



Cloning, characterization, *in vitro* and *in planta* expression of a necrosis-inducing Phytophthora protein 1 gene *npp1* from *Phytophthora cinnamomi*

Ivone M. Martins^{1,2} · Sofia Meirinho^{1,3,4} · Rodrigo Costa^{1,5} · Alfredo Cravador⁶ · Altino Choupina¹ 

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Abstract

The soil-borne oomycete *Phytophthora cinnamomi* is a highly destructive *Phytophthora* species associated with the decline of forest. This pathogen secretes a novel class of necrosis-inducing proteins known as Nep1-like proteins (NLPs). In this work, we report the sequencing and molecular characterization of one of these proteins, more specifically the necrosis-inducing Phytophthora protein 1 (NPP1). The ORF of the *npp1* gene (EMBL database AM403130) has 768 bp encoding a putative peptide of 256 amino acids with a molecular weight of approximately 25 kD. In order to understand its function, *in vitro* gene expression was studied during growth in different carbon sources (glucose, cellulose, and sawdust), and at different times of infection, *in vivo* by RT-qPCR. The highest expression of the *npp1* gene occurred in glucose medium followed by sawdust. *In vivo* infection of *Castanea sativa* roots with *P. cinnamomi* revealed a decrease in *npp1* expression from 12 to 24 h; at 36 h its expression increased suggesting the existence of a complex mechanism of defense/attack interaction between the pathogen and the host. Expression of recombinant *npp1* gene was achieved in *Pichia pastoris* and assessed by SDS-PAGE analysis of the protein secreted into the culture supernatant, revealing the presence of the NPP1 protein.

Keywords *Castanea sativa* · HE-TAIL PCR · *npp1* · *Phytophthora cinnamomi* · *Pichia pastoris* · RT-qPCR

Introduction

The European chestnut (*Castanea sativa* Miller) culture is extremely important in Europe, especially in the northern Iberian Peninsula where the sweet chestnut production has a high economic value. Furthermore, its contribution to forest diversity and to prevent soil degradation has particular ecological significance. Its extensive replacement that is currently taking place, by other forest species like pine or eucalyptus more vulnerable to forest fires, render the use of soil unsustainable in the medium term. Reduction in productivity and yield of chestnut has been occurring due to the ink disease whose most common symptoms are leaf chlorosis, root and lower stem necrosis, and reduced root growth, which invariably leads to the death of the trees. Its causal agent is the soil-borne oomycete *Phytophthora cinnamomi*, also responsible for the decline and death of many other plant species in Europe and worldwide [1–5]. This pathogen secretes a novel class of necrosis-inducing proteins, known as Nep1 (necrosis- and ethylene-inducing peptide 1)-like proteins (NLPs), more specifically necrosis-inducing

✉ Altino Choupina
albracho@ipb.pt

- ¹ CIMO-Mountain Research Center, Department of Biology and Biotechnology, Agricultural College of Bragança, Polytechnic Institute of Bragança, Campus Santa Apolónia, 5301-855 Bragança, Portugal
- ² CEB - Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
- ³ ICVS - Life and Health Sciences Research Institute, School of Health Sciences, University of Minho, 4710-057 Braga, Portugal
- ⁴ ICVS/3B's – PT Government Associate Laboratory, 4710-057 Braga/Guimarães, Portugal
- ⁵ Department of Microbiology and Genetics, CIETUS-IBSAL, University of Salamanca/CSIC, Plaza de los Drs. de la Reina s/n, 37007 Salamanca, Spain
- ⁶ Centre for Mediterranean Bioresources and Food, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Phytophthora protein 1 (NPP1), that causes necrosis on leaf and roots, ultimately leading to the plant death [6].

Several secreted pathogen proteins called elicitors are recognized by the plant defense system, being this system, consisting usually of products from the host resistance (*R*) genes. These proteins are involved in a transduction cascade that causes cell death of infection agent at the site of infection, thereby limiting the spread of the pathogen. Although this necrosis prevents the progression of biotrophic pathogen cells, the induction of such condition can be beneficial to saprotrophic pathogens, and some have acquired the ability to manipulate plant cell death to their own advantage [7–9].

Among cell-death-inducing secreted proteins of plant, pathogens are the Nep1-like proteins (NLPs), first detected in culture filtrates of the vascular wilt fungus *Fusarium oxysporum* [10]. NLPs are microbial elicitors of plant necrosis and are widespread among bacteria, fungi, and oomycetes including pathogens and non-pathogens, but absent in the plant and animal kingdom. Typically, they possess a conserved domain, referred to as the NPP1 domain (PFAM domain PF05630) and reported in studies describing the plant necrosis-inducing activity of *Phytophthora parasitica* NLP [11, 12]. NLPs comprise a family of relatively small proteins of approximately 24–26 kDa sharing a conserved heptapeptide motif -GHRHDWE in the central region, not present in other proteins [12, 13]. Depending on the presence of either two or four conserved cysteine residues, NLPs are divided into two groups, type I and II, and both types may occur in the same species [13].

Low concentration of purified NLPs can induce callose apposition, accumulation of reactive oxygen species (ROS) and ethylene as well as the activation of genes involved in stress and defense responses [14–16]. At higher concentrations, NLPs induce cell death at the site of application [10, 14].

The plant cell responses to NLPs have been extensively described in many work publications that used biochemical, cytological and transcriptomic approaches. Such studies have demonstrated that NLP treatment induces the accumulation of calcium and ROS, increases the activity of mitogen-activated protein (MAP) kinase and the production of ethylene, phytoalexins and pathogenesis-related proteins, changes the K⁺ and H⁺ channel fluxes and cell respiration, and triggers nuclear DNA fragmentation and programmed cell death (PCD) [12, 15–19].

The present work on the *npp1* gene from *P. cinnamomi*, disclosing its DNA sequence and describing its in vivo and in vitro patterns of expression, contributes significantly to the understanding of the molecular mechanism of plant necrosis induced by this oomycete during the early stages of infection of the high susceptible, and economic and ecologically valuable host *C. sativa*, helpful condition for the

implementation of control strategies of the decline and ink disease.

Materials and methods

Biological material

The *P. cinnamomi* strain pvbp was isolated from a soil sample collected in the rhizosphere of a chestnut tree affected by the ink disease from the region of Trás-os-Montes (north-east of Portugal). This isolate was further characterized by molecular methods and deposited in the Spanish Type Culture Collection (CECT) with the CECT 20919 code.

The growth conditions for this strain were at 22–25 °C in Potato-Dextrose Agar (PDA) medium in the dark for 4–6 days. Total genomic DNA from *P. cinnamomi* mycelium was extracted as described by Raeder and Broda [20]. *C. sativa* chestnuts were surface sterilized, germinated in sterile vermiculite and grown in a greenhouse until their root length reached 5–6 cm. For *C. sativa* infection, the roots were covered with fully colonized V8 agar medium and incubated for 12, 24 and 36 h in the dark at 25 °C.

Plasmids

pIB2 (Addgene) is a 5550 bp *Pichia pastoris* expression vector containing the ampicillin resistance gene and regulated by the GAP promoter.

pA1 is a 6.3 kb pIB2 based construction containing an expression cassette consisting of the *npp1* gene present as a 768 bp *EcoRI-PstI* fragment. This was obtained by polymerase chain reaction (PCR) amplification of a 768 bp ORF of the *npp1* gene from *P. cinnamomi* DNA, using the primers FNPP 5'-**GAA TTC** ATG GTA TCG GCT GTG CTC G-3', and RNPP 5'-**CTG CAG** CTA AAA CGG ATA GGT GCT ATT G-3' (with insertion of restriction sites *EcoRI* and *PstI*, respectively in bold). This fragment was digested with *EcoRI-PstI* and inserted into pIB2 at the *EcoRI-PstI* site. To prepare the pIB2 vector for the insertion this was digested to remove its original *EcoRI-PstI* DNA fragment and purified by electrophoresis in a low melting point (LMP) agarose gel and Wizard® SV Gel and PCR Clean-Up System (Promega), following the manufacturer's instructions. The positive clone resistant to ampicillin, was checked by electrophoresis for band size comparison was sequenced to confirm the correct integration of the insert. The sequencing of the DNA fragments was carried through in an automatic sequencer ABI Prism 377TM from Applied Biosciences (Foster City, CA, USA).

Plasmids were propagated in *Escherichia coli* DH5α cells [21], and their DNA was extracted/purified with the

Wizard® Plus SV Minipreps DNA Purification System (Promega), according to manufacturer's instructions.

Amplification of a short fragment of the *npp1* gene

A 600 bp fragment of the *npp1* gene was amplified by PCR. Degenerate oligonucleotide primers NPP1 (5'-CCG TTC GCC CAA CCC ACA (AG)CC A) and NPP2 (5'-CCC AGA CGA C(GC)A CGT (AG)CT CCC AG) were designed based on homology with previous published *Phytophthora* sp. NPP's sequences from EMBL databases, namely from *Phytophthora infestans* (AY961417), *P. infestans* (AF356840) and *P. parasitica* (AF352031). The following PCR cycling conditions were applied: initial 94 °C/5 min, followed by 36 cycles of 94 °C/1 min; 63 °C/1 min; 72 °C/30 s, and ending with 72 °C/5 min. PCR was performed on a volume of 50 µl containing 0.8 mM dNTP's, 0.2 µM of each primer, 100 ng genomic DNA and 1 U Taq DNA polymerase in the appropriate buffer. Aliquots of the PCR reactions were separated on 0.8% w/v agarose gel electrophoresis and stained with ethidium bromide, to check the presence of the expected amplicon.

Amplification of unknown genomic DNA sequence of *npp1*

The full-length *npp1* gene, 1328 bp long (GenBank: AM403130.1), was obtained by High-Efficiency Thermal Asymmetric Interlaced-PCR (HE-TAIL PCR), an efficient method to amplify unknown genomic DNA sequences adjacent to short known regions by flanking the known sequence with asymmetric PCR [22]. In this procedure gene-specific primers NPP1, NPP2, NPP3 (5'-GGG ATC TGC GTG GGG GTC CCA GG), NPP4 (5'-CGG GCC ACC GCC ACG AGT GGG AG), NPP5 (5'-CCT GGG ACC CCC ACG GAG ATC CC) and NPP6 (5'-CGA GCC GTC CGC CTG AAC GGC AGG) were used. Degenerate primers were applied, based on the IUPAC nucleotide code: R1 (5'-NGT CGA SWG ANA WGA A), R2 (5'-GTN CGA SWC ANA WGT T), R3 (5'-WGT GNA GWA NCA NAG A) and R4 (5'-NCA GCT WSC TNT SCT T). Three rounds of PCR were performed using the product of each previous PCR as a template for the next round. A detailed cycler program is shown in Table 1.

The primary PCR was performed in a 50 µl volume containing 80 ng genomic DNA, 0.2 mM of primers NPP3 or NPP4, 2 mM of a random primer (R1, R2, R3, R4), 0.2 mM of each dNTP and 1U Taq DNA polymerase in the appropriate buffer. The secondary PCR was performed with primers NPP5 or NPP6 (0.2 mM) and the same random primer R (2 mM) as used in the primary reaction. 1 µl 1/50 dilution of the primary PCR was used as a template. Single-step annealing-extension PCR consisting of a combined annealing and extension step at 65 °C or 68 °C was used in primary and

Table 1 HE-TAIL PCR cycle settings

Reaction	Number of cycles	Thermal settings
Primary	1	93 °C (1 min); 95 °C (5 min)
	5	94 °C (30 s); 62 °C (1 min); 72 °C (2 min 30 s)
	1	94 °C (30 s); 25 °C ramping 72 °C (3 min); 72 °C (2 min 30 s)
	15	94 °C (20 s); 65 °C (3 min 30 s); 94 °C (20 s); 65 °C (3 min 30 s); 94 °C (30 s); 42 °C (1 min); 72 °C (2 min 30 s)
Secondary	1	72 °C (5 min); 4 °C hold
	12	94 °C (20 s); 65 °C (3 min 30 s); 94 °C (20 s); 65 °C (3 min 30 s); 94 °C (30 s); 42 °C (1 min); 72 °C (2 min 30 s)
	1	72 °C (5 min); 4 °C hold
Tertiary	30	94 °C (30 s); 42 °C (1 min); 72 °C (2 min 30 s)
	1	72 °C (5 min); 4 °C hold

secondary PCR reactions. The tertiary reaction was carried out with 1 µl of 1/10 dilution of the secondary reaction, 0.2 mM primers NPP3 and NPP4, 0.2 mM random primer R (the same as used in the previous cycles), 0.2 mM each dNTP, 1U DNA Taq polymerase in the appropriate buffer. To exclude nonspecific amplification, a tertiary control reaction R–R was set up without adding gene-specific primers.

Amplification of *npp1* ORF

A PCR was used to amplify the 768 bp ORF of the *npp1* gene using the primers described under the Plasmids section. The PCR cycling conditions were: 95 °C/3 min, followed by 40 cycles of 94 °C/1 min, 61 °C/1 min, 72 °C/3 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 MgCl₂ and 0.05 U Taq DNA polymerase in the 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 expected amplicon.

Assessment of expression

For functional studies, heterologous expression of NPP1 was performed in *P. pastoris* GS115 yeast (secretory expression plasmid pPIC9 K, Multi-Copy Pichia Expression Kit, Invitrogen). The recombinant plasmid pA1 was digested with *Stu*I and transformed by electroporation into the *P. pastoris* strain. Positive clones were screened by HIS4 selectable marker in RDB plates and by PCR with the oligonucleotides

FNPP (5'-GAA TTC ATG GTA TCG GCT GTG CTC G-3') and RNPP (5'-CTG CAG CTA AAA CGG ATA GGT GCT ATT G-3'), with insertion of restriction sites *EcoRI* and *PstI*, respectively in bold.

The genetic engineered strains pIB2-pA1/GS115, pIB2/GS115 and GS115 were cultured in a YEPD (yeast extract-1%, peptone-2% and dextrose-2%) medium at 30 °C, with agitation (250 rpm) and 2 ml aliquots were removed every 2 h to evaluate growth rate.

Analysis of NPP1 production

The expression of the 25 kDa NPP1 protein encoded by *npp1* gene in *P. cinnamomi* was checked by SDS-PAGE in spent culture medium. Briefly, *P. cinnamomi* mycelium was grown for 5 weeks at 24.5 °C in the dark, in a sucrose-asparagine synthetic medium described in [23] [41], in standing culture. After 5 weeks the mycelium was filter/sterilized and 1/10 of the volume was passed through an 8 kD cut-off membrane (Millipore Biomax Pelicon XL filter). The liquid was concentrated 25 times, divided into four tubes and further concentrated in a speedvac Savant™ SPD131DDA (ThermoScientific). The resulting concentrated growth medium was precipitated with trichloroacetic acid (TCA) and resuspended in 20 µl of Tris (pH 9.5). The secreted proteins were separated by SDS-PAGE (15% w/v) and visualized with silver nitrate. We used silver nitrate stain as it is more sensitive and allows to analyse a wider range of expression levels.

Expression of *npp1* 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 three different carbon sources [2% (w/v) glucose, 0.2% (w/v) cellulose and 0.2% (w/v) sawdust] and the relative expression of *npp1* gene was assessed at 2, 4, 6 and 8 days of growth. Control cells were grown in dextrose-containing medium (PDA). The expression level of the *npp1* gene was measured relative to the expression level of the housekeeping gene *actin2*.

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, along with all external mycelia growth. The roots were examined for the presence and extent of necrosis and then frozen to –80 °C. The expression level of the *npp1* gene was measured relative to that of the reference gene *actin2*. Results were normalized to *actin2* gene and calculated using the $\Delta\Delta C_t$ method, as fold change relative to control cells [24].

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 iScript™ cDNA Synthesis Kit (BioRad) primed with oligo (dT), following the manufacturer's instructions. The qPCR was performed with IQ™ SYBR® Green Supermix (Biorad) 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 MgCl₂, 20 nM SYBR Green I fluorescein, and stabilizers) and 1.25 µM of each primer. The reactions were run in triplicate, initially 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.

The assays were repeated three times. Amplification primers were targeted to the coding regions of *P. cinnamomi* *actin2* and *npp1* genes (Table 2).

Results

Amplification and characterization of the *npp1* gene of *Phytophthora cinnamomi*

In the present work we report the sequencing of a class of genes coding to necrosis-inductor *P. cinnamomi* proteins that specifically cause necrosis in plant leaves and roots. DNA was generated using the HE-TAIL PCR (High-Efficiency

Table 2 Sequences of primers used to amplify *actin2* and *npp1* genes in the RT-qPCR experiments

Target gene	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
<i>actin2</i>	GGCCTCGAGAAGAGCTACG	CTTCATGATGGTCTGGAACG
<i>npp1</i>	GTGCTCGACAGTCGTTTCAA	GGTACTTGATAGCTGCCTCTCG

Thermal Asymmetric Interlaced-PCR). A small fragment of 600 bp of the *npp1* gene was first extracted from *P. cinnamomi* strain pvbp DNA, by PCR amplification using degenerate primers designed based on the homology with the open reading frames of other NPPs from *P. infestans* and *P. parasitica*. The full-length DNA of the gene (1328 bp, GenBank Accession No. AM403130), was obtained by flanking the known sequence with HE-TAIL PCR. This full-sequence comprises a 199 bp promoter and a 359 bp terminator with a poliA tail. The translated ORF of *P. cinnamomi npp1* codifies a 256 amino acid protein, with a predicted molecular weight of 25 kDa.

Figure 1 shows the alignment of the *P. cinnamomi* NPP1 sequence with NPP's from other *Phytophthora* species, and a NPP1 super-family conserved domain from oomycetes, fungi and bacteria, sharing a conserved heptapeptide motif -GHRHDWE in the central region, not present in other proteins [12, 13].

Assessment of expression and protein production assay

The expression of the *npp1* gene was analyzed in *Pichia pastoris* in order to study the *npp1* gene and to produce the NPP1 protein heterologously in *P. pastoris*, useful for further studies. The *npp1* gene does not seem to affect the general growth of *P. pastoris*, only a decrease in the lag phase was observed (Fig. 2).

The expression of the expected 25 kDa NPP1 protein encoded by the *npp1* gene of *P. cinnamomi* in the spent culture medium, after 5 days of standing culture was confirmed by SDS-PAGE. A band of approximately 25 kDa was observed, indicating the NPP1 protein was produced (Fig. 3).

Protein quantification was not evaluated, as the aim at this stage was not to optimize the protein expression but to

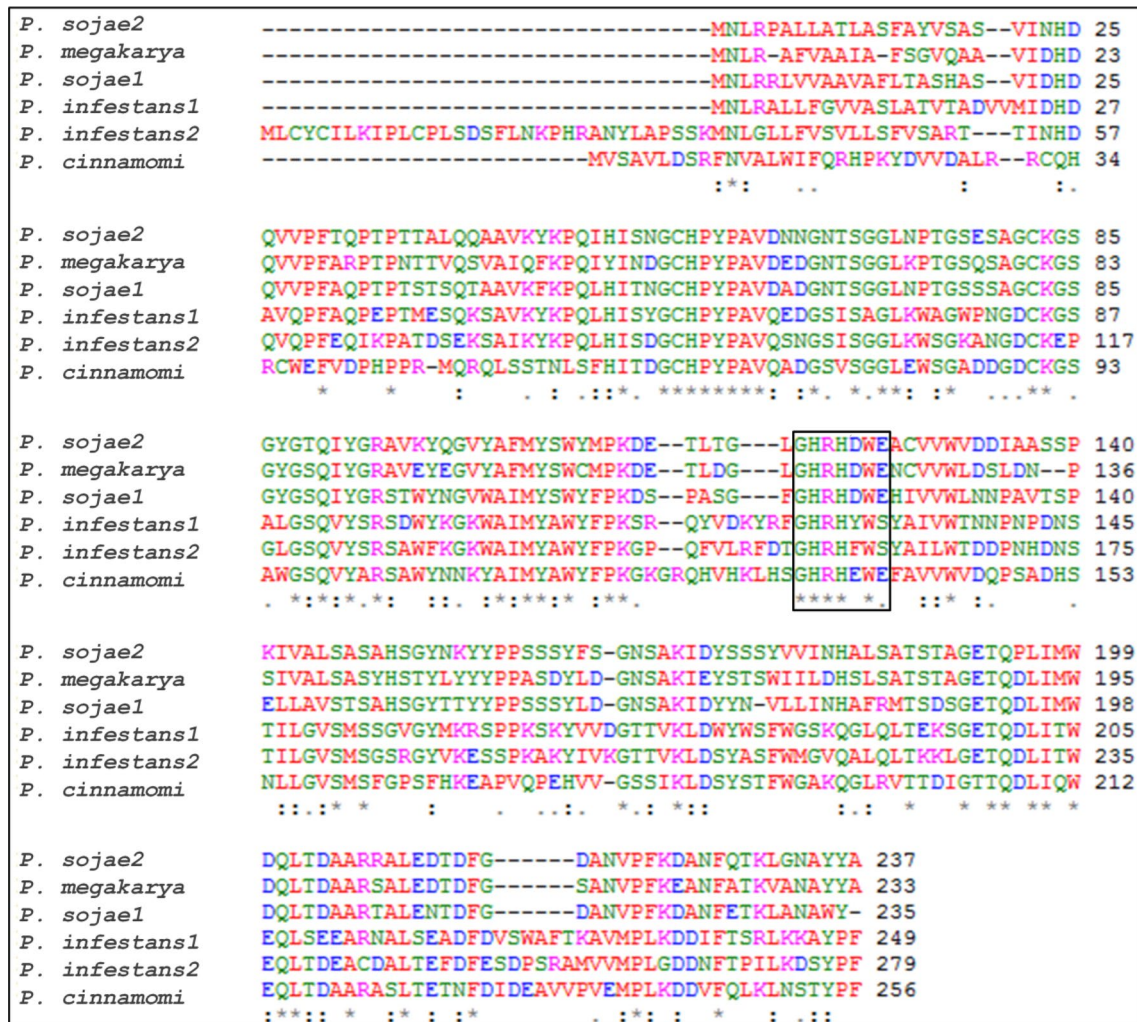


Fig. 1 Sequence alignment of NPP1 proteins. In the central region, the conserved heptapeptide motif GHRHDWE is indicated by the black box

Fig. 2 Expression of *npp1* gene in *Pichia pastoris*. GS115—*P. pastoris* strain; pIB2/GS115—*P. pastoris* carrying pIB2; pIB2-pA1/GS115—*P. pastoris* expressing *npp1*

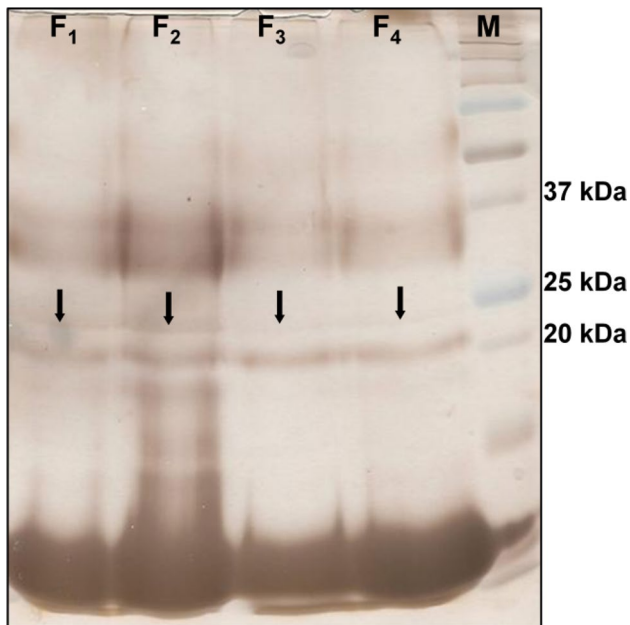
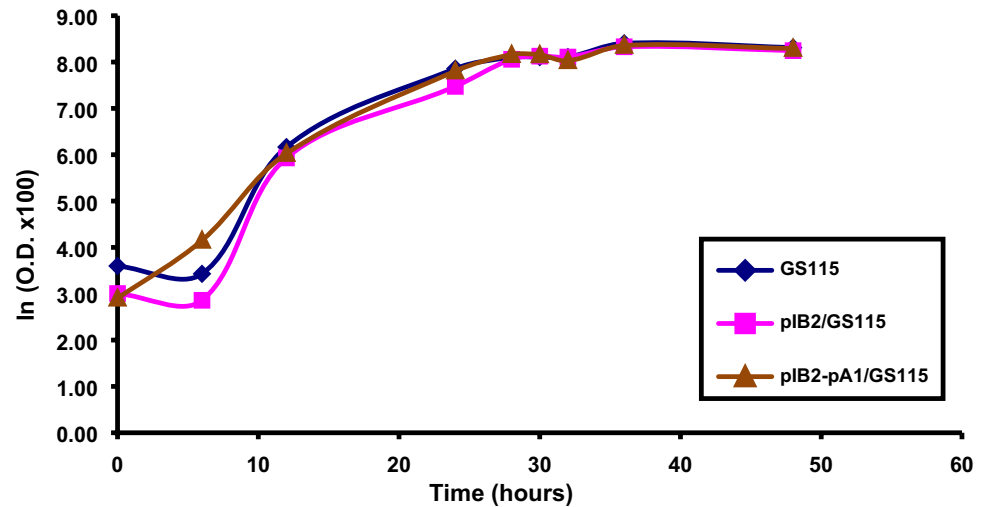


Fig. 3 Expression of NPP1 protein in exhaust culture medium, separated by SDS-PAGE. The 25 kDa NPP1 protein is shown with an arrow in four precipitated and concentrated fractions (F1–F4) of exhaust culture medium, recovered after 5 days of standing growth. Silver staining at 0.2% for 16 h. **M**—precision plus protein ladder (BioRad)

show that the system works, that there is expression using the more direct construction.

Infection of *Castanea sativa* roots

Castanea sativa roots were inoculated with mycelium of the *P. cinnamomi* pvbp strain and necrosis caused by the infection was evaluated at three (12 h, 24 h and 36 h) post-inoculation time points (hpi). The first necrotic lesions appeared

at about 12 hpi, visible in areas in direct contact with the inoculum. By 24 hpi, the original lesions had extended along the root and by 36 hpi the root necrosis had spread toward the non-suberized region of the root (Fig. 4).

Quantification of *npp1* transcripts

We followed the procedure described in [25] to study the expression of the *npp1* gene during the growth of *P. cinnamomi* in different substrates.

Protein expression is directly related to the type of substrate in which *P. cinnamomi* grows. Therefore, two simple and one more complex carbon sources were selected for *P. cinnamomi* growing: 2% glucose, 0.2% cellulose and 0.2% sawdust respectively as described in “Materials and methods”. The expression level was measured over 8 days of growth relative to the expression of the constitutive gene *actin2* [26, 27]. 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.

Analysis of the expression levels of the *npp1* gene in *P. cinnamomi* mycelia grown in glucose and sawdust shows that these carbon sources induce a small and progressive increase at 2, 4 and 6 days, and a significant increase of the expression level at 8 days (Fig. 5). In 2 day cultures, gene expression level in glucose and sawdust was very similar. In all-days cultures, *npp1* expression levels in glucose and in sawdust were much higher than in cellulose reaching an eightfold and tenfold increase, respectively, relative to cellulose.

We then proceed to analyze the expression of the infection of *P. cinnamomi* in the host *C. sativa* following a procedure reported in [25]. The analysis of the expression levels of *npp1* transcripts *in planta*, after infection of *C. sativa* roots at three (12, 24 and 36 h) hpi, is represented in Fig. 6.



Fig. 4 Necrotic effect of *Phytophthora cinnamomi* in *Castanea sativa* roots. The roots were covered with fully colonized V8 agar and incubated for 12, 24 and 36 h in the dark at 25 °C. Necrotic tissue

is indicated by arrows. **a** Non infected root–control; **b** infected root after 12 h of incubation; **c** infected root after 24 h of incubation and **d** infected root after 36 h of incubation

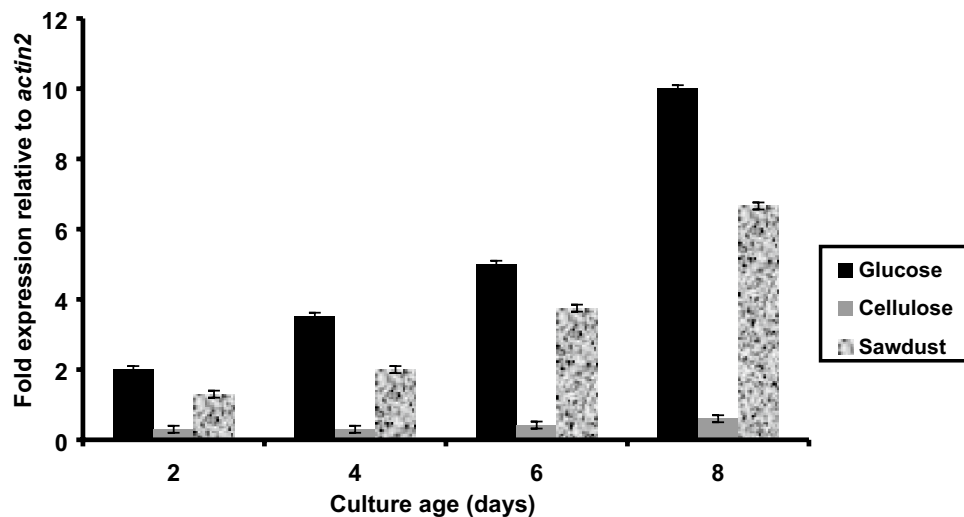


Fig. 5 Effect of glucose, cellulose and sawdust on the expression levels of the *npp1* gene of *Phytophthora cinnamomi*. Mycelia of the wild type *P. cinnamomi* pvbp strain were exposed to glucose 2% (w/v), cellulose 0,2% (w/v) and sawdust 0,2% (w/v) for 2, 4, 6 and 8 days. RNA was isolated from these cells, cDNA was synthesized and the

expression levels of the *npp1* 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 \pm standard deviation (SD) of three experiments done in triplicate

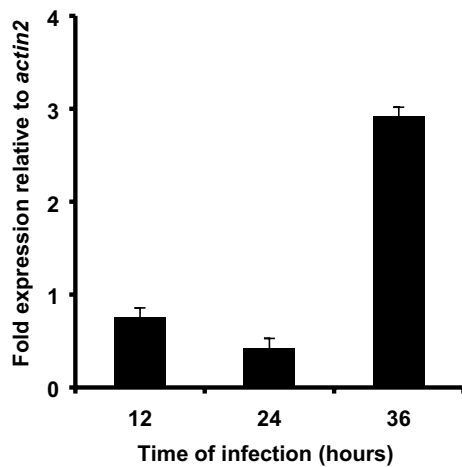


Fig. 6 Expression levels of the *npp1* gene in *Phytophthora cinnamomi* during infection of *Castanea sativa* roots. The roots were exposed to mycelia of the wild type isolate *P. cinnamomi* for 12, 24, 36 h. RNA was isolated from these cells, cDNA was synthesized and the expression levels of the *npp1* 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 \pm standard deviation (SD) of three experiments done in triplicate

The expression observed at 12 hpi is followed by a significant decrease at 24 hpi and then by a significant increase at 36 hpi.

Discussion

The main goal of this work was to contribute to a better understanding of the mechanisms by which the infectious process of oomycetes and particularly *P. cinnamomi*, affect forest trees, an essential condition for the implementation of control strategies.

Like other plant pathogens [17, 18], *P. cinnamomi*, one of the most aggressive and destructive pathogens, uses necrosis-inducing proteins as part of a mechanism of infection against which plants usually respond activating genes involved in stress and defense responses. It secretes, in particular, a necrosis-inducing Phytophthora protein 1 (NPP1) whose role in the infection of *C. sativa*, one of the most susceptible and economic valuable host, is important to study.

Transcripts of *npp1* were detected as early as 12 hpi, coinciding with the formation of primary lesions. The fact that *npp1* was expressed very early during infection suggests that the protein may contribute to trigger plant cell death during early stages of lesion development. However, at this stage we cannot discard the possibility that only non-active copies of the gene were measured by qRT-PCR.

In vitro, a steady increase in expression over time is observed, especially regarding the expression on glucose and

sawdust; however, *in planta*, there are variations reflected in an oscillatory expression, which may be due to the mechanisms of plant defense. For a pathogen to colonize a host successfully, it must develop mechanisms to evade detection or, failing that, to subvert defense responses. Models have been proposed in which pathogen-derived effector molecules interfere with elicitor binding, signal transduction, gene activation, or other activities of the defense responses [8, 17–19, 28–32].

It has been reported in similar studies using glucose sawdust and cellulose as carbon sources for *P. cinnamomi* mycelia grown that the medium has a visible effect on the level of expression of the *gip* (glucanase inhibitor protein, also known to influence plant–pathogen interactions) gene [25]. Over time all media showed an increase in the expression level of the *npp1* gene. The medium with glucose is a richer medium, so it was not unexpected that over time it showed a greater induction compared to the other media. In contrast, with cellulose a significantly lower level of expression was achieved because it is a poorer carbon source. This result is consistent with the fact reported in other studies that the level of expression is favored by the adaptation of the pathogen to the culture media closest to its natural environment [25].

The analysis of the expression of the infection of *P. cinnamomi* in the host *C. sativa* showed that the expression values are generally lower than those found in growth media with different carbon sources (Fig. 5), what can be explained by an inhibition due to a factor secreted by the plant. This oscillation of values of expression during infection also suggests a complex mechanism of interaction related to the defense response [32, 33].

Different families of phytopathogenic fungi and oomycetes have the NLP gene. This indicates that this gene has an important role in the interaction of plant pathogens with plants, especially for pathogens that have a necrotrophic lifestyle. Moreover, the presence of this gene among oomycetes and pathogenic plant fungi as well as plant-associated bacteria shows that it plays a very important role in the parasitic plant lifestyle [14, 34] and induces complex defense and cell death responses in their hosts. When the response mechanism starts the plants identify and respond to the chemical effects and mechanical signals accompanying the invasion [8, 28, 35]. Their ability to quickly and accurately detect their pathogens is essential to build an effective defense response.

Summarizing, *P. cinnamomi npp1* gene showed a 1328 bp sequence comprising a 768 bp ORF encoding a protein with 256 aa; NPP1 protein expression reveals a band of about 25 kDa; heterologous expression of *npp1* does not affect the general growth of *P. pastoris*, only a decrease in the lag phase was observed; the *npp1* gene is expressed in vitro, at the highest level in the medium with glucose as compared

with sawdust and cellulose. In vivo, after an expression increase at 12 h, a small decrease within 24 h was observed followed by a greater increase in expression at 36 h. This increase observed after 36 h of infection is related with the observed necrotic lesions in vivo in the roots upon inoculation by *P. cinnamomi*. In any case, the values of expression are generally lower than those found in growth media with glucose and sawdust.

Although the present work sheds some light on the current understanding of the mode of action of NLPs and gives some hints of how the plant responds, further studies are needed to fully understand the mechanisms underlying defense mechanisms against *P. cinnamomi* involving necrosis-inducing proteins, an important requisite to the development of control measures and resistant crops.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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