GABRIEL ALVES

SUBSTITUTION OF AMINO ACID RESIDUES IN THE CYTOCHROME B PROTEIN FROM *Phytophthora infestans*: FUNCTIONAL IMPLICATIONS AND AZOXYSTROBIN SENSITIVITY.

Dissertation submitted to the Plant Pathology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*

Adviser: Eduardo Seiti Gomide Mizubuti

Co-adviser: Marcelo Depolo Poleto

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Assent:

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Totus tuus

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ABSTRACT

ALVES, Gabriel, M.Sc., Universidade Federal de Viçosa, July, 2023. **Substitution of amino acid residues in the cytochrome b protein from *Phytophthora infestans*: Functional implications and azoxystrobin sensitivity**. Adviser: Eduardo Seiti Gomide Mizubuti. Co-adviser: Marcelo Depolo Poleto.

Late blight is caused by the oomycete *Phytophthora infestans* and is one of the main biotic factors that compromise yields of potato and tomato crops. Management of late blight is almost exclusively dependent on the application of oomicides. Azoxystrobin is a site-specific active ingredient that has "transkingdom" action, once it can be effective against fungal and oomycete plant pathogens. Unfortunately, reports of both fungal and oomycetes resistant to azoxystrobin are relatively common. Nevertheless, to date, there is no report of resistance in populations of *P*. *infestans* to azoxystrobin. The use of computational methods of molecular modeling applied to the study of proteins can help in the evaluation of the reasons why events of resistance to azoxystrobin have not been observed so far. For *P*. *infestans*, all positions in cytochrome b protein (cytB) that correspond to the loss of efficiency to azoxystrobin are set back one position in relation to the sequences of cytB of other microorganisms, with position 142 being the most important for the loss of efficiency of the molecule. The region around the site of interaction with the oomicide in *P. infestans* cytB is slightly variable, supporting less than 33% of the total number of possible substitutions. Position 142 can support a large number of residues without compromising stability, but which lead to resistance to azoxystrobin, which highlights the high risk of resistance events to this molecule. The non-occurrence of resistance to azoxystrobin in *P. infestans* seems to be related to a genomic factor that governs the stability of the cytochrome b gene sequence, since from the biophysical point of view there is no impediment to resistance to azoxystrobin. Such an approach may favor the understanding of the rules that govern the oomicide-protein molecular target interaction, serving as a theoretical subsidy for predicting the probability of loss of sensitivity in the plant pathogen population, as for other molecules that target the same protein. It is also expected that the adoption of this approach may help in the rational design of other oomicides and fungicides, and also control efficiency monitoring.

Keywords: Molecular modeling. Oomicides. Protein diversity.

RESUMO

ALVES, Gabriel, M.Sc., Universidade Federal de Viçosa, julho de 2023. **Substituição de resíduos de aminoácido no citocromo b de *Phytophthora infestans*: Impactos funcionais e na sensibilidade a azoxistrobina**. Orientador: Eduardo Seiti Gomide Mizubuti. Coorientador: Marcelo Depolo Poleto.

A doença conhecida como requeima, causada por *Phytophthora infestans,* é um dos principais fatores bióticos que comprometem a produtividade da batateira e do tomateiro. A aplicação de oomicidas é o principal método de controle desta doença. Curiosamente, a molécula azoxistrobina, utilizada para o controle de doenças causadas por fungos e oomicetos, da qual se tem relatos de resistência em diferentes fitopatógenos, não possui relato de resistência em populações de *P. infestans*. A utilização de métodos computacionais de modelagem molecular aplicados ao estudo de proteínas, podem auxiliar no entendimento do porquê eventos de resistência à azoxistrobina não foram observados na população de *P. infestans* até o momento. Observamos que para *P*. infestans a posição 142, na proteína citocromo b, corresponde a 143, relatada em outros fitopatógenos como mais importante para a resistência à azoxistrobina. A região do citocromo b, correspondente ao entorno do sítio de interação do oomicida é ligeiramente variável, suportando menos de 33% do total do número de substituições teoricamente possíveis. A posição 142 pode suportar um elevado número de resíduos sem comprometer a estabilidade proteica mas que levam a resistência a azoxistrobina. A não resistência de *P. infestans* a azoxistrobina parece estar relacionada a eventos que governam a estabilidade da sequência do DNA mitocondrial responsável pela síntese do citocromo b, uma vez que do ponto de vista de biofísica da proteína, não há impedimento à ocorrência de resistência à azoxistrobina. O uso de métodos computacionais de modelagem molecular pode favorecer a compreensão das regras que governam a interação entre oomicida-proteína servindo de subsídio teórico para predição da probabilidade da perda de sensibilidade em populações de fitopatógenos. É esperado que esta abordagem possa ajudar no design racional de outros oomicidas e fungicidas, e no monitoramento da eficiência no campo.

Palavras-chave: Modelagem molecular. Oomicida. Diversidade proteica.

SUMMARY

1. GENERAL INTRODUCTION

Among the approaches that can be used to monitor resistance, the study of specific substitution events of amino acid residues in the protein targeted by the fungicide or oomicide is a promising strategy. The relationship between residue substitutions and the loss of efficiency of molecules from different groups (benzimidazoles, myosin inhibitors, sterol demethylation inhibitors, external quinone inhibitors, succinate dehydrogenase inhibitors, anilinopyrimidines, carboxylic acid amides, and OSBPI inhibitors) in different species of plant pathogenic fungi and oomycetes is frequently reported (YIN et al, 2023), but apparently superficially studied and not fully understood. New approaches that explore features at the atomic level such as protein biophysics up to the population scenario such as molecular evolution studies may provide much broader insights related to resistance management as well as can be helpful for the design of new molecules.

The general objective of this dissertation was to study the propensity of point-substitutions of amino acid residues, through computational method, in the cytochrome b protein and their impact on the sensitivity of *Phytophthora infestans* to azoxystrobin. The specific objective was to use the saturation mutagenesis approach to investigate why resistance of *P. infestans* to azoxystrobin has not been observed. The study has the potential to provide valuable information on the evolution of oomicide resistance in *P. infestans*, as well as of other molecules, in other plant pathogens. Additionally, the approach used here may be helpful for the development of new antimicrobial compounds to be used in agriculture.

2. Literature review

2.1. Phytophthora infestans: Agricultural relevance, disease management and the problem of resistance to chemical compounds.

Phytophthora infestans (Mont.) de Bary is the causal agent of potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) late blight. Potatoes and tomatoes are the main vegetables cultivated in Brazil and late blight is one of the major biotic factors that hamper yield and profitability of both crops. Historically, late blight caused The Great Famine, which was devastating in many European

countries, especially in Ireland, in the mid-19th century (WEBSTER & WEBER, 2007. Late blight epidemics were severe, the weather conditions were favorable, the potato varieties were susceptible and there was no chemical compound that could be applied to potato fields (HAVERKORT et al, 2008; WEBSTER & WEBER, 2007). All factors combined led to an extreme shortage of potato supply and millions starved to death (Woodham-Smith, 1962). Up to this day the social and political damages are remembered. Unfortunately, late blight epidemics continue to be destructive in potato and tomato crops and many research groups dedicate considerable resources to develop more effective ways to cope with the disease (BERHAM, 2021; WEBSTER & WEBER, 2007).

In potato crops, long-distance spread of the pathogen can occur by infected seed tubers (BERHAM, 2021; FRY, 2016). The introduction of variants into a population or the movement of several individuals of a clonal lineage of the pathogen can be accompanied by negative economic and social impacts (FRY, 2016). Once the disease is established in an area, late blight can quickly compromise large proportions of plant tissue, partly due to the short latent period associated with the asexual cycle (5-7 days) and also to the easiness of pathogen spread (BERHAM, 2021).

Currently, there are no varieties that combine agronomic characteristics of interest and resistance to late blight. Therefore, the application of chemical compounds such as fungicides and oomicides, that is, chemical compounds with specific action against oomycetes (GOVERS, 2001) is the most effective management strategy (BERHAM, 2021). Disease control is challenging because it requires proper timing of oomicide application, and does not allow for errors, as late blight epidemics have high rates of progress. In Brazil, there are 208 agrochemicals registered for the control of late blight in potato crops and 214 for tomato crops (AGROFIT, 2023). The high number of products reinforces the dependence on chemical control for late blight management and the effectiveness of these compounds.

The inappropriate use of oomicides, however, can result in the selection of resistant individuals that will eventually constitute populations of *P. infestans* that will not be affected by chemical compounds. There are reports of resistance in *P. infestans* to metalaxyl, mefenoxam, propamocarb, and recently, to mandipropamid (GRÜNWALD & FLIER, 2005; MÖLLER et al, 2009; ABULEY et al, 2023).

Interestingly, there are no known reports of resistance of *P. infestans* to some active ingredients that are used in the management of late blight, such as azoxystrobin, cymoxanil, fluopicolide, cyazofamid, dimethomorph, fluazinam, and oxathiapiprolin (Saville et al, 2015; GRÜNWALD et al, 2006; FRAC, 2023a; FRAC, 2023b). On the other hand, in Mexico, even before the beginning of the use of metalaxyl, isolates of *P. infestans* resistant to this active ingredient already occurred (GRÜNWALD & FLIER, 2005). Baseline and periodic monitoring of pathogen sensitivity to different oomicides in populations are crucial to protect molecules from evolution of resistance (YIN et al, 2023).

Among the approaches that can be used to monitor resistance, the study of specific substitution events of amino acid residues in the protein targeted by the fungicide or oomicide is a promising strategy. New approaches that explore features at the atomic level such as protein biophysics up to the population scenario such as molecular evolution studies may provide much broader insights related to resistance management as well as can be helpful for the design of new molecules.

2.2. Nuclear and mitochondrial genome of *Phytophthora infestans*: Organization, transmission, and diversity.

The total (tDNA), nuclear (nDNA), and mitochondrial (mtDNA) genomes of *P. infestans* have intriguing features. The estimated size of the tDNA is 240 Mb, which is considerably larger than the genome of some *Phytophthora* species, such as *P. soyae* (95 Mb) and *P. ramorum* (65 Mb) (HAAS et al, 2009). The tDNA has a high percentage of repetitions, 74%, a G+C content of about 51%, approximately 17,797 genes predicted by computational method, and large intergenic regions (HAAS et al, 2009). The gene density distribution is considered to be discontinuous and unusual. Greater density of genes, which are conserved in the genus *Phytophthora*, is found in regions rich in repetitions. In these regions, for example, effector genes are present. The high plasticity of the region and, consequently, of the effector genes, as well as their number, is apparently associated with the specificity of the pathogen and the ability to quickly overcome resistance in plants (HAAS et al, 2009).

The sequencing of the genomes of two individuals of *P. infestans* from South Korea produced divergent results from previous work (HAAS et al, 2009). The Korean isolates were sequenced using the long read and short read methods and for

one of the individuals a smaller genome size was estimated; and the other individual had a genome larger than those previously sequenced; lowest number of repeats, and highest number of genes (LEE et al, 2020). This result, which differs from the work of Haas and collaborators, may be conditioned by the genome assembly method, gene prediction or the natural diversity of the pathogen. However, an assertive answer for this difference in the tDNA of the different sequenced individuals has not yet been reported in the literature.

Reproduction plays a central role in tDNA transmission and diversity. In a study involving isolates of *P*. *infestans*, mating types A1 and A2, from Mexico, the occurrence of recombination in matings and a chromosomal pattern consistent with diploid individuals was verified. Additionally, a high rate of oospore germination and self-fertile individuals from the crosses were recovered (SHATTOCK et al, 1986). In 1987, Tooley and Therrien, using Feulgen's method, evaluated the ploidy of isolates from Mexico, the United States, Holland, Ireland, Wales, and Italy. The Mexican population was mostly diploid and the non-Mexican population had variable ploidy with diploid, triploid, tetraploid and aneuploid individuals; with the suspicion that tetraploid individuals, in practice, function as diploids (TOOLEY & THERRIEN, 1987).

Research involving the mitochondrial genome of *P. infestans* and the inference of genes present in mitochondrial DNA (mtDNA) began just over 25 years ago. These were of fundamental importance to elucidate phylogenetic questions, mainly the reclassification of oomycetes as not belonging to the kingdom Fungi (PAQUIN et al, 1997).

Still in the field of phylogenetic studies, full sequence of *cox1* and partial sequence of the *cox2* genes (cytochrome oxidase genes) are useful for sorting *Phytophthora* species. From the 27 species of the genus *Phytophthora*, including *P. infestans*, there was little intraspecific diversity, but greater interspecific variation. Thus, the cox1 and cox2 regions are considered useful for phylogenetic studies within the genus (MARTIN & TOOLEY, 2003).

The mtDNA of *P. infestans* resembles the mitochondrial DNA of bacteria and chloroplasts. All genes can be translated following the universal genetic code. However, the tRNA for tryptophan must be imported from the cytoplasm. Furthermore, no introns were observed in the mitochondrial genome and there are no double-hairpin elements (PAQUIN et al, 1997).

The mtDNA of *P. infestans* can be divided into four haplotypes: Ia, Ib, IIa and IIb. Haplotypes differ from each other in terms of genome size: 37,922bp (Ia), 39,870bp (IIa), 37,957bp (Ib), and 39,840bp (IIb) (Avila-Adame et al, 2005). The main difference reported between haplotypes I and II is the size of the region (≈1.8 kbp) between the *trny* gene and *rns*, considered of high variability. Considering the sequence identity between genomes Ia and Ib and between IIa and IIb, the value is close to 99.99%. All haplotypes have less than 23% of G+C content and a coding region greater than 90%. As for the annotation, 61 genes with known functions are reported and genes may occur on both mtDNA strands. Six ORFs are described for haplotype I and 13 for haplotype II whose functions remain unknown (Avila-Adame et al, 2005).

When we consider mitochondrial diversity, the mode of mtDNA transmissivity to offspring can explain the scarce mtDNA diversity in a population. In fungi, mitochondrial heredity is mostly uniparental, with less frequent biparental inheritance (TAYLOR, 1986). In *P. infestans*, through the study of mature (3 weeks) and immature (5 days) oospores, from mating types A1 and A2, the uniparental inheritance of mtDNA and the absence of segregation and recombination were verified, but which parent provided the mtDNA was not identified (WHITTAKER et al, 1993). In *P. parasitica*, uniparental maternal inheritance of mtDNA is credited (FÖRSTER & COFFEY, 1990). Speculating, the fact that *P. parasitica* and *P. infestans* belong to the same genus may indicate similar transmissibility behavior.

Considering the inheritance pattern of mtDNA, according to Hill (2020), an accumulation of deleterious mutations in this genome would be expected, however, such expectation has not been met. The mitonuclear compensatory coevolution hypothesis is a possible explanation for the non-accumulation of mutations in the mtDNA. According to this hypothesis, genes in the nDNA have evolved to compensate for the effects of deleterious alleles on mtDNA. In this sense, one of the functions of genes derived from nDNA is to provide stabilizing proteins for mitochondrial electron transport chain (ETC) complexes (HILL, 2020). The author also presents the term mitonuclear coadaptation to refer to the coordination of functions of mitochondrial and nuclear genome products for the efficiency of mitochondrial oxidative phosphorylation (HILL, 2020)

Mutation of a nucleotide in mtDNA that leads to substitution of an amino acid residue in the protein are frequent in oomycetes, fungi, and mammals. Genomic

rearrangements, mutations in large regions, and recombination do not necessarily occur in all species and with the same frequency in fungi and oomycetes (TAYLOR, 1986).

In a study of the diversity of mtDNA and nDNA of *P. capsici*, *P. citrophthora*, P*. megakarya*, *P. palmivor*a, *P. citricola* and *P. parasitica*, there was greater conservation and lower mutation rate of mtDNA in relation to nDNA (FÖRSTER et al, 1990), due to the studied species sharing the same genus of *P. infestans*, such behavior may also occur in this pathogen.

In addition to reproduction, the mutation rate is an interesting variable to study about the mtDNA. Identifying the difference in mutation rate between mtDNA and nDNA may provide an explanation for the observed diversity of these two genomes in the *P. infestans* population. Furthermore, one can assume that the probability of transmissibility of desired traits or the occurrence of undesired phenotypes, such as those that lead to resistance to fungicide and oomicide molecules, must be taken into account when envisioning a broad analysis of antimicrobial resistance in crops.

2.3. Cytochrome bc1 complex: Constitution, function, and evolution.

Cytochrome bc1 is a protein complex that is present in complex III of the mitochondrial electron transport chain (ETC) in eukaryotes, and also in the energy transduction membrane in prokaryotes (FISHER et al, 2020). In eukaryotes, cytochrome bc1 is responsible for the oxidation of ubiquinol and the reduction of semiquinone through the transfer of one electron for each of the processes, and four protons for each electron transferred to the intermembrane space. This hypothesized transfer system of protons and electrons in the bc1 complex is called the Q cycle and this event allows the correct functioning of the ETC culminating in the synthesis of ATP (FISHER et al, 2020; XIA et al, 2013).

The bc1 complex in eukaryotes is formed by three main protein subunits (Figure 1) that form the catalytic motor of the enzyme responsible for the transfer of protons and electrons, namely: cytochrome b, iron-sulfur protein (ISP), and cytochrome c1 (FISHER et al, 2020; XIAO et al, 2014). Such proteins are linked together in this order. The other subunits of the bc1 complex vary in number among eukaryotes, from eight to nine, and are called supernumerary subunits (Figure 2).

The main function of such subunits is credited to stabilizing the bc1 complex, despite not acting directly in the transport of protons and electrons (XIA et al, 2013).

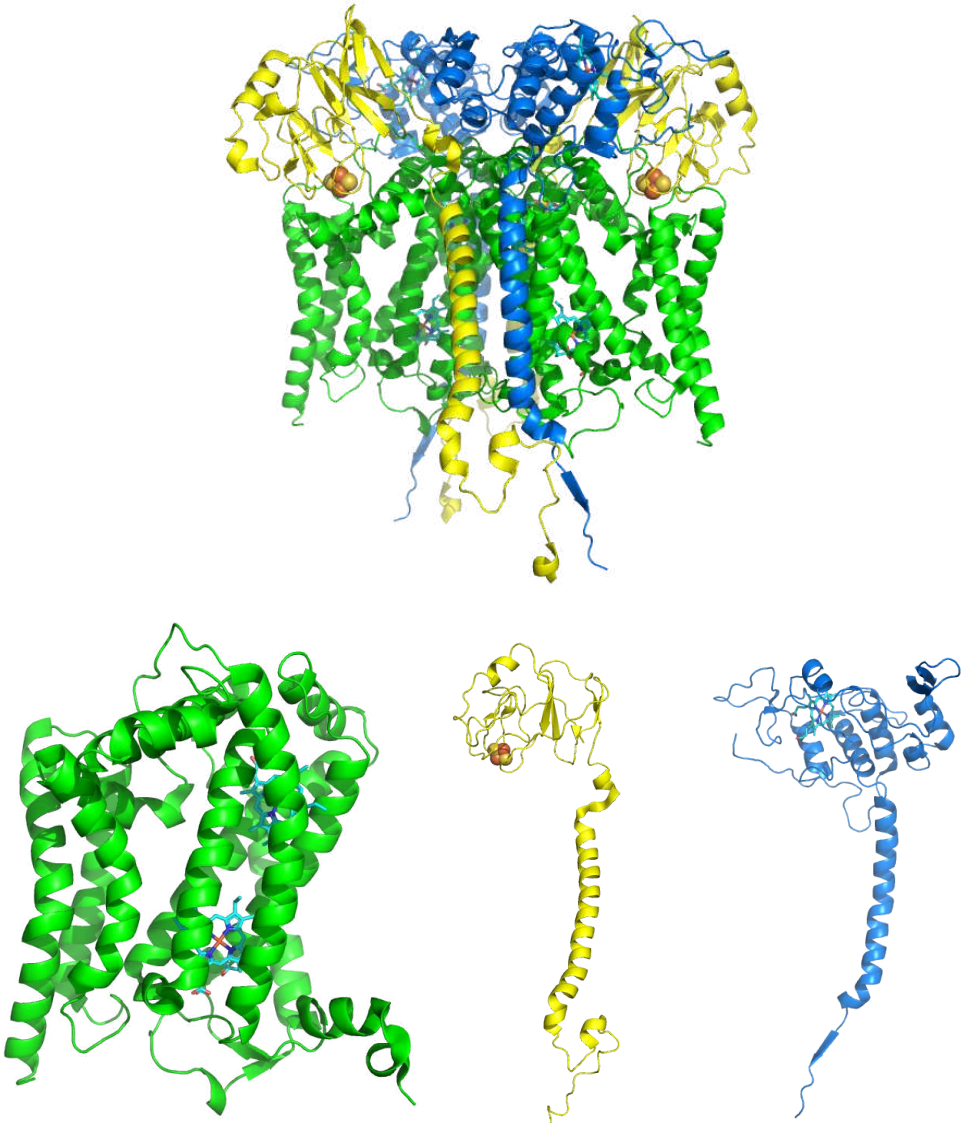

Figure 1. Cartoon representation of cytochrome protein of *Phytophthora infestans*. In the upper part of the figure the components are depicted as a complex: cytochrome b (green), iron-sulfur protein (yellow) and cytochrome c1 (blue). In the lower part of the figure, the components are depicted separately: cytochrome b (green), iron-sulfur protein (yellow) and cytochrome c1(blue).

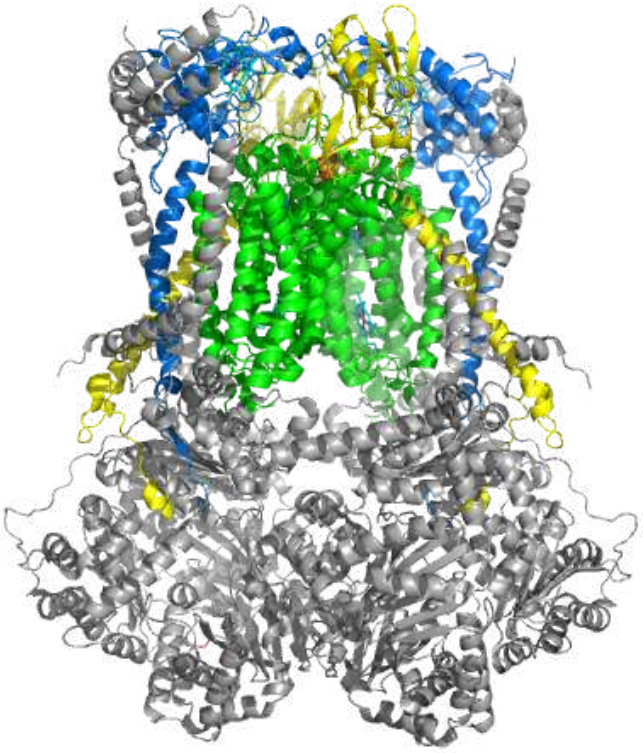

Figure 2. Cartoon representation of the cytochrome bc1 complex, cytochrome b (green), iron-sulfur protein (yellow), cytochrome c1 (blue) and supernumerary subunits (gray).

The genes that encode the proteins of the bc1 subunits are mostly found in nDNA, but the one responsible for the synthesis of cytochrome b is present in the mtDNA (FISHER et al, 2020). It is reasonable to think that such organization of the bc1 genes may be related to the mitonuclear compensatory coevolution hypothesis (HILL, 2020).

Problems associated with the functioning of the complex III of the ETC play a central role in the survival of an organism given that the bc1 is the complex with the greatest participation in the formation of the mitochondrial intermembrane proton gradient, crucial for the phosphorylation of ADP to ATP. Substitutions of amino acid residues in the bc1 can, for example, increase electron transport, and therefore, favor the escape of electrons to oxygen molecules, which can increase the content of superoxide anions with potential negative effect for the organism (XIA et al, 2013). The substitution of amino acid residues at position 155 of cytochrome b (Figure 3) is postulated to be capable of inactivating the ETC complex III, due to alterations in the interaction of cytochrome b with ISP, by limiting the movement of the alpha helix of the protein (XIA et al, 2013). In this sense, evaluating cytochrome bc1 from an

evolutionary point of view allows us to understand the limitations that substitutions of amino acid residues can impose on its correct functioning.

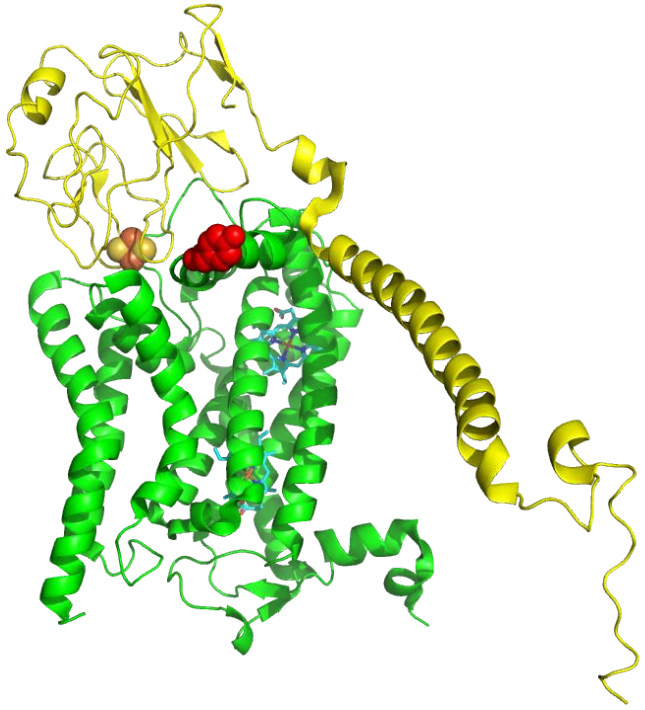

Figure 3. Cartoon representation of cytochrome b (green) and iron-sulfur protein (yellow), in dots (red) amino acid residue position 155 of cytochrome b.

Studying protein evolution, in general, is not a trivial process. Recent developments in the areas of genomics, transcriptomics, phenotyping and structural biology have enabled advances that allowed better understanding of molecular evolution (LASKOWSKI et a, 2009). Considering the bc1 complex, it can be described as a permanent obligate protein heterocomplex due to constitution by different proteins, and by the non-transient protein-protein interaction (NOOREN & THORNTON, 2003). Additionally, it is important that it be characterized in terms of the stability of the oligomeric complex state.

The oligomeric state requires that all interactions of a complex be driven by the concentration of the protein components of this complex and its free energy relative to the alternative states. In this sense, the protein-protein interactions in a complex can be controlled by changing the local concentration of the protein components that form the complex, or by the influence of binding affinity, which in turn is determined by the physical chemistry and geometrics properties of the interaction interface (NOOREN & THORNTON, 2003). The controller of the

oligomeric state is called “encounter” (NOOREN & THORNTON, 2003), where the association between proteins depends on the interaction of the surfaces, requiring co-location in time and space, is the likely controller associated with bc1.

In any protein complex, it is of fundamental importance to characterize the protein-protein interaction surface, regarding the hydropathic index, hydrogen bonds, and also regarding the planarity, size and shape of the interaction surface (JONES & THORNTON, 1996). Such indicators help to elucidate the evolution of the complex. In the case of oligomers maintained by evolution, the increase in interaction stability will not necessarily be the selected factor. In fact, the functional improvement of the complex seems to be the determinant factor (NOOREM & THORNTON 2003).

The evolution of a protein complex is determined by the evolution of one, or several, subunits of the complex. Thus, the study of protein evolution, restricted to one or a few proteins, deserves attention. Events as duplication of a gene followed by change in sequence, the activity of transposable elements, replacement of amino acid residues, and even the process of intron excision, can be considered as a "toolkit" for the evolution of proteins (LASKOWSKI et al, 2009). Due to the abundance of information, the study of the evolution of enzymatic proteins, favors the understanding of the evolution of proteins in general.

For enzymatic proteins, the characterization of substrate specificity (high specificity, group specificity, binding specificity and low specificity), and the so-called modes of evolution, are important to understand their evolution (TYZACK et al, 2017; COPLEY, 2017). Three modes of evolution, creeping, leaping, and *de novo*, are associated with protein enzymes (TYZACK et al, 2017). The first is characterized by modifications generated by point mutations or domain fusion, causing specificity to the substrate. In the second case, the changes in the protein are greater, such as changes in catalytic residues, being related to changes in the function of the enzyme (TYZACK et al, 2017). In the third case, there is gene duplication, followed by gain of function due to the replacement of one or a few residues, and fixation of the new protein in the population due to fitness improvement (SIDDIQ et al, 2017). Additionally, the study of protein homology and structural similarity, associated with the information inherent to the protein family, allow us to understand, in parts, the path of evolution (RISON & THORNTON, 2002).

The bc1 complex can be considered as highly specific in light of its function. The structural core of a protein with enzymatic function is rarely altered in the

evolutionary process and substitutions of amino acid residues in the active site located in flexible regions favor the evolution of the protein, in key residues for the function, substitutions can have a negative effect such as loss of function (TYZACK et al, 2017). Based on evidence available, the evolution of the main subunits of the complex, cytochrome b, ISP, and cytochrome c are likely to play a fundamental role in maintaining the function and elucidating the evolutionary behavior of bc1.

In addition to the impact of amino acid residue substitutions, natural organic molecules such as strobilurin can inhibit complex III (KRAICZY et al, 1996). The impact of natural inhibitors aroused interest in the study and development of synthetic compounds, consisting of an important tool in the management of fungi and oomycetes of agricultural importance (YIN et al, 2023;FISHER et al, 2020; XIAO et al, 2014; ESSER et al, 2014; GRASSO et al 2006; GRÜNWALD et al, 2006; ISHII et al. 2001; GISI et al, 1997; DEGLI ESPOSTI et al 1994; LINK et al 1993;). Despite the efficiency of synthetic cytochrome b inhibitors, Hollomon (2015) classified them as high risk for the development of resistance in oomycetes, based on the occurrence of point substitutions of amino acid residues that interfere with the efficiency of the inhibitor. However, the lack of resistant individuals in some oomycetes such as *P. infestans* (YIN et al, 2023, FRAC 2023b) is an intriguing fact. It is worth paying attention to studies of the evolutionary dynamics of cytochrome b, which could help maintain the efficiency of such inhibitors. Furthermore, considering this knowledge, the synthesis of new inhibitors of the bc1 complex should target less variable regions in order to limit the risk of resistance.

2.4. Azoxystrobin: Action mechanism and resistance.

Azoxystrobin is a molecule with anti-fungal and anti-oomycete action, it is part of the ETC complex III inhibitors of the group of external quinone (QoI) inhibitors or proximal bl inhibitors (FISHER et al, 2020) or even inhibitor of the Qp site. Functionally and structurally, azoxystrobin disrupts electron transfer from cytochrome b to the ISP, in the ubiquinol oxidation site (FISHER et al, 2020, LINK et al, 1993). From co-crystallization data of azoxystrobin with cytochrome b from *Gallus gallus* (PDB-3L71), the methoxyacrylate group of the oomicide forms hydrogen bonds with the amino acid residue at position 272 and the group known as the aromatic bridge appears to interact with residues at positions 271 and 143 (Figure 4) (ESSER et al,

2014). Very similar patterns of interaction were found in the co-crystallization of the same molecule with cytochrome b from *Bos taurus* (PDB-1SQB): The aromatic bridge interacting with the residues at positions 270 and 142 and the hydrogen bonding with the residue at position 271 (Figure 5).

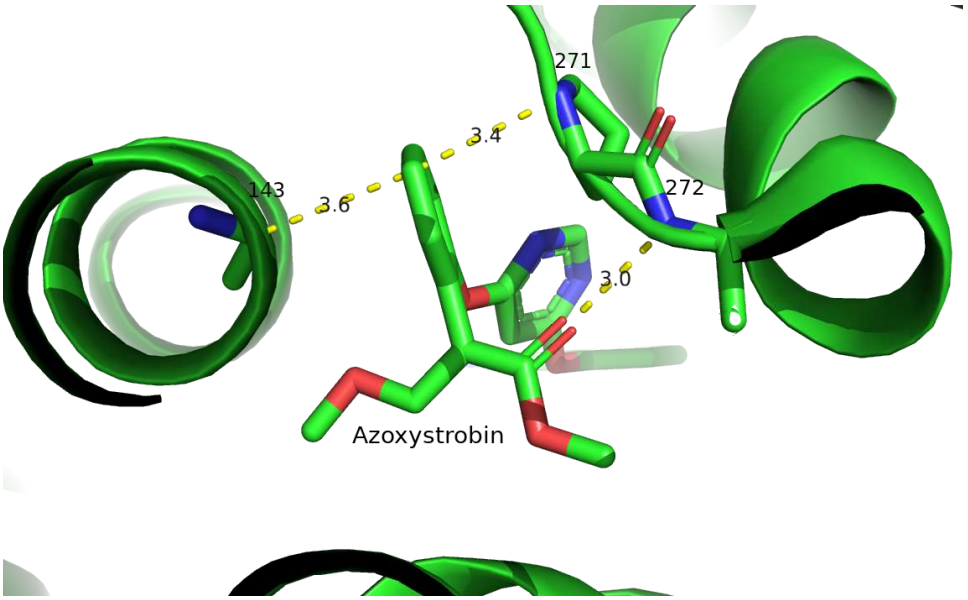

Figure 4. Cartoon representation of the interactions between cytochrome b (green) and azoxystrobin at residues 143, 271, and 272 represented in stick. Dashed lines (yellow) represent distance (Å) between the oomicide (azoxystrobin) and the protein.

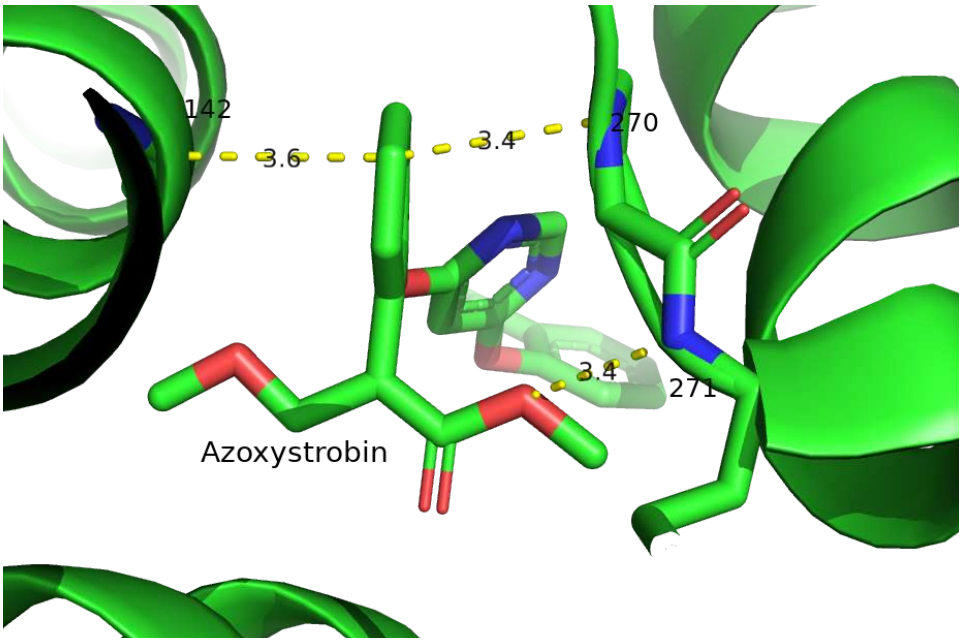

Figure 5. Cartoon representation of the interactions between cytochrome b (green) and azoxystrobin at residues 142, 270 and 271 represented in stick. Dashed lines (yellow) represent distance (Å) between oomicide (azoxystrobin) and protein .

Despite interfering in a key process, respiration, and consequently energy production, some plant pathogens evolved resistance to the azoxystrobin and this active ingredient became ineffective for the control of the disease they cause. In other words, there was certainly selection for resistance in individuals of plant pathogenic fungi and oomycetes. Probably, resistance to azoxystrobin was mediated by the substitution of amino acid residues in the cytochrome b at specific positions that allow or “tolerate” such changes. Selection by azoxystrobin increased the frequency of certain alternative forms of the protein in the population. Therefore, resistant individuals outnumbered sensitive ones, which coincided with the loss of control efficiency previously achieved by the fungicide/oomicide. Positions 128, 129, 137 and, specially, 143 (Figure 6), which presents a very characteristic replacement of the glycine residue (G) by alanine (A) (G143A), so far have had greater impacts on the phenotype, with G143A conferring the highest degree of resistance (FISHER et al, 2020, GRASSO et al, 2006).

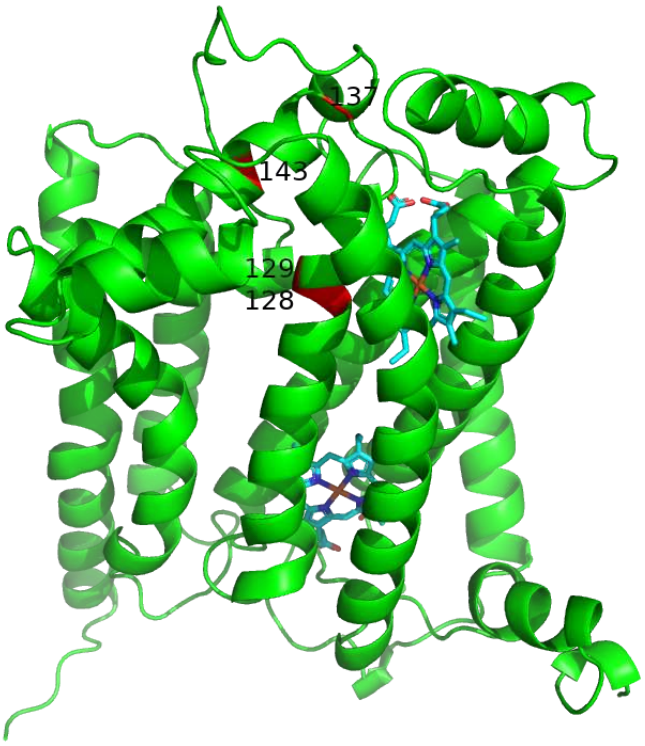

Figure 6. Cartoon representation of cytochrome b (green) and residues 128, 129, 137, and 143 (red). These residues are reported to be associated with the loss of azoxystrobin control efficiency.

Strobilurin A, mixothiazole, and mucidin are molecules that share the same target site as azoxystrobin and mutations associated with resistance to these

chemicals are also known. Therefore, such mutations may increase the list of substitutions of amino acid residues that will lead to the loss of efficiency of the molecule or to changes in the functioning of cytochrome b. Theoretically, up to about 60 amino acid substitutions may lead to the loss of efficiency of QoI's or to loss in the function of cytochrome b (KRAICZY et al, 1996; BRASSEUR et al, 1996; LINK et al, 1993). This apparently large number of possibilities is conditioned on observations in a heterogeneous sample of eukaryotes and does not necessarily apply to a single species. Furthermore, some substitutions make the protein non-functional, which imposes a dead-end in evolutionary terms.

An interesting aspect is the interaction of strobilurin with fungi that naturally produce these compounds and from which synthetic analogues used as fungicides in agriculture were developed. The basidiomycete *Strobilurus tenacellus*, one of the organisms from which strobilurin A was extracted, does not have A143 neither the other substitutions at positions 128, 129 and 137, but even so it is resistant to strobilurin A. In *Mycena galopus*, another basidiomycete from which strobilurin was obtained, A143 is the wild type residue, but the sensitivity to strobilurin A was higher than that of *S. tenacellus* (KRAICZY et al, 1996). On the other hand, the ascomycete *Alternaria solani*, the causal agent of potato and tomato early blight, has an intron after the codon for the amino acid that occupies position 143 in cytochrome b. The intron does not allow the substitution G143A. Therefore, substitutions in other positions are of greater importance for the loss of sensitivity of this ascomycete to QoI, such as F129L (GRASSO et al, 2006).

Considering the oomycetes, *Plasmopara viticola* is an interesting species to study of sensitivity to QoI, since G143A and other important substitutions for loss of efficiency of azoxystrobin occur in *P. viticola* (MASSI et al, 2021). As far as *P. infestans* is concerned, reports of resistance to azoxystrobin are unknown to date (FRAC, 2023b; YIN et al, 2023). This is a curious fact, and its elucidation can provide valuable insights into the development of resistance to azoxystrobin, and to other molecules, not only for *P. infestans* but also for fungal plant pathogens. Given the facts mentioned above, *S. tenacellus*, *A*. *solani* and *P*. *viticola* are contrasting in relation to the phenotype of *P*. *infestans* and an analysis of the biophysics of cytochrome b of these four organisms may allow the acquisition of valuable information on how resistance to the azoxystrobin can develop.

2.5. Importance of the three-dimensional structure of proteins and prediction by computational methods.

The knowledge of the sequence, the three-dimensional structure and the biophysics of a protein can be of great value and practical applicability. As an example, it is possible to have a better understanding of: the protein function (XIA et al, 2013); the relative proportion of amino acid residues that compose it (GASTEIGER et al, 2005); the characteristics of the side chain regarding the hydropathic index (KYTE & DOOLITTLE, 1982); the interaction in protein-protein complexes (JONES et al., 1996); protein evolution (MITCHELL, 2017; LASKOWSKI et al, 2009); important hotspots for protein-ligand interaction (CURRAN et al, 2020); the principles that should guide rational design and discovery of new drugs (LEACH et al, 2011); the prediction of protein-ligand interaction poses and estimation of interaction energy (ELOKELY et al, 2013); the prediction of amino acid residue substitutions that disfavor protein-ligand interaction (FREY et al, 2010; CAO et al, 2005); the prediction of amino acid residue substitutions that can be supported by a protein without a structural compromise from an energetic point of view (Lei et al, 2023; SCHYMKOWITZ et al, 2005); and the prediction of protein cavities (GUERRA et al, 2023).

The Protein Data Bank (PDB) is an important three-dimensional protein structure database that is widely used for many purposes. Data obtained from experimental methods such as X-ray crystallography, NMR spectroscopy and 3D electron microscopy are deposited in the PDB, which allows access to a secure source of information. In addition to the predicted structures, the PDB also presents quality indicators (BERMAN et al, 2020). However, despite the relevance of the PDB, the number of organisms represented is restricted. Thus, small natural variations between the structure of a deposited protein and the same protein from another organism under study may not be included in the database. In view of these restrictions, the use of a computational method for predicting the 3D structure of proteins can be of great value.

Different computational approaches can be used to predict the three-dimensional structure of a protein. The choice of prediction method depends, in part, on the availability and quality of information associated with the protein, specifically, the sequence of amino acid residues and the three-dimensional

structure. Based on this information, a choice is made between using template-based modeling or a template-free technique (Kuhlman and Bradley, 2019). Three situations can be envisaged when starting the prediction process: 1. Data on the three-dimensional structure of the protein under study are available, but predicted for another biological species; 2. There is no such data available, but it is possible to search for proteins from the same family of the protein of interest that has been preferentially characterized in the same species; and 3. It is impossible to use the first two options, in this case, a search is made in databases for proteins, or protein fragments, that are similar to the protein under study, or a template-free method is used (Kuhlman and Bradley, 2019; Verli, 2014). The percentage of similarity can be used as a determinant of the most appropriate method to predict the structure of the protein under study (Verli, 2014).

From the fulfillment of one of the three conditions above, the best method and software for computational prediction of the protein structure is chosen. If the similarity between the protein under study and a template is greater than 30%, comparative modeling may be the best approach. For similarity values between 20 and 29%, the “threading” modeling method should be used. For values below 20%, *de novo* or *ab initio* methods are usually employed (Verli, 2014). Despite these "guidelines", it is important to assess the situation case-by-case, and there may be exceptions in the previously presented "guidelines".

Considering the *ab initio* method, a software based on neural networks called AlphaFold was recently developed (SENIOR et al, 2020). Due to the accurate prediction capacity of the three-dimensional protein model this software received great attention. The results obtained with AlphaFold are considered comparable to the resolution of the structure obtained by non-computational experimental methods (SENIOR et al, 2020).

2.6. Importance of predicting amino acid residue substitution in proteins and the impact on the study of sensitivity to chemical molecules.

Usually, predictions by computational methods of the replacement of amino acid residues are done based on force fields or neural networks algorithms and different softwares can be used for these purposes (BLAABEJERG et al, 2023; THIEKER et al, 2022; ÖHLKNECHT et al, 2021; BEMEJEE and MITRA 2020;

DELGADO et al, 2019; FREY et al, 2010). Apparently, currently, the two most commonly used software are FoldX
(DELGADO et al, 2019; RADUSKY et al, 2022; Lei et al, 2023; TIBERTI et al, 2022) and Rosetta (THIEKER et al, 2022; VALANCIUTE et al, 2022). In this review, the focus will be on FoldX.

FoldX is a tool to assess the propensity of a given amino acid residue to occupy certain positions in the three-dimensional structure of proteins. The program is accurate, has low computational cost and it is possible to use protein models obtained by computational methods as an input file (VALANCIUTE et al, 2022). FoldX calculates the change in free energy (ΔΔG) between the protein with the native amino acid residue (wild-type) and the protein with the substituted residue (mutant) and estimates the propensity of different residues to occupy certain positions in the three-dimensional structure. The calculation of ΔΔG takes into account: Van der Waals interactions; the contribution of the solvent with hydrophobic groups; solvent with polar groups; water molecules that make more than two hydrogen bonds with water; contributions from hydrogen bonding; contributions from electrostatic interactions between atoms of different polypeptide chains; the entropy penalty of conformation; the entropic cost associated with side chain attachment; and, finally, the steric overlap between atoms in the protein structure (SCHYMKOWITZ et al, 2005). Based on the calculated value of ΔΔG, it is possible to predict stabilizing and destabilizing mutations of the protein. With regard to stabilizing substitutions, FoldX allows inferences about the theoretical diversity that a given protein can assume, favoring the study of protein evolution.

The complexity of assessing the resistance of plant pathogens to fungicides/oomicides requires the use of multiple strategies. Traditional laboratory and greenhouse tests are often sensitive to only a portion of the theoretical diversity of the population, taking the risk of poorly representing reality. Broad studies that evaluate biochemistry and molecular biology aspects of fungicide/oomicide resistance under laboratory, combined with greenhouse tests that allow the estimation of fitness cost associated with mutations and the structure of the proteins involved would constitute an ideal scenario to properly address fungicide resistance (HOLLOMON, 2015).

The approach mentioned above would allow access to the theoretical diversity of a protein and the consequences it brings to the evolution of fungicide/oomicide

resistance. As an example, the use of comprehensive approach could have avoided scenarios such as Metalaxyl resistance in *P. infestans* in Mexico, prior to the use of the oomicide (GRÜNWALD & FLIER, 2005) and could also have helped predict the recent mandipropamid resistance event (ABULEY et al, 2023) before its occurrence. These two events impose the need to assess the propensity for resistance in a population even before the launch of the active ingredient on the market. Once the population's theoretical protein diversity is accessed, computational methods for predicting the protein-ligand interaction, estimating the interaction energy, and predicting substitutions that disfavor the protein-ligand interaction (ELOKELY et al, 2013; FREY et al, 2010; CAO et al, 2005, ZHOU et al, 2015) can be used as an additional strategy to estimate the risk of loss of efficiency of a molecule and serve as a guide for its rational use.

The study of the evolution of cytochrome b of *P*. *infestans*, regarding the propensity to punctual substitutions of amino acid residues and the impact on the sensitivity to azoxystrobin, and why no resistance has been observed in *P*. *infestans* to this QoI, will be able to provide valuable information on the development of resistance in this oomycete, as well as in other plant pathogens, in addition to serving as a theoretical subsidy for the synthesis of future cytochrome b inhibitors.

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CHAPTER 1 SUBSTITUTION OF AMINO ACID RESIDUES IN THE CYTOCHROME B PROTEIN FROM *Phytophthora infestans*: FUNCTIONAL IMPLICATIONS AND AZOXYSTROBIN SENSITIVITY.

Abstract:

After almost three decades of usage of Azoxystrobin for late blight management, there is no report of resistance of *P. infestans* to this molecule. This is an intriguing fact since there are several cases of other Peronosporaceae, including other *Phytophthora* species for which resistant individuals have been reported. In this work we used amino acid residue substitution saturation method to evaluate the theoretical diversity of the cytochrome b protein (cytB) of *P. infestans*, *Plasmopara viticola* and *Strobilurus tenacellus*, and the impact of residue substitution in the cytB - azoxystrobin interaction. The three species support less than 33% of the total theoretical number of substitutions in the region of interaction between azoxystrobin and the cytB. Additionally, among the critical positions for the interaction, position 142 for *P. infestans* and position 143 for *P. viticola* and *S. tenacellus* can support many residues, most of which can interfere with the loss of sensitivity to azoxystrobin. Based on biophysics approaches and on the analysis of the mutation landscape, resistant isolates were highly likely to have been reported. Position 142 can support a large number of residues, without compromising stability and compromising sensitivity to azoxystrobin, in *P. infestans*. The non-occurrence of resistance to azoxystrobin in *P. infestans* seems to be related to a genomic factor that governs the stability of the cytochrome b gene sequence, once from the biophysical point of view there is no impediment to resistance to azoxystrobin. Although the approach used in this study could not explain the scenario of *P. infestans* resistance to azoxystrobin, the utilization of computational tools to assess the theoretical diversity of a target protein before the release and wide usage of a pesticide is a potential strategy for rational resistance management.

1 . Introduction

Antimicrobial resistance is of great concern in agriculture, particularly for diseases that require intensive application of fungicides or oomicides (chemical compounds specific for Oomycetes - GOVERS, 2001). Potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most destructive biotic stresses of plants known to date and synthetic fungicides and oomicides must be intensively used to prevent or reduce crop losses. Consequently, resistance of *P. infestans* to oomicides has profound impacts for growers and for companies when it comes to marketing issues. Late blight epidemics are destructive to potato and tomato crops and many research groups dedicate considerable resources to develop more

effective ways to cope with the disease (BERHAM, 2021; WEBSTER & WEBER, 2007). Currently, there are no varieties that combine agronomic characteristics of interest and resistance to late blight. Therefore, the application of chemical compounds such as fungicides and oomicides is the most effective management strategy (BERHAM, 2021), requiring proper timing application due to the fact that late blight epidemics have high rates of progress (FIORINI et al, 2010).

The inappropriate use of single-site oomicides can result in the selection of resistant individuals that will eventually constitute populations of *P. infestan*s that could not be managed with the available pesticides made from an active ingredient. There are reports of resistance in *P. infestans* to metalaxyl, mefenoxam, propamocarb, and recently, to mandipropamid (GRÜNWALD & FLIER, 2005; MÖLLER et al, 2009; ABULEY et al, 2023). However, there are no known reports of resistance of *P. infestans* to some single-site active ingredients that are used in the management of late blight, such as azoxystrobin, fluopicolide, cyazofamid, and oxathiapiprolin (Saville et al, 2015; GRÜNWALD et al, 2006; FRAC, 2023a; FRAC, 2023b).

Azoxystrobin is a synthetic fungicide that was developed based on a naturally-occurring compound, strobilurin A, that is produced by some fungi of the Basidiomycota phylum, such as *Strobilurus tenacellus* (Pers) Singer (ANKE et al, 1977). The fungicide inhibits the mitochondrial electron transport chain at complex III, it is classified as a member of the group of outside quinone inhibitors (QoI), or proximal bL inhibitors (FISHER et al, 2020) or even inhibitors of the Qp site (ESSER et al, 2014). Functionally and structurally, azoxystrobin disrupts the electron transfer from cytochrome b (cytB) to the iron-sulfur protein (ISP) at the ubiquinol oxidation site (FISHER et al, 2020; ESSER et al, 2014; LINK et al, 1993). Azoxystrobin has been in use for management of late blight for approximately 30 years, but there are no reports of resistance to the compound (FRAC, 2023b). The no occurrence or the extremely low frequency of azoxystrobin-resistant isolates of *P. infestans* deserves attention due to the fact of several cases of resistance in other oomycetes and in fungi (YIN et al, 2023; MASSI et al, 2021; GRASSO et al, 2006; KRAICZY et al, 1996). Curiously, there is high similarity in the nucleotide sequence of the cytochrome b gene among organisms of evolutionary distant groups, Fungi (Kingdom) and the supergroup SAR (Stramenopila, Alveolata, and Rhizaria), share a specific amino acid substitution at position 143 from glycine (G) to alanine (A) (G143A) is associated

with resistance to azoxystrobin in plant pathogenic fungal species of the phyla Ascomycota and Basidiomycota and also in species of the family Peronosporaceae, class Oomycetes, in which *P. infestans* is classified (YIN et al, 2023; KRAIEZY et al, 1996).

There are two situations related to resistance to azoxystrobin that are noteworthy due to their contrasting consequences to the phenotype: 1. In the position 143 of cytB some fungi of the phyla Ascomycota and Basidiomycota, and Oomycetes, the wild-type state is a glycine and the phenotype is sensitivity to azoxystrobin; 2. *Strobilurus tenacellus*, a Basidiomycota fungus, also has a glycine residue, but it is naturally resistant to azoxystrobin. Therefore, the dynamics of residue substitutions may impact the response of the organism to the application of an active ingredient and accurate predictions of the consequences of such substitutions can help increase the lifetime of a product and also to analyze future scenarios of evolution of chemical resistance before the product is made available in the market.

Predictions by computational methods of the replacement of amino acid residues allows the estimation of the theoretical diversity of a protein and can help understand the impact on the fitness of an organism (BLAABEJERG et al, 2023; THIEKER et al, 2022; TIBERTI et al, 2022; ÖHLKNECHT et al, 2021; BEMEJEE and MITRA 2020; DELGADO et al, 2019; FREY et al, 2010). This concept has been applied lately to assess the impacts of substitutions in protein interactions of interest, either involving targets of drugs developed to be used in human diseases or for the understanding of ligand - protein or even protein - protein interactions in diseases such as cancer, Influenza, and Covid (TIBERYI et al 2022; LEI et al, 2023, TENG et al, 2021). In several cases, a positive relationship between protein stabilizing substitutions and fitness was observed (LEI et al, 2023) suggesting that these substitutions are likely to be maintained in the population and the associated phenotype is expected to be detected at high frequency.

The dynamics of amino acid residue substitutions, by mutation saturation, supported in the cytochrome b of *P. infestans*, *P. viticola*, and *S. tenacellus* was estimated to assess the impacts on the resistance to azoxystrobin. The variation in free energy (ΔΔG) in the probable positions of interaction with azoxystrobin and in nearby residues was calculated. The free energy was estimated for substitutions at the interaction sites of interest under two scenarios: cytB and cytB + azoxystrobin.

The first scenario was constructed to analyze the effects of substitutions on the cytB alone, without interacting with azoxystrobin. The second scenario was designed to assess the magnitude of ΔΔG when azoxystrobin is interacting with cytB; in other words, to assess the impact of the ligand on mutational saturation. This study can provide valuable insights into the development of resistance to azoxystrobin, and to other molecules, not only for *P. infestans* but also for other oomycetes and fungal plant pathogens.

2. Material and methods

2.1. Cytochrome b sequences, alignment, and protein sequence.

The cytB sequences of *P. infestans* (AY894835, NC002387, AY898627, AY898628, MH286887,MH286886, MH286885, MH286884 and U17009), *P. viticola* (DQ459459) and *S. tenacellus* (X88000) were obtained from the National Center for Biotechnology Information (NCBI). One dataset comprised only of *P. infestans* sequences aligned in Clustal W in the Clustal Omega web server (https://www.ebi.ac.uk/Tools/msa/clustalo/). Another dataset was formed with the *P. infestans* sequence NC002387 aligned with sequences of *P. viticola* and *S. tenacellus*. The three sequences (NC002387, DQ459459, and X88000) were analyzed in ProtParam tool web server (https://web.expasy.org/protparam/) for the number of amino acid residues, amino acid residue composition, aliphatic index and stability index.

2.2. Protein homology modeling.

The modeling of three-dimensional cytB structure of NC002387, DQ459459, and X88000 sequences was predicted by comparative modeling by spatial constraint using Modeller 10.3 (WEBB and SALI, 2016) software running on a personal computer dual core (2,53 Ghz), 4 Gb ram and Windows 7. Two cytB templates were obtained from the RCSB Protein Data Bank (https://www.rcsb.org/): PDB-3L71, cytochrome bc1 from *Gallus gallus* (Ggal), and PDB-1SQB, cytochrome bc1 from *Bos taurus* (Btau). The templates were aligned on the Clustal Omega web server to the cytB sequences NC002387 (*P. infestans*), DQ459459 (*P. viticola*), and X88000

(*S*. *tenacellus*) to verify percentage identity. Due to the co-crystallization data of azoxystrobin with cytochrome b and percentage identity greater than 48% (Table 1) both templates, PDB-3L71 from *G*. *gallus* and PDB-1SQB from *B*. *taurus*, were selected to construct the cytochrome b protein of *P. infestans*, *P. viticola* and *S. tenacellus* by homology method .

Modeller was used with the default parameters. In the production model step, 100 models were created. The best model for each sequence-template combination was selected using the GA341 method, models with values around or equal to 1 and of good-fit, and of the energy profile according to the discrete optimized protein energy (DOPE) graph, especially in the region of interaction with azoxystrobin, were selected (WEBB and SALI, 2016).

For the selection step, structural alignment was conducted in PyMOL. Model selection was based on the values (low) of the root-mean-square deviation (RMSD) and visual search for problems, such as great differences between the template structure and models, or discontinuous three-dimensional structures in the predicted cytB. Two three-dimensional cytB protein structure were selected for each of the combinations: *P. infestans* - (cytB_Pi_Btau and cytB_Pi_Ggal), *P. viticola* - (cytB_Pv_Btau and cytB_Pv_Ggal), and *S. tenacellus* - (cytB_St_Btau and cytB_St_Ggal).

2.3. Saturation mutagenesis without azoxystrobin.

The impact of a single amino acid substitution in the protein stability was assessed by the substitution saturation approach based on the variation in free energy (ΔΔG). The analyses were conducted using the MutateX (TIBERTI et al, 2022), a pipeline front-end that automates the use of the FoldX 5.0 software (DELGADO et al, 2019) used to predict the ΔΔG associated with the mutation of each residue in a protein.

The software PyMOL was used to view regions of molecular interactions between azoxystrobin and cytB, and its surroundings, which could be important for the initiation or subsequent maintenance of the interaction. Data sets were constructed for the different PDB templates of interest: cytB_Pi_Btau, cytB_Pi_Ggal, cytB_Pv_Btau, cytB_Pv_Ggal, cytB_St_Btau, and cytB_St_Ggal. After this step, the

amino acid residues at the interaction sites of azoxystrobin were visually inspected and selected to be analyzed by mutate simulation (Figure 1).

All the 20 natural amino acids were simulated to be replaced in each position. Heatmaps were constructed to depict the results of the simulation analysis. When the calculated value of ΔΔG was equal to or less than the values associated with the wild-type amino acid, the substitution was considered to be "stable" and no fitness penalty was set. Energy values higher than those presented by the wild-type were considered destabilizing and negative substitutions from the point of view of adaptive value. Simulations were performed on a workstation with Ubuntu 22.04 operating system, 24 Gb of Ram and Intel® Xeon(R) CPU E5-2407 quad-core 2.20Ghz processor.

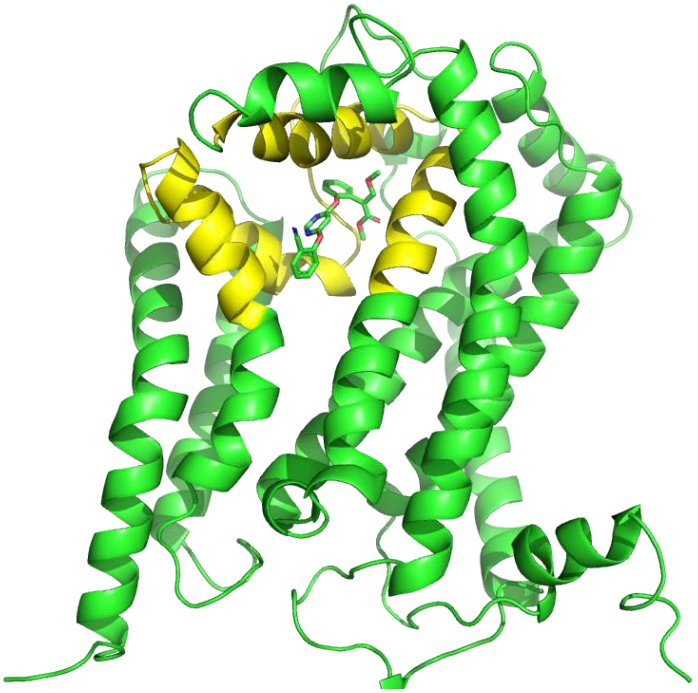

Figure 1. The region of interaction (yellow) between azoxystrobin (represented by a stick model) placed inside the cytochrome b protein (cartoon representation) of *Phytophthora infestans* used for the saturation mutagenesis analysis in MutateX.

2.3. Saturation mutagenesis with azoxystrobin in the interaction site.

To assess the impact of saturation substitution in the interaction between azoxystrobin and cytB of the three species, the molecule was parameterized with the Python pyFoldX library (RADUSKY et al, 2022). The presumably key-positions for the interaction between azoxystrobin and cytochrome b were identified based on

literature information and visualized in PyMOL. Positions 128, 136, 142, 270, and 271 were chosen for *P. infestans* and positions 129, 137, 143, 271, and 272 for both *P. viticola* and *S. tenacellus*. The variation in ΔG for both, with and without azoxystrobin, to each model, was recorded in terms of variation in total energy, electrostatic force, entropy side chain, entropy main chain, polar and hydrophobic solvation, entropy complex cost, side chain hydrogen bond, and variation clashes in: Van der Waals, backbone, and energy torsion. All simulations were performed on a workstation with Ubuntu 22.04 operating system, 24 Gb of Ram and Intel® Xeon(R) CPU E5-2407 quad-core 2.20Ghz processor.

3. Results

3. 1. Alignment analysis and characterization of protein sequence.

All cytB sequences from *P. infestans* were identical, thus, only sequence NC002387 was used in the analyses. When comparing among the three species, *P. infestans* and *P. viticola* were 93.2% similar while the similarity between *S. tenacellus* and *P*. *infestans* or *P*. *viticola*, was around 57% (Table 1). When the cytB sequences were compared in ProtParam, there were differences in the number of residues and in their composition (Table S1). *P. infestans* and *P. viticola* are similar in the constitution of residues, but both differ in relation to *S. tenacellus* mainly in the number of isoleucine, leucine, serine, tyrosine, and valine. On the other hand, the number of arginine, asparagine, histidine, phenylalanine, proline, and tryptophan is similar among species, which may indicate the importance of this characteristic in protein structure and functionality. The total number of residues in the cytB sequences of *P. infestans* and *P. viticola* were similar: 383 and 384 (Figure 2), respectively.

Table 1. Percent identity matrix created by Clustal 2.1 for the templates and proteins used in the modeling step.

Code		*G. gallus*	*B. taurus*	*S. tenacellus*	*P. infestans*	*P. viticola*
PDB 3L71	*Gallus gallus*	100.00	74.67	48.94	55.17	54.76
PDB 1SQB	*Bos taurus*		100.00	52.12	54.91	53.70
X88000	*Strobilurus tenacellus*			100.00	57.18	57.29
NC002387	*Phytophthora infestans*				100.00	93.21
DQ459459	*Plasmopara viticola*					100.00

```
X88000          MRLLKNNIILRLLNSYMVDSPQPANLTYLWNFGSLLGICLVLQILTGCFLAMHFTPHAEM      60
NC002387        -MRWNKKSLFAVINNHLIDYPTPINLNYFFGFGSLAGIMLVVQILTGIFLAMHYTPHIDL      59
DQ459459        MIRWNKKSLFTVINNHLIDYPTPINLNYFYGFGSLAGIMLVVQILTGIFLAMHYTPHIDL      60
                    ::: :: ::*.:::* * * **.*::.**** ** **:***** *****:*** ::

X88000          AFNSVEHIMRDVQSGWIVRYTHANVASFFFIFVYAHIGRGLYYNSYKSPRVLLWSIGVII      120
NC002387        AFNSVEHIMRDVNNGWLIRYTHANGASFFFIVVYIHIFRGLYYGSYITPREAIWCSGVII      119
DQ459459        AFNSVEHIMRDVNNGWLIRYIHANGASFFFIVVYIHIFRGLYYGSYITPREALWCSGVII      120
                ************:.**::** *** ******.** ** *****.** :**  :*. ****

X88000          LVLMMAIGFLGYVIPFGQMSLWGATVITNLLSAIPVFGQDIVELIWGGFSVSNATLNRFF      180
NC002387        FILMMATAFMGYVLPWGQMSFWGATVITNLFSAIPLIGKDIVDWLWGGFAVDNPTLNRFF      179
DQ459459        FILMMATAFMGYVLPWGQMSFWGATVITNLFSAIPLIGKEVVDWLWGGFAVDNPTLNRFF      180
                ::**** .*:***:*:****:*********:****::*:::*: :****:*.* ******

X88000          SLHYILPFVLAALVVAHFMALHIHGSNNPNGVTSNTDRYPMYPYFIFKDLVTIFAFFWIL      240
NC002387        SLHFTLPFIIVGAVLVHLILLHEVGSNNPLGITLKTENIPFYPYFYTKDLFGLMVLFLVF      239
DQ459459        SLHFTFPFVIVGAVLIHLILLHEVGSNNPLGITLKTENIPFYPYFYTKDLFGLIVLFLIF      240
                ***: :**::.. *: *:: **  ***** *:* :*:. *:****  ***. ::.:* ::

X88000          SVIVFFYPNLMGHQDNYIPADPMVTPASIVPEWYLLPFYAILRSIPDKLLGVVAMFGSLL      300
NC002387        FIFVFYYPNTLGHPDNYIEANPMKTPLHIVPEWYFLPFYAILRSIPNKIGGVVAMFGSLI      299
DQ459459        FIFIFYYPNTLGHPDNYIEANPMKTPLHIVPEWYFLPFYAILRSIPNKIGGVIAMFGSLI      300
                 :::*:*** :** **** *:** **  ******:***********:*: **:******:

X88000          ILLVLPLTDLSRIRGNQFRPAMKFFFWFFVVNFIMLFWLGSQHPNTPYLEIGQLSTTFYF      360
NC002387        ILLTIPFTNSSEIRSTAFRPIFKVCYWLLVIAFLILGWIGQCPVEYPYTEIGIISMIYYF      359
DQ459459        ILLTIPFTNSSEIRSTTFRPIFKVCYWLLVVAFILLGWVGQCPVEYPYTEIGIISMIYYF      360
                ***.:*:*: *.**.. *** :*. :*::*: *::* *:*.   : ** *** :*  :**

X88000          SFFLVIVPFTGLVENTLLDLNIKELDLNL     389
NC002387        FFFIIIIPFLGKFEAYLVRYSINK-----     383
DQ459459        SFFLIIIPFLGKIETYLIRYKCNK-----     384
                 **::*:** * .*  *:  . ::
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Figure 2. Alignment of the cytochrome B protein from *Phytophthora infestans* (NC002387), *Plasmopara viticola* (DQ459459), and *Strobilurus tenacellus* (X88000). * = identical (conserved) residue at a position, . = conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix, and : = conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

3.2. Homology modeling of cytochrome b protein of *P. infestans*, *P. viticola* and *S. tenacellus*.

All models chosen for *P. infestans* and *P. viticola* had GA341 values equal to 1 (best value for this index). For *S. tenacellus*, the GA341 values were 0.99998 and 0.99964 when 1SQB and 3l71 templates were used, respectively. The DOPE plot was used to guide selection and the models were chosen based on the best fit between template and model in the azoxystrobin interaction region. An example of the DOPE graph for *P. infestans* is presented and the region of of interaction with azoxystrobin is highlighted (Figure 3). The chosen protein models had low RMSD value when aligned with the respective templates in PyMOL reflecting adequate quality for further analysis (Table 3, Figure 4). Furthermore, all predicted protein structures were visualized in PyMol and no errors were detected.

Table 3. Root mean squared-deviation (RMSD), values in Angstrom (Å) for different templates used in the models for the different species under study.

	Templates RMSD Values in Å	
Model	**1SQB**	**3l71**
P. infestans	0.147	0.137
P. viticola	0.129	0.147
S. tenacellus	0.125	0.141

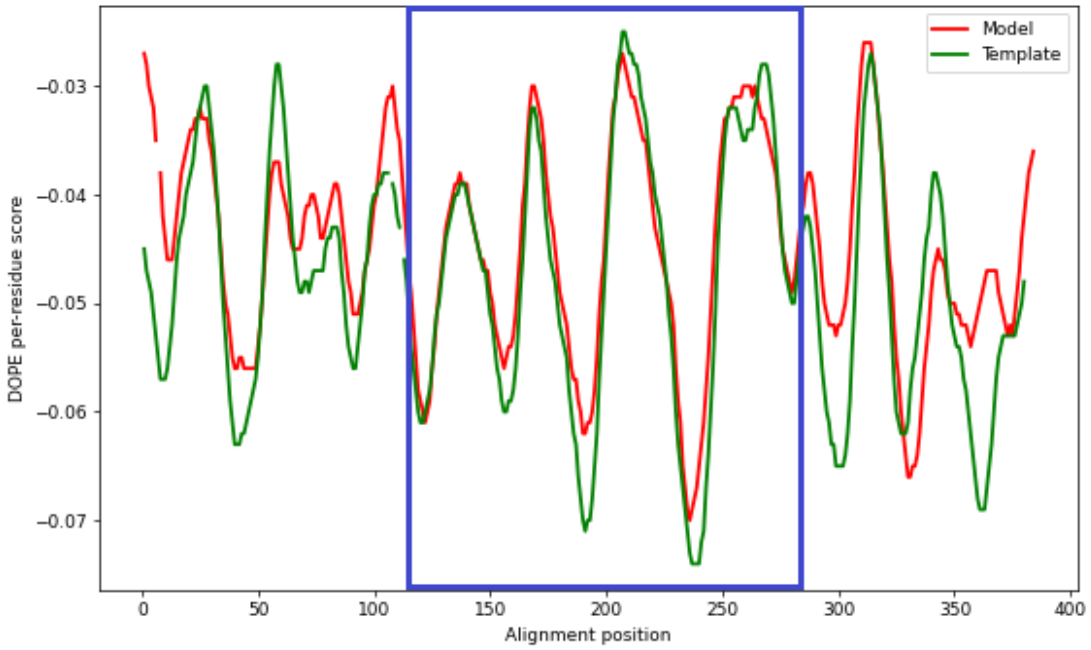

Figure 3. DOPE plot of the cytochrome b model of *Phytophthora infestans* against the 1SQB template. In the blue box, positions around the region of azoxystrobin interaction site.

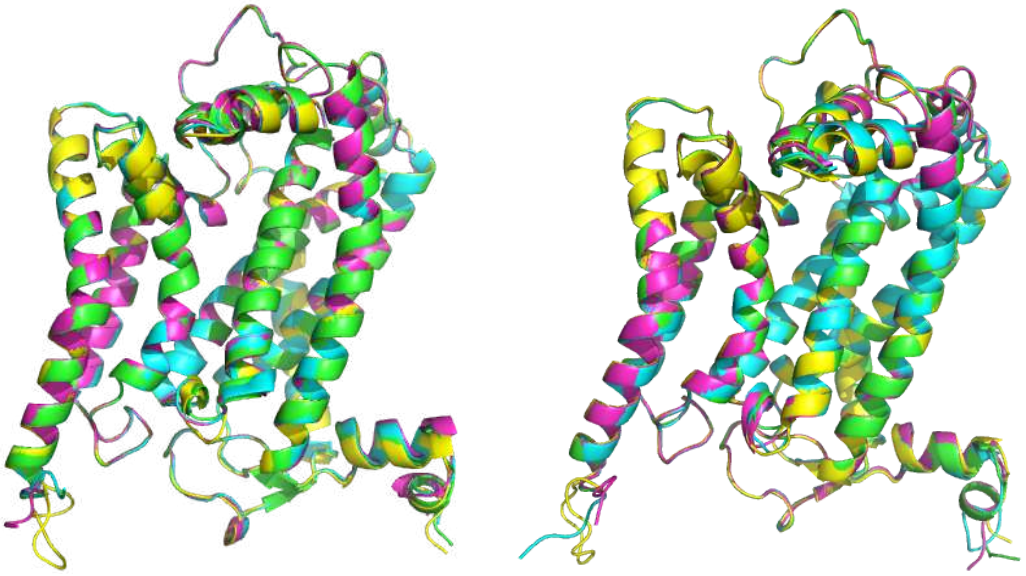

Figure 4. Structural alignment model and template. *Phytophthora infestans* (blue), *Plasmopara viticola* (pink), *Strobilurus tenacellus* (yellow), and the 1SQB template (left); models and 3l71 template (right). The templates on both sides of the figure are represented in green.

3. 2. Determination of substitution tolerance.

The number of positions selected for the cytB study in MutateX was 62, 64, and 65 for *P. infestans*, *P. viticola*, and *S. tenacellus*, respectively, due to the shape and size of the interaction site of azoxystrobin and the surrounding region (Figure 1). The number of positions varied among species. To improve visualization and interpretation, the heatmaps have different y-axis scales associated with the range of ΔΔG values. Considering the substitution tolerance, 29 of the 62 positions for the cytB_Pi_Btau model were stabilizing substitutions, supporting from 1 to 4 different amino acids, including the wild-type residue. Twelve of the 29 positions supported only the wild-type residue (Figure S1). Seventeen out of the 62 positions were considered as "promiscuous" and supported more than 10 substitutions.

Considering substitutions that are energetically favorable for stability (ΔΔG ≤ wild-type residue), of the 1240 possible substitutions (considering wild-type residues) 403 contributed to stability (Figure 5). For cytB_Pi_Ggal 42 positions were restricted, supporting from 1 to 4 substitutions. Out of the 42 positions, 17 supported only the wild-type and 11 positions were “promiscuous” (Figure S2). In total, 298 substitutions contributed to stability (Figure 6).

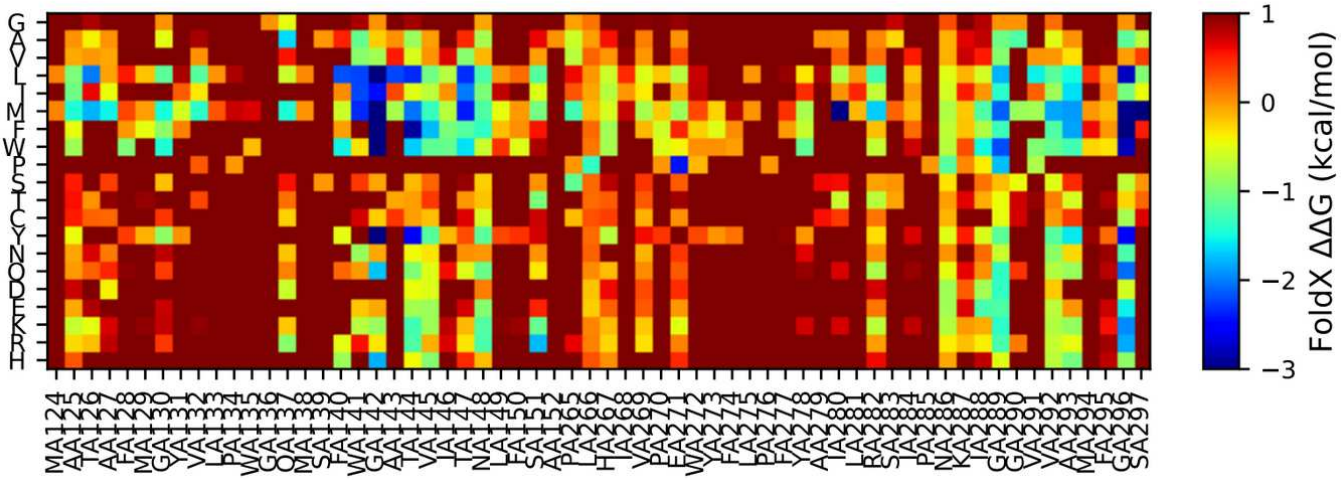

Figure 5. Residue substitution impact in the cytB_Pi_Btau model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference varied −3 to 1 kcal/mol.

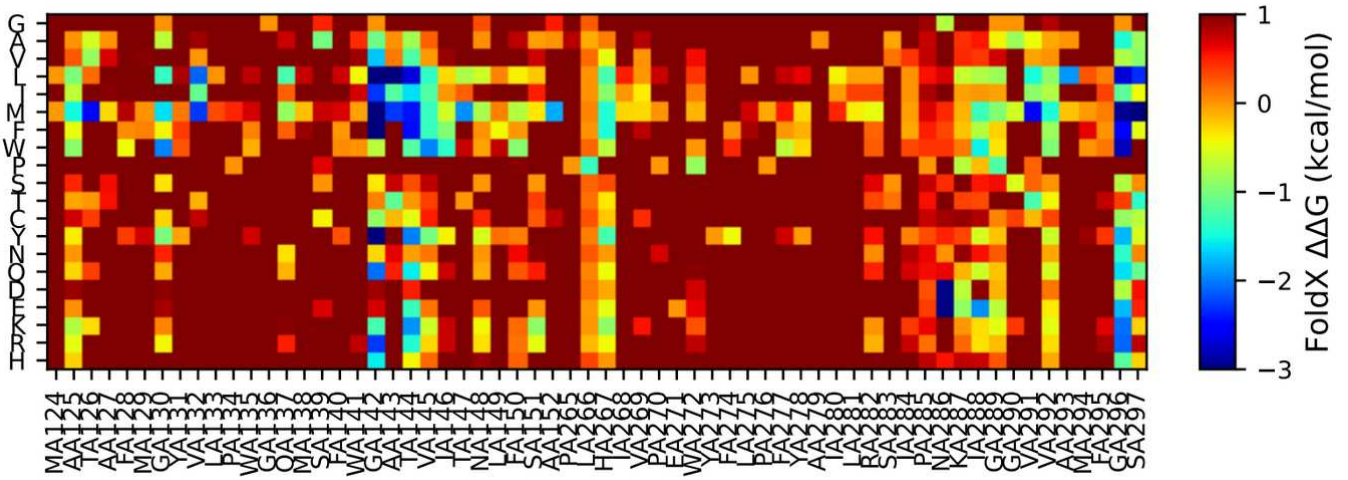

Figure 6. Residue substitution impact in the cytB_Pi_Ggal model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference varied −3 to 1 kcal/mol.

For the cytB_Pv_Btau (Figure S3) and cytB_Pv_Ggal (Figure S4) 64 positions were evaluated. Thirty seven for the first model and 40 for the second, supported from 1 to 4 substitutions, considering the wild-type residue. In 20 and 21 positions respectively, only the wild-type residue was supported. Fourteen “promiscuous” regions were detected for the first model and nine for the second model. These “promiscuous” positions supported at least 10 substitutions. Of the 1280 possible substitutions, 362 for the first model (Figure 7) and 297 for the second (Figure 8) are favorable for protein stability.

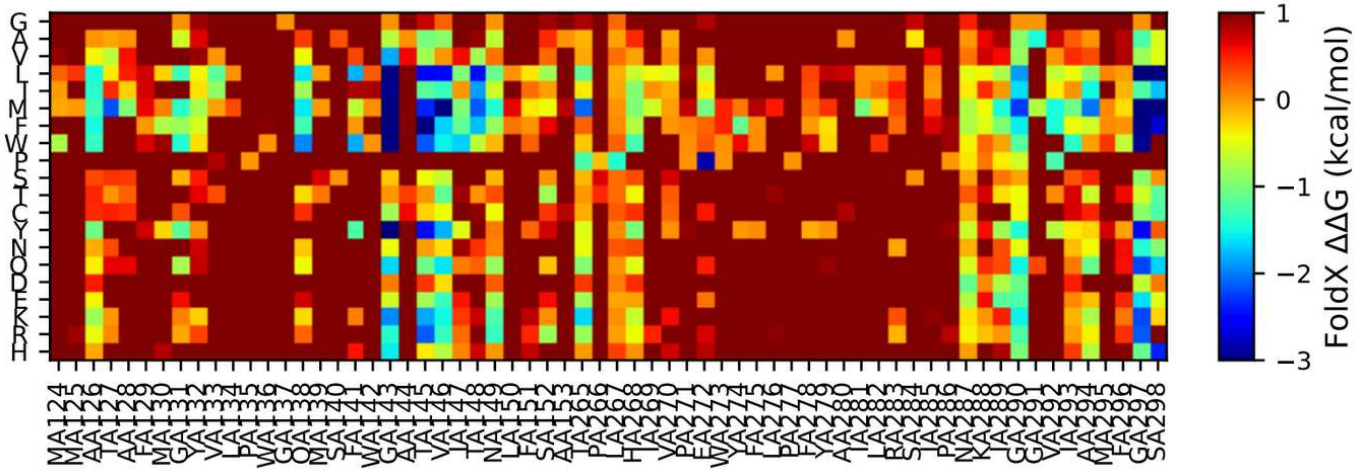

Figure 7. Residue substitution impact in the cytB_Pv_Btau model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference varied from −3 to 1 kcal/mol.

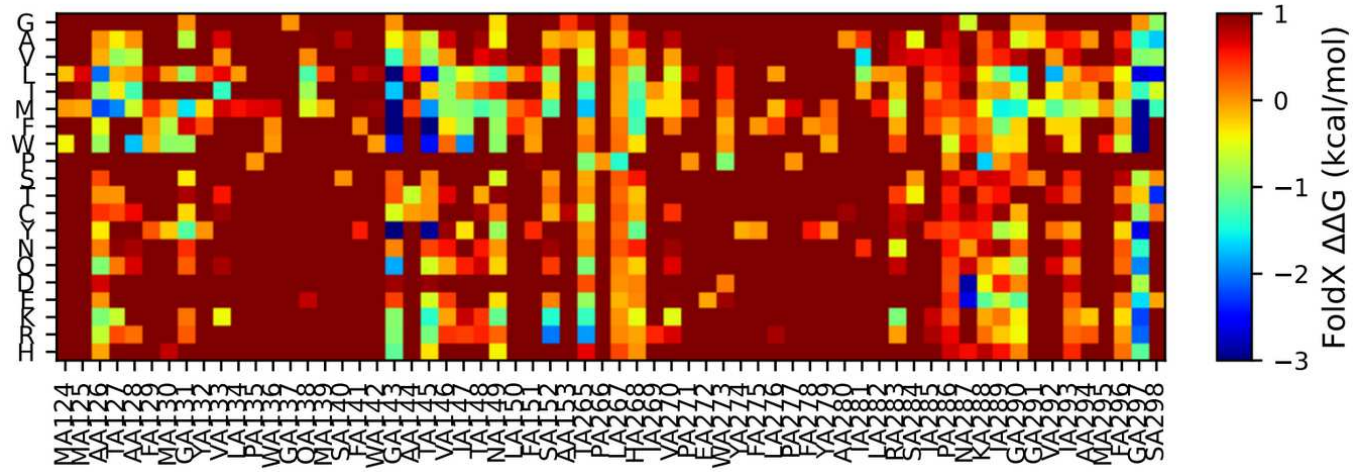

Figure 8. Residue substitution impact in the cytB_Pv_Ggal model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference varied from −3 to 1 kcal/mol.

Finally, for both cytB_St_Btau (Figure S5) and cytB_St_Ggal (Figure S6) 65 positions were evaluated, of which 33 for the first and 39 for the second supported from 1 to 4 substitutions considering the wild-type residue. For both models, 15 positions supported only the wild-type residue. For the first and second models, 13

and 12 “promiscuous” regions, respectively, were identified. Of the 1300 possible substitutions, 369 for the first model (Figure 9) and 345 for the second (Figure 10) favored protein stability.

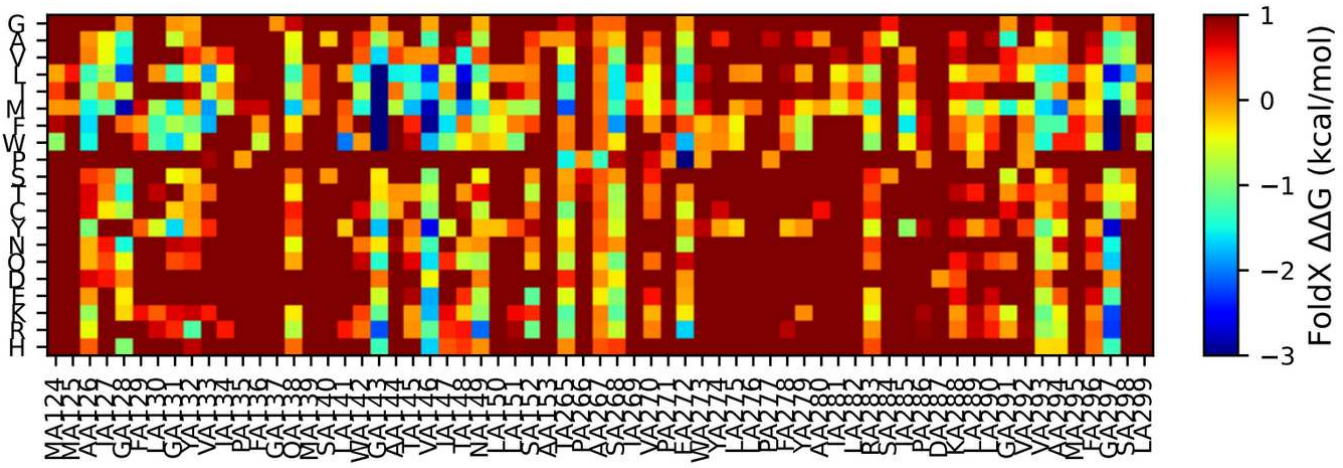

Figure 9. Residue substitution impact in the cytB_St_Btau model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference varied from −3 to 1 kcal/mol.

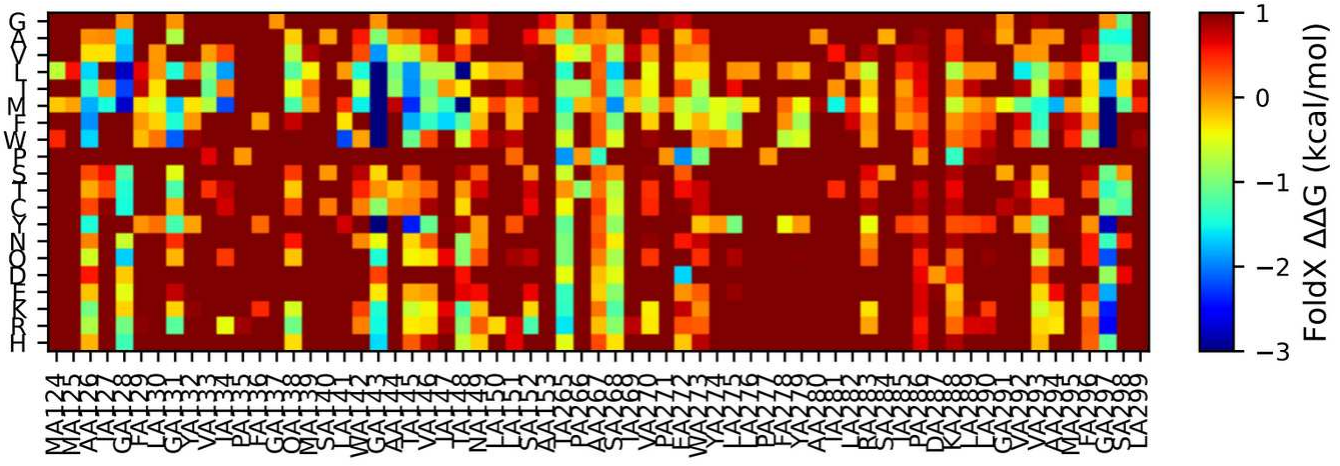

Figure 10. Residue substitution impact in the cytB_Pv_Ggal model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference varied from −3 to 1 kcal/mol.

3.3. Substitution tolerance at azoxystrobin interaction site.

For the pyFoldX analyses, only the substitutions predicted as not penalizing protein stability were analyzed. For the cytB_Pi_Btau and cytB_Pi_Ggal models, 33 and 21 substitutions were analyzed, respectively (Table 4). For both models, five positions were evaluated, 128, 136, 142, 270, and 271 considering the interaction with azoxystrobin and the published data available. Point-mutations in these positions caused alteration in the sensitivity to azoxystrobin (YIN et al, 2023; FISHER et al, 2020; ESSER et al, 2014; GRASSO et al, 2006). The G142A substitution in *P. infestans* corresponds to G143A in many other pathogens (Figure 2).

For the cytB_Pi_Btau model, alanine(A) (Figure 11) at position 142 had a small Van der Waals and backbone clash as major components in the variations of protein energy in the presence of azoxystrobin. Valine (V), serine (S), and cysteine (C) in position 142 also produced a small clash. On the other hand, arginine (R), asparagine (N), cysteine (C), glutamine (Q), glutamic acid (E), histidine (H), isoleucine, (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), serine (S), tryptophan (W), and tyrosine (Y) (Figure 12), returned a large clash with approximate minimum value of 16.64 Kcal/Mol and maximum of 55.00 Kcal/Mol. Great clash of Van der Waals was identified for F, L, M, and W in the 270 position. Small Van der Waals clash was detected in I and L at the 271 position, but a large clash was detected for W at this position. Large variations in Van der Waals were not detected for other interactions.

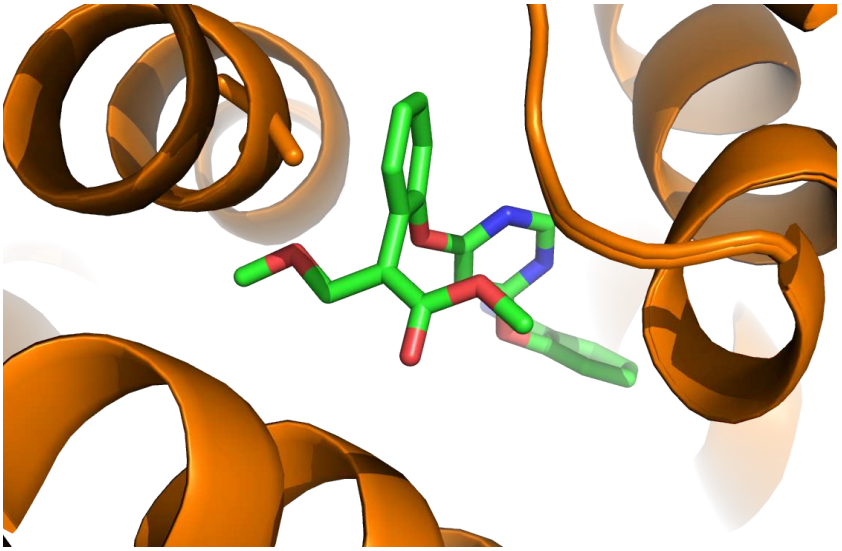

Figure 11. Residue substitution impact in cytochrome b azoxystrobin interaction. Cytochrome b (orange cartoon), alanine at position 142 (orange stick) and azoxystrobin (stick in center).

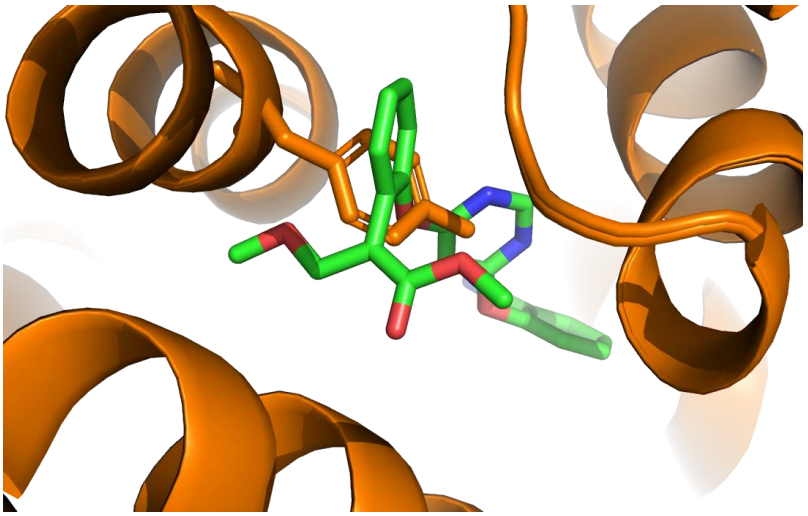

Figure 12. Residue substitution impact in cytochrome b azoxystrobin interaction. Cytochrome b (orange cartoon), tyrosine at position 142 (orange stick) and azoxystrobin (stick in center).

For the cytB_Pi_Ggal model, A, H, I, K, L, M, N, Q, R, F, C, S, V, W, and Y at the position 142 increased the clash of Van der Waals. In the other positions for this model no large variations in Van der Waals interactions were identified.

Table 4. Residuos evaluated at each position in two models.

	Position				
Model	**128**	**136**	**142**	**270**	**271**
cytB_Pi_Btau	F, L, W	G	G, A, C, E, F, H, I, K, L, M, N, Q, R, S, V, W, Y	P, F, L, M, W	E, I, K, L, M, P, W
cytB_Pi_Ggal	F, W	G	G, A, C, F, H, I, K, L, M, N, Q, R, S, V, W, Y	P	E

For *P. viticola*, 31 and 20 substitutions were considered for the models cytB_Pv_Btau and cytB_Pv_Ggal, respectively (Table S2). For both models, five positions were evaluated considering the interaction with azoxystrobin: 129, 137, 143, 271, and 272. For the cytB_Pv_Btau model, R and S produced Van der Waals clashes in position 137. The residues E, F, H, I, K, L, M, N, Q, R, T, W, and Y produced Van der Waals clashes in position 143. The alanine in position 143 had a small clash and C, S, and V had an even smaller clash than A. For the cytB_Pv_Ggal model, C, V, and A substitution at the 143 position caused Van der Waals clashes. The strength of the clashes increased in the order in which residues were listed. At this position (143), residues F, H, I, K, L, W, M, Q, R, V, and Y produced greater clash of Van der Waals. At position 129, only W increased Van der Waals clash. For the other positions, for both models, no large or considerable variations in Van der Waals or other terms of interaction were detected.

For the cytB_St_Btau and the cytB_St_Ggal models, 34 and 30 substitutions were analyzed, respectively (Table 5). For both models, five positions were evaluated considering the interaction with azoxystrobin: 129, 137, 143, 271, and 272. For the cytB_St_Btau model, E, P, H, I, K, L, M, N, Q, and R produced a great Van der Waals clash in 143 position. The residues A, C, and T in the same position produced a little clash and residues S and V produced even a smaller clash than A. For the 272 position only R and W produced a Van der Walls clash, but only for R it produced an increase in total energy. In the cytB_St_Ggal model, C, S, V, and A, increased in this order the Van der Waals clash in 143 position. At the same position, residues E, F, H,

I, K, L, W, M, N, Q, R, W, and Y produced great clash of Van der Waals. At the 129 position only W increased energy torsion clash. For the 272 position L, M, and F increased the total energy and had a small Van der Walls clash. For the other positions in both models there was no considerable variation in Van der Waals or other terms of interaction.

4. Discussion.

Although cytochrome b is a well-known target for controlling fungi and oomycetes, the biophysical constraints on protein and antimicrobial resistance evolution are not entirely clear. In this study, the saturation mutagenesis method was used to investigate the cytochrome b mutational fitness scenario with and without azoxystrobin. The cytB was conserved among the three species under study, and a restricted number of residue substitutions was supported in the evaluated region. Less than 33% of residues, considering the wild-type, may vary across the evaluated region. These data agree with the expected stability required for the functional maintenance of a protein (TYZACK et al, 2017). It is important to note that the predicted difference between the two models for each species does not imply an error, but rather the well-known variation inherent to the sensitivity of FoldX to the input model (TIBERTI et al, 2022). However, from an energetic point of view, the three species behave similarly, supporting a rather equivalent number of substitutions. Interestingly, when the available sequence data of cytB of *P. infestans* are analyzed, even among the different haplotypes (Avila-Adame et al, 2005) of the pathogen, there is no variation in the gene that codes for this protein. The non-occurrence of resistance in *P. infestans* seems not to be related to the protein capabilities of supporting the substitutions. Additionally, introns that are known to prevent mutations in some fungal species are not present in the mtDNA of *P. infestans* (GRASSO et al, 2006; PAQUIN et al, 1997). It is, thus, hypothesized that the non-occurrence of resistance to azoxystrobin may also be justified due to factors associated with the inheritance mode and low levels of mutation rates of the mtDNA.

In *P. infestans*, the uniparental inheritance of mtDNA and the absence of segregation and recombination were verified, but which parent provided the mtDNA was not identified (WHITTAKER et al, 1993). In *P. parasitica*, uniparental maternal inheritance of mtDNA is credited (FÖRSTER & COFFEY, 1990). Therefore, it is

possible that the phylogenetic proximity may indicate a similar transmission pattern in *P*. *infestans*. For *P. capsici*, *P. citrophthora*, *P. megakarya*, *P. palmivora*, *P. citricola*, and *P. parasitica*, there was greater conservation and lower mutation rate of the mtDNA in relation to the nuclear DNA (FÖRSTER et al, 1990). Again, it is speculated that a similar pattern may be found in *P*. *infestans*. Overall, both factors, the pattern of heritance and the low variability in mtDNA, could explain the low diversity and conservation of the cytochrome b gene and the lack of resistance in *P*. *infestans*.

When we analyze the substitutions at the positions of interaction with azoxystrobin, the large number of residues that, due to Van der Waals clash, possibly disrupt the protein-azoxystrobin interaction, triggers an alert. Jointly considering the combination of two plant pathogens under study, *P*. *infestans* and *P*. *viticola*, and the two models (cytB_Pi_Btau, cytB_Pi_Ggal, cytB_Pv_Btau, and cytB_Pv_Ggal) at the positions 142 for *P*. *infestans* and 143 for *P*. *viticola*, there are at least 11 substitutions that would lead to the loss of efficiency of the molecule. It is worth noting that G143A substitution is the most important substitution in terms of the azoxystrobin resistance phenotype in both fungi and oomycetes (FISHER et al, 2020, GRASSO et al, 2006). Curiously, in the *P*. *infestans* mtDNA, the wild-type residue glycine is located in the position 142. Thus, G142A is anticipated to be involved in resistance to azoxystrobin in *P. infestans*. Considering the theoretical diversity of cytB in this position, azoxystrobin is only effective when a glycine is in the 142 position. As A142 corresponds to the most important substitution with alteration in the sensitivity phenotype, the Van der Waals clash and backbone clash may represent an important variable to explain the inefficiency of azoxystrobin in the substitution, in this sense, any substitution with Van der Waals clash or backbone clash similar or greater than A142, capable of disturbing the interaction of azoxystrobin with the protein will lead to loss of efficiency of the oomicide. At least 15 possible substitutions, estimated by the cytB_Pi_Btau, cytB_Pi_Ggal models, may cause an increase in ΔΔG, which can reduce the stability of the fungicide/oomicide interaction with the protein. Due to this fact, for *P. infestans*, the risk of these 15 energy-altering substitutions that do not negatively impact on the stability of cytB is expected to be high. Consequently, the risk of resistance to external quinone inhibitors can increase, as foreseen by Hollomon (2015). On the other hand, the lack of the other substitutions in field isolates of *P*. *infestans* may be related to sampling or a possible penalty on the protein due to the stability that these residues cause in cytB.

Not all gains in energy stability confer advantage to the organism. In the case of oligomers maintained by evolution, the increase in interaction stability will not necessarily be under selection, but rather, the functional improvement of the complex seems to be the preponderant factor (NOOREM & THORNTON 2003). Due to this, the list of substitutions that can be supported in cytB may be shorter than those known to date. Therefore, an important complementary analysis is the study of the "wild-type system": ubiquinol at its interaction sites; i.e. without azoxystrobin. The simulation of ubiquinol at its site is important to assess the stabilizing substitutions in the functional context of cytochrome b, and may help to assess the impact on protein flexibility and function.

The F129L substitution where L at position 129 is reported as involved in the resistance to azoxystrobin in different plant pathogens, including *P. viticola* (YIN et al, 2023; GRASSO et al, 2006; SIEROTZKI et al, 2005). In the present study this substitution is not a cytB stabilizer. This suggests that the substitution penalty for a protein destabilizing residue can be overcome and will be a benefit dependent on the selection event. Paradoxically, the regions that support only the wild-type residue, due to the proximity to the site where azoxystrobin binds to and the lower possibility of substitution, can serve as a potential target for the design of new inhibitors, decreasing the risk of resistance due to mutations that coincide with residue replacement.

5. Conclusion

The fact that the lack of resistance to azoxystrobin in *P. infestans* cannot be explained by the analyses carried out in this study sheds light on the difficulty of studying resistance and the need for the use of different approaches. To the best of our knowledge, the present study is one of the first in the area of plant pathology that used the saturation mutagenesis approach to predict the consequences of mutations in target genes. The current study calls attention to the use of protein biophysics tools in the study of the diversity of protein targets in plant pathogens, to improve the design of molecules and management so that control efficiency is maintained. In the short term these and other structural biology tools of proteins may become more useful among plant pathologists.

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SUPPLEMENTARY MATERIAL

Table S1. Total number of residues (To) and distribution of each amino acid (one-letter code, A to W) of the cytochrome b protein of *Phytophthora infestans* (Pi), *Plasmopara viticola* (Pv), and *Strobilurus tenacellus* (St). Alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V	To
Pi	19	10	21	8	3	3	10	27	11	49	41	10	12	40	23	17	19	10	24	26	383
Pv	17	10	21	7	4	3	11	26	11	51	42	11	11	38	23	17	21	10	25	25	384
St	21	12	23	11	2	9	7	23	11	33	56	6	15	38	22	24	17	9	18	32	389

Table S2. Residuos evaluated by position in each model.

	Position				
Model	**129**	**137**	**143**	**271**	**272**
cytB_Pv_Btau	F, L	G, R, S	G, A, C, E, F, H, I, K, L, M, N, Q, R, S, T, V, W, Y	P	D, I, L, H, P, V, E
cytB_Pv_Ggal	F, L, W	G	G, A, C, F, H, I, K, L, M, Q, R, V, W, Y	P	E
cytB_St_Btau	F	G	G, A, C, E, P, H, I, K, L, M, N, Q, R, S, T, V, W, Y	P	E, A, C, G, I, K, L, M, N, P, R, V, W
cytB_St_Ggal	F, M, W	G	G, A, C, F, E, H, I, K, L, M, N, Q, R, S, V, W, Y,	P	E, A, D, F, H, L, M, P

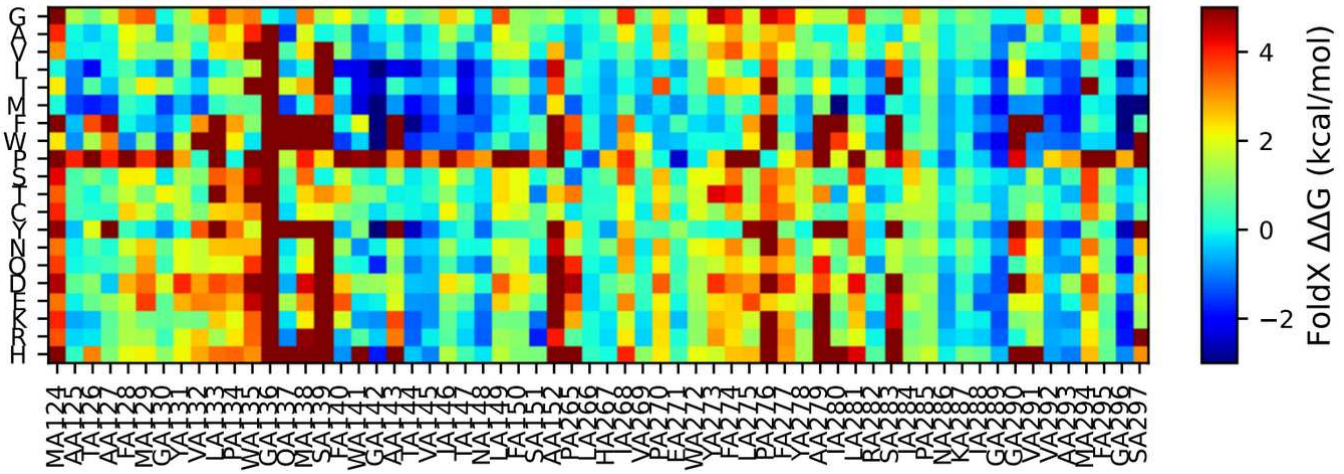

Figure S1. Residue substitution impact in the cytB_Pi_Btau model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference ranging from −3 to 5 kcal/mol.

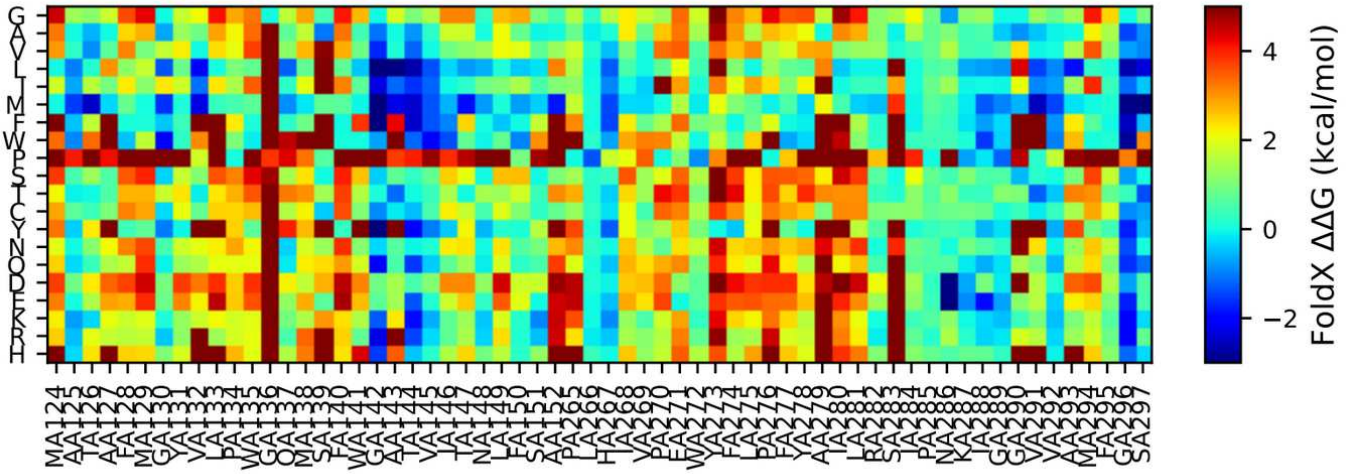

Figure S2. Residue substitution impact in the cytB_Pi_Ggal model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference ranging from −3 to 5 kcal/mol.

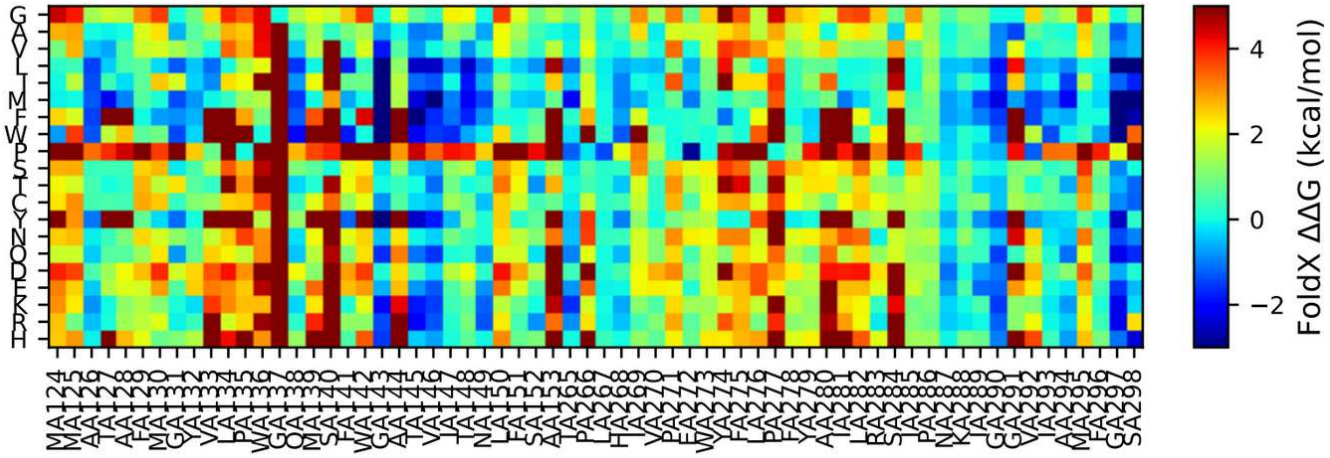

Figure S3. Residue substitution impact in the cytB_Pv_Btau model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference ranging from −3 to 5 kcal/mol.

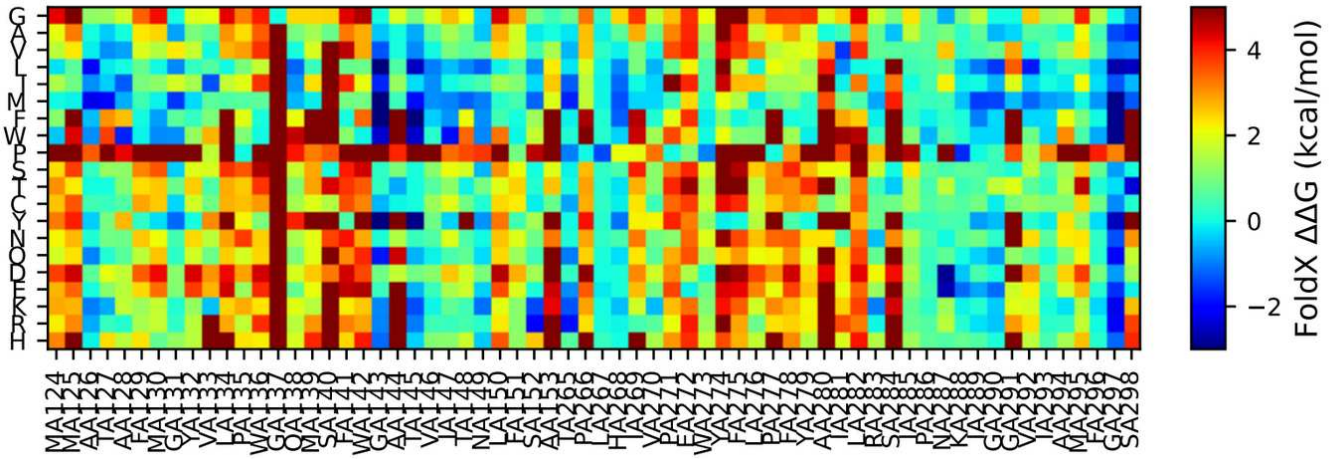

Figure S4. Residue substitution impact in the cytB_Pv_Ggal model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference ranging from −3 to 5 kcal/mol.

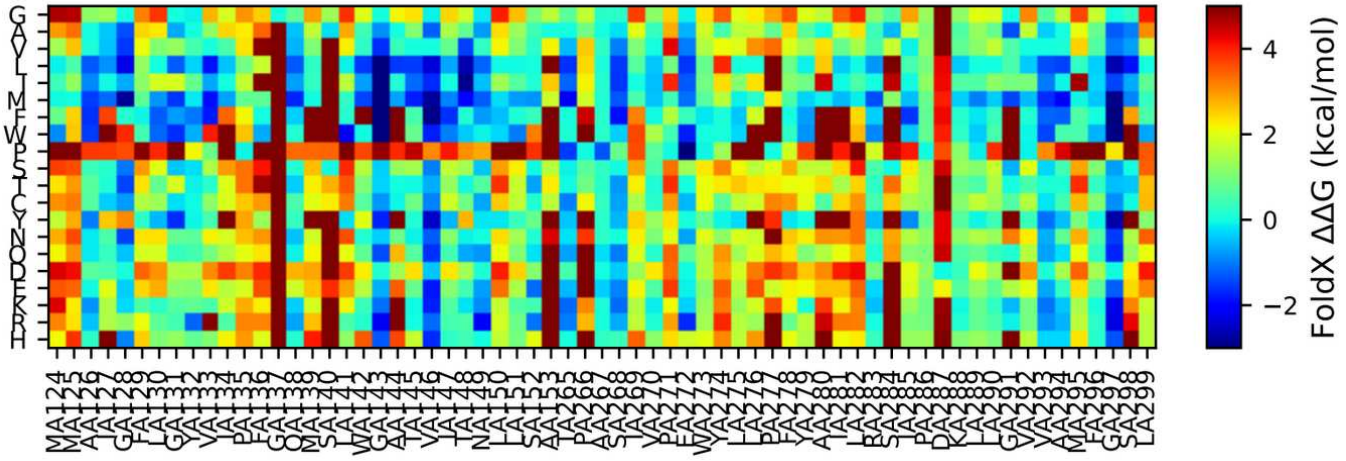

Figure S5. Residue substitution impact in the cytB_St_Btau model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference ranging from −3 to 5 kcal/mol.

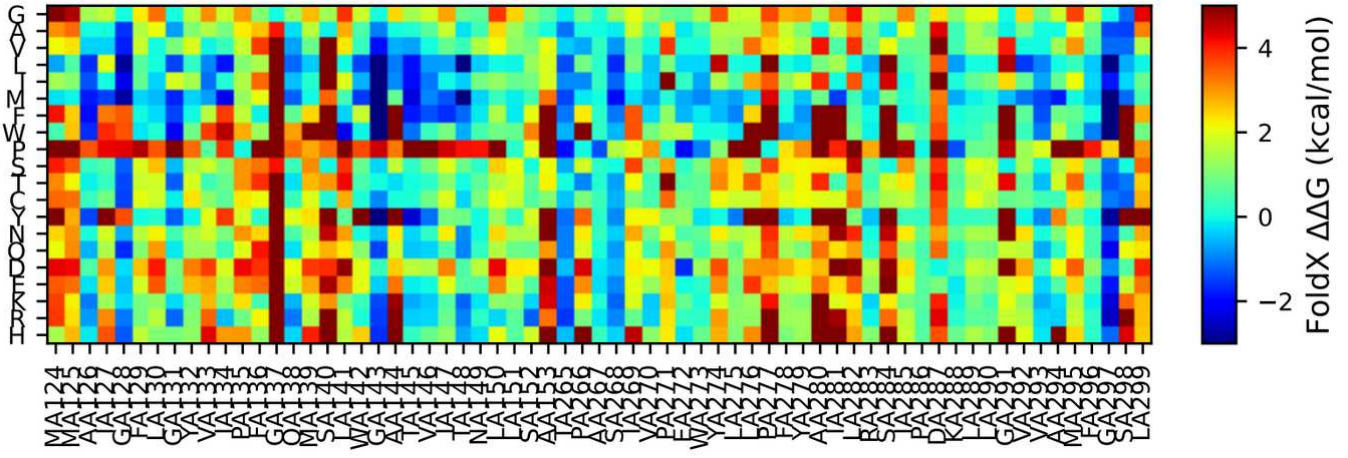

Figure S6. Residue substitution impact in the cytB_Pv_Ggal model. Values of free-energy variation (ΔΔGs) estimated for a substitution by an amino acid (y-axis) at a given position (x-axis) using MutateX. The values of free energy difference ranging from −3 to 5 kcal/mol.