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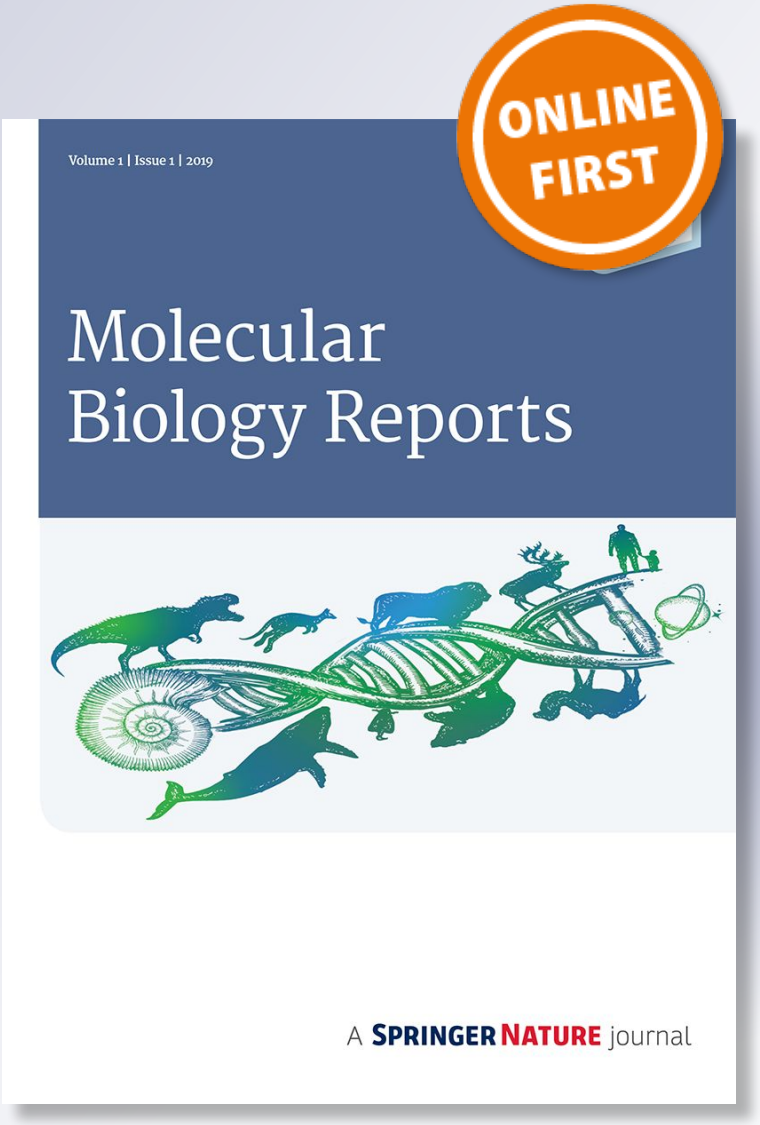

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ORIGINAL ARTICLE

Cloning and expression analysis of an *endo-1,3-β-D-glucosidase* from *Phytophthora cinnamomi*

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Abstract

*Phytophthor*a is considered one of the most destructive genus for many agricultural plant species worldwide, with a strong environmental and economic impact. *Phytophthora cinnamomi* is a highly aggressive *Phytophthora* species associated with the forest decline and responsible for the ink disease in chestnut trees (*Castanea sativa* Miller), a culture which is extremely important in Europe. This pathogenicity occurs due to the action of several enzymes like the hydrolysis of 1,3-*β-glucans* at specific sites by the enzyme *endo-1,3-β-D-glucosidase.* The aim of this work to analyze the heterologous expression in two microorganisms, *Escherichia coli* and *Pichia pastoris*, of an *endo-1,3-β-D-glucosidase* encoded by the gene ENDO1 (AM259651) from *P. cinnamomi.* Different plasmids were used to clone the gene on each organism and the real-time quantitative polymerase chain reaction was used to determine its level of expression. Homologous expression was also analyzed during growth in different carbon sources (glucose, cellulose, and sawdust) and time-course experiments were used for *endo-1,3-β-D-glucosidase* production. The highest expression of the *endo-1,3-β-D-glucosidase* gene occurred in glucose after 8 h of induction. In vivo infection of *C. sativa* by *P. cinnamomi* revealed an increase in *endo-1,3-β-D-glucosidase* expression after 12 h. At 24 h its expression decreased and at 48 h there was again a slight increase in expression, and more experiments in order to further explain this fact are underway.

Keywords *Castanea sativa* · *Endo-1,3-β-D-glucosidase* · Heterologous expression · Homologous expression · *Phytophthora cinnamomi* · RT-qPCR

Introduction

Oomycetes of the genus *Phytophthora* are responsible for major economic losses around the world. Different agricultural and forest species of plants have been declining with the diseases caused by *Phytophthora cinnamomi Rands* [1]. *Phytophthora cinnamomi* has the broadest host range of all Phytophthora species and is considered an aggressive forest pathogen worldwide being considered a very serious problem in Australia. For example, the European chestnut tree *Castanea sativa*, which is extremely important in Europe, especially in the northern Iberian Peninsula where the sweet chestnut production has a high edaphic, ecological and economic value; has suffered substantial damage through chestnut ink disease caused by *P. cinnamomi* that consumes the roots leading to the subsequent death of the tree [2].

This oomycete completes its life cycle under humid conditions and warmer temperatures. It can persist in moist soil for several years. These conditions favor their establishment, propagation, and survival. It is known that asexual sporulation requires a liquid environment, both for the formation of sporangia and for the release and activity of motile zoospores. That is why; disease development is incremented after heavy rain and in waterlogged soils [3, 4].

Different steps such as the manipulation of biochemical and physiological processes occur during plant infection. Many virulence molecules known as effectors play an important role in the infection process [5–8]. These effectors are known to elicit a defense response in hosts known as the hypersensitive response (HR) that causes the destruction of invading cells [7–9]. These strategies of attack and defense

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in plant–oomycete interactions, carried out by molecular factors, have been studied in different *Phytophthora* especially *Phytophthora infestans* and *Phytophthora parasitica*. Other effectors are more effective and promote plant infection by reducing defense and increasing the symptoms of disease in the plant. Some of these *Phytophthora* effectors have been studied in various pathosystems [10–12].

Some of these effectors induce defense responses in both susceptible and resistant plants and are referred to as general elicitors. Elicitor molecules from structural components or metabolites secreted by oomycetes have been isolated and identified, including oligosaccharides, proteins, glycoproteins, and enzymes [13].

The wall glucan elicitor of the phytopathogenic oomycete *Phytophthora sojae* is one of the most studied due to causing stem and root rot of soybean plants [14, 15].

In pathogens such as *P. cinnamomi* the process of germination, sporulation and cell growth have been reported with the change of the cell wall structure. This pathogenicity occurs due to the hydrolysis of *1,3-β-glucans* at specific sites by the enzyme *endo-1,3-β-*D*-glucosidase* [15].

The role of *endo-1,3-β-*D*-glucosidase* in the biology of *P. cinnamomi*, is not fully understood. The identification, study, and analysis gene expression involved in the biological development of *P. cinnamomi* and their interaction with hosts is a fundamental step in understanding important molecular-biochemical events [15–17].

The *P. cinnamomi endo-1,3-β-*D*-glucosidase* gene (based on the NCBI sequence data, duly assigned the accession number AM259651) has a 1005 bp ORF encoding a putative peptide of 334 deduced amino acids. This gene was identified, isolated, and characterized by asymmetric polymerase chain reaction (PCR), using conserved primers and the whole genomic sequence with 2586 bp was obtained by amplifying the previous sequence by asymmetric PCR [18].

The purpose of this study was to clone and analyze the heterologous expression in two systems, *Escherichia coli* and *Pichia pastoris*, of the *endo-1,3-β-*D*-glucosidase* encoding gene ENDO1 produced by *P. cinnamomi*. Different plasmids were used to clone the gene on each organism, and RT-PCR was used for the analysis level of expression. The expression analysis of the protein *endo-1,3-β-*D*-glucosidase* was studied during growth in different carbon sources (glucose, cellulose, and sawdust). The expression levels of endo-1,3-β-D-glucosidase transcripts in plants were also studied after infection of *C. sativa* roots and analyzed at different times.

Materials and methods

Biological material

Isolated Pr120 of *P. cinnamomi* was kindly provided by Eugénia Gouveia from Instituto Politécnico de Bragança. It was isolated from soil samples associated with a *C. sativa* tree contaminated with ink disease in Trás-os-Montes region (northeast Portugal). The strain was grown in the dark for 4–6 days at 22–25 °C in potato-dextrose agar (PDA) medium.

Total genomic DNA from *P. cinnamomi* mycelium was obtained as described in [19].

Castanea sativa chestnuts were surface sterilized, germinated in sterile vermiculite and grown in a greenhouse until their root length reached 5–6 cm.

Amplification of *endo-1,3-β-*D*-glucosidase* ORF

PCR was used to amplify the 1005 bp ORF of the *endo-1, 3-β-*D*-glucosidase* gene. The PCR cycling conditions were 95 °C/5 min, followed by 40 cycles of 94 °C/1 min, 71,4 °C/1 min, 72 °C/1,10 min, and ending with 72 °C/10 min. Each 25 µl PCR contained 1.6 mM dNTP, 0.2 mM of each primer, 100 ng genomic DNA, 1.5 mM $MgCl_2$ and 0.05 U Taq DNA polymerase in appropriate buffer. Aliquots of the PCR reactions were separated on 0.8% w/v agarose gel electrophoresis and stained with ethidium bromide, to check for the presence of the amplicon.

Plasmids

pET-28a(+) (Novagen), is a 5369 bp *E. coli* expression vector containing the *kanamycin* resistance gene. It is regulated by the T7 promoter (present as a 16 bp fragment) and by the T7 terminator (present as a 46 bp).

pET-28 recombinant, a 6374 pb construct (based on pET28a(+)), contains an expression cassette consisting of the *endo-1,3-β-*D*-glucosidase*, present as a 1005 bp *Hin*dIII-*Xho*I fragment. This was obtained by PCR amplification of a 1005 bp ORF of *endo-1,3-β-*D*-glucosidase* from *P. cinnamomi* strain pr120 DNA, using primers M18 (5′-GCGACA TCCAAGCTTCACCATGGTG-3′, insertion of restriction site *Hin*dIII in bold) and M19 (3′-GGCGTCCGAGGCTCG AGCTATCCGCG-5′, insertion of restriction point *Xho*I in bold). The fragment was digested with *Hin*dIII-*Xho*I and cloned into *Hin*dIII-*Xho*I digested pET-28a(+). The DNA digested vector band was purified in a low melting point (LMP) agarose gel and Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions. Clones were selected by restriction analysis with *Apa*I

and *Sal*I. The positive clone obtained was sequenced to confirm the correct integration of the insert. The sequencing of the fragments of the DNA obtained was carried through in an automatic sequencer ABI Prism 377TM from Applied Biosciences (Foster City, CA, USA).

pPICZα is a 3600 pb *P. pastoris* expression vector containing the *zeocin* resistance gene. This one has a 5′ fragment containing the *AOX1* promoter for tightly regulated, methanol-induced expression of the gene of interest, α-factor secretion signal for directing secreted expression of the recombinant protein and have the a C-terminal peptide containing the *c-myc* epitope and a polyhistidine (6xHis) tag for detection and purification of a recombinant fusion protein.

pPICZα recombinant, a 4605 pb construct (based on pPICZα), contains an expression cassette consisting of the *endo-1,3-β*-D-*glucosidase*, present as a 1005 bp *Eco*RI-*Xho*I fragment. This was obtained by PCR amplification of a 1005 bp ORF of *endo-1,3-β*-D-*glucosidase* from *P. cinnamomi* strain pr120 DNA, using as primers M17 (5′-GAC ATCCAAGCGAATTCATGGTGAACG-3′, insertion of restriction site *Eco*RI in bold) and M19 (3′-GGCGTCCGA GGCTCGAGCTATCCGCG-5′, insertion of restriction point *Xho*I in bold), digesting this fragment with *Eco*RI-*Xho*I and finally cloning it into *Eco*RI-*Xho*I digested pPICZα. The DNA digested vector band was purified in a LMP agarose gel, Wizard® SV Gel, and PCR Clean-Up System (Promega), following the manufacturer's instructions. Correctly oriented clones were selected by restriction analysis with *Sac I* and *Sal I*. The positive clone obtained was sequenced to confirm the correct integration of the insert. The sequencing of the fragments of the DNA obtained was conducted using an automatic sequencer ABI Prism 377TM from Applied Biosciences (Foster City, CA, USA).

Plasmids pPICZα with the of 1005 bp of the *endo-1,3-β*-D-*glucosidase* ORF were linearized with Pme I enzyme under the conditions: *Pme I* (1 U/µl), 2 µl reaction buffer (1×), 2 µl (10 µg/µl) BSA, 1 µg/µl DNA for a final volume of 20 µl. The digestion product was visualized on a LMP 0.8% (w/v) agarose gel and purified by the commercial PCR Clean-Up System kit and later integrated into the yeast *P. pastoris* X-33 (Invitrogen®).

Bacterial and fungal transformation and DNA extraction

Plasmids were propagated in *E. coli* (DH5α cells) following the procedure described by [20] and plasmid DNA was extracted-purified with the Wizard® Plus SV Minipreps DNA Purification System (Promega), following the manufacturer's instructions.

The transformation of *P. pastoris* was done by electroporation. The total volume of 40 µl of *P. pastoris* competent cells and 1 µg/µl linear plasmid DNA was mixed. The mixture was transferred to a 2 mm cold cuvette and incubated on ice for 5 min. The mix was electroporated at 400 V for 1 s. 1 ml of 1 M cold sorbitol was immediately added to the cuvette. The contents of the cuvette were transferred to a sterile tube (1.5 ml). The tube was incubated at 28 °C for 2 h and plated in YPDS (agar, yeast extract, peptone, and sorbitol) agar medium with zeocin at 28 °C for 3 days.

Analysis of *endo-1,3-β*-D-*glucosidase* production

The pET-28 recombinant clone was harvested in LB with *kanamycin* and protein expression was induced by adding 1 mM IPTG at 0, 4 and 7 h. Secreted proteins were concentrated by precipitation with trichloroacetic acid (TCA 8% v/v), from the supernatant and with a lysis solution (50 mM Tris–HCl pH 7.5, 50 mM EDTA e 1 mM PMSF) from *E. coli* cells.

Protein quantification was performed using the protein quantification kit-general use (Fluka), following the manufacturer's instructions. After quantification, secreted proteins at all induction time points (0, 4 and 7 h) were separated by SDS-PAGE (15% w/v). The membrane was stained with Coomassie brilliant blue BG-250 0.25% (BioRad) and then was washed in a solution of methanol 45% (v/v) and acetic acid 10% (v/v).

The pPICZα recombinant clone was harvested in methanol induction medium during different times of protein expression. Cell lysis was performed using the vortex through lysis solution *breaking lysis buffer* and glass beads (0.5 mm). The breakdown of the cell wall of oomycete cells was performed by SDS loading buffer (2×) procedure. Protein quantification was performed using the protein quantification kit-general use (Fluka), following the manufacturer's instructions. After quantification, secreted proteins were analyzed using the same method as for pET28.

Expression of *endo-1,3-β*-D-*glucosidase* gene

A real-time quantitative PCR (RT-qPCR) assay was performed in order to analyze gene expression in vitro and in vivo.

In vitro

Phytophthora cinnamomi was grown in different carbon sources (2% (w/v) glucose, 0.2% (w/v) cellulose, and 0.2% (w/v) sawdust) and the expression of *endo-1,3-β*-D-*glucosidase* gene was evaluated at 2, 4, 6 and 8 days of growth of *P. cinnamomi*. Control cells were grown in PDA medium. The expression level of the *endo-1,3-β*-D-*glucosidase* gene was expressed relative to the expression level of the housekeeping gene *actin2*. Expression was

measured at four different times of induction with glucose, cellulose and sawdust.

In vivo

Castanea sativa roots were covered with fully colonized PDA and incubated in the dark at 25 °C for 12, 24 and 36 h. Negative controls were provided by roots in contact with non-colonized agar. After the incubation period, the agar was removed manually, with tweezers and stilettos, under sterile conditions, along with all external mycelia growth. The roots were examined for the presence and extent of necrosis and then frozen at − 80 °C. The assays were repeated three times. The expression level of the *endo-1,3-β*-D-*glucosidase* gene was expressed relative to the level measured in the reference gene *actin2*. Results were normalized to *actin2* gene and calculated using the $^{\Delta\Delta}$Ct method, as fold change relative to control cells.

Total RNA was isolated from *P. cinnamomi* mycelia using the Rneasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. Residual DNA was removed by DNase I (Qiagen) treatment, following the manufacturer's instructions. The integrity of the RNA was assessed by formaldehyde agarose gel electrophoresis (1.5% agarose). 1 µg of RNA was reverse transcribed with the aid of the iScript™ cDNA Synthesis Kit (BioRad) primed with oligo (dT), following the manufacturer's instructions. The qPCR was performed with IQ™ SYBR® Green Supermix (Bio-Rad) Real Time using a MiniOpticon™ Real-Time PCR Detection System (BioRad). Each 25 µl reaction contained 100 ng RNA, as well as 12.5 µl IQ SYBR Green Supermix (100 mM KCl, 40 mM Tris–HCl pH 8.4, 0.4 mM of each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, 20 nM SYBR Green I fluorescein, and stabilizers) and 1.25 µM of each primer. The reactions were run in triplicate and incubated at 95 °C for 3 min, followed by 40 cycles of 95 °C/30 s, 61 °C/30 s and 72 °C/30 s. The endogenous control was normalized to the expression levels of *actin2* gene within the same sample. Amplification primers (Table 1) were targeted to the coding regions of *P. cinnamomi actin2* and *endo-1,3-β*-D-*glucosidase* genes.

Table 1 Primers sequences to amplify *actin2* and *endo-1,3-β*-D-*glucosidase* genes in the RT-qPCR experiments

Act2	Forward: 5′-GGCCTCGAGAAGAGCTACG-3′
	Reverse: 5′-CTTCATGATGGTCTGGAACG-3′
Endo1	Forward: 5′-CGCTGACTATCGCTGACATC-3′
	Reverse: 5′-GCGCTGGTAAAAGTGGTCTG-3′

Results and discussion

This work reports the cloning of *endo-1,3-β*-D-*glucosidase*, using the HE-TAIL PCR (high-efficiency thermal asymmetric interlaced-PCR) [16] related to the glycoside hydrolases of family 17 that are secreted by *P. cinnamomi* and that are specifically included among the group of pathogenesis-related proteins due to the many forms that are rapidly induced during a fungal invasion.

We obtained the 1005 pb ORF of the *endo-1,3-β*-D-*glucosidase* gene. The cloning was successful in the two vectors of expression and we were able to express the protein in *E. coli* and *P. pastoris.*

The use of these two systems for the expression of proteins is interesting because the use of both provides a more comprehensive analysis of the study of protein expression. *Escherichia coli* system provides a variety of cloning vectors, allows easy control of gene expression and easy growth with high protein yields. The *P. pastoris* is a eukaryotic organism, thus the production of heterologous proteins in this system is more complex than in *E. coli*. This yeast has gained wide acceptance as a host organism for the production of heterologous proteins because it has advantages such as having strong and inducible promoters, intra- and extracellular production, purification and detection tags for high levels of protein expression and post-translational modifications.

The *endo-1,3-β*-D-*glucosidase* gene encodes a 334 aa protein that has a molecular function for catalysis of the hydrolysis of any *O*-glycosyl bond. This sequence presents the conserved domain *Exo-beta-1,3-glucanase* (COG5309), and homology with a glycoside hydrolase family 17 (InterProSan: EMBL).

The amino acid sequence was submitted to the database of protein sequences (http://www.uniprot.org/blast/uniprot/) and there was a higher homology with the putative glycosyl hydrolase family 17 protein (G5AGU4) from *P. sojae* (90%).

Protein production assay

Expression of the *endo-1,3-β-glucanase* protein in *E. coli*

The aim of this assay was to study the heterologous expression of the endo-1,3-β-D-glucosidase gene in *E. coli*. The expression of the *endo-1,3-β*-D-*glucosidase* gene of *P. cinnamomi* was induced in *E. coli* transformed with the plasmid pET-28 recombinant resulting from the insertion of the 1005 bp endo-1,3-β-D-glucosidase ORF in the plasmid pET28a(+). Positive transformation, integration of the ORF and their orientation were confirmed by enzymatic digestion and sequencing of the extracted plasmid DNA.

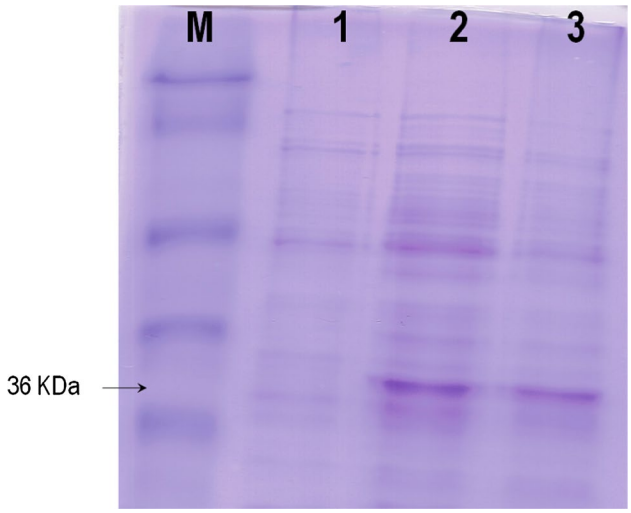

Fig. 1 Separation of the expressed endo-1,3-β-D-glucosidase protein in a polyacrylamide/SDS gel (15%) expressed in *E. coli. M* protein marker kDa, *1 E. coli* without addition of IPTG, *2* protein expressed in *E. coli* after 4 h of induction, *3* protein expressed in *E. coli* after 7 h of induction

The band corresponding to the 36 kDa *endo-1,3-β-*D-*glucosidase* protein is shown with the arrow, 7 h after induction (Fig. 1).

It can be observed that the protein had its highest production after 4 h of induction. At 7 h of induction, a decrease in its production was detected, possibly due to an increase in its degradation (Fig. 1).

Expression of the endo-1,3-β-glucanase protein in *Pichia pastoris* X-33

The use of the pPICZαA vector was intended to study expression in a eukaryotic organism because it presents advantages over *E. coli*. Positive transformants, integration of the ORF and their correct orientation were confirmed by enzymatic digestion and sequencing of the extracted plasmid DNA as had already been done in *E. coli*. Expression systems in eukaryotic cells allow the production of recombinant proteins in their native form. This makes it possible to make post-translational modifications, essential for maintaining the original function of these proteins.

After growth under vigorous stirring at 220 rpm at 28 °C for 18 h, reaching an absorbance 4 at a wavelength of 600 nm in culture liquid medium (MG) for biomass generation, a sample for the negative control and the samples for the transformants at different induction times were analyzed. Cell lysis was achieved through vortex agitation and using glass spheres (0.5 mm), and a Breaking Buffer solution. Glucanases are easily trapped in the cell wall and for this reason, soluble proteins were extracted from the cell wall by SDS loading buffer (2×). SDS-PAGE polyacrylamide gel electrophoretic separation analysis was performed for the negative control and for the samples at 3 induction times: 12, 24 and 48 h.

The profile of bands shows a fragment corresponding to the *endo-1,3-β-glucanase* protein (estimated size 36 kDa) (Fig. 2).

As we can see in Fig. 2, there was an expression of the protein during all times assayed in the transformed sample, observing a considerable increase of the intensity of the glucanase band with induction times suggesting that the level of *endo-1,3-β-glucanase* expressed is related to the growth of the organism. This expression had already been observed at 4 h of induction in *E. coli* and proves that our approach is correct.

Infection of *C. sativa* roots

We followed the same procedure described in [21] for infection of the host roots. This procedure was performed with mycelium of the *P. cinnamomi* isolate Pr120. The infection process of *C. sativa* roots of 5–6 cm length by *P. cinnamomi* was carried out under the same conditions as in previous studies by us [21]. Roots of *C. sativa* not infected with *P. cinnamomi* were cultured in vitro in sterile vermiculite for 12, 24 and 36 h like negative control. The infection was performed by wrapping cellophane paper contaminated with *P. cinnamomi* in V8 medium in the roots of *C. sativa*. The infected root was then cultured in vitro at room temperature in flasks with sterile vermiculite for 12, 24 and 36 h. These spaced observations permitted a better examination of the progression of the infection in the plant. The first necrotic lesions appeared after about 12 h in those areas in direct

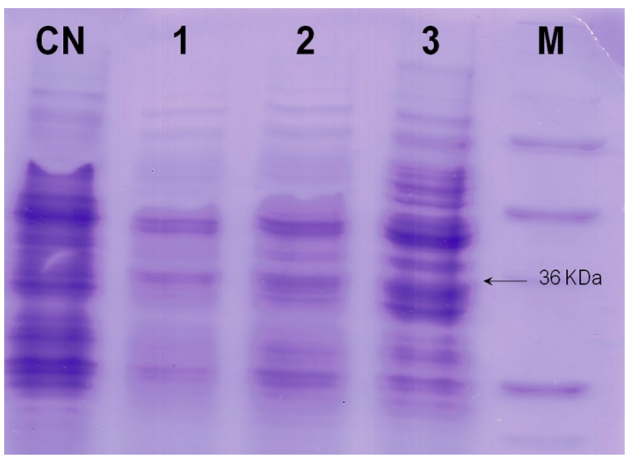

Fig. 2 SDS-PAGE of *endo-1,3-β-glucanase* expressed in *Pichia pastoris*. *CN P. pastoris* untransformed, *1* expression in *P. pastoris* at 12 h of induction, *2* expression in *Pichia pastoris* at 28 h of induction, *3* expression in *Pichia pastoris* with 48 h of induction, *M* kDa protein marker

contact with the inoculum. By 24 h, the original lesions had extended along the root (Fig. 3). By 36 h post-inoculation, the root necrosis had spread and was localized in the other non-suberized regions of the root (Fig. 3).

Quantification of *endo-1,3-β-D-glucosidase* transcripts

We followed the same procedure described in [21] for the study of the expression of the *endo-1,3-β-glucanase* gene during the growth of *P. cinnamomi* in different substrates. Likewise, analysis of the expression of the protein for *P. cinnamomi* infection in the *C. sativa* host was also performed.

The expression of *endo-1,3-β-D-glucosidase* gene in response to glucose, cellulose and sawdust was studied in *P. cinnamomi* cells using gene-specific primers (Table 1). The expression levels of actin2 gene were used as internal cDNA loading controls [22]. Mycelia of *P. cinnamomi* were incubated either with: 2% (w/v), cellulose 0.2% (w/v) or sawdust 0.2% (w/v), for 2, 4, 6 and 8 days. Those three substrates were chosen considering the plant–pathogen system: Glucose is a universal substrate, the main product of photosynthesis, an energy source, and metabolic intermediate; Cellulose is one of the major constituents of the cell walls of plants including the ones infected by *P. cinnamomi*; Sawdust was obtained by grinding roots of *C. sativa* and is, therefore, an approximation of the natural substrate of *P. cinnamomi*. The choice of actin mRNA as a stable endogenous control to normalize the amount of sample RNA was validated by evaluation of the oomycete actin mRNA levels in in vitro and *in planta* conditions. In RNA extracted from various in vitro cultures, both bands (18S and 28S) had equal intensity, showing that an identical quantity of *Phytophthora* RNA was present. The intensity of actin mRNA bands was similar in all samples, showing that actin mRNA levels were also similar [23]. Total RNA was extracted from *P. cinnamomi* cells grown in the three media mentioned above, cDNA was synthesized and gene expression measured by qRT-PCR. The results were normalized to actin2 gene and calculated as fold change relative to control cells (Fig. 4).

During 2, 4, 6 and 8 days the expression levels of the *endo-1,3-β-D-glucosidase* gene in *P. cinnamomi* were analyzed for different substrate types. This analysis showed that all carbon sources can induce increased expression levels of the *endo-1,3-β-D-glucosidase* protein and show differences in percentage growth from one substrate to another. In the early stages of the analysis, 2, 4 and 6 days glucose was the substrate that showed the highest induction. However, the expression at 8 days was superior in the cellulose substrate and even higher in the substrate sawdust. We can explain this result by a better adaptation of the pathogen to the substrate most similar to the substrate found in the natural environment of *P. cinnamomi.* The type of medium had a visible effect on the level of expression of the *endo-1,3-β-D-glucosidase* gene-it is significantly higher in sawdust than in glucose or cellulose and incremented in all media as the culture aged. This fact may be related to the adaptation of the pathogen at the culture media. Also, both glucose and cellulose are simple carbon sources compared with sawdust,

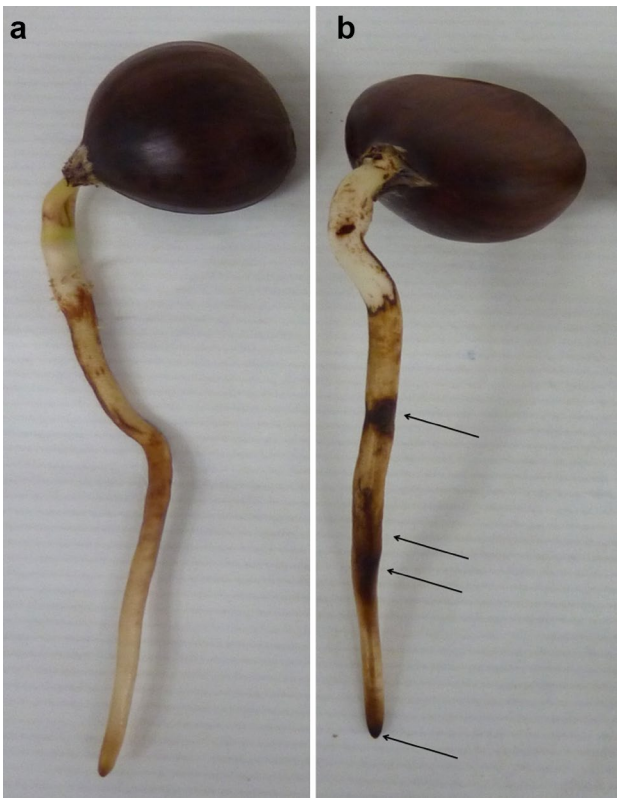

Fig. 3 Necrotic effect of *P. cinnamomi* in *C. sativa* roots. *C. sativa* roots were covered by fully colonized V8 agar by *P. cinnamomi* and incubated for 24 h in the dark at 25 °C. Necrotic tissue is indicated by arrows. **a** Control, non-infected root with *P. cinnamomi* and **b** infected root by *P. cinnamomi* [21]

Fig. 4 Effect of glucose, cellulose, and sawdust on the expression levels of the *endo-1,3-β-D-glucosidase* gene of *P. cinnamomi*

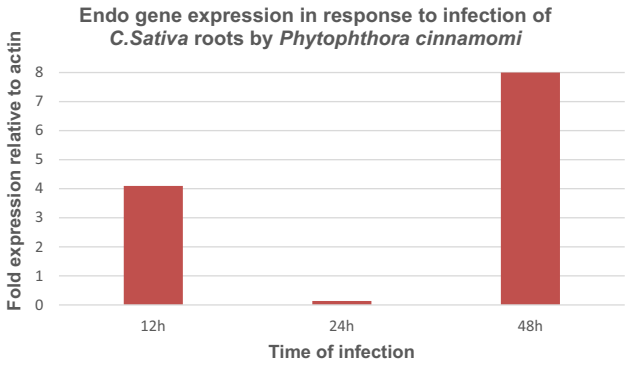

Fig. 5 Expression levels of the *endo-1,3-β*-D-*glucosidase* gene in *P. cinnamomi* during infection of *C. sativa* roots. *C. sativa* roots were exposed to mycelia of the wild type isolate Pr120 for 12, 24 and 48 h. RNA was isolated from these cells, cDNA was synthesized and the expression levels of the *endo-1,3-β*-D-*glucosidase* gene were analyzed by qRT-PCR, using specific primers. Values were normalized to the expression levels of actin2 gene determined in the same sample and are shown as relative mean values ± standard deviation (SD) of three experiments done in triplicate

hence easily degraded by the pathogen. In this work, sawdust is the substrate that presents more similarity with the natural substrate used by the pathogen and protein expression is definitely related to this fact. Similar results have already been observed for the *P. cinnamomi* Gip gene under the same experimental conditions [21].

The expression levels of *endo-1,3-β*-D-*glucosidase* transcripts in planta after infection of *C. sativa* roots, analyzed at different times, are represented in Fig. 5. Expression significantly decreases from 12 to 24 h and then significantly increases at 48 h.

The expression values of the protein in the infection over time showed an important oscillation, different from the constant increase in the expression in the carbon sources. After 12 h of increase in expression levels of the protein, there was a considerable decrease at 24. Then, at 48 h, a significant increase in expression levels of the *endo-1,3-β*-D-*glycosidase* protein (Fig. 5) was registered. This can be explained by pathogen–host interactions in which the plant is responsible for secreting inhibitory factors for the expression of the genes that produce fungal attack proteins, and subsequent response of the fungus to those factors, then increasing levels of expression at subsequent stages, such as a molecular battle.

This oscillation in expression values during infection suggests a complex mechanism of interaction involving *P. cinnamomi* and a defensive response of the plant [21, 26]. Effector molecules, such as *Hm1* gene revealed that it encoded an NADPH-dependent reductase that inactivates the toxin produced by the invading fungus, have been reported to interfere with plant defense activities [2, 21, 24]. Plants have the ability to quickly and accurately perceive their invaders and identify and respond to the chemical effects and mechanical signals that accompany the attack. The results of this expression analysis suggest the existence of an interaction between molecular effectors by the plant *C. sativa* (Fig. 5) and molecular suppressors by the inverse fungus, which lead to the considerable decrease of the expression at 24 h followed by a rise at 48 h [21, 25].

These suppressors are determinants in the pathogenicity process because they induce the local susceptibility of the infection and participate in the general suppression of plant resistance. Some *glucosidases* have also been classified as suppressors. However, future studies may reveal that *endo-1,3-β*-D-*glucosidase* from *P. cinnamomi* can be classified as elicitor and suppressor molecule [21, 26].

Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest. Also, are indebted to the careful and constructive criticisms of the reviewers.

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