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Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*

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ABSTRACT

The hemibiotrophic basidiomycete *Moniliophthora perniciosa* causes witches' broom disease of *Theobroma cacao*. Analysis of the *M. perniciosa* draft genome led to the identification of three putative genes encoding necrosis and ethylene-inducing proteins (MpNEPs), which are apparently located on the same chromosome. MpNEP1 and 2 have highly similar sequences and are able to induce necrosis and ethylene emission in tobacco a'd cacao leaves. MpNEP1 is expressed in both biotrophic and saprotrophic mycelia, the protein behaves as an oligomer in solution and is very sensitive to temperature. MpNEP2 is expressed mainly in biotrophic mycelia, is present as a monomer in solution at low concentrations (<40 μM) and is able to recover necrosis activity after boiling. These differences indicate that similar NEPs can have distinct physical characteristics and suggest possible complementary roles during the disease development for both proteins. This is the first report of NEP1-like proteins in a basidiomycete.

Introduction

Witches' broom disease (WBD) of cacao (*Theobroma cacao*) is one of the most important phytopathological problems to afflict the Southern Hemisphere in recent decades (Griffith *et al.* 2003). In Brazil, the disease is endemic in the Amazon region, and in 1989 was introduced into southern Bahia, the largest area of cacao production in the country (Pereira *et al.* 1996). This resulted in a severe drop in the production of this commodity and Brazil shifted from the second largest cacao exporter to a cacao importer.

Moniliophthora perniciosa, the causal agent of WBD, is a basidiomycete and has a hemibiotrophic life cycle (Aime & Phillips-Mora 2005; Purdy & Schmidt 1996). Initially, uninucleated

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basidiospores, dispersed by wind and rain at night, germinate on rapidly growing tissues, such as meristems, initiating the biotrophic phase of the disease. This is characterized by the presence of monokaryotic intercellular mycelia that lack clamp connections. Recent studies have shown that it is possible to maintain biotrophic-like mycelia '*in vitro*', and that the conditions that prevent phase change are a low nutrient content and the presence of glycerol as the only carbon source (Meinhardt *et al.* 2006). When emerging branches are infected, the disease is characterized by a spectacular growth of new shoots (green broom), which seems to be a nutritional sink (Purdy & Schmidt 1996; Scarpari *et al.* 2005). Eight to twelve weeks after the initial infection, the infected tissues begin to senesce and are colonized inter and intracellularly by the saprotrophic mycelia of *M. perniciosa*, which are dikaryotic and have clamp connections (Delgado & Cook 1976; Evans 1980; Griffith & Hedger 1994). It is important to note that transition from biotrophic to necrotrophic involves changes not only in the plant, but also in the biology of the vegetative mycelia. The senescing brooms turn brown, forming the typical necrotic dry broom structures of this disease, which remain attached to the plant. After a latent period of three to nine months, the dry brooms begin to produce fruiting bodies (basidiocarps), which release basidiospores, thus completing the life cycle (Purdy & Schmidt 1996; Wheeler & Suarez 1993).

Due to the complexity of the witches' broom disease, we started a *M. perniciosa* genome project (www.lge.ibi.unicamp.br/vassoura) in 2001 with the goal of identifying genes encoding proteins that are potentially involved in the disease. A series of related studies were also undertaken to provide the technical (Gesteira Ada *et al.* 2003; Lima *et al.* 2003), biological (Rincones *et al.* 2006; Rincones *et al.* 2003), biochemical (Scarpari *et al.* 2005) and cytological basis (Meinhardt *et al.* 2006) to investigate and interpret the sequence data.

Several secreted pathogen proteins, called elicitors, are recognized by the defense system in the plant, which normally consists of products from the R genes. These proteins are connected to a transduction cascade that causes cell death at the site of infection, thereby limiting the spread of the pathogen (Nimchuk *et al.* 2003). 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 (Mayer *et al.* 2001; Qutob *et al.* 2002).

Nep1 (necrosis and ethylene-inducing peptide) is a peptide representing a new class of necrotic elicitors. This extracellular protein, initially identified from the culture filtrate of *Fusarium oxysporum*, has the ability to induce necrosis in several plants, including cacao (Bailey 1995; Bailey *et al.* 2005; Verica *et al.* 2004). Over the last ten years a number of Nep1-like proteins (NLPs) have been found in a diverse group of microorganisms, such as bacteria, fungi, and in particular, oomycetes (Gijzen & Nurnberger 2006; Pemberton & Salmond 2004). In several cases, a species can have more than one copy of NLPs; as in *Phytophtora sojae* and *P. ramorum*, where 50–60 loci are involved, and it is believed that several of these copies are pseudogenes (Gijzen & Nurnberger 2006; Tyler *et al.* 2006). In *P. megakarya*, a devastating cacao pathogen that causes black pod disease (BP) in Africa, nine orthologous have been found; most of them are organized in clusters and at least six of them seem to be expressed (Bae *et al.* 2005).

It is very interesting to note that although NLPs are present in phylogenetically distant organisms, their sequences remained remarkably conserved throughout evolution (Pemberton & Salmond 2004). There is a heptapeptide (GHRHDWE) and some conserved cysteine residues present in all sequences. These residues allow for the classification of the NLPs into two groups, according to the cysteine number and position (Gijzen & Nurnberger 2006). Some organisms, like *Magnoportha grisae*, have copies from both groups (Dean *et al.* 2005). Initially, it was though that this conservation could be related to a common phytopathogenic strategy, but homologues of this gene have also been found in non-pathogens, such as the bacteria *Vibrio pommerensis* (Jores *et al.* 2003) and the ascomycete fungus *Neurospora crassa* (Galagan *et al.* 2003). To date, no homologues has been reported in plants, animals, or protists (Pemberton & Salmond 2004; Win *et al.* 2006)

Gijzen & Nurnberger (2006) summarized the common characteristics of the NLPs: they are active only on dicotyledonous plants; the necrosis activity is heat labile and is not reproduced using only derived peptides; the protein acts outside of the plant cell, suggesting the existence of cell wall receptors for this elicitor; and the NLPs cause rapid activation of the cell defense response, which ultimately leads to cell death and tissue necrosis.

The exact mechanism by which the NLPs cause necrosis is not clear. In tobacco leaves, Nep1 caused an increase of ethylene emission (Bailey *et al.* 1997; Fellbrich *et al.* 2002; Jennings *et al.* 2000), suggesting that necrosis could be an indirect effect of this hormone. However, in some plants necrosis induction was not accompanied by ethylene production (Bailey 1995; Bailey *et al.* 1997), indicating that other mechanisms may be involved. Nep1 has also been linked to the increased production of superoxide anions and with the expression of the salicylic acid-dependent resistance gene, PR1 (Fellbrich *et al.* 2002). Studies with *Pythium aphanidermatum* showed that this protein can induce the development of programmed cell death (PCD) (Veit *et al.* 2001). Additionally, several Nep1-induced genes were identified in different plant species and most were related to stress/defense responses (Bailey *et al.* 2005; Keates *et al.* 2003; Verica *et al.* 2004). Remarkably, it was observed that the expression of the *P. sojae* necrosis-inducing protein, PsojNIP, in soybean hypocotyls parallels the transition from the biotrophic to the saprotrophic phase, suggesting that this protein could facilitate the colonization of host tissues during the necrotrophic phase of pathogen growth (Qutob *et al.* 2002).

In this work, we show the presence of NLPs in the genome of *M. perniciosa* (MpNEPs), and this is the first report of NLPs in a basidiomycete. MpNEPs have the ability to induce ethylene emission and necrosis in cacao and tobacco tissues, indicating that they can play an active role in witches' broom disease. Despite the high sequence similarity, MpNEP1 and MpNEP2 showed different structural features, and MpNEP2 activity was resistant to high temperatures. Finally, the genes are differentially expressed in the two fungal phases, and we discuss the significance of this regulation to the disease development.

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Material and methods

Biological material and growth conditions

The strain used in this work was derived from a monosporic culture of *Moniliophthora perniciosa* isolate CP02 described elsewhere (Rincones *et al.* 2003). Genomic DNA was isolated from saprotrophic mycelia grown at 28 °C in medium containing glucose (1 %), $NH_4H_2PO_4$ (1 %), KCl (0.2 %), $MgSO_4.7H_2O$ (0.2 %), yeast extract (0.5 %), $CuSO_4.5H_2O$ (0.05 %) and $ZnSO_4 \cdot 7H_2O$ (0.001 %). This genomic DNA was used for the amplification of the genes by PCR. For the MpNEPs expression analysis, RNA was isolated from biotrophic-like mycelia from spores germinated on a special medium (Meinhardt *et al.* 2006), which can sustain fungal growth as mononucleated hyphae without clamp connections and from saprotrophic mycelia also inoculated on the same medium.

Southern blot analysis

Genomic DNA was isolated from mycelia according to Specht *et al.* (Specht *et al.* 1982) and quantified spectrophotometrically (A260/A280). Approximately 10 μg DNA was digested with appropriate restriction enzymes (*Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI, *Bgl*II, *Cla*I and *Xho*I) and submitted to ordinary Southern blotting (Ausubel *et al.* 1998). Hybridization was carried out at 65 °C in a buffer containing 2 % SSPE, 1 % (w/v) SDS, 0.5 % Blotto, 10 % (w/v) dextran sulphate, and 0.5 mg ml^{-1} salmon sperm DNA. As a probe we used the complete MpNEP1 ORF, labelled by random primers in the presence of [^{32}P]γATP (Feinberg & Vogelstein 1983). The MpNEP1 ORF was amplified from genomic DNA using the primers MpNEP1-F and MpNEP1-R (Table 2). MpNEP1 probe was also used to hybridize with the chromosomes of *M. perniciosa*. Chromosomes were separated through pulsed-field gel electrophoresis (PFGE) with a DRII CHEF gel apparatus (BioRad), transferred to a membrane and hybridized as previously described (Rincones *et al.* 2006; Rincones *et al.* 2003).

Sequence analysis

Sequences in Table 1 were identified by BlastP and tBlastN (Altschul *et al.* 1990; McGinnis & Madden 2004) using MpNEP1 as a reference. Sequences were aligned by ClustalW using the default options (Higgins *et al.* 1996), except when the transition matrix PAM was used. Phylogenies were constructed using the NJ algorithm, using the transition matrix PAM (Dayhoff *et al.* 1972), and the branch support was verified using 1K BSs (Felsenstein 1985). Removal of the signal peptide, the most variable region of this protein class, did not affect the tree topology. The signal peptide region was predicted using the program Signal P3 (Bendtsen *et al.* 2004). All phylogenetic inferences were obtained using the program MEGA 3 (Kumar *et al.* 2004). Blast tools (tBLASTN and tBLASTX) were used to analyse the presence of homologous MpNEPs in other basidiomycetes, particularly those with completed genome sequences or sequencing near completion: *Cryptococcus neoformans* (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=5207), *Ustilago maydis* (http://www.broad.mit.edu/annotation/genome/ustilago_maydis/Home.html), *Phanerochaete chrysosporium* (http://genome.jgi-psf.org/whiterot1/whiterot1.info.html), *Coprinus cinereus* (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Info.html), and *Laccaria bicolor* (http://genome.jgi-psf.org/Lacbi1/Lacbi1.info.html). The codon usage of *M. perniciosa* genes was determined using a 'cusp' program from the European Molecular Biology Open Software Suite (EMBOSS) (http://bioweb.pasteur.fr/seqanal/interfaces/cusp.html). A 'syco' program (http://bioweb.pasteur.fr/docs/EMBOSS/syco.html) was used to compare codon usage of MpNEPs with *M. perniciosa* and *Phytophthora infestans* genes (http://www.oardc.ohio-state.edu/phytophthora/codon.htm).

Table 1 – Nep1-like proteins used in the phylogenetical analysis

Organism	Access number	Experimental evidence of necrotic activity[a]	e-Value[b]
Aspergillus nidulans (1)	gi|67525506	N	3e-31
A. nidulans (2)	gi|67525754	N	0
A. fumigatus (1)	gi|70985545	N	1e-30
A. fumigatus (2)	gi|70986079	N	0.002
Bacillus halodurans	gi|47118318	Y	3e-27
B. licheniformis	gi|56160984	N	1e-29
Moniliophthora perniciosa (1)	EF109894	Y	-
M. perniciosa (2)	EF109895	Y	-
Pythium aphanidermatum	gi|5834378	Y	5e-36
P. affinis	gi|37183405	N	5e-36
P. middletonii	gi|37183403	N	5e-35
P. monospermum	gi|37183401	N	5e-35
Phytophthora parasitica	gi|13346473	Y	5e-38
P. sojae (1)	gi|12698671	Y	5e-39
P. sojae (2)	gi|21327104	N	5e-39
P. sojae (3)	gi|21327103	N	5e-39
P. infestans	gi|89888600	Y	7e-37
P. megakarya (1)	gi|59939261	N	1e-36
P. megakarya (2)	gi|59939265	N	1e-35
P. megakarya (3)	gi|59939255	N	1e-32
P. megakarya (4)	gi|59939253	N	3e-29
Fusarium oxysporum	gi|2697131	Y	2e-25
Magnaporthe grisea (1)	gi|39947090	N	3e-26
M. grisea (2)	gi|39969844	N	4e-26
M. grisea (3)	gi|39968478	N	2e-11
Verticillium dahliae	gi|42742372	Y	3e-29
Gibberella zeae (1)	gi|46123278	N	8e-31
G. zeae (2)	gi|46115103	N	4e-14
G. zeae (3)	gi|46126818	N	2e-10
Streptomyces coelicolor	gi|24418961	Y	7e-16
Erwinia carotovora	gi|49609491	Y	6e-05
Vibrio pommeresis	gi|14331088	Y	0.02
Neurospora crassa	gi|85093778	N	2e-07

a Indicates existence (Y) or not (N) of experimental evidence of necrotic activity for the proteins.
b The e-values were obtained comparing every sequence with that of MpNEP1.

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Analysis of MpNEP1 and MpNEP2 expression

Total RNA was obtained from biotrophic and saprotrophic mycelia using Trizol (Ausubel *et al.* 1998). The total RNA from infected (45, 60 and 90 DAI) and control cacao tissues were extracted accordingly to the protocol recently developed (Gesteira Ada *et al.* 2003). Ten micrograms of each sample were reverse transcribed using a PCR-select cDNA subtraction kit according to manufacturer's instructions (Clontech). PCRs were set up using approximately 1 μg aliquots from each cDNA reaction, which were also diluted ten or 50-fold. cDNA dilutions were amplified with 1 unit Taq polymerase in the buffer provided by the manufacturer (Invitrogen). Primer sequences are described in Table 2 and were used at 15 pmol/reaction. As an internal control, primers MpAc-F and MpAc-R were used to amplify a 216 pb fragment of the *Moniliophthora perniciosa* actin cDNA. The presence of contaminating genomic DNA in the cDNA preparations was assessed using the size of the amplified actin fragment, as the actin primers (MpAc-F and MpAc-R) are placed at different exons. Therefore, amplification from genomic DNA generates a larger fragment (384 bp) in comparison with cDNA amplification (216 bp). As an internal control to monitor the amount of *Theobroma cacao* cDNA used for the amplification of *M. perniciosa* genes in infected tissues, we amplified a fragment of the 18S ribosomal gene using the primers TC18S-F and TC18S-R.

The relative *ex plant* expression of actin, MpNEP1 and MpNEP2 genes was estimated by semi-quantitative PCR. PCR aliquots were analysed every five cycles, starting from cycle ten and used as dot-blot samples. Membranes were hybridized overnight (43 °C) in a buffer containing 50 % formamide; 0.12 M Na_2HPO_4, pH 7.2; 0.25 M NaCl; 7 % (w/v) SDS; 1 mM EDTA; 10 % PEG 8000 and 0.2 mg salmon sperm DNA. The corresponding probes (MpNEP1 and MpNEP2 genes) were obtained by labelling with random primers as described above. The membranes were washed according to manufacturer's instructions. In all cases, amplification products were analysed by gel electrophoresis to confirm that they were single bands of the correct size.

Expression and purification of recombinant proteins

The MpNEP1 and MpNEP2 genes without signal peptides were amplified from genomic DNA with specific primers (Table 2). NPP1 was amplified by the primers NPP1-F and NPP1-R using *Phytophthora parasitica* genomic DNA as the template. After digestion of the fragments with enzymes that recognition sites introduced into the primer sequences, the PCR products were inserted into the corresponding sites of an expression vector. MpNEP1 was cloned into pET15b+ and MpNEP2 and NPP1 were cloned into pET28a (Novagen). The *Escherichia coli* strain BL21(DE3)pLysS was transformed with the recombinant plasmid constructions containing MpNEP1 or MpNEP2 gene and BL21(DE3)pTrx with pET28-NPP1. Protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 3 h at 28 °C, producing proteins with his-tags. Cells were harvested by centrifugation, resuspended in 0.01 volume of buffer 10 mM Tris–HCl, pH 8; 500 mM NaCl, 5 mM imidazole, 2 % Triton X-100, and a protease inhibitor cocktail (1 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin). After two freeze–thaw steps, 0.01 volumes of the same buffer without Triton X-100 were added and cells were sonicated. Afterwards, the solutions were treated with 2 % streptomycin sulphate for 30 min at 4 °C. Cell debris was removed by centrifugation at 38,000 *g* for 1 h at 4 °C. The supernatant was then loaded into a Hi-Trap chelating column (1 ml), charged with Ni^{2+} ions according to the manufacturer's protocol (Amersham) and equilibrated with binding buffer (10 mM Tris–HCl, pH 8; 500 mM NaCl, 5 mM imidazole), at a flow rate of 1 ml min^{-1}. After washing with the same buffer, a 30–200 mM imidazole gradient was performed until the elution of the protein. Fractions containing pure protein were pooled and dialysed against 10 mM Tris–HCl, pH 8; 100 mM NaCl buffer (TNB). The purity of the protein was confirmed with SDS PAGE.

Assay of necrosis activity

MpNEP1, MpNEP2 or NPP1 in TNB buffer were infiltrated into leaves of three-month-old plants of *Nicotiana tabacum* and four-month-old seedlings of *Theobroma cacao*. Protein and buffer solutions were injected near the central vein of tobacco leaves using 1 ml plastic syringes. The same procedure was used for cacao leaves without success. In view of these results, cacao leaves with freshly cut petioles were dipped into a 100 μl solution of each protein and sealed in flasks. The concentration of each protein used was 1 μM.

MpNEP1, MpNEP2 and NPP1 were also placed into a boiling water-bath (100 °C) for a half hour. At 2 min intervals after this step, similar amounts of each protein (1 μM) were inoculated into tobacco leaves using the syringe method described above.

Table 2 – Primers used for cloning and amplification

Gene name	Sequences (5′to 3′)	Restriction sites
MpNep1	F: GGAATTCCATATGGCTCCACATCAGCTTCC	*Nde*I
	R: CGGGATCCTTACTACCACATCCAAGCCC	*Bam*HI
MpNep2	F: CGTCTCAGGATCCATTGCCGGC	*Bam*HI
	R: CCAAGCTTTCACTACTACCACATCCAAGCC	*Hind*III
NPP1 (AF352031)	F: GGAATTCCATATGGACGTGATCTCGCACGATGC	*Nde*I
	R: CGGGATCCTTACTAAGCGTAGTAAGCGTTGCC	*Bam*HI
Mp-Actin (EF066485)	F: CCACAATGGAGGACGAAGTCG	
	R: CCCGACATAGGAGTCCTTCTG	
TC-18S	F: CAAGCGATCTTTTCGTAGGC	
	R: CGAAGATAAAATCCGAGCTTGT	

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As experimental controls we used the native protein (not boiled) and TNB buffer boiled and not boiled.

Ethylene measurement

Tobacco and cacao leaves of comparable sizes were sealed in flasks containing 100 µl (1 µM) of the purified protein solutions. Following the incubation period, 0.5 ml gas samples were withdrawn from the flasks with a syringe and analysed for ethylene using an analytical gas chromatograph (Shimadzu GC-14B) equipped with HayeSepT column and flame-ionization detector. Column temperature was set at 80 °C and hydrogen was used as the carrier gas at a flow rate of 20 ml min^{-1}. A standard curve (1 ppm) was prepared using pure ethylene and acetylene from White Martins. Results were analysed statistically by analysis of variance and significance between means by Tukey test (5 %) using Origin software.

Analytical gel electrophoresis

Two gel systems, SDS-PAGE and non-denaturing PAGE, were employed. Protein separation was accomplished by applying 0.2 µM of each denatured protein to a 12 % SDS-PAGE following the Laemmli method (Laemmli 1970). Studies on the aggregation state of the NPLs were developed by resolving native proteins in non-denaturing PAGE conditions using the Laemmli buffer system without SDS.

Dynamic light scattering

Dynamic light scattering (DLS) measurements were conducted on a temperature controlled DynaPro DLS instrument (Protein Solutions). Data were analysed using the DYNAMICS, version 6, software from Protein Solutions. Each measurement consisted of at least 500 independent readings, with each reading being 10 s in duration. A 100 l aliquot of each protein [MpNEP1 (23 µM); MpNEP2 (40 µM) and NPP1 (40µM)] in 10 mM Tris–HCl (pH 8) containing 180 mM KCl was centrifuged (10,000 *g*, 1 h, 4 °C), and a 60 µl aliquot of the supernatant was loaded into a quartz cuvette. All measurements were made at 25 °C.

Circular dichroism spectroscopy

Circular dichroism measurements were carried out on a JASCO J-810 spectropolarimeter, equipped with a Peltier-type temperature controller and a thermostated cell holder, interfaced with a thermostatic bath. The far-UV spectrum was recorded in a 0.1 cm path length quartz cell at a protein concentration of 6 µM, and near-UV spectrum was recorded in a 1 cm path length quartz cell at a protein concentration of 20 µM. The proteins were in 5 mM Tris–HCl buffer at pH 8 containing 50 mM NaCl. Five consecutive scans were accumulated and the average spectra stored. The data were corrected for the baseline contribution of the buffer. Thermal unfolding experiments were performed by increasing the temperature from 20 to 95 °C at 1 °C min^{-1}, allowing temperature equilibration for 5 min before recording each spectrum.

Results

Sequence analysis of the necrosis-inducing protein

In a preliminary experiment, we observed that the filtrate of the saprotrophic phase of *Moniliophthora perniciosa* cultures growing in liquid medium was able to induce a strong necrosis in tobacco leaves (data not shown). This result suggested that this fungus was able to secrete necrosis-inducing factors.

To date, the *M. perniciosa* genome initiative has generated approximately 75M bp of shotgun sequences (around 2.5× coverage) and around 11K ESTs. Analysis of the sequences at the beginning of the project revealed a 696 bp intronless open-reading frame (ORF) with significant similarity to genes encoding NEPs (Pemberton & Salmond 2004). The high similarity suggested that this gene belongs to this group of conserved proteins and therefore it was named MpNEP1.

In order to determine the number of copies of NEPs in the *M. perniciosa* genome, we carried out southern blot analyses. The *M. perniciosa* genomic DNA was digested with restriction enzymes that cleave inside (*Cla*I, *Bgl*II and *Xho*I) or outside (*Eco*RI, *Eco*RV, *Bam*HI and *Hind*III) the MpNEP1 ORF sequence. Hybridization was accomplished using the complete sequence of the MpNEP1 ORF as a probe, which was obtained by PCR using the oligos MpNEP1F and MpNEP1R. With the digestion of the genomic DNA with inside cutting enzymes, more than one band hybridized. Genomic DNA cut with *Bam*HI and *Eco*RV generated two and three strong bands, respectively, indicating that several copies of MpNEP could be present in the genome (Fig 1A). Conversely, hybridization of the digestions produced by the other non-cutting enzymes (*Eco*RI, and *Hind*III) generated only a single high molecular weight band. This suggests a close physical proximity of the copies in the genome. To test this possibility, the *M. perniciosa* chromosomes (Rincones *et al.* 2006; Rincones *et al.* 2003) were probed with MpNEP1, producing a positive signal on chromosomal band 6 (Fig 1B), indicating that all MpNEP genes are located on the same chromosome.

The progression of the genome sequencing reinforced this view and two further homologous sequences were found (see schema in Fig 1C). One of them, still incomplete (MpNEP3), is in tandem with MpNEP1. A third complete copy was identified in an independent cluster and named MpNEP2. This sequence was also found in a cDNA library derived from a compatible cacao–*M. perniciosa* interaction (GenBank accession EF114673). Comparison of the cDNA with its genomic counterpart showed that this gene does not have introns, similarly to MpNEP1.

MpNEPs sequence comparison

Initially, we selected all GenBank sequences presenting significant similarity to the conserved domain (CD) NPP1 (pfam 05630) and to MpNEP1 sequence. In Table 1 these sequences are shown together with information about the experimental evidence of necrosis induction. As detailed alignments of NLPs have been shown elsewhere (Bae *et al.* 2005; Fellbrich *et al.* 2002; Pemberton & Salmond 2004), we restricted our sequence alignment to the comparison between MpNEP1 and

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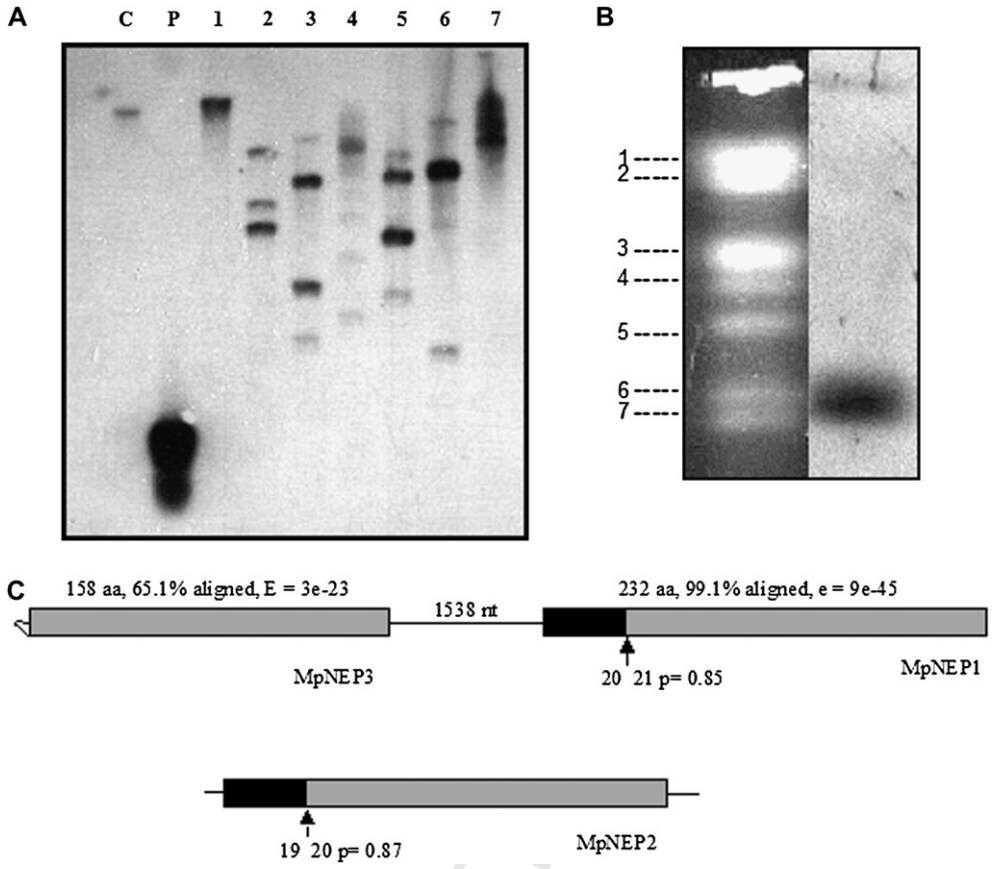

Fig 1 – Analysis of the MpNEP copies in the *Moniliophthora perniciosa* genome. (A) Total DNA was cut with the enzymes *Eco*RI (1), *Eco*RV (2), *Hin*dIII (3) or *Bam*HI (4), which do not cut inside the MpNEP1 ORF, and *Bgl*II (5), *Cla*I (6) or *Xho*I (7), which cut once inside the ORF. Molecular weight markers were defined with λ DNA cut with *Hin*dIII; C- Genomic DNA without any restriction analysis; P – 1 ng of the MpNEP1 DNA fragment used as a probe. (B) The *M. perniciosa* chromosomes were separated by pulse-field gel electrophoresis and hybridized with the same probe used in A; *M. perniciosa* chromosomal bands were numbered (1 to 7). (C) General scheme showing the three ORFs identified with significant similarity to the conserved domain (CD) NPP1 (pfam 05630), with indication of number of amino acids and alignment extension and e-value for the comparison of each ORF against the NPP1 CD. MpNEP3 was only partially cloned and is in tandem with MpNEP1. Black rectangles indicate the predicted position of the signal peptides. Arrows indicate predicted cleavage site (numbers correspond to amino acids positions in the predicted protein) according to SignalP3 (Bendtsen *et al.* 2004).

MpNEP2 (Fig 2A). This analysis showed that, excluding the peptide signal region (identified by the program SignalP 3; Bendtsen *et al.* 2004), there are 16 amino acid differences between these proteins, with one indel and four conservative substitutions (Fig 2A). Moreover, most of the differences were concentrated at the N-terminal region.

The NLPs are classified in two groups, type I and type II, depending on the presence of two or four cysteine residues at conserved positions, respectively (Gijzen & Nurnberger 2006). Both MpNEPs belongs to type I with cysteine residues at positions 53 and 79 in MpNEP1 and at positions 52 and 78 in MpNEP2 (Fig 2A).

The phylogenetic relationship between these sequences was analysed. Initially we tested the construction of rooted trees, employing *Bacillus* or *Streptomyces* as an outgroup (data not shown). In this case, the results were inconsistent. The tree was then rooted between type I and II NEPs, producing a more consistent topology (Fig 2B). As expected, the MpNEPs grouped together with other type I proteins.

Moreover, we analysed, by tBlastN, the possible occurrence of sequences encoding NEPs in other basidiomycetes species, five with completed (*Cryptococcus neoformans*) or nearly completed genome sequences (*Ustilago maydis*, *Phanerochaete chrysosporium*, *Coprinus cinereus* and *Laccaria bicolor*). No potential homologues were been found in this analysis.

Finally, we compared the GC content and codon usage of MpNEPs with other ORFs of known genes identified in the *M. perniciosa* genome (data not shown). As a result, we identified that MpNEPs retains the same codon usage as the other genes from *M. perniciosa*, but have a significant higher GC content (56 % *versus* 46 % on average for other ORFs).

MpNEPs have necrosis ability

MpNEPs and NPP1 were expressed in *Escherichia coli*, purified (the sequences were confirmed by mass spectrometry) and inoculated into tobacco leaves by hypodermic injection. Fig 3A (top left) shows that MpNEPs were able to induce necrosis in

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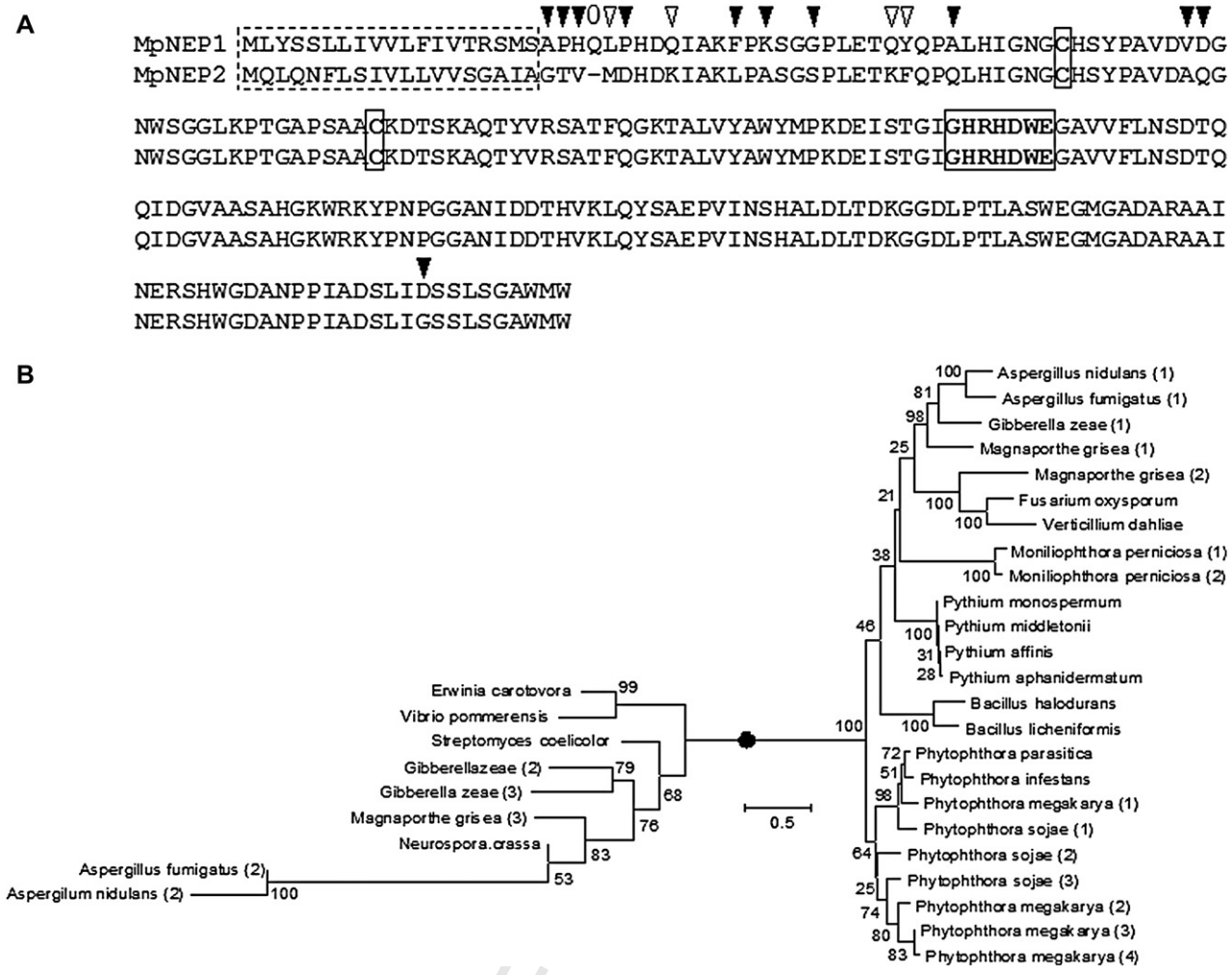

Fig 2 – Sequence comparison and phylogenetical analysis of NEP1-like proteins. (A) MpNEP1 and MpNEP2 primary sequence alignment. Dotted rectangle indicates the signal peptide. Black and white arrows show non-conservative and conservative substitutions, respectively. Conserved cysteines and the typical heptapeptide are indicated in boxes; (B) Minimum evolution (ME) tree of NEPs type I and II. The root (black dot) was placed between the two types. BS values are shown on the branches.

tobacco, similarly to NPP1, the effect of which has been previously described (Fellbrich *et al.* 2002). With the protein concentration used in the experiment, the symptoms could be clearly observed after 18 h, with maximum necrosis observed after 36 h. The three proteins showed a similar effect with no apparent differences in their necrosis efficiency. As negative control, the same volume of TNB buffer was infiltrated immediately below the point of the NEPs inoculation (indicated by white arrows).

A similar experiment was performed with cacao leaves of the sensitive variety 'Catongo'. In this case, the leaves were physically resistant to infiltration. To overcome this mechanical problem, the recombinant proteins were infiltrated into cacao leaves using a different methodology: cacao leaves with freshly cut petioles were dipped into a 100 µl protein solution (1 µM). All three proteins caused necrosis in cacao leaves, with a similar pattern (Fig 3A, middle): the symptoms were evident 15 d after the infiltration and began at the bottom of the leaves, propagating upward. In most cases, after 20 d the leaves were completed necrotic. The experiment was repeated several times, alway using three independent samples for each treatment and three negative controls (leaves incubated with buffer only). In the controls, no necrosis was observed during the time considered for the experiment.

The recombinant MpNEP1 protein was also infiltrated in the cacao meristems, which allowed inoculation by needles (Fig 3A, bottom). Necrosis symptoms started 4 h after infiltration, which were clearly visible after 24 h with complete necrosis after 48 h. As a control, TNB buffer was inoculated under the same conditions as the proteins and caused no necrosis.

Ethylene emission

To verify the MpNEPs able to induce the synthesis of ethylene, tobacco and cacao leaves were inoculated as described above for the cacao leaves and sealed in 14 ml flasks (Fig 3B). Evaluation of ethylene emission started immediately after sealing

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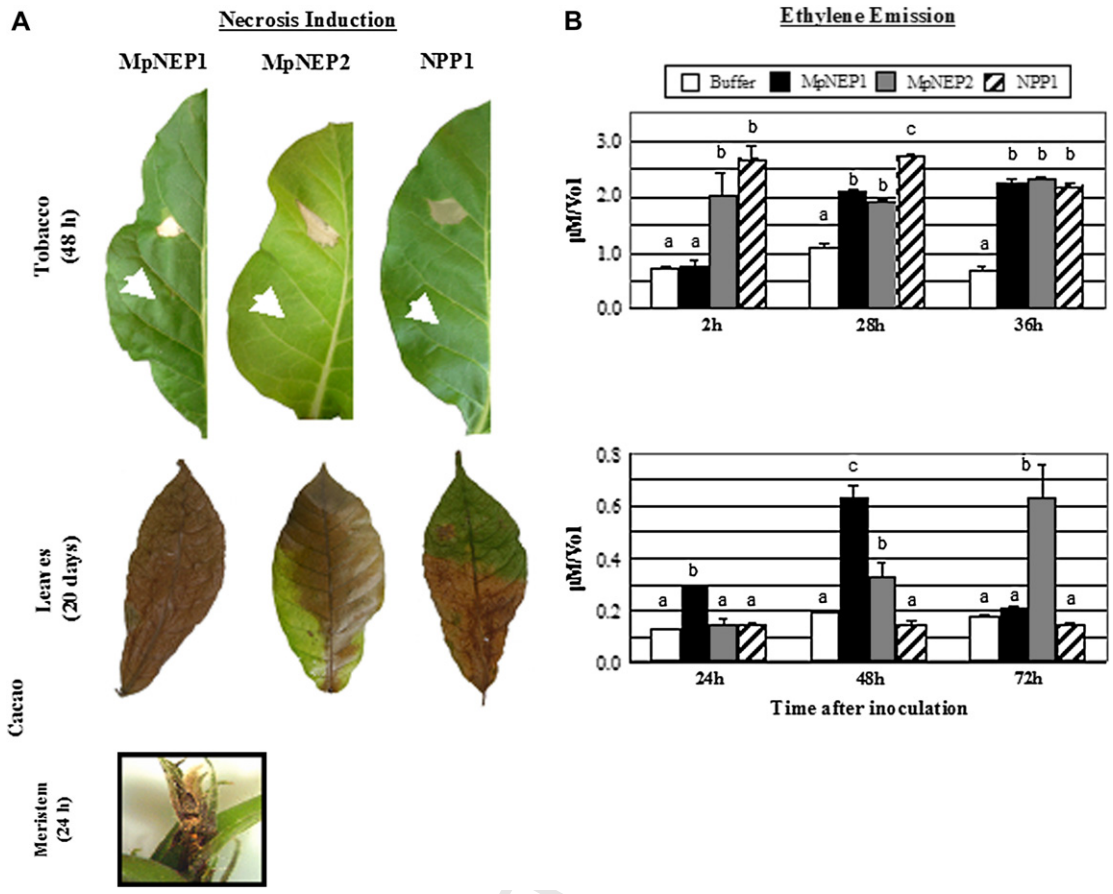

Fig 3 – Necrosis activity and ethylene emission induced by NEPs. (A) NEPs induced necrosis in tobacco and cacao leaves and cacao meristems. Top: Ninety day-old tobacco leaves were infiltrated with 20 µl TNB buffer containing 1 µM of the each recombinant NEPs protein or only with the TN buffer (in the area indicated by white arrow). The picture was taken 72 h after infiltration. Middle: Cacao leaves were inoculated with NEPs at the same concentration used for the tobacco experiment and evaluated 20 d after inoculation. Bottom: Induction of necrosis in *Theobroma cacao* meristems. One hundred and twenty day-old plants of a susceptible cacao variety 'Catongo' were infiltrated with 20 µL of a solution containing 1 µM of the recombinant MpNEP1 protein. After 48 h the meristems were completely necrotic. The experiments were repeated on at least ten independent meristems. (B) Ethylene emission induced by NEPs in tobacco and cacao leaves. Tobacco and cacao leaves with freshly cut petioles were dipped into 100 µl of the same concentration of protein solutions. Leaves were sealed in flasks. Mean of three repetitions ± standard error. a, b or c represents significant difference between treatments (* $P < 0.05$).

the flasks. In 2 h this gas could be detected in tobacco leaves treated with MpNEP2 and NPP1. After 28 h, similar emission levels could be detected for all three treatments, which remained stable for at least 36 h. With cacao leaves the ethylene emission was much less intense than with tobacco leaves (compare graphic scale in Fig 3B), and the emission profile was more specific for each protein. With MpNEP1, ethylene was detected after 24 h; the emission increased for at least 48 h and virtually disappeared at 72 h. The MpNEP2 inoculation revealed that ethylene was detected only after 48 h and increased at 72 h. Remarkably, NPP1 induced only very low amounts of ethylene from cacao leaves, which were not significantly different from the control experiment.

NLP form oligomers

We performed an electrophoretic analysis of MpNEP1, MpNEP2 and NPP1 under non-denaturing conditions. In this experiment, MpNEP2 produced only one band, whereas the other two proteins produced a scale with at least three bands (Fig 4A). The same experiment was performed under denaturing conditions, and all three proteins presented only one band, with the expected molecular weight for the monomer (Fig 4B). These results suggested that MpNEP1 and NPP1 exist as oligomers in solution, whereas MpNEP2 is predominantly a monomer.

To elucidate this oligomeric state, we conducted experiments with a more accurate technique, DLS. With this technique we obtained the Radius of gyration for the proteins and an estimation of their molecular weight in solution. The radius of gyration obtained for MpNEP2 was 2.4 nm, which is compatible with the molecular weight of the monomer; 26 kDa. In the case of MpNEP1 and NPP1 the radius of gyration was 3.7 nm, corresponding to a spherical protein with a molecular weight of 74 kDa (table at the bottom of Fig 4A). As both proteins have a monomeric molecular weight of about 26

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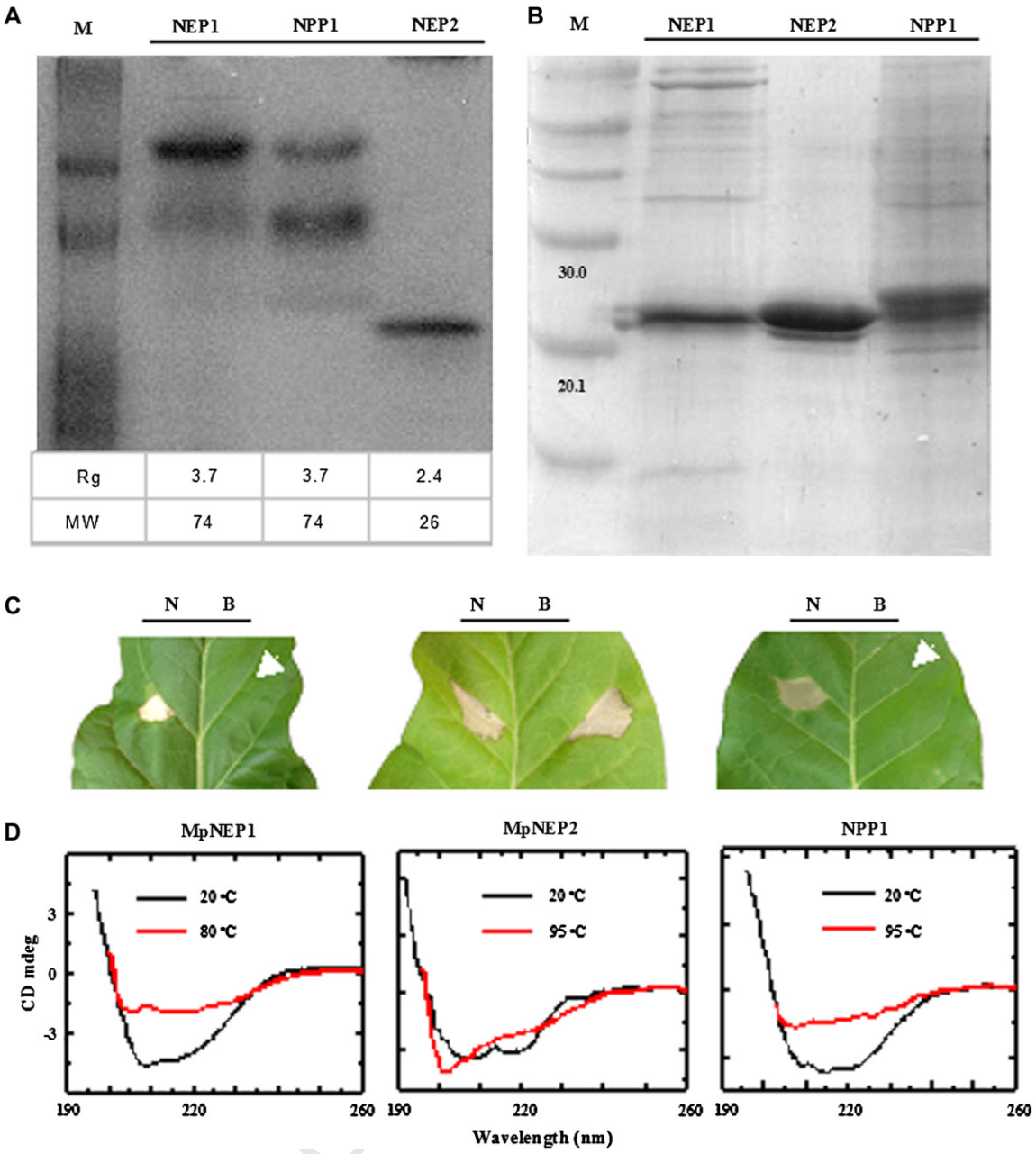

Fig 4 – Physical properties of NEPs. (A) Non-denaturing PAGE analyses suggesting formation of aggregate for NPP1 and MpNEP1, which are confirmed by the different radius of gyration estimated by DLS for each protein (table on the bottom). (B) SDS PAGE of NEPs. (C) Necrosis activity of NEPs in native state (N) and 2 min after boiling (aB). (D) Circular dichroism of NEPs at different temperatures.

kDa, these results suggest that they are present as oligomers. Preliminary experiments using small angle X-ray scattering, SAXS, showed that at low concentrations MpNEP1 is a dimer, whereas MpNEP2 remains as monomer (data not shown).

Effect of the temperature on the necrotic activity and structure

Previous studies showed that NLPs activity is heat labile, losing the necrosis-inducing ability after being exposed to 65 °C for 15 min (Fellbrich *et al.* 2002; Gijzen & Nurnberger 2006). To investigate whether NEPs from *Moniliophthora perniciosa* were also sensitive to temperature, thermal treatments were performed. The proteins were incubated at 100 °C for 30 min, left at room temperature for 2 min, and then inoculated into tobacco leaves. Unexpectedly, MpNEP2 retained its total activity, causing necrosis exactly as with the untreated protein (Fig 4C). The other two proteins precipitated, at around 40 °C, and were unable to cause necrosis after the treatment (Fig 4C).

In order to verify resistance of MpNEP2 to denaturation, we analysed the behaviour of the proteins by circular dichroism spectroscopy (Fig 4D). Indeed, both MpNEP1 and NPP1 lost the signal at higher temperature, whereas MpNEP2 kept the signal despite the fact that it was denatured by the temperature. Therefore, we concluded that this protein has the ability

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to rapidly renaturize when shifted from high to low temperatures.

Expression analysis

Though the proteins encoded by MpNEPs presented activity, this is not sufficient to show that the MpNEP genes are effectively expressed. To verify this, we performed gene expression analysis *ex planta* and *in planta* (Fig 5). For *ex planta* testing, we applied a semi-quantitative dot blot (Fig 5A). Total RNA from the biotrophic and saprotrophic phases of *Moniliophthora perniciosa* cultures was collected, quantified by spectrophotometry and agarose gel, and the same amount of RNA from each sample was treated with DNaseI and converted to cDNA, which was then quantified by spectrophotometry. To assure a more accurate quantification of the template for PCR reactions, a serial dilution of cDNA from each sample was used to amplify the *M. perniciosa* actin gene (MpActin). The samples from different PCR cycles were blotted onto a membrane and hybridized with the actin probe (Fig 5A, left). Concentrations of cDNA producing a similar MpActin expression profile in both samples were considered equivalent, assuming that this gene is similarly expressed in both biotrophic and saprotrophic mycelia. With this information, these cDNA samples were amplified using specific primers for MpNEP1 and MpNEP2 genes and aliquots from different amplification cycles were blotted and hybridized with probes from the corresponding genes. As a result, we observed that MpNEP1 is similarly expressed in both mycelia, while MpNEP2 is predominantly expressed in biotrophic mycelia.

As previously mentioned, MpNEP2 was also identified in a cDNA library produced from total RNA of infected cacao tissue (GenBank accession EF114673), demonstrating that this gene is expressed by the fungus during disease development. To verify that MpNEP1 is also expressed *in planta*, total RNA was collected from healthy and infected branches and PCR was performed using primes for MpNEP1 and MpActin. As an additional internal control, we used primers for a *Theobroma cacao* gene encoding a ribosomal protein (18S). Fig 5B shows that both MpActin and MpNEP1 were expressed in infected cacao tissues.

Discussion

In this study, for the first time, representative members of the NLPs were found in a basidiomycete. The genes are present in multiple copies, at least three, have no intron, seem to be located in a close proximity in the genome, and two of them, MpNEP1 and MpNEP3, are located in tandem (Fig 1). The genes with completed sequences, MpNEP1 and MpNEP2, were analysed in detail.

Initially, we verified that their sequences are highly similar (Fig 2A), with most of the differences concentrated in the N-terminal region, predominantly in the secretion signal. As this domain is removed in the mature peptides, these sequence discrepancies may play no role in their activities. Analysis of secondary structure also showed no significant differences, with the domains placed at the same relative position, which were very similar to the combinations of alpha-helix and beta-sheet found for other NLPs (Qutob *et al.* 2002), in particular NPP1 (data not shown).

Phylogenetic comparison with other sequences grouped MpNEP1 and MpNEP2 together, as expected, but put them along with sequences from very diverse organisms, such as oomycetes and bacteria (Fig 2B). No monophyletic fungal group has been formed. The only clear separation was between sequences that have two or four conserved cysteines, which were recently defined as group I and II, respectively (Gijzen & Nurnberger 2006). The MpNEPs belong to group I, along with all NEP sequences from oomycetes.

The odd phylogenetical distribution form NEP sequences have been indicated for several authors. One possible explanation for this would be the occurrence of horizontal gene transfer (HGT) (Gijzen & Nurnberger 2006; Pemberton & Salmond 2004). Indeed, the inspection of sequences from other basidiomycetes species, including some with completed genome sequence (*Cryptococcus neoformans*) or sequencing near completion (*Coprinus cinerea*, *Phanerochaete chrysosporium*,

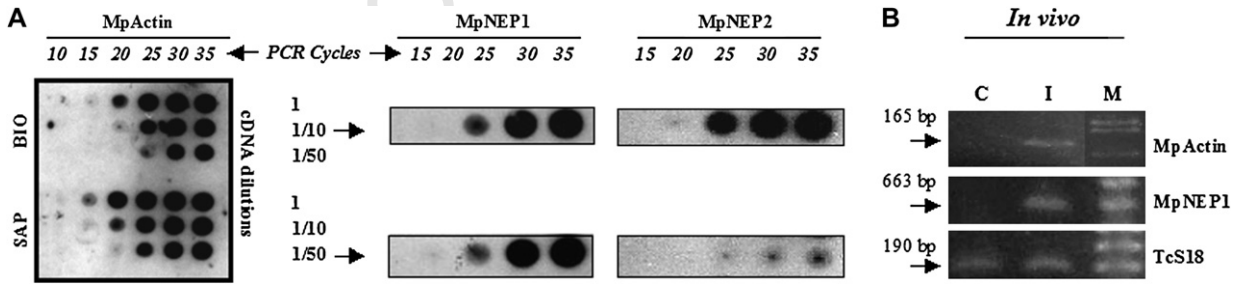

Fig 5 – Expression analysis. (A) Dot-blot experiment showing MpNEPs expression '*ex planta*' in different life phases of *Moniliophthora perniciosa*. Left: cDNA normalization by actin expression. The RNA was extracted from the biotrophic (Bio) and saprotrophic (Sap) mycelia and converted to cDNA. Approximately 1 g of cDNA along with two dilutions (ten and 50 fold) was employed as template for RT-PCR using actin primers. Aliquots of the reactions were taken after a specific number of PCR cycles, immobilized on a membrane, and hybridized against the actin probe. Amplification products with equivalent signals were employed for the evaluation of MpNEPs expression, as indicated. (B) RNA was extracted from healthy (C) and infected (I) cacao branches, converted to cDNA and amplified with *M. perniciosa* (*Mp*) specific primers for actin and MpNEP1. Amplification of *Theobroma cacao* (*Tc*) 18S gene as employed as internal control for the relative amount of cDNA applied for the reactions.

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Ustilago maydis and *Laccaria bicolor*), failed to detect NEP homologues, indicating discontinuity in the evolution of these sequences. Moreover, although the codon usage of the MpNEPs is similar to other *M. perniciosa* genes, the GC content was much higher (56 % *versus* 46 %), suggesting that these sequences were recently introduced in the species and were not completely adapted. On the contrary, *Phytophthora* spp. NEP genes have a GC content (56 %, the same as in *M. perniciosa* NEPs) and codon usage that are the same as most of the other genes, suggesting that these coding sequences are ancient in these species. Taken together, these data suggest that the presence of the MpNEPs could be the result of HGT.

The HGT possibility is indeed very attractive in the case of cacao pathogens. It is very common to see cacao fruits infected at the same time by *Phytophthora palmivora*, which causes pod rot (Flament *et al.* 2001), and *M. perniciosa*. Thus, this co-infection and the general tissue degradation opens the possibility that free DNA and intact cells could have come in contact, enabling natural transformation and possibly HGT.

An alternative explanation would be that NLPs are ancient sequences, which have duplicated and evolved into the two types (I and II) before the division of cells in eukaryotes and prokaryotes. As the function of the protein is achieved apparently by only a few essential conserved positions (Pemberton & Salmond 2004), which are possibly responsible for the maintenance of a defined architecture (Fellbrich *et al.* 2002), the rest of the sequence could undergo a rapid evolution. Conversely, mutation of only few positions would lead to the loss of the function (Fellbrich *et al.* 2002). Therefore, organisms that did not use these proteins such as protozoa, plants and animals, may have had rapid erosion of their sequences. Even organisms that benefit from the NLPs could have lost one of the types, with the function being assumed by the remaining type. In this case, the importance of the protein is strongly suggested by the expansion observed in most species, in particular *Phytophthora* spp., which show over 40 NEP copies in the genome (Tyler *et al.* 2006). The expansion observed in *M. perniciosa*, with at least three copies, also suggests the importance of this protein for this species.

The recurrence of duplications and the rapid evolution of the copies suggest that these individual sequences could have acquired additional properties. In order to verify this possibility, we analysed the characteristics of MpNEP1 and MpNEP2. Initially, we verified that both proteins are able to cause necrosis in tobacco and cacao plants, as well as the induction of ethylene in vegetative tissues (Fig 3). Although both proteins induce necrosis after a comparable time (18 h for tobacco and 15 d for cacao) when applied at similar concentrations, the induction of ethylene emissions showed distinct profiles. In tobacco, MpNEP2 induces ethylene very rapidly, comparable to the NPP1 effect. However, this situation was reversed in cacao leaves and here the ethylene induction by NPP1 was very low (sometimes detectable, but not in the experiment shown in Fig 3B). Furthermore, the necrosis observed in cacao was only evident several days after the cessation of the ethylene emission. It is possible that MpNEPs initially induce ethylene and that this hormone prepares the tissue for later necrosis. Indeed, ethylene has been associated with hypertrophy (Orchard *et al.* 1994), degradation of chlorophylls (Trebitsh *et al.* 1993), and epinasty of leaf petiole and stems (Woodrow *et al.* 1989), symptoms that are present during *M. perniciosa*–cacao interaction and correlate with an increase in the ethylene emission (Scarpari *et al.* 2005).

The differences in the profiles of ethylene induction by MpNEP1 and MpNEP2 suggested that the proteins, even with high sequence similarity, could have significant differences in their action. Compelling evidence of differences emerged with the native gel electrophoresis, the DLS experiments (Fig 4A and B) and evaluation by SAXS (data not shown). It was clear that MpNEP2 tends to be a monomer in solution, while MpNEP1 is present as an oligomer (dimer or trimer), similarly to NPP1. Furthermore, although both MpNEP1 and NPP1 precipitated during heating, which resulted in a loss of their necrosis-inducing ability, MpNEP2 remained in solution and recovered its necrosis ability even after a prolonged boiling period that led to a probable transient denaturation (Fig 4C and D). Heat resistance had been previously detected in Nep1 (Bailey 1995), but this is the first time that such a variation has been described for NLPs in the same organism, indicating that these proteins could have specificities and may result in a complemented cellular function.

In agreement with this hypothesis, we detected that MpNEP2 is primarily expressed in biotrophic mycelia (Fig 5A), which are present in low density in the initial stages of the witches' broom disease. At this phase, it is highly probable that the fungus is subject to oxidative stress by the plant (Low & Merida 1996; McDowell & Dangl 2000). If so, the expression of an isoform that works as a monomer and is more resistant to stress may allow the protein, at low concentration, to accomplish its mission. On the other hand, the saprophytic phase is accompanied by a significant increase in the mycelial density. As MpNEP1 is constitutively expressed, increase in the mycelia means increase in the protein concentration, a condition that seems to be more appropriate to the oligomer formation.

We have also identified MpNEP expression *in planta*. MpNEP2 has been isolated in a cDNA library produced from total RNA of infected tissues (data not shown) and MpNEP1 could be detected by RT-PCR in the same tissues (Fig 5B).

As mentioned previously, NLPs are currently regarded as elicitors, inducing cell death (Gijzen & Nurnberger 2006). However, although a common mechanism may be behind the NLPs, significant variability in the time for necrosis according to the leaf age and nutritional status has been observed (Bailey 1995). Indeed, it is highly interesting that the MpNEPs are expressed in biotrophic mycelia, which are present in cacao tissues for weeks without causing any apparent necrosis (Purdy & Schmidt 1996). This could indicate that the protein needs to accumulate or reach a threshold concentration to work in cacao tissues.

Preliminary results of immunolocalization show that MpNEPs are effectively present in infected tissue, in the apoplast. Moreover, they seem to accumulate externally in the cacao cell wall and are concentrated in dead cells, suggesting that the protein could play a role in this process (not shown). Another hypothesis for the mechanism of NLPs would be that they could degrade cell wall components and necrosis could be a consequence of the cellular response to this process. It has been demonstrated that the degradation of cell wall can

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release signalling molecules, like linear oligogalacturonides, which can elicit necrosis (Boudart *et al.* 2003; Ridley *et al.* 2001). We are currently testing this hypothesis.

In summary, we report here, for the first time, that NLPs are present in a basidiomycete, *Moniliophthora perniciosa*, and that these paralogous proteins can have diverse physical properties and be differentially expressed. It is probable that the MpNEPs play a role in the witches' broom disease, as the genes are expressed *in planta* and the proteins can be detected in the infected tissues. We are currently studying the structure of the MpNEPs, the necrosis mechanisms, and developing protocols to knock out these genes in *M. perniciosa*.

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