# Experimental confirmation of *Bursaphelenchus xylophilus* survival and propagation in birch logs

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**Summary.** The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, introduced from North America to Asia, has caused extensive pine wilt disease of coniferous forests in China, Japan, and the Republic of Korea. PWN has not been detected in Russia, but there is a threat of possible introduction of this pathogen from these neighbouring countries. Transfer of nematodes from an PWN-infested tree to a healthy tree is achieved by *Monochamus* longhorned beetles during maturation feeding on coniferous twigs. The white mottled sawyer (WMS) *M. urussovii* beetles infested with pinewood nematodes attack birch trees *Betula* spp., leading to the hypothesis that the resulting nematodes may also infect birch. Nevertheless, is it possible for the PWN to survive in birch wood? To address this question, an experiment was conducted to investigate whether PWN could survive and reproduce in the logs of silver birch *B. pendula*. Birch and pine (*Pinus sylvestris*) logs were inoculated with 500 PWN per log. The nematodes multiplied in pine and birch logs during the entire study period (80 days). Nematode numbers increased 171-fold in pine logs and 5-fold in birch logs compared to the initial inoculum. The highest average nematode abundance in birch logs (8-fold increase in number) was observed on the 45th day of experiment. The authors intend to continue their research on the possibility of PWN transmission and survival in living birch trees.

**Key words:** *Betula pendula, Monochamus urussovii,* pine wilt disease, pinewood nematode.

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer), is one of the world's most dangerous conifer pathogens. The original PWN distribution is North America where native conifer species are resistant/tolerant to the pathogen (Rutherford *et al*., 1990; Sutherland, 2008). PWN was introduced to Asia in the early 20th century and spread to China, Japan and the Republic of Korea causing an epidemic of pine wilt disease in pine forests (Futai, 2008; 2021; Shin, 2008; Zhao, 2008). The situation is similar in Portugal, with the first PWN outbreak detected in 1999 (Mota *et al.,* 1999) and has continued to spread across most of the entire Portuguese territory (Mota & Paulo, 2008; Vicente *et al*., 2011; Fonseca *et al*., 2012; Rodrigues *et al*., 2015). Subsequent, outbreaks of PWN have been detected in Spain (Abelleira *et al.,* 2011; Robertson *et al*., 2011; De la Fuente & Saura, 2021). *Bursaphelenchus xylophilus* is included in quarantine lists of most countries of the world including Russia (EPPO, 2022).

The transfer of PWN from tree to tree is carried out by longhorn beetles of the genus *Monochamus* Dejean during their maturation feeding and egg-laying period. The life cycles of these two organisms, are closely related. The fourth dispersal juvenile stage (JIV, ‘dauer juvenile’) of PWN enters through the spiracles and localizes mainly in the tracheal system of the beetles. Nematodes leave the beetles during their maturation feeding period on twigs of healthy trees and penetrate the wood of the tree. Within a healthy tree, PWN feed on epithelial cells of resin canals, rapidly multiply and disperse throughout the tree, causing wilt disease culminating in tree mortality (Kuroda, 2008; Futai, 2013). In the wood of a dead tree, PWN feeds on the mycelia of fungi that occur within the dead tree (Mamiya, 1983, 1984; Vicente *et al.,* 2022). Trees killed by PWN are further substrate for egg-laying

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wood-boring insects, including sawyer beetles that attack weakened trees or logs. Usually, *Monochamus* beetles do not lay eggs on healthy trees (Izhevskii *et al.*, 2005). A symbiotic relationship exists between PWN and *Monochamus* spp. utilising the same host species. It is assumed that all *Monochamus* spp. associated with conifers are potential vectors of PWN (Linit, 1988; Akbulut & Stamps, 2011; Bonifácio *et al*., 2015), so it is presumed that *M. urussovii* (Fischer), can also transmit PWN. Adults of *M. urussovii* have been found in Hokkaido, Japan, as vectors of the *B. mucronatus* nematode, a closely related species to PWN (Togashi *et al*., 2008).

PWN has not been detected in Russia, but pest risk analysis have shown that PWN could become established in significant areas of the country (Kulinich & Kolosova, 1995; Kulinich *et al.,* 2020). In Russia there are coniferous species susceptible to PWN, such as Scots pine (*Pinus sylvestris* L.), Siberian larch (*Larix sibirica* Ledeb.) and Korean pine (*Pinus koraiensis* Sieb.). Seven species of *Monochamus* longhorn beetles are associated with conifers in Russia. These are *M. galloprovincialis* (Oliv.), *M. impluviatus* Motsch., *M. nitens* (Bat.), *M. saltuarius* Gebl., *M. sartor* (F.), *M. sutor* (L.), and *M. urussovii.* Principle host trees of *Monochamus* longhorn beetles are conifer species. *Monochamus urussovii* often attacks birch trees when conifers are scarce (Yanovsky & Baranchikov, 1999; Izhevsky *et al.,* 2005; Grodnitsky & Gurov, 2017). This longhorn beetle species is widely distributed in Russia and neighbouring countries, including Mongolia, China, Republic of Korea, Kazakhstan and Japan, and is also found in northern and parts of interior Europe (Izhevskii *et al.,* 2005; EPPO, 2023).

The life cycle of *M. urussovii* varies from 1-3 years but is usually completed in 2 years and the flight period occurs from late May through to September. The beetle infests wilting and felled trees of spruce (*Picea* spp.), fir (*Abies* spp.), larch (*Larix sibirica* Ledeb., *L. gmelinii* (Rupr.) Kuzen., *L. olgensis* (A. Henry) Ostenf. & Syrach, *L. kamtschatica* (Rupr.) Carriere, and pines including *Pinus sibirica* (Du Tour) and *P. koraiensis* Siebold & Zuccarini. It may also colonise birch trees during maturation feeding (Yanovsky & Baranchikov, 1999; Izhevsky *et al.,* 2005).

In maturation feeding experiments of *M. urussovii* different tree species, Grodnitsky and Gurov (2017) noted that among deciduous species, silver birch, *Betula pendula* (Roth) is ranked first as a host plant for this beetle, preferred over *Pinus sibirica*. Silver birch is widespread throughout Russia. The tree grows in a variety of soils within various climatic conditions, from the far North to the Southern regions of Russia. Birch is often used in landscape design, urban greening, reforestation and in land reclamation. Birch wood is used for construction and furniture production.

Due to the significance of silver birch, this study aimed to examine the hypothesis concerning the survival and reproduction of the pinewood nematode in birch wood when white mottled sawyer carrying PWN juveniles colonise *B. pendula* logs. An experiment was conducted to investigate the survival of PWN in silver birch logs, a non-specific host species for PWN.

## MATERIALS AND METHODS

**PWN survival in birch wood.** To investigate the survival and reproduction of the pinewood nematode in silver birch, logs from this hardwood and from Scots pine for comparison were obtained from the experimental site of All-Russian Plant Quarantine Center, Moscow region, Russia (GPS 55.641464, 38.103010). Experiments on PWN survival in logs were conducted in the laboratory of this institution from May to September, 2022. Scots pine logs were used as a control in the experiment. Fifteen sawed logs of pine and birch with an average diameter of 7-10 cm and a length of approximately 25 cm were collected for the study. All 30 birch and pine logs used in the experiment were checked for the presence of any nematode species. The ends of the logs were sealed with paraffin to reduce drying. At a distance of 10 cm from the end of each log, two holes (5 mm diam.) were drilled to the core of the log using a battery-powered drill (Makita DHP451RFE LXT). Using a Sartorius pipette dispenser, 100 µl of a suspension containing 500 PWN nematodes (BxAm) of different stages was injected into each log, averaging 50 ± 14 nematodes (100 g)$^{-1}$ wet weight of wood for each PWN-infested log.

To infect the logs, a PWN population was propagated on *Botrytis cinerea* fungus. After introducing the nematode inoculum into the holes, the logs were covered with Parafilm, then wrapped in plastic wrap and kept in a thermostatically controlled environment (Panasonic MIR-274 PE) at 27°C for 2.5 months.

The study PWN survival within inoculated birch and pine logs was conducted in laboratory of the forest quarantine department. Treatments were evaluated 21, 30, 45 and 80 days following nematode inoculation of logs. Wood chips were taken from each log using a cordless drill (Makita) and a 16 mm diameter drill bit. Nematodes were isolated using the Baermann funnel technique, after 24 h of wood chips immersion at a temperature 20°C.

The logs had different weight and masses, so when comparing the nematode infestation of pine and birch logs, the average number of extracted nematodes was recalculated per 100 g of wet weight of wood. Nematode abundance data were processed in MS Excel. Significant difference was set at $P <$ 0.05.

**Maturation feeding of *Monochamus urussovii* on birch.** Logs of Scots pine with signs of egg-laying by *Monochamus* sp. beetles were sawn from fallen trunks of pine in the forests in the Moscow region. The feeding larvae of the longhorned beetle in the log were making a characteristic sound. Several pine logs were split to extract the insect larvae to confirm their presence. The logs were placed in a tightly closed 60 × 60 cm entomological cage (BioQuip). Two each of 1- and 2-year-old twigs (0.5 – 1.5 cm diam.) of silver birch and Scots pine were placed in the same cage. White mottled sawyer behaviour was observed including emergence from the logs and the period of maturation feeding from May to September, 2022.

## RESULTS

Testing of pine and birch logs prior to PWN inoculation revealed that they were not infested with any nematode species. Nematode counts in Petri dishes during the control periods on days 21, 30, 45 and 80 visually revealed no other nematode species except *B. xylophilus*. The study results indicate that the nematode population density in PWN-infested birch and pine logs at the conclusion of the experiment on the 80th day of observation was significantly higher compared to the original inoculum inoculated in the wood.

**Pine logs.** PWN multiplied most intensively in pine logs. At 21 days after infestation of the logs, the average nematode abundance increased 10-fold, and at 30 days it increased more than 100-fold. There was a direct proportional relationship: nematode abundance increased depending on the duration of log incubation in the thermostatically controlled environment. The total number of nematodes in pine logs on day 80 of the study increased 171 times (8565 nematodes $(100\ g)^{-1}$) wet weight of wood compared to the initial inoculum of 50 ± 14 nematodes $(100\ g)^{-1}$).

**Birch logs.** Nematodes in birch logs multiplied significantly less than in pine. Their numbers after 21 days were the same as in the initial inoculum (50±14 nematodes $(100\ g)^{-1}$) wet weight). At 21 days the number of juveniles was 17% of their total number (Fig. 1).

On day 30 of the study, nematode abundance in birch logs exceeded the volume of inoculum by a magnitude of 1.6. The average abundance of PWN had increased by a magnitude of 8 on day 45 after inoculation totaling 402 nematodes $(100\ g)^{-1}$) of wet weight of wood ($P < 0.05$). At the conclusion of the study, day 80 of the experiment, mean PWN abundance decreased compared to the previous day 45 observation totaling 261 nematodes $(100\ g)^{-1}$) wet weight of wood (Fig. 1) ($P < 0.05$). However, this was significantly higher (a magnitude of 5) compared to nematode abundance in the initial inoculum (Table 1).

Thus, nematodes multiplied significantly in birch wood. On day 21 from the original inoculation, adult nematode abundance reached 83% compared to the juvenile stage, 17%. However, on day 30 of the study, the ratio of juveniles to adults was similar for the remainder of the experiment. (Fig. 1).

**Maturation feeding of *Monochamus urussovii*.** Observation of *M. urussovii* beetles in laboratory cages with birch and pine twigs revealed that 17 adult WMS beetles emerged from logs from June to August, 2022. Maturation feeding of WMS beetles on pine and birch twigs was observed from June to the end of September, 2022. Figure 2 displays signs of WMS beetles feeding on twigs of birch and pine.

## DISCUSSION

The results of the experiment revealed that PWN can survive and reproduce in silver birch logs. Nematodes multiplied significantly for the first 45 days of observation but decreased at the 80-day observation. Although PWN numbers decreased after this period, they were significantly higher compared to the original inoculum.

PWN is a plant-parasitic nematode and feeds on healthy conifer wood on epithelial cells of the resin canals (Mamiya, 1983, 1984; Kuroda, 2008). In dead conifer wood, PWN feeds on hyphae of fungi that are commonly present in wood (Wingfield & Blanchette, 1983; Back *et al*., 2024). These are mostly fungi of the genera *Fusarium, Botrytis, Ophiostoma sensu lato* etc. (Vicente *et al*., 2022) The PWN are able to multiply on some of other fungal species (Fukushige, 1991; Pimentel *et al*., 2020; Vicente *et al*., 2022). Live epithelial cells of resin canals are absent in birch logs, so is hypothesised that nematodes multiplied in birch logs on fungal hyphae, but this must be confirmed through the isolation of fungi both from the wooden

**Table 1.** Average pinewood nematodes number in birch (n=15) and pine logs (n=15) at sampling periods of the experiment (nematode individuals $(100\ g)^{-1}$) wet weight of wood).

| Tree species (logs) | Nematode numbers in the inoculum[1] | Nematode numbers in wood following inoculation | | | |
|---|---|---|---|---|---|
| | | 21 days | 30 days | 45 days | 80 days |
| ***Betula pendula*** | 50±14 | 27±19 | 81±34 | 402±85 | 261±86 |
| ***Pinus sylvestris*** | | 519±42 | 5458±68 | 5771±2773 | 8565±6671 |

[1]- Day 1 (PWN infection of logs).

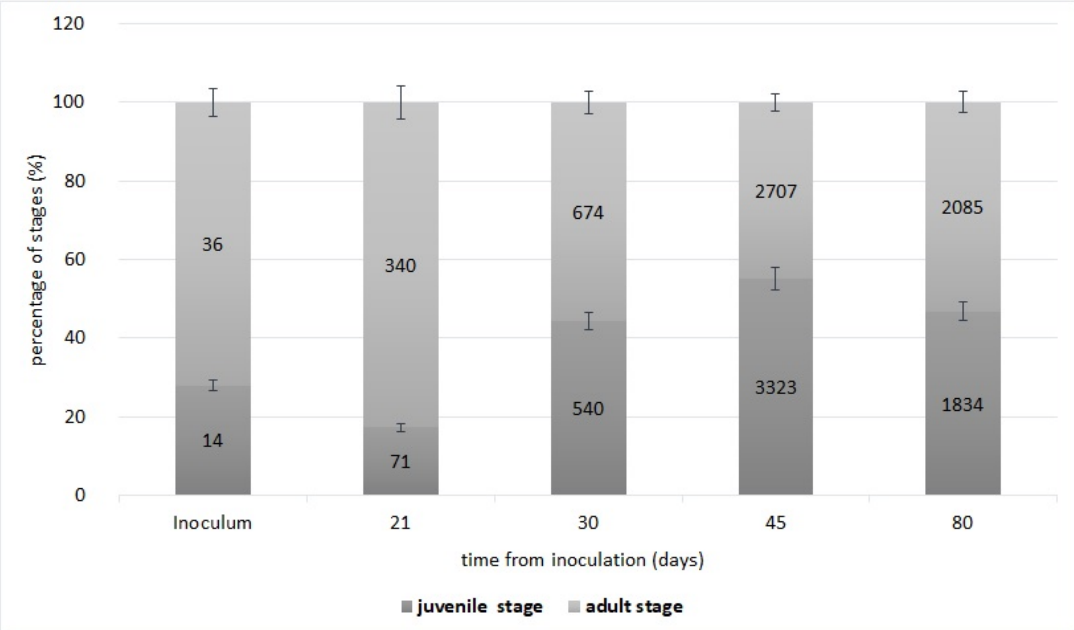


**Fig 1.** Ratio of juvenile and adult nematode *Bursaphelenchus xylophilus* in *Betula pendula* birch logs at different observation periods after PWN infection (%).

material.

There are no data on the association of PWN with deciduous species, but there is information on the association of the closely related species *B. mucronatus* with deciduous trees in previous experiments (Ryss *et al*., 2018). Except for the virulence, *B. mucronatus* and *B. xylophilus* have similar biology and life cycle, and their host plants are coniferous trees. Their tree-to-tree vectors are the same beetle species in the genus *Monochamus*. However, compared to PWN, *B. mucronatus* is widely distributed worldwide and is considered non-pathogenic or weakly pathogenic for conifers (Kulinich *et al*., 2010; Dayi & Akbulut, 2011). The longhorn beetle *M. urussovii* has been noted as a vector of *B. mucronatus* (Togashi *et al*., 2008).

Experiments on the survival and pathogenicity of *B. mucronatus* in different trees were conducted by Ryss (Ryss *et al*., 2018; Ryss 2023). Cuttings of different coniferous and deciduous plants placed in soil were infested with *B. mucronatus* nematodes. An increase in nematode abundance was found only in *P. sylvestris*. On rooted cuttings of Norway spruce, *Picea abies*, and *Betula pendula* a significant decrease in *B. mucronatus* abundance was observed compared to the inoculum. Wilting symptoms were completely absent in deciduous plants. In our studies, the closely related species *B. xylophilus* survived and reproduced in the wood of *B. pendula*. Our study conclusion does not contradict the conclusion of Ryss (2023) about the specificity of host plants and nematodes of *Bursaphelenchus* spp. Our experiments also indicate that *B. xylophilus* reproduces significantly better in Scots pine than in *B. pendula*. In conclusion, PWN can survive and reproduce in silver birch wood in controlled conditions. However, whether PWN can survive in living *B. pendula* trees and what factors favour its reproduction in dead *B. pendula* wood requires far more studies.

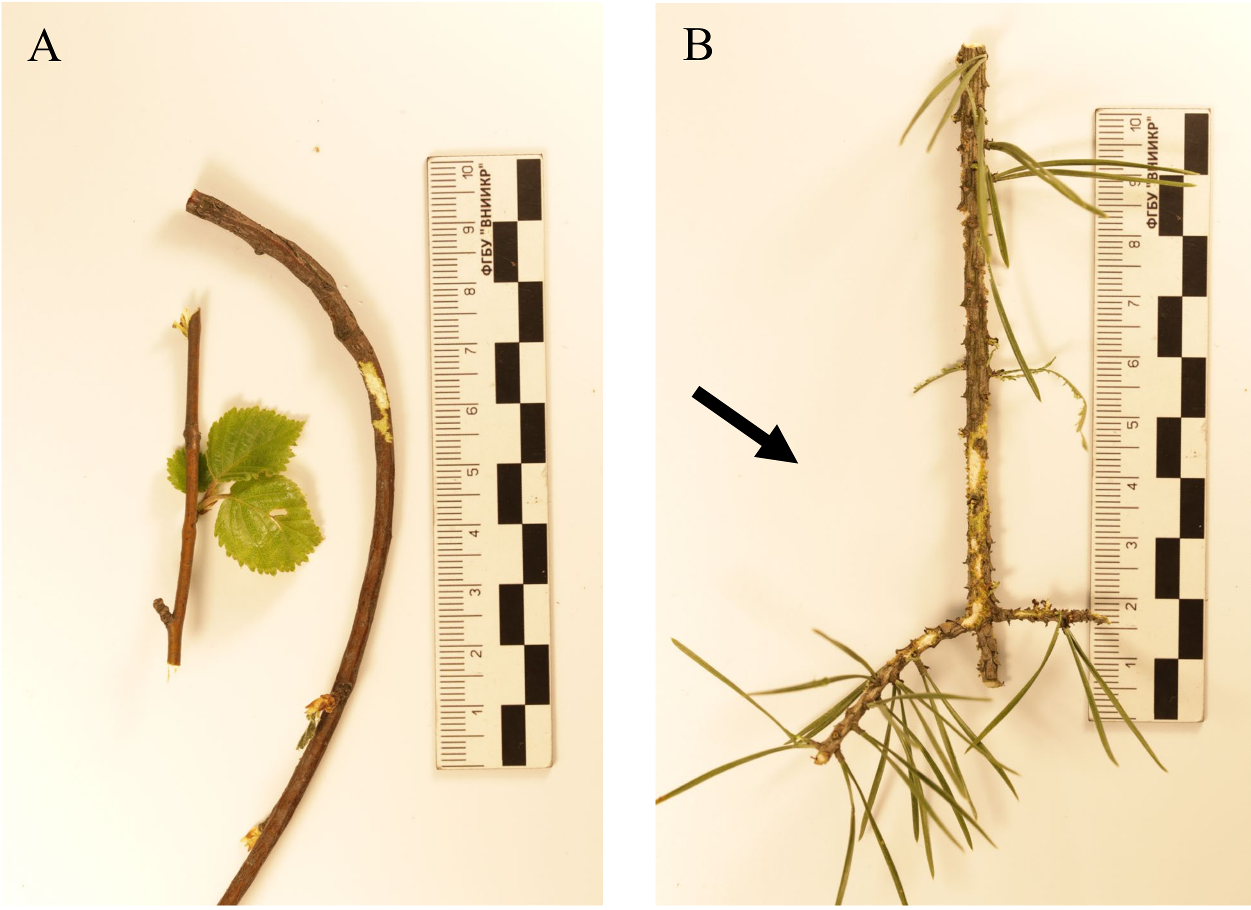


**Fig. 2.** Signs of maturation feeding by the white mottled sawyer, *Monochamus urussovii* on (A) silver birch *Betula pendula*, and (B) on Scots pine, *Pinus sylvestris*. (Scale bar in cm.)

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**О.А. Кулинич, Е.Н. Арбузова, А.А. Чалкин, и Н.И. Козырева.** Экспериментальное подтверждение выживаемости и размножения *Bursaphelenchus xylophilus* в берёзовых бревнах.

**Резюме.** Сосновая стволовая нематода *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, занесенная из Северной Америки в Азию, вызвала массовое увядание хвойных лесов в Китае, Японии, Республике Корея. В России *B. xylophilus* не выявлена, но существует угроза возможного заноса этого патогена из этих стран. Перенос *B. xylophilus* с дерево на дерево осуществляется усачами рода *Monochamus.* Нематоды покидают жука в период откладки яиц и дополнительного питания в кроне хвойных деревьев. Усач *M. urussovii,* широко распространенный на территории России, кроме хвойных пород также может заселять березу. Если усач, зараженный *B. xylophilus*, заселит березу, то предполагается, что вышедшие из него нематоды также проникнут в древесину березы, но возникает вопрос - смогут ли эти нематоды выжить в этой древесине? Для ответа на этот вопрос был проведен эксперимент по изучению возможности выживания и размножения *B. xylophilus* в поленьях березы повислой *Betula pendula*. Поленья березы и сосны (как контроль) были заражены *B. xylophilus* (50 нематод/100 г сырого веса древесины). В результате установлено, что нематоды размножались в древесине сосны и березы в течение всего периода исследования (80 дней): численность нематод в поленьях сосны увеличилась в 171 раз, а в березе в 5 раз в сравнении с исходным инокулюмом. Наибольшая средняя численность нематод в березе (увеличение в 8 раз) отмечена на 45 день наблюдений. Предполагается, что размножение *B. xylophilus* в древесине березы происходило за счет дереворазрушающих грибов, на которых питаются нематоды в мертвой древесине. Авторы намерены продолжить исследования по изучению возможности выживания *B. xylophilus* в живых растениях березы.

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