CHANGES IN ULMUS AMERICANA IN RESPONSE TO INFECTION BY CERATOCYSTIS ULMI

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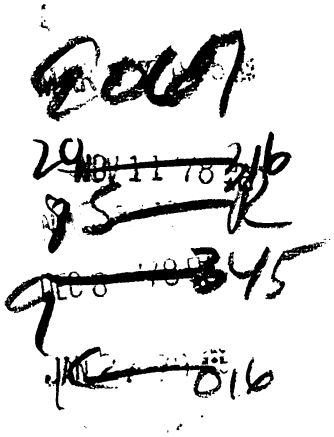

ABSTRACT

CHANGES IN ULMUS AMERICANA IN
RESPONSE TO INFECTION BY CERATOCYSTIS ULMI

By

William Ronald Landis

Experiments were designed to study the response of pathogen-free tissue of American elms, Ulmus americana L. infected with the Dutch elm disease pathogen, Ceratocystis ulmi (Buism.) C. Moreau. Development of disease following artificial inoculation was compared with disease development in natural infections.

Oxygen uptake of pathogen-free leaf discs began to increase 5-11 days after inoculation of trees, and reached a maximum between 11 and 22 days, when oxygen uptake was as much as 80 percent higher than in controls. Thereafter, oxygen uptake decreased until it was less than controls 20-26 days after inoculation. Increased oxygen uptake was not caused by water stress since plants which had water stress had lower oxygen uptake than did normally watered controls. Conductivity of aqueous leachates from leaves was greater for inoculated than for control plants; this increased loss of electrolytes was correlated with increased oxygen uptake until maximum oxygen uptake was reached. When oxygen uptake began to decrease, conductivity began a rapid increase, reaching over 300 percent of controls when oxygen uptake was at its minimum of 30-50 percent of

controls. Reduction of triphenyltetrazolium chloride demonstrated that twigs (from which the leaves were harvested for respiration and conductivity experiments) contained living parenchyma.

Microscopic observation demonstrated that the twig, midvein, and larger veins in blades from leaves of inoculated trees were not visibly occluded during respiration and conductivity studies. Attempts to isolate C. ulmi from these tissues were not successful.

By using symptom development and presence of the pathogen from leaves, twigs, and stems as criteria, colonization of naturally infected and artificially inoculated trees was studied. When trees were inoculated with approximately 400 spores, the extent of colonization was similar to that of naturally infected trees. When large numbers of spores were used in inoculation, colonization was more rapid and extensive.

This work demonstrates that physiological changes occur in pathogen-free tissue of U. americana soon after inoculation with C. ulmi. It suggests that occlusion and subsequent inability of vessels to transport water may occur relatively late in disease development, and that physiological changes may be due to a toxin in the transpiration stream.

CHANGES IN ULMUS AMERICANA IN RESPONSE TO INFECTION BY CERATOCYSTIS ULMI

by

William Ronald Landis

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INTRODUCTION

Vascular occlusions casued by gums and tyloses occur in American elm (Ulmus americana L.) infected by Ceratocystis ulmi (Buism.) C. Moreau (9, 20, 44). Several toxic substances which occur in crude culture filtrates of the fungus elicit a disease syndrome said to be similar to that of Dutch elm disease (18, 22, 24, 53, 70, 72). The relative importance of vessel occlusion and toxic substances is not known in Dutch elm disease.

Systemic effects in host or non-host tissues which did not contain the pathogen have been reported for other vascular diseases of plants. Veinclearing (23), increases in respiration (16, 55), increases in permeability (16), and discoloration of parenchyma (54) occurred in pathogen-free tissues of tomato infected with Fusarium oxysporum f. lycopersici (Sacc.) Snyd. and Hans. Non-host scions of intergeneric (17) and intrageneric (37, 38) grafts responded to invasion of the host stock with necrosis. Photosynthesis decreased in pathogen-free blades of cotton plants infected with Verticillium albo-atrum Reinke and Berthold (39). These data suggested that some substance was moving in advance of the pathogen, disrupting the normal metabolism of the host.

The purpose of this research was to determine whether or not there are physiological changes in trees with Dutch elm disease which might be caused by a toxin. If so, at

what time in the disease syndrome do the physiological changes occur? Can changes be expressed in advance of the invading fungus, or do they occur only at the site of infection? What is the pattern of fungus distribution within the host in relation to possible changes occurring in tissues? Additional experiments were designed to compare the extent of colonization of the host under conditions of natural infection and artificial inoculation with reference to symptom expression and inoculation technique.

LITERATURE REVIEW

Dutch elm disease was first described in France in 1918 (28). The pathogen was carried in elm burl logs to North America (5) where it was identified in Ohio in 1930 (40). Since reaching North America, the pathogen has spread throughout most of the natural range of the principle host, the American or white elm (62). The range of this tree is demarked by the Atlantic Ocean, the Gulf of Mexico, and a line from Cape Breton Island, Nova Scotia westward through Ontario, northwest through eastern Saskatchewan, south through the Dakotas, and south-southeast through middle Kansas and eastern Texas (27). The total number of wild elms in North America when the disease was introduced was estimated at one billion. In addition to the wild trees there were approximately 500 million elms growing as ornamental trees in cities (14).

The smaller European elm bark beetle (*Scolytus multistriatus* Marsham) and the American elm bark beetle *Hylurgopinus rufipes* Eichoff) are the principal vectors of *C. ulmi* (15). *S. multistriatus* first was found in North America in 1904 and has since become established throughout most of the range of the American elm (68). This insect is the most important vector of *C. ulmi* throughout most of the United States (46), but in Ontario, *H. rufipes* is the primary vector (47). When elms larger than 7 inches diameter

breast height (dbh) are growing within a few feet of each other, as in a street planting, root grafts between trees are common. The fungus spreads from tree to tree through these grafts (67).

Trees are most commonly inoculated by S. multistriatus contaminated with C. ulmi spores when the adults feed in twig crotches of healthy trees (15, 48). This process is of low efficiency with approximately 3-8 percent of the feeding wounds resulting in infection (45). Under favorable conditions, spores which reach the xylem germinate and initiate infection (35, 44, 65). The pathogen spreads throughout the tree by means of yeast-like spores (1) or microendospores (43, 69) which are carried in the transpiration stream. The host usually dies two or three years after inoculation, but may die the year of inoculation if the pathogen moves into the main trunk and becomes systemic (12, 41). When non-infected elms are pruned late in the summer, they are attractive to S. multistriatus as brood trees. If the females are contaminated with C. ulmi spores, they commonly inoculate the tree when preparing their egg gallery (30). These trees usually die the year following infection (31).

Many factors affect the development of Dutch elm disease. Pomerleau (47), Smalley (60), and Banfield (4) reported that the disease develops more readily in the spring and early summer. This was explained by the more rapid

movement of spores in the large springwood vessels (2, 4, 11). The prevention or inhibition of disease development by chemicals which inhibit the development of springwood or initiate occlusion of vessels is consistent with this view (7, 8, 10, 19, 59). The number of spores used for inoculation does not affect the incidence of disease, but does affect the time required for symptom development (2, 58, 72). Banfield (2) reported that C. ulmi spores are distributed to the uppermost branches of large trees rapidly when many spores were injected into the base of the trees. Pomerleau and Mehran (49) found spores distributed into leaves within 24 hours of inoculation and Pomerleau and Pelletier (50) reported that the pathogen was easily obtained from leaves. In the latter report (50), the inoculation technique was not described, but in the former (2, 49) large numbers of spores were used. Campana (12) and Neely (41) have criticized reports of rapid spore movement. When trees were artificially inoculated at twig crotches, C. ulmi usually colonized only the inoculated branch during the year of inoculation. After the pathogen reached the main trunk, colonization of the tree was more rapid. Experiments which demonstrated rapid colonization have been conducted by injecting large number of spores, suspended in large volumes of water, into trees. Spores are rapidly distributed to terminal branches following these injections, but the technique is entirely artificial and does not measure the rate

of natural colonization.

Smalley (60) reported that increased nitrogen lengthened the susceptible period of young trees by lengthening the period of succulent growth. Zentmyer and Wallace (71) reported that nitrogen reduced susceptibility of large trees to disease by increasing the vigor of the host. Schreiber (57) reported that trees 1-5 months old were immune to Dutch elm disease, but Smalley (60) was able to inoculate young trees in the two leaf stage. He attributed the general lack of success of others to inoculating the trees too late in the season or to lack of nitrogen. Smucker (61) and Banfield et al. (3) reported that trees which are less than 1 inch in diameter do not have recurrence of disease the season following inoculation. In trees larger than 15 inches dbh, disease recurrence was 100 percent (3). The rare recurrence in small trees was associated with growth of the fungus into the current year's growth ring (4, 61). Although C. ulmi was able to invade the new growth of stems in some cases, most disease recurrence occurred in roots where vessels of different growth rings were often contiguous (4).

There are variations in susceptibility of elm clones (33, 34, 63) and in virulence of isolates of C. ulmi (24, 32, 64). The age of spore inoculum also affected disease development (71). Disease developed much slower when trees were inoculated in twigs than when spores were injected into the bole (11, 12, 13). This apparently was associated with

slow movement of C. ulmi proximally from the inoculation point.

The mechanism by which C. ulmi induces symptoms is not understood. Many workers have suggested that a toxin is involved in symptom development. A water soluble substance obtained from the culture filtrate of C. ulmi caused symptoms similar to those of Dutch elm disease on elm, tomato, maple, and snapdragon (70). Dimond (18) isolated two fractions from culture filtrates; a polysaccharide which caused upcurling of leaves, and an alcohol-soluble, ether-insoluble fraction which caused interveinal necrosis. Feldman et al. (21, 22) and Frederick and Howard (24) found no correlation between the ability of an isolate to cause disease and its polysaccharide production in culture. Even though the polysaccharide component could cause wilting, they did not believe it to be the primary wilt inducing agent because removal of the polysaccharide did not reduce phytotoxicity of the filtrate. They found that the highest toxin production occurred at pH 4.25 in 7 days, and that a higher pH prevented toxin formation and deactivated preformed toxin. C. ulmi always lowered the pH of culture media, and the affect of pH on the pathogenicity of the fungus was not tested. Frederick and Howard (24) found no correlation between toxin titre and pathogenicity of different isolates of C. ulmi upon elm seedlings. These reports (18, 21, 22, 24, 70) were based upon crude culture filtrates of C. ulmi.

Salemink et al. (53), using more precise techniques, isolated two substances from culture filtrates which were toxic to elm cuttings. The first substance, a glycoprotein, reproduced Dutch elm disease symptoms alone or in combination with the second. The second component was not isolated in pure form; therefore, its phytotoxicity is unknown. Resistant controls were not tested.

Beckman (6) found that pectin depolymerase and cellulases were produced, but that polygalacturonases were not produced in culture. He suggested that gums and tyloses observed in vessels could be a result of the action of these enzymes, but in vivo experiments were not conducted. Beckman (9) later suggested that Dutch elm disease, as well as other vascular diseases, was the result of vascular dysfunction caused by gum and tylose formation behind the fungus in the transpiration stream. However, Beckman only studied resistant late summer wood. Elgersma (20) studied resistant and susceptible elm clones, and found that the resistant clones had gel formation in vessels, which caused a localization of infection soon after inoculation. Susceptible plants had extensive colonization of the vascular system, followed by vessel occlusion.

The first symptom of disease observed by Kerling (36) was discoloration of parenchyma. Her photographs indicated that gums occurred where many spores had lodged, and that tyloses formed where fewer spores were found. She related these events to the amount of hypothetical toxin which these

spores might produce. Gagnon (26) also found that early changes occurred in xylem parenchyma, but that these were preceeded by yellowing of pits in xylem walls. He found that gel plugs which were formed in vessels contained pectin as a main constituent, and that the plugs were associated with thickening of vessel walls (25). Wilson (69) found that isolated areas of the xylem were blocked by gums, tyloses, and hyphae, but that only a small portion of the vascular system was blocked when foliar symptoms became apparent. He stated that the fungus was found primarily in the parenchyma tissue, and that this may be the tissue upon which resistance is based. Banfield (4) however, found C. ulmi only rarely in parenchyma until disease symptoms were severe.

MATERIALS AND METHODS

Trees inoculated in 1967 were growing approximately 2.5 km southwest of Laingsburg, Michigan or on the Mighigan State University Department of Botany and Plant Pathology farm. Trees inoculated in 1968 were growing approximately 2.5 km south of East Lansing, Michigan.

C. ulmi was isolated from three different naturally infected trees in February 1967 and in April 1968. These six cultures were grown in shake culture at 22°C in a medium containing (g/l): glucose, 50; asparagine, 2; malt extract, 12; KH_2PO_4, 1.5; $MgSO_4 \cdot 7\ H_2O$, 0.5; $FeCl_3$, 0.01. Most growth under these conditions was bud cells with little hyphal growth. Inoculum was prepared by mixing bud cells from three isolates (approximately 10^6 cells per ml), or by diluting this mixture to a concentration of approximately 8000 cells per ml.

Each of thirty American elm trees (2.5-4.0m tall) was inoculated with one drop (approximately 400 cells) of diluted bud cell suspension of C. ulmi on one of the following days: May 23, June 7, 17, and 27, 1967 and May 15, June 5, and July 5, 1968. Fifteen trees were water injected on each of these dates to serve as controls. The drop was placed in a twig or branch axil 70-130 cm above ground level, where the trees were 3-6 cm in diameter. The bark was then pierced through the drop with a No. 11 Bard-Parker lance.

When the lance was withdrawn, the drop of spore suspension was rapidly drawn into the transpiration stream. Two inoculated trees and one non-inoculated tree were harvested daily or every other day during the 30 days following inoculation. Terminal leaves and twigs from these trees were used in respiration studies, the uppermost lateral leaves and adjacent twigs were used in conductivity studies, and the next lower lateral leaves and adjacent twigs were sectioned and stained.

The extent of colonization of trees inoculated with 400 spores per tree was compared to that of naturally infected trees and to trees inouclated with 5×10^4 spores per tree. To study distribution of C. ulmi in naturally infected trees, one or two branches which bore leaves showing early symptoms (slight chlorosis or necrosis) were cut from different large (>25 cm dbh) American elms at intervals throughout the spring and summer of 1967 and 1968. Leaves from these branches were divided into three symptom categories: symptomless (Figure 1 a-f), chlorotic (Figure 1 g-i), and necrotic (Figure 1 j). Leaves which contained more necrotic tissue than shown in Figure 1 j were included in the third category. Leaves and sections of the twigs bearing the leaves were placed without surface sterilization on the surface of elm extract agar in petri plates. Tissues were considered free of the fungus when no synnemata characteristic of C. ulmi were present after 14 days at 22°C.

Figure 1. Symptomless, chlorotic, and necrotic leaves from Ulmus americana infected with Ceratocystis ulmi (a-f symptomless, g-i chlorotic, j necrotic).

To study distribution of C. ulmi in trees inoculated with 5×10^4 spores per tree, each of five to ten American elm trees (2.5-4 m tall) were inoculated with one drop (approximately 5×10^4 cells) of a concentrated bud cell suspension on one of the following days: May 15, June 5, and July 5, 1968. These trees were harvested after some of their leaves began to show yellowing or browning and colonization was studied by the same techniques used for naturally infected trees.

RESULTS

Distribution of C. ulmi in naturally infected large trees and in inoculated small trees - The rate and extent of colonization of U. americana by C. ulmi varied with different inoculation techniques (2, 12, 41, 49, 50). When many spores ($>10^6$) were used, colonization was more rapid and extensive than with natural colonization (2, 49, 50). Therefore, an attempt was made to compare colonization of trees inoculated with few spores with colonization of naturally infected trees.

The number of attempted isolations from leaves and twigs with each type of symptom and the percentage of attempted isolations which contained C. ulmi are shown in Table 1. Data are presented for naturally infected large trees, and for small trees inoculated with 5×10^4 spores per tree.

A comparison of these data with those obtained from trees inoculated with 400 spores per tree (Table 2) indicates that the extent of colonization of trees inoculated with 5×10^4 spores per tree was more rapid and extensive than the colonization of naturally infected trees or trees inoculated with 400 spores per tree. When trees were naturally infected or when trees were inoculated with 400 spores per tree, symptomless leaf blades rarely contained C. ulmi (0.6 and 0.0 percent respectively). Eight percent of symptomless leaves from trees inoculated with 5×10^4 spores per tree contained

Table 1. Recovery of *Ceratocystis ulmi* from leaves and twigs of *Ulmus americana* showing various degrees of symptom development.

Leaf Symptoms	Naturally Infected			Inoculated with 5 X 10^4 Spores/Tree		
	Blade	Petiole	Assoc.[a] Twig	Blade	Petiole	Assoc.[a] Twig
Symptomless						
No. Positive[b]	2	16	54	61	87	170
No. Tested	356	356	167	759	759	330
% Positive	0.6	4.5	32.3	8.0	11.5	51.5
Chlorotic						
No. Positive[b]	1	9	27	16	30	53
No. Tested	111	111	76	140	169	81
% Positive	0.9	8.1	35.5	11.4	17.8	65.4
Necrotic						
No. Positive[b]	9	29	96	69	107	182
No. Tested	184	184	186	390	390	251
% Positive	4.9	15.8	51.6	17.7	27.4	72.5

[a] A portion of the twig within 2 cm of the node to which the test leaf was attached.

[b] Number of sections from which *C. ulmi* was isolated.

Table 2. Recovery of *Ceratocystis ulmi* from *Ulmus americana* inoculated with approximately 400 spores per tree[a].

Area isolated (cm above point of inoc.)	Surface Sterilized			Not Surface Sterilized		
	Number[b] Positive	Total[c]	%[d]	Number[b] Positive	Total[c]	%[d]
0-30	42	97	43.3	23	24	95.8
30-60	34	97	35.1	16	24	66.7
60-90	28	97	28.9	14	24	58.3
90-120	22	97	22.7	10	24	41.7
Twig	8	97	8.2	4	24	16.7
Petiole	4	194	2.1	0	48	0.0
Blade	0	194	0.0	0	48	0.0

[a] Isolations were attempted from the 121 trees used in oxygen uptake and conductivity studies. All leaves from which isolations were attempted were symptomless.

[b] Number of sections from which *C. ulmi* was isolated.

[c] Number of sections which were sampled for the presence of *C. ulmi*.

[d] Percentage of sections from which *C. ulmi* was isolated.

the pathogen. A similar situation occurred with chlorotic leaf blades where 0.9 percent of the lamina from natrually infected trees contained the fungus whereas 11.4 percent of the chlorotic leaf blades from trees inoculated with 5×10^4 spores per tree contained the fungus.

Colonization of trees inoculated with 400 spores more closely resembled natural colonization than did colonization of trees inoculated with 5×10^4 spores. Therefore, respiration and conductivity studies were conducted on trees which were inoculated with 400 spores per tree.

Oxygen uptake - If physiological changes occurred in host tissue before marcoscopic symptoms developed, they could be interpreted as being caused by a toxin moving in the transpiration stream in advance of the pathogen. Such changes might be reflected in oxygen uptake by diseased tissue. Therefore, an attempt was made to determine whether or not oxygen uptake changed during development of Dutch elm disease.

Oxygen uptake was determined with a Warburg apparatus using standard manometric technique (66). Four to six terminal leaves from each tree were selected for uniformity in size and appearance. Eighty to 100 leaf discs 7 mm in diameter or 80 to 100 twig sections 5 mm long were harvested from the terminal leaves and twigs of each tree. The discs and sections from each tree were placed on moistened filter paper in separate petri dishes until they were put into manometric flasks (approximately 1 hr after trees were cut). Twenty leaf

discs or stem sections from each petri dish were randomly selected and placed in each of three manometric flasks on a filter paper circle moistened with 0.2 ml distilled water. Center wells contained KOH (20 percent) to absorb evolved CO_2. After a 20 min equilibration period at 25°C, oxygen uptake was recorded for 1-2 hr with readings taken at 15 or 20 min intervals. Experiments were run in either reduced light or in darkness to prevent interference from photosynthesis. Leaf discs and twig sections were removed from the flasks, dried at 95°C for 48 hr, and then weighed. Data were expressed as µl oxygen uptake per mg dry weight of tissue per hr, and as a percentage of the oxygen uptake by the non-inoculated control tree used on each day.

A least squares regression analysis was performed on oxygen uptake increases using the Michigan State University Agricultural Experiment Station STAT Series Description Number 7. Input values were obtained by dividing the amount of oxygen (µl per mg) taken up in each flask by the tissue from inoculated trees by the mean amount of oxygen taken up by tissues from non-inoculated trees harvested on the same day. An analysis of variance was performed between trees harvested each day using the Michigan State University Agricultural Experiment Station STAT Series Description Number 13. Input values consisted of the amount (µl per mg) of oxygen taken up by the tissue contained in each flask.

Oxygen uptake by leaf discs from inoculated trees was

similar to that of control trees for the first five to eleven days after inoculation (Figures 2-8, Table 3). At this time oxygen uptake by the discs from inoculated plants began to increase until it reached a maximum 11-22 days after inoculation. Maximum oxygen uptake by leaf discs from inoculated trees was as much as 80 percent higher than uptake by healthy control tissues. Oxygen uptake then began to decrease, until it was as little as 30-50 percent of control oxygen uptake when the experiments were terminated. Oxygen uptake of healthy tissue was much higher (3-5 μl per mg dry weight per hr) soon after bud break than when the leaves were fully expanded (0.8-1.5 μl) (Table 3). Oxygen consumption was highly variable between trees and between days in late May and early June (Figures 2-4, Table 3) in both inoculated and control trees, but showed much less variability in later experiments (Figures 5-8, Table 3).

The significance level between inoculated and control plants was <0.05 when there was more than a 20 percent change in oxygen consumption. Least squares analysis demonstrated that change in oxygen uptake curves followed the general equations $y=a+bx+cx^2+dx^3$ of $y=a+bx+cx^2$ where x is days after inoculation (Figures 2-8). In all cases but one, the significance probability of the curve was <0.0005 and r values were >0.72. The experiment begun on June 7, 1967 (Figure 4) had a low (0.52) r value and a significance probability of

0.017. The poor fit and relatively high significance level of this experiment are probably due to the high oxygen uptake (2.5 and 2.9 µl per mg dry weight per hr) of the non-inoculated trees on days 6 and 7. This was a twofold increase over non-inoculated trees harvested on days 5 and 8. The factor responsible for this great increase in oxygen uptake is unknown.

In 1967, a seasonal effect on oxygen uptake occurred. Plants inoculated on May 23 had a significant (0.05 level) increase in oxygen uptake 7 days after inoculation (Figure 3), those inoculated on June 7 had a significant increase after 9 days (Figure 4), those inoculated on June 19 had a significant increase after 18 days (Figure 6), and those inoculated on June 27 had a significant increase after 16 days (Figure 7). This is consistent with reports (47, 60) that Dutch elm disease develops more rapidly in spring and early summer. In 1968, oxygen uptake increase did not occur as quickly after inoculation in May as after inoculation in June and July. Dutch elm disease development has been shown previously to vary with soil moisture (35, 47, 72). Lack of a seasonal affect in 1968 could have been due to the amount of rain received in the spring and early summer of 1968. While 8.97 inches fell during May-July, 1967, 15.13 inches fell during May-July, 1968.

Oxygen uptake of twigs was highly variable between trees and in no case did a discernible pattern develop.

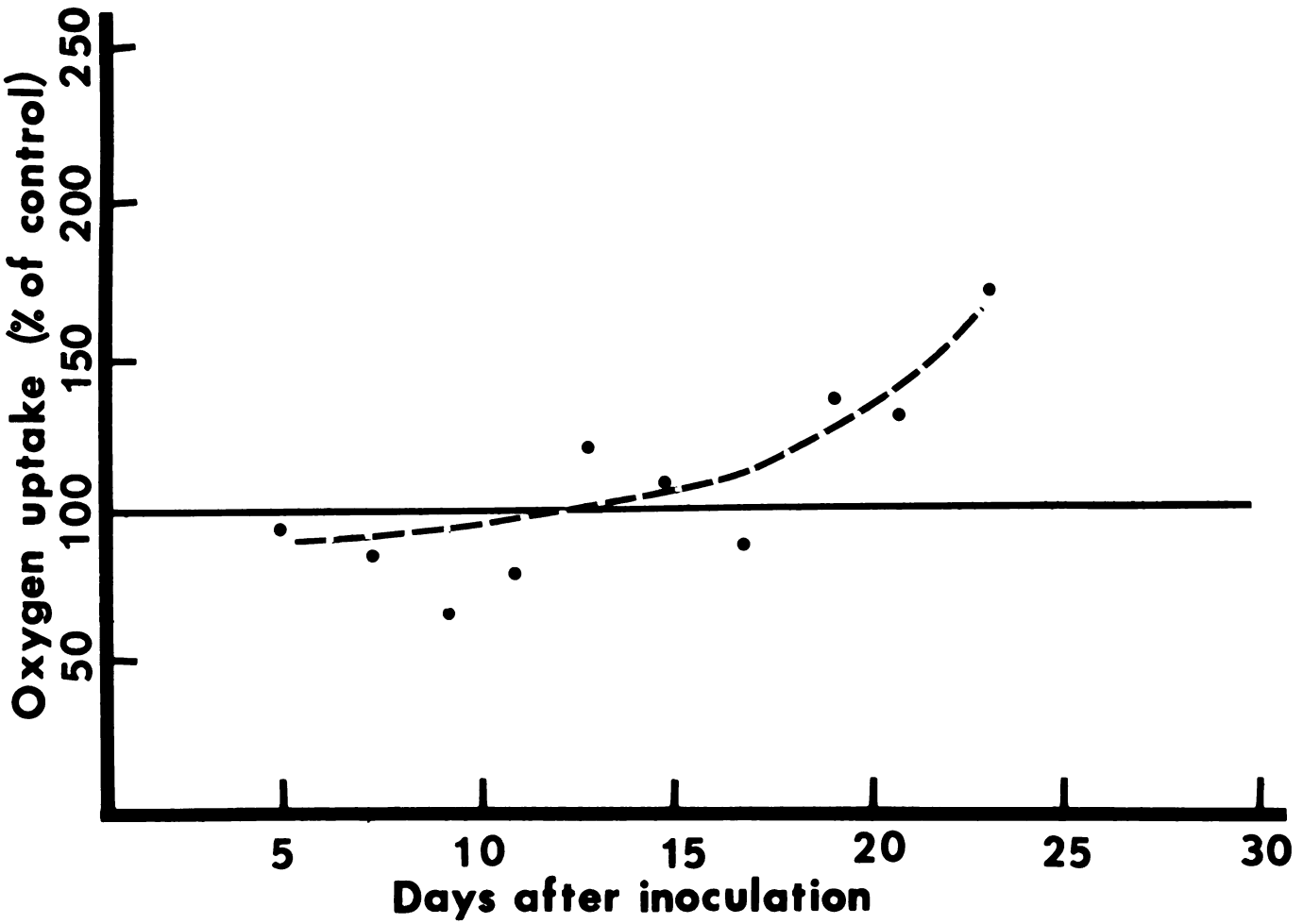

Figure 2. Oxygen uptake by leaf discs from Ulmus americana inoculated on May 15, 1968 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=0.954-0.049x+0.004x^2$, $r=0.82$. Twenty percent difference from the control is significant (0.05 level).

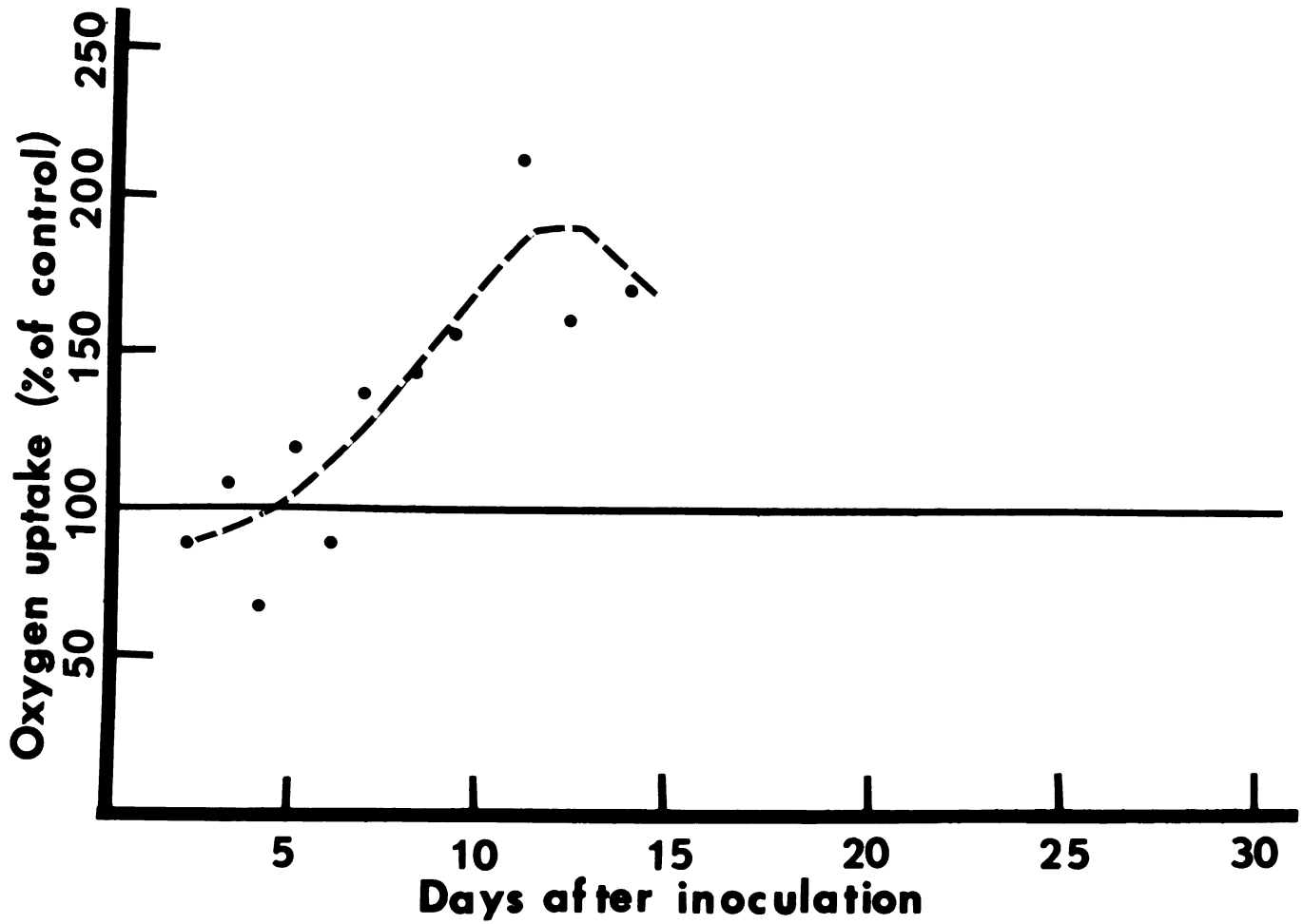

Figure 3. Oxygen uptake by leaf discs from Ulmus americana inoculated on May 23, 1967 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=1.329-0.307x+0.062x^2-0.003x^3$, $r=0.75$. Twenty percent difference from the control is significant (0.05 level).

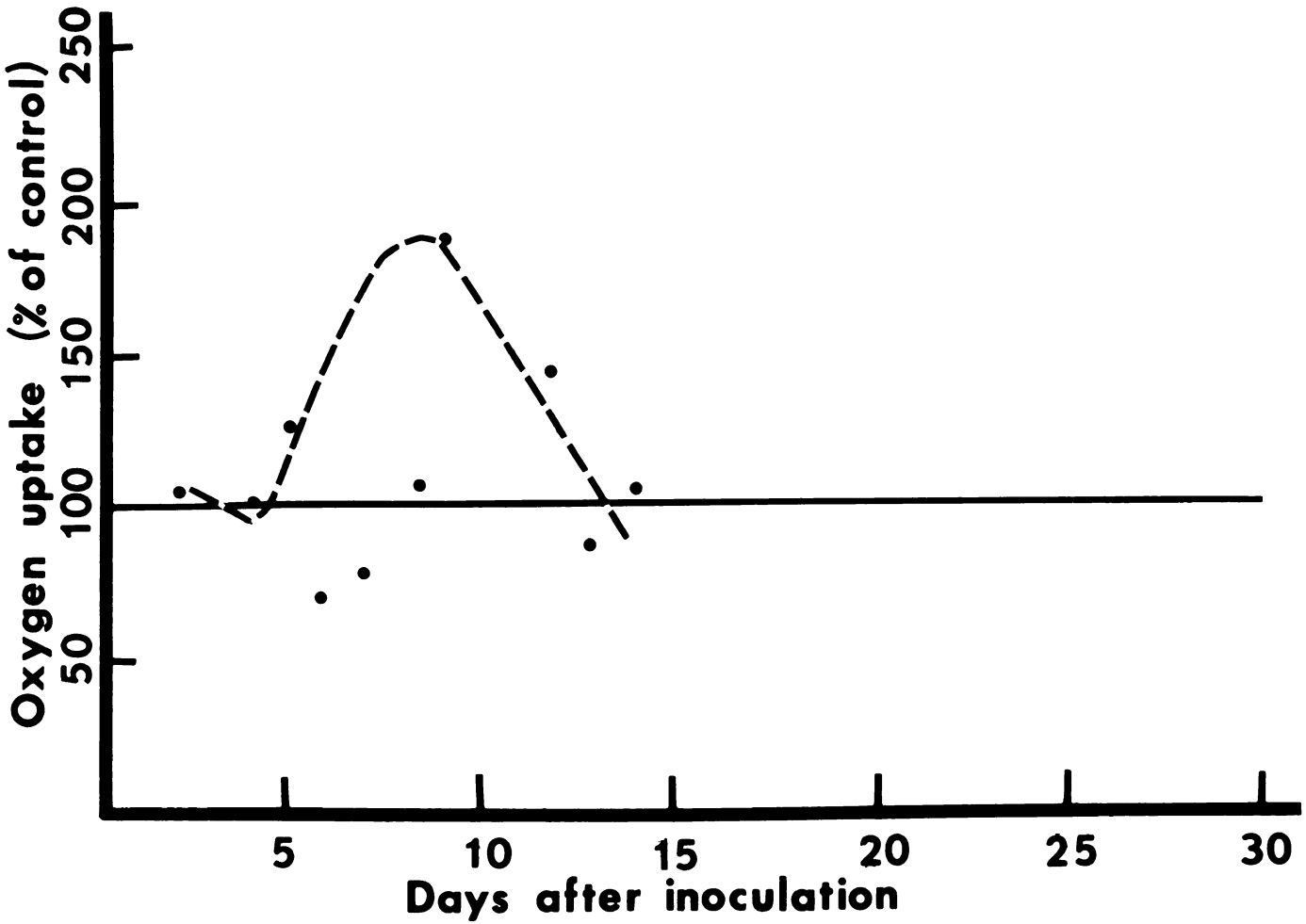

Figure 4. Oxygen uptake by leaf discs from Ulmus americana inoculated on June 7, 1967 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=1.864-0.545x+0.091x^2-0.004x^3$, $r=0.52$. Twenty percent difference from the control is significant (0.05 level).

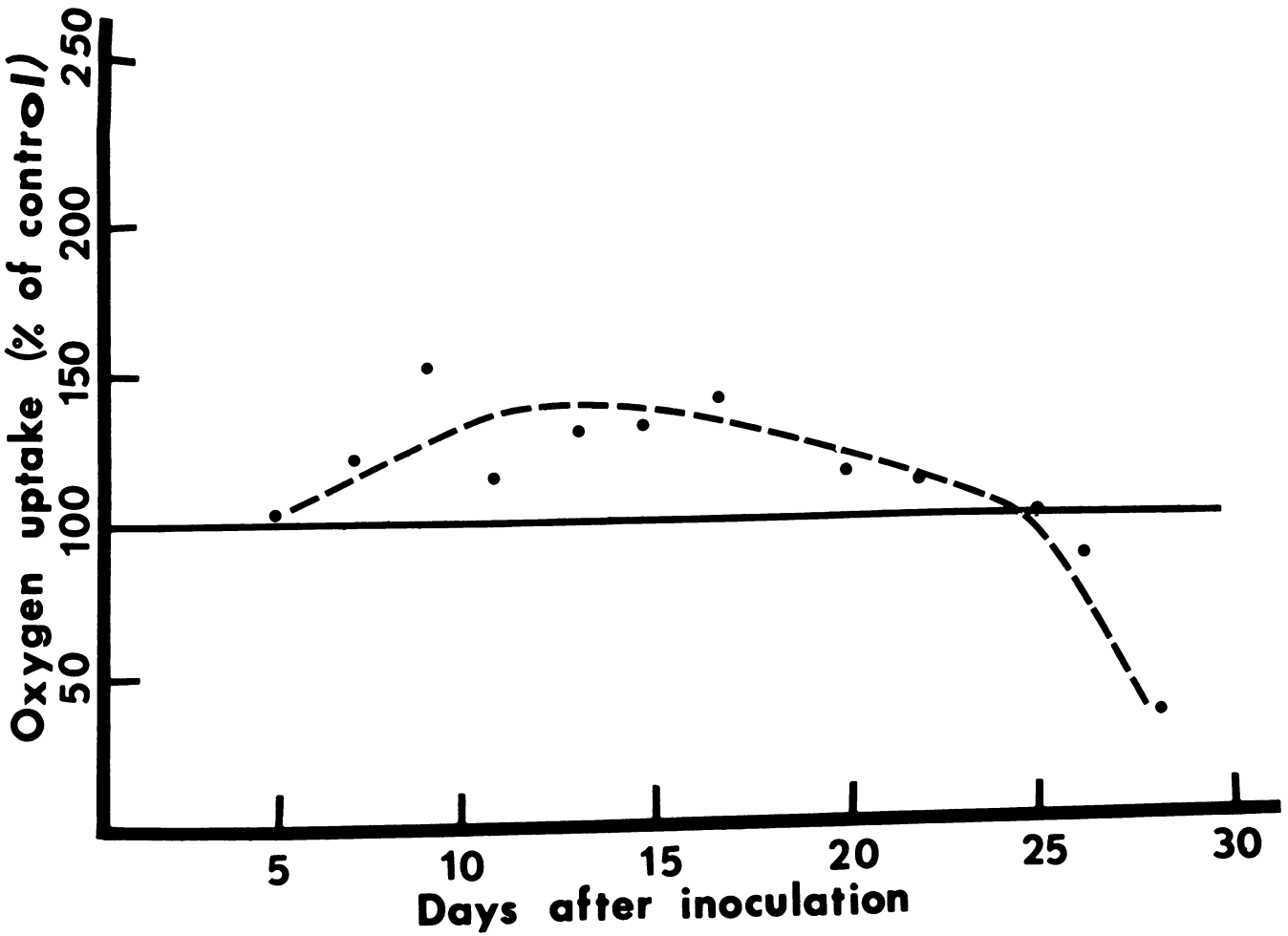

Figure 5. Oxygen uptake by leaf discs from Ulmus americana inoculated on June 5, 1968 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=0.669+0.104x-0.004x^2$, $r=0.88$. Twenty percent difference from the control is significant (0.05 level).

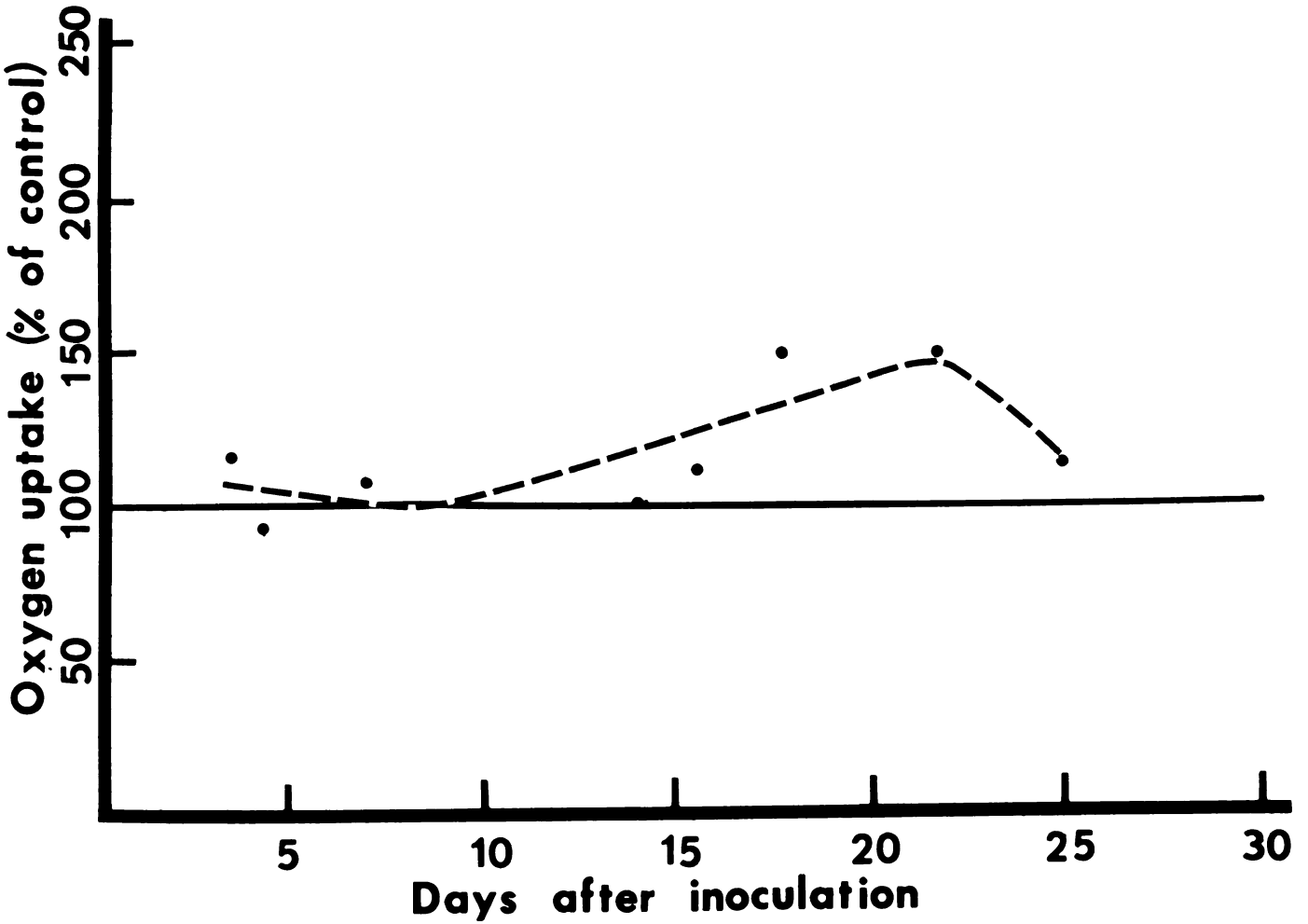

Figure 6. Oxygen uptake by leaf discs from Ulmus americana inoculated on June 19, 1967 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=1.481-0.173x+0.016x^2$, $r=0.72$. Twenty percent difference from the control is significant (0.05 level).

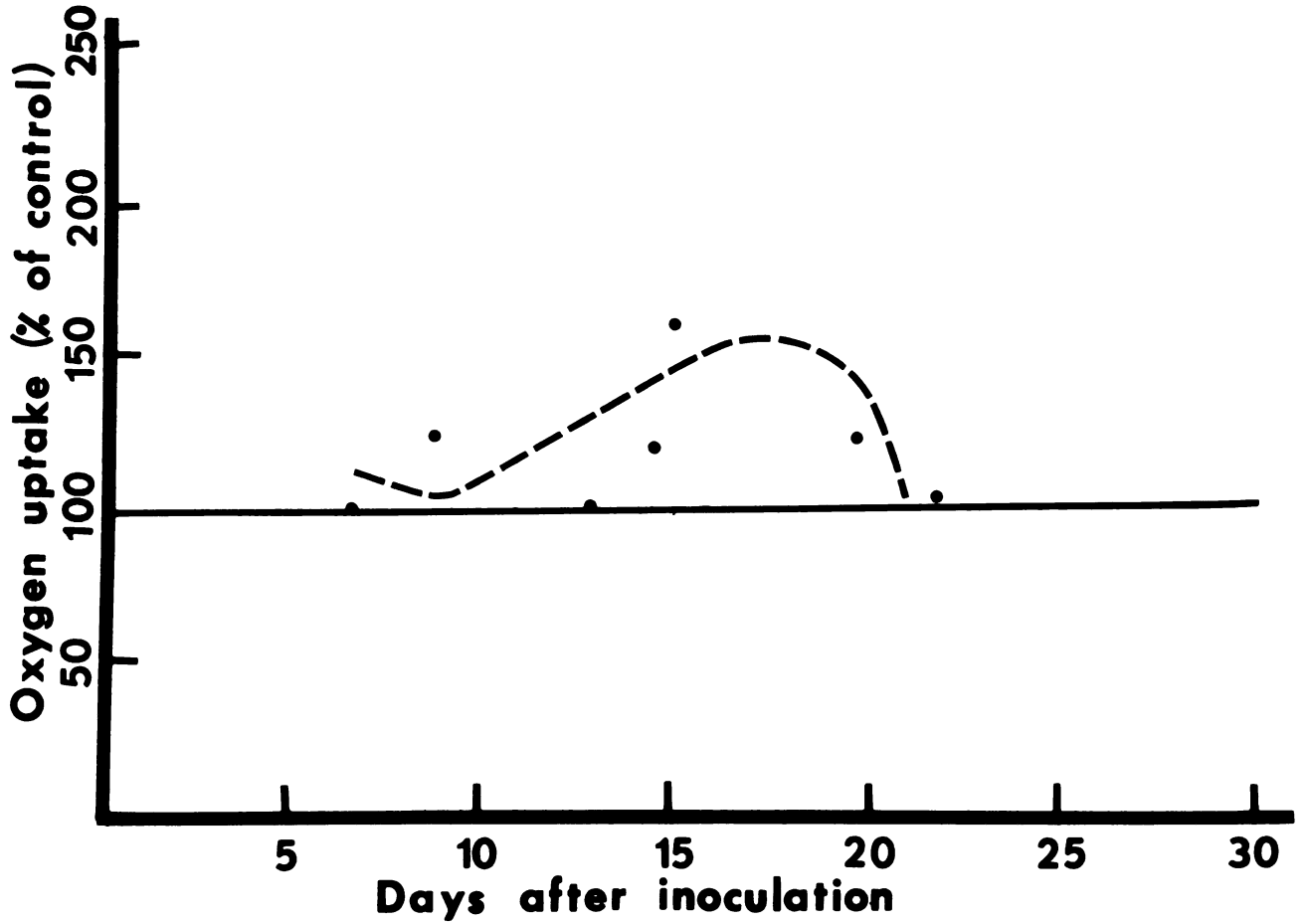

Figure 7. Oxygen uptake by leaf discs from Ulmus americana inoculated on June 27, 1967 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=3.550-0.698x+0.060x^2-0.002x^3$, $r=0.76$. Twenty percent difference from the control is significant (0.05 level).

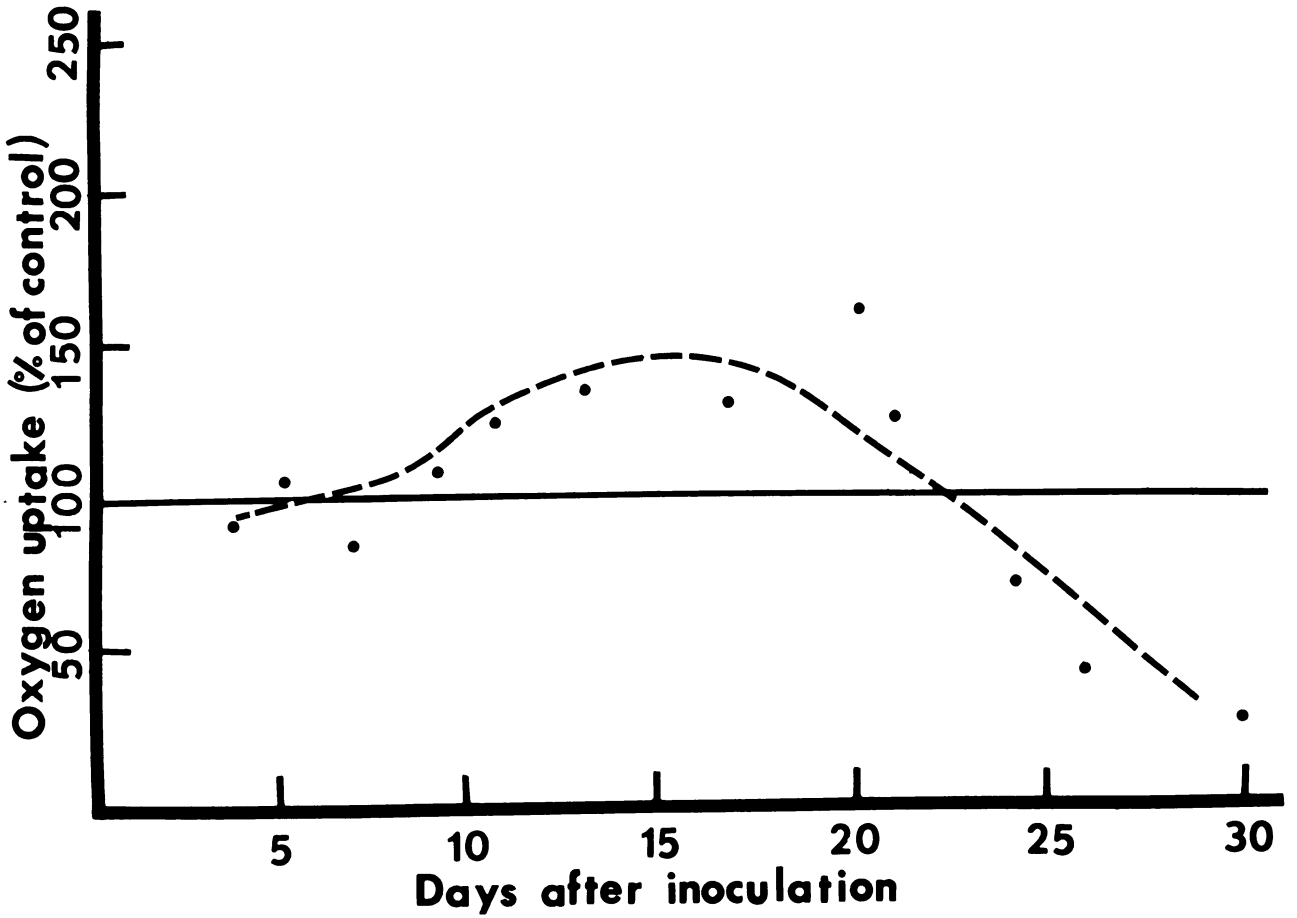

Figure 8. Oxygen uptake by leaf discs from Ulmus americana inoculated on July 5, 1968 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=0.384+0.138x-0.005x^2$, $r=0.81$. Twenty percent difference from the control is significant (0.05 level).

Table 3. Oxygen uptake by leaf discs from *Ulmus americana* inoculated with *Ceratocystis ulmi* or injected with water.

Date Inoculated	Days after Inoculation	Oxygen uptake[a] inoculated[b]	control[c]	% of control
May 23, 1967	2	2.7	3.2	84.4
		3.2		100.0
	3	3.4	3.2	106.3
		-		-
	4	3.6	5.2	69.2
		3.5		57.3
	5	4.4	3.2	137.5
		3.3		103.1
	6	3.1	3.7	83.8
		3.5	3.7	94.6
	7	4.5	3.2	140.6
		4.4		137.5
	8	4.3	2.9	148.3
		4.1		141.4
	9	3.8	2.5	152.0
		-		-
	11	4.1	2.0	205.0
		4.3		215.0
	12	3.3	2.0	165.0
		3.0		150.0
	14	3.3	1.5	220.0
		1.8		120.0
June 7, 1967	2	1.6	1.6	100.0
		1.7		106.0
	4	2.5	2.2	113.6
		1.9		86.4
	5	1.5	1.2	125.0
		-		-
	6	1.7	2.5	68.0
		1.8		72.0
	7	2.3	2.9	79.3
		2.5		86.2
	8	1.4	1.4	100.0
		1.5		107.1
	9	3.3	1.5	220.0
		2.3		153.3
	12	1.7	1.2	141.7
		1.7		141.7
	13	1.0	1.2	83.3
		1.1		91.7
	14	1.2	1.2	100.0
		1.3		108.3
June 19, 1967	3	1.0	0.9	111.1
		1.1		122.2
	4	0.9	0.9	100.0
		0.8		88.9

Table 3 continued

Date Inoculated	Days after Inoculation	Oxygen uptake[a] inoculated[b]	control[c]	% of control
	7	1.0	1.0	100.0
		1.1		110.0
	14	0.7	0.9	77.8
		1.1		122.2
	16	0.8	0.7	114.3
		0.8		114.3
	18	1.4	0.9	155.6
		1.3		144.4
	22	0.9	0.6	150.0
		0.9		150.0
	25	0.7	0.6	116.7
		0.7		116.7
June 27, 1967	7	0.7	0.8	87.5
		0.9		112.5
	9	0.8	0.8	100.0
		1.2		150.0
	13	0.8	0.9	88.9
		1.0		111.1
	15	1.0	0.8	125.0
		0.9		112.5
	16	1.5	0.9	166.7
		1.4		155.6
	20	1.1	0.9	122.2
		1.1		122.2
	22	0.8	0.9	88.9
		1.0		111.1
May 15, 1968	5	5.5	4.8	114.6
		3.8		79.2
	7	5.2	5.1	102.0
		4.0		78.4
	9	3.7	3.7	74.0
		2.9		58.0
	11	3.5	3.7	94.6
		2.6		70.3
	13	2.7	2.6	103.8
		3.3		126.9
	15	3.2	3.3	97.0
		3.9		118.2
	17	3.2	3.5	91.4
		3.3		94.3
	19	3.5	2.3	152.2
		2.8		121.7
	21	2.2	1.5	146.7
		1.7		113.3
	23	4.2	2.2	190.9
		3.2		145.5
June 5, 1968	5	1.8	1.7	105.9
		1.8		105.9

Table 3 continued.

Date Inoculated	Days after Inoculation	Oxygen uptake[a] inoculated[b]	control[c]	% of control
	7	2.2	1.9	115.8
		2.4		126.3
	9	2.2	1.6	137.5
		2.6		162.5
	11	1.5	1.4	107.1
		1.8		128.6
	13	1.7	1.3	130.8
		1.7		130.8
	15	1.9	1.4	135.7
		1.8		128.6
	17	1.6	1.2	133.3
		1.8		150.0
	20	1.6	1.6	100.0
		2.1		131.3
	22	1.6	1.4	114.3
		1.6		114.3
	25	1.5	1.7	94.1
		1.8		105.8
	26	1.2	1.4	85.7
		1.2		85.7
	28	0.7	1.8	38.9
		0.6		33.3
July 5, 1968	3	1.7	1.7	100.0
		1.6		94.1
	5	1.3	1.2	108.3
		1.2		100.0
	7	1.1	1.3	84.6
		1.2		92.3
	9	1.2	1.1	109.1
		1.2		109.1
	11	1.1	0.9	122.2
		1.1		122.2
	13	1.4	1.2	116.7
		1.8		150.0
	17	1.3	1.0	130.0
		1.3		130.0
	20	1.3	0.7	185.7
		1.0		142.9
	21	1.5	1.3	115.4
		1.8		138.5
	24	0.8	1.2	66.7
		1.0		83.3
	26	0.8	2.1	38.1
		1.2		57.1
	30	0.4	1.3	30.8
		0.3		23.1

[a] µl per mg dry weight per hr. There was 50-70 mg leaf tissue per flask.

[b] Mean of three samples per tree, trees inoculated with 400 spores.

[c] Mean of three samples per tree, trees injected with water.

Because changes in oxygen uptake might be due to lack of water in leaves and twigs (42, 56), two experiments were conducted on droughted trees grown in the greenhouse. American elms 1 m tall were transplanted from the field to 25 cm clay pots and held in a non-airconditioned greenhouse without supplementary light. The soil in which the greenhouse plants were growing was brought to field capacity by daily watering unless otherwise noted. In the first experiment, test plants were not watered after oxygen uptake measurements were begun. In the second, plants were watered daily with approximately one-half (200 ml) the water given to controls, beginning 10 days before oxygen uptake measurements were started. This had no visible effect upon the plants. They were given an additional 200 ml 7 days after measurements were begun. Oxygen uptake was determined every other day on randomly collected leaves from four test and four control plants by standard manometric technique (66).

Plants with water stress did not have increased oxygen uptake compared to non-water-stressed plants. In both experiments, by the third day after watering was stopped, oxygen uptake of leaf tissue had decreased to approximately 80 percent of that of leaf tissue of normally watered plants. Oxygen uptake continued to decrease until 14 days after watering was stopped, when it was approximately 50 percent of that of normally watered plants. After 16 days, some of the leaves had fallen off of the water-stressed plants, and the

experiments were terminated.

Results of oxygen uptake experiments indicate that pathogen-free leaves from trees inoculated with C. ulmi respond to infection by increases in oxygen uptake. Leaves from inoculated plants did not show visible symptoms until after oxygen uptake had reached a maximum. Oxygen uptake increases were not due to water stress within the leaves. Therefore, changes in oxygen uptake must be an early response to infection by C. ulmi and must be due to some factor other than water stress. Seasonal variation in oxygen uptake corresponds to seasonal development of disease and suggests that rate of oxygen uptake is closely related to disease development.

Location of the pathogen in test trees - To determine the location of C. ulmi in trees used in respiration and conductivity studies, isolations were made at various locations throughout the trees. Isolations were made 0-30, 30-60, 60-90, and 90-120 cm above the point of inoculation, from terminal twig sections, and from two leaves below those used in the respiration and conductivity studies. Tissues explants were placed directly on elm extract agar (29) in petri plates or they were dipped in alcohol, flamed, and placed on elm extract agar. Plates were observed after 10 days, and if negative, a second time after 14 days. Samples were considered negative unless synnemata characteristic of C. ulmi were present after 14 days.

In all experiments conducted in 1967 and in the first experiment in 1968, all tissues were flame sterilized before they were placed on elm extract agar. Percentage of recovery of C. ulmi from these tissues was lower than expected (Table 2). Therefore, throughout the second and third experiments of 1968, isolations from one of the two inoculated trees were attempted without prior sterilization. Isolations were attempted from the second inoculated tree with prior sterilization. C. ulmi was recovered from approximately twice as many samples when the tissues were not sterilized (Table 2). With the exception of four petioles, C. ulmi was not recovered from leaf tissue.

A general pattern of colonization relative to oxygen uptake changes was found (Table 4). The fungus was restricted to the inoculated area until oxygen uptake increased over control oxygen uptake. The maximum distance above the point of inoculation from which the pathogen was isolated varied between 60 and 120 cm after oxygen uptake increases began but before maximum oxygen uptake was reached. After maximum oxygen uptake increase, the pathogen was found in terminal twigs.

Attempted isolations demonstrated that C. ulmi was not present in leaf discs used in respiration studies. Colonization of trees inoculated with 400 spores was much slower than has been previously reported for trees inoculated with larger numbers of spores (2, 49).

Dehydrogenase activity in terminal twigs - If a toxin was

Table 4. Relation between extent of colonization of *Ulmus americana* by *Ceratocystis ulmi* and oxygen uptake of leaf discs.

Oxygen uptake (relative to control)	Days after Inoculation	Maximum height above inoculation point from which *C. ulmi* was isolated
Equal to control	0 to 5-11	30 cm
Increasing	5-11 to 11-22	60-120 cm
Maximum	11-22	120 cm-terminal twigs
Decreasing from maximum	11-22 to 20-26	Terminal twigs
Less than control	20-26 to 20	Rarely into petioles

present in the transpiration stream, cells in terminal twigs might be killed in advance of the pathogen. Dehydrogenase activity, a characteristic of living cells, was used as a criterion to determine whether cells were living or dead.

Bark was peeled from two-three terminal twigs from each tree used for respiration and conductivity studies. Four or five pieces 1 cm long were removed from the debarked twigs and put into stoppered test tubes containing 5 ml aqueous triphenyltetrazolium chloride (TTC) (1 percent). Test tubes were placed in a 25°C incubation chamber without light. After 24 hours the pieces were removed and hand sections were prepared for microscopic observation. Sections were placed in water under a coverglass and observed at 100 and 430 X magnification. Cells were considered to be alive if they turned red in 24 hours, indicating reduction of the TTC to the formazan form. Twigs killed by autoclaving served as checks.

Throughout the respiration and conductivity studies, cells in terminal twigs had dehydrogenase activity. The red color of formazan was localized in xylem parenchyma tissue. Apparently cells in terminal twigs were not killed during the conductivity and oxygen uptake experiments. However, no quantitative estimates of activity were made and activity could have been reduced. Autoclaved twigs did not reduce TTC.

Loss of electrolytes from leaves and twigs - If host

cell walls or membranes were damaged in the development of disease, a change in loss of electrolytes might occur in host tissue. If conductivity of aqueous leachates increased before macroscopic symptoms developed, the effect might be attributed to a toxin moving in the transpiration stream in advance of the pathogen. Therefore, conductivity of aqueous leachates from pathogen-free tissues was measured during development of disease.

Loss of electrolytes was determined by measuring the conductivity of aqueous leachates from leaves and twigs. An Industrial Instruments Inc. Model RC-16Bl conductivity bridge with a conductivity cell having a cell constant of 1.0 or 0.1 was used. Leaves adjacent to those used in respiration studies were selected for uniformity in size and appearance, washed twice in distilled water, and three times in deionized glass distilled water. Six leaves were harvested per tree. Two of these leaves were put into each of three 120 ml beakers which contained 40 ml of deionized glass distilled water or 40 ml of an aqueous solution of chloramphenicol. Chloramphenicol (3 ppm) did not increase the conductivity of the bathing solution. The leaves were placed in the leaching fluid as soon as the last leaves were washed (approximately 2 hr after trees were cut). Twigs were prepared in a similar manner, with five pieces 3 cm long per beaker. Beakers were maintained at 22°C on a reciprocal shaker (6.5 cm stroke, 60 cycles per min) to insure wetting;

after 8 hours, readings were taken.

Leaf and twig sections were then dried 40 hr at 95°C for determination of dry weights. Conductivity was measured after 8 hr and expressed as micromhos per 100 mg dry weight of tissue and as a percentage of loss of electrolytes from healthy trees.

A least squares regression analysis was performed on conductance increases using the Michigan State University Agricultural Experiment Station STAT Series Description Number 7. Input values were obtained by dividing the conductance (mhos per mg) of the aqueous leachate in each beaker containing tissue from inoculated trees by the mean conductance of leachates containing tissue from non-inoculated trees harvested on the same day. An analysis of variance was performed between trees harvested each day using the Michigan State University Agricultural Experiment Station STAT Series Description Number 13. Input values consisted of conductance measured for each beaker in mhos per mg.

Conductivity of leachates from leaves was highly variable between trees and between days when water alone was used as the leaching fluid. This may have been due to multiplication of bacteria within or upon host tissue during the 8 hr test period. When chloramphenicol was added to the leaching fluid, variability between trees was reduced, but remained great (Table 5). A difference of 30 percent between values for inoculated and uninoculated plants was required for statistical significance (0.05 level). The results of the

Table 5. Conductance of aqueous leachates of leaves from *Ulmus americana* inoculated with *Ceratocystis ulmi* or injected with water.

Date inoculated	Days after inoculation	Conductance[a] inoculated[b]	Conductance[a] control[c]	% of control
June 5, 1968	5	2.15	3.37	63.8
		3.96		117.5
	7	2.31	3.82	60.5
		3.36		88.0
	9	5.28	4.78	110.5
		6.32		132.2
	13	2.91	2.25	129.3
		4.02		178.7
	15	4.31	3.35	128.7
		5.01		149.6
	17	2.57	2.33	110.8
		3.44		147.6
	20	5.22	3.00	174.0
		4.20		140.0
	25	6.44	1.80	350.0
		4.60		250.0
	26	21.15	3.58	590.8
		4.17		116.5
	28	3.56	1.26	282.1
		4.29		339.7
July 5, 1968	3	4.23	2.97	142.4
		2.66		89.6
	5	6.33	4.38	144.5
		4.90		111.9
	7	4.73	4.14	114.3
		4.78		115.5
	9	4.31	3.35	128.7
		5.01		149.6
	11	2.78	2.53	109.9
		5.10		201.6
	13	9.10	6.15	149.3
		6.32		102.8
	17	5.50	5.09	108.1
		5.23		102.8
	20	4.04	2.32	174.1
		3.11		134.0
	21	4.28	3.70	115.6
		5.28		142.7
	24	4.80	2.47	194.3
		10.97		444.1
	26	2.60	148	175.7
		6.35		429.1

[a] Mhos X 10^{-6} per g dry weight per 8 hr.

[b] Mean of three samples per tree, two trees each day. Trees were inoculated with 400 spores.

[c] Mean of three samples per tree, one tree each day. Trees were injected with water.

two experiments in which chloramphenicol was used were very similar to each other (Figures 9, 10).

Electrolyte loss of leaves from inoculated plants closely followed that of control plants for eight days after inoculation, then it increased to about 150 percent of that of the controls in 11 or 13 days. The increase was followed by a decrease to 129 percent or 105 percent 17 days after inoculation. Electrolyte loss then increased until it was greater than 300 percent of that of controls 24 or 26 days after inoculation. Least squares analysis demonstrated that changes in electrolyte loss followed the general equation $y=a+bx+cx^2+dx^3$ where x is days after inoculation. In both cases, the significance probability of the curve was <0.0005. No seasonal effect on loss of electrolytes was noted.

No statistically significant changes in conductivity of aqueous leachates from twigs of inoculated plants could be detected due to great variability between individual trees.

Results of conductivity experiments indicate that leaves from trees inoculated with C. ulmi respond to infection by increased loss of electrolytes. Leaves from inoculated plants did not show visible symptoms until the conductivity of aqueous leachates from inoculated plants was at least 50 percent greater than that from healthy plants. A rapid increase in conductivity of aqueous leachates from leaves simultaneous with a decrease in oxygen uptake by leaf discs (Figures 11, 12) occurred as chlorosis and browning of leaves developed.

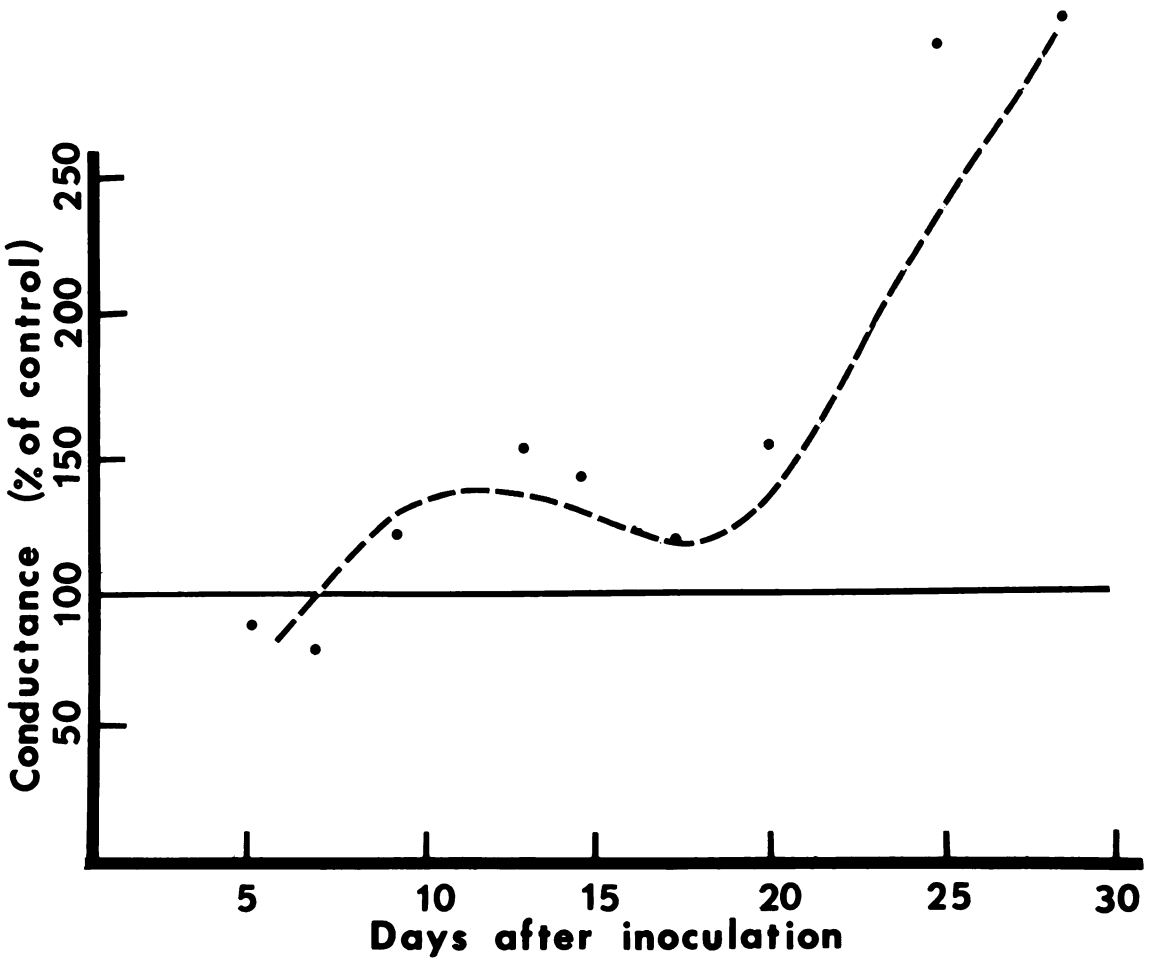

Figure 9. Conductance of leaves from Ulmus americana inoculated on June 5, 1968 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=1.735+0.697-0.050x^2+0.001x^3$, r=0.61. Thirty percent difference from the control is significant (0.05 level).

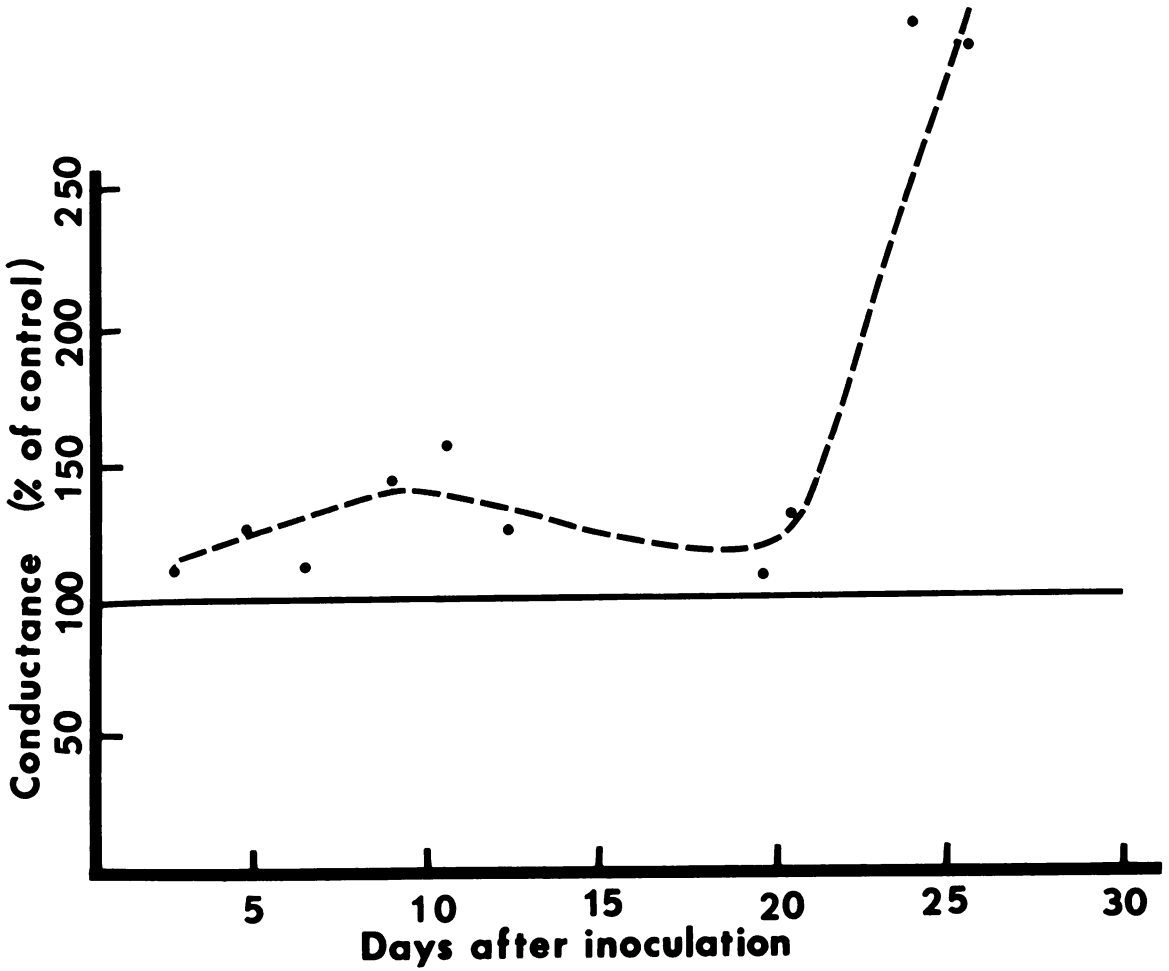

Figure 10. Conductance of leaves from Ulmus americana inoculated on July 5, 1968 with Ceratocystis ulmi. Each point represents the mean of two trees with three replicates per tree. $y=0.544+0.243x-0.022x^2+0.001x^3$, $r=0.52$. Thirty percent difference from the control is significant (0.05 level).

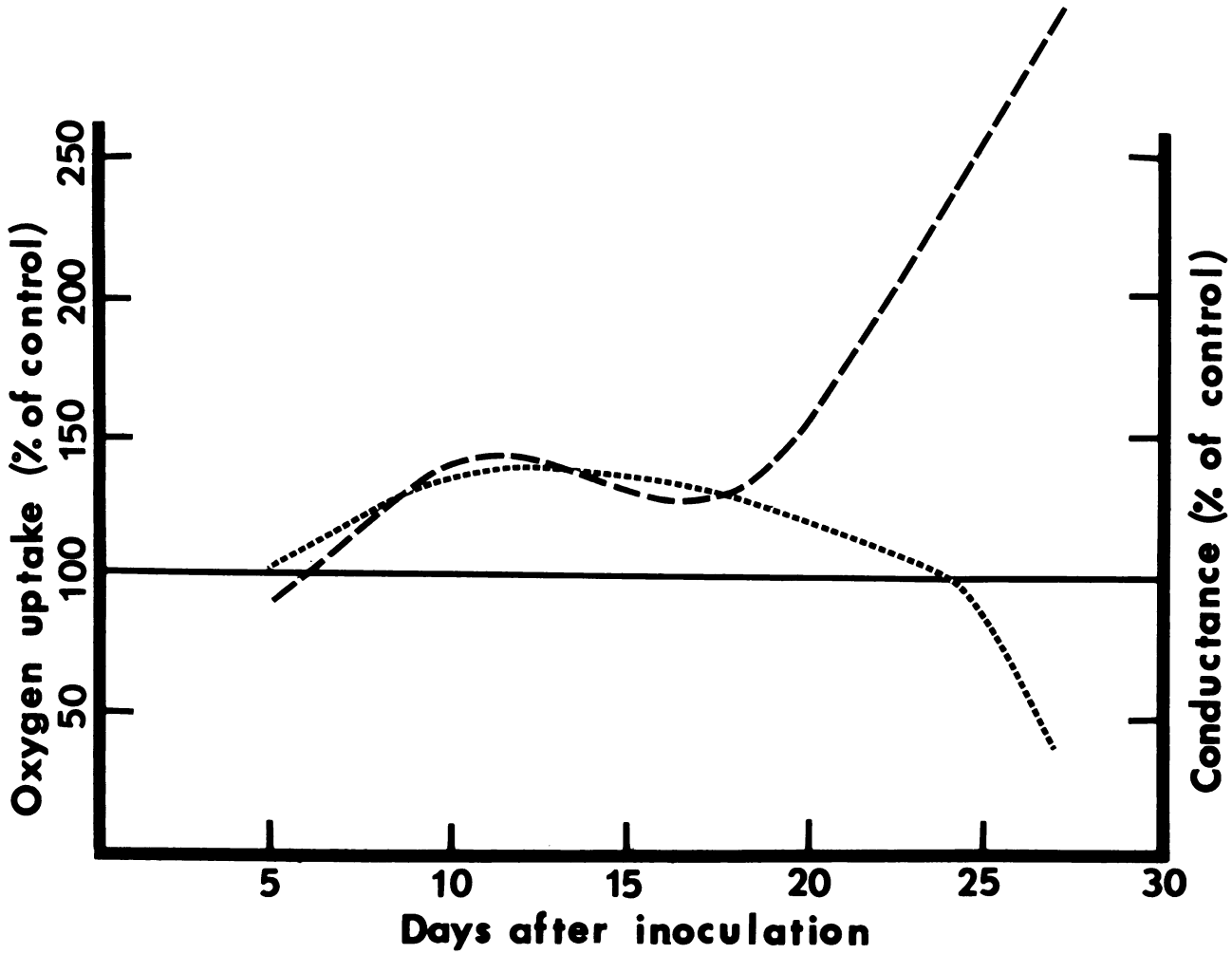

Figure 11. Oxygen uptake and conductance of leaves from Ulmus americana inoculated with Ceratocystis ulmi on June 5, 1968.

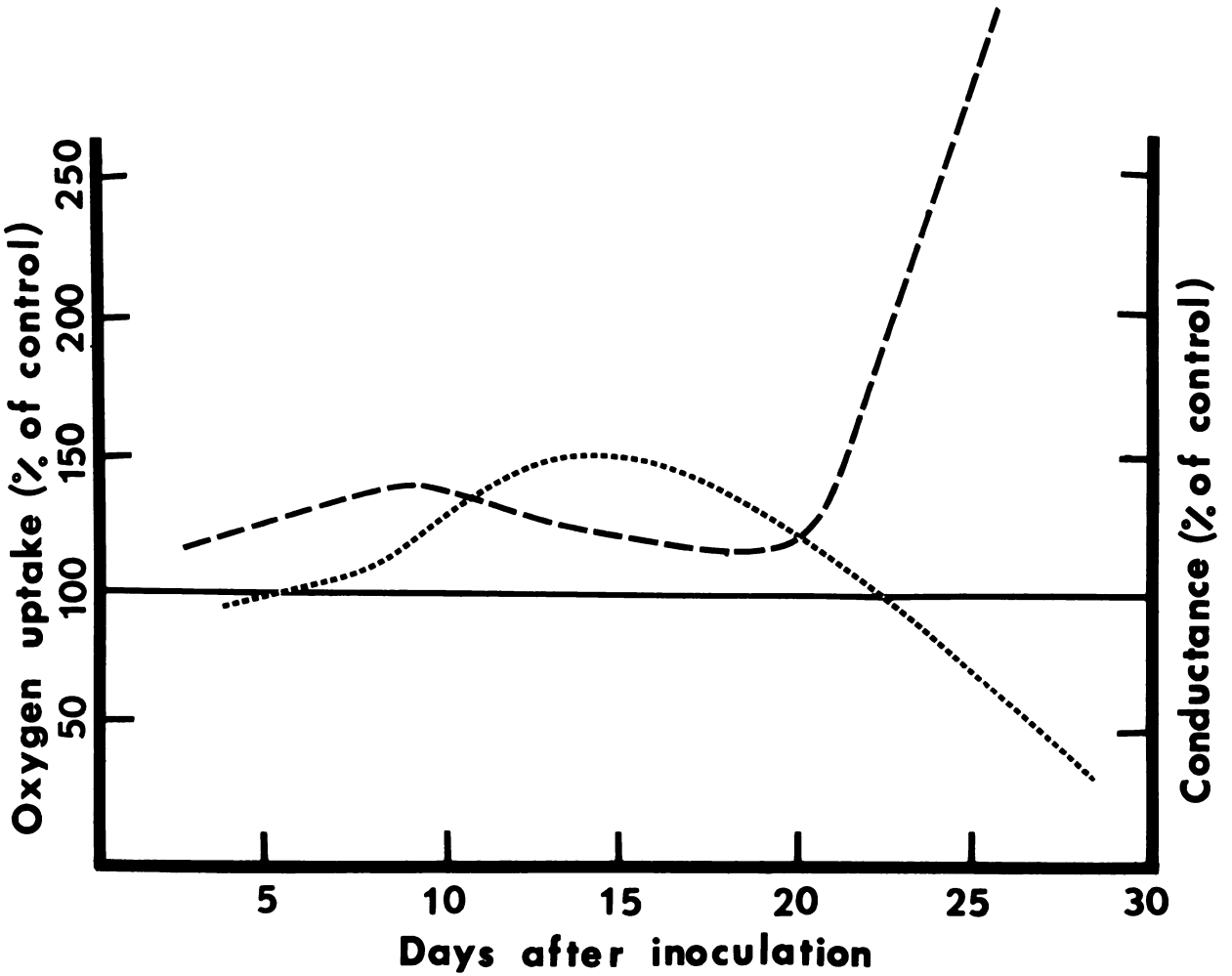

Figure 12. Oxygen uptake and conductance of leaves from Ulmus americana inoculated with Ceratocystis ulmi on July 5, 1968.

This phase of the disease was probably due to a general disorganization of leaf cells and is additional evidence for physiological changes preceding the presence of the pathogen.

Tissue discoloration and vessel occlusion - If Dutch elm disease symptoms were caused by a toxin, damage to cells and tissue discoloration might occur in terminal leaves and twigs before macroscopic symptoms appeared. If symptoms were caused by vascular occlusion, such as gums and tyloses, these might be detected by microscopic observation. Therefore, sections from leaves and twigs proximal to those used in respiration and conductivity studies were observed under a microscope.

Possible discoloration of terminal leaves and twigs and visible vessel occlusion were studied by clearing sectioned leaves and twigs using a modification of Scheffer and Walker's method (54). Leaves and twigs from all trees used in respiration and conductivity studies conducted in 1967 were cut into 2-3 cm long pieces with a razor blade, vacuum infiltrated with aqueous basic fuchsin for 5 min, then bathed in aqueous basic fuchsin for 24 hr to stain the xylem. The sections were cleared in a sequence consisting of hot 85 percent ethanol for 4-5 min, 95 percent ethanol for 15-20 min, anhydrous ethanol for 30 min, and three changes of lacto-phenol over a 72 hr period. The sections were then preserved in xylol until further sectioning. Ten to 20 transverse and longitudinal sections were prepared from each tree with a sliding microtome

at 20-120 μ, or by hand with a razor blade. Sections were mounted in diaphane under a coverglass and observed at 50-970 X magnification.

Leaves from inoculated trees developed macroscopic symptoms approximately 20 days after inoculation, when they became chlorotic. Parenchyma and vessels of twigs and leaves from inoculated trees could not be distinguished from those of control trees during the 30 days after inoculation. Vessels of twigs, petioles, and midveins were free from visible occlusions with one exception. One petiole from an inoculated tree was found in which all vessels were blocked by a brown granular mass. In no case was the fungus detected by microscopic observation.

Vessel occlusions and the fungus might have been removed by sectioning and staining technique. The possibility of mechanically removing occlusions was reduced, however, by preparing thick sections. These observations indicated that visible vascular occlusions in the small twigs and leaf petioles were not responsible for symptom development.

Isolation of toxic substances - Respiration, conductivity and colonization studies suggested that a toxic substance might be causing physiological changes in pathogen-free tissue. Therefore, an attempt was made to isolate a toxic substance from culture filtrates of C. ulmi.

A modification of the method of Pringle and Braun (51) was selected. Three isolates of C. ulmi used for respiration

and conductivity studies were grown separately in still or shake culture at 22°C for 10 or 20 days in 250 ml flasks, each containing 50 ml of the medium used in preparation of inoculum or 50 ml of yeast extract supplemented (1.0 g) Fries No. 3 basal medium (51). The shaker was set for a 6.5 cm stroke and 60 cycles per min. In one experiment, medium from 10 day cultures grown in still culture in modified Fries medium was tested. In a second, media from 20 day cultures of the three isolates grown in medium used in preparation of inoculum contained in shaken and non-shaken flasks were combined before testing.

Culture media were filtered three times with suction through Whatman No. 1 filter paper on a Buchner funnel. Filtrates were concentrated *in vacuo* to 0.1 original volume at 32°C, an equal volume of methanol was added, then the concentrated filtrate and methanol were stored at 0-1°C for 40 hr. The precipitate which formed was discarded and methanol was removed from the remaining filtrate *in vacuo* at 32°C. Five ml of the remaining crude concentrate was further purified by gel filtration.

Ten g dry spherical polyacrylamide (Bio Gel P-2, 200-400 mesh) was hydrated by soaking in water 20 hr at 22°C. The gel was periodically stirred and water was replaced several times. After packing a K 15/30 Sephadex column, the water was brought even with the top of the gel bed. Five ml of the crude culture concentrate was carefully placed on top of the gel bed, the concentrate was brought

even with the top of the bed, water was carefully added to the top of the column, and the column was sealed. The column was developed with water at 1-2 ml per min. The effluent was collected in 14 five ml fractions. Each fraction was diluted 1:10 and 1:50 for testing.

Possible toxicity of the fractions was tested on U. americana and Celtis occidentalis L. in two experiments and on U. americana and Acer saccharum Marsh. in the third. Succulent cuttings (10 cm) were collected from large trees and placed in 4 ml of each dilution of each fraction. A water control was maintained. An additional amount of the appropriate diluted fraction was added to each test tube to maintain the fluid level.

None of the fractions collected from culture filtrates of C. ulmi caused any distinct symptoms on host or non-host cuttings. Some of the cuttings, including controls, developed curled leaves, but this was usually associated with increased turbidity of the fraction, and could have been due to multiplication of bacteria in the fraction.

Bio Gel P-2 would have excluded the 25,000 MW compound isolated by Salemink et al. (53) which was toxic to cuttings of U. hollandica Mill. Cv. belgica. They were also unable to recover the toxic principle by gel filtration using a gel with an inclusion range of 4000-150,000 MW.

DISCUSSION AND CONCLUSIONS

Experiments were designed to determine if physiological changes occurred in host tissues early in disease development, before macroscopic symptom expression began, and to determine the distribution of the pathogen within the host at the time of these events. If physiological changes occurred in pathogen-free tissue, particularly if the pathogen was distant from the site of change, a toxic substance which was mobile in the transpiration stream might be responsible for the changes.

Physiological changes which occurred in pathogen-free tissues have been reported with several vascular diseases. Foster (23) found that vein-clearing occurred after inoculation with Fusarium wilt of tomato before other symptoms were observed. Davis (17), working with intergeneric grafts and F. oxysporum f. lycopersici, found non-host scions responded to infection in the stock by necrosis. Keyworth (37, 38) worked with multiple grafts between Fusarium wilt-resistant and susceptible tomato plants and found discoloration in pathogen-free resistant tissues. Collins and Scheffer (16) worked with the same disease, and found respiration and permeability changes in pathogen-free leaves. Scheffer (55) however, did not believe that respiration changes had a major role in disease development because disease could develop normally with essentially no change in oxygen uptake.

Mathre (39) found that photosynthesis decreased in pathogen-free blades of Verticillium-infected cotton plants. Roberts (52) demonstrated an early increase in transpiration in both susceptible and resistant elms following inoculation with C. ulmi.

The present work demonstrated that changes in loss of electrolytes and oxygen uptake occurred in pathogen-free tissues of U. americana in response to C. ulmi infection. Oxygen uptake by leaf discs from inoculated plants increased to as much as 180 percent of controls 11-22 days after inoculation. Conductance of aqueous leachates of leaves increased to approximately 150 percent of controls in 11-13 days after inoculation. After reaching a maximum, oxygen uptake decreased to 30-50 percent of controls. A decrease in oxygen uptake was accompanied by a rapid increase in conductivity. Leaf chlorosis became evident during this phase of disease development, and indicates that general disruption of host metabolism occurred. These changes are interpreted as occurring in response to some substance which is transported through the transpiration stream to the site of action in terminal leaves. However, these data do not prove the involvement of a toxin in disease development. This can only be established by isolation and purification of the substance and demonstration that it has a role in disease development.

One alternative, that water stress was the cause of the observed changes, was tested and was found not to be involved.

Oxygen uptake increased after inoculation with C. ulmi, but decreased in healthy plants subjected to water stress. There are, however, other factors which could elicit a host response in non-infected tissues. The observation that oxygen uptake and conductivity changes occur soon after inoculation, before any morphological or anatomical symptoms occur seems to imply that these are early effects in the disease syndrome, but it does not imply that these are necessary for disease development. Conductivity and oxygen uptake changes may indicate that general cell disorganization is occurring.

Observations on spore movement and subsequent disease development in American elm have often been made on plants which received enormous numbers of spores, often in large volumes of fluid (2, 4, 20, 49, 52, 64). Comparison of these results with natural infection has been criticized (12, 41). The data reported herein substantiate these criticisms. C. ulmi was not isolated from twigs of small trees in less than 11 days after inoculation when the trees were inoculated with approximately 400 spores; nor was the fungus isolated from leaf blades and only rarely (1.6 percent) from petioles in the 30 day test period. Comparable (symptomless) leaf blades and petioles from naturally infected trees contained the pathogen in 0.6 percent and 4.5 percent of the attempts. By contrast, 8.0 percent of the leaf blades and 11.5 percent of the petioles from trees which were inoculated with a large spore load contained C. ulmi. The number of spores herein

considered to be a large spore load (5×10^4 spores per tree) was much smaller than those used in other colonization studies (2, 4, 49). Experiments which demonstrated rapid distribution of spores throughout trees and into leaves within 24 hours of inoculation (49) are not representative of natural colonization.

Vessels in twigs and in petioles and midveins of leaves on inoculated trees were not visibly occluded when the leaves were taking up oxygen at maximum rate. Sectioning and preparation of tissues for microscopic observation could have removed occluding gums. However, control of Dutch elm disease can be obtained by chemically-induced occlusion of vessels (59). Control was obtained by preventing spore movement in the vessels and thus preventing systemic infection. To understand how occlusion can be responsible for both symptom development and resistance, especially since vessel occlusion is complete in resistant-treated trees, is difficult.

Most previous work has been done on that phase of the disease during which visible symptoms occur or with tissue containing the pathogen. Leaf symptoms occur concurrently with decreases in oxygen uptake and large increases in loss of electrolytes, and on this basis is rather late in disease development. Many changes have probably occurred in the host by the time leaf symptoms occur. Leaf symptoms may be the manifestation of general cellular disorganization: in this event, all physiological changes which led to disorganization

of host cells would have preceeded symptom expression. Therefore, future research should be concentrated on the early events in disease development, those which occur before macroscopic symptoms are observed.

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