SYMPTOMATIC, RESPIRATION AND BIOCHEMICAL RESPONSES OF CUCUMBER TO SCAB (CLADOSPORIUM CUCUMERINUM ELL. AND ARTH.) INFECTION

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SYMPTOMATIC, RESPIRATION AND BIOCHEMICAL RESPONSES OF CUCUMBER TO SCAB (CLADOSPORIUM CUCUMERINUM ELL. AND ARTH.) INFECTION

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CLYDE LEAON BURTON

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CLYDE LEAON BURTON ABSTRACT

Environmental, respiration and chromatographic studies of Cladosporium cucumerinum infection in cucumber leaf tissues were made to obtain information leading to an understanding of the mechanisms of disease resistance. Susceptible (Yorkstate and inbred CS2W) and resistant (Maine No. 2 and inbred CS2M) cucumber plants were grafted in all stock-scion combinations. After graft unions were firm, the plants were inoculated. Within 5 days all new growth on susceptible scion parts showed scab symptoms. Resistant-type scion material was resistant.

All attempts to demonstrate specific anti-parasitic compounds in resistant plants before and after infection gave either negative or inconclusive results.

Under winter conditions, infection of the first true leaf of susceptible individuals was 2 days slower than in summer. Both respiratory changes and tissue necrosis were affected. Plants grown under 300 ft-c artificial light also showed scab symptoms 2 days later than those under 900 ft-c.

In systemic or local respiration experiments, respiration increases in the leaf tissue were noted in most cases whenever extensive damage occurred to the vascular supply of the leaf. When the lower half of the leaf was infected and respiration was measured on the infected portions of the same leaf, increases were observed.

Only slight increases in respiration occurred in the upper non-infected portion of this leaf. No increases were noted, however, in respiration of the lower half if only the upper half of this same leaf was infected. This would indicate that respiration increases can be initiated by blocking the flow of transitory metabolites between the leaf and the root system. This blocking may be accomplished either by severing the vascular elements mechanically or as a result of infection.

Attempts to locate the source of the respiration increases biochemically were negative. Respiratory increases induced by DNP were similar in diseased and non-diseased tissue and it was concluded that respiration uncoupling was not a feature of infection with scab. Similarly it was concluded that the succinic dehydrogenase system was not differentially involved. Infiltration of radioactive carbon labelled glucose (C_1 and C_6) in leaves showed that the oxidative pathway was not changed in scab infection.

Leaf respiration studies were conducted at 24 hour intervals on inoculated and noninoculated plants for a 5 day period. Chromatographic studies of leaf tissue free amino acids were run in parallel with respiration studies for possible leads as to the function of these materials in disease resistance or susceptibility. Sixteen ninhydrin positive amino acids and amides were identified in cucumber leaves and 2 of them, asparagine and glutamine, were reduced or absent in severely diseased plants. Pipecolic acid was found to be

a common nitrogenous product in cucumber plants. It was found that respiration increases coincided with the disappearance of asparagine and glutamine as well as with the appearance of visible symptoms.

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INTRODUCTION

Scab, "spot" or "spot rot" disease has long been an economically important disease of the cucumber. It was first reported by Arthur (3) in 1889 as a fungus inciting scabby lesions on the fruit of the cucumber. The symptoms on cucumber foliage were described in 1893 (22).

Since parasitism of the cucurbits by Cladosporium cucumerinum is favored by low night temperatures and high humidity the disease is essentially one of the cucumber growing sections in the northern states, parts of Canada bordering on these states and northwestern Europe.

The causal organism is an imperfect fungus Cladosporium cucumerinum Ell. and Arth. It is most conspicuous on susceptible young cucumber fruits where it produces discrete lesions. Leaf lesions are similar but often disregarded since they cause little direct loss. The lesion is characterized by limited cell destruction and discoloration of the immediate infected area. Olive green masses of spores developing subsequently on the surface of this restricted area are inocula for infections on other plants or new growth. Spread may be by wind, water, and cultural operations such as picking.

The pathogen is a facultative parasite, overwintering in the soil and on organic matter in the field. Control of such a well adapted endemic organism is difficult with chemicals and no very

successful fungicides, capable of full protection of the many rapidly developing fruits, has been marketed. The development of scab resistant pickling cucumber varieties in recent years has been the salvation of this industry. Although most cucumber varieties are susceptible to limited invasion by the pathogen, incompatibility of the host tissue restricts the lesion greatly and makes the resistant variety a success.

This type of host-parasite relationship and the facultative nature of the organism provides an excellent opportunity to investigate the disease under various conditions and on several hosts varying in susceptibility. Invasion of both resistant and susceptible tissue is the same but some unknown defensive mechanism in the resistant variety generally limits development of the parasite. The degree of development and severity of disease in susceptible tissue is a function of the interaction between host and parasite, and this function is directly dependent on the environment.

The purpose of this research was to investigate the disease in resistant and susceptible cucumber plants under different environments in an attempt to find or define any physiological or biochemical factors of disease resistance. To this end a number of approaches were utilized. Studies were made on respiration modifications, free amino acid variations, as well as other plant constituents that might differ in the resistant and the susceptible host. Observations on symptomatic responses occurring under various environmental

conditions and their coincidence with other responses were integral in the entire investigation.

This approach was chosen because disease involves a modification in the physiological constitution of the host. The reaction of the host to the invading parasite induces biochemical reactions which may destroy the metabolic equilibrium of the host cells. An understanding of changes taking place within inoculated and noninoculated resistant and susceptible tissue is necessary before a general understanding of the host-parasite relation can be deduced.

REVIEW OF THE LITERATURE

Many references in the literature indicate that plant disease may be controlled or modified by changes of the external environment and mechanical or structural features of the host. The effect of the environment on the host's metabolism and its morphology is limited by the genetic constitution of the plant.

Literature reviews bearing directly on the biochemical and physiological nature of parasitism, and various defensive mechanism found in infected plants have been reported by Gäumann (20); Allen (2); Walker and Stahmann (42); and Uritani and Akazawa (40), and no attempt will be made to expand on them in this connection. Literature pertinent to cucumber scab is cited hereafter where required to illustrate the general aspects of the problem. Other literature with a direct and distinctive relationship to a part of the investigation is discussed or cited elsewhere.

Despite many diligent investigations relatively little is known of the basic nature of disease resistance in plants and less is known in particular of cucumber scab resistance. Why one variety may be resistant and another susceptible to scab, remains obscure. Genetic resistance in cucumber varieties to scab was shown to be controlled by a single gene pair dominant for resistance (41).

Walker (41) inoculated cucumber plants at different stages of development. He concluded that the optimum temperature for disease

development was 17° C, being lower than the optimum for either the host (25° C) or the parasite on agar (21° C). Schultz' (33) and Behr's (6) findings were different from Walker's as to the optimum temperature for disease development, both stating that 25° C appeared to be the more favorable temperature.

Incubation periods ranging from 2 to 6 days for complete disease expression have been reported (6, 29, 34, 41). Differences in culture, variety, pathogenic environment, and interpretation of results have undoubtedly been responsible for most of the disagreement reported.

Pierson (30) traced the disease development in susceptible and resistant cucumber tissues inoculated with C. cucumerinum. He found in susceptible varieties that penetration was direct by well defined appressoria and occurred within 48 hours. Initially the pathogen was intercellular and developed along the vascular strands and in the intercellular spaces of the cortex of stems and petioles within a short time. The pathogen assumed an intracellular habit approximately 96 hours after inoculation. The same rapid progress was also noted in leaf tissue. Hyphae were seen between the cells of the epidermis and the palisade mesophyll within 48 hours after inoculation. Twenty-four hours later the hyphae had reached veinlets and vein endings. At the end of 96 hours the hyphae was observed to have reached the vascular bundles in all parts of the leaf and intracellular habit had been assumed in the parenchyma cells adjacent to them.

Phloem, phloem parenchyma and xylem parenchyma had broken down. Pierson concluded that cells were killed in advance of the hyphal invasion by an enzymatic action. He further stated that penetration was the same in resistant and susceptible varieties, but later progress of the fungus in the resistant varieties was arrested by a series of host-parasite reactions associated with cell wall thickening and cell necrosis.

Husain and Rich (23) recently offered an explanation of dissolution of cell walls by the pathogen. They stated that C. cucumerinum produced both cellulolytic and pectic enzymes extracellularly. Their results, however, are based on activity in vitro. Fouad (17) working with the same organism and a susceptible variety, demonstrated, by using staining techniques, that the parasite secreted a pectinolytic enzyme which destroyed parts of the middle lamella of the cell wall.

The metabolism of green plants and some fungi is closely keyed to the light supplied and there are many ramifications of the light-disease relationship. When photosynthesis is checked, respiration is altered, thus effecting carbohydrate and nitrogen metabolism. Both the kind and amount of host metabolites are probably greatly influenced by the inter-action between host genotype and extremes in light intensities as well as photoperiods. Bonner (9) stated, for example, that the asparagine and glutamine concentration increased in plants during the night.

The host cell contents appear to be critical in determining the relation of the host to the invading parasite. It is thus important

to examine the cell contents, of both resistant and susceptible plant material, for properties that might suggest anti-parasitic action, or lacking that, some variations in essential metabolites. Specific inhibitory substances have been demonstrated, for example, in tomato genetically resistant to Cladosporium fulvum (36). Presumably this was an alkaloid, although the exact composition of the material was not determined. Cook and Taubenhaus (12) correlated instances of disease resistance with tannin formation in cells. Recently, Garber and Houston (19) found an unidentified partly diffusable fungistat in verticillium wilt-resistant cotton seed. Other reports of resistance factors is sap of resistant plants that are active in culture have been reviewed by Ranker (31); Walker and Stahmann (42).

It has long been debated that plants may have a system of inhibitors that would not be set into play unless excited to do so by certain types of aggressive parasites (10, 28). This defensive mechanism operates only after an initial injury by a pathogen. The parasite may excite the cell upon penetration by disturbing the host metabolic balance through the interaction of the 2 biological systems coming in contact with each other. A plant that is inherently resistant to the invader would contain only the potential for this defense, but could quickly bring about a great influx of the inhibiting material. Brown (10) found, by laying drops of distilled water on the intact surface of a variety of plant parts, that in many cases solutes diffusing from the tissues markedly stimulated the germination of spores. Other solutes had the opposite effect.

In recent years there has been an interest in the amino acid content of plants. There is reason to believe that these constituents of plant cells play a role in plant resistance mechanisms. Although there are limitations to the methods employed, some contributions were made by studying amino acid composition of healthy and diseased plants. Benedict and Hildebrand (7) reported that soybeans, which were highly susceptible to infection by Diaporthe phaseolorum, earlier in their development, became less susceptible as they grew older. They attributed this to the increases in the amino acid content of the plant as it grew older. Laloraya and Jee (25) noted increases in certain water-soluble or free amino acids in the case of virus infected Nicotiana leaves. Fuchs and Rohringer (18) reported leaves from certain wheat varieties such as Marquis and Vernal, infected by P. graminis tritici showed losses of several amino acids when compared with the healthy plants. Kuc, et al. (24) showed that infusions of D and DL isomers of phenylalanine, as well as other amino acids, into the growing shoots via leaf midribs, increased the resistance of certain apple varieties to attack by Venturia inaequalis. Various other studies established similar changes in amino acids between diseased and healthy plants (11, 15, 38). The evidence from the literature appears insufficient to hypothesize any special amino acid resistance relationship applicable to all plant diseases.

One of the most interesting changes that takes place in the metabolism of diseased tissue is the alteration of respiration. There has been little work on the physiology and biochemistry of

diseases caused in facultative parasites such as C. cucumerinum, however, the rust and mildew diseases, both obligate parasites, have been investigated extensively from this standpoint (1, 2, 13, 14, 16, 27, 32, 35).

Allen (2) proposed limited evidence that respiration increase caused by wheat infected with Puccinia graminis could result from parasitic toxins which uncouple respiration from energy-requiring processes of the host tissue. Shaw and Samborski (35) concluded that stimulation of O_2 uptake by DNP was just as great in rust-infected wheat leaves as in the healthy. Daly, Sayre, and Pazure (13) compared the rate of evolution of radioactive CO_2 from leaf disc infiltrated with C_1-labelled or C_6-labelled glucose. They indicated that the direct oxidative pathway is of increased importance in plants infected with rust. Farkas and Kiraly (16), using P. graminis tritici infected wheat leaves, found that inhibition of respiration in healthy tissue was about twice that of rust-infected tissue. By-passing of the usual succinate-fumarate oxidation was evident.

MATERIALS AND METHODS

Generally, unless a special environment was needed, the plants were grown in the greenhouse using the best available horticultural practices. The cucumber plants were raised in permanent benches, or in clay pots, or in flats, depending on the nature of the study. The soil was a freshly steam-sterilized, sandy-loam with approximately 10 percent peat added. Ordinarily, no fertilizer was added to the soil mixture.

Cultures used for inoculations were grown in Petri dishes on potato-dextrose agar at room temperatures. The spores were harvested from the culture mat 7 to 10 days after the seeding by adding distilled water to the plates and rubbing with a rubber tipped glass rod. The suspension of detached spores together with mycelial fragments was filtered through a fine sieve to make an atomizable inoculum. Preliminary experiments showed that spore concentration, could vary within wide limits without affecting disease development; because of this no precise adjustment of number of spores per unit volume was made. The same approximate concentration of spores was maintained for all experiments, however, by diluting the suspension to about the same visual turbidity.

The inoculum was sprayed on the plants until the leaves were dripping. Several types of sprayers were used; a small paint gun, a DeVilbiss atomizer, and a hand atomizer. In certain experiments

spores were applied by "painting" them on with a small brush, or swabbed on the plant surfaces with cotton applicators.

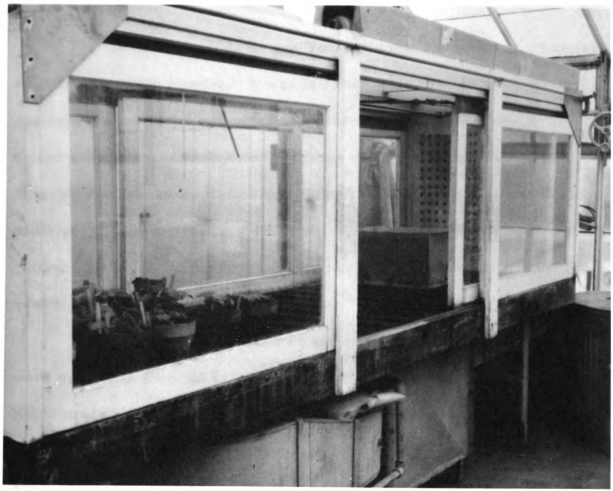
The controlled temperature-humidity chamber used for most incubation after inoculation was a large, 12-flat capacity, glass enclosed bench (Figure 1). Temperature below that in the greenhouse was maintained within $\pm$ 1° F. The humidity was maintained by mist from air-water spray jets (Spraying Systems Inc.). Plants in the chamber were protected by additional plastic covers to insure isolation and to prevent wash-off of inoculum.

Sources of Cucumber Varieties

Four pickling type cucumber varieties, of definite genetic constitution and known scab reaction under both field and greenhouse test conditions were used throughout the investigation. Yorkstate and CS2W varieties are susceptible to scab, whereas Maine No. 2 and CS2M varieties are resistant. Yorkstate is a blackspined, mosaic resistant, scab susceptible pickling variety developed by H. M. Munger from crosses involving Chinese Long, Early Russian, and National Association Pickling. Maine No. 2 is a white spined, mosaic susceptible, scab-resistant variety, released by the Maine Agricultural Experiment Station in 1939 (4).

Since phenotypic characters depend on the interaction of the genotypes and the environment, it was very important that the varieties used in this study respond alike in every way except disease development to a given set of conditions.

FIGURE 1. --The controlled temperature-humidity chamber. Through the open glass door can be seen one of the plastic covers used to insure isolation and to prevent wash-off of spores.



In early growing stages Yorkstate and Maine No. 2 varieties are phenotypically similar. It was felt that while interaction between genotype and environment might not distort the disease picture a closer breeding was desirable. Knowing the genetic and phenotypic differences between Yorkstate and Maine No. 2 varieties, it was decided to adopt two new sister lines which would, as nearly as practical, be uniform for all the characters evident, excepting susceptibility to scab in several environments.

Scab susceptible (CS2W) and scab resistant (CS2M) plants were developed from an original cross between Yorkstate and Maine No. 2 made by Isbit and deZeeuw.[a] The sister lines were selected after several generations of backcrossing and homozygous resistant and susceptible line preserved. These lines labelled CS2W and CS2M were increased by the author to obtain sufficient seed for the projected experiments.

Sources of the Pathogen

Four isolates of C. cucumerinum were tested for their suitability as inoculum for this study. The original cultures were obtained from Michigan, Wisconsin, Illinois and England (Table 1).

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[a]Personal communication by D. J. deZeeuw on Experiment Station Project No. 156.

TABLE 1.--Sources of cultures of Cladosporium cucumerinum used in experiments

MSU-5	D. J. deZeeuw, Michigan State University, East Lansing
Wis-3	C. F. Pierson, University of Wisconsin, Madison
Libby	Libby, McNeill and Libby, Blue Island, Illinois
Ches. A	F. Joan Moore, Harpenden, Herts, England

Single conidial isolates were made from the individual cultures and sub-cultures from these were studied under various combinations of environmental conditions. Phenotypic differences between the 4 isolates were evident in the amount of sporulation and in color of the spore mass. Exploratory experiments were made to find out if the isolates displayed the same degree of virulence. It was considered that cultures behaving alike in virulence under varying conditions of the environment such as light, temperature, etc., would be equally useful and uniform in demonstrating more subtle interactions of the host-parasite and environment. The 4 isolates were grown on potato-dextrose agar and spores sprayed on the host tissue under optimum conditions for development of the desease. The cultures showed the same high degree of virulence.

Respiration Techniques

Oxygen uptake by leaf tissue was measured in a Warburg respirometer, using standard manometric techniques (39). Leaf samples were washed in distilled water, and discs were cut with a

sharp 6-mm cork borer. Twenty leaf discs from each set of plants were placed in Warburg flasks with bottom liners of a slightly moistened filter paper. Each treatment was duplicated. The temperature of the water bath was 30° C and the entire mechanism was covered with a black cloth to prevent photosynthesis. Respiration readings were taken 30, 60, and 120 minutes after equilibration. After the O_2 uptake data was taken the leaf discs were removed from the vessels and dried for 48 hours in a 100° C oven and weighed. The data calculated were usually in terms of microliters of gas (taken up or evolved) per hour per mg dry weight of plant tissue.

In the experiments involving the use of 2, 4-dinitrophenol (DNP), malonic acid, and labelled compounds, the leaf discs were submerged in the various solutions. The solutions were then vacuum infiltrated into the host tissue. Control tissue was infiltrated with M/30 potassium phosphate buffer (pH 6.0).

Glucose-1-C^{14} and glucose-6-C^{14} solutions used in estimating the direct oxidation pathway in plant respiration were prepared by diluting them to approximately the same activity with non-labelled glucose solutions. Each flask of glucose-1-C^{14} had 50,501 cpm and each flask of glucose-6-C^{14} had 46,361 cpm. The reaction solution per flask contained 10 u moles glucose (including C^{14}-labelled glucose) and 66 u moles KH_2PO_4 in 2.0 ml. For quantitative determination of the conversion of carbohydrates to CO_2, the center well of the flask contained 0.2 ml of 20 percent CO_2-free NaOH. The

recovery period of respiration CO_2 was three hours. The respiratory CO_2 was precipitated as $BaCO_3$ by the addition of $BaCl_2$. The $BaCO_3$, after aging overnight, was filtrated onto a weighed 24 mm paper disc held by an aluminum planchet. The radioactive carbon was determined by employing a sensitive counter and the data recorded on an ultra-scaler.

Grafting Techniques

It was very difficult to obtain successful grafts unless special conditions were provided for the plants. A mist chamber constructed of clear plastic was placed on the greenhouse bench. The bottom of the chamber was lined with thermostatically controlled heating coils buried in wet sand. A high pressure atomizer air-water jet was used to keep the relative humidity near 100 percent. The grafted plants were kept in the chamber at 27° C for 5 to 10 days. After the graft union was firm, the humidity was reduced to about 70 percent and the temperature to that prevailing in the greenhouse. After a few days of conditioning, the plants were moved to a shaded area on a greenhouse bench. At first the plants were subjected to short periods of sunlight. The short light periods were continued until the plants were able to withstand normal greenhouse conditions. The grafted plants able to survive without showing any unusual physiological symptoms, were used in the inoculation tests.

Chromatography Techniques

For the determination of the free amino acids within the cells of the leaf tissue of healthy and diseased cucumber plants,

modifications of techniques discussed by Block (8) and Hrushovetz (21) were employed. The general procedure for the separation of the free amino acids in preparation for spotting was to thoroughly homogenize frozen leaf samples in a blender with sufficient 95 percent ethanol to give a final ethanol concentration of approximately 80 percent. The resulting homogenate was placed in a 60° C oven until the alcohol evaporated. The dried material was finely ground and stored in air tight bottles. One hundred milligrams of the leaf extract powder was mixed with 20 ml of 80 percent ethanol. This solution was refrigerated for 24 hours. The contents were stirred several times during this period. The samples were then filtered to remove the insoluble residue using an additional 5 ml of 80 percent ethanol as wash. To each volume of filtrate, three volumes of chloroform were added. After a thorough mixing, the resulting aqueous layer, which contained the water soluble amino acids, was removed and saved. This material was used for spotting.

Aliquots of the undiluted free amino acid solution were applied to Whatman No. 1 chromatographic filter paper (18 x 22 inches) using untra micro-pipettes. The samples were applied to the paper as spots confined within small specified circles marked a standard distance from the edge of the paper. One- and two-dimensional descending techniques were used.

A standard size stainless steel chromatocab designed to hold 10 full-size papers was used. Large chromatojars with the

capacity of eight 4 x 22 inch papers were also used. The mobile phases for the two-dimensional runs were n-butanol:acetic acid: water (250:60:250 V/V/V) and phenol and triple distilled water (80:20). The above solvents were also used for one-dimensional analysis.

As far as possible all of the necessary precautions that are recognized for obtaining reliable separation were used. Some of these procedures included equilibration of the papers, controlled temperatures, running times, pure chemicals, etc. At least 4 standards, made up from known amounts of amino acids of varying concentrations, were included with each run. The extracts from the 4 varieties, during the same time interval, were run at the same time within the same chamber so as to eliminate as many outside variations as possible within each run.

The chromatograms were developed by spraying with a 0.25 percent V/V solution of ninhydrin in acetone. The color areas were outlined with pencil and identified 24 hours after developing. The different samples were compared and color density of the spots noted. Preliminary examinations of chromatograms by ultra-violet light before development with ninhydrin were found unsuitable for the materials analyzed.

EXPERIMENTAL RESULTS

Effect of Some Environmental Factors on Disease Development and Respiratory Responses to Infection

Effect of Humidity on Symptom Expression

Since several disease variations were observed that were believed caused by fluctuations in humidity, an experiment was designed to investigate the importance of this factor. One hundred and eight plants from each of the 4 varieties were kept 6 days at a temperature of 17° C and in a dry atmosphere. An equal number of seedlings from each variety were maintained in the same environment without inoculation. At intervals of 24 hours for 6 days after being placed in the chamber, leaf tissue from all the varieties in the 2 treatments were collected for respiration studies.

The results of this study indicated that there was no significant respiration variation between either the varieties or the treatments in the absence of infection. In later respiration studies on diseased tissue subjected to low temperatures and high humidity, it will be noted that there were significant variations in the respiration of healthy and diseased tissue. A temperature below the host optimum and a prolonged period of free moisture on the leaf surfaces is necessary for good disease development.

Effects of Photoperiod on Symptom Expression

There was a seasonal variation of 2 days between the time it took susceptible varieties to fully express disease symptoms. During the long days of summer 4 days were required for expression of disease symptoms at 17° C; 5 days during fall and spring and 6 days during the short days of winter.

Because attempts were planned to disclose biochemical and physiological differences between resistant and susceptible plants, any cultural variability in symptom expression should be known. With both genetic constitution and temperature factors stabilized, symptomatic differences or differences in incubation period could be the result of variation in light intensity or photoperiod. Experiments were conducted to investigate these factors.

The first experiment in this series was a short term study of photoperiodism and its effect upon the host-parasite interaction of resistant and susceptible cucumbers. Plants were held under different photoperiods prior to inoculation, during inoculation, and after inoculation.

The 4 standard varieties were used in the single leaf seeding stage. The treatments consisted of:

A(1). Pre-inoculated growth under continuous light. Daylight supplemented by incandescent lamps. Continuous light during 48 hours in the inoculation moist chamber. Normal

light on the greenhouse bench (average length of day - 10 hours, intensity 300 ft-c).

A(2). As in A(1) but kept in complete darkness after removal from chamber.

A(3). As in A(1) but kept under continuous light after removal from chamber.

B(1). Pre-inoculation growth under normal greenhouse light. Complete darkness during the 48 hour moist chamber period. Normal light after removal from the chamber.

B(2). As in B(1) but kept in complete darkness after removal from the chamber.

The susceptible varieties, Yorkstate and CS2W, displayed disease symptoms 6 days after inoculation (Table 2). About 50 percent of the plants were severely diseased. Although initially there appeared to be a correlation for severity of disease and rapid death with post-inoculation darkness--treatment A(2) and B(2)--this was later attributed to the high humidity maintained in the metal chambers used. All susceptible individuals of this treatment except one Yorkstate seedling died within 6 days after inoculation. It was found that the disease was more severe when inoculated susceptible plants were kept under conditions of high humidity after being removed from the chamber.

A second experiment was made in the late spring when the normal greenhouse light was higher to study the effects of abnormal periods of light and darkness and variations in temperature on the host and development of disease.

TABLE 2. --The environmental conditions used in photoperiod study on parasitism of resistant and susceptible cucumbers by scab

Treatment	Number[a] plants	Environmental conditions controlled		
		Pre-inoculation	Inoculation	Post inoculation
A-1	48	Continuous light (daylight plus incandescent)	Continuous light	Normal daylight
A-2	48	"	"	Continuous darkness
A-3	48	"	"	Continuous light
B-1	48	Normal daylight	Complete darkness	Normal daylight
B-2	48	"	"	Complete darkness
Control	12	"	Normal daylight	Normal daylight

[a]All 4 varieties were used, and each treatment was represented by 48 plants from each variety.

Two varieties (CS2M and CS2W) were each treated by maintaining part of them in darkness at 29° C for the 4 days prior to inoculation and the other part at 29° C in continuous light. Inoculated plants were incubated at 17° C. The plants that had the pre-inoculation dark period were again darkened and held at 23° C after removal from the moist chamber. The light conditioned plants were returned to continuous light at 23° C. A second group was treated in the same manner except the moist chamber temperature was maintained at 23° C for the 48 hour inoculation period (Table 3).

No alteration of disease expression was induced by pre-inoculation darkness or continuous light treatment at high temperature

TABLE 3. --Environmental conditions used in the photoperiod study on cucumber varieties CS2M and CS2W

Variety	Number plants	Pre-inoculation condition	Inoculation condition	Post inoculation condition
CS2M	24	Darkness 4 days 29° C	Normal light 17° C	Darkness 23° C
CS2W	24	"	"	"
CS2M	24	Continuous light 4 days, 29° C	"	Continuous light 23° C
CS2W	24	"	"	"
Control	12	Normal light	Normal light	Normal light
CS2M	24	Darkness 4 days 29° C	Normal daylight 23° C	Darkness 23° C
CS2W	24	"	"	"
CS2M	24	Continuous light 4 days, 29° C	Normal light 23° C	Continuous light 23° C
CS2W	24	"	"	"

in comparison to the normally grown controls. All the susceptible plants displayed good symptoms 4 1/2 days after inoculation if moist chamber incubation was performed at 17° C. No disease developed in either susceptible or resistant plants when this incubation was at 23° C.

The third experiment involving photoperiodism is also related to nutrition of the fungus. Observations made during preliminary investigations indicated that cotyledons of susceptible plants would greatly restrict the development of the organism's growth after a short period

of time. The mycelium was able to spread in the surface tissues of the cotyledon for only a few days then become static and sporulated. The pathogen would remain viable as long as the cotyledons remained healthy. After 6 weeks of minimal growth the pathogen was still static and did not spread or invade the rest of the plant even when the plant was placed in an environment optimal for disease development. The spores were found to be viable when cultured, however.

This phenomenon conceivably resulted from either a nutritional deficiency in the cotyledonary tissue after sufficient growth of the fungus or the development of metabolic changes, possibly inhibitory substances, as a result of parasitism. These ideas were examined indirectly. The first approach was to find out the importance of chlorophyll in the nutritional value of the resistant and susceptible plants in disease development.

The 4 cucumber varieties were used to study the effect of etiolation on susceptibility of cotyledonary tissue. The treatments consisted of:

A. Plants grown 10 days after emergence in total darkness at 22° C before inoculation. Incubation, after inoculation, at 17° C in darkness for 48 hours. Post incubation at 26° C in total darkness at high relative humidity.

B. Plants grown 14 days after emergence in darkness before inoculation. Incubation at 17° C in darkness for 48 hours. Post incubation at 20° C in darkness as above.

C. Plants grown 18 days after emergence in darkness before inoculation. Incubated at 17° C in darkness for 48 hours. Post incubation at 20° C in darkness as above.

D. Controls were plants given normal greenhouse light exposure.

Although the 10 day old seedlings were held at a higher post-inoculation temperature and humidity than the 14 day old seedlings, the latter nevertheless were suitable for disease development. Thus it is assumed that in the 18 day old plants there was not enough food for the organism to develop, whereas there was sufficient food for parasitic development in the seedlings etiolated for only 10 days. It was additionally noted that the 10 day plants exposed to the 26° C post-inoculation temperature were very weak. Some decomposition of the tissue before inoculation could have provided a growing medium for the fungus living semi-saprophytically. If the latter explanation were valid it would appear that length of etiolation time was immaterial, and the nutrients involved in truly parasitic invasion do not develop in the host in the absence of light.

Results obtained from the experiments on etiolated seedlings were variable. Where etiolated seedlings were inoculated 18 days after emergence, there were no visible disease symptoms, although the controls were normally diseased. When the seedlings were inoculated 10 days after emergence, the susceptible CS2W variety was infected.

The stems broke down after 6 days, approximately 1 inch below the cotyledons. Some cotyledons were also affected in areas next to the stem. The inoculations of the 14 day etiolated seedlings were inconsistent. Only part of the infected plants developed scab symptoms.

Effect of Light Intensity on Disease Development and Respiratory Responses to Infection

Symptom expression during the winter was approximately 2 days later than initial symptoms during the early and mid-summer. A 4 day period was required for complete breakdown of the seedlings during the summer, whereas at the same temperature it required 6 days during the winter. In each case incubation in the moist chamber for 48 hours was at 17° C and post inoculation growth at 23° C. Photoperiod, as reported above, was not responsible for this large difference. Since it was noticed that winter grown plants were less vigorous than those cultivated during the summer season, they may also have provided a poorer nutritional environment for the parasite and thus retarded pathogeneses. Light intensity is higher in the summer and also affects host metabolites at least quantitatively. An experiment was designed to test directly or indirectly the association of high light intensity with more rapid invasion and tissue breakdown. Susceptible CS2W seedlings were grown under 2 levels of fluorescent lighting. Eighty plants were grown at 900 foot-candles and 80 at 300 foot-candles. The illumination measurement was taken 1 inch above the pots. A 16 hour photoperiod

was used and no supplemental natural illumination admitted. The 300 ft-c illumination level is roughly equivalent to that supplied by the greenhouse incandescent lighting used to extend the winter photoperiod to 16 hours. The 900 ft-c illumination is well above the average incident on plants in the greenhouse during a Michigan winter. The temperature under light ranged from 26° C to 29° C and held at above 24° C in the dark period.

Two week old seedlings grown under 900 ft-c were a deep green color; the stems were thick; and the plant had a generally vigorous appearance. The 300 ft-c plants were in the same stage of leaf development but were pale green in color; the stems elongated; and the plant generally weak in appearance. The 900 ft-c plants were visually representative of the type of seedling grown under natural light in the summer months while the 300 ft-c plants were comparable to the usual winter seedlings.

One-half of the plants of each treatment was inoculated by spraying with a suspension of spores and held in the moist chamber at 17° C for the next 6 days. The controls were similarly treated without inoculation. During this period all plants received the same unsupplemented low level illumination that filtered into the chamber from the greenhouse proper.

Samples for respiration studies were taken from representative first leaves of each treatment at 24 hour intervals after inoculation The experiment was repeated 2 additional times under the same

conditions. Yorkstate was used as an additional variety in one of the replications.

It was found that there were no significant changes in respiration rates between treatments until 72 hours (Table 4). At that time there was a slight increase in respiration of the inoculated 900 ft-c plants coinciding with the earliest symptoms, a water-soaked appearance of the leaf veins.

TABLE 4. --Effect of light (900 vs. 300 ft-c) on respiratory response of tissue infection

Tissue	Pre-inoculation light (ft-c)	O_2 Uptake[a]					
		24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
Healthy	900	6.7	5.8	6.6	6.2	6.5	6.3
Disease		7.2	6.4	8.3	10.1	13.6	12.1
Healthy	300	7.1	5.5	6.4	6.8	7.1	6.3
Disease		7.2	6.1	6.6	6.5	8.7	9.5

[a] ul O_2/mg dry weight per 2 hours.

At 96 hours the diseased 900 ft-c tissue was respiring at a substantially higher rate than the healthy tissue and considerable vein soaking was noted. By 120 hours the diseased tissue was respiring 100 percent greater than the healthy tissue. The plants were badly diseased and the petioles and leaf surfaces were covered with water-soaked

spots. Tissue destruction was nearly complete at 144 hours, when it was difficult to obtain good representative samples of living leaves. Cotyledons were severely infected.

The first indication of any disease symptoms on the 300 ft-c samples was at 120 hours. This was a barely discernable vein soaking. There was no indication of a respiration increase. At 144 hours only a small amount of vein soaking was noted. The emerging second true leaf, however, was badly infected, especially the petiole. Respiration increase in the infected 300 ft-c plants at 144 hours was comparable to that obtained for the 900 ft-c plants at 72 hours.

An additional observation of the inoculated 300 ft-c plants at 168 hours showed that although many first true leaf petioles were severely diseased, the leaf lamina remained fairly intact. There was no significant damage to the cotyledons.

Further experiments on the varieties including Yorkstate gave identical results. Another trial on CS2W in which an inoculum--carborundum mixture was rubbed on the leaves showed wounding the tissue did not promote a more rapid or severe disease development.

The importance of inoculum load (spore concentration) was investigated in relation to symptom development in plants grown at 300 and 900 ft-c. When plants were sprayed with either the standard spore suspension or a one-tenth dilution no measurable differences in time of symptom development or disease severity could be found.

Effect of Nitrogen Nutrition
on System Development

The experiments on high and low light intensities lead to an experiment on the effect of supplemental nitrogen on development of the disease in seedlings. Ten pots each containing 3 to 5 plants of each of the 4 varieties of cucumbers were planted during the winter using regular potting soil. Five pots from each variety were watered with a solution containing 45 percent urea supplement (1 tablespoon per liter of water) every 4 days. The remaining pots were watered with tap water. The nitrogen-fed plants were noticeably deeper green than the ones supplied only with water. When the plants were in the first true leaf stage, nearly 3 weeks after planting, they were all transferred to the temperature control moist chamber at 17° C, inoculated and held there for 4 days. After this incubation period the pots were returned to the greenhouse bench.

The dark green plants again showed increased susceptibility in agreement with the results obtained by using higher light. The susceptible varieties, given extra nitrogen, were badly diseased within 4 days, whereas the same varieties without the nitrogen broke down only after 5 1/2 days. The nitrogen amended resistant varieties when inoculated showed the flecking or hypersensitive reaction ordinarily obtained on the more vigorous, dark green resistant individuals in which the pathogen has made rapid invasion but no extensive growth.

Symptom Development and Respiratory Response in Cotyledons

The results obtained from the experiment involving etiolated seedlings were not conclusive. There were, however, indications that some unknown conditions of nutrition, depletion, or inhibitory elements prevailed in the cotyledonary tissue which restricted disease development.

Susceptible CS2W and Yorkstate seeds were planted in fifty 5-inch clay pots, 3 to 5 seeds per pot. The seeds in 15 pots of each variety were immediately watered with a dilute suspension of spores taken from 7 day old plate cultures. These plants were kept at greenhouse conditions for 10 days. Approximately two-thirds of the plants had small scab lesions on the surface of the cotyledons later developing into greyish-green spore mats. Both diseased and control plants were held for 10 days in a dry atmosphere at 17° C to prevent rapid growth. At the end of this time the scab lesion remained confined and the cotyledons had stopped growing.

The plants were then placed in the moist chamber at 19° C and held there for 6 days. No evidence that the disease had progressed could be seen. The lesions were indexed for the pathogen by plating bits of tissue and spores on potato-dextrose agar and shown to be viable.

From the above cotyledon samples, material was chosen to study the respiration differences between the treatments. Twenty discs from each variety were collected from healthy plant cotyledons;

from the lesioned areas on the diseased cotyledons; and from areas closely surrounding the lesions, but not including them. The experiment was performed 3 times using duplicate flasks for each treatment.

Results indicate that lesions will not expand unless tissue is expanding. There were no significant respiration differences between the 3 types of cotyledonary tissue used in the study nor between the varieties. This is in contrast to a preliminary study of respiration in actively growing inoculated CS2W cotyledons. In the CS2W cotyledons a very significant respiratory increase was coincident with infection. Respiration of fungus tissue present in the inoculated lesions was as low in activity as the mature host cells it replaced.

Studies on the Nature of the Respiratory Response to Infection

Are Respiratory Responses Systemic or Local?

In order to further test the localization of resistance, a series of respiration experiments were designed to find out what metabolic effects infected cells may have on non-contiguous cells. It was also planned to find out how the primary leaf lamina is affected when other parts such as stems, cotyledons and petioles are diseased. The objective was thus to obtain evidence of movement of metabolic disease material in advance of the pathogen.

Plants of the susceptible variety CS2W were inoculated by spraying a suspension of spores on designated parts of the seedlings

(Table 5). Non-inoculated comparable plant parts were sprayed with distilled water for controls. Samples for respiration studies were taken by punching discs from both upper and lower halves of 10 representative leaves unless otherwise specified (see Table 5, numbers 3, 4 and 5). Samples were taken 72, 96, and 120 hours after inoculation.

Respiration studies showed that there was slightly more oxygen uptake in the lower portion of the healthy leaf than the upper half of the leaf. This same small difference was also found in the completely inoculated plants (number 5). At 72 hours a significant increase in respiration was recorded in the lower half of the leaf in situations where the lower half of the leaf, petiole, cotyledons and stem areas were inoculated (number 4a). A slight respiration increase was noted in the upper noninoculated half of the same leaf. An increase in respiration was observed in the lower half of the leaf when only the lower half of the leaf was inoculated (number 3a). A significant increase in respiration at 96 hours occurred in the completely inoculated plants (number 5); the plants in which the petiole, cotyledons and stem were inoculated (number 2) as well as the cases mentioned above. These increases were noted especially where there were indications of visible breakdown.

At 120 hours, a considerable respiration increase was noted in the inoculated lower half of the leaves, in comparison with the noninoculated upper half of the same leaf. When only the top half of the leaf was inoculated (number 6), respiration of the upper half

TABLE 5. --Respiration analysis of leaf tissue from cucumber plants submitted to various restricted inoculations with Cladosporium cucumerinum

	Treatment		O_2 Uptake[a] Percent of control		
	Parts inoculated	Parts noninoculated	72 hour	96 hour	120 hour
1.	Petiole	Leaf, cotyledons, stem	96	114	103
2.	Petiole, stem, cotyledons	Leaf	96	140	117
3.	(a) Lower half of leaf		112	144	146
		(b) Top half of leaf petiole, cotyledons, stem	100	108	102
4.	(a) Lower half of leaf, cotyledons, stem, petiole		120	153	127
		(b) Top half of leaf	102	113	105
5.	Complete plant		118	150	126
	Lower half of leaf				115
	Top half of leaf				105
6.	Top half of leaf				138
		Lower half of leaf, petiole, cotyledons, stem			100

[a]Respiration made only on leaf tissue. Composite samples taken from both top and lower halves of leaves as indicated in 1, 2, and the first part of 5.

at 120 hours showed a considerable increase in oxygen uptake. There was no increase in respiration in the lower portion of this leaf compared to the lower half of the control.

Respiration rate increases were only of significance in areas that showed damage caused by the pathogen. There was no evidence of any toxin diffusing from the infected site, able to cause respiration increases in the sound tissue near the point of damage.

Responses of the plant to disease under these conditions are local in nature. Each point of spore penetration involves only a few cells but greater leaf damage may develop from multiple infections near the main conduction system of the leaf. Respiration rate increases thus result from cumulative cell destruction whether caused directly by the pathogen or indirectly by disruption of its vascular supply.

To substantiate this idea, respiration studies were made on CS2W leaves 120 hours after inoculation in comparison to healthy leaves that had been cut from plants 24 and 48 hours previously. The non-inoculated excised leaves were kept in Petri dishes on moistened filter paper and under the same general environment as the inoculated intact plants. When sampled for the respiration study the leaves in the Petri dishes were sound and showed no signs of wilting. The controls were noninoculated intact plants. Table 6 shows the respiration differences between samples of mechanically severed, noninoculated attached, and attached inoculated leaves.

TABLE 6. --Comparative respirations of attached and severed cucumber leaves

Treatment	Oxygen Uptake[a]
Detached 48 hours--not inoculated	10.74
Detached 24 hours--not inoculated	9.65
Attached 120 hours--inoculated	9.85
Attached 120 hours--not inoculated	6.30

[a] ul O_2 mg dry weight per 2 hours.

Respiration of inoculated leaves at 120 hours was less than that of noninoculated leaves that had been detached for 24 hours (9.85 compared to 10.74). All detached or inoculated leaves respired at approximately 50 percent greater rate than the controls. This experiment helps to explain why respiration of leaves from completely inoculated plants was generally greater than that of tissue from plants with restricted inoculations. In ordinary inoculation extensive damage to the petiole, with attendant vascular disruption, usually occurs. The respiration rate increase noted in diseased tissue could be a secondary response caused by the prior damage to the host. Conditions such as mechanical injury of the host cells, or deficiency in certain necessary plant constituents can cause respiration rate increase.

Respiration errors in these trials introduced by the presence of fungus hyphae in tissue were not controlled. Since respiration did not rise until the host responded visibly, although the fungus hyphae

were previously active, it is assumed that host respiration was measured.

Possible Quantitative Changes in Respiration after Infection

Respiration studies reported above have demonstrated a marked increase in activity coincident with infection of cucumber by the scab fungus. Further, it was concluded from studies of restricted inoculation and grafting experiments that if there are metabolites diffusing from the parasite, they do not exert their action on adjacent sound tissue but rather on the cells directly parasitized. As respiration increases represent some disruption of the normal metabolic cycle it should be possible to locate the site of disruption eventually by producing similar effects with chemicals whose activity has been already defined. Under these conditions, the action on noninoculated tissue should be parallel to that produced by pathogenesis. Ideally if disruption of respiration by the pathogen is at a single site, the known respiratory reactant will have no additive effect on parasitized cell respiration.

Uncoupling Effects?

Low concentrations of 2, 4-dinitrophenol (DNP) are known to inhibit the uptake of inorganic phosphate by intact cells and uncouple respiration from the energy-requiring activities of the cell. This type of uncoupling was first suggested by Allen (1). Accordingly experiments were designed to compare the effect of DNP on respiration of cucumber with that produced by C. cucumerinum. Five groups of experiments

involving a total of 18 individual experiments using CS2W healthy and diseased leaf tissue were carried out using standard Warburg manometry. Respiration studies on tissues infiltrated with 10^{-4}, 2×10^{-4}, 4×10^{-4}, 10^{-5}, 2×10^{-5}, and 4×10^{-5} M DNP were made at 24 hour intervals after inoculation. The experiments, run on duplicate or triplicate samples were concluded 6 days after inoculation. The controls were similarly infiltrated with a 30/M KH_2PO_4 solution.

There was considerable increase in respiration caused by the presence of DNP (10^{-5}M). Its effect on inoculated tissue, however, was as great as on noninoculated tissue (Table 7). This indicates that the action of the metabolites responsible for the respiration stimulation in scab diseased tissue is not the same as the uncoupling agent, DNP. Only the quantitative increase in respiration is the same.

Changes in Citric Acid Cycle?

Malonic acid is a known inhibitor of succinic dehydrogenase, a vital enzyme in the citric acid cycle, upon which the carrier activity of the succinate-fumarate system depends. Malonate combines with the enzyme similarly to succinate to reduce the rate of oxidation. Instead of yielding reaction products like the enzyme-succinate complex, however, it gives the more or less inactive enzyme-malonate complex and contributes nothing to the reaction velocity. If malonic acid, infiltrated into the leaf, inhibits respiration of healthy tissue but does not inhibit in the diseased tissue, it would indicate that in the diseased tissue there is respiration taking place by means other than the normal pathway.

TABLE 7.--Effect of 2, 4-dinitrophenol (10^{-5} M) in stimulating O_2 uptake in inoculated, and noninoculated cucumber (CS2W) leaves susceptible to Cladosporium cucumerinum

Treatment	O_2 uptake[a]					
	24 hour	48 hour	72 hour	96 hour	120 hour	144 hour
Noninoculated						
30/M KH_2PO_4	10.74	9.45	10.33	9.87	11.17	10.91
DNP	15.15	13.74	14.54	13.41	14.53	14.19
Percent increase	41.0	45.5	40.8	36.0	30.5	30.0
Inoculated						
30/M KH_2PO_4	10.25	10.24	11.76	11.76	13.15	15.62
DNP	15.17	13.65	15.96	15.61	16.09	18.79
Percent increase	48.0	33.2	35.8	33.0	22.2	20.2

[a] ul O_2/mg dry weight per 2 hours.

It could possibly be that the respiration responses are due to metabolites from the parasite.

Methods similar to those used with DNP were followed except that malonic acid was used in concentrations of 10^{-2}, 5×10^{-3}, and 10^{-3} M. As in the preceding experiments 30/M KH_2PO_4 was used for the control infiltrations. Determinations were made at 96 and 120 hours after inoculation.

Respiration values obtained from malonic acid infiltrated leaf tissue indicated that this part of the citric acid cycle is not by-passed

in scab infection. Respiration responses of healthy and diseased tissues infiltrated with 10^{-2} concentration of the inhibitor indicated that the oxidation of succinate to fumarate via the succinic dehydrogenase enzyme system is of major consequence in both types of tissue (Table 8).

Is Direct Oxidation of Carbohydrates Being Stimulated?

Shaw and Samborski (35); and Daly et al. (13) compared the rate of evolution of radioactive CO_2 from leaf discs infiltrated with C_1-labelled or C_6-labelled glucose. They speculated that the direct oxidative pathway is of increased importance in plants infected with rust. The Embdem-Meyerhof-Parnas pathway (EMP) is thus compared to the direct oxidation pathway in the cells of scab infected cucumber plants. If only the EMP pathway contributes to CO_2 production, the ratio of radioactive CO_2 from C_6 and C_1, respectively (the C_6/C_1 ratio), would be 1.0; if only direct oxidation is involved in CO_2 production, the ratio would be zero; a combination of the two routes would yield a ratio between these extremes (2, 5).

It would be of interest to determine whether or not direct oxidation of carbohydrates is stimulated in all diseased plants, since this pathway could provide the precursors of phenolic compounds, which are said to function in certain resistant reactions (40). Studies employing radioactive tracer techniques were made on both inoculated and noninoculated CS2W primary leaf tissue. Respiration was

TABLE 8. --Respiration of diseased and healthy scab-susceptible (CS2W) leaf tissue as influenced by infiltration with 10^{-2} M malonic acid

Treatment	96 hour		120 hour	
	O_2 uptake	%inhibition	O_2 uptake	%inhibition
Healthy				
KH_2PO_4	15.23		10.55	
Malonic	10.18	33	2.50	76
Diseased				
KH_2PO_4	22.37		17.01	
Malonic acid	12.38	54	7.51	56

determined at 24 hour intervals for a total of 4 days after inoculation. The leaf discs were vacuum infiltrated with glucose labelled either in the first or sixth carbon.

The C_6/C_1 ratio of inoculated tissue was essentially the same as that of noninoculated tissue at the 24, 48, and 72 hour samplings. There was a significant respiratory increase in the inoculated samples at the 72 and 96 hour manometric study (Table 9). The ratio of radioactive C_6/C_1 for both healthy and diseased tissue was between 0.25 and 0.34 at 24, 48, and 72 hours. At 96 hours the ratio changed slightly, and the healthy tissue had a ratio of 0.31. The disease tissue ratio was approximately 0.53.

TABLE 9. -- Respiration analysis of the experimental material used in the study of radiochemical tracers infiltrated into susceptible, diseased and healthy cucumber tissue. C_6/C_1 ratios obtained for the same test period

Treatment	Total O_2 uptake[a]			
	24 hour	48 hour	72 hour	96 hour
Healthy				
Glucose 6-C^{14}	10.37	10.86	11.53	10.94
Glucose 1-C^{14}	10.12	9.08	10.87	10.78
Diseased				
Glucose 6-C^{14}	10.17	10.94	14.48	14.45
Glucose 1-C^{14}	9.97	10.42	12.93	14.28
	CO_2 evolved[b]			
Healthy				
Glucose 6-C^{14}	530	692	533	475
Glucose 1-C^{14}	1533	2745	1768	1524
Ratio	0.34	0.25	0.30	0.31
Diseased				
Glucose 6-C^{14}	525	661	890	913
Glucose 1-C^{14}	1678	2283	3500	1713
Ratio	0.31	0.29	0.25	0.53

[a] ul O_2/mg dry weight per 3.0 hours.

[b] Average counts per minute of precipitated $BaCO_3$.

Are Materials of Fungus Origin Causing Respiration Increases?

An experiment was designed, finally, to find out whether extracts from freshly ground spores and mycelium contained any materials, possibly enzymes, capable of inducing host symptoms associated with parasitism.

Ten day old mycelial mats grown on liquid potato-dextrose nutrient were washed in cold, distilled water and ground in a large mortar with sea sand and a small quantity of water. The grinding, filtering through sintered glass, and homogenizing was done at approximately 2° C. The filtrate was homogenized in a blender with glass beads for 10 minutes. The homogenate was tested for viability of fragments by seeding PDA in Petri dishes. Only 5 colonies developed in 15 plates. The homogenate was kept at 2° C until applied variously to the leaves of the 4 cucumber varieties in the greenhouse. Two incubation conditions were used. Part of the seedlings were placed in the 17° C moist chamber and part were left on the open greenhouse bench at 20 to 24° C. The treatments used on each variety at the 2 temperature conditions were as follows:

1. Cotton swabs, dipped in the homogenate, were rubbed freely over the top and bottom surfaces of the leaf.
2. As No. 1 but with the addition of carborundum.
3. Controls. Rubbed with water-carborundum.
4. Inoculated with viable spores by rubbing a spore-water carborundum mixture over the leaf surfaces.

5. Inoculated by rubbing spores in water on the leaf. No carborundum.

6. The homogenate was injected into the stem tissue of the plants with a hypodermic syringe.

7. Control. Water only injected.

Each treatment unit consisted of 15 plants. There were 5 pots per treatment and 3 plants in each pot. The same treatments were repeated at 17° C only.

No evidence of metabolites capable of producing scab symptoms under the test conditions was found (Table 10). Disease developed normally for each variety only when viable spores were applied under 17° C moist conditions but traces of faint infection occurred on susceptible plants treated with the homogenate since a few viable fragments of mycelium or spores were present. No symptoms of disease developed in any treated host incubated at the higher temperature. Some slight superficial injury resulted from the carborundum abrasion but was not to be confused with scab lesions. Slight mechanical injury resulted alike from injection of either water or homogenate.

Are There Exotoxins Formed in Fungus Filtrates?

Diseased and healthy CS2W cucumber leaves were infiltrated variously at the 120 hour stage with (a) dilute nutrient solution (10 ml potato juice made from 200 grams potato per liter, 10 grams dextrose, made to 1,000 ml with water), (b) the same solution after supporting

TABLE 10.--Effects produced by various applications of non-viable mycelium- and spore-homogenates of Cladosporium cucumerinum to resistant and susceptible cucumber seedlings

Variety	Incubation[a]	Symptoms resulting from application of:[a] Carborundum only[c]	Homogenate only	Homogenate plus carborundum	Viable spores only	Spores plus carborundum	Homogenate injected[b]	Water injected[b]
Yorkstate	17°	-	F	F	S	S	-	-
(susceptible)	20°	-	-	-	-	-	-	-
CS2W	17°	-	F	F	S	S	-	-
(susceptible)	20°	-	-	-	-	-	-	-
Maine No. 2	17°	-	-	-	R	R	-	-
(resistant)	20°	-	-	-	-	-	-	-
CS2M	17°	-	-	-	R	R	-	-
(resistant)	20°	-	-	-	-	-	-	-

[a]Explanation of symbols: "-" no disease, "F" occasional lesion, "S" typical susceptible lesion, and "R" typical resistant flecks.

[b]17° C incubation in moist chamber; 20° to 24° C incubation in open greenhouse.

[c]Superficial injury.

growth of C. cucumerinum for 3 weeks, and (c) a filtered extract of the fungus itself made up to 5 percent (W/V). Respiration was measured on these to determine the activity contributed by such things as blocked carbohydrates, nitrogenous materials, fungus metabolites, and fungus hyphal material.

There was an increased respiration of from 50 to 90 percent attributable to the added nutrients from the broth or the fungus itself (Table 11). The depleted nutrient broth, however, did not produce any additional respiration in either infected or non-infected leaves.

TABLE 11.--Respiration responses to infiltration of cucumber (CS2W) leaves with potato-dextrose broth, spent culture broth, and fungus extract

Treatment	O_2 uptake[a] 120 hours after inoculation	
	Healthy tissue	Infected tissue
30/M KH_2PO_4	8.57	13.77
Potato-dextrose nutrient[b]	17.51	21.20
Culture filtrate[c]	9.07	13.59
Fungus filtrate[d]	11.77	16.01

[a] ul O_2/mg dry weight per 2 hours.

[b] 10 ml potato juice made from 220 grams potato per liter, 10 grams dextrose, made to 1,000 ml with water.

[c] Cell free PD broth after 3 weeks growth of C. cucumerinum.

[d] Homogenized fungus mat, filtered and made to 5 percent (W/V) extract.

Correlation of Respiratory Response with Changes in Free Amino Acids

Analysis of the free amino acids in cucumber were made as a part of the study on the biochemical nature of resistance. It is fully realized that this is neither the sole group of metabolites involved in disease resistance nor necessarily the most important. Such materials, however, may supply a source of nitrogen nutrient for the parasite in susceptible tissue that is unavailable in resistant tissue or be utilized in enzymes required for pathogenesis (37). At present many of the amino acids can be identified readily and with some precision in small amounts of plant tissue by paper chromatography. Because of the importance of these metabolites either to the parasite or as a reflection of parasitic action, it was decided to investigate their presence in healthy and diseased cucumber plants differing in resistance to scab.

Plants of the 4 varieties were grown to the beginning of the 2 leaf stage under the prevailing greenhouse conditions. Half of them were inoculated and incubated at 17° C in the moist chamber and the remainder similarly treated without inoculum. Approximately 100 plants from each treatment were selected and samples taken for 5 days at 24 hour intervals. Immediately after harvesting part of the leaves were transferred to a freezing unit where they remained until removed for amino acid analysis and another part used for a parallel respiration experiment.

The varieties CS2M and CS2W were considered to be the most important because of their genetic similarity, and were studied in more detail. Respiration determinations were begun within 30 minutes of collecting the leaves. All sampling for both amino acid and respiration determinations was completed before mid-morning to partially control diurnal effects.

Chromatographic analysis of the 4 cucumber extracts indicated there were many ninhydrin-positive nitrogenous compounds. Soon after undertaking this study it was realized that the methods contained many limitations. Difficulty was experienced in obtaining closely reproducible results and for this reason only 24, 72, and 120 hour samples were replicated enough times to be considered highly reliable. The two-dimensional chromatograms made from the alcoholic extract were compared with standard amino acid test chromatograms for identification of the components. At the 24 hour sampling 16 ninhydrin spots were identified as typical of all varieties and treatments. One of these and a 17th spot was thought to be a separation of glutamic acid (Table 12 and Figure 2). The leucines were not separated by the method used and were considered as a unit. The amino acid and amide array of the noninoculated plants of all varieties remained unchanged throughout the experiment.

When the 4 inoculated varieties were analyzed it was noted that their amino acid and amide constitutions at 24 hours were the same as the controls. At 72 hours, however, the inoculated

susceptible varieties showed reduced amounts of glutamine and asparagine compared to all noninoculated varieties or the resistant varieties. Chromatograms obtained from inoculated susceptible material, at 120 hours, had no identifiable ninhydrin reactions for glutamine or asparagine. Other amino acids appeared to be unchanged in concentration in all cases regardless of variety, inoculation or stage of incubation.

TABLE 12. --Amino acids determined by paper chromatography from extracts of inoculated and noninoculated Maine No. 2, CS2M, Yorkstate, and CS2W cucumber seedling leaves. Twenty-four, 72 and 120 hours after inoculation

1.	Aspartic acid	9.	Histidine or Arginine
2.	Serine	10.	Citrulline
3.	Glutamic acid	11.	a-Alanine
4.	Glycine	12.	g-Aminobutyric acid
5.	Asparagine[a]	13.	a-Aminobutyric acid
6.	Lysine	14.	Pipecolic acid
7.	Glutamine[a]	15.	Valine
8.	Threonine	16.	Leucine (s)

[a]Began to disappear at 72 hours and not detectable at 120 hours in susceptible, inoculated plants.

FIGURE 2. --Two-directional paper chromatogram of amino acids from extracts of inoculated and noninoculated Maine No. 2, CS2M, CS2W and Yorkstate cucumber seedling leaves. Butanol-acetic acid-water was the first solvent. 1 - aspartic acid, 2 - serine, 3 - glutamic acid, 4 - glycine, 5 - asparagine, 6 - lysine, 7 - glutamine, 8 - threonine, 9 - histidine or argine, 10 - citrulline, 11 - a-Alanine, 12 - g-Aminobutyric acid, 13 - a-Aminobutyric acid, 14 - pipecolic acid, 15 - valine, 16 - leucine(s). Asparagine (5) and glutamine (7) began to disappear at 72 hours and were not detectable at 120 hours in susceptible, inoculated plants.

16
15
14
13
12
11
8
3
10
4
2
7
9
6
5
1
14
12
10
8
6
4
2
← PHENOL WATER
2
4
6
8
10
12
14
16
N-BUTANOL ACETIC ACID WATER →

Pipecolic acid, a relatively uncommon plant amino acid except in legumes, was found in all varieties. Its characteristic presence in cucumber is not revealed, however, when phenol is used as the first solvent for two-dimensional work.

Asparagine and glutamine are important in protein synthesis. The failure to find these two amides on the 120 hour chromatograms might possibly be explained if; (1) the pathogen was using them in its metabolic processes; (2) the metabolism of the host diverts them, or their precursors for other purposes. Number 2 appears to be more logical.

No evidence of new or different amino acids characteristic of diseased plants was found. Because of this the possibility of toxic amino acids diffusing from the hyphae of the pathogen or induced breakdown of more complex plant nitrogenous materials into amino acids was not considered.

Respiratory responses were correlated closely, in time, with quantitative and qualitative differences of glutamine and asparagine in host extract material. Respiration increase of inoculated susceptible plants was evident at 72 hours as was the first demonstrable reduction in asparagine and glutamine (Figure 3 and Table 12). At 96 and 120 hours the response was even clearer.

The respiration response of resistant varieties after inoculation is unique. In experiments using the 4 varieties, it was found that there was an initial increase in respiration of the resistant,

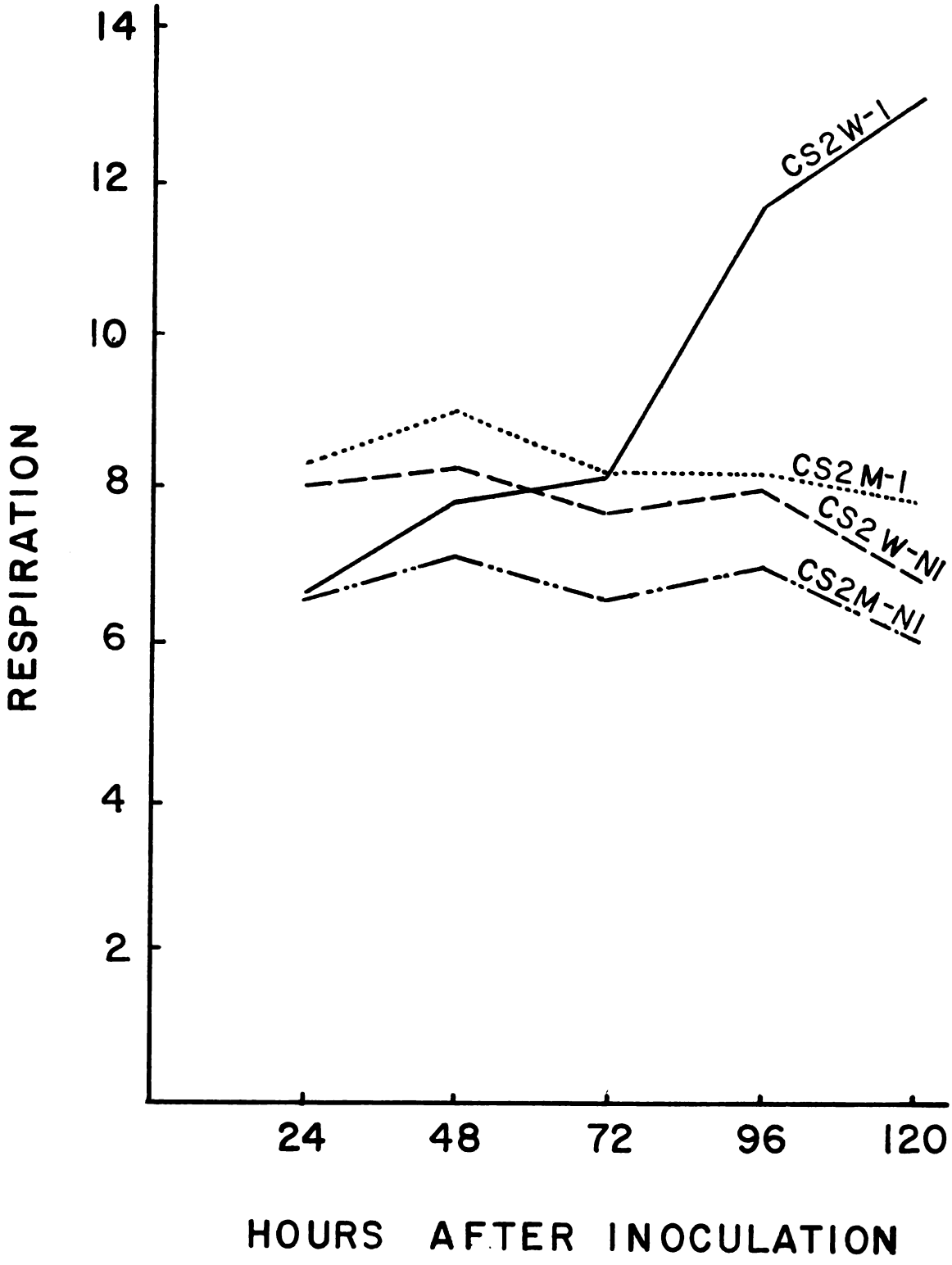
inoculated plants and this was always greater than its noninoculated counterpart and temporarily greater than for inoculated susceptibles. Once at this level, the respiration remained relatively constant. It was finally surpassed at 72 hours by the susceptible, inoculated plants but never by the resistant noninoculated ones. These results were confirmed in succeeding trials using the sister varieties, CS2M and CS2W, under closely controlled conditions. Since resistant inoculated plants react typically under these conditions by production of a necrotic fleck or hypersensitive spot, it is probable that such a consistent respiratory response is related to the defense mechanism of the host.

Experiments on the Nature of Disease Resistance

Are There Pre-formed Resistance Factors in Cucumber?

A series of experiments was undertaken to either substantiate or eliminate the possibility of scab inhibiting substances being present in genetically resistant cucumber tissue. Preliminary data obtained in preparation for this work showed that the pathogen was able to grow well on a variety of commonly used natural and synthetic media. The experimental efforts reported here were directed toward finding evidence of cell sap constituents inhibitory to the parasite rather than determination of missing essential plant materials. The latter aspect is treated elsewhere.

FIGURE 3. --Respiration response of resistant (CS2M) and susceptible (CS2W) cucumber leaves to infection by Cladosporium cucumerinum. The varieties Maine No. 2 (resistant) and Yorkstate (susceptible) followed the same pattern. Respiration in ul O_2/mg dry weight per 2 hours.


14
12
10
8
6
4
2
RESPIRATION
CS2W-1
CS2M-1
CS2W-NI
CS2M-NI
24
48
72
96
120
HOURS AFTER INOCULATION

Specific inhibitory materials occurring before infection and acting directly against an invading fungus might be located or defined by in vitro tests. If, however, the relationship is indirect and dependent on the living interactions of the specific parasitism a more refined approach would be required. Although indications are that many plant defensive mechanisms are extremely complex it was deemed advisable to investigate this approach to the problem.

The first of these experiments was designed to test activity of cucumber leaf juice incorporated in a culture medium. Samples of 100 leaves from each of the 4 varieties were washed in distilled water and ground immediately in a Waring blender with 25 ml of distilled water. The pulp then was divided into 2 aliquots. One was centrifuged (3, 500 r. p. m.) for several minutes to remove extraneous material and filtered through a sterilized Seitz filter. It was immediately refrigerated until used one-half hour later. The other aliquot, not filtered, was autoclaved for 15 minutes at 15 pounds pressure.

Twenty-five ml of water agar, cooled to 40° C was mixed in Petri dishes with either 0. 5 ml, 0. 1 ml, 2. 0 ml, or 3. 0 ml of the 8 cucumber juice extracts. One ml of a standard spore suspension was added immediately and the plates swirled to mix the contents. Reference plates were prepared with water agar alone using different spore concentrations. Comparative observations were made at 4, 7 and 14 days but only the readings of the 7th day are recorded (Table 13).

TABLE 13. --Effect of extracted sap incorporated in agar on the growth of C. cucumerinum

Concentration ml juice/25 ml agar	Mycelial growth[a]	Remarks
0.5	1	Poor growth, nearly the same as controls
1.0	2-3	Mycelial development scant
2.0	4	Fair development, some sporulation
3.0	4-5	Fair to good development, plates nearly covered with growth, fair sporulation

[a]All extracts of the 4 varieties, either heat sterilized or Seitz filtered, gave growth directly proportional to concentration of extract in the medium. Mycelial growth was rated as 0--no visible growth; 1--spore germination, very poor growth; 2--more than trace growth; 3--scant; 4--fair growth, some spore formation; 5--good mycelial growth, fair spore production.

There were no consistent differences in development of mycelium on media containing extracts from any of the four varieties. Both resistant and susceptible leaf extract material supported the growth of mycelium more or less in direct proportion to the concentration of extract in the medium. Spore germination on water agar was good, but subsequent growth was nil.

These data show that no thermostable or oxidation stable inhibitor was present in crude preparations of resistant cucumber leaves. Since half of the experiment included samples from filtered material never subjected to temperatures above 41° C, no enzyme constituent

that can be thus preserved is likely to be implicated. Observations at 4, 7, and 14 days suggested that there was no delay of mycelial growth early in the development of the pathogen, when being incubated on material from resistant plants. The nutritional value of the autoclaved plant extract was not disturbed, since equal mycelial growth was noted for both the heat sterilized and non-autoclaved plant juices.

Cucumber fruit juices were tested for presence of specific inhibitor using the same method employed with leaf juice. Hand pollinated fruit, of the four varieties, grown in the greenhouse, were selected from the different varieties at 10, 20, and 30 days of age. Different age fruits were used because older leaves develop a type of resistance and the age of fruit might be a factor. The fruit was thoroughly washed; the seeds removed; and the fruit ground without adjuvants. After grinding, the pulp samples (pH 6.8 to 7.1) were divided into 2 equal parts; one being filtered through cheese cloth and a sterile Seitz filter; the other autoclaved for 15 minutes at 15 pounds of pressure. Only the one concentration of juice, 3.0 ml, was used and all plates were incubated for growth of the fungus at room temperature. Water agar and PDA control plates were included.

Age of the fruit from which the extracts were made was not a factor in growth or inhibition of the scab organism. Growth on the extract media of fruits of different genetic resistance or age was better than on the leaf medium but not equal to that on PDA. Water agar controls supported spore germination only.

The following experiment was designed to compare the nutritional values of the 4 varieties when alcoholic leaf extracts are used as media for growth of the organism. Tests were also conducted in this manner on previously parasitized leaves to see if the nutritional value was reduced.

Leaves of the various test samples of seedling cucumbers were washed in distilled water and ground in a Waring blender with just enough 95 percent ethyl alcohol to facilitate the grinding. The alcoholic extract was then dried in a 60° C drying oven, stirring occasionally. The dried preparation was ground to a fine powder and stored in oven-dried glass containers. Two grams of the powder was placed in each 250 ml Erlenmeyer flask, mixed with 100 ml of glass distilled water, and autoclaved for 15 minutes at 15 pounds pressure. After the contents of the flask had cooled, 0.1 ml of spore suspension was seeded to each flask. The fungus was allowed to develop for 3 days on a mechanical shaker at 23° C. Following this the cultures were grown for 3 weeks at 23° C without agitation. The thick, black, floating mycelial mat was removed from the flask intact, washed in running distilled water, ground in 95 percent ethanol, and dried in a 60° C oven for 48 hours. Weights of dried mycelium were obtained from preparations of 4 varieties of noninoculated cucumber and of 2 varieties, CS2M (resistant) and CS2W (susceptible), that had been inoculated 96 hours prior to collecting (Table 14).

TABLE 14. --Weights of mycellium of Cladosporium cucumerinum grown in liquid cultures of alcoholic leaf extracts from resistant and susceptible cucumber leaves

Varieties	Resistance	Inoculation	Replication	Dry weight average
CS2M	R	NI	5	0.1965
Maine No. 2	R	NI	5	0.1838
CS2W	S	NI	5	0.1693
Yorkstate	S	NI	5	0.1554
CS2M	R	I	5	0.1866
CS2W	S	I	5	0.1633

These data indicate that extracted leaf material supported growth of the organism. Disease resistance or susceptibility was found to be not controlled by the supply of organic food. The 6 leaf powders tested were all excellent nutrient sources for the fungus. It can also be seen that growth of the pathogen on leaves for 96 hours did not seriously deplete the nutrients used by the same fungus in culture. No readily isolated heat-stable inhibitor could have been responsible for disease resistance since growth in culture, without exception, was somewhat better on preparations from the resistant varieties.

Are Resistance Factors Formed after Invasion?

A series of experiments were designed to see if there was a passive exosmosis of electrolytes from the tissue into drops of

water or spore solutions placed on the surfaces of the leaves and if these substances contained stimulators of inhibitors of the pathogen as reported by Brown (10) and Müller (28).

The first experiment was designed to test for defensive anti-parasitic metabolites eluted into water droplets around the area of attack. Seedlings of the 4 varieties, in first leaf stage were chosen. They were washed carefully with distilled water and grown for another 24 hours. They were then placed in the 17° C moist chamber under protective covers. Plants of each of the 4 varieties were sprayed with distilled water to the point where many small droplets remained on the leaf surface. Other plants were similarly sprayed with the standard spore suspension from a group of 7-day cultures.

After inoculation, droplets were removed from the leaf surfaces with micro-pipettes and pooled in small vials by treatment and variety. Collections were made daily for 5 days. Microscopic examination of the diffusates immediately after removal revealed there were very few detached spores, nearly all having germinated and adhered to the leaf. Mycelium was observed in plant epidermal cells 24 hours after inoculation. At this time there was no apparent difference in penetration of germ tubes or amount of mycelial growth in any of the resistant or susceptible varieties. The collected diffusates did not require filtration because of the few spores present. To each vial, which contained approximately 2 ml of diffusate, was added 2 drops of a fresh spore suspension in 0.5 percent dextrose. All the

seeded diffusates were examined at 24 hours for signs of spore germination inhibition or stimulation.

No evidence of diffusible, parasite-induced inhibitors of C. cucumerinum could be substantiated in this experiment. Neither resistance of host, presence of specific spores in the diffusion drop, nor age of infection was of significance in this respect. In all cases the ratio of germinated to nongerminated spores was approximately 3:1. Under the conditions described there was no diffusion of inhibitor to the leaf surface.

A similar experiment was undertaken using leaves of the resistant variety, CS2M cut from the plants and placed on moistened filter paper in Petri dishes. One-third of the leaves had drops of concentrated spore suspension placed on their upper surface; one-third had a 1:10 dilution of the spore suspension similarly applied and the remainder served as controls with distilled water only. The incubation temperature was 17° C and the relative humidity nearly 100 percent.

At 48 and 120 hours the diffusate was collected as before. Each collection was additionally filtered through sintered glass to remove spores and foreign material. Assay was made by preparing Petri plates each containing 20 ml of PDA (20 grams agar, 5 ml potato juice made from 200 grams potato per liter, and 5 grams dextrose, made to 1,000 ml with water). Sterilized one-half inch filter paper bioassay discs were placed on the surface of the agar, or wells

were cut out of the agar using a 6-mm cork borer. To each of the discs or wells was added two drops of the particular test diffusate. Distilled water was used as a control. Observations were made daily for 7 days.

The results were negative as in the preceding experiment. Apparently inhibitory substances, if present, were unable to diffuse from the interior of the infected leaf tissue into droplets on the leaf surface. Growth of the mycelium on the assay plates was uninhibited by the diffusate and had covered the agar completely by the seventh day. It was further determined that neither concentration of spores used to produce the diffusate or time of sampling the diffusate after inoculation were factors in inhibition. Progress of the leaf infection under the spore drops was essentially normal and the typical resistant necrotic flecking of resistant varieties was seen at 5 days.

The possibility remained that an inhibitory material induced by invasion of the pathogen in resistant tissue was present in such low concentration as to be ineffective in the tests used. An attempt was made to increase its concentration.

Leaf samples of CS2M were taken at 48 and 120 hours after inoculation. The samples were washed in distilled water and ground in a Waring blender using a minimum amount of 30/ M KH_2PO_4 buffer to facilitate grinding. Precautions were taken to prevent heating of the material during this operation. The leaf extract was filtered successively through a Buchner funnel and sterilized Seitz filters.

The filtrate was then reduced to approximately one-tenth volume in a flash evaporator at a temperature below 30° C.

The following tests for inhibitors were made with the concentrate:

a. Five bioassay discs soaked in the plant extract were placed on seeded low nutrient PDA, as described above, in each of 5 Petri dishes.

b. Three plates were prepared by mixing the PDA with 1 ml of concentrate to which 0.1 ml of spore suspension had been added.

c. One plate was supplied 4 ml of concentrate and 0.1 ml of spore suspension was added.

d. Controls consisted of the same low nutrient 2 percent PDA seeded with spores but without the plant juice concentrate.

As in previous trials, there was no indication of specific inhibitors in the plant material. The heaviest mycelial growth appeared in 5 days in the area immediately surrounding and over the bioassay discs in the case of both 48 and 120 hour samples. Where the plates contained the mixture of extract, spores, and PDA, growth was equal to or better than the control.

These results, and the results from the experiment using alcoholic leaf extract powder demonstrated that leaf material of resistant plants may offer an excellent source of nutrients for growth

and development of the pathogen. Since all the essential nutrients and none of the inhibitors appear to be present, resistance to the pathogen must be achieved by a dynamic host.

Is Resistance Influenced by Grafting Susceptible and Resistant Plants Together?

A different experimental approach to the possibility that certain plant metabolites may restrict, or aid the development of a pathogen was made through use of grafting techniques. Hypothetically, the sap of a resistant host mingling in the natural living state with the sap of a susceptible plant would carry translocatable inhibitors. If the latter were found to be less susceptible on inoculation the hypothesis of extra cellular transportable inhibitors would be strengthened. The plausibility of the reverse situation is also assumed, that is, the resistant host plant fluid may be attenuated by the intermingling of the susceptible host juices, resulting in less resistant plant tissue.

To investigate this hypothesis various grafts were made with several stock-scion combinations of the 4 different cucumber varieties. Successful cleft, approach, and other modifications of grafts were made using the 4 varieties of cucumber plants (Table 15). Most grafts were made at nodes. Grafts involved either components of the same age or stocks 2 weeks older than scion.

In most instances the plants were never developed beyond the third leaf stage. At times the cotyledons and 1 or 2 of the leaves

TABLE 15. --Grafts of scab-resistant and scab-susceptible cucumbers

Stock	Genetic resistance	Scion	Number of successful grafts
Maine No. 2	R	Yorkstate	8
		CS2W	9
Yorkstate	S	Maine No. 2	12
		CS2M	6
CS2M	R	Yorkstate	11
		CS2W	11
CS2W	S	Maine No. 2	5
		CS2M	13

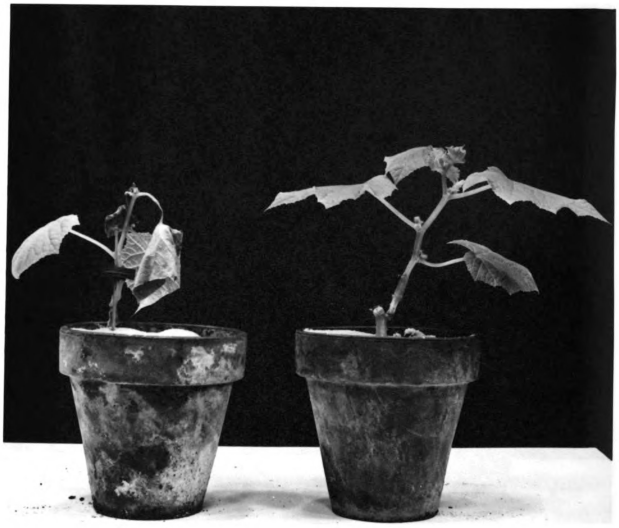
were removed. When the graft unions were complete, the stock part above the union was ordinarily removed. In making some of the grafts the stock plant was left intact, however. Some grafts were made with both root systems intact, and the scion's root system was not disturbed until there was evidence of a good take." Tongue and groove grafts were also made in which a tag section of the scion below the graft was left. This portion was placed in a vial of water to aid in graft establishment (Figure 4).

Because grafted plants had to be maintained in susceptible condition for several weeks before being inoculated, it was necessary to provide additional fertilization by using a complete nutrient solution at weekly intervals. Suitable control plants of the same age were included for comparison of inoculation. All the grafted and non-grafted plants were held after inoculation in the 17° C moist chamber for 2 weeks and observed twice daily for symptoms.

FIGURE 4. --Example of tongue type graft. The scion tag end was inserted in a water vial to aid in graft establishment.

FIGURE 5A. --Inoculation of grafted plants. (left) Susceptible scion on resistant stock. (right) Resistant scion on susceptible stock.

FIGURE 5B. --(left) Resistant scion on a susceptible stock. (right) Susceptible stock (top remaining on left side) and resistant scion (top on right side).



A

B

The young growing terminals of the susceptible tissue all showed excellent disease symptoms within 5 days (Figure 5A, 5B). Most of these newly formed terminals were badly wilted, but the older more mature leaves of the susceptible portion of the graft remained unaffected. The young terminal tissue from the resistant varieties showed hypersensitive lesions. The unique genetic resistance type was expressed in every graft combination used and was affected neither qualitatively nor quantitatively by the graft partner.

Since one graft component had no apparent influence on resistance of the other, it is possible to conclude that any possible inhibitory substance reacting to the invasion of the pathogen, is either quickly neutralized when translocated to susceptible tissue or, more reasonably, the resistance factor is entirely intracellular. Conversely, toxic substances produced in the susceptible plants by the pathogen could be quickly neutralized by the resistant tissue.

DISCUSSION

Cladosporium cucumerinum lends itself well to a variety of experimentation, being a facultative organism without obvious nutritional fastidiousness. It produces clear cut evidence of disease on seedling plants of genetically susceptible cucumber under predictable environmental conditions. Two of the test varieties, furthermore, were inbreds approaching the same genetic constitution but differing sharply in resistance to scab. This genetic similarity has thus made it possible to minimize the variable that different varieties generally introduce into studies of infection.

Experiments with various amounts of resistant and susceptible plant juice extracts from cucumber fruits and leaves treated in different ways to provide sterilized test samples, showed that the pathogen grew well on all the materials. Neither resistant or susceptible plant juices contained inhibitory elements which prevented the pathogen from making good growth regardless of concentration. Thus pre-infectional antipathogenic materials do not seem likely.

Experiments were designed to measure possible host cell defense reactions, operating to produce anti-pathogenic chemicals in the presence of the fungus. No substances could be found that would inhibit the development of the pathogen. No measurable anti-parasitic chemical product was produced that could be removed from the resistant host's cell and probably inactivation is not a consequence of a simple

chemical factor unique in resistant tissue. If there are such substances synthesized due to host-parasite interaction, they must come from a living dynamic cell substrate and be very subtle in their effect. Grafting experiments gave convincing evidence that no plant metabolites from resistant tissue diffused into the vascular system and flowed past graft unions into susceptible host cell tissue to cause this tissue to react differently than an intact susceptible plant to infection. The converse was true for the action of susceptible plant sap in resistant tissue, that is, the resistant part of the graft would not respond as susceptible tissue. This is further evidence that the defensive mechanism of host tissue lies within the cell proper and only performs when the pathogen is present.

The data clearly showed a typical steady increase in the rate of oxygen uptake in tissue infected with the scab. This rate change in respiration normally occurred about three days after inoculation. The increase continued until the leaf tissue was so badly damaged 5 or 6 days after inoculation that no further tests were possible. As was demonstrated, the pathogen produced marked increases in respiration of susceptible cucumber seedlings, but only accompanying pronounced disease symptoms. It was determined that the vascular systems of the host had been considerably affected and the greatest respiration increases were observed when susceptible plants were completely inoculated. The greatest increases were noticed just before collapse of the petiole. In controlled inoculation

experiments it was demonstrated that this type of breakdown was not nearly so evident if the area at the base of the leaf next to the petiole was protected from infection. Since this specific area of the leaf is prone to hold a greater volume of inoculum, the most severe infection will occur at this point. The main vascular system carrying metabolites to and from the leaf area is thus greatly disturbed.

In the systemic or local respiration experiments, respiration increases in the leaf tissue were apparent whenever extensive damage occurred to the vascular system between the stem and lower half of the leaf. When the lower half of the leaf was infected and respiration was measured on the infected portions of the same leaf, increases were observed. No increase was noted, however, in respiration of the lower half if only the upper half of this same leaf was infected This would indicate that respiration increases can be initiated by blocking the flow of transitory metabolites between the leaf and the root system. This blocking may be accomplished either by severing the vascular elements mechanically or as a result of infection. There may be a build up of cell metabolic materials, such as sugar, amino acids, etc., which serve as energy sources for increases in the respiration rate. Normally these metabolites would diffuse into the vascular elements and be transported downward and this is prevented if the pathogen has destroyed the natural arteries. Any wilting that should be caused by the corresponding blockage of flow from roots to the leaves would be obscured by the rapid primary symptoms of disease.

It was demonstrated that, within practical limits, varying the photoperiod before and after inoculation had little effect on disease development. If, however, the susceptible plants were subjected to different light intensities for relatively long periods before inoculation, disease development progressed at a different rate. A light intensity of 900 ft-c induced disease symptoms 2 or more days sooner than those grown under 300 ft-c. Severity of infection was also greater with the more intense illumination. These results strongly suggested a dependence of disease development and stimulated respiration on availability of nutrients elaborated most effectively under higher light conditions. This hypothesis was further strengthened by producing similar intensification of disease in urea fertilized plants. The reduction of disease intensity and increased incubation time in artificially etiolated seedlings also points this way. Depleted cotyledonary tissue was also shown to be unsuitable for rapid pathogenic development of the fungus. When cotyledons were old and no longer growing actively, neither symptoms or respiration effects were typical of those developed in the usual susceptible tissue.

Susceptible host cells in low light do not react violently to the pathogen. When the plant develops under an environment more nearly ideal for the host, this being similar to the 900 ft-c conditions, the host cells, being more resistant, react strongly against the pathogen. Due to their inherent make up, however, they never approach the more ideal hypersensitive reaction expressed in the truly resistant host cells.

Susceptibility of the host is influenced by its general state of health. It appears in this case that the less thrifty the plant, the more unfavorable it is to the parasite. The correlation between vigor of host and virulence of the pathogen is inverse.

If the nutrient solution was used for the growth of the organism and then infiltrated into the healthy tissue, no additional increase in respiration occurred. Diseased and healthy leaf tissue infiltrated with such materials as dilute potato-dextrose nutrient, or a filtrate made from the fungus, however, showed increased respiration. This is additional evidence that fungus secretions or ordinary growth by-products do not directly induce host cell respiration increases. The data suggest that respiration increases may arise secondarily as a result of demands for energy brought about by a disturbance of the metabolic processes of the host.

The disappearance of asparagine and glutamine in infected susceptible leaves was coincident with respiration increases and initiation of visible symptoms. The relationship must be of significance in parasitism not only because of the importance of these 2 nitrogenous compounds in the plant's metabolism generally but also because the fungus is not selective in its utilization of the amino acids and amides *in vitro*.[a] It is doubtful that asparagine and glutamine are used selectively

[a] A preliminary chromatographic study was made on spent filtrates of fungus growth on cucumber (CS2M and CS2W) leaf extracts after 3 weeks. Growth was good on all the extracts. It was found that most of the ninhydrin active compounds were lost and the others reduced substantially compared to the controls.

as nutrients by the parasite. Neither the behavior of the fungus in vitro nor chromatographic results of affected plants tend to support such a static mechanism. No other change could be found in the ninhydrin staining active compounds of diseased versus non-diseased tissue.

Although the relationship between the two missing amides and the physiological respiration increase remains obscure, it is conceivable that their removal from the free amino acid pool, or use in other processes, would necessitate an increase in metabolic processes of the plant cell to restore a normal balance.

Glutamine and asparagine play an important part in nitrogen metabolism of plants by preventing the accumulation of free ammonia (26). They appear in plants when large quantities of ammonium ions are being absorbed by the roots and when reserve proteins in young seedlings are being digested to supply energy for growth. They are also evident in instances of carbohydrate starvation, when amino acids and amides are oxidized during respiration, setting free large quantities of ammonia. This free ammonia is utilized in amide synthesis. This suggests that disappearance of glutamine and asparagine results when the pathogen acts in any way to prevent the movement or availability in the leaf of ammonium ions.

Respiration increases reflecting metabolic changes in diseased cells of plants is an accepted phenomenon (1, 2). In the case of scab infection of cucumber leaves this respiration increase was also

noted but was not evident until visible cell damage was detected. These alterations in host cell respiration could result from direct action of pathogen metabolites on the host tissue, or from a more indirect action via interferance with one of the regulatory mechanisms. These could include controlled photophorylation, alteration in the tricarboxylic acid cycle, etc. Experiments were made to define some of these possible causes of respiration alteration in disease plant tissue.

A comparison of the effects produced by a known respiratory uncoupler, DNP, on diseased and healthy leaf tissue showed that the chemical induced essentially the same respiration increase in both inoculated and noninoculated susceptible tissues. This evidence made it appear improbable that uncoupling agents similar to DNP accounted for the respiration increases that occur in the diseased cucumber leaf tissue.

Leaf tissues, infiltrated with malonic acid, an inhibitor of succinic dehydrogenase, demonstrated that healthy as well as diseased tissues were unable to function properly if this enzyme system was blocked. This part of the citric acid cycle appears to be vital in diseased tissue if oxygen uptake is to continue, thus the oxidation of succinate to fumarate must occur via the succinic dehydrogenase enzyme system.

Studies involving application of radioactive tracer techniques were designed to give a quantitative estimation of the catabolic pathways

involved in the conversion of carbohydrates to respiratory CO_2 in plant tissue. C_6/C_1 ratios were the same in diseased and healthy tissues, indicating no change in carbohydrate breakdown pathway.

Most of the evidence leads to the conclusion that a high percentage of the respiration rate increases may result from mechanical blocking or the destruction of the downward diffusion routes necessary for flow of cell end-products. The possibility may exist that the pathogen furnishes materials directly usable for the host energy requirements by the fractionation of complex nitrogenous and carbohydrate compounds to simpler substances. The plant's cells would then use these compounds for energy.

The hypothetical mechanism of parasitism and of host resistance in the scab disease of cucumber should be recapitulated briefly. The initial parasitism must be counteracted by excitation of some host cell defense mechanism for effective expression of resistance. A chain of unknown events, possibly formation of antitoxins or other anti-metabolites, could either repel the invader or prevent some vital process in its metabolism. When successful pathogenesis occurs the pathogen could reorganize essential constituents within the host to aid in its own development. Interaction of dynamic metabolites of both host and parasite is required to decide which will be the successful survivor.

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