Effect of Simulated Acid Rain on *Bursaphelenchus xylophilus* Infection of Pine Seedlings[1]

R. I. Bolla and K. Fitzsimmons[2]

Abstract: White, Scots, and Austrian 3-year-old pine seedlings were treated with conditions simulating acid rain and inoculated with the white pine specific pathotype of *Bursaphelenchus xylophilus*, VPSt-1. Oleoresin concentration increased slightly and carbohydrate concentration decreased in all seedlings treated with simulated acid rain (SAR). The changes were significantly increased after inoculation of SAR-treated white and Scots pine seedlings with VPSt-1. Wilting was delayed and nematode reproduction decreased in SAR-treated white pine seedlings inoculated with VPSt-1. SAR-treated Austrian pine seedlings were resistant to VPSt-1, but SAR-treated Scots pine seedlings lost tolerance to VPSt-1 and wilted 50–60 days after inoculation.

Key words: abiotic stress, acid precipitation, acid rain, Austrian pine, *Bursaphelenchus xylophilus*, carbohydrate, host response, nematode, oleoresin, pathology, pathotype, pinewood nematode, pine wilt disease, *Pinus nigra, P. strobus, P. sylvestris,* resistance, Scots pine, susceptibility, white pine.

Acid rain is a common environmental pollutant in developed nations where burning of fossil fuel releases sulfur and nitrogen oxides into the atmosphere (10,15). In northern Europe and the eastern United States, the acidity of rain and snow is pH 4.0 or greater (15). Effects of precipitation of atmospheric sulfur and nitrogen on plant growth, survival, and resistance to stress is difficult to demonstrate and is probably complex (10). Conifers may be resistant to acute effects of low pH rains; however, Johnson et al. (13) demonstrated a positive statistical correlation between precipitation pH and decreased radial growth of pitch (*Pinus rigida* Mill.), shortleaf (*P. echinata* Mill.), and loblolly (*P. taeda* L.) pines in plantations and native stands. Productivity of other pine species increases when the acidity of precipitation increases from pH 4.0 to pH 3.0 (16,20,26). Germination and survival of white (*P. strobus* L.) and Scots (*P. sylvestris* L.) pine are not affected at pH 4.0 to 3.0; however, needle length of Scots pine is decreased and that of white pine is increased. Scots pine growth is inhibited at acidity greater than pH 3.6, whereas white pine growth is stimulated (10,20). The positive effect of acid rain on pine productivity may relate to increased nitrogen and available nutrient cations in acid weathered soils (16,26,29). Acid rain reduces mycorrhizal formation on roots of forest trees (23,27). Because of the importance of mycorrhizae for growth, development, general physiology, disease resistance, and survival of pines, acid rain may seriously affect disease resistance (20,23,28,29). Stroo and Alexander (29) suggest that the effect of acid rain on mycorrhizal formation is a plant-mediated process involving decreased allocation of carbohydrates to the roots. Although not clearly defined, an increase in the acidity of rainfall apparently stresses pines.

Pines initiate a hypersensitive response to biotic and abiotic stress (14,21). This response involves increased production of ethylene, decreased rates of transpiration and photosynthesis, biosynthesis of unique stress phytochemicals, including terpenes and phenols, and changes in susceptibility to pathogens (8,9,19,24,25).

Bursaphelenchus xylophilus (Steiner and Buhrer) Nickle, the causal agent of pine wilt disease, is widespread in North America, but incidence of the nematode is not always associated with pine wilt disease (1,2,18,30). This nematode is most patho-

genic to exotic pines planted outside their native range and to drought-stressed or overwatered pines, and it accelerates decline of pines infected with other pathogens or attacked by insects (1,2,7,30). *B. xylophilus* infection of susceptible pines induces decreased water conduction, oleoresin flow and carbohydrate concentration, biosynthesis of infection-unique phytochemicals, and leaf chlorosis (3–5,17,18,25). Myers (19) suggested that pines initiate a hypersensitive response to *B. xylophilus,* and that this response includes tissue lignification and increased synthesis of phenolic compounds and terpenes. Metabolism is altered when a plant initiates a hypersensitive response; photosynthate and storage carbohydrates become limited, and response to a second stress is compromised (9,15).

Acid precipitation occurs in northern Europe where imported pine products contaminated with *B. xylophilus* have been found and where this nematode may become a threat to stability of the forest ecosystem (22). The goal of this research was to investigate whether pine seedlings exposed to simulated acid rain (SAR) undergo physiological changes that alter susceptibility to a host-specific pathotype of *B. xylophilus.*

MATERIALS AND METHODS

Nematode culture and seedling inoculation: The white pine specific pathotype, VPSt-1, (5) was maintained by serial passage on cultures of *Botrytis cinerea* Pers. ex Fr. on PDA. White pine seedlings were infected with VPSt-1 at 3-month intervals, and the nematodes recovered from these seedlings were used to reestablish cultures to assure continued host specificity and virulence of the nematode population (6). VPSt-1 recovered from fungal cultures and treated with antibiotics (5) were used for inoculation. Bacterial and fungal contamination of nematodes for inoculation was determined by standard microbiological procedures, and any contaminated populations were discarded.

Three-year-old seedlings of white pine, a susceptible host for VPSt-1, Scots pine, a tolerant species, and Austrian pine (*P. nigra* Alt.), a resistant species, were transplanted into 30-cm-d pots filled with a mixture of equal parts peat moss, vermiculite, perlite, and sand in the greenhouse 6 months before use. The seedlings were maintained at an average daily temperature of 24.5 ± 4.5 C and an average relative humidity of $75 \pm 12\%$ on a 12-hour light–dark cycle.

Simulated acid rain conditions: Seedlings (120 of each species) were sprayed twice weekly with 0.1 N H_2SO_4 in deionized water (pH 3.6) until leaves were saturated to the drip point. This pH was used because it is within the pH range in which conifer growth is affected and it is a low mean pH for precipitation in northern Europe and the eastern United States (15). Control seedlings (120 of each species) were sprayed with deionized water. One month later 60 SAR-treated and 60 control seedlings of each species were inoculated with 5,000 *B. xylophilus* VPSt-1 (19). The remaining seedlings were sham inoculated with distilled water. SAR or water treatment was continued throughout the course of the experiment. Experimental groups were SAR-treated seedlings; SAR-treated, inoculated seedlings; unstressed seedlings; and unstressed, inoculated seedlings for each pine species.

Symptom development: Seedlings were observed daily for leaf chlorosis cessation of oleoresin flow and inhibition of bud development. At 15-day intervals after inoculation, 30 seedlings of each species from each group were harvested by carefully washing the soil away from the roots with a stream of water.

Water movement in conducting tissues was determined by incubating three seedlings of each species from each group in 200 ml of 0.05% aqueous acid fuchsin at 30 C for 10 hours with continuous light (25). The seedlings then were sectioned (0.2 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.

Rate of seedling dehydration was estimated gravimetrically by determining the fresh weight of 5-cm-long stem sections and then freezing the sections in liquid N_2 for lyophilization. The dried sections were weighed, and the fresh weight–dry weight ratio was calculated (5). Ten seedlings of each species from each group were used for each experimental point.

Oleoresin flow and concentration: Oleoresin flow was estimated at harvest by the amount of resin that accumulated at a cut on the stem of the seedling during a 15-minute period. The accumulation of oleoresin was rated from 0 to +4 to indicate no flow to copious flow. Five seedlings of each species from each treatment group were used at each experimental point.

Twenty grams fresh weight of seedling wood and leaves were extracted by the $CHCl_3$-base procedure (5). The final $CHCl_3$ fraction was evaporated to dryness, weighed, and resuspended in $CHCl_3$ for analysis by thin layer chromatography (5). Chromatography was on 250-μm-thick silica gel plates developed with either ethylacetate or $CHCl_3 : CH_3OH$ (19:1, v:v). Chromatograms were sprayed with 50% H_2SO_4 and heated to locate terpenes. Five seedlings from each experimental group were extracted at each experimental point.

Carbohydrate analysis: Sections of inoculated seedlings from which nematodes were extracted and of uninoculated seedlings were frozen in liquid N_2 and lyophilized. The sections were triturated in liquid N_2 in a Waring blender and suspended in distilled water. Carbohydrate was extracted by reflux boiling (6). Debris was removed by centrifugation at $1,000\ g$ for 15 minutes, and total carbohydrate was estimated in the aqueous phase by reaction with anthrone using glucose as a standard (11). A sample of the aqueous phase was adjusted to 6 N KOH, boiled for 1 hour, and cooled. Nonreducing carbohydrate was then determined in aliquots of this phase with anthrone (11). Soluble starch, treated in parallel, was used as a standard. Reduc-

ing carbohydrate concentration was obtained by taking the difference between total and nonreducing carbohydrate. Five seedlings from each experimental group were assayed in triplicate.

Nematode populations: Sections (2 cm long) were cut from the seedlings at the inoculation site and from a 20-cm area around this site and chipped; nematodes were extracted in a Baermann funnel. Numbers of adults and juveniles were scored in triplicate from 10 separate samples from five seedlings.

RESULTS

Visual symptoms: Oleoresin flow to the site of a wound ceased and leaf chlorosis began by day 7 after VPSt-1 inoculation of untreated white pine seedlings, and all seedlings wilted within 28–32 days. Untreated Scots and Austrian pine seedlings inoculated with VPSt-1 did not wilt. Oleoresin flow decreased initially in untreated, VPSt-1-inoculated Scots and Austrian pine seedlings before increasing by day 30. Oleoresin flow ceased by day 30 after inoculation of SAR-treated white pine and Scots pine seedlings. Leaf chlorosis developed between 45 and 50 days after inoculation of SAR-treated white and Scots pine seedlings, and the seedlings wilted by day 60. Wilt symptoms did not develop in SAR-treated, VPSt-1-inoculated Austrian pine seedlings.

Water relations: Seedling dry weight of untreated Scots and white pine seedlings increased significantly by day 30 after inoculation (Fig. 1). Dry weight continued to increase in white pine seedlings, but this increase was only transient in inoculated, untreated Scots pine seedlings. Similar changes did not occur in control seedlings; in inoculated, untreated Austrian pine seedlings; or in uninoculated, SAR-treated seedlings (Fig. 1). Dry weight increased significantly 30–45 days after inoculation of SAR-treated white and Scots pine seedlings with VPSt-1 and continued until the seedlings wilted between day 55 and day 60. The dry weight–fresh weight ratio did not change in SAR-treated Austrian pine seedlings inoculated with VPSt-1 (Fig. 1).
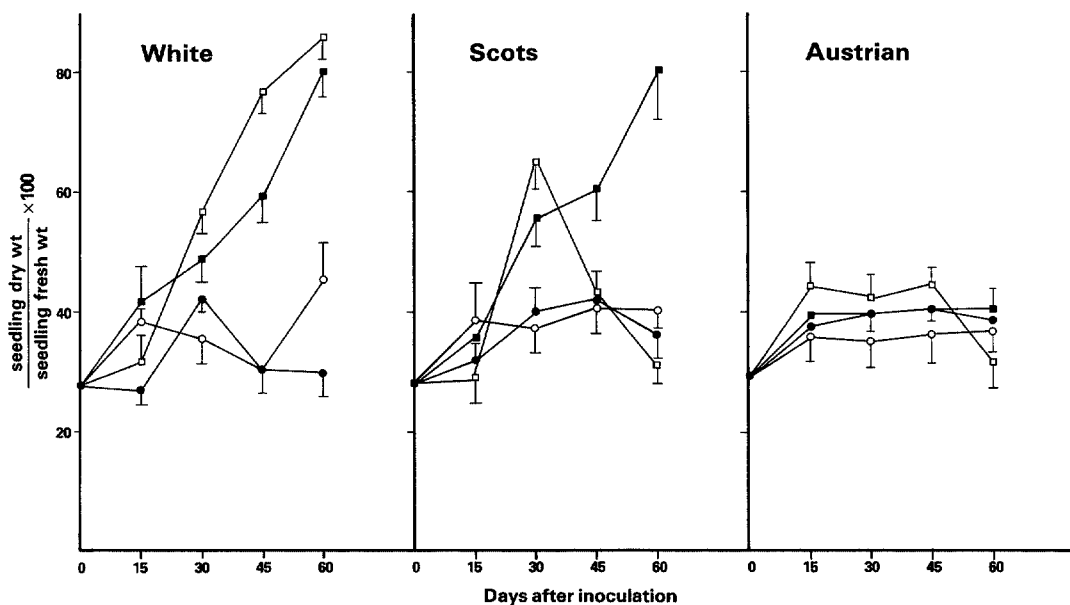
FIG. 1. Effect of simulated acid rain (SAR), SAR and VPSt-1 inoculation, or VPSt-1 inoculation only on the dry weight–fresh weight ratio of pine seedlings. □ = control seedlings inoculated with VPSt-1, ■ = SAR-treated seedlings inoculated with VPSt-1, ○ = SAR-treated seedlings, and ● = control seedlings. Data are reported as dry weight–fresh weight × 100 ± standard error, N = 10.

Water movement was not inhibited in conducting tissues in untreated control or control, SAR-treated seedlings. Acid fuchsin was excluded from 48 ± 6% of the stem cross sectional area of SAR-treated white pine seedlings beginning 30 days after inoculation with VPSt-1. After 45–60 days, 80 ± 10% of the stem cross sectional area was white wood. Similar changes occurred in untreated white pine seedlings inoculated with VPSt-1. Dye was excluded from 11 ± 2% of the stem cross sectional area in inoculated, untreated Scots pine seedlings after 15–30 days and did not increase. Water movement in SAR-treated Scots pine seedlings was inhibited by 87 ± 11% by day 50 after inoculation with VPSt-1. In Austrian pine seedlings, inoculation with VPSt-1 and SAR treatment did not affect water movement.

Oleoresin synthesis: Concentration of $CHCl_3$-base extractable oleoresins increased significantly in inoculated, untreated white pine seedlings by day 15 after inoculation and continued until wilting (Fig. 2). Oleoresin concentration initially increased following inoculation of control

Scots and Austrian pine seedlings with VPSt-1, but decreased 45–60 days after inoculation (Fig. 2). In SAR-treated, VPSt-1-inoculated white pine seedlings, oleoresin concentration was approximately four times that in control seedlings by day 30 after inoculation (Fig. 2). After 30–60 days oleoresin concentration in SAR-treated, inoculated white pines was approximately one-half that in inoculated control white pine seedlings. By day 45 after inoculation, oleoresin concentration in SAR-treated Scots pine seedlings increased to twice that seen in inoculated control seedlings and to six times that in uninoculated control seedlings (Fig. 2). Oleoresin concentration did not change after the seedlings wilted. SAR treatment alone did not affect oleoresin concentration in Austrian pine seedlings; however, oleoresin concentration increased by day 30 after inoculation of these SAR-treated seedlings. The concentration then returned to control levels after 45–60 days (Fig. 2). Monoterpenes unique to *B. xylophilus*-infected pines were identified in extracts of VPSt-1-inoculated, untreated white pine seedlings and from VPSt-1-
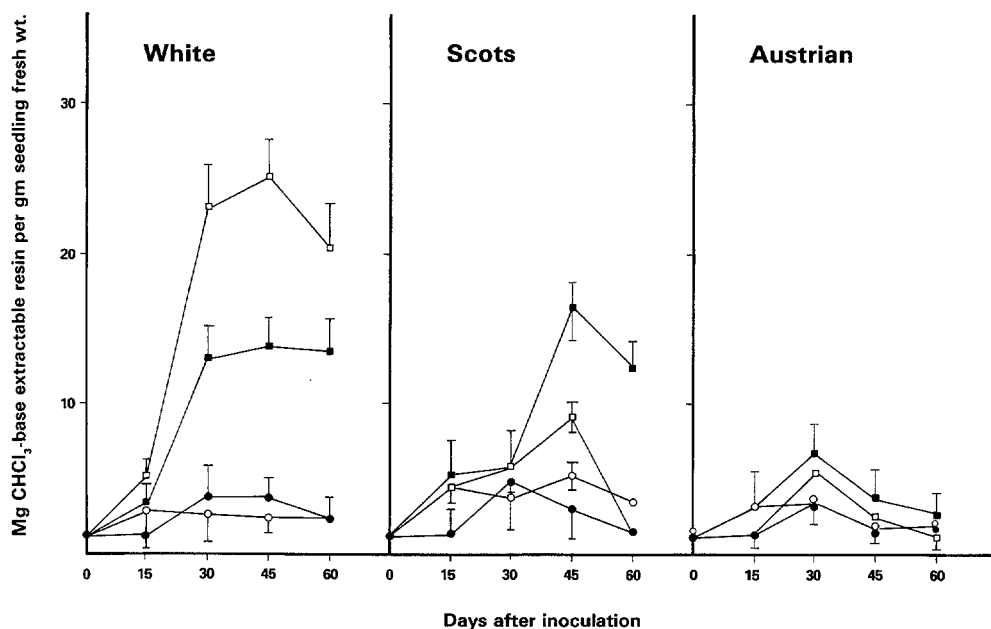
FIG. 2. Effect of simulated acid rain (SAR), SAR and VPSt-1 inoculation, or VPSt-1 inoculation only on the concentration of nonacidic $CHCl_3$ extractable oleoresins from pine seedlings. □ = control seedlings inoculated with VPSt-1, ■ = SAR-treated seedlings inoculated with VPSt-1, O = SAR-treated seedlings, and ● = control seedlings. Data are reported as milligrams extractable oleoresin per gram seedling fresh weight ± standard error, N = 5.

inoculated, SAR-treated white and Scots pine seedlings. These monoterpenes were absent from SAR-treated, uninoculated seedlings, VPSt-1-inoculated, untreated Austrian and Scots pine seedlings, and from SAR-treated, VPSt-1-inoculated Austrian pine seedlings.

Carbohydrate concentration: Reducing and nonreducing carbohydrate concentrations decreased significantly by 15–30 days after VPSt-1 inoculation of untreated white, Scots, and Austrian pine seedlings (Fig. 3). In untreated, VPSt-1-inoculated white pine seedlings, carbohydrates decreased to approximately 12% of the control as the seedlings wilted. In untreated, VPSt-1-inoculated Scots and Austrian pine seedlings, the concentration of reducing carbohydrate decreased through day 30 after inoculation; nonreducing carbohydrate decreased through day 15 after inoculation. By day 60 the concentration of these carbohydrates was 80–100% of the control (Fig. 3).

At the time of VPSt-1 inoculation of

SAR-treated seedlings, reducing carbohydrate concentration was decreased in all seedlings relative to that in untreated seedlings (day 0, Fig. 3). By day 60 after inoculation, reducing carbohydrate concentration in SAR-treated white pine seedlings increased to 50% of that in untreated controls. Reducing carbohydrate in SAR-treated Scots and Austrian pine seedlings was nearly equal or equal to the concentration in untreated control seedlings by day 60 after inoculation (Fig. 3).

During the preinoculation period of SAR treatment, nonreducing carbohydrate concentration in all seedling species decreased to 20–40% of that in the untreated control seedlings and then remained unchanged for the next 60 days (Fig. 3). When the SAR-treated white and Scots pine seedlings were inoculated with VPSt-1, nonreducing carbohydrate concentration decreased further so that by day 30 after inoculation this concentration was approximately 10% of that in untreated control seedlings. Nonreducing carbohydrate con-
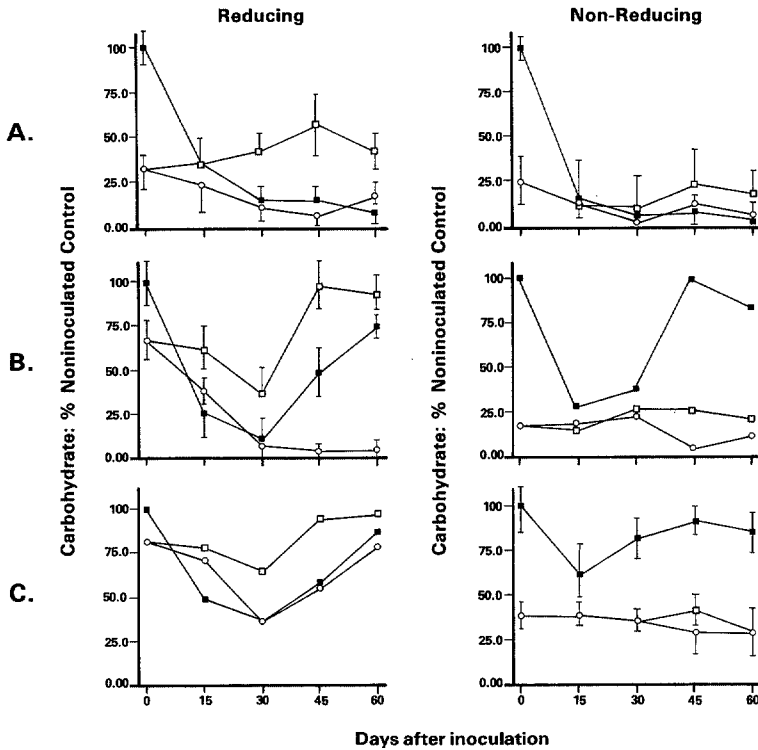
FIG. 3.   Effect of simulated acid rain (SAR), SAR and VPSt-1 inoculation, or VPSt-1 inoculation only on the concentration of reducing and nonreducing carbohydrates of white pine (A), Scots pine (B), and Austrian pine (C) seedlings. □ = control seedlings inoculated with VPSt-1, ■ = SAR-treated seedlings inoculated with VPSt-1, ○ = SAR-treated seedlings. Data are reported as concentration of carbohydrate in experimental seedlings : carbohydrate concentration in control seedlings × 100 ± standard error, N = 10.

centration in these seedlings then re-mained unchanged through 50–60 days after inoculation when leaf chlorosis became evident.

Concentration of reducing carbohydrates in SAR-treated, VPSt-1-inoculated Austrian pine seedlings increased toward control levels between 30 and 60 days after inoculation, but nonreducing carbohydrate concentration in these seedlings remained unchanged at approximately 40% of the control (Fig. 3).

Nematode populations: Largest VPSt-1 populations were seen in untreated white pine seedlings at day 30 after inoculation (Fig. 4). Nematode populations initially declined in untreated Scots pine seedlings, increased through day 30, and declined after 30–45 days. The maximum population size in untreated Scots pine seedlings was one-sixth that in untreated white pine

seedlings (Fig. 4). By day 60 after inoculation, 70% of the nematodes in Scots and white pine untreated, control seedlings were juveniles, of which 80% were dauer juveniles. VPSt-1 development in SAR-treated Scots pine seedlings was comparable to that in untreated Scots pine seedlings; however, tolerance to VPSt-1 was lost and the seedlings wilted. Maximum nematode populations in SAR-treated, inoculated white pine seedlings were not attained until day 60 after inoculation at a level approximately one-third that in untreated white pine seedlings (Fig. 4). VPSt-1 did not develop in either SAR-treated or untreated Austrian pine seedlings.

DISCUSSION

Tolerance and susceptibility of pine seedlings to a host-specific pathotype of *B.*
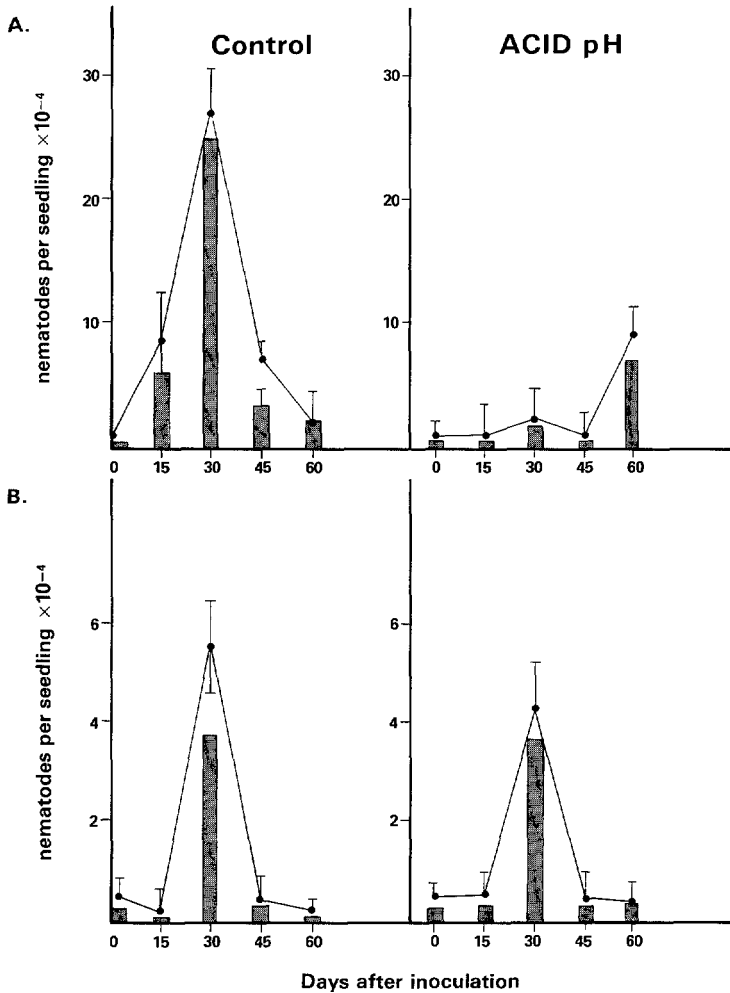
FIG. 4. Effect of acid pH on development of VPSt-1 populations in white (A) and Scots pine (B) seedlings. Bars indicate juvenile population, and O = total nematode population. Data are reported as mean number of nematodes ± standard error, N = 20.

xylophilus was affected by simulated acid rain conditions. Symptom development was delayed and reproduction of the nematode was decreased in susceptible seedlings. The change in pathogenesis and reproduction of the pathogenic nematode might not be unexpected, since Shriner (28) demonstrated decreased plant–pathogen interactions and severity of disease in plants exposed to precipitation at pH 3.2–5.0. Reproduction of *Meloidogyne hapla* on field-grown kidney beans exposed to pH 3.6 was inhibited by 66% because of the effect of acid precipitation on host physiology.

Oleoresin synthesis is altered in stressed pines, and these pines are more susceptible to pathogens (16,20). Lodgepole pine attacked by mountain bark beetle becomes highly susceptible to blue stain fungi (21). Myers (19) proposed that pines respond to inoculation with *B. xylophilus* by initiating a hypersensitive response, including synthesis of phenolic compounds and terpenes, and lignification of tissues at the inoculation site and that, in susceptible pines, this reaction extends throughout the pine as the nematode becomes systemic. Increased or altered oleoresin synthesis in conifers requires increased supplies of acetyl CoA, mevalonic acid and other intermediates, and products of catabolism as precursors for terpene biosynthesis (8,9).

This need for precursors for oleoresin synthesis suggests diversion of de novo synthesized and stored carbohydrates away from pathways of energy production to pathways for synthesis of a chemical response. Use of energy reserves to respond to stress could alter the pine's ability to maintain energy homeostasis, particularly if the stress compromised the photosynthetic capability of the pine, and could decrease the pine's ability to respond to another stress.

Acid pH alters leaf physiology, reduces the ability of plants to resist pathogens (10,12), and might influence the response of pines to pinewood nematode. In Scots and white pine, SAR treatment alone causes decreased nonreducing carbohydrates and increased concentration of oleoresins. Energy reserves, therefore, may not be sufficient for a response to the nematode. Changes in nonreducing carbohydrates in uninoculated, SAR-treated Scots pine are what would be expected in seedling growth. That these changes did not occur in VPSt-1-inoculated, SAR-treated Scots pine seedlings suggests decreased photosynthesis associated with initiation of a phytochemical response, decreased water metabolism, and leaf chlorosis (5,17,19). In either case nematode inoculation alters energy homeostasis of pines. Why resistant Austrian pine seedlings did not respond in a similar manner is unknown, and these seedlings, even after SAR treatment, were resistant.

Physiological relationships between response of pine seedlings to an abiotic stress and the susceptibility to infection by *B. xylophilus* is not completely understood. It is apparent, however, that whereas some pine species tolerate inoculation with some pathotypes of *B. xylophilus*, they become susceptible when subjected to acid pH. Acid pH suppresses reproduction of the nematode and delays wilting of susceptible pines. These experiments have recently been repeated with comparable results.

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