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Investigations on pathogenicity, invasion biology and population dynamics of the Pine Wood Nematode *Bursaphelenchus xylophilus* (Steiner und Buhrer 1934) Nickle 1970 in European conifers

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Investigations on pathogenicity, invasion biology and population dynamics of the Pine Wood Nematode *Bursaphelenchus xylophilus* (Steiner und Buhrer 1934) Nickle 1970 in European conifers

The objectives of the present study were to identify potential European host trees for the Pine Wood Nematode (PWN) and to investigate the interrelationship between PWN, host trees and temperature.

Inoculation experiments with a life stage mixture of 2400 to 4000 *B. xylophilus* per sapling were conducted with three to four year old saplings in greenhouse and climate chambers adjusted at 25 C. To evaluate the development of Pine Wilt Disease (PWD), symptoms were assessed in six wilt classes and water content of wood and needles were recorded. Nematodes were extracted from shoots and roots using a modified Baermann funnel technique.

To determine the effect of *B. xylophilus* on mortality of *P. sylvestris*, nematodes were inoculated in 140 saplings with seven densities: 100, 300, 800, 2400, 4000, 6000, 10000. Nematodes were extracted four and twelve weeks after inoculation or at plant death if that occurred before end of experiment. Inoculum densities had no influence on mortality rate which was in general high (70-90 %). Higher population densities in saplings after four weeks were related to higher inoculum densities.

After screening 13 conifer species, *P. sylvestris*, *P. cembra*, *P. nigra, P. strobus*, *P. pinaster*, *P. radiata, P. mugo* and *L. decidua* could be identified as potential sensitive and susceptible hosts for *B. xylophilus* isolates from Portugal, China and North America. *L. kaempferii* was moderately susceptible and *P. pinea* was only susceptible against the isolate from Portugal. *A. alba*, *P. abies* and *P. halepensis* were no hosts for *B. xylophilus*.

Migration and population dynamics of PWN was studied on *P. sylvestris* saplings. Plants were divided into 17 segments and nematodes were extracted from each segment at nine sampling dates within a 27 day period after inoculation. The nematode population density per sapling reached three population peaks, 12, 19 and 27 days after inoculation. PWN could be isolated from all 17 segments six days after inoculation. Results indicate that *B. xylophilus* first migrate rapidly throughout the host before building up a high population level. Nematodes were found to develop several overlapping populations in time depending on the area of the sapling. Four consecutive stages of nematode invasion were observed: (1) Early migration, (2) Distribution and colonisation of all plant parts, (3) Population build up and (4) Retreat into the root-system.

The effect of temperature on population dynamics of PWN and pathogenicity towards *P. sylvestris*, *L. decidua* and *P. abies* was studied by inoculation of sapling with 4800 nematodes. Experiments were carried out in climate chambers at 15°C, 20°C and 25°C. Nematodes were extracted from shoots and roots at seven sampling dates during a 61 day period. Temperature had a major effect on the population dynamics of *B. xylophilus* in both susceptible conifer species. Temperature had no influence on the pathogenicity of PWN, as maximum mortality in *P. sylvestris* and *L. decidua* was reached when temperature exceeded 20°C. However no wilt symptoms were detected at 15°C in any conifer species. The population in *P. sylvestris* increased to approximately 4000 nematodes per gram dry matter in shoots at 25°C. At 20°C the maximum population density in shoots was approximately 2500 nematodes per gram dry matter. A threshold population density of *B. xylophilus* must be reached for induction of irreversible wilt in *P. sylvestris*.

Untersuchungen zur Pathogenität, Invasionsbiologie und Populationsdynamik des Kiefernholznematoden *Bursaphelenchus xylophilus* (Steiner und Buhrer 1934) Nickle 1970 in Europäischen Koniferen

Ziel der Arbeit war es, den Wirtspflanzenstatus Europäischer Koniferen gegenüber *B. xylophilus* zu klären und die Wechselbeziehung zwischen Nematoden, Wirtsbäumen und Temperatur zu untersuchen.

Inokulationsversuche wurden mir einer Population von 2400 bis 4000 Nematoden pro Pflanze an drei bis vier Jahre alten Schösslingen im Gewächshaus und in Klimakammern durchgeführt. Die Versuche erfolgten bei einer Temperatur von 25°C, sofern nicht verschiedene Temperatureinflüsse untersucht wurden. Um die Entwicklung der Kiefernwelke zu bestimmen, wurde die Welke in sechs Klassen bonitiert und zusätzlich die Wassergehalte von Spross und Nadeln gemessen.

Um den Einfluss von *B. xylophilus* auf die Mortalität von *P. sylvestris* zu untersuchen, wurden insgesamt 140 Schösslinge mit sieben unterschiedlichen Populationsdichten inokuliert: 100, 300, 800, 2400, 4000, 6000, 10000. Die Inokulumdichte hatte keinen Einfluss auf die Mortalitätsrate. Sie war mit 70 % bis 90 % generell hoch. Vier Wochen nach Inokulation konnte ein Zusammenhang zwischen hoher Inokulationsdichte und hoher Populationsdichte in Schösslingen festgestellt werden.

Im Wirtspflanzen-Screening von 13 Koniferenarten konnten die Arten *P. sylvestris*, *P. cembra*, *P. nigra, P. strobus*, *P. pinaster*, *P. radiata, P. mugo* and *Larix decidua* als Wirte für die *B. xylophilus*-Isolate aus Portugal, China und Nord Amerika bestätigt werden. *L. kaempferi* war moderat anfällig gegen *B. xylophilus*. *P. pinea* wies eine moderate Anfälligkeit für das Portugiesische *B. xylophilus*-Isolat auf. *A. alba*, *P. abies* und *P. halepensis* waren keine Wirte für den Kiefernholznematoden.

Migration und Populationsdynamik von *B. xylophilus* wurden in *P. sylvestris* Schösslingen untersucht. Dazu wurden die kompletten Pflanzen in 17 Segmente unterteilt. Nematoden wurden nach Inokulation aus Schösslingen an neun Probenterminen während eines Zeitraumes von 27 Tagen aus allen Segmenten extrahiert. Nach sechs Tagen konnten in allen Pflanzenteilen Nematoden nachgewiesen werden. Nematoden im Schössling erreichten drei Populationsspitzen 12, 19 und 27 Tage nach Inokulation. Die Ergebnisse deuten darauf hin, dass *B. xylophilus* sich zunächst im Wirt ausbreitet und danach eine Population aufbaut. Vier aufeinanderfolgende Stadien der Invasion wurden unterschieden: (1) Frühe Ausbreitung (2) Verbreitung und Besiedlung aller Pflanzenteile, (3) Populationsaufbau und (4) Rückzug in das Wurzelsystem.

Für Untersuchungen zum Einfluss der Temperatur auf die Populationsdynamik von *B. xylophilus* und auf die Pathogenität gegenüber *P. sylvestris*, *L. decidua* and *P. abies* wurden Schösslinge mit Nematoden inokuliert. An sieben Probenterminen wurden Nematoden innerhalb eines Zeitraumes von 61 Tagen aus Spross- und Wurzelteilen extrahiert. Die Versuche wurden in Klimakammern bei Temperaturen von 15°C, 20°C und 25°C durchgeführt. Die Temperatur hatte einen Einfluss auf die Populationsdynamik von *B. xylophilus* in beiden anfälligen Wirtsarten. Nematoden entwickelten sich nicht in *P. abies*. Temperatur hatte keinen Effekt auf die Pathogenität des Nematoden. Die maximale Mortalität wurde bei Überschreiten von 20°C in *P. sylvestris* und *L. decidua* festgestellt. Keine der Koniferenarten zeigte Welkesymptome bei 15°C. Die Population in *P. sylvestris* erreichte mit 4000 Nematoden pro Gramm Trockenmasse höhere Dichten bei 25°C als bei 20°C. Daraus kann abgeleitet werden, dass die irreversible Welke in *P. sylvestris* durch eine Schwellenpopulation von *B. xylophilus* induziert wird.

Contents

1 Introduction

The Pine Wilt Nematode (PWN) is considered to be the main causative organism of Pine Wilt Disease (PWD), which primarily affects conifer species of the genus *Pinea.* *Bursaphelenchus xylophilus* (Steiner and Buhrer 1934; Nickle 1970) belongs to the order *Aphelenchida* (Hunt 1993). The genus *Bursaphelenchus* (Fuchs 1937) includes mainly mycetophagous nematodes that are closely associated with insects (phoresy) and can be found in decaying wood of primarily conifers (Ryss et al. 2005). *B. xylophilus* and *B. cocophilus* (Cobb 1919) are currently known to be the only two species within this genus that are truly plant parasitic (Penas et al. 2006).

Although Pine Wilt Disease (PWD) was first reported in the early 1940's in Japan it took roughly thirty years before the causal agent of this disease, the Pine Wood Nematode (PWN), *Bursaphelenchus xylophilus* was identified in 1971 (Tokushige and Kiyohara 1969; Kiyohara and Tokushige 1971). Soon after longhorn beetles of the genus *Monochamus* had been determined as most important vectors for PWN fundamental studies on the ethiology, biology and epidemiology of *B. xylophilus* followed thereafter and were reviewed by Mamiya (1973, 1975, 1976, 1983 and 1984).

B. xylophilus is known to be endemic to North America where it lives in association with indigenous pine species (Sutherland and Peterson 1999). Incidences of PWD in North America (the Mid West) only occur occasionally in exotic and susceptible pine species that were introduced due to ornamental or commercial use (Dwinell and Nickle 1989). Since the outbreak of the *B. xylophilus* epidemics in Japan (Kishi 1995), the PWN spread further to Nanjing, China in 1982, Taiwan in 1985 and Korea in 1988 (Suzuki 2004). Reports on discoveries of *B. xylophilus* in Mexico (Dwinell 1993) were not confirmed. The PWN was established in Portugal in 1999 (Mota et al. 1999) where it affects the maritime pine (*P. pinaster*) on an area of presently 510.000 ha (Schröder and Pfeilstetter 2007).

The pathogenicity of *B. xylophilus* towards conifers is primarily controlled by temperatures above 20°C (Mamiya 1983; Rutherford and Webster 1987, Melakeberhan et al. 1992; Braasch 2000). Under these conditions, wilt symptoms develop rapidly followed by the physiological collapse of the water supply. Another main factor responsible for the occurrence of PWD mainly in dry areas (Kishi 1995) is a limiting water availability, which could be also simulated by inoculation experiments (Fukuda and Suzuki 1988; Ge and Xu 1999; Xu et al. 1996).

On a pathological context it was difficult to determine whether physiological reaction of the tree should be regarded as a response towards the activity of the PWN in the host (Ikeda et al. 1990; Ikeda and Kiyohara 1995) or if the breakdown of physiological activity of the host should be seen as a precondition for the population increase in the host (Suzuki 1984). This is evidently the case for the break down of oleoresin flow which is one of the pre wilt symptoms of PWD in host trees (Kishi 1995).

Physiological reactions that occur before symptoms become visible include an increase of ethylene concentration which is often associated with cambial death in susceptible trees (Fukuda et al. 1994). Furthermore reduction of transpiration occurs at early stages of the PWD (Fukuda and Suzuki 1988; Ikeda 1996a; Ikeda et al. 1990) caused by the blockage of water supply in tracheids. This blockage could be observed as excessive cavitations of the affected xylem by stained tissues (Kuroda et al. 1988; Kuroda et al. 1991; Ikeda and Kiyohara 1995; Ikeda 1996b; Ikeda 1999) or by non destructive magnetic resonance microscopy (Utsuzawa et al. 2005). Those cavitations of the xylem are generally accepted as the primary cause of wilt and therefore the key symptom of PWD. In inoculation experiments axial and radial expansion of cavitated xylem areas in the tree was observed in close relation to the dispersal of virulent *B. xylophilus* isolates, whereas avirulent *Bursaphelenchidae* were restricted to the inoculation area (Odani et al. 1985; Ichihara et al. 1999; Ichihara et al. 2000a). Both, the spread of virulent *B. xylophilus* isolates and an intensive population build up were always observed in susceptible and sensitive host trees. Consequently migration and reproduction ability of PWN was regarded as key factors of their virulence.

Early investigations on histopathology of the PWD were conducted by Mamiya and Kiyohara (1972) and Mamiya (1980) who showed that during the initial stage after inoculation with PWN into host trees, nematodes are mainly concentrated in the epithelial cells of the resin channels. Myers (1988b) hypothesised that invasion and migration of *B. xylophilus* through host tissues invoke an innate hypersensitive defence mechanism which leads to tree death as a consequence of the xylem blockage with oleoresin and toxic substances. This hypothesis was supported by Fukuda et al. (1992) who found that cavitations in tracheids and cytological changes of xylem parenchymatous cells occurred long before nematodes entered these tissues. These changes did not occur with avirulent *B. xylophilus*. It was also hypothesised that regeneration of tracheids is irreversible due to the destruction of the cambium by PWN (Fukuda et al. 1992; Myers 1986). As the impact of *B. xylophilus* on the PWD is believed to be largely indirect there is an ongoing discussion about the role of bacteria which could be isolated from host trees in the presence of *B. xylophilus* (Kawazu et al. 1998; Kawazu et al. 1999; Zhao et al. 2003; Zhao and Lin 2005).

Originally pathogenicity of *B. xylophilus* was expressed as the proportion of dead trees that carry live nematodes (Bedker et al. 1987; Bakke et al. 1991; Caroppo et al. 2000). This method was recognized to give a poor implication on pathogenicity of PWN, due to the fact that PWN affect trees most at an early stage of PWD (Panesar and Sutherland 1989; Melakeberhan and Webster 1990). The early stage of PWD is the period in which pathological interrelation between PWN and hosts mainly occur as physiological reaction of the plant.

After introduction of PWN to Europe and based on the potential pest risk of *B. xylophilus* for overall Europe (Evans et al. 1996), a collaborative EU project was initiated in 2003 entitled “Development of an improved Pest Risk Analysis technique for quarantine pests using pinewood nematode *Bursaphelenchus xylophilus* in Portugal as a model” (PHRAME). The aim of the project was to generate an improved computer based pest risk analysis for Central and Southern Europe which should interlink distribution of host trees, climatic data, the interaction between PWN and European long horn beetles as well as interrelation between PWN and host plants.

The Portuguese isolate of *B. xylophilus* was selected as a model organism. The presented thesis was conducted as part of the PHRAME project at the Dept. of National and International Plant Health, Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany.

The objectives of the thesis were to:

1. Identify potential European conifer hosts for *B. xylophilus* isolates from Portugal, China and North America
2. Clarify the relation between inoculum of *B. xylophilus*, population development in the plant and mortality of the host *P. sylvestris*
3. Determine the exact temporal and spatial distribution of *B. xylophilus* to understand the relation between population distribution and population density in the tree
4. Investigate the effect of temperature on the population dynamics of *B. xylophilus* in host trees
5. Examine the relation between sensitivity of hosts and population dynamics of *B. xylophilus*

2 Material and methods

2.1 General material and methods

This chapter describes nematode isolates and general methods used to inoculate, extract and evaluate nematode populations in conifer saplings. General methods used to prepare and handle samples are described. Furthermore methods used to gain data by counting, weighing and visual assessment are presented.

2.1.1 *Bursaphelenchus xylophilus* isolates

The term isolate in this study refers to nematode-isolates. The nematode isolates are identified by geographic origin, host or host material from where it was originally extracted, the date of isolation and the history. The history of the isolates corresponds to their *in vitro* culturing. References to the specific isolates used are described in the respective chapters. All *B. xylophilus* isolates used for inoculation trials were reared as part of the reference culture on *Bursaphelenchidae* at the Department for National and International Plant Health, Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany.

According to objectives of the PHRAME project the main focus of this study was on the Portuguese isolate of *B. xylophilus* PT 3 (w) and its interrelation with Central European conifers. The isolate PT 3 (w) was provided by Dr. E. Sousa (Instituto Nacional de Investigação Agrária, Portugal).

2.1.2 Identification of nematodes

Morphological identification was conducted using a high power microscope Axioscope 40 (Zeiss, Germany) with the identification keys and published descriptions by: Hunt (1993); Mamiya and Kiyohara (1972); Nickle et al. (1981). All isolates used for inoculation trials were confirmed as morphologically *B. xylophilus*. Additional molecular identification was carried out by Dr. W. Burgermeister and co-workers

(Institute for Plant Virology, Microbiology and Biosafety, Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany) using ITS RFLP (Burgermeister et al. 2005). This method was applied to isolates of *B. xylophilus* during the studies in 2003 and 2004 before inoculation and after re-isolation from host trees.

2.1.3 Nematode extraction

The Baermann-funnel technique originally was developed to extract hookworm larvae from soil samples (Beane and Hopps 1983). This technique has been modified to extract plant parasitic nematodes for various applications in phytonematology (Decker 1969).

In this study the method was modified as follows:

1) Plastic (PVC) funnels with different diameters to fit the variety of sample sizes (⌀ 100 mm, 120 mm and 150 mm, ∠ 50 °).
2) The outlet consisted of a silicon pipe and a clip acting as a valve.
3) The samples were placed on two layers of a commercial cotton milk-filter (⌀ 140 mm – 200 mm).
4) The funnel was then filled with water until the entire samples were submerged in water.
5) The H_2O was enriched with oxygen by using a hard jet of water to fill the beaker.

Wood and root samples were incubated for 48 hours. After this time, nematodes were collected by opening the clip and letting off a 10 ml aliquot into a vial that was labelled previously. All funnels were arranged on a bench like frame which could carry 18 funnels. This arrangement was designed for wood samples and root samples of saplings and tested for extraction efficiency and selectivity (males, females and juveniles) for *B. xylophilus* (chapter 3.1).

2.1.4 Rearing and multiplication of nematodes

All isolates of *B. xylophilus* were reared using a "sporulating" or a "non-sporulating" form of the grey mold rot fungus *Botrytis cinerea* (de Bary) as feeding source.
Both forms belong to the anamorphic state of the fungus. The two fungal forms were cultured on 1.5 % Malt Extract Agar medium (MEA: 15 g of Agar, 15 g of Malt Extract, 750 ml Aqua dest., 7.0 pH) in Petri dishes. The fungi were propagated by transferring either spores or pieces of agar from established culture plates to freshly prepared plates. Freshly inoculated Petri dishes were then left in an incubator at 24 °C for seven days to enhance the growth of mycelium.

The "sporulating" form was characterized as having abundant hyaline conidia (asexual spores) borne on grey branching tree-like conidiophores. Nematodes that were maintained in the reference culture were reared on the "sporulating" form because it continuously supplied a good food source.

When nematodes were multiplied for inoculation trials they were cultured exclusively on a "non-sporulating" form of *B. cinerea*. The "non- sporulating" form does not produce spores and survives exclusively by vegetative mycelia growth. The "non-sporulating" form mutated from the original "sporulating" form and was first used by Dr. M.A. Harmey (formerly Department of Botany, University College Dublin, Ireland; pers. communication Dr. H. Braasch). The reason for using the "non-sporulating" form was to exclude spores from adhering to the cuticle of nematodes used for inoculation of plants which then could enter trees via nematodes and could interfere with pathogenicity. *Botrytis cinerea* is a known pathogen on many hosts including *P. sylvestris* (Capieau et al. 2004) and other conifers. However pathogenicity tests with *B. cinerea* on *P. sylvestris* showed that the used fungal form was not pathogenic towards pines. The nematode population for inoculation trials were built up over a period of about eight weeks. One piece of agar (with nematodes) from nematode inhabited plates was transferred to fresh culture plates (with mycelium) for multiplication. On the average 15.000 nematodes could be collected from one culture plate three weeks after transmission at room temperature. To insure inoculating with predominantly vital and active individuals the nematodes were extracted using a

modified Baermann-funnel technique and collected 24 hours later. Nematodes were controlled for general vitality (movement). The movement of nematodes was observed with a binocular at magnification 40 to 80 times.

2.1.5 Preparation of nematodes for inoculation

Depending on the experiment nematodes were inoculated at different densities per sapling in a suspension of 300 µl of sterile tap water (Tab 2.1). Nematodes extracted from several plates were combined into a 500 ml beaker and concentrated by letting the nematodes settle for 24 hours and siphoning off the supernatant (Fig. 2.1). The beaker was then placed on a magnetic stirrer to homogenise the suspension. Nematodes densities were then determined by individuals in two 100 µl aliquots. Water was added until the desired density was obtained. The nematode suspension was portioned out in closable Eppendorf tubes (Eppendorf, Hamburg, Germany) containing the 300µl inoculum of nematodes per plant and used the same day.

Tab. 2.1: Code of *Bursaphelenchus xylophilus* origin of each isolate, inoculum density per sapling in the respective trial

Isolate	Origin	Inoculum	Trial
US DE 2 (w)	North America	4000	Pathogenicity of *Bursaphelenchus xylophilus* isolates towards European conifer species
NE 12/02	China	4000	
PT 3 (w)	Portugal	4000	
		100,300,800,2.400, 4.000,6000,10000	Effect of inoculated *Bursaphelenchus xylophilus* population on mortality
		2400	Invasion Biology of *Bursaphelenchus xylophilus*
		4000	Effect of temperature on *Bursaphelenchus xylophilus*-host interaction

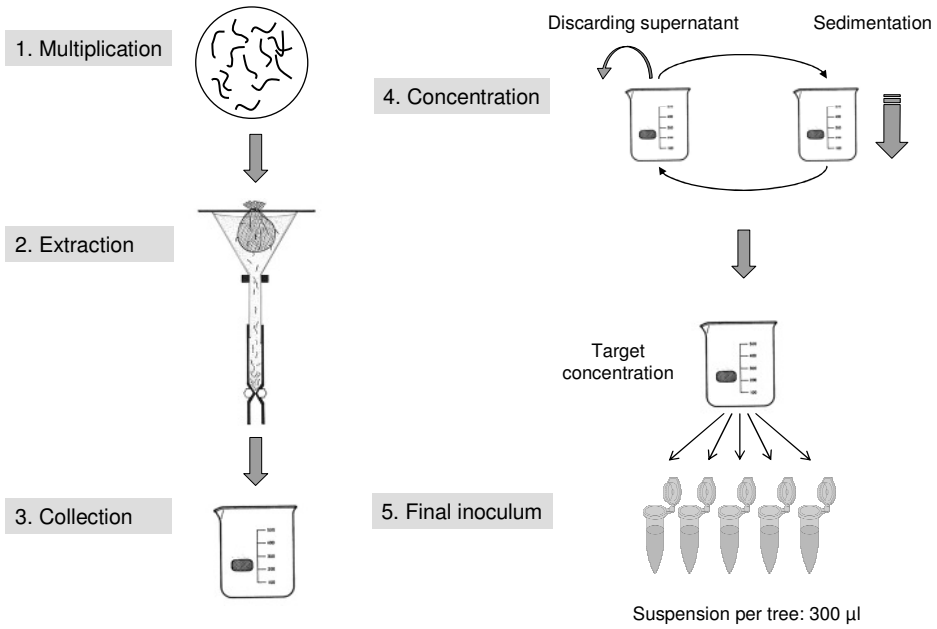


Fig. 2.1: Preparation of nematodes for inoculation; (1) multiplication on Malt Extract Agar plates on *Botrytis cinerea*, (2) Baermann extraction and (3) collection of nematodes from culture plates, (4) repetitive concentration of nematodes suspension until target concentration and (5) preparation of inoculum per sapling

(1) Following extraction by the Baermann method, nematodes were collected in a beaker

(2) The nematodes suspension was left for about five hours at room temperature to let nematodes settle to the bottom of the beaker.

(3) The supernatant was then carefully removed by using a water jet siphon pump.

(4) An 80 °C solution of TAF (concentrated fixative: 560 ml Aqua dest., 10 ml Triethanolamine and 100 ml Formaldehyde solution, 35 %) was added to kill and preserve the nematodes.

(5) Prior to counting the fixation solution was washed by two times removing the supernatant with the water jet siphon pump and adding to water.

(6) Counting of *B. xylophilus* was either done of the total 5 ml suspension or of 50 µl or 1 ml aliquots in case of high nematode densities.

(7) Each sample was counted twice under an inverse microscope (Axiovert 25, Zeiss, Germany).

2.1.6 Fixation and counting of nematodes

In experiments with a large number of samples nematodes could not be counted within an appropriate time, so nematodes were preserved. The procedure of preservation was as follows (Fig. 2.2):

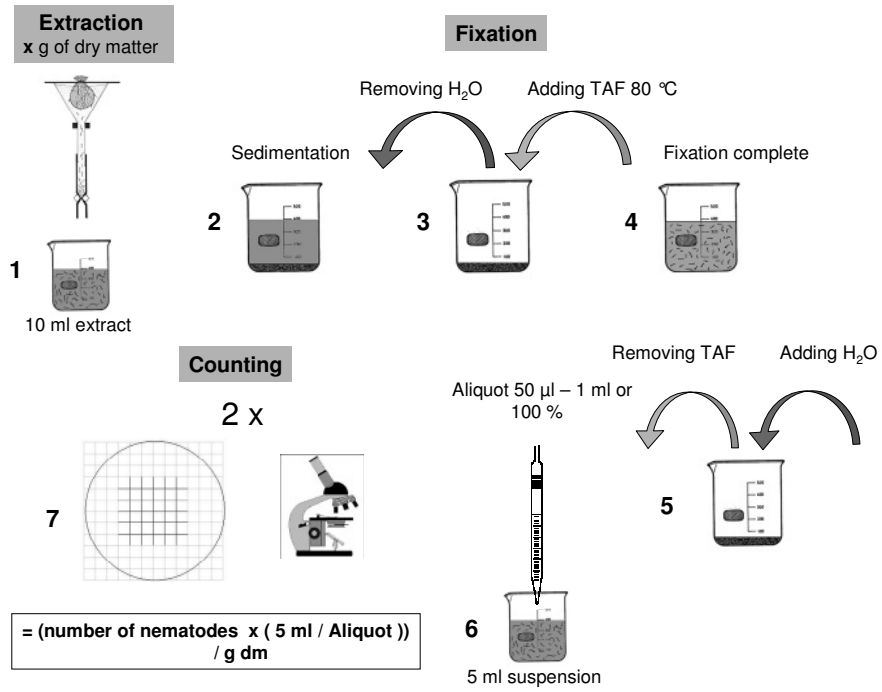


Fig. 2.2: Extraction, fixation and counting of nematodes; (1) Baermann extraction and collection of nematodes in a 10 ml aliquot, (2-4) fixation of nematodes with hot TAF, (5) exchange of TAF through H_2O for counting, (6) removing an aliquot from a homogenized 5 ml suspension, (7) counting and computing number of nematodes on the basis per gram of dry matter

2.1.7 Bark inoculation technique

An inoculation technique was modified allowing *B. xylophilus* to enter its host in a more or less natural manner. In nature, nematode transmission is vectored by beetles of the

genus *Monochamus*. Two possible means of entry exist for the nematodes when beetles come into contact with host trees: First, during oviposition and second, during maturation feeding. Maturation feeding will be exclusively considered here. By destroying the bark of young twigs of host trees during maturation feeding the beetle gives free access to all tissues up to the cambial layer. The technique was modified to fit the test conditions in my studies. Because *Monochamus* beetles in nature feed in the crown of pines, bark inoculation focused on the previous year's shoot of the sapling. The bark inoculation technique in general was used for all inoculations carried out in this study. The technique itself as it was applied consisted of several steps: (Fig. 2.3).

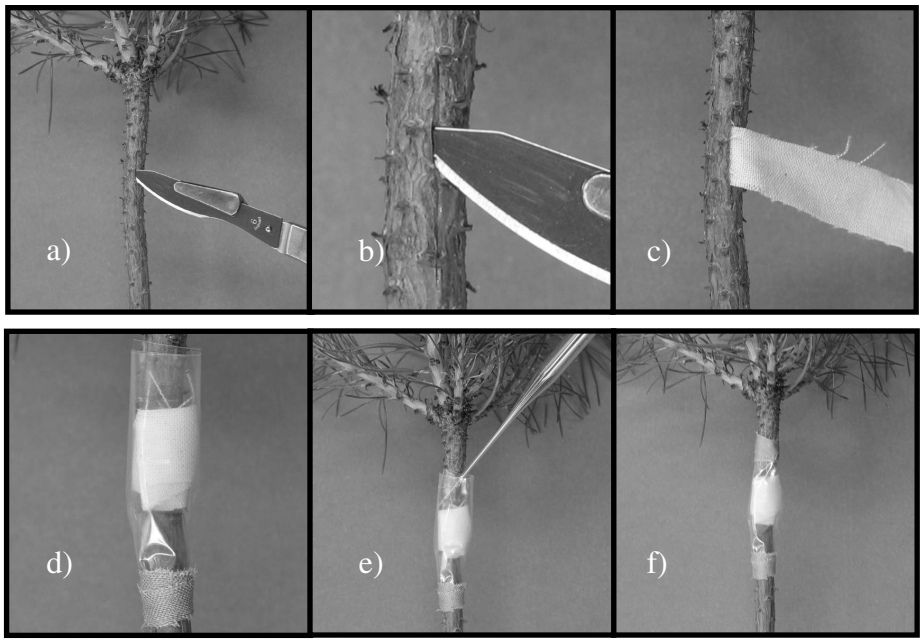


Fig. 2.3: Technique used to inoculate *Bursaphelenchus xylophilus* suspension in three - four year old *Pinus sylvestris* saplings; a) – b) Cutting an I shaped slit in the previous year shoot, c) inserting a cotton stripe, d) enclosing the cotton, e) transferring nematodes onto the cotton, f) sealing the inoculation site with plastic tape

1) The needles at the inoculation position were removed
2) The bark was cut with a scalpel to separate bark and inner cortex without disturbing the cambial layer

3) A cotton stripe (9 x 1 cm) was inserted into the cut in order to give contact between cotton and inner cortex. From preliminary testing the size of the cotton was selected because it was suitable to absorb precisely the amount of 300 µl suspension that was used for inoculum per sapling. The stripe was folded and fixed by a small piece of plastic that was wrapped around the stem and then sealed underneath with tape (Leucoplast®, Hamburg, Germany),
4) The nematode-inoculum was pipetted onto the folded cotton stripe
5) The upper side of the plastic was sealed with tape to prevent drying out of the inoculum.

The position of the inoculation point depended on the shape of host tree species. For *P. sylvestris* inoculation was performed on the previous year's shoot below the youngest whorl. An overview of the position of the inoculation point in all tree species used in the inoculation trials is given in the Appendix (Fig. A1-2).

2.1.8 Assessment of wilt symptoms and mortality

The visual evaluation of wilt symptoms was based upon six wilting classes and described in (Tab. 2.2). Wilt classes were expressed as percentage of symptom coverage in relation to the whole foliage and associated with vitality of the plant. This assessment scheme was carried out in all the inoculation trials and applied to all conifer species studied.

Tab. 2.2: Assessment scheme for the evaluation of wilt symptoms in six classes and associated physiological conditions of plants

Wilt Class	Coverage of Symptoms in %	Physiological Condition
0	0	vital
1	1 - 25	Pathogenesis of PWD
2	26 - 50	
3	51 - 75	
4	76 - 99	
5	100	dead

2.1.9 Sampling and handling of wood, needle and root samples

The sampling of saplings was carried out similarly between the trials. In general shoots and root parts were sampled and processed separately (Fig. 2.4). The cutting site always was the intersection between root collar and first stem part.

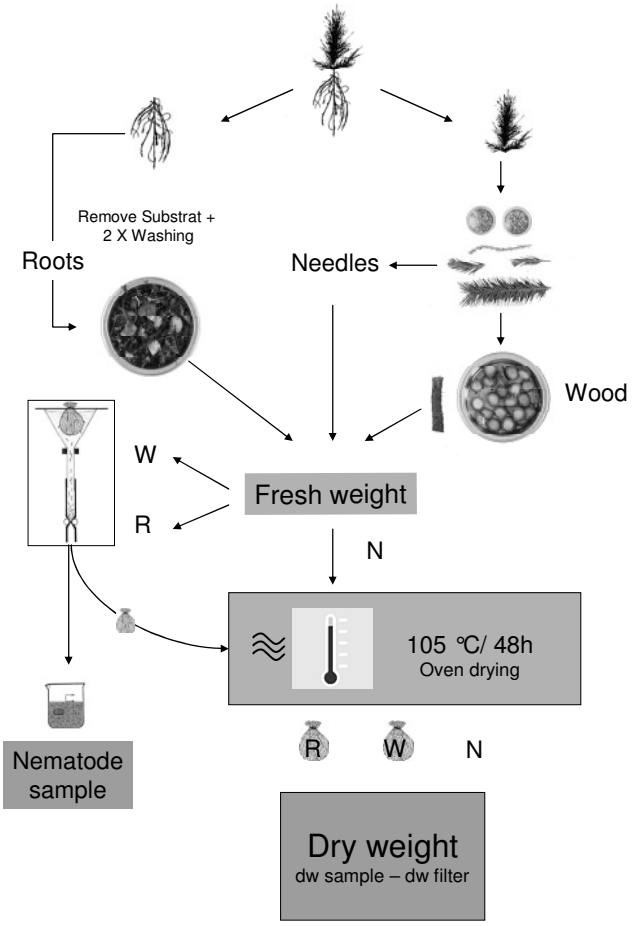


Fig. 2.4: Processing of wood (W), root (R) samples and needles (N) from conifers for the determination of nematode density, fresh and dry weight of samples; Samples were processed in the order: 1. Separation of wood /needles and roots, 2. Determination of fresh weight (W, R and N), 3. Baermann extraction of nematodes (W,R), 4. Drying at 105 °C for 48 h and 5. Determination of dry weight (W, R, N)

All plant segments were then cut into 5 mm to 10 mm pieces using commercial pruning shears. The resulting samples were then weighted to obtain the fresh weight. Then the samples were submitted to the Baermann extraction procedure for 48 hours. Roots were carefully cleaned in tap water. Afterwards root parts were cut into 5 mm to 10 mm pieces and processed like other samples.

2.1.10 Determination of fresh weight, dry weight and relative water content

Determination of dry weight was carried out in accordance with the method proposed for the determination of dry weight and water content of woody plants DIN ISO 11465 described in the handbook on analytics in forestry (BMVEL 2005). Samples were oven dried at 105 °C until weight consistency was attained which was usually reached after 48 hours. The relative water content was only taken for shoots and needles. Fresh weight of roots was taken after removing soil substrate from the root surface by washing. To measure the relative water content separately old (> one year old) and young (< one year old) needles were dried separately at 105 °C for 24 hours directly after sampling. The water content was calculated in the formula:

$$H_2O\,[\%] = \frac{fres\ weight - dry\ weight}{fresh\ weight} * 100 \qquad (2.1)$$

2.2 Test on extraction efficiency

The test was conducted as described in chapter 2.1.3. Extraction selectivity for female, male or juveniles also was investigated as they differ distinctively in their morphological characteristics. Body shape was thought to interfere with the mesh size in the cotton filter. Funnels were inoculated with prepared suspension of 1.000 nematodes of a known proportion of male, females and juveniles. Nematodes were collected and counted after 24 hours and 48 hours. In addition the remaining water inside the funnel was also checked for nematodes and all life stages were counted separately. Three adaptations of the Baermann method were tested with 10 replications:

(1) Two layers of cotton milk filter
(2) One layer of cotton milk filter
(c) Without filter

2.3 Effect of *Bursaphelenchus xylophilus* on *Pinus sylvestris* – mortality

2.3.1 Experimental set up

The aim of the investigation was to determine the effect of different inoculum densities on the population dynamics of *B. xylophilus* and the relation between population density and mortality of pines. This should give an adequate base line on the inoculation densities for further trials with this pathosystem. To assess these effects, *B. xylophilus* PT 3 (w) was inoculated in *P. sylvestris* at densities of 100, 300, 800, 2.400, 4.000, 6.000, 10.000 per sapling.

Altogether 140 three to four year old saplings of comparable shape and size were inoculated. *B. xylophilus* was applied by the bark inoculation technique as described in chapter 2.1.7. Saplings treated with sterile tap water served as control. Total number of replications was 20. All saplings were placed in a climate chamber adjusted to 25 °C and 50 % rH. Light duration was adjusted to 12 h at 4000 lux. The saplings were watered as needed.

2.3.2 Development of wilt symptoms, mortality and nematode reproduction

Inoculated and control saplings were assessed for PWD symptoms according to the scheme described in chapter 2.1.8. Wilt intensity of 10 saplings for each inoculation density were recorded on a weekly basis for a period of 12 weeks. Mortality of saplings was determined as part of the wilt assessment.

Ten saplings of each inoculation density were sampled for determination of nematode reproduction after four weeks and another 10 saplings after plant death or if plants survived after 12 weeks. Nematodes were extracted form saplings according to the procedure described in chapter 2.1.3.

2.4 Pathogenicity of *Bursaphelenchus xylophilus* isolates towards European conifers

The objective of the following study was to determine the host status of European conifers and the potential risk of different isolates of *B. xylophilus* from Portugal, China and North America.

2.4.1 Host trees

An overview of tree species and respective number of saplings involved in this pathogenicity trial, as well as of age classes and geographic distribution is provided in Tab.2.3.

Tab. 2.3: Geographic distribution, age, size and circumference of tree species used in the greenhouse trial on pathogenicity of three *Bursaphelenchus xylophilus* isolates

Tree Species	Geogr. Distribution	Age Years	Height cm	Stem Ø cm	Number of Saplings
Pinus sylvestris	North Eastern Europe	2-3	61.9	9.9	140
Pinus nigra austrica	Southern Europe	3-4	56.5	11.7	140
Pinus mugo	Central (mountainous) Europe	3-4	24.4	7.9	140
Pinus cembra	Central (mountainous) Europe	3-4	15.1	9.6	140
Pinus halepensis *	Western Mediterranean	2-3	54.1	6.1	140
Pinus strobus	North America	3-4	34.5	7.6	140
Pinus pinaster	Mediterranean	2	48.1	5.7	140
Pinus radiata	South-West USA	2-3	41.0	6.1	140
Pinus pinea	Mediterranean	3-4	84.4	9.9	140
Picea abies	North East Central Europe	3-4	38.3	6.1	140
Abies alba	South East (mountainous) Europe	3-4	38.3	11.7	140
Larix decidua	Central (mountainous) Europe	2-3	56.7	6.4	140
Larix kaempferi	Northern Coastal Europe	3-4	81.1	9.1	140

The majority of tree species could be found in the genus *Pinus*, as it is known to include susceptible hosts for *B. xylophilus*. Tree species slightly varied in age and development. Conifer species were selected according to occurrence, distribution and importance in Europe. Test saplings in general were not older than four to five years when being inoculated.

2.4.2 *B. xylophilus* isolates

The identities of all isolates used in this trial are given in Table 2.4. The different isolates were selected according to their origin and their known pathogenicity. Pathogenicity was known for the Portuguese isolate PT 3 (w) and the Chinese isolate Ne 12/2 and suggested for the North American isolate US DE 2 (w). The Portuguese and the Chinese isolates were isolated from host trees that suffered from severe PWD, whereas the isolate US DE 2 (w) came from wood packaging. All isolates were collected in 2002 or 2003.

Tab. 2.4: History, origin, collector and number of nematodes used for *Bursaphelenchus xylophilus* stock cultures

Code	Origin	Date of Isolation	Original Location	Geographic Origin	Initial Population	Collector
PT 3 (w)	*P. pinaster*	29. Apr. 2003	Lezirias	Portugal	270	Bravo M.A.
US DE 2 (w)	wood package	15. Nov. 2002	Germany	North America	50	Anonymous
Ne 12/ 02	*P. thunbergii*	30. Okt. 2002	Nanjing	China	40	Schröder T.

2.4.3 Experimental design

Forty saplings of each tree species were inoculated with 4.000 nematodes per sapling of each nematode isolate. In addition saplings were inoculated with sterile tap water (control). Inoculation was carried out according to the method described in chapter 2.1.7. Due to the variation in plant shapes between the various species, nematodes had to be inoculated at different positions within the sapling (Fig A1 - A2, Appendix).

Due to the high number of saplings and tree species inoculation of all saplings could not be realized within a short period of time. Therefore inoculation was carried out from the third to the twenty-second July in 2003. All saplings of one conifer species were inoculated within 12 hours. After inoculation all saplings were placed on tables in two greenhouse compartments. Each compartment was equipped with shading and maintained at a temperature of 25 °C ± 2 °C. The saplings were grouped within each species in a random block design. Saplings were watered as needed.

2.4.4 Susceptibility of conifers and nematode population development

Nematodes were extracted from 20 saplings of each combination of conifer species and nematode isolate four weeks after inoculation to determine the multiplication rate of *B. xylophilus* and its distribution within the conifers. Corresponding to the literature (Braasch 1997) nematode densities were considered to vary greatly between individual saplings. To compensate far variation five saplings were aggregated to one sample. Therefore the total number of replications was four. Nematodes were extracted using a modified Baermann-funnel (chapter 2.1.3). Roots were not included in the investigation. Saplings were sampled for nematodes following the scheme in chapter 2.1.9. From the 20 remaining saplings nematodes were extracted similarly after plant death or in case of plant survival at the end of 12 weeks. Population densities were expressed in number per gram fresh weight.

Twenty nematode treated saplings and twenty control saplings of each species were assessed for wilt symptoms or mortality at weekly intervals for 12 weeks according to the assessment scheme in chapter 2.1.8. Each sapling was assigned to a wilt class until mortality occurred. Mortality was calculated as the percentage of dead saplings.

2.5 Invasion biology of *Bursaphelenchus xylophilus* in *Pinus sylvestris*

To investigate if migration and multiplication are complementary or independent key factors of virulence of *B. xylophilus*, the invasion biology of the Pine Wood Nematode in a susceptible host under optimum development conditions was investigated. The number and selection of the plant parts to be studied was a compromise between

coverage of the whole plant but also providing information about different plant parts. The latter should also provide data if nematodes were aggregated or randomly distributed inside the sapling.

2.5.1 Segmentation of *Pinus sylvestris* saplings

Pinus sylvestris was chosen as a representative susceptible host tree. The saplings were divided into 17 segments as shown in Fig. 2.5 a-b. Further information about the segmentation and their position in the plant is given in Table 2.5

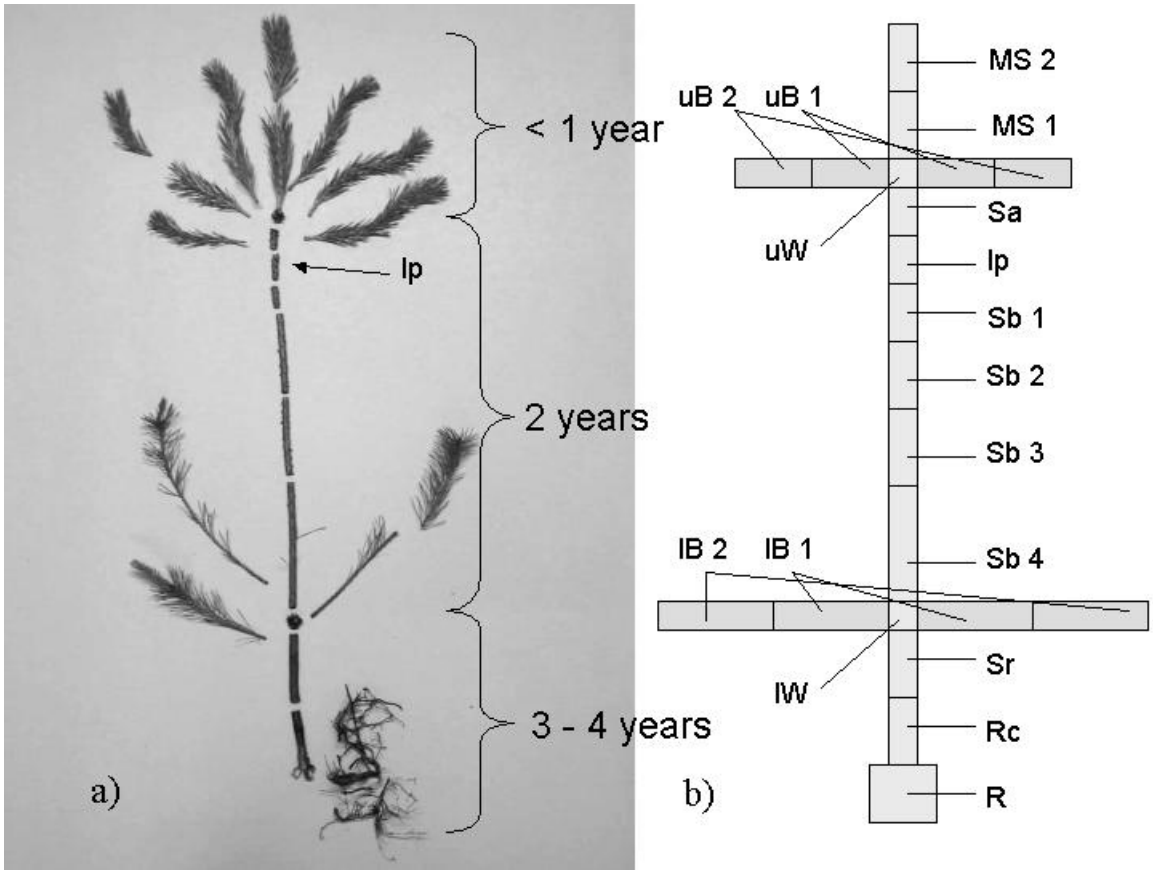


Fig. 2.5 a-b: a) Morphological parts of three to four year old *Pinus sylvestris* saplings and site of nematode inoculation; b) Basic segmentation consisting of 17 segments according to morphological parts of the sapling ; MS 1 +2 (main shoot), uB 1+2 (upper branches), uW (upper whorl), Sa (Stem above inoculation point), IP (inoculation point), Sb 1-4 (stem below inoculation point), lB 1+2 (lower branches), lW (lower whorl), Sr (stem above root collar), RC (root collar), R (roots)

Tab. 2.5: Segmentation and coding of morphological plant parts of *Pinus sylvestris*. Adjacent segments in larger parts of the sapling having the same code but different numbers; Upper and lower branches represent aggregated samples of all respective branches

Segment Number	Segment	Morphological Part of the Sapling	Aggregated	Position
1	**MS 1**	Main Shoot		Basis
2	**MS 2**			Terminal
3	**uB 1**	Upper Branches	x	Basis
4	**uB 2**		x	Terminal
5	**uW**	Upper Whorl		
6	**Sa**	Stem above Inoculation Point		
7	**Ip**	Inoculation Point		
8	**Sb 1**	Stem below Inoculation Point		
9	**Sb 2**			
10	**Sb 3**			
11	**Sb 4**			
12	**lB 1**	Lower Branches	x	Basis
13	**lB 2**		x	Terminal
14	**lW**	Lower Whorl		
15	**Sr**	Stem above Root Collar		
16	**RC**	Root Collar		
17	**R**	Root		

Lateral branches in the top and basis of the saplings were aggregated to one representative sample each and further divided by a basis and terminal part. The same aggregation was applied to the lower branches and the main (leader) shoot.

2.5.2 Experimental design

The Portuguese isolate PT 3 (w) of *B. xylophilus* was used in this trial. Altogether 120 four to five year old saplings were used that had similar length of stem parts and crown circumference and comparable positions of old and young whorls. Saplings were selected from outdoor nurseries and placed in a climate chamber (25 °C, 50 % rH, 12 h light). Saplings were kept there for two days before inoculation, allowing them to adapt

to the slightly different climate. Approximately 4.000 nematodes were inoculated per sapling.

To cover all phases of population development for *B. xylophilus* nematodes were extracted at nine sampling dates over 27 days period and described in Tab. 2.6.

Tab. 2.6: Time of inoculation, sampling interval, sampling date and cumulative number of samples arising from the segmentation of three to four year old saplings during the inoculation trial on migration and distribution of *Bursaphelenchus xylophilus* in *Pinus sylvestris* saplings

Days after Inoculation	Sampling Interval (Days)	Sampling Date	Cumulative Number of Samples (10 Saplings * 17 Segments)
2	2	1	170
4		2	340
6		3	510
9	3	4	680
12		5	850
15		6	1,020
19	4	7	1,190
23		8	1,360
27		9	1,530

The end of the experiment was determined by the death of saplings. The first three samplings were taken at two days interval, the next three samplings at three days interval and the last three samplings at four days interval. At each sampling date ten saplings were examined. Saplings were randomly arranged on a table in the climate chamber. During the experiment all saplings were watered according to their demand.

When sampled, shoots were separated into the 15 segments and labelled individually. The two root parts were separated from the substrate by carefully washing. The inoculation segment and the neighbouring segments were washed before nematode extraction. To exclude nematodes from extraction that remained on the surface of the

inoculation segment. Segments were then placed on the Baermann-funnel for nematode extraction. In cases of very small segments (upper whorl, stem above inoculation point, inoculation point and stem below inoculation point), segments were enclosed in commercial cotton filters (4 x 5 cm), and placed in a 10 ml beaker, filled with H_2O. After 48 hours sample plus filter were removed and nematodes in the solution were counted. Dry and fresh weights of all samples were determined as described in chapters 2.1.9 and 2.1.10.

2.5.3 Relative water content of shoot parts and needles

Each segment (except root collars and roots) was measured for relative water content at nine sampling dates over a 27 day period. To calculate a representative value for the relative water content of shoots, values of the following segments were aggregated to one value: MS 1-2, uB 1-2, Sb 2-4, lW and lB 1-2 (see also Fig. 2.5 a-b. and Tab. 2.5).

There was a high probability of losing material between weightings of very small segments namely uW, Sa, Ip, Sb 1. As a result these small segments produced measurement artefacts (Fig. A3 – A5, Appendix) and therefore were excluded from measurement.

2.5.4 Distribution and migration of nematodes in the host

The number of adults and juveniles in each segment was counted. This data was computed to nematodes in one gram of dry weight for each segment and represents the population density.

Population densities were calculated (1) for each single segment or (2) summarized to present certain areas of the plant, (3) for shoots only or (4) for plant roots. To display the distribution of nematodes inside the saplings, population densities were depicted in special tree diagrams (see also Fig. 2.5 a-b) The percentage distribution of nematodes per sapling in the comparison drawn between two major plant parts were based upon absolute numbers of nematodes. These numbers were summed from all segments and

related to the total nematode count per sapling. Differences between the two plant parts (R, Rc, Sr vs. rest of the plant) were statistically analyzed.

2.5.5 Nematode population dynamics in different parts of the host

Population dynamics were displayed over time based on the population densities of *B. xylophilus* calculated for one gram of dry matter over a period of 27 days. Population dynamics were presented for certain areas of the plant: (1) segments around the inoculation point, (2) segments from lateral branches and the plant top, (3) segments from the basis of the plant, (4) for shoots only or for plant roots only.

2.6 Influence of temperature on *Bursaphelenchus xylophilus* – host interaction

Considering 15 °C, 20 °C and 25 °C as the August isotherms for the European Union (Evans et al. 1996) this investigation aimed at a better understanding of the nematode - plant interrelation in sensitive and tolerant hosts as effected by temperature.

2.6.1 Host trees

Pinus sylvestris (L.) and *L. decidua* (Mill.) were selected as susceptible conifer species and *P. abies* (L.) as a tolerant species (Tab. 2.7).

Tab. 2.7: Geographic distribution, age, size and circumference of saplings of *Pinus sylvestris*, *Larix decidua* and *Picea abies* used to investigate the influence of *Bursaphelenchus xylophilus* on Pine Wilt Disease in climate chamber trial

Tree Species	Geogr. Distribution	Age Years	Heigth cm	Stem Ø cm	Number of Saplings
Pinus sylvestris	South-West Germany	3-4	58.4	8.7	160
					110
					360
Larix decidua	South-West Germany	3-4	64.9	7.7	360
Picea abies	South Germany	3-4	39.6	6.2	360

The major host tree species was *Pinus sylvestris* as it is broadly distributed in Germany and Northern Central Europe up to Scandinavia and down to the Mediterranean (Kindel 1995). *Larix decidua* was selected because it is a frequently planted forest tree in mixed stands in Germany and widely distributed throughout Central Europe especially in Alpine regions (Erlbeck et al. 2002). *Picea abies* was integrated into the trial to investigate the stability of tolerance under Central European temperatures. Economically it is the most important conifer species in Germany that has been planted for many decades in pure stands especially in areas like Bayerischer Wald in Southern Germany (Schmidt-Vogt 1991).

2.6.2 Experimental design

To examine the population dynamics of *B. xylophilus* in conifers the Portuguese isolate PT 3 (w) was chosen. Experiments were conducted in climate chambers at three temperatures 15 °C, 20 °C and 25 °C.

Three to four year old saplings were inoculated with 2400 nematodes / sapling, whereas controls were inoculated with sterile tap water. Saplings that belong to one temperature regime were inoculated within the same day. Saplings were placed in the climate chambers in a randomized design. The following parameters were investigated: 1) wilt symptoms, 2) population development of *B. xylophilus* and 3) relative water content in wood and needles. The trial design combined three factors: Conifer species (three species), temperatures (three temperatures) and time (seven sampling dates, weekly interval). Each temperature regime comprised 120 saplings for each conifer species. Assuming that non treated control saplings would not change their relative water content during the experiment, saplings were taken only at the end of experiment. All saplings were watered by demand.

2.6.3 Effect of temperature on the progression of wilt

The assessment of wilting symptoms was examined on 20 inoculated and control saplings. The saplings were assessed on a weekly basis (7, 15, 21, 28, 32, 46 and 60 days after inoculation) in concordance with the sampling dates of the other parameters.

The assessment of wilt symptoms (chapter 2.1.8) provided a set of 20 ordinal values for treated and control saplings at each date. These values were expressed as median that stood for a representative wilting class of the respective date. Comparisons for plant death were carried out for day 32, 46 and 61 after inoculation but not between assessment dates. Relative water content of wood and needles was determined for every sampling date.

2.6.4 Effect of temperature on nematode population dynamics

Population densities of nematodes were recorded at: 7, 15, 21, 28, 32, 46 and 60 days after inoculation. Population densities of nematodes were calculated from the number of nematodes extracted from the entire shoot or the roots. Ten root and shoot samples were taken from 10 saplings at one sampling date for each tree species and temperature. Population dynamics were presented as median of population densities in time for each tree species and each temperature.

2.7 Statistical analysis

All statistical calculations were computed with the software STATISTICA 6.0 (Statsoft, Tulsa, USA).

Descriptive statistics

Whereas the mean is the preferred representative for samples that show normal distribution and a homogeneous variance it is not recommended for samples which are characterized by extreme values and a restricted number of replications (Lozan 1992). A suitable parameter in this case is the median. The median is a measure of the central tendency of a sample. It divides the sample into two equal parts. When ranked, 50 % of the values will be below and 50 % will be above the median. Depending on the number of values (n) in one sample (x) its position is defined differently (Richter 2004).

An uneven number of values in a sample is exactly in the centre of a sample which is expressed in the formula:

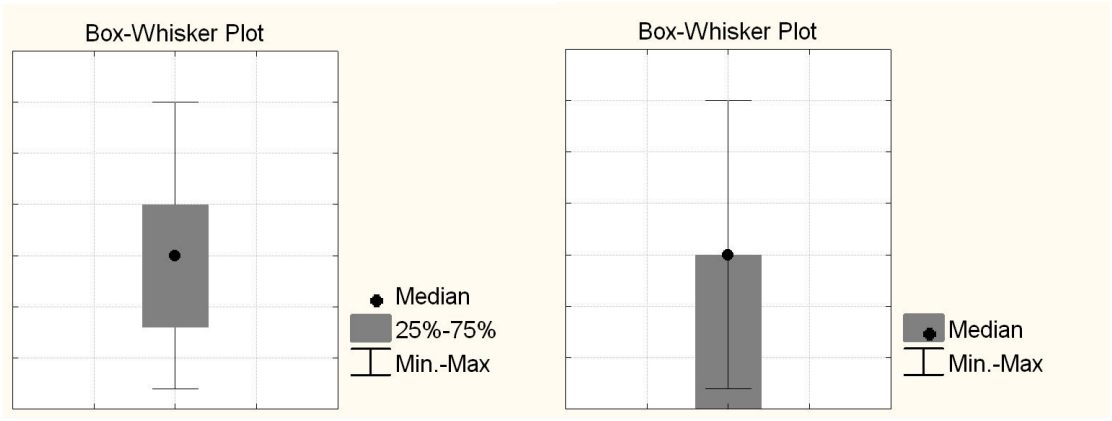
$$Median = x_{((n+1)/2)} \tag{2.2}$$

If the number of values is even the two central values of a sample are taken and their arithmetic mean is computed in the formula:

$$Median = \frac{x_{(n/2)} + x_{((n/2)+1)}}{2} \tag{2.3}$$

The median has the advantage of being tolerant to extreme values. The higher the number of samples with a normal distribution and homogeneity of variance, the more the arithmetic mean and the median are similar (Sachs 1992). In this study the median was the main descriptive parameter for all nematode population related evaluations. As the median is the suitable parameter for ordinal scales of data it also was used to evaluate the assessment of wilt classes (chapter 2.1.8). The arithmetic mean was only used to explain the relative water content of wood, roots, needles, when the values were normally distributed and variance was homogeneous.

The dispersion of values was characterized by the minimum, maximum, 25 % quartile and 75 % quartile. The 25 % quartile contains 25 % of all values of a sample and the 75 % quartile analogically. The central 50 % dispersion of a sample is characterized by the distance between those quartiles. The median and the parameters of dispersion are presented as Box-Whisker plots with two modifications (Fig 2.6 a-b). The modification b) was used when the box was replaced by a bar.



a) b)

Fig. 2.6 a-b: a): Box Whisker plot to present Median (point), 25 % Quartile to 75 % Quartile span (grey box) and Minimum to Maximum span (Whisker) of values; b): Box Whisker plot to present Median (point) and Minimum to Maximum span (Whisker) of values

Statistical analysis

Only non parametric tests were applied. To compare two dependent variables the Wilcoxon matched pairs test at $\alpha \leq 0.05$ was used. It is a nonparametric alternative to the *t*-test. In order to compare two independent variables the Mann-Whitney U test ($\alpha \leq 0.05$) was used as an alternative to the *t*-test for independent samples. Both procedures assume that the variables under consideration were measured on a scale that allows the rank ordering of observations based on each variable and that allows rank ordering of the differences between variables.

The median test was used as a nonparametric alternative to the between-groups one-way analysis of variance procedure (ANOVA). The Software STATISTICA determined the number of cases in each treatment that fell above or below the common median, and computed the χ^2 value for the resulting 2 x 2 samples contingency table. Under the null hypothesis (all samples come from populations with identical medians) the software expected approximately 50% of all cases in each sample to fall above or below the common median. The median test considered differences to be significant at $p \leq 0.05$.

In order to evaluate differences between the frequencies of values observed versus expected the χ^2 procedure was applied. The χ^2 test considered frequencies to be significantly different at $p \leq 0.05$. Also the 2 x 2 table was used to compare frequencies of two dichotomous variables computing χ^2 values at $p \leq 0.05$.

3 Results

3.1 Extraction efficiency and selectivity

More nematodes were extracted from the modified Baermann funnel equipped with one or two cotton filters than without using a filter (Tab. 3.1).

Tab. 3.1: Mean number of *Bursaphelenchus xylophilus* and standard deviation (SD) taken after extraction by a modified Baermann technique after 24 and 48 hours in comparison to the number of nematodes remaining in the funnel

	Number of Nematodes							
	After 24 h		After 48 h		Remaining		Efficiency (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
No Filter	417	75	193	100	397	132	61.30	11.00
1 Filter	670	116	52	56	287	100	72.17	10.00
2 Filters	640	125	41	44	310	137	68.71	17.00

n = 10

The majority of nematodes were extracted within 24 hours. After 48 hours roughly a third of the introduced nematodes still remained inside the funnel. The extraction efficiency of the modifications with filter varied insignificantly but efficiency without filter was reduced at 10 % compared to both other modifications. Extraction in general showed a low standard deviation, thus the extraction appeared to be consistent.

The selectivity of the various Baermann funnel modifications is presented in Tab. 3.2. The percentage of juveniles and adults was computed based on 400 to 600 individuals being counted. The introduced population of approximately 1000 individuals contained a high percentage (76.5%) of juveniles. Female to male rate was 1: 0.8. This relation did not change after extraction by using either one or two filters. When no filter was used, there were about 10 % more males extracted and 16 % less juveniles.

Tab. 3.2: Selectivity of different filter-modifications of a Baermann extraction technique

		Proportion in %			
Populations		♀	♂	Larvae	♀ : ♂
No Filter	1)	17.2	22.4	60.4	1 : 1.3
1 Filter	2)	15.5	11.9	72.6	1 : 0.8
2 Filters	3)	18.7	11.1	70.2	1 : 0.6
Initial	4)	13	10.5	76.5	1 : 0.8

1) 477 Individuals counted
2) 478 Individuals counted
3) 571 Individuals counted
4) 620 Individuals counted

3.2 Effect of *Bursaphelenchus xylophilus* on *Pinus sylvestris* - mortality

3.2.1 Pine Wilt Disease as effected by different inoculation densities

None of the control saplings did develop symptoms throughout the experiment. Symptom development of saplings at the different inoculation levels expressed as the median of wilting classes is given in Fig. 3.1.

Development of symptoms appeared more or less simultaneously at inoculation levels 2400, 4000, 6000 and 10000 after 27 to 35 days post inoculation. Symptom development from class two to four at 2400 and 4000 nematodes per sapling appeared slightly earlier than at 6000 and 10000 nematodes per sapling. Low inoculation levels resulted in a delay of symptom development which was more pronounced at 100 and 300 nematodes per sapling than at 800. Mortality of saplings was high (70% - 90%), regardless of the number of nematodes inoculated (Tab. 3.3). Low inoculation densities at 100 and 300 nematodes resulted in mortality rates of 70 %. Inoculum densities of more than 800 nematodes showed mortality rates of 80 % to 90 % at the end of experiment. There were 60% of test saplings where *B. xylophilus* could be re-isolated at the end of experiment when 100 nematodes were inoculated. This was less than the proportion of 85% – 90% at all other inoculation densities.

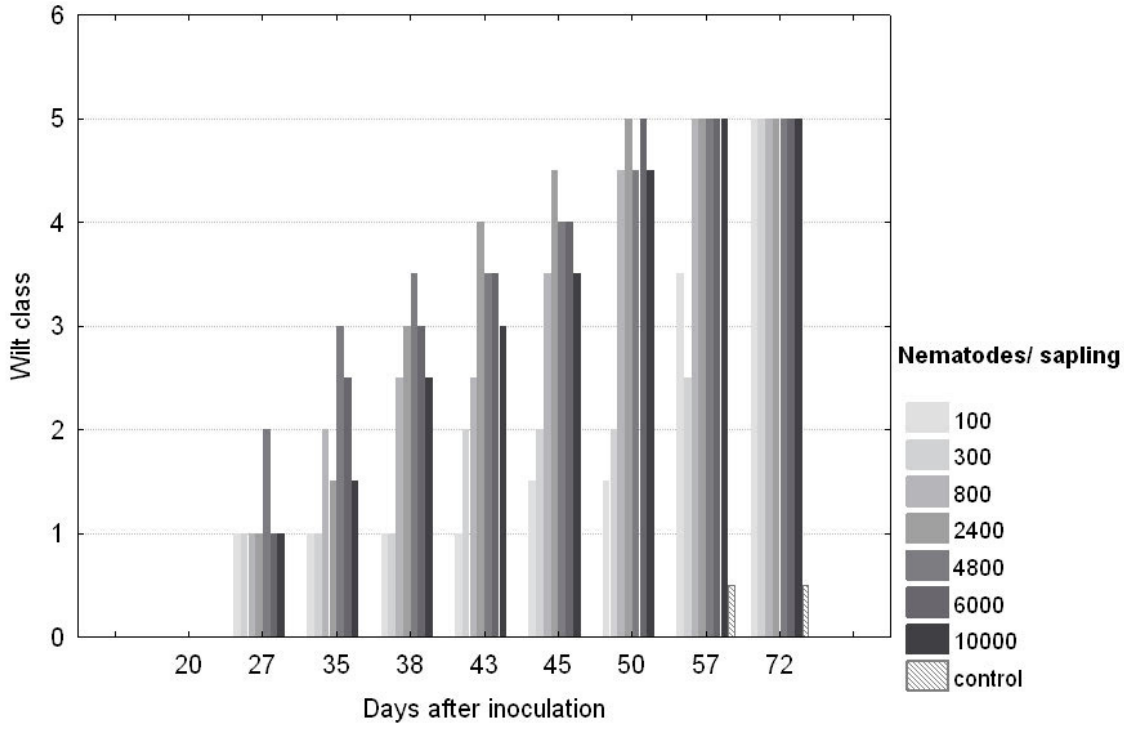


Fig. 3.1: Median of symptom development in *Pinus sylvestris* saplings after inoculation with *Bursaphelenchus xylophilus* with 100, 300,800, 2400, 4800, 6000 or 10000 nematodes per sapling and control (n = 10)

Tab. 3.3: Mortality rate of *Pinus sylvestris* and inoculation success achieved at different inoculum sizes of *Bursaphelenchus xylophilus*

Variation	Mortality in %	Inoculation Success in %
100	70	60
300	70	85
800	80	90
2400	90	95
4000	80	90
6000	90	95
10000	90	95
control	0	

n = 10

The relative water content of the wood of the healthy saplings in the control variation was above 70 % (Tab. 3.4). Infestation with *B. xylophilus* reduced the relative water content. Reduction of water content to around 30% was highest at 2400 nematodes per sapling followed by inoculation density at 4000 nematodes.

Tab. 3.4: Mean relative water content and standard deviation (SD) detected in wood of *Pinus sylvestris*, 12 weeks after inoculation with 100, 300, 800, 2400, 4000, 6000 and 10000 *Bursaphelenchus xylophilus* per sapling

	H_2O in %	
Variation	Mean	SD
100	48.2	18.2
300	45.8	17.7
800	44.6	12.9
2400	30.2	11.8
4000	38.5	14.6
6000	43.3	17.2
10000	39.3	13.1
Control	72.8	6.9

n = 10

3.2.2 Population development as effected by different inoculation densities

Nematodes were extracted from *Pinus sylvestris* saplings at two different sampling times. One group of 10 saplings was sampled after four weeks considering the time it takes for population build up. A second group of another 10 saplings was sampled at plant death (assessed with wilt class five) or if they survived at the end of experiment to confirm presence of *B. xylophilus* in those saplings. Inoculation success was determined as the proportion of sapling that carried nematodes in relation to the number of saplings inoculated.

Taking all saplings (n=20) that were inoculated inoculation success in each inoculation level was high. Inoculation success in the inoculation density of 100 nematodes per sapling was low (60 %), when compared to 90 to 95 % at the higher inoculation densities.

Population development of *B. xylophilus* in *P. sylvestris* gave contrary results between both sampling times (Fig. 3.2 – 3.3). The number of nematodes that was extracted from the roots and shoots after four weeks increased with inoculation density until an inoculation density of 6,000 nematodes per sapling and then decreased. Population densities of nematodes extracted after four weeks were equivalent between inoculation densities of 800 and 10000 nematodes per sapling. The overall population densities of *B. xylophilus* in shoots and roots in saplings that died were comparable between the inoculation densities. The highest median number of nematodes was below 1000 nematodes per gram dry matter. Saplings that survived at the end of experiment contained less than 50 nematodes per gram dry matter or were not present. Population densities in general showed a high variability at both sampling dates

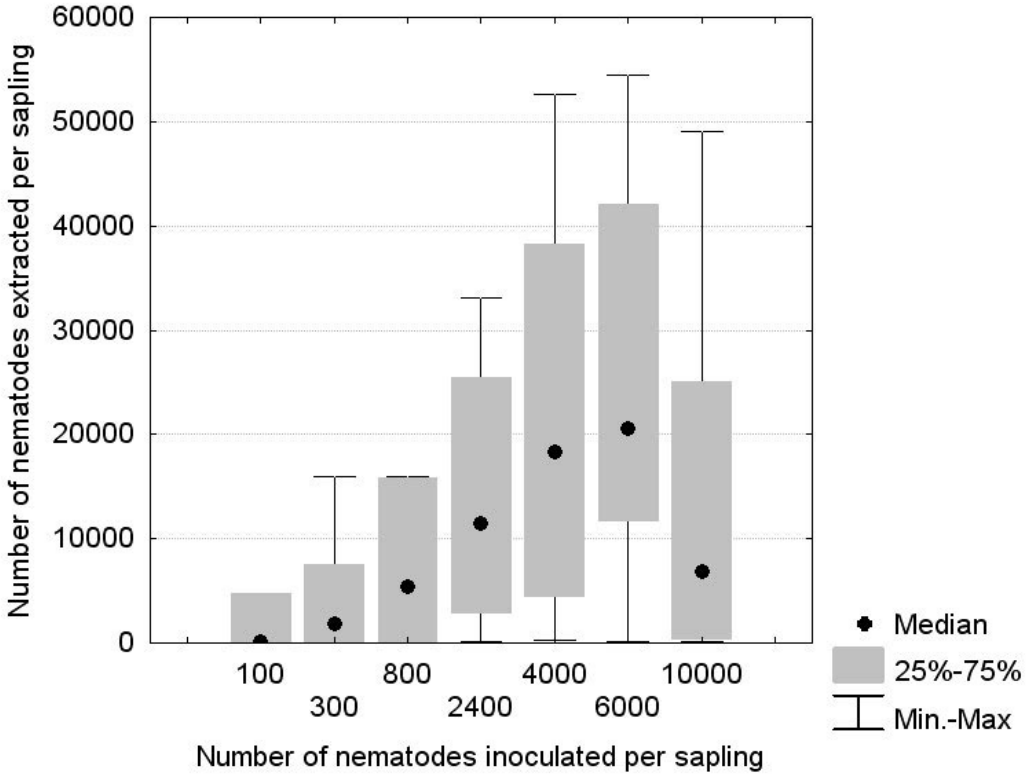


Fig. 3.2: Median of total number of *Bursaphelenchus xylophilus* extracted four weeks after inoculation from roots and shoots of *Pinus sylvestris* (n = 10)

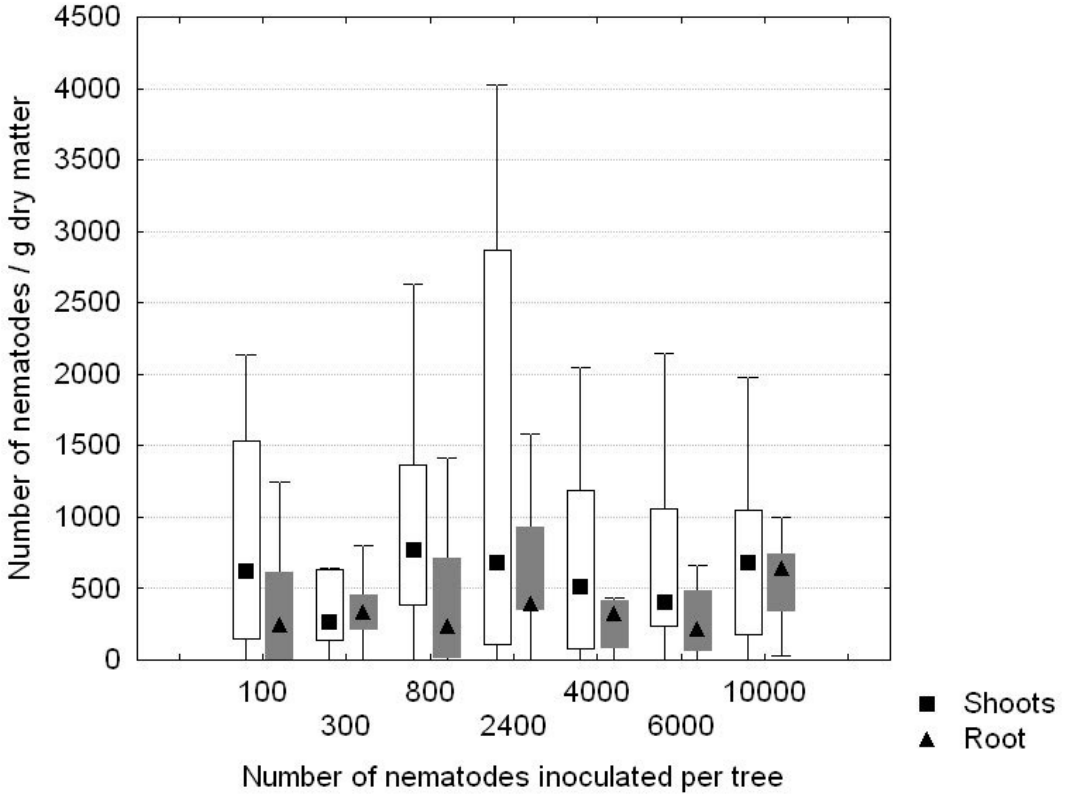


Fig. 3.3: Median of number of *Bursaphelenchus xylophilus* extracted from roots and shoots of *Pinus sylvestris* of dead saplings.

3.3 Pathogenicity of *Bursaphelenchus xylophilus* isolates towards European conifers

3.3.1 Development of Pine Wilt Disease

Differences of sensitivity of 13 European conifer species towards the *B. xylophilus* isolates PT 3 (w) from Portugal, Ne 12/2 from China and US DE 2 (w) from North America were assessed based on plant mortality within a period of 12 weeks.

According to their mortality (Tab. 3.5) three qualitative groups can be differentiated: (1) a highly sensitive group comprised of *Pinus sylvestris*, *Pinus nigra*, *Pinus cembra* and *Larix decidua* with mortality-levels of 100 %, (2) a moderate to highly sensitive group including *Pinus mugo*, *Pinus strobus*, *Pinus pinaster*, *Pinus radiata* and *Larix kaempferi*

with mortality rates ranging from 55 % to 100 % and (3) an insensitive group including *Picea abies*, *Abies alba* and *Pinus halepensis* ranging from 0 % to 15 % mortality.

Tab. 3.5: Mortality rate of 13 European conifer species inoculated with three isolates of *Bursaphelenchus xylophilus* from North America - US DE 2 (w), China – Ne 12/02 and Portugal - PT 3 (w) after 12 weeks

		Bursaphelenchus xylophilus Isolates		
Tree species	Control	North America	China	Portugal
		US DE 2 (w)	Ne 12/02	PT 3 (w)
		Mortality in % of 20 saplings		
Pinus nigra	0	100	100	100
Pinus sylvestris	5	100	100	100
Pinus cembra	0	100	100	100
Larix decidua	15	100	100	100
Pinus strobus	0	90	100	95
Pinus radiata	5	95	75	95
Pinus pinaster	0	90	95	75
Pinus mugo	0	85	80	55
Pinus pinea	10	10	25	60
Larix kaempferi	5	55	85	60
Pinus halepensis	0	5	0	15
Abies alba	10	5	0	0
Picea abies	0	5	5	0

n = 20

A distinct difference in conifer species mortality between nematode isolates only occurred in *P. pinea* and *P. mugo*. In *P. mugo* the isolates Ne 12/2 (China) and US DE 2 (w) (North America) triggered mortality to a level of 80 – 85 %, whereas the isolate PT 3 (w) caused a distinctively lower mortality. In contrast, *P. pinea* showed 60 % mortality when inoculated with the isolate PT 3 (w) in comparison to 10 to 20 % mortality when inoculated with isolates Ne 12/2 and US DE 2 (w).

With the exception of the tolerant conifers *P. abies*, *A. alba* and *P. halepensis* wilt symptoms in general occurred two to three weeks after inoculation with *B. xylophilus* and increased over time. The first *L. decidua* saplings (three saplings) died three weeks after inoculation and 60 % to 75 % of the saplings exhibited severe to complete wilt (class four or five) after four weeks (Fig. 3.4). In comparison to *L. decidua,* the first *P. sylvestris* (Fig 3.5) sapling died later four to five weeks after inoculation and less saplings showed severe wilt (class four or five). The conifer species *P. mugo* and *P. pinea* that showed differences in mortality between nematode isolates also showed different wilt patterns between the isolates (Fig. 3.6 – 3.7). Wilt in the control variations of each conifer species rarely exceeded class one to two.

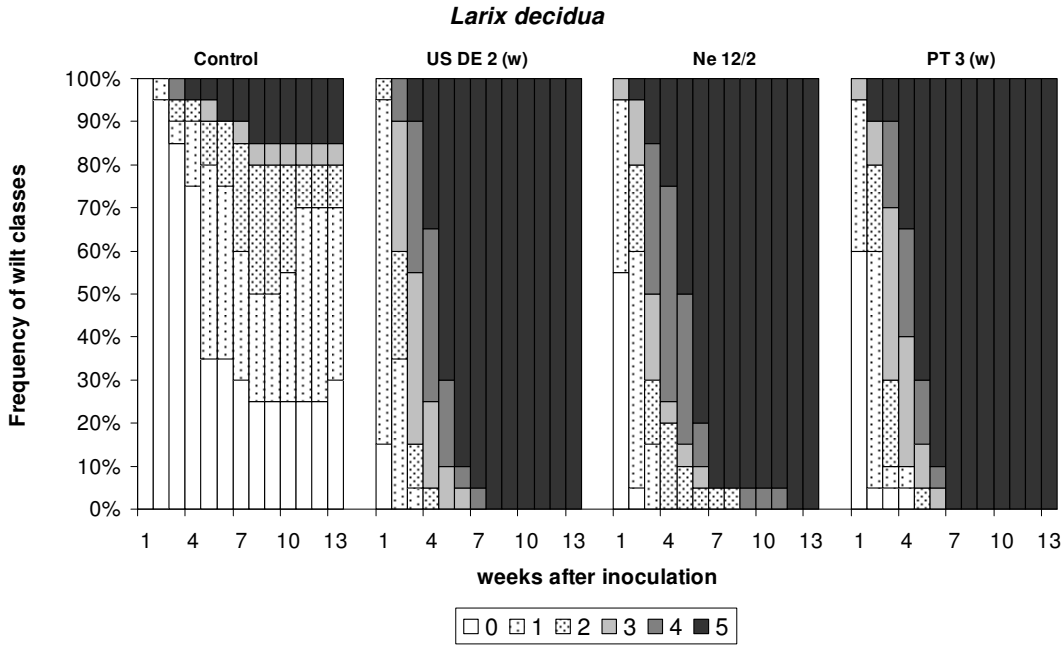


Fig. 3.4: Frequencies of wilt classes of *Larix decidua* (0: no symptoms and wilt class: 1 - 5) inoculated with *Bursaphelenchus xylophilus* isolates from North America -US DE 2 (w), China - Ne 12/02 and Portugal - PT 3 (w).

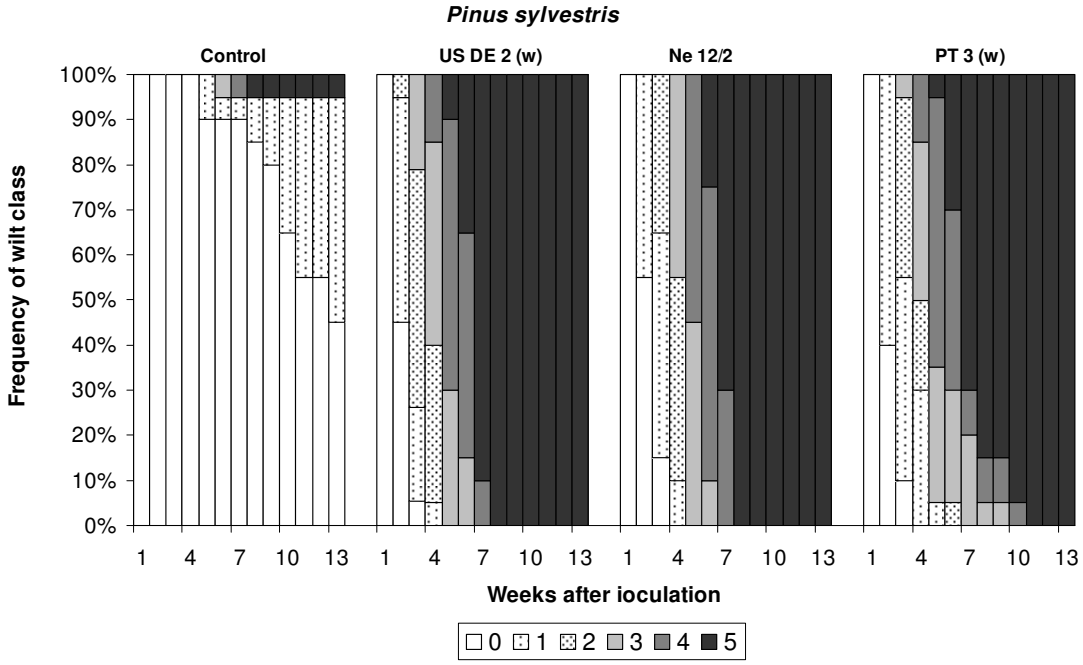


Fig. 3.5: Frequencies of wilt classes of *Pinus sylvestris* (0: no symptoms and wilt class: 1 - 5) inoculated with *Bursaphelenchus xylophilus* isolates from North America -US DE 2 (w), China - Ne 12/02 and Portugal - PT 3 (w)

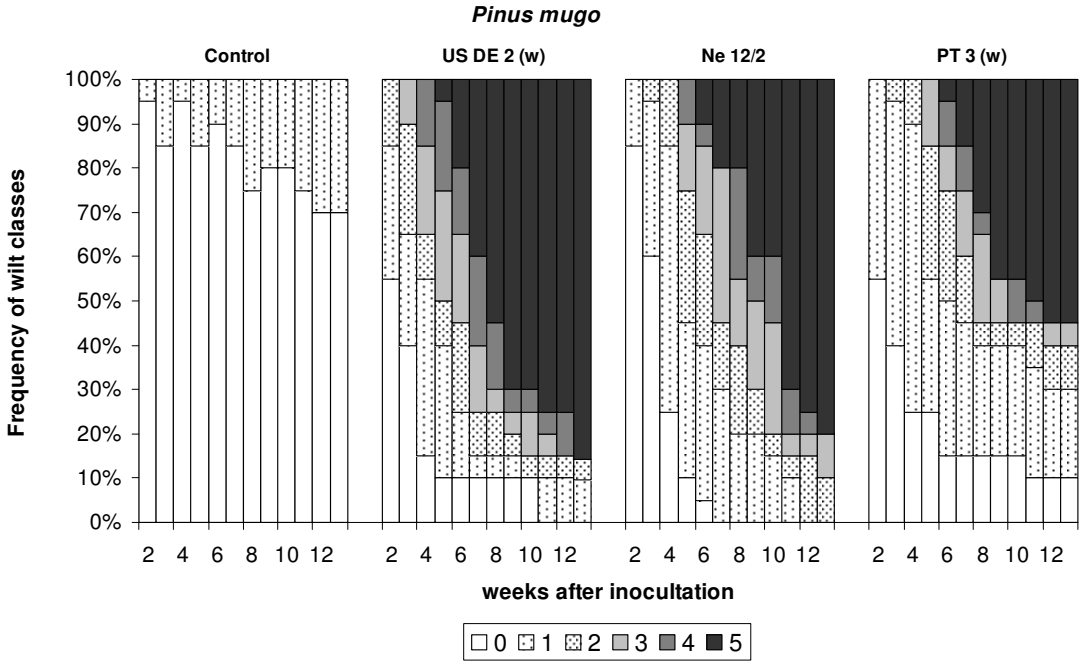


Fig. 3.6: Frequencies of wilt classes of *Pinus mugo* (0: no symptoms and wilt class: 1 - 5) inoculated with *Bursaphelenchus xylophilus* isolates from North America -US DE 2 (w), China - Ne 12/02 and Portugal - PT 3 (w)

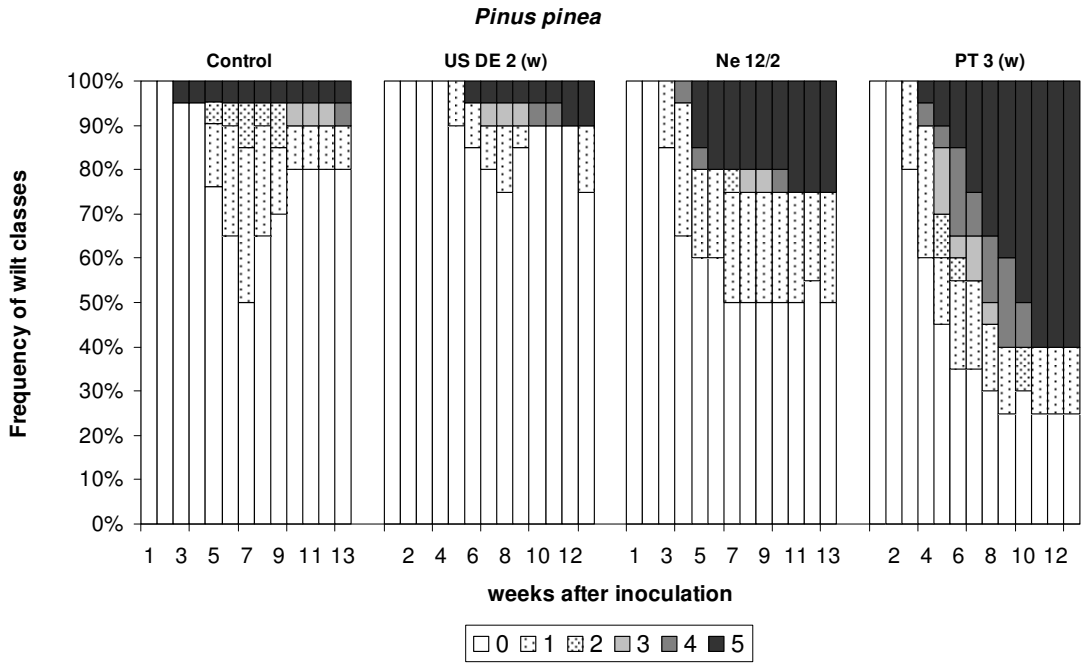


Fig. 3.7: Frequencies of wilt classes of *Pinus pinea* (0: no symptoms and wilt class: 1 - 5) inoculated with *Bursaphelenchus xylophilus* isolates from North America -US DE 2 (w), China - Ne 12/02 and Portugal - PT 3 (w)

3.3.2 Development of nematode population densities

B. xylophilus could not be identified in extractions from saplings of the control variation of the respective conifer species. Likewise *A. alba* saplings which were inoculated did not carry *B. xylophilus* after four weeks or after saplings died (Tab. 3.6). *B. xylophilus* was extracted in densities below 10 nematodes per gram fresh matter from *P. halepensis* and *P. abies*. All other tree species carried *B. xylophilus* isolates in higher numbers after four weeks as well as after death.

In general, the highly sensitive hosts *P. sylvestris*, *P. nigra*, and *L. decidua* contained a lower population density ranging from a median of 263 to 801 nematodes per gram dry matter than the less sensitive species *P. strobus* or *P. pinaster* inhabiting a range of 944 to 2,985 nematodes per gram dry matter four weeks after inoculation.

Tab. 3.6: Population densities of *Bursaphelenchus xylophilus* the isolates from Portugal - PT 3 (w), China - Ne 12 /02 and North America - US DE 2 (w) extracted four weeks after inoculation from shoots of 13 European conifer species

		Nematodes / g fresh matter		
		PT 3 (w)	Ne 12 /02	US DE 2 (w)
Pinus sylvestris	Median	647.83	423.21	664.74
	Min	96.23	170.31	578.81
	Max	2,062.98	1,077.54	753.16
Pinus nigra	Median	426.27	796.78	801.44
	Min	218.85	538.79	509.20
	Max	1,639.97	1,013.68	1,183.48
Pinus strobus	Median	1,135.94	2,110.56	1,534.05
	Min	568.00	1,446.81	474.75
	Max	1,492.45	4,053.19	2,873.42
Pinus mugo	Median	134.08	481.01	86.06
	Min	53.54	4.48	10.08
	Max	696.97	995.96	405.25
Pinus halepensis	Median	9.53	0.00	6.72
	Min	1.29	0.00	0.00
	Max	24.17	1.02	68.36
Pinus pinea	Median	26.98	7.47	2.42
	Min	11.41	0.92	0.00
	Max	47.32	17.45	54.56
Pinus pinaster	Median	944.19	1,063.52	2,985.43
	Min	663.04	541.22	1,439.56
	Max	1,352.70	1,621.40	5,185.19
Pinus radiata	Median	244.32	175.30	583.03
	Min	149.78	33.13	342.11
	Max	702.90	711.44	1,493.67
Pinus cembra	Median	5104.86	3,046.47	3,039.01
	Min	2,966.42	1,920.75	1,854.65
	Max	8,021.21	4,620.32	5,000.00
Larix decidua	Median	263.18	434.36	596.73
	Min	85.77	85.84	199.47
	Max	881.63	949.83	983.33

		Nematodes / g fresh matter		
		PT 3 (w)	Ne 12 /02	US DE 2 (w)
Larix kaempferi	Median	22.43	49.64	39.16
	Min	5.67	26.81	22.18
	Max	80.83	91.03	80.78
Picea abies	Median	3.06	1.25	4.25
	Min	0.00	0.00	1.63
	Max	3.93	2.11	15.21
Abies alba	Median	0.00	0.00	0.00
	Min	0.00	0.00	0.00
	Max	0.40	1.13	0.00

n = 4 (each replication consists of five saplings)

Nematode numbers are divided by the inoculated isolate and related to the median of four replications (consisting of five trees each) and respective Minimum and Maximum

The lowest population density after four weeks was detected in the species *L. kaempferi* and *P. mugo* ranging from 22 to 134 nematodes. *P. cembra* contained the highest nematode density ranging from a median of 3039 to 5104 nematodes in comparison to all other conifer species after four weeks.

Population densities among the three isolates in general were not significantly different after four weeks. In *P. pinea* slight differences occurred in the population densities of the three isolates. Dead saplings inoculated with the isolate Ne 12/2 carried 299 nematodes per gram fresh matter. If inoculated with the isolate PT 3 (w) dead saplings carried 150 nematodes per gram fresh matter and if inoculated with the isolate US DE 2 (w) saplings only contained 31 nematodes per gram fresh matter.

Looking at saplings that were dead (wilt class five) the following situation was detected (Tab. 3.7). In dead saplings population densities of *B. xylophilus* isolates in *P. sylvestris* and *L. decidua* saplings remained at a comparable level in comparison to densities in living saplings after four weeks.

Tab. 3.7: Population densities of *Bursaphelenchus xylophilus* extracted from a number (n) of dead saplings of conifer species after inoculation of three isolates: Portugal - PT 3 (w). China – Ne 2 /02 and North America - US DE 2 (w)

		Nematodes / g Fresh Matter					
		PT 3 (w)	n	Ne 12 /02	n	US DE 2 (w)	n
Pinus sylvestris	Median	546.7	20	658.7	20	642.08	20
	Min	36.6		50.8		17.70	
	Max	2,385.7		3,137.7		2,086.29	
Pinus nigra	Median	2,660.56	20	11,30.21	20	1,214.51	20
	Min	375.00		111.11		152.54	
	Max	7,110.62		8,976.00		5,039.60	
Pinus strobus	Median	1,641.03	17	5,157.89	20	1,611.51	18
	Min	362.20		43.48		168.42	
	Max	3,283.58		5,157.89		6,587.30	
Pinus mugo	Median	925.93	11	871.79	15	1,250.00	15
	Min	191.49		105.26		442.31	
	Max	2,000.00		1,582.09		6,465.75	
Pinus pinea	Median	155.96	9	299.23	4	30.52	2
	Min	7.94		166.67		13.70	
	Max	518.87		545.02		47.34	
Pinus pinaster	Median	1,440.00	14	1,525.60	20	3,088.81	18
	Min	250.00		142.86		222.22	
	Max	3,958.33		4,290.32		12,931.03	
Pinus radiata	Median	416.10	20	351.85	15	126.40	18
	Min	44.44		62.50		25.00	
	Max	1,777.78		3,396.83		958.33	
Pinus cembra	Median	455.18	18	849.06	19	1,129.03	17
	Min	69.44		83.33		90.91	
	Max	8,758.62		4,307.69		6,000.00	
Larix decidua	Median	216.22	17	168.67	20	568.63	19
	Min	16.95		36.36		19.23	
	Max	913.04		800.00		3,657.53	
Larix kaempferi	Median	216.22	11	168.67	18	568.63	12
	Min	16.95		36.36		19.23	
	Max	913.04		800.00		3,657.53	

In contrast population densities in *P. nigra* were higher and in *P. cembra* clearly lower in dead saplings than in living sapling after four weeks. Population densities of *B. xylophilus* were higher in dead saplings in *P. mugo*, *P. pinea*, *P. pinaster* and *L. kaempferi* in comparison to densities four weeks post inoculation. Saplings that showed only partial wilt contained negligible numbers *of B. xylophilus* after 12 weeks.

The min- max span of nematode population densities was wider in dead saplings than in those sampled after four weeks.

3.4 Invasion biology of *Bursaphelenchus xylophilus* in *Pinus sylvestris*

3.4.1 Migration and distribution

Population densities of *B. xylophilus* determined for 17 segments of *Pinus sylvestris* (chapter 2.5.1) were calculated on the basis of one gram of dry matter. The average dry weight of segments was calculated from 10 saplings at nine sampling dates (Tab. 3.8).

Tab. 3.8: Mean dry weight and standard deviation of segments computed from the total number samples

	Dry Weight in g				
Segments*	Mean	SD	Segments*	Mean	SD
MS 1	0.32	0.10	Sb 3	0.62	0.12
MS 2	0.25	0.08	Sb 4	1.63	0.46
uB 1	0.74	0.23	IB 1	0.90	0.31
uB 2	0.60	0.16	IB 2	0.67	0.23
uW	0.14	0.07	IW	0.46	0.14
Sai	0.12	0.06	Sr	1.49	1.03
Ip	0.15	0.06	RC	1.37	0.39
Sb 1	0.14	0.09	R	1.74	0.54
Sb 2	0.58	0.11	Sum	11.92	

n = 90

* MS 1 +2 (main shoot). uB 1+2 (upper branches). uW (upper whorl). Sa (Stem above inoculation point). IP (inoculation point). Sb 1-4 (Stem below inoculation point). IB 1+2 (lower branches). IW (lower whorl). Sr (Stem above root collar). RC (root collar). R (roots)

Most segments had dry weights below one gram. There were only four segments (´Sb4´, ´Sr´, ´Rc´ and R) that together showed a range in between 1.37 g to 1.74 g which made up the major weight proportion of the whole saplings. Corresponding to natural deviations in growth of individual saplings, the deviation of dry weight among the segments appeared to be high.

The distribution of *B. xylophilus* in *Pinus sylvestris* was determined for each of the 17 segments per sapling. The population densities of the segments were measured at nine sampling dates. Distribution of nematodes in saplings was displayed utilizing tree diagrams that stand for the natural arrangement of segments. Each diagram stands for one sampling date. The population density within each segment is represented by key colours (see Fig. 3.8). Altogether five categories cover the overall span of the population densities that were defined with: 0, < 50, 51-500, 501-2000 and > 2000 nematodes / g dry matter. Without exception population densities were expressed as median values. A reference table containing all population densities and min-max spans of each segment at each sampling date is given in Appendix (Tab.A1 – A2). In general, the span between minimum and maximum values of population densities in segments deviated extremely between single saplings. This could be observed in initial samplings of the segments ´Sa, ´Ip´ and ´Sb1´ and from day twelve after inoculation onwards in the rest of the segments.

Early migration and distribution

Two days after inoculation *B. xylophilus* were recovered in the host with 33 nematodes /g dry matter with of range in between 27 to 45 nematodes /g dry matter. Most nematodes were still concentrated at the inoculation point (Ip) two days after inoculation with 1500 nematodes /g dry matter and its neighbouring segments with 130 and 280 nematodes /g dry matter, whereas densities in the other segments remained clearly below 50 nematodes /g dry matter. The lower branches, ´lB1´ and ´lB2´, as well as root parts (´R´ and ´Rc´) were not invaded two days after inoculation. During the first week nematodes mainly migrated into neighbouring parts, starting from the inoculation point and dispersed further downwards in the saplings. Six days after inoculation nematodes were present throughout the entire sapling.

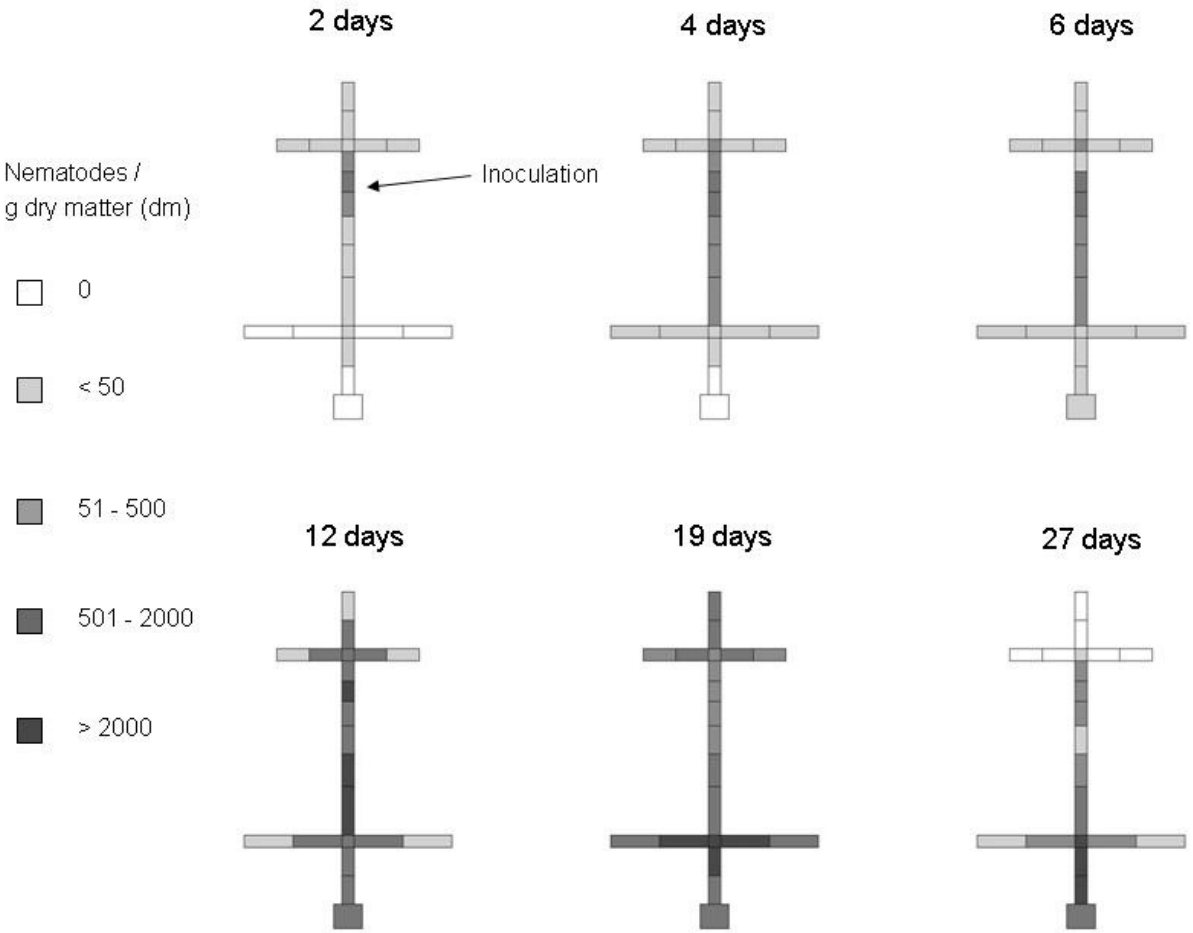


Fig. 3.8 Distribution of *Bursaphelenchus xylophilus* in 17 segments of 3-4 year old *Pinus sylvestris* saplings presented for 6 dates at a 27 day period after inoculation at 25 °C; Tree diagrams show the position of segments in the sapling; Population densities of nematodes (median) presented in grey color charts in categories 0< 50, 51-500, 501-2000 and > 2000, n=10

Once the nematodes passed barriers like the upper whorl ´uW´, nine days after inoculation, they could establish higher densities in the crown parts (´uW´, ´uB 1-2´ and ´MS 1-2´). Considering maximum values two days after inoculation (Appendix, Tab. A1 - A2) there were 3 of 10 saplings that contained nematodes in the terminal part of the main shoot ´MS 1´as well as in the root collar ´RC´.

Population build up

Having invaded all segments nine days after inoculation, nematode densities increased in all segments, which resulted in a density of 1820 nematodes /g dry matter in shoots and roots. The main population growth in shoots and roots was detected between day 9 and day 12 after inoculation. This overall population growth occurred simultaneously in all segments. Population growth in the main stem including the inoculation point of the sapling (segments: Sa, Ip, Sb 1-4) attained its maximum and reproduction was stopped after 12 days. Termination of population growth in the segments of the crown parts (´MS 1-2´, ´uB1-2´) and the lower branches occurred 19 days after inoculation. At the same time the highest nematode density was achieved in the root collar with 7177 nematodes /g dry matter, followed by the stem above root collar ´Sr´ with 3301 nematodes /g dry matter, basis of the main shoot ´MS 1´ with 2003 nematodes /g dry matter and lower whorl ´lW´ with 2109 nematodes /g dry matter.

Retreat of nematodes and concentration

After 27 days a distinctive polarization in the distribution of nematodes occurred. Whereas nematodes were undetectable in the crown and only appeared in low densities around the inoculation point they were heavily concentrated in the basis of the sapling. This was the area, which was formed by the lower whorl ´uW´, all segments around the lower whorl, the root collar ‘RC’ and stem above the root collar ‘Sr’. The stem above the root collar at this final date showed a density of 5073 nematodes /g dry matter.

Change of centre of population

Two regions of the sapling were defined:

(1) ‘Plant basis’ consisting of root segments (´RC´, ´R´and ´Sr´)

(2) ‘Shoots’ (Rest of segments without ´RC´, ´R´ and ´Sr´).

The proportion of *B. xylophilus* in each region and total number of nematodes per sapling is given in Fig. 3.9.

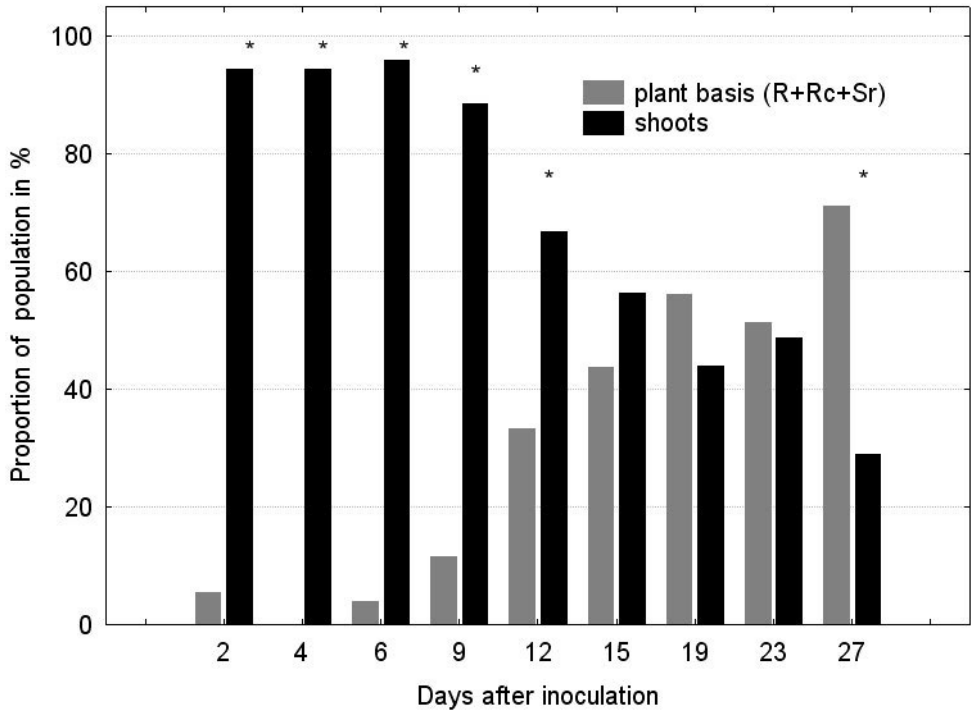


Fig. 3.9: Proportion of *Bursaphelenchus xylophilus* population after inoculation in *Pinus sylvestris* saplings in the plant basis (stem above root collar, root collar and roots) and shoots of saplings at 25 °C; * significant differences (Wilcoxin-test, $\alpha = 0.05$)

All proportions in the respective plant parts at each sampling date but not between sampling dates were tested by Wilcoxin test at $\alpha \leq 0.05$. Until day nine more then 90 % of the nematodes were distributed in 'shoots'. Differences between the proportions of both regions were significant before and after the overall population density per sapling was at its maximum with 36.809 nematodes in between day 9 and 12. The proportion in 'shoots' gradually decreased to 70 % after 12 days and dropped to 44 % after 19 days. At the end of the experiment, 27 days after inoculation the 'plant basis' contained a significantly higher proportion of nematodes (71 %) than the 'shoots'.

3.4.2 Population dynamics

Population dynamics of *B. xylophilus* in pines were related to certain parts of the host trees with regard to the development of the nematode population in root parts or shoots.

Shoots

The population dynamics of nematodes per gram dry matter in the host trees in shoots is given in Fig. 3.10 a-b. The population in shoots showed three distinctive peaks 12 days (1840 nematodes /g dry matter), 19 days (2431 nematodes/g dry matter) and 27 days after inoculation (1569 nematodes /g dry matter).

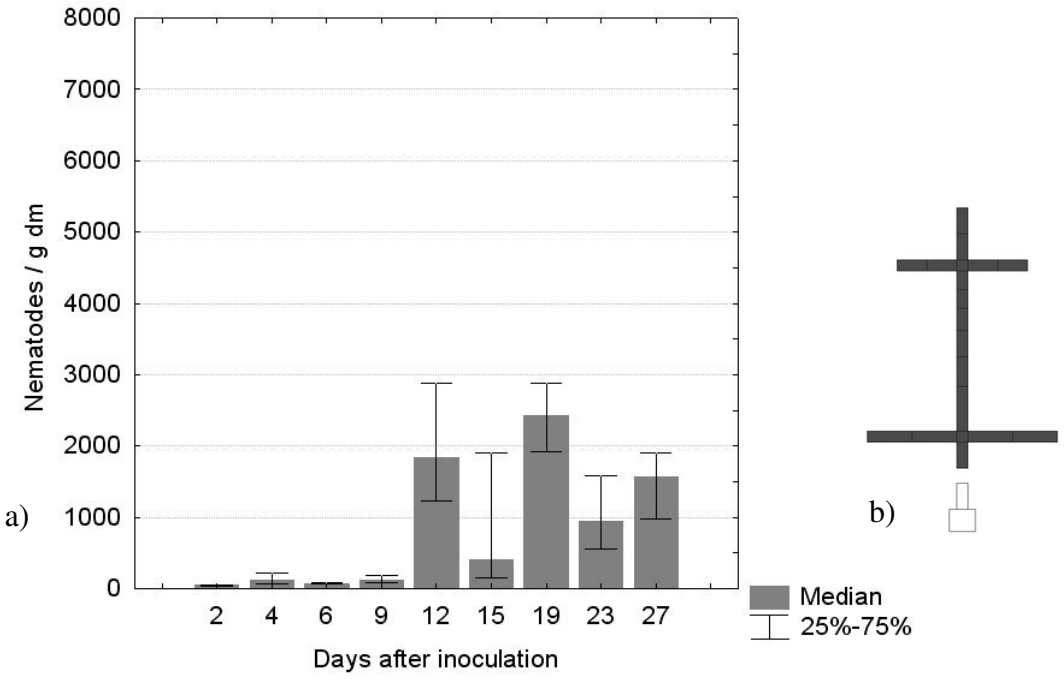


Fig. 3.10a-b a) Population dynamics of *Bursaphelenchus xylophilus* in shoots of three to four year old *Pinus sylvestris* saplings detected as median of number of nematodes / g dry matter during a 27 day period after inoculation at 25 °C, n = 10, b) Segments of the sapling to which population dynamics is related

Roots

The population dynamics of nematodes per gram dry matter in the host trees in root parts is given in Fig. 3.11 a-b. The population in roots grew continuously until nematode densities reached a peak of 4915 nematodes /g dry matter, day 19 after inoculation. This was roughly two weeks after they were observed in roots at day six. The population declined again at day 23. However another peak at day 27 (2093 nematodes /g dry matter), which was moderately higher than achieved in shoots was detected.

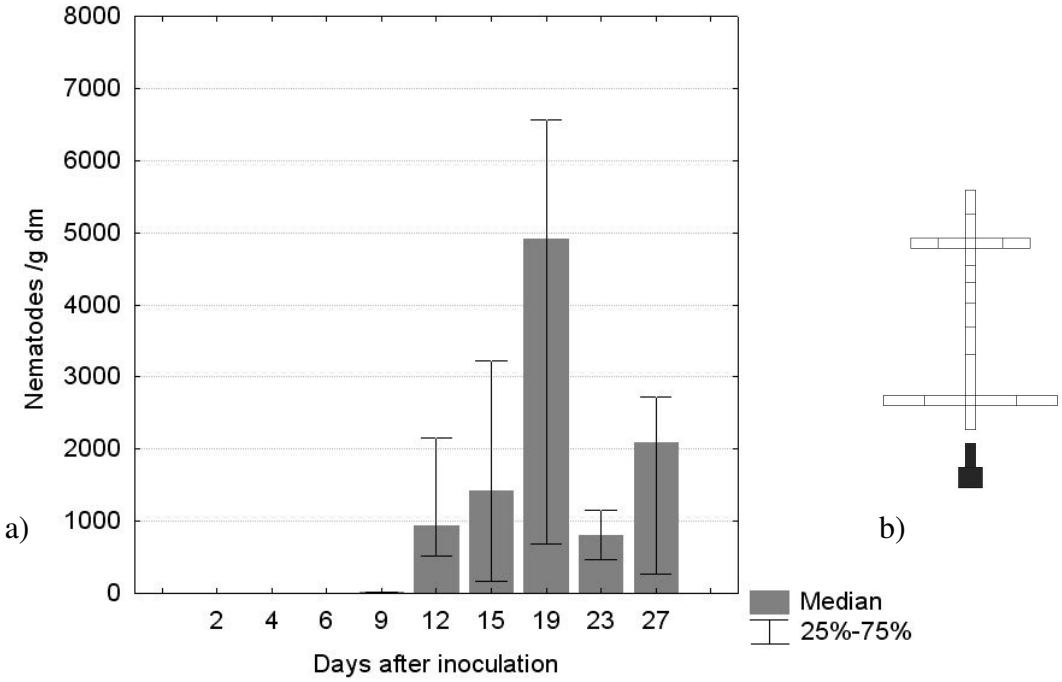


Fig. 3.11a-b: a) Population dynamics of *Bursaphelenchus xylophilus* in roots of three to four year old *Pinus sylvestris* saplings detected as median of number of nematodes / g dry matter during a 27 day period after inoculation at 25 °C, n = 10,b) Segments of the sapling to which population dynamics is related

Regions in the sapling

Different segments of the sapling independently showed similar population dynamics of *B. xylophilus*, but at slightly deviating periods during the experiment. Altogether three regions could be identified that distinctively showed similar population dynamics (Fig. 3.12 a-b – 3.14 a-b).

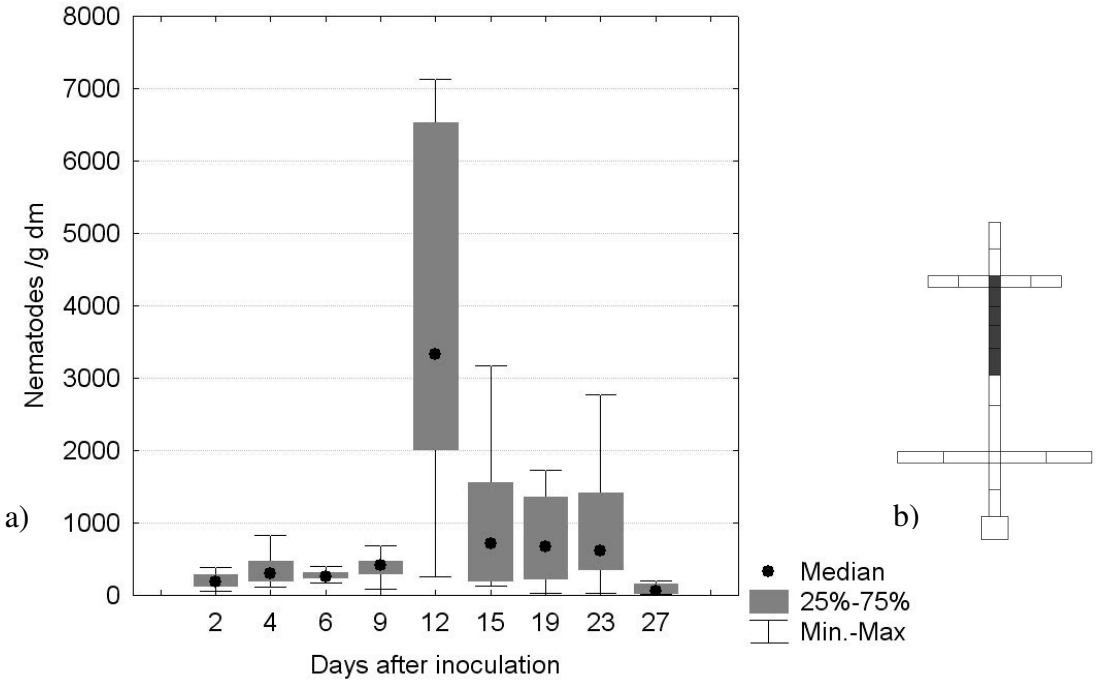


Fig. 3.12 a-b: a) Population dynamics of *Bursaphelenchus xylophilus* in the area around the inoculation point of *Pinus sylvestris* saplings detected as median of number of nematodes / g dry matter during a 27 day period after inoculation at 25 °C, n = 10; b) Position of segments around the inoculation point

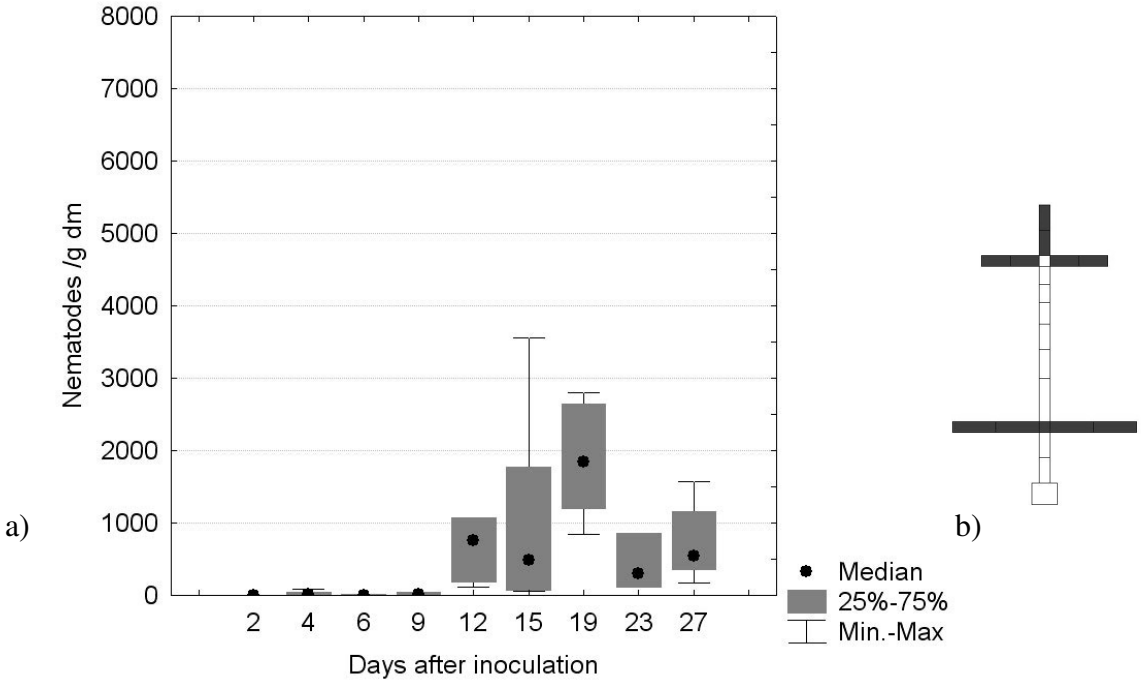


Fig. 3.13 a-b: a) Population dynamics of *Bursaphelenchus xylophilus* in the upper and lower branches of *Pinus sylvestris* saplings detected as median of number of nematodes / g dry matter during a 27 day period after inoculation at 25 °C, n = 10; b) Position of segments of upper and lower branches

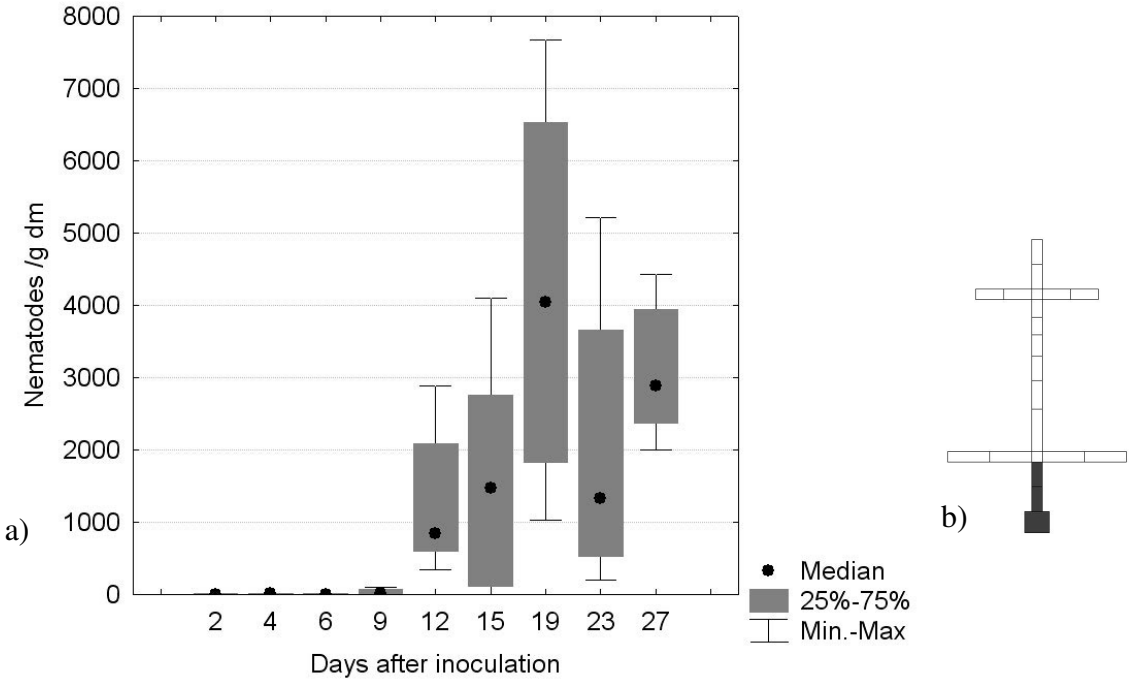


Fig. 3.14 a-b: a) Population dynamics of *Bursaphelenchus xylophilus* in the stem basis, root collar and roots of *Pinus sylvestris* saplings detected as median of number of nematodes / g dry matter during a 27 day period after inoculation at 25 °C, n = 10; b) Position of stem basis, root collar and root segments

Region 1: The maximum population was reached 12 days after inoculation in the stem segments around the inoculation point. This was the only distinctive peak (3331 nematodes /g dry matter) that occurred in this region during the experiment (Fig. 3.12 a-b).

Region 2: A second population maximum 19 days after inoculation was detected in the crown parts and the area around the lower branches. Nematode density in these segments reached 1846 nematodes /g dry matter. A slight rise in densities was also observed for day 12 (752 nematodes /g dry matter) and day 27 (541 nematodes /g dry matter) but not as clearly as on day 19 (Fig. 3.13 a-b).

Region 3: The last increase in nematode population was detected in the plant basis (root parts and stem above root collar). Unlike the other regions there were two peaks: A maximum at day 19 (4043 nematodes /g dry matter) and again at day 27 (2892 nematodes /g dry matter). None of the other segments or their aggregations showed this

third peak in the nematode population as clear as this region. When comparing the plant basis to the crown parts plus lower branches the second population peak was more distinctive in the basis (Fig. 3.14 a-b).

3.4.3 Relative water content in shoot parts and needles

Two fractions were defined: Stem basis (segment 'Sr') and shoots (aggregated segments MS 1-2, uB 1-2, Sb 2-4, lW and lB 1-2). The Chi^2 test was applied to median values of the relative water contents of both fractions: Stem basis and shoots (Tab. 3.9).

Tab. 3.9: Comparative test (χ^2 Test) between mean relative water content in the segments stem basis and shoots with branches of *Pinus sylvestris* at different days post inoculation with *Bursaphelenchus xylophilus*

	H_20 in (%)					
Days after inoculation	Shoots Mean ± SD		Stem basis Mean ± SD	*Chi²*	*FG*	*p*
2	68.58 ± 1.40		65.55 ± 5.74	5.41	8	0.73
4	66.34 ± 2.36		63.20 ± 2.28	1.60	7	0.97
6	66.70 ± 2.68		61.33 ± 6.03	8.48	9	0.49
9	68.98 ± 1.10		64.13 ± 2.15	3.82	9	0.92
12	58.20 ± 7.30		57.73 ± 3.79	7.12	9	0.62
15	55.00 ± 9.16		59.03 ± 11.18	18.24	9	0.33
19	33.11 ± 6.89	*	51.52 ± 10.33	153.38	9	0
23	29.57 ± 8.50	*	55.11 ± 17.39	407.62	9	0
27	24.82 ± 8.37	*	54.34 ± 6.70	441.58	9	0

* significant at $\alpha = 0.05$ n = 10

The relative water content of both fractions is shown in Fig. 3.15. Relative water content in shoots and Stem basis remained between 60 % and 70 % for the first 12 days after inoculation. From day 15 on the relative water content in shoots decreased from 55 continuously to 25 %. The relative water content in the stem basis decreased slightly and remained at a level above 50 %. From day 19 to 27, water content in stem basis was significantly higher than in shoots.

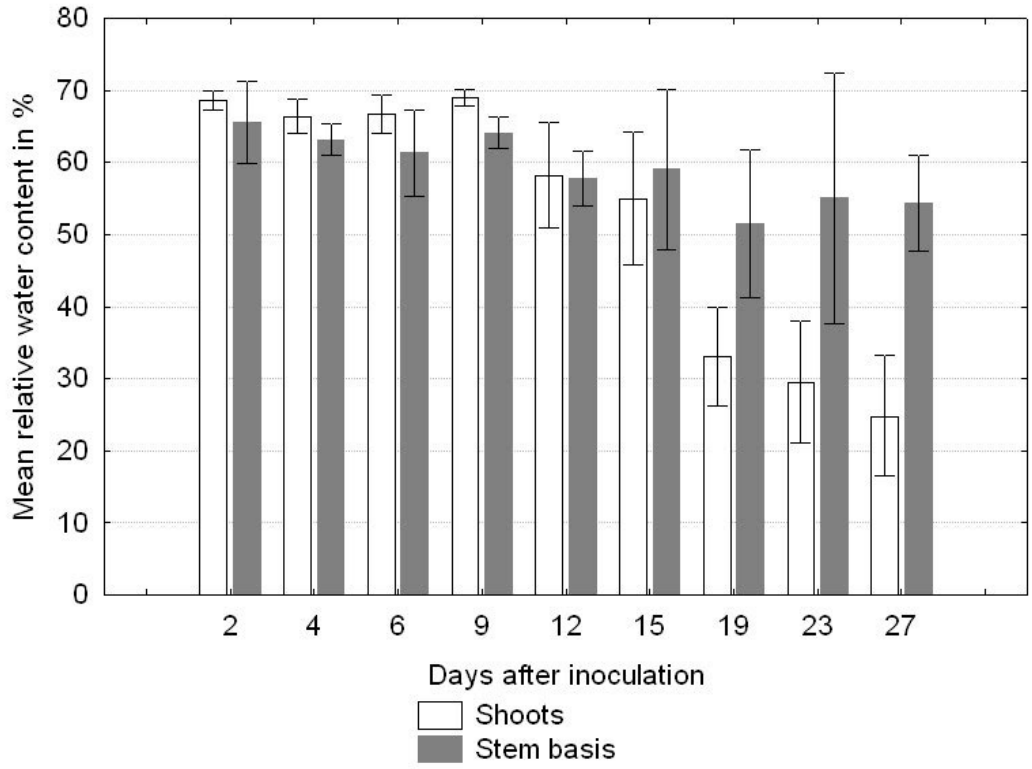


Fig. 3.15: Relative water content of wood in shoots and stem basis (Sr) of *Pinus sylvestris* saplings during a 27 day period after inoculation with *Bursaphelenchus xylophilus* at 25 °C, n = 10

The relative water content of young needles was about 10 % higher than in old needles, when focusing on the first period until day 12 after inoculation (Fig. 3.16). The difference increased from day 12 on as relative water content started to decrease stronger in old needles than in young ones. Old needles followed a similar trend in reduction water content like in wood. At the end of the experiment needles in general reached a relative water content of 14 % to 15 %. Within a period of 12 days (from day 15 to the end after 27 days) young needles in the crown part of the saplings lost about 50 % water and old needles roughly 40 % in the same period.

Although wilt classes were not assessed in this trial the first appearance of chlorotic needles was observed at day 12. Between day 19 and day 23, wilting rapidly progressed. Needles dried out and turned completely brown.

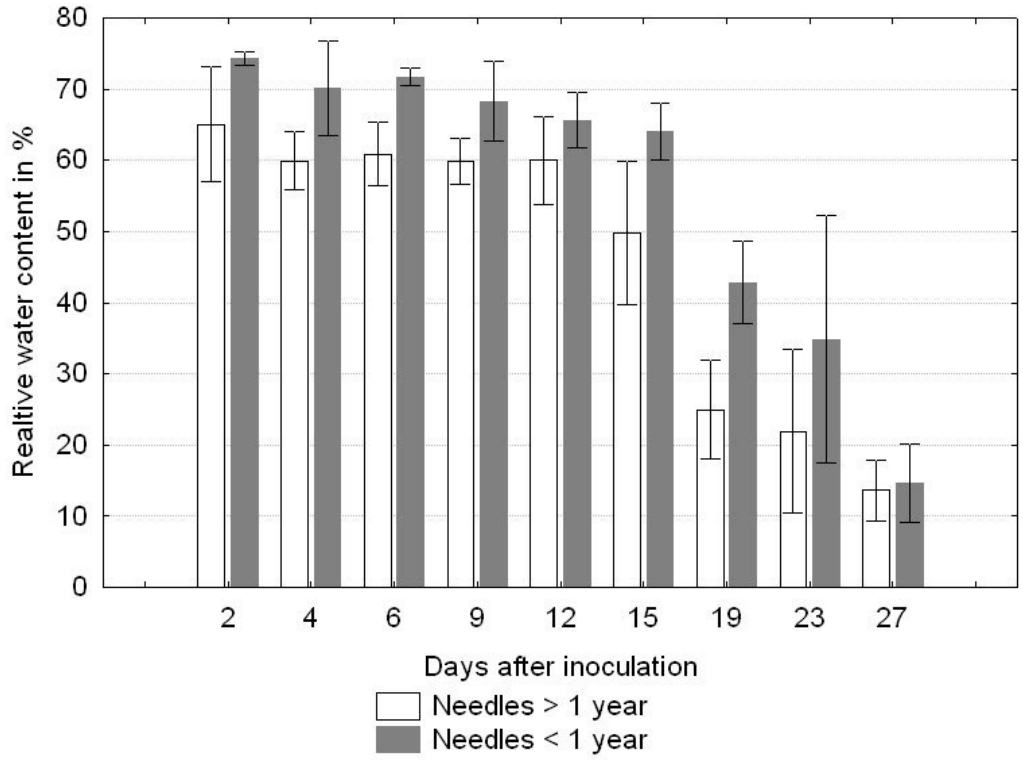


Fig. 3.16: Relative water content of young (< 1 year) and old (> 1 year) needles of *Pinus sylvestris saplings* during a 27 day period after inoculation with *Bursaphelenchus xylophilus* at 25 °C, n = 10

3.5 Influence of temperature on *Bursaphelenchus xylophilus* – host interaction

3.5.1 Wilt symptom development

Comparing final wilt classes 61 days after inoculation (Tab. 3.10), only *Pinus sylvestris* and *Larix decidua* showed significant differences in relation to the control at temperatures 20 °C and 25 °C. Saplings of both species at 15 °C did not develop distinctively different symptoms than the control saplings. *P. abies* also did not show any significant differences of wilt classes between inoculated saplings and controls at all temperatures.

Tab. 3.10: Final wilt classification as median of *Pinus sylvestris*, *Larix decidua* and *Picea abies* saplings achieved 61 days after inoculation with *Bursaphelenchus xylophilus*

Wilt classes (median) and χ^2 values 61 days after inoculation

	25 °C			20 °C			15 °C		
	B.x.	control	Chi ^{2}	B.x.	control	Chi ^{2}	B.x.	control	Chi ^{2}
Pinus sylvestris	5.0	0	112.0 *	5.0	0	84.2 *	2.5	1.0	32.0
Larix decidua	5.0	0	51.4 *	5.0	0	203.1 *	1.0	0	7
Picea abies	2.0	0	0	0.5	0	0	0	0	0

* significant differences at $\alpha = 0.05$, n = 10. FG = 19.; χ^2 test of differences between the distributions of wilt classes in the comparison between inoculation with *Bursaphelenchus xylophilus* and control at 61 days post inoculation, B.x. = *Bursaphelenchus xylophilus*

P. sylvestris

The medians of wilt classes of *P. sylvestris* saplings at the three temperatures in time are presented in Fig. 3.17. Saplings inoculated at 25 °C developed symptoms earlier than at 20 °C. Progression of wilt appeared similar between the both temperatures. As a consequence temperature had a clear effect on the development of symptoms of *P. sylvestris* comparing 15 °C on the one hand and the 20 °C and 25 °C variation on the other hand.

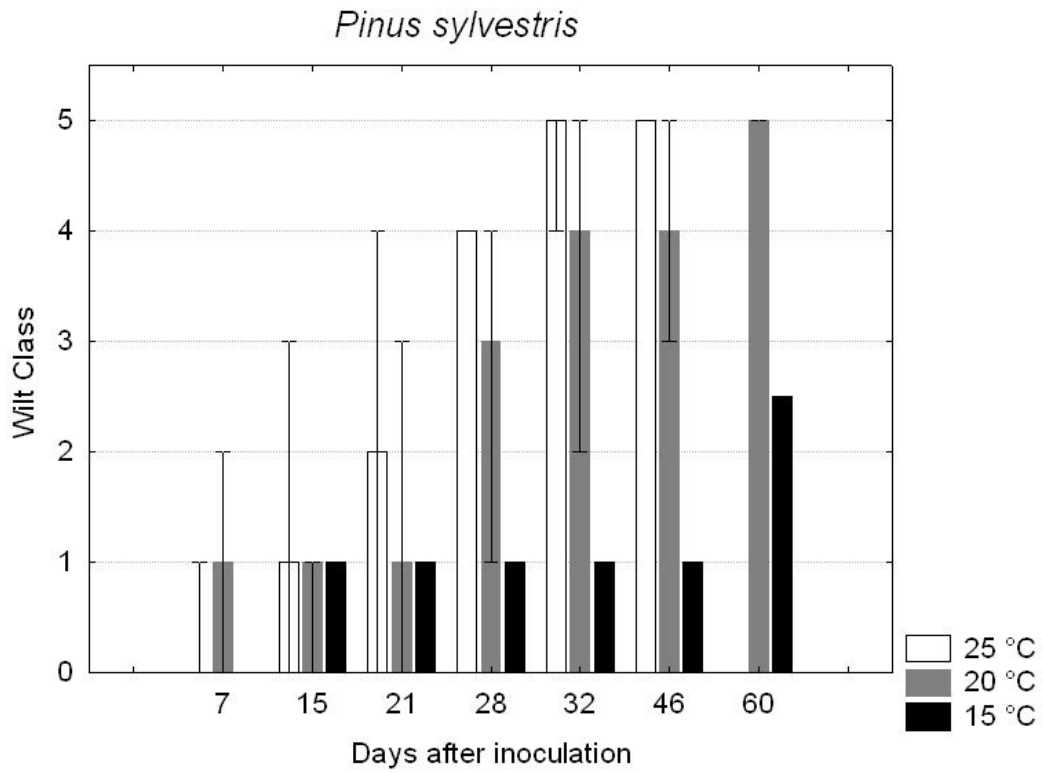


Fig. 3.17: Median of wilt classes (0 - 5) of *Pinus sylvestris* saplings inoculated with *Bursaphelenchus xylophilus* at 25 °C, 20 °C and 15 °C assessed during a 60 day period

L. decidua

Like in *P. sylvestris* the development of wilt appeared similar in *L. decidua* Fig. 3.18. There was no obvious reaction in wilting of *L. decidua* at 15 °C but severe wilt did occur at 20 °C as well as at 25 °C. Again saplings at 25 °C reached wilt class four, one week earlier then did saplings at 20 °C. At this temperature symptoms developed rapidly. No differences were detectable between 20 °C and 25 °C from day 28 on. Temperatures therefore had a distinct effect on wilting when comparing 15°C and the temperatures above 20°C.

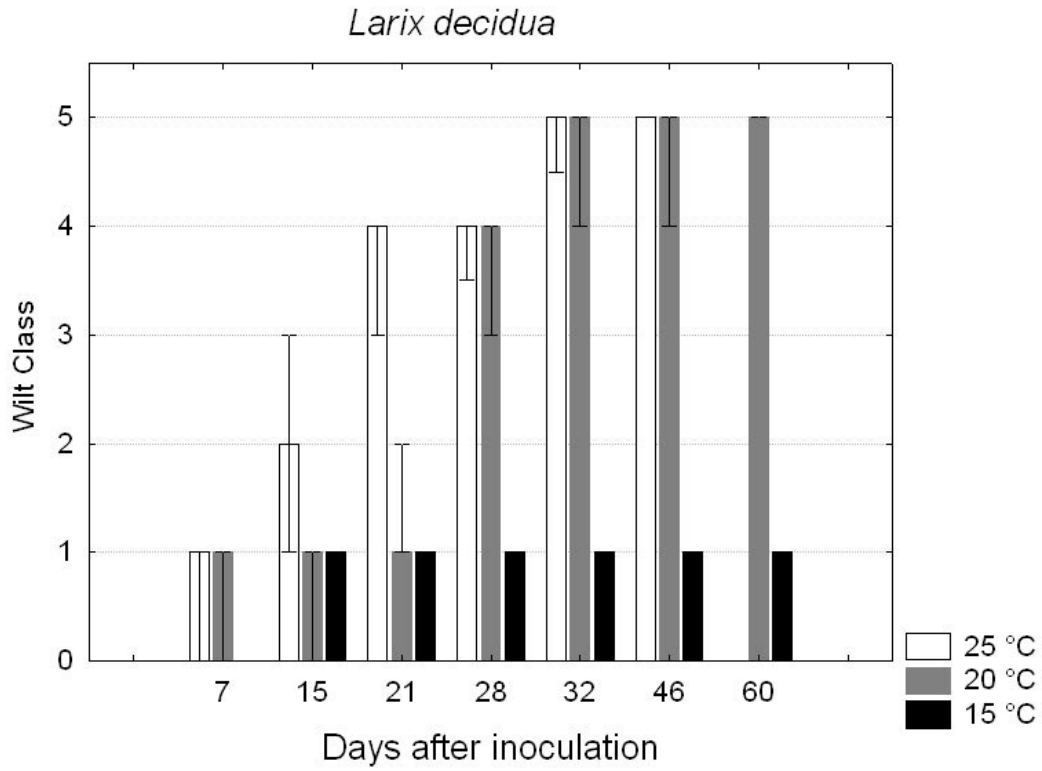


Fig. 3.18: Median of wilt classes (0 - 5) of *Larix decidua* saplings inoculated with *Bursaphelenchus xylophilus* at 25 °C, 20 °C and 15 °C assessed during a 60 day period

Picea abies

Picea abies showed a slight but insignificant development of wilt symptoms towards the end of the experiment at 25 °C. Temperature had no clear effect on wilting of *Picea abies*.

Comparing *P. sylvestris* and *L. decidua* that were inoculated with *B. xylophilus*, there was a tendency in *L. decidua* to develop symptoms earlier at both 20 °C and 25 °C than in pines. Furthermore *L. decidua* showed a similar development of symptoms between 20 °C and 25 °C, whereas symptoms of *P. sylvestris* at 20° C deferred from saplings at 25 °C.

3.5.2 Mortality

Mortality is presented as the number of saplings related to 20 reference saplings that reached wilt class five over time in Fig. 3.19. Comparisons were only made between the dates 32, 46 and 61 days after inoculation. The statistical parameters are given in Tab. A3 (Appendix). Regardless of species none of the saplings died at 15 °C within the period of investigation. Comparing mortality between *P. sylvestris* and *L. decidua* at 25 °C there was no evident difference at any time. Temperature had a significant influence on mortality of *P. sylvestris* between 20 °C and 25 °C after 32 and also after 46 days. Regardless of being subjected to 20 °C or 25 °C, *B. xylophilus* caused 100 % mortality in *P. sylvestris* and *L. decidua*. Only 4 of 20 *P. abies* saplings died after 61 days at 20 °C and 25 °C.

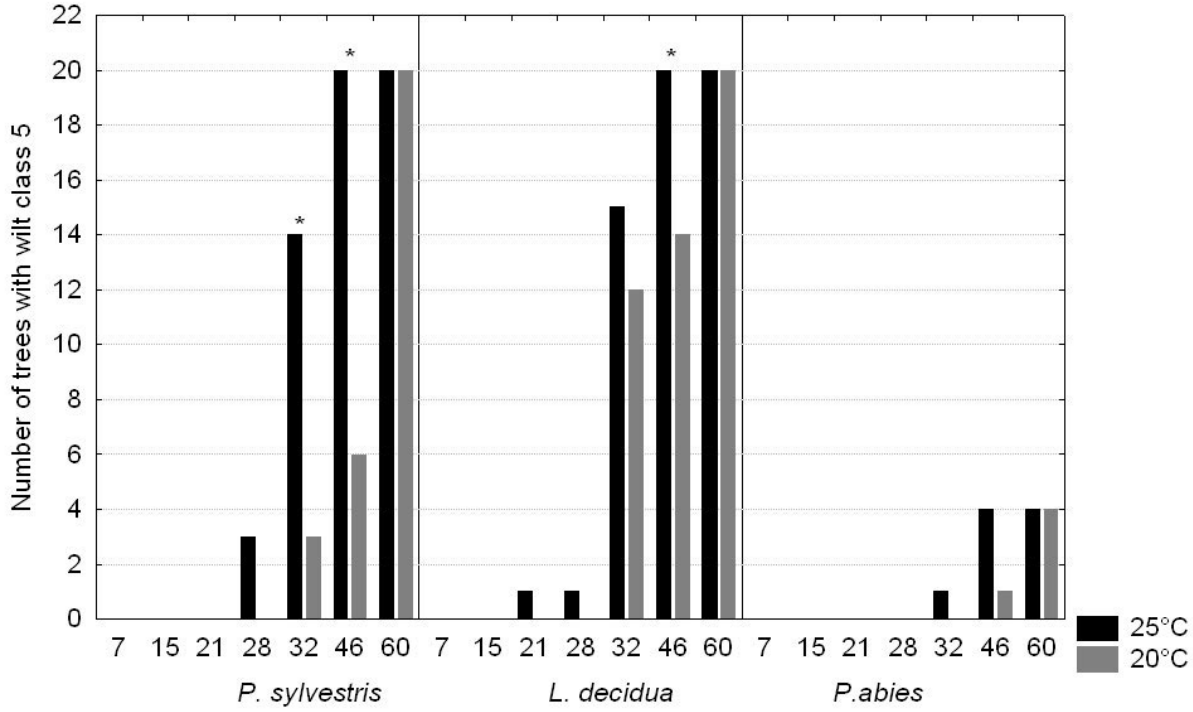


Fig. 3.19: Number of dead *Pinus sylvestris*-, *Larix decidua*-. *Picea abies*-saplings at 20 °C and 25 °C during a 60 day period after inoculation with Bursaphelenchus xylophilus, n = 20; * indicate significant differences (χ^2 –test. $\alpha = 0.05$) between temperatures within the same assessment dates post inoculation.

3.5.3 Relative water content in wood and needles

At the end of the experiment water content in wood of *P. sylvestris*, *L. decidua* and *P. abies* remained in between 58 % and 62 % in the controls irrespective of the temperature. The relative water content of needles remained above 60 % in *P. sylvestris*, *L. decidua* and *P. abies*.

The evident Z-values of the Mann-Whitney U-test of the comparison between controls and saplings which were inoculated with nematodes are given in Tab A4 (Appendix). Differences in the relative water content in needles between the temperatures of all tree species inoculated with *B. xylophilus* are given in Fig. 3.20 - 3.22 and for wood in Fig. A9 – A11 (Appendix).

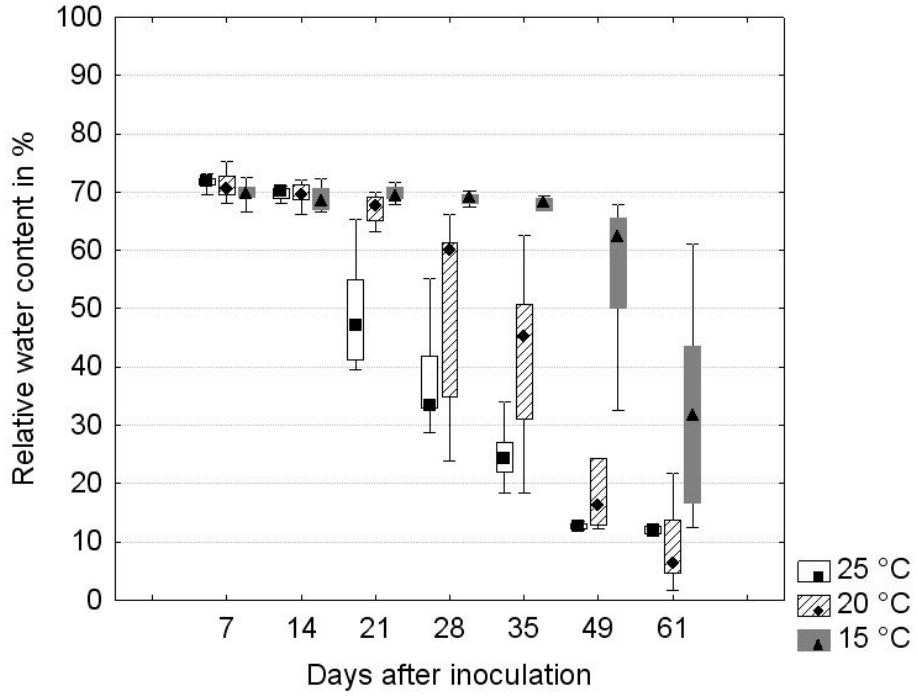


Fig. 3.20: Relative water content of needles of *Pinus sylvestris* saplings inoculated with *Bursaphelenchus xylophilus* at 25 °C, 20 °C and 15 °C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

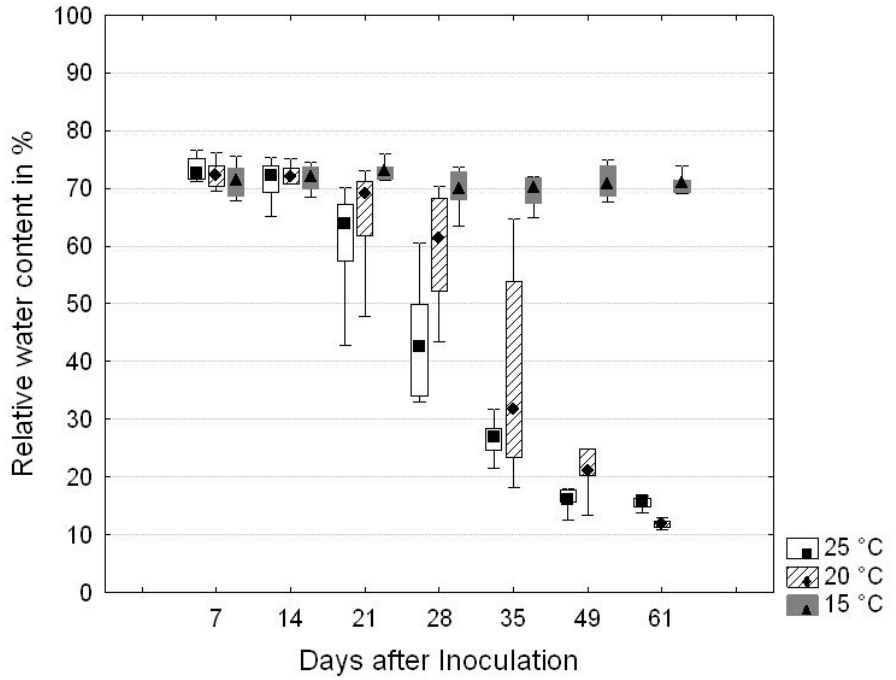


Fig. 3.21: Relative water content of needles of *Larix decidua* saplings inoculated with *Bursaphelenchus xylophilus* at 25 °C. 20 °C and 15 °C. n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

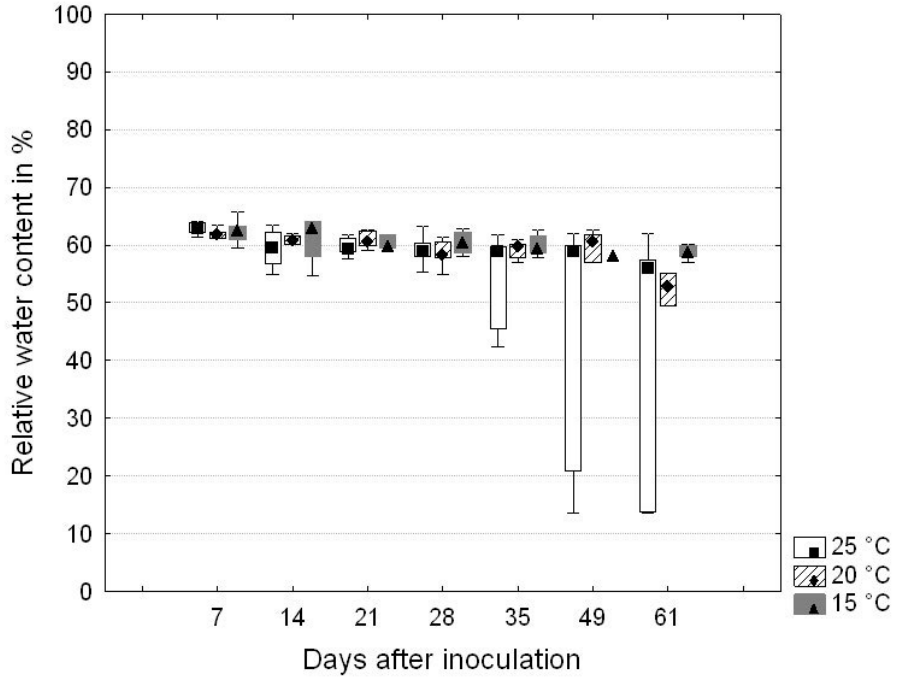


Fig. 3.22: Relative water content of needles of *Picea abies* saplings inoculated with *Bursaphelenchus xylophilus* at 25 °C, 20 °C and 15 °C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

P. sylvestris

B. xylophilus significantly altered the relative water content of wood and needles of inoculated *P. sylvestris* at all temperatures compared to the controls at the end of experiment. The relative water content in wood of *P. sylvestris* was not different between the temperatures. Water content at 61 days after inoculation reached 47 % to 52 % water content at all temperature.

A different situation occurred when regarding the relative water content of needles of *P. sylvestris* (see also Fig. 3.20). The reduction in water content of needles was significantly different between all temperatures. The decline of relative water content started after 21 days at 25 °C and one week later at 20 °C. At 15 °C the relative water content of needles decreased 49 days after inoculation. After 61 days, when the experiment was terminated the needles at all temperatures reached their minimum water content level ranging from 10 % to 15 %.

L. decidua

A significant difference of the relative water content in wood was detected in inoculated *L. decidua* saplings in comparison to the control when kept at 20 °C and in needles at 20 °C as well as in 25 °C. The relative water content in wood of *L. decidua* showed an overall decrease over time and was similar to that in *P. sylvestris*. Significant differences were observed between 15 °C and 25 °C. The final relative water content of wood above 20 °C in *L. decidua* reached at 51 % to 52 %.

The decrease of water content in needles at 20 °C and 25 °C as wilt progressed appeared as distinct as in *P. sylvestris* (see also Fig. 3.21). The water content was significantly different between all temperatures throughout the period of investigation. There was no change in the relative water content in needles of *L. decidua* when exposed to 15 °C. Similar to *P. sylvestris* needles of *L. decidua* reached a minimum water content of 10 % to 15 % when a majority of saplings was dead after 61 days at 20 °C and 25 °C.

P. abies

B. xylophilus also had a significant influence on the relative water content of wood Fig. A11 (Appendix) and also of needles of *P. abies* at 25 °C (Fig. 3.22). The deviation in water content of wood of *P. abies* right at the beginning of the experiment is most likely an artifact as water content at all temperatures showed similarly high values thereafter. Therefore the fist date was excluded in further evaluations. In doing so *P. abies* did not show an alteration of relative water content in wood at 15 °C or 20 °C. A significant difference is the result of a decline in the 25 °C variation at the end of the experiment.

3.5.4 Nematode population dynamics

B. xylophilus could not be identified in any sapling of the control variations of *P. sylvestris*, *L. decidua* or *P. abies*. Population dynamics were observed in two areas of the sapling: shoots and roots. Population densities of *B. xylophilus* are specified in Tab. A5 – A6 (Appendix). These are given as median and min-max spans of population densities per gram dry matter for shoots and roots of all species, at all temperatures and for all sampling dates. The Wilcoxin test was applied to all population densities throughout all sampling dates for the respective plant part and between the temperatures. Test specific Z values are given in Tab. A7 a-b (Appendix)

P. sylvestris

Population dynamics of *B. xylophilus* in *P. sylvestris* was strongly influenced by temperature in the shoot (Fig. 3.23). Population densities of nematodes were significantly higher at 25 °C than at 20 °C and both higher than at 15 °C. The population densities in roots of *P. sylvestris* in general were lower than detected in the shoots at any date of investigation. Population dynamics of nematodes in roots at 20 °C and 25 °C were not significantly different (Fig. 3.24). Population densities in roots at these temperatures reached a comparably maximum level of 745 nematodes /g dry matter at 20 °C and 1070 nematodes /g dry matter at 25 °C. At 25 °C the growth phase, the peak and the regression of the two population dynamics in roots and shoots were similar over time. This was not as distinctive at 20 °C.

Generally characteristic phases of the population development of *B. xylophilus* (growth, maximum and regression) in shoots at 20 °C appeared slower than at 25 °C. There was a slight increase in the population densities in the shoots as well as in roots at 15 °C towards the end of the experiment, 61 days after inoculation. *B. xylophilus* densities of approximately 200 nematodes /g dry matter in both areas were detected at this lowest temperature.

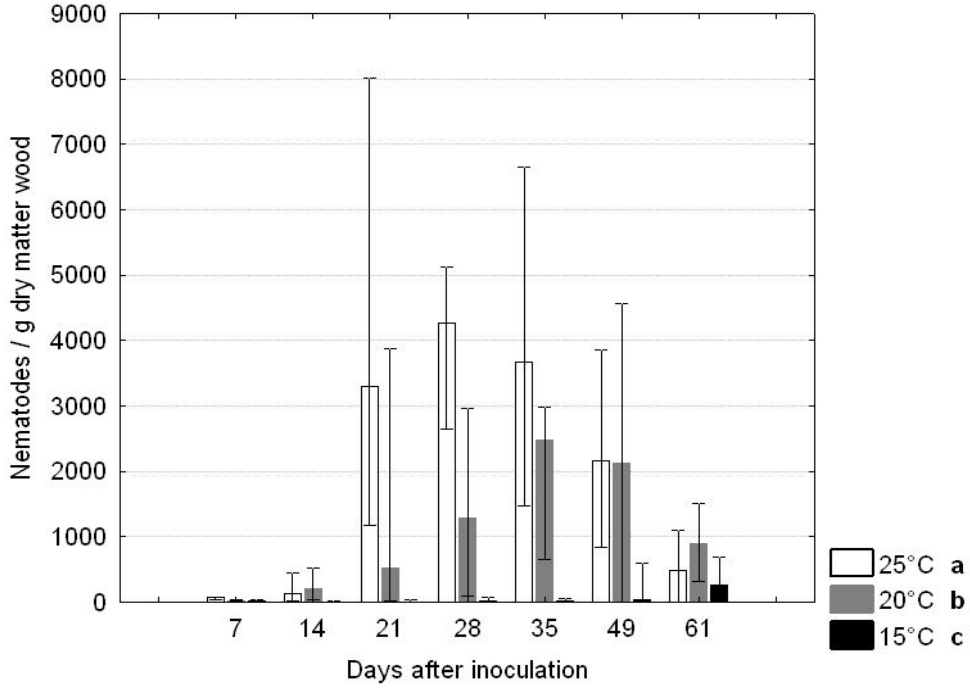


Fig. 3.23: Population dynamics of *Bursaphelenchus xylophilus* in the shoots of *P. sylvestris* saplings detected as median of number of nematodes / g dry matter during a 60 day period after inoculation at 25 °C, 20 °C and 15 °C; n = 10; Different letters indicate significant differences between temperatures according to Wilcoxin-test ($\alpha = 0.05$)

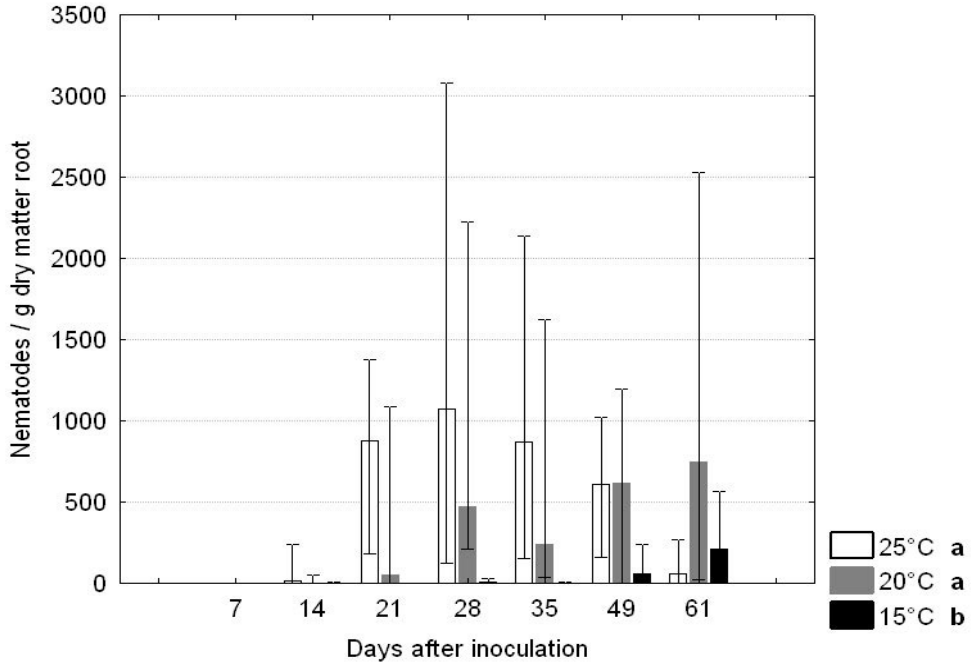


Fig. 3.24: Population dynamics of *Bursaphelenchus xylophilus* in the roots of *P. sylvestris* saplings detected as median of number of nematodes / g dry matter during a 60 day period after inoculation at 25 °C, 20 °C and 15 °C; n = 10; Different letters indicate significant differences between temperatures according to Wilcoxin-test ($\alpha = 0.05$)

L. decidua

Temperature had no significant effect on the population densities in both, shoots and roots compared between 20 °C and 25 °C (Fig 3.25 – 3.26) throughout the period of investigation. The population density reached a maximum level of 2691 nematodes /g dry matter 28 days after inoculation in the shoots subjected to 25 °C. The maximum level of the population density was detected in shoots 35 days after inoculation at 20 °C with 2,370 nematodes /g dry matter. Population densities in roots at 20 °C and 25 °C were lower than in shoots. The maximum of population density in roots reached 382 nematodes /g dry matter at 20 °C and 450 nematodes /g dry matter at 25 °C. *B. xylophilus* did not reproduce in saplings at 15 °C although some nematodes were found in very low numbers in shoots and roots as well.

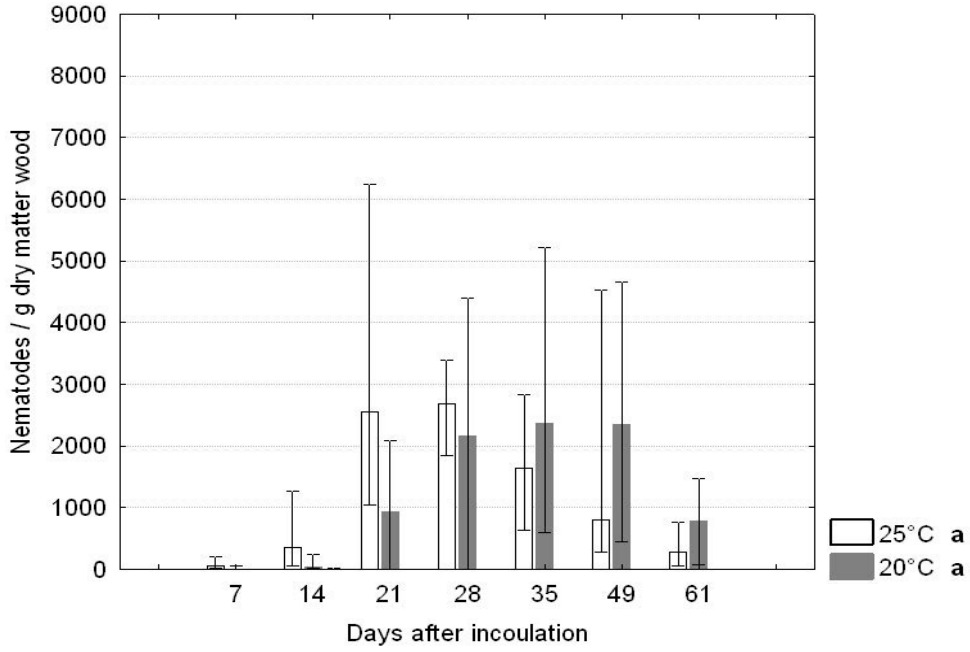


Fig. 3.25: Population dynamics of *Bursaphelenchus xylophilus* in the shoots of *L. decidua* saplings detected as median of number of nematodes / g dry matter during a 60 day period after inoculation at 25 °C, 20 °C and 15 °C. n = 10; Different letters indicate significant differences between temperatures according to Wilcoxin-test ($\alpha = 0.05$)

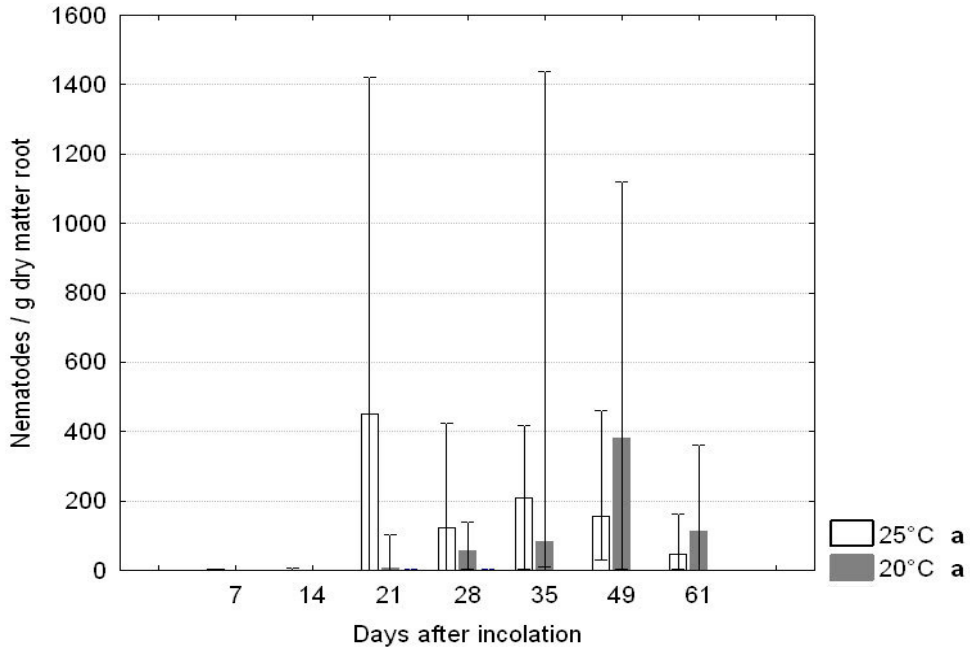


Fig. 3.26: Population dynamics of *Bursaphelenchus xylophilus* in the roots of *L. decidua* saplings detected as median of number of nematodes / g dry matter during a 60 day period after inoculation at 25 °C, 20 °C and 15 °C. n = 10; Different letters indicate significant differences between temperatures according to Wilcoxin-test ($\alpha = 0.05$)

P. abies

In general the population density of *B. xylophilus* in shoots of *P. abies* was very low at all temperatures and lower in roots. The nematode densities however were significantly different among the temperatures in shoots (Fig. 3.27). Temperature also influenced the population of *B. xylophilus* in *P. abies* in both shoots and roots. Nematode densities of 355 nematodes /g dry matter were reached in shoots 28 days after inoculation at 25 °C which was higher than in shoots at 20 °C. Nevertheless the Wilcoxin test distinguished both populations to be significantly different which is related to the occurrence of higher population densities in two to three saplings (of 10) only.

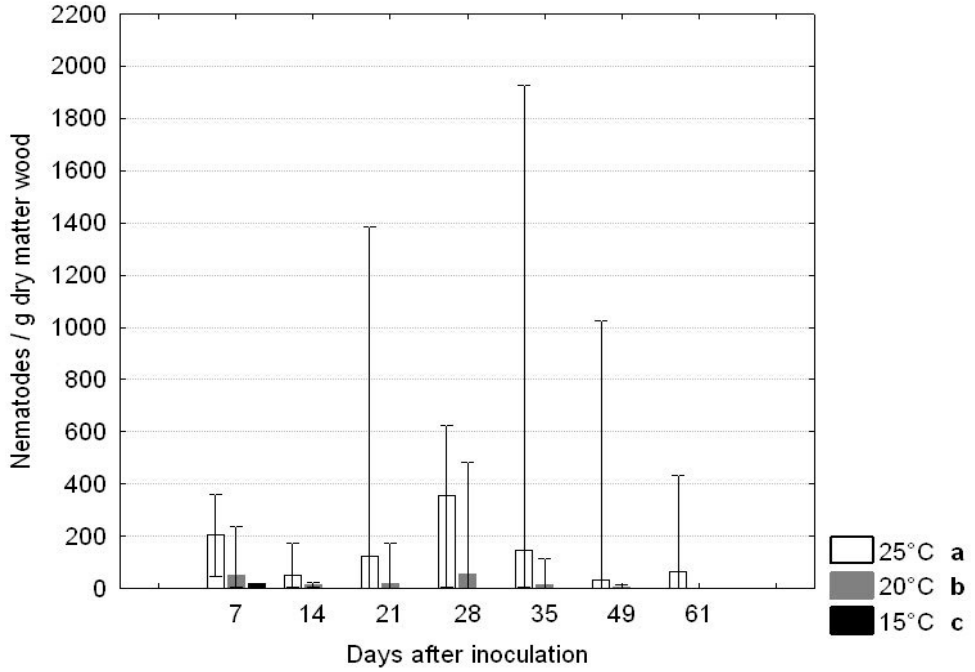


Fig. 3.27: Population dynamics of *Bursaphelenchus xylophilus* in the shoots of *P. abies* saplings detected as median of number of nematodes / g dry matter during a 60 day period after inoculation at 25 °C, 20 °C and 15 °C. n = 10; Different letters indicate significant differences between temperatures according to Wilcoxin-test ($\alpha = 0.05$)

The median test was applied to compare population dynamics of *B. xylophilus* between the investigated tree species over time (Tab. 3.11 a-b). However, in shoots population dynamics were not significantly different between *P. sylvestris* and *L. decidua* at 20 °C

and 25 °C but were significantly different at 15°C. In roots population dynamics were tested being significantly different between *P. sylvestris* and *L. decidua* at all temperatures.

Tab. 3.11a-b: Median test of differences between the population dynamics of *Bursaphelenchus xylophilus* at all sampling dates in shoots and roots after inoculation in *Pinus sylvestris*, *Larix decidua* and *Picea abies* saplings at temperature 25 °C, 20 °C or 15 °C

a)

Population dynamics in wood

χ^2 values

		P. sylvestris			*P. abies*		
		25	20	15	25	20	15
P. sylvestris	25				22.40 *		
	20					45.71*	
	15						55.31*
L. decidua	25	1.03			22.40*		
	20		0.07			32.29*	
	15			77.14*			2.85

n = 60 α = 0.05 FG = 1 * significant

b)

Population dynamics in roots

χ^2 values

		P. sylvestris			*P. abies*		
		25	20	15	25	20	15
P. sylvestris	25				29.26*		
	20					31.57*	
	15						50.81*
L. decidua	25	11.43*			11.43*		
	20		5.54*			17.28*	
	15			38.53*			7.00*

n = 60 α = 0.05 FG = 1 * significant

Temperature therefore had a significant effect within tree species between 15 °C to 25 °C. *P. sylvestris* and *L. decidua* showed a comparable population dynamics. In both species characteristic phases of the dynamic (growth, maximum and depression) appeared temporarily delayed in 20 °C in relation to 25 °C. *B. xylophilus* reached its maximum population in shoots 28 days after inoculation at 25 °C and after 35 days in *P. sylvestris* and *L. decidua* at 20 °C. *B. xylophilus* could achieve a higher population maximum in *P. sylvestris* than in *L. decidua* at 25 °C. Although this was not significantly different in shoots, it was in roots between *P. sylvestris* and *L. decidua*. *B. xylophilus* could not establish in *L. decidua* at 15 °C but showed a tendency to build up a population in *P. sylvestris*. *Picea abies* was not susceptible to *B. xylophilus* in general but showed a tendency for a reduction of its invulnerability at the temperature tested. This was observed in some single saplings where maximum population density (Tab. A5 – A6, Appendix) could reach population densities above 1,000 nematodes /g dry matter in wood irrespectively at 20 °C and 25 °C.

4 Discussion

4.1 Effect of *Bursaphelenchus xylophilus* on mortality of *Pinus sylvestris*

The aim of this study was to test the effect of the inoculated *B. xylophilus* population on mortality of *P. sylvestris.* In previous investigations the number of nematodes inoculated in conifers to investigate pathogenicity or other nematode-host relationships varied greatly among numerous studies. These variations depended on host tree species, age class of trees, *Bursaphelenchus* species and isolates used (Bergdahl and Halik 1999; Braasch 2000; Kishi 1995; Kawaguchi et al. 1999; Melakeberhan and Webster 1990; Riga et al. 1991; Schauer-Blume 1990). Only a part of the inoculated nematodes enters the host due to callus formation of the tree. Therefore the initial inoculum must contain a sufficient number of nematodes that is able to sustain and to build a stable population (Braasch 1997). Depending on the interaction between the *B. xylophilus* isolate and hosts, inoculation success can be altered according to the experimental condition of temperature and water regime.

Increasing inoculum densities from 100 – 10000 nematodes per sapling had no clear effect on symptom development or mortality of *P. sylvestris*. The occurrence of first visible symptoms was detected roughly at the same period four weeks after inoculation independent of the inoculum density. Sensitivity of *P. sylvestris* in general was high at all inoculation densities tested. There is a restricted amount of literature comparing inoculum density and its effect on PWD (Pine Wilt Disease). For example, Chang (1999) found 100 % mortality in *P. thunbergii* when *B. xylophilus* was inoculated with 600 to 1600 nematodes/tree but a reduced mortality of 30 to 40 % at inoculum densities of 100 to 200 nematodes. This is in accordance with the results of the present study which showed that low inoculum densities of 100 to 300 nematodes per sapling caused a lower mortality which most likely was affected by a low inoculation success.

In the present study no relation between a wide range of nematode densities in the host after four weeks and development of PWD could be detected. The onset of wilt symptoms roughly appeared at the same time. The induction of PWD therefore was already caused by a very low population density at this time, when considering the reaction of *P. sylvestris* towards an inoculation density of 100 nematodes per sapling. In this case the induction of PWD appears to be rather specific. However Kishi (1995) gave an overview of several inoculation studies that were conducted in Japan and mentioned that in some cases inoculation of *B. xylophilus* with 10-30 nematodes/tree caused dead or severe wilt symptoms.

In the present study the nematode population development inside the sapling was strongly affected by the number of nematodes inoculated. Taking the population densities that were extracted four weeks post inoculation, nematodes obviously had reproduced during this period with exception of the lowest inoculum density of 100 nematodes/sapling. The reproduction within four weeks after inoculation increased with the number of nematodes that were inoculated until reaching a maximum at an inoculum density of 6000 nematodes/sapling. Using 10000 nematodes per sapling inverted this relation. A similar situation was reported by Melakeberhan and Webster (1990) who found a higher reproduction of *B. xylophilus* in *P. sylvestris* saplings 28 days after inoculation with 2500 nematodes per sapling in comparison to a lower reproduction at 10000 to 20000 nematodes/sapling. The authors assumed a population density dependent factor to be present in *B. xylophilus*. It is well known that population growth of nematodes is determined by density dependent factors (Mc Sorly and Duncan 2004) which was the basis for the early development of population growth models for sedentary and migratory nematodes (Steinhorst 1967). The density dependence is also relevant for the invasion of hosts by plant parasitic nematodes and their establishment thereafter. For example, the density of infective stages of the sedentary cyst nematodes *H. schachtii* and *H. avenae* and final population densities are dependent (Moltmann et al. 1985).

Another factor which might determine the invasion and consequently the development of *B. xylophilus* afterwards is the defence of the host. A higher initial defence response

of two to three year old *P. sylvestris*, *P. strobus*, *P. nigra*, and *P. taeda* was observed by means of $CHCl_3$ extractable resin when *B. xylophilus* was inoculated with higher densities at 25000 nematodes per tree in comparison to 5000 nematodes (Bolla et al. 1986). Accordingly an interrelationship between population density and host reactions was reported by Kosaka et al. (2001) who observed a higher induced resistance against virulent *B. xylophilus* using higher densities of avirulent *B. xylophilus* for pre-inoculation.

In the present study nematodes that were extracted from dead saplings were found in densities of several hundred nematodes per gram dry matter in shoots as well as in roots. Comparing the densities reached in dead saplings after inoculation with 100 – 10000 nematodes/sapling, no effect of inoculum density could be detected. As a consequence all saplings that died or showed severe wilt symptoms more or less contained the same population densities. Furthermore the nematodes were distributed similarly throughout the roots and shoots. Distribution and high densities of *B. xylophilus* in dead trees were commonly observed in other inoculation trials with *B. xylophilus* in different conifers (Futai 1980; Braasch 1996; Bedker et al. 1987). In accordance to my results Melakeberhan and Webster (1990) found no relation between number of inoculated *B. xylophilus* per tree and mortality of *P. sylvestris*. They observed that due to higher reproduction, lower initial population densities present at early wilt stages achieve the same densities at late wilt stages as were reached by high initial population densities. This development dependent on population density was already discussed earlier in this chapter but seems also to be relevant during late wilt, too. The high reproductive capacity and distribution of *B. xylophilus* are considered to be the key factors in its pathogenicity towards pines (Iwahori and Futai 1996; Iwahori and Futai 1995; Odani et al. 1985). Nevertheless it is unknown if there exists a certain threshold capacity that induces irreversible wilt. Therefore it remains unclear whether a low population density already allows the outbreak of PWD in *P. sylvestris*. To determine the presence of a threshold density for *B. xylophilus*, nematode development inside the tree needs to be interrupted without affecting tree physiology. This would require a careful selection of application times and the method or agents to use.

However, a prerequisite for irreversible wilt is the establishment of *B. xylophilus* in the host which in this study could be achieved at any inoculation density between 100 and 10000 nematodes per sapling. Reproduction and distribution of *B. xylophilus* as a key factor for its pathogenicity was investigated by application of inoculation trials which are discussed in chapter 4.3 - 4.4.

4.2 Pathogenicity of *Bursaphelenchus xylophilus* isolates towards European conifers

The three isolates of *B. xylophilus* from Portugal, North America and China were shown to be pathogenic towards most of the conifer species examined. The largest group of species belonged to the genera *Pinea* which could be divided into three groups according to mortality detected at the end of the experiment three months after inoculation:

1) Mortality of 100 % (*P. sylvestris*, *P. cembra*, *P. nigra*)
2) Mortality between 70 and 100 % (*P. strobus*, *P. pinaster*, *P. radiata, P. mugo*) and
3) Less than 20 % mortality (*P. halepensis* and *P. pinea)*, with the exception of the Portuguese isolate where 60 % mortality was detected.

High levels of mortality of pine species with special reference to *P. sylvestris* were reported from other inoculation trials (Tomminen 1993; Braasch 2000). Other highly sensitive and susceptible pines are *P. thunbergii* and *P. densiflora*, *P. luchuensis* and *P. masoniana* in Asia (Suzuki 2002). The maritime pine *P. pinaster* is the predominant susceptible pine that currently is the main host for *B. xylophilus* in Portugal which until now is the only European country affected by *B. xylophilus*.

The two *Larix* species showed an unexpected high rate of mortality: *L. decidua* (100 %) and *L. kaempferi* (>50 %). Both *Larix* species contained distinctively lower population densities of the nematode than the pine species. Symptoms in *L. decidua* however still developed slightly faster than symptoms in *P. sylvestris*. *L. decidua* therefore showed a

high sensitivity towards *B. xylophilus* which confirm results obtained by Braasch (1997).

P. abies was not a host for *B. xylophilu*s, as it carried a negligible number of *B. xylophilus* and only in the first four weeks after inoculation. Only one sapling out of ten contained a higher number of nematodes (2000 nematodes/ sapling) 77 days after inoculation. The very low susceptibility of this conifer in the present tests confirmed results of other studies (Braasch 1997; Futai and Sutherland 1989; Sutherland et al. 1991).

A. alba was resistant to *B. xylophilus*. It was not a suitable host for any isolate tested as there was neither symptom development nor evidence of nematode multiplication.

Picea rubens (Red spruce) and *Abies balsamea* (Balsam fir) that were inoculated with different isolates of *B. xylophilus* from North America were found susceptible (Sutherland et al. 1991).

P. pinea, *P. mugo* and *L. kaempferii* were the only conifer species that showed differences in sensitivity towards *B. xylophilus* between the isolates from North America, China or Portugal in the present study. However, these conifer species contained population densities of nematodes that were more or less in a comparable range between the isolates. The results from pathogenicity tests of selected *B. xylophilus* isolates in the literature are variable. Bolla et al. (1986) found distinctive pathogenicity of isolates of *B. xylophilus* from different locations in the USA, isolated from *P. sylvestris* or *P. strobus.* Comparing the pathogenicity of five isolates from USA and Japan towards *P. sylvestris* Riga et al. (1991) could not detect any difference. Other factors might overlay the pathogenicity of *B. xylophilus.* When comparative trials were conducted at different dates Panesar and Sutherland (1989) found that pathogenicity of isolates from North America changed with the season, when they were inoculated. Thus differences in the pathogenicity between the isolates detected by the authors in March were inconsistent with those differences detected in May or September.

Inoculation of immature trees under greenhouse conditions to evaluate pathogenicity of *B. xylophilus* has been criticized, because many conifer species that were confirmed as highly susceptible with this approach were not confirmed with mature trees. In two studies the inoculation of *B. xylophilus* into immature *P. resinosa* (Wingfield et al. 1986) and *P. elliotti* (Dwinell 1985) resulted in high susceptibility of the tree species in the greenhouse but there was no susceptibility in mature trees in outdoor experiments. Pathogenicity tests with *Bursaphelenchus mucronatus*, *B. sexdentati*, *B. leoni* and *B. hellenicus* which are thus far not known to be pathogenic in nature were tested for pathogenicity towards immature trees of various pine species (Skarmoutsos and Michalopoulos-Skarmoutsos 2000; Braasch et al. 1999; Braasch 1996) and *B. sexdentati* as well as *B. mucronatus* were verified to be virulent under greenhouse conditions in young pines. The occurrence of *B. xylophilus* in mature tree species under natural conditions is not necessarily a requirement for studying pathogenicity of the nematode in artificial environments. Trees that are known as natural hosts like *P. sylvestris* show the typical symptoms of PWD and a clear reproduction of the nematode when inoculated on immature trees. In principal, tree species that were known from the literature to be moderately resistant like *P. halepensis* (Evans et al. 1996) clearly could be separated from highly susceptible pines species with the used inoculation method.

Another factor that directly controls the infestation of trees in nature is the presence of a beetle vector for *B. xylophilus*. This factor is technically excluded when nematodes are inoculated artificially. The moderately high mortality of *P. pinea* towards the Portuguese isolate of *B. xylophilus* detected in the current study, demonstrates that *P. pinea* was a good host even without a vector. Due to the non host status of *P. pinea* to the vector beetle *Monochamus galloprovincialis*, this tree species has not yet been observed to be naturally infested by *B. xylophilus.*

It is not clear whether the high susceptibility of *L. decidua* would imply a risk for Central European forests as in the case of *P. sylvestris.* The Cerambicid beetle *Tetropium gabrieli* is a potential vector beetle for the transmission of *Bursaphelenchidae* into Larch trees and is abundant throughout Central Europe. The epidemiology of the Coleoptera species is closely related to the growing stress status of

Larch populations particularly in Austria and Switzerland (Krehan and Cech 2004; Meier et al. 2004). The very effective interrelation between the beetle vector *Monochamus* spp. and blue stain fungi in pines which is a prerequisite for the survival and transmission of *B. xylophilus* also exists between *Tetropium* and blue stain fungi in *Larix* (Jacobs and Kirisits 2003). In principal Bowers et al. (1992) discovered *B. xylophilus* in mature *L. laricina* in North America.

Evaluating susceptibility of inoculated conifers is difficult due to the uncertainty of the population dynamics of *B. xylophilus* in the tree at different wilt stages. It is not clear if the nematode population had reached its maximum at the end of the experiment in those susceptible tree species that showed mortalities less than 100 %, as seen in the distinct increase of the population in *L. kaempferi*. It is possible that further population growth 12 weeks after inoculation would have resulted in higher mortality at later stages. This uncertainty is basically true for all conifer species that show low or moderate mortality detected in the early stages. As a result, finding a suitable stage of wilt to evaluate susceptibility is not resolved. Pathogenicity studies with *B. xylophilus* often related susceptibility of the hosts to the final population density of the nematodes in dead trees or at the end of a defined period (Braasch et al. 1999; Moon et al. 1993; Bakke et al. 1991; Riga et al. 1991; Sikora and Malek 1991; Caroppo et al. 2000).

Braasch (1997) computed a relative host susceptibility index (RHS) which related the population densities of nematodes detected in trees when they were assigned to be dead to the number of successfully inoculated trees. No obvious correlation between the population density of a certain isolate and mortality of a host has been detected (Melakeberhan and Webster 1990; Bolla et al. 1986; Riga et al. 1991). Under ideal conditions at 25 °C, *B. xylophilus* were shown to achieve generation cycles within four days when feeding on *Botrytis cinerea* (Wang et al. 2005). In pathogenicity trials the common period of investigation exceeds several weeks and populations often were extracted at the end of a preset period or from dead trees. In both cases the PWN may have completed many cycles and therefore it is impossible to predict true population dynamics from one or two extractions. Extracting nematodes even after four weeks could reach population growth at progression, maximum or regression. This might

explain the observed dissimilarities between population densities and mortality in the present study.

Another unknown component is the lack of knowledge concerning the relation of *B. xylophilus* to its hosts. *B. xylophilus* shifts from a phytophagous behaviour in living hosts to a mycophagous stage in dying hosts (Wingfield 1987). Populations of *B. xylophilus* that are extracted from dead wood give poor estimates of pathogenicity towards their hosts (McNamara 2004).

The present investigation is one of the first studies focusing on a Portuguese isolate in comparison with other isolates. Some unique observations with this particular isolate were made in the course of the present studies. The Portuguese isolate showed a tendency towards extreme deviations and extraordinary high population densities compared to the other two isolates. A recent comparative study on Portuguese and Japanese isolates indicated distinctively higher mortality rates caused by Portuguese isolates (Mota et al. 2006). In their study this isolate achieved evidently higher population densities four weeks after inoculation in Japanese black pines than the Japanese isolate. The authors also observed variability between Portuguese isolates in their cytogenetical characteristics and also in their pathogenicity.

Nevertheless sensitivity of most *Pinea* and *Larix* species tested in the present study could be clearly detected. It is evident that all *B. xylophilus* isolates could multiply to extremely high population densities among the pine and larch species tested. However susceptibility of immature trees detected needs to be reconfirmed by inoculation of the nematode into mature trees before evaluating the relevant pathogenicity of *B. xylophilus*. In this respect the pathogenicity of *B. xylophilus* detected in the present study should be understood as a “screening” of possible European hosts. Further pathogenicity tests for European conifers should focus on mature larch and pine species. *Larix decidua* should be seriously considered in further pest risk analysis as the potential prerequisites for the establishment of *B. xylophilus* in this species seems to be given.

4.3 Invasion biology of *Bursaphelenchus xylophilus* in *Pinus sylvestris*

Migration and population build up in a very short time was determined to be a key factor of pathogenicity of the Pine Wood Nematode. This hypothesis on pathogenicity was formulated in different studies after restriction in the distribution of avirulent *B. xylophilus* and *B. mucronatus* in trees were found, whereas virulent *B. xylophilus* moved free inside inoculated pine seedlings and caused severe symptoms (Futai 1980; Odani et al. 1985; Kosaka et al. 2001). My study clearly confirmed a rapid spread and intensive population growth in a short period of 27 days after inoculation in *P. sylvestris* at 25 °C. The invasion of *P. sylvestris* by PWN passed four more or less overlapping stages:

(1) Early migration

(2) Distribution and colonisation of all plant parts

(3) Population build up

(4) Retreat into the root-system

The objective of this investigation was to explain the relation between the development of the nematode population inside the host and the time when wilt symptoms appeared. This was conducted to explain whether reproduction of *B. xylophilus* or migration is the factor which induces wilt. Another aim was to understand the phases of invasion which may give possible approaches for finding crucial resistant mechanisms in non susceptible pine species.

Early migration

Only two days after inoculation *B. xylophilus* could be found in most segments of the pine saplings. This was apparently the time after which the nematodes successfully entered tissues via inoculation wound and started to disperse systemically into the main stem and the crown. At this stage the main population of *B. xylophilus* was characteristically concentrated inside the inoculation site and population density decreased with distance from the inoculation point. Mamiya (1984) reported that rapid spread of *B. xylophilus* from the inoculation point could be shown 24 hours after inoculation. Concentration of nematodes around the inoculation point and rapid spread thereafter was also observed in other studies (Fukuda et al. 1992; Odani et al. 1985). The initial spread of nematodes after inoculation is considered to be mainly enabled by

axial resin channels in the cortex and to a lesser extend in the xylem where they probably feed on epithelial cells (Mamiya 1985; Ichihara et al. 2000b; Suzuki 2004). This was particularly demonstrated by Ichihara et al. (2000a) who found the majority of nematodes to be present in the axial resin channels throughout the entire stem after inoculation. The driving force for the early spread of *B. xylophilus* is still unknown. In tests the closely related non pathogenic nematode species *B. mucronatus* was shown to achieve similar migration speed like *B. xylophilus* (Iwahori and Futai 1995). Instead of migrating *B. mucronatus* remains in the inoculation area which was discussed in the context of inhibiting resistance factors of the tree by Fukuda et al. (1992) and Kuroda and Kuroda (2004).

In the current study the population density of *B. xylophilus* in the inoculation segment was distinctly higher than in the neighbouring segments until the nematodes had completely distributed in the sapling six days post inoculation. A density dependent factor might also induce an early spread of the nematode population, if inhibiting factors of the tree can be avoided by PWN. In this context the study of Iwahori and Futai (1995) showed an interesting correlation between the population density of nematodes and their migration speed. The species, *B. xylophilus* and *B. mucronatus* increased their migration speed until a threshold density was reached, where movement speed decreased. This relation reflects evidently the density dependence of movement speed which might be triggered by other stimuli inside trees.

The bark inoculation technique which was applied in this study (Chapter 2.1.7) was criticized by Ichihara et al. (2000a) to cause an early introduction of nematodes into tissues, where they unlikely appear right after entrance into the tree and therefore facilitate the early spread of *B. xylophilus*. However, natural introduction by the beetle vector in principal also access entry into the xylem, as maturation feeding of the *Monochamus* vectors typically destroys the cambium of one year old shoots (Kishi 1995; Tsutsumi and Furukawa 2000).

Distribution and colonisation of all plant parts

In the present investigation *B. xylophilus* had completely colonized the entire plant six days post inoculation. At this stage the population density started to increase in each individual segment. Similar results were achieved with PWN in Japanese red and black pines (Fukuda et al. 1992; Ichihara et al. 2000a). In four to five year old *Pinus densiflora* Kuroda and Kuroda (2004) found the *B. xylophilus* population initially to increase in segments beneath the inoculation downwards the plant base in an outdoor experiment. This corresponds to an early increase of population densities between four and nine days after inoculation in lower stem segments detected in this study and also might be associated with a downwards movement of nematode population at this stage. The complete distribution of nematodes in hosts seems to be a critical stage that decides if nematodes can establish in the host or not. It was shown that avirulent *B. xylophilus* in susceptible hosts as well as virulent *B. xylophilus* in resistant trees do not reach this stage of development and disappear from the tree in time (Fukuda et al. 1992; Kuroda and Kuroda 2004).

Myers (1988a) hypothesized that a hypersensitive reaction in susceptible pines is responsible for tree death. Later it was shown that wilt inducing embolism in xylem vessels is indirectly caused by the PWN which possibly provokes cell death in tree areas where nematodes occur (Fukuda 1997). Thus *B. xylophilus* in this stage expanded the reaction zone inside the tree (Giblin-Davis 1993). According to observations made by Kuroda et al. (1988) a population of *B. xylophilus* above 100 nematode/g dry matter in wood of Japanese pines was the nematodes density that induced wilt. This conclusion was drawn from an enlargement of dysfunctional area and xylem desiccation. In the present study the overall population density in wood was 124 nematodes/g dry matter nine days after inoculation. In terms of distribution and population density *B. xylophilus* had established inside *P. sylvestris* at this stage and might already have induced the wilt process. However, no reduction of relative water content in needles could be detected at this stage.

Population build up

In the present study the *B. xylophilus* population in general started to increase distinctively from day 12 onwards after which it colonised all parts of the sapling. The period and the maximum population density in this study corresponded to observations made in various inoculation trials with *B. xylophilus* in *P. sylvestris* and other susceptible pine species (Melakeberhan and Webster 1990; Bolla et al. 1986; Kiyohara and Suzuki 1978). However, exponential growth models were assumed for PWN populations *in vivo* and *in vitro* (Iwahori and Futai 1995; Futai 1980; Mamiya and Furukawa 1977; Melakeberhan and Webster 1990; Wang et al. 2005). The growth pattern is primarily a result of a short generation cycle of four days at 25 °C. Thus, *B. xylophilus* during its plant parasitic stage was considered as belonging to nematodes of the r-strategy type which is characterised by very high reproduction, short life span and sharp decline of population (Decker and Fritzsche 1991).

The population dynamics of *B. xylophilus* detected in shoots and partly in roots showed three distinctive peaks at day 12, 19 and 27 post inoculation. The reliability of the existence of three consecutive population peaks is suggested, as it simultaneously occurred in segments which were sampled and submitted to Baermann extraction separately. It was always neighbouring segments in certain plant areas that showed a similar dynamic. The used method in this study could not distinguish between population increase as a matter of nematode reproduction or as a matter of migration of nematodes from other parts of the sapling. The population density in shoots and roots increased 12 days and further increased until 19 days after inoculation but decreased again towards the end of experiment. Therefore it is assumed that the population increase of *B. xylophilus* in the respective segments of *P. sylvestris* was primarily caused by reproduction at day 12 and 19 post inoculation and by migration from other plant areas at day 27 after inoculation.

The time shift in the population development was distinctive between the stem around the inoculation site after 12 days and branches after 19 days. The branches were invaded by *B. xylophilus* later then the stem part. The whorls that connected the main stem and

the branches in the base and the top of the pine saplings possibly act as a mechanical barrier for the distribution of *B. xylophilus* inside the tree. The same conclusion was drawn by Kuroda and Kuroda (2004) who found a restricted distribution of *B. xylophilus* and discussed the role of disconnections and diminutions of resin channels inside these whorls in resistant Japanese red pines as a barrier. In fact, in this study the population of nematodes detected in the whorl segment achieved a distinctively higher density then in adjacent branch segments before the nematodes started to establish in branches.

In the present study first discoloration of needles could be observed 12 days post inoculation and one week later the water content in needles was severely reduced. Onset of physiological wilt appeared synchronously with the highest reproduction of the population thereafter. However, the development of wilt symptoms of pine seedlings was reported to start at different stages of the population dynamics of *B. xylophilus* by various authors. Wilt was observed to start

before - (Mamiya 1984),

at - (Melakeberhan and Webster 1990; Kiyohara and Suzuki 1978)

after - (Kishi 1995) nematode populations reached their maximum density in pine seedlings.

Retreat into the root-system

In the current study the concentration of *B. xylophilus* in the base of the plant (root collar and roots) was significantly higher than in the rest of the plant, from where nematodes apparently vanished at the end of experiment. The driving factor for this behaviour was assumed to be the relative water content which was significantly higher in the root collar of the pine sapling than in the other segments of the shoot. A similar retreat of the PWN into roots was observed by Futai (1980) at relative water content in wood between 20 to 40 %.

It was already discussed that migration possibly contributed to a larger extent than reproduction to the increase of population in the base of the *P. sylvestris* sapling, detected towards the end of the experiment. This would require that *B. xylophilus* could actively move in the wood parts which showed relative water content of 25 % at this stage. Plant parasitic nematodes invading aerial parts normally loose their body water regulation at the same time, the plants exceed the permanent wilting point (Robinson 2006). This explains the disappearance of *B. xylophilus* from the entire crown part of the plants toward the end of experiment in this study. In the actual study the population structure was not determined but it might be assumed that a majority of the population was compiled of the dispersal of third stage juveniles of *B. xylophilus*. This survival stage is typically observed in trees after population declines during the advanced wilt process until tree dead (Mamiya 1984; Bolla et al. 1986). Due to the thick cuticle and the storage of glycogen granules in the nematode body (Kondo and Ishibashi 1978) this survival stage could withstand dry conditions, but it was found to concentrate in wood with higher water content (Futai et al. 1986). Another reason for the aggregation of nematodes in roots was provided by Melakeberhan and Webster (1990), who found high concentrations of nematodes in roots. As a result they hypothesized that nematodes start to invade roots in search of a toxic free environment or availability of food.

It is broadly accepted that migration and reproduction of *B. xylophilus* are key factors in its pathogenicity as it is distinctly different between the virulent and avirulent *B. xylophilus* or *B. mucronatus* (Kosaka et al. 2001). The invasion stages of *B. xylophilus* observed in this study particularly featured spatial and temporal aspects which were reflected by the migration and the reproduction of the nematode. In this study it was evident that both aspects could not be regarded separately, as both interrelated with each other. In fact both factors were observed to appear simultaneously in susceptible trees, as was described in earlier studies (Ichihara et al. 2000a; Iwahori and Futai 1996; Fukuda et al. 1992; Kiyohara and Bolla, I 1990).

The short sampling interval of two, three and four days uncovered extraordinary dynamic population development that otherwise would not have been observable. Segmentation and short interval sampling was an essential method to follow these peaks

for the development of several populations inside *P. sylvestris* saplings. Furthermore the high resolution in 17 segments showed that invasion by *B. xylophilus* was characterised by a successive process of migration, colonisation and population increase in different tree parts which has not been reported on a systematic whole plant approach to date. The results reflect the rapid life cycle of the Portuguese isolate PT 3 (w) of *B. xylophilus* on the one hand and its rapid invasion ability in *P. sylvestris* on the other hand.

4.4 Influence of temperature on *Bursaphelenchus xylophilus* – host interaction

Influence of temperature on wilt symptoms and mortality

The development of Pine Wilt Disease (PWD) in the tested species confirmed earlier results of the pathogenicity trial which was conducted in the greenhouse (chapter 3.3). Both, *P. sylvestris* and *L. decidua* were highly susceptible at temperatures above 20°C. *P. abies* in general was tolerant against the Portuguese isolate of *B. xylophilus* which was used in this trial. At 20°C or 25°C all *P. sylvestris* or *L. decidua* saplings inoculated with *B. xylophilus* died after a relative short period of 60 days. However, no plant death was registered at 15 °C. These results confirmed those obtained in other studies that focused on pathogenicity of *B. xylophilus* towards *P. sylvestris* at similar temperatures (Braasch 2000; Melakeberhan et al. 1992). Mortality may be altered by temperatures depending on the temperature optimum of the respective *B. xylophilus* isolate that is inoculated. For example, Braasch (2000) found that the pathogenicity of two North American isolates of *B. xylophilus* was strongly influenced by temperatures ranging between 15°C and 30°C.

The development of PWD appeared accelerated by temperatures above 20°C in susceptible conifers, as was also observed by Sikora and Malek (1991). In my study relative water content of needles decreased earlier with rising temperatures in *L. decidua* and *P. sylvestris*. In both tree species similar low relative water content was

observed, when maximum mortality was detected. In this respect relative water content of needles was a very useful predictor of PWD.

This was not the case for the relative water content of wood. Despite the very early wilt reaction of *L. decidua* at 20°C and 25°C, the water content of wood of infected saplings remained relatively stable, even though a majority of saplings was already dead 32 days after inoculation. Due to high variation of values, a reduction of relative water content in *P. sylvestris* towards the end of experiment could not be confirmed.

Influence of temperature on population dynamics

Although population in *P. sylvestris* did not achieve its maximum at 20°C, mortality of saplings between 20 °C and 25 °C was not affected. Thus a threshold population density of *B. xylophilus* which induced wilt in *P. sylvestris* was already achieved at 20 °C. The existence of a threshold population density was suggested by Braasch (1997), as well as Kuroda and Kuroda (2004). The stimulating effect of temperature on development of *B. xylophilus* also was observed on Agar plates using *B. cinerea* as a food source (Futai 1980). According to Wang et al. (2005) temperature mainly affects the embryonic development of *B. xylophilus*. A strong positive correlation between temperature and life stage development is also known from sedentary nematodes like the sugar beet cyst nematode *Heterodera schachtii* (Griffin 1988).

Ichihara et al. (2000a) found that temperatures between 20 °C and 30 °C did not affect the early migration of *B. xylophilus* into Japanese black pines. This is in accordance with the findings of Iwahori and Futai (1995), who found little alteration of the migration speed of *B. xylophilus* between 20 °C and 25 °C. This provides another explanation for the results obtained in the present study that the pathogenicity of *B. xylophilus* in both susceptible conifers was not altered by a temperature between 20 °C and 25 °C. In contrast Rutherford et al. (1992) found a significant difference in migration speed of *B. xylophilus* between 10 °C, 20 °C and 30 °C.

Melakeberhan et al. (1992), formulated three different hypothesises on the influence of temperature on *B. xylophilus* populations and the physiological response of the host tree to infection:
(a) If temperature primarily alters reproduction of the nematode, *B. xylophilus* would increase in number above the threshold temperature level and infected pines should die.
(b) If the effect of temperature is only on host physiology, there should be no relationship between nematode numbers and pine death with increasing temperature.
(c) If the effect of temperature is on both a correlation between them could be expected with increasing temperature.

When applying these hypothesises to my findings the effects observed indicate an intermediate affect of temperature on both population development of the nematode and on the physiology of the saplings as outlined in hypothesis (c). Population development of *B. xylophilus* was clearly inhibited at 20 °C compared with 25 °C. The same was true for wilt symptoms and also for the relative water content of needles in both susceptible conifers. Nevertheless, at 20 °C mortality of *L. deciuda* saplings was significantly higher 32 days after inoculation than in *P. sylvestris* saplings. Likewise, wilt symptoms reached a higher wilt class earlier in *L. decidua* than in *P. sylvestris*. In this respect *L. decidua* appeared more susceptible than *P. sylvestris*. Reproduction clearly accelerated effect with increasing temperature in *P. sylvestris* and *L. decidua* as well as in *P. abies*.

Some *P. abies* saplings showed severe wilt symptoms and reduced water content of needles at 25°C and harboured a density of more then 1800 nematodes/g dry matter in shoots. This was observed only at 25 °C and did not occur in the majority of *P. abies* saplings at 15 °C or 20 °C. The reason for the occurrence of this sensitivity is not clear. If predisposition to PWD in *P. abies* is due to genetic or environmental conditions studies on PWD should be conducted with caution because in that case it would be of importance for pest risk analysis.

The development of the nematode in *P. sylvestris* at 15°C is not considered to be a temperature that allows development of *B. xylophilus* in susceptible conifer species. In my study, *B. xylophilus* showed a tendency towards higher population growth at the end

of the experiment 60 days after inoculation at 15°C. At this time nematodes were distributed throughout the whole plant without any symptom development. In other studies *B. xylophilus* was observed to persist many years in asymptomatic *P. sylvestris* in outdoor inoculation experiments conducted in Canada (Bergdahl and Halik 1999, 2004). Futai (2003) in particular found that asymptomatic carrier trees play an important role in epidemic spread of pine wilt which he studied under field conditions in Japan. Therefore asymptomatic trees that are known to be susceptible should be considered in PWN monitoring procedures.

In conclusion the three major effects of temperature on the population dynamics of *B. xylophilus* and Pine Wilt Disease in the present study were:

(1) It influenced maximum population development in *P. sylvestris*
(2) It affected the emergence of the population dynamic
(3) It altered the development of wilt symptoms and water content of needles.

The present study showed that temperatures above 20 °C are a general requirement for the occurrence of PWD even in host trees that belong to different genera like *P. sylvestris* and *L. decidua*. Temperatures differently affected susceptibility of the investigated host trees but the sensitivity of both host species in general was very high when 20°C was exceeded.

5 Conclusion

Effect of *Bursaphelenchus xylophilus* population on *Pinus sylvestris* mortality

One of the objectives of my study was to investigate the relation between inoculation density of PWN, nematode development and mortality of the sensitive host *P. sylvestris*. It was shown that inoculation density in the range 100 – 10000 nematodes per sapling always caused high mortality of three to four year old *P. sylvestris* saplings. This finding indicates that PWN is pathogenic even at very low inoculation densities and that pathogenicity was not determined by inoculated number of nematodes. The reproduction of PWN in *P. sylvestris* before the wilt symptoms became visible was clearly affected by the inoculation density.

Pathogenicity of *Bursaphelenchus xylophilus* towards European conifers

A range of pine species with a wide geographical distribution in Europe were confirmed to be susceptible and highly sensitive against *B. xylophilus* isolates from North America, China and Portugal: *P. sylvestris*, *P. cembra*, *P. nigra, P. strobus*, *P. pinaster*, *P. radiata* and *P. mugo*. The species *P. halepensis, P. abies* and *A. alba* were considered tolerant and not susceptible to PWN (Pine Wood Nematode).

As a screening method to identify potential host trees, bark inoculation method was demonstrated to be a useful predictor for sensitivity or tolerance in tree species and to distinguish pathogenicity or non- pathogenicity of PWN. There is a need for inoculation field experiments with mature trees to verify the results on sensitivity. This is especially important for *P. cembra*, *P. mugo* and *P. radiata*, as these pines are not yet reported to be susceptible to PWN. Furthermore a small amount of information on inoculation experiments with mature trees exists.

Differences in the degree of plant mortality were due to the variability in the data. The variability was caused by accuracy of the methods used but also by the high genetic variability of tolerance and resistance that occur naturally in the plant material used in the experiments. Thus clear differences in the pathogenicity of the nematode isolates from Portugal, North America and China could not be detected in the majority of the host trees. As a consequence, screening of conifer species for resistance against specific isolates of *B. xylophilus* clearly requires an extended number of replications and the use of genetically identical trees.

In my study it was confirmed that *Larix decidua* is a susceptible and highly sensitive host tree to *B. xylophilus*. The results of the inoculation experiments also showed that *L. decidua* was more sensitive to PWN than *P. sylvestris*. Therefore *L. decidua* is a potential risk factor in Central European forests for PWN and should not be planted in high risk areas in the future. However there is a gap in our present knowledge of the level of risk with regard to sensitivity, susceptibility and latency of PWN in mature *L. decidua*, as in this study only saplings were used. The suitability of the Cerambicid beetle *Tetropium gabrieli* for PWN as vector is also in need of further studies.

Invasion, distribution and population dynamics of *Bursaphelenchus xylophilus*

According to the accepted theory migration and reproduction inside trees are the key factors influencing pathogenicity of PWN. My studies on the interrelation between population dynamics and distribution of the nematode in a susceptible tree over time, concentrate on consecutive stages of the invasion process in *P. sylvestris.* The following four stages were examined:

(1) Early migration
(2) Distribution and colonisation of all plant parts
(3) Population build up
(4) Retreat into the root-system

It was shown that in good hosts at optimal temperature the PWN can spread and establish in saplings within a view days. Population increase inside the sapling was clearly evident only after the nematodes were distributed in all plant parts. This implies that a small number of nematodes first spread into distant areas of the plant. In my study migration and reproduction was shown as a process. The results suggested that migration is a precondition for the population build up of PWN in host trees. To verify these findings additional investigations on epidemiology of virulent *B. xylophilus* in host trees are needed.

An important interrelationship between migration and population dynamics of PWN was made in the present study. It was shown that population clusters of PWN in *P. sylvestris* saplings developed independently of each other in different areas of the same plant. Although the method used could not distinguish between reproduction and migration of nematodes into the respective plant areas, populations increased first in the area close to the inoculation site and appeared later in distant areas.

It was shown that significant concentrations of nematodes appeared in the root system, where the water content was significantly higher than anywhere else inside the sapling during the later stages of the Pine Wilt Disease development. The results indicate that the nematodes retreat at some point in time from the shoot to the roots.

Due to the intensive segmentation of the whole plant and the extraction of nematodes in short intervals it could be demonstrated that reproduction of *B. xylophilus* in hosts can be best detected by extraction of nematodes at the following PWD stages:

(1) Two to four days after inoculation (initial density)

(2) At onset of wilt symptoms (maximum nematode population growth-phase)

(3) At terminal wilt symptoms (final density)

The detection of the shifting occurrence of the population in restricted plant parts helps to understand PWN population dynamics when being related to the whole plant. Therefore it is advisable to standardize extraction times and to extract PWN separately from root collar and shoots.

Effect of temperature on pathogenicity and population dynamics of *Bursaphelenchus xylophilus*

Temperature is known to affect the development of PWD in nature and under controlled climate conditions. It is also known that increasing temperature enhances the development of all life stages of *B. xylophilus* on Agar medium. The temperatures 15 °C, 20 °C and 25 °C were used as they reflect current summer isotherms for Central Europe. The influence of temperature on pathogenicity and on the population development of PWN in tolerant (*P. abies*) and sensitive (*P. sylvestris* and *L. decidua*) conifer hosts was investigated.

Higher temperatures were shown to increase the development of wilt and PWN development inside the susceptible conifer species.

The temperature did not affect tree mortality which was always highest at temperatures above 20°C. This temperature appeared to be a limiting factor for PWD. The lowest temperature of 15 °C did not induce wilt development in any tree species tested. This was also true for the development of PWN in the susceptible species *L. decidua* at 15 °C. However, nematodes were present in the shoots and roots of *P. sylvestris* saplings and indicated the presence of PWN in asymptomatic host trees at lower temperatures.

The PWN in *P. sylvestris* had reached higher densities at 25 °C than at 20 °C, whereas tree mortality was not different in both cases. Therefore the results demonstrated a threshold population density of *B. xylophilus* for the induction of irreversible wilt in *P. sylvestris*.

With the exception of a few saplings it was also shown that *Picea abies* remained tolerant at all temperatures.

Additional methodological conclusions of the present study are:

- Inoculation experiments for tree susceptibility to PWN require a high number of test saplings and the availability of a viable inoculum of a virulent nematode population.

- Inoculation trials need to be conducted during the season that is suitable for PWD which is the summer period from June to early August under Central European conditions.

- *P. sylvestris* with known susceptibility and sensitivity confirmed in many published inoculation trials should be used as a control.

- The validation of the results from saplings or seedlings on mature trees has to be done with caution.

- The methods used led to high variability of population density. Using the median which was useful in the determination of nematode distribution should be considered.

- Inoculation of *B. xylophilus* in conifer saplings was a successful method for observation of migration and population dynamics of the PWN. Using saplings allowed the sampling of the whole plant, whereas mature trees need sub-sampling that might not cover the present nematode population.

- The segmentation of the whole saplings was suitable for determining the characteristic phases of population growth, population maximum and population decline.

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7 Appendix

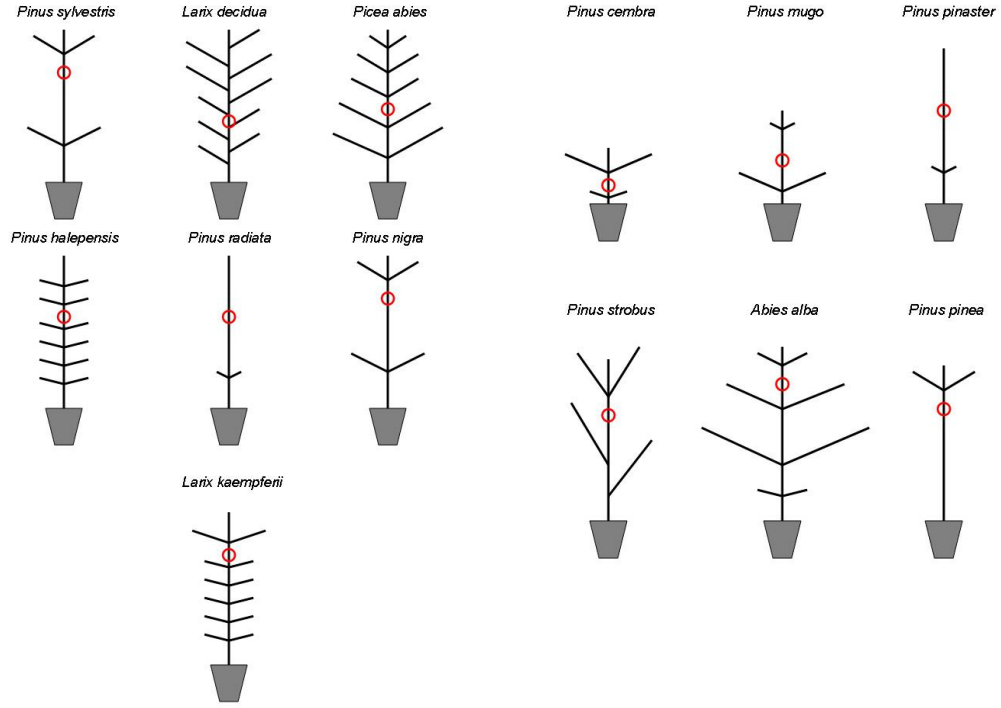

Fig. 7A 1-2: Point of inoculation of *Bursaphelenchus xylophilus* into potted conifer species in a greenhouse inoculation trial

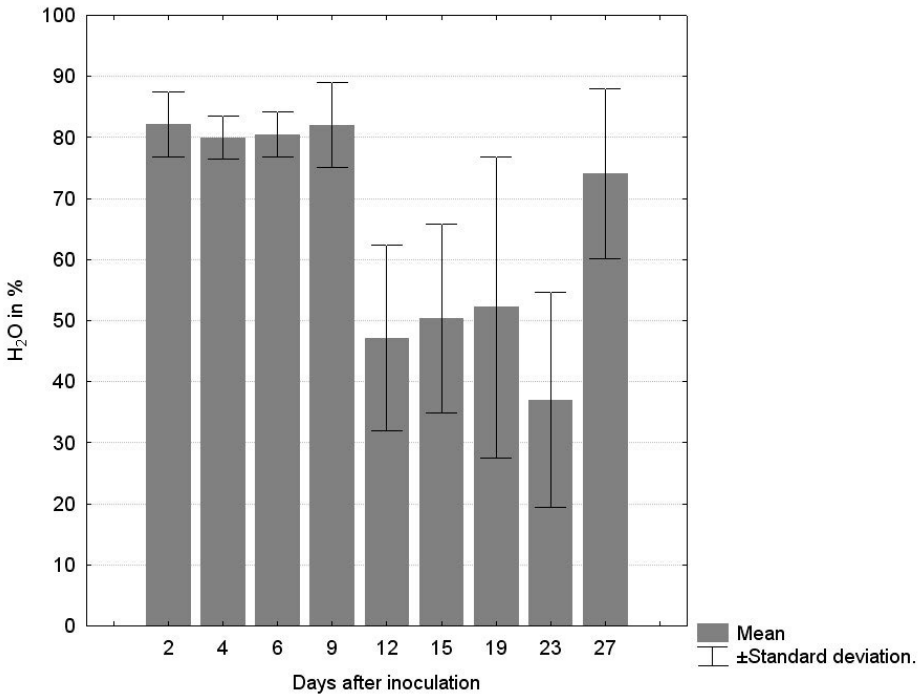

Fig. A 3: Relative water content of upper whorl (uW) of *Pinus sylvestris* saplings at nine sampling dates during a 27 day period after inoculation with *Bursaphelenchus xylophilus* at 25 °C, n = 10; Box-whisker plot: Box and Point (mean), Whisker (standard deviation)

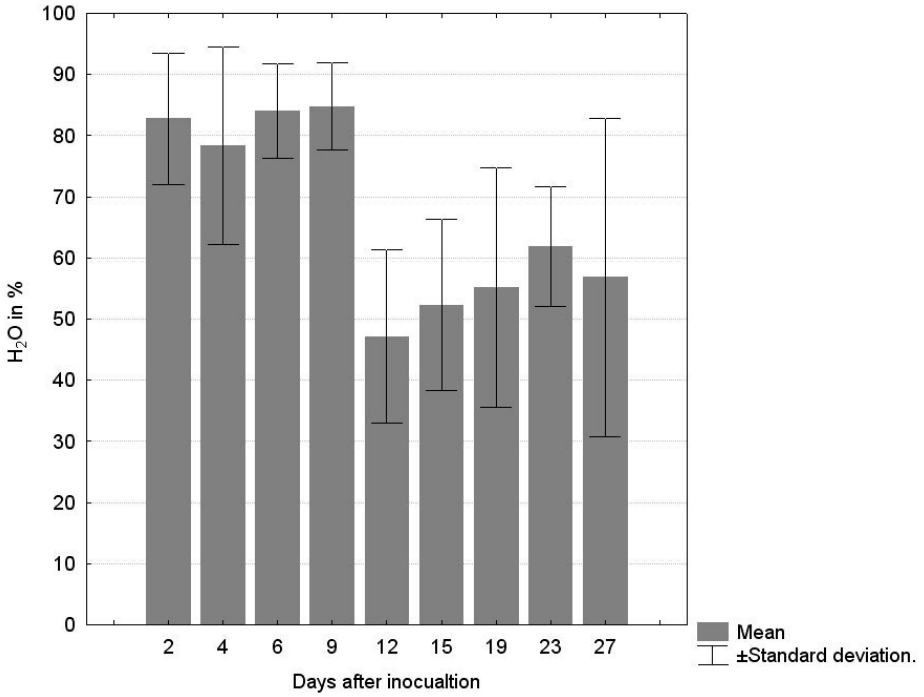

Fig. A4: Relative water content of the stem above the inoculation point (Sa) of *Pinus sylvestris* saplings at nine sampling dates during a 27 day period after inoculation with *Bursaphelenchus xylophilus* at 25 °C, n = 10; Box-whisker plot: Box and Point (mean), Whisker (standard deviation)

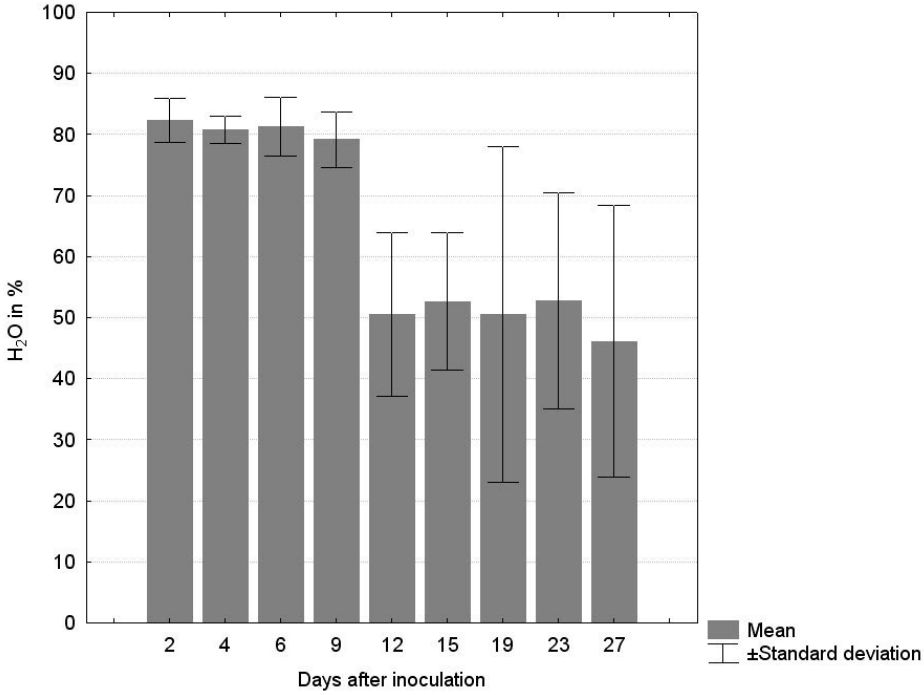

Fig. A5: Relative water content of the stem below the inoculation point (Sb1) of *Pinus sylvestris* saplings at nine sampling dates during a 27 day period after inoculation with *Bursaphelenchus xylophilus* at 25 °C, n = 10; Box-whisker plot: Box and Point (mean), Whisker (standard deviation)

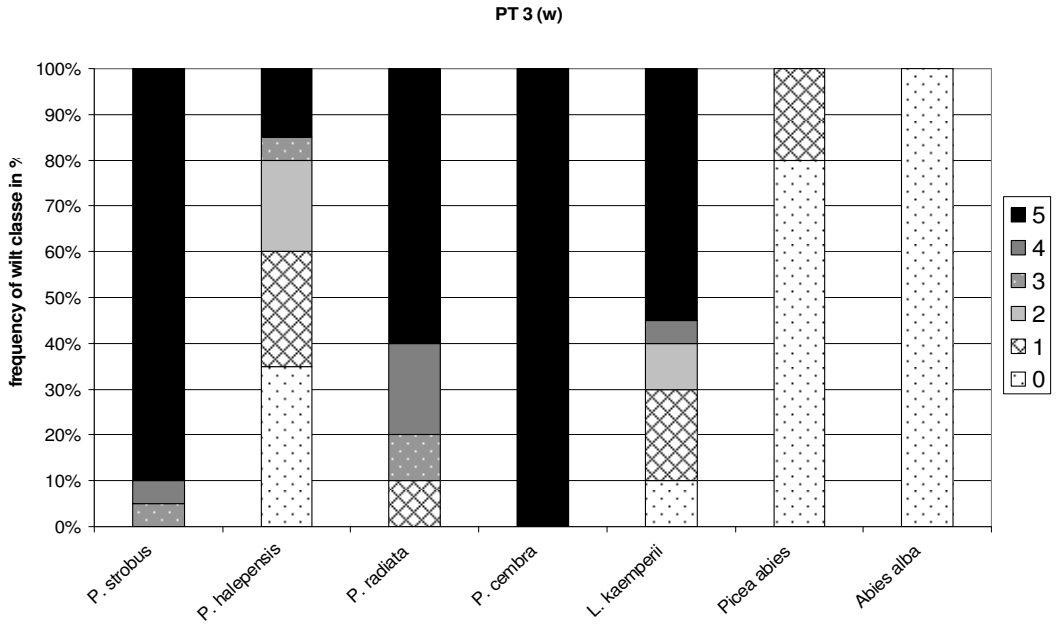

Fig.A 6: Frequencies of wilt classes of European conifers. (0: no symptoms and 1 - 5) inoculated with the *Bursaphelenchus xylophilus* isolate PT 3 (w) (Portugal) 12 weeks after inoculation; Each bar represents 100 % of saplings and the respective proportion of saplings belonging to one class

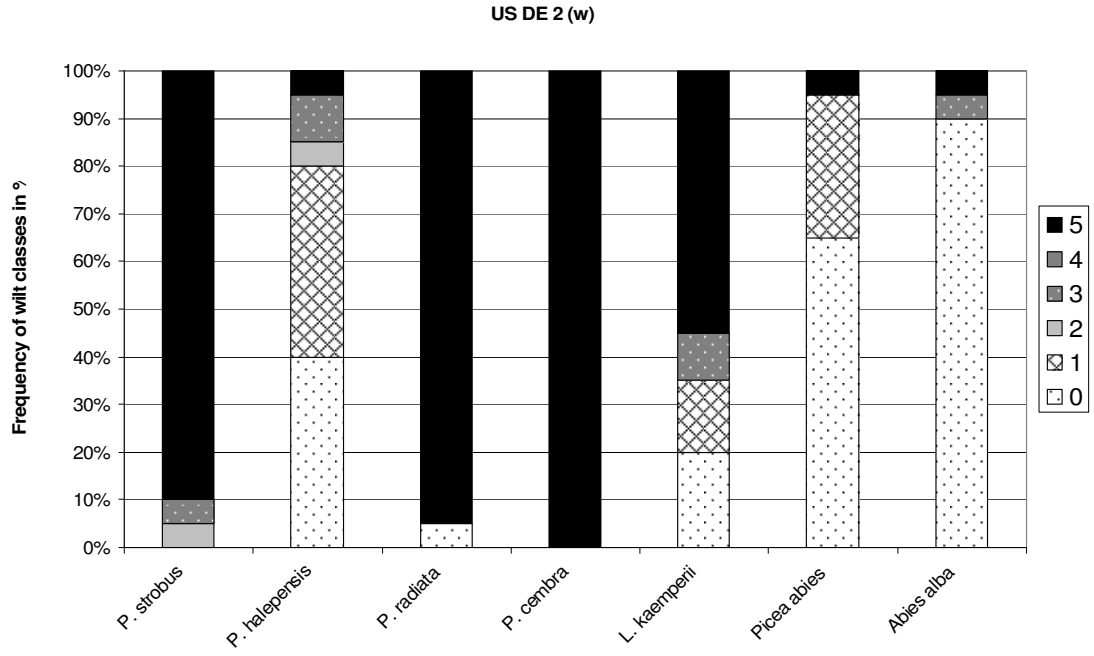

Fig.A7: Frequencies of wilt classes of European conifers. (0: no symptoms and 1 - 5) inoculated with the *Bursaphelenchus xylophilus* isolate US DE 2 (w) (North America) 12 weeks after inoculation; Each bar represents 100 % of saplings and the respective proportion of saplings belonging to one class

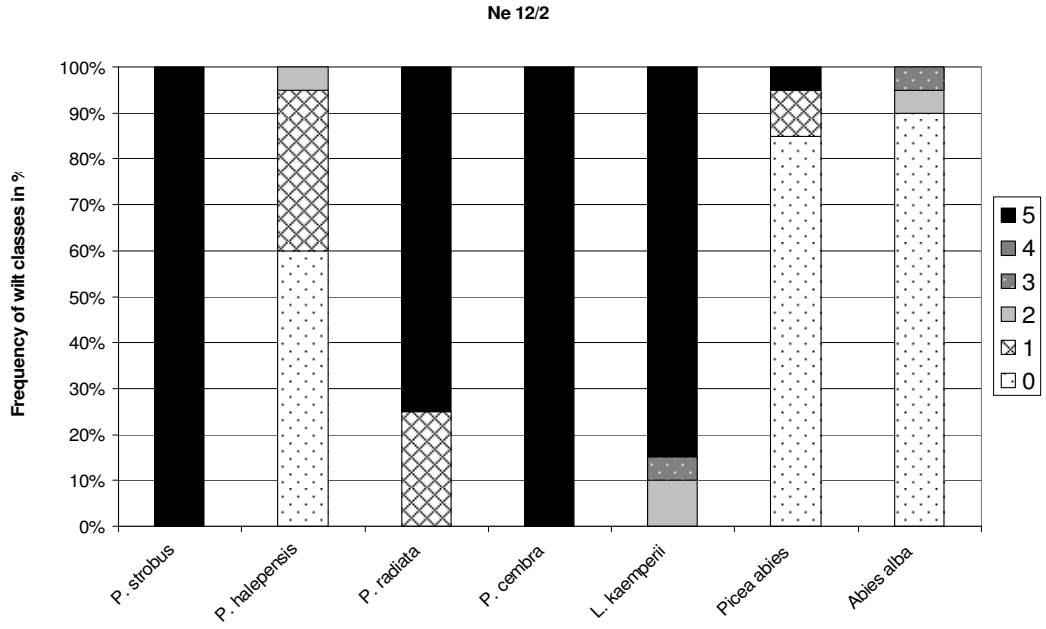

Fig.A8: Frequencies of wilt classes of European conifers. (0: no symptoms and 1 - 5) inoculated with the *Bursaphelenchus xylophilus* isolate Ne 12/02 (China) 12 weeks after inoculation; Each bar represents 100 % of saplings and the respective proportion of saplings belonging to one class

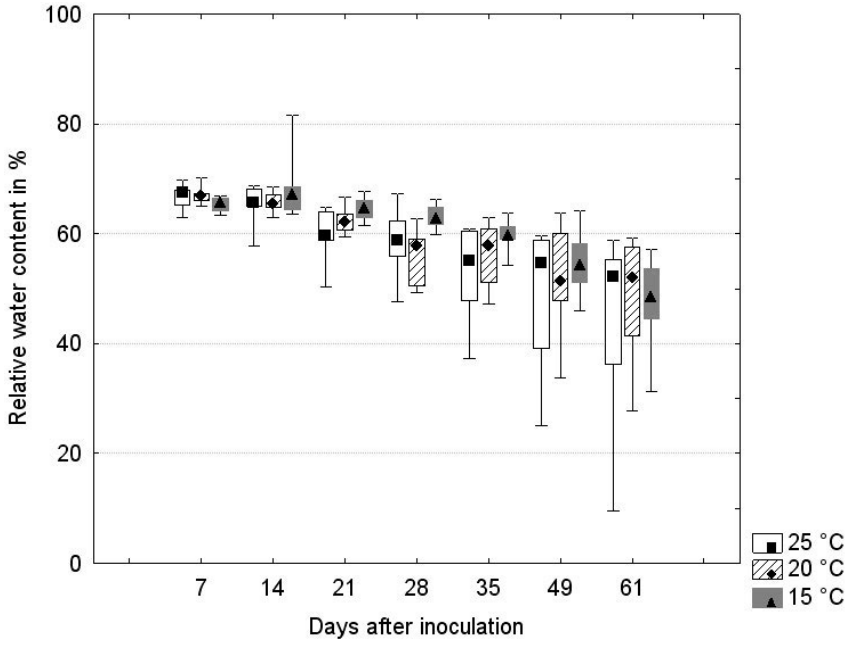

Fig. A9: Relative water content of wood of *Pinus sylvestris* saplings inoculated with *Bursaphelenchus xylophilus* at 25 °C, 20 °C and 15 °C, n = 10; Box-whisker plot: Point (median). Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

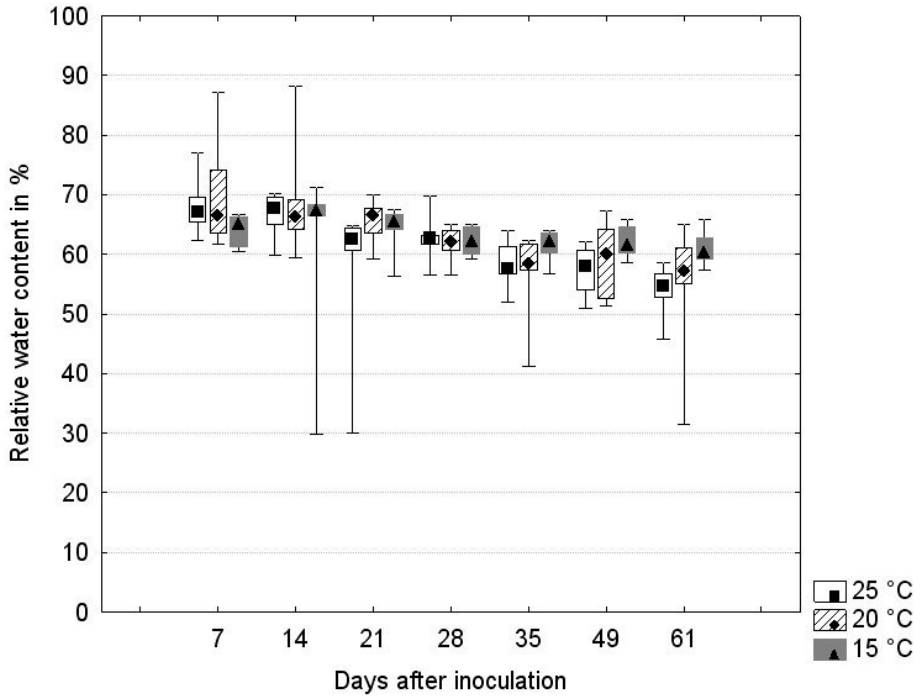

Fig. A10: Relative water content of wood of *Larix decidua* saplings inoculated with *Bursaphelenchus xylophilus* at 25 °C, 20 °C and 15 °C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

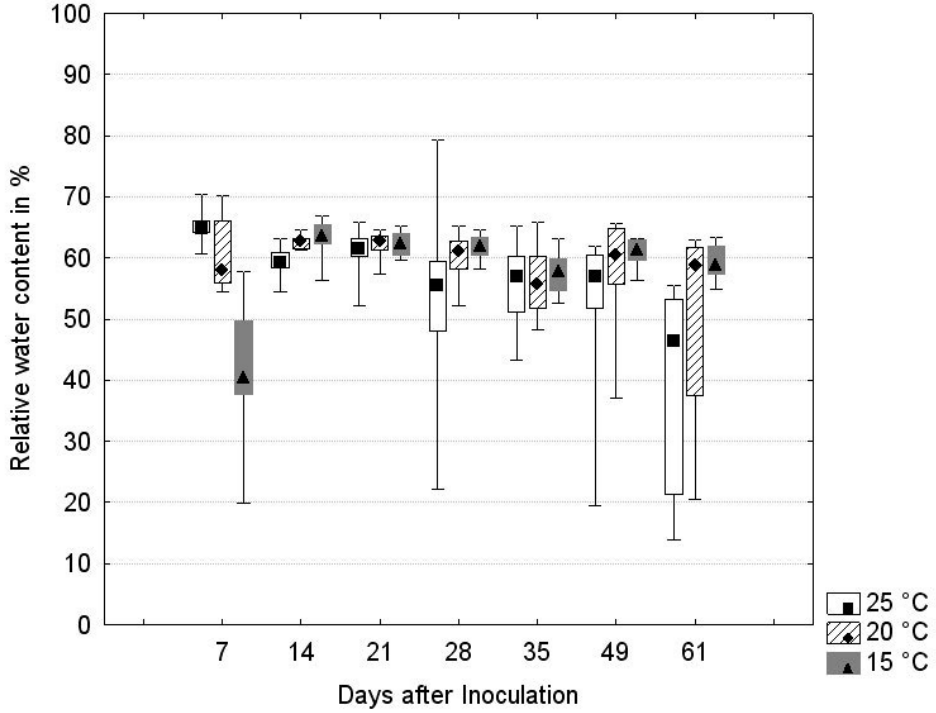

Fig. A11: Relative water content of wood of *Picea abies* saplings inoculated with *Bursaphelenchus xylophilus* at 25 °C, 20 °C and 15 °C, n = 10; Box-whisker plot: Point (median), Box (25 % - 75 % quartiles of values), Whisker (min-max span of values)

Tab.A1: Population densities of *Bursaphelenchus xylophilus* per g dry matter in segments of three - four year old P*inus sylvestris* sapling

	Number of nematodes /g dry matter (median)								
Days after inoculation	2 days	4 days	6 days	9 days	12 days	15 days	19 days	23 days	27 days
MS 1	8	20	10	20	1366	352	2003	70	0
MS 2	6	3	7	10	132	84	1016	29	0
uB 1	2	15	21	26	1374	62	1207	49	0
uB 2	2	3	5	12	133	30	459	25	0
uW	47	63	135	367	1979	199	139	27	14
Sa	130	280	545	882	1220	336	505	138	60
Ip	1502	1595	1755	1902	3773	1189	322	1140	68
Sb 1	281	699	264	1124	1474	353	468	111	71
Sb 2	46	95	64	164	1680	407	256	170	7
Sb 3	24	116	30	100	3104	363	915	517	90
Sb 4	25	129	27	63	2956	806	1288	538	1205
lB 1	0	21	0	9	351	108	2662	282	645
IB 2	0	5	1	25	108	70	706	96	30
lW	7	34	23	22	1407	299	2109	468	3044
Sr	9	20	14	39	1120	1148	3301	1795	5073
RC	0	0	5	34	1041	1996	7177	1132	3353
R	0	0	5	6	624	705	860	428	317
Plant	46	122	63	124	1840	406	2431	938	1569
Roots*	0	0	5	15	931	1416	4915	803	2093

n= 10; Values are expressed as median of the densities detected at 9 sampling dates during 27 day period after inoculation; Population densities of (1) the merged area Plant refers to segments: MS 1 +2 (main shoot). uB 1+2 (upper branches). uW (upper whorl). Sa (Stem above inoculation point). IP (inoculation point). Sb 1-4 (Stem below inoculation point). lB 1+2 (lower branches). lW (lower whorl). Sr (Stem above root collar); (2) of the merged area Roots to the segments: RC (root collar). R (roots)

Tab.A2: Maximum and Minimum values of population densities of *Bursaphelenchus xylophilus* per g dry matter in segments of three to four year old *Pinus sylvestris* sapling

	Number of nematodes /g dry matter (min - max)								
Days after inoculation	2 days	4 days	6 days	9 days	12 days	15 days	19 days	23 days	27 days
MS 1	0 - 34	0 - 311	0 - 44	0 - 259	12 - 8924	19 - 7689	0 - 5577	0 - 663	0 - 3
MS 2	0 - 26	0 - 24	0 - 43	0 - 108	0 - 1514	0 - 1410	0 - 2331	0 - 494	0 - 0
uB 1	0 - 36	4 - 69	0 - 95	0 - 70	32 - 4443	17 - 3055	0 - 2378	4 - 201	0 - 10
uB 2	0 - 29	0 - 40	0 - 43	0 - 44	4 - 8583	5 - 1256	0 - 2603	2 - 210	0 - 2
uW	6 - 243	0 - 1891	21 - 434	14 - 976	76 - 13584	107 - 1596	0 - 12982	0 - 1788	0 - 103
Sa	0 - 1400	0 - 5077	0 - 5432	188 - 1889	226 - 6739	0 - 1099	0 - 14698	10 - 16627	9 - 1571
Ip	500 - 1949	507 - 5105	346 - 3839	351 - 10410	631 - 17236	107 - 3204	130 - 9608	13 - 9315	21 - 490
Sb 1	114 - 981	409 - 1796	0 - 1662	161 - 5371	285 - 8075	56 - 4582	37 - 10960	0 - 36508	0 - 1371
Sb 2	14 - 102	17 - 916	10 - 232	11 - 695	95 - 10616	50 - 3901	10 - 4517	8 - 1883	0 - 39
Sb 3	0 - 99	59 - 409	0 - 101	0 - 817	176 - 11965	18 - 4050	25 - 2782	51 - 1793	0 - 1964
Sb 4	0 - 95	31 - 410	2 - 95	14 - 275	222 - 3947	25 - 3821	101 - 7030	81 - 3222	125 - 8105
lB 1	0 - 12	0 - 193	0 - 15	0 - 103	0 - 1755	13 - 6115	557 - 6413	20 - 12709	13 - 5185
IB 2	0 - 0	0 - 102	0 - 19	0 - 55	3 - 625	0 - 2314	46 - 2394	0 - 1119	0 - 679
lW	0 - 56	4 - 434	0 - 87	3 - 311	57 - 10759	0 - 6060	155 - 13810	27 - 35708	664 - 39484
Sr	0 - 50	4 - 96	0 - 37	0 - 384	38 - 4475	14 - 2698	628 - 7591	174 - 29767	1993 - 37598
RC*	0 - 11	0 - 16	0 - 36	0 - 135	275 - 5555	6 - 7692	164 - 26440	391 - 5272	16 - 9350
R*	0 - 0	0 - 0	0 - 6	0 - 43	0 - 4882	1 - 5559	181 - 4642	85 - 5440	121 - 1598
plant	37 - 62	59 - 386	47 - 119	24 - 351	107 - 3379	68 - 2751	179 - 3577	246 - 5841	532 - 9257
roots*	0 - 5	0 - 6	0 - 20	0 - 70	125 - 5200	3 - 5110	366 - 8333	226 - 5369	89 - 3401

n= 10; Values are related to densities detected at 9 sampling dates during 27 day period after inoculation; Population densities of (1) the merged area Plant refers to segments: MS 1 +2 (main shoot). uB 1+2 (upper branches). uW (upper whorl). Sa (Stem above inoculation point). IP (inoculation point). Sb 1-4 (Stem below inoculation point). IB 1+2 (lower branches). IW (lower whorl). Sr (Stem above root collar); (2) of the merged area Roots to the segments: RC (root collar). R (roots)

Tab. A3: χ^2 Values of differences in mortality rates of *Pinus syvestris*, *Larix deciduas*, *Picea abies* at 25 °C, 20 °C or 15 °C after 32, 46 and 60 days post inoculation

Days after inoculation	Comparisons*		χ^2
60	Ld 25	Pa 25	26.67 *
	Ld 20	Pa 20	
	Ps 25	Pa 25	
	Ps 20	Pa 20	
46	Ps 20	Ld 20	6.40
	Ps 25	Ps 20	21.54 *
	Ld 25	Ld 20	7.06
	Pa 25	Pa 20	2.06
32	Ps 20	Ld 20	8.64
	Ps 25	Ps 20	12.38 *
	Ld 25	Ld 20	1.03

n = 10, α = 0.05

*Ps (*Pinus sylvestris*), Ld (*Larix decidua*), Pa (*Picea abies)*

Tab.A4: Mann - Whitney U-test of differences between the relative water content of wood or needles of *Pinus sylvestris*, *Larix decidua* and *Picea abies* saplings inoculated with *Bursaphelenchus xylophilus* and control at 61 days after inoculation at temperatures at 25 °C, 20 °C or 15 °C

Mann-Whitney U-test					
	Variation	Wood Z value		Needles Z value	
P. sylvestris	25 °C	3.70	*	-3.78	*
	20 °C	3.77	*	-3.78	*
	15 °C	-3.77	*	-3.78	*
L. decidua	25 °C	-1.13		-3.43	*
	20 °C	-2.19	*	-2.82	*
	15 °C	0.45		-0.90	
P. abies	25 °C	-3.17	*	-1.56	
	20 °C	-0.03		-1.56	
	15 °C	0.37		-1.63	

n = 10 α = 0.05
* significant

Tab.A5: Population densities of *Bursaphelenchus xylophilus* per g dry matter in shoots and roots of three to four year old *Pinus sylvestris*, Larix decidua and Picea abies at 25 °C, 20 °C or 15 °C

			Number of nematodes /g dry matter					
Tree species	Temperature	Plant part	7 days	14 days	21 days	28 days	35 days	49 days
P. sylvestris	25	Shoots	70	122	3290	4275	3674	2171
		Roots	1	18	875	1070	868	613
	20	Shoots	27	204	528	1286	2480	2124
		Roots	1	3	49	471	243	619
	15	Shoots	26	6	9	19	25	47
		Roots	nd	1	1	11	3	60
L. decidua	25	Shoots	67	350	2558	2691	1640	806
		Roots	2	1	450	123	209	157
	20	Shoots	8	29	934	2166	2370	2346
		Roots	0	0	6	56	82	382
	15	Shoots	1	0	1	5	0	1
		Roots	nd	0	0	2	0	0
Picea abies	25	Shoots	205	50	123	355	148	31
		Roots	0	1	15	28	20	2
	20	Shoots	53	15	20	54	14	3
		Roots	0	0	0	1	1	3
	15	Shoots	18	2	1	2	1	0
		Roots	nd	0	0	0	0	0
	n = 10							

Values are expressed as median of the densities detected at seven sampling dates during 61 day period after inoculation; nd= not

Tab.A6: Maximum and minimum *Bursaphelenchus xylophilus* population densities in the shoots or roots of three to four year old *Pinus sylvestris*, Larix decidua and Picea abies at seven sampling dates over a 61 day period at 25 °C, 20 °C or 15 °C

			Min - max number of nematodes /g dry matter						
Tree species	Temperature	Plant part	7 days	14 days	21 days	28 days	35 days	49 days	61 days
P. sylvetris	25	Shoots	29 - 385	16 - 3555	1180 - 8014	52 - 8825	1480 - 6658	846 - 3851	9 - 1096
		Roots	0 - 10	2 - 1012	184 - 5336	120 - 3081	153 - 2135	163 - 1025	0 - 269
	20	Shoots	15 - 94	31 - 1619	15 - 3885	91 - 4426	645 - 2984	9 - 4568	313 - 3378
		Roots	0 - 2	0 - 1057	0 - 1088	212 - 3547	39 - 3528	0 - 2608	24 - 2529
	15	Shoots	40	1 - 27	5 - 70	9 - 109	8 - 284	3 - 606	67 - 1076
		Roots	nd	0 - 6	0 - 4	0 - 28	0 - 102	5 - 500	41 - 566
L. decidua	25	Shoots	11 - 593	56 - 2025	1040 - 6249	597 - 8513	640 - 2833	280 - 4532	50 - 759
		Roots	1 - 14	1 - 1089	0 - 2839	2 - 169	2 - 418	31 - 1252	2 - 163
	20	Shoots	0 - 57	14 - 246	5 - 2095	3 - 4041	590 - 5223	439 - 4657	80 - 1479
		Roots	0 - 5	0 - 3	0 - 104	3 - 634	9 - 1438	2 - 3410	0 - 3810
	15	Shoots	0 - 4	0 - 0	0 - 9	0 - 11	0 - 11	0 - 12	0 - 38
		Roots	nd	0 - 0	0 - 5	0 - 5	0 - 4	0 - 7	0 - 15
Picea abies	25	Shoots	48 - 682	3 - 873	1 - 1386	6 - 1690	3 - 1927	1 - 1503	1 - 435
		Roots	0 - 0	0 - 39	0 - 784	0 - 416	0 - 465	0 - 83	0 - 144
	20	Shoots	3 - 239	6 - 77	0 - 2685	0 - 3289	0 - 863	0 - 1078	0 - 550
		Roots	0 - 2	0 - 1	0 - 40	0 - 414	0 - 304	0 - 76	0 - 436
	15	Shoots	4 - 50	0 - 11	0 - 18	0 - 18	0 - 8	0 - 1	0 - 1
		Roots	nd	0 - 9	0 - 7	0 - 0	0 - 1	0 - 1	0 - 3
	n = 10								

Values are expressed as median of the densities detected at seven sampling dates during 61 day period after inoculation, nd= not determined

Tab.A7 a-b: Wilcoxin test (Z values) for differences between population densities in wood (a) and roots (b) of conifers at 25 °C, 20 °C or 15 °C

Population dynamics in wood

		Z values	
		Temperature in °C	
		20	15
P. sylvestris	25	3.08**	6.80***
	20		6.76***
L. decidua	25	1.04	7.27***
	20		7.16***
P.abies	25	3.59***	7.27***
	20		5.90***

n = 70 α = 0.05
** significance moderate
*** significance high

Population dynamics in root

		Z values	
		Temperature in °C	
		20	15
P. sylvestris	25	2.17*	5.21***
	20		4.14***
L. decidua	25	0.91	6.71***
	20		6.08***
P. abies	25	2.17*	5.21***
	20		4.14***

n = 70 α = 0.05
significance low ** significance moderate
*** significance high

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