ILLINOIS

Natural History Survey

BULLETIN

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Root Infection of Woody Hosts with Verticillium albo-atrum

Gerald L. Born

VERTICILLIUM WILT is a plant disease caused by the fungus *Verticillium albo-atrum* Reinke and Berthold. This pathogen is peculiar in that it does not confine its attacks to one host, or a few closely related hosts, as is so frequently the case with most other pathogenic fungi; it attacks a large number of widely unrelated plants, many of which are of economic importance. The disease does not often occur in forest stands, but it is becoming increasingly prevalent in plantings of ornamental trees and shrubs, particularly in temperate regions of the world.

Symptoms of Verticillium wilt on woody hosts are variable and often difficult to recognize. Usually the first visual symptom is sudden wilting of foliage on one or several twigs on a branch. A yellowing of foliage sometimes precedes wilting. Most plants exhibit leaf symptoms in early July, but some trees may first show symptoms in early spring or late fall. Leaves on affected ash species may drop while still green and before noticeable yellowing or wilting has occurred.

Other symptoms suggesting Verticillium wilt are decline in current twig growth, stunting, and dieback of individual twigs and branches. Occasionally trees such as maple and tulip tree develop elongated dead areas of bark on the diseased branches or trunk. Water-soaked areas sometimes develop under the killed bark.

Trees that develop a limited amount of branch wilt during the summer may show additional wilt and dieback the following year, and others may recover and not wilt in succeeding years. Trees that have extensive wilt throughout the crown usually die before the end of the summer.

The present study initiated in 1970 and completed in 1972 deals with (1) the influence of root wounds and age of wounds on infection, (2) penetration and development of the fungus in susceptible and resistant woody hosts, (3) analysis of the growth response of young tree seedlings after root infection, (4) the influence of temperature and heat treating of soil on development of *V. albo-atrum* in excised roots, and (5) laboratory and greenhouse evaluation of fungicides against *V. albo-atrum*.

This report is adapted from a thesis submitted to the University of Illinois in partial fulfillment of requirements for the degree of Doctor of Philosophy in Plant Pathology.

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LITERATURE REVIEW

The first reference to a wilt disease was made by Reinke & Berthold (1879). They isolated a fungus from potato plants with the Krauselkrankheit disease which they named *Verticillium albo-atrum*. Their investigations were not appreciated until 30 years later when *Blattrollkrankheit* and *Krauselkrankheit* were causing severe losses in the potato fields of Europe.

Van Hook (1904) described a typical case of wilt in ginseng (*Panax quinquifolium* L.) which he attributed to *Acrostalagmus albus* Preuss. However, this name is synonymous with the earlier name *Verticillium* which was established by Nees von Esenbeck (1816). Corda (1838) did not describe the genus *Acrostalagmus* until 1838.

Klebahn (1913) isolated a *Verticillium* from dahlia plants which he considered distinct from *V. albo-atrum*, and he named this fungus *V. dahliae* Kleb. Since 1913 the relationship between *V. albo-atrum* and *V. dahliae* has been the subject of much controversy. Many investigators have disagreed in their interpretations of the drawings and descriptions found in previous reports. Wollenweber (1929), Rudolph (1931), Presley (1941), Wilhelm & Taylor (1965), and Van den Ende (1958) maintained that the fungi that produce sclerotia and resting mycelium are members of a large variable species. Others, e.g., Klebahn (1913), Van der Meer (1925), Isaac (1949), and Smith & Walker (1930), have preferred to treat them as separate species.

In 1957 Verticillium wilt was reported as affecting plants in at least 18 orders, 38 families, 98 genera, and 137 species in the temperate climates of the world (Caroselli 1957).

Numerous papers in the past have dealt with factors that influence the incidence of Verticillium wilt. Nutrition, soil type, soil moisture, soil and air temperature, and light have all been shown to have an effect on the incidence of this disease (Arndt 1957; Edgington & Walker 1957; Gallegly 1949; Gilman 1916; Ludbrook 1933; Wilhelm 1950).

Although many workers have mentioned wounds as a source of entry by pathogenic fungi into the root system, little work has been done on the problem. Selman & Buckley (1959) suggested that root injury facilitated fungus invasion and that deliberate injury with a scalpel was less harmful than normal transplanting. Their study made with transplanted seedlings, or with plants damaged by cutting, gave clear evidence that exposing injured roots to a conidial suspension of the fungus resulted in rapid systemic invasion of the host. Selman & Pegg (1957) failed to show any appreciable increase in infection as a result of deliberate root damage but, in these cases, the "undamaged" control plants had been transplanted. Under normal conditions of root growth in soil, where the inoculum potential of the fungus may be expected to be relatively low, it seems probable that entry into the xylem vessels occurs largely through wounds.

Armstrong & Armstrong (1958) found that a high incidence of infection occurred when roots of herbaceous hosts were cut prior to inoculation with *Fusarium* spp. Also, the average number of days for wilt to occur significantly decreased when roots were cut immediately prior to inoculation. Fulton (1952) showed that more infection occurred when the canes or roots of raspberry were injured prior to inoculation.

Little has been reported on the anatomy of woody hosts affected with Verticillium wilt. Extensive histological work has been done on herbaceous hosts attacked by this pathogen. Ru-

dolph (1931) reported that the fungus was found only in the xylem in the early wilt stage, and later invaded the pith, cambium, and cortex in the advanced wilt stages. McWhorter (1962), working with *Pelargonium* infected with *V. albo-atrum*, found only traces of mycelium in tissues that had considerable discoloration. He rarely found large amounts of mycelium in diseased tissue. Therefore, the amount of mycelia in the vessels is not always indicative of the severity of wilt.

Talboys (1958) observed that acute symptoms of hop wilt were associated with extensive development of mycelium in the xylem vessels but sparse production of tyloses; conversely, mild symptoms were associated with the development of limited mycelium but abundant tyloses in the vessels. Talboys (1964) suggested a simple explanation of the inverse correlation he had found between density of mycelium and frequency of tyloses in infected xylem vessels of the hop plant by postulating that a low concentration of fungal metabolites in the xylem stimulates the formation of tyloses but that a high concentration inhibits formation.

Until recently, spread of the fungus throughout the plant has received little attention. Sewell & Wilson (1964) concluded that *V. albo-atrum* conidia are transported in xylem sap of hops and occasionally they become lodged in vessels where they germinate and produce more conidia. In cotton and tomatoes, conidia may spread throughout the plant in 12 hours to 6 days following inoculation (Garber 1957; Green 1954).

Many earlier workers noted that the hyphae are very slender and reduced in diameter at the point where they pass through the cell walls, but once through they swell to a much greater size (Garber 1957; Garber & Houston 1966; Klebahn 1913; Reinke & Berthold 1879). In the vascular system, the fungus moves from one vessel element to another through pits (Garber 1957; Garber & Houston 1966; Green 1954). There is an apparent inability of the mycelium to penetrate new cellular growth lateral to the invaded cells as rapidly as the new cells are formed (Green 1954).

Klebahn (1913) and Rankin (1914) reported microsclerotia in the vessels of infected plants. Talboys (1958) observed that penetration of vascular tissue of hop by *V. albo-atrum* depended on the amount of suberin in the endodermal cell walls. Garber & Houston (1966) observed gum-like deposits in tolerant cotton plants which impeded the fungus from penetrating the vascular element. They reported that the splitting apart of cells was a mechanical process and not enyzmatic although they observed enzymatic action on the middle lamella of cell walls when the inoculum potential was high.

Symptom appearance is variable, requiring days to many weeks after infection for expression. Yellowing of foliage and sudden wilting are usually the first visual symptoms. General stunting accompanied by shortening of the internodes may accompany wilt. Young tomato plants infected with *V. albo-atrum* may show neither leaf yellowing nor wilting in the initial stages, but only a stunting of the whole plant (Selman & Pegg 1957).

Selman & Pegg (1957) found that 8 weeks after inoculation the dry weights of tomato leaves, stems, and roots were decreased by 72, 70, and 65 percent respectively. Of the growth characteristics studied, leaf area was most reduced by infection and this was due to a failure of the leaves to expand rather than to a reduction in leaf production.

After infection, symptom development, and necrosis, the fungus may overwinter within the plant as microsclerotia. Benken & Khakimov (1964) observed abundant microsclerotia of *V.*

albo-atrum in veins and petioles of overwintering cotton leaves. The fungus spread unchecked in the field within the necrotic tissues of infected cotton seedlings and sporulated freely over the surface of the stems for a short distance above ground level, eventually forming numerous microsclerotia in stems and roots. Nadakavukaren (1965) observed that *V. albo-atrum* microsclerotia survived best at low temperatures and high moisture levels. Heale & Isaac (1963) reported that resting mycelium remained viable for 9 months in pieces of necrotic lucerne buried 12 inches (30 cm) in soil. Brinkerhoff (1969) observed that microsclerotia were elongated in leaves incubated at 28 to 30 C and round in leaves incubated at 18 C. *V. albo-atrum* survived for relatively long periods in cotton tissue, and infested debris constituted a ready source of inoculum when incorporated into either sterile or nonsterile soil. Evans et al. (1966) suggested that further colonization by *V. albo-atrum* was arrested when cotton plants were plowed under prior to microsclerotial formation in the tissues.

Many recent papers have shown the value of systemic fungicides for the control of vascular wilts. Most of the work has been done with Benlate (benomyl) and thiabendazole (TBZ). Schreiber et al. (1971) found that benomyl was taken up equally well when either applied as a drench or incorporated directly into the potting media. The planting medium affected the concentration as well as the rate of accumulation of benomyl. Highest levels of accumulation of the fungitoxicant were in seedlings grown in media that had the lowest content of organic matter and the highest pH. Heat sterilization of soil prior to benomyl treatment resulted in greater accumulation of benomyl in elm seedlings than when the plants were grown in nonsterile soil.

Erwin et al. (1971) found that the addition of thiabendazole to soil reduced the incidence and severity of cotton wilt in plants subsequently inoculated with *V. albo-atrum*. Rawlins & Booth (1968) reported that the addition of surfactant Tween 20 increased the effectiveness of benomyl and thiabendazole against *V. albo-atrum*, probably by increased absorption of the fungicide by the roots. Erwin et al. (1968) found that thiabendazole not only translocates from the roots to the stems of cotton plants but also can be detected in the bark. They concluded that thiabendazole diffused laterally from the xylem to the bark.

Soil treatment, or seedling root dips with difolatan (Bankuti 1964) gave good control of *Fusarium oxysporum* f. sp. *lycopersici* and *V. albo-atrum* on tomatoes in greenhouse and field tests. Complete protection against *V. albo-atrum* was provided for seedlings planted up to 140 days in soil treated with difolatan.

Applying systemic fungicides to the foliage and allowing the chemical to be translocated downward may be the method used in the future. However, this method presents many problems. Many fungicides, such as benomyl, are extremely insoluble in water. Hock (personal communication) has been able to solubilize benomyl using inorganic acids, heat, and constant stirring. Buchenauer & Erwin (1971) found that benomyl and thiabendazole induced curative effects when sprayed on inoculated cotton plants that showed initial symptoms of Verticillium wilt. Both fungicides were detected by bioassay and chemical analysis in xylem tissue and in nontreated stems and leaves above the place of application.

CODE OF VERTICILLIUM ALBO-ATRUM ISOLATES

MATERIALS AND METHODS

All isolates used throughout this study were obtained from actively wilt-

ing hosts in Illinois. They were maintained on freshly prepared potato dextrose agar (PDA) tube slants and transferred periodically. An isolate used to inoculate a particular species was obtained earlier from another of the same species. Resistant species were inoculated with a mixture of all isolates. Below are the code numbers used in this study to identify each isolate. Also, the host, date of isolation, and location of host plant in Illinois are given for each isolate.

Code	Host	Date	Place
1	Sugar maple	1969	Urbana
2	Russian olive	1956	Wheaton
3	Redbud	1960	Urbana
4	Green ash	1961	Decatur

RELATIONSHIP OF ROOT WOUNDS & AGE OF WOUNDS ON INFECTION

MATERIALS AND METHODS

Two hundred twenty each of 2-year-old bare-rooted sugar maples (*Acer saccharum* Marsh.) and redbud (*Cercis canadensis* L.) seedlings were selected as test plants. The plants were breaking dormancy when received from a commercial nursery. The average height was 45 to 60 cm. The roots of each plant were washed with tap water and rinsed with distilled water prior to planting. The plants were potted in a medium-grade perlite and fertilized biweekly with a balanced liquid fertilizer.

Isolate 1 was used to inoculate sugar maple and Isolate 2 was used to inoculate redbud.

Type of Wound

Two weeks after potting, 100 plants were removed from the perlite and treated. Treatments immediately preceding inoculation included: (1) no wound, (2) abrasion, (3) puncture, and (4) vascular incision. Wounds were made on the primary root approximately 5 cm below the ground line. With the abrasion-type wound, the root surface was injured by rubbing moist 400 grade carborundum against the root surface. Puncture wounds were made by forcing a balsam wood block, in which five pins were embedded, against the root. This produced pin prick wounds 3 mm deep into the root. The vascular incisions were made by cutting a V-shaped wedge approximately 0.5 cm deep into the root. When no wound was made, a mycelial disc was placed against the root surface.

All treated plants were inoculated with a mycelial disc and the wound area covered with vinyl grafting tape to prevent moisture loss. The control plants were treated identically except that a sterile agar disc was placed on the wounded area and covered with grafting tape. Twenty plants of each species were used for each treatment.

After 30 days, all plants were removed from the pots and isolations were attempted from the plant roots and stems.

Age of Wound

In an additional experiment 120 plants of each species were tested to determine the importance of wound age on infection. Two weeks after potting, the plants were gently removed from the potting medium and V-shaped wounds were made on each plant approximately 5.0 cm below the soil line on all plants. Fifteen plants were inoculated with a mycelial disc immediately after wounding and the wounds were covered with vinyl grafting tape. All other wounds were wrapped with vinyl grafting tape and the wounded plants replaced in perlite. At intervals of 1, 2, 4, 8, 16, and 32 days, 15 plants were removed from the potting mixture, inoculated at the wound site, rewrapped with grafting tape, and planted back in perlite. Thirty days after each inoculation date, the plants were removed from the pots and isolations were made from the roots and stems of each plant.

RESULTS

Type of Wound

No infection occurred on unwounded roots. Root wounds were a prerequisite for fungus entry into the plant (Table 1). Any disruption of the periderm on the older roots which allowed the fungus to by-pass these tissues was suitable to fungal entry. The percentages of infection for abrasive, puncture, and vascular wounds were 75, 80, and 85 respectively on redbud, and 50, 55, and 80 on sugar maple. The most efficient wound on both hosts was a vascular wound which placed the pathogen in direct contact with the vessel members.

Age of Wound

Root wounds remained as infection courts up to 32 days on redbud and 16 days on sugar maple seedlings (Table 2). As the age of the wound increased the number of plants infected through wounds decreased. Only 13 percent of the redbud plants became infected when inoculated at wound sites that were 32 days old and no infection occurred through wound sites 32 days old on sugar maple.

Thirty two-day-old wounds had several layers of dead cells which were occluded with heavily pigmented materials. This condition was a barrier against penetration by the fungus. Many vessel members adjacent to wounds were occluded with tyloses and wound reaction materials. Callus was beginning to form at the margins of the wound after 32 days.

Wounds were not made on other areas of the root. Therefore, location of the wounds may have some significance because wounds made on older secondary tissue may require a longer time for initiation of repair tissue. Younger tissue, i.e., at the root tip or lateral roots, may heal faster and reduce the time a wound remains as an infection court.

DISCUSSION AND CONCLUSIONS

The periderm consists of the phelloderm, phellem, and phellogen which completely surrounds the vascular cylinder of woody plant roots. The cells of the phelloderm are parenchyma and remain alive and active. The cells of the phellem become suberized, which renders them virtually waterproof, and at maturity they die, forming a rather impervious, protective layer around the outside of the root.

The fungus gains entrance through

Table 1.—The effect of root wounds on number of redbud and sugar maple plants infected with **Verticillium albo-atrum.**

Type of Wound[a]	*Number of Plants Infected*		
	Roots Only	*Roots and Stems*	*Total*
Redbud — 2 years old			
No wounds	0	0	0
Abrasion	10	5	15
Puncture	9	7	16
Vascular incision	12	5	17
Noninoculated (controls)	0	0	0
Sugar maple — 2 years old			
No wounds	0	0	0
Abrasion	6	4	10
Puncture	8	3	11
Vascular incision	11	5	16
Noninoculated (controls)	0	0	0

[a] Twenty plants were used per treatment.

Table 2.—The effect of age of root wounds prior to inoculation with **Verticillium albo-atrum.**

Age of Wound at Inoculation[a]	*Number of Plants Infected*
Redbud — 2 years old	
Immediate	10
1 day	8
2 days	7
4 days	4
8 days	3
16 days	2
32 days	2
Noninoculated (controls)	0
Sugar maple — 2 years old	
Immediate	11
1 day	10
2 days	6
4 days	3
8 days	3
16 days	1
32 days	0
Noninoculated (controls)	0

[a] Fifteen plants were used for each treatment.

root wounds into the vascular system while by-passing the periderm. Any injury acts as an infection court but a wound deep into the stele places the fungus in direct contact with the vessel, thus the infection court is more conducive for penetration by the fungus. Moisture and temperature optima may interact with age of wounds for maximum infection.

The older the wound the less chance for infection by the fungus. This may be correlated with growth responses by the plant at the wound site. Following wounding, a layer of dried cells forms on the pruned surface. These cells die as the result of injury by the knife. Adjacent to the dead cells is a zone which becomes infiltrated with wound substances. The trachieds remain intact and ultimately become occluded with wound substances. Tyloses develop in vessel members adjacent to the wound site. This growth response results in a barrier that prevents the fungus from invading the functional vessel members. The sequence of wound healing may take place much faster on young root tissue. Vigor of the host plant will affect the time in which root wounds heal over.

Good cultural practices should be followed when planting susceptible hosts in soil that may be infested with *V. albo-atrum*. When digging plant material, care should be taken to keep wounds to a minimum. Digging a ball larger than normal may decrease the chances of severing large roots. Root pruning should be avoided. After planting, the application of fertilizer and water will decrease transplanting shock and increase plant vigor. If the vigor of the plant can be increased, root wounds will heal more quickly and this will decrease the chances of infection.

PENETRATION AND DEVELOPMENT OF V. ALBO-ATRUM IN ROOTS OF WOODY HOSTS

MATERIALS AND METHODS

Redbud and green ash [*Fraxinus pennsylvanica* Marsh. var. *subintegerrima* (Vahl.) Fern.] are hereafter designated as susceptible, and honey locust (*Gleditsia triacanthos* L.) and sycamore (*Platanus occidentalis* L.) are hereafter designated as resistant. The susceptible species were selected from a list of susceptible hosts of *Verticillium albo-atrum* as reported by Himelick (1969). The resistant hosts were so designated from unpublished work of E. B. Himelick (personal communication). Isolates 3 and 4 were used to inoculate redbud and green ash respectively. A mixture of Isolates 1, 2, 3, and 4 was used to inoculate honey locust and sycamore. The soil used in the greenhouse was a 1:1:1 ratio by volume of loam soil, peat, and river sand, steamed for 4 hours at 100 C.

To obtain seedlings, seeds were collected in early fall and cold-stratified in sand for 90 days at 5 C. The stratified seeds were immersed for 2 minutes in a 10 percent sodium hypochlorite

solution and germinated in perlite under glass. Twenty seedlings of each species in the 2-leaf stage were inoculated by dipping the roots into an approximate 1 x 10^4/ml conidial density of *V. albo-atrum,* by placing 3-mm blocks of PDA containing the fungus on selected areas, and by placing a *Verticillium*-infested oat seed adjacent to a selected area. After inoculation, the seedlings were placed horizontally in 150-mm petri dishes containing sterile peat moss or planted in sterile soil in pots in the greenhouse.

Selected seedlings were sectioned for microscopic examination at intervals after inoculation. The seedlings were removed from the petri dishes or soil and the portions to be sectioned were killed and fixed in FAA, dehydrated in tertiary butyl alcohol, embedded in paraffin, and sectioned at 12 to 15μ using the technique described by Johansen (1940). The sections were stained with thionin in phenol and counterstained with orange G. in absolute alcohol (Stoughton 1930), then examined under the microscope.

RESULTS

Fungus Growth on Root Surface

The four genera of hosts used were essentially alike morphologically and no differences were detected in the way the fungus penetrated them (Table 3).

The fungus colonized the exterior surface of the epidermis (Fig. 1). The fungal growth was appressed over the entire epidermal surface with conidiophores arising at right angles from the surface. Tissue around the area of penetration became necrotic. Brown

Table 3.—Root colonization of susceptible redbud and green ash, and of resistant honey locust and sycamore seedlings, with **Verticillium albo-atrum.**

Days Exposure to Inoculum	*Host*	*Intensity of Colonization[a] in Susceptible and Resistant Plants in Specified Regions of Penetration*					
		Root Tip	*Epidermis*	*Outer Cortex*	*Inner Cortex*	*Xylem*	*Phloem[b]*
1	Redbud	3	3	0	0	0	0
	Green ash	3	3	0	0	0	0
	Honey locust	3	2	0	0	0	0
	Sycamore	3	2	0	0	0	0
2	Redbud	3	3	2	1	0	0
	Green ash	3	3	2	1	0	0
	Honey locust	3	3	2	1	0	0
	Sycamore	3	3	2	1	0	0
4	Redbud	3	3	3	2	1	0
	Green ash	3	3	3	2	1	0
	Honey locust	3	3	3	2	0	0
	Sycamore	3	3	3	2	0	0
6	Redbud	3	3	3	3	2	0
	Green ash	3	3	3	3	2	0
	Honey locust	3	3	3	3	1	0
	Sycamore	3	3	3	3	1	0
8	Redbud	3	3	3	3	3	0
	Green ash	3	3	3	3	3	0
	Honey locust	3	3	3	3	1	0
	Sycamore	3	3	3	3	1	0

[a] Symbols shown represent infection intensity as follows: 0 = no colonization; 1 = slight colonization; 2 = moderate colonization; and 3 = severe colonization.

[b] Passage of the fungus through the phloem into the vessel members occurred but no phloem colonization occurred.

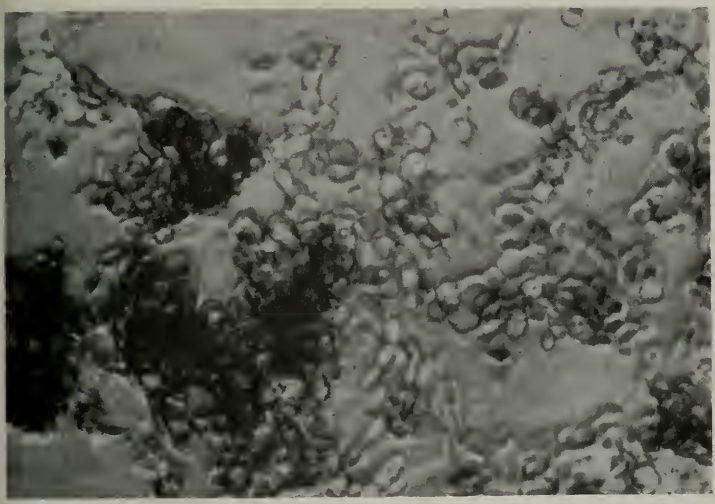


Fig. 1.—Verticillium microsclerotia completely colonizing the exterior surface of a green ash root (X 250).

necrotic flecks could be seen extending above but not below the point of infection.

Root Tip Penetration

The fungus penetrated the root cap within 48 hours. The hyphae penetrated both intercellularly and intracellularly, but intracellular penetration was most common. There was no tendency for the cells to separate, which might have occurred if a weakening of the middle lamella took place, unless an extremely high inoculum potential occurred on the root surface.

In the region of root elongation and maturation, the fungus penetrated through the epidermis. Penetration was either direct through the cell wall or between the epidermal cells. The hyphae or germ tubes produced appressorium-like swellings over the epidermis within 48 hours. A penetration peg developed from the appressorium and was smaller in diameter than the parent hypha.

Penetration in Root Hair Region

In the epidermal area between the root hairs, the fungus penetrated at random, both inter- and intracellularly. Germ tubes developed over the root hairs but none was seen penetrating the root hairs. The base of the root hair frequently was penetrated but no further growth occurred.

Penetration in Area of Lateral Root Formation

Another avenue for fungus penetration into roots is the area of lateral root formation. Rupture of the primary root tissue did not occur until the lateral root primordia were well developed. The fungus penetrated the torn areas where the lateral root emerged. Mycelia could be seen in the cortical layers of the lateral root but none was observed in the xylem. At this point in the process of invasion no differences were detected between the susceptible and resistant hosts.

Cortical Invasion — Susceptible Hosts

Most mycelial growth in the cortex was intracellular. Mass penetration resulted from a high inoculum potential at the invading point, and the mycelial development was centripetal (Fig. 2). Many hyphae at the point of penetration formed appressorium-like swellings against the cortical cell wall and penetrated to the next cell layer (Fig. 3a). Other hyphae that penetrated the cortical cells were constricted in diameter at the point of penetration (Fig. 3b).

When the invasion of the inner cortical layers was limited to a few hyphae, no marked centripetal alignment of hyphal strands occurred. Hyphal strands sometimes deviated from the centripetal development and developed tangentially and intercellularly for several cell layers and then penetrated directly through the wall.

Cortical Invasion — Resistant Hosts

Most mycelial growth in the cortex was intracellular. The mycelium was hyaline but became heavily pigmented after 3 days. After 8 days, most hyphae were dark brown, regularly septate, and swollen between the septa so as to appear torulose. These hyphal strands gave rise to microsclerotia by repeated budding (Fig. 4a). Microsclerotia varied in shape, from elongate to irregularly spherical, and varied in size, from 15 to 75μ in diameter. These microsclerotia continued to enlarge, which caused cortical cells to be separated or expanded many times their normal size (Fig. 4b).

Penetration of Vascular Region of Susceptible Hosts

If the fungus penetrated the cortical cells of the susceptible hosts, it in-

Fig. 2.—Longitudinal section of redbud cortex showing mass penetration of cortical cells resulting from a high inoculum potential at the invasion point (X 400).

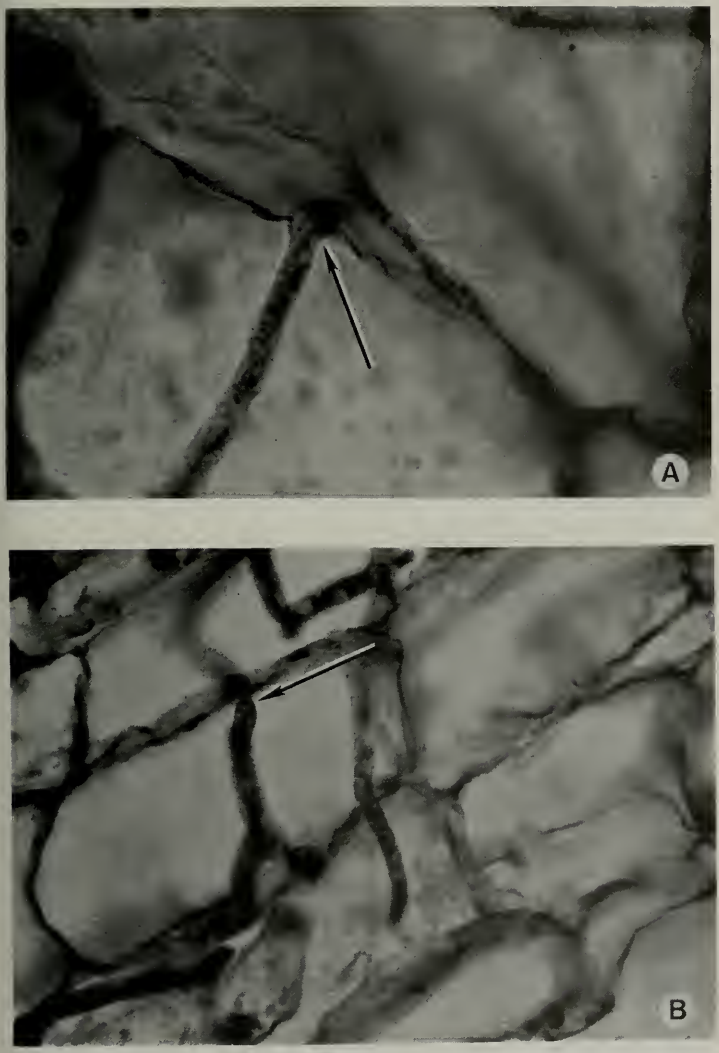


Fig. 3.—Cortical cells in longitudinal section. A) Appressorium-like swellings against the cortical cell wall (X 2500). B) Hyphal constriction in diameter at the point of penetration through a cortical cell wall (X 2000).

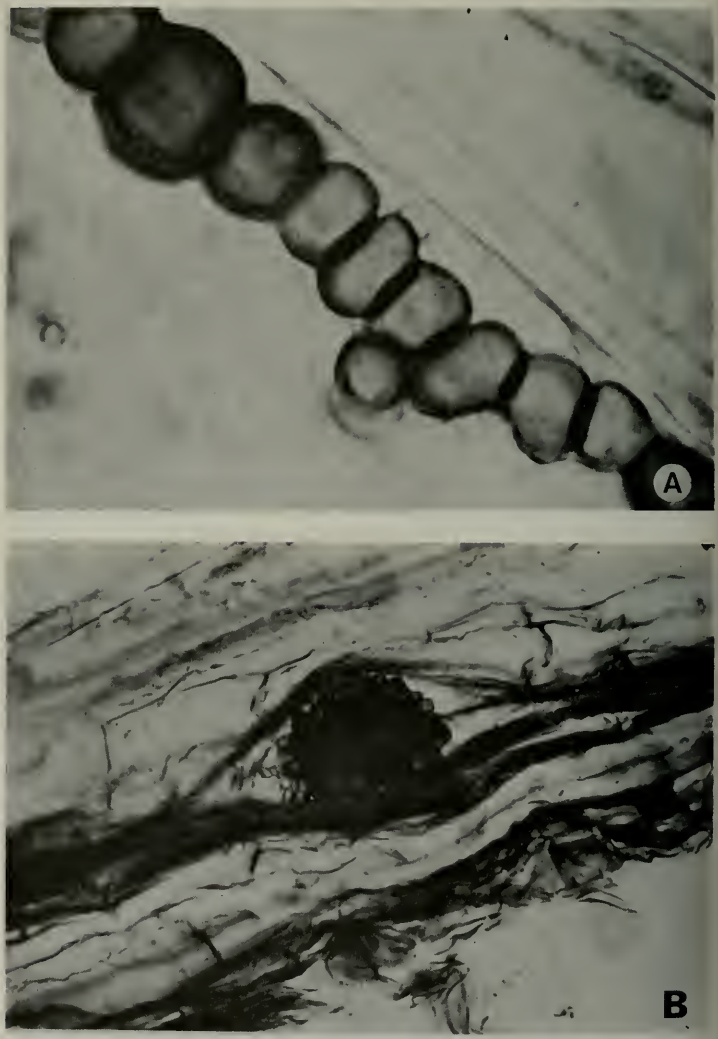


Fig. 4.—Cortical cells of honey locust in longitudinal section. A) Dark brown, septate, budding hypha (X 2200). B) Microsclerotia causing cortical cells to be separated or expanded many times their normal size (X 250).

variably penetrated the endodermis and vessel members. The fungus grew to the endodermal layer within 4 days. The quantity of vessel members invaded appeared related to the number of points of entry and to the mass of mycelia that developed from the points of entry.

The hyphae that penetrated the endodermis usually penetrated the vessel members through pits. The hypha narrowed to a thin, peg-like projection as it grew through the pit. Hyphae did not necessarily stop at the first vessel member contacted, but in many instances they grew out through a pit on the wall of the vessel into an adjacent vessel on the side opposite the entry point (Fig. 5). The mycelium was generally unbranched, hyaline, 3.5μ in width. No typical conidiophores were observed.

Verticillium conidia were observed in the xylem 8 days after inoculation. In most cases, the conidia appeared to be free-floating in the xylem stream and in no way connected with the mycelium present (Fig. 6 and 7). The conidia often were found lodged at the end walls of the vessel members (Fig. 8). No defense mechanism such as tyloses or gum deposits was observed in the xylem members. The lack of a defense mechanism on susceptible hosts is in complete disagreement with other workers' data on hops and cotton (Table 4).

Penetration of Vascular Region of Resistant Hosts

Although the fungus penetrated the cortical cells, few hyphae penetrated the endodermis and vessel members. The quantity of vessel members in-

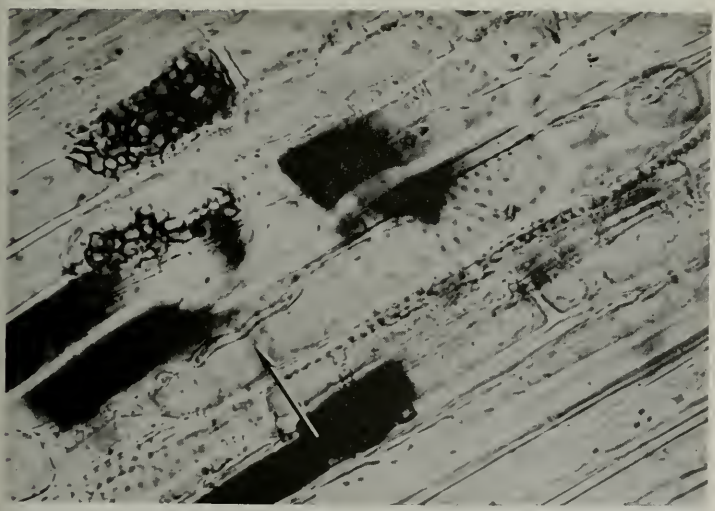


Fig. 5.—Longitudinal section through the vascular cylinder of redbud showing a hypha within a vessel member (X 850).

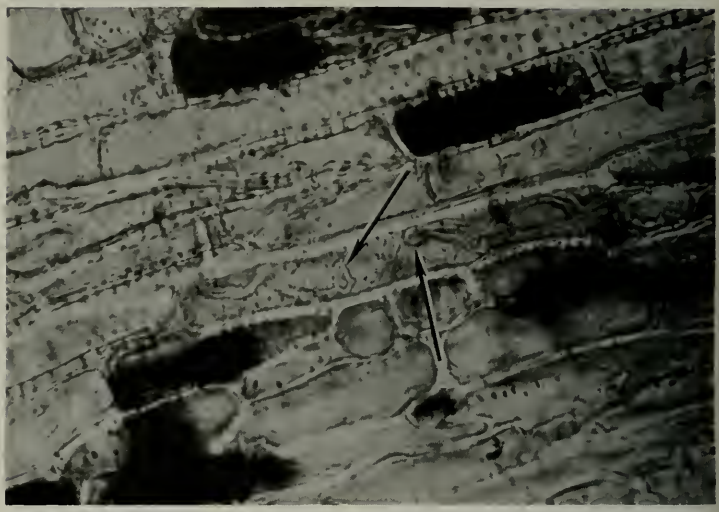


Fig. 6.—Free-floating conidia of **V. albo-atrum** in a vessel member of green ash (X 500).

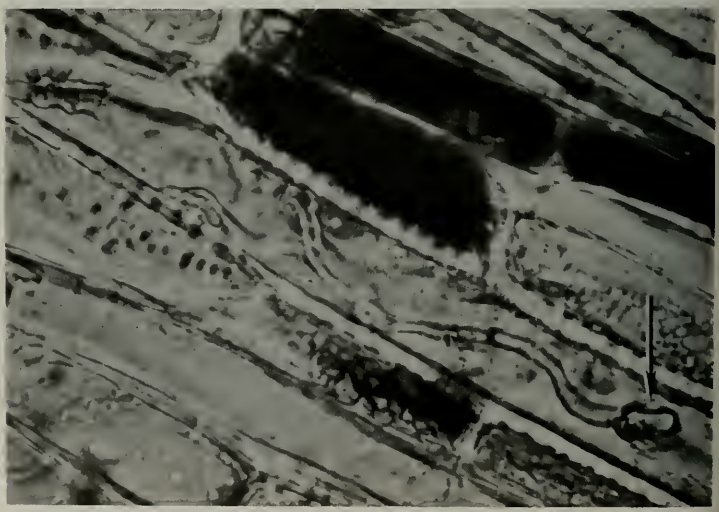


Fig. 7.—Longitudinal section through a vessel member of a redbud root showing a **V. albo-atrum** condidium germinating (X 1700).

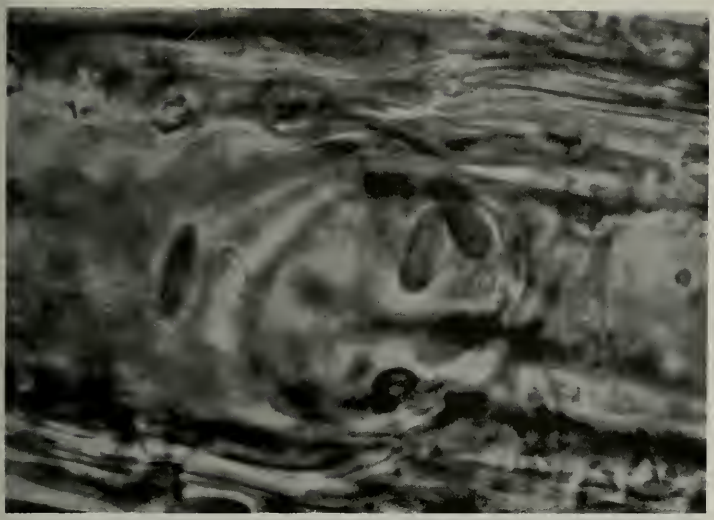


Fig. 8.—Longitudinal section showing lodged conidia at the end walls of the vessel member of a redbud root (X 2300).

Table 4.—A comparison of penetration and development of **Verticillium albo-atrum** in roots of herbaceous and woody hosts.

	Intensity of Colonization in Susceptible (S) and Resistant (R) Plants[a]							
	Hops[b]		*Cotton*[b]		*Woody Ornamentals*[c]			
	S	*R*	*S*	*R*	*S*	*S*	*R*	*R*
Region of Infection and Signs of Disease	*Fruggle*	*OR/55*	*Daltapine 15*	*Acata 4-42*	*Redbud*	*Green Ash*	*Honey Locust*	*Sycamore*
Epidermis	3	3	3	3	3	3	3	3
Root hairs	2	2	2	2	0	0	0	0
Lateral roots	0	0	0	0	1	1	1	1
Cortical colonization	3	3	3	3	3	3	3	3
Endodermis	3	3	3	3	3	3	1	1
Phloem colonization	0	0	0	0	0	0	0	0
Xylem colonization	3	2	3	2	3	3	1	1
Conidia (xylem)	3	1	3	2	3	3	0	0
Microsclerotia (cortex)	0	0	0	0	0	0	3	3
Mechanical plugging (xylem)	1	2	2	2	0	0	1	1

[a] Symbols shown represent infection intensity as follows: 0 = no colonization; 1 = slight colonization; 2 = moderate colonization; and 3 = massive colonization.

[b] Data on hops from Talboys (1958); data on cotton from Garber & Houston (1966).

[c] Redbud and green ash are susceptible; honey locust and sycamore are resistant.

vaded did not appear related to the number of points of entry or to the mass of mycelia that developed from the points of entry.

The hyphae that penetrated the endodermis and vessel members did so through pits in the same manner as in the susceptible hosts. Few hyphae

were observed in the vessel members. The mycelium was hyaline, unbranched, and 2.7μ in diameter. The mycelia did not ramify throughout the vessel members as they did in the susceptible hosts. No conidia could be seen in the xylem members although mycelium was present.

Frequently, microsclerotia developed in the vessel members, completely plugging the vessel members (Fig. 9a). They arose from single hyphae by repeated budding of heavily pigmented, thick-walled cells. The microsclerotial cells often grew through pit pairs and moved into adjacent vessel members where repeated budding took place (Fig. 9b and 9c). Germinated microsclerotial cells were also observed that grew through pit pairs into adjacent vessel members.

The ray parenchyma was heavily colonized with microsclerotia. Germ tubes from microsclerotia grew from one parenchyma cell to another through pit pairs or plasmodesmata (Fig. 9d). This may be an avenue for lateral growth of the fungus outward from the central vascular cylinder.

DISCUSSION AND CONCLUSIONS

Conidia of *V. albo-atrum* germinated on the surface of both the susceptible and resistant roots and grew in random directions. Some germ tubes grew away from the host; others penetrated the epidermis. Although germ tube penetration occurred, most epidermal penetration was by either hyphae or germinated microsclerotia. Intercellular and intracellular penetration occurred within 48 hours after inoculation. Nelson (1950) found that *V. albo-atrum* penetrated peppermint roots 6 hours after inoculation. Reid (1958) reported intercellular penetration but observed no intracellular penetration of melon roots by *F. bulbigenum* Cook and Massee.

According to Anderson & Walker (1935), *F. conglutinans* Wollenw. penetrated the cell walls of cabbage plants by mechanical pressure. Talboys (1958) found that the splitting apart of hop cells by *V. albo-atrum* was a mechanical rather than an enzymatic process. My evidence through visual observation did not suggest that an enzyme was involved in either epidermal penetration or cortical invasion unless the cells were invaded by a mass of hyphae. This is in agreement with Garber & Houston (1966) on *Verticillium* invasion of cotton. Direct penetration was either by constriction of a hypha as it passed through the wall or by a peg-like projection of an appressorium-like swelling. Garber & Houston (1966) noted similar structures in cotton cells invaded by *Verticillium*.

I did not observe penetration of root hairs although it has been reported by Smith & Walker (1930) for *Fusarium* invasion of cabbage roots and by Garber & Houston (1966) for *Verticillium* invasion of cotton roots.

The areas of lateral root emergence were not important as infection courts. The fungus penetrated the lateral root and ramified throughout the cortical tissue, but no mycelia were found invading the vascular tissues. Many uninjured roots were invaded to the same cortical layers. Smith & Walker (1930) reported similar observations; however, Reid (1958) suggested that penetration of emerging lateral roots might provide a mechanism for a vascular pathogen to avoid the penetration barrier of the endodermis.

The progress of infection in the susceptible green ash and redbud and the resistant honey locust and sycamore was identical after the point of cortical colonization. The species were alike in morphology and were penetrated by the fungus in a comparable fashion. Differences in fungus growth were noted immediately as the fungus progressed beyond the initial cortical colonization.

In the susceptible species, mycelia

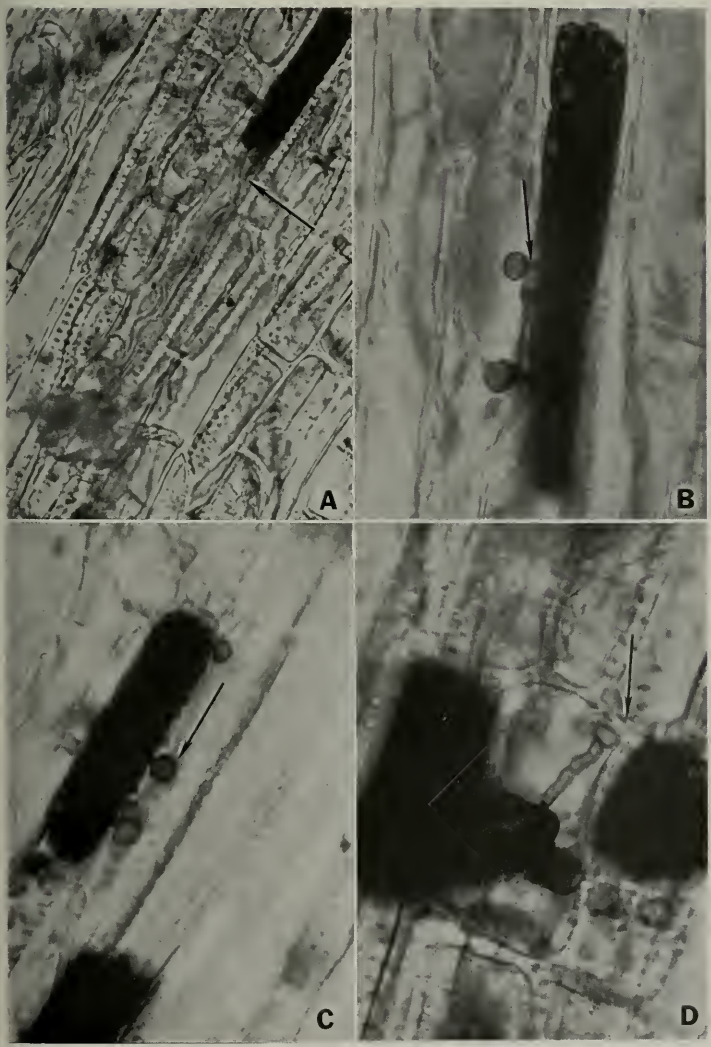


Fig. 9.—Longitudinal section through the vascular cylinder of a sycamore root. A) Germinating microsclerotium of **V. albo-atrum** which has completely plugged a vessel member (X 550). B, C) Budding cells growing through pit pairs into adjacent vessel members (X 1000). D) Ray parenchyma heavily colonized with microsclerotia and microsclerotium germinating (X 1000).

ramified throughout the tissues and reached the endodermis and xylem elements within 4 days. Conidia were found in the vessels of roots 8 days after inoculation. Lack of mycelial connections between fungus parts present in the xylem and conidia at secondary sites higher in the root system can be explained by conidial movement. It is reasonable to assume that free-floating conidia moved to secondary infection sites and provided for rapid fungus dispersal throughout the plant. In some susceptible cotton plants Schnathorst et al. (1967) found that 30,000 conidia/ml of tracheal fluid were present 96 hours after inoculation.

In the resistant species, microsclerotia were produced in abundance in the cortex. These structures enlarged by repeated budding and ruptured the walls of the cortical cells. Few hyphae penetrated the endodermis and reached the xylem members. Few hyphae were found in the xylem members and conidia were not observed. Schnathorst et al. (1967) found that tolerant varieties of cotton depressed conidial numbers more than 20 fold.

Talboys (1964) postulated that the xylem defense-response is much the same in different species and cultivars of plants. Since it is a generalized response to physical damage and infection, the difference in host resistance to vascular infection is constituted by a difference in response of the extravascular tissue at the early stage of infection. This I found to be only partially true. The endodermis prohibited mycelial penetration to some extent in the resistant hosts. However, a xylem-defense response took place after penetration of the vessel members. Few hyphae were found in the vessel members after penetration and no conidial production occurred. Beckman et al. (1962) inoculated bananas with *Fusarium* by means of a standard dose of microconidia introduced into the xylem elements and found a highly significant difference between the xylem-defense response of the resistant Lacatan and susceptible Gros Michel bananas. Therefore, Talboys' postulate should be expanded to include the infection sequence in the vascular system in trees.

EFFECT OF ROOT INFECTION ON GROWTH RESPONSE OF REDBUD & GREEN ASH SEEDLINGS

MATERIALS AND METHODS

Redbud and green ash seeds were collected and germinated as previously described. After 3 weeks, 80 seedlings of each species were removed from the germination beds and the roots dipped in inoculum for 5 minutes. After root-dipping, 5 plants were planted in each of 32 No. 10 potting cans.

Isolates 3 and 4 were used to inoculate redbud and green ash respectively. Each isolate was grown on PDA for 14 days at 24 C. The fungus and agar were macerated with water in a Waring blendor to produce a thick suspension of inoculum. An equal number of control plants were root-dipped in a PDA blended suspension which did not contain the fungus and potted as described above.

The plants were inoculated on March 29. The first samples of healthy and infected plants were taken on April 12 and at 2-week intervals thereafter until July 19. Ten plants per treatment were sampled on eight occasions making a total of 160 redbud and 160 green ash plants. The following data were obtained from each treatment: stem height, leaf area, total number of leaves produced, fresh and dry weights, water content of leaves, and nitrogen content of stems, leaves, and roots.

Dry weights were obtained by drying the plant parts in an electric oven at 80 C for 72 hours. Leaf areas were determined by weighing a specific

known leaf area as compared to the weight of the whole leaf.

Micro-Kjeldahl determinations for toal nitrogen were made on bulk samples of leaves, stems, and roots from healthy and infected plants.

All data for stem height, leaf area, and dry weight were analyzed statistically using a one-way analysis of variance and student "T" tests.

RESULTS

Symptoms

Fourteen days after inoculation, young inoculated plants were retarded in growth but no wilt symptoms were apparent. Two weeks later the plants were stunted and the leaves had failed to expand.

Sectioned roots and stems showed extensive invasion of the vessel members by the fungus. The hyphae were confined to the primary xylem vessel members 16 weeks after inoculation.

Dry Weight

Infection markedly reduced dry-matter production on both redbud and green ash seedlings. The mean values for the dry weight of whole plants for controls and infected plants are shown in Table 5 and Fig. 10. All weight data for leaves, stems, and roots were analyzed statistically and the mean values for the dry weights on all sampling periods after inoculation are given in Tables 6 and 7 and Fig. 11 and 12. When comparing healthy and infected plants, a significant difference in dry weight was evident for leaves and stems of redbud and leaves of green ash 14 days after inoculation. A significant difference in dry weight of roots of both hosts occurred 28 days after inoculation. On July 19, 112 days after inoculation, the percentage differences for healthy and infected plants were 45, 53, and 47 for leaves, stems, and roots of redbud, and 36, 17, and 24 for leaves, stems, and roots of green ash, respectively.

Leaf Number

The mean values for the number of leaves for healthy and infected redbud and green ash plants are given in Table 8 and Fig. 13. The infected plants showed limited leaf production 28 days after inoculation, and thereafter the rate of leaf production differed little in the two groups.

Stem Height

The mean values for stem height of healthy and infected redbud and green ash plants are given in Table 9 and Fig. 14. A significant difference in stem height of redbud and green ash was not evident until 42 days and 28 days after inoculation, respectively. The initial reduction in growth due to infection

Table 5.—The dry weight of redbud and green ash seedlings infected with **Verticillium albo-atrum.**

Days After Inoculation	*Mean Dry Weight (g per plant)[a] (10 Plants)*			
	Redbud		*Green Ash*	
	Noninoculated	*Inoculated*	*Noninoculated*	*Inoculated*
14	.23	.16*	.22	.16*
28	.75	.22**	1.20	.44**
42	.77	.30**	1.49	.50**
56	2.29	.51**	2.87	1.11**
70	3.36	2.20**	3.89	2.13**
84	4.62	2.96**	5.25	3.04**
98	7.12	3.56**	7.23	4.09**
112	8.21	4.29**	10.54	7.20**

[a] An asterisk denotes a significant difference (0.05) between noninoculated and inoculated means, and two asterisks denotes a highly significant difference (0.01).

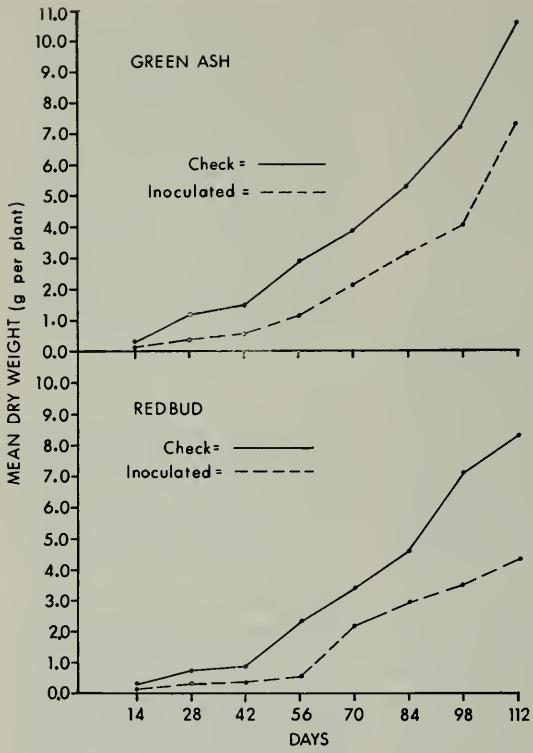
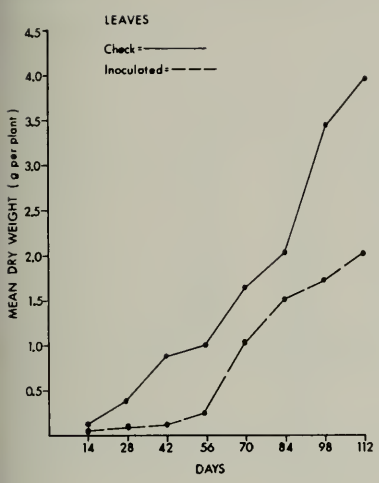


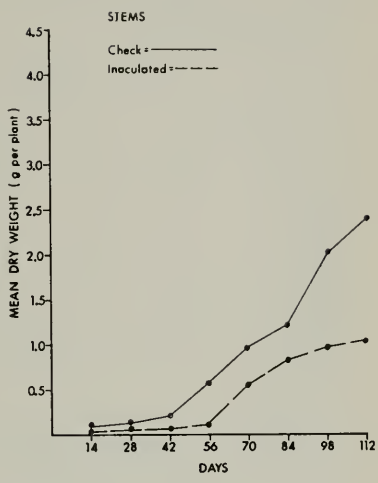
Fig. 10. — The dry weights of green ash and redbud seedlings after inoculation with **V. albo-atrum.**

Table 6.—Dry weights of leaves, stems, and roots of redbud seedlings infected with **Verticillium albo-atrum.**

	Mean Dry Weight (g per plant)[a] *(10 Plants)*					
	Leaves		*Stems*		*Roots*	
Days After Inoculation	*Noninoculated*	*Inoculated*	*Noninoculated*	*Inoculated*	*Noninoculated*	*Inoculated*
14	.11	.06*	.04	.01**	.07	.05
28	.36	.10**	.14	.07**	.11	.07*
42	.82	.13**	.22	.05**	.16	.08**
56	1.02	.25**	.67	.15**	.58	.11**
70	1.61	1.07**	.97	.64**	.82	.49**
84	2.17	1.53**	1.23	.73**	1.16	.69**
98	3.45	1.70**	1.99	.95**	1.68	.90**
112	3.97	2.18**	2.36	1.10**	1.91	1.01**

[a] An asterisk denotes a significant difference (0.05) between noninoculated and inoculated means, and two asterisks denotes a highly significant difference (0.01).





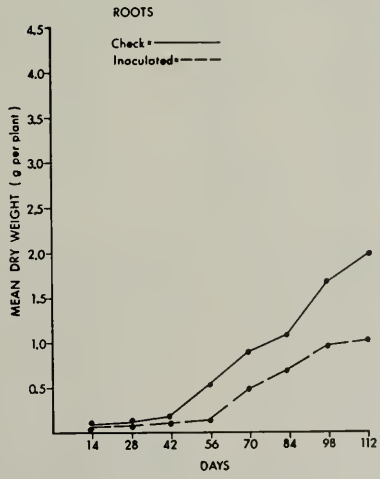


Fig. 11.—The dry weights of leaves, stems, and roots of redbud seedlings after inoculation with **V. albo-atrum.**

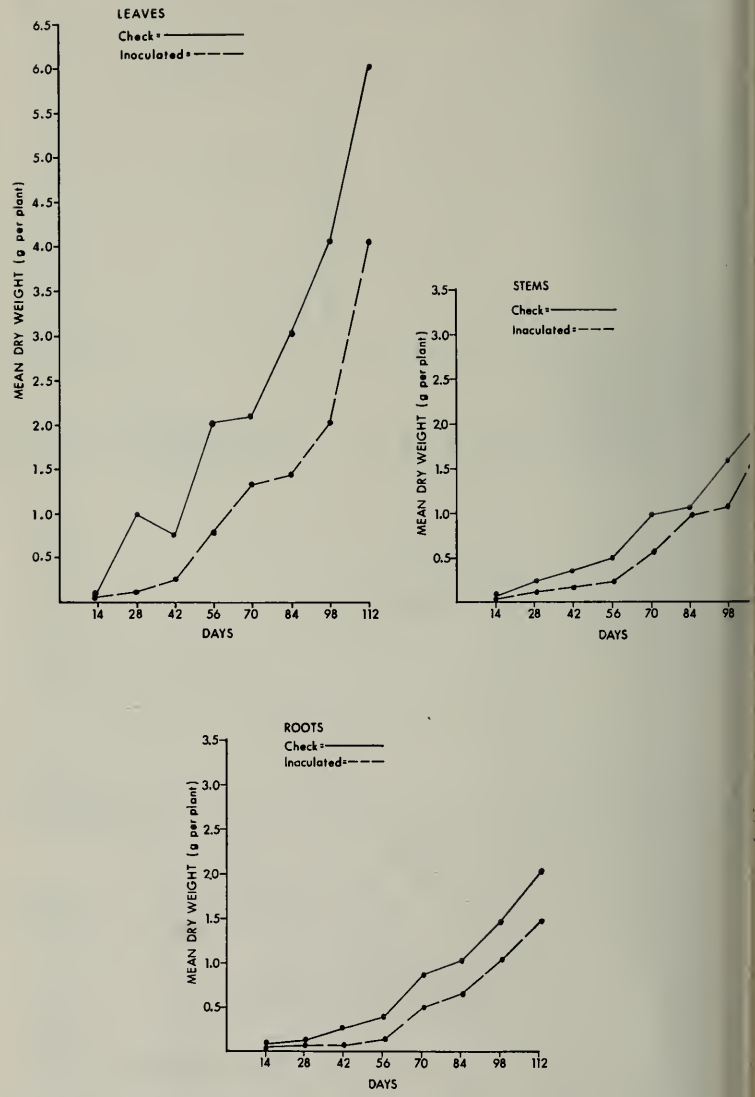


Fig. 12.—The dry weights of leaves, stems, and roots of green ash seedlings after inoculation with **V. albo-atrum.**

Table 7.—Dry weights of leaves, stems, and roots of green ash seedlings infected with **Verticillium albo-atrum.**

	Mean Dry Weight (g per plant)[a] *(10 Plants)*					
	Leaves		*Stems*		*Roots*	
Days After Inoculation	*Noninoculated*	*Inoculated*	*Noninoculated*	*Inoculated*	*Noninoculated*	*Inoculated*
14	.11	.06*	.03	.02	.04	.03
28	1.04	.16**	.21	.11**	.15	.07*
42	.74	.25**	.35	.12**	.25	.07**
56	2.08	.77**	.49	.20**	.35	.14**
70	2.13	1.30**	.90	.56**	.80	.47**
84	3.08	1.43**	1.18	.92**	1.00	.69**
98	4.02	2.05**	1.67	1.08**	1.45	1.04*
112	6.26	4.01**	2.11	1.76**	1.91	1.45**

[a] An asterisk denotes a significant difference (0.05) between noninoculated and inoculated means, and two asterisks denotes a highly significant difference (0.01).

Table 8.—Influence of root infection of redbud and green ash seedlings on total number of leaves produced per plant.

	Mean Number of Leaves Per Plant (10 Plants)			
	Redbud		*Green Ash*	
Days After Inoculation	*Noninoculated*	*Inoculated*	*Noninoculated*	*Inoculated*
0	3.20	3.50	7.56	7.68
14	4.80	4.02	10.00	8.50
28	7.60	5.37	14.35	10.20
42	8.60	6.20	16.27	12.73
56	10.08	7.48	18.75	14.88
70	10.90	8.65	20.95	16.90
84	12.20	10.06	22.67	19.06
98	13.10	11.30	23.80	20.80
112	14.00	12.20	24.00	22.80

was slight, but further growth of the inoculated plants was reduced. The difference in stem height between healthy and infected plants 112 days after inoculation was 37.5 percent for redbud and 30 percent for green ash.

Nitrogen Content

The nitrogen content percentages of redbud and green ash leaves, stems, and roots of healthy and infected plants are given in Table 10. There was 26 percent less N in infected redbud stems and 31 percent less in infected green ash stems when compared with the controls 112 days after inoculation. The N content in the leaves and roots was higher in the infected plants than in the healthy controls.

Water Content of Leaves

From the fresh-weight and dry-weight data, the percentage water content of leaves was determined. The leaf data for healthy and infected plants are given in Table 11.

There was no definite pattern of water content between infected and healthy redbud or green ash seedlings. Frequently (but not consistently) the water content of the infected seedlings was above that of the healthy controls. Wilt symptoms did not occur at any time during the experiment. No cor-

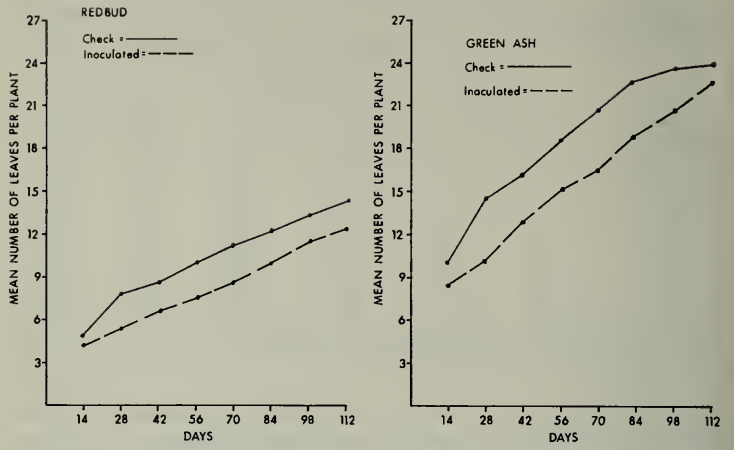


Fig. 13.—Influence of root infection of redbud and green ash seedlings on total number of leaves produced per plant.

Table 9.—Influence of root infection on stem height of redbud and green ash seedlings.

Days After Inoculation	Mean Stem Height (cm per plant)[a] (10 Plants)			
	Redbud		Green Ash	
	Noninoculated	Inoculated	Noninoculated	Inoculated
0	6.20	6.01	6.31	5.96
14	6.90	6.35	7.58	6.80
28	8.37	7.30	13.45	9.04**
42	9.25	7.51**	15.16	11.58**
56	9.75	7.64**	19.86	13.46**
70	12.00	8.02**	22.53	15.20**
84	13.50	8.70**	25.67	16.83**
98	15.00	9.15**	28.25	18.75**
112	16.00	10.00**	32.00	22.40**

[a] An asterisk denotes a significant difference (0.05) between noninoculated and inoculated means, and two asterisks denotes a highly significant difference (0.01).

relation could be made on water content between healthy and infected seedlings due to sampling time or greenhouse watering maintenance

Leaf Area

The mean values for leaf area of redbud and green ash are given in Table 12. A significant difference in leaf area of healthy and infected redbud and green ash was found 14 days after inoculation. A highly significant difference occurred on both hosts after 28 days. Although leaf area was less in infected plants, deformity of the leaves was not observed.

DISCUSSION AND CONCLUSIONS

The presence of *V. albo-atrum* might be expected to affect the metabolism of the host in any or all of the following ways: a) obstruction to water absorp-

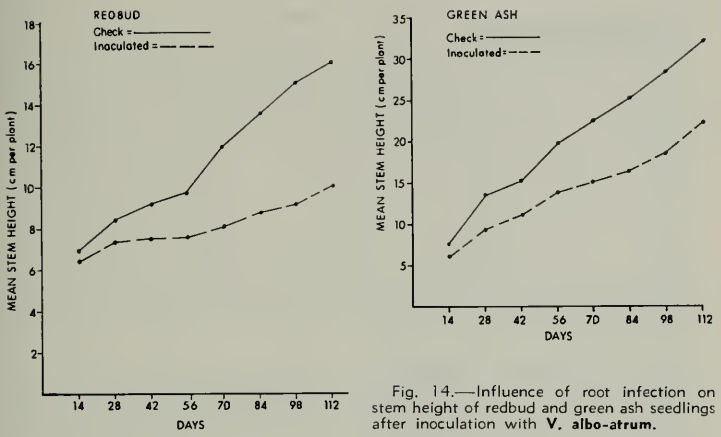


Fig. 14.—Influence of root infection on stem height of redbud and green ash seedlings after inoculation with **V. albo-atrum.**

Table 10.—Influence of root infection on total nitrogen content of leaves, stems, and roots of redbud and green ash seedlings.

Days After Inoculation	*Total Nitrogen (percent dry weight)*			
	Redbud		*Green Ash*	
	Noninoculated	*Inoculated*	*Noninoculated*	*Inoculated*
Leaves				
14	2.61	3.26	4.00	3.50
56	2.00	3.63	5.45	4.98
112	2.87	3.41	3.03	4.11
Stems				
14	1.71	1.23	4.50	4.10
56	2.17	1.36	4.34	3.56
112	1.47	1.09	4.04	2.75
Roots				
14	2.13	1.86	3.02	3.31
56	1.64	2.00	2.36	2.50
112	1.50	1.69	2.10	3.95

tion and movement; b) obstruction to the uptake and translocation of mineral nutrients; and c) production of toxic substances. The data have been examined in the light of these hypotheses.

Infection leads to a drastic reduction in dry-matter production of all parts of the plant. The greatest effect of infection was a reduction in stem height and leaf area. Total leaf area decreased significantly in the inoculated plant when compared with the control. The number of leaves per plant exhibited only a slight initial reduction and thereafter was little affected.

Nitrogen is one of the most important major nutrients affecting leaf expansion, but there was no evidence of a reduction in the uptake of nitrogen. Frequently, the nitrogen content was higher in the infected plants than in the control plants. The results are surprising since the root system is the first part of the plant to be affected by the

Table 11.—The water content of redbud and green ash leaves in response to root infection with **Verticillium albo-atrum.**

Days After Inoculation	Percentage Water Content of Leaves[a]			
	Redbud		Green Ash	
	Noninoculated	Inoculated	Noninoculated	Inoculated
14	70	71	78	75
28	60	73	74	84
42	59	63	78	75
56	52	63	55	62
70	53	50	67	58
84	52	48	60	71
98	52	50	62	70
112	51	54	55	52

[a] Percentage water content was calculated from the difference between the dry weight and the fresh weight.

Table 12.—Influence of root infection on leaf area of redbud and green ash seedlings.

Days After Inoculation	Mean Value of Leaf Area Per Plant (cm^2)[a]			
	Redbud		Green Ash	
	Noninoculated	Inoculated	Noninoculated	Inoculated
14	32.62	22.65*	29.84	21.32*
28	72.70	36.21**	63.81	31.37**
42	99.04	40.41**	108.43	65.72**
56	107.54	54.75**	141.01	83.20**
70	138.18	109.64*	186.39	103.21**
84	145.23	129.88*	207.46	136.81**
98	190.31	147.14*	265.78	183.21**
112	203.08	157.86*	298.76	201.21**

[a] An asterisk denotes a significant difference (0.05) between noninoculated and inoculated means, and two asterisks denotes a highly significant difference (0.01).

fungus, and thus mineral absorption might be impaired. No other mineral nutrients were determined but it would seem unlikely that infection would interfere with their uptake or translocation.

A low water supply might be expected to account for the general stunting which occurred. However, the water content in this experiment was approximately the same for the infected plants and controls, and there were no symptoms of general wilt.

The results of the growth analysis may be interpreted in terms of a toxin theory. The reduction in growth may be initiated by toxins entering the stems and leaves at concentrations below the level that would cause wilt or death. This could affect cell extension or reduce photosynthesis. Therefore, at the meristems the toxins may interfere with stem elongation and thus reduce internode growth. The fact that general wilting was never observed would indicate a greater tolerance of toxin by young plants.

EFFECT OF TEMPERATURE & HEAT TREATING ON DEVELOPMENT OF V. ALBO-ATRUM IN ROOTS

MATERIALS AND METHODS

Redbud and green ash seeds were collected and germinated as previously described. At the 2-leaf stage, plants were removed from the germination bed and root-dipped in inoculum for 5 minutes.

Isolates 3 and 4 were used to inoculate redbud and green ash respectively. Each isolate was grown on PDA for 14 days at 24 C. The fungus mycelia and agar were macerated with water in a Waring blendor to produce a thick suspension of inoculum. The control plants were root-dipped in a PDA solution without the fungus.

After the roots were dipped, the plants were potted in perlite and allowed to grow for 14 days. The plants were then removed from the perlite and the roots were excised at the ground line.

To study the effects of temperature on microsclerotial development, the excised roots were incubated at continuous temperatures ranging from 5 to 35 C at 5-degree intervals. The roots were wrapped in moist paper towels and then placed in capped bottles to maintain a moist atmosphere.

Cultures of the fungus on PDA were grown at the same range of temperatures. Observations were made on the production of microsclerotia.

The influence of the soil microflora on microsclerotial formation was determined by incubating whole roots in sterile and nonsterile soil in capped bottles. Two soil-moisture levels were used. One level approximated field capacity; the other approximated one-half field capacity. The temperature was maintained at 25 C for the 28-day test.

Both tests had four root systems per treatment replicated three times. Observations were made at 7-day intervals for 35 days. For microscopic observation, roots were cut into small pieces, sectioned on a freezing microtome, and stained in cotton blue.

RESULTS

Effect of Temperature

Abundant microsclerotia were observed in roots after 14 days incubation at 15, 20, 25, and 30 C. Microsclerotia did not develop at 35 C and were not observed in roots incubated at 5 and 10 C until after 35 days. The microsclerotia tend to develop as compact balls of dark-walled cells (Fig. 15). At the lower temperatures, individual microsclerotia tended to be elongated, and some were reduced to single strands of rounded, dark-walled cells.

Although the fungus failed to form microsclerotia on PDA at 35 C, a limited amount of mycelial growth occurred. After 14 days' growth, abundant microsclerotia were produced (Fig. 16) at 15, 20, 25, and 30 C. Fewer microsclerotia developed at 30 and 10 C. Little growth occurred on PDA after 14 days at 5 C, but measurable hyphal growth occurred after 35 days. Thus, microsclerotial development on PDA closely paralleled development in moistened roots at similar temperatures.

Effect of Heat Treating of Soil

Microsclerotia developed in dead roots incubated in both steamed and nonsteamed soil. Moisture levels near the field capacity of the soil were more favorable for microsclerotial development. Relatively few microsclerotia developed in nonsterile soil at the low moisture level. Although microsclerotia developed uniformly and more abundantly in steamed soil, appreciable numbers of microsclerotia were found in nonsteamed soil.

DISCUSSION AND CONCLUSIONS

The microsclerotia of *V. albo-atrum* develop rapidly at 15 to 30 C in excised green ash and redbud roots after being incubated at high moisture levels. Microsclerotia were produced at 5 C, but a longer incubation period was required. Temperature requirements for microsclerotial development on PDA and in dead host tissue were similar.

The development of microsclerotia at low temperatures is important in inoculum increases in overwintering de-

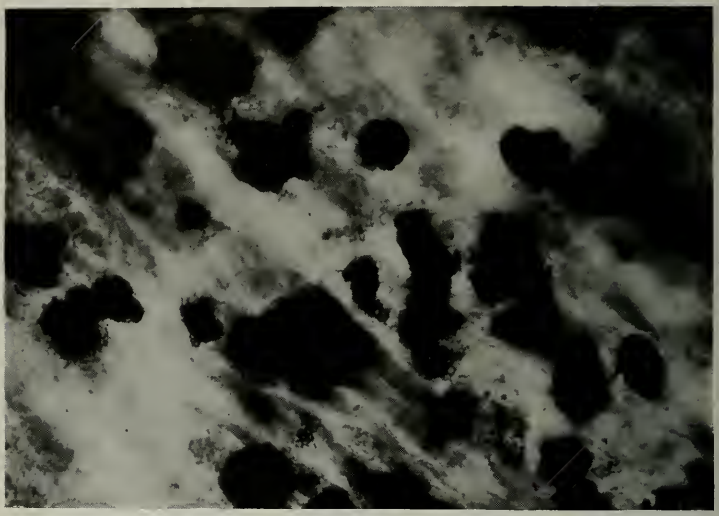


Fig. 15.—**V. albo-atrum** microsclerotia consisting of compact balls of dark-walled cells on dead root tissue (X 250).

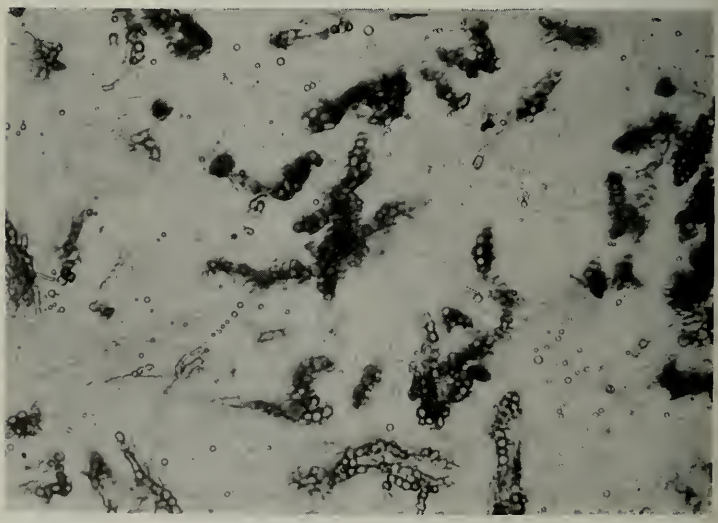


Fig. 16.—**V. albo-atrum** microsclerotial development on PDA (X 250).

bris. Evans et al. (1966) found large numbers of microsclerotia in overwintering cotton stalks where fall and winter weather temperatures were relatively low and there was sufficient moisture. The range of temperatures at which microsclerotia form permits the fungus to compete favorably with organisms that decompose root debris. Born (1971) found that heat-treating the soil increases symptom development because of a decrease in competition with other fungi.

The present study indicates that with high temperature and low soil moisture prior to microsclerotial development, the inoculum level was significantly reduced.

EVALUATION OF SYSTEMIC FUNGICIDES AGAINST V. ALBO-ATRUM

MATERIALS AND METHODS

V. albo-atrum Isolates 1 and 2 were used throughout this study. Inocula for laboratory studies were prepared by growing the fungus for 14 days at 24 C in petri dishes containing PDA.

Greenhouse experiments were initiated in March and ran through June. The day and nighttime temperatures were approximately 25 and 16 C, respectively. The soil consisted of a mixture of equal parts by volume of loam, peat, and river sand, steamed at 100 C for 4 hours. Soil pH varied from 6.5 to 7.2.

Inocula for infesting soil were produced by growing the fungus for 14 days at 24 C in petri dishes containing PDA. The fungus mats, containing both microsclerotia and conidia, were fragmented in tap water in a Waring blendor for 2 minutes. The fungus was added to the soil at the rate of one culture mat in 100 ml of water/20,000 g of soil. The soil was stirred after adding the inoculum to distribute the fungus uniformly throughout the soil. To determine the inoculum potential in the soil, the soil mixture was air-dried and screened to break up large particles. One-g samples were diluted with sterile water to 10^6 g ml, and 1 ml aliquots plated out on PDA + streptomycin. This measured an inoculum potential of 250,000 propagules/g of dry soil.

When plants were inoculated directly, a V-shaped wound was made with a scalpel on the primary root approximately 5 cm below the soil line. A 5-mm mycelial disc was inserted under each flap, pressed in place, and wrapped with vinyl grafting tape to prevent drying of the inoculum and wound area.

The fungicides subjected to laboratory and greenhouse evaluation were: Benlate 50 percent WP [Methyl-1-(butylcarbamoyl) 2-benzimidazolecarbamate]=benomyl; Thiabendazole 60 percent WP [2-4-(thiazolyl) benzimidazole]=TBZ; Bravo-6F 54 percent (tetrachloroisophthalonitrile); and Vitavax 75 percent WP (5, 6-dihydro-2-methyl-1, 4-oxathiin-3-carboxanilide).

Laboratory Studies

The four fungicides were tested in vitro to determine the antifungal activity of each against *V. albo-atrum*. Concentrations of 1,000, 500, 100, and 10 μg ml (active ingredient) aqueous suspension of each fungicide were prepared and 10-mm Whatman filter discs were soaked for 5 minutes in each concentration. Sterile PDA culture plates were seeded with a conidial suspension and discs from each fungicide were placed on the seeded culture plates, two per plate. This was replicated four times using a factorial arrangement of treatments (4 trials x 4 fungicides x 4 levels) in a completely random design with four plates per treatment combination. Zones of inhibition were measured after 4 or 5 days, at which time growth on control plates had entirely covered the agar surface.

Laboratory bioassays were conducted on plant materials used in fungicide

tests in the greenhouse. The plants were severed at the base of the stem and divided into three regions: (1) terminal, characterized by fully expanded terminal leaves; (2) center; and (3) bottom, located about 5 cm above the severed base of the stem. Leaf discs (9 mm diameter) and wood and bark sections (100 mm long) from each region were frozen at −10 C for 24 hours prior to being placed into petri dishes which contained 15 ml PDA seeded with a conidial suspension of *V. albo-atrum*. After the plates were incubated at 24 C for 7 days, the diameters of the zones of inhibition were measured to determine the relative concentration of fungitoxicant present in the sample.

Greenhouse Studies

SOIL DRENCHES.—Three hundred twenty seedlings each of sugar maple and Russian olive were used as plant material. The seedlings were 2 years old, bare-rooted, and 45–60 cm in height. The seedlings had no previous treatment and were just breaking dormancy. Plants wound inoculated or placed in infested soil were potted 2 weeks prior to fungicide treatment. Control plants were treated identically, but without the fungus. Eight different treatment combinations for each of the four fungicides were tested; with 36-treatment combinations arranged as a 4 x 3 x 3 factorial [four fungicides x three levels (two rates and a control)] x three infestations (with and without fungus) in a completely randomized design giving a total of 320 observations per species. The eight treatments were: (1) infested soil, nontreated plants; (2) wound inoculated, nontreated plants; (3) infested soil, plants treated with 1,500 μg/ml; (4) infested soil, plants treated with 500 μg/ml; (5) wound inoculated, plants treated with 1,500 μg/ml; (6) wound inoculated, plants treated with 500 μg/ml; (7) noninfested soil, plants treated with 1,500 μg/ml; (8) noninfested soil, plants treated with 500 μg/ml. Each plant was placed in a No. 10 potting can. In each pot, 200 ml of the fungicide at the designated concentration were applied as soil drenches three times at weekly intervals. Water was applied and the soil kept moist by watering when required. All fungicide treatments had 10 plants per treatment except that the benomyl treatments had 25 plants per treatment. Disease control was calculated by using the following formula.

$$\text{Percent disease control} = \frac{\text{Disease incidence in control} - \text{Disease incidence in treated}}{\text{Disease incidence in control}} \times 100$$

The noninfested treated pots were used for detection of fungicide phytotoxicity on seedlings.

FOLIAR TREATMENTS.—A benomyl derivative was applied to the foliage of sugar maple and Russian olive seedlings to evaluate its effectiveness as a foliar fungicide. Solutions of the benomyl derivative were prepared as follows: benomyl (5.0 g of active chemical) was dissolved in 100 ml of 85-percent concentrated lactic acid over heat and brought up to a liter with distilled water (5,000 μg/ml); benomyl (5.0 g of a.c.) was dissolved in a liter of distilled water over heat in which 2 ml of concentrated sulfuric acid had been added (5,000 μg/ml); benomyl (5.0 g of a.c.) was suspended in a liter of distilled water (5,000 μg/ml). The pH of the benomyl-lactic acid-water solution was 1.2–1.5, and of the benomyl-sulfuric acid-water solution was 2.5–3.0.

Each formulation of the benomyl derivative was applied to an equal number of plants 2 weeks prior to soil infestation. Another group of plants was treated with each formulation 2 weeks after soil infestation. Foliage was dipped twice to run-off in late afternoon to retain moisture on the foliage as long as possible. The fungicide was

prevented from contaminating the soil by the placing of a cardboard cover on the top of each can before dipping.

To determine if the benomyl derivative could be translocated from the place of application to new growth in sugar maple seedlings, foliar dips were applied to localized areas. A benomlylactic acid-water solution was prepared as previously described. Treatments with 5,000 μg/ml were applied in three different ways — to the top three leaves, applied to leaves on the lower two branches, and applied to all leaves on one side of the plant.

The agar diffusion bioassay method was used to detect fungitoxic chemicals in 9-mm leaf discs above and below the area of treatment or in 10-mm sections of xylem tissue.

ROOT TREATMENTS.—Benomyl, thiabendazole, Bravo-6F, and Vitavax were applied as root dips to evaluate each fungicide as a prophylactic against root penetration by the pathogen. Four liters of each fungicide were formulated at 1,500 μg ml in distilled water. Ten plants of each species were allowed to stand in each fungicide for 5 minutes. Only the roots were covered with the fungicide. After 5 minutes each plant was removed from the dip, shaken to remove excess liquid, and planted in infested soil. Each plant was potted in a No. 10 potting can. Data on phytotoxicity and symptom development were recorded.

RESULTS

Symptoms

Initial wilt symptoms occurred within 7–10 days on both sugar maple and Russian olive seedlings after being inoculated by the wound method. When the plants were placed in infested soil, symptoms occurred within 12 to 14 days. The progression of symptom development was the same regardless of the inoculation method. The leaves rapidly lost their turgidity within 2–3 days. Browning of the leaves and premature leaf drop occurred soon after the leaves had wilted. Unlike larger trees where only a branch or several branches may wilt, these seedlings wilted quickly and completely.

Laboratory Studies

With the paper disc bioassay in vitro, benomyl and TBZ were highly inhibitory at a concentration of 10 μg/ml (Table 13). Vitavax was somewhat less fungitoxic, and Bravo-6F was much less active. As the concentration of each fungicide decreased the zone of inhibition decreased proportionately (Fig. 17). The minimum concentration of benomyl and TBZ that inhibited growth was 0.01 and 0.1 μg/ml, respectively. The minimum concentration of Vitavax was 0.1 μg ml and for Bravo-6F it was 1 μg/ml.

In PDA plates containing benomyl or TBZ, conidia germinated but failed to grow more than a few microns in

Table 13.—Paper disc bioassay of fungicides against **Verticillium albo-atrum** in vitro.

Concentration[a] μg/ml	*Fungicide*			
	Benomyl	*Thiabendazole*	*Bravo-6F*	*Vitavox*
	Diameter of zone of inhibition (mm)[b]			
1000.000	48	55	12	47
500.000	41	53	8	41
100.000	30	43	6	18
10.000	25	39	5	15
1.000	11	8	1	7
0.100	3	1	..	2
0.001	1	..	..	..

[a] μg/ml based on weight of active ingredient of fungicide.
[b] Zone of inhibition computed as average of four trials with four replications per trial.

length. When single conidia were transferred from these plates to PDA slants after 10 days, more than 90 percent gave rise to established colonies.

Greenhouse Studies

SOIL DRENCHES.—When benomyl, TBZ, Vitavax, and Bravo-6F were ap-

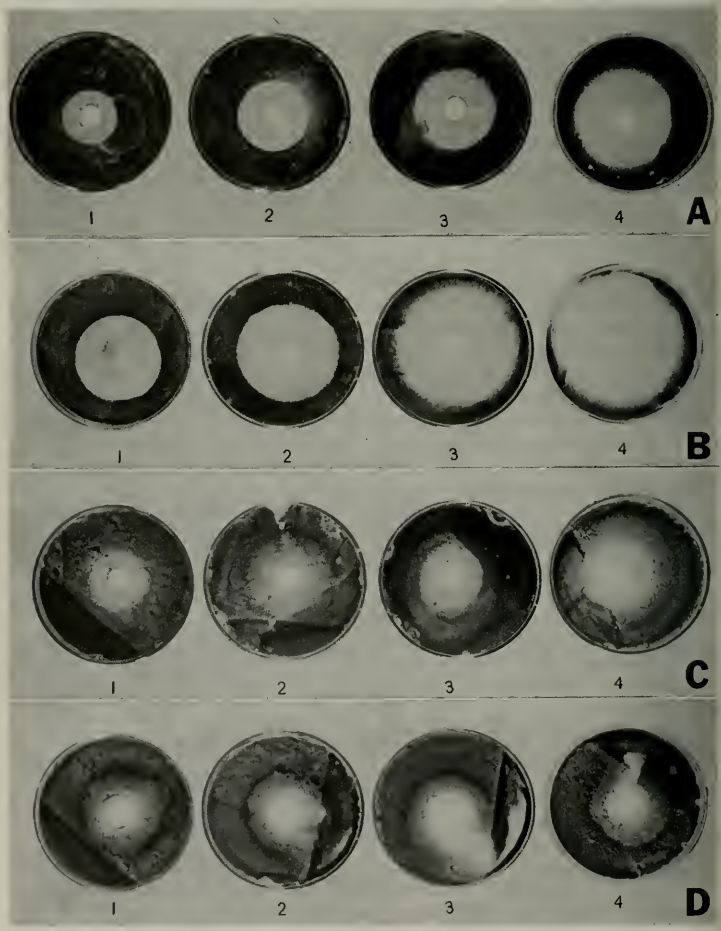


Fig. 17.—Filter paper disc bioassay of four fungicides for the control of **V. albo-atrum** illustrating zones of inhibition outward from filter discs. A) Benomyl. B) Thiabendazole. C) Bravo-6F. D) Vitavax (1 = 10 μg/ml; 2 = 100 μg/ml; 3 = 500 μg/ml; 4 = 1,000 μg/ml).

plied as soil drenches to sugar maple and Russian olive seedlings, each gave some degree of disease control except Bravo-6F at 500 μg/ml (Table 14). Benomyl, TBZ, Vitavax, and Bravo-6F, in descending order, were effective when applied 2 weeks after soil infestation. Benomyl at 1,500 μg/ml gave the best control of Verticillium wilt of both sugar maple and Russian olive seedlings. The fungicide concentration, whether at 1,500 μg/ml or 500 μg/ml, at the time of application made little difference in the percentage of disease control. Benomyl at 1,500 μg/ml and 500 μg/ml gave 47.5 and 42.5 percent disease control, respectively, on sugar maple seedlings. Differences were noticed when comparisons were made between fungicides and fungicide concentrations. On Russian olive seedlings, TBZ at 500 μg/ml gave the same amount of control as Vitavax at 1,500 μg/ml. Benomyl at 500 μg/ml gave less control than TBZ at 1,500 μg/ml. Therefore the rate of soil application of any one fungicide is important in the control of the disease.

Bioassay of terminal, center, and lower leaves of plants treated with a soil drench with each fungicide showed the highest accumulation of the fungi-

Table 14.—Effect of soil drenches for the control of Verticillium wilt of sugar maple and Russian olive seedlings.

Fungicide and Concentration[a]	*Number of Plants*	*Intensity of Infection*[b]		
		Plants With Wilt Symptoms	*Plants Without Wilt Symptoms*	*Percent Disease Control*
Sugar maple				
Benomyl				
1,500	50	21	29	47.5
500	50	23	27	42.5
Thiabendazole				
1,500	20	10	10	37.5
500	20	11	9	31.2
Bravo-6F				
1,500	20	15	5	6.3
500	20	16	4	0.0
Vitavax				
1,500	20	12	8	25.0
500	20	14	6	12.0
Control	20	16	4	0.0
Russian olive				
Benomyl				
1,500	50	19	31	55.0
500	50	24	26	43.5
Thiabendazole				
1,500	20	9	11	47.0
500	20	10	10	41.1
Bravo-6F				
1,500	20	15	5	12.0
500	20	17	3	0.0
Vitavax				
1,500	20	10	10	41.1
500	20	11	9	35.3
Control	20	17	3	0.0

[a] Fungicides applied three times as a soil drench at the rate of 200 ml of aqueous suspension per pot. Plants bioassayed 30 days after last treatment. Concentration at μg/ml active ingredient-aqueous suspension.

[b] Data on symptom development taken 30 days after last treatment.

toxicant in the lower leaves and stems (Tables 15 and 16). Benomyl was detected in higher concentrations than all other fungicides in both leaves and stems whether it had been applied at 1,500 or 500 μg/ml. Bravo-6F could

Table 15.—Effect of soil drenches on uptake and translocation of fungitoxic materials by sugar maple seedlings.

Fungicide and Concentration[a]	*Tissues and Portions of Plant Sampled*[b]					
	Leaves			*Wood*[c]		
	Terminal	*Center*	*Lower*	*Top*	*Center*	*Bottom*
	Diameter of zone of inhibition (mm)					
Benomyl						
1,500	27	34	41	21	19	23
500	18	23	24	13	17	17
Thiabendazole						
1,500	18	23	25	14	16	18
500	13	16	17	9	11	11
Bravo-6F						
1,500	0	0	0	0	0	0
500	0	0	0	0	0	0
Vitavax						
1,500	19	25	28	16	21	23
500	16	15	18	14	13	12
Control	0	0	0	0	0	0

[a] Fungicides applied three times as a soil drench at the rate of 200 ml of aqueous suspension per pot. Plants bioassayed 30 days after last treatment. Concentration at μg/ml active ingredient-aqueous suspension.

[b] Leaf disc (9 mm diameter); wood sections (10 mm long).

[c] Top (characterized by fully expanded terminal leaves); bottom (5 cm above severed base of stem).

Table 16.—Effect of soil drenches on uptake and translocation of fungitoxic materials by Russian olive seedlings.

Fungicide and Concentration[a]	*Tissues and Portions of Plant Sampled*[b]					
	Leaves			*Wood*[c]		
	Terminal	*Center*	*Lower*	*Top*	*Center*	*Bottom*
	Diameter of zone of inhibition (mm)					
Benomyl						
1,500	18	19	22	13	15	15
500	12	13	16	8	11	12
Thiabendazole						
1,500	16	16	18	11	13	14
500	9	9	11	5	6	6
Bravo-6F						
1,500	0	0	0	0	0	0
500	0	0	0	0	0	0
Vitavax						
1,500	10	11	13	6	6	7
500	8	10	10	5	6	6
Control	0	0	0	0	0	0

[a] Fungicides applied three times as a soil drench at the rate of 200 ml of aqueous suspension per pot. Plants bioassayed 30 days after last treatment. Concentration at μg/ml active ingredient-aqueous suspension.

[b] Leaf disc (9 mm diameter); wood sections (10 mm long).

[c] Top (characterized by fully expanded terminal leaves); bottom (50 mm above severed base of stem).

not be detected in any plant tissue above ground. A higher concentration of the fungitoxicant accumulated in the sugar maple seedlings than in the Russian olive seedlings. The bioassay of foliage and wood from the sugar maple produced zones of inhibition approximately twice as large as those from Russian olive seedlings.

FOLIAR TREATMENTS.—A foliar application of benomyl, dissolved in lactic acid or sulfuric acid, 2 weeks prior to soil infestation gave the best control (Fig. 18). Benomyl suspended in water gave less control than either application of benomyl dissolved in acid. When the application of benomyl was delayed for 2 weeks after soil infestation, little control occurred. All foliar applications, regardless of formulations, gave better control if they were applied prior to soil infestation.

Benomyl, or a benomyl derivative, was detected moving upward to areas of new growth after it had been applied to localized areas at 5,000 μg/ml. After applications had been made to the top three leaves of sugar maple seedlings, a fungitoxic material could be detected in the treated leaves, but no fungitoxic material was found moving downward in the wood. After a benomyl derivative was applied to the lower two branches and leaves, a fungitoxic material was found in the foliage and vascular wood of the treated area, and also in the untreated foliage and wood above the point of application. When applications were made to leaves on one side of the plant, a fungitoxic material was found adjacent to the treated area and upward in the nontreated areas. Therefore, a benomyl derivative was translocated from the treated areas to adjacent nontreated areas above the point of application. No fungitoxic materials were detected below the point of application.

ROOT TREATMENTS.—Root infection of sugar maple and Russian olive seedlings can be reduced and symptom expression delayed by dipping the roots with fungicides before placing them in infested soil (Table 17). Benomyl, TBZ, Bravo-6F, and Vitavax all gave some degree of control against Verticil-

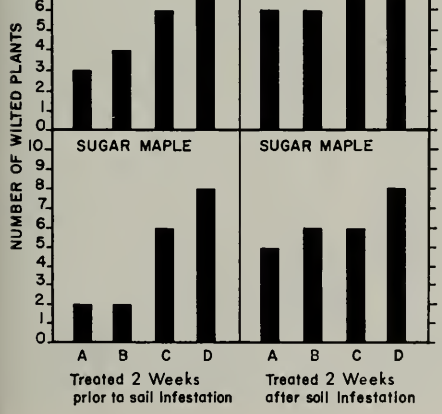


Fig. 18.—Degree of Verticillium wilt control with various foliar applications of benomyl: A) Benomyl/lactic acid/water; B) Benomyl/sulfuric acid/water; C) Benomyl/water; D) Control.

Table 17.—Effect of root treatments on symptom expression of sugar maple and Russian olive seedlings planted in **Verticillium albo-atrum**-infested soil.

Fungicide[a]	*Plants Treated*	*Days After Treatment for Initial Symptom Expression*	*Percentage of Plants Wilted*
Sugar maple			
Benomyl	10	28	40
Thiabendazole	10	21	60
Bravo-6F	10	14	70
Vitavax	10	18	50
Control	10	7	80
Russian olive			
Benomyl	10	17	30
Thiabendazole	10	14	50
Bravo-6F	10	12	60
Vitavax	10	13	50
Control	10	8	70

[a] Fungicides applied as a root dip for 5 minutes at the rate of 1,500 μg/ml.

lium wilt. On sugar maple and Russian olive seedlings, benomyl was much more prophylactic in protecting against root infection than the other fungicides tested. All fungicides used as a prophylactic delayed initial symptom development. Symptoms on sugar maple seedlings treated with benomyl developed 21 days later than symptoms on the control. Initial wilt symptoms on benomyl-treated Russian olive seedlings occurred 9 days later than those on the control.

DISCUSSION AND CONCLUSIONS

The control of a vascular wilt pathogen is extremely difficult. Systemic fungicides which can be applied to the soil, taken up by the root system, and translocated throughout the plant are the most feasible. Fungicides applied prior to infection may serve as a barrier which will kill or arrest the fungus before it becomes established. The application of a systemic fungicide, which will inhibit growth of the fungus after symptoms appear, may be a more logical control measure.

The in vitro assay for fungicide toxicity appears to be quantitative using the agar diffusion method. There is a proportional increase in size of the zone of inhibition with increase in quantity of the fungicide. The difference in inhibition may not be related as much to differences in toxicity of the chemical as to solubility and diffusibility. The in vitro data indicate that these fungicides are fungitoxic at low concentrations and that benomyl or its toxic breakdown product exists in plant tissue at a point beyond the place of application.

Benomyl, TBZ, and Vitavax, when applied as soil drenches, reduced symptom development after plants had become infected. The inability of any fungicide to give 100 percent control may be due to the tyloses and gum-like materials which inhibit the fungitoxicant from being translocated to the foliage. Recovery of the fungitoxicant from wilting plants that showed vascular plugging was limited. If the fungicide was applied before vascular plugging took place, the fungitoxicant readily moved throughout the plant and could be assayed in the above-ground parts. Fungicides, such as Bravo-6F, which show no systemic action are of little value in controlling Verticillium wilt when applied as a soil drench.

Benomyl dissolved in either an organic or inorganic acid and applied to the foliage gave better control than a benomyl suspension in water. With

the addition of acids, the fungitoxicant was water soluble, and could be taken up more readily and translocated throughout the plant. The fungitoxicant must be localized in the plant parts before the host-pathogen interaction produces gums and tyloses, blocking the upward movement of the fungitoxicant. Once wilt symptoms occurred and the vascular system was occluded, translocation of the fungitoxicant was reduced regardless of the formulation.

The time of fungicide application is critical for the fungitoxicant to be distributed throughout the plant before the fungus can become established. The critical time of application was similar for foliar treatments and soil drenches.

When fungicides were tested as prophylactic root dips, each gave some degree of control. Initial wilt symptoms were delayed as much as 3 weeks with benomyl. The delaying of root infection may allow wounds to be occluded with wound material before the fungus can become established at the wound site. This method of control may be of value when used on bare-rooted nursery materials.

More work is needed to determine how fast these systemic fungicides will move in the plant and how long they will remain active. Additional work is needed to determine the critical time of application and if higher concentrations will be more effective but not phytotoxic to the host.

SUMMARY

Verticillium albo-atrum Reinke and Berthold is a widespread and destructive vascular pathogen. It is peculiar in that it does not confine its attack to one host, or a few closely related hosts, but attacks a large number of widely unrelated plants, many of which are of economic importance.

The wound most conducive to infection was a vascular wound which allowed the pathogen to come in direct contact with the vessel members. No infection took place unless a wound was present on the root. Root wounds remained as infection courts up to 32 days on redbud and 16 days on sugar maple seedlings. As the age of the wound increased, the number of plants infected through wounds decreased sharply.

In the susceptible hosts, the pathogen rapidly colonized the cortex, endodermis, and vessel members. Conidia were produced in abundance within 8 days. The pathogen in the resistant hosts readily colonized the cortex, but few hyphae were found in the vessel members. Conidia were not present in the vascular system. Microsclerotia were found in both the cortex and vascular cylinder of the resistant hosts.

Infection leads to a significant reduction in dry-matter production, stem height, and leaf area of the plants. The nitrogen content was lower in infected redbud and green ash stems, but higher in leaves and roots. There was no definite pattern of water content between infected and healthy redbud and green ash seedlings. Frequently the water content of the infected seedlings was above that of the healthy controls, but not consistently.

Abundant microsclerotia were observed in roots after 14 days when incubated at 15, 20, 25, and 30 C. Microsclerotia were observed after 35 days at 5 and 10 C, but no microsclerotia were observed at 35 C. Microsclerotia developed in dead roots incubated at 25 C in both steamed and nonsteamed soil. A moisture level near the field capacity of the soil was more favorable for microsclerotial development than was a lower soil moisture.

The in vitro assay of the toxicity of the fungicides by the agar diffusion method appears to be quantitative. There is a proportional increase in the size of the zone of inhibition with increase in quantity of the fungicide.

Benomyl, TBZ, and Vitavax, when applied as soil drenches, reduced symptom development after plants had become infected. The inability of any fungicide to give 100-percent control may be due to the host-pathogen interaction producing tyloses and gum-like material which prevents the fungitoxicant from being translocated to the foliage. Fungicides which show no systemic action are of little value in controlling Verticillium wilt when they are applied as a soil drench.

Benomyl which had been solubilized in either an organic or inorganic acid and applied to the foliage gave better control than a benomyl suspension in water. With the addition of acids, the fungitoxicant was water soluble, and could be taken up more readily and translocated throughout the plant.

When the fungicides were tested as prophylactic root dips, all delayed symptom expression and gave some degree of control. Benomyl delayed initial wilt symptoms as much as 3 weeks. The delaying of root infection may allow wounds to be occluded with wound material before the fungus can become established at the wound site.

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