Wilt Development in Red Oak Seedlings: A New System for Studying Oak Wilt

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The authors thank S. A. Vicen for his assistance in preparing the figures.

Research was cooperative with the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and the Agricultural Research Service, U. S. Department of Agriculture.

Accepted for publication 25 June 1975.

ABSTRACT

Young red oak seedlings developed foliar wilt, plugging of vessels by tyloses and gums and resistance to water movement when infected by *Ceratocystis fagacearum*. In addition, the host range, the slower average rate of symptom development in white oak seedlings, and the effects of temperature and inoculum dosage on disease development were all similar to those reported for woodland trees and older (1-3 year-old) seedlings. Young seedlings are readily adapted for study under controlled environments. Reproducible wilt development was obtained under standardized conditions (28-day-old seedlings, 10^4 conidia per seedling, 26 C). The technical advantages of seedlings, and the similarities between their disease development and that of woodland oaks, indicate that young seedlings are a valid model system for detailed studies of oak wilt.

Phytopathology 65:1381-1386

Additional key words: Quercus spp., resistance to oak wilt.

Our understanding of pathogenesis in oaks infected with *Ceratocystis fagacearum* (Bretz) Hunt has expanded little since the early work of Beckman et al. (2), Parmeter et al. (16) and Struckmeyer et al. (18). From their observations and experiments with large woodland oaks, they concluded that this wilt pathogen induces the formation of tyloses and gums which occlude the vessels, impair water transport and result in the death of the tree from foliar wilt. In contrast, Wilson (22) has argued that death results from fungal invasion and killing of parenchyma tissues, and not from a lack of water caused by vessel occlusion. He suggested that wilt symptoms reflect the desiccation of the dying tree. In addition, fungal metabolites able to cause desiccation and discoloration of oak leaves and cuttings have been demonstrated in culture filtrates. However, they have not been found in diseased trees and their potential role in pathogenesis remains unknown (13, 21).

A major barrier to the further testing of these hypotheses is the lack of an adequate plant system for definitive studies. Mature trees are unsuitable because of their size, extreme variability, and seasonal changes in susceptibility (15). The lack of environmental control and knowledge of possible factors predisposing oaks to wilt are further difficulties which prevent precise studies with woodland trees. One- to three-year-old seedlings, though having many of the same disadvantages, have been used for a few oak wilt studies under controlled environments (9, 10). Also, 1-year-old paired ramets were used to obtain genotypic control in one experiment (8).

That juvenile tissues are susceptible to the pathogen and that young, succulent shoots often show striking symptoms when infected prompted an investigation into the reaction of very young seedlings to *C. fagacearum*. Red oak seedlings 4 to 5 weeks old were found to be highly susceptible to the pathogen and suitable for growth

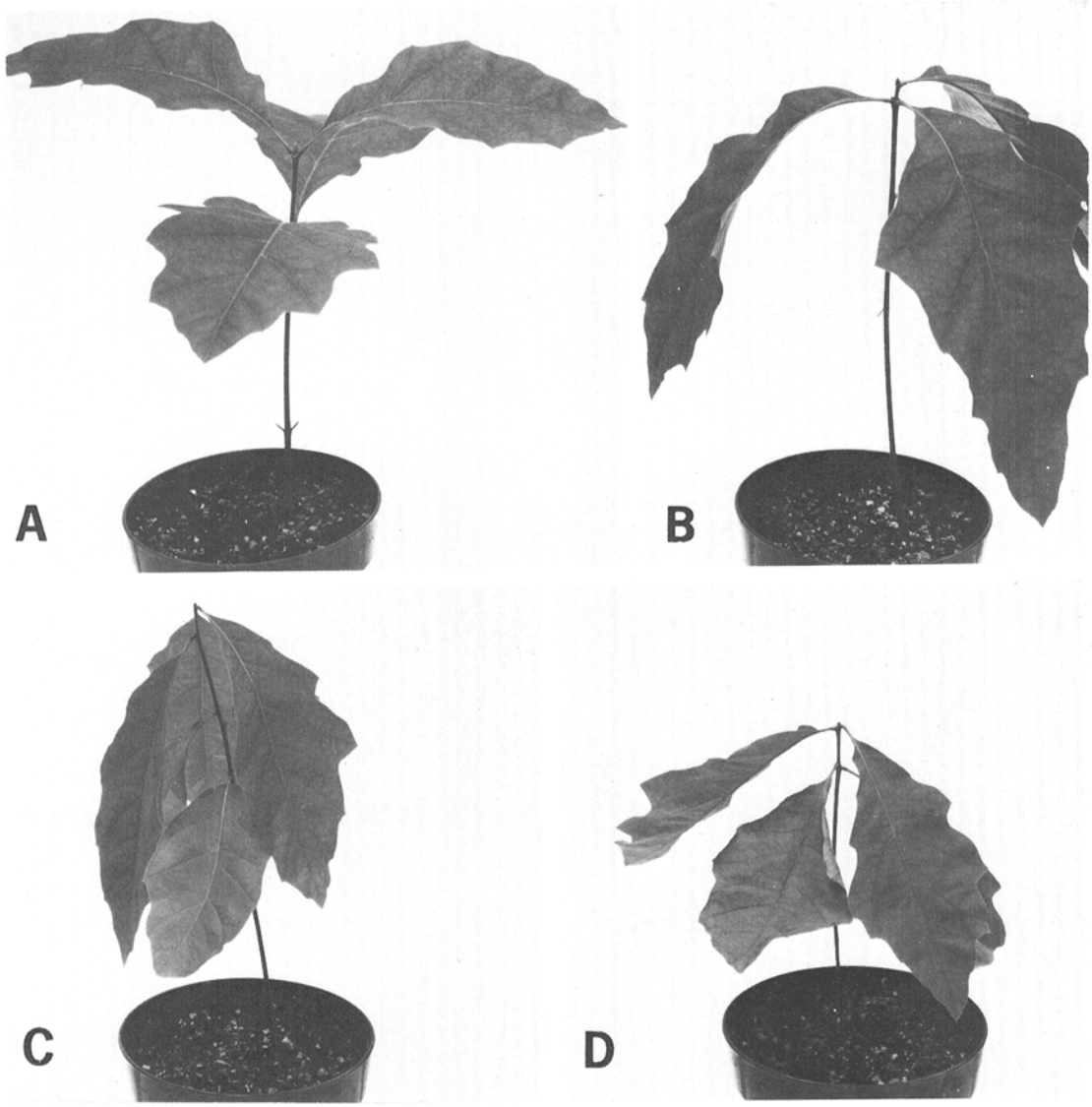

Fig. 1-(A to D). Symptoms of *Ceratocystis fagacearum* infection in red oak seedlings inoculated with 10^4 conidia each and incubated at 26 C, 5,350 lux and 50% relative humidity. **A)** Control, treated with sterile distilled water. **B)** Permanent drooping of leaves of an infected seedling 7 days after inoculation. **C)** Severe drooping of flaccid leaves 8 days after inoculation. **D)** Inward curling and drying of wilting leaves 8 days after inoculation.

and study under controlled conditions (5, 7).

This paper describes disease development in such seedlings and compares the results to the wilt syndrome reported in older trees. The objectives of this study were: (i) to determine whether disease development in young seedlings is a valid system for studying oak wilt, and (ii) to evaluate certain factors affecting disease development in seedlings so that conditions could be established for reproducible wilt development.

MATERIALS AND METHODS.—Seeds of *Quercus rubra* L. were collected in central and southern Wisconsin. Most collections represented pooled seed from two to six adjacent trees. Seeds were stratified in moist peat moss for 12-16 weeks at 2 C before planting. Seeds of other species were collected locally or obtained from appropriate sources and, if necessary, treated for breaking dormancy by standard methods (20).

Germinating red oak seeds were dusted with Captan 50% WP (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) and planted in 10-cm diameter plastic pots containing a 1:1 (v/v) mixture of vermiculite and peat moss. From the time of planting all seedlings were watered daily with Hoagland's solution to ensure that adequate levels of inorganic nutrients required for early growth and leaf development were present (6).

Growth studies (5) showed that seedlings grew well under a broad range of conditions; however, to ensure rapid and uniform growth most studies were conducted

under controlled environments. Seedlings were grown in a growth room (University of Wisconsin Biotron) under 5,350 lx of mixed cool-white fluorescent and incandescent lighting (0.017 cal/cm^2/minute) on a 15-hour photoperiod at 26 ± 0.5 C and 50 ± 5% relative humidity. Some experiments were conducted in a growth chamber, where conditions were altered to 6,030 lx, 26 ± 2 C, but no humidity control. Under these conditions seedlings with fully expanded leaves were obtained in 24-30 days. A few experiments were conducted in a greenhouse (21-30 C) under a 16-hour photoperiod maintained with incandescent lighting.

All inoculations were made with an isolate of *C. fagacearum* obtained in 1969 from a naturally infected red oak. Stock cultures were maintained on potato-dextrose agar (PDA) slants at 4 C. This highly virulent, "A" mating type isolate produced abundant conidia on all media tested and showed optimum growth on PDA at 24-28 C. to produce inoculum, a mycelial plug from a 10- to 15-day-old culture was placed into 50 ml of potato-dextrose broth in a 125-ml flask. Log-phase conidia were harvested after 5-7 days of incubation on a shaker at 27 C in the dark. The culture was strained through four layers of cheesecloth and the filtrate was centrifuged (1,000 *g*) for 10 minutes. The conidial pellet was resuspended in sterile distilled water, and the conidia washed twice by centrifugation. Conidial numbers were determined by means of hemacytometer counts at appropriate dilutions made with sterile distilled water.

An inoculation procedure adapted from that described by Bugbee and Presley (3) was found to be the most effective. On the lower part of the stem, a 0.01-ml drop containing a known number of conidia was placed with a syringe fitted with a 26 G (regular point) needle. While the drop was held by surface tension, the needle point was carefully forced through the drop and into the stem at a 45-degree angle. When the needle was moved from side to side or withdrawn, the drop was drawn into the stem tissues by xylem suction, thus giving direct evidence of successful inoculation. Controls were treated similarly with sterile distilled water.

For reisolation, tissues were surface sterilized in 0.64% NaOCl, washed in sterile water, and pieces were plated on acidified PDA.

Tissues for histological examination were fixed in a formalin, acetic acid, and ethanol mixture, embedded in paraffin, sectioned at 10 μm, stained with safranin-aniline blue according to the methods of Jensen (11).

RESULTS.—*Symptom development.*—In the controlled environments, inoculated seedlings began to develop wilt symptoms after incubation from 6-11 days. The first symptom was a permanent drooping of the leaves at the pulvinus (Fig. 1-B). At this stage the leaves appeared turgid and of normal color. However, within a few hours they drooped severely, and the leaf blades became flaccid (Fig. 1-C). The pulvinal tissues often blackened and wilting leaves rarely abscised. The leaves then rapidly died and curled inward from the margins, and the lamina lost their sheen and became light green or greenish-brown in colour (Fig. 1-D). Several days after leaf wilt, the stems became dry and necrotic. This sequence of symptoms was similar under the different environments, but the timing was closely related to the environmental conditions during incubation. In the controlled environmental regimes, symptom development, from initial drooping until drying of the leaves, occurred over a period varying from 18-30 hours. Under greenhouse conditions, the same sequence was prolonged at low temperatures and high humidity, whereas at high temperatures and low humidity leaves often curled and dried without showing any earlier symptoms.

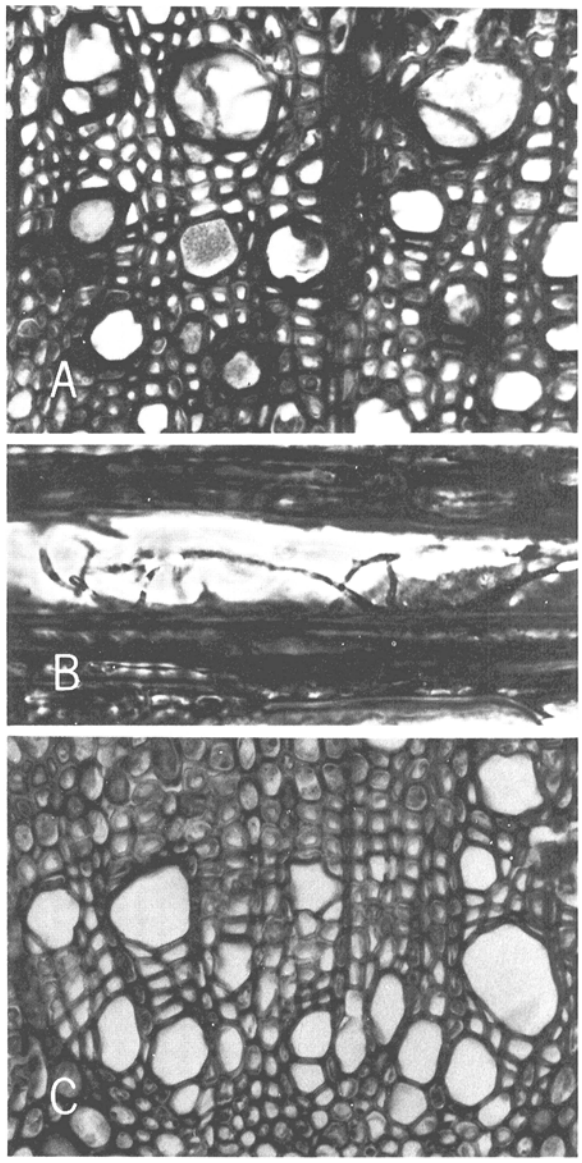

Fig. 2-(A to C). **A)** Cross section of stem tissues showing occlusion of vessels by tyloses and amorphous materials 7 days after inoculation (× 360). **B)** *C. fagacearum* mycelium in the vessels of a stem 8 days after inoculation (× 570). **C)** Cross section of stem tissues of a control seedling. Vessels are free of occluding materials (× 360).

Plugging of the vessels by tyloses and amorphous materials always was observed in stem and root sections from wilting plants (Fig. 2-A). Occasionally they were found as soon as 4 days after inoculation; i.e., at least 2

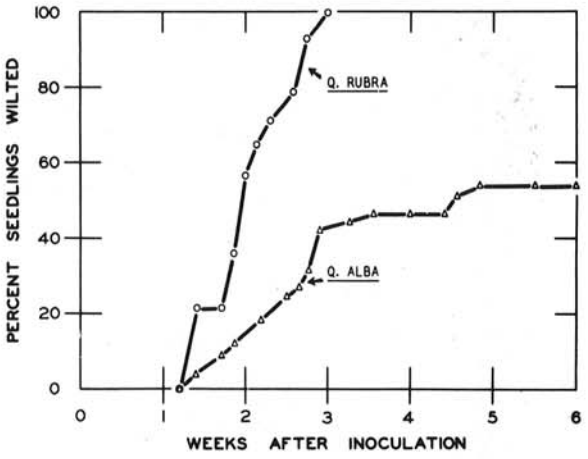

Fig. 3. Curves showing cumulative wilt development in red oak versus white oak seedlings under greenhouse conditions. Forty seedlings of each species were inoculated.

days prior to the first evidence of foliar symptoms. Mycelia were observed often in the vessels of wilting seedlings (Fig. 2-B).

In several experiments, stem tissue from more than 200 wilting seedlings was plated on acidified PDA. The pathogen was recovered from 94% of these seedlings; it also was recovered readily from the roots and petioles.

Water movement.—Resistance to water movement was tested by introducing a drop of 1.0% aqueous acid fuchsin into the xylem about 1 cm above the inoculation wound by the inoculation procedure described above. By recording the time required for the dye to appear as a red coloration in the leaf midveins, and the distance travelled, rates of movement were calculated and used as a basis for comparison. The dye had no overt toxic effect on the seedlings. Dye was administered to different groups of five control and 10 inoculated seedlings at 0, 2, 4, 7, and 10 days after inoculation. In the control seedlings at all sampling times and in the inoculated seedlings at 0, 2, 4 days after inoculation, the dye moved at an average rate of 32.4 cm/minute (± 17.8 cm/minute standard deviation). In contrast, in symptomless seedlings, 7 days after inoculation, the dye moved at 11.1 cm/minute (± 12.5 cm/minute SD) while in wilting seedlings the average rate was 1.0 cm/minute (± 0.2 cm/minute SD). After 10 days, dye moved with an average rate of 4.6 cm/minute (± 0.4 cm/minute SD) in symptomless seedlings and at 3.0 cm/minute (± 4.5 cm/minute SD) in wilting seedlings. These differences in dye movement indicated that resistance to water flow occurred in infected seedlings prior to, and during foliar wilt.

Specificity of Ceratocystis fagacearum.—Inoculation trials were conducted to determine whether *C. fagacearum* could incite a disease in young seedlings of other tree species. Fifteen to 40 greenhouse-grown individuals of each species and 5-10 red oaks which served as inoculation checks were inoculated with approximately 10^4 conidia each. Seedlings inoculated with water served as controls. After 6 weeks under greenhouse conditions favourable for disease development there were no symptoms on the following species, although the pathogen was recovered from stem tissues near the inoculation wound in some seedlings (3-78%) of each species: box elder, *Acer negundo* L. (38%); black maple, *A. nigrum* Michx. f. (53%); sugar maple, *A. saccharum* Marsh. (44%); white birch, *Betula papyrifera* Marsh. (60%); shagbark hickory, *Carya ovata* (Mill.) K. Koch (71%); hackberry, *Celtis occidentalis* L. (78%); American beech, *Fagus grandifolia* Ehrh. (40%); white ash, *Fraxinus americana* L. (16%); honey locust, *Gleditsia triaconthos* L. (50%); staghorn sumac, *Rhus typhina* L. (15%); and American elm, *Ulmus americana* L. (3%). Inoculated seedlings of apple, *Malus sylvestris* Mill. developed vascular browning and chlorosis of the

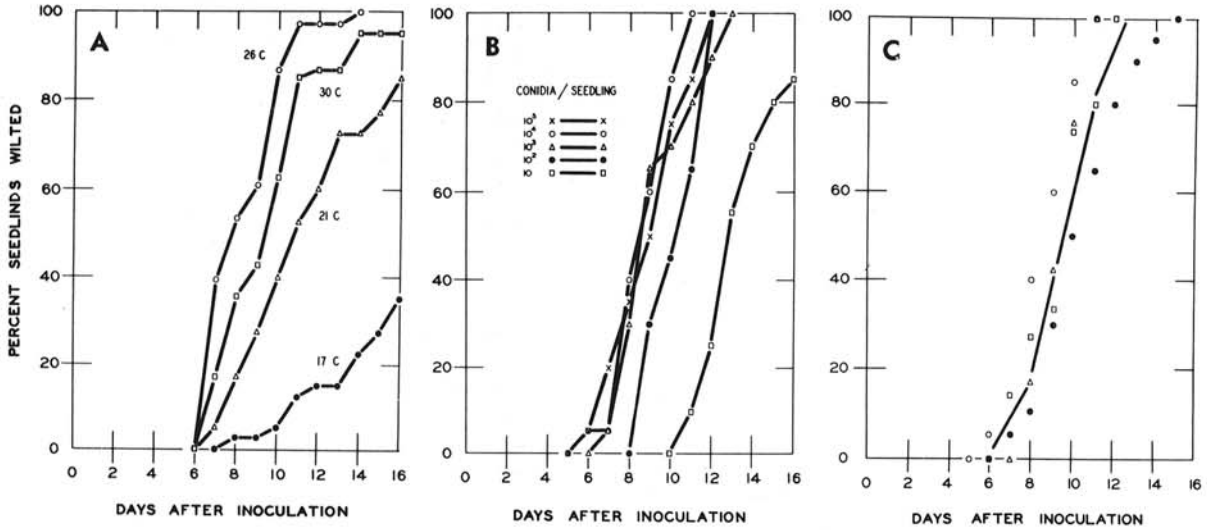

Fig. 4-(A to C). Curves showing the cumulative effects of: **A)** incubation temperature, and **B)** inoculum dosage on wilt development in groups of red oak seedlings at various days after inoculation. **C)** Reproducibility of wilt development in four groups of red oak seedlings. Each type of symbol represents wilt development in one group of 20 seedlings.

older leaves. The fungus was reisolated from 95% of these seedlings after 6 weeks. Wilt symptoms developed on all inoculated seedlings of the following members of the Fagaceae and infection was confirmed by recovery of the fungus: chestnut hybrid, *Castanea dentata* (Marsh.) Borkh. × *C. mollissima* Blume; European chestnut, *C. sativa* Mill.; coast live oak, *Quercus agrifolia* Neé; northern pin oak, *Q. ellipsoidalis* E. J. Hill; Oregon white oak, *Q. garryana* Dougl.; bur oak, *Q. macrocarpa* Michx.; water oak, *Q. nigra* L.; *Q. torjana* Webb; and black oak, *Q. velutina* Lam.

Although the sequence of symptoms in inoculated white oak (*Q. alba* L.) seedlings was similar to that in red oak, the former consistently developed foliar wilt more slowly. This difference in rate of symptom development between red and white oak seedlings was observed in two semi-controlled greenhouse experiments and to a lesser extent in the controlled environment chamber. In one greenhouse experiment (Fig. 3), all red oak seedlings had wilted in 3 weeks, whereas only 54% of the white oak seedlings had wilted after 6 weeks. The pathogen was recovered from 57% of the remaining symptomless white oaks. Contaminant microorganisms growing from the plated stem tissues prevented the determination of the presence of the pathogen in the remaining white oak seedlings.

Initial experiments in the greenhouse and growth chamber revealed considerable variation in the speed and uniformity of symptom development among different experiments. Observations of inoculated seedlings of mixed ages indicated that older seedlings (40 days old or older) tended to have longer incubation periods than younger seedlings (24-30 days). However, as much variation appeared within seedlings of the same age as among seedlings of different ages. Also, within an experiment, incubation periods varied from seedling to seedling, ranging from 6 to 11 days in the controlled environments. Experiments were designed to determine the effect of different factors on these variations, with the objective of selecting a standard set of conditions that would result in reproducible wilt development.

Temperature.—Forty red oak seedlings, inoculated with 10^4 conidia each, were placed at each of the following temperatures: 17, 21, 26, and 30 C (± 2C). Temperature treatments were maintained simultaneously in a temperature gradient room, where all seedlings received equal lighting of 5,350 lx for 15 hours. The results showed that the rate of symptom development was most rapid at 26 C (Fig. 4-A). The slopes of the cumulative curves also showed that there was less variation in the length of the incubation period when seedlings were incubated at 26 C than at the other temperatures tested.

Inoculum dosage.—Groups of 20 red oak seedlings each were inoculated with 10, 10^2, 10^3, 10^4, or 10^5 conidia per seedling and placed at 26 C and 5,350 lx. Increasing the dosage from 10 to 10^3 conidia decreased the average incubation period from 13 to 8.5 days (Fig. 4-B). Above 10^3 conidia per seedling, a "saturation" effect was observed in that the incubation period was not shortened by higher inoculum levels. Similar results were obtained in two other experiments. The slopes of the cumulative curves also showed that increasing the inoculum dosage did not eliminate the seedling-to-seedling variation in the incubation period.

Genotypic effects.—If genotype is important in determining the length of the incubation period, genetically identical red oak seedlings should show nearly identical incubation periods while open-pollinated progeny from single parent trees and genetically unrelated seedlings should show increased variation in their incubation periods, all other conditions being equal. To test this hypothesis, paired ramets (12), pairs of open-pollinated progeny from single parent trees, and pairs of genetically unrelated seedlings were inoculated with 2×10^3 conidia each and placed in the growth chamber at 26 C, 6,030 lx, with a 15-hour photoperiod. The length of the incubation period for each seedling in each pair was recorded and the difference between the two seedlings in each pair was calculated. In two tests where: (i) 25 paired ramets were compared to 25 pairs of progeny from single trees, and (ii) 25 paired ramets were compared to 25 genetically unrelated pairs, the within-pair differences ranged from 0-8 days, but there were no significant differences between the pairs in the different tests (*t*-test at $P = 0.05$) (17). The average incubation period for the genetically identical ramets was 10.6 days. For the progeny from single parent trees and genetically unrelated seedlings the average incubation periods were 10.1 and 10.5 days respectively.

To determine if there were major differences among incubation periods for red oak seedlings derived from different open-pollinated parent trees, seed from four individual trees were collected and four families established, each containing 30-70 seedlings. All seedlings were inoculated with 10^4 conidia each and incubated at 26 C, 5,350 lx with a 15-hour photoperiod. The length of the incubation period was recorded for each seedling and the mean for each family was calculated. The data for each family were found to fit statistically a normal distribution ($P = 0.05$). Incubation periods for the families averaged from 8.8 to 9.1 days; however, no significant differences were found among the means for the four families (analysis of variance $P = 0.05$) (17).

Reproducibility.—On four separate occasions, groups of twenty 28-day-old red oak seedlings were randomly selected, inoculated with 10^4 conidia each, and placed in the growth chamber. Symptom development was recorded. Under these conditions, satisfactory reproducibility was obtained among the experiments (Fig. 4-C).

DISCUSSION.—Oak wilt development in young red oak seedlings shares the following important similarities to its development in large woodland trees and old seedlings. Foliage symptoms are preceded by and associated with vessel occlusion and resistance to water movement (2); *C. fagacearum* is pathogenic on oaks and related genera, can cause a disease in apple trees, but is not a pathogen of other species (1); disease development in white oak is slower, often more limited, and less dramatic than in red oak (16, 19); the optimum temperature for symptom expression corresponds to that for pathogen growth (10); and incubation periods partially depend on inoculum dosage (14).

In addition, by their nature, young seedlings lack many of the problems; e.g., size, variability, seasonal susceptibility, and dormancy, shown by woodland oaks and older seedlings. Their small size and rapid growth make them adaptable for study under controlled

environments, where the effect(s) of an individual variable can be studied, and possible predisposing factors can be identified and controlled. Because young red oak seedlings have a determinant type of shoot growth; i.e., shoot elongation occurs in distinct flushes, seedlings in the same stage of development can be recognized easily and selected for uniformity with respect to age and size. Also, under standardized conditions of seedling age (28 days), inoculum dosage (10^4 conidia per seedling), and incubation temperature (26 C) we have found disease development to be readily reproducible. The technical advantages of seedlings together with the similarities between their disease development and that of woodland oaks indicate that young seedlings constitute a valid model system for detailed studies of oak wilt.

In addition to its advantages for basic investigations of disease mechanisms, a standardized seedling system may find a practical application as the first step in a screening program for identifying resistance to oak wilt (5, 7). Cech (4) has recently emphasized the need for such a seedling system as a major component of current oak improvement programs.

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