# THE INITIAL PHASE OF PATHOGENESIS IN VICTORIA BLIGHT OF OATS

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ABSTRACT

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by Olen C. Yoder

The early events in invasion of oat tissue by Helminthosporium victoriae were studied by histological and biochemical methods. H. victoriae is selectively pathogenic to certain cultivars of oats. It produces a substance which is required for pathogenicity and which is toxic only to susceptible plants. An attempt was made to correlate invasion of host tissue, as determined histologically, with the presence of toxin at the site of invasion.

Conidia of a pathogenic H. victoriae isolate germinated, produced appressoria, and penetrated susceptible leaves within 8 hours after inoculation. By 12 hours many penetration pegs extended into the mesophyll cells and a few long hyphae traversed the entire width of the leaf. The fungus continued to develop and by 48 hours the leaf tissue was full of hyphae. Yet there was little or no apparent effect on host cells 48 hours after inoculation. H. victoriae appears to have an initial compatible relationship with its host which is typical of the host-parasite relations of many plant diseases.

Spore germination and hyphal growth sequences on the surface of H. victoriae resistant oat leaves were similar to those observed on susceptible leaves. However, there were fewer penetrations of resistant leaves and less fungal growth in resistant tissue 8-12 hours after inoculation. Therefore, resistance is expressed less than 12 hours after inoculation. In resistant leaves, penetration pegs were most commonly found in the epidermal

cells, although invasion of the first or second layer of mesophyll cells was not unusual. The amount of fungal growth in resistant tissue after 48 hours was the same as that found after 12 hours.

In most cases, resistance was detected as early limitation of fungal growth. Occasionally, after 24 or 48 hours, epidermal cell wall thickenings below appressoria or cells filled with granular amber material were observed. It was concluded that these visible host responses occurred too late and too infrequently to account for resistance to this disease. Similar host reactions have been observed in many other disease systems which is another indication that Victoria blight development is similar to development of many other diseases.

When spores of a nonpathogenic H. victoriae isolate or spores of H. carbonum, a pathogen of corn, were placed on oat leaves, a typical resistant reaction occurred. When 1.6 $\mu$g/ml HV-toxin was present in the infection drops on susceptible leaves, a typical susceptible reaction occurred. In resistant leaves the reaction to these isolates was not changed by the presence of toxin. These results indicate that toxin confers pathogenicity to the fungus and that toxin is required in the initial stage of disease development.

Ascospores of Neurospora tetrasperma, a saprophyte, germinated and produced a large amount of mycelium on the surface of H. victoriae-susceptible or -resistant oat leaves. However, N. tetrasperma did not attach itself to the leaf surface and did not attempt to penetrate by 48 hours after inoculation. There was no effect on leaf tissue. When ascospores were applied to susceptible leaves in the presence of toxin, there was no attachment of the fungus to the leaf after 24 hours but after 48 hours the fungus

had colonized the leaf tissue and the leaf cells had collapsed. There was no effect on resistant leaves when they were exposed to N. tetrasperma in the presence of toxin. The action of a pathogen differs from the action of a saprophyte if toxin is available to both. H. victoriae appears to be more than a saprophyte which lives on tissues killed by its toxin.

Some early biochemical effects of infection were studied and compared with the known effects of toxin. Increased electrolyte loss from infected susceptible tissues was detected 6 hours after inoculation, which suggests that nutrients from the host are available to the fungus at or before the time of penetration. Since the primary action of the toxin is thought to be on the cell membrane, this indicates that toxin is necessary for initial invasion of tissue by the fungus. Incorporation of $C^{14}$ labelled amino acids was inhibited in susceptible leaves 8 hours after inoculation. Cell permeability and amino acid incorporation effects of infection parallel the known effects of toxin. Although $C^{14}$ amino acid incorporation was inhibited soon after infection, there was a substantial amount of incorporation after 12 hours which indicates that the synthesizing machinery was at least partially active. Activity of dehydrogenase enzymes was not affected in susceptible tissues 12 hours after inoculation but activity was greatly reduced after 24 hours. The capacity for metabolic activity may be maintained for some time after the cells are initially affected.

The data on dehydrogenase activity and amino acid incorporation suggest that cells are not dead or metabolically inactive either before or soon after they become infected with the fungus.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued) Page

LIST OF FIGURES AND TABLES

LIST OF FIGURES AND TABLES (Continued) Page

# INTRODUCTION

Victoria blight of oats, caused by Helminthosporium victoriae Meehan and Murphy, has been developed as a model disease for studying pathogenesis and the nature of disease resistance. This disease is exceptional because a highly active host specific toxin has been isolated from cultures of the causal fungus. The toxin has been shown to reproduce all the known visible and biochemical effects caused by the fungus. There are several lines of evidence which indicate that the toxin is the primary determinant of the disease and that resistance to the disease is equivalent with resistance to the toxin (69). In this model, the host-parasite relationship is greatly simplified since the complication of two interacting metabolic systems is avoided.

This study was initiated with four principle objectives. First, the effects of the causal fungus on its host were investigated by histological methods and compared to host-parasite relationships in other plant diseases. Second, experiments were designed to determine whether or not toxin is required by the fungus for initial colonization of the host. Third, microscopically observable host responses to fungal attack were analyzed and an attempt was made to determine their role in disease resistance. Fourth, biochemical changes which occur very soon after infection were studied and were compared with the early changes which result from toxin treatment.

Victoria blight of oats has not been accepted as a generally valid model for plant disease development. It has been discounted as being atypical (79, 83), and doubts about the ability of toxin to confer pathogenicity to the fungus have been raised (35). Such questions are reconsidered in some of my experiments and discussions.

# LITERATURE REVIEW

H. victoriae was first recognized as the incitant of Victoria blight in 1946 by Meehan and Murphy (44). They later reported highly active metabolic by-products in culture filtrates which were selectively toxic to oats derived from the cultivar Victoria (45). This result was confirmed by Luke and Wheeler (42) and the toxin was isolated and purified by Pringle and Braun (58). The literature on HV-toxin has been reviewed extensively several times (60, 64, 69, 83). Histological studies of host-parasite interactions will receive major emphasis in my review, since previous reviews covering the host-specific toxins have given little attention to this. I am also reviewing additional materials on disease resistance.

Visible disease symptoms, as described by Meehan and Murphy (44), are necrosis of basal portions of the stem, root rot, and striping or reddening of the leaves. Leaf spots can occur under certain conditions (7). The toxin, in the absence of the fungus, can reproduce all these symptoms (41). Biochemical effects which have been described for both toxin and infection are increased respiration, increased ascorbic acid oxidation, and increased permeability of cell membranes (27, 82). Physiological effects known to be caused by the toxin are reduction in uptake and incorporation of $P^{32}$ into organic compounds, decrease in uptake and incorporation of

$C^{14}$ amino acids, loss of ability of root hair cells to plasmolyze, increase in apparent free space, lysis of cell wall free protoplasts, inhibition of seed germination, and inhibition of gibberellic acid induced ∝-amylase production (64).

The histology of infection by H. victoriae was studied in detail by Paddock (57). Spore germination and growth of germ tubes were similar on susceptible and resistant oat leaves. There were fewer appressoria and attempts to penetrate on resistant than on susceptible leaves, indicating that resistance was expressed even before penetration occurred. In susceptible tissues, the hyphae grew freely and abundantly with little or no host response during the early stages. About 48-72 hours after infection the host cells began to collapse, even in advance of the hyphae. The fungus penetrated epidermal cells of resistant leaves and stopped there without inducing a host response, or grew 2-3 cells farther and caused a hypersensitive reaction. The host response differed somewhat from one cultivar to another. Sometimes cell wall thickenings were observed below appressoria within 18 hours after inoculation. Thus the histological evidence indicates that H. victoriae attacks its host in a manner similar to that of many other fungi.

The notion that facultative and obligate parasites behave differently in their respective hosts was suggested early (12) and still persists in spite of known similarities (71). According to the early views, obligate parasites have an initial compatible relationship with susceptible hosts and cause a hypersensitive reaction in resistant hosts. Hypersensitivity was thought to

account for resistance to obligate parasites. Facultative parasites were thought to kill susceptible cells on contact or in advance of the growing hyphae. Resistance was thought to be due to a number of causes such as morphological barriers or production of fungitoxic compounds. These concepts are not backed by conclusive evidence in all cases.

Many histological studies have shown that there is a typical pattern followed by most fungal plant pathogens in the inception of infection. Both obligate and facultative parasites have an early compatible phase in susceptible tissue (73). Some representative examples are listed.

a. Helminthosporium gramineum was shown to penetrate barley coleorhiza after formation of appressoria and to ramify through parenchyma for some time before a host response occurred (70).

b. Puccinia graminis tritici and Erysiphe graminis hordei grew for 7 days in their respective susceptible hosts without causing any collapse of tissue (71, 84).

c. Bean hypocotyls were initially compatible with invading hyphae of Thielaviopsis basicola (15).

d. Rhizoctonia solani penetrated bean hypocotyls from infection cushions and quickly invaded all tissues, forming a delimited water soaked lesion in which cells showed no signs of collapse for 32-36 hours (78).

e. Zoospores of Phytophthora infestans germinated on potato leaves, formed appressoria, and penetrated epidermal cells within 2 hours

after inoculation (61). Hyphae ramified through tissue with no apparent host response for 24-48 hours.

f. The safflower rust fungus, Puccinia carthami, formed appressoria and penetrated in 4-5 hours but host cells were not affected until sporulation occurred at 240 hours (86).

g. The infection patterns of three Helminthosporium leaf blights of corn have been compared (31). Germination and penetration occurred 12-18 hours for all three. However, H. turcicum elicited no host response until 14 days after inoculation whereas H. maydis and H. carbonum had only brief compatible periods before host cells collapsed.

h. Puccinia coronata, causal fungus of oat crown rust, penetrated leaves within 12 hours, formed haustoria and sporulated in 160 hours, but had no visible effect on host cells until after 216 hours (62).

i. Puccinia sorghi formed haustoria in corn leaves 6-10 hours after inoculation but there were no visible symptoms until 24-48 hours (28).

j. A compatible relationship was maintained between hyphae of Gymnosporangium juniperae-virginianae and apple leaf cells for 10 days before pycnia were formed and host cells collapsed (53).

These examples illustrate that many fungi, including both obligate and facultative parasites, attack their susceptible hosts in a similar manner and exist compatibly for varying but definite periods before effects on the host are evident.

The resistant reaction varies more than does the susceptible reaction. Events occurring on the surface of the host, such as spore germination, growth of the germ tube, and appressorium formation, are usually identical on both susceptible and resistant varieties but there is a great deal of variation as to the time after penetration that resistance is expressed. There are also a variety of ways in which a resistant host responds to attack; some of these are illustrated in the following examples.

a. Six days after infection, barley resistant to H. gramineum formed a granular reaction zone beyond which the fungus did not pass (70).

b. Stakman (71) noticed that hyphae of P. graminis were surrounded by dead cells 3.5 days after inoculation and proposed that this hypersensitive type of reaction was responsible for resistance.

c. Allen (5) found that there were fewer penetrations of resistant than of susceptible wheat by P. graminis, which suggests that resistance was expressed even before penetration occurred.

d. The incompatible reaction between P. sorghi and resistant corn killed 2 to 500 host cells before the fungus was stopped (28). It is interesting that this same type of incompatible reaction sometimes occurred in susceptible varieties.

e. Resistance of oats to P. coronata was expressed by few penetrations and poor development of haustoria. Host cells died sometime after penetration occurred (62).

f. Erysiphe graminis elicited a variety of resistant responses, depending on the host cultivar, ranging from no penetration and no host response to limited haustorial development and collapse of mesophyll in the area of infection (84).

g. Corn, resistant to P. graminis, allowed fewer fungal penetrations than did susceptible wheat about 8 hours after inoculation (38). The first host response in corn was a darkening of cell walls about 24 hours after inoculation.

h. Hyphae of Phytophthora infestans did not advance beyond the epidermal cell in resistant potato tissue. The host response was death of a few cells below the penetrated cell (61).

i. Susceptible and resistant reactions of the three Helminthosporium leaf blights of corn appeared to be similar except that the resistant reaction affected fewer cells. In both cases, chloroplasts and cell walls broke down and the cells finally collapsed (31).

j. Hyphae of the cedar-apple rust fungus penetrated 1-2 cells in resistant apple leaves and stopped. Later, a few host cells around the infection site collapsed (53).

k. Flentje (26) proposed that resistance to Pellicularia filamentosa was due to one of four phenomena: failure of hyphae to attach to host; failure of appressorium or infection cushion formation; thickening of host cell walls; or hypersensitive response of the host.

Many of the histological studies appear to be aimed at understanding disease resistance. However, the investigators either made no further attempt to explain the cause of resistance (28, 31, 62, 86) or proposed hypotheses which have not or cannot be proved. The proposals to explain disease resistance can be divided into three general categories: structural barriers; substances present in the resistant plant before attack; and substances produced by the host in response to attack. Disease resistance has been reviewed many times (2, 3, 8, 35, 73, 75, 80, 85). I will discuss a few current concepts which attempt to explain the nature of disease resistance.

Structural barriers cannot explain disease resistance in general because there are many systems in which they do not occur. However, it has long been known that fungi can generate enough mechanical force to penetrate artificial membranes, provided they are not too thick (13). Therefore, it is possible that a thick cuticle or epidermal cell wall could contribute to resistance. Adams et al (1) proposed that Ginkgo biloba protects itself with epidermal cell wall thickenings through which fungi cannot penetrate. Conant (16) reported that tobacco plants resistant to Thielaviopsis basicola formed cork layers in response to attack by the fungus. Using seven different varieties, he found that the amount of cork formation was correlated with resistance to infection. Cunningham (22) observed that certain plants form a cicatrice following fungus attack or wounding. However, this phenomenon could not be correlated with disease resistance. Melander and Craigie (46)

could not find a correlation between relative resistance and thickness of the cuticle or epidermal cell wall. They concluded nevertheless that resistance may be partially due to morphological barriers but that physiological factors are also involved. There is a recent report (30) suggesting that resistance of sweet potato stems to Ceratocystis fimbriata is due to formation of callus tissue. Akai (2) has published a detailed review of structural barriers as factors in disease resistance.

Phenolic compounds present in healthy plants have often been implicated as primary resistance factors. This concept became popular following the report of Link and Walker (40) that protocatechuic acid and catechol, two phenolic compounds toxic to fungi in vitro, could be isolated from scales of pigmented resistant onions. The toxic components were not found in susceptible non-pigmented onions. This is the first and only report to date of a definite correlation between resistance to a plant disease and presence of a specific compound in the resistant host. However, this is an unusual case since resistance factors are located in the dead outer scales of the onion.

Other attempts to correlate resistance with constitutive phenols have not been successful (21, 24). For example, Newton and Anderson (51) reported that the total phenol in the cell sap of wheat susceptible to P. graminis was less than that found in a resistant variety. They proposed that resistance to rust was "due to liberation of phenols in the host cell upon entrance of the fungus." However, Anderson (6) retracted this claim after carefully studying several susceptible and resistant varieties. He found no correlation

between resistance to rust and the inhibitory effect of cell sap on spore germination and germ tube growth. This work was confirmed using more sophisticated techniques (32). More evidence against the constitutive phenol theory was presented by Samaddar (66) who found that there was no significant difference in the phenol content of oats susceptible and resistant to H. victoriae. In general, it has been found that susceptible and resistant varieties often contain equal amounts of phenol (75), which presents a fundamental weakness in the constitutive phenol theory. An investigation of healthy potato peel has revealed the presence of two nonphenolic steroid-glycoalkaloids which are fungitoxic in vitro (4). The concentration of these compounds in the tissue is high enough to inhibit fungal growth but their role in disease resistance has not been investigated. Walker (80) has disclaimed the idea "that the mere presence of a material in the extract of host tissue which is toxic to a pathogen of that host, contributes to the resistance of the host."

Various kinds of phenolic compounds are produced in response to attack. Fungitoxic quinones occur when host phenols are acted upon by polyphenoloxidase from the host tissue or from the fungus (24). Toxic phenols such as chlorogenic and caffeic acids can be produced by a metabolic pathway of the host which is triggered by phenylalanine ammonia lyase (87). Most compounds classed as phytoalexins are derived from host phenols but are not detectable in healthy plants (19). The hypersensitive reaction is reported to be a response to attack which involves localization of host phenols,

phytoalexins, and other abnormal metabolites which may be fungitoxic (24, 75). The literature on dynamic responses to attack is voluminous (35) so the discussion here will deal only briefly with a few examples.

Investigations of potato tuber and carrot root tissue have revealed that chlorogenic and caffeic acids (36) and isocoumarin derivatives (18) are produced in response to attack by nonpathogens of these tissues. In the case of carrot, a pathogen did not induce significant increases of phenol (17). Another report (55) has shown that rice leaves resistant to H. oryzae quickly accumulated large amounts of phenol when attacked by the fungus. Phenol accumulation in susceptible was slower than in resistant tissue.

The so-called hypersensitive reaction was first recognized by Stakman (71) as a quick, violent host response to attack by an obligate parasite. Disease development was arrested through death of a few host and a few pathogen cells. This phenomenon was later reported for facultative parasites (26, 54, 57). After the proposal of the phytoalexin theory (50), it was suggested that a combination of the hypersensitive reaction and phytoalexin was responsible for resistance (49). Tomiyama (76) has found that the cut surface of potato petioles, when inoculated with P. infestans 15-20 hours after cutting, will respond hypersensitively within 40-60 minutes. The hypersensitive host response occurred very late when cells were inoculated immediately after the petiole was cut.

The proposed involvement of phytoalexins in disease resistance has been adequately reviewed (19, 20, 21, 48). Müller has maintained that the

rate of phytoalexin production after attack determines the susceptibility or resistance of a given plant to a given disease (48). Cruickshank (20) modified this proposal by suggesting that susceptible and resistant reactions are determined by the differential sensitivity of pathogens and nonpathogens to the toxic phytoalexins. Theoretically, a resistant reaction occurs when the host produces a phytoalexin concentration above the level of tolerance of the parasite. A susceptible reaction results if the fungus is unable to induce phytoalexin production or if the fungus is tolerant of the level produced. There is some evidence which supports this hypothesis but there are also exceptions. In spite of the exceptions, it is clear that phytoalexins do exist since several have been identified (20). Current reports of new phytoalexins may lend support to the theory (39, 77).

Compounds produced in plants after infection have had much study, but in no case have they been shown conclusively to have a causal role in disease resistance. In fact, Kuć (35) has proposed that resistance is due to a combination of factors. Several studies have provided evidence for skepticism that dynamically produced compounds have a primary function in resistant reactions. There is evidence that a nonpathogenic isolate of Ceratocystis fimbriata did not cause sweet potato to produce more chlorogenic acid and ipomeamarone than a pathogenic isolate (30). It has been reported that phenol accumulation in rice plants infected with the blast fungus is not a direct cause but a result of the resistant reaction (63).

The role of the hypersensitive reaction in apple leaves resistant to Venturia inaequalis has been questioned because it did not occur in all resistant varieties (54). Müller's claim (47) that greater resistance is due to a more rapid host response was refuted by Ferris (25). She found a very small difference in the times required for susceptible and resistant varieties to respond to attack and concluded that the rate of response may not be involved in resistance. Several incongruities have been found in the theory that the hypersensitive reaction is responsible for resistance of wheat to stem rust (11). There was no consistent relationship between the area of a rust colony and the amount of hypersensitive tissue present; susceptible plants sometimes produced more necrotic tissue than did resistant plants. Rings of necrotic tissue ahead of advancing mycelium failed to halt fungal growth. It was suggested that necrotic tissue in resistant hosts indicates sensitivity to disturbance and that the hypersensitive reaction is a result, not a cause, of resistance. Rust resistant safflower produced a hypersensitive response 12 hours after inoculation (33) but the fungus continued to grow for 3 days, which could mean that some other factor is involved in resistance. Potato petiole cells resistant to P. infestans were dead 40-60 minutes after inoculation but fungal growth did not stop for 2.5 hours (76). Another study has shown that a high phenol concentration does not contribute to the resistance of potato tubers to nonpathogenic bacteria (88). Other examples which tend to discredit the role of phenols and the hypersensitive reaction in resistance have been reported (21, 73).

There is at least one disease system in which a phytoalexin was found but could not be correlated with resistance. Nishimura and Scheffer (52) found that susceptible and resistant oat leaves produced equal amounts of a fungus inhibitor in response to both pathogenic and nonpathogenic H. victoriae spores. Both the pathogen and nonpathogen were affected equally by the inhibitor, and the inhibitor was not produced early enough to account for resistance in this system. Furthormore, HV-toxin, the primary determinant of this disease, did not induce phytoalexin production when applied to resistant or susceptible leaves. There is also evidence that a quantitative increase in phenol after infection is not responsible for resistance to H. victoriae (66). Cruickshank (21) concedes that cases of extreme host specificity are not easily explained by the phytoalexin theory.

If post-infectionally produced antibiotics are not the cause of resistance, then what is their function? A logical suggestion is that they are involved in normal wound healing processes (75). It is known that plants respond to wounding, mechanical pressure, and application of injurious chemicals with similar cellular changes (10). Antifungal compounds have been shown to be present in wound sap less than 10 minutes after wounding (9). Possible function of such substances in wound healing has not been determined.

There have been other proposals designed to explain the nature of disease resistance. For instance, Thatcher (74) proposed that susceptible host cells have a lower osmotic pressure than fungus cells. Thus nutrients

would diffuse from host to pathogen. Resistant cells therefore would have higher osmotic pressure than fungus cells. Leach (37) has proposed that resistant cells lack a specific nutrient required by a specific fungus. There is no supporting evidence for these and many other hypotheses.

Ward (81) first suggested that disease resistance is physiological as well as structural, leading many workers to attempt to prove the dynamic nature of disease resistance. The basis of resistance has been proven only for onion smudge, an unusual case of constitutive resistance. Perhaps a new approach to the problem of disease resistance is in order. The discovery of host specific toxins has provided the basis for a new hypothesis. Scheffer and Pringle (68) have postulated that susceptible cells have toxin receptor sites and that the basis for resistance is simply a lack of receptor sites in resistant cells. Samaddar has presented evidence that the primary site of toxin action is in the cell membrane (65) and that nutrients leak out of susceptible cells within minutes after exposure to toxin (66). There is no leakage from resistant cells. It is possible that fungal growth stops in resistant cells because the fungus lacks the ability to obtain nutrients from these cells. It is also possible that after fungal growth is stopped, competition for nutrients between fungus and host results in the death and disintegration of the fungus.

The data of Burrows (14) has been interpreted as an indication that nutrition is not a factor in resistance. Using "leaf sandwiches" of susceptible and resistant leaves, he showed that the fungus took up radioactive

label from the susceptible leaf, advanced into the resistant leaf and stopped. Label was found in the resistant host only in areas where the fungus had penetrated. This evidence could be used to support the idea that the resistant host actually removes nutrients from the fungus.

# MATERIALS AND METHODS

Plant material - Oat cultivars Park, Clinton and Cornellian were used. Park is susceptible and Clinton is resistant to Helminthosporium victoriae and to its toxin. Cornellian has been reported to be intermediate in resistance to H. victoriae in field tests (57) but in a seedling bioassay its resistance to HV-toxin equals that of Clinton. Seeds were planted in vermiculite, watered with White's solution, and incubated in the laboratory at 22° C under Sylvania Gro-Lux lamps for 9 to 11 days. Primary leaves were 6-8 inches long when they were removed from seedlings and used as experimental material.

Fungi and spore production - A highly virulent, high toxin producing isolate of H. victoriae was used. The nonpathogenic isolate used was a mutant from the pathogenic isolate. Some experiments required H. carbonum and Cercospora apii, which are pathogens on corn and celery respectively, and Neurospora tetrasperma, a saprophyte.

Conidia of Helminthosporium sp. were produced following a method modified from that of Lukens (43). Fungi were grown in 25 ml Fries medium supplemented with 1% yeast extract (59) in 125 ml flasks on a reciprocal shaker (100 strokes/minute). After 3-4 days, the fungal mycelium was collected on cheesecloth, transferred to distilled water in a

blender and comminuted for 2 minutes. Mycelial fragments were washed twice by centrifuging from distilled water at 1500 x g for 10 minutes. Pellets were suspended in 0.02 M phosphate buffer pH 6.4. Two ml aliquots were dispersed on dry 9 cm filter paper in petri dishes. Spores appeared in 3-5 days and retained their viability after being stored in the petri dishes for three months. Spore production was increased if petri dishes were incubated in continuous light.

C. apii produced conidia on V-8 juice agar in 2-3 weeks when exposed to alternating 12 hour periods of light and dark.

N. tetrasperma grown on yeast-maltose agar in a petri dish (34) produced orange conidia and black asci on the agar surface. Ejected ascospores adhered to the lid of the petri dish. The lid was removed, the ascospores were suspended in water and allowed to sediment. Contaminating conidia remained in suspension and were removed by decanting. Ascospores were stored in water at 4° C.

Inoculation procedure - Spore suspensions were prepared by adding water or toxin solution to a petri dish culture and rubbing the surface with a glass rod. The spore concentration was adjusted so that when drops were applied there were 20-25 spores/$mm^2$ of leaf surface. N. tetrasperma ascospores were activated by heating them in a water bath for 20 minutes at 58° C.

Oat leaves were rubbed gently with the fingers (so droplets would adhere to the leaf surface) and attached to glazed clay plates with rubber bands. The

plates were placed horizontally over water in a glass chamber with the cut ends of the leaves below the water surface. Droplets containing spores were placed on the upper leaf surfaces and the chamber was closed. Spore suspensions were incubated in water on glass slides as germination controls.

Toxin preparation and bioassay - The toxin preparation, which had a dry weight of 16 mg/ml, was a partially purified acetic acid eluate from an alumina column prepared by the method of Pringle and Braun (58). The oat seedling bioassay, described previously (58), showed that this preparation completely inhibited root growth of Park oats at a concentration of 0.016 μg/ml but had no effect on Clinton seedlings at a concentration of 160 μg/ml. A 640 μg/ml solution of toxin was partially inactivated by adjusting to pH 9.5 and autoclaving for 30 minutes. The solution was readjusted to pH 5.0 and assayed with the seedling bioassay. Growth of susceptible (cv. Park) seedling roots was inhibited 50% by a concentration of 160 μg inactivated toxin/ml. Growth of resistant (cv. Clinton) seedling roots was not affected.

Sectioning, staining, and photography - Cross sections of leaves were cut 20-60 μ in thickness with Hooker's fresh tissue microtome (29). Sections were collected in a pan of water and transferred to a water drop on a glass slide with a wooden toothpick. Water was removed and the sections were flooded with 0.1% cotton blue in lactophenol. The stain was immediately replaced with lactophenol and the sections were mounted under a cover glass and examined under the microscope. All photomicrographs were taken with Kodak Ektachrome X film.

Ion leakage from oat leaves - Oat leaves were washed by gentle shaking for 2 hours in 3 changes of glass distilled water which had 650,000 ohms resistance. The leaves were then anchored to glazed clay plates and drops of water, toxin solution, or spore suspensions were applied 2-3 mm apart. Spore suspensions contained 50-75 spores/$mm^2$ of leaf surface. After incubation, drops were removed with streams of distilled and glass distilled water. Approximately 50% of the spores and hyphae remained attached to the leaves. Leaves were cut into one-inch pieces and placed in a cheesecloth bag. The bag was washed 0.5 to 2 hours in three 50 ml changes of glass distilled water. Hourly readings of the 50 ml ambient solution were taken with a model RC 16B1 Industrial Instruments conductivity bridge.

Dehydrogenase activity in oat leaves - Oat leaves (100 mg) were cut in 5 mm pieces and placed in a test tube. Three ml of triphenyl tetrazolium chloride (TTC) solution (0.6% w/v TTC in 0.05 M $Na_2HPO_4$-$KH_2PO_4$ buffer, pH 7.3) was added, the leaves were vacuum infiltrated for 10 minutes, and incubated at 30° C for 2 hours. The TTC solution was decanted and the leaves were washed twice with water. Formazan was extracted by adding 7 ml 95% ethanol and boiling in a water bath for 5 minutes. The volume was made to 10 ml with 95% ethanol and the optical density at 530 mμ was determined with a Beckman DB spectrophotometer (72).

$C^{14}$ amino acid incorporation in oat leaves - Tissue samples (150 mg) were vacuum infiltrated for 10 minutes with solutions of L-leucine-1-$C^{14}$ or L-valine-1-$C^{14}$ containing 0.5 μc/ml. Samples were then incubated for

4 hours at 22° C and extracted 3 times with hot 80% ethanol. Tissues were ground in a mortar with sand and 80% ethanol. The homogenate was centrifuged for 5 minutes at 15,000 x g and the pellet was washed 3 times with 80% ethanol. The pellet was suspended in cold 5% trichloroacetic acid (TCA) and held on ice at 4° C for 15 minutes, then centrifuged at 0°. The resulting precipitate was washed twice with 5% TCA in the cold, once with 80% ethanol, once with 100% ethanol and twice with hot ethanol : ether (3:1 v/v). The pellet was suspended in 1 ml 1 N NaOH, incubated for 1 hour at 90°, and centrifuged (56). Aliquots (0.1 ml) of the supernatant were spread evenly on ground glass planchets, dried, and counted in a gas flow detector.

# RESULTS

## Histological studies of tissue invasion

General observations - There was approximately 95% germination of conidia of Helminthosporium sp. used in this study. Germination and hyphal growth sequences were the same for all types of spores on both susceptible and resistant tissue prior to penetration. Excised and intact leaves gave comparable results in all types of experiments. Hyphae on the leaf surface sometimes appeared granular, non-staining, and in a state of degeneration between 24 and 48 hours after inoculation in all host-pathogen combinations used (Figure 11).

Development of pathogenic H. victoriae in susceptible oats - The first penetration of the upper epidermal cells was observed 8 hours after inoculation (Figure 1). By 12 hours there were many penetrations, numerous hyphae growing inter- and intracellularly in the mesophyll (Figure 2), and an occasional hypha approaching the lower epidermis of the leaf. The mesophyll contained many hyphae at 24 hours and by 48 hours the leaf was saturated with hyphae (Figure 12). There was no definite effect on the host cells until 48 hours after inoculation, when chloroplasts sometimes began to lose their green color and appear slightly yellow.

Development of pathogenic H. victoriae in resistant oats - Cornellian and Clinton oats were used in all experiments involving resistant tissue and both gave identical results. A few epidermal cells were penetrated by 12 hours after inoculation (Figure 3), but the number of penetrations was less than with susceptible oats. Fungal growth usually stopped in the epidermal cell or in the first or second layer of mesophyll cells (Figure 5). After 48 hours most penetration pegs looked just as they had at 12 hours (Figure 4). On rare occasions thin hyphae were seen growing half way or more through either Clinton or Cornellian leaves 48 hours after inoculation. By 24 hours a few appressoria had caused epidermal cell wall thickenings below their point of attachment (Figure 6). However, in the majority of cases, there was no host response to either appressorium formation or penetration even after 48 hours (Figures 4 and 5).

Development of H. carbonum and nonpathogenic H. victoriae in oat tissue - These isolates were potential pathogens of susceptible and resistant oats since spores and germ tubes attached to the leaf surface, appressoria were formed, and penetration occurred. H. carbonum made many penetrations by 12 hours after inoculation. Penetration by nonpathogenic H. victoriae was rare or absent after 12 hours but it was evident by 24 hours. Initial penetration was similar to that shown in Figure 3. After 48 hours hyphal growth by both isolates was limited to epidermal cells or to the first two layers of mesophyll cells (as shown in Figures 4 and 5). In most cases there was no visible host response (as shown in Figures 4 and 5).

Figure 1. Susceptible (cv. Park) oat leaf 8 hours after inoculation with pathogenic H. victoriae. Spore, germ tube, appressorium, penetration peg, and hypha in epidermal cell are shown.

Figure 2. Susceptible (cv. Park) oat leaf 12 hours after inoculation with pathogenic H. victoriae. A hypha is growing inter- and intracellularly in the mesophyll.

Figure 3. Resistant (cv. Cornellian) oat leaf 12 hours after inoculation with pathogenic H. victoriae. There are two penetration hyphae in an epidermal cell.

Figure 4. Resistant (cv. Clinton) oat leaf 48 hours after inoculation with pathogenic H. victoriae. Growth of penetration hyphae is limited to the epidermal cell and substomatal cavity.

Figure 5. Resistant (cv. Cornellian) oat leaf 48 hours after inoculation with pathogenic H. victoriae. Penetration hypha is advancing to second layer of mesophyll cells.

A few epidermal cells of both susceptible and resistant varieties were filled with a deep amber granular material by 48 hours (Figures 7 and 8). This reaction was believed to be similar to the hypersensitive reaction described for other diseases. Hyphae were sometimes observed to grow beyond the area of the hypersensitive reaction (Figure 8). Epidermal cell wall thickenings like those shown in Figures 6 and 8 were occasionally seen below appressoria. However, most appressoria and penetration pegs induced no visible host reaction (as shown in Figures 3, 4, and 5). Epidermal cell wall thickenings and hypersensitive reactions were unusual occurrences.

The effect of HV-toxin on development of H. carbonum and nonpathogenic H. victoriae in H. victoriae susceptible oats - Spores for inoculum were suspended in water containing 1.6 μg HV-toxin per ml and applied to leaves of H. victoriae susceptible oats as described in the methods section. Results were nearly identical to those obtained when pathogenic H. victoriae was used to inoculate susceptible oats. Hyphae (similar to those shown in Figure 2) were observed within the leaf tissue 12 hours after inoculation. Abundant fungal growth, comparable to that made by pathogenic H. victoriae in susceptible tissue (Figure 12) was observed at 24 and 48 hours. There was no visible effect on the host tissue even after 48 hours.

To further test the effect of toxin, an experiment with inactivated toxin was included. Conidia of the nonpathogenic H. victoriae isolate were suspended in a solution containing 1.6 μg inactivated HV-toxin per ml and applied to susceptible leaves in the usual manner. Conidia suspended in

water or in a solution containing 1.6 μg active HV-toxin per ml served as controls. After 48 hours, leaves treated with spores suspended in inactivated toxin or in water had hyphae in the epidermal cells or occasionally in the first or second layer of mesophyll cells. This was the usual resistant response (Figures 4 and 5). Epidermal cell wall thickenings and hypersensitive reactions (such as those shown in Figures 6, 7, and 8) were occasionally present, although resistance was evident in most cases without a visible host response. Conidia in the active toxin solution produced abundant hyphae (as shown in Figure 12) in the tissue by 48 hours. Active HV-toxin appears to be required for fungal development in susceptible tissue.

The toxin concentration required to confer pathogenicity to the nonpathogen was determined by serial dilutions. Conidia of the nonpathogenic H. victoriae isolate were suspended in solutions containing 1.6, 0.16, 0.016, and 0.0016 μg HV-toxin per ml and applied to susceptible leaves. Conidia suspended in water were used as controls. After 48 hours, the leaves treated with 1.6 μg/ml were full of hyphae (as in Figure 12) and there were no host responses. The 0.16 μg/ml treatment also showed many hyphae in the leaf (as in Figure 12) but there was an occasional epidermal cell wall thickening in response to appressorium formation (as in Figure 6). Spores suspended in more dilute toxin solutions (0.016 and 0.0016 μg/ml) did not invade the tissue beyond the initial stage, and a resistance sequence occurred. Fungal growth was limited to the epidermis or sometimes the first two mesophyll layers (as in Figures 4 and 5) and an occasional hypersensitive reaction

Figure 6. Resistant (cv. Cornellian) oat leaf 24 hours after inoculation with pathogenic H. victoriae. There is an epidermal cell wall thickening below the appressorium.

Figure 7. Susceptible (cv. Park) oat leaf 48 hours after inoculation with nonpathogenic H. victoriae. The penetrated epidermal cell is filled with amber material.

Figure 8. Susceptible (cv. Park) oat leaf 48 hours after inoculation with nonpathogenic H. victoriae. Shown are an epidermal cell wall thickening below an appressorium, and a hypha growing through and beyond amber epidermal cells.

Figure 9. Susceptible (cv. Park) oat leaf 24 hours after inoculation with N. tetrasperma in the presence of 1.6 $\mu$g toxin/ml. The stomate is invaded.

Figure 10. Susceptible (cv. Park) oat leaf 48 hours after inoculation with N. tetrasperma in the presence of 0.16 $\mu$g toxin/ml. There are many hyphae in the leaf tissue. Leaf cells are falling apart and disintegrating.

(as in Figures 7 and 8) was seen in addition to a few cell wall thickenings (as in Figures 6 and 8). It should again be emphasized that host responses were the exception and that usually hyphae penetrated without a host response (as in Figures 3, 4, and 5).

The effect of HV-toxin on development of H. carbonum and nonpathogenic H. victoriae in H. victoriae resistant oats - The addition of toxin to spores of nonpathogenic H. victoriae had no effect on the ability of the fungus to attack H. victoriae resistant tissue. Results of this experiment were identical to those for nonpathogenic H. victoriae without toxin on susceptible or resistant oat tissue.

When toxin was added to H. carbonum infection drops, penetration occurred 12 hours after inoculation. After 24 and 48 hours, long thin hyphae were frequently seen in the mesophyll (Figure 13). Thus H. carbonum grew in H. victoriae resistant oat tissue somewhat more with than without toxin. Cell wall thickenings and amber cell responses (such as those in Figures 6, 7, and 8) were occasionally evident after 48 hours. These reactions had no effect on fungal growth. Most appressoria and hyphae were not accompanied by a host response.

Inoculation of oat leaves with Neurospora tetrasperma - The dormancy of N. tetrasperma ascospores was broken by heat treatment before spore suspensions were applied to leaves. Germination occurred in about 5 hours and a thick network of hyphae developed on the leaf surface. These hyphae were easily washed from the leaf, since they did not become attached as the

hyphae of pathogens did. There was no appressorium formation, no attempt at penetration, and no change in the host tissue after 48 hours.

Effect of HV-toxin on growth of N. tetrasperma in oat tissue - When spore suspensions containing 1.6 $\mu$g toxin per ml were applied to susceptible leaves, there was a little fungal growth in the leaf tissue after 24 hours (Figure 9), but by 48 hours the host tissue was full of hyphae and completely disintegrated (Figure 14). If 0.16 $\mu$g toxin per ml was used, there was no invasion at 24 hours but by 48 hours there were many hyphae in the leaf and much of the tissue appeared to be falling apart (Figure 10). There was no host response.

When resistant leaves were used, germination and germ tube growth occurred on the leaf surface but there was no hyphal attachment to the leaf or attempt at penetration by 48 hours. There was no effect on the leaf cells and no host reaction.

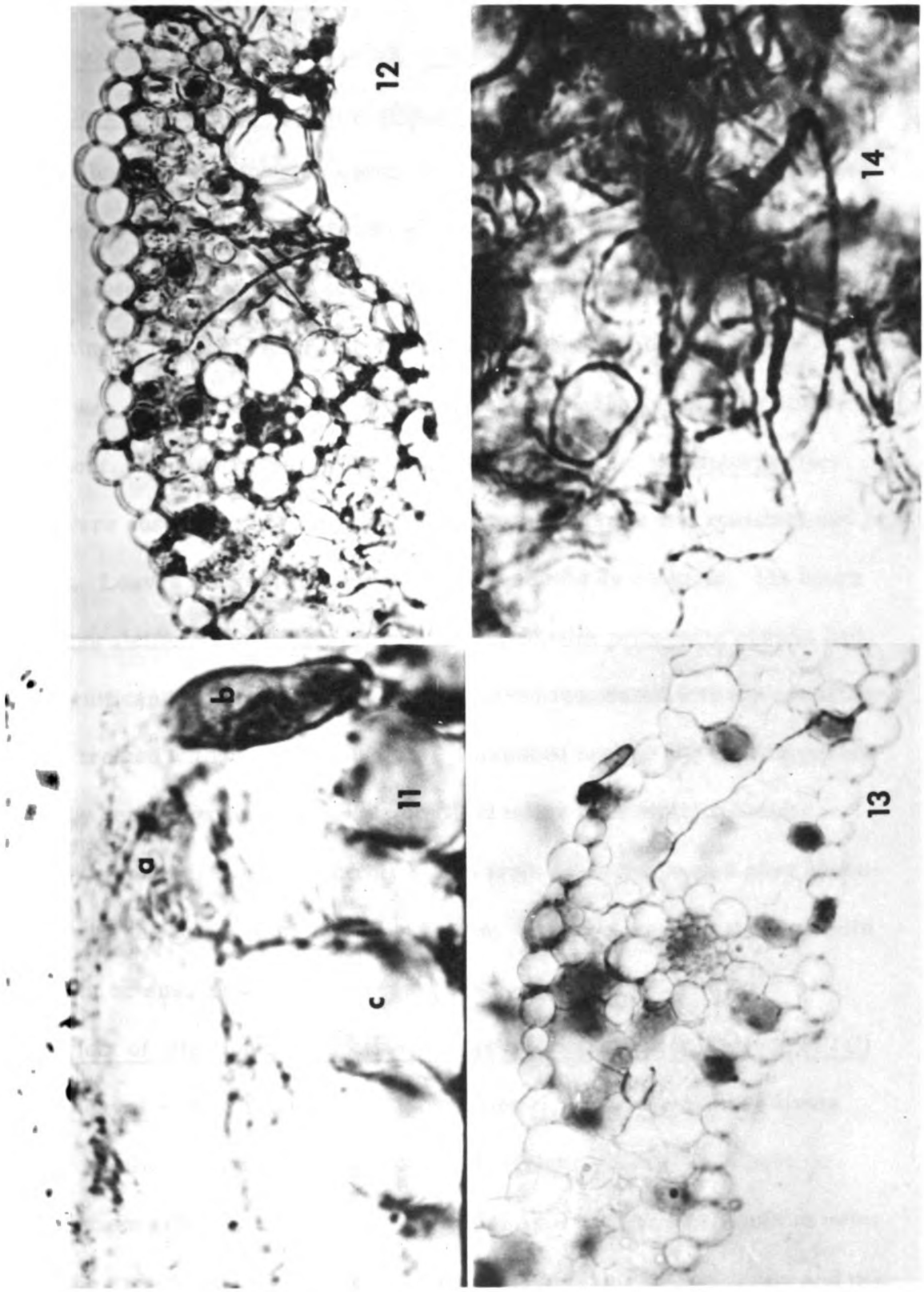
Inoculation of oat leaves with Cercospora apii - Conidia suspended in water or in 1.6 $\mu$g HV-toxin per ml germinated and germ tubes grew well after attaching to the leaf surface. However, there was no penetration, no effect on the leaf, and no host response of susceptible or resistant leaves 48 hours after inoculation. C. apii, a leaf pathogen of celery, was expected to attempt penetration. Perhaps a longer incubation period was needed.

Figure 11. Resistant (cv. Cornellian) oat leaf 48 hours after inoculation with pathogenic H. victoriae. A mass of degenerating hyphae (a) appears on the surface of the leaf. Also visible are a spore (b) and an epidermal cell (c).

Figure 12. Susceptible (cv. Park) oat leaf 48 hours after inoculation with pathogenic H. victoriae. There are many hyphae in the leaf tissue but there is no apparent effect on host cells.

Figure 13. Resistant (cv. Clinton) oat leaf 48 hours after inoculation with H. carbonum in the presence of 1.6 µg toxin/ml. A long hypha is growing in the leaf tissue.

Figure 14. Susceptible (cv. Park) oat leaf 48 hours after inoculation with N. tetrasperma in the presence of 1.6 µg toxin/ml. There are many hyphae in the leaf and the leaf tissue is completely collapsed and disorganized.


12
14
b
11
a
c
13

Physiological studies of the initial effects of H. victoriae on oat tissue

Effect of infection on loss of electrolytes from susceptible and resistant oat leaves - HV-toxin is known to cause host cells to leak electrolytes. An attempt was made to determine whether or not H. victoriae infection causes a similar loss. In a preliminary experiment, drops of a solution containing 0.16 μg HV-toxin/ml were placed on susceptible oat leaves. There was a significant loss of electrolytes from leaves after a 15 minute treatment. Conidia of the pathogenic and nonpathogenic H. victoriae isolates were suspended in water and applied to susceptible and resistant oat leaves. Leaves treated with drops of water served as controls. Six hours after inoculation, susceptible leaves inoculated with pathogenic conidia had lost significantly more electrolytes than leaves inoculated with the nonpathogen or treated with water (Figure 15). Unwashed spores and spores washed twice by centrifugation from glass distilled water gave similar results. There was no increase in electrolyte loss from resistant leaves after inoculation with the pathogen or the nonpathogen. The loss was the same as with resistant or susceptible water treated controls.

Effect of infection on reduction of triphenyl tetrazolium chloride (TTC) by oat leaves - It is frequently asked whether H. victoriae invades living tissue or kills in advance and invades dead tissue. Living cells have dehydrogenase activity, a characteristic which was used in an attempt to determine the extent of damage to cells. Spore suspensions of pathogenic and nonpathogenic H. victoriae were placed on susceptible and resistant oat leaves;

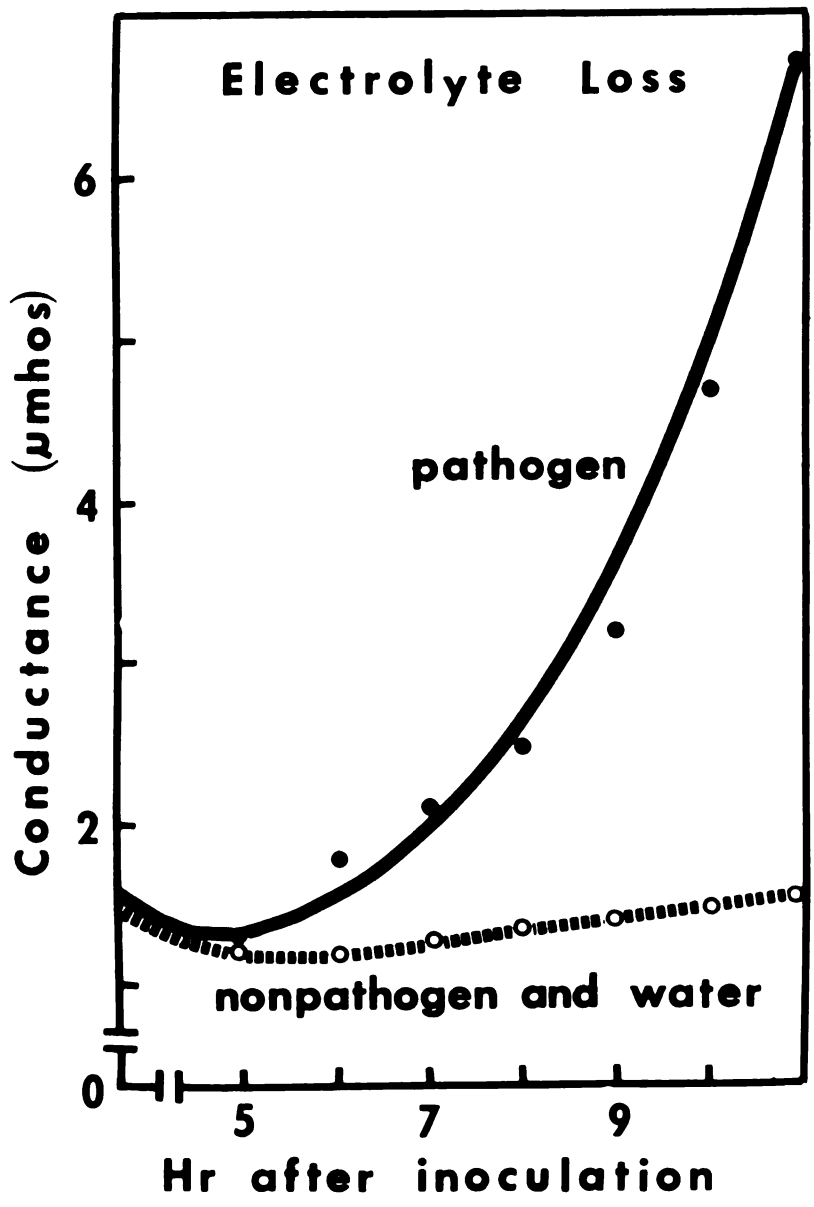



Figure 15. Effect of infection on loss of electrolytes from Helminthosporium victoriae susceptible leaves. Tissue samples (0.5 g) of inoculated leaves were washed and suspended in glass distilled water. Electrolyte loss was determined from conductivity of the water. • = leaves inoculated with pathogenic H. victoriae; o = water control leaves or leaves inoculated with nonpathogenic H. victoriae.

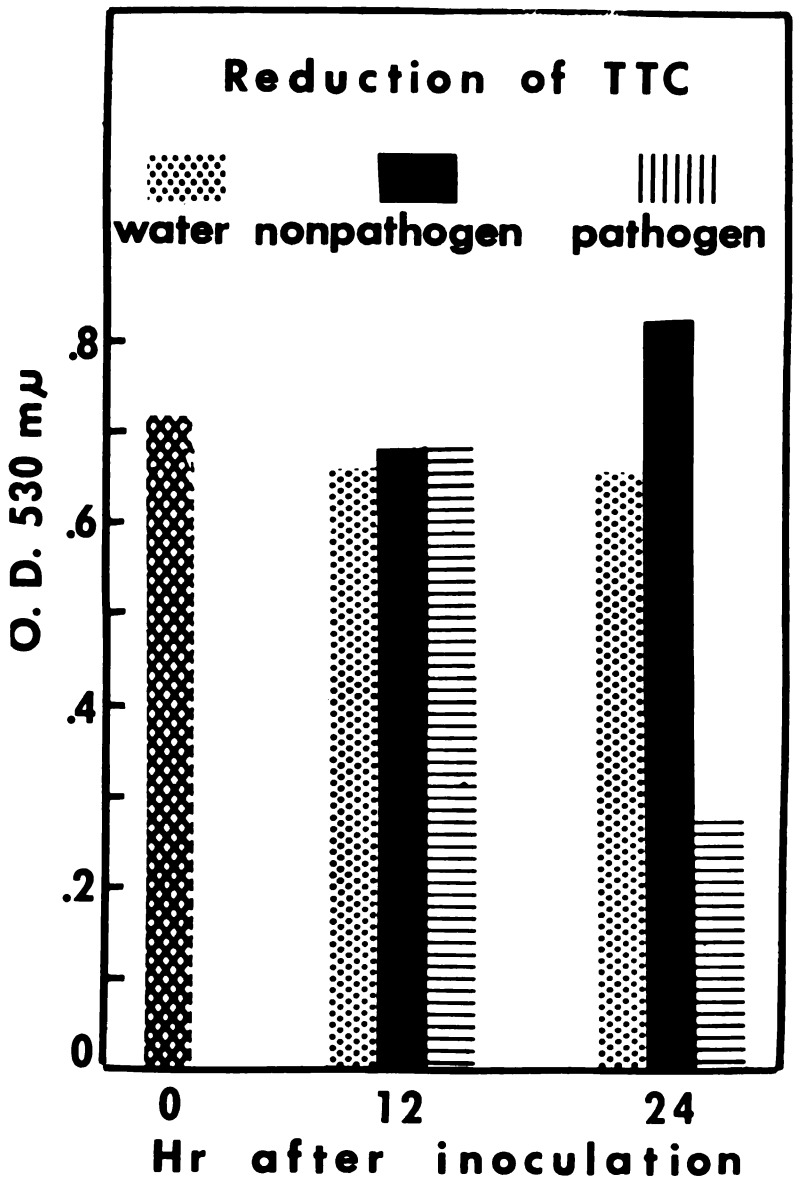



Figure 16. Effect of infection on reduction of triphenyl tetrazolium chloride (TTC) by Helminthosporium victoriae susceptible leaves. Tissue samples (100 mg) of inoculated leaves were vacuum infiltrated for 10 minutes with TTC and incubated at 30° C for 2 hours. Formazan was extracted in 95% ethanol and detected by the optical density of the solution at 530 mμ.

water drops placed on other leaves served as controls. After incubation for several time periods, leaves were treated with TTC and the amount of formazan formed was determined. Zero time and boiled controls were included. Results showed that dehydrogenase activity 12 hours after inoculation was the same for resistant and susceptible leaves and their water and 0 time controls (Figure 16). After 24 hours, activity of leaves treated with water was unchanged, activity of leaves treated with nonpathogenic spores had increased somewhat, and activity of leaves treated with pathogenic spores was greatly reduced. By 48 hours, dehydrogenase activity in leaves treated with pathogenic spores was greater than in leaves of the other two treatments. However, this activity might be traced to the growing fungus. All treatments of resistant leaves caused no change in dehydrogenase activity after 48 hours. Boiled controls reduced an insignificant amount of TTC.

Effect of infection on incorporation of $C^{14}$ amino acid into the trichloroacetic acid (TCA) precipitable fraction of susceptible oat leaves - Another characteristic of living cells is the ability to synthesize macromolecules. This also was used as a criterion of damage immediately following infection by H. victoriae. Water drops and spore suspensions of pathogenic and nonpathogenic H. victoriae isolates were incubated on susceptible oat leaves, then vacuum infiltrated with $C^{14}$ leucine. Results showed that $C^{14}$ leucine incorporation by leaves inoculated with the pathogen was greatly reduced at 8 and 12 hours (Table 1). By 24 hours, incorporation in leaves treated with the pathogen was slightly higher than incorporation by non-inoculated

control leaves. Another experiment which included 4 hour and 36 hour incubation times showed similar amino acid incorporation in all treatments after 4 hours, but incorporation by leaves inoculated with the pathogen was 100% more than by the controls after 36 hours. Similar results were obtained with $C^{14}$ valine. The recovery in synthetic ability may be the result of fungal growth in the tissue. Inoculation with washed and unwashed spores gave similar results. A boiled leaf control did not incorporate amino acids.

Table 1. Effect of H. victoriae infection of susceptible oat leaves on incorporation of $C^{14}$ leucine into the trichloroacetic acid precipitable fraction.

| Inoculum[a] or treatment | Incubation time[b] | CPM[c] x 100 |
|---|---|---|
| Pathogenic H. victoriae | | 5 |
| Nonpathogenic H. victoriae | 8 hours | 24 |
| Water control | | 19 |
| Pathogenic H. victoriae | | 8 |
| Nonpathogenic H. victoriae | 12 hours | 20 |
| Water control | | 19 |
| Pathogenic H. victoriae | | 19 |
| Nonpathogenic H. victoriae | 24 hours | 16 |
| Water control | | 16 |

[a] Inoculum contained approximately 50-75 spores per $mm^2$ of leaf surface. Drops containing spores were placed 2-3 mm apart on the leaf surface.

[b] Period from inoculation to vacuum infiltration with $C^{14}$ leucine. Leaves were then incubated with $C^{14}$ leucine for 4 hours.

[c] CPM (above background) per 0.1 ml fractions of NaOH hydrolysate.

# DISCUSSION

The validity of Victoria blight as a model for studying plant disease development is an issue of basic importance. It has been considered by some workers as an atypical disease. For example, Meehan and Murphy (45) concluded that "H. victoriae is primarily a facultative soil and seed-borne saprophyte that possesses a low order of phytopathogenicity," and that it is "too weak a parasite to establish infection in healthy tissue, even of susceptible oat varieties, without the help of toxic secretion in advance." Walker (79) questioned the parasitic nature of H. victoriae. Wheeler and Luke (83) indicated that Victoria blight is an unusual disease which should not be used as a general model. Kuć (35) has questioned the requirement of toxin for fungal growth in tissue, the involvement of toxin in symptom expression, and the importance of toxin in the expression of resistance. It is possible that such reservations are based on negative evidence, which is never conclusive, or on an incomplete analysis of the disease. I will attempt to evaluate this model on the basis of recent data.

A detailed histological study of H. victoriae in susceptible and resistant oat leaf tissue was completed by Paddock (57). All of Paddock's observations that I reexamined were confirmed. Penetration occurred

quickly after spores germinated. Hyphae ramified freely through the tissues for 2 days with little or no apparent effect on the host cells. Paddock found that susceptible cells collapsed 48-72 hours after inoculation. Thus the relationship between H. victoriae and its host appears characteristic of many diseases.

H. victoriae susceptible leaves were colonized by germinating spores of a pathogenic isolate or by spores of a nonpathogenic fungus to which toxin was added. In either case, toxin is known to be present at the site of infection, since toxin is released when pathogenic spores germinate (52). In both cases, histological observations showed that the host-fungus relationships were similar. There was an initial period of compatibility between host and parasite, followed by collapse of the tissue (57). When spores of a nonpathogenic H. victoriae isolate were suspended in inactivated toxin and applied to susceptible leaves, the results were identical to those obtained when spores were suspended in water. Apparently the toxin breakdown products and impurities in the toxin solution do not confer pathogenicity to the fungus. A threshold amount of toxin was required by the nonpathogenic H. victoriae isolate for successful invasion of susceptible tissue.

Disease resistance was expressed as limited fungal growth in host tissues. A typical resistant response occurred in H. victoriae resistant leaves under all conditions used. A resistant response also occurred in H. victoriae susceptible leaves when toxin was absent from infection sites as was the case when H. carbonum or nonpathogenic H. victoriae spores (52)

were used as inoculum. Visible responses of resistant cells, which included epidermal cell wall thickenings and apparent hypersensitive reactions, appear to be secondary effects of resistance. There were sometimes hints of cell wall thickenings below appressoria as early as 12 hours after inoculation of resistant leaves, but these indications were not distinct enough for positive identifications. The thickenings became evident between 12 and 24 hours after inoculation. Hypersensitive reactions were not yet visible at 24 hours. Resistance was first expressed as fewer penetrations and less hyphal growth in resistant than in susceptible tissue between 8 and 12 hours after inoculation. Therefore, the earliest visible host response occurs too late to account for resistance to the fungus. Phytoalexin production by oat tissue also appears to occur too late to account for resistance (52).

Another indication of the secondary role of visible host responses in resistance was the lack of correlation between fungal growth and host response. Only a small percentage of the appressoria or penetration pegs induced a cell wall thickening or a hypersensitive reaction. These host responses appear to be similar to those described for other diseases (2, 75), and support the belief that Victoria blight is similar to many other diseases. In some cases, hyphae were observed beyond the area of amber cells, indicating that the host response did not inhibit growth of hyphae. Most fungal growth, either on the surface of the leaf or in the tissues, caused no visible reaction even after 48 hours.

An apparent exception to the typical resistant response occurred when H. carbonum was placed on H. victoriae resistant oats in the presence of toxin; after 24 and 48 hours hyphae were frequently seen growing all the way through a leaf. H. carbonum did not make such growth in either resistant or susceptible leaves when no toxin was present. The long hyphae of H. carbonum in resistant leaves in the presence of toxin were thinner and stained less darkly than did hyphae growing in susceptible leaves in the presence of toxin. As in the usual reaction of resistant cells, a few cell wall thickenings and amber areas were present but they had no obvious effect on the abnormally long hyphae.

Fungal degeneration on the surface of the leaf, which was observed in all host-fungus combinations, occurred between 24 and 48 hours after inoculation. Careful observations were made to determine whether this phenomenon was correlated with resistance. No such correlation could be made. In some instances there appeared to be more hyphal degeneration on resistant than on susceptible leaves, but no consistent, clear distinction was evident.

Many types of fungi are known to grow on the surface of green leaves without colonizing the tissue. Saprophytic fungi quickly colonize yellowing or dead leaves (23). Ascospores of N. tetrasperma, a saprophyte, germinated and hyphae grew copiously on the surface of oat leaves, but there was no colonization and no visible effect on the leaf tissue. When a small amount of toxin was added to N. tetrasperma ascospores, colonization occurred in H. victoriae susceptible but not resistant leaves. However, three aspects

of the development of N. tetrasperma in the presence of toxin differed from that of potential pathogens under the same conditions.

1. Hyphae did not attach to the leaf surface, produce appressoria, or penetrate the epidermis.
2. Depending on the toxin concentration, 12-36 hours longer was required for hyphae to gain access to leaf tissue.
3. There was neither a typical susceptible nor a typical resistant relationship with oat tissue.

N. tetrasperma had no period of compatibility with H. victoriae susceptible tissue when toxin was present, and induced no host responses when toxin was absent. When toxin was present, host tissue began to disintegrate as soon as hyphae entered the leaf. The saprophyte destroyed toxin damaged tissue more effectively than did the potential pathogens. The atypical action of N. tetrasperma can be explained on the basis of differences between potential pathogens, which can invade living tissue, and saprophytes, which can colonize only dead tissue. The saprophyte was completely harmless to oat leaves until toxin had killed the cells. Once the fungus colonized toxin-killed tissue, its powerful macerating enzymes caused rapid collapse and disintegration of the host cells. The suggestions that H. victoriae is a saprophyte which lives on toxin-killed tissue are unfounded since the mode of action of H. victoriae is strikingly different from that of a saprophyte. In addition, evidence that host cells are not dead in the early stages of infection is provided by the physiological experiments.

An attempt was made to correlate histological observations with some known biochemical effects of the toxin. HV-toxin and H. victoriae infection produce similar physiological effects in susceptible oat tissue. There are increases in respiration, ascorbic acid oxidation, and permeability in infected and in toxin treated tissues (27, 82). Increased loss of electrolytes and decreased amino acid incorporation occur very quickly after toxin treatment (64, 67). A study of such changes in infected tissue was undertaken for comparative purposes and to determine the viability of cells after infection. The rate of metabolic decline in infected cells may be important in determining the ability of H. victoriae to invade living tissue.

The earliest known response of susceptible tissue to HV-toxin is increased loss of electrolytes (64). Ion leakage from susceptible cells occurs within minutes after exposure to toxin (66). When drops containing toxin were placed on intact leaves, increased electrolyte leakage occurred after a 15 minute treatment. An increase in electrolyte loss from infected tissues was detected 6 hours after inoculation. This was somewhat earlier than epidermal penetration, which was evident in some cells 8 hours after inoculation. Spores are known to release toxin on germination (52). Therefore, it is conceivable that the fungus received nutrients from the host before penetration occurred, which could give the fungus an advantage in its conquest of host tissues. However, increased electrolyte loss is an indication of an effect on cell membranes, but is not necessarily an indication of cell death or metabolic inactivity.

Dehydrogenase activity is one parameter of metabolic activity. My data show no change in dehydrogenase activity of infected tissue 12 hours after inoculation, but after 24 hours the activity was greatly reduced. This is an indication that the capacity for metabolic activity was maintained after membrane alteration occurred. It is probable that the majority of leaf cells were exposed to toxin 12 hours after inoculation, since hyphae could grow all the way through a leaf within 12 hours.

Another criterion of a living cell is its ability to synthesize macromolecules. $C^{14}$ amino acid incorporation in infected tissue was significantly decreased 8 hours after inoculation. This could mean that the protein synthesizing machinery was inhibited or it could mean that the labelled amino acid did not reach the site of synthesis, since the cell membrane was altered soon after inoculation. It is important that a significant amount of amino acid incorporation occurred as late as 12 hours after inoculation. This suggests that the synthesizing machinery was still at least partially viable and that cells were not dead.

## SUMMARY

Histological observations indicated that the host-parasite relationship of H. victoriae and oats is typical of many plant diseases. Addition of toxin to infection drops of a nonpathogenic mutant of H. victoriae and to fungi pathogenic to other species resulted in typical invasion of H. victoriae susceptible oat leaves. This suggests that toxin is required for pathogenicity.

Resistance was observed as a limitation of fungal growth in the tissue. Resistance was expressed in H. victoriae resistant tissue and when toxin was absent from infection sites of H. victoriae susceptible tissue. Visible host responses do not appear to be primary factors in disease resistance. Development of a saprophyte in the presence of toxin on H. victoriae susceptible tissue differed from development of potential pathogens under the same conditions. Physiological experiments showed that the early effects of infection parallelled the early effects of toxin treatment and that host cells were not dead before or soon after infection occurred.

These results support the thesis that Victoria blight is a useful model for the study of plant disease development.

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