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Infestation of pine (*Pinus sylvestris* L.) seedlings with the pinewood nematode *Bursaphelenchus xylophilus* Steiner and Buhrer (Nickle) through wood sawdust

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Abstract

Key message There are various pathways for infesting pine trees with the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. As a result of the experiment, we determined that sawdust infested with the nematode can pose a phytosanitary risk. Nematodes actively moved from infested sawdust into root or stem damaged pine seedlings.

Context The spread of PWN, *Bursaphelenchus xylophilus* in pine plantations and forests is caused by beetles of the genus *Monochamus*, but the nematode can also be introduced via different coniferous commodities.

Aims The study objective was to assess the possibility that injured roots and stems of *Pinus sylvestris* seedlings could be infested with PWN through nematode-infested sawdust.

Methods Experiments of PWN infestation of pines were conducted in a climatic chamber at a temperature of 26 °C and moisture content of 60–70%. After a month in the climatic chamber, the seedlings were exposed to PWN through infested sawdust.

Results It was determined that PWN actively penetrated seedlings with injured stems when directly exposed to PWN-infested sawdust (83% of seedlings ($p < 0.012$)). Similar results were obtained in the trial in which during planting, seedlings with damaged roots were exposed to PWN infested sawdust (50% of seedlings ($p < 0.008$)). The nematodes could not infest seedlings if the sawdust occurred on the soil surface at a distance of 2.5 cm from the seedling stem.

Conclusion Our results indicate nematode infestation of pine trees can occur through PWN-infested sawdust.

Keywords Pine wilt disease, Non-vector spread, Scots pine, Stem, Root, Soil

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This article is part of the topical collection on Advances in the understanding of the pine wilt disease and in its management strategy.

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1 Introduction

The pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, causes pine wilt disease (PWD) under suitable climatic conditions (Mamiya 1984, 1988; Rutherford et al. 1990; Evans et al. 2009; Futai 2008, 2021). PWN is native to North America and widely distributed on that continent. Native conifers in North America are resistant to PWN (Sutherland, 2008), but when the pathogen was introduced to other continents, it began causing mass mortality of pines susceptible to the nematode. It is believed that PWN was introduced to Asia from North America in the early twentieth century, where it spread widely in Japan, Republic of Korea, Taiwan, and China (Shin 2008; Zhao 2008; Futai 2021).

The spread of PWN in coniferous forests is initiated by the beetles of the genus *Monochamus*, that carry nematodes from PWN-infested trees to other uninfested hosts. Long distance spread of the nematode can occur from transport of coniferous commodities. PWN can be introduced from plants for planting, round wood, sawn wood, wood chips, wood residues, bark, and wood packaging materials used for transporting different commodities (EPPO, 2018b; Douma et al. 2017).

Despite phytosanitary measures taken worldwide to prevent PWN introduction, the pathogen is gradually spreading throughout the world into uninfested suitable environments. In 1999, PWN was detected in Portugal, where it was presumably introduced with wood packaging materials and has now spread throughout most of the country (Mota and Paulo, 2008; Vicente et al., 2012; Sousa et al., 2015). In 2008, outbreaks of PWN were detected in Spain, where it was probably introduced by *Monochamus* spp. vectors (EPPO, 2010; Zamora et al., 2015). The PWN outbreak in Spain is under eradication, but nematodes are intercepted annually by the National Plant Protection Organizations (NPPOs) in Europe and elsewhere in the world during consignment inspections.

Wood chips infested with PWN in trade were first intercepted in Europe in 1984 when they were imported from the United States (Rautapää, 1986). Subsequently, PWN has been intercepted many times in imported consignments, mainly in wood packaging materials from countries where the nematode is widespread (Kulinich et al., 2013, 2022a; Douma et al., 2017; EFSA, 2020). A high pest risk in trade may be posed by conifer commodities, which may contain live long-horn beetles of the genus *Monochamus* infested with the PWN transmissive dauerlarvae (EPPO 2018a). At the same time, there is a risk of PWN introduction even if the wood contains only nematodes of the propagative generations without the vector. It has been found that PWN can migrate independently from infested wood to healthy wood if these wood blocks are in contact with each other (Sousa et al., 2011).

PWN poses a high pest risk to European and Asian forests (Robinet et al. 2011; Bergseng et al. 2012; Soliman et al. 2012; Tuomola et al. 2021) and is listed as a quarantine pest in many countries (EPPO 2022). Therefore, most countries banned the import of conifer plants for planting from regions where PWN is known to be present and require that sawn wood must be subjected to special phytosanitary treatments (EPPO, 2018b). Previous experimental tests indicate that plants can be infested with PWN via wood chips, if chips were produced from PWN-infested logs (Halik and Bergdahl, 1992; Panesar et al. 1994; Hopf-Biziks et al. 2017). Wood chips are widely used as a fuel source and are a frequently traded commodity between countries.

Wood sawdust can be referred to as a commodity and may contain various pathogens, including PWN, if the sawdust is produced from infested wood. It was previously thought that sawdust cannot contain pathogen vectors and therefore does not pose a threat to plants (EPPO, 2015). However, nematodes are capable of moving short distances, thus they do not always require a vector.

According to the International Standards for Phytosanitary Measures (ISPM 15), sawdust, together with wood shavings and wood wool, are classified as low pest risk products and are not covered by the Food and Agriculture Organization of the United Nations (FAO) international standard as wood packaging material (FAO 2019). Given that sawdust and wood shavings are not considered wood packaging materials, sawdust could be used for shipment packaging.

Sawdust is generally used to cover paths in garden plots and as mulch for cultivating agricultural and ornamental plants or as a carbon source in compost piles. If the wood sawdust comes from a facility where PWN-infested wood is processed (e.g., China or Portugal), the following question arises: could this PWN-infested sawdust be a source of plant infestation if it was placed on/in soil where conifer trees are growing?

The objectives of this study were to test the hypothesis about the possibility of *B. xylophilus* nematode transfer through infested sawdust into injured stems and roots of uninfested plants. A laboratory experiment was designed to determine if PWN-infested sawdust placed in various combinations in and on the soil of pine trees under climatic conditions similar to natural growing conditions could infest uninfested host plants.

2 Material and methods

2.1 Preparation of pine seedlings for the experiment

To study non-vector spreading of pinewood nematode *B. xylophilus* through sawdust, 4-year-old seedlings of *P. sylvestris* were used for the experiment. Seedlings were carefully (without root injury) transplanted (15.05.2019) from the nursery in 2-L pots with soil. The pots with seedlings were kept outdoors at the site of the All-Russian Plant Quarantine Institute in Bykovo, Russia, for 1 year. Soil consisted of raised peat, lowland peat, sand, limestone (dolomite) flour, and Azophoska complex mineral fertilizer (macroelements: N—350, P—30, K—400 mg/l, pH 5.5—6.5).

A year later (11.05.2020), pots with seedlings were placed (in a climatically controlled room at a temperature of 26 °C and humidity of 60–70%. The seedlings in the study were watered periodically as needed. Seven trials were included in the study, each with 12 pine seedlings.

To infest pine seedlings, we used an isolate of *B. xylophilus* (US1-Bx) that was extracted from wood packaging materials in Finland during inspection. The isolate was kept in the All-Russian Plant Quarantine Institute (Bykovo, Russia) for approximately 10 years. During this period, the nematodes propagated on the fungus *Botrytis cinerea* and were periodically placed in pine logs or pine seedlings. For our tests, nematodes *B. xylophilus* were propagated on the fungus *B. cinerea*.

2.2 Production of sawdust infested with nematodes *B. xylophilus*

To obtain sawdust, 14 pine logs of *P. sylvestris* 30–40 cm long, from 5 to 10 cm in diameter and with moisture contents ranging from 70 to 78% were prepared. Each log end was sealed with paraffin, three holes, 5 mm in diameter were drilled at regular intervals every 10 cm to the core of the log and 100 μl of inoculum (suspension with nematodes) was injected by pipette into each log.

One hundred micriliters of suspension contained approximately 4500 nematodes of mixed age stages. After inoculation, the holes were sealed with paraffin. The logs were wrapped in 20 μm plastic film and kept at 27 °C for 5 weeks.

Each log was checked for live nematodes before the experiment. For this purpose, wood samples were taken from each log using a "Makita" battery-powered drill with 14 mm "Bosch" wood drill bit. After determining that the infested logs contained live PWN, the previously bark peeled logs and with paraffin coated ends removed the logs were sawn using a battery-powered Greenworks chainsaw creating smaller wood pieces and sawdust (Fig. 1). The largest wood piece produced was 11 ± 0.5 mm long, 5.8 ± 0.3 mm wide, and 1.3 ± 0.1 mm thick. The wood sawdust produced was checked for *B. xylophilus*. All sampled sawdust (about 7.5 kg) was infested with the *B. xylophilus* nematodes. The average number of nematodes in 100 g of sawdust was 1550 live *B. xylophilus* specimens. Species, belonging to other nematode genera, were absent in the sawdust mixture.

The nematodes were extracted using the Baermann funnel technique by a metal sieve 105 mm diameter comprised of 0.16 mm diagonal mesh. Wood tissues were exposed to water for 24 h at a room temperature of 22–23 °C. The resulting water suspension was then studied under a microscope to observe if nematodes were present.

Fig. 1 Sawdust infested with the pinewood nematode *Bursaphelenchus xylophilus* and used in the experiment

2.3 Experiments of PWN infestation of pine seedlings through sawdust

All treatments described below were conducted in a climatically controlled room at a temperature of 26 °C and with a moisture content of 60–70%. After a month (11.06.2020) in the climatic controlled room, the seedlings were infested with PWN according to the diagram in Fig. 2. One hundred grams of sawdust infested with PWN was added to each two-liter pot with pine seedlings. The study was conducted over a 20-week period from June 11 to October, 29, 2020. The seven treatments are described below:

Seedling stems (uninjured and injured)

- I Uninjured stem + PWN-infested sawdust. The PWN-infested sawdust is placed on the soil contacting the uninjured stem.
- II Injured stem + PWN-infested sawdust. The PWN-infested sawdust is placed on the soil contacting the injured stem (Fig. 3).
- III Injured stem + PWN-infested sawdust is 2.5 cm away from the stem. The PWN-infested sawdust is placed on the soil 2.5 cm distant from the injured stem.

Seedling roots (uninjured and injured)

- IV. Uninjured roots + PWN-infested sawdust in soil. Seedlings with uninjured roots are placed in the soil mixed with PWN-infested sawdust.
- V. Injured roots + PWN-infested sawdust in soil. Seedlings with injured roots are placed in the soil mixed with PWN-infested sawdust (Fig. 3).
- VI. Injured roots without sawdust. Seedlings with injured roots are placed in the soil without sawdust.

Control

- VII. Control (without sawdust). Uninjured seedlings without sawdust.

Wilting symptoms were examined visually once a week, using a wilting class scheme presented in Table 1. The seedlings were tested for nematodes in the 4th and 5th classes of wilting (Fig. 4).

2.4 Extraction of nematodes from seedlings and soil

Scots pine seedlings were cut above the soil or sawdust layer at a height of 0.5 cm and tested for PWN presence in stems and branches. Root portions were checked for nematodes after thoroughly removing soil by applying running water to the roots. All sample plant parts (roots and stem) were cut with a hand pruner and weighed before nematode extraction using the Baermann funnel technique (Fig. 5). Sawdust collected from the pot was weighed and tested for nematodes. Nematodes were also isolated from the soil (100 cm^3) taken from the pot at the conclusion of the test.

The exposure time of wood tissues and soil in water was 24 h at room temperature (22–23 °C), after which

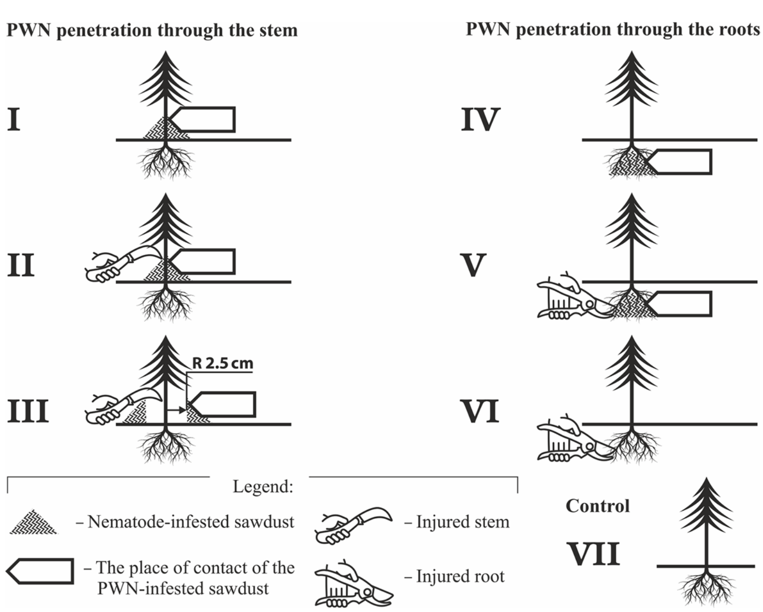

Fig. 2 Design of the experiment with pine seedlings infestation with the *Bursaphelenchus xylophilus* nematode through PWN-infested sawdust

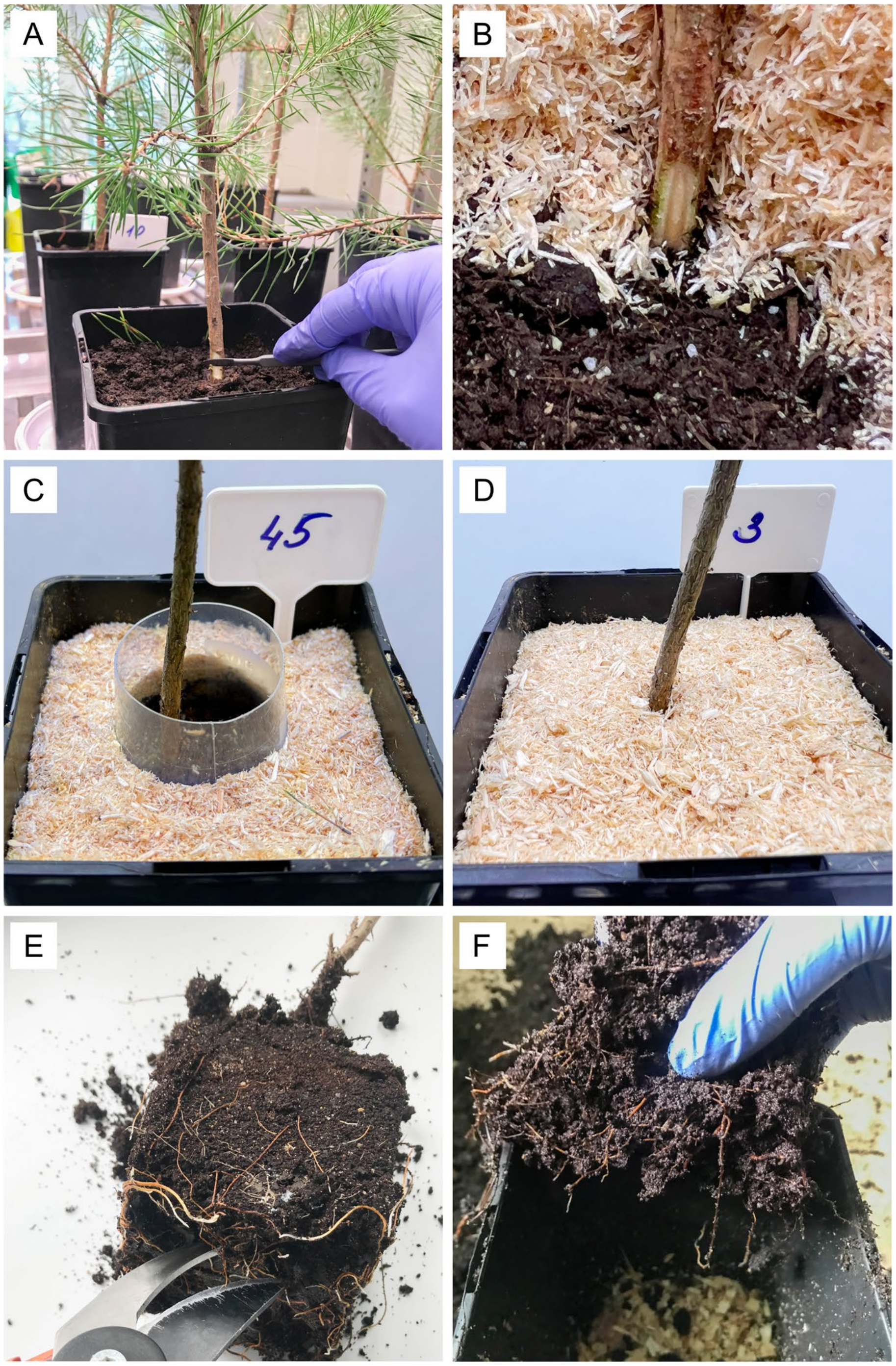

Fig. 3 Preparation of pine seedlings (*Pinus sylvestris*) for the experiment. **A** Injuries to the stem of seedlings. **B** Trial II. **C** Trial III. **D** Trial I. **E** Trial V. **F** Trial IV

the obtained suspension was studied under a microscope. The extracted nematodes were fixed with 39% formaldehyde at 60 °C.

2.5 Statistical analysis

The Pearson's criterion $\chi 2$ was used for pairwise comparison of number of PWN-infested seedlings between

Table 1 Class of wilting of nematode-infested seedlings

Wilt class	Physiological condition of seedlings
0	Healthy
1	Yellowing of needles up to 25%
2	Yellowing of needles up to 50 %
3	Yellowing of needles up to 75 %
4	Yellowing of needles up to 95 %
5	Yellowing of needles up to 100%

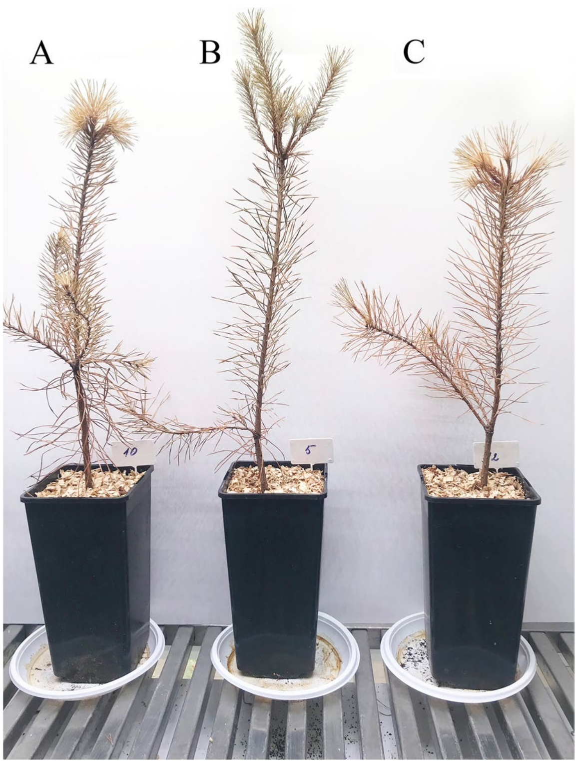

Fig. 4 Pine seedlings depicting different classes of wilting symptoms (**A**, **C** 5th; **B** 4th wilt class)

all trial treatments (uniform frequency distribution test). The mean value (M) and standard deviation (S) of nematode numbers in infested seedlings per 100 g wet weight of substrate were calculated to assess the levels of infestation. The mean number of nematodes in infested seedlings was compared by a resampling method, since the frequency distribution did not correspond to the normal frequency distribution in all samples (Lilliefors test with $p < 0.05$). We used Monte Carlo randomization (MCR-test) with the number of iterations $B = 1000$. The significance of differences p in this approach is the adjusted proportion of null-model combinations (empirical difference between means not greater than the randomized one, |Mobs| ≤ |Mran|) from the total number of iterations B. The standard value of $\alpha = 0.05$ was taken as the critical value for p (Shitikov, Rosenberg, 2013). The data was processed in MS Excel and R 4.0.1 (R Core Team, 2021) using basic functions.

3 Results

3.1 Wilting dynamics of the seedlings during the experiment

At the beginning of the experiment, all pine seedlings in each treatment were healthy with no visible display of wilting symptoms. The first wilting symptoms (needle discoloration/yellowing needles), characteristic of pine wilt diseases (PWD), appeared in eight seedlings in the 2nd week of the experiment. PWD symptoms of the 3rd wilt class were observed in three seedlings in trial II and five seedlings in trial V in week 6, where PWN-infested sawdust was in direct contact with the injured stem or root. Seedling mortality of PWN-infested seedlings (5th wilt class) was recorded 9 weeks following nematode exposure (three seedlings in trial II and three seedlings in trial V).

In trial I, where nematode-infested sawdust was in contact with the uninjured stem, the first symptoms of PWD were observed only in two seedlings in week 7. The seedlings died at week 20.

In the control trial (trials VI and VII) the seedlings remained healthy without PWD symptoms throughout the experiment period.

3.2 *Bursaphelenchus xylophilus* infestation of pine seedlings through the stem

Intensive infestation of pine seedlings with *B. xylophilus* nematodes was observed in trial II, where the treatment involved direct contact of infested sawdust with the injured stem. Nematodes were found in 83% of seedlings in trial II (Table 2). The number of PWN-infested seedlings in trial II did not differ significantly from trial V with injured roots ($\chi2 = 1.00$, df = 1, $p = 0.242$) but was higher than in all other test trials ($\chi2 > 5.33$, df = 1, $p < 0.012$). The average number of *B. xylophilus* nematodes in the seedling stem after 20 weeks of observation was the highest in trial II in comparison with other trials (3263 ± 2171 nematodes per 100 g wet weight of the wood). The first symptoms of PWD (1st wilt class) appeared the 2nd week, and six seedlings mortality (5th wilt class) occurred at the 9th week of the experiment (Fig. 6). Nematode infestation of two seedlings did not occur in trial II.

In trial I where there was direct contact of PWN-infested sawdust with an uninjured stem, nematodes did not penetrate the stem of ten seedlings. However, *B. xylophilus* was detected in two seedlings (Table 2). This number of PWN-infested seedlings was significantly lower than in trial II with injured bark ($\chi^2 = 5.33$, df = 1, $p =$

Fig. 5 Nematode tests: **A** Using the Baermann funnel technique. **B** Soil. **C** Stems. **D** Roots. **E** Mixture of soil and PWN-infested sawdust

Table 2 Trial results of pine seedling infestation (*Pinus sylvestris*) with *Bursaphelenchus xylophilus* through PWN-infested wood sawdust

Trials	**Number of PWN-infested seedlings**[1]		**PWN number in infested seedlings, per 100 g wet weight ($M \pm S$)**		
	n	**%**	**Sawdust**	**Stem**	**Roots**
Nematode infestation through the stem					
I. Uninjured stem + PWN-infested sawdust	2^{ab}	17	469 ± 470^{a}	275 ± 58^{a}	0
II. Injured stem + PWN-infested sawdust	10^{cd}	83	378 ± 206^{a}	3263 ± 2171^{b}	554 ± 530^{a}
III. Injured stem + PWN-infested sawdust 2.5 cm away from the stem	0^{a}	0	1096 ± 847	0	0
Nematode infestation through the roots					
Trials	*n*	%	Soil + sawdust[2]	Stem	Roots
IV. Uninjured roots + PWN-infested sawdust in the soil	0^{a}	0	116 ± 78	0	0
V. Injured roots + PWN-infested sawdust in the soil	6^{bc}	50	237 ± 278^{a}	715 ± 227^{b}	376 ± 131^{a}
VI. Injured roots without sawdust	0^{a}	0	0	0	0
Control					
VII. Control: seedlings without sawdust	0^{a}	0	0	0	0

Note. *n* number of plants, *M* mean value, *S* standard deviation. Different letters indicate significant differences in the columns (numbers of PWN-infested seedlings, $\chi^2 > 5.3$, $df = 1$, $p < 0.012$) and rows (mean numbers of nematodes, MCR-test: $p < 0.003$)

[1] All *B. xylophilus*-infested seedlings died at the end of the experiment

[2] Average number of PWN in soil + sawdust (nematodes/100 cm^3)

0.012) and did not differ significantly from other test trials ($\chi^2 < 2.00$, df = 1, $p > 0.104$).

Where PWN infested sawdust was not in direct contact with the seedling stem and placed 2.5 cm from the seedling (trial III), *B. xylophilus* infestation did not occur. The nematodes could not move from the PWN-infested sawdust, through soil without wood substrate, and further into the roots or into the stem of the plant. In trial VII where no PWN infested sawdust was placed in the pot, no seedling in this trial was infested with *B. xylophilus*. The experiment has therefore demonstrated that *B. xylophilus* can move from PWN-infested sawdust to pine seedlings if there is damage to the bark at the point of contact between the seedling stem and sawdust.

3.3 *Bursaphelenchus xylophilus* infestation of pine seedlings through the roots

When mixing PWN-infested sawdust with soil, nematode infestation of seedlings occurred only in trial V, where the roots were injured during planting. Nematodes were detected in 50% of all seedlings with PWD symptoms (Table 2). The number of infested seedlings in this trial was not significantly different from trials I and II ($\chi^2 < 2.00$, df = 1, $p > 0.104$) but more significant compared to other trials ($\chi2 = 6.00$, df = 1, $p = 0.008$).

Fig. 6 Seedlings of *Pinus sylvestris* showing symptoms of wilt via direct contact of PWN-infested sawdust with injured stem (6th week)

Thus, the possibility of PWN infesting root damaged seedlings via nematode infested sawdust mixed with soil was demonstrated in this experiment.

3.4 Nematode density in pine seedlings and other substrates

The study of pine seedlings at week 20 of the study period showed that all PWN-infested seedlings died. Nematode concentrations were greatest in the seedling stems (Table 2, Fig. 7). The highest number of nematodes were extracted from the injured stems of ten dead plants that directly contacted PWN-infested sawdust (trial II). The average number of PWN in trial II was 3263 ± 2171 nematodes per 100 g wet weight of wood. In trial I, where PWN infested sawdust was in contact with uninjured stems, only two seedlings died due to PWN infestation. The average concentration of PWN in the stems of both plants was twelve times lower than in trial II with only 276 ± 58 nematodes per 100 g of wet weight of wood (difference of the means is significant, MCR-test: $p = 0.034$). Seedling roots were infested only in trial II, where the number of PWN was similar to PWN numbers in the sawdust in trial V (MCR-test: $p = 0.350$).

The high number of PWN persisted in sawdust in trials I, II, and III at the conclusion of the experiment, where sawdust was placed on the soil surface. This experiment demonstrates that PWN can survive in sawdust at a temperature of 26 °C and a moisture content ranging from 60 to 70% when placed on the soil for at least 3 months. The average density of nematodes in sawdust in trial III was significantly higher than in trials I and II (MCR-test: $p < 0.040$).

In trials IV and V, where sawdust was mixed with soil, PWN was extracted from stems and roots only in trial V, where roots were injured during planting (Fig. 8). The results of infestation are generally similar to trial II, but the average nematode density in the stem was significantly lower (MCR-test: $p = 0.009$).

PWN was also found in the soil. In trials I, II and III *B. xylophilus* was found in the soil as single specimens, but in trials IV and V, where the PWN-infested sawdust was mixed with the soil before planting, *B. xylophilus* numbers (adults and larvae) were 116 ± 78 and 237 ± 278 nematodes per 100 cm^3 of soil, respectively. In addition to *B. xylophilus*, nematodes of the genera *Mononchamus*, *Dorylaimus*, *Eudorylaimus*, *Mesodorylaimus*, *Rhabditis*, *Diplogaster*, *Protorhabditis*, *Wilsonema*, and *Cephalobus* of different age stages were also found in the soil. Species of these genera are often present in soil and decomposed plant substrate. PWN density in the soil was 68–95% of the total number of all nematodes. After 3 months, *B. xylophilus* persisted in the soil mixed with sawdust (Table 2). Explanations are provided in Fig. 8.

This study implies that *B. xylophilus* nematodes are able to infest seedlings with damaged stems or roots and can survive for long periods in the soil when mixed with PWN-infested sawdust.

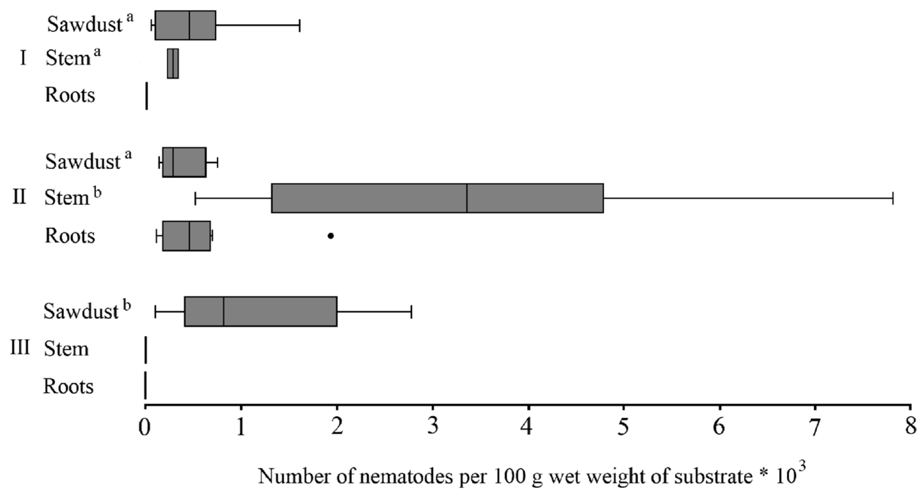

Fig. 7 Distribution of *Bursaphelenchus xylophilus* population in trials I, II, and III. The vertical borders of the box indicate half of all variability in the sample and the center line marks the median value. Lines extending from each box cover the range of remaining data. The dots in the figure indicate outliers. Different letters indicate significant differences between sawdust and stems in different trials of the experiment (mean number of nematodes, MCR test: $p < 0.040$)

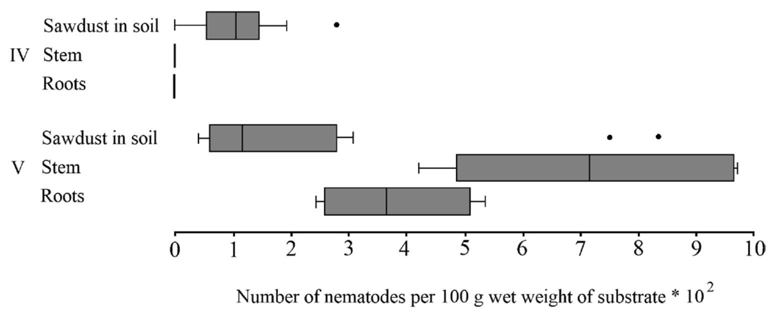

Fig. 8 Distribution of number of *Bursaphelenchus xylophilus* in trials IV and V of the experiment. The mean numbers of nematodes in sawdust in the soil between the two trials of the experiment is not significant (MCR-test: $p = 0.165$)

4 Discussion

The study objective was to determine if pine seedlings could be infested with *B. xylophilus*, through PWN-infested sawdust. Our results imply that nematodes can move actively from PWN infested sawdust into conifers if the roots or stems of seedlings are injured. Similar results have been obtained by researchers studying the possibility of pine infestation via wood chips (Halik and Bergdahl, 1992; Hopf-Biziks et al. 2017). Their experiments also implied that nematodes can actively move from wood chips into pine seedlings if the stem is injured at the point of contact with the infested woody substrate.

This pathway is similar to the natural spread of PWN, in which nematodes penetrate trees during the maturation feeding of long-horn beetles, *Monochamus* spp. or during egg-laying by females. During these processes, the insects damage the bark of branches or the trunk of trees (Linit 1990; Edwards and Linit, 1992; Togashi 2008). Considering this, phytosanitary requirements in many countries prohibit the import of conifers for planting and logs from countries where PWN is widespread because infestation of trees with *B. xylophilus* at the beginning of pine wilt disease (PWD) is undetectable and has no symptoms. In addition, conifers resistant to *B. xylophilus* may have no symptoms of PWD (Halik and Bergdahl, 1992).

Can PWN penetrate trees through healthy stems? In our experiment (trial I) with an intact stem, only two seedlings out of 12 were found to be infested with PWN. We do not exclude that both seedlings may have had micro wounds on the stem, which are difficult to determine visually. In their wood chip experiments, Hopf-Biziks et al. (2017) also identified two PWN-infested seedlings (out of 80 uninjured seedlings) and derived the same conclusion. Thus, if plants appear healthy when visually inspected, but may have been in contact with PWN-infested wood substrate (chips, shavings, sawdust), nematodes may be able to infest these trees through micro cracks. As our and other experiments have shown, when a healthy uninjured tree bole or seedling stem comes into direct contact with PWN-infested sawdust, shavings, or chips, the risk of *B. xylophilus* penetration into the tree is extremely low, but it is present (Halik and Bergdahl, 1992; Hopf-Biziks et al. 2017; Kulinich et al., 2022).

Infestation of pine can also occur through soil, if PWN-infested sawdust or wood chips are present in the soil. In this case, the nematodes usually penetrate the plants through damaged roots. In our experiment and those of Hopf-Biziks et al., 2017, about 50% of the seedlings in the test trial were infested with PWN through damaged roots. Similar results were obtained by Halik and Bergdahl (1992, 1994) in the trial with wood chips. In a study by Mamiya and Shoji (1989) using an aqueous suspension with PWN, it was found that mortality of pine seedlings was 100% if the roots were damaged. However, there was no seedling mortality if the roots were not damaged. It should be noted that often planted seedlings have damaged roots.

Another relevant question is whether PWN can survive in soil. Studies by Mamiya and Shoji (1989) indicate that *B. xylophilus* nematodes do not usually survive in just soil for long periods of time, no more than 72 h. In our studies, PWN persisted in the soil in considerable numbers (up to 20 weeks), but this soil was mixed with PWN-infested sawdust. We found that the length of nematode survival in the soil was due to the presence of PWN-infested sawdust within the soil.

Our study demonstrates that PWN can penetrate coniferous seedlings through damaged stem and roots if exposed to infested wood sawdust. This is another anthropogenic pathway for *B. xylophilus* nematode in the environment. Sawdust is used in a variety of human activities: in agriculture, in industry or can be used as wood packaging materials to transport commodities. The highest risk using PWN-infested sawdust poses may be associated with its use as mulch on agricultural plots. However, this paper does not address how often sawdust is used in human activities. This issue requires a separate study.

5 Conclusion

This research presents the results of a study on *B. xylophilus* nematode infestation of pine seedlings through PWN-infested sawdust. Our results indicate that the highest phytosanitary risk occurs when there is direct contact of PWN-infested sawdust with an injured seedling stem or injured roots. This non-vectored (*Monochamus* beetles) spread of PWN is not the natural pathway for *B. xylophilus*, but, considering anthropogenic factors, this is the pathway of spread that is often the most significant worldwide (Evans et al. 2009). Our results demonstrate that sawdust infested with PWN should also be considered in the pest risk analysis of PWN spread. Wood sawdust is often used for soil mulching on agricultural plots and for covering garden paths, it can be used as fuel and bedding for animals or for other purposes. Sawdust can be used as packaging material when moving commodities and the international standard ISPM 15 does not prohibit the use of sawdust as wood packaging material (FAO, 2019). Results obtained from the experiment indicate an additional pest risk assessment should be considered regarding possible spread of PWN through infested sawdust.

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Code availability
Not applicable.

Authors' contributions
Oleg Kulinich, Andrei Chalkin, and Elena Arbuzova: conceptualization. Viacheslav Gorbach: research and data analysis. Oleg Kulinich and Elena Arbuzova: writing—original draft preparation. Andrei Chalkin, Natalia Kozyreva and Alexander Ryss: writing—review and editing. Elena Arbuzova, Viacheslav Gorbach: resources. The authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and analyzed during the current study are available in the Zenodo repository, https://doi.org/10.5281/zenodo.7248148

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable while no humans and animals were used for the experiments.

Competing interests
The authors declare that they have no conflict of interest.

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