222223
	6-4	0F112222223	0F12223

non-fertilized, acetone and Tween-80 sprayed, and untreated plants) as well as the scores of wt on W64A, W117N, W117T, 5002XN, and 5002XT.

Data assembled in this manner from different scoring dates were much less meaningful than data compared to wt and control scores on a particular date and using a particular batch of plants. For example, on 4-29 it took the wt only 3 days to achieve a rating of 3 at 30° C on W64A, yet it took the same wt isolate 9 days to achieve the same rating on 3-6 and 5-26. By the same token, it was noted that an uninoculated control plant scores a 3 at high temperature within 7 days. A numerical rating system was therefore devised to evaluate any given isolate's reaction on any date in comparison to any other isolate. The ratings of all 76 tested isolates, analyzed as explained in Materials and Methods, are presented in Table 6.

The temperature in the incubation chamber on two occasions rose during the day to well above 30° C; these are the 5-26 and 6-4 study dates. The 22° column of Table 6 of both these dates represents a temperature downshift, for the exposure to 30° began the day of innoculation, and was not ended until two days later, upon transfer to the incubation chamber. The 30° column then, represents a relatively steady temperature state. This situation is the exact reverse of the six previous sets of data in the table, where the 30° columns represent temperature downshift experiments, while the 22° columns represent steadily held temperatures of 22°.

Mutations to increased virulence.---Four mutant isolates were discovered which produced disease symptoms much faster than wt: TS-23, TS-26, TS-27, and TS-75 (Table 6).

Table 6.---Rated and summarized scores of all *ts* mutants. "NSD" = not sufficient data; "+" = scores equivalent to the *wt*; "-" = no growth; "0" = no flecking observed; "F" = flecking observed; "F/0" = flecking observed which later disappeared; "0/F" = 0 score followed by flecking; "-?" = plant never achieved a score of 3 during the experiment; "+1", "+2", etc., = number of days a score of 3 was achieved in advance of the *wt*; "-1", "-2", etc., = number of days a score of 3 was achieved after the *wt*. "++" = rating of 3 achieved much faster than *wt*; mutants so marked are considered to have mutations to increased virulence.

Table 6

On agar Summary		Name	3-6		3-15		4-8		4-29		5-13		5-22		5-26*		6-4*		On host Summary	
22°	30°		22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°
		Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
+	+	WT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	TS- 1	-?	0															NSD	
+	+	2	+6	+3			+	+											+	+
+	-	3	+5	0			0	0											-	-
+	+	4	+1	-1									-?	+4	-?	+			-	+
+	+	5	+4	+1															NSD	
+	-	6	-?	+3			-?	+							-?	+3	F/0	+	-	+
+	+	7	-?	-?															NSD	
+	-	8	-?	+			-2	+									0/F	0	+	+
+	-	9	+1	+1			0	0											+	+
+	-	10	+6	+			-3	+									0/F	0	+	+
+	+	11	+3	-?															NSD	
+	+	12	+6	+6															+	+
+	+	13											-3	+5					NSD	
+	+	14											+5	+4					+	+
+	+	15											+	+5					+	+
+	+	16											-1	+					+	+
+	+	17					-3	+					-?	+					-	+
+	-	18					-1	+					-3	+4	0	0	+3	+	-	+
+	-	19					-2	+									0	0	+	+
+	-	20													0	0	0	0	NSD	
+	-	21													0	0			NSD	
+	-	22											-?	0			0	0	-	-
+	+	23					+6	+3					+5	+5					++	++
+	+	24			-?	+													NSD	

*The 30° temperature was held on these two dates from the time of innoculation. On all other dates, the 30° column represents an upshift in temperature from 22°. By the same token, the 22° column on these two dates represents a downshift in temperature from 30°, while on all other dates the 22° temperature held relatively steady from the time of innoculation. All temperature shifts occurred 48 hours after innoculation.

Table 6.---(Cont.)

On agar Summary		Name	3-6	3-15		4-8		4-29		5-13		5-22		5-26*		6-4*		On host Summary	
22°	30°			22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°
+	+	TS-25		-?	-1							+	+3					+	+
+	+	26		+	+							+5	+5					++	++
+	+	27		+	+							+5	+5					++	++
+	+	28		+	+							+	+2					+	+
+	+	29		+	+							-4	+5					+	+
+	+	30		+	+1							+3	+					+	+
+	+	31		+2	-1	-2	+					+5	+4					+	+
+	+	32		+	+	-1	F/0					-6	+5					?	?
+	-	33		F/0	-1	-2	+1					-2	+5					-	+
+	+	34		+	-1							+2	-1					+	-
+	+	35		+	+													+	+
+	+	36		+	-1													NSD	
+	+	37		-?	-1													NSD	
+	+	38		-?	-1													NSD	
+	+	39		+	-1													NSD	
+	-	40		F/0	+	-1	+							0	0	0	0	-	+
+	+	41		+2	-1													NSD	
+	+	42		F/0	-?													NSD	
+	-	43		F/0	-1	-1	+					0	+					-	+
+	+	44						+	+									+	+
+	+	45						+	+									+	+
+	+	46						+1	+									+	+
+	+	47						-3	+									NSD	
+	+	48						+2	+									+	+
+	+	49				F/0	+	-10	-6							+	+	-	+
+	+	50						-3	+							+1	+3	-	+
+	+	51				+6	+3	-	-3									+	+
+	+	52						+	-1									NSD	
+	-	53						-7	-3							+3	+2	+	+
+	-	54				+	+	-3	+							0	0	+	+
+	+	55				+7	+3	+1	+									+	+

Table 6.---(Cont.)

On agar Summary		Name	4-8		4-29		5-13		5-22		5-26*		6-4*		On host Summary	
22°	30°		22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°	22°	30°
+	+	TS-56			-6	+									NSD	
+	+	57													NSD	
+	+	58			-5	-3									NSD	
+	+	59			+1	+									+	+
+	+	60													NSD	
+	-	61			-10	-4	-4	-2			F/0	+2	-4	+2	-	+
+	+	62			-3	-5	-4	-2							-	-
+	+	63			-3	-5	-4	-3							-	-
+	+	64			-3	-5	-7	-2							-	-
+	+	65					0	0			0	0			-	-
+	-	66					-4	-1			F/0	+			-	+
+	-	67									0	0			NSD	
+	+	68					-1	-2			+2	+3			+	+
+	+	69					0	0			+2	+5			+	+
+	+	70					-6	-1			F/0	-2			-	-
+	-	71					-6	-1			-1	+			-	+
+	+	72					0	0			-2	-2			-	-
+	+	73					0	0			+2	+4			+	+
+	-	74									-3	+			NSD	
+	+	75					-1	+			+6	+7			++	++
+	-	76					-1	+			-?	-4	0/F	0/F	-	-

DISCUSSION

Even though care was taken with the UV treatments to avoid photoreactivation, the low yield of stable auxotrophs and even presumptive auxotrophs (less than 1%) suggested that either repair processes were highly operant [photoreactivation or photo-preactivation (10)], or the selection procedure was missing many of the mutants. The probability that more than one nucleus per conidia was present---indicated by the multiple hit kinetics shown in the survival curves, and supported by the Fuelgen staining of 4-6 objects per conidia---suggested a change in procedure which would help isolate individual nuclei. The new procedure, single hyphal tip isolation, while certainly more tedious than the layer plate selection method, has the presumed advantage of isolating the mitotic descendents of a single mutated nucleus, regardless of how many were in the conidial spore to begin with. And indeed, a mutation rate of about 4% is normally reported in the literature (30). NG was used as a mutagen with this procedure, however, and how much of the increased mutant production is attributable to the selection method, and how much to the NG, is unknown.

The evidence for 4-6 nuclei per conidial spore, while not definitive (it would be if a different DNA stain showed the same 4-6 objects), is at least strongly indicative. Both survival curves (replicated six times in the case of the complete media curve) show multiple hit kinetics; a selection procedure which retains all nuclei

in a single clump is very poor in providing mutations; and a selective DNA stain reacts with 4-6 objects per conidium. If these indications are confirmed, then P. maydis can generate a great deal of variation through segregation of dissimilar nuclei in the germination of its asexual spores.

This study was undertaken with the purpose of obtaining linkage data on auxotrophic mutants. The difficulties encountered in obtaining crosses of these mutants could be the result of other mutations in developmental pathways involved in forming the perfect stage; these might well be expected since it is considered that most of the Imperfect Fungi have evolved from the Ascomycetes by loss of sexual reproductive function. Another distinct possibility is that P. maydis is not entirely homothallic, as has been indicated by the inability to obtain fertile asci through three generations in the wild type. All work with P. maydis reported here was performed on cultures derived from a single ascospore isolate; that isolate---this same one being used in all mutagenic treatments---upon being passed through a second perfect stage in the wild type produced very few pseudothecia. A single ascospore was isolated from one of these few, however, and all attempts to pass this isolate through the perfect stage a third time have failed. This indicates a restriction or limitation on homothallism for this fungus.

Because the mutants had difficulty growing on the corn leaves alone, the positively complementing auxotrophs were expected to overcome their nutritional difficulties together, which they did. Since the basic

genetic operation that defines a gene involves determining whether or not two mutations complement one another in phenotype (13), the complementation data should be able to at least determine whether or not the ten auxotrophic mutants are of the same or different genes. NG-36 and UV-53, both arginine requirers, are probably mutations of the same gene. NG-35, a leucine requiring, and NG-30, a lysine requiring mutant, are probably not mutants of the same gene, even though they do not complement. It is a widely observed and largely unexplained phenomenon in the fungi that distant or unlinked markers will frequently not complement one another (12).

The main purpose of the attempt to demonstrate complementation was to determine whether or not P. maydis could use heterokaryosis as a source of variability. Of course, proof that heterokaryosis is possible does not mean that it naturally could or does occur---an important consideration in terms of pathogenic capability. If genes could be exchanged between races of P. maydis through heterokaryosis without involving the perfect stage, then those populations which lose sexual competence would not be reproductively isolated. This would favor and help explain the loss of the perfect stage, as well as the tendency to homothallism, as one distinct advantage of the ascigerous stage is its ability to build up large numbers of spores overwinter in corn debris, and then to forcibly discharge this innoculum onto newly planted corn. On the other hand, it is not yet certain that various isolates used and reported as P. maydis are indeed one and the same species, since it is unknown whether or not these isolates as populations can naturally exchange genes (11).

The temperature-sensitive mutants provided the greatest support for the idea that an avirulent gene has some function not easily dispensed with by the pathogen. The three basic types of temperature-sensitive mutations expected---those temperature-sensitive on both agar and the host, those temperature-sensitive on agar only, and those temperature-sensitive on the host only---are designated by the roman numerals I, II, and III, respectively, and are broken down by subscripts to accomodate the possible combinations to be found in Table 7. By far the greatest numbers of ts mutants fell into two categories, both of which are temperature-sensitive on the agar: Ib and II. This is not surprising, as the original screening was for mutants ts on agar, but what is surprising is the total lack of the expected type Ia. These were a priori expected simply because a mutant with a restrictive temperature on agar of 30° might experience the same functional difficulties at the same temperature on the host. Perhaps because all ts auxotrophs were discarded, most metabolic mutants were eliminated. However, mutants defective in mitotic nuclear division, easily obtained of A. nidulans at a frequency of 5% of the total number of ts mutants (30), would be expected to follow the Ia pattern. One mutant would be expected, if the 5% rate holds for P. maydis.

Type Ib, the most frequent class encountered, is most easily explained if one assumes that the temperature-sensitive mutations in these cases are of genes which have essential function to the pathogen, at least as saprobes. They are not related to nutritional deficiencies, since ts auxotrophs were screened for and discarded. (Or noted in Table 7

Table 7.---*A priori* scoring expectations of temperature-sensitive mutants; list of mutants fitting the expectations.

Type[a]	Summary Scores[b] Agar 22°	Agar 30°	Host 22°	Host 30°	Ratings Expected[c] Steady 22°	Steady 30°	Upshift 22°---30°	Downshift 30°---22°	Mutants Obtained
Ia	+	-	+	-	+	0	F/0 or -?	0 or 0/F	None
Ib	+	-	-	+	F/0 or -?	+ or 0	+ or ++	0 or F/0	TS-6,18,33,40,43, 49,53,61,66,71,76.
II	+	-	+	+	+	0	+	0 or 0/F	TS-8,10,19,20,21, 22,54.
IIIa	+	+	+	-	+	F/0 or -?	F/0 or -?	+	TS-34
IIIb1	+	+	-	+	F/0 or -?	+ or ++	+ or ++	F/0 or -?	TS-4,17
IIIb2	+	+	-	+	+	++	++	+	TS-50

[a]The three basic types of temperature-sensitive mutations expected---those temperature-sensitive on both agar and the host, those temperature-sensitive on agar only, and those temperature-sensitive on the host only---are designated by the roman numerals I, II, and III respectively, and are broken down by subscripts to accomodate the possible combinations to be found.

[b]"+" = growth; "-" = no growth.

[c]"+" = growth equivalent to wild type; "0" = Neither growth nor flecking;"F/0"= flecking which disappeared;"0/F" = 0 score followed by flecking; "-?" = plant which never achieved a score of 3 during the experiment.

as having excaped the original screening. Significantly, though, none of these were found to be temperature-sensitive on the host.) When applied to the host, the spores may or may not germinate at 30°, depending on whether or not spore germination per se is affected by the mutation; in any case, they will continue to infect, once started, a plant held at high temperature, and will cease to infect, or slow greatly, at low temperature. This type of reaction is similar to the P6/Sr6 system in wheat stem rust, as well as to a type III reaction, except that in this latter type, the spores are not temperature-sensitive on the agar.

A type III reaction may be regarded as involving some active interaction of parasitism, but not directly related to basic life processes, as in type I. A type IIIa, which shows inactivation at the high temperature, may be regarded as a mutation affecting the basic ability to parasitize. If the nonpermissive temperature is assumed to be the high temperature (an assumption warranted on the basis of general experience with ts mutations in this and other organisms), then some gene or gene product which confers ability to promote primary infection on corn is non-functional at the high temperature. By the same token, a type IIIb reaction, non-functional at the high temperature, indicates the dysfunction of the specific interaction necessary for incompatibility, i.e., the inability to parasitize. Type IIIb thus follows the gene-for-gene interaction. This type closely resembles type Ib, except in this case, the parasite gene does not appear to function in support of intrinsic viability of

the pathogen.

The type IIIb reaction is divided into two subgroups, based upon the similarity or lack of it to the wild type reaction at low temperature. One troubling factor in both the Ib and IIIb1 reactions is the difficulty in interpreting their low-temperature reactions, as compared to wild type. If the temperature-sensitivity is truly at the high temperature, why does the low-temperature reaction differ from the wild type? Far more satisfactory (convenient?) is the type of score represented by IIIb2, where low temperature reaction is unaffected by the mutation(s), and high-temperature reaction shows increased compatibility, due to inactivation of the specific interaction necessary for incompatibility---presumably, an avirulence gene(s). With types IIIb1 and Ib, multiple mutations which in general slow down the metabolic machinery of the fungus could account for the generally increased incompatibility these classes show compared to wt at low temperature, but separation of these effects from those which just affect gene-for-gene interactions would be more satisfactory.

The mutant types were fairly evenly distributed. The seven type II mutations, which are temperature sensitive on agar, and still are able to parasitize well, but which show no temperature-sensitivity on the host, should facilitate study of materials transferred from host to parasite. To be sure, four of these have turned out to be auxotrophic, but whatever they require is not provided by any of the nutrients used to supplement the minimal media in the screenings, and remain unknown. The other three, however, do not score as auxotrophic. It is possible

that these are the missing Ia mutant types---after all, there is only one IIIa type, and it grows so poorly that it was almost missed---it may be difficult to score a mutant as temperature-sensitive at the high temperature on corn due to the very rapid onset of a 3 score. (Compare how fast a 3 is achieved on uninoculated plants at 30°).

Consider the single example of type IIIa, TS-34. Since it is not temperature-sensitive on agar, reaction at 30° proceeded without difficulty through germination (Refer to Table 6). In comparison to control data, the high temperature score of TS-34, even though achieving a score of 3 rapidly, did so less rapidly than the wt., and only somewhat more rapidly than the uninoculated control. By comparison, the rating at 22° followed that of the wt closely, with attainment of scores of 2 and 3 simultaneously. Further work, using a slightly lower high-temperature setting (say 28°) may help clarify certain of these reactions.

The four mutants to increased virulence are probably a result of the loss of function of an avirulence gene(s) which is not harmful to the pathogen. These mutants may be useful in isolating individual resistance factors in host plants, in the absence of natural isolates able to do so. Mutations have been used with effect to obtain dominant resistance factors in the host (37)., and mutations to virulence in the pathogen such as these should be helpful in identifying and isolating newly discovered, and newly created, resistance factors. They should also help avoid the accidental loss, through inbreeding of naturally variable host plants, resistance factors

which would otherwise go undetected---overshadowed by other factors.

The ease with which the mutations to increased virulence were obtained supports the hypothesis that a gain in virulence represents a loss of function, and that specificity of gene-for-gene interactions is for incompatibility, not compatibility. If specificity were for compatibility, mutations to increased virulence should have been difficult to obtain. In addition, the ease with which ts mutations to increased virulence at the restrictive temperature were obtained supports the same hypothesis: the loss in function occurs at the restrictive temperature and translates into a gain in virulence. If specificity were for compatibility, then ts mutations to increased virulence at the restrictive temperature should have been difficult to obtain.

SUMMARY

Preliminary work on P. maydis to determine its suitability for more extensive genetic analyses has been done. Both auxotrophic and temperature-sensitive mutations were obtained with facility when care was taken to overcome the apparently multi-nucleated condition of the conidia. Reports of homothallism must be amended in light of indications that the homothallism, while primary, was restricted. These restrictions made classical genetic analysis difficult, and prevented use of a single ascospore background for analysis in the present study. Parasexual analysis may be more profitable.

Temperature-sensitive mutations supported previous indications that P. maydis follows a gene-for-gene pattern of interaction on N cytoplasm corn, where toxin production appeared to be useless. All three expected types of ts mutations were found: those temperature-sensitive on agar only, those temperature-sensitive on host plants only, and those temperature-sensitive on both.

Four mutants to increased virulence were found, and should be useful in screening for new or hidden resistance factors. The ease with which the mutations (temperature-sensitive and non-temperature-sensitive) to increased virulence were obtained supports the hypothesis that a gain in virulence represents a loss in function, and that specificity of gene-for-gene interactions is for incompatibility, not compatibility.

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