

# Study of symptoms and gene expression in four *Pinus* species after pinewood nematode infection

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## Abstract

Pine wilt disease, caused by the pinewood nematode *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle, is originating severe infections in pine trees. The disease is detected when external symptoms appear (e.g. needle chlorosis), but trees could remain asymptomatic for long periods and serve as a long-term host. The primary goal of this study was to assess the effect of inoculation with an avirulent isolate of *B. xylophilus* (C14-5) on different *Pinus* spp. seedlings (*P. sylvestris*, *P. nigra*, *P. pinea* and *P. pinaster*). At the same time, seedlings were also inoculated with a virulent strain, HF, in order to compare the phenotypic and genomic results of the two types of inoculations. The effect of inoculation was determined in terms of expression of various *Pinus* genes potentially involved in the response to the disease. The results suggest that *P. pinea* and *P. nigra* are more resistant to infection by the nematode than *P. sylvestris* and *P. pinaster*. The phenotypic and genetic differences were more marked among *P. pinea* and *P. pinaster*.

**Keywords:** *Bursaphelenchus xylophilus*; genetic expression; *Pinus* spp

## Introduction

Recently, pine wilt disease (PWD), caused by the pinewood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle, has become a major threat to the European forests, with an estimated mortality risk of 50%. In Portugal, PWN was first detected in 1999 (Mota *et al.*, 1999), and in 2008, the entire continental Portugal was demarcated as PWN-infested. Its insect vector is *Monochamus galloprovincialis* and, once infected, most plants cease resin production and show the symptoms of needle chlorosis. Infection usually becomes a fatal condition in just a few months (Fukuda, 1997). However, it is not known how the

involved genes are regulated in trees with differential disease resistance after attack by nematodes with varying degrees of virulence (Kosaka *et al.*, 2001; Kuroda *et al.*, 2004). Thus, a targeted gene expression approach was taken in order to investigate the infection mechanisms in commercially important pine species, namely *P. sylvestris*, which is the most threatened species in northern/central Europe, *P. nigra* and *P. pinaster*, that are being affected in the central/southern areas and *P. pinea* that is thought to be resistant to the infection (OEPP/EPPO, 2001). Symptoms of infection with a virulent strain of PWN (HF, isolated from Setúbal, Portugal) and with an avirulent strain (C14-5, described by Takehushi *et al.*, 2006) were also monitored and evaluated.

## Materials and methods

Seeds of *P. pinaster*, *P. pinea*, *P. sylvestris* and *P. nigra* were sterilized, germinated in 1% water agar (Agar no. 1,

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**Table 1.** Development of symptoms in *P. pinaster* (PP), *P. pinea* (PPi), *P. nigra* (Pni), *P. sylvestris* (Psy), before ( $T_0$ ), and 10 ( $T_{10}$ ) and 20 d ( $T_{20}$ ) after inoculation with sterile water ( $H_2O$ ), avirulent strain (A) and virulent strain (HF)

| Treatment conditions | Incubation time (d) |          |          |
|----------------------|---------------------|----------|----------|
|                      | $T_0$               | $T_{10}$ | $T_{20}$ |
| PP $H_2O$            | I                   | I        | II       |
| PP A                 | I                   | II       | III      |
| PP HF                | I                   | III      | IV       |
| PPi $H_2O$           | I                   | I        | I        |
| PPi A                | I                   | II       | II       |
| PPi HF               | I                   | I        | II       |
| Pni $H_2O$           | I                   | I        | I        |
| Pni A                | I                   | II       | II       |
| Pni HF               | I                   | II       | II       |
| Psy $H_2O$           | I                   | I        | II       |
| Psy A                | I                   | II       | II       |
| Psy HF               | I                   | II       | III      |

I, Healthy plant; II, partial needle discolouration; III, partial needle discolouration, necrosis and reduction in the resin production; IV, total discolouration, necrosis and seedling death.

Lab M) and incubated for 2 weeks at 25°C, with a photoperiod of 8h light–16h dark. Once germinated, seedlings were individually incubated vertically for 4 months under the same conditions described earlier and supplied with 10 ml of nutritive solution Murashige and Skoog basal medium (Sigma).

*B. xylophilus* strains were grown on barley seeds with *Botrytis cinerea* at 26°C, in the dark, and extracted using the Baermann funnel technique. A total of 20 seedlings of each pine species were inoculated with 500 avirulent or virulent nematodes in a 100  $\mu$ l sterile water suspension (Asai and Futai, 2002).

To evaluate the genetic expression, samples were taken at 0, 10 and 20 d after inoculation and stored at – 80°C. Total RNA was extracted according to Le Provost *et al.* (2007) and purified with Turbo DNA-free kit (Applied Biosystems, Foster city, CA, USA), according to the

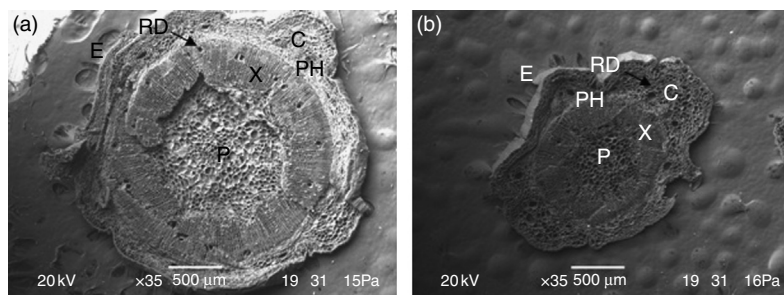
manufacturer's instructions. Gene expression was determined using 100 ng of RNA, with the conditions and program presented in Supplementary Table S1 (available online only at <http://journals.cambridge.org>), using an MJ Mini Gradient Thermal Cycler (Bio-Rad Laboratories, PA, USA). The gene *18S* was used as internal control.

Scanning electron microscope was used to examine the morphology of *P. pinea* and *P. pinaster* stems. Thin, manual cuts were made with a scalpel. Each sample was attached to a support with double-sided duct tape and placed in a desiccator until the samples were dehydrated. Samples were analysed following the user manual of SEM JSM5600LV, operating at 20 kV.

## Results and discussion

Symptoms of disease were only detected 10 d after inoculation. *P. pinea* and *P. nigra* seem less susceptible to the infection since their symptoms did not develop beyond stage II; *P. pinaster* appears to be the most susceptible species, as some seedlings died 20 d after the inoculation. Also, inoculation with the avirulent nematode resulted in seedlings with some degree of needle discolouration. (Table 1) These differences of infection may be due to the blocking of the vascular system with resin produced by cells from radial parenchyma, which are damaged by the nematode, resulting in needle chlorosis and plant death some time after infection (Jones *et al.*, 2008).

Anatomical differences among genotypes might be on the basis of differential resistance to PWN, therefore *P. pinaster* and *P. pinea* were examined with scanning electron microscope (SEM). Visual inspection of the resulting photographs (Fig. 1) indicates that the round shape of the stem is better maintained in *P. pinea* than in *P. pinaster* after manual cross-sectioning. This may be due to higher lignin content in *P. pinea* and may be related to increased resistance to PWD in this species. Lignin has a recognized role in plant defence, and constitutive lignin has already been related with



**Fig. 1.** SEM imaging of *P. Pinea* (a) and *P. pinaster* (b) stem cross-sections showing morphological differences between the two species. E, epidermis; C, cortex; RD, resin duct; PH, phloem; X, xylem; P, pith.

defensive mechanisms against nematodes in other species (Fogain and Gowen, 1996). Experiments regarding lignin quantification are ongoing. Furthermore, the diameter in cortical resin ducts seems larger in *P. pinaster* stem. This may contribute to the susceptibility of this genotype to PWD, since PWNs progress inside the plant through resin ducts (Fukuda, 1997). Increased number and diameter of resin ducts have already been associated with PWD susceptibility (Kawaguchi, 2006).

Biotic and abiotic factors stimulate the plant's defence response, diminishing the negative impacts of the pathogenic attack. The genes of interest tested in this work were found to be associated to osmotic stress, oxireductive processes and cell death, among others, which are important in the defence response of *P. densiflora* (Japanese Red Pine) against the nematode (Shin *et al.*, 2009).

Pathogenesis-related proteins 4 expression was detected in all treatments (Supplementary Figs. S1–S4, available online only at <http://journals.cambridge.org>). PR proteins are induced as response to pathogen attacks (Osmond *et al.*, 2001) and can be factors of hypersensitive response to nematode infection (Meins and Ahl, 1989; Shin *et al.*, 2009). *ATTRX1*, a protective gene against oxidative stress, was also expressed in all treatments and pine tree species (Supplementary Figs. S1–S4, available online only at <http://journals.cambridge.org>), suggesting that different types of defences may be activated. There is a reported relationship between metallothionein being expressed in the presence of intensive oxidative stress (Mir *et al.*, 2004).

Ethylene is an important component of conifer response against pathogens (Miller *et al.*, 2005), hence it can induce cell defence. In *P. pinaster* (Supplementary Fig. S1, available online only at <http://journals.cambridge.org>), the expression of *MAT2/SAM2* (ethylene production) was only detected at the end of 20 d, which in the case of virulent *B. xylophilus* treatment corresponded to plant death; in *P. pinea* (Supplementary Fig. S2, available online only at <http://journals.cambridge.org>), the inoculations with both avirulent and virulent nematodes originated the same type of response as water-inoculated plants (control). On the other hand, in seedlings of *P. nigra* (Supplementary Fig. S3, available online only at <http://journals.cambridge.org>), *MAT2/SAM2* and *SHEPERD* (water reduction) gene expression was only verified in nematode-inoculated plants. This can be explained by the increasing release of volatile compound production, which alters water transportation (Jones *et al.*, 2008). Finally, *P. sylvestris* seedlings (Supplementary Fig. S4, available online only at <http://journals.cambridge.org>) demonstrated that virulent strains of *B. xylophilus* did not cause any *MAT2/SAM2* gene expression.

It must be noted that some of the primers used (Supplementary Table S2, available online only at

<http://journals.cambridge.org>) were not specifically designed for the species in question. Thus, it could explain the absence of genes that normally would be expressed. Also, plant mechanisms vary during the time of a day, so the time when the sampling was made may also have influenced the results.

This is the first report of inoculations with virulent and avirulent *B. xylophilus* strains in various pine species, and though infection mechanisms of both PWN were not clear, this study suggests that inoculation with virulent nematode can trigger a phased systemic response that differs from the avirulent strain. However, it is necessary to identify other factors that may be responsible for the plant defence when it is attacked by the different pathogens.

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## References

- Asai E and Futai K (2002) Promotion of the population growth of pinewood nematode in 4-month-old. *Journal of Forestry Research* 7: 113–116.
- OEPP/EPPO (2001) *Bursaphelenchus xylophilus*. *PM 7/4 (1) OEPP/EPPO Bulletin* 31: 61–69.
- Fogain R and Gowen SR (1996) Investigations on possible mechanisms of resistance to nematodes in *Musa*. *Euphytica* 92: 375–381.
- Fukuda K (1997) Physiological process of the symptom development and resistance mechanism in pine wilt disease. *Journal of Forest Research* 2: 171–181.
- Jones J, Moens M, Mota M, Li H and Kikuchi I (2008) *Bursaphelenchus xylophilus*: opportunities in comparative genomics and molecular host–parasite interactions. *Molecular Plant Pathology* 9: 357–368.
- Kawaguchi E (2006) Relationship between the anatomical characteristics of cortical resin canals and migration of *Bursaphelenchus xylophilus* in stem cuttings of *Pinus thunbergii* seedlings. *Journal of the Japanese Forest Society* 88: 240–244.
- Kosaka H, Aikawa T, Ogura N, Tabata K and Kiyohara T (2001) Pine wilt disease caused by the pine wood nematode: the induced resistance of pine trees by the avirulent isolates of nematode. *European Journal of Plant Pathology* 107: 667–675.
- Kuroda K (2004) Inhibiting factors of symptom development in several Japanese red pine (*Pinus densiflora*) families

- selected as resistant to one wilt. *Journal of Forestry Research* 9: 217–224.
- Le Provost G, Herrera R, Paiva J, Chaumeil P, Salin FF and Plomion C (2007) A micromethod for high throughput RNA extraction in forest trees. *Biological Research* 40: 291–297.
- Meins F and Ahl F (1989) Induction of chitinase and [beta]-1, 3-glucanase in tobacco plants infected with *Pseudomonas tabaci* and *Phytophthora parasitica* var. *nicotianae*. *Plant Physiology* 61: 155–161.
- Miller B, Madilao L, Ralph S and Bohlmann J (2005) Insect-induced conifer defense, White pine weevil and methyl jasmonate induce traumatic resinosis, *de novo* formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiology* 137: 369–382.
- Mir G, Demenech G, Huguet G, Guo W, Goldsbrough P, Atrian S, Molinas M (2004) A plant type 2 metallothionein (MT) from cork tissue responds to oxidative stress. *Journal of Experimental Botany* 55: 2483–2493.
- Mota MM, Braasch H, Bravo MA, Penas AC, Burgermeister W, Metge K and Sousa E (1999) First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* 1: 727–734.
- Osmond R, Hrmova J, Fontaine F, Imberty A and Fincher G (2001) Binding interactions between barley thaumatin-like proteins and (1,3)- $\beta$ -d-glucans. *European Journal of Biochemistry* 268: 4190–4199.
- Shin H, Lee H, Woo K-S, Noh E-W, Koo Y-B and Lee K-J (2009) Identification of genes upregulated by pinewood nematode inoculation in Japanese red pine. *Tree Physiology* 29: 411–421.
- Takehushi Y, Kanzaki N and Futai K (2006) How different is induced resistance against the pine wood nematode, *Bursaphelenchus xylophilus*, by two avirulent microbes? *Nematology* 8: 435–442.