Effect of Watering Regime on Disease Development in *Pinus sylvestris* Seedlings Inoculated with *Bursaphelenchus vallesianus* and *B. mucronatus*

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ABSTRACT

Polomski, J., and Rigling, D. 2010. Effect of watering regime on disease development in *Pinus sylvestris* seedlings inoculated with *Bursaphelenchus vallesianus* and *B. mucronatus*. Plant Dis. 94:1055-1061.

Several *Bursaphelenchus* spp. have been detected in declining pine trees in Europe during intensive monitoring for the pine wood nematode *B. xylophilus*. We investigated the pathogenicity of *B. vallesianus* and *B. mucronatus*, isolated from declining Scots pine (*Pinus sylvestris*) forests in Valais (Switzerland), in relation to drought stress. Four isolates of *B. vallesianus* and two isolates of *B. mucronatus* were inoculated into 3-year-old *P. sylvestris* trees, which were subjected to different watering treatments (50, 100, 150, and 250 ml of water per pot, biweekly). Disease symptoms, plant mortality, nematode population density, and nematode distribution in dead plants were assessed. Both *Bursaphelenchus* spp. proved highly pathogenic to the seedlings and watering treatment affected disease development in the inoculated pine trees. With decreasing water supply, we observed faster disease progress and higher pine mortality for both *Bursaphelenchus* spp.. The overall mortality 70 days after inoculation was 60, 92, 95, and 100% for *B. vallesianus* and 40, 95, 100, and 100% for *B. mucronatus* in the 250-, 150-, 100-, and 50-ml watering treatments, respectively. Both nematode species multiplied in the inoculated plants; however, *B. mucronatus* had higher population densities than *B. vallesianus* in all watering treatments (on average, 33,159 versus 14,702 nematodes/dead plant compared with the initial inoculum density of 6,000 nematodes/plant). The highest nematode density was found in the lower part of the stem. About 7 to 16% of the nematodes were extracted from the roots. This study demonstrated that *B. vallesianus* has a pathogenicity potential comparable with that of *B. mucronatus* and provided experimental evidence that drought stress can result in increased symptoms caused by either *Bursaphelenchus* sp.

Numerous species of the nematodes in the genus *Bursaphelenchus* have been detected in coniferous forests worldwide (1,2,6,10,30,36,61). Research has focused mainly on one species, the highly pathogenic *Bursaphelenchus xylophilus*. This species, commonly known as pine wood nematode, is a causal agent of pine wilt disease (PWD) and has occurred epidemically in pine forests in Japan, China, and other Asian countries for many years, causing substantial economic losses (15,56,61). In Europe (Portugal), *B. xylophilus* was first recorded in 1999 on *Pinus pinaster* (42). This finding initiated an intensive search in the pine forests of several European countries for *Bursaphelenchus* nematodes, which were found to occur frequently in Europe. The most common species are *B. mucronatus* and various members of the *B. sexdentati* group (12,14,16,41,53). In Valais (Switzerland), *B. vallesianus* and *B. mucronatus* were found in almost half of the recently

dead pine trees (*P. sylvestris*), probably as secondary invaders after insect attacks (48,60). A decline of the pine forests has been observed for decades in Valais but, within the past 10 years, nearly 50% of local pine trees have died (5,20,49). Although the causes of pine decline appear very complex, increased summer temperatures and drought episodes seem to be important initiating factors (49).

In trees, drought stress and reduced water supply to the leaves causes a decrease in the photosynthesis rate followed by a reduction in water potential, which may severely weaken the plants (24–26,45). A low water potential may induce cavitation and, consequently, embolism of tracheides by breaking the water column in xylem and impairing the water transport in the plant (58,62). Several reports indicate that plants subjected to drought stress become more susceptible to pathogens and insect pests (17,19,45,51,55). Drought may also affect trees indirectly by interacting with wilt-inducing vascular pathogens, reducing tolerance and promoting disease outbreak. Epidemiological investigations of *B. xylophilus* suggested that drought stress in combination with high temperatures (mean >20°C) were key factors in the establishment and spread of PWD in Asian countries (27,36,52). *B. xylophilus* has rapidly killed pine trees under such favorable con-

ditions in Japan or China, whereas pine wilt remains latent during cold and rainy weather for months or years (27,36). Several inoculation experiments indicated that other *Bursaphelenchus* spp. were pathogenic (3,4,9,11,38,41,47). *B. mucronatus* and *B. sexdentati*, for example, killed 2- to 4-year-old pine seedlings, although the mortality rate varied greatly depending on the host species, origin of nematode isolates, and climatic factors such as temperature and water status (9,11,50). *B. mucronatus* was also inoculated into larger trees but did not cause significantly mortality and disease symptoms when compared with *B. xylophilus* (38). However, other Japanese isolates of *B. mucronatus* also proved to have similarly low pathogenicity in the inoculated pine seedlings (38).

Even though the occurrence of various *Bursaphelenchus* spp. in declining pine forests in Europe has frequently been reported, their role in the decline process remains largely unknown. Few experimental studies have been conducted to evaluate the interaction between the nematodes and the environmental factors thought to be involved in pine decline. The aim of the present study was to assess (i) the pathogenicity to Scots pine of *B. vallesianus* and *B. mucronatus* isolated in Valais and (ii) the effect of watering treatments on disease development by the two *Bursaphelenchus* spp.

MATERIALS AND METHODS

Plant material and growth conditions.

Seedlings of *P. sylvestris* (3 years old; provenance: Tschlin, Switzerland) were used for the inoculation experiment. In April 2005, bare-root plants were transplanted into 3.7-liter pots filled with an organic substrate consisting of 67% garden mould (bark humus, peat, and wood fiber) and 33% wood fiber. The potted pine seedlings were kept outdoors and watered as required. Four weeks before inoculation, the pots were brought into a greenhouse and placed in water-filled trays for 48 h to saturate the substrate with water. The pots were placed on water-free trays and allowed to drain for 3 days to achieve field water capacity (FWC). According to Mückenhausen (43), FWC was assumed to be 80% of the total water capacity (TWC) in organic soils, and was calculated as 59% of water volume in the substrate used in our experiment. TWC of the growth substrate was determined from three water-saturated substrate cores (150 cm³) dried at 105°C to a constant

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Accepted for publication 27 April 2010.

weight and expressed as the percentage of water volume per substrate volume. Available water content (AWC) was assessed during the course of the experiment in extra pots in three replications for each watering treatment. From each of these pots, core samples (25 cm³) were taken twice a week before watering. The substrate was dried at 60°C for 24 h and AWC was expressed as the percentage of water volume per substrate volume taken (Vol%).

Temperature in the greenhouse fluctuated with the outside sunshine and temperature conditions. The daily mean temperature (average of maximum and minimum temperatures) in the greenhouse was 25°C in July, 23°C in August, and 22°C in September. The mean maximum temperature was 30°C in July, 28°C in August, and 27°C in September and the mean minimum temperature was 20, 17, and 17°C, respectively.

Watering treatments. After FWC was reached in the substrate as described in the previous section, the plants were watered with tap water biweekly with one of four volumes per pot: 50, 100, 150, and 250 ml. The watering treatments corresponded, on average, to an AWC of 39, 42, 46, and 53% in the growth substrate, respectively (mean of all samples per watering treatment). These values were above the “permanent wilting point”, which is 20% AWC in an organic substrate (43), but below the estimated 59% FWC.

Nematode inoculation. The *B. vallesianus* and *B. mucronatus* isolates used in this study were extracted from declining pine trees (*P. sylvestris*) at two sites, Salgesch and Stalden in Valais (Switzerland), in March 2005 as described by Polomski et al. (48). Four isolates of *B. vallesianus* (three from Salgesch and one from Stalden) and two isolates of *B. mucronatus* (one from Salgesch and one from Stalden) were used. The isolates were identified to species by the standard internal transcribed spacer–restriction fragment length polymorphism technique (11). To produce inoculum, the isolates were reared on *Botrytis cinerea* mycelium grown on malt agar in 9-cm petri dishes at 25°C in the dark. Nematodes were extracted in distilled water in Baermann funnels (57) 1 day before inoculation. The plants were inoculated between 18 and 22 July 2005, as described by Braasch et al. (11). A longitudinal slit about 1 cm long was cut with a scalpel in the bark at the base of the previous year’s main shoot, and a sterilized cotton strip was inserted into the slit. Each plant was inoculated with 0.5 ml of distilled water containing 6,000 nematodes. This inoculum level was chosen based on previous studies in which 6,000 to 10,000 nematodes per plant were applied (9,27,29,31,54). The inoculation site was also adopted from other studies (11,18,54) to allow better comparison of the results. The nematode suspension was dropped

onto the cotton strip and the inoculation site was wrapped with polyethylene plastic foil to retain moisture. Each nematode isolate was inoculated into 10 plants per watering treatment. Two pots were accidentally lost during inoculations, resulting in a total of 159 plants inoculated with *B. vallesianus* and 79 plants with *B. mucronatus*. Ten plants per treatment (in total, 40 plants) were inoculated with distilled water and served as controls. Additionally, 10 control plants were inoculated with an extract from a nematode-free *B. cinerea* culture to examine the impact of fungus alone on the plants.

Assessment of disease symptoms. Needle discoloration and plant wilting were evaluated twice a week for 10 weeks on four distinct segments of each plant: (i) the stem above the inoculation site, (ii) the branches above the inoculation site, (iii) the stem below the inoculation site, and (iv) the branches below the inoculation site. The severity of needle discoloration was classified for each plant segment according to the following scale: class 1 = 0 to 20%, class 2 = 21 to 50%, class 3 = 51 to 80%, and class 4 = 81 to 100% discolored needles. The plant was considered dead if needle discoloration had reached class 4 on all the plant segments.

Nematode extraction and reproduction potential. Dead plants were harvested immediately and cut into three segments: (i) plant segment above the inoculation point, (ii) branches, and (iii) stem below the inoculation point. The nematode distribution pattern was determined in more detail in the plants subjected to the 50-ml watering treatment, in which the stem part below the inoculation site was cut in half (resulting in a middle and lower part of stem) and coarse roots (>2 mm in diameter) were included in the nematode extraction. Nematodes were extracted from each plant segment separately on Baermann funnels (57). The reproduction potential of the nematodes was expressed as the number of reisolated nematodes per plant (NN). The nematode density was calculated as the number of nematodes per gram fresh weight (NC). The reproduction ratio was calculated by dividing the number of nematodes isolated at harvest by the number of nematodes inoculated per plant (reproduction rate [RR]).

Statistical analysis. All statistical analyses were performed with the program Data Desk (version 6.1; Data Description Inc., Ithaca, NY). Logistic regression and Scheffe post-hoc tests were used to test for differences in plant mortality among *Bursaphelenchus* spp., isolates, and watering treatments. In this analysis, plant mortality was the dependent binary variable, expressed as dead = 1 and alive = 0 for each seedling. The explanatory (independent) variables were *Bursaphelenchus* sp. and isolate (nested in the variable “species”) that were inoculated into the seedlings, and

Fig. 1. Progress of needle discoloration in 3-year-old Scots pine (*Pinus sylvestris*) seedlings inoculated with *Bursaphelenchus mucronatus* in the 50-ml watering treatment. Symptom development was assessed as the percentage of seedlings showing needle browning for each of four discoloration classes (class 1 = 0 to 20%, class 2 = 21 to 50%, class 3 = 51 to 80%, and class 4 = 81 to 100% discolored needles). Data were the means of 10 replications and were pooled over two isolates ($n = 20$). **A**, Stem above the inoculation sites. **B**, Branches above the inoculation sites. **C**, Stem below the inoculation sites. **D**, Branches below the inoculation sites.

watering treatment. Differences in nematode population in the dead plants, calculated as NN, NC, and RR among species, isolates, and watering treatments, were evaluated with analysis of variance (ANOVA) and Scheffe post-hoc tests. NN, NC, and RR were the dependent variables and species, isolates, and watering treatment were the explanatory variables in this analysis. The distribution of *Bursaphelenchus mucronatus* and *B. vallesianus* in the dead plants was examined by comparing the NC (= dependent variable) among the different plant segments (= explanatory variables) with ANOVA and Scheffe post-hoc tests. Regression analysis was used to assess the relationship between the nematode population (NN and NC = dependent variables) and the plant size (stem diameter and plant height = independent variables). Data for NN, NC, and RR were log-transformed before these analyses. The significance level for all statistical tests was set at $P < 0.05$.

RESULTS

Symptom development. *B. vallesianus* and *B. mucronatus* induced serious symptoms in the inoculated pine seedlings. Damage appeared as a yellowing and browning of the needles on the stem and branches and, finally, resulted in wilting of the whole plant. No differences were observed in symptom expression and spread between the two *Bursaphelenchus* spp. A typical pattern of symptom development was observed for pine seedlings inoculated with *B. mucronatus* and given the 50-ml watering treatment (Fig. 1). The first symptoms became visible about 7 days after inoculation (on average, 11 days after inoculation for both species; Table 1) and progressed rapidly, primarily on the older needles below the inoculation site (Fig. 1C and D) and later on the needles above the inoculation site (Fig. 1A and B). On average, half of the plants manifested a total needle discoloration (class 4) on the stem below the inoculation site within 14 days of inoculation, on the branches below the inoculation site within 21 days of inoculation, and on the plant segments above the inoculation site 1 week later (i.e., within 28 days of inoculation) (Fig. 1). Low watering accelerated the onset of symptom development. Needles discolored earlier in the 50-ml than in the 250-ml treatment; on average, 4 days earlier for *B. vallesianus* and 12 days earlier for *B. mucronatus* (Table 1). The control plants in all watering treatments remained symptomless throughout the 70-day observation period. No symptoms were observed on the control plants inoculated with *Botrytis cinerea*, which verifies that the plant symptoms were solely nematode-induced.

Mortality. The final mortality rate of all inoculated pine seedlings was very high for both *Bursaphelenchus* spp.; on average, *Bursaphelenchus vallesianus* killed 86% of

the plants and *B. mucronatus* killed 84% (Table 1). Logistic regression analysis showed no significant differences in mortality rates between species ($F = 0.265$, $P = 0.607$) and among isolates within species (two isolates of *B. mucronatus*: $F = 0.065$, $P = 0.803$; four isolates of *B. vallesianus*: $F = 0.059$, $P = 0.622$). The progress and rate of plant mortality, however, were significantly affected by the watering treatments (Table 1). The plants subjected to the 50-ml treatment showed first mortality 16 days earlier for *B. mucronatus* and 8 days earlier for *B. vallesianus* than the well-watered plants (250 ml). The mean time required for *B. mucronatus* to kill 25% of the inoculated plants was nearly three times less at low watering conditions: only 22 days in the 50-ml treatment compared with 64 days in the 250-ml treatment. For *B. vallesianus*, the difference

was not so great but the mean time still increased from 27 days in the 50-ml treatment up to 40 days in the 250-ml treatment (Fig. 2A and B). The mean mortality rate was 100% in the 50-ml treatment and was also high in the 100- and 150-ml treatments (92 to 100%); it decreased significantly, to 60% for *B. vallesianus* and 40% for *B. mucronatus*, in the 250-ml treatment (Table 1; Fig. 2A and B).

NC in dead seedlings. Both *Bursaphelenchus* spp. reproduced in the inoculated plants but *B. mucronatus* had a significantly higher mean RR than *B. vallesianus* over all treatments (viz., 5.7 and 2.5, respectively; Table 2). Likewise, the NN and NC were much higher for *B. mucronatus* than for *B. vallesianus* in all watering treatments (Table 2). There was no effect of watering treatments on the population densities of both *Bursaphelenchus* spp.

Table 1. Effect of watering treatments on disease development and mortality in 3-year-old *Pinus sylvestris* inoculated with *Bursaphelenchus vallesianus* (four isolates) and *B. mucronatus* (two isolates)

Isolates	Treatment (ml) ^y	n ^z	Onset (dai) of ^w		Tree mortality ^x	
			Symptoms	Mortality	n	Percent
<i>B. vallesianus</i>						
bvsa136	50	10	12	24	10	100
bvsa(136/8)	...	10	12	24	10	100
bvsa8	...	10	12	31	10	100
bvst179	...	10	9	24	10	100
Sum or Mean	...	40	11	26	40	100 a
bvsa136	100	10	12	24	9	90
bvsa(136/8)	...	10	12	24	10	100
bvsa8	...	10	12	27	10	100
bvst179	...	10	14	24	8	80
Sum or Mean	...	40	13	25	37	95 a
bvsa136	150	10	19	42	9	90
bvsa(136/8)	...	10	12	38	9	90
bvsa8	...	9	12	34	8	89
bvst179	...	10	21	31	10	100
Sum or Mean	...	39	16	36	36	92 a
bvsa136	250	10	12	34	7	70
bvsa(136/8)	...	10	12	34	7	70
bvsa8	...	10	19	31	6	60
bvst179	...	10	15	31	4	40
Sum/Mean	...	40	15	33	24	60 b
Total	...	159	14	30	137	86
Sum/Mean						
<i>B. mucronatus</i>						
bmsa(240/205)	50	9	14	22	9	100
bmst80	...	10	7	19	10	100
Sum or Mean	...	19	11	21	19	100 a
bmsa(240/205)	100	10	14	19	10	100
bmst80	...	10	7	22	10	100
Sum or Mean	...	20	11	21	20	100 a
bmsa(240/205)	150	10	14	26	10	100
bmst80	...	10	14	26	9	90
Sum or Mean	...	20	14	26	19	95 a
bmsa(240/205)	250	10	19	36	4	40
bmst80	...	10	26	39	4	40
Sum or Mean	...	20	23	38	8	40 b
Total	...	79	15	26	66	84
Sum/Mean						
Control	All	40	no	no	0	0

^w Onset at days after inoculation (dai).

^x Tree mortality 10 weeks after inoculation. Mean mortality rates within species followed by different letters are significantly different among watering treatments ($P < 0.05$) according to logistic regression model and Scheffe post-hoc tests.

^y Watering treatment, performed biweekly and measured in milliliters per pot; All = 50, 100, 150, and 250 ml/pot.

^z Number of plants. Each nematode isolate was inoculated into 10 plants per watering treatment and 10 control plants per watering treatments were inoculated with water.

The total NN and the RR tended to be lower in the low- versus high-watering treatments but this difference was only statistically significant for *B. vallesianus*. No relationship was found between NC and plant size (plant height and stem diameter) for either *B. vallesianus* or *B. mucronatus* but the total NN seemed to be dependent on the plant height ($R^2 = 13.1\%$, $P = 0.0016$ for *B. mucronatus* and $R^2 = 14.9\%$, $P < 0.0001$ for *B. vallesianus*).

Nematode distribution in dead seedlings. Nematodes migrated upward and downward from the inoculation site throughout the plant and were recovered from the branches, stem, and roots of the dead plants (Fig. 3). The distribution pattern was very similar for both nematode species and for all isolates, and was also

comparable among the watering treatments. On average, 75 and 79% of the *B. mucronatus* and *B. vallesianus* population was concentrated in the stem below the inoculation point, whereas the lowest proportion of nematodes was found in the branches (16%) and in the upper part of the plants (9 and 5%, respectively). More detailed examination of the nematode distribution in the plants subjected to the 50-ml treatment confirmed that the nematode population was highest in the stem below the inoculation site and lowest in the upper part above the inoculation site (Fig. 3). The NC in the coarse roots of the dead pine trees reached 219 and 203 nematodes/g fresh weight for *B. vallesianus* and *B. mucronatus*, respectively. When considering the total number of nematodes in

each plant segment, the coarse roots in the 50-ml treatments contained relatively high proportions of the two nematode populations; namely, 16% of *B. vallesianus* and 7% of *B. mucronatus*. Nematodes were also found in the fine roots (<1 mm in diameter). From the total of seven fine root samples tested, six samples contained *Bursaphelenchus* spp., yielding population densities between 0.3 and 1.3 nematodes/g fresh weight. No nematodes were found in the soil samples collected around the dead plants.

DISCUSSION

In this study, we attempted to assess the pathogenicity of *B. vallesianus* and *B. mucronatus*, the two most common *Bursaphelenchus* spp. found in declining pine trees in Valais. The results show that both species are highly pathogenic to pine seedlings, killing, on average, 84 and 86%, respectively, of the inoculated plants. The watering regime proved to be an important factor for disease development in the inoculated pine trees. With decreasing water supply, we observed faster disease progress and higher pine mortality for both *Bursaphelenchus* spp. The effect of watering was particularly evident in the well-watered plants (250 ml), where significantly fewer pine trees were killed by either *B. vallesianus* or *B. mucronatus* than in the other treatments. As Fukuda (23) and Braasch (9) suggested, moist conditions seem to be unfavorable for the establishment and progression of PWD. Drought stress, in contrast, may facilitate these processes in a tree by increasing the negative pressure in the xylem and inducing a cavitation process (22,25,26,58,62). An interaction between drought and vascular pathogens, such as fungi (17,19,22,45) or *Bursaphelenchus* nematodes, could amplify the cavitation and cause the hydraulic conductivity to break down irreversibly (23,31). Indeed, *B. xylophilus* has been observed to induce xylem cavitation during the early disease stage before external symptoms become visible (33,59).

There were no significant intraspecific differences in symptom expression and mortality rate among the four isolates of *B. vallesianus* and the two isolates of *B. mucronatus*. This result may be explained by the close proximity of the sampling areas and the putatively close relatedness of the isolates within species. Isolates that originated from different regions and countries have been found to vary considerably in their pathogenicity, as studies of *B. mucronatus* and *B. sexdentati* have shown (4,8,9,11,39,50,54).

B. mucronatus was somewhat more virulent than *B. vallesianus*, particularly under low watering conditions, although the mean mortality rates and the overall symptoms were very similar for both species. *B. mucronatus* caused a more rapid onset and rise of mortality in the 50- to

Fig. 2. Progress of mortality of Scots pine seedlings (*Pinus sylvestris*) inoculated with **A**, *Bursaphelenchus mucronatus* and **B**, *B. vallesianus*. Mortality is shown as the cumulative percentages of dead seedlings in the four watering treatments: 50, 100, 150, or 250 ml of water per pot, biweekly. Data represent means of 10 replications and were pooled over the two isolates for *B. mucronatus* ($n = 20$) and four isolates for *B. vallesianus* ($n = 40$). Ten control plants per watering treatment that had been inoculated with distilled water showed no mortality.

150-ml watering treatment than *B. vallesianus*. This could be explained by the significantly higher reproduction potential of *B. mucronatus* found in each watering treatment. The ability of *Bursaphelenchus* spp. to multiply rapidly and develop high population densities in host plants has been regarded as an important factor promoting disease development and mortality (4,9,18, 21,40).

The higher pathogenicity potential of *B. mucronatus* may be due to its close similarity to *B. xylophilus*. Based on both morphological and phylogenetic analysis, *B. mucronatus* is the closest relative to *B. xylophilus*, whereas *B. vallesianus* is clearly distinct (10).

However, the consistently lower number of *B. vallesianus* in the dead plants could also mean that this nematode species requires lower population levels to cause mortality than *B. mucronatus*. It is also interesting to note that *B. vallesianus* was more aggressive than *B. mucronatus* in the 250-ml watering treatment. This observation could indicate that *B. vallesianus* is better at causing disease in well-watered plants.

In contrast to the large difference in the reproduction potential of the two species, no major differences were observed in the symptom expression induced by *B. vallesianus* and *B. mucronatus* manifested as needle discoloration and necrosis above and below the inoculation site of the inoculated plants. Moreover, the symptoms were very similar to those manifested on various pine species inoculated with *B. xylophilus* (28,29,31,35,37,59). It seems to be typical of *Bursaphelenchus* spp.-induced symptoms under comparable experimental conditions to have a rapid spread and increase in the nematode population density, which results in needle discoloration and plant wilt within a short period of 3 to 4 weeks after inoculation.

The minimum population level required to trigger disease and to spread symptoms could not be determined definitely in previous studies (7,18,21,40), nor was it clear in our results. However, our statistical analysis was hampered by high variation in the NC among plants, with a mean standard deviation ranging from 50 to 150%. Other studies have also found high variability in the population densities of nematodes in pine seedlings inoculated with *B. mucronatus*, *B. sexdentati*, or *B. xylophilus* (4,9,18,38,54). In addition, the reproduction dynamic of the nematodes during the

first days after inoculation, not examined in our experiment, could be more decisive for disease development than the final nematode population estimated in the dead seedlings. Melakeberhan and Webster (40) found a dramatic decline of the initial nematode population immediately after inoculation followed by an exponential reproduction of the nematodes associated with wilting symptoms.

The nematode distribution followed a very similar pattern for both species studied and was comparable with that observed for *B. xylophilus* in inoculated pine seed-

Fig. 3. Distribution of *Bursaphelenchus vallesianus* and *B. mucronatus* in the Scots pine (*Pinus sylvestris*) seedlings subjected to the 50-ml watering treatment (milliliters of water per pot, biweekly) at the time of pine death. The nematode population in each plant segment is expressed as nematode density. Data were pooled over two isolates for *B. mucronatus* and four isolates for *B. vallesianus*. Bars marked with the same letter within the nematode species indicate mean values that are not significantly different (analysis of variance and Scheffe post-hoc tests, $P < 0.05$).

Table 2. *Bursaphelenchus vallesianus* and *B. mucronatus* populations in 3-year-old Scots pine trees (*Pinus sylvestris*) at the time of pine death in four different watering treatments^y

Treatments (ml) ^y	n	No. of nematodes/plant ^w				Nematode density (no./g fresh weight) ^x			
		Min.	Max.	Mean	SD (%)	Min.	Max.	Mean	RR ^z
<i>B. vallesianus</i>									
50	40	3,429	103,770	19,026 a	92	105	2,118	464 a	3.2 a
100	37	1,750	44,500	17,629 ab	75	34	1,873	657 a	2.9 ab
150	36	1,705	31,020	9,798 ab	69	81	1,108	385 a	1.6 bc
250	24	764	79,700	12,353 b	151	85	4,195	614 a	2.1 c
Mean		1,912	64,747	14,702	97	76	2,323	530	2.5
<i>B. mucronatus</i>									
50	19	5,041	113,775	43,623 a	71	370	2,211	1,051 a	7.3 a
100	20	9,100	55,825	28,542 a	53	225	2,304	1,201 a	4.5 a
150	19	6,155	75,110	31,945 a	68	327	5,274	1,450 a	5.3 a
250	8	1,910	75,970	36,525 a	105	32	2,187	1,105 a	6.1 a
Mean	...	7,904	80,170	33,159	74	238	2,994	1,202	5.7

^y Means within a column and species followed by the same letters are not significantly different ($P < 0.05$) among watering treatments (analysis of variance and Scheffe post-hoc tests).

^w Number of nematodes per plant. Minimum (Min.), maximum (Max.), and mean values ($\pm$ percent standard deviation [SD]) are given.

^x Number of nematodes per gram fresh weight. Min., Max., and Mean values are given.

^y Watering treatment, performed biweekly and measured in milliliters per pot. Data were pooled over four isolates of *B. vallesianus* and two isolates of *B. mucronatus* for 10 replicates per watering treatment.

^z Reproduction rate (RR) = number of reisolated nematodes in the dead plant/number of inoculated nematodes per plant (6,000).

lings (18,33). It is not clear which factors control nematode migration in the plant. Kuroda (31) and Utsuzawa et al. (59) reported that *B. xylophilus*, for example, moved as cavitation was spreading in the xylem and that anatomical features of the host wood strongly affected nematode migration (32,33). The water gradient along the plant could also affect the nematode distribution. Daub (18) found a positive correlation between the density of *B. xylophilus* and the water content in the stem base and the roots of inoculated *P. sylvestris*. We detected the presence *B. vallesianus* and *B. mucronatus* in both the coarse and the fine roots (<1 mm in diameter) of plants. Several investigators (11,18,29,40,44) have detected *Bursaphelenchus* nematodes in plant roots but it is not clear whether the occurrence of *Bursaphelenchus* spp. in the roots is biologically relevant. Blakeslee et al. (6), hypothesized that *Bursaphelenchus* nematodes could be transmitted from tree to tree via root grafts. Root grafts are relatively common in mature pine forests and have been suggested as a potential conduit between trees for transmitting water and mineral nutrients but also certain pathogens (46). Vascular pathogens, such as *Ophiostoma ulmi* (cause of Dutch elm disease) or *Ceratocystis fagacearum* (cause of oak wilt), are known to spread through root grafts, which can result in extensive tree mortality in areas where host trees are closely spaced (13,34). Whether root-to-root transmission is also relevant for *Bursaphelenchus* nematodes needs to be shown.

This study has demonstrated the ability of *B. vallesianus* and *B. mucronatus* to destroy living plant tissue and induce the death of pine seedlings under experimental conditions. It has also shown that watering regimes affect the degree of mortality. Our results suggest that the symptom expression and disease pattern induced by the two *Bursaphelenchus* spp. are very similar and comparable with those described for *B. xylophilus* in inoculated pine seedlings.

However, we cannot claim, based on our experiments alone, to have the final verdict about the ability of nematodes other than *B. xylophilus* to kill mature pine trees in the forest. First, the adverse effect of nematodes on younger trees can be more severe than on older trees (39). Second, trees are artificially inoculated with nematodes in such studies whereas, in the forest, insect vectors transmit nematodes (36). Thus, disease epidemics are influenced not only by the pathogenicity and virulence of nematodes but also by the life cycle and population dynamics of vectors and the efficiency of nematode transmission (both *B. mucronatus* and *B. xylophilus* are dispersed by *Monochamus* sp. [38,53], whereas the vector of *B. vallesianus* is unknown). Nevertheless, from our findings showing that *B. vallesianus* and *B. mucro-*

natus are both pathogenic to pine seedlings, we cannot reject the hypothesis that these two *Bursaphelenchus* spp. are involved in the observed pine decline in the Swiss Valais. Even though the nematodes might not be a primary cause of the pine decline, they could contribute to the decline process as secondary pathogens.

Moreover, the observed relationship between water regime and mortality in our experiment corresponded well to field observations reporting that pine mortality increases after hot and dry summers (49). However, pine decline in the Valais seems to be a complex phenomenon involving multiple abiotic and biotic factors (5,49). Besides nematodes, other biotic agents possibly contribute to pine decline, including bark- and wood-boring insects (60), blue-stain fungi (U. Heiniger and D. Rigling, unpublished results), and mistletoes (20).

ACKNOWLEDGMENTS

We thank H. Braasch and Ute Schönfeld for their introduction into the rearing of nematodes and the inoculation procedure, U. Heiniger for suggestions and discussions in the course of manuscript preparation, H. Blauenstein and R. Graf for their help with sample processing, W. Burgermeister and E. Buchbach-Woldt for internal transcribed spacer-restriction fragment length polymorphism analysis, and S. Dingwall for English corrections.

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