

## The insignificance of the energy requirements of *Bursaphelenchus xylophilus* as a causal factor in *Pinus sylvestris* seedling mortality

Haddish MELAKEBERHAN \* and John M. WEBSTER

Department of Biological Sciences, Simon Fraser University, Burnaby, Vancouver, B.C., Canada, V5A 1S6.

Accepted for publication 9 April 1991.

**Summary** — The relationship between nematode size and energy consumption by *Bursaphelenchus xylophilus* population density, and death and dry matter accumulation of 8-month-old *Pinus sylvestris* was examined over a period of 5 weeks. Nematode fresh weights from juvenile to adult stage, calculated as a function of length and width were 0.02-0.5 µg, equivalent to 0.000043-0.0011 calories per nematode, respectively. Pine death and dry matter accumulation were measured on *P. sylvestris* that received either no wound (check), wound, wound and water, wound and *B. xylophilus* culture filtrate, or wound and 400, 4000 or 40 000 *B. xylophilus*. The medium and high nematode treatments resulted in 30 % and 50 % pine death, respectively, but nematodes accounted for less than 4 calories per plant of food consumption. There was no difference in dry matter accumulation by the pine seedlings in any of the treatments whether the pines were dead or symptomless at the time of harvest. The data suggest that food consumption by *B. xylophilus* is not a significant factor in the cause of pine death.

**Résumé** — *L'insignifiance des besoins en énergie de Bursaphelenchus xylophilus en tant que facteur causal de la mort des plants de Pinus sylvestris* — Les relations entre taille du nématode et consommation d'énergie par *Bursaphelenchus xylophilus* en population dense, et la mort et l'accumulation de matière sèche de *Pinus sylvestris* âgés de huit mois ont été étudiées sur une période de 5 semaines. Les poids frais du juvénile et de l'adulte — calculés d'après la longueur et le diamètre du nématode — sont de 0,02 et 0,5 µg, équivalant à 0,000043 et 0,0011 calories par nématode, respectivement. La mort des pins et l'accumulation de matière sèche ont été mesurées sur des *P. sylvestris* intacts (témoins), simplement blessés, blessés et arrosés, blessés et ayant reçu un filtrat d'élevage de *B. xylophilus*, et blessés ayant reçu 400, 4000 ou 40 000 *B. xylophilus*. Les deux derniers traitements provoquent 30 et 50 % de mortalité, respectivement, mais les nématodes n'interviennent que pour moins de 4 calories par plant en ce qui concerne la prise de nourriture. Il n'a été observé aucune différence dans l'accumulation de matière sèche entre les différents traitements, que les pins soient morts ou ne présentent aucun symptôme à la fin de l'expérience. Ces données suggèrent que la prise de nourriture par *B. xylophilus* est un facteur dénué de toute signification dans la cause de la mort des pins.

**Key-words** : *Bursaphelenchus*, *Pinus*, energy demand, dry matter.

When a nematode invades a host plant it causes damage directly by breaking down cell structure and consuming cell contents and/or indirectly by disrupting physiological processes (Melakeberhan & Ferris, 1988). If plant cell destruction during feeding by *Bursaphelenchus xylophilus* on pine trees is an important cause of plant death, a relative decrease in dry matter accumulation by the plant could be expected. If such a decrease does not occur, the effect of the nematode on the host is more likely to be due mainly to the modification of physiological processes. *Bursaphelenchus xylophilus* is one of the few plant parasitic nematodes to act as a primary pathogen and kill a mature host, and it has been suggested that in addition to modification of the physiological processes (Melakeberhan *et al.*, 1991) the number of host cells damaged by *B. xylophilus* may be a factor in pine mortality (Melakeberhan & Webster, 1990b). It may be that the energy (food) requirements of the *B. xylophilus* population in an infected pine is a significant factor in pine death. One of the ways to determine the energy requirements of small nematodes such as *B.*

*xylophilus* is to measure the volumetric increase in nematode biomass (Andrássy, 1956). Energy requirement is function of individual nematode size and the nematode's reproduction potential (Melakeberhan & Ferris, 1988). Although mature *B. xylophilus* is smaller than mature *Meloidogyne incognita* which has high energy requirements (Melakeberhan & Ferris, 1988), *B. xylophilus* has a high reproductive potential and completes its life cycle in about 5 days at 25 °C (Melakeberhan & Webster, 1990b). Therefore, we hypothesized that the large increase in numbers of *B. xylophilus* would result in a significant food demand in young *Pinus sylvestris* seedlings. This paper tests the hypothesis and helps our understanding of the mechanisms leading to pine wilt disease and death of the pine.

### Materials and methods

Approximately 300 *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, of all stages and both sexes were fixed in FA 4.1 and permanent slides were prepared. Length and width of individual nematodes were mea-

\* Present address : Department of Entomology, Michigan State University, East Lansing, MI 48824, USA.

sured from photographs of 155 selected nematodes and nematode weights were calculated according to Andrásy's (1956) formula :

$$Wt = (W^2 \times L)/(16 \times 10^5)$$

where  $Wt$  = fresh weight in  $\mu\text{g}$ ,  $W$  = greatest width in  $\mu\text{m}$ , and  $L$  = length ( $\mu\text{m}$ ) excluding the tail. Nematode developmental stages were separated by size. In order to calculate the amount of energy consumed by nematodes, fresh weights were converted into calories on the basis of 2.152 calories per mg fresh weight and dry weights as 25 % of fresh weights (Yeates, 1979). Energy consumed by nematodes is defined as the amount of energy equivalent to a nematode size.

Each of 24, 8-month-old potted *Pinus sylvestris* L. seedlings were treated with either wound, wound and water, wound and *B. xylophilus* culture filtrate, and wound and 400, 4000 or 40 000 *B. xylophilus*, or unwounded checks as described by Melakeberhan and Webster (1990b). With the exception of the wound and check, all treatments received a 75  $\mu\text{l}$  aliquot with or without nematodes, and all pines were arranged randomly in a growth chamber at 25 °C (Melakeberhan & Webster, 1990b).

The pine seedlings were examined daily and disease symptoms recorded. When pines died (Melakeberhan & Webster, 1990b), they, together with three or four symptomless (healthy) pines from all treatments, were harvested. This was done so as to allow comparison of dry matter accumulation, the parameter selected to test the hypothesis, between and within treatments. Shoots were dissected longitudinally, vascular necrosis noted, fresh weights recorded and cut into lengths of approximately 1 cm. All of the plant tissue except for 0.2 g of the shoot fresh weight was oven-dried for 24 h at 75-80 °C and dry weights recorded.

Nematode populations in pine seedlings were estimated after 24 h of extraction on a modified Baermann funnel of 0.2 g shoot fresh weight sub-samples (Melakeberhan & Webster, 1990b). Nematode developmental stages were not separately counted. Hence, the mean weights and calorific equivalents of all developmental stages and both sexes were used for calculations of energy consumed by nematodes.

Changes in dry matter accumulation up to 37 days after inoculation, or up to the time of recorded plant death for each treatment, were recorded. Nematode dry weights were subtracted from that of the host (Melakeberhan & Ferris, 1989). Host dry matter was converted into calories based on 4.7 kcal per g dry weight (Leith, 1968). Calculations of differences in dry matter accumulation between nematode-infected dead and symptomless pines and control treatments were similar to those described by Melakeberhan and Ferris (1989). Total dry matter accumulation of dead and symptomless nematode-infected plants, with or without nematodes, were compared with all the controls.

Regression and ANOVA methods were used to analyze the data (Anon., 1985).

## Results

The range of nematode width and length from the second-stage juvenile to the adult was 5-28  $\mu\text{m}$  and ca 200-1000  $\mu\text{m}$  (Fig. 1) with fresh weight values of 0.02-0.50  $\mu\text{g}$  (Fig. 2), respectively. Because of the degree of overlapping between the sexes, a mean of 0.1499  $\mu\text{g}$  fresh weight per nematode was used for energy equivalent calculations. The corresponding values for dry weights and calorific equivalents from the second-stage juvenile to the adult (which are direct conversions of fresh weight) were 0.005-0.13  $\mu\text{g}$  and 0.000043-0.0011 calories, with means of 0.03778  $\mu\text{g}$  and 0.000305 calories per nematode, respectively.

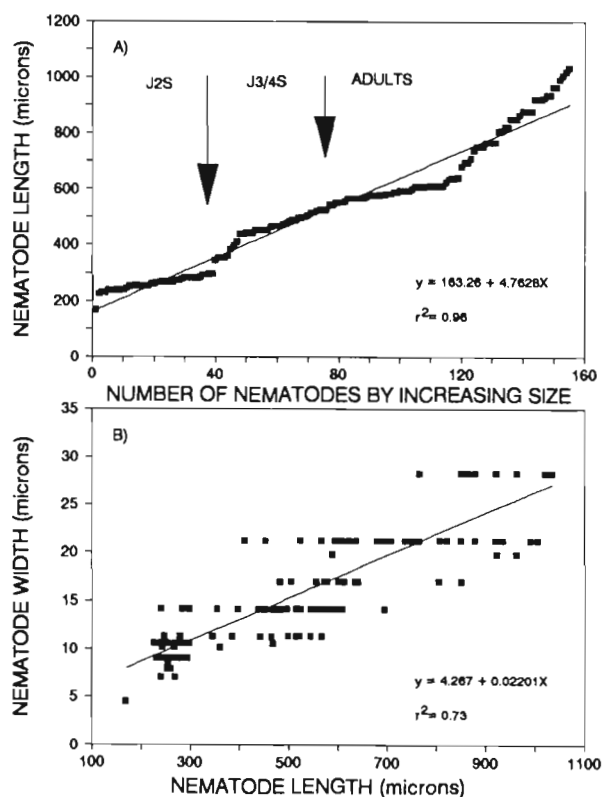
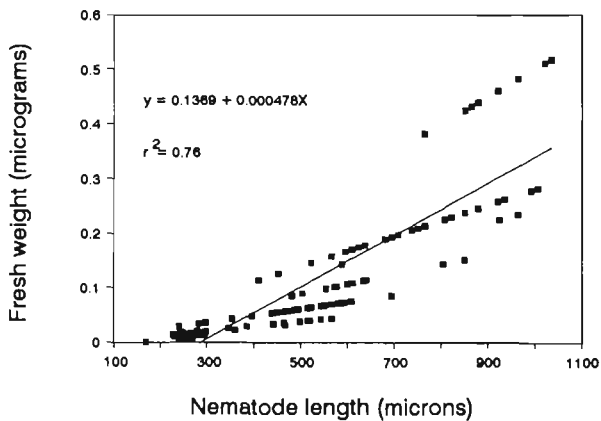


Fig. 1. The relationship between (A) developmental stage and body length and (B) body length and width of 155 nematodes *Bursaphelenchus xylophilus*. Changes in nematode developmental stage, as it relates to length, is shown by arrows.

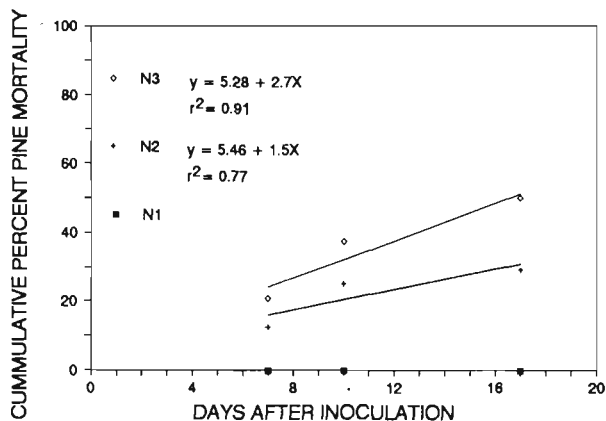
Pine death was preceded by chlorosis and wilting, and dead pines almost invariably had ca a 2-4 cm long necrotic lesion in the vascular tissue around the inoculation sites. Symptomless pines from the medium and high nematode inoculum treatments had necrotic vascular lesions but not as severe as in the dead pines. At 7, 10 and 17 days after inoculation, three, three, and one



**Fig. 2.** The relationship between body length and fresh weight in *Bursaphelenchus xylophilus*. Dry weight is a function of fresh weight  $\times 0.25$ , and calorific equivalents fresh weight  $\times 0.002152$ . Thus, they will have an exact same trend as the fresh weight figure.

pinus in the medium, and five, four, and three pines in the high nematode inoculum treatments, respectively, died (Fig. 3). Three to four randomly selected symptomless pines from all treatments were harvested at 7, 10, 17, and 30 days after inoculation. At 37 days after inoculation, a similar number of symptomless pines were harvested from all but the highest nematode inoculum treatments by which time there were no pines left and so the experiment was terminated.

Although the estimated amount of food consumed by nematodes decreased with time (Fig. 4), it was generally proportional to the level of inoculum and it was slightly more in the nematode-infected dead than in the nematode-infected but symptomless pines (Fig. 4). However, the maximum amount of food consumed by nematodes was less than 4 calories (Fig. 4) and there was no significant difference in dry matter accumulation with or

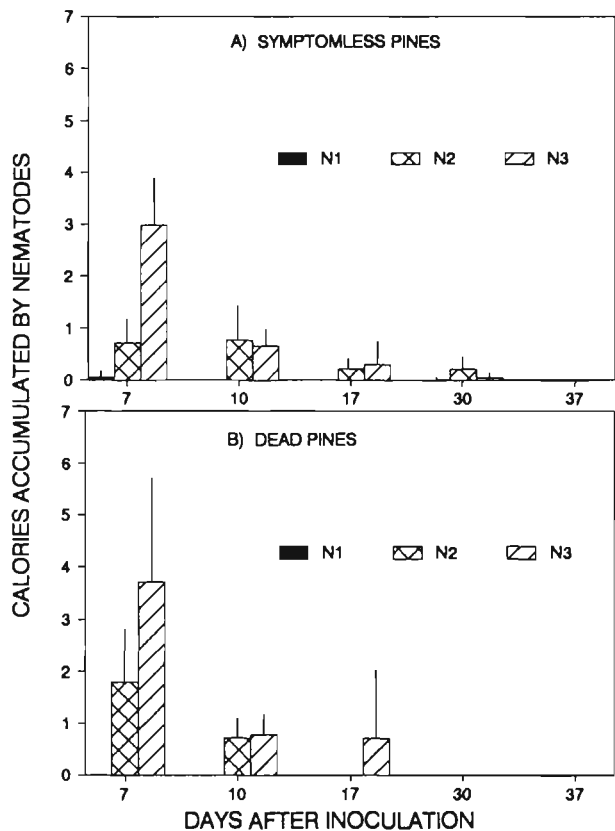


**Fig. 3.** Cumulative percent pine mortality of 8-month-old *Pinus sylvestris* seedlings infected with wound + 400 (N 1), or 4000 (N 2), or 40 000 (N 3) *Bursaphelenchus xylophilus*. Pine death occurred in only N 2 and N 3.

without the food consumed by nematodes whether the pines were dead or symptomless at the time of harvest (Table 1).

### Discussion

Pine death occurred in only the two highest *B. xylophilus* treatments. Although sampling error may have contributed to the decrease in the number of nematode population density with time, the number of seedlings dying was proportional to the level of inoculum. The fact that the two highest nematode treatments resulted in pine death at the same time and no pines died in the lowest nematode treatment suggests that a minimum population level of nematodes must occur in the host to cause pine death. The recovery of a larger number of



**Fig. 4.** The number of calories consumed per *Bursaphelenchus xylophilus* over 30 days in (A) symptomless and (B) dead 8-month-old *Pinus sylvestris* seedlings infected with wound + 400 (N 1), or 4000 (N 2), or 40 000 (N 3) *Bursaphelenchus xylophilus*. (Bars indicate standard errors.)

nematodes from wilted pines than from symptomless pines at the time of harvest and that the latter had less necrotic tissue than the former suggest that the degree, location and type of cell damage might be important in determining the time of pine death (Melakeberhan & Webster, 1990b).

**Table 1.** Comparison of total dry matter accumulated (kcal) by the check (C), wound (WD), wound and water (WDW) and wound and *Bursaphelenchus xylophilus* culture filtrate (WDCF) with increasing number of nematodes extracted per pine, and with (+) or without (-) nematodes on dead pines at 17 days and on symptomless pines at 17, 30 and 37 days after inoculation. x = comparison does not include the highest nematode treatment (see Methods). There was no significant difference between any of the treatments.

Days after inoculation	C		WD		WDW		WDCF	
	+	-	+	-	+	-	+	-
DEAD								
17	9.44	9.48	9.31	9.97	9.46	9.50	9.81	9.85
SYMPTOMLESS								
	+	-	+	-	+	-	+	-
17	9.00	9.00	9.32	9.32	9.01	9.01	9.24	9.24
30	9.79	9.79	9.48	9.48	9.25	9.25	9.26	9.26
37	10.08	9.74	9.58	9.23	9.26	8.91	9.04	8.69

There was no significant difference in dry matter accumulation between *B. xylophilus*-infected (dead or symptomless) and non-infected pines. If the working hypothesis is true, *i*) *B. xylophilus*-infected pines should have had smaller biomass than uninfected controls of equivalent age and development, *ii*) nematode food consumption should have accounted for the difference in the amount of dry matter accumulation between infected and uninfected pines, and *iii*) pines may or may not have died. However, the amount of energy consumed by *B. xylophilus* accounted for less than 4 calories (Fig. 4) or ca 0.0001 % of the total dry matter accumulated by the pines (Table 1) despite pine seedling mortality of 30-50 % (Fig. 3). Since there was no difference in biomass accumulation between nematode-infected and uninfected pines but highly infected pines died, the data suggest that the hypothesis is not true and show that *B. xylophilus*-infected pine seedlings die due to causes other than energy consumed by the nematodes. This pattern contrasts with the *M. incognita* infection of grape where both the nematode energy demand and the effect on host physiology are important in host response (Melakeberhan & Ferris, 1989) and in the *M. incognita*-grape situation the host plant does not die.

In conclusion, measuring the increase in nematode biomass allows for the calculation of *B. xylophilus* weights and energy equivalence and for the separation of the nematode food requirements from the effect on host physiology. This helps our understanding of how *B. xylophilus* kills its host. Food demand of the *B. xylophilus* population does not seem to be a significant factor in causing the death of young pine seedlings. Rather, the large *B. xylophilus* population appears to influence *P. sylvestris* seedling health by means other than energy requirements. Physiological studies seem to suggest that pine death is a function of a combination of factors such as toxins (Oku *et al.*, 1979, 1980) and/or nematode-

induced host metabolic modifications that result in altering water relations (Tamura *et al.*, 1987; Kuroda, 1989; Ikeda *et al.*, 1990; Melakeberhan *et al.*, 1991) which, in turn, affect other related physiological processes (Melakeberhan & Webster, 1990a).

#### Acknowledgments

We thank Susan Chow for technical assistance, and the Natural Sciences and Engineering Research Council of Canada and the Canadian Forestry Service for financial support.

#### References

- ANDRÁSSY, I. (1956). Die Rauminhalts- und Gewichtsbestimmung der Fadenwürmer (Nematoden). *Acta Zool. Acad. Sci. hung.*, 2 : 1-15.
- ANON. (1985). *SAS Institute Inc. Statistics User's Guide*. Cary, North Carolina, USA : 433-506.
- IKEDA, T., KIYOHARA, T. & KUSUNOKI, M. (1990). Changes in water status of *Pinus thunbergii* Parl. inoculated with species of *Bursaphelenchus*. *J. Nematol.*, 22 : 132-135.
- KURODA, K. (1989). Terpenoids causing tracheid-cavitation in *Pinus thunbergii* infected by the pine wood nematode (*Bursaphelenchus xylophilus*). *Ann. Phytopath. Soc. Japan*, 55 : 170-178.
- LIETH, H. (1968). The measurement of calorific values of biological material and the determination of ecological efficiency. In : Eckardt, F. E. (Ed.). *Functioning of Terrestrial Ecosystems at the Primary Production Level*. Proceedings of the Copenhagen Symposium, UNESCO : 233-241.
- MELAKEBERHAN, H. & FERRIS, H. (1988). Growth and energy demand of *Meloidogyne incognita* in susceptible and resistant *Vitis vinifera* cultivars. *J. Nematol.*, 20 : 545-554.
- MELAKEBERHAN, H. & FERRIS, H. (1989). Impact of *Meloidogyne incognita* on physiological efficiency of *Vitis vinifera*. *J. Nematol.*, 21 : 74-80.
- MELAKEBERHAN, H., TOIVONEN, P. M. A., VIDAVER, W. E., WEBSTER, J. M. & DUBE, S. L. (1991). Effect *Bursaphelenchus xylophilus* on the water potential and water-splitting complex of photosystem II of *Pinus sylvestris* seedlings. *Physiol. mol. Plant Path.*, 38 : 83-91.
- MELAKEBERHAN, H. & WEBSTER, J. M. (1990a). Effect of *Bursaphelenchus xylophilus* on the assimilation and translocation of <sup>14</sup>C in *Pinus sylvestris*. *J. Nematol.*, 22 : 506-512.
- MELAKEBERHAN, H. & WEBSTER, J. M. (1990b). Relationship of *Bursaphelenchus xylophilus* population density to mortality of *Pinus sylvestris*. *J. Nematol.*, 22 : 297-302.
- OKU, H., SHIRAIISHI, T., OUCHI, S., KUROZUMI, S. (1979). Participation of toxin in wilting of Japanese pines caused by a nematode. *Naturwissenschaften*, 66 : 210.
- OKU, H., SHIRAIISHI, T., OUCHI, S., KUROZUMI, S., & OHTA, H. (1980). Pine wilt toxin, the metabolite of bacterium associated with a nematode. *Naturwissenschaften*, 67 : 198-199.
- TAMURA, H., MINEO, K. & YAMADA, T. (1987). Blockage of water conduction in *Pinus thunbergii* inoculated with *Bursaphelenchus xylophilus*. *Jap. J. Nematol.*, 17 : 23-30.
- YEATES, G. W. (1979). Soil nematodes in terrestrial ecosystems. *J. Nematol.*, 11 : 213-229.