ENVIRONMENTAL EFFECTS AND TRANSFER EVENTS DURING PRIMARY INFECTION OF WHEAT BY ERYSIPHE GRAMINIS

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ABSTRACT

ENVIRONMENTAL EFFECTS AND TRANSFER EVENTS DURING PRIMARY INFECTION OF WHEAT BY ERYSIPHE GRAMINIS

by Mark Samuel Mount

The objective of this research was to define more precisely the various components of primary infection of wheat by E. graminis. On the basis of morphological changes and differential sensitivity to environmental conditions, the components of primary infection of powdery mildew were determined. They are: 1) germination, 2) formation of appressorial initials, 3) maturation of appressoria, 4) penetration, 5) haustorial development, 6) formation of secondary hyphal initials, and 7) formation of functional or elongating secondary hyphae.

The degree of a successful host-parasite relationship was correlated with the number of functional secondary hyphae and haustoria formed. The critical environmental condition necessary for synchronizing the rate of formation of functional secondary hyphae is that the inoculated plants be subjected to darkness from 6-20 hr after inoculation. It is during this dark period that penetration and haustorial development take place. Approximately 60% of the mature appressoria produced

penetration pegs by 10-12 hr after inoculation.

High light treatments given at various hours after inoculation clarified further by differential sensitivity that secondary hyphal initials and functional secondary hyphae were indeed separate stages of the primary infection process. Ultraviolet light was used as a tool in determining the time after inoculation that the haustorium is "functional" within the epidermis. Experiments showed that the epidermis could function as a protective barrier against UV radiation. The parasite began losing its sensitivity to UV radiation at the 14th hr after inoculation, indicating that the haustorium had developed sufficiently in the host to be protected by the host. If the haustorium had not developed sufficiently within the epidermal cell, UV radiation killed the fungus. Nuclear migration into the haustorium was first observed 16 hr after inoculation. Appendages formed at the ends of the haustorial bodies by 18 hr after inoculation.

The determination of isotope transfer from host to parasite further defined the initial infection process. An interpretation of the data suggested that possibly 2 mechanisms of ^{32}P and ^{35}S transfer (one, a low transfer from 12-16 hr, and the other, a high transfer from 16-24

hr after inoculation) exists with a susceptible host-parasite interaction. A high transfer of radioactivity was first detected at the 18th hr which corresponded with the formation of appendages on the haustoria.

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by

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TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	3
MATERIALS AND METHODS	16
Culturing of Powdery Mildew	16
Methods of Inoculation	17
Environmental Conditions for Experiments	18
Examination of Fungal Structures	20
Ultraviolet Experiments	21
Histological Studies	21
Parasite Removal with Parlodion	22
Uptake of Phosphorus-32	22
Detection of P-32 and S-35 in the Parasite	24
Statistical Analysis	27
RESULTS	28
Germination of Conidia and Formation of Appressorial Initials, Mature Appressoria, and Secondary Hyphal Initials	28
Effect of Light on the Development of Secondary Hyphal Initials	34
Effect of Temperature and Relative Humidity on the Formation of Secondary Hyphal Initials	42
Development of Functional Secondary Hyphae	45
Effect of High Light on the Formation of Functional Secondary Hyphae	54
Formation of Penetration Pegs	54
Effect of Ultraviolet Radiation on the Formation of Functional Secondary Hyphae	59
Haustorial Development and Nuclear Staining	72

Table of Contents (cont) Page

Uptake of P-32 by Wheat Seedlings 72
Transfer of P-32 and S-35 from Host to Parasite . . 86

DISCUSSION 94

SUMMARY 105

LITERATURE CITED 108

LIST OF TABLES

Table		Page
1.	The ^{32}P activity (CPM) per centimeter leaf section from uninoculated wheat grown in Hoaglands and KNO_3-Tes buffer solution.	83
2.	Comparison of ^{32}P uptake between uninoculated and inoculated wheat plants	84
3.	Uptake of ^{32}P (100 uc/ml) by wheat plants 4, 17, 20, and 24 hr after being placed in the radioactive solution	85
4.	Uptake of ^{32}P (100 uc/ml) by wheat plants that were cut at the crown and placed into the radioactive solution	87
5.	Activities (CPM) of parlodion strips containing mildew conidia	89
6.	CPM of parlodion strips containing mildew conidia	90

LIST OF FIGURES

Figure		Page
1.	Wheat leaves placed in 6 mm tubes containing water and 100 uc/ml of P-32	25
2.	Kinetics of the formation of 4 stages of the primary infection process	29
3.	Drawings illustrating (a) conidial body, (b) mature appressoria, (A:c) a secondary hyphal initial, and (B:d) functional secondary hypha .	32
4.	Effect of 2 hr low light treatments (240 fc) on the formation of secondary hyphal initials .	35
5.	Effect of either 2 or 4 hr medium light treatments (1400 fc) on the formation of secondary hyphal initials	38
6.	Effect of 1.5 hr high light treatments (2400 fc) on the formation of secondary hyphal initials	40
7.	Effect of 15 min high light treatments (2400 fc) on the formation of secondary hyphal initials	43
8.	Effect of 1/2 hr temperature treatments (31 C) on the formation of secondary hyphal initials .	46
9.	Effect of 1/2 hr treatments of high relative humidity (ca. 100%) on the formation of secondary hyphal initials	48
10.	Percent formation of functional secondary hyphae using various periods of darkness after inocuation	52

List of Figures (cont) Page

11. Effect of 15 min high light treatments (2400 fc) on the formation of functional secondary hyphae 55

12. Percent formation of penetrations pegs as determined from spores embedded in parlodion . 57

13. Pictures of fungal units showing penetration pegs at the ends of the mature appressoria . . 60

14. Effect of 5 sec UV radiation on the development of functional secondary hyphae 62

15. Effect of 30 sec UV radiation on the development of functional secondary hyphae 65

16. Effect of 60 sec UV radiation on the development of functional secondary hyphae 67

17. Effect of 60 sec UV treatments on development of mildew pustules on wheat 6 days after inoculation 70

18. Effect of 60 sec UV radiation 10 hr after inoculation on the formation of functional secondary hyphae with and without a protective epidermal strip over the inoculated wheat leaves 73

19. Development of the haustorium at various hr after inoculation 75

20. Counts per minute of 1 cm long leaf sections 20 hr after roots of wheat plants were placed in medium containing P-32 78

21. Uptake of P-32 (20 uc/ml) into wheat plants 1, 14, 24, and 48 hr after roots were placed in the radioactive medium 80

22. Transfer of P-32 and S-35 from leaf to parasite 92

23. Comparison of the kinetics of formation of mature appressoria, A, and functional secondary hyphae, C, with the kinetics of sensitivity to 60 sec UV treatments, B 99

INTRODUCTION

Powdery mildew of wheat is caused by Erysiphe graminis f. sp. tritici. This fungus is an obligate parasite which is most common and destructive in the wheat growing regions of the Pacific coast, Great Lakes, and Atlantic seaboard states (42).

Conidia are most abundant during the growing season and are the principle secondary inoculum. They germinate under favorable conditions and invade only the epidermal cells of the host. Conidia germinate and produce appressoria. Pegs produced on appressoria penetrate into the epidermal cells where haustoria are formed. Further development of the fungus is exterior to the epidermis (42, 43) except for haustoria in the epidermal cells.

The effects of environment on the development of the mildew fungus has been studied extensively (5, 13, 34, 43). Most of these reviews list results which are different, probably due to the differential requirements of the various mildews tested. Differential effects were

determined, in most cases, after the mildew had sporulated and were not concerned with the initial interaction between the host and parasite. Recent studies demonstrated, however, that the initial infection process on susceptible wheat could be divided into several components on the basis of the differential responses to various environmental treatments in the different stages of primary infection (21, 27, 29, 31).

The objective of this research was to define more precisely the various components of primary infection. More specifically, experiments were designed to: 1) determine the effects of various environmental treatments (light, temperature, and relative humidity) on the development of secondary hyphae; 2) determine optimal environmental conditions to obtain synchronized growth rates for the formation of functional secondary hyphae; 3) study the effects of UV exposure on the host-parasitic interaction and to determine the degree of synchrony in the development of haustoria; 4) examine the migration of the nucleus from the mature appressorium into the developing haustorium to gain insight as to when the haustorium is "actively" functional; and 5) determine the time after inoculation that a transfer of substances from the host to the parasite occurs.

LITERATURE REVIEW

According to Yarwood (43) over 3000 articles have been written on powdery mildews since 1900. Earlier studies on parasitism by the powdery mildews and rusts can be divided into 2 main categories - 1) the effects of environmental conditions on germination of fungus spores and overall development of disease, and 2) changes in metabolism in infected leaves. In most cases these studies did not attempt to investigate changes occurring during the initial host-parasite interaction.

The effects of temperature, light and relative humidity on spore germination has been examined extensively (3, 5, 11, 13, 29, 31, 32, 34, 43). Several investigators have found that germination can occur over a wide range of relative humidities (3, 43). The conidia of Erysiphe sp. can germinate from near 0-100% relative humidity. Conidia produced in moist chambers contain about 60-70% of their weight as water (38), and this probably accounts for their ability to germinate at low relative humidities. In studies

dealing with high efficiency of germination of E. graminis f. sp. tritici on wheat (31), it was found that an initial relative humidity of approximately 100% was more favorable than 65%. Germination of mildew spores of several species was inhibited at a saturated atmosphere (34), however a marked increase in the percentage of germination occurred when the relative humidity was between 95-99% (34). Variability in germination studies might be attributed to the differences and variation of the mildew species tested (34, 43).

Powdery mildew fungi will develop under a wide range of temperature (43). Optimum temperatures have been found to be between 11-28 C (5, 13, 43). In one study (13), the optimal temperature for germination of powdery mildew conidia was 12 C (66% germinated) and the optimal temperature for growth was 21 C. Others have shown that the germination of E. graminis conidia was fairly good between 3-20 C, however, infection and development of mildew took place best under alternating temperatures (5).

Penetration of wheat by E. graminis occurred at temperatures from 10-30C (32). The best temperature for the development of powdery mildew on red clover was considered to be 24 C (39). It has also been demonstrated

that changes in environmental conditions were necessary for the optimal development of various stages (germination, mycelial growth, and sporulation) (21,39).

Research on the effects of light on germination and disease development has given variable results (5, 10, 11, 32). The conclusion from one study (5) was that light had no direct effect in the development of E. graminis. Experiments indicated that infection by powdery mildew proceeded in darkness as well as in the sunlight (5). In fact, darkness for 4 days did not appear to retard mildew development. There are observations that in total darkness, the mildew spores penetrated epidermal cells, but later died (32). If wheat plants were illuminated for 3 hr each day, infection was heavy. Infection was reduced if plants were illuminated for 1 hr each day.

In studying the effects of light on the germination of urediospores of Puccinia graminis var. tritici, it was shown that germination was reversibly inhibited when inoculated water agar plates were incubated for 2 hr under 400 fc of light (11). Over long incubation periods the percent germination of light-treated urediospores as compared to those left in darkness was essentially the same. Germination of urediospores of P. cynodontis was inhibited when light

intensities were above 200 fc (41). Light was a critical factor and was necessary for the development of this disease. Urediospores of P. graminis var. tritici formed appressoria, penetration pegs and vesicles within 6 hr when grown on various artificial substrates at 80-85 F in a saturated atmosphere with light intensities between 2000-5000 fc (10). Infection hyphae developed if the parasite was subjected to darkness for 16-18 hr after the initial 6 hr period.

The differential response of the different stages in the infection process of the stem rust fungus to environmental treatments has also been studies (33). Results were as follows: 1) Under dew conditions at 21 C in the dark, germination of the urediospores occurred during the first 4 hr and appressorial formation between 4-8 hr after inoculation. 2) The appressoria remained dormant unless the plants were slowly dried at 65 F and then exposed to intense light at 85 F. Under this change of conditions vesicles and infection hyphae developed.

It can be seen from this brief review that effects of various environmental conditions upon either mildew or rust fungi are unclear. Before beginning a study of the host-parasite relations, it was considered necessary to

determine the optimal environmental conditions for each stage of primary infection. If the initial infection process can be precisely defined, then quantitative data can better be obtained and interpreted for problems concerning the host-parasite interactions (21). The following studies have contributed to the definition of the kinetics of infection of susceptible wheat and barley by E. graminis (20, 21, 22, 23, 27, 28, 29, 31, 37).

The effects of light, relative humidity, and temperature on the germination of conidia of E. graminis f. sp. tritici on wheat were examined (29, 31). Spore germination was found to be dependent on temperature and relative humidity, but insensitive to the range of light treatments given. Maximum germination occurred when spores were held for 1 hr at 17 C with 100% relative humidity followed by 65% relative humidity at 22 C. Under these conditions over 90% of the conidia on the leaf germinated by the 4th hr after inoculation.

Two to 4 hr after germination germ tubes began to swell to form appressorial initials (29). The formation of appressorial initials appeared to be independent of changes in temperature, relative humidity, and low light intensities in the ranges tested. A subsequent stage, the

maturation of appressoria, was light dependent for optimal development (21, 29). At 22 C and 65% relative humidity, 96% of the applied conidia formed appressorial initials 8 hr after inoculation (20, 21, 29). Light intensities were important in the formation of mature appressoria (29). Darkness or high light conditions (2800 fc) delayed the formation of mature appressoria. The optimal light condition tested was 240 fc started 2 hr after inoculation. With this light condition 86% of the total conidial population formed mature appressoria by 8 hr after inoculation.

Secondary hyphae form at the bend of the mature appressorium. When the secondary hypha is first detected as a bud-like knob, it is called a secondary hyphal initial. The first appearance of this stage was 16 hr after inoculation. High temperatures (30 C) drastically reduced the percentage of parasite units forming secondary hyphal initials (20, 21). High relative humidity (100% given during the 8-16th hr after inoculation) inhibited the development of secondary hyphal initials. Optimal conditions for the formation of secondary hyphal initials included darkness between 6-16 hr after inoculation followed by low light (240 fc). These studies (20, 21, 29, 31) defined and synchronized at least 4 stages of the primary infection

process - (a) germination, (b) formation of appressorial initials, (c) maturation of appressoria, and (d) formation of secondary hyphal initials. Approximately 90% of the applied conidia proceeded through each stage of development within a 4-6 hr period.

A fifth phase of the infection process is the formation of functional secondary hyphae (20, 27). Earlier work suggested that the formation of secondary hyphal initials could be used as a criterion for the establishment of a compatible host-parasite relationship (29). By using various barley cultivars containing genes for resistance (20, 22), it was demonstrated that the number of functional (elongating) secondary hyphae formed corresponded to the number of haustoria formed within the epidermal cells. The formation of secondary hyphal initials was not controlled by the major genes for resistance in 4 barley lines, whereas these genes did control the formation of functional secondary hyphae (23). The effects of different genes for resistance in 4 wheat lines in relation to the development of functional secondary hyphae of E. graminis showed a 4-8 fold decrease in the percentage of functional secondary hyphae formed. The reduction in percent of functional secondary hyphae was dependent upon the particular gene

for resistance (37). Therefore, it was hypothesized that either the formation of functional secondary hyphae or haustoria could be used as a criterion for the establishment of a compatible relationship.

In this "defined" model system, penetration of the epidermal cells of wheat and barley began approximately 10 hr after inoculation (22, 28). The development of a haustorium almost always led to the establishment of functional (elongating) secondary hyphae (20). Failure to produce a haustorium has meant failure to produce elongating secondary hyphae and secondary haustoria. The production of elongating secondary hyphae was considered, therefore, to be indicative of the establishment of a successful infection, i.e., a compatible, functional host-parasite relationship.

The period of darkness starting 6 hr after inoculation appeared to be critical for regulating the formation of functional secondary hyphae. Since the formation of functional secondary hyphae was considered to be the first morphological indication for a compatible relationship, it was of great importance to define more fully the events which occurred during this dark period (27, 28).

Ultraviolet radiation can inhibit the development of powdery mildew (4, 7, 15, 26, 28). When spores of powdery mildew were irradiated for 10 minutes, only 0.4% germinated as compared to the control of 15% (7). Inoculated wheat plants irradiated for 3, 10, or 15 minutes each day over a period of 14 days remained relatively free from mildew and without any apparent harmful effects on the host (15). If the UV treatments were stopped, the plants eventually became mildewed. Buxton et al. (4) found that a 20 sec UV treatment was necessary to decrease germination of E. graminis conidia by 50%. For Botrytis fabae, UV irradiation 7 hr after inoculation had no apparent effect, probably because the spores had germinated and the parasite had penetrated into the host, and was shielded from UV radiation. Less macrosopically visible damage was done to E. graminis when irradiated 70 rather than 20 hr after inoculation. Data (26) concerning the effects of UV light on E. graminis f. sp. hordei indicated that germination decreased as the exposure time increased from 0-50 sec. Pustule development was reduced considerably as exposure of UV was increased from 30 to 50 sec. The fungus lost its sensitivity to UV as the time between inoculation and UV exposure increased. It was concluded

that the host-parasite relationship was more tolerant to UV when the mildew fungus had established a well-developed, compatible relationship with the host.

The physiological studies of the host-parasite (mildews and rusts) interaction have mainly been concerned with changes which took place after the parasite has established a compatible relationship with the host. Metabolic changes during the initial infection process are, for the host part, unknown. The following is a brief review of some aspects of these metabolic changes which occur during disease development.

At the time of sporulation of powdery mildew on wheat, there was an increase in sucrose synthesis in the mildewed leaves as compared to healthy controls (1). Only slight differences were found between healthy and mildewed leaves in the synthesis of fructose. There are also data which demonstrated a high concentration of protein, carbohydrates and large sized nuclei in haustoria 72 hr after inoculation (40). This indicated an active center of metabolic activity.

There was also an increase in the size of the host nucleus and in the synthesis of RNA and protein in diseased tissue (35). There was no apparent increase in RNA content

for a resistant reaction. It was speculated that there may be an exchange of mRNA between host and parasite (14, 35). However, no evidence of mRNA exchange is available.

No differences in the total soluble protein from mildewed and healthy barley plants were found (16). There were also no changes in the total activity of malate dehydrogenase during development of mildew. There was evidence that multiple forms of this enzyme were present 12 days after inoculation (16).

Phosphatase was reported to be concentrated around the haustoria (2), and it was speculated that fungal enzymes may play an important part in the transfer of metabolites between host and parasite. Attempts to reproduce the data with acid phosphatase have failed (20, 22). As soon as haustoria are detectable there was an increase in succinate dehydrogenase activity inside and around the haustoria (20, 22). This indicated an early interaction between the host and parasite.

Haustorial bodies were full-sized by 18 hr after inoculation (20). The haustorium of the mildew is surrounded by a sheath (9) and does not penetrate the plasma membrane of the epidermal cell, but rather causes an invagination of this membrane (24). There is a space

between the plasma membrane and the haustorium. It is surmised to be filled with a nutrient solution which would seem to be responsible for transport between host and parasite.

Several workers (8, 12, 36, 44) have shown that there is an accumulation of radioactive substances at or near sori. The first studies (12) to demonstrate accumulation of an isotope showed the accumulation of ^{32}P in wheat leaves infected with stem rust. After inoculated wheat was grown in isotope solutions for several days, there was movement of ^{32}P to the infection sites of P. graminis. A large accumulation of radioactivity at the infection sites 3-4 days after inoculation was detected when ^{14}C xylose was fed to inoculated wheat plants (36). Similar results were obtained with radioactive glucose and ribose. Since accumulation of these radioactive substances was much greater with susceptible than resistant-type reactions, it was suggested that the rate of movement of substrates toward infection sites possibly could play an important role in determining susceptibility to disease (36). Rust-infected bean plants, allowed to absorb ^{32}P for 1 hr, showed an accumulation of more ^{32}P than the uninoculated control (8). Since ^{32}P accumulation has been

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found with obligate parasites, it was suggested (12) that maybe pathogens require phosphorylated intermediates of host metabolism. An increased accumulation of ^{35}S into infected areas on bean leaves was also shown (44).

When ^{35}S labeled urediospores were inoculated onto oat plants, there was a release and translocation of the ^{35}S within the host at the time of flecking (17). This demonstrated that there was a transfer of radioactive substances from parasite to host between inoculation to flecking. Germinating urediospores of P. coronata released amino acids (18). The exchange of metabolites is probably not unidirectional but also includes a transfer of materials from the host to the parasite.

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MATERIALS AND METHODS

Culturing of powdery mildew -- The strain of Erysiphe graminis D.C. f. sp. tritici Em Marchal (incitant of powdery mildew on wheat) was collected in Michigan and maintained on susceptible wheat (Triticum aestivum L. 'Little Club'). Wheat plants were grown in 4-inch pots and were inoculated when they were 7 days old. Different sets of wheat plants were inoculated daily by dusting conidia produced 7 days after inoculation onto the leaves of the susceptible wheat. Inoculated plants were maintained in a controlled environment chamber provided with adequate air circulation and controlled relative humidity (65±5%) and tempterature (18±2 C). The stock cultures were kept under approximately 450 fc of combined incandescent and fluorescent light for 15 hr per day. Plants were watered daily and the relative humidity during the dark hours approached 100%. Mycelial growth of the cultures was first macroscopically evident 3-4 days after inoculation. Conidia for experimental uses were abundant by 6 days after

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inoculation. Conidia produced 6 days after inoculation were used in experiments. Trial experiments indicated a significant difference between 6 and 7 day old conidia with respect to the kinetics of infection.

Methods of inoculation -- Two methods of inoculation were employed. For experiments involving certain environmental and ultraviolet treatments, single 5 or 6 day old susceptible wheat plants grown in 2-inch pots were inoculated on the lower leaf surface by the "rolling method" (30). This consisted of collecting conidia on a glass slide and transferring them to a host plant with a cotton swab. This method of inoculation caused a breakage of the conidial chains and provided a uniform distribution of single conidia over the leaf surface. The quality of the conidia could be estimated by the percentage of collapsed spores on the glass slide. If more than an estimated 10% of the conidia were collapsed, the experiment was discontinued and fresh conidia were collected at a later time. The number of conidia per centimeter of inoculated leaf ranged between 75-150. The kinetics of infection were the same whether plants were inoculated on the upper or lower leaf surface. However, for facilitation of observations, all experiments performed involved the inoculation of the

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lower surface of the leaf. The lower epidermal surface was easier to strip from the plant for making direct observations of the haustoria.

In experiments on the transfer of ^{32}P and ^{35}S from the host to the parasite, the method of inoculation was by lightly dusting conidia on the lower leaf surface. Although some of the conidia were in chains and the efficiency of infection was reduced, the kinetics of infection appeared to be consistent with experiments using the rolling method.

Environmental conditions for experiments -- All experiments were carried out in Scherer-Gillet Model CEL 512-37 growth chambers. The conditions necessary for synchronous growth for each developmental stage of the infection process were as follows:

1. Inoculated plants were kept in a moist chamber near 100% relative humidity at 18$\pm$1 C in darkness for the first hour after inoculation.
2. For 1-6 hr after inoculation, they were placed under 240 fc (three 60-watt incandescent bulbs and two 40-watt fluorescent tubes) of light (termed as low light) with 65$\pm$5% relative humidity at 22$\pm$1 C.

3. In some experiments the plants were kept in darkness with 65±5% relative humidity at 22±1 C from 6-16 hr after inoculation. Later this condition was modified so that darkness was from 6-20 hr after inoculation.
4. From 20 hr after inoculation until the completion of the experiments, all conditions remained the same as in 3 except that 240 fc of light were employed.

The inoculated plants were placed approximately 3 ft from the light source and the intensity in fc was measured at this distance.

Recordings of temperature and relative humidity were made with a hygrothermograph calibrated with a sling psychrometer.

Temperature, light and relative humidity treatments were given between 6 and 16 hr after inoculation (during the "dark period"). Temperature treatments consisted of short exposures (0.5 hr) of the inoculated plants to high temperature (32 C). Light treatments were of three types. These consisted of low light (240 fc), medium light (1400 fc) and high light (2400 fc). These treatments were given during the "dark period" for various periods of time. For

the relative humidity treatments, 100% relative humidity was given for a period of 1/2 hr at 7 and 9 hr after inoculation.

The kinetics of the formation of secondary hyphae (initials and functionals) were observed and used as an indication of the sensitivity of the parasite or the host-parasite interaction to the various treatments.

Examination of fungal structures -- Observations of the infection stages were made with a light microscope. At various hours after inoculation an estimated 1 cm long section of an inoculated wheat leaf (not including the tip of the leaf) was cut and placed on a slide for examination. All of the parasitic units on this cut portion were examined and the percentages of each developmental stage determined. These stages consisted of (a) germination, (b) appressorial initials, (c) mature appressoria, (d) secondary hyphal initials, (e) haustoria, and (f) functional secondary hyphae. After each count of a particular phase of the infection process, the inoculated plant was discarded and any subsequent readings were made from other inoculated plants. Percentages of the various stages (a-d and f) were determined by the above method. Haustoria (e) were observed by stripping the lower epidermis from a leaf

section and placing on a glass slide with an appropriate stain. Immediately after staining the haustoria were examined directly under the light microscope (125x and 594x). Examination of these various mildew structures were made from the start of the experiments (zero hour) until approximately 3 days after inoculation.

Ultraviolet experiments -- Ultraviolet light was used as a tool to determine the time after inoculation that the nucleus has divided and a nucleus has entered the haustorium. The formation of functional secondary hyphae was used as a criterion for sensitivity to UV. Plants were irradiated by placing the inoculated surface of the leaf 1 ft from a General Electric G30T8 germicidal lamp (short wavelength, 254 mμ). These plants were irradiated between 6-20 hr after inoculation (dark period) for durations of 5, 30, and 60 sec. The percentages of functional secondary hyphae were determined between 22 and 50 hr after inoculation.

Histological studies -- The only structure which developes within the host is the haustorium. The presence of a nucleus in a haustorium was determined by stripping the epidermis from an inoculated plant, immediately fixing in 0.5% osmic acid for 1-3 minutes, and staining in Mayer's

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Haemalum (Haematoxylin) for 15 minutes (25). After staining, the epidermis was washed in distilled water to remove excess haematoxylin. This procedure for staining was quite reliable in detecting the nucleus with minimum distortion to the haustorial body. If the fixing process was longer than 5 minutes, the haustorium, as observed under the light microscope, appeared to develop a granular appearance. A minimum of 15 minutes was necessary to obtain reproducibly dark staining nuclei.

Parasite removal with parlodion -- A 2% parlodion solution was prepared by dissolving parlodion strips in equal amounts of ethyl alcohol and ether. At various hours after inoculation, wheat leaves were cut and laid flat on a glass slide. A few drops of parlodion was spread onto the leaf's lower surface. After several minutes the alcohol-ether solution evaporated and left a thin parlodion film intact with the leaf. The parasitic units which were on the surface of the leaf were embedded in the parlodion. In experiments dealing with the formation of penetration pegs, this film containing the parasite was removed with forceps, placed on a slide, and examined under the microscope.

Uptake of phosphorus-32 -- Several types of

preliminary studies were undertaken to determine the most efficient means of saturating the leaf with ^{32}P in the shortest time. Wheat plants were grown in sand and after 6 days the roots were washed. These washed roots were immediately submerged into a 25 ml Erlenmeyer flask containing a total volume of 5 ml of 0.05 M KNO_3, 0.05 M Tes buffer (pH 7), and $H_3P^{32}O_4$ (various activities of 190, 100, and 20 μc/ml). After the wheat seedlings were grown in the radioactive solution for 20 hr, the first centimeter at the tip of the plant was removed and discarded. The next 2, 1-cm sections were separately homogenized in Ten Broeck tissue grinders containing 10 ml of cold 1M PO_4. These radioactive solutions were counted in an annular-well Geiger-Mueller detector and recorded on a Nuclear-Chicago Model 186 counter.

Phosphorus-32 uptake was also determined after various hours in which the roots were in the liquid media containing 20 μc/ml of ^{32}P. Four 1-cm sections per leaf, excluding the tip, were homogenized and counted in 10 ml of phosphate solution. The leaves were sectioned after the roots were in the radioactive media for 1, 14, 24, and 48 hr. Comparisons were made between uninoculated plants that were grown in Hoaglands (19) and KNO_3-Tes solutions

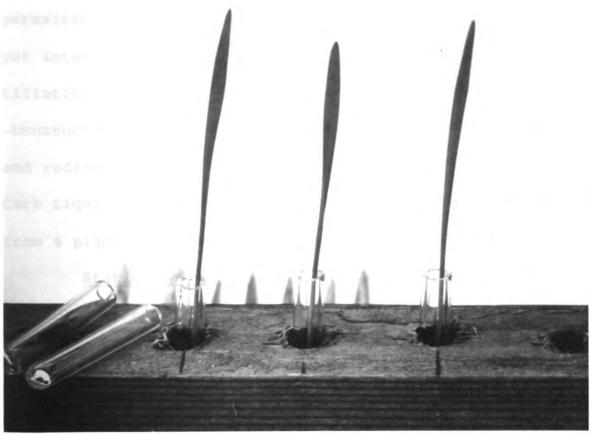
after 48 hr in the ^{32}P.

Experiments were also performed to determine if there was a greater uptake of ^{32}P (20 and 28 μc/ml) in inoculated than uninoculated plants. Different sets of wheat plants were placed in KNO_2-Tes buffer solutions (pH 7) of ^{32}P 6 and 12 hr after inoculation. Counts per minute were recorded with an annular-well G-M tube on the first 2 cm sections below the discarded tip at 24 and 30 hr after inoculation.

Another method of studying ^{32}P uptake into the wheat seedlings was to cut the plant at the crown and immediately place it into a small tube (6mm) containing water and 100 μc/ml of ^{32}P (Fig. 1). Two 1-cm leaf sections were homogenized after 4, 17, 20, and 24 hr and counted in an annular-well G-M tube.

Detection of ^{32}P and ^{35}S in the parasite -- Two methods of determining radioactivity were employed. Wheat plants were grown in the radioactive KNO_3-Tes solution for at least 20 hr. After the isotope "feeding" they were inoculated and at 10, 12, 24, 28, and 30 hr after inoculation the parasitic units were removed with 2% parlodion. The parlodion strips were dissolved and heated to dryness on planchets. Radioactivity on the planchets was determined

Fig. 1. Wheat leaves placed in 6 mm tubes containing water and 100 μc/ml of ^{32}P.



with an open end G-M tube. Controls consisted of duplicating the same procedure except with uninoculated plants.

With the second method, inoculated leaves were placed in 6 mm diameter test tubes (Fig. 1) containing 100 μc/ml of either ^{32}P or ^{35}S for a minimum of 5 hr. The parasitic units were removed from the host with parlodion, put into scintillation vials and dried overnight. Scintillation fluid (POPOP-1,4-bis- [2-(5-phenyloxazolyl)] -benzene and PPO-2,5-diphenyloxazole in toluene) was added and radioactivity determined in a Packard Model 3003 Tri-Carb Liquid Scintillation Spectrometer. Parlodion strips from 6 plants were used as a single sample per vial.

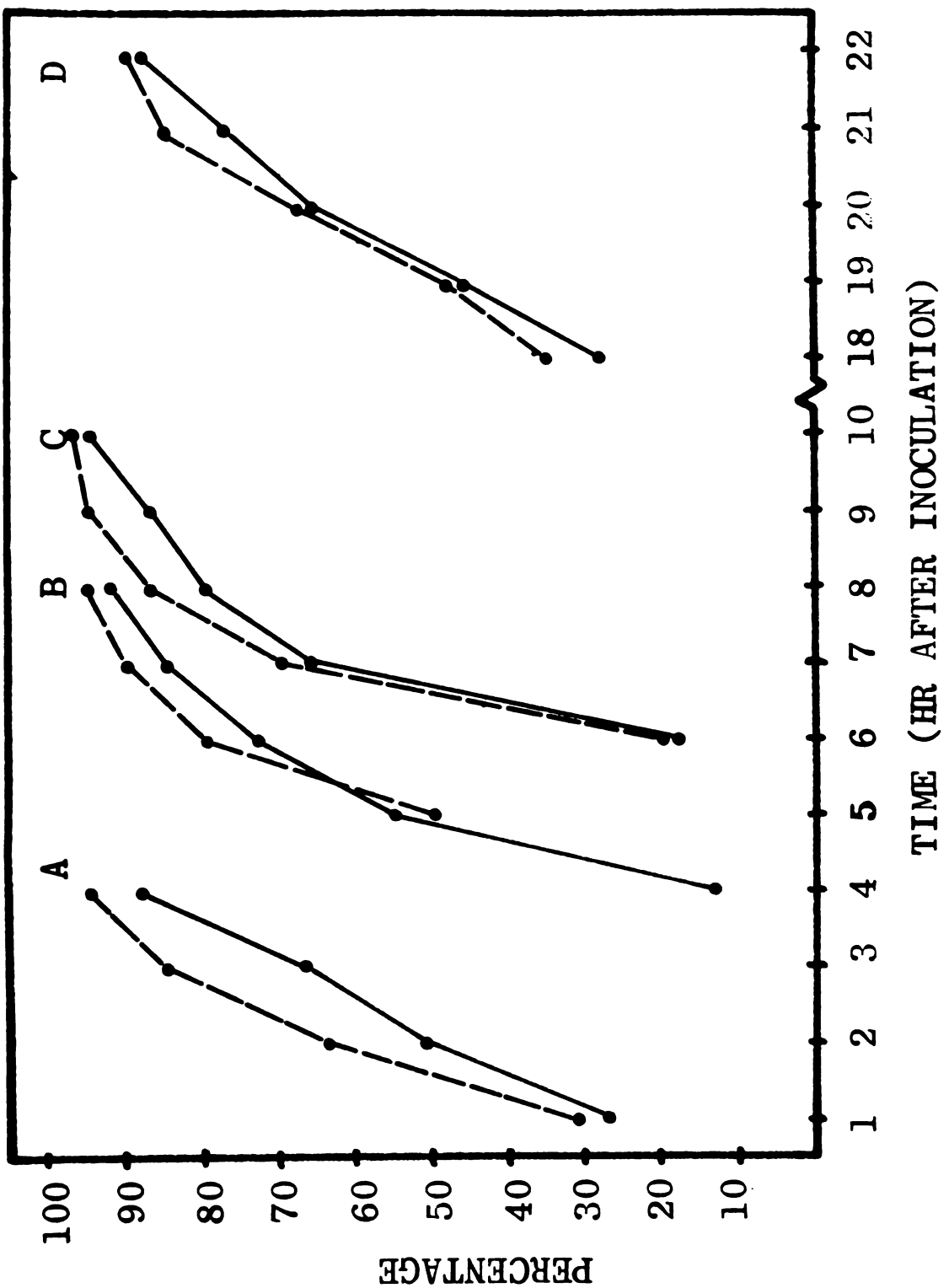
Statistical Analysis -- The two-way analysis of variance was used witn 5% level of significance.

RESULTS

Germination of conidia and formation of appressorial initials, mature appressoria, and secondary hyphal initials -- Preliminary experiments dealt with repeating earlier work on the stages of the primary infection process (germination, formation of appressoria, maturation of appressoria, and formation of secondary hyphal initials). When inoculated plants were maintained in a relative humidity of 100% at 18 C in darkness for 1 hr after inoculation followed by 3 hr under 240 fc of light with 65% relative humidity at 22 C, the germination percentages at 1, 2, 3, and 4 hr after inoculation were in close agreement with those obtained previously (20) (Fig. 2). Four hr after inoculation 88% of the total conidial population germinated as compared to previous results of 94%.

When the germ tube enlarged at the distal end, i.e., became club-shaped, the structure was called an appressorial initial. Approximately 13% of the applied conidia formed appressorial initials 4 hr after inoculation

Fig. 2. Kinetics of the formation of 4 stages of the primary infection process. A) Germination, B) Appressorial initials, C) Mature appressoria, and D) Secondary hyphal initials. (-----) Masri (20), (———) repeat of Masri's work.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PERCENTAGE
1 2 3 4 5 6 7 8 9 10 18 19 20 21 22
TIME (HR AFTER INOCULATION)

and by the 8th hr, 92% had developed these structures (Fig. 2).

Six hr after inoculation 18% of the applied conidia had formed mature appressoria. This stage of development was characterized by the "hook-shaped" or bending appearance of the appressorial initials. At 7 hr 65% of the applied spores had formed mature appressoria. Ninety-five percent of the total parasite units had formed mature appressoria 10 hr after inoculation (Fig. 2). Inoculated plants were subjected to darkness 8 hr after inoculation. In later experiments this "dark period" was extended from 8-16 hr to 6-16 hr after inoculation. In either case the percentages of mature appressoria formed remained the same.

The 4th stage of the infection process was the formation of secondary hyphal initials. These were characterized by the development of a small bud-like protrusion from the mature appressorium (Fig. 3). It was observed that the secondary hyphae could develop anywhere along the full length (approximately 30 μ) of the mature appressoria. However, under the environmental conditions used in these experiments, most of the secondary hyphae developed from the "hooked" portion of the mature appressorium. Secondary hyphal initials were first detected 17 hr after inoculation

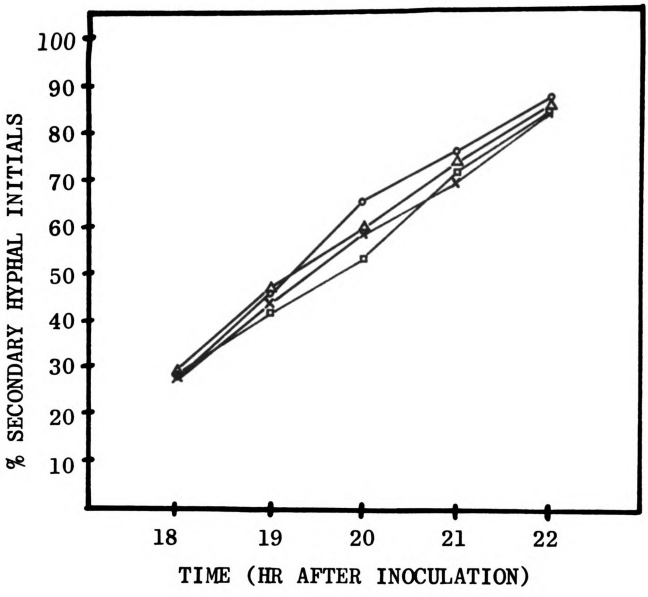
Fig. 3. Drawings illustrating (a) conidial body, (b) mature appressoria, (A:c) a secondary hyphal initial, and (B:d) functional secondary hypha.

and by 18 hr 28% of the applied conidia had formed secondary hyphal initials. This percentage increased so that 89% of the infectious units had formed secondary hyphal initials 22 hr after inoculation if low light was given beginning with the 17th hr.

The slopes of the curves for the development of these 4 stages were similar and indicated that the parasite units were proceeding through each stage of development in a somewhat synchronous manner under the environmental conditions used. Having defined the kinetics of the initial infection process (20, 21), any deviation in kinetics of a particular developmental stage due to different environmental treatments at various hours would indicate the time after inoculation that a possible change in fungal development or host-parasite interaction had occurred. Therefore, it was during the "dark period" that inoculated plants were subjected to various environmental treatments.

Effect of light on the development of secondary hyphal initials -- Low light (240 fc) given for 2 hr periods between 10 and 16 hr after inoculation had essentially no effect on the formation of secondary hyphal initials (Fig. 4). A statistical analysis of the results showed there were no significant differences between the treatments and

Fig. 4. Effect of 2 hr low light treatments (240 fc) on the formation of secondary hyphal initials. (o——o) No light treatment (control). (Δ—— Δ) Light treatment from 10-12 hr, (□——□) 12-14 hr, and (x——x) 14-16 hr after inoculation.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TIME (HR AFTER INOCULATION)

the control.

Inoculated plants were radiated with medium light (1400 fc) for durations of 2 and 4 hr 8-16 hr after inoculation (Fig. 5). Fifty-five to sixty percent of the total population of parasitic units appeared to be stimulated in that they formed initials earlier than the control. The remaining 40-45% of the parasite units were slightly inhibited as compared to those kept in darkness.

High light (2400 fc) was initially given for 1.5 hr beginning 7 1/4, 7 3/4, 8 1/4, and 8 3/4 hr after inoculation. Treatments beginning at 7 1/4 and 7 3/4 hr after inoculation significantly delayed the formation of secondary hyphal initials by approximately 2 hr (Fig. 6). The total population of the inoculated conidia did, however, reach similar percentages of the control after the period of delay. On plants subjected to high light at 8 1/4 and 8 3/4 hr after inoculation, a slight stimulatory effect was observed in the development of secondary hyphal initials for some of the applied conidia. The remaining, approximately 50%, did not appear to be affected by the high light treatments.

To pinpoint more precisely the time after inoculation the high light treatments affect the development of

Fig. 5. Effect of either 2 or 4 hr medium light treatments (1400 fc) on the formation of secondary hyphal initials. (o——o) No light treatment (control), (●——●) light treatment 8-10 hr, (x——x) 10-12 hr, (□——□) 12-14 hr, and (Δ——Δ) 12-16 hr after inoculation.

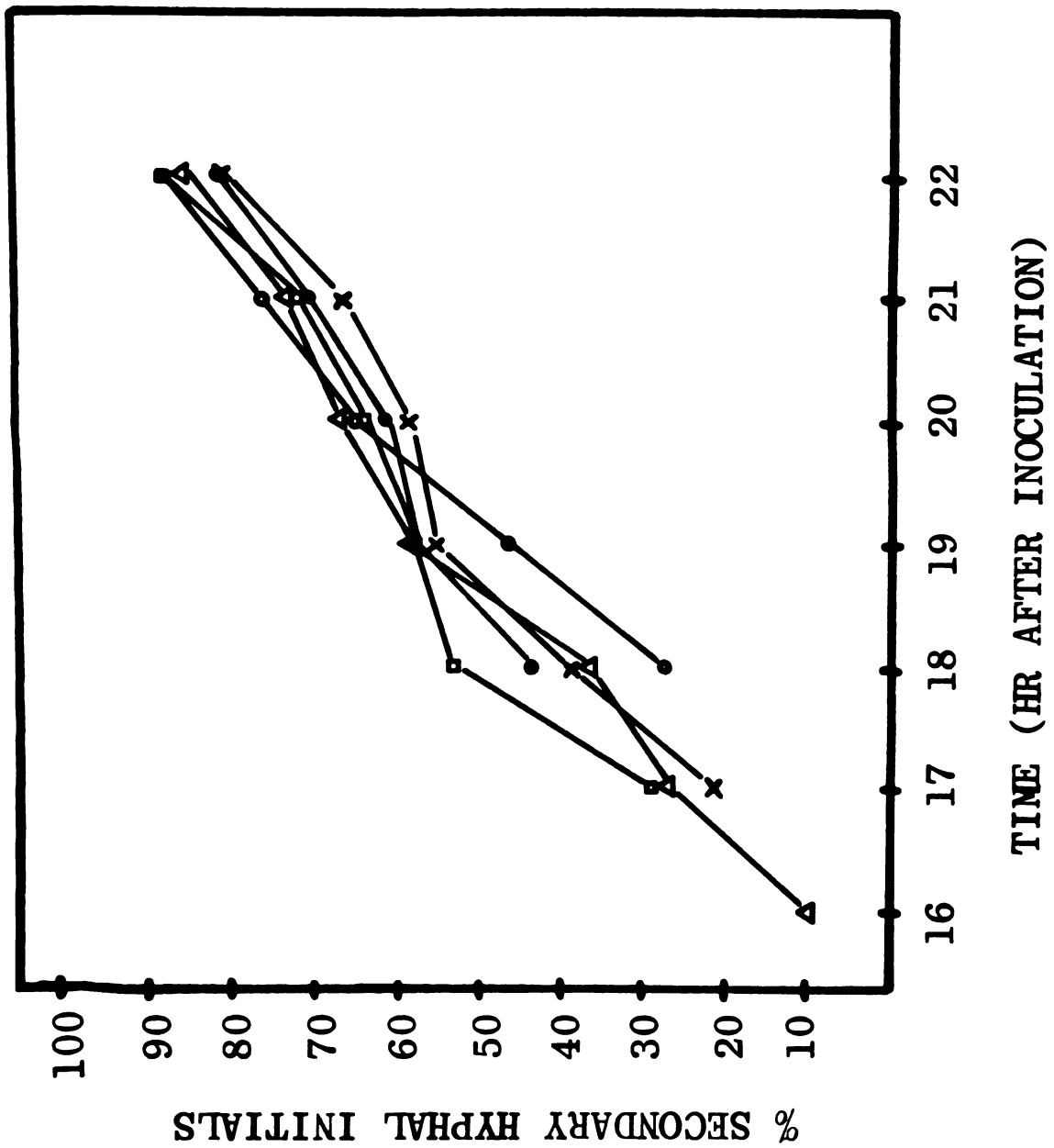
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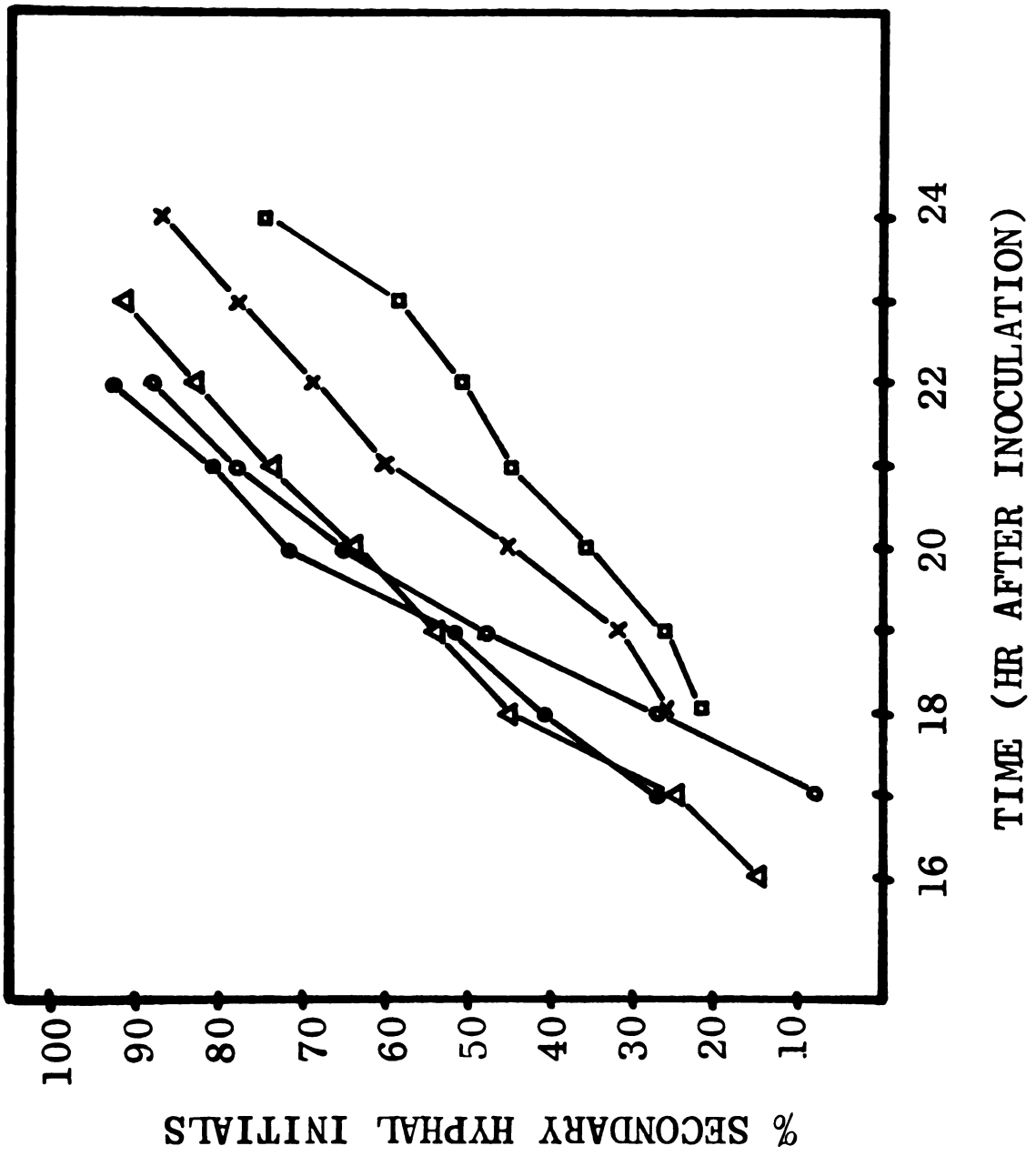
Fig. 6. Effect of 1.5 hr high light treatments (2400 fc) on the formation of secondary hyphal initials. (o——o) No light treatment (control). Light treatment begun 7 1/4 (□——□), 7 3/4 (x——x), 8 1/4 (Δ——Δ), and 8 3/4 (●——●) hr after inoculation.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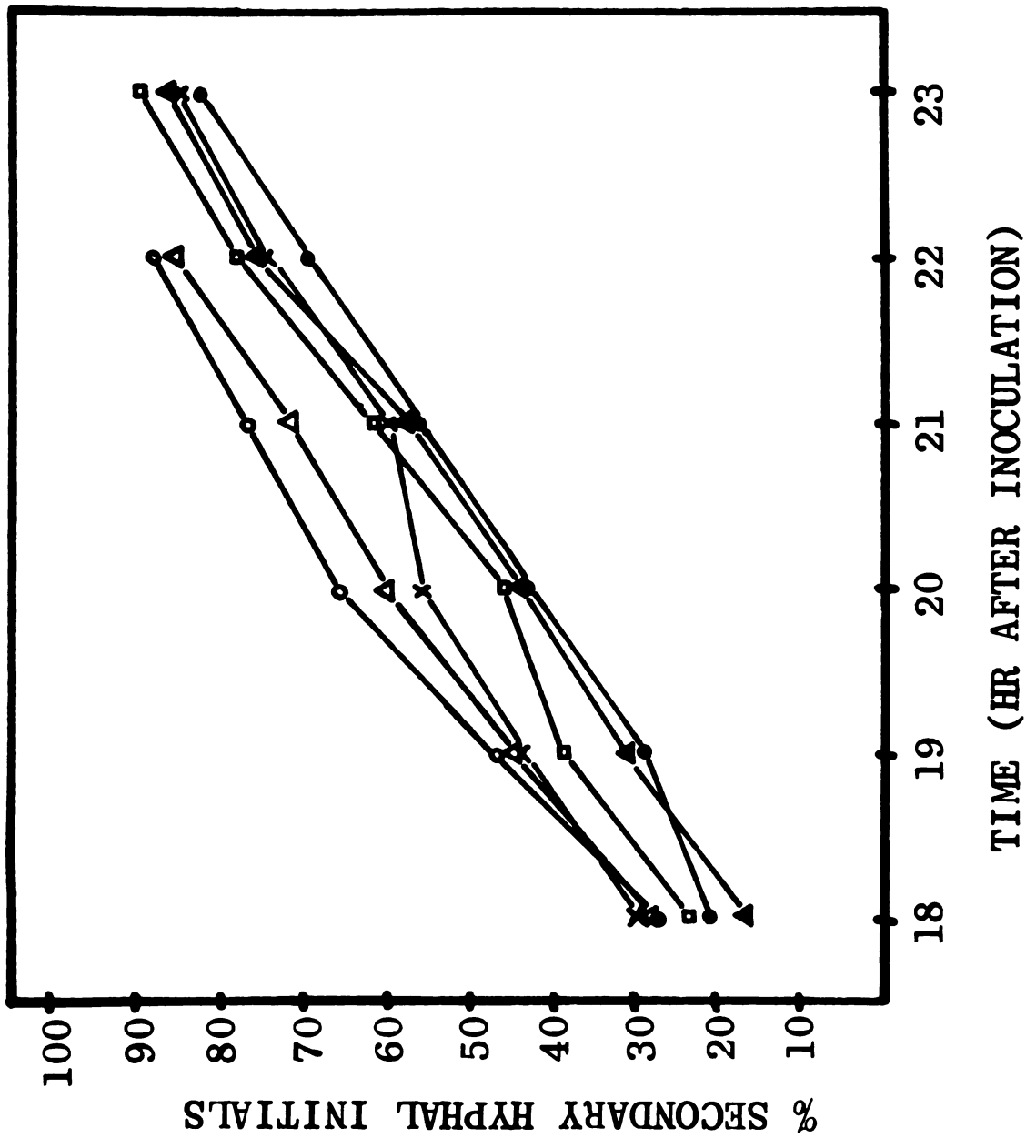
TIME (HR AFTER INOCULATION)

secondary hyphal initials, high light treatments were given for 15 minutes at various hours after inoculation. When inoculated plants were placed in high light at either 6, 7, or 8 hr after inoculation, there was a significant delay of 1-1.5 hr in the formation of secondary hyphal initials (Fig. 7). When the high light treatment was given at the 9th hr after inoculation, approximately 50% of the applied parasitic units were not inhibited in their development of secondary hyphal initials. The remaining 50% were inhibited to the same extent as with earlier light treatments. No differences were detected between the 10th hr high light treated plants and the control. Therefore, in using the formation of secondary hyphal initials as a criterion for evaluating the light effect, the data suggest that the parasite units move through a stage of light sensitivity to light insensitivity. Here, approximately 50% of the parasite units have passed from one stage to the next 9 hr after inoculation.

Effect of temperature and relative humidity on the formation of secondary hyphal initials -- Only a brief study was conducted concerning the effects of high temperature and high relative humidity on the development of

Fig. 7. Effect of 15 min high light treatments (2400 ft-c) on the formation of secondary hyphal initials. (o——o) No light treatment (control), light treatments given at 6 (□——□), 7 (●——●), 8 (▲——▲), 9 (x——x), and 10 (Δ——Δ) hr after inoculation.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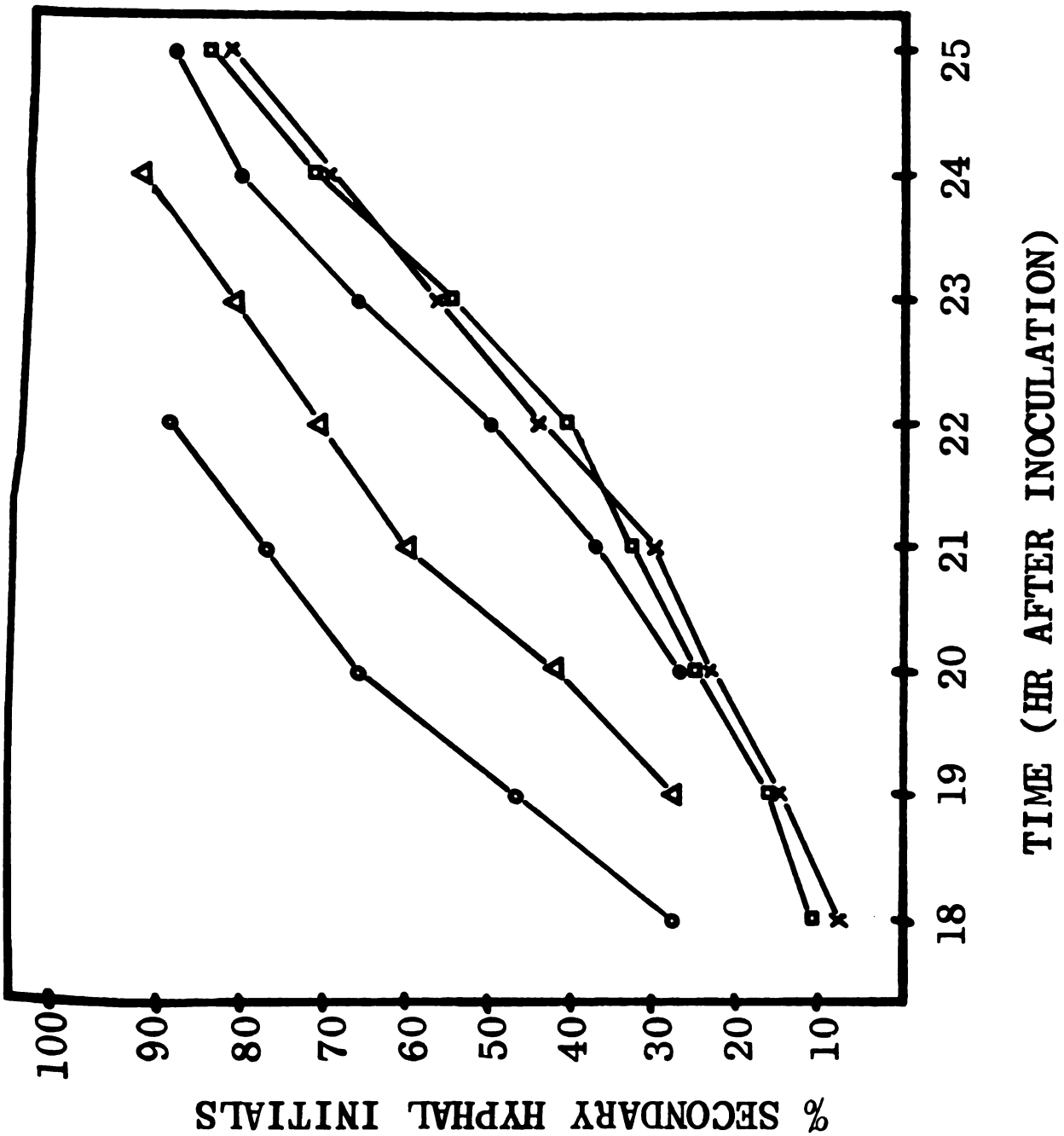
TIME (HR AFTER INOCULATION)

secondary hyphal initials. Temperature treatments (31 C) were given for durations of 0.5 hr at either 6, 7, 9, or 11 hr after inoculation. In each set of experiments dealing with a different treatment there was a significant difference in the percentage of secondary hyphal initials formed at a given hr in comparison to inoculated plants not subjected to high temperatures. The formation of secondary hyphal initials as a result of the 6, 7, or 9th hr heat treatments was delayed from 2-4 hr (Fig. 8). The 11th hr treatment resulted in a shorter delay of about 1.5 hr compared to the control. In all high temperature treatments there were no complete inhibitory effects observed. With each treatment between 80-90% of the applied conidia eventually formed secondary hyphal initials.

Inoculated plants were placed in a growth chamber with near 100% relative humidity for 1/2 hr periods at various times after inoculation (7 and 9 hr). The 7th treatment was not significantly different from the control (Fig. 9). A significant difference was obtained in experiments when inoculated wheat was subjected to high relative humidity 9 hr after inoculation.

Development of functional secondary hyphae -- A functional secondary hypha was defined as an elongating

Fig. 8. Effect of 1/2 hr temperature treatments (31 C) on the formation of secondary hyphal initials. (o——o) No temperature treatment (control), temperature treatment given at 6 (x——x), 7 (□——□), 9 (●——●), and 11 (Δ——Δ) hr after inoculation.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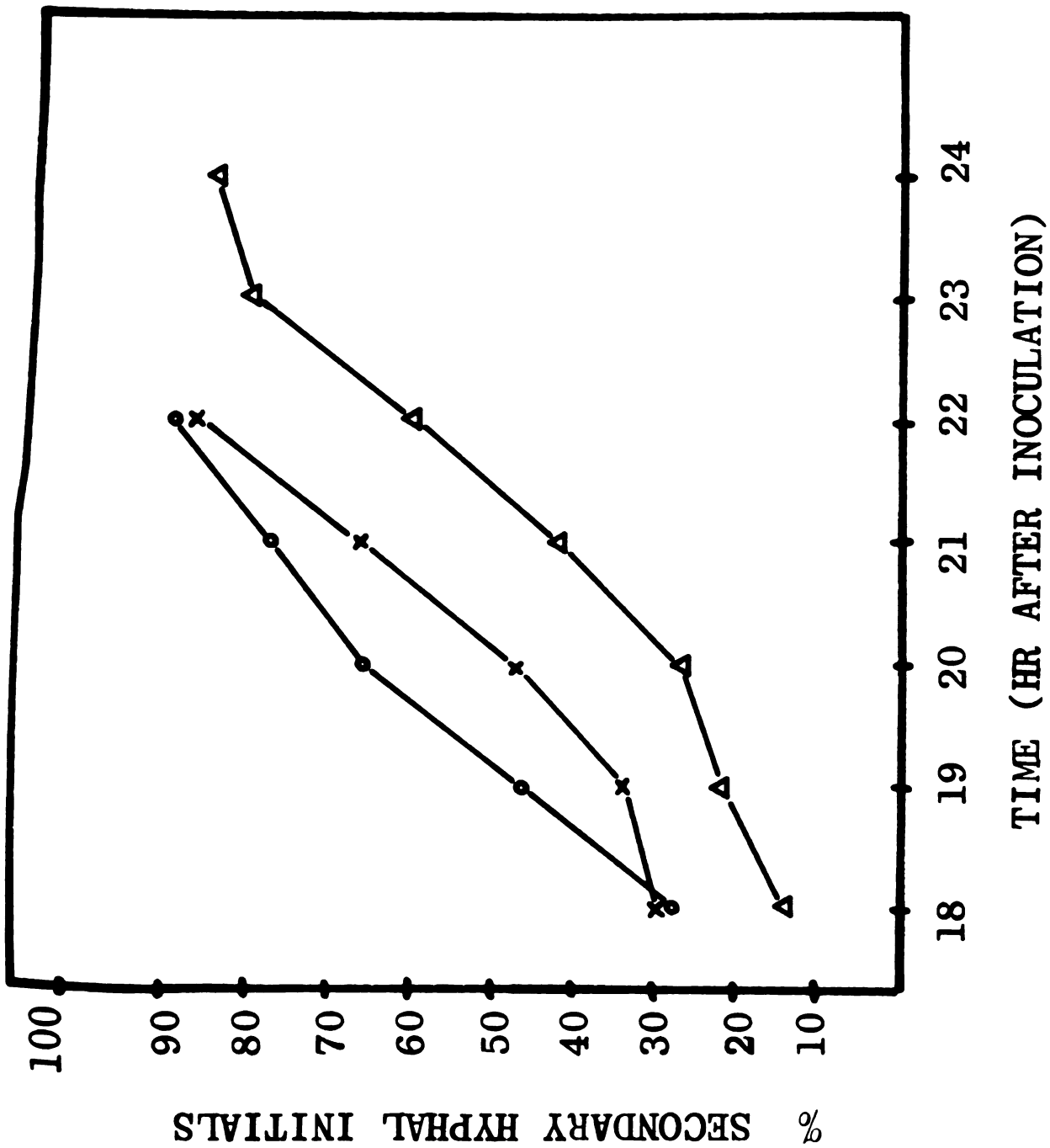
TIME (HR AFTER INOCULATION)

Fig. 9. Effect of 1/2 hr treatments of high relative humidity (ca. 100%) on the formation of secondary hyphal initials. Standard conditions (control) (o——o), relativity humidity treatments given at 7 (x——x) and 9 (Δ——Δ) hr after inoculation.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secondary hypha. Evidence was presented in earlier studies (20, 21) to show that a distinction could be made between secondary hyphal initials and functional secondary hyphae. Two criteria were used in distinguishing a functional from an initial secondary hypha. First, the functional hypha had to be at least 2 μ in length and, second, it had to be clearly demonstrated that the hypha was actively growing or elongating (Fig. 3). Interpretation of later experiments (20, 22, 23) indicated that the development of functional secondary hyphae could be used to demonstrate the establishment of a successful infection.

In earlier studies the final percentage of parasite units which formed functional secondary hyphae was approximately 80% with a compatible host and parasite, and somewhat less in the presence of a gene for resistance. The fact that functional secondary hyphae formed over many hr indicated a greater lack of snychrony in this stage of the development of the fungus. Since a high degree of synchrony of the parasite population for each stage of development is considered necessary for these studies, experiments were begun in which the environment was manipulated in an attempt to get the formation of functional secondary hyphae as synchronized as other developmental

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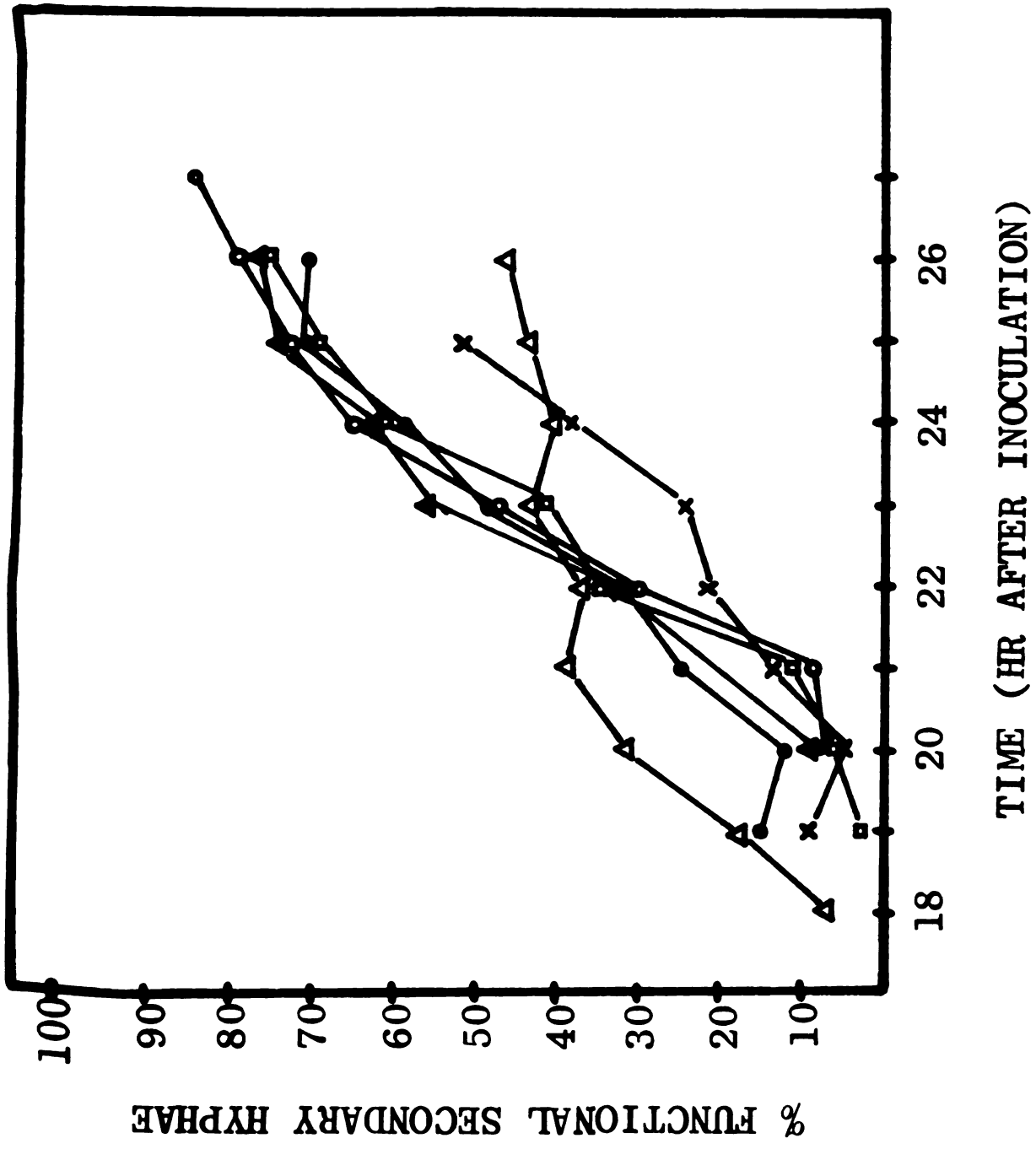
The length of the "dark period" was assumed to be the principle factor involved in determining the rate of development of functional secondary hyphae. The condition found for the best development of secondary hyphal initials (darkness, 6-16 complete hr after inoculation) was not optimal for the rapid formation of functional secondary hyphae. When the period of darkness was shortened from 6-16 hr to 6-14 or 4-12 hr after inoculation, the percentage of the applied conidia that formed functional secondary hyphae was low (ca. 45-50%) (Fig. 10). By extending the darkness from the 16th to the 17, 18, or 20th hr after inoculation, a higher percentage of the total parasitic units formed functional secondary hyphae. Other periods of darkness were tested but were abandoned since they altered previously established developmental stages of primary infection. The condition considered optimal for the formation of functional secondary hyphae was darkness from 6-20 hr after inoculation followed with 240 fc of light (Fig. 10). Approximately 7% of the applied conidia formed functional secondary hyphae at 20 hr and increased at a fairly rapid rate to 84% by 27 hr after inoculation. This curve is represented by the averages of 11 replicates.

Fig. 10. Percent formation of functional secondary hyphae using various periods of darkness after inoculation. Dark period 4-12 (△——△), 6-14 (x——x), 6-16 (●——●), 6-17 (□——□), 6-18 (▲——▲), and 6-20 (o——o) hr after inoculation.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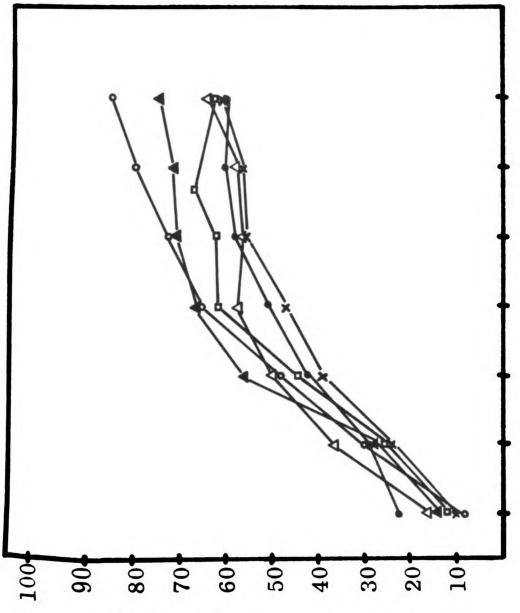
Effect of high light on the formation of functional secondary hyphae -- Short exposures (15 min) to high light (2400 fc) between 8 to 13 hr after inoculation caused differential inhibitory effects on the formation of functional secondary hyphae (Fig. 11). The greatest inhibition occurred when high light treatments were employed at 9, 10, or 11 hr after inoculation. With these light treatments there was a decrease of approximately 20-25% as compared to the control. The percentage of functional secondary hyphae formed with the light treatment given at the 13th hr was essentially the same as the control. These light effects upon the development of functional secondary hyphae were substantially different from the light effects upon the formation of secondary hyphal initials (Fig. 7).

Formation of penetration pegs -- By counting the knob-like protrusions (penetration pegs) on the underside of the mature appressoria embedded in the parlodion strips, it was possible to estimate a percentage of the applied conidia which had penetrated or started to penetrate the epidermis (Fig. 12). Of the 75-90% of the applied spores which were recovered in the parlodion strips, approximately 60% exhibited these penetration knobs between 10 and 12 hr after inoculation. Spores that were separated from the

Fig. 11. Effect of 15 min high light treatments (2400 ft-c) on the formation of functional secondary hyphae. No light treatment (control) (o——o), light treatments given at 8 (□——□), 9 (x——x), 10 (●——●), 11 (Δ——Δ), and 13 (▲——▲) hr after inoculation.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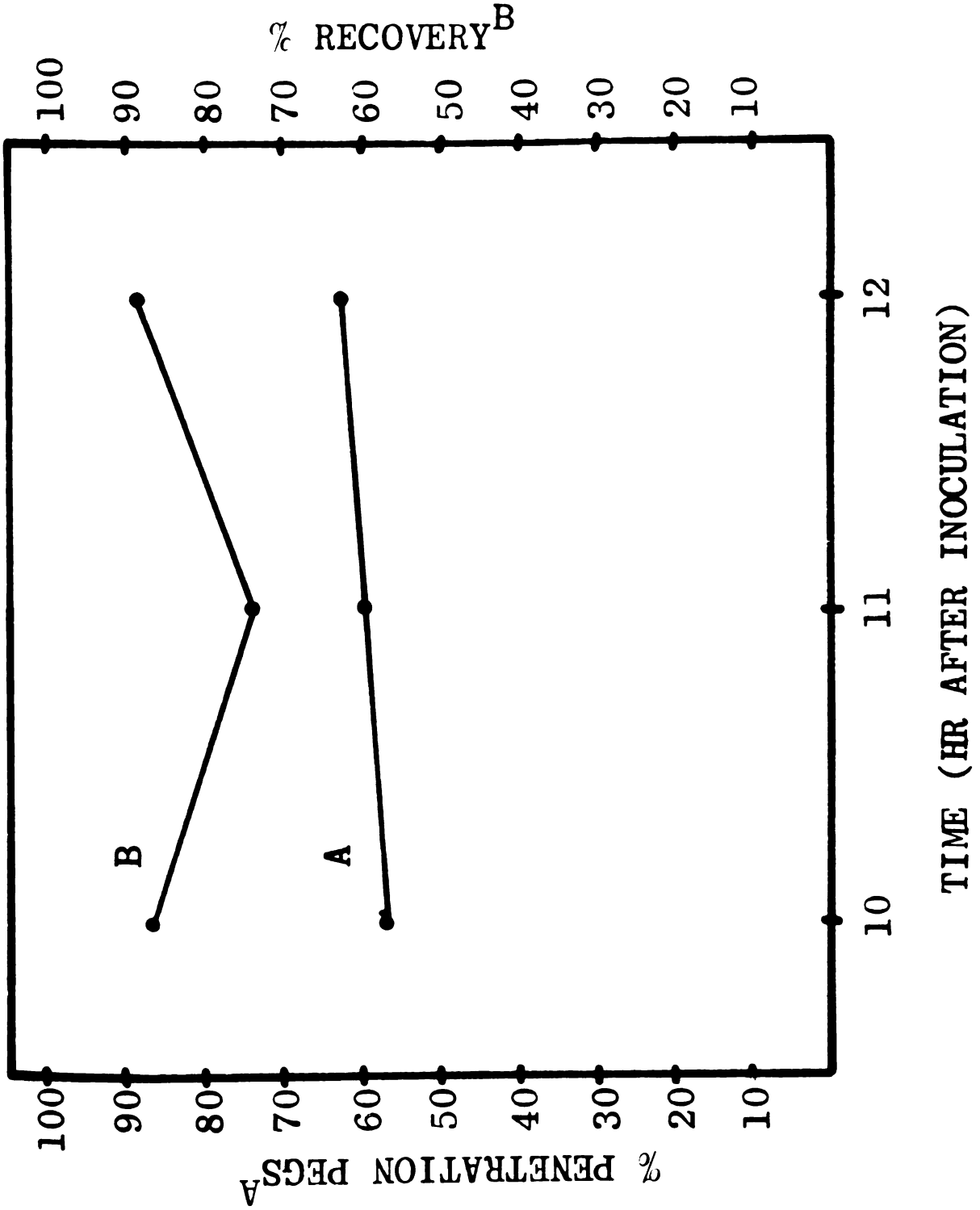
TIME (HR AFTER INOCULATION)

Fig 12. A) Percent formation of penetration pegs as determined from spores embedded in parlodion.

B) Percent recovery is a percentage of the total applied conidia that was embedded in the parlodion.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TIME (HR AFTER INOCULATION)

host by this method detached themselves from the haustorium which was left in the epidermal cell (Fig. 13).

Effect of ultraviolet radiation on the formation of functional secondary hyphae -- The period between 6 and 20 hr after inoculation appeared to be critical for the optimal development of functional secondary hyphae. It is during this "dark period" that penetration and haustorial development take place (20). The effects of treatments during this period on the formation of functional secondary hyphae was determined. The relationship between UV sensitivity to the appearance of nuclei in the haustoria was also determined.

Figure 14 presents the percentage of functional secondary hyphae formed after inoculated plants were irradiated with UV for 5 sec at various times between 3 and 20 hr after inoculation. Plants irradiated 7 hr after inoculation showed a marked inhibition in the formation of functional secondary hyphae. The degree of inhibition decreased as the length of the dark period prior to the irradiation increased. When UV treatments (5 sec) were given at the 3rd hr, only a slight inhibition resulted. Since inoculated plants were under low light illumination at the 3rd hr, there exists the possibility of

Fig 13. Pictures of fungal units showing penetration pegs at the ends of the mature appressoria (A) 10 and (B) 24 hr after inoculation.

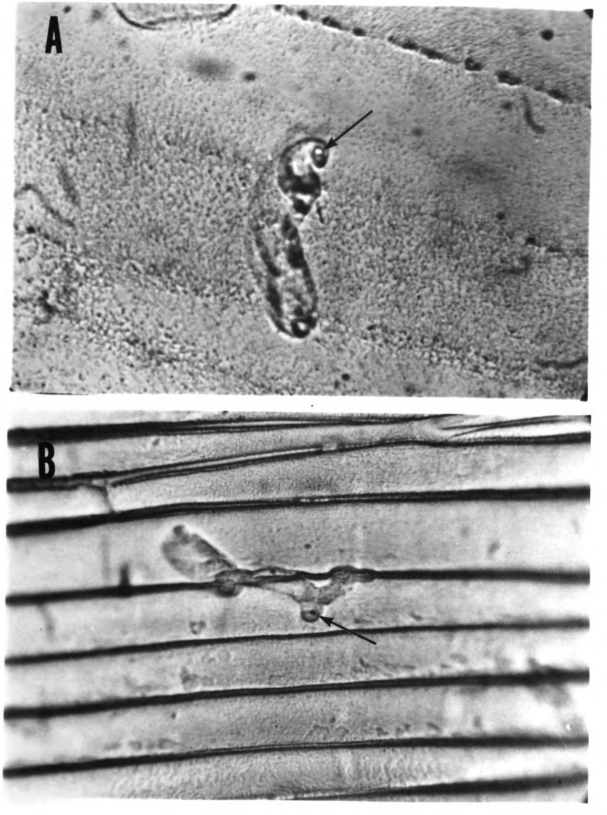
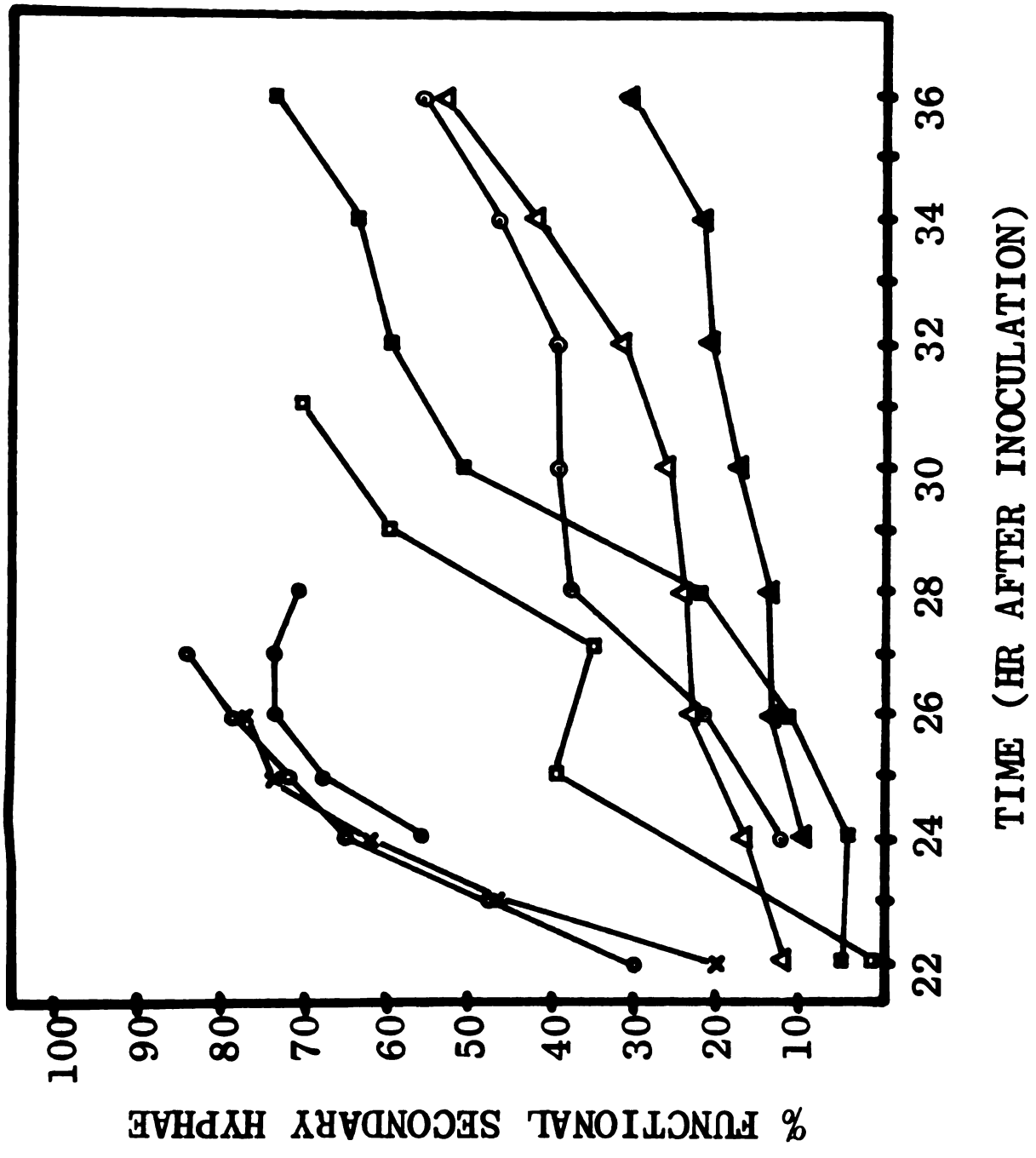

A
B

Fig 14. Effect of 5 sec UV radiation on development of functional secondary hyphae. Control (o—— o) had no UV treatment. Inoculated plants were irradiated 5 sec at 3 (●—— ●); 7 (▲—— ▲); 9 (Δ—— Δ); 11 (⊙—— ⊙); 13 (■—— ■); 18 (□—— □); and 20 (x—— x) hr after inoculation.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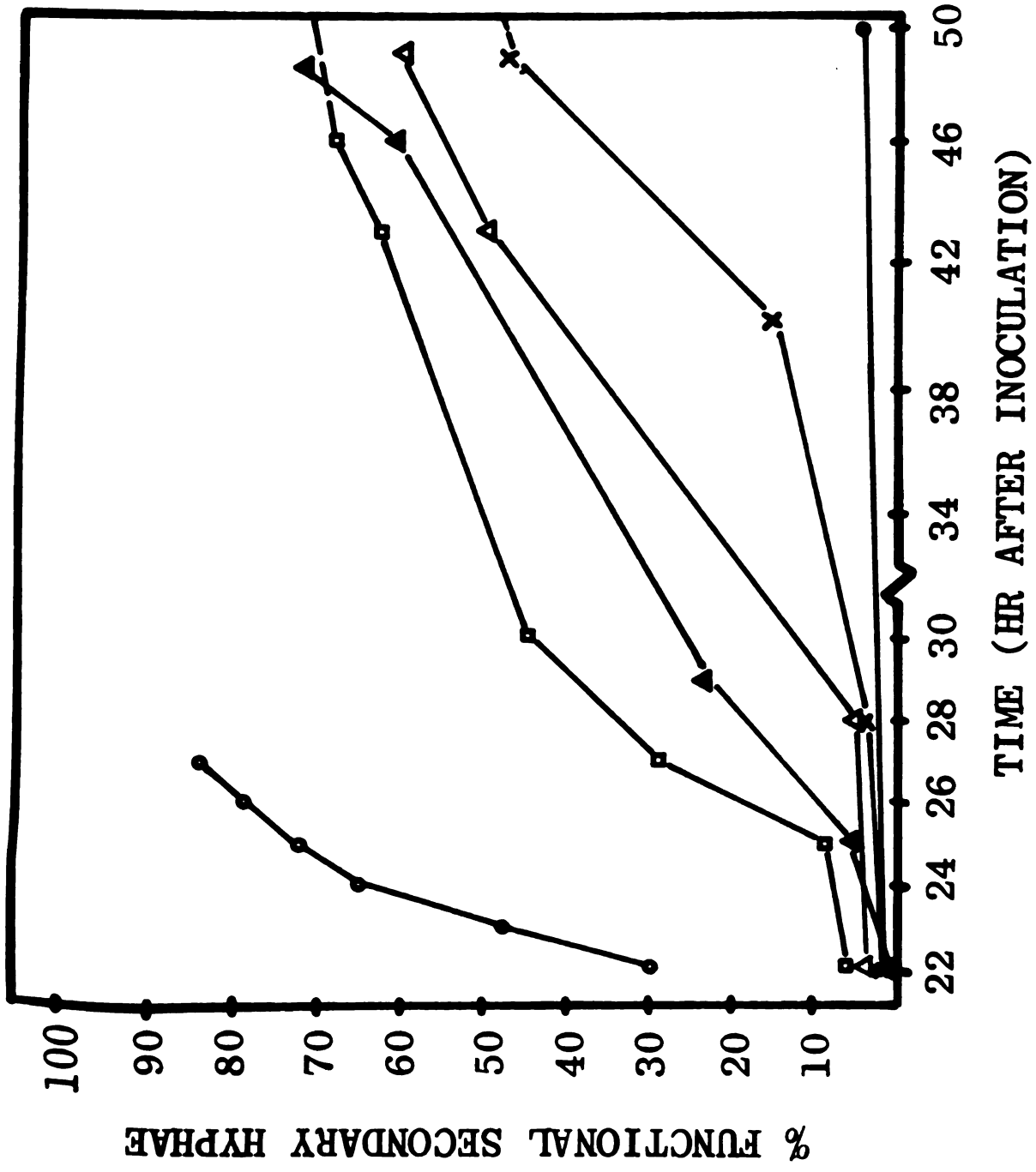
TIME (HR AFTER INOCULATION)

photoreactivation masking the effects of the UV treatments. Photoreactivation may also explain the increase in the percentage of functional secondary hyphae formed at other hr UV treatments were given since this reversible process can occur subsequent to the time of UV radiation. Twenty hr after inoculation, when plants were switched from darkness to low light (240 fc), the UV treatment had no apparent effect. When plants from all UV treatments were allowed to develop for 6 days, mildew pustules were evident on all plants. Therefore, 5 sec of UV radiation was not sufficient to kill the fungus on the leaf's surface.

The UV doses at various hr after inoculation were increased to 30 and 60 sec. Plants that were irradiated at 6, 8, 10, or 12 hr after inoculation (either 30 or 60 sec UV treatments) exhibited almost complete inhibition in the formation of functional secondary hyphae (Fig. 15 and 16). When inoculated plants were irradiated at the 14th hr for 30 sec, approximately 45-50% of the parasitic units formed functional secondary hyphae by 50 hr after inoculation. This percentage decreased to 20-30% when plants were irradiated for 60 sec at the 14 hr. When inoculated wheat plants were irradiated for 30 or 60 sec at 16 hr after inoculation, 60 and 50%, respectively, formed functional

Fig. 15. Effect of 30 sec UV radiation on the development of functional secondary hyphae. Control (o——o) had no UV treatment. Inoculated plants were irradiated for 30 sec at 14 (x——x), 16 (△——△), 18 (▲——▲), and 20 (□——□) hr after inoculation. Inoculated plants irradiated at 6, 8, 10, or 12 hr after inoculation (●——●).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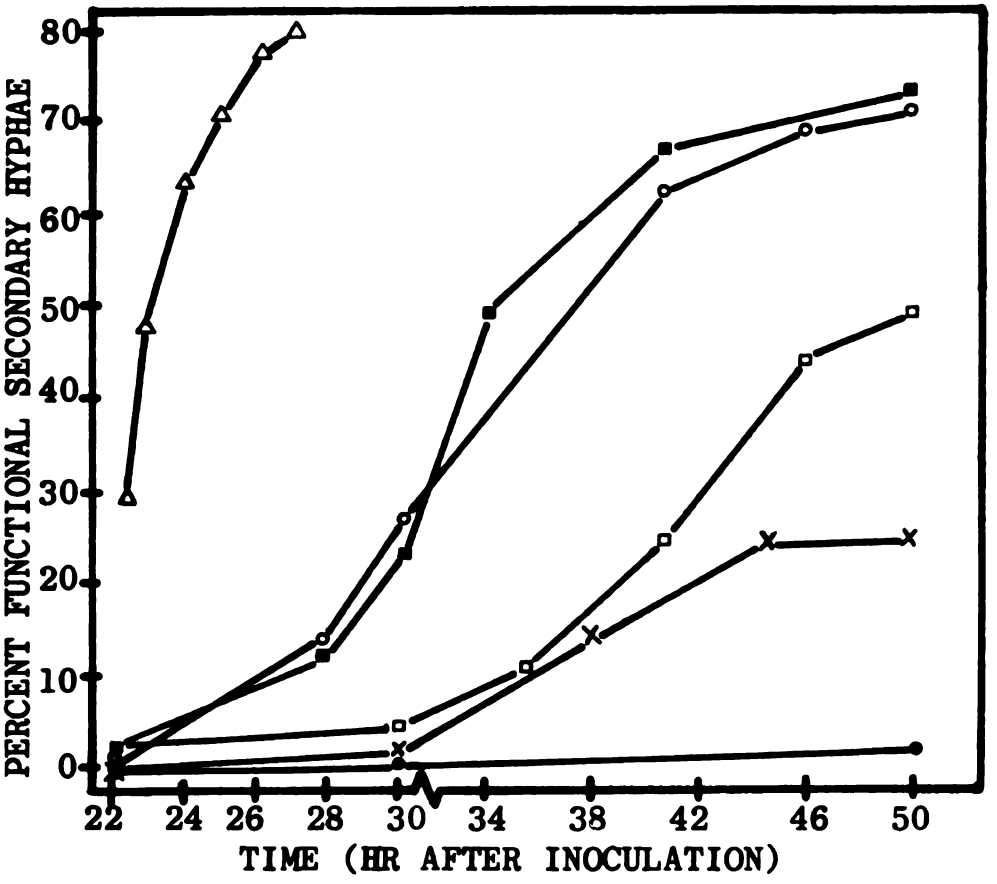
TIME (HR AFTER INOCULATION)

Fig 16. Effect of 60 sec UV radiation on the development of functional secondary hyphae. Control (Δ—— Δ) had no UV treatment. Inoculated plants were irradiated for 60 sec at 14 (x——x), 16 (□ —— □), 18 (o—— o), and 20 (■ —— ■) hr after inoculation. Inoculated plants irradiated at 6, 8, 10, or 12 hr after inoculation (●——●).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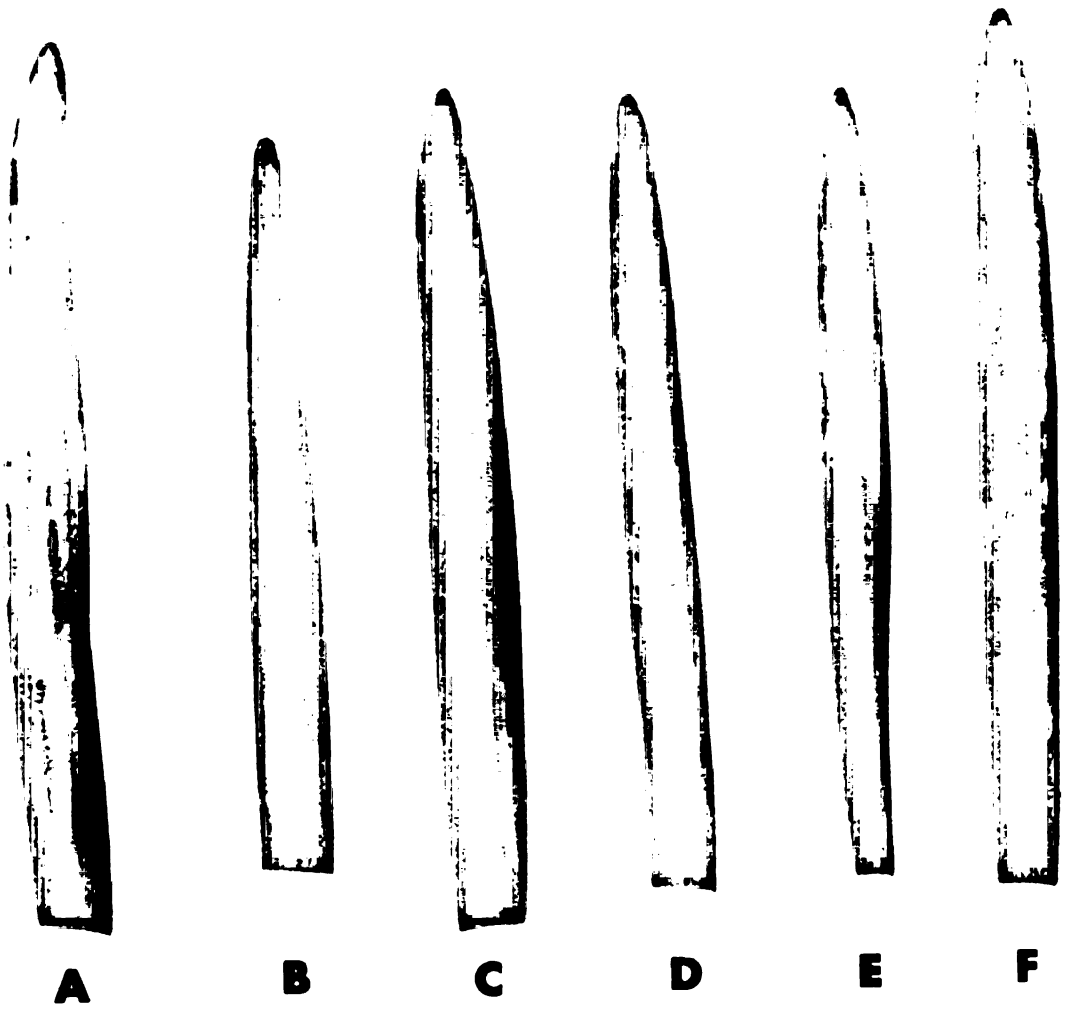
50
TIME (HR AFTER INOCULATION)

secondary hyphae after 49 and 50 hr. The percentage was greater when inoculated plants were irradiated at 18 or 20 hr after inoculation. No further increase in percent functional secondary hyphae were observed after 50 hr following the 60 sec UV treatments. Though the formation of functional secondary hyphae was delayed approximately 10-15 hr for all 60 sec treatments as compared to the control, the percent of the parasite units which developed secondary haustoria and eventually sporulated was greater as the time after inoculation the UV treatment was given increased. It appeared that UV treatments caused different degrees of injury to the parasite depending upon the hour after inoculation that plants were irradiated. The decrease in kill with the 60 sec UV treatments at 18 and 20 hr after inoculation may be due to the fact that a portion of the parasite (the haustorium) is now in the host cell and physically protected by the host cell. When UV irradiated plants were allowed to develop until 6 days after inoculation, there was a marked difference in the pustule development of the mildew (Fig 17). No pustule formed on plants irradiated 10 or 12 hr after inoculation. Few pustules formed on plants irradiated 14 hr after inoculation. Almost normal mildew development was found on plants

Fig. 17. Effect of 60 sec UV treatments on development of mildew pustules on wheat 6 days after inoculation. A) No UV treatment (control). Wheat irradiated for 60 sec at 10 (B), 12 (C), 14 (D), 16 (E), and 18 (F) hr after inoculation.


A
B
C
D
E
F

irradiated 18 hr after inoculation.

An experiment was performed to see if the epidermis could function as a barrier to UV radiation and protect the developing parasite. When an epidermal strip from an uninoculated plant was placed between the UV lamp and the inoculated portion of the test plant, the percentage of functional secondary hyphae was only slightly reduced as compared to no UV radiation. No functional secondary hyphae were observed on the inoculated, unprotected plants irradiated for 60 sec 10 hr after inoculation (Fig. 18).

Haustorial development and nuclear staining -- A young developing haustorium was first detected 12 hr after inoculation (Fig. 19A). The haustorium increased in size until the haustorial body attained maximum size by 18 hr after inoculation (Fig. 19B-F). The first haustorium observed with a nucleus was 16 hr after inoculation (Fig. 19C and 19D). In Figure 19E-H (18 and 20 hr), the nucleus was quite distinct and appendages had started to form at the ends of the haustorial body. The time of appearance of nuclei in haustoria is correlated with the decrease in sensitivity to UV light.

Uptake of ^{32}P by wheat seedlings -- Experiments were performed to determine the time required to get a measurable

Fig. 18. Effect of 60 sec UV radiation 10 hr after inoculation on the formation of functional secondary hyphae with (o——o) and without (●——●) a protective epidermal strip over the inoculated wheat leaves. Control plants (□——□) had no UV treatment.

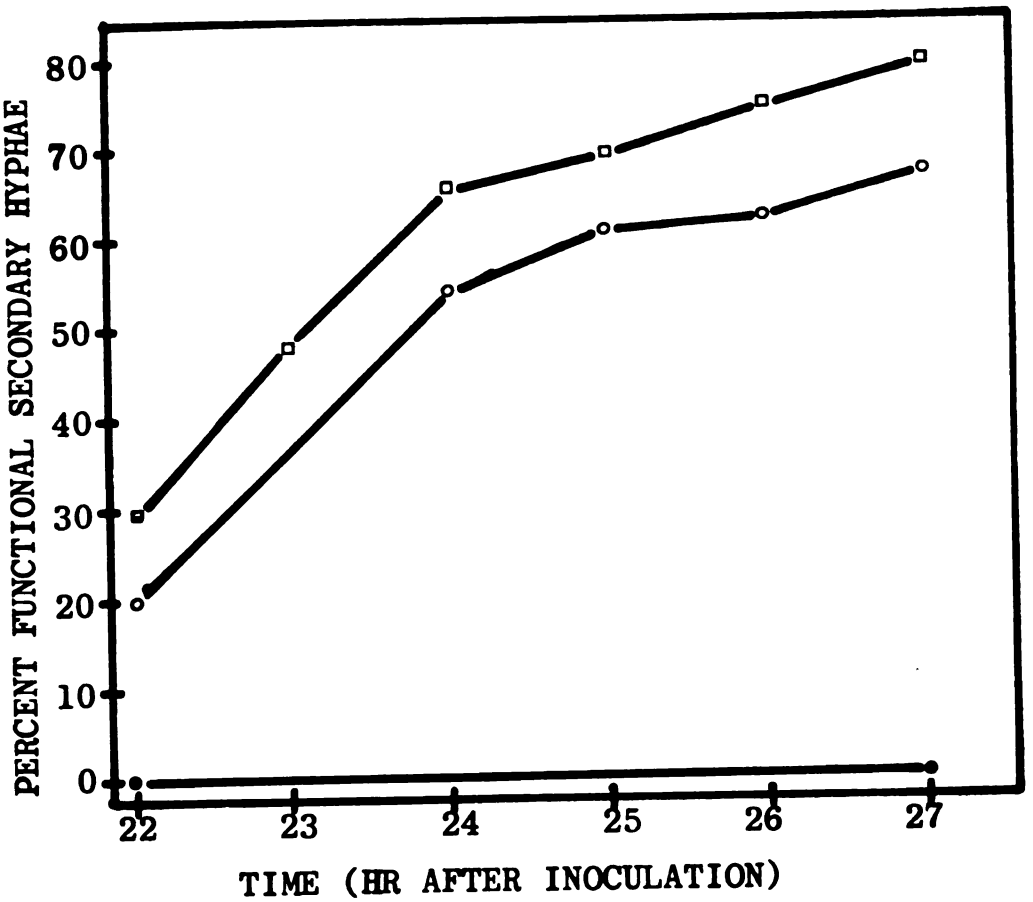

PERCENT FUNCTIONAL SECONDARY HYPHAE
80
70
60
50
40
30
20
10
0
22
23
24
25
26
27
TIME (HR AFTER INOCULATION)

Fig. 19. Development of the haustorium at various hr after inoculation. A) 12, B) 14, C) and D) 16 (note the presence of a nucleus within the haustorium), E) and F) 18 (note appendages beginning to form), and G) 20 hr after inoculation. H) Haustorium (a), mature appressorium (b), and conidial body (c) 20 hr after inoculation. (956-1434X)


A
B
C
D
E
F
G
H
a
b
c

amount of label (^{32}P) into the leaf, to show the effect of medium on the amount of ^{32}P uptake, to demonstrate the variability which exists from plant to plant, and to compare ^{32}P uptake into inoculated and uninoculated wheat plants.

Figure 20 presents the data for ^{32}P uptake by wheat seedlings 20 hr after the roots were placed in the medium containing ^{32}P. Plants that were grown in 190 uc/ml of ^{32}P had an activity between 1.9×10^3 to 32.2×10^3 counts per minute (CPM) in the first and second sections of one plant, and 66.6×10^3 to 69.0×10^3 CPM in the 2 sections of another. There was approximately a 2-3 fold difference in the uptake of ^{32}P by the 2 plants. The ranges for the wheat grown in 100 uc/ml and 20 uc/ml were 10.3×10^3 to 13.9×10^3 CPM and 2.4×10^3 to 4.0×10^3 CPM, respectively. In most cases the second centimeter of leaf contained higher activity than the first centimeter section.

The time required to detect various amounts of radioactivity in the leaf was determined by growing plants 1, 14, 24, and 48 hr in a ^{32}P medium. A small but significant amount of uptake was detected after 1 hr feeding (Fig. 21). The uptake of ^{32}P into the wheat seedling continued to increase until 24 and 48 hr. There was a 2-fold difference

Fig. 20. Counts per minute of 1 cm long leaf sections 20 hr after roots of wheat plants were placed in medium containing ^{32}P. Section 1 of the leaf is the first 1 cm after the tip had been discarded. Plant numbers 1 and 2 were grown in 20 uc/ml, numbers 3 and 4 in 100 uc/ml, and numbers 5 and 6 in 190 uc/ml of ^{32}P.

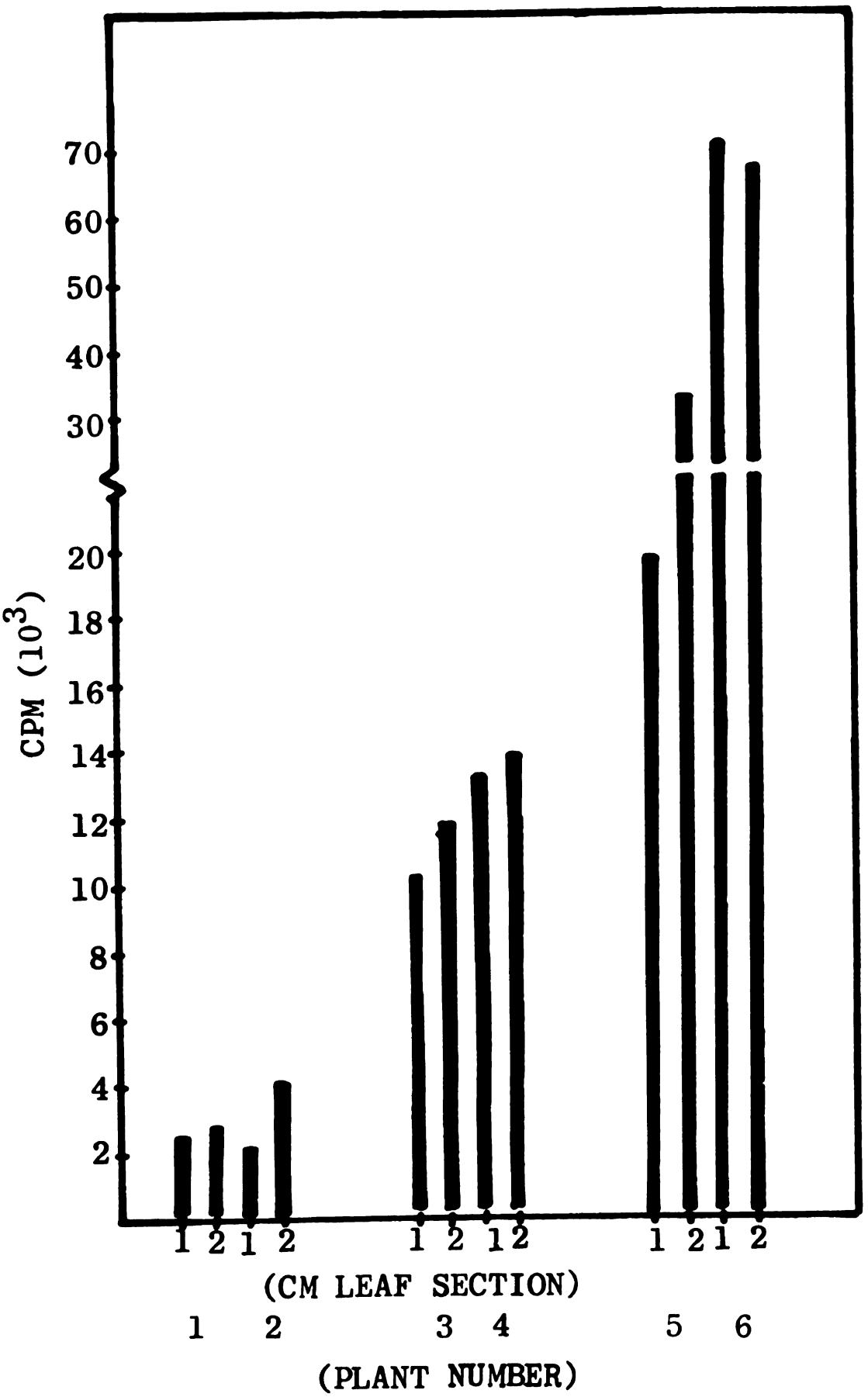
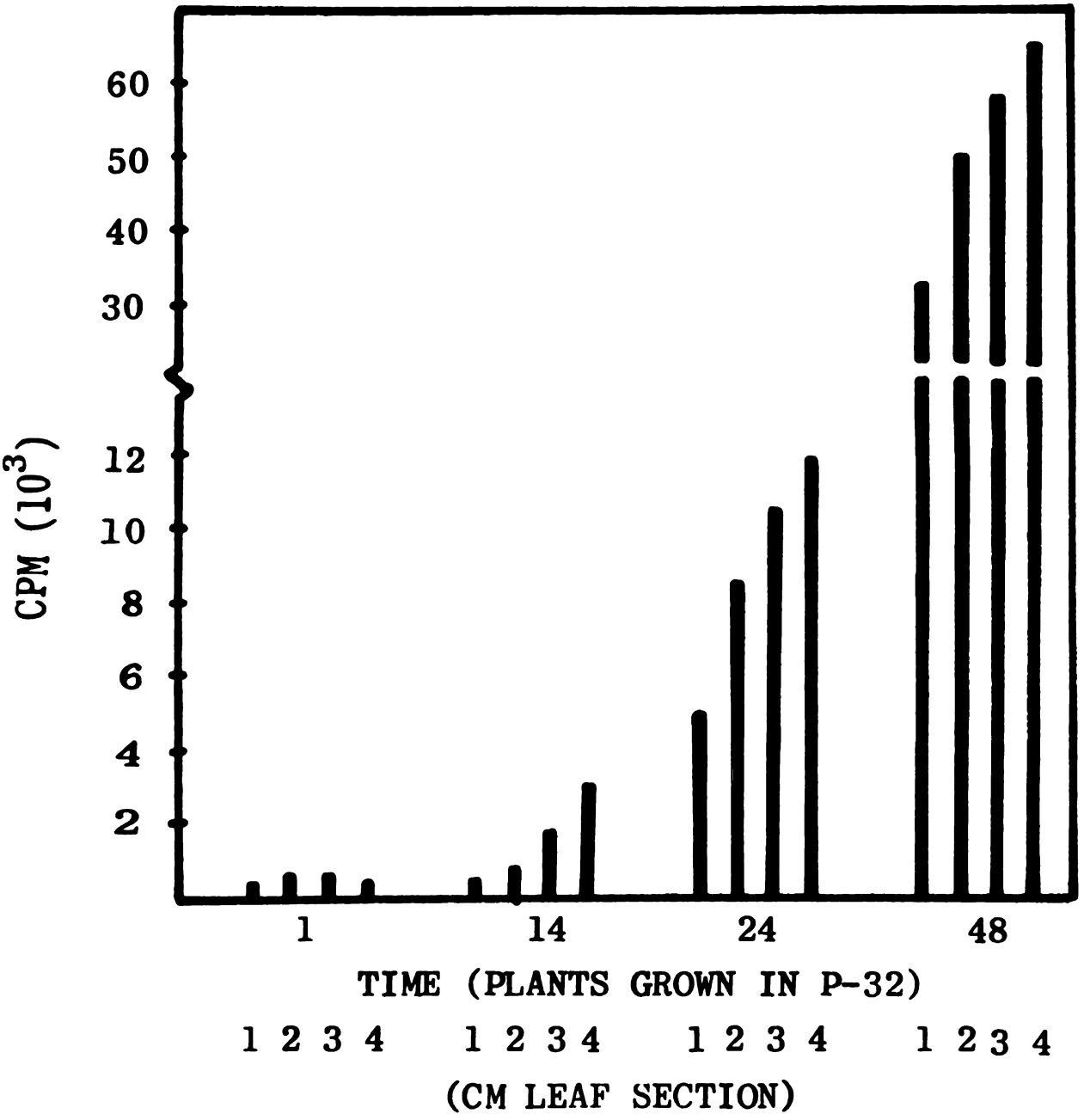

70
60
50
40
30
20
18
16
14
12
10
8
6
4
2
CPM (10^3)
1 2 1 2 1 2 1 2 1 2 1 2
(CM LEAF SECTION)
1 2 3 4 5 6
(PLANT NUMBER)

Fig. 21. Uptake of ^{32}P (20 uc/ml) by wheat plants 1, 14, 24, and 48 hr after roots were placed in the radioactive medium. Activity recovered is expressed as counts per minute (CPM). The first centimeter section of leaf is the distal one after the tip had been discarded.


60
50
40
30
12
10
8
6
4
2
CPM (10^3)
1
14
24
48
TIME (PLANTS GROWN IN P-32)
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
(CM LEAF SECTION)

be

Ho

a

32

Ho

KN

su

int

lat

dif

pla

you

cor

acu

ho

ap

pr

32

32

between the 1st and 4th leaf sections after a 48 hr feeding.

The uptake of ^{32}P by the wheat seedlings grown in Hoaglands solution was less than uptake of ^{32}P by wheat in a medium containing KNO_3 and Tes buffer (pH 7) (Table 1). ^{32}P activity of centimeter sections from wheat grown in Hoaglands solution was markedly less than plants grown in KNO_3 and Tes buffer.

Experiments were done to see if there might be a substantial increase in the uptake of ^{32}P early in the interaction of the host and parasite as compared to uninoculated wheat. Results indicated there wasn't a significant difference of ^{32}P uptake between inoculated and uninoculated plants (Table 2).

^{32}P in leaf sections increased very rapidly when young wheat leaves (Fig. 1) were placed into the medium containing ^{32}P (100 uc/ml) (Table 3). After 4 hr the activity was about 36×10^3 to 37×10^3 CPM. At subsequent hours (17, 20, and 24 hr) there did not appear to be an appreciable increase in the ^{32}P activity. This was interpreted to mean that the plant was probably saturated with ^{32}P after 4 hr in the solution.

To increase the efficiency of detecting uptake of ^{32}P into cut wheat leaves (Fig. 1), radioactivity of leaf

TABLE 1. The ^{32}P activity (CPM) per centimeter leaf section from uninoculated wheat grown in Hoaglands and KNO_3-Tes buffer solutions

		Medium					
		Hoaglands			KNO_3Tes		
		Plant Number					
		1	2	3	4	5	6
leaf section[a]	1	147	138	1,754	12,404	17,451	13,166
	2	134	131	1,695	18,022	28,677	21,057
	3	151	195	2,498	25,062	35,381	28,400
	4	124	303	3,946	29,234	40,146	28,965

[a]First 4, 1-cm sections excluding the tip. The first centimeter section of leaf (1) is the distal one after the tip had been discarded.

TABLE 2. Comparison of ^{32}P uptake between uninoculated and inoculated wheat plants.

Plant Number	Hr ^{32}P Added	Activity[a]	Hr of Reading	(CPM) Leaf Section[b] 1	2
		Uninoculated Plants (Control)			
1	6	28	24	5,241	6,322
2	6	28	30	6,825	8,826
3	12	28	24	4,298	4,501
4	12	28	30	11,121	10,793
5	6	20	24	2,281	3,433
6	6	20	30	5,733	8,951
7	12	20	24	3,489	3,596
8	12	20	30	5,204	6,720
			Total	44,192	53,142
		Inoculated Plants			
9	6	28	24	7,821	8,370
10	6	28	30	3,536	5,884
11	12	28	24	3,759	4,416
12	12	28	30	13,432	15,132
13	6	20	24	3,973	4,685
14	6	20	30	5,576	7,227
15	12	20	24	2,897	3,273
16	12	20	30	3,150	5,063
			Total	44,144	54,050

[a]Activity (uc/ml)

[b]First 2, 1-cm sections excluding the tip. The first centimeter section of leaf is the distal one after the tip had been discarded.

TABLE 3. Uptake of ^{32}P (100 uc/ml) by wheat leaves 4, 17, 20, and 24 hr after being placed in the radioactive solution.

Leaf Section[a]	Time (hours in ^{32}P solution) 4	17	20	24
1	37,702[b]	41,198	44,589	22,587
2	36,227	37,200	54,968	17,907

[a]First 2, 1-cm sections excluding the tip. The first centimeter section of leaf is the distal one after the tip had been discarded.

[b]CPM determined in an annular-well G-M tube.

sections was determined in a Packard Liquid Scintillation counter. The amount of time in which the plants were fed ^{32}P ranges between 0-5 hr. Only the first centimeter section below the tip of the leaf was counted. The uptake of the ^{32}P was rapid over a 5 hr period (Table 4). After 5 hr the activity was quite high, ca. 6.7×10^4 to 7.6×10^4 CPM. There was, on the average, about a 120-fold increase over the control (zero hour) in the activity of the centimeter section tested after 1 hr of being in the ^{32}P solution. After the wheat had been growing in the labeled medium for 2 hr, one of the leaves had an extremely high activity as compared to another (248×10^3 and 3.9×10^2 CPM). Since this activity was not detected in the following 2 hr, it was assumed that contamination may have contributed to the high counts obtained. The important information obtained from these experiments was that 5 hr in ^{32}P was considered sufficient to obtain considerable activity of ^{32}P in the upper portions of the leaf.

Transfer of ^{32}P and ^{35}S from host to parasite -- An attempt was made to detect an initial interaction between the host and parasite with the use of ^{32}P and ^{35}S. As indicated earlier, a haustorium was evident by 12 hr after inoculation. A distinct nuclear body was seen in

TABLE 4. Uptake of ^{32}P (100 uc/ml) by wheat plants that were cut at the crown and placed into the radioactive solution.

Plant Number	Hour[b]	Uptake of ^{32}P[a] CPM
1	0	38
2	0	42
3	1	8255
4	1	1331
5	2	248158
6	2	3939
7	3	43836
8	3	40147
9	4	122947
10	4	100772
11	5	677303
12	5	760225

[a] ^{32}P uptake was a measure of the 1st cm section below the tip. CPM determined with a Packard Liquid Scintillation Counter.

[b] Indicates amount of time that cut plant was in the ^{32}P solution.

the haustorium by the 16th hr. The development of appendages at the ends of the haustorial bodies was first observed 18 hr after inoculation. Masri (20) found that there was an increased succinate dehydrogenase activity in the vicinity of the haustorium around 12 hr after inoculation. Therefore, some time between the onset of penetration and 24 hr after inoculation, it should be possible to demonstrate transfer of ^{32}P and ^{35}S from host to parasite. Inoculated "labeled" wheat leaves were stripped with parlodion to separate the fungus from the host. The parlodion was analized to determine if the parasite had obtained any labeled material from the host.

In preliminary experiments wheat plants were placed in ^{32}P (20 uc/ml) 20 hr prior to inoculation. The mildew on the leaf surface was removed in parlodion strips 10-30 hr after inoculation, dried on planchets, and CPM determined with an open end G-M tube (Table 5). No clear evidence of transfer or ^{32}P from host to fungus on the leaf surface was obvious.

Plants that were inoculated at the same time the roots were placed in the ^{32}P (20 uc/ml) solution gave somewhat similar results (Table 6). The parlodion strips made from plants 10 hr after inoculation had essentially the

TABLE 5[a]. Activities (CPM) of parlodion strips containing mildew conidia. Parlodion was used on 4 wheat plants per sample.

	Background Counts	Control[b]	Time (Hr after inoculation) 10	12	24	28	30
	13	17	19	15	28	25	23
	20	24	17	14	34	14	35
	18	12	11	19	34	25	27
	20	15	24	21			
	14	31	19	19			
CPM	15	15	15	17			
	15	18					
	18	20					
	20	17					
		30					
		27					

[a]Wheat roots were placed in ^{32}P (20 uc/ml) solution 20 hr before inoculation.

[b]Parlodion strips were made at 10-30 hr on labeled uninoculated wheat plants.

TABLE 6[a]. CPM of parlodion strips containing mildew conidia. Each sample contained parlodion strips from 4 wheat plants.

Background Counts	Control[b]	Time (Hr after inoculation) 10	24
22	13	17	46
14	21	18	53
15	18	22	53
24	23	27	35
23	25	19	40
22	22	22	38
14	25	18	39
17	11	27	40
16	29	21	44
	24	20	
	14	22	
	28	20	
	23		

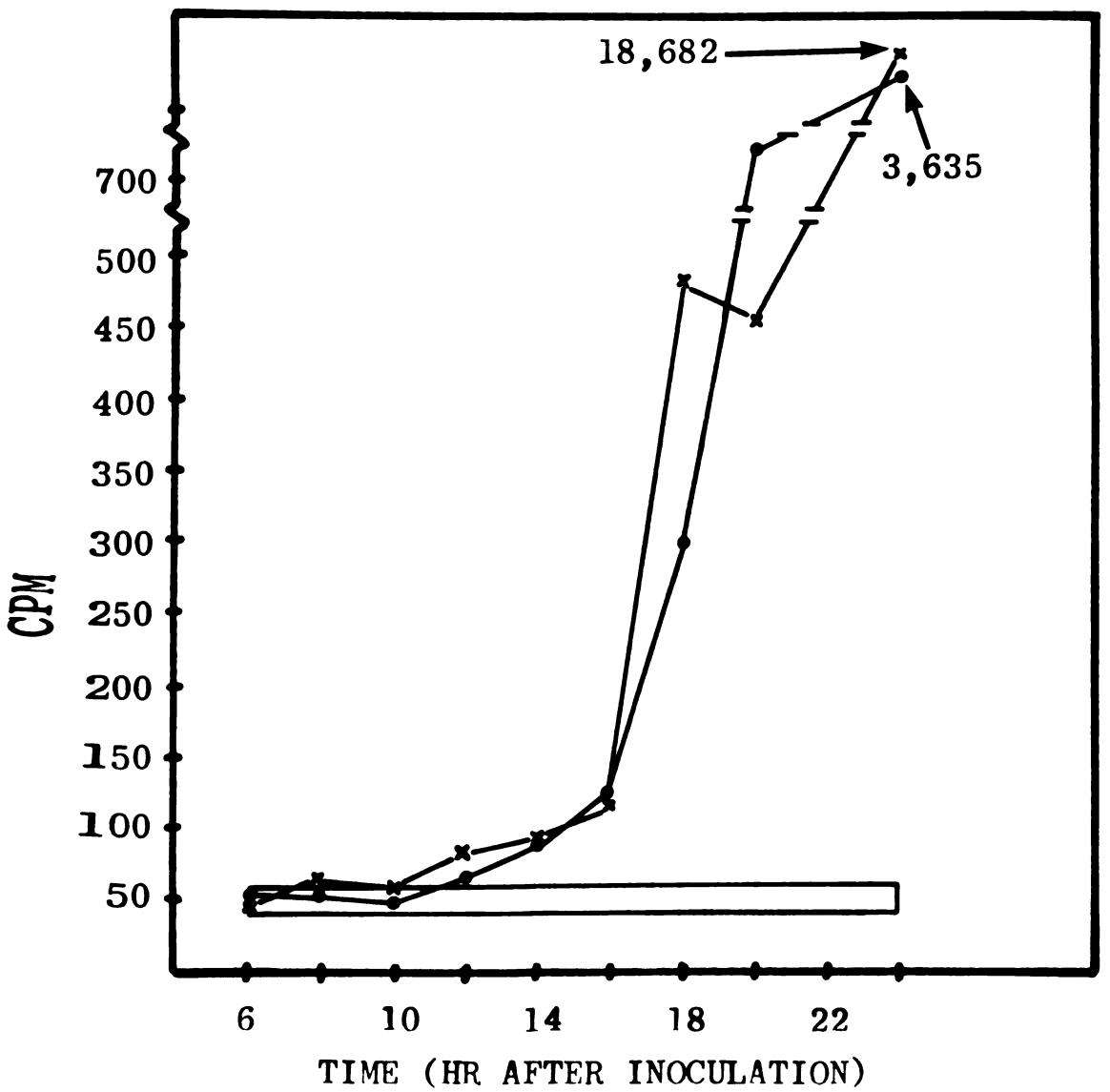
[a]Wheat plants were inoculated at the same time that the roots were placed in the ^{32}P (20 uc/ml) solution.

[b]Parlodion strips were made at 10 and 24 hrs on labeled uninoculated wheat plants.

same CPM as parlodion strips from uninoculated plants. However, the results in Table 6 indicate more ^{32}P activity in parlodion strips made from plants 24 hr after inoculation. This is interpreted to be evidence for transfer of ^{32}P from the host to the parasite by the 24th hr after inoculation.

In order to increase the efficiency of detecting isotope transfers, a higher ^{32}P activity (100 uc/ml) was used, cut leaves were placed in radioactive solutions 5 hr prior to removal of the parasite in parlodion, and radioactivity in parlodion strips was determined in a liquid scintillation counter. The transfer of ^{35}S (100 uc/ml) from host to parasite was also studied with the same methods. There was no apparent difference between the kinetics of transfer for ^{32}P and ^{35}S (Fig. 22). A slight ^{32}P and ^{35}S activity was first detected in the parlodion strips containing the parasite about 12 hr after inoculation. Before the 12th hr essentially no activity above background was detected. Sixteen hr after inoculation there was approximately a 2 to 3-fold increase in activity as compared to the control. Hours subsequent to the 16th exhibited a marked increase in ^{32}P and ^{35}S activities (ca. 3×10^2 to 18.7×10^3 (CPM).

Fig. 22. Transfer of ^{32}P (x——x) and ^{35}S (●——●) from leaf to parasite.


18,682
3,635
700
500
450
400
350
300
250
200
150
100
50
CPM
6
10
14
18
22
TIME (HR AFTER INOCULATION)

DISCUSSION

The events that take place during the early interaction which determines whether a functional secondary hypha will form, and consequently whether a compatible relationship will develop, apparently occur sometime between 6-20 hr (dark period) after inoculation. The effects of environmental treatments (mainly high light intensities) during this dark period were used to indicate the times after inoculation which were critical for the development of secondary hyphae and to further clarify the kinetics of the infection process.

Low light treatments (240 fc given for 2 hr periods) had no significant effect upon the development of secondary hyphal initials. With light intensities of 1400 and 2400 fc (for 2-4 hr) there was a partial stimulatory effect on a portion of the total population of developing fungal units while the remaining portion was either unaffected or inhibited in the development of secondary hyphal initials. Although there were statistically significant differences,

the biological significance of these results is not known. Possibly the effects of the light were on the host in supplying nutrients for the development of the parasite. Other effects may be a result of the light inactivating or delaying chemical processes necessary for the development of secondary hyphae. The formation of secondary hyphal initials was inhibited more with 15 min high light treatments (2400 fc) at the 7th or 8th hr, while development of functional secondary hyphae was inhibited most by applying high light at 9 or 10 hr after inoculation. Since there was a differential response of secondary hyphal initials and functional secondary hyphae to high light, it further substantiated that these 2 components of the infection process were indeed separately induced stages. The greatest effects upon the formation of secondary hyphae occurred early during the "dark period." It is difficult to assess the biological significance of the differential effects of high light with our present knowledge.

A high percentage of the appressoria had formed penetration pegs by 10 hr after inoculation (Fig. 12). Wehther these pegs were just small protrusions on the underside of the mature appressoria or whether they had actually penetrated into the epidermal cell was not determined.

Since a high percentage of conidia were in the initial stages of penetration by the 10th hr, the high light treatments may have affected this penetration process, thereby delaying the formation of secondary hyphae.

The development of haustoria has been studied microscopically (22). Young, developing haustoria were rarely seen prior to 12 hr after inoculation. The development of a haustorium almost always led to the establishment of functional secondary hyphae. Failure to produce a haustorium has meant failure to produce functional secondary hyphae and secondary haustoria.

Sensitivity to UV radiation was used to further define the stages of development of mildew in the establishment of compatible, functional relations with the wheat host. During development of the parasite in primary infection, the nucleus in the appressorium divides and a daughter nucleus migrates into the haustorium. The nucleus in the haustorium should be more protected from UV than the nucleus (or nuclei) in the appressorium. Thus, if an appropriate dose of UV is given during primary infection, prior to the migration of a nucleus into the haustorium, most of the parasitic units should be killed. After entry of a nucleus into the haustorium, physical protection by

the epidermis should allow the fungus to survive.

Developing thalli of the mildew fungus were protected by an epidermis from another plant during UV treatments, indicating that the epidermis was an effective barrier against UV radiation. The percentage of functional secondary hyphae was used as a basis for determining sensitivity to UV radiation. The fact that essentially no functional secondary hyphae are formed after UV irradiation at 12 hr after inoculation suggests that the parasite has not developed sufficiently in the host to be protected by the host. The 20-30% of applied conidia which formed functional secondary hyphae after UV irradiation at 14 hr is taken to mean that 20-30% of the fungal units have developed sufficiently to be protected by the host. Almost all parasitic units are protected by 18 hr after inoculation. Further evidence for the protection of the parasite by the host at the various hours of UV treatments is demonstrated by the amount of pustule development 6 days after inoculation. The formation of pustules appeared to be proportional to the final percentage of the delay involved in the formation of functional secondary hyphae.

The decrease in sensitivity to UV is correlated with the appearance of nuclei in haustoria. It seems

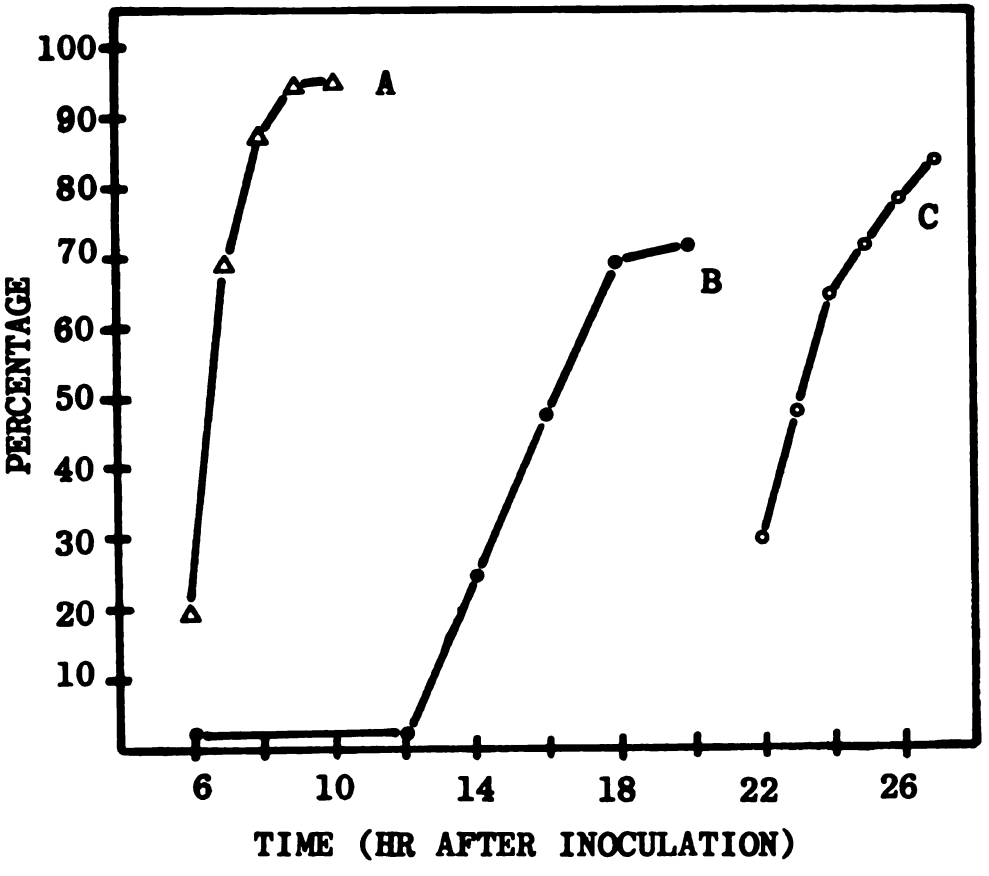
logical, therefore, though not proven, that the site of action of the UV radiation is the nucleus, which is protected in the haustorium from the 30 and 60 sec UV treatments. This eventually leads to the production of secondary hyphae.

As shown in Figure 23 the kinetics of UV sensitivity followed essentially the same pattern as the formation of mature appressoria and functional secondary hyphae. This indicates a separate, synchronized phase in the development of the mildew fungus and allows, quantitatively, the prediction of what proportion of the conidial population is in a particular developmental stage.

There was greater delay in the formation of functional secondary hyphae if the inoculated plants were irradiated 14 of 16 hr as contrasted to 18 or 20 hr after inoculation, or the control. The delay is probably related to such factors as the degree of injury to the various cellular components, the proportion of each affected cellular component protected in the epidermal cell and the number of lesions required to inactivate each component.

Most of the results and interpretations pertained to changes in morphological events -- namely, the formation of the different stages of the infection process. Only

Fig. 23. Comparison of the kinetics of formation of mature appressoria, A, and functional secondary hyphae, C, with the kinetics of sensitivity to 60 sec UV treatments, B. Sensitivity to UV treatment at the various hrs recorded as the percent of functional secondary hyphae formed 50 hrs after inoculation.


100
90
80
70
60
50
40
30
20
10
PERCENTAGE
A
B
C
6
10
14
18
22
26
TIME (HR AFTER INOCULATION)

preliminary work has been devoted to the physiology of the host-parasite interaction. This involved the transfer of ^{32}P and ^{35}S from the host to the parasite. Both ^{32}P and ^{35}S transfers were similar. It appeared that possibly 2 mechanisms were in operation for the transfer of the isotopes: One, between the 12-16th hr (slight amount of transfer) and the other between the 16-24th hr (high transfer) after inoculation. The first indication of a high transfer of radioactive substances corresponded to the time when appendages were beginning to form at the ends of the haustorial bodies, and to the appearance of a distinct nucleus within the haustorium.

One question, as of yet not fully determined, was whether this so called "transfer" of radioactivity was (1) actually a transfer from the host cytoplasm to the haustorium and then transported up into the external fungal structures, (2) whether the parlodion strips were contaminated as a result of the isotope leaking through the penetration area, or (3) whether, upon stripping the parasite from the host, the haustorium was pulled out of the epidermal cell and became lodged within the parlodion. Data which supports the first idea is illustrated in Figure 13. By using parlodion to separate the developing

parasite from the host, it was possible to demonstrate that the haustoria were left within the epidermis upon stripping the external structures from the leaf's surface. Occasionally, a globular body located at the end of the mature appressorium could be extracted from the epidermis. This was a rare event only found during the very early interactions between the host and parasite (ca. 10-12 hr after inoculation) and was never observed in later hours. If there was a leakage of isotopes through the penetration sites, it was assumed that this would have been detected early during the parasitic interaction. However, there was a high percentage of appressoria which exhibited penetration knobs (pegs) between 10 and 12 hr after inoculation (Fig. 12). Since radioactivity was not prominant in the parlodion strips 10-12 hr after inoculation, it was assumed that leakage to the external environment was not a major problem. It appeared, therefore, that there was a legitimate transfer of ^{32}P and ^{35}S from the host via haustorium to the external structures.

In previous studies (20), it was found that penetration occurred early during the host-parasite interaction (ca. 10th hr). The results presented here confirm and define this penetration process more quantitatively. In

resistant reactions functional haustoria are not formed (20), but penetration is attempted by the fungus. In these studies haustoria were first detected 12 hr after inoculation. It would therefore appear that either susceptibility or some resistance is expressed sometime around 10 hr and not later than 12 hr after inoculation. Since this time period is fairly well-defined, it would appear that possibly changes of susceptibility or resistance could be brought about by changes in specific environmental conditions during these early interactions.

A future experiment which may also give an insight into the mechanism or time after inoculation of gene expression would be to study the kinetics of isotope transfer with plants that carried different genes for resistance. If these genes are acting early and at different times during the host-parasite interaction, then one might expect a change in the transfer mechanisms. This possibly could be used as a means for identifying and characterizing certain genes for resistance.

Also of interest would be to pulse-label susceptible and resistant wheat with specific amino acids and determine the radioactivity in protein fractions from inoculated epidermal strips. Here, one might speculate that differences

in labeled protein fractions would be found, presumably at the onset of penetration throughout the development, or lack of development, of haustoria. If differences could be detected in protein fractions for plants carrying various genes for resistance, then it would appear that these differences could be used for further characterization of a particular host-parasite interaction.

The various stages of the initial infectious process must be precisely defined in order to make quantitative analyses of the host-parasite interactions, and it is believed that the clarification of this process is necessary in understanding cause and effect relationships.

SUMMARY

After the author repeated previous work (20, 21, 22), and on the basis of morphology and sensitivity to various environmental conditions, there is good evidence that there are several distinct components of the initial infection process of E. graminis on wheat. They are: 1) germination, 2) formation of appressorial initials, 3) maturation of appressoria, 4) formation of haustoria, 5) formation of secondary hyphal initials, and 6) formation of functional secondary hyphae. Optimal environmental conditions were determined for the development of functional secondary hyphae. The development of these stages was synchronous under the environmental conditions used.

The formation of functional secondary hyphae was used as a criterion for compatibility between the host and parasite. Darkness (6-20 hr after inoculation) was necessary for the synchronous development of secondary hyphae. It was therefore during the "dark period" that various environmental treatments were given in order to better understand

the establishment of powdery mildew with its host.

Low light treatments (240 fc) had no observable effect upon the development of secondary hyphal initials. Medium light treatments (1400 fc) exhibited both stimulatory and inhibitory effects upon different portions of the total population of mildew spores. Fifteen minute high light treatments (2400 fc) gave differential responses between the development of secondary hyphal initials and functional secondary hyphae. This suggested that they were 2 separate components of the infection process.

High temperature and high relative humidity treatments both inhibited the development of secondary hyphal initials. However, the significance of these results was not determined.

By embedding mildew conidia in parlodion, it was determined that approximately 60% of the spores had developed penetration pegs by the 10-12th hr after inoculation. Haustorial buds were first observed at the 12th hr after inoculation. Inoculated plants irradiated with UV light indicated that a portion of the applied conidia had developed sufficiently and were protected within the epidermal cell by 14 hr after inoculation. The migration of a nucleus into the haustorium was first detected by 16 hr, while appendages

formed at the ends of haustorial bodies by 18 hr after inoculation. Pustule development 6 days after inoculation also indicated the degree of haustorial development within the epidermis. If the haustorium had not sufficiently established itself within the epidermal cell, UV radiation killed the fungus and pustules were not formed.

The uptake of ^{32}P into wheat plants was measured and the kinetics of transfer of radioactive substances from the host to the parasite were determined. Interpretation of the results indicated that 2 mechanisms of transfer were involved - one, which occurred from 6-16 hr and the other from 16-24 hr after inoculation. The first indication of a high transfer of either ^{32}P or ^{35}S was at the 18th hr, and corresponded with the formation of appendages on the haustorial bodies.

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