Biotechnological strategies for resistance induction to the pinewood nematode (PWN) in *Pinus pinaster*

Rui Rodrigues, Andreia Reis, Albina Franco, Carla Santos, Maciej Markowski, Marta W. Vasconcelos

Escola Superior de Biotecnologia -Universidade Católica Portuguesa

ABSTRACT

Bursaphelenchus xylophilus, also known as the Pine Wood Nematode (PWN), is responsible for the yearly loss of millions of pine trees worldwide due to the disease it causes, the Pine Wilt Disease (PWD)[1]. Having originated in North America, the disease has spread to Portugal[1], seriously threatening European native pine forests and economy. Symptoms are wilting of needles, reddish brown foliage and lack of resin exudation. Disease development can be rapid, with tree death occurring in about three months depending on environmental conditions[1,2].

Field observations of areas where *P. pinae* and *P. pinaster* coexist produced the hypothesis that the first is resistant to the infection with PWN. In order to test this hypothesis we have conducted inoculation trials with virulent and non-virulent nematodes in one year old seedlings, and determined resistance to the disease by symptom observation and gene expression studies.

We propose the identification of genes responsible for the reported natural resistance of *P. pinae* in order to better understand the molecular mechanisms of tree susceptibility and resistance.

MATERIALS AND METHODS

A) Cultures

i. **Nematode culture and extraction**
- ✓ *Botrytis cinerea* (Fig. 1);
- ✓ Virulent (HF) or non-virulent isolates (C14-5) (Fig. 2.);

ii. **Pine seedlings**
- ✓ Eight month old *P. pinea* and *P. pinaster* (Fig. 3 and 4).

B) Inoculation Trial
- ✓ Plants were inoculated with 2000 nematodes/ml and water (control) and samples were collected at 0h, 3h, 9h, 10d and 20d.

C) RNA Extraction and Purification
- ✓ RNA was cleared of DNA with Turbo-DNA (Ambion);
- ✓ Quantification/QC: Nanophotometer (Implen) and gel electrophoresis.

D) cDNA synthesis and RT-PCR
- ✓ cDNA synthesis (Applied Biosystems);
- ✓ RT-PCR

E) Gene Expression Analysis
- ✓ RT-PCR of samples collected at 3h, 9h, and 20d was conducted using the primers for *Peroxidase and Mat/sam* with *Actin* as loading control.
- ✓ At 9h, 10d and 20d *ATTRX1, PORA, PMonPR10, PsylSupOxDis, VvinTh, PsylAp1, PLDAlfha1, E2F1, P450, PR4, SEP1, glychase, HSP60, MSD1, PR5, PsylGAPDH, PtaeMSyn* with *18S* as internal control.

Fig.1 *Botrytis cinerea* growing on barley

Nematode inoculation

Fig.2 Fungus-depleted nematode culture

Fig.3 *P. pinaster* and *P. pinae* seedlings pre- inoculation

Plant inoculation

Fig.4 Inoculated *P. pinae* seedlings

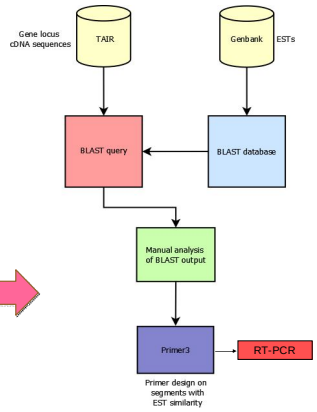

Fig.5 Primer design workflow

RESULTS AND DISCUSSION

	9h								10d								20d							
	P.Pinaster				*P.pinae*				*P.pinaster*				*P.pinae*				*P.pinaster*				*P.pinae*			
	0	H20	HF	C14-5	0	H20	HF	C14-5	0	H20	HF	C14-5	0	H20	HF	C14-5	0	H20	HF	C14-5	0	H20	HF	C14-5
ATTRX1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0
PORA	0	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	0	0
PLDALPHA1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0
PmonPR10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	0	1	0
PsylAP1	0	0	1	0	1	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
PsylSupOxDis	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
VvinTh	0	1	1	1	1	1	0	1	0	1	1	1	1	0	0	0	0	1	1	1	1	1	0	0
18s	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Table 1. Gene expression at 9 hours, 10 days and 20 days in *P. pinaster* and *P. pinea* with different condicions (0, water, virulent and non-virulent), *18S* was used as internal control.

- ❑ *Peroxidase* expression (Table 2) was only observed 3h and 9h after inoculation, which may indicate the gene is expressed on the initial steps of the infection. However, it was not detected on *P. pinaster* inoculated plants with virulent strain, suggesting that the presence of different NWP virulent strain is sufficient to induce a stress response by the plant;
- ❑ Other stress induced genes were detected on both plants (Table 2), such as genes expressed during plant cell death (*Mat/sam*) and oxidative stress (*ATTRX1*). However, in *P. pinea* plants after 20 days of inoculation, the genes ceased to be expressed. Also, in *P. pinaster* plants with non-virulent NWP strain, *ATTRX1* expression stopped.

- ❑ *ATTXR1* (Table 1), an important gene in oxidative stress response, was expressed in both species at all time points, except with C14-5 (non-virulent strain) in the last two time points;
- ❑ There was no expression of *PORA* (photosynthetic factor) in *P. pinaster*, but in *P. pinea* it was expressed at 9h with water, and at 10d when plants were inoculated with C14-5;
- ❑ *PLDAlpha1*, a gene activated by abcisic acid as a response to plant stress which generates the acidification of plant cells, was only detected 10 days after inoculation in all *P. pinea* treatments. No expression was observed in *P. pinaster* plants, indicating that plant species may have a differentiated stress response to the same pathogenic attack;
- ❑ In *P. pinaster*, *PsylAP1* gene (cell death) only expresses at 9h with HF. Moreover, *P. pinae* at 9h doesn't express with HF;
- ❑ *PsylSupOxDis*, (gene associated with oxidative stress factors) had no expression in *P. pinaster* at 9h. In *P. pinae* it doesn't express at 9h and 20d with C14-5;

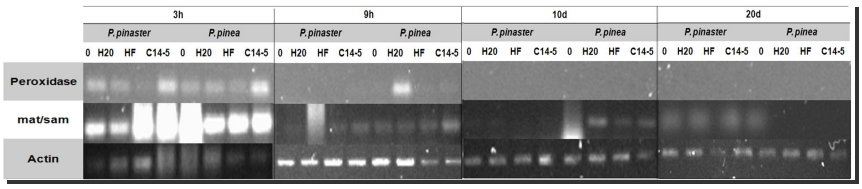

Table 2. Gene expression at 3 hours, 9 hours, 10 dayss and 20 days in *P.pinaster* and *P. pinea* with different condicions: Control (O), Water (H_2O), Virulent NWP (HF) and Non-virulent NWP (C14-5). Actin was used as internal control.

CONCLUSIONS AND FUTURE WORK

- ✓ The variable expression of several stress related genes during the time course (*PsylSupOxDis* , *PMonPR10*, and *PsylAP1*) may indicate that the plant generated a cascaded signalling response over time;
- ✓ Also, *P. pinaster* and *P. pinea* may have different stress induced mechanisms, as suggested by the presented results.
- ✓ SSH and 454 Pyrosequencing studies are underway to collect data that should shed light on the defence mechanisms underlying species specific resistance to the PWD.
- ✓ Metabolomic and proteomic studies will follow, providing a wider perspective on the disease response model.

References:

[1] Zhao, Bo Guang *et al*, 2008, Pine Wilt Disease, Springer ISBN: 978-4-431-75654-5

[2] Fraedrich, Bruce R., 1999, Pinewood Nematode (Pine-Wilt Disease): Technical Report, Bartlett Tree Research Lab, Charlotte NC

Acknowledgments:

Autoridade Florestal Nacional, Dr. Hajme Kosaka, Dr, Takuya Aikawa, Dr. Manuel Mota