Tree vaccination of pine wilt disease: resistance induction in *Pinus* spp. with a non virulent nematode strain

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INTRODUCTION

Bursaphelenchus xylophilus (Steiner and Buhrer) Niclke is a pine wood nematode (PNW) responsible for causing pine wilt disease (PWD) in pine trees. Resistance to plant pathogens has been widely addressed over the last decade [1]. However, it is not known how and which genes are induced and trigger plants defense mechanism, to overcome the infection, producing a systematic defence or a local response to the virulent agent [1, 2]. 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]. Moreover, this induced resistance can also provide an experimental system to clarify physiological interactions between the PNW and pine trees [1,3]. The aim of this work was to assess the resistance induction to pine wilt disease on young pine seedlings, under sterile conditions. Subsequent inoculations were induced by (i) non-virulent isolate of *B. xylophilus* (C14-5) and (ii) virulent nematodes strains (20, HF and 80), on *Pinus pinaster* and *P. pinea*. seedlings. The resistance mechanisms of PWN were assessed and the effect of seedling inoculation was determined in terms of the expression of several *Pinus* genes potentially involved in the disease response. Finally, PWN progression was also monitored to confirm successful seedling inoculation, and the possibility for full tree nursery seedlings resistance induction will be addressed.

MATERIALS AND METHODS

A) Nematode inoculums preparation:

✓Nematode strains (C14-5, HF, 20 and 8A) were grown on sterilized barley medium with *Botrytis cinerea*;
✓Nematode cultures were extracted using the Baermann funnels technique;

Nematode culture

B) Experimental design:

P. pinaster (PP) and *P. pinea* (PPi) seedlings were inoculated with 4000 C14-5. Non-inoculated controls were also established

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6 days after inoculation, the % of C14-5 nematode viability was determined

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Seedlings were re-inoculated 30 days after first inoculation with 2000 Nematode virulent strains (HF, 20 and 80). Non-inoculated controls were made

- Inoculation technique:

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.

C) RNA Extraction and Gene expression:

✓ Total RNA was extracted (Provost *et al.*, 2007), and contaminant DNA removed using the Turbo-DNA free kit (Ambion). RNA quantification was made on a spectrophotometer Nanophotometer (Implen);
✓c-DNA was synthesis and expressed genes of interested determined (18S internal loading control);
✓PCR products were loaded on 1,5% agarose gels, for 45 minutes at 120V in TAE buffer;

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

RESULTS AND DISCUSSION

➢ *Pinus pinaster* and *P. pinea* seedling plant health

▪C14-5 survival % on the inoculated pine seedlings was determined before re-inoculation with virulent strains [0% on *P. pinaster* (PP) and 70% on *P. pinea* (PPi)];

▪ No seedling death or needle discoloration was observer between the inoculation with non-virulent strain and the re-inoculation with the virulent ones;

▪However, resin exudates stopped on the 6th day after the re-inoculation with virulent strains on both pine seedlings;

▪Plant health was reevaluated 12d after virulent inoculation. *P. pinaster* seedlings showed needle necrosis and discoloration on 90% of the inoculated seedlings. As for *P. pinea*, virulent inoculation showed no visual effects;

▪Results suggest *P. pinaster* is more susceptible to virulent strains than *P. pinea*.

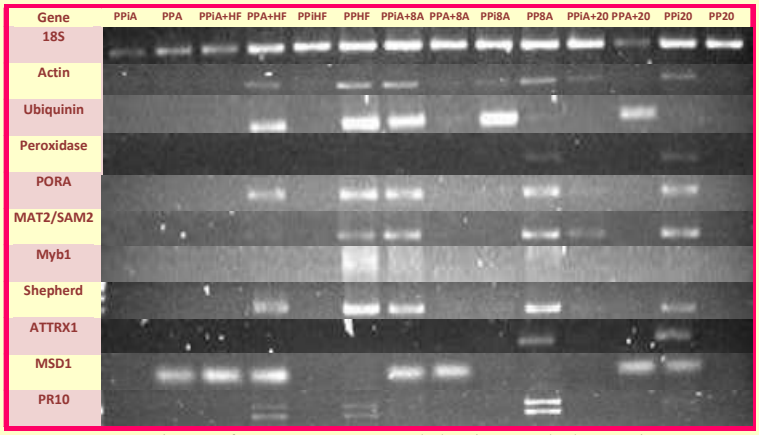

Figure 1 – Expressed genes of interest on *P. pinaster* (PP) and *P. pinea* (PPi). Legend: PPiA – *P. pinea* inoculated with C14-5 strain; PPA - *P. pinaster* inoculated with C14-5 strain; PPiA+HF - *P. pinea* inoculated with C14-5 and HF strains; PPA+HF - *P. pinaster* inoculated with C14-5 and HF strains; PPiHF - *P. pinea* inoculated with HF strain; PPHF - *P. pinaster* inoculated with HF strain; PPiA-8A - *P. pinea* inoculated with C14-5 and 8A strains; PPA+8A - *P. pinaster* inoculated with C14-5 and 8A strains; PPi8A - *P. pinea* inoculated with 8A strain; PP8A - *P. pinaster* inoculated with 8A strain; PPiA+20 - *P. pinea* inoculated with C14-5 and 20 strains; PPA+20 - *P. pinaster* inoculated with C14-5 and 20 strains; PPi20 - *P. pinea* inoculated with 20 strain; PP20 - *P. pinaster* inoculated with 20 strain.

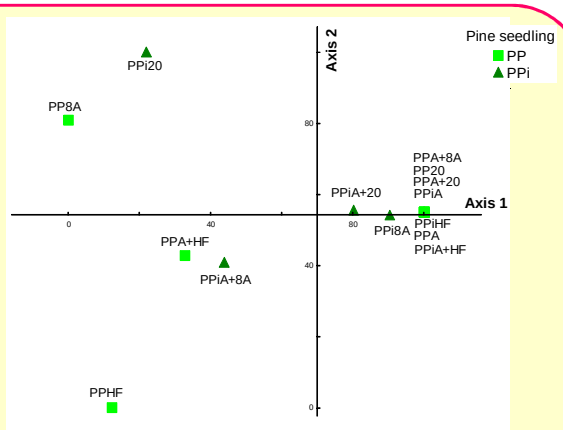

Figure 2- Principal component analysis of the expressed genes (Actin, Ubiq, Perox, Pora, Sep1, Mat/Sam, myb1, myb2, pr4, PsylAP4, Shep, ATTRX1, TT7, MSD1, PR10) in *Pinus pinaster* (PP) and *Pinus pinea* (PPi). Axis 1 and 2 explain 79% of the variation observed (P<0.05).

➢ *Expressed genes*

▪ Target gene expression were analyzed according to the effect vaccination produced on *P. pinaster* and *P. pinea* seedlings inoculated with non-virulent and virulent strains;

▪ Ordination analysis showed that *P. pinaster* re-inoculated with virulent strains (20 and 8A) had the same gene expression than *P. pinea* seedlings, under the same conditions;

▪Results suggest that a "vaccination" effect may have been induced on *P. pinaster* seedlings. However, the induced mechanisms are not yet clear.

Conclusions and future work:

❑ The presented results suggest that plant vaccination is possible and may be an important tool on plant disease control.
❑Vaccinated seedlings will be continually monitored. Further proteomic and metabolomic studies will be conducted.

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.*