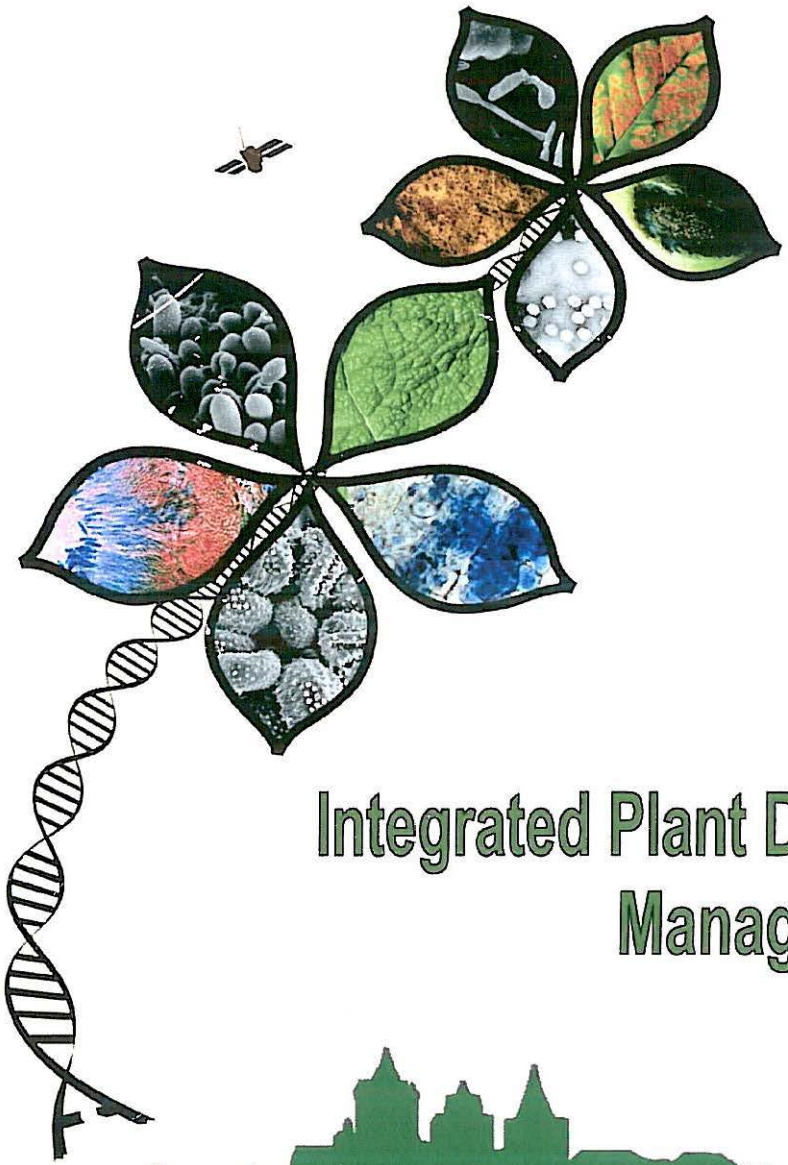
Book of Abstracts

9th Conference of the European Foundation for Plant Pathology

6th Congress of the Sociedade Portuguesa de Fitopatologia



Integrated Plant Disease Management

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9TH CONFERENCE OF THE EUROPEAN FOUNDATION FOR PLANT PATHOLOGY AND 6TH CONGRESS OF THE SOCIEDADE PORTUGUESA DE FITOPATOLOGIA

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Cloning and expression analysis of glucanase genes from *Phytophthora cinnamomi*

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Summary

Phytophthora cinnamomi is a soil-borne pseudofungus belonging to the Class Oomycetes or 'water moulds' in the Kingdom Chromista (**Figure 1**). Is one among the most destructive species of *Phytophthora* associated to the decline of forestry, ornamental and fruit species, as well as of some 900 other woody perennial plant species Associated with this oomycete is the ink disease of *Castanea Sativa* Mill.

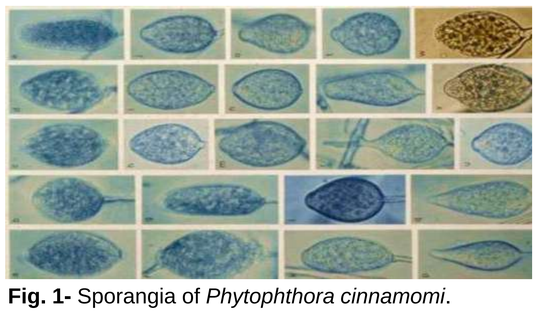


Fig. 1- Sporangia of *Phytophthora cinnamomi*.

Glucan endo-1,3-β-D-glucosidase (EC 3.2.1.39) catalyzes de hydrolysis of 1,3-β-D-glucoside linkages in callose, laminarin and several carbohydrates found in the cell wall of plants and fungi. It is generally thought that glucanases play a role in plant defense by digesting wall components of the fungal pathogen. In yeast, 1,3-β-glucanases have been studied for their role in germination, sporulation, mating and cell growth since they are regulated in cell cycle dependent manner, and are differentially expressed during vegetative growth, mating and the late stages of sporulating diploid. In plant, 1,3-β-glucanases have been characterized for their major role in plant defence, as well as for their involvement in germination, microsporogenesis and embryogenesis. In oomycetes, glucanases have been studied on a biochemical level for their possible role in hyphal tip growth and branching where there is thought to be a delicate balance between cell wall synthesis and hydrolyses.

In the present work, we obtained a fragment with 1231bp of the endo-1,3-β-glucanase gene by standard PCR, using conserved primers and the whole genomic sequence with 2586 bp was obtained by amplifying the previous sequence by asymmetric PCR. The gene expression was studied during growth in different carbon sources and was also performed a time course of endo-1,3-β-D-glucosidase production.

Material and Methods

***P. cinnamomi* growth conditions:** *P. cinnamomi* mycelium for genomic DNA extraction were obtained by growth in cellophane-PDA medium. The C-PDA plates were inoculated by placing a plug of mycelium (0.5cm in diameter) on new plates and incubating in the dark for 4-6 days at room temperature (22-25ºC).

PCR Amplification: The polymerase chain reaction (PCR) was used to amplify a 1231bp fragment of the glucan endo-1,3-β-D-glucosidase gene. The conserved primers Gendo1F and Gendo1R were applied (McLeod *et al*., 2003).

TAIL-PCR Procedure: Gene-specific primers SP1, SP2, SP3 , were used for flanking the 5' region and SP4, SP5 and SP6 for flanking the 3' region. A degenerated primer R3 was also applied (**Figure 2**). Three rounds of PCR were performed using the product of the previous PCR as a template for the next.

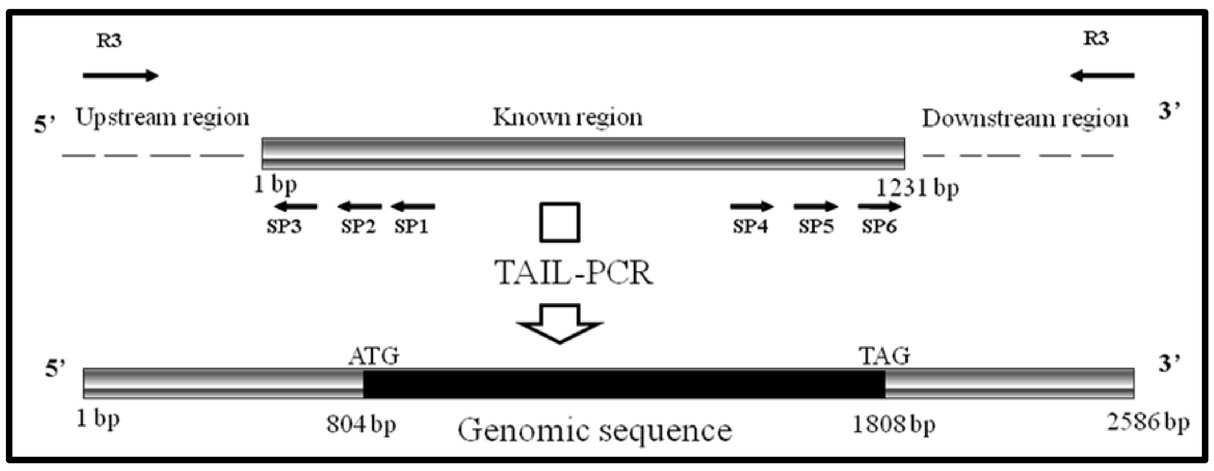


Fig. 2- Schematic representation of TAIL-PCR amplification.

DNA Sequencing and Sequences Analysis: DNA fragments were sequenced in an automatic capillary sequencing and the "European Bioinformatics Institute" was accessed to extract the sequences from the databases (EMBL), and from the "National Center of Biotechnology Information" (NCBI). These servers allowed the comparison of DNA sequences and of proteins by several programs: Clustal, Fasta and Blast. The BioEdit, Sequencher and DNASTAR™ (SeqMan and EditSeq) programs were used to analyze the sequences.

Gene Expression: The expression was studied during growth in different carbon sources and was also performed a time course of endo-1,3-β-D-glucosidase production. To analyze gene expression was performed RT-qPCR using the *SyBr Green* method and the actin gene *Act2* as a endogenous control.

Results

The fragments amplified by TAIL-PCR were isolated and sequenced. In the upstream region a fragment of approximately 800 bp was obtained while in the downstream region one of approximately 600 bp was collected (**Figure 3**).

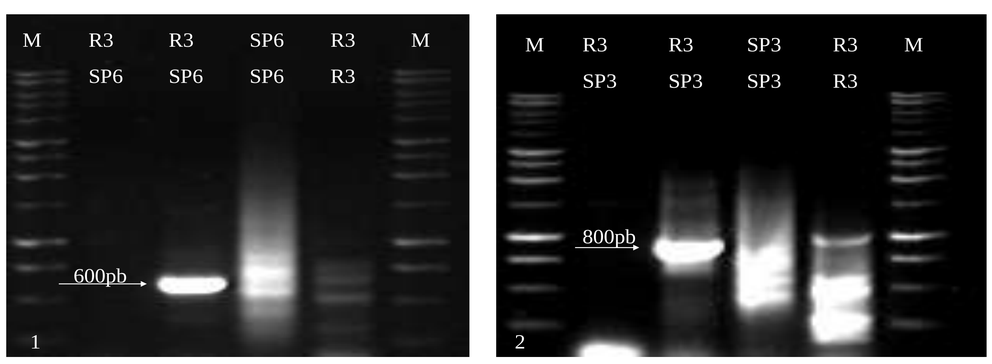


Fig. 3- Products of the tertiary TAIL-PCR with controls. **[1]** Agarose gel analysis using degenerated primer R3 to isolate the upstream region of the *endo1* gene of *P. cinnamomi*. The gene-specific primer used was the SP3. **[2]** Agarose gel analysis using degenerated primer R3 to isolate the downstream region of the *endo1* gene of *P. cinnamomi*. The gene-specific primer used was the SP6. **M** is a Promega 1kb DNA Ladder. Lanes I and V- reaction control without DNA. Lanes II and VI- amplification product. Lanes III, IV, VII and VIII- tertiary reaction control.

The complete sequence of the glucan endo-1,3-D-glucosidase gene was compared with the sequences deposited in the NCBI and EMBL databases. According to the BLASTX search program, the glucan endo-1,3-D-glucosidase gene of *P. cinnamomi* has homology with a putative endo-1,3-beta-glucanase of *P. infestans* (E-value = $8e^{-135}$), an endo-1,3-beta-glucanase of *Saprolegnia parasitica* (E-value = $7e^{-56}$), an endo-beta-1,3-glucanase of *Tetrapisispora phaffii* (E-value = $8e^{-25}$) and an endo-beta-1,3-glucanase of *Saccharomyces cerevisiae* (E-value = $1e^{-24}$). This sequence presents the conserved domain Exo-beta-1,3-glucanase an carbohydrate transport and metabolism(COG5309), andhomology with a glycoside hidrolase family 17(InterProSan – EMBL).

Alignment of glucan endo-1,3-D-glucosidase gene with several proteins endo-1,3-β-glucanases from GH family 17 revealed that contain the conserved domain of this family ([LIVMKS] -X- [LIVMFYWA](3)- [STAG] -E- [STACVI] -G- [WY] -P- [STN] -X- [SAGQ]), (**Figure 4**).

In the dendrogram obtained by phylogenetic analyzes (**Figure 5**), we observed that the glucan endo-1,3-D-glucosidase of *P. cinnamomi* is very similar to the endo-1,3-beta-glucanase of *P. Infestans*.

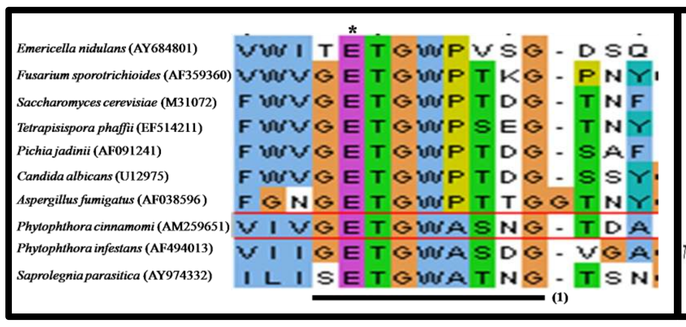


Fig. 4- Alignment of the amino acid sequences of glycoside hydrolase family 17 fungal endo-1,3-β-glucanases and plant endo-1,3-β- and 1,3; 1,4-β-glucanses, as well as an endo-1,3-β-glucanases of *P. infestans* and *P. cinnamomi*. (*) Is an active site residue. (1) Characteristic domain of GH family 17 ([LIVMKS]-X-[LIVMFYWA](3)-[STAG]-E-[STACVI]-G-[WY]-P-[STN]-X-[SAGQ]).

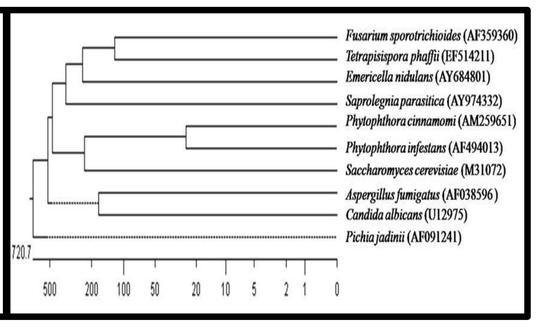


Fig. 5- Phylogram of glycoside hydrolase family 17 fungal endo-1,3-β-glucanase and oomycete endo-1,3-β-glucanase.

Promoters from non-oomycete species do not function in *Phytophthora*, suggesting the presence of a unique transcriptional machinery. Canonical eukaryotic elements, such as the TATA box, were not always detected in oomycete promoters, as occasionally reported for fungal been determined and are typically only 50 to 100 bp upstream from the star codon. This suggests that oomycetes bear highly compact transcripts, with some of the shortest untranslated regions noted for eukaryotes. A conserved 16-bp sequence motif, GCTCATTYBNCAWTTY, surrounds the transcriptional start site of many oomycete genes (Kamoun, S., 2003). This motif has facilitated the prediction of transcribed regions from genomic sequences. The conserved sequence motif is present in the 5' flanking regions of the Pcendo1 gene, 68 to 83 nt upstream from the ATG start codon (**Figure 6**).

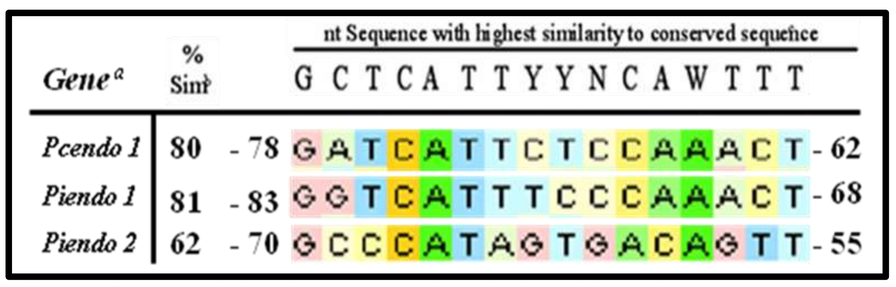
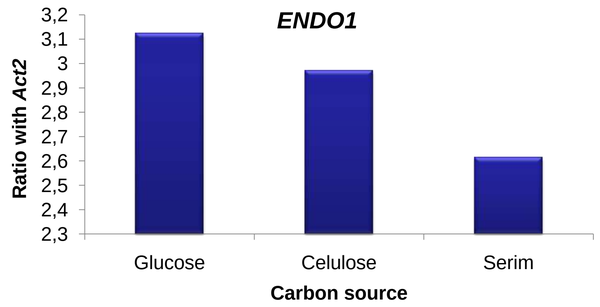


Fig. 6- Alignment of the 5' upstream region of *Phytophthora* glucanases genes to a conserved sequence GCTCATTYYNCAWTT found in a promoter region of oomycete genes. **[a]** *Piendo1* (AF494013), *Piendo2* (AF494017), *Pcendo1* (AM259651). **[b]** The promoter region of each glucanase gene was searched upstream from the ATG for highest similarity with the consensus sequence GCTCATTYYNCAWTT (where Y=C or T; N= and nucleotide; W= A or T). **[c]** Nucleotides that are similar to the consensus sequence are shaded.

Fungal cell wall degrading enzyme production is influenced by a number of factors including the type of strain, the culture conditions and substrate type. The expression of our gene was studied during growth in different carbon sources and was also performed a time course of endo-1,3-β-D-glucosidase production. Different plasmids were used to clone the gene on each organism and we used RT-qPCR analysis to examine its expression. The major expression levels occurred at the medium with glucose as carbon source (**Figure 7**).



Currently we are silencing the endo-1,3-β-D-glucosidase encoding gene *ENDO1* produced by *P. cinnamomi* to undertake studies of heterologous expression, in *Pichia pastoris*, as well as its phenotype through infection with the wild and transformed lineage in *Castanea sativa*.

Conclusions

The *P. cinnamomi* glucan 1,3-beta-D-glucosidase gene (*PcENDO1*) was closely related to glycoside hydrolases of family 17 because it also contained the conserved domain of this family (**Figures 4** and **5**). A putative conserved 16 nt core sequence (GCTCATTYYNCAWTT) possibly used as an initiation of transcription point in oomycetes, has been also identified in *Pcendo1* (**Figure 6**). The major levels of expression of the *ENDO1* gene occurred at the medium with glucose as carbon source (**Figure 7**).

Acknowledgements: The Project COMBATINTA/SP2.P11/02 Interreg IIIA – Cross-Border Cooperation Spain-Portugal, financed by The European Regional Development Fund, and the Project "Identification, characterization and role of molecular factors associated with the mechanisms of infection of *Fagaceae* species by *Phytophthora cinnamomi*" (PTDC/AGR-AAM/67628/2006) FCT, supported this work.