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Pathogenicity of selected isolates of the quarantine pinewood nematode *Bursaphelenchus xylophilus* to Scots pine (*Pinus sylvestris* L.)

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Abstract: The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease (PWD). This nematode is considered to be an indigenous to North America and was introduced to Japan in the late 19th century. Subsequently, it has spread throughout Japan and in many other countries, China, Taiwan, and South Korea. In 1999, *B. xylophilus* was discovered in Portugal, and in 2008 in Spain. So far the studies have revealed that the pathogenicity of *B. xylophilus* varies between different isolates. The conducted study compared the pathogenicity of five isolates of *B. xylophilus*, originating from different parts of Japan, to 3-year-old *Pinus sylvestris*, and their ability to reproduce in the seedlings. The results revealed diverse virulence of *B. xylophilus* resulting in plant mortality. Three isolates S10, Ka4, and T4 caused 100% mortality of plants within three months while at the same time, the other two isolates, C14-5 and OKD-1 did not cause any disease symptoms on plants. After seven months, some dieback occurred on two seedlings, but similar symptoms were also found on the control plant. Moreover, a significant positive correlation was found between nematode virulence and the number of nematodes reproducing on pine seedlings.

Key words: *Bursaphelenchus xylophilus*, pathogenicity, pinewood nematode, *Pinus sylvestris*

Introduction

The pinewood nematode (PWN), *Bursaphelenchus xylophilus*, is the causative agent of pine wilt disease (PWD) (Kiyohara and Tokushige 1971). This nematode is considered to be a native species to North America and it was introduced to Japan in the late 19th century (Dropkin *et al*. 1981; Rutherford *et al*. 1990; Furuno *et al*. 1993). After 1980, it spread to other Asian countries (China, Taiwan, and South Korea). Despite the phytosanitary measures provided by the European and Mediterranean Plant Protection Organization (EPPO) and the European Union (EU) to prevent the accidental introduction of *B. xylophilus* with wood products imported from infested areas, *B. xylophilus* was detected in Portugal in 1999, and subsequently, in Spain in 2008 (Mota *et al*. 1999; Filipiak 2008; Robertson *et al*. 2011). There is a risk of further spread of this pest to other countries in Europe, including Poland. In Poland, pine trees occupy 60% of the country's area (GUS 2012). The spread of this pest additionally increases due to the presence of at least five *Monochamus* spp. (*M. galloprovincialis, M. sartor, M. sutor, M. saltuarius*, and *M. urussovi*). These species are natural vectors of *B. xylophilus* in other countries (Kozłowski 2003). In Japan, the annual loss of pines caused by pine wilt, reached a maximum value of 2,430,000 m^3 in 1979 (Togashi and Shigesada 2006). Nowadays, pine wilt disease annually kills 2,000,000 m^3 of pine trees worldwide (EPPO 2015). In Portugal, up till now, *B. xylophilus* has destroyed more than 1,000,000 ha of pine forests (Robertson *et al*. 2011). The pinewood nematode is recognised worldwide as one of the most important pests in the forestry industry and is listed as a major plant quarantine objective for most countries in the world (Li 2008).

So far, studies have revealed that the pathogenicity of *B. xylophilus* associated with conifers, varies between different isolates (Mota *et al*. 2006). The nematode populations isolated from different coniferous host plants and/or from different regions have been demonstrated as having different virulence on *Pinus* spp. Virulence ranges from 0 to 100%. However, the virulence of nematode isolates was found not to vary within a single pine or vector insect (Kiyohara and Bolla 1990; Jiao *et al*. 1996). Virulence of nematode isolates is generally evaluated by the mortality of pine seedlings (mainly more than one year old) inoculated with nematodes (Aikawa and Kikuchi 2007). However, until now, naturally occurring avirulent isolates have been isolated only in Japan (Kiyohara and Bolla 1990).

The main objectives of the study were to compare the pathogenicity of five isolates of *B. xylophilus*, originating from different parts of Japan, to 3-year-old *Pinus sylvestris* L. plants, and to compare the ability of these isolates to reproduce in the seedlings.

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Materials and Methods

In this study, five isolates of *B. xylophilus* originating from Japan, were examined: C14-5, Ka4, OKD-1, S10, and T4. The isolates were kindly supplied by Dr Yuko Takeuchi from Kyoto University, Japan (the Main Inspectorate of Plant Health and Seed Inspection gave permission to import them for research purposes). The isolates C14-5 and OKD-1 are well known avirulent isolates originating from Japan, which previously have been extensively studied by Takemoto *et al*. (2005). Prior to examination, all isolates were reared on *Botryotinia fuckeliana*/malt agar (4.5%) at 25°C for ca 2 weeks. Propagated nematodes were collected by the Baermann funnel method and counted under a stereomicroscope.

The pathogenicity of isolates was assessed in relation to 3-year-old *P. sylvestris* potted at least six weeks before inoculation (pot height: 13 cm, diameter: 17 cm). The substrate contained a mixture of garden turf and sand (3 : 1). The experiment began in March when the new growth appeared on the plants. The nematodes were introduced onto plants using the standard method of artificial inoculation (Braasch 2000; Tomalak 2004). A 1-cm long slit was cut on the stem of the young plants, just below the new shoot. A small cotton strip was inserted into the slit. Then, 100 µl of water containing 2,500 nematodes were dripped into this slit. The inoculation point was wrapped with a Parafilm strip. Fifty plants were inoculated with five different nematode isolates, i.e. 10 plants per isolate, and 10 control plants were inoculated with water only. The plants were kept in a climatic chamber [lighting – 12 h per day, constant temperature – 25°C, relative humidity (RH) – 60%]. Each plant was irrigated with water twice a week. The plants were observed for symptoms once a week. The first obvious external symptom is the yellowing and wilting of the needles leading to eventual death of the tree, although the whole tree may later show symptoms. Dead plants were cut immediately above the ground. The twigs and needles were removed and the stems were chopped and extracted by the Baermann funnel technique for 24 h to get the nematodes. Plants that had not totally died off were cut, chopped, and extracted after ten months. The extracted nematodes were counted under a stereomicroscope. The number of nematodes was then determined for the wilted and healthy seedlings.

Results were analysed statistically using analysis of variance (ANOVA). The significance of differences was tested using Tukey's multiple comparison test (significance level 0.05). To achieve homogeneity of variance, a root transformation was used for the number of nematodes. The analysis of regression was performed to examine the cause and effect relationship between the number of days, after which plant death occurred, and the number of recovered nematodes. Statistical analyses were performed using Statistica software (version 10).

Results

The experiment conducted under controlled conditions revealed varied ability of *B. xylophilus* to cause plant mortality. The first wilting symptoms were observed during the first month of the experiment (Fig. 1). After 4 weeks, isolate Ka4 caused 60% plant mortality, isolate S10 – 40% plant mortality, and isolate T4 – 30% plant mortality. Further plant wilting was observed over the next month. The first complete wilting case was caused by isolate T4, and was observed in the second month of the experiment. At the same time, the other isolates: S10 and Ka4, caused an 80% mortality of the plants. There was a big increase in the number of wilted *P. sylvestris* seedlings in the third month of the experiment. The experiment also showed that 100% of the *P. sylvestris* seedlings inoculated with isolates S10 and Ka4 wilted in the twelfth week of the experiment.

During the sixth month of the experiment, the two other isolates, C14-5 and OKD-1, did not cause any symptoms of disease on the plants. Two seedlings (one for each of the isolates) showed some signs of dying after seven months, but similar symptoms were also found on a control seedling. The control plants showed a 10% mortality of the seedlings, but no nematodes were recovered from extracted wood.

In all of the dying plants, characteristic disease symptoms caused by *B. xylophilus* were observed, i.e. blockage of a sap flow, sudden discoloration of needles, and finally death of the plant.

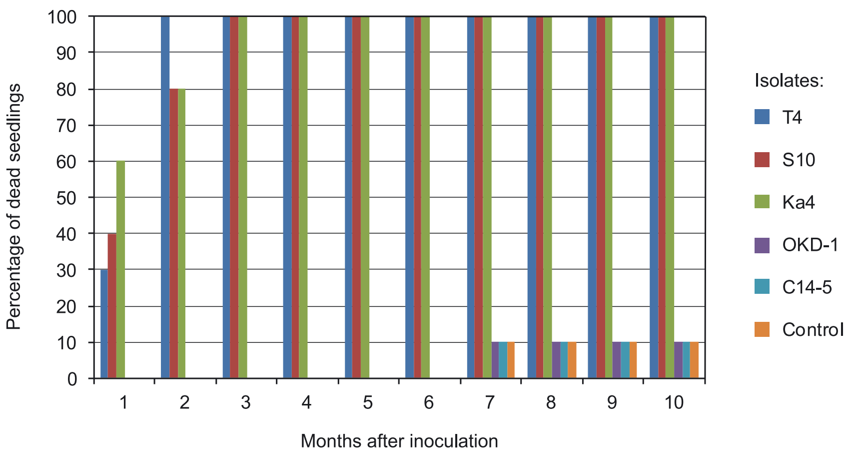

Fig. 1. Mortality of plants caused by tested isolates of *Bursaphelenchus xylophilus* in subsequent consecutive months of the growing season

Table 1. Pathogenicity test results of *Bursaphelenchus xylophilus* on 3-year-old seedlings of *Pinus sylvestris* under greenhouse conditions and results of grouping based on Tukey's test for average number of re-isolated nematodes per seedling ($\alpha = 0.05$; MS = 543.32; df = 45) and for average number of days after which plant death occurred ($\alpha = 0.05$; residual: MS = 751.80; df = 45)

Isolate	Mortality [%]	Average no. of re-isolated nematodes per seedling	Average no. of days after which plant death occurred
Ka4	100	11,051.0 b	46.3 a
T4	100	10,660.0 b	44.7 a
S10	100	9,392.0 b	52.5 a
C14-5	10	2,958.5 a	295.7 b
OKD-1	10	2,082.0 a	294.7 b

Means followed by the same letters are not significantly different

After the death of the plants or at the final end of the experiment, the numbers of re-isolated nematodes were counted (Table 1). The number of nematodes re-isolated from the seedlings ranged from 40 to over 21,000 specimens. The average number of nematodes re-isolated was the highest in the *P. sylvestris* seedling inoculated with isolate Ka4 (Fig. 2). The lowest average number of nematodes was recovered from the seedling inoculated with isolate OKD-1. The highest number of nematodes, amounting to 21,000, was recovered from a single seedling inoculated with isolate T4. The average number of nematodes re-isolated was higher in dead seedlings than in healthy seedlings. The average number of nematodes re-isolated from wilted seedlings inoculated with all the five isolates was almost identical, and a similar trend was observed among the healthy seedlings. The highest number of nematodes recovered from a healthy seedling was 3,400 from a plant inoculated with isolate C14-5. A significant positive correlation was shown between nematode virulence and the number of nematodes reproduced on pine seedlings.

The analysis of variance was conducted for a number of days after which plant death occurred and for the total number of recovered nematodes (a root of the number of nematodes based on a root transformation). The analysis of variance revealed significant differences between the tested combinations (respectively: $F_1 = 244.3$; df = 4, 45; $p < 0.001$; $F_2 = 15.5$; df = 4, 45; $p < 0.001$). Isolates T4, Ka4, and S10 were significantly different from isolates C14-5 and OKD-1 and formed two homogeneous groups (Table 1).

The results of the analysis of variance for regression showed a highly significant effect of the number of days after which plant death occurred on the number of recovered nematodes (F = 54.9; df = 4.8; $p < 0.001$) (Fig. 3).

Discussion

Previous studies demonstrated that the virulence level of *B. xylophilus* varies greatly among isolates and that there are differences in the reactions of pine trees to infection by virulent and avirulent *B. xylophilus* isolates (Ikeda and Suzuki 1984; Kiyohara and Bolla 1990; Aikawa and Kikuchi 2007). Until now, non-pathogenic isolates of *B. xylophilus* have been confirmed only from Japan, while naturally occurring avirulent isolates of *B. xylophilus* have not yet been isolated in the United States or in Europe. Virulence varies greatly among isolates of this nematode from pines within the same stand and different stands in Japan. These differences could arise because of host or geographic isolation. The differences may also have been influenced by environmental stress, including the range of seasonal temperatures (Rutherford and Webster 1987; Kiyohara and Bolla 1990).

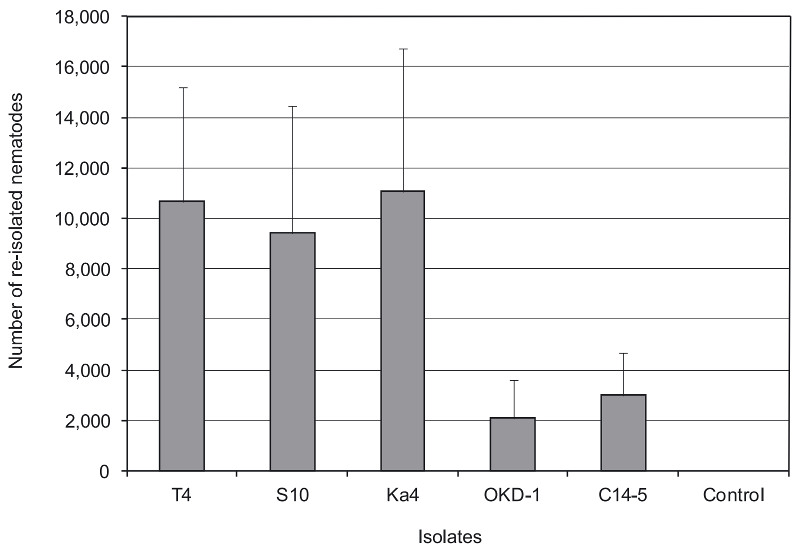

Fig. 2. The ability to reproduce different *Bursaphelenchus xylophilus* isolates in inoculated seedlings (mean±SD)

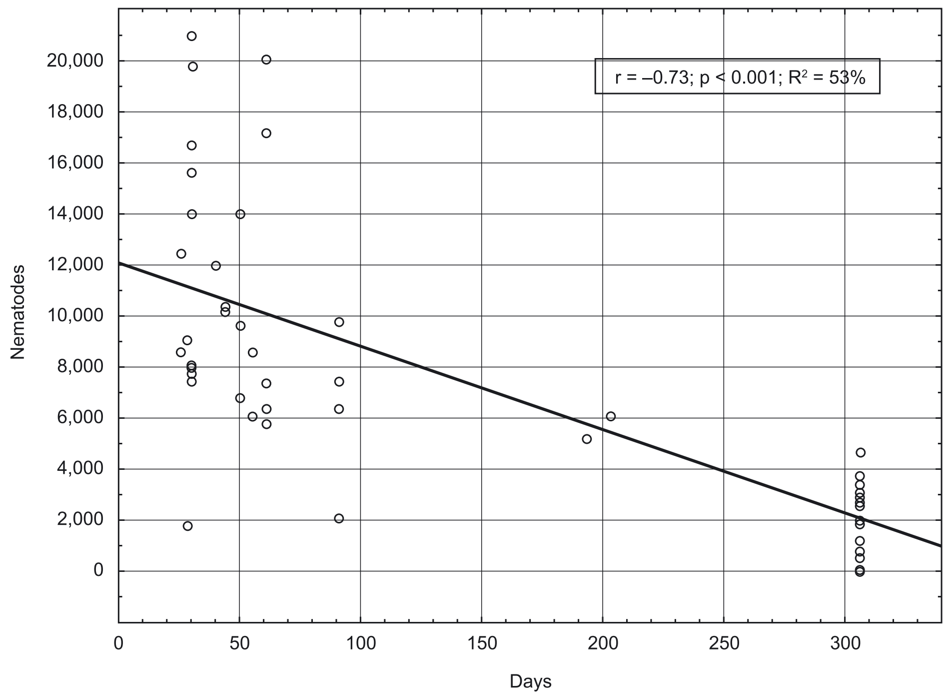

Fig. 3. A scatterplot with a fitted regression line (the number of re-isolated nematodes with respect to the changing number of days)

In this study, the nematode virulence and reproduction were investigated using *P. sylvestris*. The species, *P. sylvestris*, is one of the most susceptible species to *B. xylophilus,* and it is the most widely spread conifer species in central and northern Europe (Futai and Furuno 1979; Caroppo *et al*. 2000). The conducted study confirmed the different levels of pathogenicity of *B. xylophilus* to *P. sylvestris* seedlings. The examined isolates Ka4, S10, and T4 were highly pathogenic and caused a 100% mortality of plants during the three months of the experiment, while the C14-5 and OKD-1 isolates seemed to be nonpathogenic to *P. sylvestris* seedlings. During the study only one wilted seedling was observed for each of these isolates.

To date, several studies have reported on the reproductive ability of *B. xylophilus* isolates, with different levels of virulence, under *in vitro* and *in vivo* conditions. The studies indicated a significant positive correlation between virulence and the reproductive ability of *B. xylophilus* on the *B. fuckeliana* culture (Aikawa and Kikuchi 2007). Virulent isolates reproduced four times as abundantly as avirulent ones when cultured on fungal mats on *B. fuckeliana* at 25°C for 6 days (Kiyohara and Bolla 1990; Wang *et al*. 2005; Aikawa and Kikuchi 2007). In the *in vivo* situation, when virulent and avirulent isolates separately inoculated *P. thunbergii* seedlings, nematode density in the case of the virulent isolates, increased with time after inoculation (Aikawa *et al*. 2006; Aikawa and Kikuchi 2007).

In this study, reproductive ability was examined using *B. xylophilus* isolates with different virulence. A highly positive correlation was found between virulence and reproductive ability of *B. xylophilus*. For the Ka4, S10, and T4 isolates, causing 100% mortality of plants during the three month period, significantly higher numbers of re-isolated nematodes were recorded when compared to the C14-5 and OKD-1 isolates. Moreover, the healthy seedlings inoculated with the C14-5 and OKD-1 isolates had a significantly lower number of nematodes when compared to the wilted seedlings. This suggests that nematode virulence is closely associated with its reproductive ability, irrespective of the *in vitro* or *in vivo* conditions.

However, nematode virulence cannot be explained only by its reproductive ability. The ability of nematodes to establish in pine tissues, and to feed on living cells in pine tissues, are also thought to be important factors for determining the virulence level. Nematode infection of a host tree is impossible without these attributes (Aikawa and Kikuchi 2007). It was mentioned, that composition and/or activity of the enzymes related to the degradation of the cell wall in pine tissues is one of the factors determining the virulence level of nematodes (Kojima *et al*. 1994). Moreover, some studies suggest that the secreted proteins are essential molecules for parasitism by *B. xylophilus* (Shinya *et al.* 2013; Espada *et al*. 2015).

This study compared the pathogenicity of different *B. xylophilus* isolates originating from Japan. So far, the occurrence of isolates of this nematode with varying degrees of pathogenicity has been confirmed from that region, only. In future research, it may be interesting to compare the pathogenicity of *B. xylophilus* isolates originating from Europe and North America and to elucidate the relationship between virulence and reproductive ability of the nematode using pine species other than *P. sylvestris.*

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