Study of specific genes of infection by *Bursaphelenchus xylophilus* in Portuguese pine trees

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INTRODUCTION

Maritime pine (*Pinus pinaster*) is one the most important species of south-western Europe forest, and in Portugal it occupies about one third of its forest surface. Pine Wood Disease (PWD), caused by the nematode *Bursaphelenchus xylophilus*, is a major threat to the European forests today with an estimated mortality risk of 50%. In 2008, the entire continental Portugal was demarcated as PWD-infested, and some recent isolated outbreaks occurred in Spain. The study of the interaction pine:nematode is yet incipient. Nevertheless, in previous studies of our team, we found that this pathogen attacks the xylem, and that genes of the peroxidase and ethylene biosynthesis pathway are important in the disease response. However, it is not known how these and other genes are regulated in trees with differential disease resistance and after attack by nematodes with varying degrees of virulence [1, 2]. Also, previous studies have demonstrated the induction of *P. thunbergii* tree resistance by inoculating plants with non-virulent PWN strains, suggesting that the creation of a biological control on PNW is possible ("Vaccination") [3]. A targeted gene expression approach was taken out in order to verify the infection mechanisms by *B. xylophilus* in *P. pinaster* (susceptible) and *P. pinea* (resistant) and simultaneously conduct tree vaccination. Infection with virulent (HF and 20 isolated from Setubal and 8A isolated from Portuguese center region) and avirulent (C14-5 isolated from Japan) strains was studied. Symptoms of infection were monitored and evaluated 6, 10 and 20 days after infection, according to predefined parameters.

MATERIALS AND METHODS

- Nematode isolates (C14-5, HF, 20 e 8A) were grown on sterilized barley medium with *Botrytis cinerea* and extracted using the Baermann funnels technique;
- *P. pinaster* (PP) and *P. pinea* (PPi) seedlings were inoculated with 4000 **C14-5** (non-inoculated controls were also established);
- Seedlings were re-inoculated 30 days after first inoculation with 2000 nematode virulent strains **HF**, **20** and **8A** (non-inoculated controls were made);
- 6 days after inoculation with the virulent strain a sampling was made;
- Symptoms were monitored 6 days, 10 days and 20 days after infection;
- Total RNA was extracted (Provost *et al*., 2007 [4]) and DNA removed w/Turbo-DNA free (Ambion).
- cDNA was synthesised and expression of genes of interested was determined (18S internal loading control);
- Scanning electronic microscope (SEM) was utilized to look at the morphology of *P.pinea* and *P.pinaster* stems.

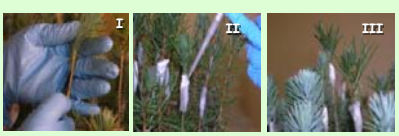


Figure 1: Seedling inoculation: (I) Cuts were made horizontally with the help of a sterile blade and a paper tissue was fixed with parafilm surrounding the wound area. (II) Nematode suspension was added to the tissue paper and closed (III) with parafilm to prevent drying out of the suspension.

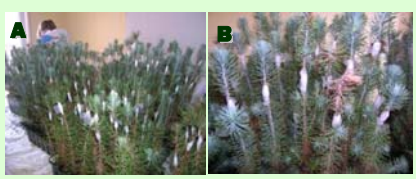
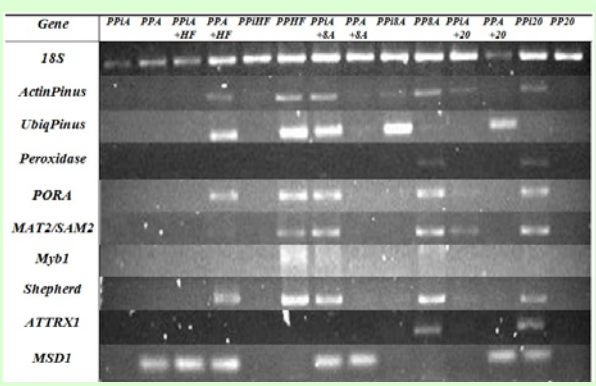


Figure 2: Inoculated seedlings of *P. pinaster* (A) and *P. pinea* (B) with non virulent and virulent nematode strains.

RESULTS AND DISCUSSION

- Transcription factor Myb was found to be important in the infection process - at 6 days of infection no expression was detected.



Treatment conditions : A - inoculation with C14-5 strain; A+HF - inoculation with C14-5 and HF strains; HF - inoculation with HF strain; A+8A – inoculation with C14-5 and 8A strains; 8A - inoculation with 8A strain; A+20 - inoculation with C14-5 and 20 strains; 20 - inoculation with 20 strain.

Figure 3 - Expressed genes of interest on *P. pinaster* (PP) and *P. pinea* (PPi).

- Expression of light captation and chlorophyll synthesis related genes was reduced, possibly due to the inoculation technique.
- Expression of ethylene production (MAT2/SAM2) and dehydration (Shepherd) associated genes was detected in about 1/3 of the samples
- Peroxidase was hardly expressed, which may indicate that the defense mechanisms were not fully activated.

Table1 – Symptom development in *P. pinaster* (PP) and *P. pinea* (PPi) 6 days (T6), 10 days (T10) and 20 days (T20) after re-inoculation with virulent strains

Treatment conditions	Incubation time (days)		
	T6	T10	T20
All treatments	I	I	III

Legend: (I) Healthy plant; (II) Partial needles chlorosis; (III) Partial needles chlorosis, necrosis and resin exudation diminished ; (IV) Pine death

- 12 days after re-inoculation, resin exsudation stopped.
- No seedling death was detected during this time course trial.
- Results suggest that *P. pinaster* is more susceptible to virulent strains than *P. pinea*.
- The obtained results show that *P. pinaster* and *P. pinea* generally had the same response to re-inoculation with virulent strains 20 and 8A.

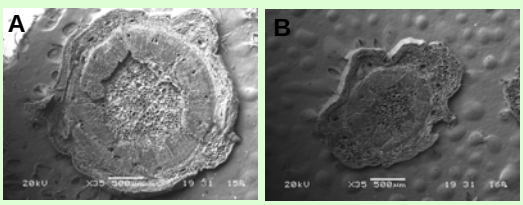


Figure 4 - SEM imaging of *P. pinea* (**A**) and *P. pinaster* (**B**) stems showing morphological differences between the two species.

CONCLUSIONS FUTURE WORK

- ❑ The presented results suggest that plant vaccination is possible and may be an important tool on plant disease control.
- ❑ SSH and pyrosequencing will be carried out in order to analyze variations in the expression of each gene as the infection progresses. Further proteomic and metabolomic studies will be conducted.
- ❑ Vaccinated seedlings will be continually monitored.
- ❑ Stem morphology of *P. pinea* and *P. pinaster* is different (Figure 4), suggesting that anatomy of the stem may be involved in the resistance mechanism

References
[1] Kosaka *et al.* (2001) *Eur. J. Plant Pathol.* ***107: 667–675;***
[2] Kuroda, K. (2008) *in,* Pine Wilt Disease, Springer;
[3] Nose & Shiraishi (2008) *in,* Pine Wilt Disease, Springer;
[4] Provost *et al.,* (2007) *Biol Res 40: 291.297.*