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Further investigations of the population dynamics and pathogenicity of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934) Nickle 1970, and its non-vector transmission to *Pinus sylvestris*

Dissertationen aus dem Julius Kühn-Institut

Julius Kühn-Institut
Bundesforschungsinstitut für Kulturpflanzen

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Bibliografische Information der Deutschen Nationalbibliothek
Die Deutsche Nationalbibliothek verzeichnet diese Publikation
In der Deutschen Nationalbibliografie: detaillierte bibliografische
Daten sind im Internet über http://dnb.d-nb.de abrufbar.

Bibliographic information published by the Deutsche Nationalbibliothek (German National Library)
The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available in the Internet at http://dnb.dnb.de.

ISBN 978-3-95547-089-0
DOI 10.5073/dissjki.2020.001

Herausgeber l Editor
Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Quedlinburg, Deutschland
Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Quedlinburg, Germany

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Further investigations of the population dynamics and pathogenicity of the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934) Nickle 1970, and its non-vector transmission to *Pinus sylvestris*

Dissertation

zur Erlangung des mathematisch-naturwissenschaftlichen Doktorgrades
"Doctor rerum naturalium"
der Georg-August-Universität Göttingen

im Promotionsstudiengang "Wood Biology and Wood Technology"
der Georg-August-University School of Science (GAUSS)

vorgelegt von

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geboren in Bad Salzungen, Deutschland

Göttingen, 2019

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Tag der mündlichen Prüfung: 16. September 2019

Dedicated to my beloved family, in particular my grandfather Karl Johannes Erbach

Acknowledgements

Many people contributed to the success of this thesis.

First and foremost, I would like to express my deep gratitude to my supervisor, Dr. Thomas Schröder (in those days at the Institute for National and International Plant Health, Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Braunschweig), and my supervisor at the time, Prof. Dr. Stefan Schütz (Forest Zoology and Forest Conservation, University of Göttingen). Together with European and Chinese project partners, Dr. Thomas Schröder successfully developed and submitted a proposal to the European research project REPHRAME called "Development of improved methods for detection, control and eradication of pinewood nematode in support of EU Plant Health policy" [KBBE.2010.1.4-09]. Based on this project, I was able to gain and contribute to knowledge in the (new to me) subject of nematology. During my job, I gained work experience outside the university at the Julius Kühn-Institut in Braunschweig for nearly 3.5 years and worked with the quarantine pest *Bursaphelenchus xylophilus*.

Beyond my dissertation, Prof. Dr. Stefan Schütz supervised my laboratory work on the vector beetle *Monochamus galloprovincialis* at the University of Göttingen. I am very thankful to both supervisors, who were also my co-authors, for their support, shared expertise, encouragement and patience during the entire process.

I wish to thank my supervisors Dr. Thomas Schröder, Prof. Dr. Wolfgang Rohe and Prof. Dr. Holger Militz for examining my PhD thesis and further members of the examination board, Prof. Dr. Ursula Kües and Prof. Dr. Niko Balkenhol, for taking part in my final examination.

Many thanks are due for the encouragement and suggestions of all my friends and colleagues at the Julius Kühn-Institut (Institute for National and International Plant Health and neighbouring institutes) in Braunschweig as well as the University (Department of Forest Zoology and Forest Conservation and Department of Wood Biology and Wood Technology) of Göttingen. I will hold you in my mind.

I extend my extra special thanks to Doreen Mybes and Silvia Urban for their vigorous technical assistance in the implementation of our nematode investigations in Braunschweig. Furthermore, for their support during my fungal and beetle laboratory work, I owe special thanks to Dr. Clovis Douanla-Meli in Braunschweig and Dr. Bernhard Weißbecker, Ulrike Eisenwiener, Sara Nicke-Mühlfeit, Thi Phuong Hoang and Dr. Gerrit Holighaus in Göttingen.

This project was funded by the European Union, which is gratefully acknowledged.

Moreover, I do not want to forget to thank my parents, my grandparents and all my teachers and professors from the school and university where I received my education for helping me reach this milestone in my life.

Cordial thanks are due to my family, including my parents, grandparents, parents-in-law, husband, and our daughter Nora, for their love, help in all life circumstances and financial support.

I want to deeply thank my grandfather Karl, who very strong believed in me and enabled my studies. Without you I would be not here, writing my PhD thesis. Moreover, I am very grateful to my husband Vladimirs. On days requiring a very large amount of practical work, he helped me a lot and also took care of our baby.

My biggest thanks go to God for everything that I have in my life.

Abstract

The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle 1970, is the causal agent of the so-called pine wilt disease in susceptible conifer species, mainly *Pinus* spp., outside its natural range (North America) and in non-native pine species. Since its introduction to Asia and Europe, it has become the most harmful plant parasitic nematode of trees. Diseased trees wilt and die. Currently affected European countries are Portugal and Spain. Because of the observed threat in infested countries, *B. xylophilus* is listed as a quarantine pest in the European Union. In this PhD thesis, which is part of a European Union research project of the European Union Commission, investigations were conducted to support the pest risk analysis, management strategies and contingency planning of European Union Plant Health policy.

The transmission of *B. xylophilus* to new host trees is commonly by vector beetles of the genus *Monochamus*. However, *B. xylophilus* have been complained in wood chips with origin North America imported to the European Union, and the demand for wood chip imports from North America is increasing. Therefore, the phytosanitary risk of non-vector transmission of *B. xylophilus* by wood chips is of interest.

Moreover, *Pinus sylvestris*, a widespread tree species in Germany and northeastern Europe, was found to be highly susceptible to *B. xylophilus* in greenhouse trials using saplings. In Europe, mature trees of this species have not yet been tested.

The third part of this thesis was an evaluation of potentially tolerant or resistant host tree provenances as an option for management of pine wilt disease in affected countries.

The long-term survival of *B. xylophilus* in wood chips and its non-vector spread from infested wood chips to non-infested trees were investigated. *B. xylophilus*-infested wood chips were produced by inoculating a nematode-tap water suspension into *P. sylvestris* logs. During the long-term storage test, the survival of *B. xylophilus* was studied in sealed and openly stored *P. sylvestris* wood chips at 15 °C and 25 °C. For the investigation of non-vector spread, *B. xylophilus*-infested wood chips were placed on *P. sylvestris* saplings under different conditions.

Investigations using seven- to eight-year-old trees were conducted to examine the significance of sapling-based analyses of the population dynamics and pathogenicity of the pest for mature *P. sylvestris* trees. The trees were artificially inoculated with *B. xylophilus* using a nematode-tap water suspension. For nematode extraction during the population dynamics investigation, the pines were divided into 48 segments. Physiological changes and the development of wilt symptoms were recorded until tree death.

The pathogenicity of *B. xylophilus* towards different German pine provenances (according to the German Legal Ordinance on Regions of Provenance) was studied. For this purpose, *P. sylvestris* saplings were artificially inoculated with *B. xylophilus* using a nematode-tap water suspension.

In the sealed wood chips, *B. xylophilus* was found for more than 1 year at 15 °C and 25 °C. This was significantly longer than the duration observed for the variant openly stored at 25 °C. Furthermore, non-vector spread through wood chips was influenced by temperature, tree condition and wood chip location. Trees with stem or root injuries plus direct contact of the wounded part with infested wood chips at 25 °C were primarily *B. xylophilus*-infested and showed clear symptoms of pine wilt disease. Moreover, for stem- and root-injured pines, direct contact with infested wood chips was not always necessary for non-vector spread. At 15 °C, one *B. xylophilus*-infested pine exhibited clear symptoms of pine wilt disease.

At the start of the population dynamics investigation, *B. xylophilus* was located at the inoculation site and in adjacent segments. Before any external wilt symptoms developed, *B. xylophilus* was located in the entire stem, adjacent branch segments, root collar and roots. Finally, *B. xylophilus* was detected in all wood and root segments in combination with an increase in pine wilt disease and high nematode densities. Shortly before tree death, the treetop was partly nematode-free, and the subjacent tree segments were highly nematode-infested. During the pathogenicity investigation, all *B. xylophilus*-inoculated pines died and exhibited a significant but variable decline in the water potential in the needles compared to a drought-stressed variant.

All tested *P. sylvestris* provenances showed a mortality of 100 %. However, significant differences in the time course of disease development were found for a few provenances.

In conclusion, long-term survival in wood chips and non-vector transmission from infested wood chips to damaged trees were clearly shown, although such establishment should be less likely than spread via vectors. These findings should be tested in outdoor trials.

P. sylvestris saplings are good indicator trees for investigations of *B. xylophilus* population dynamics and pathogenicity because the results in seven- to eight-year-old *P. sylvestris* trees were comparable to those in saplings, although delayed in reaching a population peak and developing wilt symptoms.

The phenomena of delayed symptom development and delayed tree death of some pine provenances should be more closely examined with respect to potential defence traits. An ongoing search for tolerant or resistant provenances or individuals for cross-breeding purposes is suggested as part of a long-term phytosanitary strategy against *B. xylophilus*.

Overall, the threat based on infested wood chips and the high risk for *P. sylvestris* forests must be considered in pest risk analysis, management strategies and contingency planning.

Zusammenfassung

Der Kiefernholznematode, *Bursaphelenchus xylophilus* (Steiner und Buhrer) Nickle 1970, führt in anfälligen Koniferenarten, hauptsächlich *Pinus* spp., außerhalb seines natürlichen Verbreitungsgebietes (Nordamerika) und in nicht heimischen Kiefernarten zur sogenannten Kiefernwelkekrankheit. Er ist seit seiner Einschleppung nach Asien und Europa zum schädlichsten pflanzenparasitären Nematoden von Bäumen geworden. Erkrankte Bäume welken und sterben. Derzeit betroffene europäische Länder sind Portugal und Spanien. *B. xylophilus* ist in der Europäischen Union (EU) aufgrund der zu beobachteten Gefahr in Befallsländern als ein Quarantäneschädling gelistet. In dieser Doktorarbeit, die Teil eines EU-Forschungsvorhabens der EU-Kommission ist, wurden Untersuchungen zur Unterstützung der Schädlingsrisikoanalyse, Managementstrategien und Notfallplanung der EU-Pflanzengesundheitspolitik durchgeführt.

Die Übertragung von *B. xylophilus* zu neuen Wirtsbäumen findet üblicherweise über Vektorkäfer der Gattung *Monochamus* statt. Es wurden jedoch *B. xylophilus* in importierten Holzhackschnitzeln mit Ursprung Nordamerika in der EU beanstandet und die Nachfrage nach Hackschnitzelimporten aus Nordamerika steigt. Daher ist das phytosanitäre Risiko der nicht-vektorassoziierten Übertragung von *B. xylophilus* mittels Hackschnitzeln von Interesse.
Darüberhinaus stellte sich heraus, dass *Pinus sylvestris*, eine in Deutschland und Nordosteuropa weitverbreitete Baumart, in Gewächshausversuchen mit Sämlingen höchst anfällig gegenüber *B. xylophilus* ist. In Europa wurden bisher noch keine erwachsenen Bäume dieser Art getestet.
Der dritte Teil dieser Doktorarbeit bestand aus einer Bewertung von potenziell toleranten oder resistenten Wirtsbaumherkünften als eine Managementoption der Kiefernwelkeerkrankung in betroffenen Ländern.

Das Langzeitüberleben von *B. xylophilus* in Hackschnitzeln and seine nicht-vektorassoziierte Übertragung von befallenen Hackschnitzeln zu unbefallenen Bäumen wurden untersucht. Mit *B. xylophilus* befallene Hackschnitzel wurden hergestellt, indem eine Suspension bestehend aus Nematoden und Leitungswasser in *P. sylvestris* Stämme inokuliert wurde. Während des Langzeitlagerungstests wurde das Überleben von *B. xylophilus* in versiegelten und offen gelagerten *P. sylvestris* Hackschnitzeln bei 15 °C und 25 °C untersucht. Für die Untersuchung der nicht-vektorassoziierten Übertragung wurden mit *B. xylophilus* befallene Hackschnitzel unter unterschiedlichen Testbedingungen an *P. sylvestris* Sämlinge platziert.
Es wurden Untersuchungen mit sieben- bis achtjährigen Bäumen durchgeführt, um die Aussagefähigkeit der auf Sämlingen basierenden Analysen zur Populationsdynamik und Pathgenität des Schädlings für erwachsene *P. sylvestris* Bäume zu überprüfen. Die Bäume wurden mit einer *B. xylophilus* Suspension bestehend aus Nematoden und Leitungswasser

künstlich inokuliert. Zur Nematodenextraktion während der Populationsdynamikuntersuchung wurden die Kiefern in 48 Segmente geteilt. Physiologische Änderungen und die Entwicklung von Welkesymptomen wurden bis zum Tod der Bäume aufgenommen.
Die Pathogenität von *B. xylophilus* wurde gegenüber unterschiedlichen deutschen Kiefernherkünften (gemäß der Deutschen Herkunftsgebietsverordnung) untersucht. Hierfür wurden *P. sylvestris* Sämlinge mit einer *B. xylophilus* Suspension bestehend aus Nematoden und Leitungswasser künstlich inokuliert.

In den versiegelten Hackschnitzeln wurde *B. xylophilus* bei 15 °C und 25 °C mehr als 1 Jahr lang gefunden. Dies war signifikant länger als die zu beobachtende Dauer für die Variante offen gelagert bei 25 °C. Weiterhin war die nicht-vektorassoziierte Übertragung mittels Hackschnitzeln durch Temperatur, Baumzustand und Hackschnitzelposition beeinflusst. Hauptsächlich waren Bäume bei 25 °C mit Stamm- oder Wurzelverletzungen plus Direktkontakt des verwundeten Pflanzenabschnitts mit befallenen Hackschnitzeln mit *B. xylophilus* befallen und zeigten eindeutige Symptome der Kiefernwelkekrankheit. Darüberhinaus war für stamm- und wurzelverletzte Kiefern nicht immer ein Direktkontakt mit befallenen Hackschnitzeln für die nicht-vektorassoziierte Übertragung nötig. Bei 15 °C wies eine mit *B. xylophilus* befallene Kiefer eindeutige Symptome der Kiefernwelkekrankheit auf.
Zu Beginn der Populationsdynamikuntersuchung war *B. xylophilus* in der Inokulationsstelle und den benachbarten Segmenten lokalisiert. Bevor externe Welkesymptome entstanden, war *B. xylophilus* im gesamten Stamm, den benachbarten Astsegmenten, Wurzelhals und Wurzeln lokalisiert. Schließlich wurde *B. xylophilus* in allen Holz- und Wurzelsegmenten in Kombination mit einer Zunahme der Kiefernwelkeerkrankung und hohen Nematodendichten festgestellt. Kurz vor dem vollständigen Absterben der Bäume war die Baumspitze teilweise nematodenfrei und die darunter liegenden Baumsegmente waren stark mit Nematoden befallen. Während der Pathogenitätsuntersuchung starben alle mit *B. xylophilus* inokulierten Kiefern und zeigten einen signifikanten, aber unterschiedlich verlaufenden Abfall des Wasserpotentials in den Nadeln im Vergleich zu einer trockengestressten Variante.
Alle getesteten *P. sylvestris* Herkünfte zeigten eine Mortalität von 100 %. Es wurde jedoch eine zeitlich signifikant unterschiedlich verlaufende Krankheitsentwicklung bei wenigen *P. sylvestris* Herkünften gefunden.

Schlussfolgernd betrachtet wurden das Langzeitüberleben in Hackschnitzeln und die nicht-vektorassoziierte Übertragung von befallenen Hackschnitzeln zu beschädigten Bäumen klar aufgezeigt, obwohl eine solche Etablierung weniger wahrscheinlich als eine Ausbreitung über Vektoren sein sollte. Diese Ergebnisse sollten in Freilandversuchen getestet werden.

P. sylvestris Sämlinge sind gute Indikatorbäume für Untersuchungen der Populationsdynamik und Pathogenität von *B. xylophilus*, weil die Ergebnisse in sieben- bis achtjährigen *P. sylvestris* Bäumen vergleichbar zu denen in Sämlingen waren, auch wenn das Erreichen eines Populationsmaximums und die Entwicklung von Welkesymptomen zeitlich verzögert waren.

Die Phänomene verspäteter Symptomentwicklung und verspäteten Baumtodes einiger Kiefernherkünfte sollten hinsichtlich potenzieller Abwehreigenschaften näher überprüft werden. Es wird eine fortlaufende Suche nach toleranten oder resistenten Herkünften oder Individuen für Kreuzungsvorhaben als Teil einer phytosanitären Langzeitstrategie gegen *B. xylophilus* vorgeschlagen.

Alles in allem müssen die von befallenen Hackschnitzeln ausgehende Gefahr und das hohe Risiko für *P. sylvestris* Wälder bei der Schädlingsrisikoanalyse, den Managementstrategien und der Notfallplanung Berücksichtigung finden.

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1 Chapter 1: General introduction

1.1 Bursaphelenchus xylophilus and related species

In 1934, the pinewood nematode (PWN) was first described by STEINER and BUHRER as *Aphelenchoides xylophilus*. Later, in 1970, NICKLE described this species as *Bursaphelenchus xylophilus*. Moreover, a new species, *Bursaphelenchus lignicolus* MAMIYA and KIYOHARA (1972), described in the literature was found to be *B. xylophilus* (NICKLE et al., 1981).

B. xylophilus belongs to order Tylenchida (EPPO, 2019). Within the genus *Bursaphelenchus* Fuchs, only two species are known to be plant-parasitic: *B. xylophilus* in conifers and *B. cocophilus* (Cobb) Baujard in coconut palms (GIBLIN-DAVIS et al., 2003). *Bursaphelenchus* spp. are mainly mycetophagous nematodes, meaning that they are closely associated with insect vectors and transmitted to dead, dying or healthy trees, mainly conifers (RYSS et al., 2005) but sometimes deciduous trees (such as TOMALAK et al., 2017; TOMALAK and FILIPIAK, 2019). For example, a study by VICENT et al. (2008) evaluated the appearance of *Bursaphelenchus* spp. in trap trees and the emergence of insects from these trees (pines, including *Pinus sylvestris* L.) throughout France. The isolated species included *Bursaphelenchus hellenicus* Skarmoutsos, Braasch and Michaloupolou; *B. leoni* Baujard; *B. mucronatus* Mamiya and Enda and *B. sexdentati* Rühm but not the PWN. SCHÖNFELD et al. (2001) conducted similar studies in Brandenburg, Germany, and summarised all the *Bursaphelenchus* spp. isolated in this region based on the literature. They mentioned the same and some other *Bursaphelenchus* spp. and found that *B. mucronatus* was the most frequent species. Moreover, *B. mucronatus* is very similar to the PWN in terms of morphology, with only minor differences (EVANS et al., 1996). VICENT et al. (2008) found *B. mucronatus* in several regions in France, with an average frequency of 19 %, and 27 % of *Monochamus galloprovincialis* Olivier vector beetles were infested by *B. mucronatus*.

SCHRÖDER et al. (2009) summarised methods for the detection of the PWN in trees, wood, wood products and vector beetles as well as molecular biological methods for nematode identification in wood. Classical methods for molecular identification after nematode extraction, for example, internal transcribed spacer- restriction fragment length polymorphism (ITS-RFLP), are recommended by the FAO (2016). For morphological identification of adult PWNs, EPPO (2013) and FAO (2016) diagnostic protocols can be referenced.

Under certain conditions, the PWN is the causal agent of pine wilt disease (KIYOHARA and TOKUSHIGE, 1971; RUTHERFORD et al., 1990; EVANS et al., 1996). In contrast to the “M” form of the PWN (with a mucronated tail), the “R” form of the PWN (with a rounded tail) is considered to be the causal agent of pine wilt disease. In North America, both forms occur (DWINELL and NICKLE, 1989). Moreover, avirulent PWN isolates have also been reported but are restricted in

dispersal and multiplication inside trees, as observed in the non-pathogenic species *B. mucronatus* (ODANI et al., 1985b; FUKUDA et al., 1992).

The PWN feeds primarily on the hyphae of fungi inside trees (mycophagous phase). However, the PWN can change to the phytophagous phase and feed on living tree tissues, which can result in the death of infested trees (WINGFIELD, 1987). Few PWNs (tens to a few hundreds) are required for the establishment of a PWN population inside a host (EPPO, 2009). During the propagative mode with rapid multiplication, males, females and four juvenile stages (J_1-J_4) are present (KONDO and ISHIBASHI, 1978), whereby J_2 individuals hatch from the egg. The life cycle of the PWN, for example, on fungal cultures is completed in 4-5 days at 25 °C (WANG et al., 2005). However, under unfavourable conditions, such as low temperatures (ZHAO et al., 2007), low food availability or drought, if the vector beetles develop inside the tree, the propagative mode changes into the dispersal mode consisting of other types of juveniles (J_{III} and J_{IV}), which allows the PWN to survive for a longer period inside the wood (J_{III}) and to be transmitted to new hosts (J_{IV}) by vector beetles (KONDO and ISHIBASHI, 1978). Nevertheless, J_{III} can also resume the propagative mode and develop into J_4 under improved circumstances (ZHAO et al., 2013).

1.2 Pine wilt disease

1.2.1 Factors affecting the spread of *Bursaphelenchus xylophilus* and pine wilt disease outbreak

The PWN can spread to (new) trees and become established in a country if insect vectors and host tree species are present (EVANS et al., 1996; EPPO, 2009). Details on these factors (with regard to Germany) and, as part of this thesis, the non-vector spread of the PWN are discussed in chapters 1.3-1.5.

However, the occurrence of virulent PWNs inside a host can lead to pine wilt disease only if the tree is susceptible to the PWN and the mean daily summer temperatures in July/August are higher than 20 °C (RUTHERFORD et al., 1990; EVANS et al., 1996). Chapter 1.5.2 provides more details on susceptible hosts. A further factor that increases the potential risk for pine wilt disease outbreaks is drought stress (ROQUES et al., 2015).

1.2.2 Disease process

Over time, PWNs disperse to and colonise all wood and root parts (DAUB, 2008). During the invasion of the PWN, resin canals, rays, and tracheids are affected. PWNs produce cellulase, which hydrolyses cellulose and allows the PWNs to enter host cells (ZHANG et al., 2006). The resin flow then ceases (MAMIYA, 2008). After a certain degree of wilting, the size of the

nematode population increases exponentially (MELAKEBERHAN and WEBSTER, 1990), and the destruction of wood tissues, such as through feeding on parenchyma cells, becomes faster (MAMIYA, 2008). Mainly epithelial cells of cortical resin canals are destroyed by the PWN, followed by pine cell death in the surrounding cortical tissue and periderm, xylem, pith, and finally, cambium (ISHIDA et al., 1993). Defence reactions of the tree lead to denaturation and necrosis of parenchyma cells (FUKUDA, 1997). Oleoresin leakage (ODANI et al., 1985a), accumulated vacuole content (NOBUCHI et al., 1984) and hydrophobic volatile terpenes (KURODA, 1991) occur and lead to tracheid cavitation and blockage. Additionally, the outermost xylem becomes blocked after cambium destruction and enhanced ethylene production by the tree. Finally, occlusion of water conduction throughout the xylem occurs. In parallel, the transpiration, photosynthesis, water potential (FUKUDA, 1997) and relative water content in wood and needles decline, and irreversible wilt until the death of the tree is observed (DAUB, 2008).

MYERS (1988) hypothesised in his review that the observed host toxin production, parenchyma death and oleoresin leakage into tracheids are part of the hypersensitive defence mechanism of the tree continuously triggered by small PWN numbers. Nematodes rapidly move and therefore overcome defence mechanisms, causing extended high-defence zones. Such hyperreaction could lead to symptoms such as wilt and tree death. BOLLA et al. (1984) also mentioned the possibility that phytotoxin could become systemic with the development of the infection.

Additionally, the amount of NO, the signalling molecule of H_2O_2, significantly increases and is involved in the response to nematode invasion. It might induce hyperreaction, such as programmed cell death, and is related to the development of early external symptoms (YU et al., 2012).

Moreover, toxin-producing bacteria are transmitted by PWNs throughout the tree (OKU et al., 1980). The mutualistic relationship between bacteria and PWNs is still not clear (PROENCA et al., 2017), but toxin-producing bacteria seem to also be involved in pine wilt disease (ZHAO et al., 2003; VICENTE et al., 2013). Additionally, various fungal genera were found to be associated with the PWN and its vector beetle and host (HYUN et al., 2007), but their possible involvement in pine wilt disease has not been tested.

The basic ecology of these complex interactions, including the interaction between the PWN and its vectors, is even less well understood.

At sufficiently high temperatures in nature, susceptible trees usually show disease symptoms within a few weeks and die within a year (MAMIYA, 1983), depending on the time of infestation (GRUFFUDD et al., 2016 and 2018).

1.2.3 History of spread

The native range of the PWN is North America (it is widespread in Canada and the United States of America), where the harm caused by this nematode is limited to several non-native conifer species (DWINELL and NICKLE, 1989). In 1993, the PWN was also reported in Mexico (DWINELL, 1993; EPPO, 2019). Most likely, the PWN spread from North America to Japan by overseas timber imports (KISHI, 1995). The first outbreak of pine wilt disease in native pines in Japan was reported at the beginning of the 20th century. However, only more than 60 years later (MAMIYA, 1988), *B. xylophilus* was discovered to be the causal agent of pine wilt disease in Japan (KIYOHARA and TOKUSHIGE, 1971). In 1979, the largest annual loss of timber in Japan was recorded, at nearly 2.5 million m³ (MAMIYA, 1988).

From Japan, the PWN spread most likely through timber exports to other Asian countries, including China, Taiwan and the Republic of Korea, where pine wilt disease also developed (MAMIYA, 1988, EVANS et al., 1996). In these Asian countries, the occurrence of the PWN is restricted to the Republic of Korea and some affected states of China. In the other cases, the PWN is widespread (EPPO, 2019).

The PWN has also spread to Europe, where the first pine wilt disease-affected area was detected in Portugal in 1999 (MOTA et al., 1999). In 2008, all of continental Portugal, with the exception of a 20 km buffer zone to Spain, was declared infested by the PWN (EC, 2008). Currently, the PWN has a restricted distribution in continental Portugal as well as on the island of Madeira, where it was found in 2009 (FONSECA et al. 2012; EPPO, 2019).

Since 2008, five outbreaks have been detected in Spain. Two outbreaks are undergoing eradication, and three have been successfully eradicated (EC, 2018; EPPO, 2019).

In Germany, no PWN infestation has been found during yearly surveys (HOPPE, 2018; EPPO, 2019).

1.3 Spread of Bursaphelenchus xylophilus by vector beetles

The PWN is transmitted to host trees by vector beetles without benefiting or harming these beetles (phoresy) (FIRMINO et al., 2017). In nature, the only known efficient vectors of the PWN are in the genus *Monochamus* (Coleoptera: Cerambycidae), despite several nematode-carrying genera in the Cerambycidae and Coleoptera (DWINELL and NICKLE, 1989; EVANS et al., 1996).

EVANS et al. (1996) reported the geographical distribution of all very and less efficient *Monochamus* spp. vectors for coniferous trees and potential *Monochamus* spp. vectors of the PWN in non-infested countries. *Monochamus carolinensis* Olivier and *Monochamus alternatus* Hope are major vectors of the PWN in North America and Japan, respectively (KOBAYASHI et

al., 1984; DWINELL and NICKLE, 1989; EVANS et al., 1996). In contrast, *M. galloprovincialis* is the only known vector in Portugal and Spain (SOUSA et al., 2001; ÁLVAREZ et al., 2013; SANCHEZ-HUSILLOS et al., 2013).

Similar to the pattern observed in other European countries, in addition to *M. galloprovincialis*, the species *Monochamus sutor* Linnaeus, *Monochamus sartor* Fabricius and (least likely) *Monochamus saltuarius* Gebler could be potential PWN-carrying vector species in Germany (KLAUSNITZER and SANDER, 1981; EVANS et al., 1996). *Monochamus* spp. are missing from only the United Kingdom and Ireland (EVANS et al., 1996). However, *Monochamus* spp. are differently distributed. In Germany, *M. galloprovincialis* is the most frequent species and is distributed region-wide in Brandenburg and the Palatinate Forest. *M. saltuarius*, the rarest species, is present in the Alps and Saxon Switzerland and is seldom observed flying. *M. sutor* is often found in uplands in some regions, and *M. sartor* is less abundant than *M. sutor*, with the former sometimes spread by wood transport from mountainous regions (KLAUSNITZER and SANDER, 1981; MEYER, 2003). Moreover, a monitoring programme exists in all European Union member states that attempts to record all naturally occurring and possibly introduced *Monochamus* spp. and their distribution (EU, 2012 and its actual version).

Vector species and potential vector species of the genus *Monochamus* prefer different host tree places for oviposition (SCHRÖDER et al., 2009) as well as different host tree species based on their different geographical distributions, as summarised by EVANS et al. (1996). In nature, the preferred host tree species of *M. galloprovincialis* are pine species, except the Mediterranean pine species *Pinus pinea* L. (EVANS et al., 1996; SANCHEZ-HUSILLOS et al., 2013). The other mentioned *Monochamus* spp. in Germany prefer additional conifer species (KLAUSNITZER and SANDER, 1981; EVANS et al., 1996). Although *Monochamus* spp. play an important role in pine wilt disease, they are only secondary pests (EVANS et al., 1996), and "*M. galloprovincialis* is a minor component of the bark- and wood-boring insects of pine trees" (FIRMINO et al., 2017).

M. galloprovincialis can fly between May and September/October under suitable conditions (SCHÖNFELD, 2006; HOCH et al., 2014; FIRMINO et al., 2017) and was found to have a mean flight capacity of 16 km over its lifetime (DAVID et al., 2014).

WINGFIELD (1987) illustrated the transmission cycle of the PWN to host trees by its vector beetles, which is shown in Fig. 1, modified by PWN-transmitted bacteria. During maturation feeding of the vector beetle on twigs of healthy pines as well as oviposition on dying and recently died and cut pines, PWNs are transmitted to host trees through wounds, where the inner bark is exposed (LINIT, 1990; EDWARDS AND LINIT, 1992).

In the mycophagous phase of the PWN after oviposition of the vector, no living pine tissues are necessary, and selected trees are dying or have died because of pine wilt disease or for other

reasons. Bark beetles, which are associated with and transmit fungi, such as blue-stain fungi, are also present. PWNs can use these fungi as a food source (WINGFIELD, 1987). In spring, PWNs are attracted to the pupal chambers of *Monochamus*, where they move onto the bodies of the developed beetles and enter the spiracles and tracheae to be transmitted to new healthy or dying trees (WINGFIELD, 1987; AKBULUT and STAMPS, 2012). Most often, between some hundreds and a few thousands of PWNs are carried by one insect (SOUSA et al., 2001).

In the phytophagous phase of the PWN after maturation feeding of the vector beetle, plant tissues are attacked. Due to the susceptibility of certain tree species to the PWN (WINGFIELD, 1987) and the potential influence of PWN-transmitted bacteria (ZHAO et al., 2003; VICENTE et al., 2013), PWN infestation can lead to tree death under certain conditions. These trees attract (potentially PWN-carrying) vector beetles for oviposition, which later also allows PWN spread via newly developed beetles (WINGFIELD, 1987).

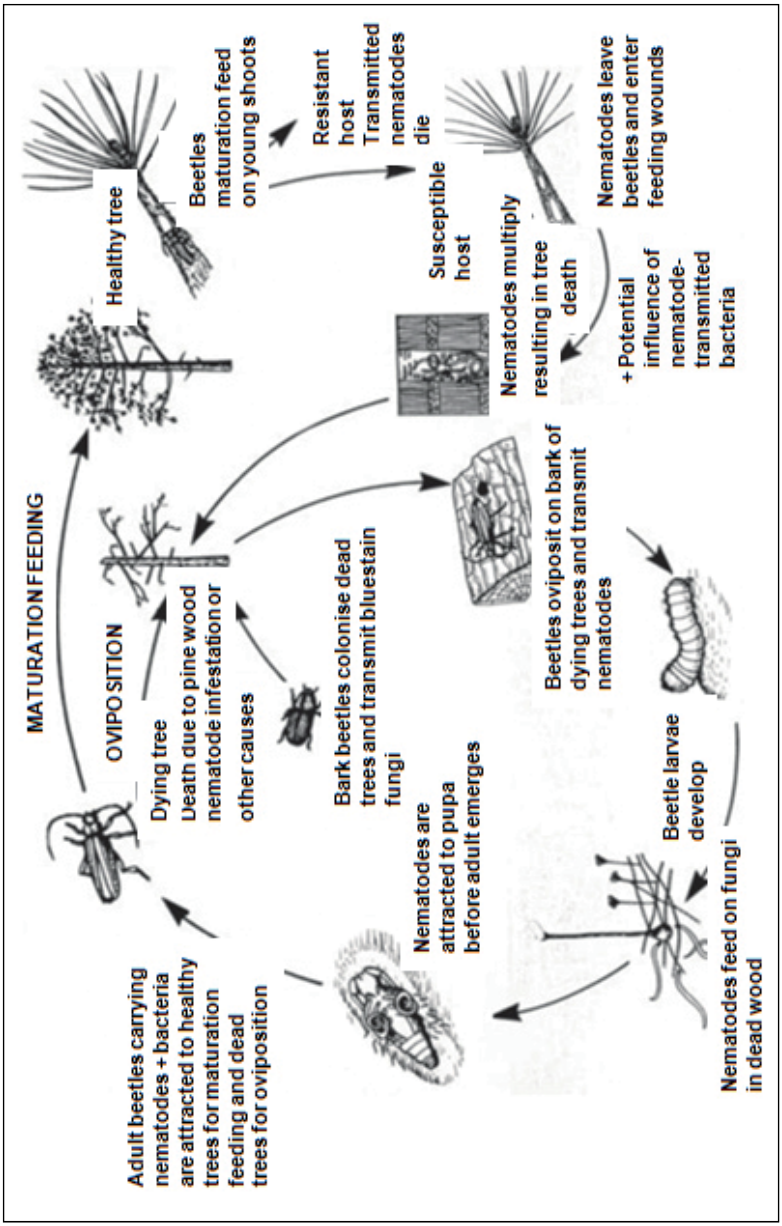

Fig. 1. Transmission cycle of *B. xylophilus* by its vector beetles in the genus *Monochamus*; *Source:* Modified after WINGFIELD, 1987

1.4 Non-vector spread of Bursaphelenchus xylophilus

Some evidence from Japanese research suggests that the non-vector spread of PWNs between adjacent trees through root grafting can occur locally (EVANS et al., 1993), although no evidence for European pine species has been published. Moreover, for *Pinus pinaster* Ait., the transfer of PWNs from a donor board to an adjacent recipient board by direct contact is possible if the wood moisture content and temperature are favourable (SOUSA et al., 2011).

Despite some experimental work, whether the PWN can be transmitted from infested boards, bark or wood chips to non-infested trees without any vector and cause new outbreaks of pine wilt disease has not been studied or not in depth. For potential non-vector transmission by wood chips, some laboratory-based or small-scale investigations, such as wounded saplings coming into contact with infested mulch or wood chips in soil (HALIK and BERGDAHL, 1987 and 1992), have been performed, but definitive and stringent investigations are still necessary. All three scenarios (boards, bark and wood chips) should be studied as part of the European research project REPHRAME: "Development of improved methods for detection, control and eradication of pinewood nematode in support of EU Plant Health policy". The last case of possible non-vector spread was part of this thesis.

Wood chips represent a potential pathway of transmission of pine wilt disease because PWNs can survive in wood chips at suitable wood moisture contents (PANESAR et al., 1994). In wood chips, PWNs might feed on fungi (HALIK and BERGDAHL, 1990; EVANS et al., 1993), whereby they differentiate between fungal species. For example, MAEHARA and FUTAI (1996) reported that wood infested with the blue-stain fungus *Ophiostoma minus* Hedgcock was a better food resource that allowed more PWN reproduction than wood harbouring other fungal species.

In the case of wood chip storage, infested wood chip piles are known to harbour living PWNs if the temperatures are not high enough to kill PWNs. This is always the case for the outer shells and for interior areas if the environmental conditions and pile construction do not ensure a natural heat development to lethal temperatures throughout the pile (DWINELL, 1986). In wood, PWNs can live for at least one year and survive shipments from North America and Asia to Europe (EVANS et al., 1996). Before pest risk management strategies and treatment recommendations were developed, PWNs were first detected in imported wood chips in the European Union in 1984 (RAUTAPÄÄ, 1986). During international trade, PWNs have been intercepted in wood and wood products on a number of occasions, including in European countries (EVANS et al., 1996). In Germany, for the first time, PWNs were intercepted in wood packing material imported from China in 2001 (BENNEWITZ, 2002). Import controls on packaging wood in China between 2003 and 2005 revealed that even imports from non-infested

countries can contain PWNs due to global circulation (GU et al., 2006). In line with FAO (2009), wood packaging material with origin in countries, where the PWN is present, is reused. Additionally, in pine wood, wood packaging material and bark from Portugal, repeated interceptions of PWNs occurred (EU, 2012).

The United States of America is a major global exporter of wood chips (JIANG et al., 2017). The international wood chip trade is growing (MARITIME NEWS, 2017) because wood chips are increasingly used for energy purposes (KOPINGA et al., 2010), pulp and paper production (JÖNSSON, 2011), wood-based panels, surface layering of paths and mulching, often around trees (EVANS et al., 1996). In the near future, the demand for wood chips for cellulosic biofuel and electricity production is expected to increase rapidly due to the renewable energy policy in the European Union (JIANG et al., 2017).

Therefore, the survival time of PWNs in wood chips is of interest, and it is necessary to clarify whether PWN-infested wood chips are a potential threat to *P. sylvestris* saplings in terms of non-vector spread under different test conditions, which would represent an additional source of nematode establishment and pine wilt disease outbreaks.

1.5 Hosts of Bursaphelenchus xylophilus

1.5.1 Non-susceptible conifers

In North America, where the PWN is native, indigenous conifers are tolerant or resistant to the PWN as a secondary associate (DWINELL and NICKLE, 1989). SUGA et al. (1993) revealed the effectiveness of nematicides and repellents in some of these species. For example, FUTAI and FURUNO (1979) showed the high resistance of *Pinus taeda* L., *P. elliottii* Engelm. and *P. rigida* Mill. to PWNs.

Based on the literature, EVANS et al. (1996) listed many North American (Canada and the United States of America), Central American (including Mexico), Euro-Mediterranean and Asian pine species according to their susceptibility (resistant, intermediate, or susceptible) and mentioned additional host conifer species, such as *Cedrus* spp. Moreover, an actual summary of all as known hosts categorised tree genera is given in EPPO (2009). Concerning European conifer species, DAUB (2008) found that tested *Pinus halepensis* Mill., *Picea abies* (L.) Karst. and *Abies alba* Mill. saplings were tolerant to the PWN. Although *P. abies* was regarded as a susceptible host in EVANS et al. (1996), NUNES DA SILVA et al. (2013) observed tolerance to the PWN in this species. It seemed to be a compatible host that could harbour PWNs for some time. Moreover, some non-native species, such as North American *Abies* spp., planted in the European Union were also found to be hosts (EVANS et al., 1996).

In Germany, in addition to *Pinus* spp., *Picea abies* is an additional host for the potential PWN vectors *Monochamus saltuarius*, *M. sartor* and *M. sutor*, and *Abies alba* is an additional host for *M. sartor* and *M. sutor* (KLAUSNITZER and SANDER, 1981).

Additionally, single trees or families of susceptible conifer species can be tolerant or resistant to the PWN and are often used in breeding programmes in Japan. Differences in suceptibility were caused by suppressed migration and reproduction of PWNs (KURODA and KURODA, 2004; MORI et al., 2008; KUSUMOTO et al., 2014; SON et al., 2015) due to numerous branches with a complex arrangement of resin canals, which served as migration barriers (KURODA and KURODA, 2004) and retarded damage expansion (especially in the cambium), most likely due to wall protein-based defences. During the provided time, the host can complete the lignification of cell walls around the damaged regions (KUSUMOTO et al., 2014).

1.5.2 Susceptible conifers

In North America, foreign pine species are susceptible to the PWN as a primary pathogen (DWINELL and NICKLE, 1989). As a result, natural dying or dead PWN-infested *P. sylvestris* trees may be found (DROPKIN and FOUDIN, 1979; DROPKIN et al., 1981; MALEK and APPLEBY, 1984). Furthermore, MYERS (1986), LINIT and TAMURA (1987) and BEDKER and BLANCHETTE (1988) published the highest mortality rates of 60-80 % from their pathogenicity investigations on *P. sylvestris* in the field in North America. Outside this native area of the PWN, the disease appears in different conifer genera, but the principal hosts are in the genus *Pinus*. EVANS et al. (1996) provided an overview of susceptible pine species and other host conifer species. In Asia, susceptible pine species include, for example, *Pinus densiflora* Sieb. & Zucc., *P. koraiensis* Sieb. & Zucc., *P. luchuensis* Mayr, *P. thunbergii* Parl. and *P. parviflora* Sieb. & Zucc. (KISHI, 1995). In Portugal and Spain, *P. pinaster* trees were found to be affected by pine wilt disease (MOTA et al., 1999; ABELLEIRA et al., 2011). Additionally, pine wilt-diseased trees of *P. radiata* D. Don in Spain (ZAMORA et al., 2015) and *P. nigra* J.F. Arnold in Portugal were recorded (INÁCIO et al., 2015). *P. pinea*, which grows in the Mediterranean area, appeared moderately susceptible during inoculation studies (EVANS et al., 1996; DAUB, 2008). However, pine wilt disease does not affect this tree species in the field (SANCHEZ-HUSILLOS et al., 2013) because *M. galloprovincialis* does not prefer this host. Nevertheless, the risk classification of *P. pinea* is highly uncertain due to the findings of vector breeding in fallen wood in Italy, the potential risk of asymptomatic PWN infestation in tolerant trees (EFSA, 2013) and the successful feeding and breeding of the vector on this tree species under laboratory conditions (SANCHEZ-HUSILLOS et al., 2013).

In addition, greenhouse investigations revealed the potential threat of several species, such as the European species *Pinus sylvestris*, *P. mugo* Turra, *P. cembra* L. and *Larix decidua* Mill. (DAUB,

2008). *P. sylvestris*, among these susceptible or highly susceptible species, often showed 100 % mortality within a few weeks during different pathogenicity investigations with saplings in climate chambers, greenhouses or outdoor boxes under quarantine conditions (such as SCHAUER-BLUME, 1990; BRAASCH, 1997; BRAASCH, 2000; DAUB, 2008).

In Germany, due to the dominance of *M. galloprovincialis*, mainly *Pinus* spp. are under potential threat, which are also hosts for all other native *Monochamus* spp. *Larix decidua* is additionally preferred by *M. sutor* (EVANS et al., 1996).

1.5.3 Distribution, relevance and application of *Pinus sylvestris*

Germany is covered by nearly one-third stocked forest land (BMEL, 2015), 23 % of which contains *P. sylvestris*, making it as the second most common forest tree species after *Picea abies* (L.) Karst. (BMEL, 2018a). However, among the conifer species in Germany that are potentially and most susceptible to pine wilt disease, *P. sylvestris* covers the largest forest area and is preferred by the main vector beetle species *M. galloprovincialis*, as described in chapter 1.3. Therefore, this thesis is based on investigations of Scots pine.

Additionally, *P. sylvestris* is one of the most common tree species in Europe, with one of the largest natural distribution areas, and can also be found in North Asia. In Europe, this species is missing only from the West and submediterranean South, except in isolated partial areas. Due to its widespread distribution, many races exist, which are adapted to the large climatic differences (GROSSER, 1998).

In addition to birch, pine is a pioneer species and dominates the landscape in some parts of Germany (GROSSER, 1998). As illustrated in Fig. 2, mainly the northeastern lowlands of Lower Saxony up to Brandenburg and Saxony, valley of the Rhine and Main, Palatinate Forest, Upper Palatinate basin and hilly land are stocked with *P. sylvestris* (BMEL, 2015). The largest amount of Scots pine resources per federal state is found in Brandenburg + Berlin (70 % of the forest area), Saxony-Anhalt (43 %), Mecklenburg-Western Pomerania (37 %), Lower Saxony (29 %) and Saxony (28 %) (THÜNEN-INSTITUT, 2014).

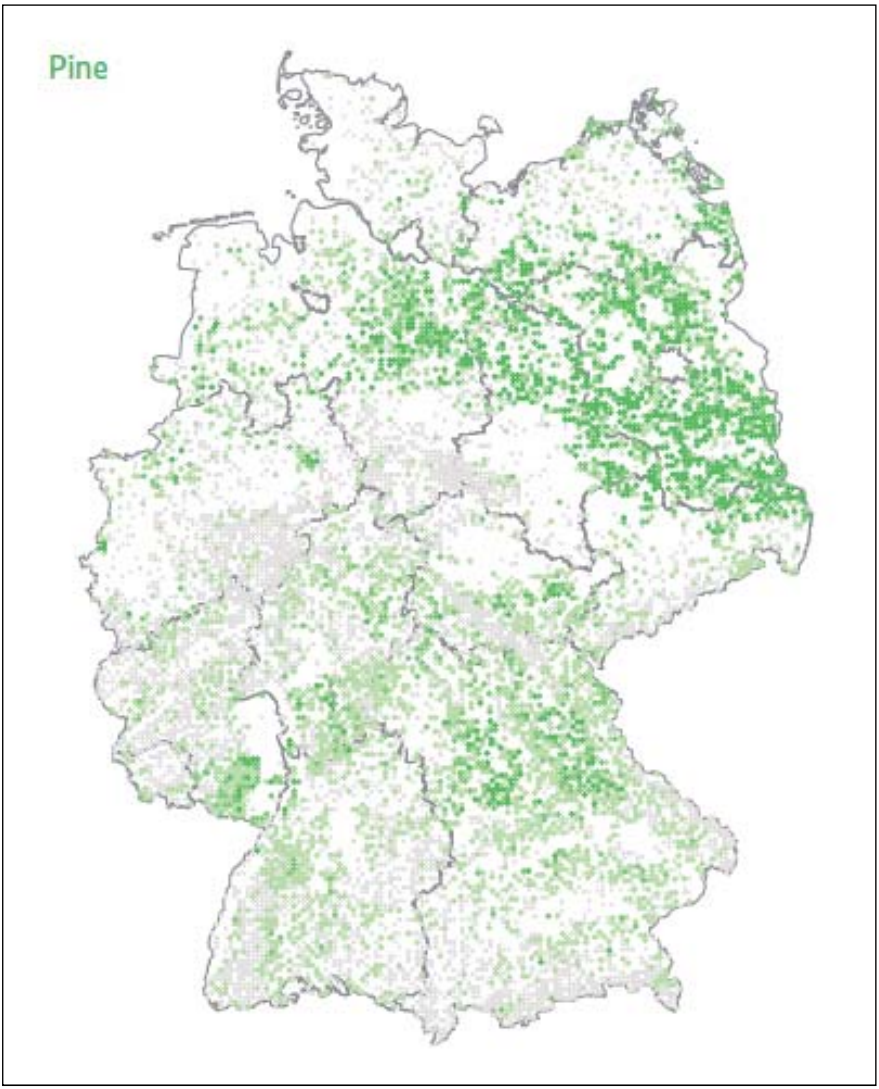

Fig. 2. Distribution of pine in Germany. Fraction of tracts: light green, up to 1/3 pine; intermediate green, more than 1/3 to 2/3 pine; dark green, more than 2/3 pine; and light grey, forest tract with other tree species. *Source*: BMEL, 2015

In 2017, approximately 12 million m³ of Scots pines was harvested in Germany, which accounted for 22 % of the total and 29 % of the coniferous wood logging (BMEL, 2018b). The common age for harvesting is 100 to 160 years, depending on the usage - as timber or high-

quality wood. Due to the colouration of the heartwood and the contrast between early and late wood, the wood is used for decorative purposes. The properties of the wood, such as its good mechanical and elasticity properties, intermediate heaviness, moderate hardness (GROSSER, 1998), moderate to low natural durability (resistance class 3-4) (NIEMZ, 2008) but good and sufficient impregnation properties of the sapwood and heartwood, respectively, allow its use in roundwood, saw wood, veneer, building and underground construction and furnishing timber applications. Examples include windows, doors, facades, stairways, indoor and outdoor floors and furniture, household appliances, masts, stakes, packaging, wood-based materials, paper and pulp (GROSSER, 1998).

For all these reasons, the loss of *P. sylvestris* forests due to pine wilt disease would cause great harm to Germany and Europe.

1.5.4 Critique of population dynamics and pathogenicity investigations

Different authors have published more or less detailed reports on the population dynamics of the PWN after inoculation into *P. sylvestris* saplings (MELAKEBERHAN and WEBSTER, 1990; DAUB, 2008; MENÉNDEZ-GUTIÉRREZ et al., 2017) and other pine species (such as FUTAI, 1980; SU and YE, 2007; XIE and ZHAO, 2008; MENÉNDEZ-GUTIÉRREZ et al., 2017). Only DAUB (2008) showed the migration and reproduction of the PWN inside the whole plant of *P. sylvestris* using detailed segmentation. He identified clear stages of nematode invasion.

Rapid dispersion and propagation, key factors for the pathogenicity of the PWN, and high dependency of the PWN on the relative water content of the host in the final stage of pine wilt disease were found (FUTAI, 1980; DAUB, 2008).

However, with the exception of a few studies, such as that of XIE and ZHAO (2008), who performed a *P. thunbergii* branch analysis, for most pine species, only saplings were tested to study the population dynamics of the PWN. This lack of knowledge has been criticised by some scientists, such as MCNAMARA (2004). Therefore, investigations are required to confirm the significance of *P. sylvestris* sapling-based population dynamics investigations of the PWN for older *P. sylvestris* trees.

The susceptibility of *P. sylvestris* and other conifer species to the PWN based on pathogenicity investigations was discussed in chapter 1.5.2.

However, as WINGFIELD et al. (1986) demonstrated for mature *Pinus resinosa* Ait. trees in outdoor trials in North America, the susceptibility of different conifer species during sapling studies cannot be confirmed in all cases by examining mature trees. Therefore, the scientific merit of sapling-based analyses, which has been doubted by some scientists, such as MCNAMARA (2004), must be studied for conifer species suspected of being susceptible. The

significance of *P. sylvestris* sapling-based pathogenicity investigations of the PWN for older *P. sylvestris* trees under European climatic conditions remains to be proven.

1.6 Pest risk analysis, management and contingency planning

A pest risk analysis is necessary for all phytosanitary regulations and, referring to this thesis, also includes the assessment of pathways and potential hosts (FAO, 2007). For *B. xylophilus* a pest risk analysis, EVANS et al. (1996), and its actual version (EPPO, 2009) were published.

The riskiest materials for spreading the PWN are wood and wood products, especially wood packaging material infested with PWNs and their vectors. In untreated wood, vectors can be killed through chipping if the chip size is small enough. Nevertheless, the possibility of non-vector spread through wood chips is also considered a possible pathway if chips come into contact with host trees. However, the risk of PWN transmission through wood chips is classified as low to moderate. In areas without vectors, permanent establishment of the PWN would be unlikely (Evans et al. 1996).

In the pest risk analysis (EVANS et al., 1996), *P. sylvestris* was evaluated as highly susceptible to the PWN. However, the pest risk analysis indicated that the relative degree of susceptibility should be interpreted with caution. Often, instead of mature trees, saplings are tested under controlled environmental conditions, especially in non-infested countries, because outdoor trials are prohibited. Nevertheless, in the field in North America, *P. sylvestris* is a pine species that also succumbs to pine wilt disease when mature (EVANS et al., 1996).

The PWN presents “a serious risk to European coniferous forests” (EVANS et al., 1996). The potential for establishment and economic impact was considered high for the whole region of the European Union where host plants are present (EPPO, 2009), including Germany (GRUFFUDD et al., 2018). Potential vector beetles are present in these locations (EVANS et al., 1996). Additionally, in cool northern climates, the potential risk for PWN infestation is due to its long-term survival inside living and asymptomatic pines at low temperatures (HALIK and BERGDAHL, 1994). However, this latent infestation, as could occur in Germany (GRUFFUDD et al., 2018), would even lead to the undetected spread of this nematode and finally to a pine wilt disease outbreak in the case of climate change accompanied by warmer temperatures and water deficits (ROQUES et al., 2015).

Efforts were made to model the potential global/European spread of the PWN and pine wilt disease (such as ROBINET et al., 2011; GRUFFUDD et al., 2018; IKEGAMI and JENKINS, 2018). For example, most critical points of entry allowing the PWN and its vectors to spread across Europe (ROBINET et al., 2011) and an increased risk under future climate scenarios were shown to be

major threats to European pine species (GRUFFUDD et al., 2018; IKEGAMI and JENKINS, 2018). Moreover, new computer models are available on the internet platform http://www.rephrame.eu/pwn.php (EU, 2019), called the "PWN tool kit", which was developed as part of REPHRAME, that allow foresters and land managers to estimate the risk for their forests.

Due to the immense harm to many forests worldwide, the PWN poses a serious ecological and economical threat. In European Union Plant Health Council Directive 2000/29/EC (EU, 2000), *B. xylophilus* is listed as a quarantine pest in the European Union. Therefore, all investigations in this thesis were conducted under quarantine restrictions.

The introduction and spread of the PWN and non-European *Monochamus* spp. in the European Union member states are prohibited (EU, 2000). In all areas where the PWN has been detected, pest risk management is vital for attempted eradication, or if eradication is not feasible, for containment the spread and impact of the PWN (EU, 2012 and 2019). An investigation of the effect of spraying a nematode-trapping fungus, a species of *Arthrobotrys* Corda, as a biocontrol agent on Japanese pine seedlings before nematode inoculation showed that attacks by the PWN could be prevented to a certain extent (SAIKI et al., 1984). Aditionally, some fungal species in the genus *Trichoderma* Persoon ex Gray were found to have nematicidal activity against the PWN (MAEHARA, 2007; YANG et al., 2012). Moreover, aerial insecticide spraying was conducted but could not entirely prevent pine wilt disease (UGAWA and FUKUDA, 2007).

An overview of actual recommended management options is available on the internet platform http://www.rephrame.eu/pwn.php (EU, 2019). Pest risk management includes two main options: vector or PWN removal. Adult vector beetles can be removed by insecticides and mass trapping with traps and lures as well as trap logs (EU, 2019). However, the application of insecticides over big forest areas is unacceptable from the ecological point of view and less successful.

For vector larvae removal, heat treatment and chipping of wood are used (EU, 2019). Potentially PWN-infested bark, wood chips and solid wood from affected countries must be heat-treated (EU, 2000 and 2019). One of the new management strategies used to prevent pines from being killed by the PWN and to diminish the vector populations in forests is injecting trunks with environmentally friendly emamectin benzoate (TAKAI et al., 2000; SOUSA et al., 2013). European Union Plant Health Council Directive 2000/29/EC (EU, 2000 and its actual version) comprises obligatory phytosanitary requirements for the introduction and movement of plants (including potentially PWN-infested *P. sylvestris*), plant products and other objects into and within the European Union member states. Potentially PWN-infested wood and wood in various forms, such as wood chips, from infested countries must be heat-treated with a minimum wood core temperature of 56 °C maintained for at least 30 min (EU, 2000 and its actual version and 2012)

and accompanied by phytosanitary certificates and an official statement that this material was protected from re-infestation (EU, 2000 and its actual version).

As observed in Portugal, in addition to short-term phytosanitary measures, long-term phytosanitary strategies, such as growing tolerant or resistant host plants, are necessary (RIBEIRO et al., 2012). Therefore, pathogenicity investigations using different German *P. sylvestris* provenances (according to the German Legal Ordinance on Regions of Provenance - GERMAN FEDERAL OFFICE FOR AGRICULTURE AND FOOD, 2013) were of interest, as performed for different Finnish or Chinese provenances of different pine species in recent decades (PANESAR and SUTHERLAND, 1989; WANG et al., 1997; XU et al., 1997).

All European Union member states are obliged to carry out annual surveys of the vectors and the susceptible plants, wood and bark for the presence of the PWN and possess contingency plans for new findings of the PWN according to EU (2012 and its actual version; surveys for Germany: HOPPE, 2018). These surveys serve to monitor the spread of the PWN but also aid in the early detection of new outbreaks (EPPO, 2018). Guidance on sampling trees, wood and beetles for the PWN regarding survey sites and methods for sampling and nematode extraction was provided by SCHRÖDER et al. (2009). Newly developed traps and lures have the aim of capturing living and mass-trapped *Monochamus* beetles (ÁLVAREZ et al., 2015; SANCHEZ-HUSILLOS et al., 2015). Moreover, *P. sylvestris* is a tree species on which the surveys primarily focus (EU, 2012; for Germany: HOPPE, 2018).

In the case of PWN presence within an European Union member state, a demarcated area must be determined, which consists of an infested zone (with all PWN-infested plants) and a buffer zone with eradication measures to prevent further spread of this quarantine pest (EU, 2012). Moreover, a clear-cut zone must be established around each plant infested with the PWN, which has a minimum radius of 500 m in the case of vector presence. In this zone, all susceptible plants (including *P. sylvestris*) must be clear cut. The buffer zone surrounds the infested zone with a width of at least 20 km, where felling and removing of susceptible plants, which are in poor health, dead or located in storm- or fire-affected areas and treatment of logs take place. In the demarcated area, intensified surveillances for the presence of the PWN must be conducted. Immediately after finding of the PWN in the buffer zone, a new demarcated area must be established (EU, 2012 and its actual version).

Restrictions on the movement of susceptible plants, wood and bark within and from demarcated areas are included. Transport to the next treatment facility, consideration of the flight season of the vector, wood transport in containers or without bark and with an insect net drenched in an insecticide, log treatment with an insecticide, heat treatment of wood and bark, plant passports

for movement in the European Union of susceptible wood and bark treated by an authorised treatment facility, wood chipping into small pieces to avoid vector contamination and burning in a designated location are obligatory measures (EU, 2012 and its actual version). For wood packaging material, specific treatment standards must be applied (FAO, 2009). According to EU (2012 and its actual version), wood waste and wood from susceptible plants that is not stripped of the bark and "left on site and ... destroyed on site shall be chipped into pieces less than 3 cm thick and less than 3 cm wide". Alternatively, susceptible wood and bark chipped into this size can be moved to the buffer zone or outside the demarcated area to be used as fuel or treated in an authorised treatment facility (EU, 2012).

To clarify the non-vector transmission of the PWN to non-infested trees and to assess the population dynamics and pathogenicity of the PWN in mature *P. sylvestris* trees as well as differences in susceptibility among different provenances of *P. sylvestris* to the PWN is vital, and if necessary, the existing pest risk analysis, management strategies and contingency plans, especially for the northern countries in Europe in terms of climate change, must be improved.

1.7 Thesis objectives

The overall objective of this thesis was to support European Union Plant Health policy by refining pest risk analysis and management and contingency planning for the quarantine pest *B. xylophilus*, with a focus on *P. sylvestris*, a highly susceptible species that is widespread in Germany and northeastern Europe. This thesis was part of the "Analysis of the potential of the pine wood nematode (*Bursaphelenchus xylophilus*) to spread, survive and cause pine wilt in European coniferous forests" of the European Union-funded research project REPHRAME [KBBE.2010.1.4-09]. Support was provided by obtaining knowledge of the PWN in terms of its potential non-vector spread to non-infested trees, the significance of sapling-based analyses for mature trees and indicators of tolerance in the species *P. sylvestris*.

For the investigations, the following specific objectives were established:

1. To assess "the survival time of the PWN in *P. sylvestris* L. wood chips for 1 year" (chapter 2: publication I).
2. To evaluate "the possibility of PWN non-vector spread from infested wood chips to non-infested *P. sylvestris* saplings" (chapter 2: publication I).
3. To study "the population dynamics and pathogenicity of the PWN in seven- to eight-year-old *P. sylvestris* trees to figure out whether the results are comparable to those published on the basis of sapling trials" (chapter 3: publication II).
4. "Using pathogenicity tests, to determine whether *P. sylvestris* provenances used in Germany for forest regeneration show any differences in susceptibility to the PWN" (chapter 4: publication III).

1.8 List of publications in peer-reviewed journals

This thesis is based on the following publications in peer-reviewed journals.

Publication I

Long-term survival and non-vector spread of the pinewood nematode, *Bursaphelenchus xylophilus*, via wood chips

HOPF-BIZIKS, A., T., SCHRÖDER, S., SCHÜTZ; For. Path. (2017), 47:e12340, DOI: 10.1111/efp.12340.

Publication II

Population dynamics and pathogenicity of *Bursaphelenchus xylophilus* in seven- to eight-year-old *Pinus sylvestris* trees

HOPF-BIZIKS, A., T., SCHRÖDER; J. Kulturpfl. (2019), **71**, 109-130, DOI: 10.5073/JfK.2019.05.01.

Publication III

The pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, and its pathogenicity to German *Pinus sylvestris* provenances

HOPF-BIZIKS, A., T. SCHRÖDER, S. SCHÜTZ; J. Plant Dis. Prot. (2016), **123**, 43-49, DOI: 10.1007/s41348-016-0005-4.

Author`s contribution

In all these papers Andrea Hopf-Biziks designed the experimental setup, was responsible for conducting the experiments and evaluated the results under supervision of her co-authors, Dr. Thomas Schröder and Prof. Dr. Stefan Schütz. She is the main-author in all of the publications.

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2 Chapter 2: Publication I: Long-term survival and non-vector spread of the pinewood nematode, *Bursaphelenchus xylophilus*, via wood chips

This chapter was published as "Long-term survival and non-vector spread of the pinewood nematode, *Bursaphelenchus xylophilus*, via wood chips" (HOPF-BIZIKS, A., T., SCHRÖDER, S., SCHÜTZ; For. Path. (2017), 47:e12340, DOI: 10.1111/efp.12340), and reused in this dissertation with the permission of the copyright owner John Wiley and Sons.

2.1 Publication I

Long-term survival and non-vector spread of the pinewood nematode, *Bursaphelenchus xylophilus*, via wood chips

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Summary

The pinewood nematode, *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease and is transmitted to new host trees by beetles of the genus *Monochamus*. The increasing interest in imported wood chips from North America for paper production and energy purposes and the corresponding phytosanitary risk of non-vector transmission of *B. xylophilus* has been discussed since 1984, the year of the first interception of *B. xylophilus* in wood chips in the European Union. The long-term survival of *B. xylophilus* in wood chips and its non-vector spread from infested wood chips to non-infested trees were studied. *Pinus sylvestris* logs were inoculated with a suspension of *B. xylophilus* to produce infested wood chips. During the long-term storage test, *B. xylophilus* in *P. sylvestris* wood chips were examined. Four variants, including sealed and openly stored wood chips at both 15 °C and 25 °C, were studied. For the test of non-vector spread, *B. xylophilus*-infested wood chips were placed on three- to four-year-old *P. sylvestris* saplings under different conditions.

Bursaphelenchus xylophilus survived for more than 1 year at both temperatures in the sealed wood chips, which was significantly longer than for the openly stored variant at 25 °C. Temperature, tree condition and wood chip location all influenced non-vector spread through wood chips. Of the 480 trees that were in contact with infested wood chips and showed clear symptoms of pine wilt disease, *B. xylophilus* were extracted from 42 pines at 25 °C and one pine at 15 °C. The highest *B. xylophilus* infestation rates resulting in clear pine wilt disease symptoms (75 %) were found in infested wood chips directly attached to stem-wounded trees at 25 °C. However, more variants exhibited *B. xylophilus* infestation at this temperature; trees with stem or root injuries plus direct contact with infested wood chips to the wounded part were primarily affected. Moreover, non-vector spread was also detected in stem- and root-injured pines without any direct contact with infested wood chips.

Our results confirmed that *B. xylophilus* can survive for long periods in wood chips and can be transmitted from infested wood chips to damaged trees, but the likelihood of such PWN

establishment should be low compared to spread through vectors. These findings must be considered in the pest risk analysis of *B. xylophilus*, and studies using outdoor trials should be carried out to complete this pest risk analysis.

1 Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, causes pine wilt disease (PWD) under suitable climatic conditions (Kiyohara and Tokushige 1971, Rutherford et al. 1990, EPPO 2009a).

PWN is native to North America, where indigenous conifers are tolerant or resistant to PWD (Dwinell and Nickle 1989), but outside of this range, this disease mainly appears on pines. PWN was most likely introduced from North America to Japan through overseas timber imports (Kishi 1995) at the beginning of the 20th century (Mamiya 1988). The pathogen was then spread to other countries, and Portugal was the first European country to be affected (Mota et al. 1999). In all of these locations, including the island of Madeira (Fonseca et al. 2012), PWN is still present and spreading; individual outbreaks in Spain (Abelleira et al. 2011) are undergoing eradication (EPPO 2014) or have been eradicated. The risk to European forests by the PWN is high (Robinet et al. 2011). According to the EU Plant Health Council Directive 2000/29/EC, *B. xylophilus* is listed as a quarantine pest (EU 2000).

It is known that PWN is transmitted to new host trees by beetles of the genus *Monochamus*, but it is still unclear whether the vector is necessary for transmission in all cases. If non-vector spread of PWN also takes place, it could be an additional factor enabling PWN establishment and PWD outbreaks in new areas or countries. Mean daily summer temperatures of more than 20 °C in July/ August are necessary for PWD outbreaks (Rutherford et al. 1990), so global climate change will also increase the risk for northern countries if PWN is present. Latent infestation can lead to the undetected spread of PWN because it can survive inside living and apparently healthy pines for several years (Halik and Bergdahl 1994). Therefore, PWN establishment without PWD symptoms would be a very dangerous situation.

Special phytosanitary requirements for the introduction and movement of plants, plant products and other objects into and within the EU member states are in place (EU 2000). Infested wood and wood products are the riskiest materials for spreading PWN over long distances, and the use of untreated wood from low-quality timber as packaging poses the highest risk for transmission due to the possibility of PWN and vector beetle occurrence inside (Evans et al. 1996, Gu et al. 2008).

Vector beetles, *Monochamus* spp., are killed during wood chipping if the chip size is small enough (Evans et al. 1996). However, PWN can survive in wood chips with suitable wood moisture contents (Panesar et al. 1994), so wood chips are a potential pathway for PWD.

Since 1984, the first detection of PWN in imported wood chips in the European Union (Rautapää 1986), the risk of PWN transmission through wood chips has been discussed. Treatment recommendations (heat treatment and fumigation) for potentially PWN-infested wood and wood in various forms, such as wood chips, from infected countries can be found in the EU Plant Health Council Directive 2000/29/EC (EU 2000). In the pest risk analysis (Evans et al. 1996), permanent establishment of PWN in new areas without vectors was considered difficult, so the potential for PWN transmission through wood chips was evaluated as low to moderate. However, there is an increasing international wood chip trade for energy purposes (Kopinga et al. 2010) and paper production (Jönsson 2011), and wood chips are used as a surface layer on paths and as a soil-covering mulch in contact with trees (Evans et al. 1996). Thus, our aim was to evaluate the survival time of PWN in *Pinus sylvestris* L. wood chips for 1 year and the possibility of its non-vector spread from infested wood chips to non-infested *P. sylvestris* saplings. To cover all possible non-vector pathways, the experimental variants consisted of all combinations of different wood chip positions with a selection of tree conditions at both 15 °C and 25 °C. Under both temperatures, wood chips were placed directly in contact with the stem, at a distance from the stem, and in the soil with direct contact to roots to mimic the influence of different types of contact with uninjured and injured stems and roots. In addition to uninjured trees, cut stumps and stem- and root-injured pines were examined to account for injuries that naturally occur in forests (e.g., storms, active root life, animals) as well as injuries caused by mechanical forestry operations.

2 Materials and methods

2.1 Production of wood chips artificially infested with *B. xylophilus*

At the end of July 2012, young *P. sylvestris* trees from a pine stand in Essehof (N 52°18.244` E 10°41.224`) near Braunschweig, Germany were felled, cut in segments and randomly checked for the presence of any nematode species. The log ends were sealed with paraffin and stored at 5 °C in a climate-controlled room for eight days. Grey mould rot fungus, *Botrytis cinerea* (Fr.) Pers. (anamorph), was cultured on 1.5 % malt extract agar medium. *B. xylophilus* isolate PT-6 (w) extracted from *Pinus pinaster* Ait. wood from Portugal was reared and multiplied in Petri dishes on non-sporulating, non-pathogenic (Daub 2008) *B. cinerea* at approximately 20 °C. Twenty-four hours before inoculation, the nematodes were extracted from the agar plates using

the Baermann funnel technique (Baermann 1917) modified for plant parasitic nematodes (described in Decker 1969).

Approximately 80 kg of the stored logs with lengths of 20 to 80 cm and diameters of approximately 8 cm were chosen. Holes with 10-mm diameters were drilled radially into the cores of the logs with a distance of approximately 10 cm between them. A 1-ml suspension of tap water containing 1,050 PWNs in mixed stages was pipetted into each hole; in total, 200 ml of suspension was used for all 80 kg of wood. After the holes were closed with wooden pegs, the logs were sealed in plastic bags and incubated at 25 °C in a climate-controlled room for 24 days. After stripping of the bark from the logs with a peeling iron, the wood chips were produced using a laboratory wood mill (RETSCH, Germany) with a two-cm sieve.

2.2 Experimental set-up of the long-term survival study

On 28th August 2012, 4,000 g of PWN-infested wood chips were mixed and stored in closed plastic bags at 25 °C in a greenhouse for five weeks until start of the long-term survival experiment. After repeated mixing, wood chips were randomly selected and divided into approximately 22-g samples, and five samples were tested for nematode density and wood moisture content (MC) at the start of the test. The samples were tested in greenhouse compartments under natural lighting and in climate chambers, and the experimental variants included PWN-infested wood chips stored openly or sealed in plastic bags at 15 °C (80 % average relative humidity (rH)) and 25 °C (60 % average rH). To avoid heating and, therefore, evaporation by sunlight, the sealed samples were covered; wood chips were exposed to 100 % rH inside the plastic bags. Every 41 days for 1 year, five wood chip samples per variant were examined for living PWNs.

2.3 Reproduction of *B. xylophilus* on a fungus isolated from PWN-infested wood chips under long-term storage

After 369 days, the last extraction date of PWNs from infested wood chips under long-term storage, all fungi were isolated. For this purpose, Petri dishes with 1.5 % malt extract agar medium treated with tetracycline-HCl (according to Oberwinkler et al. 1995) were used, and one *P. sylvestris* wood chip was placed on each plate. All of the wood chip variants with the longest PWN survival periods were tested, so the wood chips that were openly stored at 25 °C were excluded from this trial. Two treatments were applied: with and without surface sterilization of the wood chips. Two wood chips from all five samples of each experimental variant and treatment were checked for the presence of fungi. As modified after Schröder (1999), surface sterilization was performed by placing the wood chips in 70 % ethanol for 30 sec. followed by 3 % sodium hypochlorite for 2 min and 96 % ethanol for 30 sec.; chips were then washed twice

in sterilized water. The plates were stored at room temperature (approx. 20 °C) in the dark, sealed with parafilm and then checked daily to subcultivate the developing fungi. These fungi were identified by morphological methods. The most frequently isolated species was identified additionally by molecular methods (ITS-RFLP), which were performed by Dr. Benjamin Pickel and Dr. Wolfgang Maier. This fungus was tested for PWN survival and reproduction. It was cultured on 1.5 % malt extract agar medium and used as a feeding source for *B. xylophilus* (isolate PT-7 (w) extracted from Portuguese *P. pinaster* wood). Fifty microlitre of a suspension with 2,000 nematodes/ml (mixed stages in tap water) was pipetted on each fungal plate and incubated at 20 °C. Ten fungal plates were tested with *B. xylophilus* at two different time periods to monitor the first and second reproduction cycle. PWNs were extracted after 3 weeks to determine the number of living nematodes.

2.4 Experimental set-up of the non-vector spread study

Three- to four-year-old *P. sylvestris* saplings of provenance 851 04 (Middle and East German Lowland) were used for the study of non-vector spread through wood chips. The trees were carefully planted to avoid root injuries into 3-litre pots with growing medium designed for woody plants. This medium consisted of 30 % black peat, 30 % peat fibre, 20 % wood fibre, 10 % sod-based white peat and 10 % white peat (Container Substrate 1 medium, Klasmann-Deilmann GmbH, Geeste, Germany). Twenty trees were examined per experimental variant.

The trees were placed in greenhouses in a randomized block design, watered as required and pre-air-conditioned for two weeks before the start of the test. The PWN-infested wood chips were mixed, randomly selected and divided into 100-g samples. Ten samples were tested for nematode density and MC. On 29th and 30th August 2012, 100 g of prepared PWN-infested wood chip samples were placed in each 3-litre pine pot. The test was run in two greenhouses under quarantine conditions using natural lighting at 15 °C (90 % average rH) and at 25 °C (70 % average rH); the control pines were tested without wood chips. Direct contact of PWN-infested wood chips with the stem, wood chips mixed in the soil with direct root contact or wood chips on the soil surface at a distance of 3.5 cm from the stem were combined with uninjured, stem- or root-injured and felled pines (Fig. 1). Stem injuries were inflicted to mimic the damage that occurs for instance during timber harvesting in the forest, and for this purpose, a scalpel was used to remove an approximately 0.5-cm wide strip of bark at the lower end of the stem (Fig. 2a). Root injuries were produced by cutting in the root ball surface with secateurs (Fig. 2b), and felled trees were cut just above the soil (Fig. 2c). For the direct contact of wood chips to the stem (Fig. 2d) as well as the wood chips on the soil with distance from the stem (Fig. 2e), a sawn-off

ring of a drainpipe was used. Wood chips were mixed in the soil by interspersing five soil layers with four wood chip layers inside the pot (Fig. 2f).

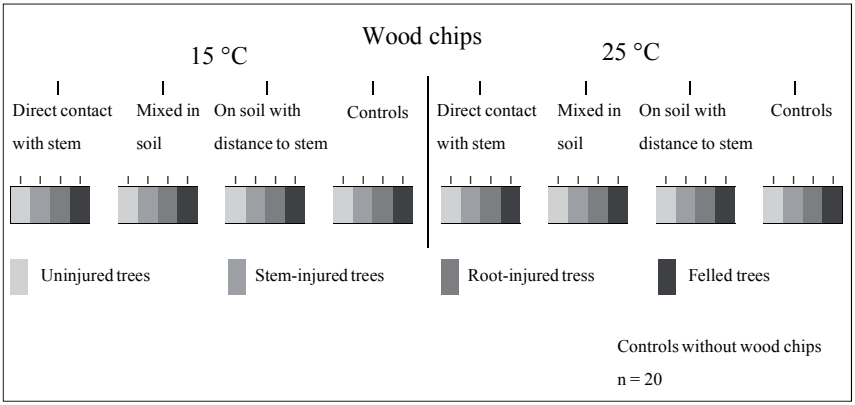
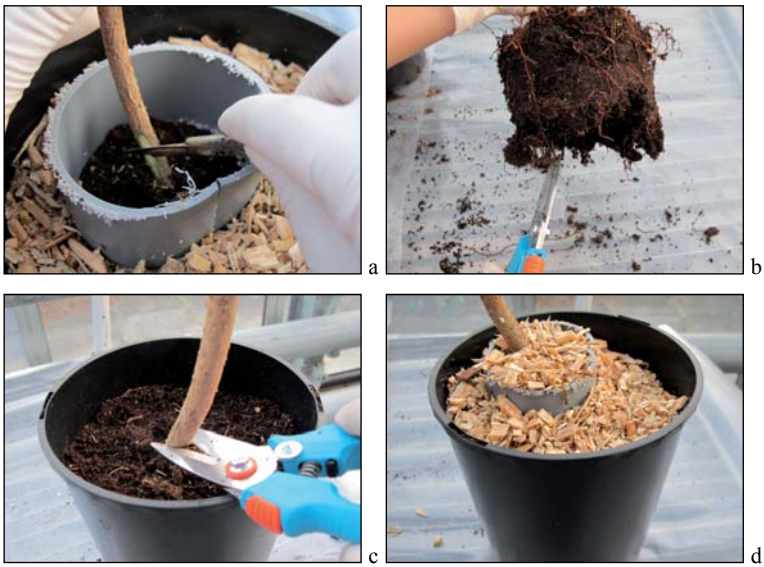


Fig. 1. Tested combinations of temperature, PWN-infested wood chips and *Pinus sylvestris* saplings




e

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Fig. 2. Examples of the tested tree and wood chip variants - a: Stem-injured pines; b: Root-injured pines; c: Felled pines; d: Direct contact of wood chips with the stem; e: Wood chips on the soil with distance with the stem; f: Wood chips mixed in the soil

2.4.1 Assessment of pine wilt

Pine wilt symptoms were visually evaluated once per week using a wilt class rating scheme (Table 1). The physiological condition of pine is related to needle discolouration, which was expressed as a percentage using wilt classes from 0 to 5. To increase the probability of extracting living nematodes, the trees were sampled when wilt symptoms became apparent at wilt class 4, or at the end of the test, week 12, whichever occurred first.

Table 1. Wilt class rating scheme for the assessment of pine wilt

Wilt class	Tree coverage by discoloured needles [%]	Physiological condition
0	0	Healthy
1	1-25	
2	26-50	
3	51-75	
4	76-99	
5	100	Dead

2.5 Sampling of plants and nematode extraction

The analysed pines were cut above the wood chip layer and the upper part of the plant was analysed after removing all of the needles. Exclusively for the felled pine variants, the root parts, including the root collar, were tested for nematodes, and the fresh weight of the roots and root collar (hereafter referred to as wood) was recorded after carefully removing the soil substrate by washing. The wood was cut into 5 mm- to 10 mm- long pieces using secateurs. After recording the fresh weight of the tree samples or the stored wood chip samples from the long-term survival

test, living PWNs were extracted for 48 h (EPPO 2009b) using the Baermann funnel technique as described above.

To determine the dry weight for the calculation of nematode density per gram dry matter, the wood was oven dried (UL 50, Universal Oven, Memmert, Germany) at 103±2 °C for 48 h. The MC was recorded according to DIN 52183 (1977):

$$MC = \frac{m_f - m_d}{m_d} * 100\ [\%]$$

MC = Wood moisture content [%]

m_f = Weight of fresh wood [g]

m_d = Weight of dry wood [g]

The nematodes were preserved in a hot, 80 °C, fixative solution (formal-acetic: 890 ml Aqua dest., 100 ml formaldehyde solution (35 %), 10 ml glacial acetic acid; modified after Dropkin 1989) for later counting.

2.6 Statistical analysis

Statistica 64 Version 10 (Stat Soft. Inc., Tulsa, USA) was used for statistical analysis of the long-term survival test and the non-vector spread study of *B. xylophilus*. For the non-vector spread trial, a chi square test ($p<0.05$) was applied to check for differences in the number of PWN-infested pines with wilt class 4 as well as the number of PWN-infested trees per independent variable. The Kruskal-Wallis test ($p<0.05$) was used in the long-term survival study to detect differences in MC, nematode density and survival time between the variants. Furthermore, the Kruskal-Wallis test was also chosen to investigate differences in MC between infested and uninfested trees and differences in the nematode density of the infested variants. In the figures and tables, significant differences are expressed by different letters.

3 Results

3.1 Long-term survival of *B. xylophilus* in wood chips

Fig. 3 and Table S1 show the development of MC per wood chip variant over time. After the start of the test, the median MC rapidly decreased in the openly stored wood chip variants from 210 % to 22 % (at 15 °C) and 18 % (at 25 °C) within 6 weeks. Over time, significant differences (Table S2, Kruskal-Wallis test: $p<0.05$) from the initial MC were recorded, but significant differences between the other extraction dates only appeared in some cases ($p<0.05$). The MC of the sealed wood chips was stable at 25 °C; no significant differences ($p>0.05$) appeared. At 15 °C, significant differences in the sealed wood chips between the extraction dates were recorded in a few cases ($p<0.05$).

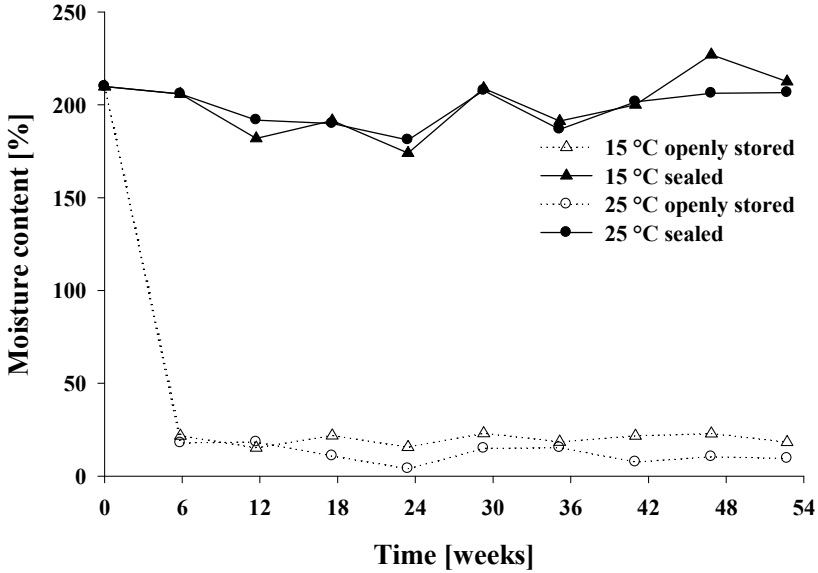

Fig. 3. Median moisture content (MC) values [%] of wood chip samples with storage time, temperature and condition; MC at start of the test: median: 210 %, min: 197 %, max: 219 %, n=5; (fibre saturation point (FSP) for woody plants: 28 to 32 % MC according to Wagenführ and Scholz 2008)

In Fig. 4 and Table S3, the development of nematode density per wood chip variant over time is shown, and the statistical analysis detected several significant differences (Table S4, Kruskal-Wallis test: $p<0.05$) in all four variants. In general, nematode density decreased after the start of the test, but different rates of decline were found. The openly stored wood chip variants exhibited the most rapid and largest declines during the first 6 weeks. On the first extraction date, less than half of the initial nematode density (187 nematodes/g dry matter) was found in the openly stored wood chip variants, but only on the second extraction date in the most samples of the closed wood chip variants. Significant differences in these rates of decline were found between the variants sealed at 15 °C and openly stored at 15 °C or 25 °C (Kruskal-Wallis test: $p<0.05$). At the end of the trial, the highest nematode density was found at 25 °C in the sealed wood chips with a median value of 56 nematodes/g dry matter after an insignificant population increase ($p=0.2471$) over half of the initial nematode density observed on the previous sampling

date. In contrast, the sealed samples at 15 °C showed a median value of only one living nematode/g dry matter, and the open variants had zero nematodes/g dry matter.

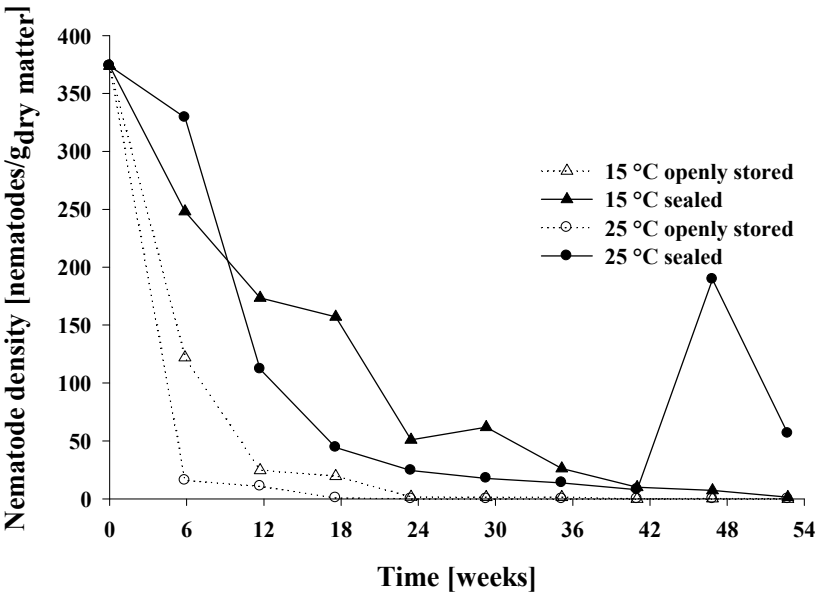

Fig. 4. Median nematode densities [nematodes/$g_{dry\ matter}$] in wood chip samples with storage time, temperature and condition; nematode density at start of the test: median: 374 nematodes/$g_{dry\ matter}$, min: 190 nematodes/$g_{dry\ matter}$, max: 487 nematodes/$g_{dry\ matter}$, n=5

The Kruskal-Wallis test detected significant differences ($p<0.05$) in MC (Fig. 3, Table S5) and nematode density (Fig. 4, Table S6) between wood chips openly stored at 25 °C and the sealed variants at both temperatures for nearly all extraction dates. Significant differences ($p<0.05$) were only found between wood chips openly stored at 15 °C and the sealed variants at both temperatures in the MC (Fig. 3, Table S5) on extraction day 2 and nematode density (Fig. 4, Table S6) on a few dates. Both the openly stored variants and the sealed variants showed no significant differences ($p>0.05$).

The PWN survival time in the variant wood chips openly stored at 25 °C was a period of 23.5 weeks, after which no nematodes could be extracted, and this was significantly shorter than the survival time in sealed wood chips at both temperatures (Kruskal-Wallis test: $p<0.05$). The sealed wood chips at both temperatures had a PWN survival time of >1 year; the final dates of successful nematode extraction are unknown due to the limited number of samples. At 53 weeks,

no nematodes could be extracted from wood chips openly stored at 15 °C, with no significant differences to the sealed variants until the end of the test.

Independent of storage condition (open/sealed), the difference in temperature caused no significant differences (Kruskal-Wallis test: $p>0.05$) in survival time, MC (Fig. 3) and nematode density (Fig. 4) throughout the experiment.

In contrast, the storage condition had a constant, significant influence on survival time ($p=0.0002$) and MC ($p=0.0002$) as well as nematode density ($p<0.05$) at both temperatures for the entire year; clearly longer survival times, higher MC (Fig. 3) and higher nematode density (Fig. 4) were observed in the sealed samples.

3.2 Reproduction of *B. xylophilus* on a fungus isolated from PWN-infested wood chips under long-term storage

All fungi isolated from wood chips of the different tested variants stored for 1 year with or without surface sterilization can be found in Table 2. Openly stored wood chips at 25 °C were not included in the test because of the shorter PWN survival period. Surface sterilization of the openly stored wood chips at 15 °C variant resulted in all samples being sterile. For the remaining combinations of wood chip variant and sterilization treatment, *Trichoderma atroviride* Karst. occurred with a frequency of 92 % among all of the isolated fungi. Moreover, *T. atroviride* appeared within these wood chip variants with the highest frequency independent of the type of sterilization. Without surface sterilization, *Rhizopus* sp. and *Penicillium* sp. were also found in the open storage at 15 °C variant, and *Graphium* sp. and *Botrytis* sp. were found on the variant sealed at 25 °C.

Table 2. Isolated fungal genera/ species and frequency of occurrence of these genera/ species per PWN-infested wood chip variant after storage for 1 year (with and without surface sterilization), n=10

Wood chip variant	Surface sterilization	Fungal genera/ species	Frequency
15 °C, openly stored	yes	Sterile wood chip	10/10
15 °C, openly stored	no	*Trichoderma atroviride*	10/10
		Rhizopus sp.	1/10
		Penicillium sp.	1/10
15 °C, sealed	yes	*Trichoderma atroviride*	9/10
		Sterile wood chip	1/10
15 °C, sealed	no	*Trichoderma atroviride*	10/10
25 °C, sealed	yes	*Trichoderma atroviride*	10/10
25 °C, sealed	no	*Trichoderma atroviride*	10/10
		Graphium sp.	1/10
		Botrytis sp.	1/10

The test for survival and reproduction of *B. xylophilus* on *T. atroviride* was negative; after an incubation time of three weeks, no PWNs were extracted. The test was repeated with a nearly five-week incubation time with the same result. In contrast to the nematodes, the fungus remained alive and could be recultured.

3.3 Non-vector spread of *B. xylophilus* via wood chips

The 100-g wood chip samples, which were placed inside the pots of each test pine, were infested with an average of 552±252 nematodes/g dry matter and had a mean MC of 194±6 %.

At the start of the test, all trees were healthy and did not show any needle discolouration. Wilt classes 1 and 2 were the most frequently observed for nearly all variants until the end of week 12. The percentage of PWN-infested trees showing wilt class 4 in each treatment at 25 °C and the significant differences (chi square test: $p<0.05$) are displayed in Fig. 5. At 25 °C, wilt class 4 could be observed for all three tree conditions and all three wood chip positions. Wilt class 4 was the most frequent class among the stem-injured pines with direct contact of wood chips with the stem and root-injured pines with PWN-infested wood chips mixed in the soil variants at 25 °C. At 15 °C, PWD symptoms were less apparent. Infestation by PWNs was found in three of the 20 stem-injured trees with direct contact of wood chips with the stem at this temperature, but only one tree exhibited wilt class 4; the others were in wilt classes 2 and 3. The earliest detection of wilt class 4 at 25 °C was in week 6 and at 15 °C in week 12. If PWNs were

able to reach the wound, tree injuries had a large influence on non-vector transmission through wood chips, especially direct contact with the wounded stem or roots. In contrast, control trees without wood chips exhibited no wilt class 4 until week 6 and beyond.

Ten per cent (47 pines) of all 480 treated trees were infested by PWN; no PWNs were extracted from the control pines. The number of PWN-infested trees (including control variants) sorted according to the independent variables of temperature, tree condition and wood chip position (at 25 °C) is shown in Table 3, and significant differences in the number of infested and uninfested pines between some variants per independent factor were found using the chi square test. At 25 °C, significantly more pines ($p<0.0001$) were affected by the spread of PWN compared to 15 °C (16 % vs. 1 %). The numbers of uninjured and felled infested pines were significantly different ($p<0.0001$) compared to the high number of infested trees with root or stem injuries at 25 °C. Moreover, at this temperature, significant differences between direct contact of wood chips with the stem, which resulted in the highest number of infested trees, and wood chips mixed in the soil ($p=0.0231$) as well as wood chips on the soil with distance to the stem ($p=0.0016$) were found. These three wood chip variants were significantly different from the control variant ($p\leq0.0037$). At 15 °C, no significant differences ($p>0.05$) between the variants per independent factor (tree condition and wood chip position) were found.

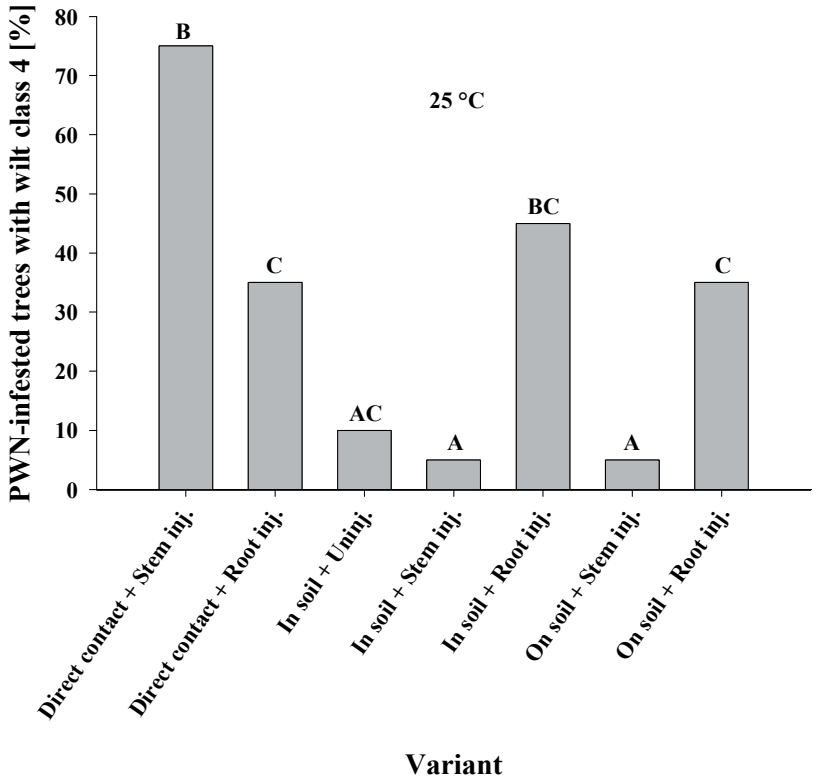

Fig. 5. Number of *Pinus sylvestris* in contact with *Bursaphelenchus xylophilus*-infested wood chips in different experimental variants at 25 °C exhibiting *B. xylophilus* infestation and wilt class 4 [%] (= needle discolouration 76-99 %) after 12 weeks; different letters indicate significant differences in the number of *B. xylophilus*-infested trees with wilt class 4 (chi square test: $p<0.05$), n=20

Table 3. Number of PWN-infested and uninfested saplings per independent variable (a: Temperature; b: Tree condition at 25 °C; c: Wood chip position at 25 °C) including control trees (15 °C results in the text); different letters indicate significant differences in the number of *B. xylophilus*-infested trees per independent variable (chi square test: $p<0.05$), n=320/80/80

Temperature [°C]	Number of trees		χ^2
	PWN-infested	Not PWN-infested	
15	3	317	A
25	44	276	B

a

Tree condition	Number of trees		χ^2
	PWN-infested	Not PWN-infested	
Uninjured	2	78	A
Stem-injured	19	61	B
Root-injured	23	57	B
Felled	0	80	A

b

Wood chip position	Number of trees		χ^2
	PWN-infested	Not PWN-infested	
Direct contact with stem	24	56	A
In soil with direct root contact	12	68	B
On soil with distance	8	72	B
Controls without wood chips	0	80	C

c

The nematode densities extracted from the upper part of the plant above the wood chip contact point of the tested variants at 25 °C are shown in Fig. 6. At 15 °C, PWNs were extracted from three stem-injured trees in direct stem contact with wood chips at densities of between 71 (wilt class 4) and 0.5 nematodes/g dry matter (wilt classes 2 and 3). Significant differences were found in nematode densities (Kruskal-Wallis test: $p<0.05$) between variants with successful non-vector transmission. The density in the wood chips with direct contact with the stems of stem-injured trees at 25 °C variant, which had the highest median value, was significantly different from that in the same variant at 15 °C as well as the wood chips in soil of uninjured or stem-injured trees and wood chips on soil of stem-injured pine variants at 25 °C.

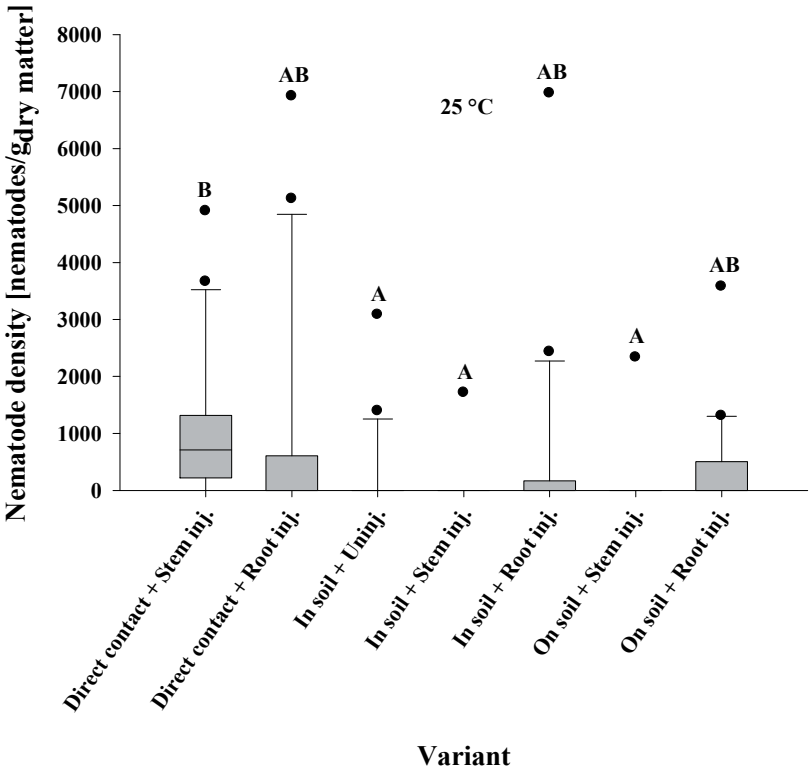

Fig. 6. Nematode density [nematodes/$g_{dry\ matter}$] in the upper part of the plant above the wood chip layer with the medians, 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers), and outliers of tested variants at 25 °C; different letters indicate significant differences in nematode densities (Kruskal-Wallis test: $p<0.05$), n=20

The successful spread of PWN led to significantly reduced MC (Kruskal-Wallis test: $p<0.001$). At the end of the test, the median MC of the uninfested pines (upper part of the plant) with wilt classes 0 and 1 was 182 %, and it was 36 % for the infested tress with wilt class 4.

4 Discussion

Our test of long-term PWN survival in wood chips resulted in significantly decreased moisture contents (MC) of the openly stored variants, while the sealed variants showed only small changes in MC. Nematode densities were significantly reduced over time, and the most rapid and largest declines were observed in the openly stored variants. The PWN survival times were

significantly different between wood chips openly stored at 25 °C (23.5 weeks) and those sealed at 15 °C and 25 °C (>1 year). Sousa et al. (2011a) observed that PWN reproduced rapidly and persisted in *P. pinaster* boards at 25 °C, and the subsequent nematode decline to zero in week 40 coincided with a decrease in MC to the fibre saturation point (FSP). In a study by Panesar et al. (1994), PWN-infested wood chips (*Tsuga heterophylla* (Raf.) Sarg. and *Pseudotsuga menziesii* (Mirb.) Franco mixture) were incubated at 20-22 °C in sealed Petri dishes, and a small number of PWNs persisted for up to 14 months (isolate 1) or 20 months (isolate 2). These two studies are consistent with our results in *P. sylvestris*. We found no significant influence of temperature between 15 °C and 25 °C in *P. sylvestris*. A drop in MC below the FSP was shown to significantly reduce the survival of PWN (Fig. 3-4), and this steep decrease in nematode density was halted at approximately 10 % of the original density, after which it continued to decrease more slowly. The extended presence of nematodes in the openly stored wood chips might be attributed to the continuing availability of some food resources, as suggested by Sousa et al. (2011b).

PWN might feed on fungi after the death of all plant cells (Evans et al. 1993), and a study by Maehara and Futai (1996) indicates that the dominant fungal species could determine the population density of PWNs in dead trees. The most frequently isolated fungus from our wood chips subjected to long-term storage was *T. atroviride*, and a study by Halik and Bergdahl (1990) of PWN-inoculated *P. strobus* wood chips also found *Trichoderma* species to be the most dominant isolated fungal genus. In that study, PWN was successfully reared on cultures of their *Trichoderma* sp., but PWN was not able to survive on *T. atroviride* in our experiment. According to Fukushige (1991), Panesar et al. (1994), Maehara and Futai (1996 and 2000), Maehara (2007) and Yang et al. (2012), differences exist between *Trichoderma* spp. in terms of nematicidal activity against PWN. For *T. atroviride* in particular, nematicidal activity against root-knot nematodes has been observed (Davies and Spiegel 2011). The persistence of the PWN at low population densities in our test of long-term survival suggests that the availability of large amounts of nutritional fungus might not have been necessary (Panesar et al. 1994, Sousa et al. 2011b). Moreover, it is possible that a less prominent fungus was serving as a nutrient source and escaped isolation. For example, Maehara and Futai (1996) found if blue-stain fungus *Ophiostoma minus* Hedgcock was inoculated in *Pinus densiflora* Sieb. & Zucc. wood blocks, the reproduction of PWN was high compared to wood blocks inoculated by *Trichoderma* spp.

In our study of the non-vector spread of PWN from infested wood chips to non-infested *P. sylvestris* saplings, significantly more saplings developed greater degrees of wilt and were PWN-infested at 25 °C compared to 15 °C. A study by Futai (1980) employing PWN on fungal

cultures showed that the nematode reproduced in 3 days at 30 °C, 6 days at 20 °C and 12 days at 15 °C. Rutherford et al. (1992) compared the temperature-mediated behaviour of PWN isolates from different geographic regions and the PWN movement rates on thin-film agar plates were recorded at 10 °C, 20 °C and 30 °C. With increasing temperature, all of the tested isolates moved more rapidly, and both activity and fecundity increased. These differences in movement speed seem to effect the transfer of PWN from donor to adjacent recipient *P. pinaster* boards by direct contact (Sousa et al. 2011a), but nematode transfer was not successful if the ambient temperature was only 10 °C. Transfer was successful at 25 °C, and Melakeberhan et al. (1992) observed that visible disease symptoms and *P. sylvestris* mortality were correlated with a high number of extracted nematodes. This might be caused by the considerable rate of nematode reproduction at this temperature, and furthermore, the physiology of the host seemed to be altered in such a way that the tree became less tolerant to PWN. In contrast, because the nematode reproduction rate was low and not correlated with visible symptoms at 15 °C, the observed pine death was not necessarily caused by PWN (Melakeberhan et al. 1992). Not all of our PWN-infested pines exhibited wilt class 4 at 15 °C at the end of the experiment, which hints at a latent PWN infestation (Halik and Bergdahl 1994). Therefore, PWN infestation through infested wood chips might be also possible in the northern countries of Europe.

Our results indicate that tree injuries had an important influence on non-vector transmission of PWN by wood chips if PWNs were able to reach the wound, and this was especially the case of direct contact with wounded roots and stems. As is known from PWN transmission to trees through feeding (Linit 1990) and oviposition wounds (Edwards and Linit 1992) inflicted by a vector beetle, the exposure of the inner bark enables PWN to enter a tree above the ground. Arakawa and Togashi (2002) found that any wound can be used by PWN, and in all of their tests carried out on *P. densiflora* logs at 25 °C, nematodes carried by male vector beetles, which were experimentally prevented from feeding on the bark, were able to enter oviposition holes. In their study, PWN establishment and reproduction in logs with artificially produced holes was successful, and the tested distances were 0, 5, 10 and 15 cm (in our test, the wood chip-stem distance was 3.5 cm). With increasing distance, the probability of successful nematode reproduction decreased, and no nematode could enter the wood at a distance of 15 cm.

Halik and Bergdahl (1987) potted five-year-old *P. strobus* seedlings after wounding the roots in a soil and PWN-infested wood chip mixture, and seven of 12 trees developed PWD over 12 weeks at 18-29 °C. PWNs could be extracted from roots and stems, and we can confirm these results for *P. sylvestris*. Additionally, in 1992, Halik and Bergdahl tested the spread of PWN from wood chips in soil to wounded *P. sylvestris* roots at 20 °C and 30 °C, and PWNs could be extracted at high rates. Seedlings with basal stem wounds were mulched with infested wood

chips in the field, and they became infested with PWNs at comparable rates and died. In our study, the spread of PWN from wood chips in and on the soil to root- and stem-injured *P. sylvestris* saplings was successful at distances of 3.5 cm at 25 °C. Mamiya and Shoji (1989) tested direct inoculation of a water suspension with PWNs into the soil adjacent to an artificially induced wound in each taproot of one-year-old *Pinus thunbergii* Parl. and *P. densiflora*. The pines were tested at a mean temperature of 26 °C and exhibited mortality rates of 94-100 %. Furthermore, PWNs could be extracted from the roots. A distance of more than 1 cm resulted in 11-30 % diseased seedlings, and the maximum time for the extraction of living PWNs from the soil was 48 hours. This indicates that PWN was affected by the soil environment and died if they could not enter the roots fast enough. In addition, Halik and Bergdahl (1992) published their studies of PWN-infested *P. strobus* wood chips, and they found that chips with soil stored in bags during 12 weeks at 12 °C and 20 °C contained significantly fewer nematodes compared to chips without soil under the same conditions. However, PWNs could still be extracted from samples with soil after 12 weeks. Evans et al. (1993) considered the scenario of wood chips coming into contact with root-wounded seedlings as being unlikely in nature. However, one has to be careful to use the term “uninjured”. Because on the one hand, uninjured plants are seldom in nature (Gardiner et al. 2016), and on the other side, despite careful handling, 100 % exclusion of root injuries cannot be guaranteed in experiments. Thus, this situation might reflect natural conditions; for example, active root life, mechanical stress due to wind, etc. can lead to micro-wounds in the roots that might explain the occurrence of infested “uninjured” trees. Moreover, Yang (1991) found evidence that wounds might not always be necessary for one population from Ontario; PWNs could immediately penetrate the roots or stem base of four-year-old *P. sylvestris*. Mamiya and Shoji (1989) observed a 3 % mortality in *P. densiflora* if a PWN suspension was inoculated in the soil adjacent to unwounded taproots.

In conclusion, PWN-infested wood chips can be a potential source of inoculum for transfer to PWD-free European countries or uninfested areas, and the threat is enhanced by the demonstrated long-term survival of PWN in wood chips. PWN from infested wood chips can establish itself in a suitable host, especially if there are stem or root injuries, direct contact with chips and suitable climatic conditions. This pathway may also be considered when planning eradication measures where infested trees are chipped on site and chips are left on the ground. However, the term “uninjured” should be used with caution as trees in nature are seldom 100 % undamaged. Our results were consistent with similar investigations found in the literature of other tree species. Moreover, latent PWN infestation through infested wood chips might be

possible at 15 °C and also increase the risk for northern countries of Europe. However, PWD symptoms in the forest can only be found at suitable climatic conditions.

Non-vector spread of PWN can lead to new isolated occurrences of PWD, but the likelihood of such PWN establishment should be low compared to spread through vectors. Quick drying of wood chips in the forest might decrease the probability of non-vector spread. However, coming into contact with thousands of trees worldwide can increase the cumulated probability of infection. The soil represents a hostile environment to PWN, but establishment although less likely is possible according to our greenhouse trials. In any case, robust outdoor trials should be conducted to confirm all these findings and to complete the pest risk analysis, taking into account that these are restricted to *B. xylophilus*-infected countries for quarantine reasons. The further spread of PWN after non-vector introduction depends on native or established vectors or the occurrence of non-vector transmissions. The knowledge of the potential threat of PWN-infested wood chips cannot be neglected and must be considered in future pest risk analysis. Trade regulations and the treatment of potentially PWN-infested wood chips from infected countries to eliminate PWN are already defined in the EU Plant Health Council Directive 2000/29/EC. From the pure scientific point of view, not all uses of wood chips of a size rendering them free of vector beetles require a treatment to avoid non-vector spread of PWN. However, the recommendations for action as part of the precautionary principle in European Union plant health regulations are to treat all wood chips from infected countries, independent from their size and the planned use, because the final use of wood chips inside a country cannot be controlled, once the material has been customs cleared.

Acknowledgements

This study was enabled by the EU-funded research project REPHRAME “Development of improved methods for detection, control and eradication of pinewood nematode in support of EU Plant Health Policy” [KBBE.2010.1.4-09] and was carried out at the Julius Kühn-Institut (JKI) in Braunschweig. The authors gratefully acknowledge the excellent help of Silvia Urban and Doreen Mybes in the laboratory. Many thanks to Dr. Benjamin Pickel (Department of Molecular Wood Biotechnology and Technical Mycology, Büsgen-Institute, Georg-August-University in Göttingen) and Dr. Wolfgang Maier (Institute for Epidemiology and Pathogen Diagnostics, JKI Braunschweig) for the molecular identification of *T. atroviride*. Moreover, our thanks go to Dr. Irina Kuzyakova (Department of Ecoinformatics, Biometrics and Forest Growth, Büsgen-Institute, Georg-August-University Göttingen) for advice on the statistical analysis and Dr. Edmundo de Sousa (I.N.I.A, Portugal) for providing the *B. xylophilus* isolates. Prof. Dr. Stephen

Woodward (The Institute of Biological and Environmental Sciences, University of Aberdeen) and both anonymous reviewers deserve many thanks for improving the manuscript.

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2.2 Publication I - Supplementary material

Table S1. Moisture content (MC) medians, minima and maxima [%] of wood chip samples with storage time, temperature and condition; MC at start of the test: median: 210 %, min: 197 %, max: 219 %, n=5

		Time [days]								
		41	82	123	164	205	246	287	328	369
Variant		Moisture content [%]								
15 °C	Median	22	15	22	16	23	18	22	23	18
open	Min-Max	19-22	14-23	19-23	13-18	21-26	16-23	18-23	19-24	16-19
15 °C	Median	206	182	192	174	209	191	200	227	213
sealed	Min-Max	191-210	181-200	188-222	158-193	201-223	188-200	196-215	209-231	203-218
25 °C	Median	18	18	11	4	15	15	8	10	10
open	Min-Max	17-21	16-21	9-16	3-8	12-16	8-16	8-9	9-11	8-11
25 °C	Median	206	192	190	181	208	187	202	206	207
sealed	Min-Max	192-209	173-200	183-193	170-201	188-213	180-191	185-208	198-215	203-220

Table S2. Significant differences (Kruskal-Wallis test: $p<0.05$) in MC in wood chip variants with different storage temperature and condition between different storage times

Variant	Significant differences between storage times [days]	p-value
15 °C open	0-82	0.0032
	0-164	0.0004
	0-369	0.0079
	164-205	0.0285
15 °C sealed	82-328	0.0094
	164-328	0.0013
	164-369	0.0106
25 °C open	0-164	0.0001
	0-287	0.0007
	0-369	0.0253
	41-164	0.0032
	41-287	0.0176
	82-164	0.0043
	82-287	0.0224

Table S3. Nematode density medians, minima and maxima [nematodes/$g_{dry\ matter}$] in wood chip samples with storage time, temperature and condition; nematode density at start of the test: median: 374 nematodes/$g_{dry\ matter}$, min: 190 nematodes/$g_{dry\ matter}$, max: 487 nematodes/$g_{dry\ matter}$, n=5

		Time [days]								
		41	82	123	164	205	246	287	328	369
Variant		Nematode density [nematodes/$g_{dry\ matter}$]								
15 °C	Median	122	25	20	2	1	1	-	0.1	-
open	Min-Max	30-173	16-51	5-28	1-8	0-4	0-4	0-1	0-0.3	-
15 °C	Median	248	174	157	51	62	26	10	7	1
sealed	Min-Max	198-312	39-194	119-188	22-69	1-117	0.4-39	2-20	4-11	0.3-2
25 °C	Median	16	11	1	-	-	-	-	-	-
open	Min-Max	10-106	7-24	0.4-2	-	-	-	-	-	-
25 °C	Median	329	112	44	25	18	14	8	190	57
sealed	Min-Max	267-370	82-189	36-51	11-31	7-24	11-70	4-153	71-253	0-151

Table S4. Significant differences (Kruskal-Wallis test: $p<0.05$) in nematode density in wood chip variants with different storage temperature and condition between different storage times

Variant	Significant differences between storage times [days]	p-value
15 °C open	0-287	0.0020
	0-328	0.0061
	0-369	0.0005
	41-287	0.0198
	41-369	0.0061
15 °C sealed	0-287	0.0121
	0-328	0.0079
	0-369	0.0002
	41-287	0.0348
	41-328	0.0233
	41-369	0.0009
	82-369	0.0439
	123-369	0.0439
25 °C open	0-164	0.0191
	0-205	0.0191
	0-246	0.0191
	0-287	0.0191
	0-328	0.0191
	0-369	0.0191
25 °C sealed	0-164	0.0474
	0-205	0.0094
	0-246	0.0274
	0-287	0.0061
	41-205	0.0198
	41-287	0.0131

Table S5. Significant differences (Kruskal-Wallis test: $p<0.05$) in MC between wood chip variants with different storage temperature and condition at different storage times

Storage time [days]	Significant differences between variants	p-value
41	25 °C open - 15 °C sealed	0.0127
	25 °C open - 25 °C sealed	0.0088
82	15 °C open - 15 °C sealed	0.0355
	15 °C open - 25 °C sealed	0.0088
123	25 °C open - 15 °C sealed	0.0017
	25 °C open - 25 °C sealed	0.0139
164	25 °C open - 15 °C sealed	0.0116
	25 °C open - 25 °C sealed	0.0021
205	25 °C open - 15 °C sealed	0.0037
	25 °C open - 25 °C sealed	0.0067
246	25 °C open - 15 °C sealed	0.0007
	25 °C open - 25 °C sealed	0.0277
287	25 °C open - 15 °C sealed	0.0025
	25 °C open - 25 °C sealed	0.0097
328	25 °C open - 15 °C sealed	0.0006
	25 °C open - 25 °C sealed	0.0327
369	25 °C open - 15 °C sealed	0.0025
	25 °C open - 25 °C sealed	0.0097

Table S6. Significant differences (Kruskal-Wallis test: $p<0.05$) in nematode density between wood chip variants with different storage temperature and condition at different storage times

Storage time [days]	Significant differences between variants	p-value
41	25 °C open - 15 °C sealed	0.0452
	25 °C sealed - 15 °C open	0.0452
	25 °C sealed - 25 °C open	0.0014
82	25 °C open - 15 °C sealed	0.0037
	25 °C sealed - 25 °C open	0.0166
123	15 °C open - 15 °C sealed	0.0452
	25 °C open - 15 °C sealed	0.0004
	25 °C sealed - 25 °C open	0.0452
164	25 °C open - 15 °C sealed	0.0007
	25 °C open - 25 °C sealed	0.0277
205	25 °C open - 15 °C sealed	0.0034
	25 °C open - 25 °C sealed	0.0301
246	25 °C open - 15 °C sealed	0.0116
	25 °C open - 25 °C sealed	0.0234
287	25 °C open - 15 °C sealed	0.0327
	25 °C open - 25 °C sealed	0.0277
328	25 °C sealed - 15 °C open	0.0197
	25 °C sealed - 25 °C open	0.0011
369	25 °C sealed - 15 °C open	0.0452
	25 °C sealed - 25 °C open	0.0452

3 Chapter 3: Publication II: Population dynamics and pathogenicity of *Bursaphelenchus xylophilus* in seven- to eight-year-old *Pinus sylvestris* trees

This chapter was published as "Population dynamics and pathogenicity of *Bursaphelenchus xylophilus* in seven- to eight-year-old *Pinus sylvestris* trees" (HOPF-BIZIKS, A., T., SCHRÖDER; J. Kulturpfl. (2019), **71**, 109-130, DOI: 10.5073/JfK.2019.05.01), and reused in this dissertation under the terms of the Creative Commons - Namensnennung 4.0 Lizenz.

Journal für Kulturpflanzen, 71 (5). S. 109–130, 2019, ISSN 1867-0911, DOI: 10.5073/JfK.2019.05.01 Verlag Eugen Ulmer KG, Stuttgart

Originalarbeit

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Population dynamics and pathogenicity of *Bursaphelenchus xylophilus* in seven- to eight-year-old Pinus *sylvestris trees*

Populationsdynamik und Pathogenität von *Bursaphelenchus xylophilus* in sieben- bis achtjährigen *Pinus sylvestris* Bäumen

Zusammenfassung

Der Kiefernholznematode, *Bursaphelenchus xylophilus*, ist der schädlichste pflanzenparasitäre Nematode an Bäumen in Asien und Europa und führt bei anfälligen Koniferenarten, hauptsächlich *Pinus* spp., zur sogenannten Kiefernwelkekrankheit. Die Krankheit ist lediglich außerhalb seines natürlichen Verbreitungsgebietes (Nordamerika) oder an nicht einheimischen Kiefernarten aufgetreten. In Gewächshausversuchen mit Sämlingen war *Pinus sylvestris* eine der anfälligsten europäischen Kiefernarten. Um die Aussagefähigkeit dieser auf Sämlingen basierenden Analysen bezüglich der Populationsdynamik und der Pathogenität für erwachsene *P. sylvestris* Bäume zu überprüfen, wurden Untersuchungen an sieben- bis achtjährigen Bäumen durchgeführt. Die Bäume wurden mit einer Suspension bestehend aus 10.000 *B. xylophilus* in 600 µl Leitungswasser pro Baum künstlich inokuliert. Für die Populationsdynamikuntersuchung wurden die Kiefern zur Nematodenextraktion in 48 Segmente geteilt. Die Entwicklung der Welkesymptome und physiologischen Änderungen wurden bis zum Tod der Bäume beobachtet.

Während der Populationsdynamikuntersuchung war *B. xylophilus* in den ersten 11 Tagen nach Inokulation in der Inokulationsstelle und den benachbarten Segmenten lokalisiert. Am Tag 16 war *B. xylophilus* im gesamten Stamm, den benachbarten Astsegmenten, Wurzelhals und Wurzeln verteilt, noch bevor äußere Welkesymptome erschienen. Mit zunehmender Kiefernwelkeerkrankung war *B. xylophilus* schließlich in allen Holz- und Wurzelsegmenten zu finden. Hohe Nematodendichten traten auf. Kurz vor dem vollständigen Absterben der Bäume zeigte die Baumspitze mehrere nematodenfreie Segmente. Die restlichen Stamm- und benachbarten Astsegmente und der Wurzelhals waren in hohem Maße mit Nematoden befallen. Während der Pathogenitätsuntersuchung starben alle *B. xylophilus*-inokulierten Kiefern innerhalb von 84 Tagen. Der signifikante Abfall des Wasserpotentials in den Nadeln war steiler und stärker mit den zunehmenden Welkesymptomen korreliert als bei einer trockengestressten Vergleichsvariante. Der Abfall des Wasserpotentials in den Nadeln trat jedoch bei der trockengestressten Kiefernvariante früher ein. Schlussfolgend betrachtet waren die Populationsdynamik von *B. xylophilus* in sieben- bis achtjährigen *P. sylvestris* Bäumen und die pathologischen Reaktionen der Kiefern vergleichbar zu denen in Sämlingsuntersuchungen, auch wenn das Erreichen eines Populationsmaximums und die Entwicklung von Welkesymptomen zeitlich verzögert waren. Aus diesem Grund sind *P. sylvestris* Sämlinge gute Indikatorbäume für *B. xylophilus* Populationsdynamik- und Pathogenitätsuntersuchungen.

Stichwörter: *Bursaphelenchus xylophilus*, Kiefernwelkekrankheit, Nematodendichte, Pathogenität, *Pinus sylvestris*, Populationsdynamik, Trockenstress, Wasserpotential

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Accepted
10 April 2019

Journal für Kulturpflanzen, 71 (5). S. 109–130, 2019, ISSN 1867-0911, DOI: 10.5073/JfK.2019.05.01 Verlag Eugen Ulmer KG, Stuttgart

Abstract

The pinewood nematode, *Bursaphelenchus xylophilus*, is the most harmful plant parasitic nematode on trees in Asia and Europe and is the causal agent of the so-called pine wilt disease of susceptible conifer species, mainly *Pinus* spp. The disease has occurred only outside its natural range of distribution (North America) or on non-native pine species. In greenhouse trials using saplings, *Pinus sylvestris* was one of the most susceptible European pine species. To examine the significance of these sapling-based analyses concerning the population dynamics and pathogenicity for mature *P. sylvestris* trees, investigations using seven- to eight-year-old trees were carried out. The trees were artificially inoculated using a suspension of 10,000 *B. xylophilus* in 600 µl of tap water per tree. For the population dynamics investigation, the pines were divided into 48 segments for nematode extraction. The development of wilt symptoms as well as physiological changes were observed until tree death.

During the population dynamics investigation, *B. xylophilus* was located at the inoculation site and in adjacent segments during the first 11 days after inoculation. On day 16, *B. xylophilus* was distributed throughout the entire stem, adjacent branch segments, root collar and roots before any external wilt symptoms appeared. With increasing pine wilt disease, *B. xylophilus* was finally found in all wood and root segments. High nematode densities appeared. Shortly before tree death, the treetop showed several nematode-free segments. The rest of the stem and adjacent branch segments and root collar were highly nematode-infested. During the pathogenicity investigation, all *B. xylophilus*-inoculated pines died within 84 days. The significant decline in the water potential in the needles was steeper and more strongly correlated with increasing wilt symptoms compared to a drought-stressed variant. However, the decline in the water potential in the needles started earlier in the drought-stressed pine variant. In conclusion, the population dynamics of *B. xylophilus* in seven- to eight-year-old *P. sylvestris* trees and the pathological reactions of the pines were comparable to those observed in assays with saplings, although delayed in reaching a population peak and developing wilt symptoms. Therefore, *P. sylvestris* saplings are good indicator trees for *B. xylophilus* population dynamics and pathogenicity investigations.

Key words: *Bursaphelenchus xylophilus*, pine wilt disease, nematode density, pathogenicity, *Pinus sylvestris*, population dynamics, drought stress, water potential

Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhrer) Nickle, is native in North America. In places outside this area and for non-native pines, PWN is the causal agent for the development of pine wilt disease (Evans et al., 1996). Following the latest research results, pine wilt disease is a complex infection process with the involvement of host, nematode, vector and possibly bacteria.

Migration of PWN within the tree tissue is connected with histological changes and wilt symptoms of the tree (Ichihara et al., 2000). In addition, PWN are associated with toxin-producing bacteria. Recent results indicate that they are involved in the disease development as well (Zhao et al., 2003; Vicente et al., 2013). Nevertheless, the mutualistic relationship between PWN and bacteria is still unclear (Proenca et al., 2017).

Requirements for the spread of PWN and development of pine wilt disease are the occurrence of conifers as host trees, mainly pines (Kishi, 1995), beetles of the genus *Monochamus* as vector for PWN transmission (Akbulut and Stamps, 2012) and suitable climatic conditions (Rutherford et al., 1990; Evans et al., 1996). Since the beginning of the 20th century, PWN has been reported outside its native range, first in Japan (Mamiya, 1988). The EPPO (2018) has listed all countries where PWN is currently present. In Europe, the first pine wilt disease affected area was confirmed in Portugal in the year 1999 (Mota et al., 1999). In Spain, over the years fife single outbreaks were found, from which three have been eradicated and two more are still undergoing eradication (EC, 2018). In northern countries, including Germany, PWN presence would also lead to pine wilt disease outbreaks if mean daily summer temperatures in July/August of more than 20 °C (Evans et al., 1996) would exist in the course of global climate change (Gruffudd et al., 2018; Ikegami and Jenkins, 2018).

According to the European Union Plant Health Directive 2000/29/EC, *B. xylophilus* belongs to the quarantine pests in the European Union (EU, 2000). Therefore, movement restrictions in trade of susceptible plants, wood and bark from PWN-affected countries and measures to eradicate PWN in European Union member states are in place (EU, 2012). Basis for all phytosanitary regulations is a pest risk analysis (FAO, 2007). In the framework of a pest risk analysis, host suitability is a key question. The use of saplings for these investigations in non-infested countries is a common method to carry out pathogenicity tests as outdoor trials are prohibited because of quarantine restrictions.

A range of European conifer species have been analysed or tested concerning their susceptibility to PWN (Evans et al., 1996; Daub, 2008). *Pinus* species would be the most endangered tree species.

In total, 23% of Germany's forest area is covered by *P. sylvestris* L. (BMEL, 2016). This pine species was found to be highly susceptible against pine wilt disease in greenhouse trials using saplings (such as Daub, 2008; Hopf-Biziks et al., 2016; Menéndez-Gutiérrez et al., 2017). However, the susceptibility of many conifer species could not be confirmed by testing mature trees, as shown by Wingfield et al. (1986) for *Pinus resinosa* Ait. in outdoor trials in North America. Based on Web of Science database research, the population dynamics of PWN in *P. sylvestris* was only tested using saplings.

In Europe, results concerning pathogenicity of PWN to *P. sylvestris* were only published about saplings, limited to climate chamber/greenhouse trials or outdoor boxes under quarantine conditions (such as Schauer-Blume, 1990; Bakke et al., 1991; Braasch, 1997; Braasch, 2000; Daub, 2008; Hopf-Biziks et al., 2016). In addition to the lack of results using mature trees under European climatic conditions, some scientists doubt that inoculating young plants with *Bursaphelenchus* spp. has any "scientific merit and provides no relevant information about pathogenicity" (McNamara, 2004) of PWN. Thus, the aim of our work was to investigate population dynamics and pathogenicity of PWN in seven- to eight-year-old *P. sylvestris* trees to figure out whether the results are comparable to those published on the basis of sapling trials. Finally, the results are aimed to support current pest risk assessment. In Germany, infestation trials with *B. xylophilus* are limited to greenhouse trials because of quarantine restrictions. Therefore, the tree height and age as well as the number of investigated trees were limited.

Materials and Methods

Materials

Seven- to eight-year-old *P. sylvestris* trees with a mean height of 2.50 m (stem base diameter: 5 cm) were used to study the population dynamics as well as the pathogenicity of *B. xylophilus*. In the spring of 2013, *P. sylvestris* were purchased in tubs (height: 47 cm, upper diameter: 58 cm) from a commercial nursery in the German provenance 851 12 (Upper Vogtland and north-east Bavarian upland). The pines were dug out from the nursery field in the autumn of 2012. Around the root ball was the clayey soil of the nursery field; a growing medium (Container Substrate 2 medium with GreenFibre + clay, Klasmann-Deilmann GmbH, Germany) designed for woody plants filled the inside of the tubs. The population dynamics investigation was conducted with ten trees (one per sampling date), which were all inoculated with PWN at the same time. For the pathogenicity investigation, two completely uninjured trees (double control), six mock-inoculated trees (control), six PWN-inoculated trees (PWN-inoculated) and six mock-inoculated trees with drought stress treatment (drought-stressed) were used.

All trees were placed in one greenhouse in a randomized block design and were acclimatized one month before the start of the experiments. The tests were run at 25 °C at an average relative humidity of 80% and natural lighting during the growing season of 2013. For the population dynamics investigation, the pines were watered as required. In contrast, for the pathogenicity investigation, strict control of the water supply was necessary to differentiate between disease symptoms and drought stress symptoms. For the estimation of the necessary amount of water and to check for drought stress, a scale (DE 150K2DL, KERN, Germany) was used for the gravimetric assessment of water in soil, and a Scholander bomb (SK-PM 1400, UP Umweltanalytische Produkte GmbH, Germany) was used to determine the water potential in the plants.

A *B. xylophilus* isolate (JKI-number: PT-7 (w)) freshly extracted from *Pinus pinaster* Ait. wood in Portugal was used for inoculation. The nematodes were reared and multiplied on grey mould rot fungus, *Botrytis cinerea* (Fr.) Pers., cultured on 1.5% malt extract agar medium. The non-sporulating fungal form was chosen to exclude the pathogenic effect of *B. cinerea* on pine trees.

For sampling of the trees for nematode extraction, a hand saw, branch shears (L98, PowerGear™, FISKARS, Finland), secateurs and a driller (DP4003, Makita Corporation, Japan) were used.

Watering regime in pathogenicity investigation

One month before the start of the test, all trees were watered until saturation of the soil and weighed to ensure the same starting condition. With a Scholander bomb, the water potential in the needles was measured (Scholander et al., 1965). According to the methods of Rust (2000), needles of the upper crown from the previous year's growth were chosen. One tree of each of the four variants was measured once per week at predawn before watering. Per test tree and measurement day, three needle pairs of the stem and three needle pairs of different one- to two-year-old main branches were selected. The common wooden end of the needle couple was carefully manually stripped off. The needle couple was placed in a high pressure head in the Scholander bomb. The watering was adjusted to maintain fixed water potential at the median of −0.5 MPa in the needles because drought stress studies of Jackson et al. (1995) in British and of Rust (2000) in German pine stands showed a predawn water potential of approximately −1 MPa and lower as an indication of drought stress.

At the start of the test, all pines of the "drought-stressed" variant were no longer watered. Four times per week, the weight of the plant + tub + soil + water was recorded. Daily until the end of the test (except Thursday because of Scholander bomb measurements being done on Fridays), all pines except the drought-stressed trees were watered to the fixed water potential using the same weight per cent of plant + tub + soil + water.

Inoculation

The population dynamics investigation began on 17th June 2013, and the pathogenicity investigation began on 26th June 2013. For each tree, 10,000 PWN of all developmental stages in 600 µl nematode suspension were used; for the mock-inoculated trees of the pathogenicity investigation 600 µl tap water was used. The nematode suspension/tap water was inoculated (for details see Hopf-Biziks et al., 2016) into the seven- to eight-year-old pines. For the population dynamics investigation, a 2–3 cm longitudinal slit was cut in the bark in the stem area of the previous year's growth below the youngest whorl (Fig. 1, segment 7). The same procedure was used for the pathogenicity investigation but with one of the two- to three-year-old main branches of the previous year below the

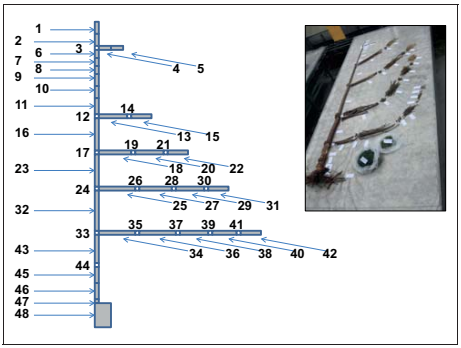

Fig. 1. Population dynamics investigation: Tree segmentation; 1–46: stem and branch segments (segment 7 with inoculation site); 47: root collar; 48: roots; whorls in white; aggregation of branch segments in the same tree height and age

youngest whorl (Fig. 1, segment 20) due to a connection with another investigation. However, pre-trials showed no difference between the inoculation sites. A cotton strip of 2 × 9 cm was inserted into the slit to absorb 600 µl nematode suspension/tap water.

Assessment of pine wilt

Wilt symptoms were evaluated according to the percentage of needle discolouration, which was expressed in six wilt classes (Table 1). For the population dynamics investigation, this evaluation was conducted according to the time scheme in Table 2. For the pathogenicity investigation, this was carried out every week for 12 weeks or until the week of tree death (wilt class 5).

Sampling of P. sylvestris trees

During the population dynamics investigation, the first six trees were cut for nematode extraction in intervals of 2–6 days (Table 2), while no symptoms occurred (wilt class 0). The next four pines were cut when symptoms corresponding to the next wilt classes (1, 2, 3 and 4) developed. To study the population density of PWN on each sampling date, one tree was sampled and divided into 46 wood segments plus the root collar and root (Fig. 1).

To examine the moisture content in parallel to the 46 wood segments, one pooled sample of needles from the test year and one from the growth of the previous year were taken. In addition, the longest stem segments of 16, 23 and 32 were cut into approximately 2 cm wide discs. The discs were alternately used for nematode extraction and cut into smaller pieces or solely used for fresh weight recording.

For the pathogenicity investigation, four stem segments were selected for a general proof of nematode infestation and moisture content analysis at tree death or after 12 weeks. One 2-cm disc of segments 16 and 23 (upper parts under the next whorls) and the complete segments of 32 and 43 were taken (Fig. 1).

Nematode extraction

All segments of the 46 wood samples, root collar and root from the population dynamics investigation and the four stem segments from the pathogenicity investigation were cut into 5–10 × 5–10 × 15–20 mm pieces as appropriate. For the population dynamics investigation, the soil substrate of the root and root collar was removed by careful washing. The root sample consisted of the main roots as well as bore-hole cuttings of the root stock using a 20 mm diameter boring bit.

For the population dynamics investigation, the fresh weight of the wood, root collar, root and needles and for the pathogenicity investigation, the fresh weight of the wood were recorded before nematode extraction. Thereafter, the living PWN were extracted from the wood, root collar and root using a modified Baermann funnel technique as described in Hopf-Biziks et al. (2016).

Following nematode extraction, all samples were oven dried (UL 50, Universal Oven, Memmert, Germany) at 103 ± 2°C for 48 h to determine the dry weight. The nematode density per gram of dry matter was calculated.

Table 1. Wilt class rating scheme for the assessment of pine wilt

Wilt class	Tree coverage by discoloured needles [%]	Physiological condition
0	0	Healthy
1	1–25	
2	26–50	
3	51–75	
4	76–99	
5	100	Dead

Table 2. Population dynamics investigation: Overview of tree sampling dates with respective time [days after inoculation] (DAI)

Sampling date	Time [DAI]
1	2
2	4
3	9
4	11
5	16
6	22
7	35
8	42
9	51
10	67

The calculation of the moisture content of wood and needles was conducted according to DIN 52183 (1977).

To enable the comparison of the results from each investigation, the relative water content was additionally calculated according to DIN ISO 11465 (1996).

The nematodes were preserved in a hot fixative solution for later counting (for details see Hopf-Biziks et al., 2016). For the population dynamics investigation, males, females and juveniles were differentiated.

Statistical analysis

Statistica 64 Version 12.7 (Stat Soft. Inc., Tulsa, USA) was applied for statistical analysis of the population dynamics and pathogenicity investigations of PWN in *P. sylvestris* trees.

In the population dynamics investigation, the Mann-Whitney U test ($p < 0.05$) was used to test the nematode number, nematode density and moisture content for significant differences between the first and last four trees. The aggregation of these trees enabled the comparison between the pines without symptoms (wilt class 0) and with wilt symptoms (classes 1–4) using the same number of trees as pseudo-replicates. The Friedman ANOVA test ($p < 0.05$) and Wilcoxon matched pairs test ($p < 0.05$) served to check for significant differences in nematode density and moisture content between different aggregated tree segments of both aggregated tree groups as well as between male, female and juvenile numbers of the aggregated tree segments. The aggregation of the above ground segments simplified the comparison of results within the tree and with the literature. The group "inoculation and adjacent segments" was defined as segments that were PWN-infested inside the first trees at least one time before intensive PWN distribution occurred.

For differences between the dates regarding the weight per cent of the drought-stressed pines in the pathogenicity investigation the Friedman ANOVA test was applied. The Fisher exact test (two-tailed; $p < 0.05$) was chosen to determine differences between pine wilt symptoms in PWN-inoculated, drought-stressed and control trees at particular wilt classes and times. The Kruskal-Wallis test ($p < 0.05$) was used to detect differences in the predawn water potential in needles of one tree each from the "PWN-inoculated", "drought-stressed", "control" and "double control" variants. Furthermore, the Kruskal-Wallis test also served to investigate differences in the moisture content between PWN-inoculated, drought-stressed and control trees.

The Pearson correlation was used to study the correlation between time and water potential as well as between water potential and wilt class for one PWN-inoculated and one drought-stressed tree. Moreover, using the Spearman correlation ($p < 0.05$ or 0.10), the correlation between water potential, nematode number, nematode density or moisture content and wilt class was tested for significance. Furthermore, the Pearson correlation was applied to study the correlation between the different parameters of the population dynamics investigation.

Results

Population dynamics investigation

In Fig. 2, the development of total number of PWN inside the pines over time is displayed. Only 16 days after inoculation (DAI) (at wilt class 0), the number of nematodes that were extracted exceeded the initial number of nematodes used for inoculation. In contrast, starting at wilt class 2, millions of nematodes could be extracted. During the test period, the maximum total number of nematodes was found at the end of the test. The nematode number significantly increased (Table S1a, Mann-Whitney U test: $p = 0.0286$) in the aggregated tree segments "inoculation and adjacent segments" (segment no. 1–3 and 6–13), "stem base" (segment no. 45–46) and "remaining stem and branch segments" (segment no. 4–5 and 14–44, except left discs of the longest stem parts 16, 23 and 32) (together "above ground segments"), root collar (segment no. 47) and roots (segment no. 48, except left root part) (together "below ground segments") between the aggregated trees 1–4 and 7–10.

Fig. S1 shows the correlation between median nematode number in the aggregated tree segments and whole tree wilt class. With increasing median nematode number in the above ground segments or "whole tree" (segment no. 1–48, except left discs of the three longest stem segments and left root part), the wilt symptoms increased. For all correlations of the population dynamics investigation, only the slope of the regression line in the phases of latency (tree no. 1–6, PWN infestation without external wilt symptoms) and early wilt was of interest. Therefore, the changed slope of the regression line starting usually at tree no. 9 or 10 during late wilt was excluded (Fig. S1–Fig. S9).

The frequency of males, females and juveniles in the whole tree per sampling date can be found in Fig. 2. Only 2 DAI, the frequency of males + females was 70%, clearly higher than that of juveniles. On almost all other sam-

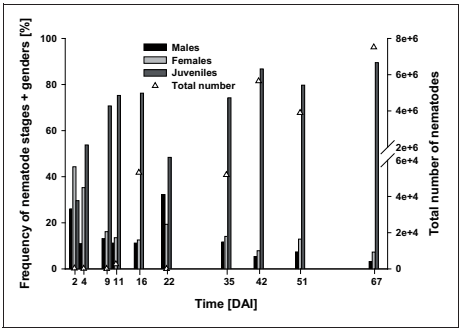

Fig. 2. Population dynamics investigation: Frequency of nematode stages (adults, juveniles) + genders [%] and total number of nematodes in the whole tree (segment no. 1–48) per sampling date [days after inoculation] (DAI); triangle symbols on x-axis: ≥ 62 nematodes (n = 1)

pling dates, the frequency of juveniles was much higher than that of adults. On average, the frequency of females compared to males was 1.6 ± 0.7 times higher. Within the aggregated trees 1–4 as well as 7–10 significant differences (Table S2, Friedman ANOVA test: $p < 0.05$) between the number of males, females and juveniles (with the highest numbers) were detected for the whole tree.

Fig. S2–Fig. S4 show for the aggregated tree segments the correlation between median nematode number and median male, female and juvenile number, respectively. With increasing median nematode number in the above or below ground segments, the median male, female and juvenile number increased. The highest median nematode, male, female and juvenile numbers were found in the stem base during the last three sampling dates (42, 51 and 67 DAI at wilt classes 2, 3 and 4, respectively) and in the root collar during the last sampling date.

In Fig. 3 and Table S3, the nematode density per gram of dry matter in the above ground segments is shown for each sampling date. Until 11 DAI and at 22 DAI (class 0), PWN were found only in the inoculation and adjacent segments. Furthermore, 16 and 35 DAI at class 0 and 1, respectively, the highest median nematode densities were extracted from the inoculation and adjacent segments. The stem base and remaining stem and branch segments were also PWN-infested. From 42 DAI (class 2), the highest median nematode densities were found in the stem base. In the inoculation and adjacent segments, only a small median nematode density remained at the end of the test. The highest median nematode density for the entire tree during the test occurred at 51 DAI (class 3), with 966 nematodes/g dry matter. The nematode density significantly increased (Table S1b, Mann-Whitney U test: $p = 0.0286$) in the above and below ground segments between the aggregated trees 1–4 and 7–10. Within the aggregated trees 1–4, the nematode density differed significantly (Table S4a, Friedman ANOVA test: $p = 0.0498$) between inoculation and adjacent segments and the other aggregated tree segments.

The highest median nematode densities were found in the stem base during the last three sampling dates and in the root collar during the last sampling date.

Fig. S5 shows for the above ground segments the correlation between median nematode density and median moisture content. In parallel to the increasing median nematode density in the above ground segments, the median moisture content decreased. In Fig. 4a and Table S5, the moisture content in the above ground segments and in Fig. 4b the moisture content in the needles are presented for each sampling date. At high moisture content (tree no. 1–3), no correlation of median moisture content with median nematode density (Fig. S5) and wilt symptoms (Fig. S6) could be observed. Therefore, these trees were excluded from further studies. Below approximately 50% median moisture content in the inoculation and adjacent segments (67 DAI), the median nematode density decreased to nearly zero nematodes/g dry matter.

The moisture content significantly decreased (Table S1c, Mann-Whitney U test: $p = 0.0286$) in the inoculation and adjacent segments (Fig. 4a, Table S5), needles younger than one-year-old and needles of the previous year (Fig. 4b) between the aggregated trees 1–4 and 7–10. Needles younger than one-year-old showed higher moisture content values than needles from the previous year, except at 67 DAI. The stem base, remaining stem and branch segments and needles from both years rapidly lost moisture content starting only between 35 DAI at wilt class 1 and 42 DAI at wilt class 2. However, the median moisture content in the stem base did not continue to fall. Therefore, at the end of the test, the highest median moisture content was found in the stem base with a three to four times higher value compared to the other aggregated tree segments. Within the aggregated trees 1–4, the moisture content differed significantly (Table S4b, Friedman ANOVA test: $p = 0.0183$) between inoculation and adjacent segments (with the highest values), the stem base and remaining stem and branch segments. Within the aggregated trees 7–10, the moisture content differed significantly (Table S4b, Friedman ANOVA test: $p = 0.0498$) between inoculation and adjacent segments, the stem base (with the highest values) and remaining stem and branch segments.

In Fig. S6 and Fig. S7, the correlation between median moisture content or median nematode density in the aggregated tree segments/needles and whole tree wilt class are shown, respectively. With decreasing median moisture content in the above ground segments or needles from the previous year, the wilt symptoms increased with a similar slope of the regression line (Fig. S6). Furthermore, with increasing median nematode density in the above ground segments or the entire tree, the wilt symptoms increased (Fig. S7). Above a median of 500 nematodes/g dry matter in the whole tree, the outbreak of pine wilt disease with wilt class 2 was observed.

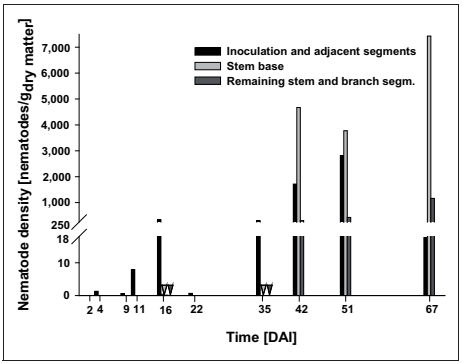

Fig. 3. Population dynamics investigation: Median nematode densities [nematodes/$g_{dry\ matter}$] in the aggregated tree segments inoculation and adjacent segments (segment no. 1–3 and 6–13), stem base (segment no. 45–46) and remaining stem and branch segments (segment no. 4–5 and 14–44) per sampling date [days after inoculation] (DAI); triangle symbols: < 0.5 nematodes/$g_{dry\ matter}$ with no visible bar (n = 1)

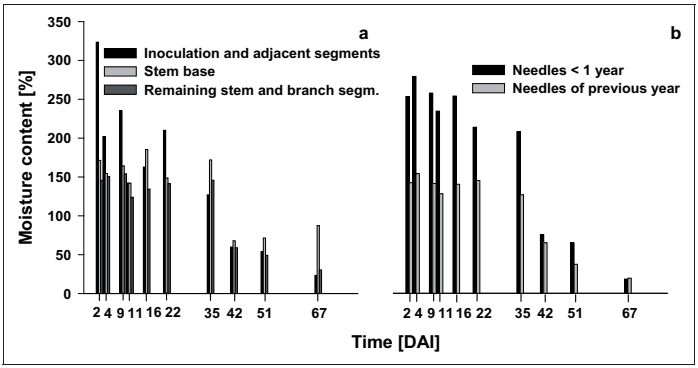

Fig. 4. Population dynamics investigation: Moisture content [%] of – a: the aggregated tree segments inoculation and adjacent segments (segment no. 1–3 and 6–13), stem base (segment no. 45–46) and remaining stem and branch segments (segment no. 4–5 and 14–44) as median values, b: needles younger than one-year-old and needles of the previous year per sampling date [days after inoculation] (DAI) (n = 1)

A detailed overview of the nematode density, wilt class and moisture content per tree segment of all trees as a pseudo-time series is illustrated in Fig. 5 and Fig. S8. After the start of the test, the inoculated PWN were mainly found with nematode densities ≤ 500 nematodes/g dry matter in the inoculation and adjacent segments. The trees showed no wilt symptoms and had, except for a few whorls, moisture content values of > 100% up to 200% or > 200% per segment. Starting at 16 DAI (class 0) and 35 DAI (class 1), a massive PWN distribution in the trees could be observed. PWN were found with ≤ 500 nematodes/g dry matter in the majority of these tree segments, including roots. On day 16, PWN were distributed in the whole stem, adjacent branch segments, root collar and roots before any external wilt symptoms appeared. At 35 DAI, wilt first occurred in the upper crown. The moisture content was not reduced below 100%. Starting at wilt class 2, most tree parts had > 500 nematodes/g dry matter and ≤ 100% moisture content. Wilt symptoms were distributed over the whole tree, except for the lower external branch segments. However, until the end of the test, all tree segments with needles showed wilt symptoms, and living PWN disappeared in the upper crown.

Pathogenicity investigation

At the start of the test, the watering of the drought-stressed pines was stopped. Weight loss over time is illustrated in Fig. 6. During the test period, the weight per cent of the plant + tub + soil + water of the drought-stressed pines significantly decreased (Friedman ANOVA: $p < 0.0001$).

The predawn water potential in the needles over time, used to monitor drought stress, is illustrated in Fig. 7 and Table S6 for all pine variants. Because of similar results between the water potential in the one-year-old needle pairs of the stems and branches, both needle types were pooled. The Scholander bomb allowed only quantitative measurements down to −4 MPa. Therefore, the decrease in water potential could not be recorded for all pine variants until the end of the test. In addition, the PWN-infested tree was harvested after its death at the 70th DAI. After a lag phase, the water potential in the PWN-inoculated and drought-stressed trees significantly decreased (Table S7, Kruskal-Wallis test: $p < 0.05$). In the PWN-inoculated tree, this decline started between 23 DAI at wilt class 0 and 30 DAI at wilt class 1. However, in the drought-stressed tree, this starting point occurred between 9 DAI and 16 DAI at wilt class 0. At 37 DAI, the water potential curves of the PWN-inoculated tree at wilt class 2 and the drought-stressed tree at wilt class 1 crossed each other and dropped to water potentials below −4 MPa at 44 DAI (at class 3) and 79 DAI (at class 3), respectively (Fig. 7, Table S8).

Fig. S9 and Fig. S10 show the correlation between time and water potential in the needles as well as water potential and whole tree wilt class, respectively. In contrast to the drought-stressed tree, the water potential decline in the PWN-inoculated tree was five times more rapid (Fig. S9). Moreover, with decreasing water potential, the wilt symptoms increased for both tree variants. However, for the PWN-inoculated tree, the steepness of this correlation curve was increased approximately 50% compared to that of the drought-stressed tree (Fig. S10). The predawn water potential of approximately −1 MPa, an indication of drought stress, was undershot at 30 DAI by the PWN- inoculated tree at wilt class 1 and at 23 DAI by the drought- stressed tree at wilt class 0 (Fig. 7).

In contrast, the water potentials in the control and double control trees remained above −1 MPa at wilt classes 0 and 1 throughout the test.

The number of trees per wilt class for all PWN-inoculated, drought-stressed and control pines over time is illustrated in Fig. 8. At the start of the test, all pines were healthy and without needle discolouration. Wilt symptoms appeared 14 DAI on PWN-inoculated and drought-stressed trees.

For the PWN-inoculated pines, starting from the initial symptoms (class 1), an increase in wilt was found for 35 days up until tree death. At 84 DAI, all PWN-inoculated trees had died, showing a significantly higher mortality (Fisher's exact test, two-tailed: $p = 0.0022$) than controls and drought-stressed trees, which showed no mortality at all. For that reason, seven- to eight-year-old *P. sylvestris*

Fig. 5. Population dynamics investigation: Nematode density [nematodes/$g_{dry\ matter}$], wilt class and moisture content [%] per tree segment of – a-e: tree no. 4, 7, 8, 9, 10 respectively; pseudo-time series of trees at given time after inoculation; whole tree wilt classes are provided (needle discolouration, turgescence); light grey, dark grey and black represent measured values, values of white segments were not recorded: segments without needles (including defoliated inoculation segment) and not recordable moisture content in roots and root collar (n = 1)

are considered highly susceptible to PWN. One-third of the drought-stressed trees developed severe wilt symptoms (class 4). The number of trees with no symptoms (class 0) and those with initial symptoms (class 1) showed significant differences (Fisher's exact test, two-tailed: $p < 0.05$) between the controls and the PWN-inoculated trees 49 DAI and between controls and drought-stressed trees 70 DAI.

In contrast, two-thirds of the control pines and one of both double control pines showed no needle discolouration until the end of the test. The rest of these trees belonged to wilt class 1.

All PWN-inoculated trees were infested, whereas no PWN were found in any other pine variant. The median nematode density in the tested stem segments was 2,846 nematodes/g dry matter.

At the end of the test, the controls with wilt classes 0 to 1 had a median moisture content in the tested stem segments of 207%, compared with 52% in the dead PWN-inoculated pines and 57% in the drought-stressed trees

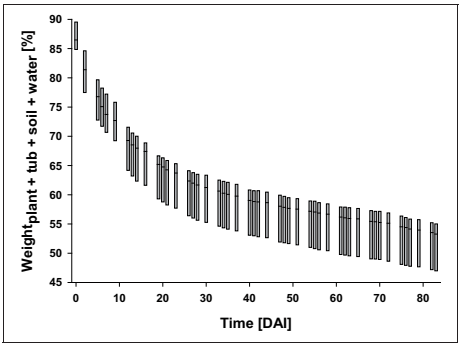

Fig. 6. Pathogenicity investigation: $Weight_{plant + tub + soil + water}$ [%] (medians, 25th and 75th percentiles (box)) over time [days after inoculation] (DAI): drought-stressed variant (n = 6)

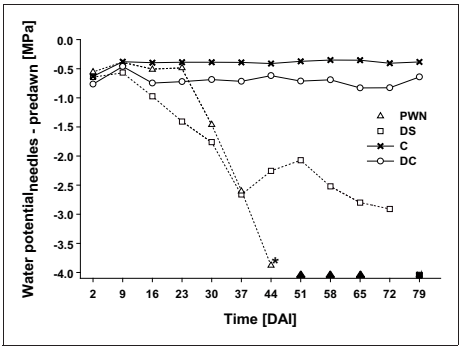

Fig. 7. Pathogenicity investigation: Median water $potentials_{needles - predawn}$ [MPa] of six needle pairs of one PWN-inoculated (PWN), drought-stressed (DS), control (C), and double control (DC) tree over time [days after inoculation] (DAI); filled symbols: without absolute values because < −4 MPa, *: partly without absolute values because < −4 MPa (n = 6)

with wilt classes 3 to 4. Compared to the control plants, the nematode infestation led to a significantly reduced moisture content (Kruskal-Wallis test: p = 0.004).

Discussion

Initial infestation of inoculated trees by PWN

Our population dynamics investigation revealed that until sampling date 4 (11 DAI, class 0) and on sampling date 6 (22 DAI, class 0), PWN were found only in the inoculation and adjacent segments, most often with nematode densities ≤ 500 nematodes/g dry matter. The trees showed moisture content values of > 100% or > 200% per segment. The population dynamics investigation of Daub (2008) is the only published scientific work about migration and reproduction of PWN in *P. sylvestris* saplings using a whole plant approach with detailed segmentation; therefore, in the following, we relate our results mainly to his findings. He inoculated his three- to four-year-old saplings with 4,000 PWN and tested them in a climate chamber at 25 °C. After different time intervals, his saplings were cut into 17 segments comparable to our large trees with 48 segments. In contrast to our results, at 2 DAI, all segments with the exception of the lower branch segments, root collar and root, contained PWN. This may have resulted from the smaller size of saplings. However, in Daub's investigation, the inoculation and adjacent segments were the centre of nematode infestation. He called this first stage of nematode invasion "early migration".

Both saplings and seven- to eight-year-old trees show a phase in which PWN is located in the entry point and adjacent tree parts before moving through the tree.

Distribution of PWN in inoculated trees and numbers of re-isolated PWN

Sixteen and 35 DAI (at wilt classes 0 and 1) marked times for which the number of nematodes re-extracted in our experiment exceeded the number used for inoculation. The same trend was shown by Melakeberhan and Webster (1990). Less than 50% of the inoculum was established in their seven-month-old *P. sylvestris* seedlings 2 DAI at 30 °C. With an inoculum of 2,500 PWN, significantly more nematodes than the inoculated amount were extracted at only > 11 DAI, which correlated with the start of chlorosis. This might result from the fact that some of the PWN died on the surface without entering the inoculation site. Another reason is that resin close to the inoculation cut becomes sticky due to evaporation and makes it difficult for PWN to migrate (Koo et al., 2013). Moreover, Bolla et al. (1989) found a changed resin acid composition with nematicidal activity in PWN-infested *P. sylvestris* saplings, and Bolla et al. (1984) reported that phytotoxins of PWN-infested *P. sylvestris* trees caused paralysis of PWN *in vitro*. In addition, Fukuda (1997) mentioned an unknown resistance mechanism to suppress the reproduction of PWN and keep them away from the cambium during the early pine wilt disease stage.

Starting with tree no. 5 (16 DAI, wilt class 0) and tree no. 7 (35 DAI, class 1), we observed intensive PWN distribution in our trees. In the majority of tree segments, including roots, PWN were found at ≤ 500 nematodes/g dry matter. The highest median nematode densities were found in the inoculation and adjacent segments. The moisture content was above 100%. Daub (2008) called this second stage of nematode invasion "distribution and colonization of all plant parts". At only 6 DAI PWN were present throughout the entire sapling, including the roots. As in our trees, but at only 9 DAI, the inoculation and adjacent segments contained more than 500 nematodes/g dry matter. No reduction in the relative water content of wood or needles was found. Moreover, similar to our results, in two-year-old *P. sylvestris* that Menéndez-

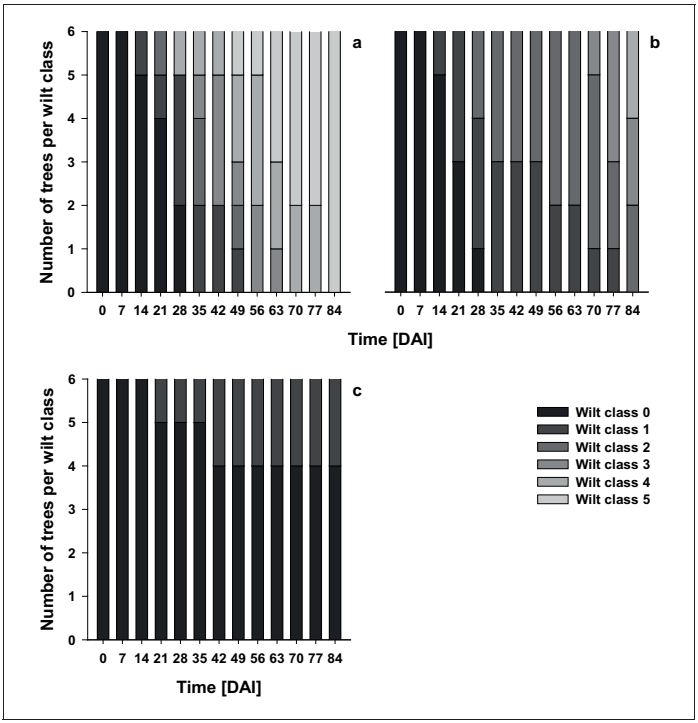

Fig. 8. Pathogenicity investigation: Number of trees per wilt class of all – a: PWN-inoculated, b: drought-stressed and c: control trees over time [days after inoculation] (DAI) (n = 6)

Gutiérrez et al. (2017) tested with 600 PWN at approximately 24°C, PWN reached the roots starting at 13 DAI. Melakeberhan and Webster (1990) reported 9 and 14 DAI until PWN were found in the roots of seven-month-old *P. sylvestris* at 30°C with inoculum densities of 10,000 or 20,000 and 2,500 PWN, respectively. This was not earlier than the appearance of wilt. Therefore, they suggested that PWN could migrate to the roots because they presented a less toxic environment and the availability of food or nutrients.

In the above and below ground segments of our big pines, the nematode density significantly increased between the aggregated trees 1–4 and 7–10. Starting at wilt class 2, millions of PWN were extracted. After the nematode population decline following inoculation, the populations in the small *P. sylvestris* seedlings of Melakeberhan and Webster (1990) increased exponentially as well. Inoculum densities of 10,000 and 20,000 PWN caused approximately equal nematode levels. Thereafter, pine wilt developed about the same time. Therefore, they assumed that the nematode reproduction rate is influenced by a population density-dependent factor.

PWN move through infested trees, and the population increases exponentially after a certain degree of wilting, which can be observed in saplings as well as in seven- to eight-year-old trees.

Densities of PWN per gram of dry matter

Starting at wilt class 2 (42 DAI) of our investigation, most tree parts contained > 500 nematodes/g dry matter and ≤ 100% wood moisture content. Wilt symptoms appeared throughout the entire tree, except for the lower external branch segments. With increasing median nematode density in the above ground segments or whole tree, the wilt symptoms increased. The outbreak of pine wilt disease with wilt class 2 appeared once a median of 500 nematodes/g dry matter was exceeded throughout the entire tree. Therefore, we propose this nematode density as a threshold for irreversible wilt under the given test parameters. Additionally, Daub (2008) suggested the presence of a threshold population density for the induction of irreversible wilt in *P. sylvestris* saplings due to unchanged mortality rates at different maximum population levels above 2,000 and 4,000 nematodes/g dry matter at 20°C and 25°C, respectively.

Daub (2008) called this third stage of nematode invasion "population build-up", which also occurred in saplings after invasion of all plant parts. This was found for his saplings at > 9 DAI, earlier than for our trees at > 35

DAI. Two-year-old *P. sylvestris* saplings studied by Vieira da Silva et al. (2015) were inoculated with 2,000 PWN; a dramatic population increase occurred at 29–35 DAI, which was also observed earlier than in our study. This earlier population increase may be correlated with the smaller tree sizes in comparison to our large trees; nevertheless, the sapling results show a similar trend as that of our large trees. As shown by Futai (1980), rapid dispersion accompanied by rapid propagation is a key factor in the pathogenicity of PWN in susceptible tree species. According to Daub (2008) and our results, both are interrelated with each other.

In Daub's (2008) study, all segments simultaneously showed a population increase. The highest nematode densities existed in the stem 12 DAI, when needle discolouration first appeared and in the branches at 19 DAI. In our case, the stem and adjacent branch segments were highly PWN-infested earlier than the rest of the branches. Daub (2008) also found the highest nematode density of a single segment at 19 DAI with > 7,000 nematodes/g dry matter in the root collar and the highest median nematode density of the entire sapling with approximately 1,800 nematodes/g dry matter. In our study, the highest nematode density of a single segment with approximately 17,500 nematodes/g dry matter was found in a segment adjacent to the inoculation site of tree no. 8 (42 DAI, class 2). For the entire tree, we observed the highest median nematode density during the test for tree no. 9 (51 DAI, class 3), with 966 nematodes/g dry matter.

In the population phase, the nematode density per gram of dry matter increases after PWN moved throughout all plant segments in saplings and seven- to eight-year-old trees.

Water supply of trees and moisture content

With increasing median nematode density in the above ground segments of our trees, the median moisture content decreased, which is in line with findings of Futai and Sutherland (1989) concerning two-year-old *P. sylvestris* saplings and those of Futai (1980) concerning three-year-old *Pinus thunbergii* Parl. seedlings. We observed that starting between 35 DAI at wilt class 1 and 42 DAI at wilt class 2, analogous to the remaining stem and branch segments, the moisture content of needles from both years rapidly decreased to similarly small moisture content values. Additionally, Daub (2008) mentioned that for sapling needles younger and older than one-year-old, the relative water content started to drop after first needle discolouration, which was 12 DAI. Needle wilting occurs in the advanced pine wilt disease stage, where PWN multiply and destroy the cambium. Together with enhanced ethylene production by the tree the outermost xylem is blocked. The water conduction is finally interrupted throughout the entire xylem. The rapid decline in transpiration, photosynthesis and water potential arise. After photosynthesis cessation, the nematode population bursts with dramatic development of symptoms (Fukuda, 1997).

By the end of our test (67 DAI, class 4), wilt symptoms were visible over all needled tree segments. In some tree segments of the upper crown, living PWN disappeared. We assume that PWN retreated into the lower plant segments because starting from sampling date 8 (42 DAI, class 2), the highest median nematode densities (maximum at the end of the test: over 7,000 nematodes/g dry matter) were on the stem base. Among the below ground segments, the root collar often showed the higher and, at the last sampling date, the highest nematode density (over 8,200 nematodes/g dry matter). Similar observations were made in Daub's (2008) detailed population dynamics investigation on saplings where, until tree death, the centre of the population changed from the stem and branches to the plant base, defined as the root, root collar and stem base. He called this stage of nematode invasion "retreat into the root system" and it appeared by 27 DAI. At this time, the stem base had the highest nematode density, with approximately 5,000 nematodes/g dry matter, followed by the root collar, similar to our results. The upper crown was nematode-free. We share his assumption that the population peaks in specific areas (in our above ground segments: on sampling date 9) occur primarily due to nematode reproduction but are related to tree death in the lower tree parts (in our below ground segments: on sampling date 10) where they occur primarily due to migration.

We observed that below approximately 50% median moisture content (= 33% relative water content) in the inoculation and adjacent segments (67 DAI), the median nematode density decreased to nearly zero nematodes/g dry matter. Additionally, Futai (1980) found that a relative water content of 20–40% caused a decline in population density in his three-year-old *P. thunbergii* seedlings. Moreover, we found that the remaining stem and branch segments and stem base rapidly lost moisture content starting only between 35 DAI at wilt class 1 and 42 DAI at wilt class 2. However, at the end of the test, the highest median moisture content was found in the stem base with a value three- to four-times higher than that in the other aggregated tree segments. Daub (2008) observed the same phenomenon for three- to four-year-old *P. sylvestris* saplings. Only from 12 DAI on, after first needle discolouration, did the relative water content of 60–70% in the remaining stem and branch segments start to decrease continuously to 25% until 27 DAI; the same trend was observed for the stem bases in which only a slight and therefore significantly lower decrease to above 50% occurred, similar to our values. We agree with Daub (2008) that the nematode shift results from the significantly lower moisture content in the upper plant parts compared to the plant base.

Our moisture content values significantly decreased in the needles younger than one-year-old and those of the previous year and in the inoculation and adjacent segments between the aggregated trees 1–4 and 7–10. Daub (2008) found that, until tree death, the needles younger than one-year-old dropped from approximately 65% to 15% in relative water content, and the needles older than one-year-old dropped from 55% to 15% in relative water content, similar to our values.

Since sampling date 3 (9 DAI) of our investigation, the frequency of juveniles was in most cases significantly higher than that of adults. This is in line with results of Malek and Appleby (1984) who reported that, during wood drying, dispersal juveniles dominated and could be recovered from the trunks of standing pines up to three years after tree death.

During pine wilt disease development in saplings and seven- to eight-year-old trees, wood moisture content decreases with increasing nematode density and leads to the movement of PWN to areas that are the last to dry out, which includes the lower part of the trunk.

Water potential pathogenicity investigation

In our pathogenicity investigation, the predawn water potential in the PWN-inoculated and drought-stressed trees significantly decreased to below −4 MPa at wilt class 3 at 44 and 79 DAI, respectively. In the case of PWN infestation, it is known that with the dysfunction of xylem water conduction, the water potential drops (Fukuda, 1997) and leads to dehydration of the tree. The occlusion of water conduction has different causes. PWN and PWN-produced cellulase were found to cause oleoresin leakage into tracheids (Odani et al., 1985). Infested trees react with the growing numbers of vacuoles in ray parenchyma cells. The vacuoles burst, and their contents, such as tanninic materials, accumulate in tracheids. Necrobiosis of ray parenchyma cells occurs (Nobuchi et al., 1984). Moreover, hydrophobic volatile terpenes of pines are produced due to PWN infestation and lead to tracheid cavitation and blockage (Kuroda, 1991).

Seven-year-old *P. thunbergii* inoculated with 50,000 PWN under field conditions lost water potential drastically starting between 20 and 23 DAI, slightly earlier than our time span (between 23 and 30 DAI). The predawn values around −0.6 MPa (similar to the controls) decreased to approximately −2.3 MPa until tree death, which started 30 DAI (Ikeda and Suzaki, 1984). Another study (Ikeda et al., 1990) under field conditions showed that for three-year-old PWN-inoculated *P. thunbergii* seedlings at only 30 DAI the water potential of the inoculated trees significantly decreased under the value of the control trees, similar to our case. The predawn water potential was < −2.0 MPa 37 DAI. Menéndez-Gutiérrez et al. (2017), in two-year-old *P. sylvestris* inoculated with 600 PWN under similar test conditions in relation to our study, showed significant losses of predawn water potentials in one-year-old needles, even if less drastic (-1.9 MPa 42 DAI) compared to our results (-3.9 MPa 44 DAI). Significant differences to the controls occurred in a similar time (28 DAI and 30 DAI, respectively). Referring to these studies, saplings and seven- to eight-year-old trees started to rapidly lose water potential over a similar time span. Only the time of death differed between both tree groups, which is discussed later.

During our investigation, in contrast to the drought-stressed tree, the water potential decline and achievement of drought stress level in the PWN-inoculated tree started later, only with the beginning of wilt, and the decline was five times steeper. Additionally, Ikeda et al. (1990) reported that the chlorosis in *P. thunbergii* needles from the previous year's growth corresponded to an abrupt decrease in water potential. In the study of Menéndez-Gutiérrez et al. (2017), the water potential decreased more than one week before any external symptoms appeared. However, physiological changes in needles, such as a decrease in water potential, were reported to occur only in the advanced pine wilt disease stage, when needle yellowing could be observed (Fukuda, 1997), which supports our results. Moreover, Utsuzawa et al. (2005) observed that xylem cavitations, after gradual enlargement in the early pine wilt disease stage, rapidly increased and fused with the cambium during the advanced pine wilt disease stage. Therefore, the drastic expansion of cavitations explained the sudden wilting of PWN-inoculated pine saplings as well as mature trees.

Wilt symptoms

The first wilt symptoms appeared 14 DAI on one of our PWN-inoculated and drought-stressed trees. Our result was comparable to a study at fluctuating temperatures sometimes above 35 °C with three-year-old *P. sylvestris* inoculated with 10,000 PWN (Bakke et al., 1991) and a study with two-year-old *P. sylvestris* inoculated with 2,000 PWN (Vieira da Silva et al., 2015). Melakeberhan and Webster (1990) found for their seven-month-old *P. sylvestris* inoculated with 2,500 PWN at 30 °C that wilt symptoms first appeared 11–12 DAI. However, several PWN-inoculated *P. sylvestris* sapling studies (Schauer-Blume, 1990; Riga et al., 1991; Melakeberhan et al., 1992; Braasch, 2000; Daub, 2008; Hopf-Biziks et al., 2016; Menéndez-Gutiérrez et al., 2017) showed shorter or longer periods (shortest: 7 DAI, longest: 28 DAI) until the first wilt symptoms appeared compared to our big trees, even in the case of a much shorter time until tree death. Moreover, Daub (2008) demonstrated that different inoculation levels (2,400–10,000 PWN at 25 °C) led to a more or less simultaneous development of wilt symptoms starting 27 DAI.

We observed significant differences between controls and PWN-inoculated trees regarding the number of trees with no (class 0) and initial symptoms (class 1) at 49 DAI and between controls and drought-stressed trees at 70 DAI. The sapling study of Hopf-Biziks et al. (2016) revealed that in addition to other factors such as temperature (Daub, 2008), pine provenance had a significant influence on the time course of disease development. The proportion of trees with wilt class 0 + 1 started to differ significantly between control and PWN-inoculated saplings at 14–28 DAI.

We found for the PWN-inoculated pines starting from the initial symptoms (class 1), an increase in wilt until tree death within 35 days. Similar results, 33 and 29 days, were found for three-year-old *P. sylvestris* after inoculation with 2,500 PWN at 28 °C (Riga et al., 1991). Schauer-Blume (1990) reported that three- to four-year-old *P. sylvestris* inoculated with 12,500 PWN at 23 °C died 28 days and those studied by Daub (2008) 25 days after the initial symptoms using 2,400 PWN at 25 °C. A high time

span of 21 or only 14 days was shown by Hopf-Biziks et al. (2016) for two- to three-year-old *P. sylvestris* of different provenances inoculated with 4,000 PWN at 25°C. Seven-month-old *P. sylvestris* inoculated with 2,500 PWN at 30°C were already dead 2–3 days after the first chlorosis (Melakeberhan and Webster, 1990).

At tree death, our nematode-infested trees had a significantly lower moisture content in the tested stem segments (median: 52%, = 34% relative water content) compared to the control trees with wilt classes 0 to 1 (median: 207%, = 67% relative water content). The same was observed for *P. sylvestris* saplings tested by Daub (2008) and Hopf-Biziks et al. (2016) at 25°C with a significant reduction from 73% to 39% relative water content compared to the control saplings and from 217% to 47% moisture content, respectively, when inoculated with 10,000 or 4,000 PWN.

Wilt symptoms develop continuously in both saplings and mature trees until tree death but are delayed for mature trees.

Mortality rates on P. sylvestris

We showed the high susceptibility of seven- to eight-year-old *P. sylvestris* towards PWN, as mentioned by Braasch (2000) and Hopf-Biziks et al. (2016) for *P. sylvestris* saplings but also by Evans et al. (1996) for mature *P. sylvestris* in the field in America. Outdoor investigations in North America with *P. sylvestris* with the highest mortality rates were published for 20- (Linit and Tamura, 1987) and 13-year-old trees (Bedker and Blanchette, 1988) as well as two- to four-year-old saplings (Myers, 1986) with values of 60%, 65% and 80%, respectively. In Europe under outdoor conditions, the highest mortality rate, 90%, was reported for three-year-old saplings in quarantine boxes in Germany (Braasch, 1997). The high susceptibility of this tree species was, for example, explained by high levels of lipid-soluble substances needed for the nutrition of PWN, low levels of certain chemical defence compounds (Menéndez-Gutiérrez et al., 2017) and the high ability of PWN to reproduce (Futai and Sutherland, 1989).

Our PWN-inoculated trees showed a significantly higher mortality of 100% compared to the controls and drought-stressed trees without dead trees at 84 DAI. In comparison to our study on big trees, sapling investigations (Dropkin et al., 1981; Malek and Appleby, 1984; Bedker et al., 1987; Schauer-Blume, 1990; Riga et al., 1991; Melakeberhan et al., 1992; Braasch, 1997; Braasch, 2000; Daub, 2008; Hopf-Biziks et al., 2016; Menéndez-Gutiérrez et al., 2017) were published for seven-month to three- to four-year-old *P. sylvestris* often at similar greenhouse/climate chamber temperatures and usually with lower inoculum levels. We found that the mortality rates between small saplings, which seldom experience less than 75% mortality, and seven- to eight-year-old *P. sylvestris* tress are comparable. Several of these investigations reported in the literature showed mortality rates of 100%, as shown in our investigation. However, the time until maximum mortality rates occurred was 16–65 DAI for saplings, which was nearly three to ten weeks shorter than observed in our study. For example, Daub (2008) tested three- to four-year-old saplings at 25°C in a climate chamber after inoculation with 2,400 PWN and observed 100% mortality 46 DAI. With 4,000 PWN inoculated into two- to three-year-old *P. sylvestris* provenances in a study of Hopf-Biziks et al. (2016) a mortality rate of 100% occurred at 35–42 DAI at 25°C in a greenhouse.

Therefore, the mortality rates of saplings and seven- to eight-year-old trees are comparable but with a delay in the timing of mortality.

Conclusions

Our greenhouse studies using seven- to eight-year-old *P. sylvestris* trees showed similar results in nematode population development, moisture content development, symptom development and tree death rates, as reported for *P. sylvestris* saplings and other pine species. The results were approximately consistent with those published on the basis of sapling trials and delayed only in time. Therefore, inoculating saplings with *B. xylophilus* has scientific merit and provides relevant information about population dynamics and pathogenicity of PWN. *P. sylvestris* saplings can be used as good indicator trees for *B. xylophilus* population dynamics and pathogenicity investigations if mature trees cannot be tested.

The classification of *P. sylvestris* as a susceptible host in the pest risk analysis was confirmed. Symptoms of pine wilt disease typical to those occurring in nature and clear stages of population dynamics could be shown with inoculations under controlled test conditions on immature as well as older *P. sylvestris* trees. In this respect, our investigations closed the information gap concerning population dynamics and pathogenicity investigations using *P. sylvestris* of European origin under European climatic conditions. Moreover, due to detailed tree segmentation and parallel recording of nematode density, nematode stages and genders, moisture content, wilt symptoms and water potential over time, we contribute to a better understanding of the progression of pine wilt disease.

Acknowledgements

This article is an output of the EU-funded research project REPHRAME “Development of improved methods for detection, control and eradication of pinewood nematode in support of EU Plant Health Policy” [KBBE.2010.1.4–09]. The work was conducted at the Julius Kühn-Institut in Braunschweig. The authors express their gratitude to Silvia Urban and Doreen Mybes for their excellent technical assistance. Many thanks to Prof. Dr. Steffen Rust (HAWK University of Goettingen, Faculty of Resource Management) for providing a Scholander bomb. We thank Prof. Dr. Stefan Schütz (University of Goettingen, Forest Zoology and Forest Conservation, Faculty of Forest Sciences) for critical reading and useful comments to improve the presentation of our results. Moreover, we are grateful

for the statistical advice of Dr. Irina Kuzyakova (University of Goettingen, Ecoinformatics, Biometrics and Forest Growth, Faculty of Forest Sciences). Both anonymous reviewers and the journal editors deserve many thanks for improving the manuscript.

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Supplementary Information

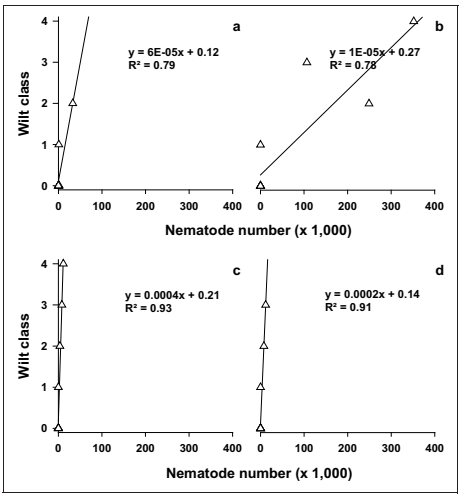

Fig. S1. Population dynamics investigation: Pearson correlation between median nematode number and whole tree wilt class of – a: inoculation and adjacent segments (segment no. 1–3 and 6–13) of tree no. 1–8, b: stem base (segment no. 45–46) of tree no. 1–10, c: remaining stem and branch segments (segment no. 4–5 and 14–44) of tree no. 1–10 and d: whole tree (segment no. 1–48) of tree no. 1–9; Spearman correlation for b-d: $p < 0.05$ and a: $p < 0.10$ (n = 8/9/10)

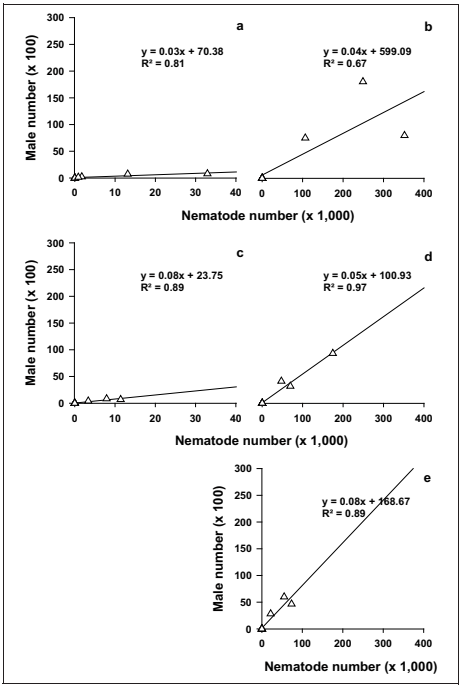

Fig. S2. Population dynamics investigation: Pearson correlation between median nematode number and median male number of – a: inoculation and adjacent segments (segment no. 1–3 and 6–13) of tree no. 1–9, b: stem base (segment no. 45–46) of tree no. 1–10, c: remaining stem and branch segments (segment no. 4–5 and 14–44) of tree no. 1–10, d: root collar (segment no. 47) of tree no. 1–10 and e: roots (segment no. 48) of tree no. 1–9; please mind the different x-axis scale (n = 9/10)

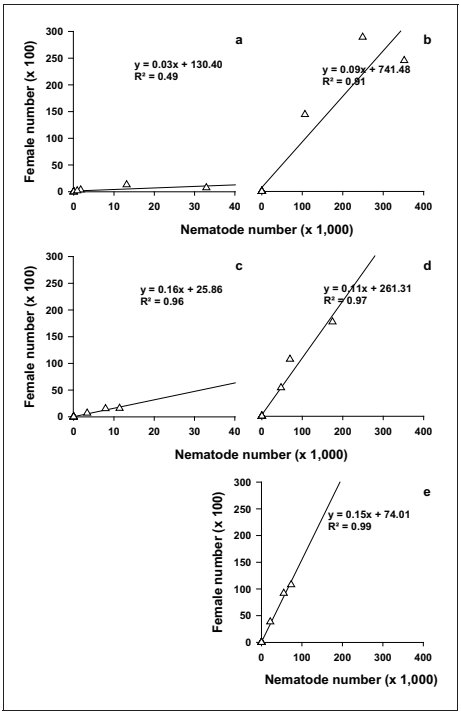

Fig. S3. Population dynamics investigation: Pearson correlation between median nematode number and median female number of – a: inoculation and adjacent segments (segment no. 1–3 and 6–13) of tree no. 1–9, b: stem base (segment no. 45–46) of tree no. 1–10, c: remaining stem and branch segments (segment no. 4–5 and 14–44) of tree no. 1–10, d: root collar (segment no. 47) of tree no. 1–10 and e: roots (segment no. 48) of tree no. 1–9; please mind the different x-axis scale (n = 9/10)

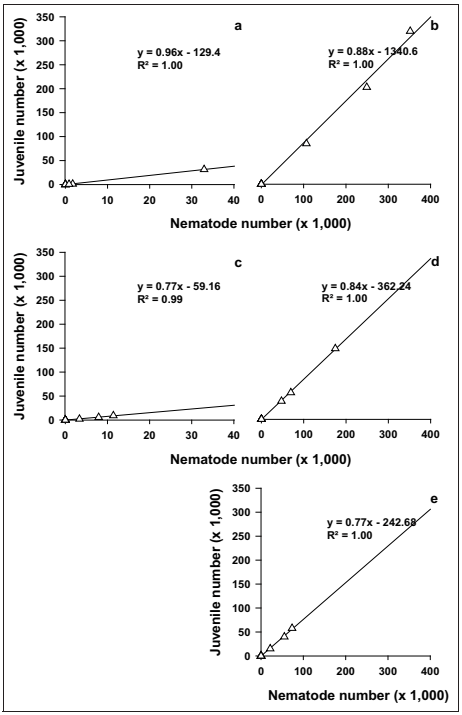

Fig. S4. Population dynamics investigation: Pearson correlation between median nematode number and median juvenile number of – a: inoculation and adjacent segments (segment no. 1–3 and 6–13) of tree no. 1–8, b: stem base (segment no. 45–46) of tree no. 1–10, c: remaining stem and branch segments (segment no. 4–5 and 14–44) of tree no. 1–10, d: root collar (segment no. 47) of tree no. 1–10 and e: roots (segment no. 48) of tree no. 1–9; please mind the different x-axis scale (n = 8/9/10)

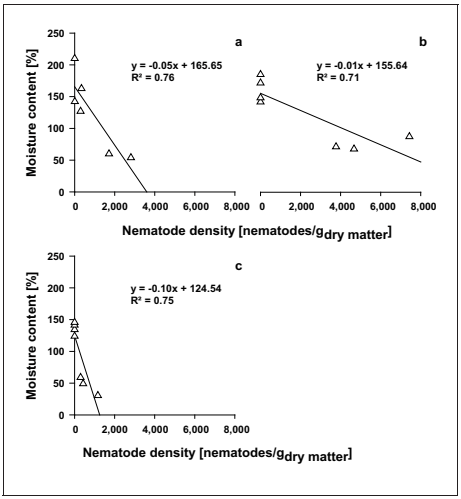

Fig. S5. Population dynamics investigation: Pearson correlation between median nematode density [nematodes/$g_{dry\ matter}$] and median moisture content [%] of – a: inoculation and adjacent segments (segment no. 1–3 and 6–13) of tree no. 4–9, b: stem base (segment no. 45–46) of tree no. 4–10 and c: remaining stem and branch segments (segment no. 4–5 and 14–44) of tree no. 4–10 (n = 6/7)

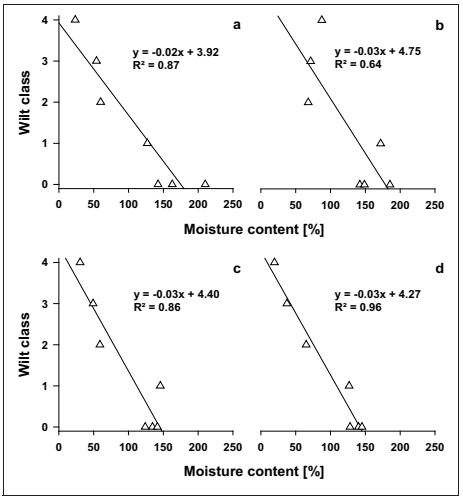

Fig. S6. Population dynamics investigation: Pearson correlation between median moisture content [%] and whole tree wilt class of – a: inoculation and adjacent segments (segment no. 1–3 and 6–13) of tree no. 4–10, b: stem base (segment no. 45–46) of tree no. 4–10, c: remaining stem and branch segments (segment no. 4–5 and 14–44) of tree no. 4–10 and d: needles of the previous year of tree no. 4–10; Spearman correlation for a and d: $p < 0.05$ and c: $p < 0.10$ (n = 7)

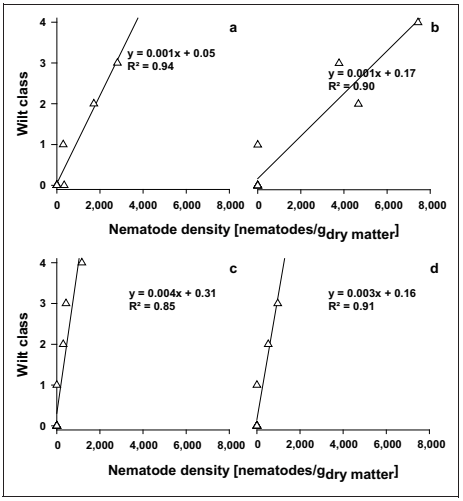

Fig. S7. Population dynamics investigation: Pearson correlation between median nematode density [nematodes/$g_{dry\ matter}$] and whole tree wilt class of – a: inoculation and adjacent segments (segment no. 1–3 and 6–13) of tree no. 1–9, b: stem base (segment no. 45–46) of tree no. 1–10, c: remaining stem and branch segments (segment no. 4–5 and 14–44) of tree no. 1–10 and d: whole tree (segment no. 1–48) of tree no. 1–9; Spearman correlation for a-d: $p < 0.05$ (n = 9/10)

Fig. S8. Population dynamics investigation: Nematode density [nematodes/$g_{dry\ matter}$], wilt class and moisture content [%] per tree segment of – a–e: tree no. 1, 2, 3, 5, 6 respectively; pseudo-time series of trees at given time after inoculation; whole tree wilt classes are provided; light grey, dark grey and black represent measured values, values of white segments were not recorded: segments without needles (including defoliated inoculation segment) and not recordable moisture content in roots and root collar (n = 1)

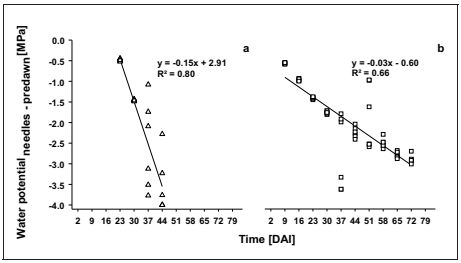

Fig. S9. Pathogenicity investigation: Pearson correlation between time [days after inoculation] (DAI) and water potential$_{needles\ -\ predawn}$ [MPa] of six needle pairs of – a: one PWN-inoculated (PWN) and b: one drought-stressed (DS) tree during time spans of significant decline in water potentials$_{needles\ -\ predawn}$ with quantitative results; filled symbols without absolute values because water potential dropped below –4 MPa (n = 6)

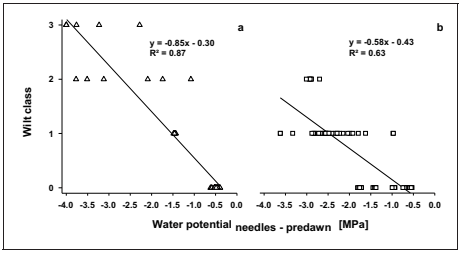

Fig. S10. Pathogenicity investigation: Pearson correlation between water potential$_{needles\ -\ predawn}$ [MPa] and whole tree wilt class of six needle pairs of – a: one PWN-inoculated (PWN) and b: one drought-stressed (DS) tree during time spans from start of the test until end of quantitative results; filled symbols without absolute values because water potential dropped below –4 MPa; Spearman correlation for both tree variants: $p < 0.05$ (n = 6)

Table S1. Population dynamics investigation: Significant differences (Mann-Whitney U test: $p < 0.05$) between the aggregated trees 1–4 and 7–10.

Aggregated tree segments	p-value
a	
Inoculation and adjacent segments	0.0286
Stem base	0.0286
Remaining stem and branch segments	0.0286
Root collar	0.0286
Roots	0.0286
b	
Inoculation and adjacent segments	0.0286
Stem base	0.0286
Remaining stem and branch segments	0.0286
Root collar	0.0286
Roots	0.0286
c	
Needles younger than one year-old	0.0286
Needles of the previous year	0.0286
Inoculation and adjacent segments	0.0286

Subtables:
a: nematode number
b: nematode density
c: moisture content
a-c tested for: the aggregated tree segments: inoculation and adjacent segments (segment no. 1–3 and 6–13), stem base (segment no. 45–46) and remaining stem and branch segments (segment no. 4–5 and 14–44) and
a-b tested for: root collar (segment no. 47) and roots (segment no. 48) and
c tested for: needles younger than one-year-old and needles of the previous year.

Table S2. Population dynamics investigation: Significant differences (Friedman ANOVA test: $p < 0.05$) in nematode number within the aggregated trees 1–4 and 7–10 between males, females and juveniles. Tested for the whole tree (segment no. 1–48), aggregated tree segments: inoculation and adjacent segments (segment no. 1–3 and 6–13), stem base (segment no. 45–46) and remaining stem and branch segments (segment no. 4–5 and 14–44), root collar (segment no. 47) and roots (segment no. 48). (Post-hoc test Wilcoxon matched pairs test ($p < 0.05$): no significant differences in all cases)

Aggregated tree segments	p-value	
	Aggregated trees 1–4	Aggregated trees 7–10
Whole tree	0.0388	0.0183
Inoculation and adjacent segments	no significant differences	0.0498
Root collar	no significant differences	0.0183
Roots	no significant differences	0.0183

Table S3. Population dynamics investigation: Nematode density medians, minima and maxima [nematodes/$g_{dry\ matter}$] in the aggregated tree segments inoculation and adjacent segments (segment no. 1–3 and 6–13), stem base (segment no. 45–46) and remaining stem and branch segments (segment no. 4–5 and 14–44) per sampling date [days after inoculation] (DAI) (n = 1)

	Nematode density [nematodes/$g_{dry\ matter}$]					
	Inoculation and adjacent segments		Stem base		Remaining stem and branch segments	
Time [DAI]	Median	Min-Max	Median	Min-Max	Median	Min-Max
2	–	0–165	–	–	–	–
4	1	0–54	–	–	–	–
9	1	0–14	–	–	–	–
11	8	0–933	–	–	–	–
16	340	1–11,035	0.4	0.2–0.6	0.1	0–276
22	1	0–15	–	–	–	–
35	298	4–1,824	0.05	0–0.1	0.02	0–296
42	1,715	0–17,528	4,671	2,348–6,993	299	0–9,002
51	2,811	2–5,916	3,773	3,503–4,043	429	4–4,093
67	18	0–7,243	7,431	6,859–8,003	1,162	0–12,670

Table S4. Population dynamics investigation: Significant differences (Friedman ANOVA test: $p < 0.05$) within the aggregated trees 1–4 and 7–10.

Significant differences between the aggregated tree segments	p-value	
	Aggregated trees 1–4	Aggregated trees 7–10
a		
Inoculation and adjacent segments – Stem base – Remaining stem and branch segments	0.0498	no significant differences
b		
Inoculation and adjacent segments – Stem base – Remaining stem and branch segments	0.0183	0.0498

Subtables:
a: nematode density
b: moisture content
a and b tested for: between the aggregated tree segments inoculation and adjacent segments (segment no. 1–3 and 6–13), stem base (segment no. 45–46) and remaining stem and branch segments (segment no. 4–5 and 14–44) and
b tested for: between needles younger than one-year-old and needles of the previous year.
(Post-hoc test Wilcoxon matched pairs test ($p < 0.05$): no significant differences in all cases)

Table S5. Population dynamics investigation: Moisture content medians, minima and maxima [%] of the aggregated tree segments inoculation and adjacent segments (segment no. 1–3 and 6–13), stem base (segment no. 45–46) and remaining stem and branch segments (segment no. 4–5 and 14–44) per sampling date [days after inoculation] (DAI) (n = 1)

	Moisture content [%]					
	Inoculation and adjacent segments		Stem base		Remaining stem and branch segments	
Time [DAI]	Median	Min-Max	Median	Min-Max	Median	Min-Max
2	324	144–387	171	171–172	146	75–346
4	202	156–292	155	154–155	151	80–291
9	235	166–396	164	164–165	154	92–383
11	142	117–169	142	140–144	124	58–245
16	163	131–281	185	181–190	134	75–394
22	210	109–275	149	140–157	142	81–250
35	127	104–164	172	169–175	146	73–253
42	60	15–68	68	56–81	59	30–165
51	54	25–85	71	62–81	49	14–89
67	23	21–44	88	82–93	30	18–61

Table S6. Pathogenicity investigation: Water potential$_{needles\ -\ predawn}$ medians, minima and maxima [MPa] of six needle pairs of one PWN-inoculated (PWN), drought-stressed (DS), control (C), and double control (DC) tree over time [days after inoculation] (DAI) (n = 6)

	Water potential$_{needles\ -\ predawn}$ [MPa]							
	PWN		DS		C		DC	
Time [DAI]	Median	Min-Max	Median	Min-Max	Median	Min-Max	Median	Min-Max
2	−0.56	−0.62–(−0,47)	−0.64	−0.75–(−0.53)	−0.63	−0.80–(−0.56)	−0.77	−0.86–(−0.73)
9	−0.42	−0.45–(−0.40)	−0.57	−0.59–(−0.55)	−0.38	−0.43–(−0.33)	−0.46	−0.50–(−0.44)
16	−0.51	−0.53–(−0.47)	−0.97	−1.00–(−0.93)	−0.40	−0.41–(−0.37)	−0.75	−0.77–(−0.65)
23	−0.49	−0.52–(−0.45)	−1.41	−1.45–(−1.38)	−0.39	−0.41–(−0.35)	−0.72	−0.76–(−0,71)
30	−1.46	−1.49–(−1.43)	−1.76	−1.81–(−1.72)	−0.39	−0.43–(−0.38)	−0.69	−0.71–(−0.66)
37	−2.61	−3.77–(−1.08)	−2.66	−3.62–(−1.79)	−0.39	−0.41–(−0.37)	−0.72	−0.85–(−0.66)
44	<−3.88	<−4.00–(−2.28)	−2.26	−2.41–(−2.04)	−0.41	−0.44–(−0.39)	−0.62	−0.66–(−0.59)
51	<−4.00	<−4.00	−2.07	−2.59–(−0.97)	−0.37	−0.43–(−0.32)	−0.71	−0.74–(−0.68)
58	<−4.00	<−4.00	−2.52	−2.64–(−2.29)	−0.35	−0.36–(−0.33)	−0.69	−0.72–(−0.68)
65	<−4.00	<−4.00	−2.80	−2.88–(−2.68)	−0.35	−0.38–(−0.34)	−0.83	−0.87–(−0.81)
72	cut tree	cut tree	−2.91	−3.01–(−2.70)	−0.41	−0.41–(−0.39)	−0.83	−0.85–(−0.79)
79	cut tree	cut tree	<−4.00	<−4.00	−0.38	−0.40–(−0.37)	−0.64	−0.68–(−0.61)

Table S7. Pathogenicity investigation: Significant differences (Kruskal-Wallis test: $p < 0.05$) in water potential$_{needles - predawn}$ of six needle pairs of one PWN-inoculated (PWN), drought-stressed (DS), control (C), and double control (DC) tree between different study times [days after inoculation] (DAI); –4 MPa tested in cases of water potentials < –4 MPa

Variant	Significant differences between study times [DAI]	p-value
PWN	9–44	0.0319
	9–51	0.0010
	9–58	0.0010
	9–65	0.0010
	23–51	0.0144
	23–58	0.0144
	23–65	0.0144
DS	2–37	0.0412
	2–65	0.0070
	2–72	0.0015
	2–79	< 0.0001
	9–37	0.0116
	9–58	0.0354
	9–65	0.0017
	9–72	0.0003
	9–79	< 0.0001
	16–72	0.0233
	16–79	0.0008
	23–79	0.0093
C	2–9	0.0382
	2–51	0.0170
	2–58	< 0.0001
	2–65	0.0003
	44–58	0.0101
	44–65	0.0445
DC	2–9	0.0016
	2–44	0.0280
	9–16	0.0382
	9–65	< 0.0001
	9–72	< 0.0001
	44–65	0.0006
	44–72	0.0011
	65–79	0.0020
	72–79	0.0034

Table S8. Pathogenicity investigation: Significant differences (Kruskal-Wallis test: $p < 0.05$) in water potential$_{needles - predawn}$ between six needle pairs of one PWN-inoculated (PWN), drought-stressed (DS), control (C), and double control (DC) tree at different times [days after inoculation] (DAI); –4 MPa tested in cases of water potentials < –4 MPa

Time [DAI]	Significant differences between variants	p-value
2	PWN-DC	0.0017
9	PWN-DS	0.0256
	DS-C	0.0001
16	PWN-DS	0.0197
	DS-C	0.0001
	C-DC	0.0197
23	PWN-DS	0.0197
	DS-C	0.0001
	C-DC	0.0197
30	PWN-C	0.0197
	DS-C	0.0001
	DS-DC	0.0197
37	PWN-C	0.0020
	DS-C	0.0010
44	PWN-C	0.0001
	PWN-DC	0.0273
	DS-C	0.0141
51	PWN-C	0.0001
	PWN-DC	0.0197
	DS-C	0.0197
58	PWN-C	0.0001
	PWN-DC	0.0197
	DS-C	0.0197
65	PWN-C	0.0001
	PWN-DC	0.0197
	DS-C	0.0197
72	DS-C	0.0197
79	DS-C	0.0014

4 Chapter 4: Publication III: The pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, and its pathogenicity to German *Pinus sylvestris* provenances

This chapter was published as "The pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, and its pathogenicity to German *Pinus sylvestris* provenances" (HOPF-BIZIKS, A., T. SCHRÖDER, S. SCHÜTZ; J. Plant Dis. Prot. (2016), **123**, 43-49, DOI: 10.1007/s41348-016-0005-4), and reused in this dissertation with the permission of the copyright owner Springer Nature. The final publication is available at https://link.springer.com/article/10.1007/s41348-016-0005-4.

The pine wood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, and its pathogenicity to German *Pinus sylvestris* provenances

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Abstract

The pine wood nematode, *Bursaphelenchus xylophilus*, can cause pine wilt disease on susceptible conifer species, mainly *Pinus* spp., outside its natural range. One of the management options for pine wilt disease in recent decades in affected countries such as Japan was the evaluation of potentially tolerant or resistant host species. In the framework of Pest Risk Assessment and Management as well in Contingency Planning in Germany, we studied the pathogenicity of *B. xylophilus* towards different German pine provenances. According to the German Legal Ordinance on Regions of Provenance, two- to three-year-old *Pinus sylvestris* saplings of eight provenances were artificially inoculated with *B. xylophilus* using a suspension of 4000 *B. xylophilus* in 300 µl of tap water per tree. No significant differences in tree death between the provenances were detected. All inoculated provenances reached a mortality of 100 %, but significant differences occurred in the time course of disease development.

Key words: nematode density, pine wilt classes, wilt coefficient, tolerance

1 Introduction

The pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, is the causal agent for pine wilt disease (PWD) if the climatic conditions are suitable (Kiyohara & Tokushige 1971, Rutherford et al. 1990, Evans et al. 1996).

In North America, the native range of PWN, indigenous conifers like *Pinus taeda* L. (Futai & Furuno 1979), *P. strobus* L. and *P. palustris* Mill. (Suga et al. 1993) are highly resistant or immune to PWD (Dwinell & Nickle 1989). Outside this native area PWD appears on conifers, mainly pines. Susceptible pine species include *P. thunbergii* Parl., *P. densiflora* Sieb. & Zucc., *P. luchuensis* Mayr, *P. parviflora* Sieb. & Zucc. and *P. koraiensis* Sieb. & Zucc. in Asia (Kishi 1995) and *P. pinaster* Ait. in Portugal (Mota et al. 1999). In addition, several species have been tested concerning this potential threat, such as the European species *Pinus sylvestris* L., *P.*

cembra L., *P. mugo* Turra and *Larix decidua* Mill. (Daub 2008), and found to be susceptible or highly susceptible in greenhouse trials.

The first outbreak of PWD, which was caused by PWN introduced to Japan, was reported at the beginning of the 20th century (Mamiya 1988). The nematode caused great harm and spread to other countries, including Portugal in 1999, the first European country affected (Mota et al. 1999). Currently, all of continental Portugal and Madeira have been declared to be infested by PWN. Four single outbreaks in Spain are under eradication (EPPO 2014). Because of the observed threat in Japan and China, *B. xylophilus* is listed as a quarantine pest in the EU Plant Health Council Directive 2000/29/EC (EU 2000), and its introduction and spread is prohibited. Based on the EU-Commission Implementing Decision 2012/535/EU (EU 2012), member states are obliged to carry out a yearly survey on the potential occurrence of PWN as a preventive measure.

Although no PWN infestation has yet been detected in Germany, several factors would allow at least an establishment of PWN in the country upon its introduction. The vector beetles of the genus *Monochamus* (Dwinell & Nickle 1989) are present in wide areas of Germany (Bense 1995, Schröder et al. 2009). *P. sylvestris* covers 23 % of the forest area in Germany, which consists of approximately 56 % coniferous species in total (BMEL 2013). *P. sylvestris* plays an important ecological and economic role and would be the most PWD-affected species in Germany. Currently climatic conditions are believed to be not suitable for PWD in Germany (Evans et al. 1996, Evans 2014 unpublished), but global climate change increases the risk for a PWD outbreak, which requires mean daily summer temperatures in July/August of more than 20 °C (Rutherford et al. 1990). If PWN should appear despite restrictions on the movement in trade of susceptible plants, wood and bark from PWN-affected countries (EU 2012), it would pose a high risk for Germany. The EU-Commission Implementing Decision 2012/535/EU (EU 2012) describes in detail measures to eradicate PWN in EU member states. Despite short-term phytosanitary measures such as the felling of infested trees, a long-term phytosanitary strategy to fight against the pest is necessary as currently demonstrated in Portugal. Growing resistant or tolerant host plants therefore might be an important part of this strategy.

Thus, the aim of this study was, using pathogenicity tests, to determine whether *P. sylvestris* provenances used in Germany for forest regeneration might show any differences in susceptibility to PWN. Based on Web of Science database research, this is the first investigation in Europe concerning differences in disease expression caused by PWN using different *P. sylvestris* provenances.

2 Materials and methods

2.1 Materials

We used *P. sylvestris* to search for possible differences in the tolerance of various host tree provenances towards *B. xylophilus*. In the spring of 2013, eight two- to three-year-old German *P. sylvestris* provenances (Fig. 1) with a root collar diameter of 1±0.2 cm and a regular branching with two whorls and approximately six new side branches were purchased from commercial nurseries. The provenances studied represent a variety to give a first overview of different German regions. The bare-root delivered pines were planted immediately in 1-l pots in growing medium designed for woody plants comprising 30 % black peat, 30 % peat fibre, 20 % wood fibre, 10 % sod-based white peat and 10 % white peat (container substrate 1 medium, Klasmann-Deilmann GmbH, Geeste, Germany). For each provenance, 20 saplings for artificial PWN inoculation and 20 control pines were used.

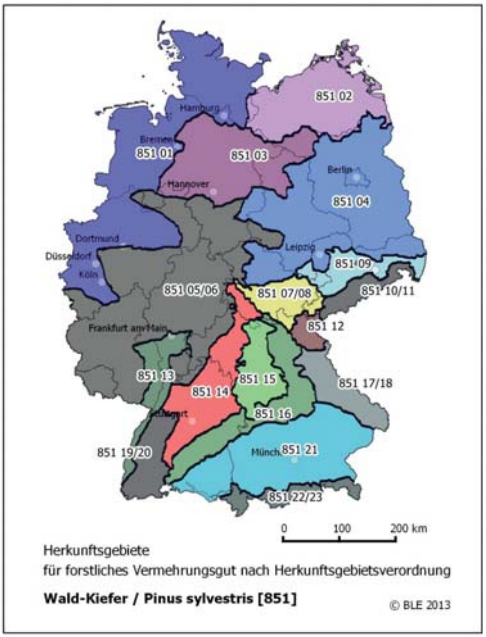

Fig. 1: Overview of German *Pinus sylvestris* provenances with their geographic distributions according to the Deutsche Herkunftsgebietsverordnung (HkG) (Forstvermehrungsgut) - German Legal Ordinance on Regions of Provenance (Forest reproductive material); Source: German Federal Office for Agriculture and Food (2013); Tested provenances:

851 02 Mecklenburg

851 03 Heide and Altmark
851 08 Vogtland, Thuringian Forest and Franconian Forest, montane level
851 13 Upper Rhine rift
851 14 Neckarland and Franconian plate
851 15 Middle Franconian hill land
851 20 Black Forest, montane level
851 22 Alps, submontane level

All saplings were placed in the greenhouse using a randomized block design and were pre-air-conditioned for two weeks before the start of the test and watered as required. The test was run at 25 °C at an average relative humidity of 80 % and natural lighting.
For the inoculation, we used the *B. xylophilus* isolate PT-7 (w) originally extracted from *Pinus pinaster* wood in Portugal. To exclude spores, a non-sporulating form of the grey mould rot fungus, *Botrytis cinerea* (Fr.) Pers. (anamorph), cultured on 1.5 % malt extract agar medium was used as a feeding source for *B. xylophilus*.

2.2 Inoculation

Bursaphelenchus xylophilus was reared and multiplied on non-sporulating, nonpathogenic (Daub 2008) *B. cinerea* in Petri dishes at approximately 20 °C.
The nematodes were extracted from the agar plates 24 h before inoculation using the modified Baermann funnel technique (Baermann 1917) modified for plant parasitic nematodes (described in Decker 1969) and collected in a beaker. The nematode concentration was determined by calculating the mean of three counted 25-µl aliquots. The living nematodes were counted using a binocular (WILD M3B Leica, Heerbrugg, Germany, magnification max. x400), a grid Petri dish and a counter (Handzähler Tally Counters, Hand-Held, neoLab®, Germany). Tap water was added until the target density of nematodes of the suspension was reached. For each tree, we used 4000 PWN of mixed stages in 300-µl nematode suspension. The control saplings were inoculated with the same volume of tap water.
The inoculations were conducted on 11 and 12 June 2013. The needles were removed from the selected shoot part. Using a scalpel, a 1-2-cm longitudinal slit was cut in the bark in the main shoot of the previous year below the youngest whorl. The inner cortex was separated from the bark without damaging the cambial layer. A cotton strip (1 x 9 cm) was with the shorter side inserted into the slit using a scalpel and then folded. A plastic strip surrounding the stem to cover the cotton was sealed at the lower end with adhesive tape. The nematode suspension/tap water was pipetted onto the cotton strip, which precisely absorbed the amount of 300 µl (Fig. 2). The top end of the plastic strip was sealed with adhesive tape.

Fig. 2: Inoculation of *Bursaphelenchus xylophilus* suspension/tap water in the bark slit of a pine shoot of the previous year below the youngest whorl

2.3 Assessment of pine wilt

A rating scheme of wilt classes (Table 1) was applied to visually evaluate pine wilt symptoms every week over 12 weeks. The six wilt classes (0 to 5) represent the percentage of needle discolouration, which is related to the physiological condition of the plant. Sampling of the trees took place in the week of tree death, which is represented by wilt class 5, or at the test end at week 12, whichever occurred first. A wilt coefficient was introduced to show the distribution of the trees between these six wilt classes over time. The wilt coefficient was calculated using equation 1.

Equation 1

$$Wilt\ coefficient = \sum_{i=0}^{n=5} \frac{WC_i}{20} * n_{pines\ with\ WCi}$$

WC_i = Wilt class 0-5

20 = Number of pines per variant

$n_{pines\ with\ WCi}$ = Number of pines with wilt class i of the investigated variant

Table 1: Rating scheme of wilt classes for assessment of pine wilt

Wilt class	Tree coverage of needle discolouration [%]	Physiological condition
0	0	Healthy
1	1-25	
2	26-50	
3	51-75	
4	76-99	
5	100	Dead

2.4 Sampling of plants and nematode extraction

For nematode extraction, the trees were cut above the root collar, and all needles were removed. Using secateurs, all remaining tree parts were cut into pieces 5-10 mm long, and the fresh weight was recorded. To extract living PWN, the Baermann funnel technique described above was applied. After 48 h, 10 ml water from each sample were released into a snap-on lid glass. The necessary extraction time for PWN from wood samples is longer than that of agar plates to enable all living nematodes to leave this complex material.

After nematode extraction, the wood was oven dried (UL 50, Universal Oven, Memmert, Germany) at 103±2 °C for 48 h to determine the dry weight for calculation of the nematode density per gram of dry matter. The wood moisture content (MC) was calculated according to DIN 52183 (1977) using equation 2.

Equation 2

$$MC = \frac{m_f - m_d}{m_d} * 100\ [\%]$$

MC = Wood moisture content [%]

m_f = Weight of fresh wood [g]

m_d = Weight of dry wood [g]

In the literature, both MC and relative water content information is used. For better comparison with results from other investigations in the discussion part, we calculated additionally the relative water content according to DIN ISO 11465 (1996) using equation 3.

Equation 3

$$H_2O = \frac{m_f - m_d}{m_f} * 100\ [\%]$$

H_2O = Relative water content [%]

m_f = Weight of fresh wood [g]

m_d = Weight of dry wood [g]

To preserve the extracted nematodes for later counting, they were killed at 80 °C in hot fixative solution (formal-acetic: 890 ml Aqua dest., 100 ml formaldehyde solution (35 %), 10 ml glacial acetic acid, modified after Dropkin 1989).

2.5 Statistical analysis

Statistica 64 Version 10 (Stat Soft. Inc., Tulsa, USA) was used for statistical analysis of the PWN inoculation effects for the different *P. sylvestris* provenances. The chi square test ($p<0.05$) was used to check for differences between the control and nematode-inoculated pines and between the pine provenances particularly for wilt classes and times. The Kruskal-Wallis test ($p<0.05$) was chosen to investigate differences in the MC between the control and nematode-inoculated trees and between the pine provenances. For differences between the provenances regarding the nematode density of the PWN-inoculated pines, one-way ANOVA and the Tukey test ($p<0.05$) were applied after log transformation log(x+1) and visual observation of normal distribution and variance homogeneity by standardized residuals against the pine provenances. In the figure, non-transformed medians are presented, and significant differences are expressed by different letters.

3 Results

The wilt coefficient of all PWN- and water-inoculated *P. sylvestris* of each of the eight tested German provenances over time is shown in Fig. 3a-b. All trees were completely healthy at the start of the test and showed no needle discolouration. Wilt symptoms on the PWN-inoculated trees had already appeared after one week. Starting from the initial symptoms (class 1+2), a rapid increase in wilt up to tree death was found within 2-3 weeks. All PWN-inoculated saplings had a mortality of 100 % and died within 5 or 6 weeks after the start of the test. Concerning the number of dead trees, no significant differences between the provenances were found (chi square test: $p>0.05$). Comparing the number of dead trees, significant differences (chi square test: $p<0.05$) between control and PWN-inoculated saplings were already found for nearly all provenances by week four. Therefore, all provenances are rated as highly susceptible to PWN. Nevertheless, the test was run until the end of the planned 12 weeks to see how the control trees developed.

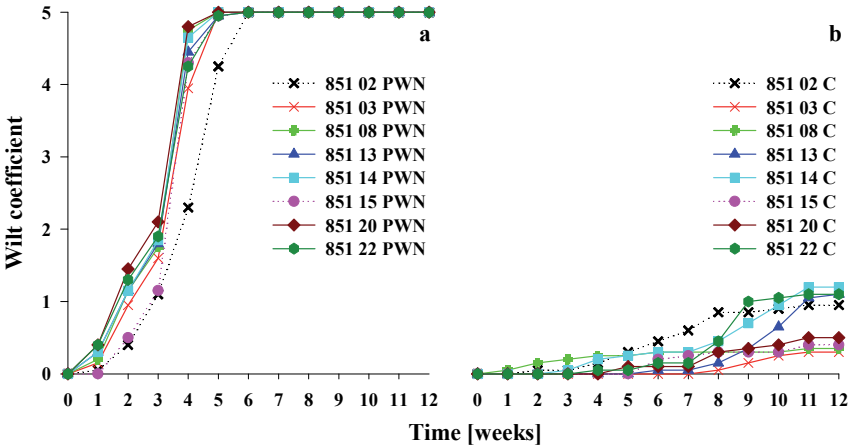

Fig. 3: Wilt coefficient of a: PWN-inoculated and b: control (C) trees for all tested pine provenances over time [weeks], n=20

The speed of wilt symptom development and death of PWN-inoculated trees differed significantly between the provenances. The number of saplings with no (class 0) plus initial symptoms (class 1) was compared using the chi square test. Significant differences between control and PWN-inoculated trees concerning the proportion of trees with wilt class 0+1 already began to show for provenances 851 14, 851 20 and 851 22 in week two, but these effects appeared later in week four for provenance 851 02. A comparison of inoculated trees showed that provenance 851 02 had significantly ($p<0.05$) more trees with wilt class 0+1 compared to nearly all other variants after two, three and four weeks. For provenance 851 15, the same result was achieved in weeks two and three. Provenances 851 08, 851 14, 851 20 and 851 22 had significantly more trees with higher wilt classes compared to the other provenances during these three weeks. Provenance 851 02 showed a significantly delayed mortality of one week compared to all other provenances.

In contrast, 60-90 % of the control trees from each provenance developed no needle discolouration until the end of the test. When wilt symptoms did occur in 10-40 % of the trees, they belonged mainly to wilt class 1. All other wilt classes were found in very low numbers. Though a few control trees showed wilt symptoms and died in exceptional cases, Fig. 3b shows that the other control trees remained stable until the end of the test. A comparison within the control trees showed that provenance 851 02 had significantly ($p<0.05$) more trees with wilt classes higher than 0+1 in week 8 compared to provenances 851 03 and 851 13.

No PWN were extracted from the control pines. Nematode densities extracted from the upper plant part in each provenance can be found in Fig. 4. Using one-way ANOVA and the Tukey test, significant differences were recorded in nematode densities between the provenances 851 03, with the highest median, and 851 08 with p=0.025, as well as between 851 03 and 851 14 with p=0.005. Despite these results, no correlation was observed between the number of PWN and dead pines because all pines died independently of the PWN load. In addition, we found no correlation between moisture content and nematode density following harvest.

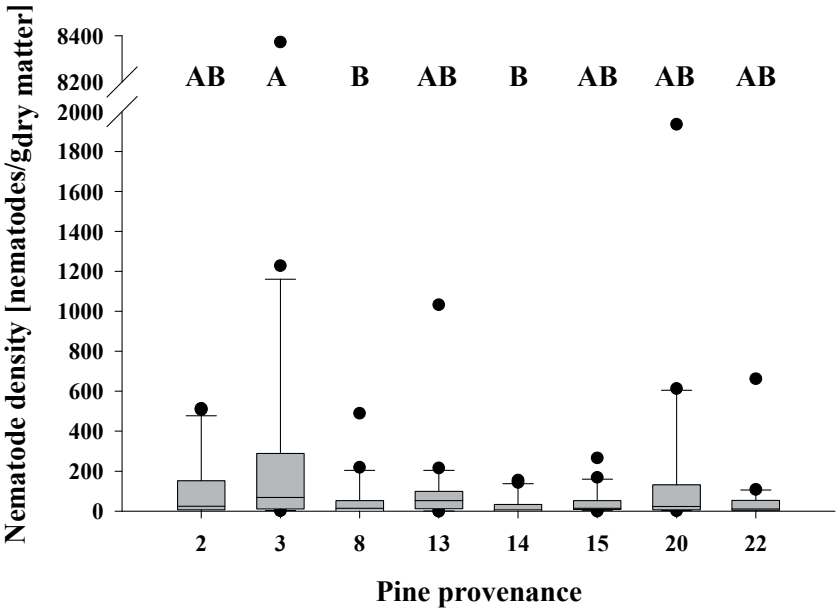

Fig. 4: Nematode density [nematodes/$g_{dry\ matter}$] in the upper plant part with medians, 25th and 75th percentiles (box), 10th and 90th percentiles (whisker), and outliers of PWN-inoculated *Pinus sylvestris* of all tested provenances; different letters indicate significant differences (One-way ANOVA and Tukey test: $p<0.05$), n=20

At the end of the test, the controls with wilt classes 0 to 1 had a median moisture content (MC) in the upper plant part of 217 %, compared with 47 % in the dead nematode-inoculated pines. The nematode infestation led to a significantly reduced MC (Kruskal-Wallis test: $p<0.001$), compared with that of the control plants. No significant difference in MC was found between the provenances ($p>0.05$).

4 Discussion

Our pathogenicity test for PWN on different German pine provenances confirmed the high susceptibility of *P. sylvestris* towards PWN, as described in Evans et al. (1996), Braasch (1997, 2000) and Daub (2008).

Different provenances were tested because they can differ genetically and physiologically from each other. These differences or adaptations in regional situations are the reason why specific provenances are only allowed to be grown in specific regions. PWN is known not to occur in Germany, as confirmed by annual surveys. Therefore, seed collection from PWN-tolerant or resistant *P. sylvestris* in nature is impossible. Because of quarantine regulations, infestation trials with PWN in Germany are limited to greenhouse trials under quarantine conditions. For our greenhouse test, we used nursery saplings of eight German *P. sylvestris* provenances from North, South, East and West Germany, as well as from different altitudes, to give an overview of different German regions.

All of the provenances were highly susceptible to PWN. Our two- to three-year-old plants showed a mortality of 100 % after five to six weeks. These findings are supported by Daub (2008), who showed a mortality of 100 % for three- to four-year-old *P. sylvestris* during a time span of eight weeks in his PWN mortality study testing different nematode densities. Moreover, these results are in line with the findings of Panesar & Sutherland (1989). The comparison of four Finnish *Pinus contorta* provenances showed no significant differences in mortality in the case of PWN infestation. The one-year-old saplings showed a mortality rate between only 25 and 60 % over a time span of one to ten weeks.

However, the time course of disease development showed significant differences between the provenances. Provenances 851 02 and 851 15 developed high wilt classes significantly later. Moreover, provenance 851 02 showed a significant delay in tree death of one week. Due to a slightly lower susceptibility of provenance 851 02 in northeast of Germany (Fig. 1) and a consequently higher susceptibility of the tested southern provenances, a slight trend to higher strength in the direction northeast can be preliminary recognized. This finding could be a first hint of differences in defence traits of pines from different regions, although a daily assessment of wilt symptoms in future studies seems to be necessary to follow a more detailed development of PWD.

Our experiment showed that PWN could be extracted from all nematode-inoculated pine variants at tree death, independent of the provenance. Significant differences in nematode densities in the upper plant part appeared among three provenances. Unfortunately, nematode density in the upper plant part could not be correlated with tree survival because the mortality was 100 %

throughout. However, two provenances, 851 08 and 851 14, which showed early symptom development, displayed significantly lower nematode densities compared to 851 03 after harvest. These nematode densities do not seem to be a good indicator of disease development. As shown by the tests of Daub (2008), a wide range in nematode densities (medians between <1000 and >20,000) did not influence the development of PWD four weeks after inoculation. Experiments with Finnish *Pinus contorta* sapling provenances showed a high range of nematode densities from approximately 400 to over 21,000 nematodes/g fresh matter after tree death (Panesar & Sutherland 1989). Significant differences in nematode densities can appear within and between trees of even the same provenance and with the same inoculum size, as described by Futai (1980) and Daub (2008). It is also known that infested trees at the final stage of PWN invasion contain PWN mainly in the root system. This pattern was connected with the significantly higher relative water content in the root collar (above 50 %) compared to the upper plant part (approximately 25 %) (Daub 2008). Futai (1980) also observed that PWN numbers begin to decrease at a relative water content in the wood of 20-40 %. At tree death, our nematode-infested trees had a significantly lower moisture content in the upper plant parts (median: 47 %, = 32 % relative water content) compared to the control trees with wilt classes 0 to 1 (MC median: 217 %, = 69 % relative water content). These results confirm the findings of other authors such as Daub (2008), who reported relative water contents above 70 % for the control pines and 39 % for the PWN-infested pines. The moisture content medians of our PWN-inoculated trees from different provenances displayed no correlation with either the occurrence of symptoms or the nematode densities extracted after tree death. Thus, it still remains unclear what mechanism caused delayed symptom development and delayed tree death. A closer examination of this phenomenon, for instance by measuring nematode densities during early and intermediate symptom development, might lead to defence traits related to tolerance or resistance to PWN infestation. Due to the immediacy and importance of this topic, research in this field has to proceed. Tolerant or resistant provenances or individuals could be used for cross-breeding purposes as a precaution to protect Central European pine forests against PWD.

Acknowledgements

This work was conducted at the Julius Kühn-Institut (JKI) in Brunswick in the framework of the EU-funded research project REPHRAME "Development of improved methods for detection, control and eradication of pine wood nematode in support of EU Plant Health Policy" [KBBE.2010.1.4-09]. Many thanks are owed to Doreen Mybes and Silvia Urban for their excellent laboratory help. We are also grateful to Dr. Irina Kuzyakova (Department Ecoinformatics, Biometrics and Forest Growth, Büsgen-Institute, Georg-August-University

Göttingen) and Dr. Doreen Gabriel (Institute for Crop and Soil Science, JKI Brunswick) for advice with the statistical analysis. We thank Dr. Edmundo de Sousa (I.N.I.A, Portugal) for providing the nematode isolate. Apl. Prof. Dr. Johannes Hallmann (Institute for Epidemiology and Pathogen Diagnostics, JKI Münster) and an anonymous reviewer deserve many thanks for improving the manuscript.

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5 Chapter 5: General discussion

5.1 General discussion

Aiming to gain knowledge of the PWN in terms of its potential non-vector spread to non-infested trees (HOPF-BIZIKS et al., 2017: publication I in chapter 2), the significance of sapling-based analyses for mature trees (HOPF-BIZIKS and SCHRÖDER, 2019: publication II in chapter 3) and indicators of tolerance in the species *P. sylvestris* (HOPF-BIZIKS et al., 2016: publication III in chapter 4), this PhD thesis supports European Union Plant Health policy by refining pest risk analysis and management as well as contingency planning.

The general discussion connects the individual discussions in publications I-III (chapters 2-4) and refers to the overall objective of this thesis. For all investigations in publications I-III presented in this thesis, *B. xylophilus* was tested in wood, saplings or mature trees of the species *P. sylvestris*. The assessment of pine wilt, sampling of plants (except detailed tree segmentation) and nematode extraction were consistent among all three publications. Additionally, in the case of PWN inoculation into trees in publications II and III, the inoculation methods (except the inoculation site in one study) were identical. These methods were already approved by and followed the standard procedures of the Julius Kühn-Institut in Braunschweig.

The meaning of the mentioned wilt classes is explained in Table 1, which shows the wilt class rating scheme used in all three publications for the assessment of pine wilt. The wilt classes are related to the percent tree coverage by discoloured needles.

Table 1. Wilt class rating scheme for the assessment of pine wilt

Wilt class	Tree coverage by discoloured needles [%]	Physiological condition
0	0	Healthy
1	1-25	
2	26-50	
3	51-75	
4	76-99	
5	100	Dead

5.1.1 Long-term survival of *Bursaphelenchus xylophilus* in wood chips

The test of long-term PWN survival in *P. sylvestris* wood chips in greenhouse compartments and climate chambers (publication I in chapter 2) revealed no significant difference between 15 °C and 25 °C. The moisture contents of the openly stored wood chips significantly decreased. Nematode densities were significantly reduced over time for the openly stored and sealed

variants. However, the most rapid and largest declines were observed in the openly stored variants. With a maximum between 23.5 weeks and > 1 year, the PWN survival times were significantly different between wood chips openly stored at 25 °C and those sealed at 15 °C or 25 °C, respectively. Results of the comparable studies SOUSA et al. (2011a) with *P. pinaster* boards and PANESAR et al. (1994) with wood chips of other conifer species were consistent with the results for *P. sylvestris* wood chips in publication I. Similar to observations in publication II, SOUSA et al. (2011a) explained the steep nematode decline by a decrease in moisture content to the fibre saturation point. Afterwards, the nematode densities in publication I halted at approximately 10 % of the original density, after which they decreased more slowly. The continuing availability of some food resources might have caused the extended presence of the PWN in the openly stored wood chips (SOUSA et al., 2011b).

PWNs might feed on fungi in the wood of dead trees (EVANS et al. 1993), whereby the population density may be determined by the dominant fungal species (MAEHARA and FUTAI, 1996). After long-term storage in publication I, the most frequently isolated fungus was *Trichoderma atroviride*, although no PWN survival was possible on this species, as observed for root-knot nematodes (DAVIES and SPIEGEL, 2011). The opposite was found by HALIK and BERGDAHL (1990) for the dominant fungal species in the genus *Trichoderma* isolated from PWN-inoculated wood chips. However, the nematicidal activity of *Trichoderma* spp. against PWN is known to differ (such as PANESAR et al., 1994; MAEHARA and FUTAI, 1996; MAEHARA, 2007; YANG et al., 2012). Moreover, in contrast to *Trichoderma* spp., other fungal species, such as *Ophiostoma minus*, are excellent for PWN reproduction (MAEHARA and FUTAI, 1996). Therefore, in publication I a less prominent fungus might serve as a nutrient source and escaped isolation, or for the persistence of the PWN at low population densities, the availability of large amounts of nutritional fungus might not be necessary (PANESAR et al. 1994, SOUSA et al. 2011b).

5.1.2 Non-vector spread of *Bursaphelenchus xylophilus* via wood chips

In the greenhouse study of the non-vector spread of PWNs from infested *P. sylvestris* wood chips to non-infested *P. sylvestris* saplings (publication I in chapter 2), significantly more pines showed greater degrees of wilt and were PWN-infested at 25 °C than at 15 °C. This can be explained by faster reproduction (FUTAI, 1980a) and movement as well as higher activity and fecundity (RUTHERFORD et al., 1992) with increasing temperature between 10 °C and 30 °C. The movement speed again seems to make the transfer of the PWN from donor to adjacent recipient pine boards by direct contact successful at 25 °C but not at 10 °C (SOUSA et al., 2011a). Moreover, in a study by MELAKEBERHAN et al. (1992), a large number of nematodes resulted in visible disease symptoms and *P. sylvestris* mortality due to fast nematode reproduction and high

susceptibility of the host at 25 °C. At 15 °C, the small number of dead pines might have appeared for reasons other than the PWN because the low nematode reproductive rate was not correlated with visible symptoms. In publication I, at this temperature, the PWN-infested pines did not always exhibit wilt class 4 at the end of the experiment as an indicator of latent PWN infestation (HALIK and BERGDAHL, 1994). Therefore, PWN-infested wood chips can also lead to infection of pines in northern European countries.

Results of publication I indicate the important influence of tree injuries on non-vector transmission by wood chips if the PWN can overcome the distance to the wound, especially by direct contact of wood chips with wounded roots and stems. The latter situation is comparable to PWN transmission through the exposure of inner bark by a vector beetle (LINIT, 1990; EDWARDS and LINIT, 1992). ARAKAWA and TOGASHI (2002) showed at 25 °C that a distance of up to 10 cm on the stem surface (in publication I, a 3.5-cm wood chip-stem distance) could be overcome and that the probability of successful nematode reproduction in logs decreased with increasing distance.

HALIK and BERGDAHL (1987 and 1992) showed successful non-vector spread from wood chips in soil to root-injured *P. sylvestris* and *P. strobus*, respectively. Additionally, mulched *P. sylvestris* saplings in the field with basal stem wounds became PWN-infested and died (HALIK and BERGDAHL, 1992). In publication I, the spread of PWNs from wood chips in and on the soil to root- and stem-injured saplings was successful at a distance of 3.5 cm at 25 °C. MAMIYA and SHOJI (1989) detected the spread of the PWN and high mortality rates in the case of direct inoculation of a PWN suspension into the soil adjacent to root-injured *P. thunbergii* and *P. densiflora* saplings. A distance of more than 1 cm resulted in reduced disease rates. The soil environment limited the survival time because living PWNs could be extracted from the soil for up to 48 hours. However, a study by HALIK and BERGDAHL (1992) revealed at least a 12-week survival period of the PWN in wood chips when mixed with soil and stored in bags. Although the scenario of wood chips coming into contact with root-injured seedlings was evaluated as being unlikely in nature (EVANS et al., 1993), uninjured plants are rare in nature (GARDINER et al. 2016). Moreover, complete exclusion of root injuries cannot be guaranteed in experiments, which could explain the occurrence of infested “uninjured” trees. However, this might reflect natural conditions, such as mechanical stress and active root life. Nevertheless, penetration of the PWN through unwounded roots of *P. sylvestris* or *P. densiflora* saplings was not excluded (YANG, 1991 and MAMIYA and SHOJI, 1989, respectively).

Referring to chapters 5.1.1 and 5.1.2, the evaluation of wood chips as a possible pathway for non-vector transmission in the pest risk analysis (Evans et al. 1996), if PWN-infested chips come

into contact with host trees, was confirmed. The risk of PWN transmission through wood chips was evaluated as low to moderate (Evans et al. 1996). However, the risk should be higher than expected if PWN-infested wood chips are blown out and left on the forest ground or on injured stems during and after eradication measures (EU, 2012 and its actual version) and if wood chips are used as mulch in contact with trees. Therefore, the potential threat must be considered in future pest risk analysis, management strategies and contingency planning.

European Union Plant Health Council Directive 2000/29/EC (EU, 2000 and its actual version) already includes trade regulations and the treatment of potentially *B. xylophilus*-infested wood chips from affected countries. These obligatory measures (EU, 2000 and its actual version), including heat treatment of potential PWN-infested wood and bark within the European Union (EU, 2012), are vital.

5.1.3 Population dynamics in seven- to eight-year-old *Pinus sylvestris* trees

The population dynamics investigation of the PWN after inoculation into seven- to eight-year-old *P. sylvestris* trees (publication II in chapter 3) revealed an initial phase in which the PWN was located at the entry point and in adjacent tree parts before moving through the tree. The same result was observed for saplings (DAUB, 2008), which were also tested in greenhouses (or climate chambers) at 25 °C. Although more tree segments were PWN-infested in saplings than in the large trees in publication II at this time of early migration in the first stage of PWN invasion, the inoculation and adjacent segments were the centre of nematode infestation (DAUB, 2008).

During the early pine wilt disease stage, the reproduction of the PWN is suppressed because of an unknown resistance mechanism that prevents the PWN from reaching the cambium (FUKUDA, 1997). The number of re-extracted PWNs is lower than the inoculated number because some PWNs might not enter the inoculation site (MELAKEBERHAN and WEBSTER, 1990), and resin close to the inoculation cut becomes sticky (KOO et al., 2013). Moreover, in PWN-infested *P. sylvestris* saplings, a change in resin acid composition with nematicidal activity (BOLLA et al., 1989) and phytotoxins causing paralysis of PWNs (BOLLA et al., 1984) were detected. In publication II, the number of re-extracted PWNs exceeded the number used for inoculation between wilt classes 0 and 1, comparable to the results of a study by MELAKEBERHAN and WEBSTER (1990) with *P. sylvestris* seedlings.

In saplings and in seven- to eight-year-old trees of publication II, starting between wilt classes 0 and 1, the PWN moved intensively throughout the trees. PWNs were distributed in the majority of the tree segments, including roots, as observed by DAUB (2008) for *P. sylvestris* saplings as a second stage of PWN invasion. The highest median nematode densities occurred in the inoculation and adjacent segments. PWNs reached the roots of *P. sylvestris* saplings several or

only a few days earlier after inoculation than in the large trees in publication II (MELAKEBERHAN and WEBSTER, 1990; DAUB, 2008), which was associated with the appearance of wilt (MENÉNDEZ-GUTIÉRREZ et al., 2017). PWNs may migrate to the roots because of a less toxic environment and the availability of food or nutrients (MENÉNDEZ-GUTIÉRREZ et al., 2017).

In *P. sylvestris* saplings (MELAKEBERHAN and WEBSTER, 1990) and in seven- to eight-year-old trees of publication II, the size of the PWN population increased exponentially after a decline following inoculation and a certain degree of wilting. Starting at wilt class 2 in the big pines in publication II, millions of PWNs were extracted. MELAKEBERHAN and WEBSTER (1990) used different inoculum densities but observed approximately equal nematode levels with subsequent pine wilt at approximately the same time. Therefore, the nematode reproductive rate might be influenced by a population density-dependent factor.

In this population phase representing the third stage of PWN invasion, the nematode density per gram of dry matter increased after PWNs moved throughout all plant segments in saplings as well as the large trees in publication II. With increasing median nematode density in the large trees in publication II, the wilt symptoms increased. Starting in wilt class 2, the wood moisture content in most tree parts was reduced to ≤ 100 %. Under the given test parameters, once a median of 500 nematodes/g dry matter as a threshold for irreversible wilt was exceeded throughout the entire large tree, the outbreak of pine wilt disease with wilt class 2 was detected. The presence of a threshold population density for the induction of irreversible wilt in *P. sylvestris* saplings was suggested by DAUB (2008) due to unchanged mortality rates at different maximum population levels.

A dramatic population increase occurred in small *P. sylvestris* saplings, for example, approximately 25 days (DAUB, 2008) or a few days (VIEIRA DA SILVA et al., 2015) earlier than in the large trees in publication II but exhibited a similar trend. According to DAUB (2008) and the results of publication II, dispersion and propagation are interrelated and together represent a key factor in the pathogenicity of the PWN in susceptible tree species (FUTAI, 1980b).

The PWN population size in saplings increased simultaneously in all segments, with the highest nematode densities first occurring in the stem (DAUB, 2008). In the large trees in publication II, the stem and adjacent branch segments were highly PWN-infested earlier than the rest of the branches; the highest median nematode density for the entire tree appeared approximately 30 days later than in the saplings of DAUB (2008) and at wilt class 3.

During pine wilt disease development, the median moisture content in the above-ground segments of the large trees in publication II decreased with increasing median nematode density. The same findings were obtained by FUTAI and SUTHERLAND (1989) and FUTAI (1980b) for *P. sylvestris* and *P. thunbergii* saplings, respectively. Furthermore, DAUB (2008) mentioned for

sapling needles that the relative water content started to drop after the beginning of needle discolouration. The needle moisture contents in the big trees in publication II dropped after wilt class 1 and analogous to the remaining stem and branch segments, but approximately 20 days later than in the saplings of DAUB (2008). FUKUDA (1997) explained the needle wilting by a rapid decline in transpiration, photosynthesis and water potential due to the multiplication of PWNs together with enhanced ethylene production by the tree in the advanced pine wilt disease stage. Finally, the entire xylem could not conduct water.

In saplings (DAUB, 2008) as well as seven- to eight-year-old pines (publication II), wilt symptoms became visible over all needled tree segments, and living PWNs disappeared in tree segments of the upper crown. We share the assumption of DAUB (2008) that PWNs retreated into the lower plant segments as a fourth stage of PWN invasion due to migration related to tree death. Until wilt class 4 (or 5), the centre of the population changed from the stem and (adjacent) branch segments to the plant base, where the highest nematode densities appeared in the stem base and root collar. This happened in the saplings of DAUB (2008) approximately 40 days earlier than in the large trees in publication II.

According to the sapling study of DAUB (2008) and the results in the large trees in publication II, PWNs might move to lower plant parts because they are the last to dry out and have significantly higher moisture content values. In line with the results for the inoculation and adjacent segments in publication II, a relative water content of 20-40 % caused a decline in PWN population density in *P. thunbergii* seedlings in the study of FUTAI (1980b). Only after the first needle discolouration, the remaining stem and branch segments and stem base of the large trees in publication II rapidly lost moisture. However, at the end of the test, the highest median moisture content was recorded in the stem base. The same phenomenon appeared in *P. sylvestris* saplings in the study of DAUB (2008) but approximately 20 and 40 days earlier, respectively.

The moisture contents in the needles of different ages significantly decreased to similar values, which were comparable to those of saplings (DAUB, 2008). Futhermore, during the test period, the frequency of juveniles became significantly higher than that of adults, which is in line with the results of MALEK and APPLEBY (1984) regarding the dominance of dispersal juveniles during wood drying.

5.1.4 Pathogenicity in seven- to eight-year-old *Pinus sylvestris* trees

The pathogenicity investigation (in the greenhouse at 25 °C) of the PWN after inoculation into seven- to eight-year-old *P. sylvestris* trees (publication II in chapter 3) revealed a significant decrease in the predawn water potential in PWN-inoculated trees as well as (water-inoculated) drought-stressed trees. The dehydration and therefore decrease in water potential in the PWN-

inoculated tree occurred because of dysfunction in xylem water conduction (FUKUDA, 1997). This is caused by oleoresin leakage into tracheids due to PWNs (ODANI et al., 1985), blocking of tracheids by hydrophobic volatile terpenes (KURODA, 1991) and the contents of burst vacuoles as well as necrobiosis of ray parenchyma cells due to the reaction of the tree (NOBUCHI et al., 1984).

Comparing the results of publication II with those from the studies of IKEDA and SUZAKI (1984), IKEDA et al. (1990) and MENÉNDEZ-GUTIÉRREZ et al. (2017), PWN-inoculated saplings and seven- to eight-year-old pines (*P. sylvestris* and *P. thunbergii*) began to rapidly lose water potential to drought stress level at similar times. In publication II, the water potential decline started in the PWN-inoculated tree only at the beginning of wilting, which was later than that in the drought-stressed tree. However, the water potential dropped five times more rapidly in the case of PWN infestation. These findings are in line with results reported by FUKUDA (1997) and IKEDA et al. (1990) for PWN-infested *P. thunbergii* seedlings. In the advanced pine wilt disease stage, UTSUZAWA et al. (2005) observed drastic expansion of xylem cavitations, which fused with the cambium. This explains the sudden wilting of PWN-infested pine saplings and mature trees.

The first PWN-inoculated and drought-stressed tree with wilt symptoms was observed 14 days after inoculation (DAI), comparable to that observed for PWN-inoculated *P. sylvestris* saplings (MELAKEBERHAN and WEBSTER, 1990; BAKKE et al., 1991; VIEIRA DA SILVA et al., 2015). Some sapling studies with *P. sylvestris* also showed shorter or longer periods until the first wilt (7-28 DAI) (such as publication III; MENÉNDEZ-GUTIÉRREZ et al., 2017).

Wilt symptoms developed continuously in the mature PWN-inoculated *P. sylvestris* trees in publication II as well as in *P. sylvestris* saplings reported in the literature until tree death but were delayed for the mature trees. The proportion of these trees with no and initial symptoms (wilt class 0+1) began to differ significantly from that of the control trees. In publication II, this occurred approximately 20 days earlier in the PWN-inoculated trees than in the drought-stressed trees and approximately 30 days earlier in PWN-inoculated saplings (publication III) than in the large pines in publication II. However, the time course of disease development is also influenced by other factors (DAUB, 2008; publication III).

The PWN-inoculated pines in publication II first exhibited the initial symptoms and then showed increased wilting until tree death within 35 days. Often, shorter time spans (such as 14 days) were found for *P. sylvestris* saplings (such as SCHAUER-BLUME, 1990; DAUB, 2008; publication III). Furthermore, at tree death, the nematode-infested large pines in publication II as well as the *P. sylvestris* saplings tested by DAUB (2008) and in publication III had a significantly lower moisture content in the tested stem segments than the control pines.

Publication II confirmed the high susceptibility of *P. sylvestris* to the PWN, as observed in the field in North America (MYERS, 1986; EVANS et al., 1996) as well as in saplings in Europe (BRAASCH, 1997 and 2000; publication III). The mortality rates of pine saplings (such as SCHAUER-BLUME, 1990; MELAKEBERHAN et al., 1992; DAUB, 2008; publication III) were comparable to those of seven- to eight-year-old trees (publication II), at 100 %, which were significantly higher than the mortality rates of the drought-stressed and control trees (publication II). However, the maximum mortality rate of the large trees in publication II occurred 84 DAI, with a delay in the timing of mortality in comparison to the saplings of approximately three to ten weeks.

5.1.5 Pathogenicity to German *Pinus sylvestris* provenances

P. sylvestris is known to be highly susceptible to the PWN (EVANS et al., 1996; BRAASCH, 1997 and 2000; DAUB, 2008). The PWN pathogenicity test of eight *P. sylvestris* provenances (publication III in chapter 4), which are used for forest regeneration in Germany and are distributed in Mecklenburg (851 02), Heide + Altmark (851 03), Vogtland + the Thuringian Forest + the Franconian Forest (851 08), the Upper Rhine rift (851 13), Neckarland + the Franconian plate (851 14), Middle Franconian hilly land (851 15), the Black Forest (851 20) and the Alps (851 22), confirmed this result. In publication II, this knowledge was enhanced by using seven- to eight-year-old *P. sylvestris* trees for the tested provenance distributed in Upper Vogtland + northeastern Bavarian upland (851 12).

Due to genetic and physiological differences, different pine provenances are adapted to various regional conditions. The selected provenances in publication III covered all four cardinal directions and different altitudes to provide an overview of German regions. In Germany, seed collection from PWN-infested trees in nature is not possible, and infestation trials with the PWN are limited to greenhouse trials under quarantine conditions. At 25 °C, all nursery saplings in publication III died 5-6 weeks after PWN inoculation, similar to the findings of DAUB (2008), who tested different nematode densities on one (unspecified) *P. sylvestris* provenance. Moreover, the comparison of four Finnish *Pinus contorta* provenances in the study of PANESAR and SUTHERLAND (1989) also revealed no significant differences in mortality in the case of PWN infestation. However, in publication III, the *P. sylvestris* provenances 851 02 and 851 15 developed high wilt classes significantly later, and the former provenance in northeastern Germany showed a significant delay in tree death of one week. Therefore, a slight trend towards higher strength against the PWN in the northeastern direction can be preliminary recognized.

PWN-inoculated pines of all tested provenances contained PWNs. Despite significant differences in nematode densities between a few provenances, no correlation was found between nematode

density in the upper plant part at tree death and tree survival, and nematode densities at tree death were not a good indicator of disease development. A study by DAUB (2008) showed a wide range of nematode densities, which did not influence the development of pine wilt disease four weeks after inoculation. Moreover, after tree death, Finnish *P. contorta* sapling provenances also showed a wide range of nematode densities (PANESAR and SUTHERLAND, 1989). In the case of nematode infestation, the relative water content in the upper plant part is significantly lower than that in control trees, which is consistent with the results of publication III. Therefore, at the final stage of PWN invasion, PWNs are mainly located in the plant base (especially the root collar and stem base) (DAUB, 2008; publication II). Moreover, the results of publication III showed no correlation between the moisture content of the PWN-inoculated pines after tree death and the occurrence of symptoms or the extracted nematode densities. According to publication II, all the correlations mentioned in publication III that were not observed at tree death were found during pine wilt disease development if the trees were still alive.

Referring to chapters 5.1.3-5.1.5, the classification of *P. sylvestris* as highly susceptible to the PWN in the pest risk analysis of EVANS et al. (1996) was confirmed. However, the pest risk analysis indicated that the relative degree of susceptibility should be interpreted with caution if only saplings of a tree species were tested. Therefore, publication II provides clear evidence that also mature *P. sylvestris* trees of European origin under European climatic conditions are highly susceptible to the PWN. Moreover, publication III with *P. sylvestris* saplings as good indicator trees for understanding the population dynamics and pathogenicity of PWNs in mature infested *P. sylvestris* trees (publication II) showed the high risk for additional eight tested German *P. sylvestris* provenances.

The management strategies and contingency planning of European Union Plant Health policy also include *P. sylvestris*, which is correct. Therefore, *P. sylvestris* trees must be felled in infested zones with PWN presence and potentially PWN-infested *P. sylvestris* plants, wood and bark from affected countries must be heat-treated (EU, 2000 and its actual version and 2012) and dealt under trade regulations (EU, 2000 and its actual version). Moreover, surveys of the susceptible plants, wood and bark for the presence of the PWN (EU, 2012) must continue to focus on *P. sylvestris*.

Concerning long-term phytosanitary strategies, in publication III no tolerant or resistant host plants of the tested German *P. sylvestris* provenances were found. However, delayed symptom development and delayed tree death were observed, serving as a possible indicator of differences in defence traits, which can be used in further studies.

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6 Chapter 6: Conclusions

- *B. xylophilus*-infested wood chips pose a threat because the long-term survival of *B. xylophilus* in wood chips and successful non-vector transmission of *B. xylophilus* from infested wood chips to non-infested *P. sylvestris* saplings, including the development of pine wilt disease, were demonstrated during greenhouse studies.
- Therefore, *B. xylophilus*-infested wood chips are a potential source of inoculum transfer to pine wilt disease-free European countries or uninfested areas, although such *B. xylophilus* establishment should be less likely than spread via vectors.
- Suitable hosts in nature are seldom 100 % undamaged, and especially in these trees with stem or root injuries in combination with direct contact with chips and suitable climatic conditions, *B. xylophilus* from infested wood chips can become established.
- Although less likely, *B. xylophilus* can also become established in trees after movement through or on the soil, which represents a hostile environment for *B. xylophilus*.
- At 15 °C, latent *B. xylophilus* infestation through infested wood chips might be possible and increase the risk in northern countries of Europe.
- Dried wood chips might be less risky for non-vector spread.
- Robust outdoor trials restricted to *B. xylophilus*-infested countries should be conducted to confirm all these findings.
- Due to the evaluation of wood chips as a possible pathway for non-vector spread in the pest risk analysis, European Union Plant Health Council Directive 2000/29/EC already includes trade regulations and the treatment of potentially *B. xylophilus*-infested wood chips from affected countries independent of their size and planned use to eradicate *B. xylophilus*.
- However, in this thesis, the necessity of the treatment regulations, which are also part of the Commission Implementing Decision within the European Union, and trade requirements for potentially *B. xylophilus*-infested wood chips from affected countries has been proven in depth.
- Moreover, during eradication measures, infested trees are allowed to be chipped on site and thereafter left on the ground, which could be critical based on demonstrated non-vector transmission.
- Therefore, knowledge of the potential threat of *B. xylophilus*-infested wood chips must be considered in future pest risk analysis, management strategies and contingency planning as part of European Union Plant Health policy.

- In the greenhouse, the development of the *B. xylophilus* population and moisture content in mature *P. sylvestris* trees as well as external wilt symptoms and tree death rates were approximately consistent with those published on the basis of *P. sylvestris* sapling trials, with the only difference being a delay in time.
- Therefore, *P. sylvestris* saplings are good indicator trees for understanding the population dynamics and pathogenicity of *B. xylophilus* in mature infested *P. sylvestris* trees.
- In the greenhouse under European climatic conditions, saplings and older *P. sylvestris* trees of European origin showed pine wilt disease symptoms similar to those occurring in nature in affected countries and clear stages of nematode invasion.
- A detailed tree segmentation and examination of nematode density, stage and gender; wilt symptoms; moisture content and water potential over time enabled a better understanding of the progression of pine wilt disease.

- In the greenhouse, all eight tested *P. sylvestris* provenances used in Germany for forest regeneration, which are distributed in Mecklenburg, Heide + Altmark, Vogtland + the Thuringian Forest + the Franconian Forest, the Upper Rhine rift, Neckarland + the Franconian plate, Middle Franconian hilly land, Black Forest and the Alps, were found to be highly susceptible to *B. xylophilus*.
- However, delayed symptom development and delayed tree death appeared for a few pine provenances, serving as a possible indicator of differences in defence traits.
- In future studies, measuring nematode densities during early and intermediate stages of symptom development in parallel with recording daily wilt symptoms could help identify possible defence traits related to tolerance or resistance to *B. xylophilus* infestation.
- As a precaution against pine wilt disease, research in this field should proceed because tolerant or resistant provenances or individuals could be used for cross-breeding purposes to protect Central European pine forests.

- Based on investigations with saplings and observations in North America, *P. sylvestris* was already correctly considered a susceptible host in pest risk analysis and included in the management strategies and contingency planning of European Union Plant Health policy.
- However, the classification of *P. sylvestris* as a susceptible host due to the high susceptibility of mature *P. sylvestris* trees of European origin under European climatic conditions as well as of *P. sylvestris* saplings of different German provenances was only confirmed in this thesis.

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