Transmission of the pine wood nematode *Bursaphelenchus xylophilus* through oviposition activity of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae)

Pedro M. Naves*, Susana Camacho, Edmundo de Sousa & José A. Quartau

Naves, P. M., Camacho, S., Sousa, E. & Quartau, J. A. 2007: Transmission of the pine wood nematode *Bursaphelenchus xylophilus* through oviposition activity of *Monochamus galloprovincialis* (Coleoptera: Cerambycidae). — Entomol. Fennica 18: 193–198.

Transmission of *Bursaphelenchus xylophilus* (PWN) by the oviposition of *Monochamus galloprovincialis* was studied in Portugal. Female insects laid eggs on 77% of pine bolts on the laboratory, of which 37% became infected with PWN, with a mean of 290 nematodes. Inside the PWN affected zone 50 *P. pinaster* trap-trees were created between May and September 2001 and 2002, of which 29 were colonised by *M. galloprovincialis* and only four became infected by the nematode. The low transmission efficiency detected both on laboratory and field suggests that nematode transmission through the vector's oviposition activity may be a secondary component of the pine wilt disease epidemiology in Portugal.

*P. M. Naves, S. Camacho & E. de Sousa, Dep. Protecção Florestal, Estação Florestal Nacional – INIAP. Quinta do Marquês 2780 – 159 Oeiras, Portugal; *corresponding author's e-mail: pedro.naves@efn.com.pt*
J. A. Quartau, FCUL, Centro de Biologia Ambiental e Dep. Biologia Animal, Campo Grande, Edifício C2, 1749-016 Lisboa, Portugal

Received 17 July 2006, accepted 20 March 2007

1. Introduction

Transmission of the pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle from its insect vector to a tree host is an essential component of the epidemiology of the pine wilt disease. Worldwide, the most important PWN insect vectors are cerambycid beetles of the *Monochamus* genus (Linit 1988, Kishi 1995), which carry the fourth-stage dispersal juvenile nematodes into new tree hosts. Nematode transmission occurs mainly in two distinct ways: through the adult's feeding activity in healthy trees or through the females' oviposition wounds (Linit 1990).

Transmission through the feedings activity occurs when the recently emerged adult insects make their maturation feeding in the bark of healthy conifer trees (Mamiya & Enda 1972, Wingfield & Blanchette 1983, Luzzi *et al*. 1984, Linit 1990). The *B. xylophilus* JIV larvae abandon the insect's body and enter the host through the wounds made the beetle when feeding on the conifer's bark. If the host is susceptible to the pine wood nematode the inoculated individuals begin to feed on the xylem tissues and reproduce, eventually interrupting the water and resin flow and killing the tree (Linit 1988). In contrast with the previous pathway, transmission through the oviposition wounds occurs when the female *Mono-*

chamus choose dead or weakened trees (or recently-cut logs) to lay their eggs (Wingfield 1983, Wingfield & Blanchette 1983, Luzzi *et al*. 1984, Linit 1989, 1990, Edwards & Linit 1992). When the female insects chew the bark to insert their eggs, the nematodes leave their bodies and enter the decayed or dead new host through the oviposition holes.

In Portugal, where the PWN was detected in 1999, the endemic pine sawyer *Monochamus galloprovincialis* (Olivier) is its sole vector (Sousa *et al*. 2001), and the recently detected nematode-vector association has been subjected to several studies, namely the characterisation of the transmission by the insect's feeding activity (Naves *et al*. 2007). The purpose of this study was to characterise a second possible transmission pathway, that is, by the oviposition activity of the female *M. galloprovincialis*, determining its occurrence and frequency under laboratory and field conditions and discussing the importance of this transmission pathway for the epidemiological cycle of the pine wilt disease in Portugal.

2. Materials and methods

2.1. Nematode transmission under laboratory conditions

Three adult maritime pine trees (*Pinus pinaster* Aiton) infected with *B. xylophilus* and *M. galloprovincialis* were cut in January 2004 near Comporta (38º 22'N; 8º 46'W), inside the pine wood nematode affected zone in Portugal. The trunks were divided into 70 cm bolts and maintained under ambient temperature on netted wood boxes until emergence of adult *M. galloprovincialis* in the following spring. Recently-emerged beetles were immediately checked for the presence of *B. xylophilus* using the methodology described by Zhang *et al*. (1995).

Fifteen female beetles found to be infected with PWN were placed isolated on transparent acrylic boxes (volume of 3100 cm^3), to which were added two adult males of similar size and age. The boxes, containing several small holes for air renewal, were kept in a room with a stable temperature of 23–25ºC, 15:9 LD and 60–75% humidity. Every two days the insects were given fresh maritime pine branches for feeding (15 cm long, 0.5–1 cm diameter) and some pine bolts (10 cm long, 4–5 cm diameter) for laying eggs. When they attained 30 days of life [age which corresponds roughly to the oviposition peak of this species (Naves *et al*. 2006)], the males were removed from the boxes and each female was given a single *P. pinaster* bolt (10 cm long, 4–5 cm diameter) for laying eggs, along with two additional 0.5–1 cm wide pine branches to feed. The feeding and oviposition bolts were obtained six days earlier from 10-year old healthy *P. pinaster* trees cut on Oeiras, outside the *B. xylophilus* affected zone.

Each bolt remained with the female for 24h, after which was replaced by a similar one, totalling four replicas (bolts) per beetle. After the removal of the last bolt, the females were measured (length of the right elytra), weighted and macerated on a Petri dish with water, counting the number of nematodes present in the water after 12 hours.

Oviposition wounds on bolts were counted and debarked, to determinate if they contained eggs. If present, feeding scars on the bark were counted and removed with a vertical electric saw, along with a piece (2–3 cm) of adjacent wood, in order to prevent the contamination of the wood with nematodes eventually introduced during the female's feeding activity. The pine bolts were then stored in closed plastic bags at 25ºC ± 1ºC and 75% RH for two weeks, to allow the reproduction of the nematodes introduced on the wood and increase the probability of extracting them, which was made using the modified Baermann technique (Southey 1986). Direct counts were made for samples lower that 500 nematodes, while for higher nematode values a counting lamellae with 1ml of the sample was used, replicating the count five times and extrapolating the mean of the five replicas to the initial sample volume.

Spearman correlations were performed to analyse the relation between insect dimensions (size and weight) and nematode load, and also between PWN abundance and number of oviposition wounds (with and without eggs), feeding wounds and insect nematode load. Statistical procedures were carried out using the software Statistica 6 (StatSoft Inc. 2003).

2.2. Nematode transmission under field conditions

Maritime pine trap-trees were set inside Tróia (38º 28'N; 8º 52'W) and Comp. das Lezírias (38º 47'N; 8º 49'W) inside the PWN affected zone, Portugal. Each trap consisted of an apparently healthy adult *P. pinaster* tree (mean 18 cm chest height diameter) debarked at waist height, removing alongside part of the cambium and xylem wood to completely stop the resin flow. The objective was to slowly debilitate and eventually kill the tree, thus turning it into a highly attractive host for oviposition by the local *Monochamus* population. An initial sample of wood from each tree was taken at breast height and analysed on the laboratory using the modified Baermann technique to ensure that the pines were nematode-free. The trap-trees were created between May and September of 2001 (two trap-trees for each month and location) and 2002 (three trap-trees for each month and location), in a total of 50 trees for both years. In November of each year the trees were cut, divided in one meter logs and taken to the Lab, where they were analysed (trunk and branches) for the presence of *M. galloprovincialis* oviposition and feeding scars. Two wood discs were collected at breast height and mid-crown from each tree and analysed for the presence of *B. xylophilus* using the modified Baermann technique.

3. Results

3.1. Nematode transmission under laboratory conditions

Of the 60 bolts analysed, 46 (77% of the total) contained a total of 124 *M. galloprovincialis* oviposition wounds, of which 66% had eggs. Despite the presence of small pine branches adequate for maturation feeding, 38% of the bolts intended for oviposition also had maturation feeding scars (Table 1).

Only 37% of the bolts contained nematodes and usually with low values, as the mean was lower that 300 individuals per bolt (Table 1). *B. xylophilus* was the only nematode present on the wood.

Table 1. Mean number of *M. galloprovincialis*' feeding and oviposition wounds per bolt and mean number of *B. xylophilus* recovered from the bolts. n = 60 bolts. Mean values ± *S.E.*

Feeding wounds	Oviposition wounds		No. PWN
	Without eggs	With eggs	
0.78 ± 0.15	0.70 ± 0.19	1.37 ± 0.22	290 ± 70

No relation was found between *B. xylophilus* wood abundance and the number of insect feeding wounds (r = 0.208, p = 0.11) or oviposition wounds without eggs (r = –0.118, p = 0.37). There was a modest (but significant) correlation between the number of oviposition wounds with eggs and nematode abundance (r = 0.575, p<0.001).

All the dissected *Monochamus* females were found to carry *B. xylophilus* on their bodies, with a mean value of 1684 ± 516 (mean ± *S.E.*) nematodes per insect. The mean length of the 15 female beetles was 15.2 ± 0.4 mm, with a weight of 0.3868 ± 0.023 g (mean values ± *S.E.*), and it was found that the smaller beetles carried a larger number of nematodes than the larger ones, as modest negative correlations were found between the nematode load and beetle size (r = –0.432, p = 0.11) and weight (r = –0.566, p = 0.028), being the second correlation significant. No relation was found between the insect's nematode load and PWN abundance on wood (r = 0.079, p = 0.55).

3.2. Nematode transmission under field conditions

All the wood samples collected initially from the pines tested negative, and thus the selected trees were considered to be nematode-free at the date of trap-tree creation. Over the two years, 29 of the 50 pines (58%) were colonised by *M. galloprovincialis*, with a total of 439 oviposition wounds detected (Table 2).

Despite the abundant presence of *Monochamus* reproductive activity on the majority of the pines, only four of the trees with sawyer pres-

Table 2. Number of trap-trees colonised by *M. galloprovincialis* (*M. g.*) on the two locations (Lezírias and Tróia), total number of *M. g.* oviposition wounds on the trees, number of trees infected with *B. xylophilus* and mean number of PWN per 100g of wood. There were two and three trap-trees in 2001 and 2002, respectively, in each month and location.

	Lezírias				Tróia			
Date (month/ year)	No. trees with *M.g.*	No. *M.g.* wounds	No. trees with PWN	Mean PWN/ 100g	No. trees with *M.g.*	No. *M.g.* wounds	No. trees with PWN	Mean PWN/ 100g
May/01	2	41	0	–	2	120	0	–
May/02	1	27	0	–	1	5	1	116
June/01	2	54	1	833	1	8	1	477
June/02	2	6	0	–	2	11	0	–
July/01	1	31	0	–	1	9	0	–
July/02	3	19	0	–	2	2	0	–
Aug/01	2	10	0	–	2	40	0	–
Aug/02	0	–	0	–	0	–	0	–
Sept/01	2	22	0	–	0	–	0	–
Sept/02	2	32	0	–	1	2	1	56
Total	17	242	1	–	12	197	3	–

ence were found containing *B. xylophilus*, with a mean of 371 nematodes per 100g of wood. The pine wood nematode was the only species of the *Bursaphelenchus* genus present on the wood, although other Aphelenchoididae were also found on some trees.

4. Discussion

The laboratory experiment confirmed that transmission of *B. xylophilus* through the oviposition activity of female *M. galloprovincialis* can occur. Some of the oviposition bolts also contained additional feeding wounds and oviposition wounds without eggs, but bolt nematode abundance appears to be related to the existence of oviposition wounds with eggs. The transmission efficiency was rather low, though, as only 37% of the laboratory bolts and 14% of the field trap-trees became PWN infected, and usually with low nematode numbers (less than 100 nematodes per bolt for the Lab experiment, despite the bolts had been kept on favourable conditions for *B. xylophilus* reproduction during 15 consecutive days). Concerning the field experiment, the percentage of trap-pines with *M. galloprovincialis* oviposition wounds (58%) and the total number of oviposition wounds detected (439) can be considered relatively low, and reflect a low abundance of *Monochamus* females reproducing on the two areas selected and/or an abundance of oviposition-suitable pine hosts, which disperse the reproductive activity of the field insects. This might also explain the very low number of eggs laid on the September trap-trees, which probably reflect a decrease in the insect population during late summer in parallel with a significant augment on the number of naturally occurring decaying and dead pine trees adequate for oviposition.

It is not possible to determinate the number of insects that visited the trap-trees to lay eggs, or know their nematode infection rate, but analysis of field collected beetles captured on both locations show that usually about 70% of the field-captured insects are infected with *B. xylophilus* (Sousa, unpubl.). As so, it is reasonable to assume that a significant part of the insects that visited the pine trees were infected by *B. xylophilus*, despite the fact that only four of the trees became contaminated by PWN. The possibility of additional nematode transmission occurring through the feeding activity of the beetles cannot be excluded, but it was impossible to evaluate as the trees had multiple bark-feeding scars made by different insect species (cerambycidae, curculionidae and scolytidae), which cannot be discriminated by species.

M. galloprovincialis has been found to successfully transmit *B. xylophilus* by the feeding activity on pine branches under laboratory conditions (Naves *et al*. 2007). This is the first record of PWN transmission via *M. galloprovincialis* oviposition under laboratory conditions, although this transmission pathway has been reported for several North American *Monochamus* species (Wingfield 1983, Wingfield & Blanchette 1983, Luzzi *et al*. 1984, Edwards & Linit 1992). Transmission via oviposition is probably an evolutionary adaptation for avirulent nematodes to persist in forest ecosystems, as the nematodes are inoculated by their vectors in already dead or weakened hosts, and thus do not need to kill the tree in which they are inoculated (Wingfield & Blanchette 1983). As for *B. xylophilus,* this transmission pathway probably evolved as an adaptation to North American pine ecosystems, where the pine wood nematode usually does not cause pine wilt on indigenous pines, being generally associated with trees suffering from the activity of other biotic or abiotic decline agents (Wingfield *et al*. 1982, Wingfield 1983, Rutherford & Webster 1987, Bergdahl 1988).

Similarly to other *Monochamus* species, female *M. galloprovincialis* only lay eggs on already dead or dying conifers, of which there is an abundant supply on the Portuguese PWN affected zone during the late summer months due to previous *B. xylophilus* infection. Other locally important pine mortality agents include cyclic heavy defoliation by the pine processionary moth [*Thaumetopoea pityocampa* (Den. & Schiff.)], forest fires, bark beetles (curculionidae and scolytidae) and drought, but, with the exception of forest fires, the other mortality agents exert their action mainly on young, dominated and low-sized pines, which are usually not colonised by *M. galloprovincialis* nor *B. xylophilus* (Sousa, unpublished results). As so, a large proportion of the *Monochamus* females lay their eggs on pines already contaminated by PWN. Even if the female insect lays eggs on nematode-free pines the transmission efficiency appears to be rather low, as found both on the Laboratory and field experiment. The results reported on this paper suggest that in Portugal, similarly to other areas of the world where *B. xylophilus* was introduced (*e.g.* Mamiya 1984), and unlike PWN transmission through feeding activity (Naves *et al*. 2007), transmission of *B. xylophilus* through oviposition wounds is an infrequent and secondary inoculation pathway and probably constitutes a minor component of the pine wilt disease epidemiology.

Acknowledgements. We thank Mr. Rui Andrade for his help in obtaining the trees and an anonymous reviewer for its comments. The work was partially funded by the Foundation for Science and Technology (Portuguese Ministry of Science and Technology) through the project Parle D, and also the European Phrame project QLK5-CT-2002-00672.

References

Bergdahl, D. 1988: Impact of pinewood nematode on North America: present and future. — J. Nematol. 20: 260–265.

Edwards, O. & Linit, M. 1992: Transmission of *Bursaphelenchus xylophilus* through oviposition wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). — J. Nematol. 24: 133–139.

Kishi, Y. 1995: The pine wood nematode and the Japanese pine sawyer. — Thomas Company Limited, Tokyo, Japan. 302 pp.

Linit, M. 1988: Nematode-vector relationships in the pine wilt disease system. — J. Nematol. 20: 227–235.

Linit, M. 1990: Transmission of pinewood nematode through feeding wounds of *Monochamus carolinensis* (Coleoptera: Cerambycidae). — J. Nematol. 22: 231–236.

Luzzi, M., Wilkinson, R. & Tarjan, A. 1984: Transmission of the pinewood nematode, *Bursaphelenchus xylophilus* to slash pine trees and log bolts by a cerambycid beetle, *Monochamus titillator*, in Florida. — J. Nematol. 16: 37–40.

Mamiya, Y. 1984: The pine wood nematode. — In: Nickle, W. R. (ed.), Plant and insect nematodes: 589–626. Marcel Dekker Press, New York.

Mamiya, Y. & Enda, N. 1972: Transmission of *Bursaphelenchus lignicolus* (Nematoda: Aphelenchoididae) by *Monochamus alternatus* (Coleoptera: Cerambycidae). — Nematologica 18: 159–162.

Naves, P., Sousa, E. & Quartau, J. 2006: Reproductive traits of *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) under laboratory conditions. — Bull. Entomol. Res. 96: 289–294.

Naves, P., Camacho, S., Sousa, E. & Quartau, J. 2007: Transmission of the pine wood nematode *Bursaphelenchus xylophilus* through feeding activity of *Monochamus galloprovincialis* (Coleoptera, Cerambycidae). — J. Appl. Entomol. 131: 21–25.

Rutherford, T. & Webster, J. 1987: Distribution of pine wilt disease with respect to temperature in North America, Japan, and Europe. — Can. J. For. Res. 17: 1050–1059.

Sousa, E., Bravo, M., Pires, J., Naves, P., Penas, A., Bonifácio, L. & Mota, M. 2001: *Bursaphelenchus xylophilus* (Nematoda; Aphelenchoididae) associated with *Monochamus galloprovincialis* (Coleoptera; Cerambycidae) in Portugal. — Nematology 3: 89–91.

Southey, J. F. 1986: Laboratory methods for work with plant and soil nematodes. – Ministry of Agriculture, Fisheries and Food, Reference Book 402, H.M.S.O., London. 202 pp.

Wingfield, M. 1983: Transmission of pine wood nematode to cut timber and girdled trees. — Plant. Dis. 67: 35–37.

Wingfield, M. & Blanchette, R. 1983: The pine-wood nematode, *Bursaphelenchus xylophilus*, in Minnesota and Wisconsin: insect associates and transmission studies. — Can. J. For. Res. 13: 1068–1076.

Wingfield, M., Blanchette, R., Nicholls, T. & Robbins, K. 1982: The pine wood nematode: a comparison of the situation in the United States and Japan. — Can. J. For. Res. 12: 71–75.

Zhang, X., Stamps, W. & Linit, M. 1995: A nondestructive method of determining *Bursaphelencus xylophilus* infestation of *Monochamus* spp. vectors. — J. Nematol. 27: 36–41.