

Structural Analysis of Cyclophilin and Inhibitory Activity of Cyclosporin A on Germination and Growth of *Moniliophthora perniciosa*

Paulo S Monzani^{1,4*}, Humberto M Pereira², Karina P Gramacho³, Fatima C Alvim⁴, Flávio V Meirelles⁵, Glaucius Oliva² and Júlio CM Cascardo^{4,†}

¹Central Avançada em Biotecnologia da Reprodução Animal, Universidade Norte do Paraná, Londrina – PR, Brazil.

²Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos – SP, Brazil

³Laboratório de Fitopatologia Molecular, Comissão Executiva do Plano da Lavoura Cacaueira, Ilhéus – BA, Brazil

⁴Departamento de Ciências Biológicas, Centro de Biotecnologia e Genética, Universidade Estadual de Santa Cruz, Ilhéus – BA, Brazil

⁵Departamento de Ciências Básicas, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga – SP, Brazil

[†]in memoriam

Abstract

Moniliophthora perniciosa is the causal agent of witches' broom disease of cacao. Cyclophilins have been implicated in a wide variety of cellular processes, including the response to environmental stresses, cell cycle control, regulation of calcium signaling and transcriptional control. The involvement of cyclophilins in pathogenicity was described both in pathogenic fungi toward animals and plants. In Yeast, calcineurin and cyclophilin are involved in fungal morphogenesis and virulence. There is evidence that cyclophilin interacts with calcineurin in the absence of cyclosporin A (CsA), and it has been proposed that the activity of CsA acts in the regulatory interaction between cyclophilin and calcineurin; also, fungistatic or fungicide actions have been observed in this activity. The *M. perniciosa* cyclophilin gene from cDNA of the fruiting body in the vector pET28a has been cloned and the protein was expressed, purified and crystallized. The cyclophilin structures in the apo and bound with CsA forms were solved at 1.85 and 1.47 Å, respectively. Comparison of structures from different organisms indicates conserved structures of cyclophilins. Small differences were found in apo and bound forms; hence, it follows that cyclophilin structures do not have accentuated modifications in the presence of the ligand. However, various hydrogen bonds between side chains of amino acids and water molecules are broken in the ligand site when CsA is bound. The germination spore assays using CsA showed a low inhibitory activity in germination, but high inhibition of the germ tube growth. These results show that cyclophilin plays an important role in the growth process, but not in the germination, hence suggesting that cyclophilin is as potential target for the fungistatic action against *M. perniciosa*.

Keywords: Characterization; Crystal structure; Cyclophilin; Cyclosporin A; *Moniliophthora perniciosa* and Witches' broom disease

Introduction

Cyclophilins are members of a highly conserved family of proteins that play a pivotal role in protein folding through enzymatic catalysis of the peptidyl-prolyl *cis-trans* isomerization reaction of the peptide bonds preceding proline residues [1]. The prolyl-isomerase enzymes consist of three structurally unrelated protein families, the cyclophilins, FK506-binding proteins (FKBPs) and parvulins [2]. Cyclophilins found to be the intracellular receptors of the immunosuppressive drug cyclosporine, whereas the complex of these molecules perturbs the signal transduction cascades through the inhibition of the protein phosphatase calcineurin, preventing T-cell activation in mammals [3, 4], and immunodeficiency virus infection in humans [5]. Besides, cyclophilins have been implicated in a wide variety of cellular processes such as cell cycle control [6], regulation of calcium signaling [7] and control of transcriptional repression [8]. Calcineurin and cyclophilin A are involved in fungal morphogenesis, virulence, hyphal branching and changes in the hyphal morphology [9, 10] of *Neurospora crassa*. These protein phosphatases are required for the regulation of morphogenesis, mating and virulence of *Cryptococcus neoformans* [11, 12] and are involved in the formation of appressorial infection structure [13] in *Magnaporthe grisea*.

The involvement of cyclophilin in pathogenicity was pointed out in fungi pathogenic toward animals or plants. Two *Cryptococcus neoformans* encoding cyclophilin A homologs (Cpa₁ and Cpa₂ proteins) play a shared role in cell growth, mating, virulence and CsA toxicity [14]. Cpa₁ mutants are attenuated for virulence, whereas Cpa₂ mutants

are fully virulent, double mutants exhibit synthetic defects in growth and virulence, and CsA active site mutants restore the growth of Cpa₁ and Cpa₂ mutants [14]. In *Magnaporthe grisea*, cyclophilin mutants show reduced virulence and are impaired in associated functions such as penetration peg formation and appressorium turgor generation [13]. CsA inhibits appressorium development and hyphal growth in a cyclophilin-dependent manner, indicating cyclophilin as a virulence factor in phytopathogenic fungi and provides evidence for a signaling required for infection structure formation by *M. grisea* [13]. The cyclophilin null mutant in *Botrytis cinerea* is able to develop infection structures, but it is in the development of symptom-altered-agents in the development of bean and tomato leaves, while calcineurin inhibition using CsA modifies hyphal morphology and prevents infection structure formation [15]. In *Saccharomyces cerevisiae*, cyclophilin A is localized to the nucleus and governs the meiotic gene expression program to promote efficient sporulation [16]. The meiotic function

*Corresponding author: Paulo S Monzani, Central Avançada em Biotecnologia da Reprodução Animal, Universidade Norte do Paraná, Londrina – PR, Brazil, Tel: 55 43 3399 4700; Fax: 55 43 4043 0888; E-mail: monzani.paulo@gmail.com

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requires the prolyl isomerase activity of the cyclophilin A and the physical association of this protein with the Set 3C histone deacetylase; Cyclophilin A is therefore involved in governing the transcriptional program required for the vegetative to meiotic developmental switch in budding yeast [16].

Basidiomycete *M. perniciosa* is the causal agent of witches' broom disease of cacao (*Theobroma cacao* L.), the chocolate tree. This disease has strongly limited cacao cultivation in many tropical regions throughout the world and represents an important factor in the economy of this crop, as it can reduce yields to up to 80-90%, hence resulting in the abandonment of many production areas around the world. Fungal life cycle began with the basidiospore release period. Formed on basidiomes, fungi are the only infective propagule of *M. perniciosa* [17] and might infect any meristematic tissues, establishing a biotrophic relationship with its host, during which the fungus is homokaryotic, intercellular and lacks clamp connections inducing a variety of symptoms on vegetative shoots, flower cushions, flowers, and pods of cocoa; the hypertrophic growth of infected vegetative meristem (broom) is the most characteristic symptom of witches' broom disease. Concurrently, the infected tissues became necrotic, whereas the hyphae became dikaryotic, clamped and saprotrophic; this fact coincides with the death of host tissue and the end of the biotrophic phase. The pathogen life cycle is completed by basidiocarp production on necrotic plant tissues (brooms and pods).

In southeastern Bahia, the cacao region of Brazil, *M. perniciosa* is considered to be the main phytopathogen to attack the cacao crops, resulting in a marked decrease in production. The use of commercial fungicides is not feasible for controlling this infection [18], and efforts to find new drug targets for controlling such plague are therefore necessary. Cyclophilin has been shown as an important virulence factor in phytopathogenic fungi and has been known as an intracellular target to CsA. The gene sequence encoding cyclophilin was obtained from the cDNA library of the *M. perniciosa* fruit body. The *M. perniciosa* cyclophilin gene was cloned, whereas the protein was expressed, purified and crystallized in apo form and in complexes with CsA. Spore germination and mycelia growth inhibition of *M. perniciosa* were evaluated using CsA.

Materials and Methods

Cloning, expression and purification

The sequence of the gene encoding cyclophilin was obtained from cDNA library of the *M. perniciosa* fruiting body, and the first strain of cDNA isolated from a fruiting body was used as a template. A forward primer including an *Nde*I restriction site (5'-GCTCATATGGCCAACGTATTCCTC-3') was designed, while the reverse primer used was the modified oligo (dT) (CDS III/3' PCR Primer - Clontech). PCR was performed using *Pfu* DNA polymerase (Promega), according to the manufacturer's instruction, considering the following cycling parameters: 5 min denaturation at 95°C, 35 cycles of 30 s at 95°C, 30 s at 50°C, 1 min at 72°C, and a final 10 min elongation at 72°C. The cyclophilin gene was cloned in the pT7-blue3 vector previously cleaved with *Eco*RV, and later incised by *Nde*I and *Eco*RI sites and subcloned into a pET28a expression vector previously cleaved with the same restriction enzymes. The gene sequence was confirmed using the T7 promoter and the T7 terminator primers (Novagen) and data was collected using an ABI PRISM 377 DNA Sequencer (Perkin Elmer - Applied Biosystems). The nucleotide sequence was deposited in GenBank with accession code FJ409218. A recombinant protein was produced in *Escherichia coli* BL21 (DE3).

Expression was induced by the addition of 0.5 mM IPTG in culture, at 0.6 OD₆₀₀ growths, and cells were cultured for 5 h, at 20°C. The cells were harvested by centrifugation at 5000 X g for 15 min, at 4°C, resuspended in 50 mM sodium phosphate buffer (pH 7.0) plus 300 mM NaCl and 20 mM imidazole, and sonicated for 7 min. Cell lysates were centrifuged at 20.000 X g for 20 min, at 4°C, and cyclophilin in soluble fraction was purified with TALON polyhistidine-tag purification resin (Clontech). The column was equilibrated with 10 column volumes, washed with 30 column volumes and eluted with 6 column volumes of buffer with the addition of 200 mM imidazole. The histidine tail was removed with bovine thrombin (Sigma) in 50 mM sodium phosphate at pH 7.2 and 100 mM of NaCl, allowing a second purification step with size-exclusion chromatography using AKTA Purifier FPLC System on a superdex 75 HR10/30 column (GE/Amersham Pharmacia Biotech, Sweden) in 20 mM Tris at pH 7.2 and 150 mM NaCl. The fractions were assessed in comparison to the retention volume of a standard sample of known molecular weight. The purified protein was concentrated to 7 mg.mL⁻¹ in 20 mM Tris at pH 7.0, using an Amicon Ultra centrifugal filter device (Millipore). Protein concentration was determined by Bradford method [19].

Crystallization, data collection and structure determination

Crystallization of the cyclophilin protein was performed using the hanging drop vapor diffusion method [20] at 20°C, by mixing 1.5 µl of the protein solution with an equal volume of a reservoir solution equilibrated against 500 µl of the crystallization solution. The solutions consisted of 30% PEG400 and 100 mM CAPS, pH 10.5 to apo form, 1.8 M ammonium sulfate, 100 mM Tris, pH 8.5 to bound form, using three-fold excess CsA than protein. The crystals grew in about six months to apo form, while the bound form grew in about two weeks. Crystals were flash-frozen in liquid nitrogen stream with the addition of 20% glycerol in the crystallization solution as a cryoprotectant. Diffraction data were collected with the Beamline W01B-MX2 at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil), at 100K, using λ=1.43 and a marmosaic 225 detector, over 1° increments in φ for a total rotation of 89° and 99° for bound and apo forms, respectively. The data set was collected at a resolution of 1.85 Å to apo form and 1.47 Å to bound form. Data were indexed and integrated using the MOSFLM program [21] and scaled using the program SCALA of CCP4 suite [22]. The initial phases were obtained through molecular replacement, using the Phaser program [23], and *Saccharomyces cerevisiae* cyclophilin, at 67% identity, as a search model (PDB code 1IST - not published) for bound form; while for apo form, in turn, the bound form here solved has been used as a model. Model refinement was initially carried out with Phenix [24], employing TLS parameters in the last stages of refinement. The model-building procedure was performed with COOT [25], using σ_a-weighted 2Fo-Fc and Fo-Fc electron density maps. A total of 178 water molecules were included using both COOT and Phenix to apo form and 402 water molecules to bound form, and CsA was added using the COOT program. The structures were refined with one or two cyclophilin molecules in the asymmetric unit for apo and bound forms, respectively. In all cases, the behavior of R_{free} was considered as the major criterion for validating the refinement protocol, whereas the stereochemical quality of the model was evaluated with PROCHECK [26] and MolProbity [27]. The structure was visualized using PyMOL [28]. Full statistics for data collection and refinement are summarized in Table 1. The coordinates and structural factor files of apo and bound cyclophilin have been deposited into the Protein Data Bank, with the accession codes 3O7T and 3PMP, respectively.

Parameters	Cyp-apo	Cyp-CsA
Data collection statistics		
Space group	I222	P42 ₂
Unit cell parameters (Å) a,b, c	37.85, 98.20, 111.68	103.97, 103.97, 61.28
Resolution range (Å)	49.08-1.85 (-1.95-1.85)	34.65-1.47 (1.55-1.47)
Measured reflections	75797 (10687)	381226 (50155)
Unique reflections	18133 (2582)	57546 (8279)
Completeness (%)	99.5 (99.5)	99.9 (99.9)
Redundancy	4.2 (4.1)	6.6 (6.1)
Average I/σ(I)	9.7 (2.2)	14.2 (2.3)
R _{meas} (%)	16.6 (64.9)	9.0 (72.3)
R _{sym} (%)	14.5 (56.8)	8.3 (66.0)
Refinement statistics		
Reflection in working set	17087	54396
Reflections in test set	868	2759
R _{work} /R _{free} (%)	17.44/20.50	17.44/20.78
R.m.s.d. bonds (Å)	0.006	0.008
R.m.s.d. angles (°)	1.005	1.857
Ramachandran plot (%)		
Favored	96.30	96.01
Allowed	3.70	3.38
Disallowed	0	0.61

The values in parentheses refer to statistics in the highest bin.

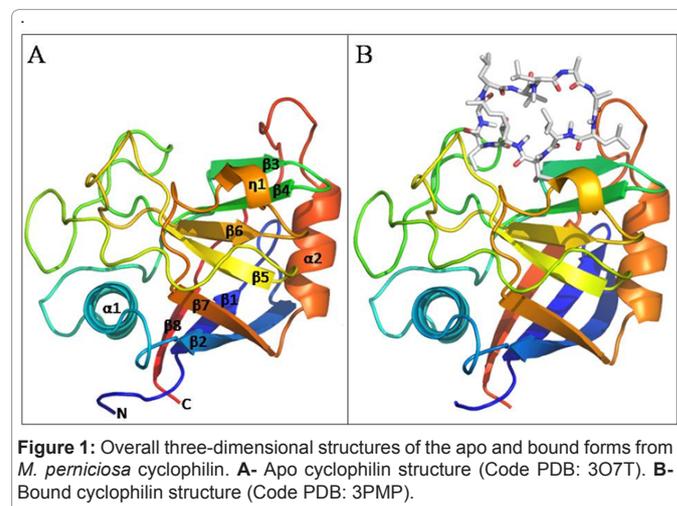
Table 1: Data collection and refinement statistics.

Assays for the Activity of Cyclosporin A. Moniliophthora perniciosa basidiospores were obtained from brooms hanged them in an illuminated (12:12 diurnal light) cabinet in which a mister sprayed brooms with deionized H₂O for 1 min every 24 h. Standard procedures to test the effect of CsA on basidiospores germination and germ tube development were adapted from previous studies on CsA inhibitory effect [13] and [15]. The effect of CsA on basidiospore germination was tested by adding 0, 1, 10, 20, and 100, and 1000 µg.mL⁻¹ CsA (Sigma) in 1% DMSO to a basidiospore suspension containing 10⁶ spores per mL before germination and germ tube were developed. This mixture was incubated at 23 ± 2°C for 30 minutes. All experiments were performed at least three times using three replicate cover slips per experiment in a completely randomized design. Three drops of the mixture were deposited on the surface of a glass cover slip coated with 1.5% agar-agar incubated in a humid environment, at 23 ± 2°C, for 4 hours. The frequency of germination was determined by counting the number of germinated basidiospores that had developed from 100 basidiospores. Germ tube lengths were measured by microscopy 4 hours after incubation and overnight.

Results and Discussion

M. perniciosa cyclophilin structure. *M. perniciosa* cyclophilin purification has shown over 95% purity based on 15% SDS-PAGE gel and the molecular mass of the purified protein estimated by gel filtration column chromatography was 21 kDa (data not shown), suggesting that it acts as a monomer in the solution. This value is similar to the predicted weight (17,675 kDa) calculated from the amino acid sequence and the predicted pI value found was 8.99. The overall three-dimensional structure of the apo and bound *M. perniciosa* cyclophilin (Figure 1) is a monomeric β-barrel formed by eight anti-parallel β-sheets and capped at either end by two α-helices, according to the observed characteristic of cyclophilins [29-31]. The amino acid sequence alignment of *M. perniciosa* cyclophilin with other organisms is shown in Figure 2, where one can observe that the regions is better stage of conservation are found in most parts of the protein, comprising all regions, since the α-helix 1 until the

β-strand 7 (residue 27 to 130), whereas the most variable regions are found in N and C-terminus. The alignment has therefore shown that cyclophilin is conserved from fungi to humans. Analysis of similarity by BLAST has shown a greater identity with cyclophilin deposited in the PDB; a 70% identity (115/162) was observed for *M. sympodialis* (PDB 2CFE), followed by *S. cerevisiae* (PDB 11ST), with 67% identity (109/162) and human (PDB 2RMA) with 67% identity (107/159). *M. sympodialis* cyclophilin appears to be responsible by hypersensitivity reactions [29], whereas *S. cerevisiae* cyclophilin governs the meiotic gene program to promote efficient sporulation [16], and human cyclophilin is essential for mitotic regulation [6]. Data from BLAST suggest that the *M. perniciosa* cyclophilin (GenBank accession code FJ409218) used in this work is similar to that cytoplasmatic cyclophilin A isoform from *S. cerevisiae* and human. *M. perniciosa* cyclophilin gene was obtained from fruit body and, due to the high similarity with *S. cerevisiae* cyclophilin, this isoform could be presumably involved in meiotic processes, as previously proposed [16]. The confirmation of



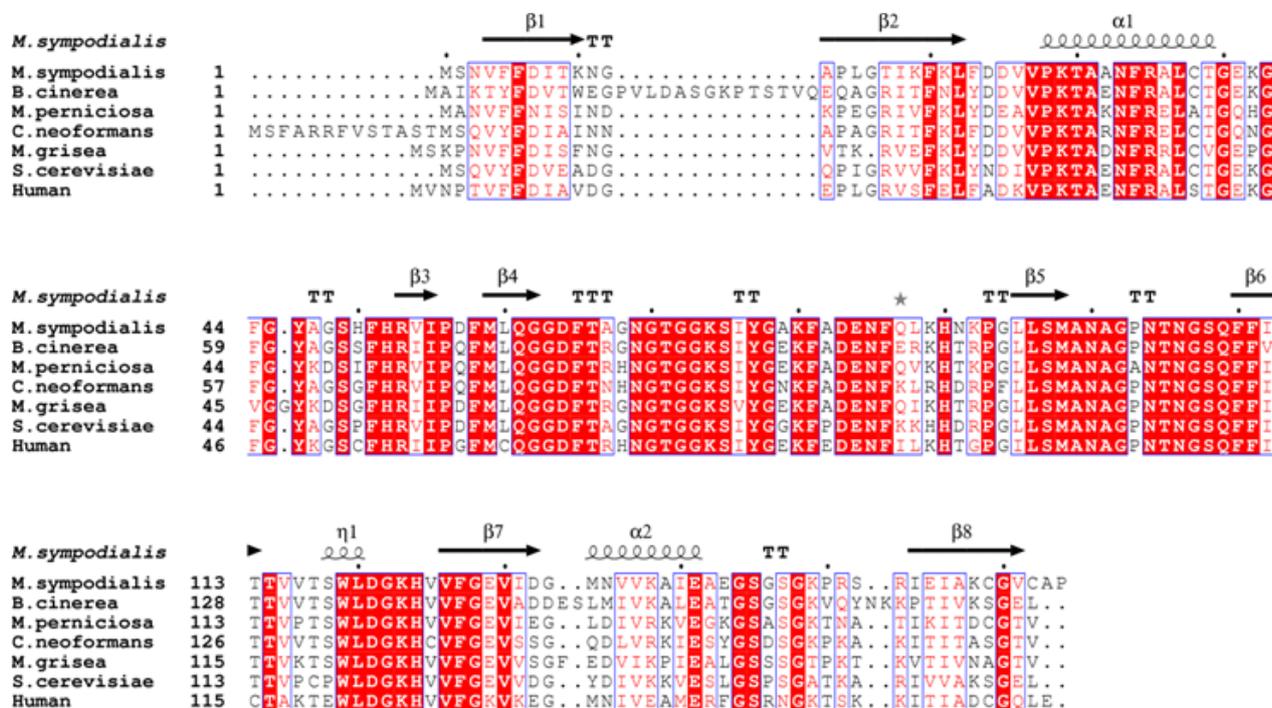


Figure 2: Amino acid sequence alignment. Alignment of amino acid sequences of the cyclophilin from fungi, yeast and human. The sequences were obtained from: *M. sympodialis* (Code PDB: 2CFE), *B. cinerea* (GenBank AL112297), *C. neoformans* (GenBank EAL22572), *M. grisea* (Genbank AF293848), *S. cerevisiae* (Code PDB: 11ST) and human (Code PDB: 2RMA).

the involvement of cyclophilin in the meiosis process of *M. perniciosa* could be an important step towards considering this protein as a possible drug target, since the products of meiosis, the haploid basidiospores, are transported by wind and air and are responsible for initiating the disease cycle [32]. Analysis of similarity by BLAST among important fungi responsible by diseases showed 74% identity (107/144) with *B. cinerea* (GenBank AL112297), 73% identity (119/162) with *C. neoformans* (GenBank EAL22572) and 69% identity (112/162) with *M. grisea* (Genbank AF293848). Cyclophilins from these fungi are involved in virulence [11-13, 15].

The structural alignment is in concordance with amino acid alignments where conserved structures of the cyclophilins (Figure 3A) have been observed. The structural superposition of apo forms with *M. perniciosa* apo cyclophilin showed a RMSD of the 0.344 Å for *M. sympodialis* (PDB 2CFE), using 135 Ca atoms aligned, 0.811 Å with human (PDB 1OCA) for 147 Ca atoms aligned, 0.409 Å with *S. cerevisiae* (PDB 11ST) using 135 Ca atoms aligned. On the other hand, the alignment of *M. perniciosa* cyclophilin in apo and bound forms has shown a RMSD of the 0.266 Å, using 136 Ca atoms aligned. The main differences were observed in the N-terminus region and in the loops involved in CsA bound (Figure 3B). This small difference observed in loops may result from the binding of CsA.

The structural alignment analysis of the CsA binding site in the apo and bound *M. perniciosa* cyclophilin has showed an accentuated change of Arg53 rotamer when CsA is bound (Figure 4A,4B). In the apo form, the amide groups (NH1 and NH2) of side chain from Arg53 point outward the binding site, whereas in the bound form, these groups are point toward the binding site. In the apo form, the amide groups of Arg53 are linked to the side chain from Gln61 and to the water molecule. The NH1 of Arg53 makes hydrogen bond with

the carboxylic group (OE1) from Gln61, whereas the amino group NE makes hydrogen bond with H₂O116 and this water makes hydrogen bonds with main-chain carbonyl group from Ser147 (Figure 4A). In the bound form, the side chain of ARG53 was found in a different conformation when compared with the apo form. The rotamer assumed by ARG53 in the bound form still conserves the bounds found in the apo form, but a new hydrogen bond was verified between NH2 from Arg53 of bound form and carboxyl group (OCY) from CsA (Figure 4B). The rotamer change of Arg53 allows a direct interaction of this residue with the ligand. The importance of this event was reported in cyclophilin structures from others organisms [30,31]. In addition

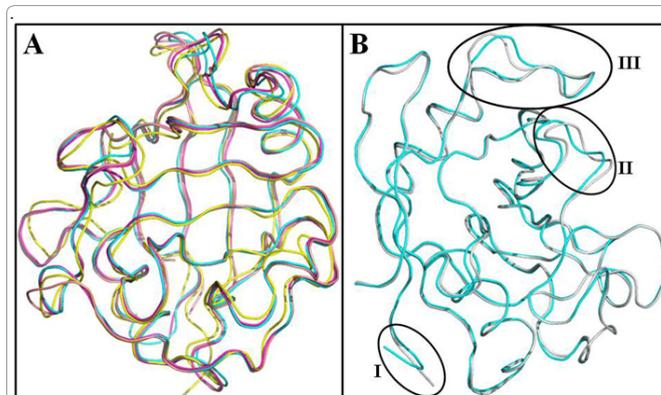


Figure 3: Structural alignment of cyclophilins. **A-** Structural alignment of apo cyclophilin from *M. perniciosa* (blue – 3O7T), *M. sympodialis* (magenta – 2CFE), human (yellow – 1OCA) and *S. cerevisiae* (pink – 11ST). **B-** Structural alignment of apo (blue – 3O7T) and bound (gray – 3PMP) forms from *M. perniciosa*. I- N-terminus; II- Loop localized between β -strand 4 and β -strand 5; III- Loop localized between α -helix 2 and β -strand 8.

to Arg53, others small structural differences between apo and bound forms have been observed. The residues Phe58 and Met59 have shown a small difference in rotamers. In the global structure, two main shifts of the backbone were found. One shift was verified in the region, from the middle to the end of the α -helix 2 (Lys139 – Ly143), including the whole loop situated between α -helix 2 and β -strand 8. The other shift was found in the ten residues after the end of β -strand 4 (Gly63 – Gly72), in the long loop localized between β -strand 4 and β -strand 5 (Figure 3B). This shift allows the loop access to the binding site, and the carboxyl of Gly70 performs hydrogen bond with the amino group of NAT residue from CsA. These two loops are located laterally, pointing towards the ends of the CsA. These smooth structural changes in the backbone might be important to CsA bound, and were observed in apo and bound forms from human cyclophilin [30]. Yet, in the *Leishmania* cyclophilin [29], the loop between α -helix 2 and β -strand 8 is furthest from the binding site for human and *M. perniciosa* cyclophilins.

In the apo structure, one can observe a formation of hydrogen bond networks, which are formed by molecules of water in the CsA binding site (Figure 5A). A network formed by Asn100(O)---H₂O45---H₂O106---H₂O100 is broken with the binding of CsA; this event should occur with the water exit the binding site and the entering of the ligand in this place, allowing interaction between the carbonyl of Asn100 with ODG of CsA (Figure 5B). Another network formed by Gln61 (NE₂)---H₂O194---H₂O172---H₂O142 is broken. In this case, the amide group of Gln61 makes hydrogen bond with O atom of CsA. In the moving of side chain from Arg53, the network formed by Arg53(NE)---H₂O116---Ser147(O) (Figure 4) is conserved, while the Arg53(NH1)---H₂O65---Gly70(O) net is broken and the Gln61(NE2)---H₂O206---H₂O279---Asn69(O) net is formed (Figure 4). The NE1 group of residue Trp119 makes hydrogen bond with H₂O102 in the apo form, whereas in the bound form, the hydrogen bond is done with OCP of CsA. A network in the apo form, H₂O103---H₂O97---H₂O180 (Figure 5B), is removed from the binding site when Arg53 assumes a new conformation. The amide group NH₂ from Arg53 occupies the place of H₂O103 and NH₁ and NH₂ groups make hydrogen bond with OCY of CsA. Another network is kept with a single substitution of the last water by hydrogen bond with CCN atom from CsA. In the apo structure, there is Ala146 (O)---H₂O75---H₂O152---H₂O122---H₂O137, and in the bound, there is Ala146---H₂O256---H₂O222---H₂O127---CCN (CsA) (Figure 5). A network of hydrogen bond formed by water molecule is preserved in both structures. In apo, it is formed by Gln57 (O)---H₂O160---

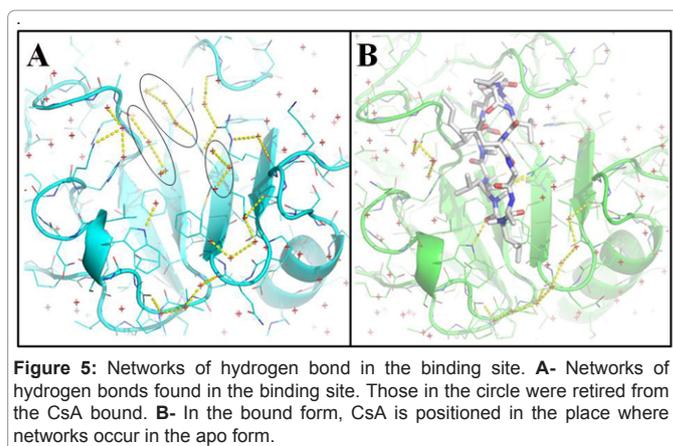


Figure 5: Networks of hydrogen bond in the binding site. **A-** Networks of hydrogen bonds found in the binding site. Those in the circle were retired from the CsA bound. **B-** In the bound form, CsA is positioned in the place where networks occur in the apo form.

H₂O139---H₂O70---Pro116 (O), whereas in the bound, it is formed by Gln57 (O)---H₂O339---H₂O283---H₂O238---Pro116(O) (Figure 5). This network in the bound form is connected to that network from Ala146 by the water molecules H₂O323 and H₂O127.

The above data suggest that, in order that the CsA binding occurs, it is necessary the breakage of hydrogen bonds between molecules of water with side chains of amino acids from the binding site (Figure 5). In conclusion, the apo form can be stabilized by a network of interactions with water molecules involving specific rotamer of the Arg53, which must be undone for the binding of CsA. Water molecules might be important for stabilization of the apo form. Structurally, there are smooth differences between apo and bound forms, and then, the main conformational changes must occur in the ligand, as previously reported [33]. It was proposed that CsA binds to cyclophilin as a transition-state analog [34].

Assays for the Activity of CsA on the germination of basidiospore and germ tube growth. It has been shown that cyclophilin encodes the cellular target for CsA in fungi [13]. Cyclophilin interacts with calcineurin even in the absence of CsA [35], and CsA thereby interferes in the natural regulation of protein-protein interactions between cyclophilin and calcineurin. The crystallographic analysis of *M. perniciosa* cyclophilin showed an interaction between cyclophilin and CsA. In consequence, the *in vivo* effect of CsA on the germination of basidiospores and germ tube growth of *M. perniciosa* has been evaluated. Basidiospore germination was first observed after 4 h of incubation at 25°C. At that time, about 75% of basidiospores had germinated on the water control experiment, showing that germination was nearly completed. Two experiments for germination inhibition were performed; in the first, the basidiospores were plated on agar-agar containing different CsA concentrations, while in the second, the basidiospores were pre-incubated during 25 minutes in CsA solutions with 1% DMSO. In both experiments, germination and germ tube development were analyzed.

The analysis of the CsA on germination and the germ tube development of *M. perniciosa* indicate that cyclophilin is an important protein involved in these processes. When plated in a high dosage of agar-agar with CsA, it was observed about 50% of germination inhibition at 1 mg.mL⁻¹ (Figure 6A, 6B), and when previously incubated with CsA during 25 minutes (Figure 6C) and plated on agar-agar without CsA, about 16% inhibition has been noted. Germination in the presence of CsA was also observed in *Botrytis cinerea* [15], suggesting that such mechanism can be altered in CsA presence, but this must not be the major point of actuation of CsA. The most striking results implicated

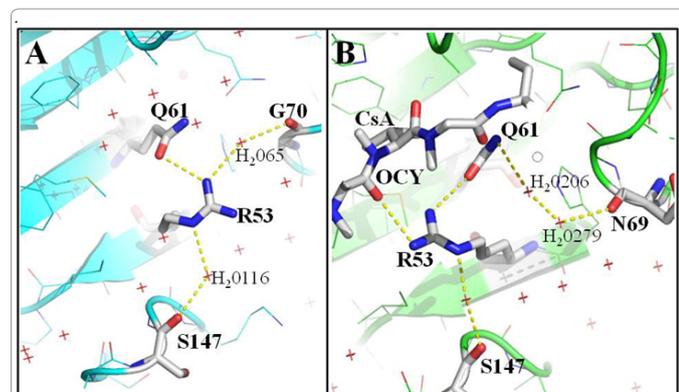


Figure 4: Change of conformational rotamer. **A-** In the apo form, Arg53 is turned out from the binding site and this conformation is coordinated by water molecules. **B-** In the bound form, Arg53 is turned into a binding site and makes hydrogen bond with CsA.

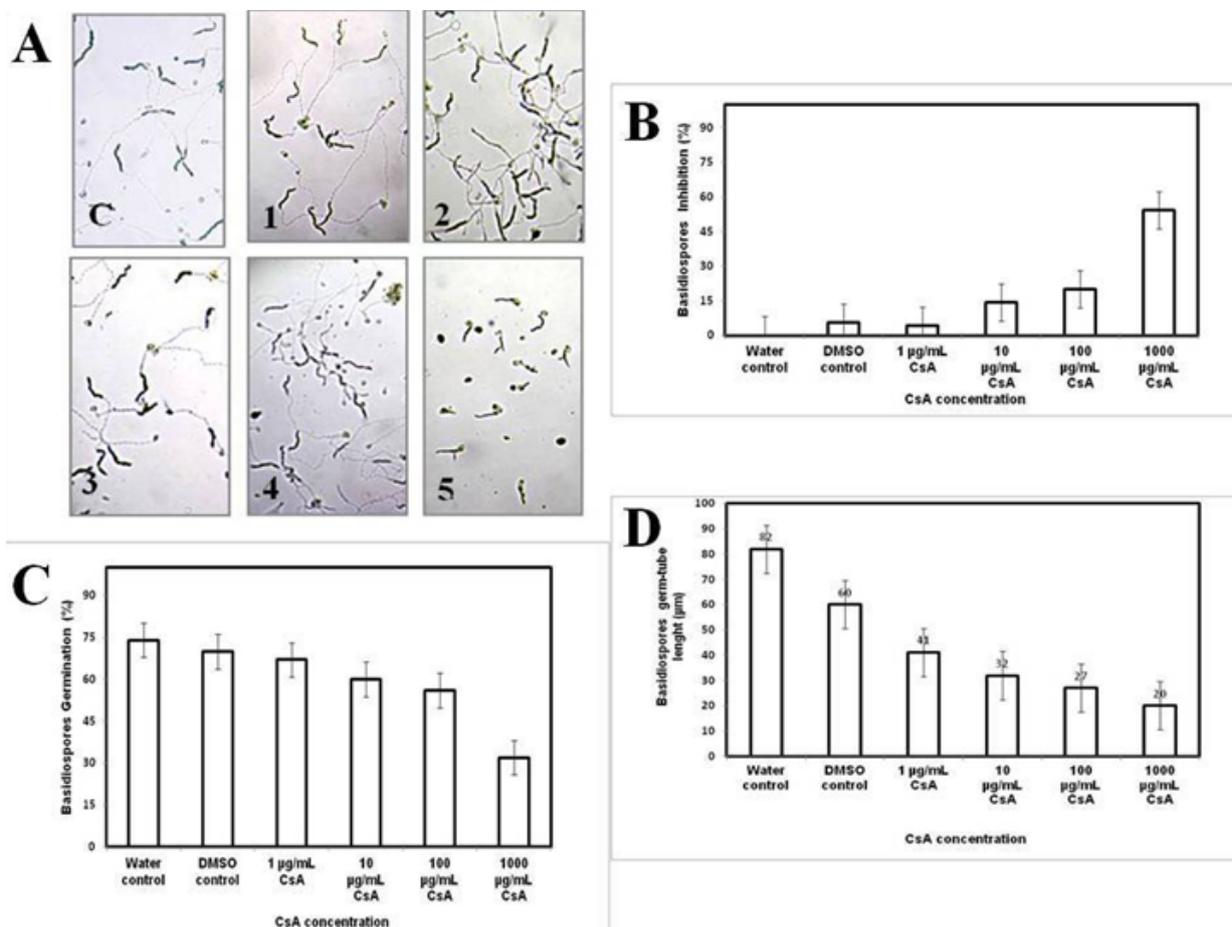


Figure 6: Inhibition of germination and germ tube development. **A-** Figure of basidiospores germination inhibition by CsA at different concentrations. **C-** control without CsA and DMSO; **1-** control in the presence of 1% DMSO; **2-** 1 µg.mL⁻¹ CsA; **3-** 10 µg.mL⁻¹ CsA; **4-** 100 µg.mL⁻¹ CsA; **5-** 1000 µg.mL⁻¹ CsA. All CsA solutions were done in 1% DMSO. **B-** Inhibition of CsA on basidiospore germination. **C-** Basidiospore germination after exposure to CsA. **D-** Effect of CsA on germ tube development after exposure to CsA. **B, C and D** - Each bar represents the mean of three independent replications of the experiment with three plates per experiment, and the error bars represent standard deviations.

that, despite CsA does not have a high inhibitory effect on basidiospores germination, it has stopped further mycelial growth (Figure D). Thus, its antifungal activity may be not fungicidal, but fungistatic, as *M. perniciosa* spore germination is possible [36]; moreover, even if the CsA is present, only 50% of germination inhibition was obtained in high concentration CsA. Yet, CsA was observed to exert an effect on *M. perniciosa* germ tube development: the incubation of basidiospores during 25 minutes with CsA at 1.0 µg.mL⁻¹ has prevented germ tube growth. Furthermore, under such CsA concentration, there was a reduction of 41%, continuing to reduce with the increase of CsA dosage, as compared to the control containing 1% DMSO (Figure 6D).

Corroborating these results, 200 nM of CsA have inhibited 50% of hyphal elongation in *Sclerotinia sclerotiorum* [37]. Tropschug and collaborators (1989) [38] have showed that, at a concentration of 1.0 µg.mL⁻¹, CsA prevents growth of *N. crassa*. The *S. cerevisiae* strains presenting the Petite phenotype were sensitive to 100 µg.mL⁻¹ CsA, whereas strains that do not present the phenotype were insensitive to CsA. The growth of most yeast strains is not inhibited by CsA, due to the lack of cyclophilin A, or express mutants that do not bind CsA [38]. The introduction of the functional cyclophilin A gene restores CsA sensitivity in yeast strains with CsA resistance, indicating that cyclophilin A is an important target to CsA toxicity [39]. However,

these authors have reported CsA-resistant strains that were not cyclophilin A mutants, and resistance may be conferred to the toxic cyclophilin A-CsA complex, possibly by calcineurin mutation [39]. The administration of CsA at a concentration of 1.0 µg.mL⁻¹ in *C. neoformans* reaches complete inhibition on *in vitro* growth [40].

The use of drugs that block the cyclosporin-calcineurin interaction may be effective in the dispersal of *M. perniciosa*, especially during the fruiting phase. Despite cyclophilin behaves as a fungistatic target, drugs that interfere with the function of this protein during the proliferation phase of the fungus may have positive effects on the increasing production of cacao.

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