The Effect of Acid Rain on the Defense Response of Pines to Pinewood Nematodes

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Abstract - We examined the invasion rate of virulent and avirulent isolates of the pinewood nematode to Japanese black pine seedlings pretreated with simulated acid rain (SAR) at pH 3 and 2. Pretreatment with SAR at pH 3 reduced the invasion rate of virulent nematodes compared to control seedlings in both juvenile seedlings and 1-year-shoot segments of 3-year-old seedlings. This suggests that acid rain at pH 3 activates some defense response(s) of pines to the nematodes.

I. Introduction

Almost 30 years have passed since the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Bühler) Nickle, was ascertained to be the pathogen causing pine wilt disease [1]. This epidemic forest disease, however, is still devastating pine forests in Japan, and is now spreading throughout the East Asian countries.

Some environmental factors, such as high temperature [1], water stress [2], or low light intensity [3] are known to increase the incidence of pine wilt disease or accelerate the disease development. Among the environmental factors affecting the epidemic of pine wilt disease, the impacts of air pollution have been the subject of debate. We have studied the effects of simulated acid rain (SAR) on the development of pine wilt disease, because acid rain is especially common to most developed countries and is one of the causes of forest decline in European and North American countries [4]. The effects of acid rain on the development of pine wilt disease are rather complex. For example, repeated exposure to pH 3 SAR retarded the appearance of symptoms after nematode inoculation, though the seedlings pretreated with SAR ceased resin exudation from the stem earlier than those pretreated with tap water [5]. Bolla and Fitzsimmons [6] reported that Scots pine seedlings lost tolerance to Vpst-1, the white pine-specific pathotype of pinewood nematode, after exposure to SAR, whereas exposure to SAR delayed the development of symptoms in white pine seedlings inoculated with the same nematode isolate. These results imply that exposure to acid rain not only increases the damage to pines caused by pine wilt disease, but delays the development of the disease.

Our previous study showed that the effects of simulated acid rain on the development of pine wilt disease could be explained by two factors: the effect on the mortality velocity of pines and on the resistance of pines to the nematodes [7]. Previous experiments suggested that seedling mortality velocity after the nematode infection depends on the population growth rate of the nematodes in the seedlings [7][8]. However, another determinant, the resistance of pines to the nematodes, still remains unclear.

Several studies give possible answers to this question. In general, resistance of plants to endoparasitic nematodes is thought to be determined by (i) whether or not the parasite can invade the plant, and (ii) the ability of the pathogen to reproduce [9]. As for pine wilt disease, Futai [10][11] found that the invasion rate of pinewood nematodes in susceptible pines was higher than that in resistant pines. Based on these results, we hypothesized that the processes of nematode invasion and establishment are possible stages in which SAR affects the resistance of pines to the nematodes. Consequently, we examined the effect of SAR on the invasion of Japanese black pine by virulent and avirulent isolates of pinewood nematodes.

II. Materials and Methods

Exp. 1. Nematode invasion of juvenile seedlings

Ten juvenile Japanese black pine seedlings, planted in plastic cylindrical vials filled with 100 mL autoclaved vermiculite, were pretreated with simulated acid rain (SAR) or distilled water (W) for two months. Acidic solution for SAR was prepared by mixing 0.5 M sulfuric acid and nitric acid at an S : N ratio of 3 : 1, and the solution was adjusted to pH 3 or 2. Seedlings were divided into the following four groups. In the pH 2-T group, only the top of the seedling was sprayed with pH 2 SAR three times a week. In the pH 2-TR and pH 3-TR groups, the top of the seedling was also sprayed with SAR (pH 2 and 3, respectively) twice a week, and the root was also exposed to SAR (pH 2 and 3, respectively) once a week. Control seedlings (W) received distilled water twice a week, and tap water once a week. Seedlings were pretreated with SAR from December 13, 1999 to February 16, 2000 in the first experiment, Exp. 1 (i). In the second experiment, Exp. 1 (ii), SAR treatment began on May 8, 2000, and finished on July 10, 2000. Seedlings were grown under a natural photoperiod during the treatment periods.

Seedlings were inoculated with 500 virulent isolate (S10) of pinewood nematodes February 17, 2000 in Exp. 1(i), or with 500 avirulent (C14-5) isolate July 11, 2000 in Exp. 1(ii). Pinewood nematodes extracted from the entire seedlings 24 hrs after the inoculation were regarded as nematodes that succeeded in invasion. Additional details of these experiments are described in our previous studies [7][8].

Exp. 2. Nematode invasion test in Petri dishes with 1-year-shoot-segments

Three-year-old Japanese black pine seedlings were used for this experiment. Twenty seedlings were exposed to diluted sulfuric acid (SAR) at pH 3. Another 20 seedlings were exposed to tap water (TW, pH 6.3) as a control. SAR pretreatment was done from June 15 to December 4, 2000. The details of this experiment are also given in our previous paper [5].

The nematode invasion test was done on December 4, 2000, using 18 randomly sampled seedlings, half of which were pretreated with SAR and the remainder with TW. The inoculum of both the virulent and avirulent nematodes we used was prepared according to Futai [12]. One-year-shoots of the seedlings which had been treated with SAR or TW were cut into 10-mm-long segments after removing their needles, and the effect of acid rain on invasion of the nematodes into these shoot segments was determined as previously described by Futai [10]: four shoot segments, two from the plants treated with SAR and two from those with TW were placed on a 1.5 % plain agar plate in 9 cm diameter Petri dishes. The four shoot segments were placed 3 cm from the center of the Petri dish, so that the segments from different treatments were at right angles to the center and the shoot segments from the same treatment were at opposite sides from the center. Fifteen hundred nematodes of each virulent (S10) and avirulent (C14-5) isolate were inoculated at the center of each of 10 Petri dishes as described above. After 12 hrs incubation at room temperature (ca. 20 °C), the nematodes were extracted separately from a 1.5-mm-thick disk cut from the bottom of each shoot segment (B), the rest of each 8.5-mm-long shoot segment (S), and from a 11-mm-diameter agar disk removed from beneath the shoot segment (A), using the Baermann funnel technique. Here, the sum of B, S, and A was regarded as the number of nematodes that aggregated to the segment, and S as the nematodes that invaded the segment. The invasion rate for the shoot segments was then determined:

$$\text{Invasion rate} = (S / (B + S + A)) \times 100\ (\%)$$

III. Results and Discussion

In Exp. 1, pretreatment with pH 3-TR significantly decreased the number of virulent nematodes isolated from Japanese black pine seedlings 24 hrs after inoculation compared to those from the control seedlings (Table 1, $p = 0.025$; Mann-Whitney U test). Pretreatment with SAR at pH 2 (both pH 2-T and pH 2-TR), however, scarcely affected the number of virulent nematodes isolated. The number of avirulent nematodes isolated was not influenced by pretreatment with SAR at any level (Table 1).

Table 1. Number of virulent and avirulent pinewood nematodes isolated from current year growth of Japanese black pine seedlings exposed to distilled water (W), or simulated acid rain (SAR) at pH 3 or 2 (pH 2-T and pH 2-TR treatment), 24 hrs after the inoculation. Seedlings received SAR on only the top part in the T-treatment, and both the top and root parts in the TR-treatment. Inoculum density: 500 nematodes/20 µL. Values are means ± S.E (n =10).

	virulent	avirulent
W(control)	58.1 ± 14.7	49.6 ± 6.0
pH 3-TR	19.5 ± 3.9	46.4 ± 7.5
pH 2-T	70.0 ± 24.5	70.3 ± 11.7
pH 2-TR	68.5 ± 27.1	56.5 ± 6.9

In Exp. 2, pretreatment with SAR at pH 3 did not affect the numbers of virulent and avirulent nematodes that aggregated to the segments. Fewer virulent nematodes invaded the segments pretreated with SAR at pH 3 compared to the control segments (Table 2), whereas there was little difference in avirulent nematode invasion between the segments pretreated with SAR at pH 3 and the control segments (Table 3). It appeared that fewer avirulent than virulent pinewood nematodes invaded the segments (Table 2, 3).

Table 2: Numbers of virulent pinewood nematodes that aggregated to or invaded the segments of Japanese black pine stem pretreated with simulated acid rain (SAR) at pH 3 or tap water (TW). Invasion rate was calculated from numbers of aggregated and invaded nematodes.

	No. of invaded nematodes	No. of aggregated nematodes	Invasion rate (%)
SAR	143 ± 22	746 ± 72	19.2 ± 2.5
TW	208 ± 56	753 ± 112	25.6 ± 3.6

Values are means ± S. E. n = 9.

Table 3: Numbers of avirulent pinewood nematodes that aggregated to or invaded the segments of Japanese black pine stem pretreated with simulated acid rain (SAR) at pH 3 or tap water (TW). Invasion rate was calculated from numbers of aggregated and invaded nematodes.

	No. of invaded nematodes	No. of aggregated nematodes	Invasion rate (%)
SAR	35 ± 13	785 ± 112	4.5 ± 1.5
TW	28 ± 11	727 ± 117	3.6 ± 0.9

Values are means ± S. E. n = 9.

Plant-parasitic nematode aggregation to a plant may reflect host preference. Futai [13] reported that the pinewood nematode preferentially aggregated to *P. thunbergii* segments than to *Quercus phyllylaeoides* segments, while

Aphelenchoides sp. and *Aphelenchus* sp. preferred *Q. phyllylaeoides* to the pine. Pretreatment with SAR at pH 3 did not influence the number of pinewood nematodes that aggregated to Japanese black pine segments, suggesting the SAR had little effect on the preference of the nematode for Japanese black pine. Kishi [14] examined the number of pinewood nematodes transmitted from Japanese pine sawyers to pine twigs for seven years, and reported that the invasion rate of the nematode ranged from 12.1 % to 35.0 % (23.2 % in average). The invasion rate of TW-treated segments by virulent pinewood nematode ranged from 7.2 % to 40.0 % (25.6 % in average) in Exp. 2. Thus, the invasion rate we observed in the present experiments seems to be similar to actual transmission in nature.

We previously discovered that the effect of SAR at pH 3 on the development of pine wilt disease was influenced by nematode inoculum density. When inoculated with 50 virulent nematodes, development of pine wilt disease was retarded by pretreatment with SAR at pH 3. However, when inoculated with 500 nematodes, the disease development was accelerated by the SAR pretreatment [7]. In our present study, the invasion of pine by virulent nematodes was suppressed by pretreatment with SAR at pH 3. This supports the results of previous experiments [5][7], and it suggests that the process of invasion (and subsequent establishment) of pine by pinewood nematode is one of the determinants of resistance of Japanese black pine to the nematode. Very few studies have examined the effect of acid rain on the defense response(s) of plants to pathogens. Bruck and Shafer [17] documented that a few applications of acid rain to loblolly pine (*P. taeda*) seedlings resulted in lower infection of fusiform rust, *Cronartium quercuum* f. sp. *fusiforme*. They also noted an increase in reaction zones (possibly accumulations of phenolics) in loblolly pines exposed to SAR treatments before and after inoculation with the rust. In Exp. V-1, SAR at pH 3 decreased the number of virulent nematodes that invaded the seedlings, whereas SAR at pH 2 did not affect this response. Our current results suggest that pretreatment with SAR at pH 3 activated some defense response(s) of Japanese black pine to the pinewood nematodes, whereas that with SAR at pH 2 did not.

Some investigators have found that invasion of pines by the pinewood nematode and/or its movement in stems is restricted when the plant-nematode relationships are incompatible [10][11][15][16]. In Exp. 2, the invasion rate of Japanese black pine segments by the avirulent isolate was lower than that by the virulent isolate, which is consistent with previous studies.

Recently, we observed that exposure to SAR at pH 3 increased proanthocyanidin (condensed tannin) content in the stem segments of Japanese black pine seedlings [18]. Tannins are known to be "wound compounds", whose concentrations increase in the tissue surrounding a wound [19], and catechin, which forms the framework of proanthocyanidin, is reported to be an effective antioxidant [20][21]. Ohyama et al. (1986) reported that pine-wilt resistant isolates of Japanese black pine, Japanese red pine, and their hybrids tended to contain higher amounts of proanthocyanidin than susceptible pines [22]. Such metabolic changes in Japanese black pine pretreated with SAR at pH 3 seems to be one of the reasons for the suppression of invasion by pinewood nematodes.

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