First molecular insights into the infection process provoked by *Neofusicoccum parvum* in *Liquidambar styraciflua* and the identification of new cysteine rich proteins in both organisms

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Abstract

Background

Neofusicoccum parvum belongs to *Botryosphaeriaceae* family that groups endophytic and latent pathogens of woody plants responsible of diseases such as cankers, dieback and blight. Is a widespread pathogen in a broad host range including agricultural, horticultural and forestry plants, therefore is relevant to characterize molecular mechanisms involved in the disease. This work, report for first time a *N. parvum* as a pathogen of *Liquidambar styraciflua*. We established an *in vitro* pathosystem using foliar tissue in order to characterize the infection process through scanning electron microscopy. Because cysteine rich proteins (CysRPs) are well described for their important functions under plant-pathogen interaction, new CysRPs were identified for these organisms, and mRNAs expression of these proteins was analyzed at early times during the interaction.

Results

Since the first 24 hours post infection, the pathogen caused visible symptoms and the microscopic analysis at 16 days post infection revealed the presence of *N. parvum* pycnidium immersed in *L. styraciflua* leaf tissue. For both organisms, two databases with transcriptomic and genomic information were analyzed and new five CysRPs were identified for each organism, the amino acid length varied between 95 and 204 and *in silico* analysis revealed that all proteins are potentially secreted. The search of conserved domains and phylogenetic analyses bring to light that all proteins are novel including two of *N. parvum* that present the well-known CFEM domain. RT-qPCR analysis was conducted at 24- and 72-hours post infection and the results showed change levels of CysRPs mRNAs for both the plant and the fungus at early times during the interaction.

Conclusions

It is recognized for first time that *N. parvum* is a pathogen of *L. styraciflua* and this work represents an approach to begin to deeply understand the molecular mechanisms involved in this interaction highlighting the potential involvement of CysRPs of both organism during this biotic stress.

Background

A current challenge in the area of plant-pathogen interaction is to deeply understand both the molecular mechanisms involved in the defense response of the plant when facing a specific pathogen, as well as the mechanisms that the causal agent uses to establish the disease. Thereby, allows to design better prevention and diagnostic strategies to improve the disease management.

Fungi are important causal agents of plant diseases and within these, the *Botryosphaeriaceae* family groups different species that are recognized as endophytic and latent pathogens of trees and other woody plants that are responsible to provoke cankers, dieback and blight even of the entire trees [1]. *N.*

parvum belongs to *Botryosphaeria* genus and has been recognized as a widespread pathogen around the world [2] provoking damage on a wide host range including agricultural, horticultural and forestry plants. Within the hosts of economical relevance are the grapevine (*Vitis vinifera*) [3], the avocado (*Persea americana* Mill.) [4–6], the blueberry (*Vaccinium* spp.) [7] the pomegranate (*Punica granatum*) [8], the peach (*Prunus persica*) [9] and the walnut (*Juglans regia*) [10] among others. The forestry host plants include the coast redwood (*Sequoia sempervirens*) [11] the Norfolk Island pine (*Araucaria heterophylla*) [12], *Eucalyptus globulus* [13, 14], and *Ginkgo biloba* [15]. It is worth mentioning that the bulk of studies have focused basically on the isolation of the fungus from symptomatic or asymptomatic tissue, followed by the molecular identification and pathogenicity testing through Koch's postulates, however, molecular information has only been generated during grapevine and *N. parvum* interaction [16–19].

The aggressive nature of *N. parvum* as a phytopathogen suggest the capacity of the fungus to secrete cell wall degrading enzymes and phytotoxins. The draft genome of the UCR-NP2 *N. parvum* isolate, revealed the presence of glycoside hydrolases, polysaccharide lyases, cutinases, lignin peroxidases and cytochrome P450 monooxygenases that might be involved in lignin degradation [20]. Regarding chemical compounds, it's already known that *N. parvum* is able to produce a variety of phytotoxic compounds that belong to chemical families of dihydrotoluquinone, epoxylactone, dihydroisocoumarin and hydroxybenzoic acid, such as (-)-terremutin, (R)-mellein and 6-methyl-salicylic acid [21]. Recently, has proved that exogenous application of (R)-mellein and (-)-terremutin in *Vitis vinifera* resulted in up-regulation of the SA-responsive genes PR1 and GST1. In contrast, the expression of JA/ET responsive genes remained weak or even down-regulated by (-) terremutin [22].

Until now there is no effective control strategies against *N. parvum* and is inferred that its transmitted horizontally [23] leading to a higher risk in a larger number of hosts, therefore it is relevant to characterize molecular mechanisms involved in the disease establishment in other important hosts, that is the case of *Liquidambar styraciflua*, a deciduous timber tree often used for reforestation, agroforestry, and landscaping [24]. *Liquidambar styraciflua* L. (Atingiaceae) is native from Americas and currently is distributed in regions localized in North and Central America [25] also has been widely introduced in eastern and central China [26]. Is also called American sweetgum and is an attractive hardwood species for potential bioenergy production [27]. This work report for the first time that *N. parvum* is a pathogen of *L. styraciflua* and through molecular approaches present knowledge of this interaction. In this sense, the objectives of this work were 1) describe for first time that *N. parvum* is a pathogen of *L. styraciflua*, 2) to establish an *in vitro* and reproducible pathosystem, 3) characterize the infection process by scanning electron microscopy (SEM) and 4) identify cysteine rich proteins (CysRPs) of both organisms that may be important during the defense response and infection process. Recent evidence indicates that pathogens effector proteins are small secrete cysteine rich proteins with a N-terminal signal peptide sequences, contain ≤ 300 amino acids and $\geq 2\%$ of cysteine content [28, 29]. Some current examples are SCR96 of *Phytophthora cactorum* [30] and SsSSVP1 in *Sclerotinia sclerotiorum* [31] where their silencing reduce the pathogen virulence respectively. However, the evidence also indicates that effector proteins are highly specific and inherent of each pathogen, and no information has been generated about the CysRPs in any specie of the *Botriosphariaceae* family. Besides, CysRPs in plants are important components of the plant

innate immune system, some examples include the α -defensins, lipid transfer proteins (LTPs), thionins, hevein- and knottintype peptides and cyclopeptide alkaloids [32].

In this work, a novel pathosystem is achieved and a first approach is made in the knowledge of CysRP in both actors of the interaction.

Results

Identification of *N. parvum* as a pathogen of *L. styraciflua*

Eleven fungi were isolated from *L. styraciflua* leaves with disease visible symptoms such as necrosis and discoloration. Its potential pathogenicity was tested in *L. styraciflua* leaves and in seedlings of the plant model *Arabidopsis thaliana* (Figs. 1 and S1). The pathogenicity screening identified the fungal Liqui 1–3 strain as the most pathogenic, since in *A. thaliana* seedlings the fungus covers all the plant tissues provoking a notorious leaves discoloration at 7 dpi, *L. styraciflua* leaves developed clear necrosis and discoloration including the principal veins and the petiole at 8 dpi. Liqui 1–02, Liqui 2–2 and Liqui 2–3 triggered clear disease symptoms in *L. styraciflua*, but in contrast with the effect produced by Liqui 1–3, no noticeable change was observed in petiole coloration. In *A. thaliana* seedlings, these three isolates did not have a pathogenic critical effect, for example, while Liqui 2–2 covered more than 50% of the seedlings, the foliar tissue showed a greater vigor compared to the control. Liqui 1-2-01, Liqui 1-2-03 and Liqui 3 – 2 at 7 dpi showed a discrete pathogenic effect. *A. thaliana* during the interaction with these isolates, developed a primary root with shorter length but increased number of secondary roots. The isolates Liqui 1–04, Liqui 1–01, Liqui 3–3 and Liqui 3 – 1 neither in *L. styraciflua* leaves nor in *A. thaliana* seedlings caused an effect.

To identified at molecular level the Liqui 1–3 strain, universal and specific primers were used (Table S1, Fig. S2). The analysis revealed that Liqui 1–3 belongs to the *Botryosphaeriaceae* family as a *Neofusicoccum parvum* species.

Establishment of the *L. styraciflua* – *N. parvum* pathosystem

Because we were interested in study the interaction between *L. styraciflua* and *N. parvum* we established the pathosystem using *L. styraciflua* leaves. We noticed that the infection progressed rapidly since at 1 and 3 dpi the inoculated leaves displayed brownish discoloration that was accentuated through time (Fig. 2), was notorious the petiole necrosis at 8 and 16 dpi, and the presence of whitish mycelium in the necrosis leaf area also was evident. To characterize the infection process in more detail, we analyzed the infected an uninfected tissue using SEM (Fig. 3). SEM images revealed that the fungus growth deeply in the leaf (adaxial) surface forming hyphae mass and leading to tissue degradation, the cuticle and the wax integrity was compromised (Fig. 3a-d). Besides, the infection provoked petiole degradation. The

transverse cut of the leaf base showed that the fungus was able to develop pycnidia, an asexual reproductive structure. The pycnidia were appear individually or as aggregates immersed in the plant tissue with thick walls that are composed of numerous cells (Fig. 3i-l). The longitudinal section of pycnidium show mature conidia. The conidiogenic cells without septa and with oval shape are localized perpendicular to the walls of the pycnidium. Because the *Botryosphaeriaceae* family members are characterized to be woody pathogens we tested the pathogenicity of Liqui 1–3 strain in fresh stems of *L. styraciflua*. As is shown in Fig S3, *N. parvum* at 7 dpi triggered notorious symptoms of disease such as discoloration and necrosis that covered a zone beyond the site of inoculation.

Detection of hydrogen peroxide in *L. styraciflua* leaves at early times of the infection process

Its already known that reactive oxygen species (ROS) accumulate in plants cells during pathogen infection and may cause oxidative damage to proteins, DNA, and lipids or also

act as signaling molecules to regulate defense response [33, 34]. One of this species is the hydrogen peroxide and by DAB staining we detected clearly the presence of a dark brown precipitate in the infected leaves at early times (1 and 3 dpi) thus showing the detection of H_2O_2 (Fig S4).

Identification of CysRPs in *L. styraciflua* and *N. parvum* and their general features

Cysteine rich proteins (CysRPs) are well described for their important functions under plant-pathogen interaction, and with the purpose of identify CysRPs in *L. styraciflua* and *N. parvum*, two databases with transcriptomic and genomic information respectively were analyzed (see Materials and Methods). For each organism, five sequences encoding CysRPs were identified (LsCysRP1-5 and NpCysRP1-5) in all of them were recognized a putative start and stop codon with the exception of LsCysRP3 sequence where a stop codon was not localized (Table 1 and Table S2). The amino acid sequence length varies between 95 and 204, the molecular weight range among 7.7 and 17.5 KDa, also the cysteine content was determined. Curiously, with the exception of LsCysRP1 that had a calculated isoelectric point (pI) of 6.04, all the calculated LsCysRPs pIs were upper than 8.67, meanwhile to the exception of NpCysRP1 that had a pI of 7.57, all the calculated NpCysRPs pIs were lower to 5.48 (Table 1). To predict SS-bonds in *L. styraciflua* and *N. parvum* CysRPs, the servers Cyscon, Disulfind, DiANNA, CYS_REC and SCRATCH were used. The results revealed that LsCysRP1, LsCysRP2, LsCysRP3, LsCysRP4, LsCysRP5 have the potential to form 3, 4, 4, 0, and 6 disulfide bonds respectively. Meanwhile in *N. parvum* 6, 4, 4, 5 and 4 disulfide bridges were estimated for NpCysRP1, NpCysRP2, NpCysRP3, NpCysRP4 NpCysRP5 respectively. A notorious discrepancy about the selection of which cysteines form the pairs is detected between the methods (Table S3).

Table 1. CysRPs amino acid sequences.

Assigned name	Target Name	Protein	Length	Total cysteines	Molecular weight (kDa)	pI	Predicted putative function
LsCysRP1	comp30850_c0_seq1	MDRLSSMRSVFWILVSFGTNAAVSELINVAQPSAEVDHQMNCRAIDDAWNV WHNDLRFAGLKEIQRPQNEVCCNLVTSCVFHWDLVSHRKSRLR	100	7	8.9	6.04	No significant similarity found
LsCysRP2	comp28855_c0_seq1	MGMALFQPVSGLQQQITAAATSKFDKGTSGVGAQCTKPCQHQMKKLCCSSY GYCNRRFRKELSYHLLVAKANHWLSCEVIFAIPVSSADLGLWLEMVKP	103	9	9.5	8.84	No significant similarity found
LsCysRP3	comp18453_c1_seq1	MASSGHLNLAQVVLVSNVLVAPHAEAAMTGGQNTSSVSPCLPYLKGASPLQPGCC NGHSLNSAAKTTHDRQAACRCLQQAASSISGNLGLASGLPGKCGSI	104	8	7.7	9.03	Lipid-transfer protein type 1
LsCysRP4	comp37785_c0_seq1	MGFTGMSPMSFLCAWCCGQPTHHLEALVSWTAAQLQLKLVKVAEDTDFFTNSFD PVEVWII SLVKYL VHKTLRKTNI GKEAALKRAKPAADIAQVVENLLIDLQS	104	4	8.9	8.8	No significant similarity found
LsCysRP5	comp31840_c0_seq3	MAIYKALLASLLVSLFVLHLVVADGMGMQITDAATSSPKSSPPKKIDQGGACSS RQQLSSRFNLCKRAGGTCCARCNQVPPGTSNEDVQPCYASMTTHGGRHKCP	107	12	8.6	8.67	Gibberellin-regulated protein 1-like
NpCysRP1	EOD50423.1	MQFKFALVSLFTALALAAPGHLEKRCANGGSCQLSQCCSGNCEYDSISGLVCK AASKKEKRCANGGACTQLAQCCSGNCEYDSISGLVCKP	95	12	7.8	7.57	hypothetical protein UCRNP_2803
NpCysRP2	EOD50922.1	MRPLYSHLVHTLPPLFSILLDPASAQDDHVPQCYYPDGTLASNDYACRLNTTES FQCTTNTVQLDNKIQCVLAPQTQVYENRGITDKTWTSDCKPKFCQQAQSPSYSGSV IRPVY	116	9	10	4.79	hypothetical protein UCRNP_2308
NpCysRP3	EOD52838.1	MRFFVAISVAATALFSTGLAASHAKCAQCIASDRGTNDAATAGMCSYVGGVMSK SNVAFEKQVATWLTSLADNTIITGVLYGKYVQGHGHELGDDAVYEACRSQK GGDTVQDSTCV	121	8	10.5	5.48	hypothetical protein UCRNP_338
NpCysRP4	EOD51105	MRSFAFAAILGYSSLALAQTLPTCAQKCVGTDRFGCSTVDKCCICANKELLTG LAQCVSTGQNAADQETVDFNAQNLCKPQGVTDLPITATGASGASSATSSASSGTAS TTASSAASASASATGSAASSASSAASSVASSAASASSAASASAATAGSGAGSC QQNAAGMGFGLVLAGLLGAL	187	11	15.5	4.53	putative CFEM domain protein
NpCysRP5	EOD44996.1	MKT SFAAVAFSLASVAYSQNSIDLPSCSLNCFVTTLGSDGCSLTDFECHCKVPGL TDEITPCVQKACSADQQTVAEQVQALCAQYGVVISVPEASSTAAPSSTAAT SAP ASSSAAATTAASSAASSAASSASASATSTETASPSATDAVISTPATPTT PGAST PA GSTPTSPQFQGAAGKTGVVGVGVGFAAAVAVAL	204	8	17.6	4.02	putative CFEM domain protein

¹ The cysteines in all the sequences are bolded

In order to know if the CysRPs have the potential to be secreted, an analysis with the TargetP-2.0 server was conducted. All sequences with the exception of LysCysRP2 have a peptide signal between 17 to 27 amino acids length. For corroborate these results, additionally analyses were performed using Protter and DeepLoc-1.0 servers, the results indicated that all CysRPs have a signal peptide and are extracellular proteins (Table 2).

For identify possible functions and similarity regions in CysRPs, BLAST and MOTIF tools were used. No significant similarity was found for LsCysRP1, 2 and 4, neither for NpCysRP1, 2 and 3. In contrast, LsCysRP3 showed similarity with a lipid transfer protein and LsCysRP5 with gibberellin-regulated protein 1-like. Interestingly, for both NpCysRP4 and 5 were identified a CFEM domain (Table 1, Fig. 6S).

Table 2. CysRPs predicted localization.

Assigned name	TargetP-2.0 Server Prediction	Cleavage site between pos	Likelihood	Protter	Cleavage site between pos	DeepLoc-1.0 prediction	Likelihood	Soluble / Membrane
LsCysP1	Signal peptide	24-25	0.9931	Signal peptide	21-22	Extracellular	0.8321	0.9813 / 0.0187
LsCysP2	Other	□□□□	0.7782	Signal peptide	18-19	Extracellular	0.8088	0.9946 / 0.0054
LsCysP3	Signal peptide	26-27	1	Signal peptide	26-27	Extracellular	0.9998	1 / 0
LsCysP4	Signal peptide	26-27	0.7135	Signal peptide	26-27	Extracellular	0.7316	0.9415 / 0.0585
LsCysP5	Signal peptide	23-24	1	Signal peptide	23-24	Extracellular	0.9973	1 / 0
NpCysP1	Signal peptide	17-18	1	Signal peptide	20-21	Extracellular	1	1 / 0
NpCysP2	Signal peptide	27-28	0.9962	Signal peptide	27-28	Extracellular	0.8773	0.9997 / 0.0003
NpCysP3	Signal peptide	20-21	1	Signal peptide	20-21	Extracellular	0.999	0.998 / 0.002
NpCysP4	Signal peptide	18-19	0.999	Signal peptide	18-19	cell membrane / extracellular / Endoplasmic reticulum	0.2925 / 0.2815 / 0.2539	0.3246 / 0.6754
NpCysP5	Signal peptide	18-19	0.9986	Signal peptide	18-19	Extracellular / Endoplasmic reticulum / Cell membrane	0.3539 / 0.2639 / 0.2186	0.4355 / 0.5645

Cysrps Phylogenetic Analyses

To obtain more information about NpCysRPs, the corresponding phylogenetic analysis for each one was conducted (Figs. 4 and 5). Clearly, all the NpCysRPs were group in the *Botryosphaeria* lineage including *Lasiodiplodia*, *Diplodia* and *Macrophomina* species, however the species in the subclade not always were the same, NpCysRP1, 2 and 5 were better related to *Lasiodiplodia theobromae* while NpCysRP3 and 4 shared the branch with *Macrophomina phaseolina*.

NpCysRP1 phylogeny revealed the existence of few orthologs sequences for this protein in databases and occurred in the *Botryosphaeriaceae* family only in the species *L. theobromae* (85.00% identity), *D. corticola* (85.42% identity) and *D. seriata* (82.29%, identity), as well as in family *Cordycipitaceae*, order Hypocreales, characterized by containing entomopathogenic fungi species as *Beauveria bassiana* and *Cordyceps confragosa* but with low identities (29.67% and 31.36% respectively) however the cysteines remain at the same site into the sequences (Fig. S5). Other species were included in the alignment as *Aspergillus leporis* and *Rhizoctonia solani*, however, they have a smaller cysteines number and lower identities.

In addition with the *Botryosphaeria* lineage, NpCysRP2 phylogenetic tree contain orthologs of family *Hypocreaceae*, order *Hypocreales* including seven different species of *Trichoderma* genus with identity that ranged from 33.04 to 38.18%. Also, members of family *Glomerellaceae* were included such as species of the well-known *Colletotrichum* phytopathogenic genus such as the species *asianum*, *nymphaeae* and *orchidophilum* with 33.04, 36.28 and 37.19% amino acid identity respectively. Interesting, in the tree is also showed species of the order *Sordariales* as the human pathogen *Madurella mycetomatis* with 33.90% identity. After performed the alignment is revealed that NpCysRP2 introduces a new cysteine rich domain with the follow consensus motif $C^1[Y/F]xPx_9-10C^2x_{6-8}C^3C^4x_4C^5x_2Nx_2C^6x_{10-23}C^7Tx_8C^9x_3C^{10}$ at the N-terminal. Also, the multiple alignment of NpCysRP2 with the ortholog sequences of *L. theobromae* (67.52% identity) and *D. corticola* (62.07% identity) showed that NpCysRP2 is a larger protein since the *L. theobromae* and *D. corticola* sequences contain 310 and 296 amino acid respectively (Fig S5X). Interesting, the proteoforms of *L. theobromae* and *D. corticola* presented a transmembrane domain crossing at carboxyl-end. Moreover, the predicted proteoforms for all the sequences used in the phylogenetic analysis (26 in total) displayed the transmembrane helix (Fig. S6).

A particular phylogeny was noted in the case of NpCysRP3 (Fig. 4C), this protein not present a well-defined clade distribution within the *Botryosphaeriaceae* family and only *Macrophomina phaseolina* orthologs (MpCysRP A, B and C) were found with 64.46, 29.17 and 30.83% identity. Interesting, *Nectriaceae* family was represented in the phylogenic tree with some members of the unique and fascinating Ambrosia fusarium clade represented by *Fusarium euwallaceae*, and *Fusarium kuroshium* that has been recognized recently as an emerging fungal pathogen [35, 36].

NpCysRP5 has a CFEM domain characterized by containing eight cysteines with the particular consensus motif $PxC^1[A/G]x_2C^2x_{8-12}C^3x_{1-3}[x/T]Dx_{2-5}C^4xC^5x_{9-14}C^6x_{3-4}C^7x_{15-16}C^8$, [37]. Interestingly, NpCysRP4

and orthologs have a CFEM-like domain with conserved extra cysteines pair (C58 and C94 in *N. parvum* sequence) forming the consensus motif $PxC^1[A/G]x_2C^2x_{8-12}C^3x_{1-3}[x/T]Dx_{2-5}C^4xC^5x_{8-13}C^6C^7x_{3-4}C^8x_{15-16}C^9x_{12-13}C^{10}$. NpCysRP4 and NpCysRP5 are well represented in the clade of the *Botryosphaeriaceae* family, but NpCysRP4 share the subclade with *M. phaseolina* with a 78.07% identity while NpCysRP5 showed an 83.01% identity with *L. theobromae*. *Cenococcum geophilum* and *Glonium stellatum* belong to *Gloniaceae* family and present orthologs for both NpCysRP4 and NpCysRP5. Interesting, for NpCysRP4 were identified various orthologs inside *Nectriaceae* family, order *Hypocreales* represented by the well-known phytopathogenic *Fusarium* species as *verticillioides* and *oxysporum* that were not found in NpCysRP5 phylogenetic tree using 33 sequences. In the case of NpCysRP5 a clear clade was identified represented by order *Eurotiales* where different *Penicillium* species were group with identities that ranged from 34.3 to 41.78%.

In the case of *L. styraciflua* CysRPs, no similar sequences were found for LsCysRP1, 2 and 4 therefore it was no possible to construct the corresponding phylogenetic trees. For LsCysRP3 the BLAST® result reveled a sequence identity of 66.98–64.15% with various *Gossypium* species and 63.81% with *Vitis pseudoreticulata*. Finally, LsCysRP5 showed 73.83–65.42% identity with different *Quercus* species, 74.77% *Castanea mollissima* and 67.29% with *Durio zibethinus*.

Expression of CysRPs mRNAs of *L. styraciflua* and *N. parvum* during early times of the infection process.

To explore if transcription of CysRPs mRNAs of *L. styraciflua* and *N. parvum* was modulated at early times of the interaction, quantitative PCR tests were performed. In Fig. 6A is showed the LsPR1 expression levels, an orthologous of *Nicotiana tabacum* gene encoding the pathogenesis-related protein 1 (PR1), a protein involved in defense response in plants and usually used as a defense marker, qPCR results showed an increase of LsPR1 mRNA at 1 and 3 dpi. An opposite profile was observed in all LsCysRPs transcripts, because the expression decreased after 1 dpi, LsCysRP2 presented the lowest level at this time post infection. At 3 dpi, LsCysRP2 presented the most significant changed and LsCysRP3 remained unchanged, while LsCysRP1, 4 and 5 presented a mild increase.

Finally, our experimental design allowed us to compare the expression of NpCysRPs transcripts between 1 and 3 dpi. Figure 6B showed that there was an increment in the expression of NpCysRP1, 2 and 5 mRNAs being the last one which showed the more significantly increased. In contrast, NpCysRP4 showed a significant decrement.

Discussion

Liquidambar styraciflua L. (Atingiaceae) is used for reforestation and landscaping issues and has been proposed as an attractive hardwood species for potential bioenergy production [27]. Because of its importance, its relevant to attend the causes that limit integrity i.e. the phytosanitary problems. In general,

is assumed that *L. styraciflua* has associated scarce pathogens [27, 38] In this sense, information collected by Hepting in 1971 [39] enlisted a *Cercospora liquidambaris*, *Septoria liquidambaris*, *Exosporium liquidambaris*, *Leptothyriella liquidambaris* and *Gloesporium nervisequum* as common foliar pathogens of the sweetgum. In the other hand, *L. theobromae* and *Botryosphaeria dothidea* have been identified responsible to provoke stem cankers and dieback in seedlings ubicated in nurseries and outplantings in the USA [38, 39]. Currently, there are no new information about the nature of the pathogenic fungi infecting *L. styraciflua*, but prevalence the incidence of symptoms associated with diseases. Here, we reported that *N. parvum* is associated with *L. styraciflua* foliar damage and the pathosystem established in this work identified the Liqui 1–3 as a very aggressive strain since provoke several symptoms at early times post infection (Figs. 1 and 2). SEM analysis showed that the fungus was able to growth and develop pycnidium in order to produce a large amount of mature spores increasing its infective potential (Fig. 3). Our results are congruent with the reported for other *Botryosphaeria* species. Amponsah and colleagues [40] reported during the interaction of grapevine and *N. luteum* a faster rate (3 h after inoculation) of conidial germination on detached and wounded leaf and shoot surfaces than on attached and non-wounded leaf surfaces, indicating that conidium adhesion, germination and development were affected by the condition of the host plants. Also, in mamey sapote stem cuttings infected with *L. theobromae* at 30 dpi was possible to identified fruiting bodies immersed in the tissue [41]. It will be interesting in future works to characterize the Liqui 1–3 strain in more detail about its capacity to produce lytic enzymes and secondary metabolites with phytotoxic effect in comparison with other strains isolated from other ecological niches. Finally, it should not take aside the identification of other fungi isolated from *L. styraciflua* that also have pathogenic potential such as Liqui 1–02 and Liqui 2–2 (Fig. 1 and Fig S1).

In addition to the phenotypic effects associated with pathogenicity caused by *N. parvum* in *L. styraciflua*, also the identification of cysteine rich proteins was considered as a part of the molecular events triggered by both organisms during infection and defense response. Five CysRPs were identified for each organism (Table 1) and bioinformatic analyses revealed that LsCysRP1, 2 and 4 are proteins with unknown function suggesting that are species-specific proteins. LsCysRP3 was recognized as lipid transfer protein (LTP), this protein is consider an antifungal protein classified as PR-14 [42] with not yet known mode of action, but *in vitro* conditions has the ability to enhance the intermembranal exchange causing a posteriori the fungal cell death [32], in barley leaves inhibit the growth of *F. solani* [43], while transgenic *Populus tomentosa* over-expressing a LTP of *Leonurus japonicus* was resistant to *A. alternate* and *C. gloesporioides* [44]. LsCysRP5 was identified as gibberellin-regulated protein 1-like, named GASA family in *A. thaliana* and Snakin in *Solanum tuberosum*. This class of proteins form 5 or 6 disulfide bonds that are necessary for the structure, in consequence for the protein function [45], moreover, the overexpression of Snakin protein 1 in potato enhances resistance to relevant pathogens [46]. Additionally, Snakin-2 of French bean may be formed a 42 kDa protein complex with a proline-rich protein and participate in the plant defense process [47]. Thus, in comparison with the reported about the orthologs of LsCysRP3 and 5, these *L. styraciflua* proteins may have antifungal activity. The expression pattern of their respective mRNA of LsCysRP3 and 5 during the interaction with *N. parvum* it is particularly interesting because of

the repressive behavior profile of both mRNAs (Fig. 6). It is well known that many pathogens have evolved to generate tools with the aim of evading the host's immune system, so it will be interesting in future studies to investigate more about this effect.

Through genomic and transcriptomic analyses, has been already recognized that the pathogenic and virulence protein arsenal of *N. parvum* potentially encompass enzymes that facilitate woody degradation and host colonization [48, 49]. In this current status of knowledge is insert our research with the aim to identify new cysteine rich proteins with potential roles in pathogenesis. After examine the genomic information of *N. parvum*, five sequences were selected with high cysteines content, the analysis identified the NpCysRP5 as a CFEM domain protein, distinguishable from other cysteine rich domains and inherent to fungi [37]. Even is already published that this class of motif is well represented in the Ascomycota phylum, the pioneers researches about CFEM domain [37, 50] did not include members of *Botryosphaeriaceae* family. In this sense, NpCysRP5 was identified for first time as orthodox CFEM protein of *N. parvum* expressing early during infection process in Liquidambar. Interestingly, NpCysRP4 (a protein apparently without a transmembrane domain) has a CFEM-like domain with two additional well-conserved cysteines (Fig. S6). The presence of these cysteines in NpCysRP4 could propitiate a disulfide rearrangement and conformation stability [51, 52] and may increase the possibility of form more proteoforms with complementary functions contributing to virulence. In accordance with the reported by Zhang and collaborators a positive correlation between CFEM domain occurrence and fungal pathogenicity was showed [37] and it seems that occurrence of CFEM domain proteins is independently of the pathogen lifestyle (i.e. biotrophic, hemi biotrophic or necrotrophic). For example, in *Botrytis cinerea* (a necrotrophic fungi), BcCFEM1 is express highly at early stages of infection in *Phaseolus vulgaris* and the gene disruption results in decreased virulence [53]. For *Magnaporthe grisea*, a hemibiotrophic filamentous ascomycete, the mutant pth11 (a CFEM transmembrane protein) is impaired in the appressorium maturation in consequence in the infection capacity [54]. However, it is important to notice that not all CFEM proteins have a role in pathogenicity, for example three CFEM-motif GPI-anchored proteins from *A. fumigatus* participate in cell wall stability but not in fungal virulence [55]. Here, we identified a CFEM motif-containing protein of *N. parvum* (NpCysRP5) that also contain a putative transmembrane helix and its expressed during the early infection stages in *L. styraciflua*. It will be exciting to elucidate additional clues about it exactly contribution in pathogenesis. Finally, the phylogenetic analyses identified orthologs of NpCysRP4 and NpCysRP5 in more *Botryosphaeriaceae* species contributing to the knowledge of this important woody pathogens (Fig. 5). Additionally, the expression analysis indicated a contrasting mRNA expression profile of NpCysRP4 versus NpCysRP5 (Fig. 6), and because only mRNA NpCysRP5 expression showed a positive correlation during early times of infection, we thought that NpCysRP5 is a better candidate with a significant role during pathogenesis, emphasizing the fact that NpCysRP4, with a CFEM-like domain, have another important functions that may be discover in future works.

Besides of the new CFEM-like motif, NpCysRP2 also introduces a new cysteine rich domain with the enlisted characteristics: 1) the consensus motif deduced is $C^1[Y/F]xPx_9-10C^2x_6-8C^3C^4x_4C^5x_2Nx_2C^6x_{10}-$

$_{23}C^7Tx_8C^9x_3C^{10}$ 2) all the cysteines are conserved 3) a transmembrane helix towards the carboxyl terminal end is also conserved. These characteristics strongly suggest the existence of a new group of proteins and because an augmented expression was observed at early times, NpCysRP2 also is a good candidate to have a role during pathogenesis. All these data support the fact that NpCysRP2 is a new protein here we named as fungal cystine rich transmembrane protein (FCRTP).

Finally, phylogenetic analyses indicated two important deductions: 1) NpCysRPs are present in various fungal families and 2) each NpCysRPs are associated with particular species, for example for NpCysRP4 the order *Eurotial* is well represented with several *Penicillium* species while for NpCysRP5 the order *Hypocrales* is represented with members of *Fusarium* genus proposing that *Neofusicocum parvum* is as a very versatile fungus.

Conclusions

In this study, we documented that *N. parvum* is an aggressive pathogen of *L. styraciflua*. Besides, we identified novel CysRPs of both organisms as the NpCysRP2 that it seems to be conserved through various fungi, contains a novel rich cysteine motif and a putative transmembrane domain. Also, NpCysRP4 was identified as a novel CFEM-like domain. In the case of LsCysRPs, three of them no presented a predicted putative function. The expression analysis during the plant-pathogen interaction revealed that all the LsCysRPs presented a repression expression profile in comparison with the expression profile of NpCysRPs, suggesting an interesting molecular mechanism during this interaction. Finally, all the CysRPs identified in this work, are suitable candidates to further investigations in order to increase the knowledge of the molecular events during the defense response of woody species against *N. parvum*.

Methods

Plant Material

L. styraciflua leaves with disease symptoms such as chlorosis and necrosis were sampled from five different points of trees located within the cloud forest Santuario del Bosque de Niebla (SBN) at Xalapa, Veracruz in Mexico (19°30'37"N 96°56'34"W). This forest is a protected area belongs to the Institute of Ecology (INECOL A.C.) and is used to collecting samples for research purposes. The identification of Liquidambar specimens was advised by the curators Carlos Duran and Sergio Avendaño in the Herbarium-XAL of INECOL A.C. The leaves and stems used for artificially inoculated purposes were collected from healthy trees also in SBN.

Fungi isolation from *L. styraciflua* leaves

The complete leaves were cut with gardening scissors and deposited in Ziploc bags and kept at 4°C. The leaves were cut in small pieces of 0.5 cm X 0.5 cm and were disinfected with ethanol (96%) and chlorine solution (20%) for 2 min each and rinsed five times with sterile water. For the fungi isolation the disinfected tissue was placed in petri dishes containing potato dextrose agar (PDA) and were incubated for 2–4 days at 25°C or 29°C under two different light conditions: at darkness or 12 h-light/12 h dark cycle. Monosporic cultures were carried out for all the fungi isolated that showed different morphology. The isolates were preserved in 10% glycerol at -80°C.

DNA extraction and molecular identification of Liqui 1–3 isolate by PCR

Each isolate was grown on PDA in 10 cm diameter Petri dishes for 9–10 days at 28°C in darkness. The mycelium was collected with a scalpel and pulverized to isolate genomic DNA according to the protocol described by Tapia-Tussell *et al.*, [56] with minor modifications. Specifically, in the lysis step we add 1 mL of SDS buffer, and the purification step was replaced with 500 µL phenol: chloroform: isoamyl alcohol (Sigma, Cat. P2069). For the molecular identification of Liqui 1–3 isolate we used primers already published that amplified regions of the β -tubulin gene [57], the internal transcribed spacer (ITS) regions [58], a portion of RNA polymerase II subunit (RPB2) [59], the locus BotF15, an unknown locus containing microsatellite repeats [60] and the portion of gene encoding translation elongation factor 1 α (EF-1 α) [59] see supplementary Table 1S. The five-region gene were amplified using the polymerase chain reaction (PCR) from genomic DNA using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen™, Cat. 11304). Each PCR was carried out in 50 µL reaction containing 20–100 ng of template DNA, 1X of High-Fidelity PCR Buffer, 100 µM of each dNTP (0.2 mM), 2.0 mM of MgSO₄ and 1 µL of each 20 µM forward and reverse primer, and 1U/rxn Platinum® Taq DNA Polymerase High-Fidelity (Invitrogen™, Cat. 11304). Optimal PCR condition on a SureCycler 8800 (Agilent Technologies, Inc.) initial denaturation at 94 °C for 1 min, followed by 32 cycles of 94 °C for 15 s, 58 °C (β -tubulin, RPB2 and Botf15) / 55 °C (ITS and EF-1 α) for 30 s, and 72 °C for 1 min with a final extension step of 72 °C for 5 min.

The sizes and concentration of amplicons were visually checked on an electrophoresis 1% agarose gel and PCR products are purified using a Wizard SV gel and PCR clean-up system (Promega, ref A9281), and the DNA concentration was measured on a NanoDrop 2000c spectrophotometer (Thermo scientific). Samples were sent to Langebio, Cinvestav, for sequencing. DNA sequences were analyzed using nucleotide collection (nr/nt) optimize for highly similar sequences (megablast) in NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Pathogenicity assay in *Arabidopsis thaliana* seedlings

Seeds of *Arabidopsis thaliana* ecotype Col-0 were surface-disinfected with 96% (v/v) ethanol for 7 min and 20% (v/v) bleach for 7 min, then rinsed five times for 5 min with distilled water and stratified for 2

days at 4 °C. Seeds were grown on each agar plates (10 seeds per plate) containing 0.2X Murashige and Skoog (1962) medium (MS basal salts mixture, Phytotechnology Laboratories, Cat. M524), 0.6% sucrose (Sucrose: Fluka Analytical, Cat. 84100) and 1% Agar Plant TC at pH 7. Plates were incubated at 21 ± 1 °C in a 12-h-light/12-h-dark cycle for 7 days. After this time, the seedlings were inoculated with 0.5 cm x 0.5 cm agar plug containing the isolate. Then, the plates were incubated for seven additional days in the same previous conditions and the photograph was taken with a Nikon camera D3200. All the experiments were carried out in triplicates.

Pathogenicity assay in *Liquidambar styraciflua* leaves and stems

Liquidambar styraciflua healthy leaves and stems were collected from the cloud forest Santuario del Bosque de Niebla at Xalapa, Veracruz in Mexico. Once they were collected, they were immediately placed in plastic bags that contained sterile water to conserve humidity. The leaves were collected and sterilized the same day that the experiment was carried out. The leaves were sterilized with 2% sodium hypochlorite for 1 minute, then washed five times with sterile distilled water. Three sterilized leaves were placed in a humid chamber. The humid chambers were prepared by placing a circle of sterile filter paper in the bottom of a Petri dish (150 × 20 mm) previously sterilized with UV light for 15 min. Approximately 4–5 mL of sterile distilled water was added to filter paper. The leaves (3) were mechanically damaged with a sterile scalpel in the base and then inoculated with a plug of approximately 0.5 × 0.5 cm of *N. parvum* (Liqui 1–3) previously grown on PDA medium. Leaves with the damage in the base of the leaf and a control with damage in the base of the leaf were used as a control. Finally, the control and inoculated leaves were incubated at 25 ± 2 °C with 80% of relative humidity in darkness in a plant growth chamber (Thermo Scientific, 3768). The photographs were taken at 0, 1, 3, 8 and 18 dpi with a Nikon camera D3200.

Stem infection experiments were carried out as followed: previously sterilized young branches of adult tree of *L. styraciflua* were cut with a scalpel of a single-edged knife into fragments of approximately 10 cm and then were cut longitudinally. The *L. styraciflua* stems were placed upward by quadruplicate inside a humid chamber that were prepared as previously described. The plant stems were infected on the center of the vascular tissue with a plug of approximately 0.5 × 0.5 cm of *N. parvum* (LSH1-083) previously grown on PDA medium. We included a negative control of a PDA plug without fungus. Finally, it was kept in darkness and incubated at 25 ± 2 °C with 80% of relative humidity in a plant growth chamber (Thermo Scientific, 3768).

Diaminobenzidine Staining

L. styraciflua leaves infected and uninfected were stained following a previously reported protocol [61]

Scanning Electron Microscopy (sem)

Sections of *L. styraciflua* leaves infected and uninfected of 1, 3 and 8 dpi were fixed in 2.5% glutaraldehyde for 12 hours and then washed in 0.1 M Sorensen's phosphate buffer. The samples were dehydrated an ethanol solutions series, from 30 to 100% in ten stages for 40 minutes each, dried in a critical point dried (Quorum K850), coated with gold (Quorum Q150RS) [62], and were observed in a scanning electron microscope (FEI Quanta 250 FEG).

Cysteine Rich Proteins Identification

For the identification of cysteine rich proteins (CysRP) of *L. styraciflua* we used the Hardwood Genomics Project database (<https://www.hardwoodgenomics.org/>) and for *N. parvum* we used the genomic information already published by Blanco et al., 10.1128/genomeA.00339 – 13. The prediction of subcellular localization for all proteins as well as signal peptide cleavage were performed with TargetP 1.1 Server [63], and Protter [64]. Predicted localization was evaluated with SignalP-5.0. [65] and DeepLoc-1.0 [66]. For infer the possible function of CysRP we followed BLAST (National Center for Biotechnology Information; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>), Motif search (GenomeNet; <https://www.genome.jp/tools/motif/MOTIF.html>) and DeepGOPlus [67] approaches.

Phylogenetic Trees

Phylogenetic analysis were construct with Mega X software [68] using the Maximum Likelihood method and JTT matrix-based model [69], Jalview software was used for perform the alignments [70], with Tcoffee method [71], and finally BLAST tool was used to find similarity of sequence in other fungus.

Rna Extraction And Gene Expression

Total RNA was extracted using the Plant/Fungi Total RNA Purification Kit (Norgen Bioteck) following the manufacturers recommendations. The amount of RNA was determined with f a spectrophotometer (NanoDrop™ 2000c; Thermo Scientific™) and its integrity was evaluated with the ratio A260nm/A280nm and by electrophoresis in 1.5% agarose gel. Later, 1 µg of RNA was treated with deoxyribonuclease I of Invitrogen™ following the manufacturer's instructions, posteriori the RNA was used as templates for synthesis of cDNA helped by the reverse transcriptase Superscript III (Invitrogen™) following the manufacturers protocol.

Quantitative Real Time-PCR (qRT-PCR) was carried out using SYBR™ Green PCR Master Mix (ThermoFisher), and the final reaction was made in 20 µL containing 10 µL SYBR™ Green, 1.0 µL at 10 mM of each primer, and 8 µL of cDNA at 50 ng or 5.0 ng. For oligos efficiency determination, curves with 50.0, 5.0, 0.5, 0.05 and 0.005 ng of cDNA were carried out. Real-time PCR was performed on a

Stratagene Mx300P system (Agilent Technologies) under the standard thermal profile design: 10 min at 95 °C followed by a total of 40 cycles of 30 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C. Oligo specificity was followed by a melting curve analysis with continuous fluorescence data acquisition during the 95 – 55 °C melt. For oligos design the primer3 software was used followed the parameters suggest by Thornton and Basu [72]. Relative expression levels for validated genes were calculated by the

$$ratio = \frac{(E_{target})^{\Delta CP_{target}(Control-sample)}}{(E_{ref})^{\Delta CP_{ref}(Control-sample)}} \text{ method [73] and oligos for Tip41, actin and ubiquitin, of } Liquidambar$$

styraciflua were evaluated as reference gene (all primers are listed in Supplementary Table S1). The geometric mean between Tip41 and actin was used as a reference gene [74].

Abbreviations

CFEM:common in several fungal extracellular membrane; CysRPs:cysteine rich proteins;
DAB:diaminobenzidine; dpi:days post inoculation; ET:ethylene; FC RTP:fungal cystine rich transmembrane protein; GST1:glutathione S-transferase-1; JA:jasmonic acid; LTP:lipid transfer protein;
LsCysRPs:*Liquidambar styraciflua* cysteine rich proteins; mRNAs:messenger RNA;
NpCysRPs:*Neofusicoccum parvum* cysteine rich proteins; PR-1:pathogenesis related protein 1;
ROS:reactive oxygen species; SA:salicylic acid; SCR96:small cysteine rich 96; SEM:scanning electron microscopy; SBN:Santuario del Bosque de Niebla; SsSSVP1:small secreted virulence-related protein in *Sclerotinia sclerotiorum*.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

EEHD and DSR conceived the idea, designed the experiments and wrote the manuscript. RVA, HVD and JBRH carried out the experiments and data interpretation. GHRS performed the experiments carried out by SEM. All authors have read and approved the final manuscript.

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Figures

Figure 1

Pathogenicity screening of the different fungi isolates in Liquidambar styraciflua leaves. *L. styraciflua* leaves were collected from adult trees and infected with different fungi isolates. The photograph was taken at 7dpi. Data are represented for at least three independent experiments.

Control

+ *Neofusicoccum parvum*

Figure 2

Neofusicoccum parvum and *Liquidambar styraciflua* pathosystem. The leaves collected from adult trees were injured with a scalpel in the leaf base before were inoculated with a PDA plug (0.5 cm X 0.5 cm) from a 7- day-old-culture of Liqui 1-3 strain. The leaves control also were injured. The photographs were taken at 0, 1, 3, 8 and 16 dpi. Data are represented for at least three independent experiments.

Figure 3

Analysis by scanning electron microscopy of *Neofusicoccum parvum* infection in *Liquidambar styraciflua* leaves. a. and c. control of adaxial leaf at 3 and 8 dpi respectively, b. and d. infected axial leaf at 3 and 8 dpi respectively. e. leaf base infection and the dashed square indicate the zone that were analyzed in f to l. f. and h. control at 3 and 8 dpi respectively, g. infected at 3 dpi, i-l. infected at 8 dpi, the arrows point out the pycnidium and conidias of *N. parvum*.

Figure 4

Phylogenetic analyses of NpCysRP1, NpCysRP2 and NpCysRP3 with orthologs from other fungal species. The phylogenetic trees were constructed using protein sequence using the Maximum Likelihood method and JTT matrix-based model. NpCysRP1, NpCysRP2 and NpCysRP3 trees with the highest log likelihood (-2656.73), (-15145.13) and (-5228.55) respectively is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. A. For NpCysRP1, amino acid sequences are from *N. parvum* (EOD50423.1), *Lasidiplodia theobromae* (KAB2569989.1), *Diplodia corticola* (XP_020126718.1), *Diplodia seriata* (KKY27788.1), *Crucibulum laeve* (TFK32468.1), *Rhizoctonia solani* (CUA75287.1), *Beauveria bassiana* (PQK10211.1), *Cordyceps confragosa* (OAA70963.1), *Aspergillus leporis* (KAB8069877.1), *Suillus luteus* (KIK46160.1), *Colletotrichum sidae* (TEA07484.1). B. For NpCysRP2, amino acid sequences are from *Trichoderma reesei* (XP_006969324.1), *T. parareesei* (A9Z42_0055550), *T. longibrachiatum* (PTB72285.1), *T. citrinoviride* (XP_024746670.1), *T. harzianum* (XP_024771023.1), *T. guizhouense* (PB44849.1), *T. arundinaceum* (RFU76343.1), *Fusarium avenaceum* (KIL85060.1), *Thermothielavioides terrestris* (XP_003656631.1) *Madurella mycetomatis* (KXX77126.1), *Thermothelomyces thermophilus* (XP_003666291.1), *Aspergillus lentulus* (KKO98531.1), *Colletotrichum asianum* (KAF0319766.1), *C. nymphaeae* (KXH38321.1), *C. orchidophilum* (XP_022481280.1), *Microdochium bolleyi* (KXJ94843.1), *Capronia epimyces* (XP_007733601.1), *Aspergillus neoniger* (XP_025474954.1), *Baudoinia panamericana* (XP_007680904.1), *Pyrenophora tritici-repentis* (XP_001934627.1), *Exophiala mesophila* (RVX68508.1), *Aspergillus terreus* (XP_001217394.1), *Pseudocercospora musae* (KXT13283.1), *N. parvum* (EOD50922.1), *L. theobromae* (KAB2578904.1), *D. corticola* (XP_020125849.1) and *D. seriata* (KKY21900.1). C. For NpCysRP3, amino acid sequences are from *Fusarium kuroshium* (RMI89680.1), *F. euwallaceae* (RTE68176.1), *F. oxysporum* (EXK86396.1), *F. avenaceum* (KIL83641.1), *Macrophomina phaseolina* B, (EKG22346.1), *Macrophomina phaseolina* C (EKG22347.1), *N. parvum* (EOD52838.1), *Macrophomina phaseolina* A (EKG20686.1), *Colletotrichum spinosum* (TDZ33147), *C. fioriniae* (EXF77634.1), *C. fructicola* (XP_031888108).

Figure 5

Phylogenetic analyses of NpCysRP4 and NpCysRP5 with orthologs from other fungal species. The phylogenetic trees were constructed using protein sequence using the Maximum Likelihood method and JTT matrix-based model. NpCysRP4 and NpCysRP5 trees with the highest log likelihood (-4619.05) and (-6976.31) respectively is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. A. For NpCysRP4, amino acid sequences are from *D. corticola* (XP_020129061.1), *D. seriata* (KKY13873.1), *L. theobromae* (KAB2571564.1), *N. parvum* (EOD51105.1), *M. phaseolina* (EKG14570.1), *Elsinoe australis* (TKX25331.1), *Sphaeloma murrayae* (PNS17704.1), *Sphaerulina musiva* (XP_016758053.1), *Passalora fulva* (AQA29231.1), *Paraphaeosphaeria sporulosa* (XP_018029092.1), *Lepidopterella palustris* (OCK84136.1), *Cenococcum geophilum* (OCK98732.1), *Glonium stellatum* (OCL13334.1), *Venustampulla echinocandica* (RDL36609.1), *Colletotrichum higginsianum* (TIC93879.1) *Fusarium langsethiae* (KPA46759.1), *F. sporotrichioides* (RGP70600.1), *F. culmorum* (PTD11477.1), *F. pseudograminearum* (XP_009256009.1), *F. longipes* (RGP60032.1), *F. oxysporum* (EXA53032.1), *F. verticillioides* (RBR06989.1), *F. nygamai* (PNP52701.1), *F. mangiferae* (CVK83605.1), *F. fujikurori* (QGI58805.1), *F. proliferatum* (XP_031075444.1). B. For NpCysRP5, amino acid sequences are from *D. corticola* (XP_020133845.1), *D. seriata* (KKY24076.1), *L. theobromae* (KAB2577423.1), *N. parvum* (EOD44996.1), *M. phaseolina* (EKG19367.1), *Cenococcum geophilum*

(OCK97775.1), *Glonium stellatum* (OCL07361.1), *Corynespora cassiicola* (PSN73263.1), *Periconia macrospinos*a (PVI08476.1), *Trichophyton rubrum* (XP_003231199.1), *Coccidioides immitis* (XP_001240075.1), *Coccidioides posadasii* (XP_003069153.1), *Paracoccidioides brasiliensis* (ODH22777.1), *Histoplasma capsulatum* (EEH05575.1), *Blastomyces parvus* (OJD22636.1), *Aspergillus calidoustus* (CEL09455.1), *A. nidulans* (XP_660049.1), *Penicillium oxalicum* (EPS32950.1), *P. steckii* (OQE27319.1), *Rasamsonia emersonii* (XP_013330685.1) *P. coprophilum* (OQE36870.1) *P. flavigenum* (OQE32686.1), *P. rubens* (XP_002567673.1), *P. arizonense* (XP_022485186.1), *P. vulpinum* (OQE12392.1), *P. italicum* (KGO74509.1), *P. nordicum* (KOS37798.1), *P. solitum* (OQD85155.1), *P. camemberti* (CRL28928.1), *P. freii* (KUM58798.1), *P. polonicum* (OQD69393.1).

Figure 6

Expression at early times of infection of CysRPs mRNAs of both *L. styraciflua* and *N. parvum*. A. LsCysRPs mRNA expression at 1 and 3 dpi. For the analysis the geometric mean between Tip41 and actin was used as a reference gene. For all the transcripts statistic significantly, difference was detected between 1 and 3 dpi at $P < 0.01$, using Student's t test. B. NpCysRPs mRNAs expression from 1 to 3 dpi.

NpCysRP3 was used as a reference gene. Data are the average of three independent inoculation experiments.

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