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Role of Phytotoxins in Pine Wilt Disease[1]

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Abstract: Characteristic rapid death of pines after infection by *Bursaphelenchus xylophilus* suggests the involvement of phytotoxins in the pine wilt disease syndrome. Crude extract from diseased pine is toxic to pine seedlings, whereas an extract from healthy pine is not. The response of seedlings to the crude toxin is more prominent in susceptible pine species than in resistant ones. Benzoic acid, catechol, dihydroconiferyl alcohol, 8-hydroxycarvotanacetone (carvone hydrate), and 10-hydroxyverbenone, which are toxic, low molecular weight metabolites, can be isolated from diseased pines. Other unidentified toxins are also found. The toxicity of some of these metabolites correlates positively to the susceptibility of pines to *B. xylophilus.* Some of these abnormal metabolites show synergistic toxicity when in combination. The D-isomer of 8-hydroxycarvotanacetone, dihydroconiferylalcohol, and 10-hydroxyverbenone inhibited the reproduction of *B. xylophilus.* Cellulase excreted by pinewood nematode also may be involved in rapid wilting.

Key words: benzoic acid, *Bursaphelenchus xylophilus,* catechol, carvone hydrate, cellulase, dihydroconiferylalcohol, 8-hydroxycarvotanacetone, 10-hydroxyverbenone, phytoalexin, synergistic effect, pinewood nematode.

Similar to Dutch elm disease in North America, pine wilt disease is one of the most devastating disease of trees in Japan. The pine wilt epidemic has become a serious social problem, not only in terms of environmental protection, but also in aesthetic aspects of natural, as well as artificial, landscape managements.

Pine wilt is caused by the nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle. This nematode, which is transmitted by *Monochamus alternatus* Hope in Japan and *M. carolinensis* Olivier in the United States, is unique in its outbreak pattern and rapidity of killing. Loss of pine wood from this disease reached a maximum of 2.4 million m^3 in 1979. Pine wilt has since decreased gradually (9), but the disease has not ceased to be a problem.

The pine wilt disease has been known in the United States for a long time, but etiological identification of the causative agent, by Dropkin and Foudin (4) with some help from Japanese exchange visitors, was not established until 1979. It has subsequently been recorded in more than 30 states (19).

The extensive forest damage in Japan is due to the high susceptibility of the indigenous Japanese red (*Pinus densiflora* Sieb. and Zucc.) and black (*P. thunbergii* Parl.) pines. Pine wilt in the United States is not as devastating as in Japan, partly because of the inherent resistance of indigenous pine species and the difference in forest composition. The disease occurs primarily in the highly susceptible, introduced Scots (*P. sylvestris* L.) and Austrian (*P. nigra* Arnold) pines in ornamental plantings, windbreaks, and plantations.

In the warmer districts of Japan, pine trees are infected during May and June when the newly emerged pine sawyers feed on growing pine shoots. Infected trees die during late summer to autumn of the same year. The characteristic rapid death of the pine, after infection, suggests involvement of phytotoxins (15), although the mechanism of wilting has been attributed to the mechanical destruction of parenchyma and epithelial cells by the invading nematodes (6,21,22). Further, as Mamiya (6) pointed out, the denaturation and death of these cells prior to invasion by the nematode also suggests the participation of toxins in the disease syndrome. This paper discusses progress toward identification of wilt-as-

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sociated toxins and clarification of their role in the syndrome.

Results of studies on pine wilt disease were comprehensively reviewed by Mamiya (7,8).

Survey of Toxic Compounds

A survey of phytotoxins in nematode-infected pines was conducted by Oku et al. (13–17) in Japan and by Bolla and colleagues (1,5,20) in the United States. An outline of the survey by Oku et al. is as follows. Three-year-old *P. thunbergii* or *P. densiflora* seedlings were inoculated with *B. xylophilus*. After wilting symptoms became visible, whole seedling shoots were cut into small pieces and immersed overnight in deionized water at room temperature to extract water soluble materials. The toxins were isolated from this extract by absorption on activated charcoal, elution with acetone, concentration, and thin layer chromatography (TLC). The TLC plates were sprayed with a suspension of conidia from *Cladosporium harbarum* (Persoon) Link and Fries in a nutrient solution. The plate was then incubated at 22 C for 2 days in a moist chamber, and the area on the TLC plate where growth of *C. harbarum* was inhibited was scratched off. The material was eluted from the support matrix and applied to cut shoots of 3-year-old *P. densiflora* seedlings. The seedlings wilted within 14 days. A similar extract was not obtained from healthy seedlings. Identical toxins were extracted from naturally infected pines, indicating that these materials were associated with pinewood nematode-infected pine.

Needles of inoculated seedlings were also surveyed for phytotoxins. A needle extract from inoculated and noninoculated 3-year-old *P. densiflora* seedlings was fractionated by high performance liquid chromatography (HPLC). Abnormal metabolites were collected by repeated HPLC and tested for toxicity on 1-month-old seedlings. At least five toxic metabolites were found in needles from diseased seedlings.

Using an extraction method comparable to that of Oku et al. (15), Bolla and colleagues (1,20) demonstrated accumulation of phytotoxins in *B. xylophilus*-infected *P. sylvestris*. In addition, they found phytotoxins in a $CHCl_3$-base extract of infected pine (3,20). Healthy wood did not contain phytotoxins (3,14,18). Experiments by both groups suggest the possibility that several phytotoxins are involved in pine wilt disease syndrome.

Identification of Phytotoxins

Isolation and characterization of wilt toxins were carried out both in Japan (13,23) and the United States (1,3).

Needles: Needles of naturally infected Japanese red pine were extracted with 70% methanol, and after solvent evaporation the extract was fractionated by silica gel column chromatography. The column eluates were assayed for toxicity using 1-month-old pine seedlings, and two fractions containing apparently different toxic compounds were obtained. Rechromatography and recrystallization of the active ingredient in these fractions gave two crystalline substances which were identified by physicochemical analyses and comparison with authentic samples as benzoic acid and catechol (23).

Wood: Branches of naturally infected Japanese red pine were chipped into small cubes, crushed, and extracted with 70% methanol. The extract was concentrated, and the residue was chromatographed on silica gel column. Three active substances (Compounds 1, 2, and 3) were obtained in crude form by repeated column chromatography from fractions that were toxic to 1-month-old seedlings. Compounds 1 and 2 were purified by recrystallization. Compound 3 was purified by repeated column chromatography to give a colorless oil. The absorbance spectra of Compound 1 was identical to that of a pure benzoic acid standard (14,23).

Pure dihydroconiferylalcohol was synthesized from ferulic acid via ethylferulate and coniferylalcohol. Physico-chemical characteristics of Compound 2 were compared to those of synthesized dihydroconiferylalcohol. Spectral characteristics of acet-

ylation products of both compounds were also compared. Based on the consensus of physico-chemical data, Compound 2 was identified as dihydroconiferylalcohol (14,23).

Spectral data from Compound 3 were compared to data obtained from pure 8-hydroxycarvotanacetone, chemically synthesized from carvone via 2,8-dihydroxy-6-p-menthene. The spectral data of synthesized 8-hydroxycarvotanacetone compared well with Compound 3 (14,23), but the absolute configuration of Compound 3 could not be determined because of insufficient quantity for determining the optical rotation.

As stated, Bolla et al. (1) and Shaheen et al. (20) obtained an extract from nematode-infected *P. sylvestris* comparable to that of Oku et al. (13). They confirmed that the toxic material could not be found in extracts from either uninfected *P. sylvestris* or trees killed by some means other than infection by the nematode. Application of the toxic extract to 1–2-year-old *P. sylvestris* seedlings caused wilting with symptoms comparable to those of natural infection.

Several components specific to infected wood (1) were obtained from an alkaline fraction of the extract ($CHCl_3$-base extract) of infected pine wood. The $CHCl_3$-base extraction method seems to have been devised to obtain phytotoxins different from those isolated by Oku et al. (18,23). Two major components of this phytotoxic extract have been identified by gas chromatography and mass spectral analysis as 10-hydroxyverbenone and carvone hydrate (8-hydroxycarvotanacetone) (3). Carvone hydrate was identified in laboratories both in Japan and the United States.

Thus, at least five phytotoxins are present in nematode infected pines—benzoic acid and catechol in needles, and benzoic acid, dihydroconiferylalcohol, 8-hydroxycarvotanacetone, and 10-hydroxyverbenone in wood (Fig. 1).

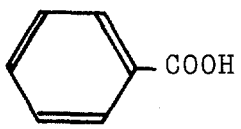
Phytotoxin Production

By incubating *B. xylophilus* with pine needle juice (50 g fresh needles were homogenized with 300 ml deionized water, filtered, and autoclaved), catechol and another toxin were detected on TLC as an agent of growth inhibition of *C. harbarum* (15–17). Catechol was shown to be produced in pine needle juice after incubation with bacteria associated with pinewood nematode (17). Catechol isolated from leaves of diseased pine may be a metabolite of the bacteria associated with the nematode. Another toxin produced in pine needle juice sometimes could be detected in the inoculated 3-year-old seedlings. This material has not yet been characterized chemically. Odani et al. (9,10) found that some cell wall degrading enzymes, especially a 1% solution of cellulase (Onozuka R-10), produced symptoms similar to those caused by *B. xylophilus*. Cellulase activity was present in the homogenate of pathogenic nematodes, and secretion of cellulase could be seen in the crawling trail of nematodes placed on carboxymethylcellulose-containing polyacrylamide gel (11,24).

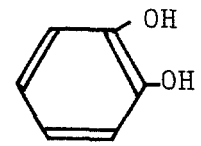
Toxicity of Phytotoxins

The toxicity of four phytotoxins isolated by Ueda et al. (23) was evaluated using 3-year-old *P. densiflora* seedlings. Among the toxins used, benzoic acid and catechol were pure reagents. Dihydroconiferylalcohol and the L- and D-isomers of 8-hydroxycarvotanacetone were synthesized by Ueda (Agricultural Chemicals Research Labs, Sankyo Co., Japan) (23). All compounds caused wilting and browning of needles when applied to cut shoots or stems. Benzoic acid (applied to a cut stem) and 8-hydroxycarvotanacetone (applied to a cut shoot) caused symptoms very similar to those induced by inoculation with *B. xylophilus*. The browning of needles was evident 4 days after application of 1,000 ppm of benzoic acid and 10 days after application of 50 mg of 8-hydroxycarvotanacetone.

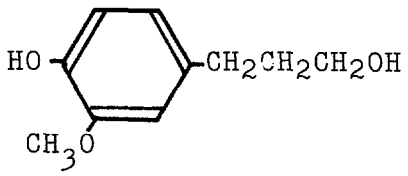
The toxicity of the four compounds was also tested on 1-month-old seedlings of four species of pine (18). These species showed different levels of tolerance to pine wilt disease. Seedlings of the most susceptible



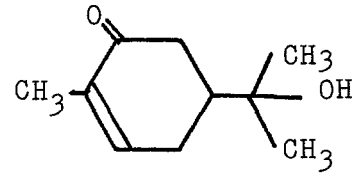
benzoic acid



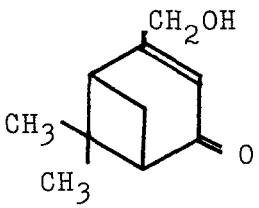
catechol



dihydroconiferylalcohol



8-hydroxycarvotanacetone
(carvone hydrate)



10-hydroxyverbenone

FIG. 1. Low molecular weight toxic metabolites isolated and identified from pine trees infected by *Bursaphelenchus xylophilus.*

species, *P. thunbergii,* were more sensitive than other species to benzoic acid, catechol, and dihydroconiferylalcohol; however, sensitivity differences were not significant enough to explain the susceptibility of each species to pinewood nematode.

A preliminary experiment using 3-year-old *P. thunbergii* seedlings indicated striking synergistic toxic effects between these compounds in combination. Application of a mixture of 5 mg L-8-hydroxycarvotanacetone, 5 mg dihydroconiferylalcohol, and 5 mg catechol to cut shoots caused severe browning and wilting of needles, whereas, when used singly, 50 mg L-8-hydroxycarvotanacetone, 50 mg dihydroconiferylalcohol, or 30 mg catechol were required to induce the same degree of symptom development. In regard to the synergistic effect, Bolla et al. (1) reported that the toxicity of 10-hydroxyverbenone was enhanced by several nontoxic abnormal metabolites unique to the nematode infected *P. sylvestris.*

Shaheen et al. (20) tested the extract from infected *P. sylvestris,* prepared by the charcoal absorbent method (modified method of Oku et al. [13]), against 2-year-old seedlings of various pine species. The response of seedlings was species, dose, and time dependent. Seedlings of the susceptible *P. sylvestris* were killed more quickly than those of resistant *P. strobus.* The immune *P. jeffreyi* seedling was not killed, even when

1,000 mg of the extract was applied. Similar results were obtained when $CHCl_3$-base extract was assayed on 2-year-old or 45-day-old seedlings (20).

The major components of the $CHCl_3$-base extract, 10-hydroxyverbenone and 8-hydroxycarvotanacetone (carvone hydrate), were synthesized and assayed for phytotoxicity by Bolla et al. (3). Both 10-hydroxyverbenone and 8-hydroxycarvotanacetone were phytotoxic, whereas their parent compounds and synthetic intermediates were not. The D-isomer of 8-hydroxycarvotanacetone was about 10 times more toxic to 45-day-old *P. sylvestris* than was the L-isomer (3). According to Oku et al. (18), the D-isomer was more toxic than the L-isomer to 1-month-old *P. rigida* seedlings, but the difference in toxicity was not as conspicuous as with *P. sylvestris*. Wilt symptoms induced by D-8-hydroxycarvotanacetone parallel those induced by the crude toxin extract or by nematode infection (3,23).

Odani et al. (11,12,24) stress the importance of the role of cellulases excreted by *B. xylophilus* in pathogenesis. They found that the early symptom, oleoresin leakage to the tracheid zone as assessed by the acid-fuchsin method, was induced by cell-wall disturbing factors and that *B. xylophilus* excrete cellulase as they migrate. They also found that the less pathogenic *B. mucronatus* also exuded cellulases. They attributed the difference in pathogenicity between *B. xylophilus* and *B. mucronatus* to the difference in cellulase isozymes and in the rate of reproduction of each nematode species in pine trees. Acceleration of ethylene production, one of the symptoms of pine wilt disease, was also attributed to the activity of cellulase excreted by *B. xylophilus* (10).

Effect of Phytotoxins

Bolla et al. (2) found that a $CHCl_3$-base extract from nematode-infected *P. sylvestris* inhibited the growth of the blue-stain fungus, *Ceratocystis ips* Davidson, and caused temporary paralysis of *B. xylophilus* in vitro. As a long-term effect, they found that the crude toxin suppressed the multiplication of *B. xylophilus*. Thus they suggested that the crude toxin had limited phytoalexin effects. Oku et al. (14,18) have also examined the effect of four toxic compounds from nematode-infected pine on nematode multiplication. A concentration of each phytotoxin solution was added aseptically to PDA medium on which *Botrytis cinerea* Persoon was cultured for 2 weeks. The compounds had no inhibitory effect on growth of *B. cinerea* at concentrations tested. Specimens of *B. xylophilus* were placed onto the cultures and incubated at 22 C, and the number of nematodes was determined 15 days later. The L-isomer of 8-hydroxycarvotanacetone inhibited multiplication of nematodes at concentrations greater than 30 μg/ml, whereas the D-isomer was not effective even at 100 μg/ml. Dihydroconiferylalcohol inhibited nematode multiplication at 10 μg/ml. This property can be referred to as phytoalexin activity, as suggested by Bolla et al. (1). Benzoic acid and catechol, which showed high toxicity to pine seedlings, did not inhibit nematode multiplication at 100 μg/ml. All compounds stimulated nematode multiplication at lower concentrations: 8-hydroxycarvotanacetone at 3 μg/ml, benzoic acid at 10–30 μg/ml, dihydroconiferylalcohol at 3 μg/ml, and catechol at 10–30 μg/ml.

Toxin Accumulation

P. densiflora (15–25 years old) were inoculated with ca. 40,000 *B. xylophilus* per tree, and the concentrations of the four compounds which accumulated in wood were determined periodically by HPLC. Pure samples were used as standards (18). The four toxic compounds were not detected in healthy wood. Both benzoic acid and 8-hydroxycarvotanacetone became detectable after 20 days in nematode inoculated trees, the time when reduction of oleoresin exudation occurred. The content of benzoic acid increased with time and reached more than 300 μg/g dry wood by 50 days. The other metabolites did not show this particular tendency. This might

be due to the fluctuation in physiological conditions among inoculated trees.

Conclusion

Toxic compounds accumulate in *B. xylophilus*-infected pines. An extract of phytotoxin from *B. xylophilus*-infected pines shows strong toxicity to susceptible pine species but not to tolerant or resistant species, suggesting that phytotoxin might be involved in the pine wilt disease syndrome. Several toxins, including catechol and benzoic acid, occur in needles of *P. thunbergii* and *P. densiflora*. Extracts of infected wood of these pine species contain the toxins, benzoic acid, dihydroconiferylalcohol, and 8-hydroxycarvotanacetone. Infected *P. sylvestris* contains 8-hydroxycarvotanacetone (carvone hydrate) and 10-hydroxyverbenone. These materials show a synergistic toxic effect. In addition, toxicity of 10-hydroxyverbenone is enhanced by adding the nontoxic abnormal metabolites unique to nematode infected *P. sylvestris*. Some of the toxins inhibit the multiplication of *B. xylophilus* at higher concentrations and stimulate multiplication at lower concentrations. These toxins actually accumulate at biologically active levels in wood of infected pine, and this further supports a role of phytotoxins in the pathogenesis of this disease complex.

Cellulase excreted by pathogenic nematodes is also toxic to pines. Further, as Oku (14) pointed out, the other unidentified toxins accumulate at an early stage of infection. Thus, the actual mechanism of rapid wilting in this disease seems to be very complicated, not as simple as in Dutch elm disease.

Further, benzoic acid and catechol show higher toxicity against pine seedling than does dihydroconiferylalcohol or 8-hydroxycarvotanacetone, and they are less toxic to nematodes than the latter two compounds. The D-isomer of 8-hydroxycarvotanacetone is more toxic to pine seedlings than L-isomer, but the toxicity to nematode multiplication is completely reversed. These differences of toxicity against nematode and pine seedling indicate the difference of mode of action against both organisms.

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