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Caracterização da enzima Oxidase Alternativa do fungo *Moniliophthora perniciosa* com o auxilio das ferramentas de bioinformática

Characterization of the enzyme Oxidase Alternative of the fungus *Moniliophthora perniciosa* with the aid of bioinformatics tools

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RESUMO
Moniliophthora perniciosa (Mp) é um fungo basidiomiceto, fitopatogênico, causador da doença vassoura de bruxa no cacau (*Theobroma cacao L.*), apresenta duas fases no ciclo de vida: a tipo biotrófica (nos tecidos vivos do hospedeiro), e fase saprofítica (na qual ocorre a morte da planta). A vassoura de bruxa é uma doença responsável por causar grandes perdas na produção de cacau. Estudos anteriores mostram que o Mp apresenta a enzima oxidase alternativa (AOX), a qual está relacionada com a virulência e resistência a fungicidas. Quando submetido a situações de estresse, pincipalmente quando há comprometimento da via principal de respiração, a AOX apresenta uma via respiratória alternativa como mecanismo de sobrevivência. Em nosso trabalho, foi realizada a caracterização da enzima AOX de Mp comparada com a AOX de outros basidiomicetos, utilizando ferramentas de bioinformática, com o intuito de conhecer melhor essa enzima que proporciona sobrevivência a este fungo. Concluimos que a AOX do Mp compartilha alta similaridade principalmente com AOX do

Moniliophthora roreri, desempenhando funções semelhantes como a via respiratória alternativa, quando estes microrganismos são submetidos a situações de estresse.

Palavras-chave: *Moniliophthora perniciosa*, *Moniliophthora roreri*, oxidase alternativa, basidiomicetos, vassoura de bruxa.

ABSTRACT

Moniliophthora perniciosa (Mp) is a basidiomycete, phytopathogenic fungus that causes witches' broom disease in cocoa (*Theobroma cacao L.*), it has two stages in the life cycle: the biotrophic type (in the living tissues of the host), and the saprophytic phase (in which the death of the plant occurs). Witch's broom is a disease responsible for causing large losses in cocoa production. Previous studies show that Mp presents the alternative oxidase enzyme (AOX), which is related to virulence and resistance to fungicides. When subjected to stressful situations, especially when there is impairment of the main airway, AOX presented an alternative airway as a survival mechanism. In our work, the characterization of the AOX enzyme of Mp was compared with the AOX of other basidiomycetes, using bioinformatics tools, in order to better understand this enzyme that provides survival to this fungus. We conclude that the AOX of Mp shares high similarity mainly with AOX of *Moniliophthora roreri*, performing similar functions as the alternative airway, when these microorganisms are subjected to stressful situations.

Keywords: *Moniliophthora perniciosa*, *Moniliophthora roreri*, alternative oxidase, basidiomycetes, witches' broom.

1 INTRODUCTION

The phytopathogen *Moniliophthora perniciosa* (Mp) is a fungus that belongs to the class Basidiomycetes, order Agaricales, family Marasmiaceae [1]. This microorganism is the causative agent of cocoa's witches' broom disease [2]. The cacao tree (*Theobroma cacao*) is a tree from a tropical region native to the Amazon basin that produces one of the most valuable agricultural spices in the world: cocoa beans. As the main ingredient in chocolate, cocoa trees are the basis of a global industry that generates approximately US $ 100 billion a year [3].

The microorganism Mp has a hemibiotrophic life cycle, with the biotrophic phases characterized by being prolonged and atypical, with morphological changes in the structure of the plant; and the necrotrophic one in which the plant dies [1,4,5].

In 2008, Modengo et., analyzed the genome of *Moniliophthora perniciosa*, and identified that this fungus encodes the gene of the alternative oxidase enzyme (AOX). This protein was purified in 1987 by Elthon et al., and it was observed that AOX is capable of directly oxidizing ubiquinol and reducing oxygen to water. Studies have shown that in the Mp fungus (AOX) is expressed exclusively during the biotrophic phase of the life cycle and plays a critical role for its virulence and survival against fungicides and stress situations imposed on the fungus [6,7].

AOX reduces oxygen to water without the involvement of complexes III and IV of the electron transport chain, providing an alternative way for the cell to, among other things, survive biotic and abiotic stress [8, 9]. The main route of electron transport in eukaryotic organisms is that carried out by the cytochrome oxidase (IV) complex, a terminal component of the respiratory chain, responsible for the reaction in which the electrons of the respiratory substrate are used to reduce molecular oxygen to water. Some organisms, however, have a second oxidase, alternative oxidase (AOX), which catalyzes the transfer of electrons and the reduction of oxygen to water, thus presenting itself as an alternative way of survival [10].

Thus, it is important to know about the alternative pathway (Alternative Oxidase Pathway) to the conventional pathway, since, when the conventional pathway is inhibited, the alternative pathway is activated in the fungus *Moniliophthora perniciosa*, presenting itself as a survival mechanism . In our work, different *in silico* analyzes were performed with the aid of bioinformatics tools to obtain information on the alternative oxidase enzyme of the fungus *Moniliophthora perniciosa* compared to AOX from other basidiomycetes.

2 METHODOLOGY

2.1 IDENTIFICATION OF AOX ON MP AND OTHER BASIDIOMYCITES

The sequence of AOX-Mp and AOX of the other basidiomycetes were obtained from the NBCI (National Center for Biotechnological Information) database. The use of databases is a relevant area in bioinformatics research, particularly molecular biology, since it allows the development of in silico models [11].

2.2 BUILDING THE PHYLOGENETIC TREE

For the construction of the phylogenetic tree, the program used was ClustalW (http://www.ebi.ac.uk/Tools/phylogen/). ClustalW is a widely used program, in which multiple alignments of nucleotide or protein sequences are performed (http://darwin.informatics.indiana.edu/yonli/I519Fall09/Lab5). Methodology: ClustalW for aligment and phylogenetic tree (using neighbor-joining). (http://www.ebi.ac.uk/Tools/phylogeny/) [12].

2.3 CONSTRUCTION OF THE 3-D STRUCTURE AND COMPARISON OF THE ALTERNATIVE OXIDASE 3-D STRUCTURE WITH THAT OF OTHER BASIDIOMYCETES

To build the 3-D structure and the alternative oxidase enzyme similarity table, BLAST (Basic Local Alignment Search Tool) tools were used, which find regions of local similarity between the sequences. This program compares sequences of nucleotides or proteins to sequence databases and calculates the statistical significance of the matches. BLAST can be used to understand functional and evolutionary relationships between sequences, in addition to helping to identify members of gene families [13].

To align and overlay the AOX-Mp sequence with a closer species whose 3D structure of its AOX was available, the PyMol algorithm was used, obtaining a molecular visualization system [14].

2.4 BUILDING THE SIMILARITY TABLE

The Swiss-PDB software was used to build the similarity table, which is an application that provides a reliable interface allowing the analysis of several proteins at the same time. Proteins can be superimposed to produce structural alignments and compare their active sites or any other relevant parts [15].
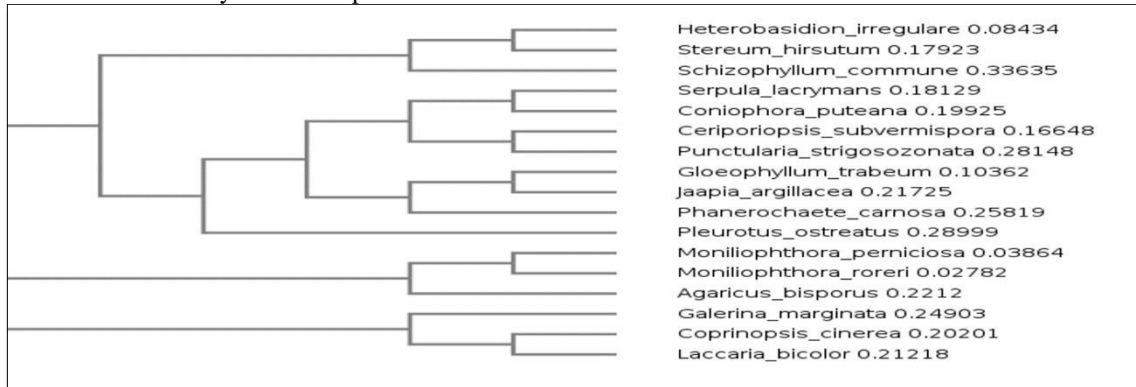
3 RESULTS AND DISCUSSION

3.1 ANALYSIS OF THE PHYLOGENETIC TREE OF MPAOX COMPARED TO THAT OF OTHER BASIDIOMYCETES

Based on the alignment of aminoacid sequences, a phylogenetic tree was built with the aid of the ClustalOmega algorithm (figure 1). With a sequence of 378 amino acids, MpAOX has high similarity with AOX protein from different species of basidiomycetes, pointing to a highly conserved function of this protein. It was observed that *Moniliophthora perniciosa* and *Moniliophthora roreri* are in the same branch, demonstrating an evolutionary proximity between the oxidases of these organisms, as well, it was demonstrated through the study of similarity between these organisms, presented in table 1. *Moniliopthora perniciosa* and *Moniliophthora roreri* are part of the same genus, which contributes to this evolutionary proximity between these basidiomycetes, thus confirming a conservation in the AOX function of these microorganisms.

The similarity between regions of proteins and conserved amino acids of Mp and *Moniliophthora roreri*, was also observed in a study by Barbosa et ,. 2018, in which a phylogenetic tree of these microorganisms was built observing many similar sequences among them [16].

Figure 1. Phylogenetic tree of the MpAOX protein compared to AOX from other basidiomycetes. The branches demonstrate the evolutionary relationships.

Heterobasidion_irregulare 0.08434
Stereum_hirsutum 0.17923
Schizophyllum_commune 0.33635
Serpula_lacrymans 0.18129
Coniophora_puteana 0.19925
Ceriporiopsis_subvermispora 0.16648
Punctularia_strigosozonata 0.28148
Gloeophyllum_trabeum 0.10362
Jaapia_argillacea 0.21725
Phanerochaete_carnosa 0.25819
Pleurotus_ostreatus 0.28999
Moniliophthora_perniciosa 0.03864
Moniliophthora_roreri 0.02782
Agaricus_bisporus 0.2212
Galerina_marginata 0.24903
Coprinopsis_cinerea 0.20201
Laccaria_bicolor 0.21218

Fonte: Os autores da pesquisa

Garmash et al., 2019, conducted a study comparing the presence of AOX in some plants, when they were subjected to stress situations, showing that the activation of AOX in species when subjected to stress, produced fewer ROSs. In this study it was also clear the presence of AOX in several species of plants, as well as in fungi, presenting the same function [17], that is, oxidative protection.

The conserved function of AOX (via an alternative to the respiratory chain) was also observed in the work carried out by Yu et., 2019, in which tobacco was subjected to salt stress, and the performance of proteins such as β-CAS and AOX was compared. The findings made in this study demonstrated results on the synergistic effect of AOX and β-CAS in protecting plants against salt stress. It was also clear from these studies that AOX helps to alleviate the toxic effect of cyanide [18].

3.2 SIMILARITY BETWEEN ALTERNATIVE OXIDASE OF DIFFERENT BASIDIOMYCETES

Table 1 shows the similarity between MpAOX with different basidiomycetes, from it it is observed that, this enzyme has a conserved function because there is a strong proximity between the fungi compared. The AOX of *Moniliophthora perniciosa* and that of *Moniliophthora roreri* showed 94% similarity, thus showing high conservation among amino acid residues and, consequently, conservation among the functions of these microorganisms.

In 2014 Meinhartd et al., compared the genome of *Moniliophthora perniciosa* with *Moniliophthora roreri* and found that these microorganisms have many conserved genome sequences, which corroborates the similarity between these microorganisms, shown in this experiment. They also did a bi-directional analysis and put together a Venn diagram of several basidiomycetes, such as *Laccaria bicolor,* Cropnopsis cinerea and compared it with *Moniliophthora roreri*, and concluded that they have less similarity, when compared to the similarity between *Moniliopthora roreri* and *Moniliophthora pernicious*, although all are basidiomycetes [19].

According to Aime et al., the similarities between MpAOX and AOX of *M. roreri*, can be justified by some characteristics that these two species share; both belong to the phylum basidiomycota, order agaracales, class agaricomycetes, family marasmiacea, and are hemibiotrophic.

Table 1- Identification of the amino acid sequence of MpAOX with the AOX of other basidiomycetes

Basidiomycetes	Genbank No	Identity with MpAOX (%)
Moniliophthora perniciosa	**ABN09948.3**	**100%**
Moniliphthora roreri	**XP-007845663.1**	**94%**
Schizophyllum commune	XP-003034454.1	71%
Agaricus bisporus	XP-006462706.1	71%
Heterobasidion irregulare	XP-009546184.1	70%
Gloeophyllum trabeum	XP-007866923.1	69%
Stereum hirsutum	XP-007304627.1	67%
Galerina marginata	KDR-82729.1	66%
Laccaria bicolor	XP-001876309.1	65%
Punetularia strigosozonata	XP-007380153.1	64%
Cropnopsis cinerea	XP-001835681-1	64%
Ceriporiopsis subvermispora	EMD-39933.1	64%
Pleurotus ostreatus	KDQ25985.1	63%
Jaapia argillacea	KDQ62279.1	63%
Serpula lacrymans	XP-007319753.1	63%
Phanerochaete carnosa	XP-007401575.1	62%
Corniophora puteana	XP-007768403.1	62%

Fonte: Os autores da pesquisa

In filamentous fungi, the presence of the alternative oxidase enzyme can also be observed, performing a function similar to that developed by AOX present in basidiomycetes. Bosnjak et., 2019, carried out a study in which the presence of the AOD-1 gene was verified in the filamentous fungus Neurospora crassa, responsible for encoding the alternative oxidase, thus confirming the presence of AOX in this fungus. In this work, it was observed that the enzyme AOX performs the same function as an alternative pathway seen in basidiomycetes [20].

3.3 TRIDIMENSIONAL STRUCTURE OF MPAOX

Using the bioinformatics tools, the overlapping regions of MpAOX compared to AOX of *Moniliophthora roreri* were determined. With the aid of the Pymol tool, it was possible to align and superimpose the AOX-Mp amino acid sequence with a closer species, whose 3D structure of its AOX was available. Thus, through the BLASTp and Swiss-PDB programs, it was possible to generate a 3D structure for AOX by *M. perniciosa* and *M. roreri* (figure 2). As a result, it is possible to observe similar structural models, where it is possible to verify that both present conformations in alpha-propellers and overlapping beta sheets. Conserved regions were found among the alternative oxidase of these basidiomycetes, which was already expected, since it plays a similar role in basidiomycetes.

Other similar works, such as that of Castro et al., 2017 also characterized the three-dimensional AOX structure of some citrus plants (*C. Clementina, C. Sinensis*), and observed the conservation of some AOX domains involved in the mitochondrial respiratory chain providing a alternative route for electron transport (via AOX alternative) [21].

The alternative route of AOX was also studied in the fungus *Aspergillus niger*, and in this research the survival of strains of this fungus with overexpression of AOX and normal strains was compared, which were submitted to respiratory chain inhibitors. The obtained result indicated that in media with respiratory chain inhibitors a higher growth rate of strains with AOX overexpression is observed, showing the effectiveness of the alternative pathway for the survival of the fungus [22].

Figure 2. 3D structure of *Moniliophthora roreri*, *Moniliophthora perniciosa*, and overlapping of conserved areas between AOX of these two basidiomycetes, respectively.
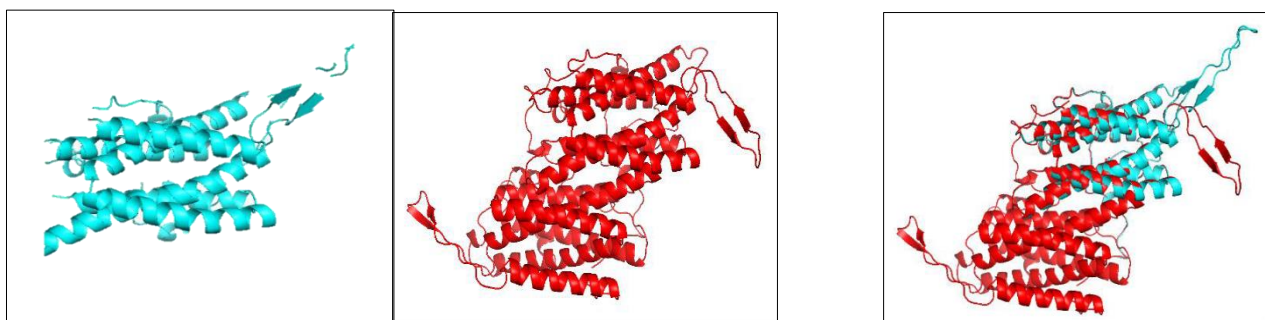
Fonte: Os autores da pesquisa

Many studies have demonstrated the expression of AOX in several fungi, both in basidiomycetes and filaments, demonstrating the alternative pathway of this enzyme, as a common factor among these microorganisms. In *Moniliophthora perniciosa* this protein presents itself as one of the main difficulties for the control of witches' broom. In order to block this alternative route, Bartoss

et al., 2019 developed a work in which it presented new perspectives to inhibit the alternative Mp oxidase [23].

4 FINAL CONSIDERATIONS

In general, the characterization of the alternative oxidase enzyme of the basidiomycete *Moniliophthora perniciosa* compared to the alternative oxidase of other basidiomycetes, demonstrated important conservation and similarity in function. This demonstrates the relevance of this macromolecule for the survival of these microorganisms when subjected to stresses.

5 VALIDATION

The Ramachandran diagram allows you to visualize all possible combinations of dihedral angles Ψ (psi) versus Φ (phi) in the amino acids of a polypeptide. This contributes to the conformation of protein structures. This diagram therefore allows us to predict which will be the secondary structure of the peptide, since there are combinations of typical angles for each structure (α-helix and β-leaf). The conformation of the peptides is defined by assigning values for each pair of corners canti, Ψi for each amino acid.

In this context, it is known that in the second quadrant are the combinations of the β sheet, in the third quadrant are the right α helix and the curves or loops; and in the first quadrant the combinations of the left α helix.

Therefore, with the aid of the RAMPAGE tool, AOX-Mp's Ramachadran was generated and it is observed that, it is in accordance with the expected structure, as as shown in figure 8, the larger composition is due to helix-type structures, located in greater number in quadrant 1 and 3 (Figure 4).

Figure 04. Ramachandran diagram
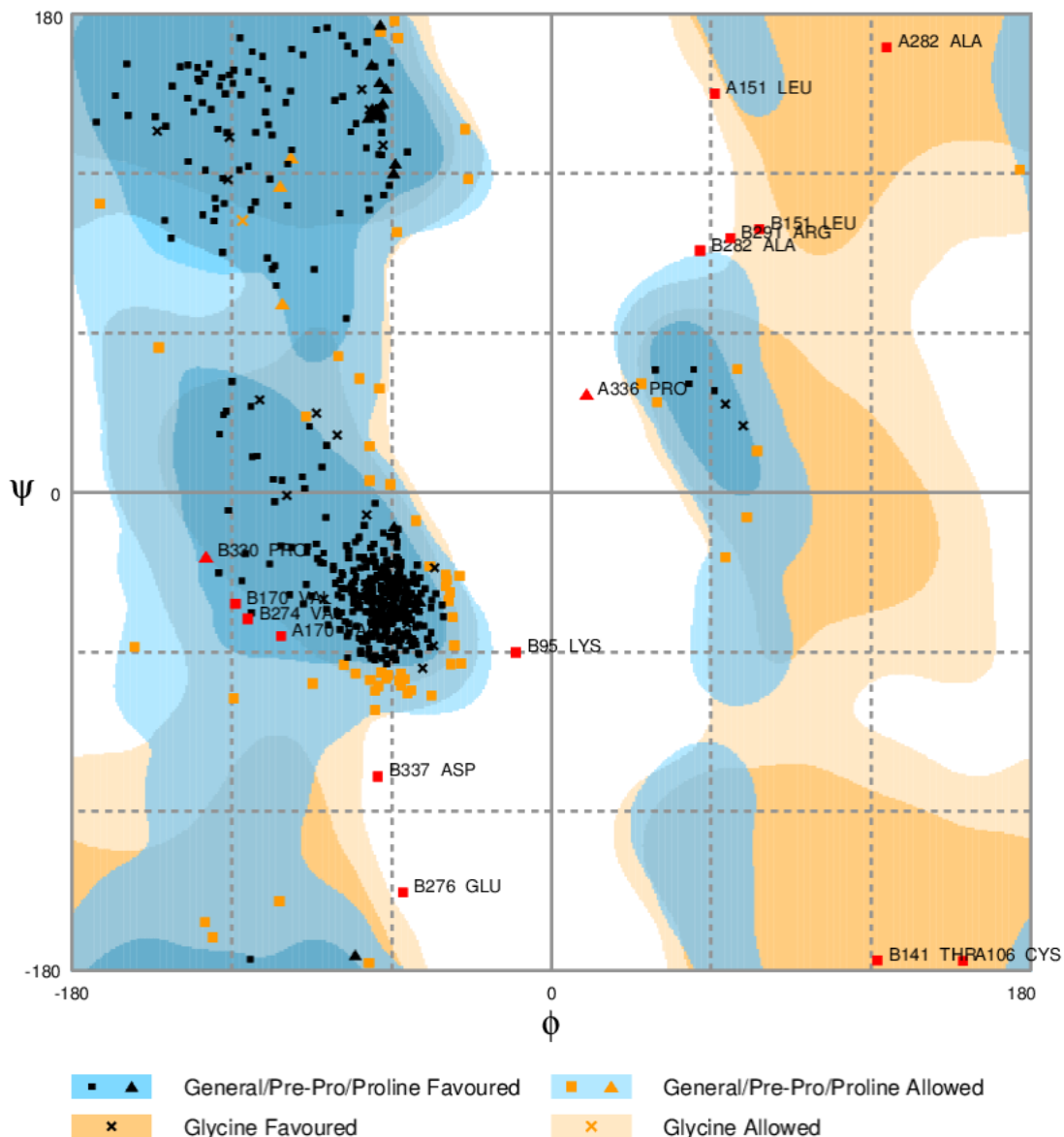
Fonte: Os autores da pesquisa

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