Physiological Changes in Pathogen-Free Tissue of Ulmus americana Induced by Ceratocystis ulmi

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ABSTRACT

The response of pathogen-free tissue of American elm, *Ulmus americana*, infected with the Dutch elm disease pathogen, *Ceratocystis ulmi*, was studied. Oxygen uptake of pathogen-free leaf discs began to increase 5 to 11 days after inoculation of trees, and reached a maximum between 11 and 22 days, when oxygen uptake was as much as 80% higher than in controls. Thereafter, oxygen uptake decreased until it was less than controls 20 to 26 days after inoculation. Increased oxygen uptake was not caused by water stress, since potted elms under 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% of controls when oxygen uptake was at its minimum of 30 to 50% of controls. Reduction of triphenyltetrazolium chloride demonstrated that twigs from which the leaves were harvested for respiration and conductivity experiments contained living parenchyma. Increased respiration and changes in permeability in pathogen-free tissues from infected plants support the hypothesis of a translocatible toxin.

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Ceratocystis ulmi, a pathogen of elm, causes a systemic vascular infection with many characteristics in common with other diseases of this type. For example, tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici* had vein clearing (5), increased respiration (1, 18), increased membrane permeability (1), and discoloration of parenchyma (19) in pathogen-free tissues. Nonhost scions of intergeneric (2) and intrageneric (9, 10) grafts became necrotic after invasion of the host stock with *F. oxysporum* f. sp. *lycopersici*. Photosynthesis decreased in pathogen-free leaves of cotton plants infected with *Verticillium albo-atrum* (12). Roberts (15) demonstrated an early increase in transpiration in both susceptible and resistant elms after inoculation with *C. ulmi*, but MacHardy (11) was unable to show such an increase in water loss. These data suggested that some substance was moving in advance of the pathogen, disrupting the normal metabolism of the host. Several toxic substances which occur in crude culture filtrates of *C. ulmi* illicit a disease syndrome similar to that of Dutch elm disease (3, 4, 6, 17, 23, 24).

The purpose of this research was to determine whether or not there are physiological changes in pathogen-free host tissue at an early stage in development of Dutch elm disease, and to correlate possible changes with development of symptoms.

MATERIALS AND METHODS.—*Ceratocystis ulmi* (Buis.) C. Moreau was isolated from three different naturally infected trees in February 1967, and April 1968. The fungus was grown in shake culture at 22 C in a medium containing (g/liter): 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 consisted of bud cells. We prepared inoculum for each year by mixing bud cells from that year's isolates.

Each of 30 elm trees (*Ulmus americana* L., 2.5 to 4 m tall) growing under natural conditions was inoculated with one drop of suspension containing ca. 400 cells of *C. ulmi* on each of the following days: 23 May and 7, 17, and 27 June 1967, and 15 May, 5 June, and 5 July 1968. Fifteen control trees were injected with a drop of water on each of these dates. The drop was placed in a twig or branch axil 70 to 130 cm above ground level, where the trunk was 3 to 6 cm in diam. The bark was then pierced through the drop with a sharp needle. When the needle was withdrawn, the drop of spore suspension rapidly disappeared into the transpiration stream. Two inoculated trees and one noninoculated tree were harvested daily or every other day up to 30 days after inoculation. Terminal leaves and twigs from these trees were used to measure respiration, the uppermost lateral leaves and adjacent twigs were used in conductivity studies, and the next lower lateral leaves and adjacent twigs were for histological studies. During the course of the study, isolations were made from 242 leaf blades and petioles and from 121 of the adjacent twigs. *Ceratocystis ulmi* was not isolated from any of the blades, but it was recovered from four petioles and 12 shoots (7). From these data, it was concluded that the leaves used in the oxygen uptake and conductivity studies were pathogen-free.

Oxygen uptake was determined with a Warburg apparatus, using standard manometric techniques (22). Eighty to 100 leaf discs 7 mm in diam or 80 to 100 twig sections 5 mm long were harvested from four to six uniform terminal leaves and twigs of each tree. The leaf discs and stem sections from each tree were placed on moist filter paper in separate petri dishes until the discs and sections were put into manometric flasks (ca. 1 hr after trees were cut). Twenty leaf discs or stem sections from each petri dish were selected at random and placed on filter paper moistened with 0.2 ml distilled water in each of three manometric flasks. Center wells contained a 20% solution of KOH. Equilibration time was 20 min; water-bath temperature was 25 C, and light was excluded from the reaction vessels. Oxygen uptake was determined for 1 to 2 hr, with readings every 15 or 20 min. After the final readings, leaf discs and twig sections were removed from the flasks, dried at 95 C for 48 hr, and weighed. Data were expressed as a percentage of the oxygen uptake by the noninoculated control tree used on the same day.

Oxygen uptake data were analyzed by the least squares regression method. Input values were obtained by dividing the amount of oxygen uptake by diseased tissues (μliter per mg per flask) by the mean oxygen uptake by tissues from noninoculated trees harvested the same day. Variation between trees at each harvest date were tested by analysis of variance using input values of the amount (μliter per mg) of oxygen taken up by the tissue contained in each flask.

Possible changes in oxygen uptake caused by water deficiency in leaves and twigs (13, 20) were tested in two experiments with water-stressed trees grown in the greenhouse. Elms 1 m tall were transplanted from the field to 25-cm clay pots and held in a greenhouse without supplemental light. Soil in the pots was brought to field capacity by a daily watering unless otherwise noted. Test plants for the first experiment were not watered after the first harvest of leaves for oxygen uptake analysis. Test plants for the second experiment were watered daily with approximately half as much water (200 ml) as that given to controls for 10 days before oxygen uptake measurements were started. They received an additional allotment of 200 ml on the 7th day after measurements began. Oxygen uptake of randomly collected leaves from four test and four control plants was measured every other day.

Loss of electrolytes was determined from the conductivity of the water on which leaves or twigs were floated. A conductivity bridge was used with cells with constants of 1.0 or 0.1. Six leaves from each tree which were uniform in size and appearance were selected from those adjacent to the ones used in the respiration studies. Leaves were washed 2 times in distilled water and 3 times in deionized glass-distilled water. As soon as the last leaves were washed (ca. 2 hr after harvest), two 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 (3 μg/ml). Chloramphenicol did not increase the conductivity of the bathing solution. Twigs were prepared in a similar manner, with each beaker containing five pieces, each 3 cm long. Samples were maintained at 22 C on a reciprocal shaker (6.5 cm stroke, 60 cycles/min) to ensure wetting; conductivity readings were taken after 8 hr of leaching. Conductivity was expressed as mhos/100 mg oven-dry weight of tissue and as a percentage of conductivity of electrolytes from healthy trees.

Increases in conductance were analyzed by the least squares regression method. Input values were obtained by dividing the conductance (mhos/mg) of the aqueous leachate in each beaker containing tissue from inoculated trees by the mean conductance of leachates from noninoculated trees harvested on the same day. Variation between trees at each harvest date was tested by analysis of variance using input values of conductance measured for each sample.

Dehydrogenase activity was used as a criterion of cell viability. Bark was peeled from 2 to 3 terminal twigs from each tree used for respiration and conductivity studies. Four or 5 pieces, each 1 cm long, were removed from the debarked twigs and put into stoppered test tubes containing 5 ml aqueous triphenyltetrazolium chloride (TTC) (1%). Tubes were incubated for 24 hr at 25 C without light. Pieces were then removed and sectioned for microscopic observation. Living cells reduced TTC to the formazan form and became red within 24 hr. Twigs killed by autoclaving served as controls.

RESULTS.—Oxygen uptake by leaf discs from inoculated trees was similar to oxygen uptake by discs from control trees for the first 5 to 11 days after inoculation (Fig. 1, 2). Oxygen uptake by discs from inoculated plants began to increase (in relation

to control values) after 5 to 11 days, and reached a maximum 11 to 22 days after inoculation. Oxygen uptake by leaf discs from inoculated trees was as much as 80% higher than uptake by healthy control tissues. After 11 to 22 days, oxygen uptake began to decrease, until it was 30 to 50% of control oxygen uptake when the experiments were terminated. Oxygen uptake of healthy tissue was much higher (3-5 μliters/mg dry weight per hr) soon after bud break than when the leaves were fully expanded (0.8-1.5 μliters/mg per hr).

At least a 20% difference in oxygen uptake between inoculated and control plants was required for a significance level <0.05. Least squares analysis demonstrated that change in oxygen uptake curves followed the general equations $y = a + bx + cx^2 + dx^3$ or $y = a + bx + cx^2$, with x representing days after inoculation (Fig. 1-2). In all cases but one, the significance probability of the curve was <0.0005, and r values were >0.72. The exception was the experiment started 7 June 1967 (Fig. 1-B), which 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 high oxygen uptake (2.5 and 2.9 μliters/mg dry weight per hr) of noninoculated trees on days 6 and 7. This was a twofold increase over noninoculated trees harvested on days 5 and 8. The factor responsible for this great increase in oxygen uptake is unknown.

There appeared to be a seasonal effect on oxygen uptake in inoculated plants in 1967. Oxygen uptake increased significantly (0.05) after 7 days in plants inoculated on 23 May (Fig. 1-A), after 9 days in those inoculated on 7 June (Fig. 1-B), after 18 days in those inoculated on 19 June (Fig. 1-C), and after 16 days in those inoculated on 27 June (Fig. 1-D). This is consistent with reports (14, 21) that Dutch elm disease develops more rapidly in spring and early summer. In 1968, oxygen uptake did not increase as quickly after inoculation in May as in June and July. Dutch elm disease development has been shown previously to vary with soil moisture (8, 14, 24). Lack of a seasonal effect in 1968 could have been due to the 15.13 inches of rain received in the spring and early summer of 1968 as compared to the 8.97 inches received during the same period of 1967.

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

Plants with water stress did not have a significant increase in oxygen uptake as compared to nonwater-stressed plants. In both experiments, by the 3rd day after watering was stopped, oxygen uptake of leaf tissue had decreased to ca. 80% of that of leaf tissue of normally watered plants. Oxygen uptake continued to decrease until 14 days after watering stopped, when it was ca. 50% that of normally watered plants. After 16 days, some of the leaves had fallen from the water-stressed plants, and the experiments were terminated.

Conductivity of leachates from leaves of different trees was highly variable when water alone was used as the leaching fluid. Bacterial growth in the preparations during the 8-hr test period may have contributed to this variation. When chloramphenicol was added to the leaching fluid, there was less variation between trees; comparable results were obtained in these two experiments (Fig. 3). A 30% difference between conductivity values by samples from inoculated and noninoculated plants was statistically significant (0.05 level).

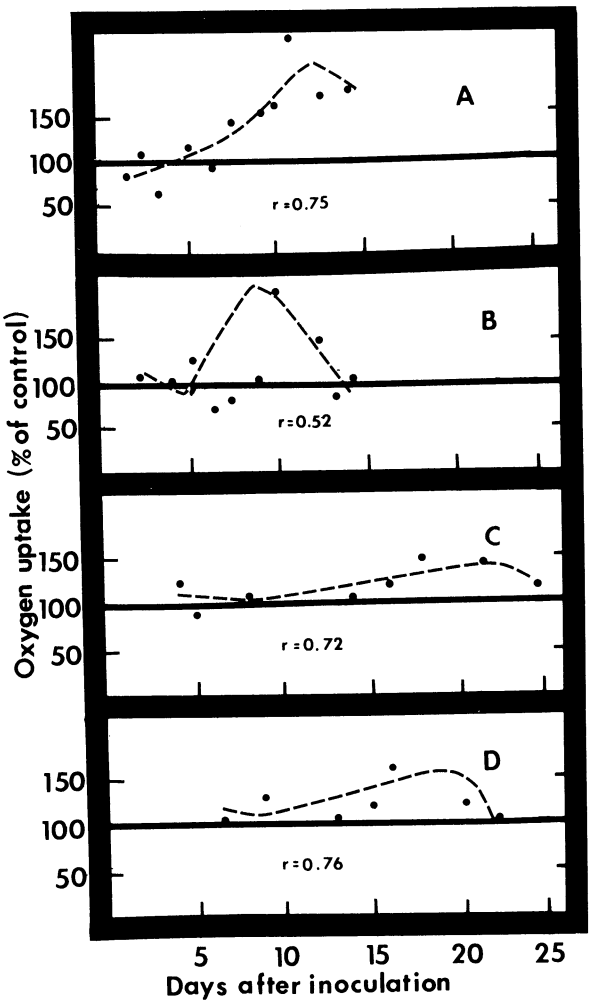

Fig. 1. Oxygen uptake by leaf discs from *Ulmus americana* inoculated on A) 23 May, B) 7 June, C) 19 June, and D) 27 June 1967 with *Ceratocystis ulmi*. Each point represents the mean of two trees with three replicates/tree (20% difference from the control is significant at the .05 level).

Losses of electrolytes from leaves of inoculated and control plants were similar for the first 8 days after inoculation (Fig. 3). Losses from inoculated plants then increased, until they were as much as 50% greater than that of the controls by 11 to 13 days after inoculation. Losses from inoculated plants then gradually fell to 29%, or 5% greater than that of control plants, at 17 days after inoculation. Electrolyte loss then increased again until it was 200% greater than that of controls 24 and 26 days

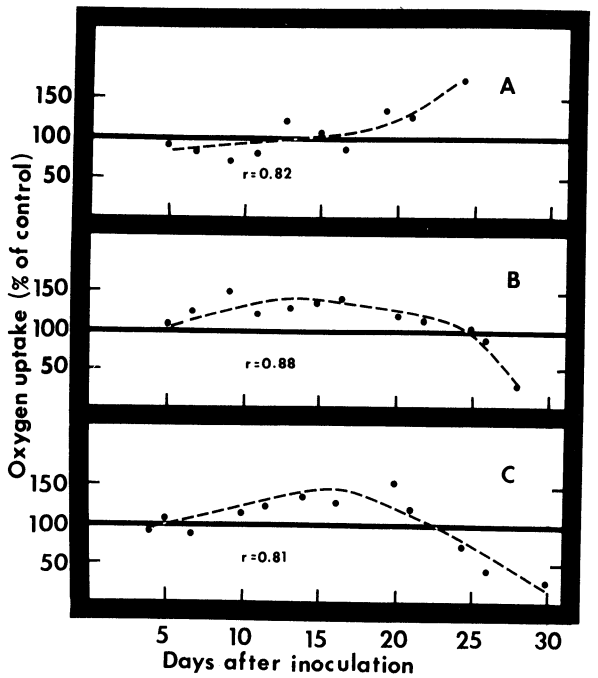

Fig. 2. Oxygen uptake by leaf discs from *Ulmus americana* inoculated on A) 15 May, B) 5 June, and C) 5 July 1968 with *Ceratocystis ulmi*. Each point represents the mean of two trees with three replicates/tree (20% difference from the control is significant at the .05 level).

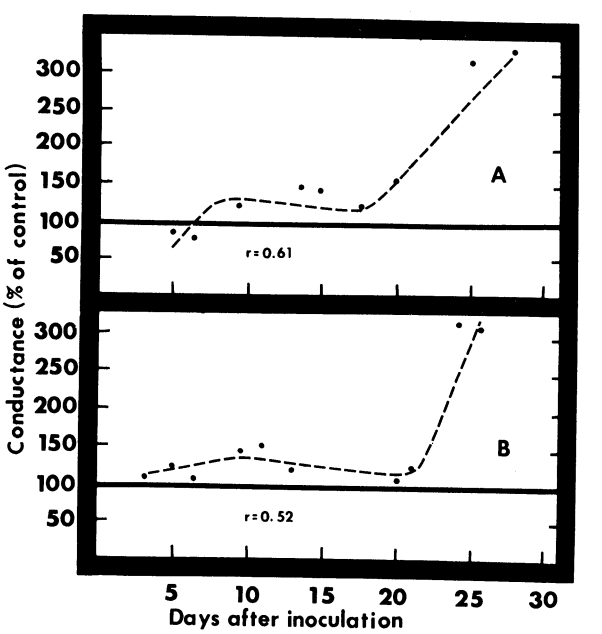

Fig. 3. Conductance of leachates from leaves from *Ulmus americana* inoculated on A) 5 June, and B) 5 July 1968 with *Ceratocystis ulmi*. Each point represents the mean of two trees with three replicates/tree (30% difference from the control is significant at the .05 level).

after inoculation. Least squares analysis demonstrated that changes in electrolyte loss followed the general equation $y = a + bx + cx^2 + dx^3$, with x representing days after inoculation. In both cases, the significance probability of the curve was $<.0005$. No seasonal effect on loss of electrolytes was noted.

No statistically significant changes in conductivity of aqueous leachates from twigs of inoculated plants were detected, because variability between individual trees was too large.

Throughout the respiration and conductivity studies, cells in terminal twigs had dehydrogenase activity, as indicated by the red color of formazan localized in xylem parenchyma tissue. The cells in terminal twigs appeared to be alive throughout these experiments. However, no quantitative estimates of dehydrogenase activity were made, and activity could have decreased. Autoclaved twigs did not reduce TTC.

DISCUSSION.—Results indicated that pathogen-free leaves from elms inoculated with *C. ulmi* respond to infection by increased oxygen uptake and increased cell permeability in a manner similar to that reported for Fusarium wilt to tomato (1). The possibility that water stress was the cause of the observed changes was tested and eliminated. Changes in oxygen uptake are an early response to infection by *C. ulmi*, and are due to some factor other than water stress. Seasonal variation in oxygen uptake corresponded to seasonal development of disease, and suggests that changes in rate of oxygen uptake were closely related to disease development. Changes in oxygen uptake were very similar to changes in CO_2 uptake (16) by leaves on inoculated plants; both increased initially before symptoms appeared, and both then decreased rapidly as symptoms began to develop (Fig. 1, 2).

These changes are interpreted as occurring in response to some substance which is transported through the transpiration stream to the leaves. Symptomless leaves on infected twigs and chlorotic or partially necrotic leaves on naturally infected trees frequently were pathogen-free (7). The observation that changes in oxygen and CO_2 uptake (16), cell permeability, and transpiration (15) occur in pathogen-free tissue soon after inoculation, before visible symptoms develop, supports the hypothesis of a systemic factor. It does not necessarily mean that the substance is required for pathogenesis. This can only be established by isolation and purification of the substance, and demonstration that it has such a role in disease development.

Most previous work has been done on late phases of disease, when visible symptoms develop, or with tissue containing the pathogen. Leaf symptoms appear after physiological changes have taken place; thus, visible symptoms are a late occurrence in disease development. Leaves from inoculated plants did not show visible symptoms until after their oxygen uptake had reached a maximum and until the conductivity of their aqueous leachates was at least 50% greater than that of healthy plants (Fig. 1-3). This was followed by decreased oxygen uptake (Fig.

1, 2) and increased conductivity of leachates (Fig. 3) 15 to 25 days after inoculation which accompanied the development of chlorosis and browning of leaves. Hence, visible leaf symptoms probably indicate general cellular disorganization. Therefore, future research should be concentrated on the early events in disease development, which occur before macroscopic symptoms are observed.

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