Effect of Copper Sulfate and Lead Acetate on Infection of Pines with *Bursaphelenchus xylophilus*[1]

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Abstract: Treatment of 3-year-old Scots, white, and Austrian pine seedlings with copper sulfate or lead acetate significantly affected energy homeostasis and oleoresin production in the seedlings but did not induce wilting of the seedlings. Inoculation of copper sulfate-treated or lead acetate-treated white, Scots, and Austrian pine seedlings with the white pine specific pathotype of *Bursaphelenchus xylophilus*, VPSt-1, caused a significant increase in oleoresin production, stressed energy homeostasis, and induced rapid wilting of the seedlings. Scots pine lost tolerance and Austrian pine lost resistance to VPSt-1 after the seedlings were treated with either copper sulfate or lead acetate. These results suggest that environmental pollution may significantly affect susceptibility of pines to *B. xylophilus* and may have a role in establishment of this nematode in uninfested areas.

Key words: *Bursaphelenchus xylophilus*, copper, heavy metal, lead, pinewood nematode, pine wilt, *Pinus nigra*, *P. strobus*, *P. sylvestris*, resistance, susceptibility.

Pine wilt disease, caused by pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, is epidemic in Japan (19). Although this nematode is widespread in North America, wilt symptoms vary and wilting occurs mainly in pines planted outside their natural range (1,2,6,28). Pine wilt disease is prevalent in the lower midwestern and southeastern United States; in other areas the nematode invades pines stressed by biotic or abiotic factors (2,6,20,28). Variable susceptibility of pine species to *B. xylophilus* is due in part to evolution of host-specific pathotypes (1,4,28). Scots pine (*Pinus sylvestris* L.) is susceptible to pathotype MPSy-1 from Scots pine in Missouri; white (*P. strobus* L.) and Austrian (*P. nigra* Alt.) pine are tolerant, and loblolly pine (*P. taeda* L.) is resistant. White pine is susceptible to pathotype VPSt-1 from white pine in Vermont, Scots pine is tolerant, and Austrian and loblolly pine are resistant (4).

Stress induces a hypersensitive reaction in conifers. This reaction includes synthesis of phenols and terpenes, mobilization of carbohydrate reserves, and synthesis of phytoalexins (7,8,11,13,15,18,20). These changes are accompanied by decreased photosynthesis, transpiration, and tree vigor and increased susceptibility to a second stress (18,22). Following inoculation of tolerant or resistant pines with *B. xylophilus*, carbohydrate concentration decreases and monoterpene oleoresin concentration increases, but as the nematode population decreases these parameters return to normal. Carbohydrate concentration continues to decrease and monoterpene oleoresin concentration continues to increase until wilting in susceptible pines inoculated with pinewood nematode (4). Compounding acid precipitation stress with stress of pinewood nematode inoculation causes the loss of tolerance of Scots pine to the white pine specific pathotype, VPSt-1 (5).

Heavy metal pollution is prominent in mining, industrial, and urban areas where copper and lead reach 200 ng/m³ and 18 µg/m³ of air (9,10,17,21,23,24). Both cop-

per and lead precipitate from rainfall, snow, or dust onto forest vegetation (7,13,15, 17,23). In the northern United States, lead accumulates at 305 g/ha each year (24). Copper accumulates to 14–65 ppm/year in jack pine (*P. banksiana* Lamb.) needles (13). Lead concentration in growing trees in urban areas exceeds that in trees growing in geologic lead deposits (25). Plants must be tolerant to heavy metal to survive around copper and lead mining sites (17). Copper and lead accumulation in trees alters root growth, induces changes in the structural integrity of leaves, inhibits plant productivity, interferes with ion balance, affects mitosis, activates metabolic enzymes, and stresses energy homeostasis (7,17,25). Photosynthesis and transpiration in loblolly pine are reduced when lead exceeds 60 μg/g dry mass (16). Synergistic interaction of atmospheric pollutants is linked to reduced resistance to pathogens, erosion of protective waxes from leaf surfaces, changes in oleoresin composition, and altered water balance in conifers (14,23).

The objective of this research was to determine the effects of copper sulfate and lead acetate on susceptibility of pines to pinewood nematode to determine if environmental stress might have an influence on introduction of PWN into noninfested areas. This becomes particularly important when considering the potential for introduction of *B. xylophilus* into Scandinavia (27).

MATERIALS AND METHODS

Nematode culture and seedling inoculation: *B. xylophilus* pathotype VPSt-1 (4) was maintained by serial passage on cultures of *Botrytis cinerea*, Pers. ex Fr. grown on PDA. At 3-month intervals, white pine seedlings were inoculated with axenized VPSt-1. Nematodes recovered from these seedlings were inoculated into fungal cultures to maintain host specificity and virulence of this pathotype (4).

Three-year-old white pine, Scots pine, and Austrian pine seedlings were transplanted 6 months before use into 30-cm-d

pots filled with a soil mixture of equal parts peat moss, vermiculite, perlite, and sand. The seedlings were maintained in the greenhouse at an average daily temperature of 25 ± 5 C and an average relative humidity of 75 ± 12% on a 12-hour light–dark cycle.

Copper sulfate and lead acetate treatment: One hundred twenty seedlings of each species were sprayed twice weekly with deionized water containing either 500 μM copper sulfate or 500 μM lead acetate until the leaves were wetted to the drip point. These concentrations simulate copper and lead concentrations in mining and urban industrial areas (7,9,10,17,21,23,24). Controls, 120 seedlings of each species, were sprayed with deionized water only. One month later, 60 heavy metal-treated and 60 control seedlings of each species were inoculated with 5,000 VPSt-1. The remaining seedlings were treated with distilled water without nematodes. Copper sulfate, lead acetate, or water treatment was continued throughout the course of the experiment. Experimental groups were: copper sulfate-treated or lead acetate-treated seedlings; copper sulfate-treated or lead acetate-treated, inoculated seedlings; unstressed seedlings; and unstressed, inoculated seedlings of each species.

Symptom development: The seedlings in each experimental group were observed daily for leaf chlorosis and cessation of oleoresin flow. At 15-day intervals after inoculation, 15 seedlings of each species from each group were harvested. The soil was carefully washed away from the roots with a stream of water.

Water relations: Three seedlings from each group were incubated in 200 ml of 0.05% aqueous acid fuchsin at 30 C for 10 hours with continuous light (3). The seedlings then were sectioned (2-mm thick) randomly from the base to the apex of the stem. Sections were observed microscopically, and the percentage of stem cross sectional area excluding dye was calculated as a function of experimental treatment and time after inoculation. Fresh weight: dry weight ratios of three 5-cm-long sections

TABLE 1. Copper and lead concentration in pine seedlings after 30 to 90 days of treatment with copper sulfate or lead acetate.

	Metal concentration (μg/g ash wt)			
	0 days	30 days	60 days	90 days
Copper				
White	< 0.1	106.5 ± 30.2	245.1 ± 40.8	566.0 ± 145.2
Scots	< 0.1	284.2 ± 13.2	369.5 ± 17.3	492.0 ± 17.2
Austrian	< 0.1	253.1 ± 9.8	329.1 ± 12.8	144.1 ± 15.0
Lead				
White	< 3.0	3,305 ± 110	3,966 ± 587	4,909 ± 103
Scots	< 3.0	1,929 ± 612	2,508 ± 794	3,257 ± 161
Austrian	< 3.0	1,730 ± 99	2,250 ± 128	3,356 ± 125

Values are reported as mean ash weight (μg/g) of sample ± standard error. Leaves from four different seedlings were sampled in triplicate at each experimental point. Zero day is the time at which the first treatment was applied.

from five seedlings of each species in each experimental group were determined gravimetrically (4,5).

Oleoresin flow: Changes in oleoresin production were assessed by estimating the accumulation of oleoresin during a 15-minute period at a cut on the stem of a seedling. The accumulation was rated from 0 to +4 to indicate no resin flow to copious resin flow (5). Five seedlings of each species from each experimental group were used at each experimental point.

Carbohydrate analysis: Concentrations of reducing and nonreducing carbohydrates were determined in sections of infected seedling wood after extraction of the nematodes (3) and in sections from noninoculated seedlings by reaction with anthrone (12). Five seedlings from each group were assayed in triplicate.

Nematode populations: A 2-cm-long section was cut from the inoculation site, and a 2-cm-long section was cut from each side of this site. The sections were chipped and the nematodes extracted (3). The number of nematodes was scored in 10 separate aliquots of each sample. Five seedlings of each species were assayed at each experimental point.

Copper and lead incorporation: Needles (ca. 5 g) were minced, dried overnight at 100 C, weighed, and ashed at 450 C. The ashed material was extracted with aqua regia for 1 hour at 65 C, the extracts were evaporated to dryness, and 1 ml concentrated HNO_3 was added. After drying, the resi-

due was suspended in 20% HNO_3 and debris was removed by filtration. The filtrate was adjusted to 1% HNO_3 with deionized water, and an aliquot was assayed for copper or lead by atomic absorption spectroscopy (26). The lamp was adjusted to 5 mA at 283.3 nm for lead analysis and to 3 mA at 324.8 nm for copper. To assay copper and lead in soil, 250 mg soil was extracted with aqua regia (26). After drying, 1 ml HNO_3 plus 1 ml concentrated HCl was added to the samples; these were then heated to 85 C for 30 minutes in a water bath. Samples were cooled to room temperature, diluted to 20% HNO_3, and allowed to stand overnight so debris could settle out. A 5-ml sample of the supernatant was diluted to 1% HNO_3 with deionized water, and the concentration of copper and lead was analyzed (26).

RESULTS

Copper and lead: Copper increased in leaves of all copper sulfate-treated seedlings and lead increased in the lead acetate-treated seedlings (Table 1). Lead accumulated to larger concentrations than did copper. There was less than 0.1 μg copper/g ash weight of needles and less than 3.0 μg lead/g ash weight of needles in control seedlings sprayed with distilled water only. Concentrations of lead in the soil in which lead-treated seedlings were grown were less than 3.0 μg/g ash weight, and copper in the soil from copper-treated seedlings ranged from 0.13 to 0.78 μg/g

TABLE 2. Oleoresin flow in seedlings treated with copper sulfate or lead acetate and inoculated with 5,000 VPSt-1 or not inoculated.

| | Day after treatment | Relative oleoresin flow | | | | | |
| | | Inoculated | | | Noninoculated | | |
		Control	Copper	Lead	Control	Copper	Lead
White	15	+3	+4	+4	+3	+4	+4
	30	+2	+4	+4	+3	+4	+4
	45	0	+1	+2	+3	+4	+3
	60	0	0/+1	0	+3	+3	+4
Scots	15	+2	+2	+4	+3	+4	+3
	30	+2	+2	+3	+3	+4	+3
	45	+3	+3	0	+3	+3	+3
	60	+3	+1	0	+3	+3	+3
Austrian	15	+2	+3	+3	+3	+3	+1
	30	+2	+3	+3	+3	+3	+2
	45	+3	0	+2	+3	+4	+2
	60	+3	0	0	+3	+3	+2

Values are based on oleoresin flow at a cut on the stem at harvest as follows: +4, copious, greater than control; +3, good, equal to control; +2, moderate, less than control; +1, poor, much less than control; 0, no resin.

ash weight of the sample. Lead and copper were not detectable in soil from untreated seedlings.

Rate of wilting: Control white pine seedlings wilted by day 35 after inoculation with VPSt-1, whereas control Scots or Austrian pine seedlings inoculated with VPSt-1 did not wilt. Copper sulfate or lead acetate alone did not induce wilting of the seedlings. All seedlings treated with copper sulfate or lead acetate and inoculated with VPSt-1 wilted. All copper sulfate-treated white pine seedlings wilted by day 40 after inoculation with VPSt-1, and all lead acetate-treated white pine seedlings wilted by day 60 after inoculation. By day 60 after VPSt-1 inoculation, 50% of the copper sulfate-treated Scots pine seedlings wilted, and all lead acetate-treated Scots pine seedlings wilted by day 45 after VPSt-1 inoculation. All copper sulfate-treated Austrian pine seedlings wilted by day 40 after inoculation with VPSt-1, whereas lead acetate-treated Austrian pine seedlings wilted by day 60. Leaf chlorosis began in control white pine within 5 days of VPSt-1 inoculation, but in inoculated copper sulfate-treated or lead acetate-treated seedlings leaf chlorosis did not begin until 5 days before the seedling wilted.

Oleoresin flow: Oleoresin production at the site of a wound increased in copper sulfate-treated and lead acetate-treated white and Scots pine seedlings and in copper sulfate-treated Austrian pine seedlings, whereas it decreased in lead acetate-treated Austrian pine seedlings (Table 2). Oleoresin flow decreased transiently in control Scots and Austrian pine seedlings inoculated with VPSt-1, whereas it continued through wilting of control white pine seedlings inoculated with VPSt-1. Oleoresin flow continued in all VPSt-1-inoculated copper sulfate-treated or lead acetate-treated seedlings until immediately before wilting (Table 2).

Water relations: Copper sulfate and lead acetate treatment alone did not alter dry weight of the seedlings. Water content of the metal-treated seedlings was 60–68% of total mass through 90 days of treatment. Water content of VPSt-1-inoculated, control Scots and Austrian pine seedlings decreased to approximately 48% of the total mass by day 30 after inoculation and then returned to control levels. The water content in VPSt-1-inoculated control white pine seedlings decreased gradually until wilting when it was 17 ± 4% of the seedling mass. Copper sulfate-treated or lead acetate-treated seedlings inoculated with VPSt-1 did not dehydrate. The water con-

tent of these seedlings was 60–68% of the total mass.

Water movement was inhibited in VPSt-1-inoculated control white pine seedlings in all VPSt-1-inoculated, copper sulfate-treated and lead acetate-treated seedlings. In control white pine seedlings inoculated with VPSt-1, water was excluded from 64 ± 10% of the stem cross sectional area 30 days after inoculation and from 92 ± 12% at the time of wilting. Water was not transported into needles of these seedlings 30 days after inoculation. Water was excluded from 19 ± 6% and 24 ± 4% of the stem cross sectional area of VPSt-1-inoculated control Scots and Austrian pine seedlings 30 days after inoculation, but by day 60 the area of exclusion was less than 2.0 ± 1% of the stem cross sectional area. Water transport was excluded from 16 to 22% of the stem cross sectional area of uninoculated copper sulfate-treated and lead acetate-treated white, Scots, and Austrian pine seedlings 30 days after the start of treatment. This did not change during the next 60 days. By day 45 after VPSt-1 inoculation of copper sulfate-treated Scots and white pine seedlings, water was excluded from 58 ± 7% of the stem cross sectional area. There was no water movement in lead acetate-treated Scots pine inoculated with VPSt-1, and water was excluded from 83 ± 12% of the stem cross sectional area of treated white pine seedlings by day 30 after inoculation. No water movement was detected in copper sulfate-treated and lead acetate-treated Austrian pine seedlings by day 45 after inoculation with VPSt-1.

Reducing carbohydrate: Reducing carbohydrates increased to 140 ± 12% of the uninoculated control by day 30 after first treatment of white pine seedlings with lead acetate, declined to approximately 95% of the uninoculated control by day 60, and increased slightly by day 90. Copper sulfate treatment had no significant effect on reducing carbohydrates in white pine seedlings (Fig. 1). In copper sulfate-treated Scots pine seedlings, reducing carbohydrate was 123 ± 14% of the control at day 60 after

first treatment then decreased slightly by day 90. Treating Scots pine seedlings with lead acetate had no significant effect on reducing carbohydrate concentration. After 30 days of treatment of Austrian pine with lead acetate or copper sulfate, reducing carbohydrate was about 60% of the control. It did not change over the next 60 days in the lead acetate-treated Austrian pine seedlings. However, when Austrian pine seedlings were treated with copper sulfate, reducing carbohydrate decreased to approximately 46% of the control by day 45 after first treatment and then increased to control levels by day 90 (Fig. 1). Reducing carbohydrate concentration decreased rapidly following VPSt-1 inoculation of copper sulfate-treated and lead acetate-treated or control white pine seedlings. It also decreased rapidly in inoculated copper sulfate-treated or lead acetate-treated Scots and Austrian pine seedlings (Fig. 1). Lead acetate-treated Scots pine seedlings had no detectable concentration of reducing carbohydrates 60 days after VPSt-1 inoculation.

Nonreducing carbohydrates: Nonreducing carbohydrate concentration in white pine was not affected by copper sulfate and lead acetate treatment alone; however, 15 days after VPSt-1 inoculation it had decreased significantly. In copper sulfate-treated Scots pine seedlings, nonreducing carbohydrate was 138 ± 14% of the control by day 30 after first treatment. The concentration decreased sharply from day 45 to day 75 and then increased slightly. Nonreducing carbohydrates did not change significantly in lead acetate-treated Scots pine seedlings until day 75 after first treatment. Nonreducing carbohydrates decreased immediately after VPSt-1 inoculation of control Scots pine seedlings but then increased to control levels (Fig. 1). When Scots pine seedlings were treated with copper sulfate or lead acetate and inoculated with VPSt-1, there was a sharp decrease in nonreducing carbohydrate. The concentration of nonreducing carbohydrate in copper sulfate-treated Austrian pine seedlings decreased by 45 days after first treatment then in-
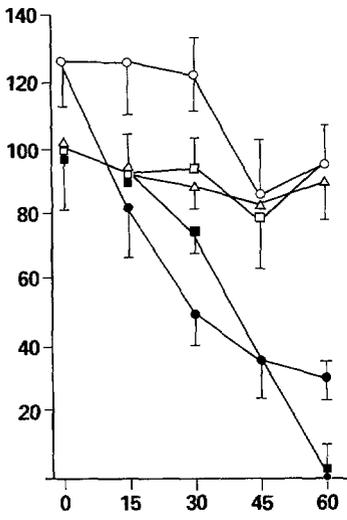
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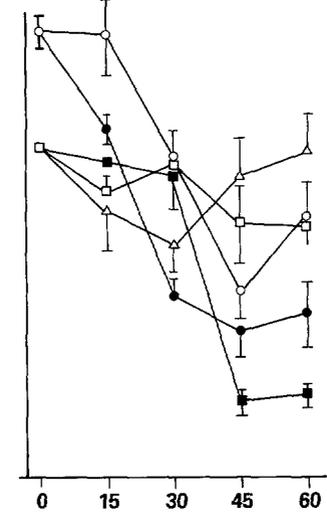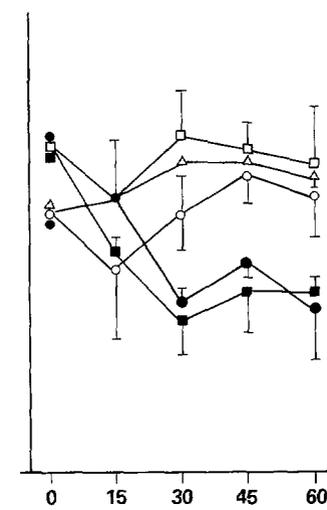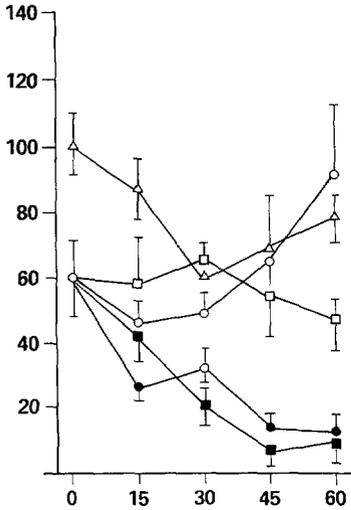**Non-Reducing**

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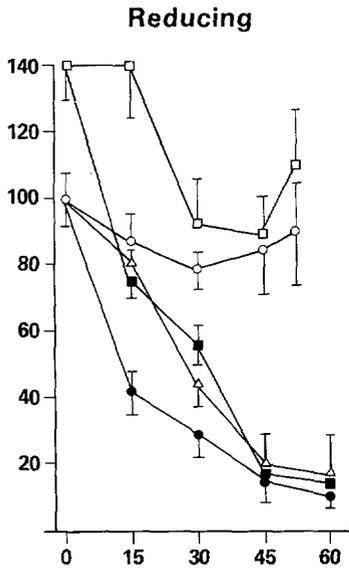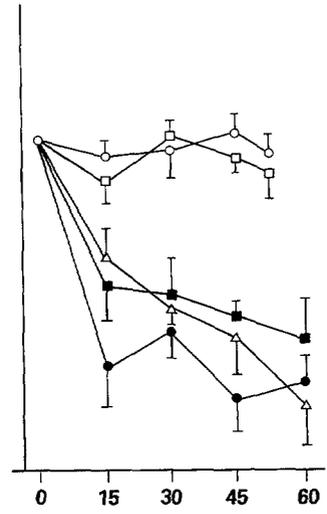
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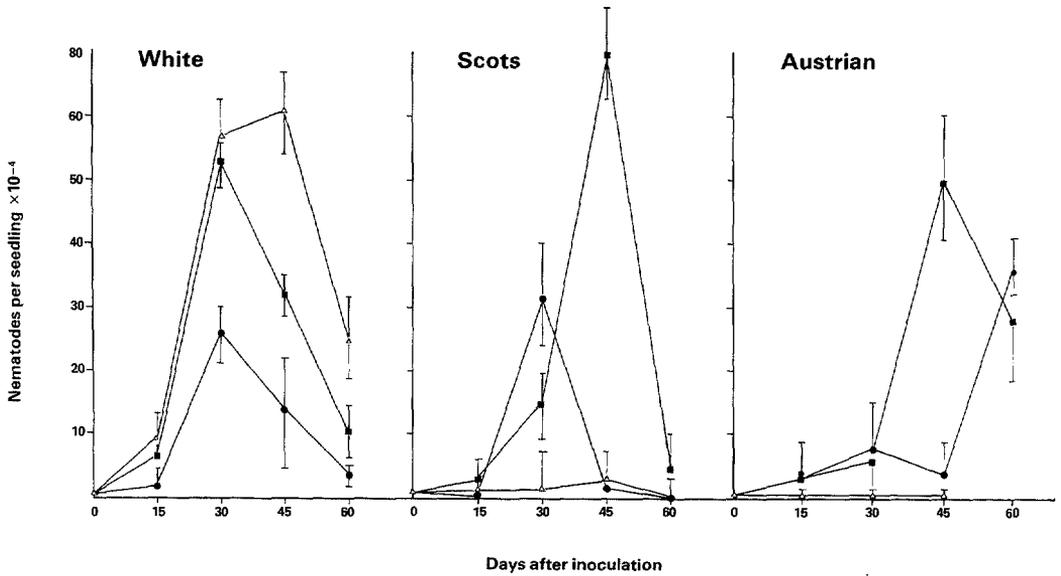
Days after inoculation

FIG. 2. Changes in VPSt-1 populations in inoculated 3-year-old pine seedlings treated with copper sulfate or lead acetate or untreated. ● = copper-treated seedlings, ■ = lead-treated seedlings, and △ = untreated seedlings. Values reported as mean ± standard error, N = 20.

creased between day 60 and day 90. When copper sulfate-treated Austrian pine seedlings were inoculated with VPSt-1, nonreducing carbohydrate concentration decreased through day 60 after inoculation. Lead acetate alone had little effect on nonreducing carbohydrate concentration in Austrian pine seedlings, but following VPSt-1 inoculation the concentration decreased rapidly. Nonreducing carbohydrate concentration in inoculated control Austrian pine seedlings was less at day 15 after inoculation but then returned to control levels (Fig. 1).

Nematode population development: Maximum populations of VPSt-1 developed 30–45 days after inoculation of control white pine seedlings. Maximum VPSt-1 populations developed 45 days after inoculation of control Scots pine seedlings, but these were 0.027× the population in white pine.

Maximum VPSt-1 populations in copper sulfate-treated and lead acetate-treated Scots pine seedlings were 20 and 68× greater, respectively, than the population in inoculated control seedlings (Fig. 2). Whereas VPSt-1 did not reproduce in control Austrian pine seedlings, copper sulfate-treated or lead acetate-treated seedlings had high populations. Maximum populations were reached 45 days after inoculation in lead acetate-treated seedlings and 60 days after inoculation of copper sulfate-treated Austrian pine seedlings (Fig. 2).

DISCUSSION

Copper sulfate and lead acetate treatment of pine seedlings affected the physiology of Scots, white, and Austrian pine seedlings and induced loss of tolerance of Scots pine and resistance of Austrian pine

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FIG. 1. Changes in concentration of reducing and nonreducing carbohydrates in 3-year-old pine seedlings exposed to copper sulfate or lead acetate and inoculated with *Bursaphelenchus xylophilus* pathotype VPSt-1. A) White pine. B) Scots pine. C) Austrian pine. ○ = copper sulfate treatment; ● = copper sulfate treatment plus inoculation with 5,000 VPSt-1; □ = lead acetate treatment; ■ = lead acetate treatment plus inoculation with 5,000 VPSt-1; and △ = untreated seedlings inoculated with 5,000 VPSt-1. Data are reported as carbohydrate concentration in experimental seedlings divided by concentration in control seedlings ×100. Mean ± standard error, N = 20.

TABLE 3. Summary of the effect of copper sulfate (Cu) and lead acetate (Pb) on inoculated seedlings of three species of pine.

	Death		Oleoresin flow		Carbohydrate content	
	Cu	Pb	Cu	Pb	Cu	Pb
White	−	−	+	+	great reduction	
Scots	±	+	− until death	+ until death	great reduction	
Austrian	+	+	±	+ until death	great reduction	

− = no effect; ± = slight increase; and + = substantial increase, compared with untreated control.

to VPSt-1 (Table 3). Loss of tolerance or resistance induced by heavy metals might be expected, since both biotic and abiotic stress affect the ability of conifers to respond to a second stress (6,8,18). Acid precipitation alters the physiology of Scots pine seedlings and they lose tolerance to VPSt-1 (5). Heavy metals compromise energy homeostasis in plants by altering photosynthesis and photosynthetic electron transport (7,14,16). Therefore, loss of tolerance and resistance may be due to the physiological state of the pine at the time of inoculation with *B. xylophilus*. Stressed pines produce a hypersensitive reaction that draws upon photosynthate and stored energy reserves for synthesis of oleoresins and phenolic compounds (13,14,20). Reduction in available carbohydrate reserves reduces the ability of the pines to respond to a second stress. Pines exposed to biotic or abiotic stress that negatively affects photosynthesis and energy production are, therefore, at a distinct disadvantage when inoculated with *B. xylophilus*, since inoculation of pines with this nematode also compromises photosynthesis and energy storage (3,5,19). Because leaf chlorosis, decreased transpiration, and inhibition of water relations accompany *B. xylophilus* infection of susceptible pines, carbon substrates available for energy production, storage or biosynthesis are limited (19). Increases in reducing carbohydrates in white pine seedlings and in reducing and nonreducing carbohydrates in Scots pine seedlings by day 30 after first treatment with the heavy metals suggest mobilization of carbohydrate reserves. There is no correlation between seedling dehydration and inhibition of water movement in the treat-

ed seedlings inoculated with VPSt-1. The tissues were sponge-like and water saturated, suggesting that retention of water might be caused by heavy metal-induced inhibition of transpiration (5,7,15,16,23).

Atmospheric pollution is a suggested cause of decline in coniferous forests in the eastern United States and Europe (7,9,10,13,17,23). Forest decline may result from synergistic interactions of pollutants or of pollutants with biotic pathogens (14,16,17,23). Atmospheric lead is alkylated by acid precipitation and is extremely toxic to plants (9,16). Loss of tolerance or resistance of pine seedlings to *B. xylophilus* following acid precipitation (5) or heavy metal stress suggests that pines planted in heavily polluted areas where *B. xylophilus* is present may affect the stability and composition of forest ecosystems. This is particularly important when considering possible introduction of *B. xylophilus* into areas where pines grow under conditions of acid precipitation, drought, heavy metal pollution, and pathogen stress (27). Our results must be viewed cautiously because they were obtained with seedlings, and similar interactions may or may not occur in a forest ecosystem.

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