The Root Rot of Black Walnut Seedlings Caused by *Phytophthora citricola**

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Introduction

Black walnut, *Juglans nigra*, L. is one of the most highly valued species of the American deciduous forest. The rich wood color, its durability and ease with which it is worked placed black walnut in high demand in both the lumber and veneer industries of the U.S. and abroad. Nearly one third of the veneer-quality walnut logs harvested annually in the U.S. come from Indiana (Blyth 1973). Because of the demand and dwindling supply of walnut trees for harvest, nursery production of seedlings in state-owned and private nurseries has more than doubled in the eastern U.s. in the past few years (Grey 1973).

Figure. 1. *Symptoms of root rot of black walnut seedlings caused by* ***Phytophthora citricola****. Infection usually restricted to root collar area of seedling.*

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Walnut seedling production is often severely curtailed both in the seedbed and in winter storage by a root rot disease. In seedbed, the symptoms include a general chlorosis and wilting of above-ground parts and the root system exhibits distinct water-soaked, greenish black lesions, usually located at the root collar (Fig. 1). The root lesions increase rapidly in size and the entire seedling turns black. Roots of infected plants in storage exhibit similar symptoms and, in both cases, mortality is almost certain.

In general, infected seedlings occur in the poorly drained areas of the seedbed. Infection centers start as isolated diseased plants which, in time, give rise to expanding areas of dead and dying plants. In storage, symptoms develop primarily from incipient infection from the seedbed, but the causal agent may spread rapidly to other plants.

In 1970, Green and Pratt implicated *Phytophthora citricola* Sawada as the causal fungus of this disease. However, other species of *Phytophthora* are also associated with root disease of black walnut (Green 1975). More recently, *Cylindrocladium scoparium* and related species have been associated with a root disease of walnut seedlings (Cordell and Matuszewski 1974). Also, critical studies have not been made of the factors of the soil environment which favor infection by *P. citricola* and the factors affecting germination of oospores of *P. citricola* in soil. For example, Banihashemi and Mitchell (1976) found that both light quality and light intensity affected germination of other *Phytophthora* species and this may be significant in the localization of root infection.

The objectives of this study were to a) ascertain whether species of *Phytophthora* or fungi other than *P. citricola* are involved in this disease, b) to determine the effect of soil environmental conditions and inoculum density on infection by *P. citricola* and c) ascertain the effect of various environmental factors on germination of oospores of *P. citricola.*

Materials and Methods

In earlier studies, Green and Pratt (1970) found it difficult to isolate consistently *P. citricola* from diseased walnut seedlings. They used both selective media and baiting techniques. We used the following selective media for isolation from diseased roots:

PNP — potato dextrose agar (PDA) + neomycin (50 ppm), penicillin G (35 ppm) and pimaracin (100 ppm). This medium is similar to that described by Eckert and Tsao (1962) for selective isolation of *Phytophthora* sp..

ENC — V-8 juice nutrient agar + neomycin (100 ppm), chloromycetin (10 ppm) and endomycin (10 ppm), for isolation of Phythiaceous fungi (Schmitthenner and Hilty 1962).

PDTA — PDA + tergitol NPX (200 ppm) and aureomycin (30 ppm) for isolation of *Cylindrocladium* sp. and other fungi (Watson 1960).

Diseased roots were washed, surface sterilized in 1% sodium hypochlorite (NaOCl) for 5 min. and tissue selections from the periphery of the root lesion

placed on the selective media. The plates were incubated at 25° C in the dark and emerging fungi isolated by hyphal tip transfer.

At least three species of *Phytophthora* cause a root disease of walnut (Green 1975). A similar, but distinct, root disease is caused by *Cylindrocladium scoparium* (Cordell and Matuszewski 1974) and related species. Black walnut seedlings were inoculated with isolates of the following fungi: *Phytophthora citricola* — (host: black walnut), *P. cactorum* — (host: apple), *P. cinnamomi* (host: rhododendron and Taxus sp). and *Cylindrocladium scoparium* — (host: black walnut).

All isolates were grown on PDA for 3 weeks at 25° C in termittent light. Walnut seedlings were grown from stratified seed in sterile sand for 4 weeks and then lifted, the roots washed gently in tap water and inoculations made in the root collar area. Root tissue was cut to a depth of ca. 1 cm and a 0.5 cm square section of mycelial mat inserted in the wound. Check plants were wounded but not inoculated. The wound area was protected with petrolatum and the seedlings incubated in sterile, moist vermiculite in the greenhouse for 3 weeks.

After incubation, the inoculated seedlings were lifted and the fleshy primary root was cut lengthwise to determine the extent of root involvement. The area of the cut surface of the primary root which exhibited symptoms was calculated as a percentage of the total cut root surface area. The comparative virulence of the various *Phytophthora* species to black walnut seedlings was determined on the basis of root involvement. The inoculated seedling roots were then surface sterilized in 1% NaOCl for 2 min. and tissue sections placed on both PNP and ENC media.

The effects of varying soil moisture and temperature regimes and inoculum density on infection of walnut seedlings by *P. citricola* were determined. Naturally infested nursery soil was used and the moisture saturation capacity (SC) was determined by the methods described by Couch and others (1967). Oospores of *P. citricola* were produced, following Honour and Tsao (1974), and added to sterile silica sand. The supplemental inoculum was incorporated in the upper 5 cm of the soil container.

Naturally infected nursery soil was placed in ceramic containers (dia 25 cm, ht 25 cm) with a drain and 5 walnut seeds with the radicle just emerging planted in each container. The containers were placed in controlled environment chambers and soil temperature, moisture and inoculum density varied as follows:

Soil temperatures — 15°C and 22.5°C, 12 hr photoperiod

Soil moisture — 100% SC and 60% SC for 24 and 72 hr, respectively, followed by free drainage

Soil inoculum — naturally infested nursery soil (NS) and nursery soil amended with 500 oospores/g soil (NS + I) in upper 5 cm

All treatments were replicated 3 times and the soil moisture adjusted daily when soil moisture was controlled. Thereafter, the soil containers were watered every 3 days for the duration of the 3-week incubation period.

The effects of culture age, temperature and light intensity and quality on germination of oospores of *P. citricola* were determined. Germination boxes (30 x 60 x 10 cm) with an open top were placed in a high light intensity, controlled environment chamber (22.5° C, 16 hr photoperiod, 3400 f.c.). The light intensity in the germination boxes was controlled with varying layers of cheese cloth (open — 3400 f.c.; 16 layers — 830 f.c.) and light quality was varied using filters of colored acetate (blue, aqua, green, yellow, and red). When light quality was varied, the light intensity under each filter was adjusted to approximately 800 f.c. Oospore germination was also compared at 25° C in total darkness and in continuous light of 300 f.c. and 3 f.c.. Ooospores from cultures 2, 4 and 7 weeks old were observed for germination after 3, 6, and 10 days.

Results

Isolation — selective media — Phytophthora citricola was recovered from 11 of the 20 diseased walnut seedlings on the selective medium PNP, but only from 2 of the seedlings on ENC. On both media, especially ENC, *Phthium* sp.and other fast growing fungi were common. This made detection of slower growing fungi as *Phytophthora* sp. difficult. Diseased root tissue on PDTA yielded numerous fungi, including *Fusarium* sp., *Penicillium* sp. and *Phythium* sp., but no known root pathogen of walnut. *Cylindrocladium scoparium*, which causes the black root rot disease of walnut seedlings, was not recovered in any isolation attempts. Thus, although successful isolation of *P. citricola* was limited (approx. 50%), no other known root pathogen of walnut was recovered. Later inoculations confirmed the virulence of *P. citricola* isolates.

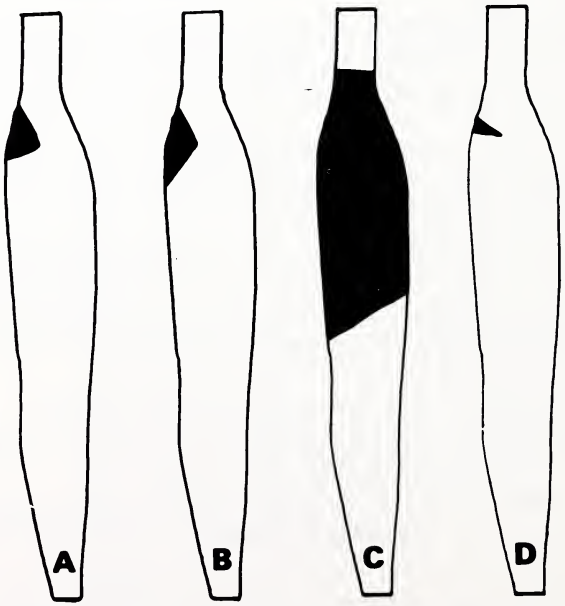

FIGURE. 2. *Comparative virulence of A)* ***Phytophthora cactorum****, B)* ***P. cinnamomi*** *and C)* ***P. citricola*** *to roots of black walnut seedlings, D) wounded, non-inoculated control.*

Inoculation — Walnut seedlings were inoculated with isolates of *P. citricola, P. cactorum* and *P. cinnamomi* to compare disease severity and symptoms. In addition, seedlings were inoculated with *Cylindrocladium scoparium*. Although all *Phytophthora* sp. induced root lesions, there was a marked difference between species in the extent of root tissue involvement. This is demonstrated in Fig. 2, which diagramatically shows the comparative root involvement by *P. citricola, P. cactorum* and *P. cinnamomi* following wound inoculation. The black area represents the average (10 seedlings) root involvement. The isolates of *P. citricola* produced extensive necrotic lesions in the root tissues, compared to infection by *P. cactorum* and *P. cinnamomi*. These differences may be due, in part, to the origin of the respective isolates, since *P. citricola* was from walnut and *P. cactorum* and *P. cinnamomi* were from other hosts. Nonetheless, only *P. citricola* produced the typical extensive root involvement associated with this disease. Also, *P. citricola* was readily reisolated from inoculated seedlings (80%), while *P. cactorum* was recovered from only 20% of the inoculated seedlings and *P. cinnamomi* was not recovered.

Walnut seedlings inoculated with *Cylindrocladium scoparium* exhibited symptoms typical of the root rot disease described for this pathogen (Cordell and Matuszewski 1974). These included longitudinal cracks and brown, necrotic lesions which were sunken in the root tissues. The fungus was readily reisolated from the inoculated seedlings on PDTA.

TABLE 1. *The effects of soil moisture, temperature, and inoculum density on infection of black walnut seedlings by Phytophthora citricola.*

Temp °C	Soil moisture[a/]	Inoc[b/]	Infect — roots
15	100% SC - 24 hr	NS	4/15
		NS + I	8/15
	100% SC - 72 hr	NS	10/15
		NS + I	13/15
	60% SC - 72 hr	NS	1/15
		NS + I	2/15
	free drainage	NS + I	1/15
22.5	100% SC - 24 hr	NS	7/15
		NS + I	10/15
	100% SC - 72 hr	NS	8/15
		NS + I	11/15
	60% SC - 72 hr	NS	2/15
		NS + I	2/15
	free drainage	NS + I	0/15

[a/]Soil moisture × 100% and 60% saturation capacity (SC) of soil for 24 and 72 hr, followed by free drainage for 3 weeks.

[b/]Inoc — NS — naturally infested nursery soil; NS + I — nursery soil + 500 oospores/g in upper 5 cm of soil.

Factors affecting infection incidence — The effects of soil temperature, soil moisture and inoculum density on infection of walnut seedlings by *P. citricola* are shown in Table 1. Infection varied markedly with soil conditions. Infection was low (0/15 — 2/15) in NS and NS+I soil if the moisture was below 100% SC.

The infection increased progressively when the soil moisture was increased to 100% SC for 24 and 72 hr, respectively, and with supplemental inoculum (NS+I). A slight increase in infection occurred in most treatments when the temperature was increased from 15°C to 22.5°C. The highest infection incidence (13/15) occurred when the soil moisture was 100% SC for 72 hr at 15°C in NS+I soil.

TABLE 2. *The effects of age, incubation time, light intensity and quality on the germination of oospores of Phytophthora citricola.*

		Germination (%)					
		Age — 2 wks.		Age — 4 wks.		Age — 7 wks.	
Temp °C	Treatment	3 da	10 da	3 da	10 da	3 da	10 da
25	dark	0.7[a]	1.3	3.7	6.0	1.3	1.3
	3 f.c.	0.7	7.3	4.7	18.0	10.0	9.0
	200 f.c.	2.0	7.3	6.0	25.3	7.3	30.0
22.5	3,400 f.c.	0	0	0	1.3	0	0
	2,300 f.c.	0	0	2.0	8.0	7.3	8.0
	1,200 f.c.	2.7	11.3	28.0	46.7	11.3	41.3
	830 f.c.	2.0	23.3	47.4	60.7	30.0	57.3
22.5	(800 f.c.)						
	blue filter	0	3.3	7.3	11.3	7.3	10.0
	green filter	0	1.3	4.7	14.7	11.3	18.0
	aqua filter	0	0	2.7	1.3	2.0	8.0
	yellow filter	0	14.7	46.0	46.7	41.7	58.7
	red filter	0	0	0	1.3	1.3	2.0

[a] Percent germination × 150 oospores counted at random.

The effects of culture age, incubation time, light intensity and light quality on germination of oospores of *P. citricola* are presented in Table 2. Oospore germination was affected by all factors, as indicated by the comparative germination rates. Germination increased with culture age, regardless of other treatments, and the data indicate that both light intensity and light quality affect germination. The highest germination occurred with oospores from cultures 4 and 7 weeks old incubated at 22.5° C, 830 f.c. and decreased sharply as the light intensity increased above 1200 f.c. Germination was almost completely inhibited at 3400 f.c. Light quality also affected oospore germination with the germination rate under a yellow filter approaching that of full light under similar intensities. Germination was reduced under all other filters with the lowest germination occurring under the red filter.

Discussion

Although isolation of *P. citricola* from diseased walnut seedlings was somewhat erratic, even with selective media, the results confirm this fungus as the causal agent of the root rot disease described. No other fungi, including other species of *Phytophthora* and *Clyindrocladium scoparium*, were consistently isolated from diseased seedlings. Inoculation trials showed that the symptoms produced by *C. scoparium* are distinct from those caused by *P. citricola*.

The inconsistency in reisolation of *P. citricola* from diseased seedlings may be related to both the relatively slow growth of this fungus and the nature of the primary root of the walnut seedling. The root is fleshy and, under soil conditions favorable for infection, is rapidly colonized by secondary organisms. In controlled inoculation studies, the reisolation of *P. citricola* was much more consistent (80%+).

The effects of soil environmental conditions on infection incidence under controlled conditions correlate with field observations. In the nursery, diseased plants occur primarily in poorly drained areas and we found that infection incidence was invariably low unless soil moisture was 100% SC for 24 hr or more, regardless of other conditions.

We also found that germination of oospores of *P. citricola* is markedly influenced by light and that both light intensity and light quality may affect the germination rate. These results compare favorably with results presented by Banihashemi and Mitchell (1976) with oospores of *P. cactorum*. They found that germination was essentially the same under blue and yellow filters (30.8% and 31%, respectively), whereas we found germination of oospores of *P. citricola* was much lower under the blue acetate filter (10%) than under the yellow filter (61.7%). However, there was good agreement on the light intensities most favorable for germination (200-1000 f.c.) and the light quality least favorable for germination (red). The differences observed in light quality may be due to innate differences in these two closely related organisms or to differences in the filter systems used. The filters we used were of unspecified wavelength transmission and comparisons based on filter color alone may be unreliable.

The dependency of the oospores of *P. citricola* on light for optimal germination may also explain the occurrence of infection almost exclusively at the root collar of walnut seedlings rather than at random over the entire root system. Since both light and soil moisture saturation are apparently required for oospore germination and subsequent infection, these conditions are met primarily at or near the soil surface. Thus, infection is restricted primarily to the root collar area of the susceptible walnut seedlings.

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