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**AVALIAÇÃO DA VIA DO CHIQUIMATO COMO ALVO PARA O
DESENVOLVIMENTO DE HERBICIDAS**

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas (área de concentração – Biologia Celular e Molecular), da Universidade Estadual de Maringá para a obtenção do grau de Doutora em Ciências Biológicas.

Orientador: Prof. Dr. Osvaldo Ferrarese-Filho

Coorientador: Prof. Dr. Wanderley Dantas dos Santos

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APRESENTAÇÃO

Esta Tese é composta de dois capítulos, redigidos no formato de artigos científicos.

O capítulo I é um artigo de revisão a respeito da via do chiquimato e a prospecção de inibidores para suas enzimas, as quais podem servir como alvo para o desenvolvimento de medicamentos e herbicidas, este artigo foi intitulado “Revisiting the shikimate pathway and highlighting their enzyme inhibitors”, a ser submetida para a revista *Phytochemistry Reviews* (Fator de impacto 2021: 7.741, Qualis 2013-2016: B1). As principais revisões acerca do tema são de Herrmann et al. (1999) e Maeda et al. (2012), não havendo atualmente nenhuma revisão atualizada.

O capítulo II é um artigo original que descreve a obtenção da estrutura tridimensional da enzima chiquimato desidrogenase e a prospecção *in silico* de um inibidor, demonstrando os seus efeitos *in vitro* e *in vivo*. Este artigo foi intitulado “6-Nitroquinazoline-2,4-diol um inibidor da chiquimato desidrogenase. Ensaios *in vitro* e *in vivo*”, que será submetido à revista *Plant Physiology and Biochemistry* (Fator de impacto 2021: 5.437, Qualis 2013-2016: B1).

RESUMO GERAL

INTRODUÇÃO – A via do chiquimato é responsável por sintetizar o precursor para a biossíntese dos aminoácidos aromáticos, o corismato. Estando presente em plantas, microrganismos e alguns parasitas apicomplexos a via exibe algumas peculiaridades. Nas plantas apresenta uma maior complexidade, seja devido a sua regulação a qual ainda não está claramente definida, ou ao fato de a partir desta serem sintetizados vários metabólitos secundários. Além disso, há relatos de que algumas enzimas também estão presentes no citosol, o que retira a sua exclusividade plastidial. A via é constituída de sete reações catalisadas enzimaticamente; nas plantas a terceira e quarta reações são catalisadas por uma enzima bifuncional, a desidroquinato desidratase (DHQD; EC 4.2.1.10)/chiquimato desidrogenase (SDH; EC 1.1.1.25). Esta enzima é uma peça chave não apenas para a via do chiquimato, mas também para a síntese dos ácidos gálico e quínico, como observado em algumas espécies de plantas. Pelo fato de estar ausente em animais, as enzimas desta via tornam-se alvos cruciais para a busca e desenvolvimento de novas moléculas com ação inibitória, visando novos medicamentos e herbicidas.

OBJETIVOS - Neste trabalho revisamos e fornecemos um background sobre as enzimas da via do chiquimato, bem como de inibidores já descritos para microrganismos e/ou plantas. Além disso, prospectamos um novo inibidor para a enzima chiquimato desidrogenase de *Arabidopsis thaliana*, e avaliamos os seus efeitos *in vitro* e *in vivo*.

MATERIAIS E MÉTODOS – Para realização do levantamento bibliográfico, nós empregamos o “nome específico da enzima” e a palavra “inibição”, utilizando de diversos buscadores (Science Direct, Web of Science e Google Scholar). A partir daí nós descrevemos as informações e peculiaridades de cada uma das enzimas, assim como os inibidores já avaliados para os mais diversos organismos. Para a prospecção de um novo inibidor, inicialmente realizamos a obtenção da estrutura tridimensional da DHQD/SDH de *A. thaliana*, por meio de ferramentas *in silico*. A partir desta estrutura, nós efetuamos a triagem virtual na Biblioteca da Sigma, a fim de obtermos uma molécula com potencial atividade inibitória. O composto selecionado, 6-nitroquinazoline-2,4-diol (NQD) foi obtido comercialmente. Para avaliação da atividade *in vitro*, a enzima DHQD/SDH de *A. thaliana* foi expressa heterologicamente em *E. coli*, purificada e submetida a atuação do NQD. Para os testes *in vivo*, plântulas de soja e milho foram incubadas por 24 a 96 horas com 500 µM de NQD, sendo avaliados o comprimento radicular e a biomassa fresca. Além disso, nós monitoramos o consumo de solução nutritiva, a depleção do NQD ao longo das 96 horas e a quantidade do composto absorvida pelas plântulas. A biomassa fresca foi utilizada para as análises dos teores de proteínas totais por espectrofotometria e perfil de aminoácidos por cromatografia líquida de alta eficiência (HPLC). A partir da biomassa seca foram quantificados os teores dos ácidos fenólicos totais por espectrofotometria e o perfil dos ácidos fenólicos por HPLC. Um segundo cultivo foi realizado para demonstrar a atuação *in vivo* do NQD sobre a SDH. Para isso, plântulas de soja e milho foram incubadas apenas com glifosato (100 e 25 µM, respectivamente) e glifosato + NQD (100 + 500 µM e 25 + 500 µM,

respectivamente). Após o período de incubação o comprimento radicular e a biomassa fresca foram avaliados e determinado o teor de chiquimato por HPLC.

RESULTADOS E DISCUSSÃO – No tocante ao levantamento bibliográfico, nós constatamos que os trabalhos mais recentes vêm empregando ferramentas de bioinformática para prospectar novas moléculas com atividade inibitória sobre as enzimas da via do chiquimato. Grande parte dos trabalhos são voltados para enzimas de microrganismos sendo um baixo percentual relacionado a ação em plantas. Isto pode estar associado com o fato da via apresentar uma maior complexidade nas plantas, onde alguns aspectos ainda não estão bem esclarecidos, o que acarreta em vultoso desafio para a localização de um candidato a inibidor. Todavia, isto não impede que pesquisas buscando novas moléculas sejam realizadas, uma vez que vivenciamos a ocorrência de plantas daninhas resistentes a herbicidas, o que tende a se agravar ao longo dos anos. A partir da obtenção da estrutura tridimensional da DHQD/SDH de *A. thaliana* foi possível iniciar a busca por uma molécula com potencial ação inibitória *in silico*. Ao final das triagens, nós selecionamos NQD como um forte candidato, pois o mesmo apresentou o melhor score em relação ao próprio substrato. Após adquirir o composto, nós iniciamos os testes *in vitro*. Para isto, a enzima DHQD/SDH de *A. thaliana* foi expressa em *E. coli* e purificada por meio de uma coluna de afinidade, sendo identificada em gel de poliacrilamida e análise em espectrometria de massas. A atividade da enzima *in vitro* foi reduzida em cerca de 33% na concentração de 250 μM de NQD a meia saturação, demonstrando sua ação sobre a SDH. *In vivo*, com cultivo hidropônico empregando uma concentração de 500 μM , o NQD reduziu o comprimento radicular de soja (50% em 24 horas) e milho (29% em 96 horas), aumentou os teores de proteínas totais e de alguns aminoácidos, mas não alterou o teor de lignina. Além disso, observamos que a partir de 48 horas para ambas as espécies de plantas ocorreu redução na captação de solução nutritiva. Entretanto, nos quesitos de depleção e absorção radicular observou-se diferenças entre as espécies avaliadas. Em milho, após 96 horas, apenas 14% do NQD presente em solução foi depletado, enquanto que em soja foi de 41%. Quanto à absorção radicular ocorreu desigualdade, ou seja, as raízes de soja absorveram 37% a mais de NQD que as raízes de milho. Estes achados podem estar relacionados com a maior redução radicular observada em soja. O conteúdo de ácidos fenólicos totais em soja aumentou após exposição com NQD. Em milho, nós constatamos um aumento expressivo de ácido gálico, o qual é em algumas plantas originado do desidrochiquimato, o substrato da SDH. No segundo cultivo com glifosato, nós objetivamos identificar se SDH, *in vivo*, era inibida pelo NQD. Como já descrito na literatura, glifosato inibe a 5-enolpiruvilchiquimato 3-fosfato (EPSP) sintase, duas reações enzimáticas após a da SDH, causando reduções no crescimento e acúmulo de chiquimato nas plantas. Em soja, ao aplicarmos 100 μM de glifosato, ocorreu acúmulo de 1,3 mg de chiquimato g^{-1} de biomassa fresca. A incubação com 100 μM de glifosato + 500 μM de NQD resultou em 0,6 mg de chiquimato g^{-1} de biomassa fresca, ou seja, houve uma redução de 54% do teor do chiquimato, evidenciando que a ação do NQD antecede a reação catalisada pela EPSP sintase. Em contraste, em milho tratado com 25 μM de glifosato, nós observamos um acúmulo de 0,7 mg de chiquimato g^{-1} de biomassa fresca; enquanto que chiquimato não foi detectado quando 25 μM de glifosato + 500 μM de NQD foi aplicado, demonstrando uma sensibilidade maior da SDH de milho perante a ação do NQD do que a SDH de soja.

CONCLUSÃO – Nós observamos que a via do chiquimato apresenta certa complexidade em plantas devido às lacunas que ainda precisam ser preenchidas, colocando em conflito a atuação de muitos compostos já identificados e dificultando a busca de novos inibidores. Todavia, com o avanço da tecnologia e da biologia molecular diversos trabalhos vêm sendo desenvolvidos, e são fundamentais para contornar os problemas atuais e futuros no quesito de plantas daninhas resistentes aos herbicidas empregados. A partir de ensaios *in silico*, nós identificamos um candidato a inibidor para SDH de *A. thaliana*, o NQD, sendo demonstrado *in vitro* e *in vivo* este potencial. Todavia, nas condições *in vivo*, o NQD atuou de maneira diferente para dicotiledônea e monocotiledônea. Este fato requer melhor averiguação visando, futuramente, uma possível ação seletiva deste composto.

PALAVRAS-CHAVE - Via do chiquimato, bioinformática, herbicidas, inibidores enzimáticos, chiquimato desidrogenase.

ABSTRACT

INTRODUCTION – The shikimate pathway is responsible for synthesizing the precursor for the biosynthesis of the aromatic amino acid chorismate. Being present in plants, microorganisms and some apicomplexan parasites, the pathway exhibits some peculiarities. In plants, it is more complex, either because of its regulation, which is not yet clearly defined, or because several secondary metabolites are synthesized from it. In addition, there are reports that some enzymes are also present in the cytosol, which removes plastid exclusivity. The pathway generally consists of seven enzymatically catalyzed reactions in plants; the third and fourth reactions are catalyzed by a bifunctional enzyme, dehydroquinate dehydratase (DHQD EC 4.2.1.10)/shikimate dehydrogenase (SDH, EC 1.1.1.25). This enzyme is a key point not only in the shikimate pathway but also in the synthesis of gallic and quinic acids, as observed in some plant species. Because it is absent in animals, the enzyme of this pathway becomes a crucial target for the search and development of new molecules with inhibitory action, targeting new drugs and herbicides.

OBJECTIVES - In this work, we review and provide a background on the enzymes of the shikimate pathway and on inhibitors already described for microorganisms and/or plants. Additionally, we proposed a new inhibitor for the enzyme shikimate dehydrogenase from *Arabidopsis thaliana* and evaluated its *in vitro* and *in vivo* effects.

MATERIALS AND METHODS - To carry out the bibliographic survey, we used the "specific name of the enzyme" and the word "inhibition" using several search engines (Science Direct, Web of Science and Google Scholar). From there, we described the information and peculiarities of each enzyme and the inhibitors already evaluated for the most diverse organisms. For the prospect of a new inhibitor, we initially obtained the three-dimensional structure of the DHQD/SDH of *A. thaliana* through *in silico* tools, from which virtual screening was obtained in the Sigma Library to obtain a molecule with potential inhibitory activity. The compound resulting from these analyses was purchased and named 6-nitroquinazoline-2,4-diol (NQD) was obtained commercially. To evaluate the *in vitro* activity, the DHQD/SDH enzyme from *A. thaliana* was *E. coli* heterologously expressed, purified, and subjected to NQD action. For the *in vivo* tests, soybean and maize seedlings were incubated for 24 to 96 hours with 500 μM NQD, and the root length and fresh biomass were evaluated. In addition, we monitored the consumption of nutrient solution, the depletion of NQD over the 96 hours, and the amount of the compound absorbed by the seedlings. Fresh biomass was used to analyze the total protein content by spectrophotometry and the amino acid profile by high-performance liquid chromatography (HPLC). From the dry biomass, the content of total phenolic acids was quantified by a spectrophotometer, and the profile of phenolic acids was quantified by HPLC. Second cultivation was carried out to demonstrate *in vivo* the action of NQD on SDH. For this, soybean and maize seedlings were incubated only with glyphosate (100 and 25 μM , respectively) and glyphosate + NQD (100 + 500 μM and 25 + 500 μM , respectively). After the incubation period, the root length and fresh biomass were evaluated, and HPLC was used to determine the shikimate content.

RESULTS AND DISCUSSION – Through the bibliographic survey, we noticed that the most recent works have been using bioinformatics tools to search for new molecules with inhibitory activity against the enzymes of the shikimate pathway. Most of the works are focused on enzymes of microorganisms, with a low percentage related to action in plants. This may be associated with the fact that the pathway presents a greater complexity in these, where some aspects are still not well clarified, which leads to a significant challenge for the location of an inhibitor candidate. Nevertheless, this does not prevent research from looking for new molecules to be carried out since we have experienced weeds resistant to commercialized herbicides that tend to worsen over the years. After obtaining the three-dimensional structure of DHQD-SDH from *A. thaliana*, it was possible to start the search for a molecule with potential inhibitory action *in silico*. At the end of the screenings, we found NQD to be a strong candidate since it presented the best score concerning the substrate. After acquiring the compound, we started the *in vitro* tests. For this, the DHQD/SDH enzyme from *A. thaliana* was expressed in *E. coli* and purified through an affinity column, identified in a polyacrylamide gel and analyzed by mass spectrometry. The enzyme activity *in vitro* was reduced by approximately 33% at a concentration of 250 μM NQD at half-saturation, demonstrating its action on SDH. *In vivo*, with hydroponic cultivation using a concentration of 500 μM , NQD reduced the root length of soybean (50% in 24 hours) and maize (29% in 96 hours) and increased the levels of total proteins and some amino acids but without changing the lignin content. In addition, we observed that after 48 hours for both seedlings, there was a reduction in the uptake of nutrient solution. However, differences were observed between the evaluated species in terms of depletion and root absorption. In maize, after 96 hours, only 14% of the NQD in the solution was depleted, while in soybean, the value was 41%. For root absorption, there was also an inequality; soybean roots absorbed 37% more NQD than maize roots. These findings may be linked to soybeans' more significant root reduction. The content of total phenolic acids in soybeans increased after exposure to NQD. In maize, we found a significant increase in gallic acid, which in some plants is indicated as being synthesized from dehydroshikimate, a substrate of SDH. In the second cultivation with glyphosate, we sought to identify whether *in vivo* SDH was inhibited by NQD. As already described in the literature, glyphosate inhibits 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, two enzymes after SDH, resulting in reductions in growth and accumulation of shikimate in plants. In soybean, when using 100 μM glyphosate, we verified an accumulation of 1.3 mg of shikimate g^{-1} of fresh biomass. When incubated with 100 μM glyphosate + 500 μM NQD, we obtained 0.6 mg of shikimate g^{-1} fresh biomass; that is, there was a 54% reduction in shikimate content, showing that the action of NQD precedes the reaction catalyzed by EPSP synthase. In contrast, in maize, when using 25 μM glyphosate, we obtained an accumulation of 0.7 mg of shikimate g^{-1} fresh biomass, but when we used 25 μM glyphosate + 500 μM NQD, no shikimate was detected, demonstrating a greater sensitivity of maize SDH to the action of NQD than soybean SDH.

CONCLUSION – We observed that the shikimate pathway presents some complexity in plants due to gaps that still need to be filled, putting into question the performance of many compounds already identified and making the search for new inhibitors difficult. However, several works have been developed with the advancement of technology and molecular

biology, several works have been developed, and they are essential to overcome current and future problems regarding weed resistance to the herbicides used. From *in silico* assays, we identified a candidate inhibitor for *A. thaliana* SDH, NQD, and this potential has been demonstrated *in vitro* and *in vivo*. However, under *in vivo* conditions, NQD showed different performance for dicotyledonous and monocots. This fact requires more investigation aiming, in the future, a possible selective action of this compound.

KEYWORDS - Shikimate pathway, bioinformatics, herbicides, enzyme inhibitors, shikimate dehydrogenase.

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CAPÍTULO I

ARTIGO:

Revisiting the shikimate pathway and highlighting their enzyme inhibitors

Este artigo será submetido a Revista *Phytochemistry Reviews*.

Revisiting the shikimate pathway and highlighting their enzyme inhibitors

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Abstract

Absent in animals and with only seven enzymatic reactions toward the synthesis of chorismate and aromatic amino acids, the shikimate pathway is a crucial target for developing antimicrobial agents and herbicides. Although this pathway has been extensively studied in microorganisms related to human health, it reveals complexities in plants, as it takes part in primary and secondary metabolism. Obtaining enzyme inhibitors is essential to circumvent the occurrence of weeds resistant to commercially available herbicides and to help control human diseases, which has challenged researchers to search for new molecules and investigate their modes of action. By applying bioinformatics tools, thousands of enzyme inhibitors of this metabolic pathway can be prospected in a brief time and at a low cost. Here, we revisit how pathway enzymes have been characterized and update the status of their inhibitors in microorganisms and plants. Their overview can be constructive in searching for enzyme inhibitors in the academic, human health, and agro-industrial fields.

Keywords: Enzymes · Bioinformatics · Antimicrobials · Herbicides · Enzymatic inhibitor.

Introduction

The shikimate pathway (Fig. 1) is a crucial metabolic route present in plants, bacteria, fungi, and some parasites but is absent in animals (Roberts et al. 1998; Herrmann and Weaver 1999). The name given to this path is because of the identification of shikimate (in Japanese, shikimi-no-ki), which was first isolated from fruits of *Illicium religiosum* (Eijkman 1885).

The shikimate pathway handles chorismate production, a common precursor for the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan), which have essential roles in all organisms (Tzin and Galili 2010). There are distinct differences among the organisms that have this pathway, such as the location of the enzymes (cytosol or chloroplast), organization of the enzymes (monofunctional, bifunctional or multifunctional complex), and metabolic regulation. Nevertheless, the formation of chorismate as an end-product of the shikimate pathway occurs in all organisms (Herrmann and Weaver 1999; Gientka and Duszkiwicz-Reinhard 2009).

The pathway originates from glycolysis and phosphate pentose pathways, and it undergoes seven enzymatic reactions (Fig. 1). First, phosphoenolpyruvate and D-erythrose-4-phosphate

are condensed into 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) by DAHP synthase (DAHPS; EC 2.5.1.54). Second, 3-dehydroquinate synthase (DHQS; EC 4.2.3.4) cyclizes DAHP to form 3-dehydroquinate. Then, 3-dehydroquinate is dehydrated by DHQ dehydratase (DHQD; EC 4.2.1.10) to produce 3-dehydroshikimate which, in turn, is reduced to shikimate by shikimate dehydrogenase (SDH; EC 1.1.1.25). In the fifth reaction, shikimate is phosphorylated to form shikimate 3-phosphate by the action of shikimate kinase (SK; EC 2.7.1.71). Catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS; EC 2.5.1.19), the sixth reaction condenses shikimate 3-phosphate and phosphoenolpyruvate to produce EPSP, releasing inorganic phosphate. Finally, EPSP is dephosphorylated by chorismate synthase (CS; EC 4.2.3.5), forming chorismate. From that point on, aromatic amino acids are produced (Marchiosi et al. 2020).

Because of the shikimate pathway, amino acid biosynthesis essentially changes carbon metabolism (Herrmann and Weaver 1999; Maeda and Dudareva 2012). To produce aromatic amino acids and other related compounds with essential roles in plants, the shikimate pathway requires approximately 30% of the fixed carbon (Maeda and Dudareva 2012; Coruzzi et al. 2015; Santos-Sánchez et al. 2019). Indeed, the carbon skeleton of the intermediates (3-dehydroquinate, 3-dehydroshikimate, and chorismate) is used for the synthesis of various compounds, including benzoic acids (quinic, gallic, and salicylic, for example) that have crucial roles in primary and secondary metabolism (Widhalm and Dudareva 2015; Marchiosi et al. 2020).

Because of their absence in animals, enzymes of the shikimate pathway are a true goldmine for discovering new antimicrobial and anti-parasitic agents and herbicides (Maeda and Dudareva 2012; Bhattacharya and Kumar 2012; Coracini and Azevedo 2014). As a straightforward example, the shikimate production, either by the action of microorganisms or extracted from plants, has great pharmaceutical value. It is used to manufacture oseltamivir (Tamiflu[®]), an antiviral drug used to treat influenza A and B infections (Singh et al. 2020). Another well-known example is the herbicide glyphosate, which inhibits EPSPS, blocking the production of aromatic amino acids and the growth of many weeds (Duke and Powles 2008). However, the resistance of microorganisms to antimicrobial agents and of weeds to herbicides are bottlenecks that challenge researchers to discover new molecules and their modes of action (Green 2014; Gatadi and Nanduri 2020; Khawbung et al. 2021). In this way, we revisited how the pathway's enzymes have been found in different tissues and highlighted current information about their inhibitors in microorganisms and plants. Peculiarly, we

believe that this mapping can aid researchers in studying enzyme inhibitors applicable to health and agro-industrial fields and fundamental scientific knowledge. Simply put, we used the "specific enzyme name" and the word "inhibition" for the bibliographic survey. All pieces of information are summarized in [Table 1](#) and described as follows.

3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS)

The shikimate pathway begins with an aldol condensation reaction of phosphoenolpyruvate and erythrose 4-phosphate forming 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP), catalyzed by DAHPS ([Figs. 1](#) and [2a](#)).

There are two types of DAHPS: I and II. The former is subdivided into I α and I β . Type I α , found in *Escherichia coli* and *Saccharomyces cerevisiae*, depends on bivalent metals and is sensitive to feedback inhibition by aromatic amino acids. The type I β , present in *Bacillus subtilis* and *Pyrococcus furiosus*, is not a metalloenzyme. The type II (>50 kDa) is found in plants and some microorganisms with subunits larger than type I (40 kDa) (Subramaniam et al. 1998; Jensen et al. 2002; Schofield et al. 2004).

The DAHPS reaction is a regulatory point for the carbon flow into the shikimate pathway. Microorganisms have isozymes DAHPS regulated by a feedback mechanism, performed by one of the aromatic amino acids (DAHPS sensitive to phenylalanine, tyrosine or tryptophan) (Jossek 2001; Wu et al. 2003). In plants, enzyme regulation is not precise (Herrmann and Weaver 1999). The expression of the *AroG* gene (phenylalanine-sensitive DAHPS) of bacteria in *Arabidopsis thaliana* changed primary and secondary metabolites; however, other enzymes may be involved in regulating primary metabolism (Tzin et al. 2012). Most plants have DAHPS isozymes with distinct levels of expression, which may be involved in regulating the path in the face of environmental variations.

From a cell suspension of *Nicotiana sylvestris*, two isoenzymes of DAHPS were detected, which had different properties for their activity (Ganson et al. 1986). In tomato (*Solanum lycopersicum*), two genes for DAHPS (*LeDHS1* and *LeDSH2*) with different expression levels depending on the organ have been described (Gorlach et al. 1993). In *A. thaliana*, at first, only two distinct genes for DAHPS were isolated (*DHS1* and *DHS2*), which showed different expressions when subjected to injury or bacterial infection (high *DHS1* while *DHS2* was not observed) (Keith et al. 1991). Subsequently, in *A. thaliana*, three genes that code for DAHPS (*At-DAHPS*) were found, and their sequences of amino acids had 75% similarity.

From homology modeling, it was seen that *At*-DAHPS belongs to the $(\beta/\alpha)_8$ TIM barrel family. The catalytic site revealed conserved residues in most of the analyzed plant species. This characteristic may be crucial to engineering transgenic plants for an herbicide whose action affects DAHPS (Bhattacharya and Kumar 2012). The three isozymes present in *Arabidopsis thaliana* (DAHPS1, DAHPS2, and DAHPS3) require a metallic cofactor for their activities but have different catalytic efficiency, higher for DAHPS1, DAHPS2, and then DAHPS3 (Yokoyama et al. 2021). Regarding the role of aromatic amino acids in the *in vitro* activity of isozymes, there are still some contradictions (Yokoyama et al. 2021; Kanaris et al. 2022). From the obtained mutants, through the insertion of T-DNA, it was found that *dahps1* mutants showed hypersensitivity to tyrosine, and *dahps3* had hypersensitivity to tryptophan. In contrast, *dahps2* did not show hypersensitivity to any of the aromatic amino acids, suggesting that DAHPS2 is tyrosine-regulated, not allosterically, but at the level of gene expression and/or post-translation. *In vivo* DAHPS2 interacts with a 14-3-3 protein, which is related to the retention of this isozyme in the cytosol and its reduction in the chloroplast after supplementation with tyrosine (Kanaris et al. 2022).

Regarding the search for molecules that inhibit DAHPS, Walker and Parker (2006) synthesized a phosphonate compound in 12 steps from D-arabinose (Table 1). The compound inhibited DAHPS from *E. coli* but with a very slow binding to the enzyme. Targeting molecules that interact with the binding site of both phosphoenolpyruvate and erythrose 4-phosphate substrates, the same authors found three compounds with good inhibitory properties (Table 1) (Walker et al. 2011).

Bisphosphonic amino-methylene acid derivatives were evaluated against DAHPS purified from *Nicotiana plumbaginifolia*. Of these, two compounds – *N*-2-(6-methyl-pyridyl) and *N*-2-(5-chloro-pyridyl)-amino-methylene bisphosphonic acids – revealed better inhibitory action (Table 1). The second compound non-competitively inhibited phosphoenolpyruvate and competitively inhibited erythrose 4-phosphate (Forlani et al. 1996). Additionally, DAHP oxime (Balachandran et al. 2016) and a phosphoenolpyruvate derivative (de Oliveira et al. 2020) inhibited DAHPS from *E. coli* and *Corynebacterium glutamicum*, respectively (Table 1).

Dehydroquinase synthase (DHQS)

In the second step of the shikimate pathway, DHQS converts DAHP to dehydroquinate (Figs. 1 and 2b). This reaction forms the first carbocyclic metabolite of the pathway because of a deletion of the β -phosphate group, and the assumed geometry would be chair-like (Widlanski et al. 1989). However, Bartlett et al. (1988) hypothesized that enolpyranose would be the product of DHQS, since the conversion of enolpyranose to 3-dehydroquinate is spontaneous, non-enzymatic, and a quick reaction. Thus, the enzyme oxidizes DAHP by eliminating the phosphate group, generating a pyranose that is released from the enzyme and quickly converted to 3-dehydroquinate (Bartlett and Satake 1988; Widlanski et al. 1989).

Until that time, the mechanism of the catalytic reaction of DHQS was not fully understood. Carpent et al. (1998) described the structure of 3-dehydroquinate and the enzyme-catalyzed reaction. At first, DHQS was identified as a supporting factor in the reaction. DHQS converts DAHP to 3-dehydroquinate in five steps (Fig. 2b). The first is the oxidation of the substrate by NAD^+ ; the second is the elimination of the phosphate group; the third is the reduction of an intermediate ketone by NADH ; the fourth is the ring-opening, and the last is the aldol condensation (Herrmann and Weaver 1999).

In higher plants, one of the first studies about DHQS was described in etiolated seedlings of *Phaseolus mungo* (Yamamoto and Minamikawa 1976). A purified extract of *P. mungo* revealed that the enzyme had a molecular weight of 67 kDa, and its activity required Co^{2+} or Cu^{2+} (Yamamoto 1980). In etiolated sorghum (*Sorghum bicolor*) seedlings, the aromatic amino acids, dehydroshikimate, shikimate, and *t*-cinnamic, *p*-coumaric, caffeic and chlorogenic acids did not interfere with the enzyme activity. Also, 3-dehydroquinate and protocatechuic acid weakly inhibited the enzyme (Saijo and Kosuge 1978). In kiwi (*Actinidia chinensis*; AchDHQS), the enzyme is a homodimeric protein including 377 amino acids, activated by divalent metal ions, requiring NAD^+ whose connection occurs between the N- and C-terminal domains. The AchDHQS has a closed conformation. It differs from that described in *Aspergillus nidulans*, which may be open or closed to prevent the early release of the substrate (Mittelstädt et al. 2013).

Some organophosphonates, such as 3-deoxy-D-arabino-heptulosonic acid 7-phosphonate (1a) and α -(2,6-anhydro-3-deoxy-D-arabino-heptulopyranosid)onate 7-phosphonate (2b) (Table 1) competitively inhibited the DHQS of *E. coli* (Myrvold et al. 1989) and pea (*Pisum sativum*) (Pompliano et al. 1989). To determine the potential herbicidal action, compound 1a was applied to the post-emergence treatment of pea, *Echinochloa crusgalli*, *Setaria viridis*, *S. halepense*, and *Avena fatua*. Except for pea, all plants suffered effects such as herbicide

(growth inhibition, discoloration, and desiccation). Compound 1a also inhibited DHQS of *E. crusgalli*, *S. viridis*, and *A. fatua*, but to a lesser degree than glyphosate, a well-known herbicide (Pompliano et al. 1989).

Carbaphosphonate (Table 1) is an irreversible inhibitor of DHQS since adding the substrate DAHP accelerated inhibition (Montchamp and Frost 1991). Despite that, in pea, *E. crusgalli*, *S. viridis*, *S. halepense*, and *A. fatua*, the foliar application of carbaphosphonate (or epimeric carbaphosphonate) did not cause any herbicidal effect (Montchamp et al. 1992).

By determining the three-dimensional structure of the DHQS of *Helicobacter pylori* (HpDHQS), two compounds (HTS11955 and RH00573; Table 1) were proposed as potential inhibitors with IC₅₀ values of 61 and 84.4 μM, respectively (Liu et al. 2008). From virtual screening, two compounds (ZINC633887 and PubChem73393; Table 1) presented the best binding energies; the molecular dynamics showed that these compounds form stable complexes with DHQS of *Mycobacterium tuberculosis*. In addition, these compounds fulfilled all the ADMET criteria, i.e., absorption, distribution, metabolism, excretion, and toxicity (Isa et al. 2018). In DHQS of *M. tuberculosis* (AroB), the *in silico* analyses revealed two compounds (lead 1 and lead 2; Table 1) with better binding affinities and energies than pravastatin and carbaphosphonate (substrate analog). These findings indicate that 1 and 2 are promising antibacterial agents (Sivaranjani et al. 2019).

Aromatic compounds having an *ortho*-dihydroxylated benzene ring (protocatechuic acid, catechol, and their derivatives) inhibited the *E. coli* DHQS (Table 1). Inhibition varied according to the metal ion used: Zn⁺² or Co⁺² (Chandran and Frost 2001). Chlorogenic acid inhibited DHQS acid from *Providencia alcalifaciens* (PaDHQS). In a virtual screening, chlorogenic acid showed the best score with PaDHQS. Its connection with the enzyme induced an open conformation, making catalysis impossible. In addition, chlorogenic acid revealed antimicrobial activity, which was reversed by aromatic amino acids (Neetu et al. 2020). Finally, the rare sugar 7-deoxy-sedoheptulose (7dSh) isolated from the cyanobacterium *Synechococcus elongates* was shown to be a potent herbicide. From 25 to 50 μM, 7dSh was similar to glyphosate, and from 130 to 260 μM, the inhibitory effect surpassed that of the herbicide (Brilisauer et al. 2019).

Dehydroquininate dehydratase – shikimate dehydrogenase (DHQD–SDH)

The third and fourth reactions are catalyzed by DHQD and SDH, respectively (Fig. 1). These enzymes are different in terms of organization; in plants, they are bifunctional; in bacteria, they are monofunctional, and in fungi, they are part of the AROM complex, a sizeable pentafunctional polypeptide (Herrmann and Weaver 1999).

Dehydroquininate dehydratase (DHQD) catalyzes the dehydration of 3-dehydroquininate to 3-dehydroshikimate (Figs. 1 and 2c). There are two types of DHQD: I and II. Type I is found in plants, fungi, and bacteria. It performs *cis* dehydration using a covalent imine intermediate (Fig. 2c). Type II is found in organisms that share this step with the quinic acid pathway, such as *M. tuberculosis*, *Streptomyces coelicolor*, and *Helicobacter pylori*. The dehydration is *trans* and with an enolate intermediate (Gourley et al. 1999). Importantly, this difference between catalyzed reactions may be helpful to prospect potent and specific inhibitors for each type of enzyme (Sann et al. 2004).

In silico analyses revealed five molecules (Zinc14981770, Zinc01147665, Zinc22910025, Zinc8442077, and PubChem72341) with inhibitory action on DHQD of *M. tuberculosis* (*MtbDHQase*) (Table 1). These inhibitors have high binding energy and no carcinogenic effect; however, some are bound to cytochrome P450 enzymes (Isa and Malik 2017). Prospected by high-throughput virtual screening, lead 1 (at 50 μ M) inhibited *MtbDHQase* by 58% (Table 1). In addition, lead 1a and 1b are promising enzyme inhibitors (IC_{50} values from 17 to 31 μ M) with low cytotoxicity (Petersen et al. 2015). From the combination of *in silico* methods usually used (virtual screening, docking molecular, and dynamics molecular) and deep learning, a new molecule with potential inhibitory action for *MtDHQD*, MOL19424, was found. Through molecular dynamics, it was observed that it makes stable and important bonds with essential amino acid residues from the active site, in addition, it can serve as a basis for the search for molecules with similar structures (Souza et al. 2022).

Flavonoids are secondary metabolites of plants that can be used as antimicrobial agents. For instance, Cheung et al. (2014) evaluated the effects of some flavonoids on *Enterococcus faecalis* type I DHQDS (*efDHQase*) activity. Virtual screening was performed on a library with flavonoids and derivatives, aromatic acids, and coumarins. Six flavonoid glycosides (gossypin, rutin, homoorientin, luteolin-3'-7-diglucoside, luteolin-7-glucoside, and rhoifolin) and two coumarins (7-hydroxycoumarin and aesculin) inhibited *efDHQase*. Two flavonoids (datisctin and naringenin) and two chalcones (phloretin and marein) inhibited the growth of *E. faecalis*. However, only marein (Table 1) was able to inhibit *efDHQase* and *E. faecalis* growth. In the virtual screening for *MtbDHQase*, naringin and hesperidin had the best anti-

tuberculosis activity (MIC of 100 µg/mL). Docking simulations showed that these molecules had interactions similar to the crystallized inhibitor–*Mtb*DHQase complex (Lone et al. 2017).

Shikimate dehydrogenase (SDH) catalyzes the reversible reduction of dehydroshikimate to shikimate, at the expense of NADPH (Figs. 1 and 2d). The SDH of methicillin-resistant *Staphylococcus aureus* (*Sa*SDH) was modeled by homology and used for virtual screening. Three compounds (894, 766, and 238) proved to be suitable enzyme inhibitors (Table 1) (Avitia-Domínguez et al. 2014). Based on the structural similarity of these inhibitors, new compounds have been proposed in other virtual libraries, and four of them were assessed for the *in vitro* activity of *Sa*SDH (Table 1). These compounds showed different inhibitions, and they conducted essential interactions in the enzyme. In addition, four new compounds (1 to 4; Table 1) were found to inhibit *Sa*SDH. All these compounds bind with other proteins, but further structural modifications could reduce toxicity (Enríquez-Mendiola et al. 2019).

In plants, the bifunctional DHQD–SDH complex catalyzes the third and fourth reactions. The DHQD–SDH structure of *A. thaliana* comprises two distinct functional domains. The DHQD domain, common in type I DHQD, has an (α/β)₈ TIM barrel structure with an alternating α/β secondary structure. The SDH domain has a nucleotide-binding domain in the C-terminal region and the substrate in the N-terminal. The DHQD active site is at the top of the TIM barrel, while the SDH site is among the domains. DHQD–SDH has a concave shape, with the active sites face to face guaranteeing the flow to the shikimate pathway (Singh and Christendat 2006, 2007). While *A. thaliana* has only one gene for DHQD–SDH, tobacco has two: one plastidial and another cytosolic. It was revealed after silencing plastidial DHQD–SDH by RNA interference (RNAi), resulting in decreased growth, aromatic amino acid content, chlorogenic acid, and lignin. However, cytosolic DHQD–SDH accumulated shikimate (Ding et al. 2007).

Populus trichocarpa encodes five putative genes for DHQD–SDH (*Poptr1* to *Poptr5*) and *in vivo*, two functionally distinct groups were found. *Poptr1* and 5 are DHQD–SDH, while *Poptr2* and 3 are quinate dehydrogenases (QDHs), using quinate as the preferred substrate and residues of SDH (Guo et al. 2014). In birch, an NADP⁺-dependent dehydroshikimate dehydrogenase (DSDG) produces gallic acid from 3-dehydroshikimate acid (Fig. 2i) (Ossipov et al. 2003). In vine berries (*Vitis vinífera*), four SDH proteins (*Vv*SDH), were identified: one with the so-called "classic" activity (*Vv*SDH1), two related to gallic acid formation (*Vv*SDH3 and *Vv*SDH4), and another possibly related to quinic acid biosynthesis (Fig. 2h) (Bontpart et al. 2016). Similarly, four genes were identified for DHQD–SDH in *Camellia sinensis*; one

facing the shikimate pathway (*CsDQD/SDHa*), two possibly involved in gallic acid biosynthesis (*CsDQD/SDHc* and *CsDQD/SDHd*), and another (*CsDQD/SDHb*) allegedly related to the quinic acid biosynthesis (Huang et al. 2019).

More recently, two DQD/SDHs (*EcDQ/SDH2* and *EcDQ/SDH3*), which catalyze gallate formation from 3-dehydroshikimate, were found in the aluminum-tolerant *Eucalyptus camaldulensis* (Tahara et al. 2021). In these plants, tolerance to aluminum may be linked to gallate biosynthesis since the detoxification process occurs by the complexation of aluminum with oenothain B, a hydrolysable tannin produced from gallate. In addition to these enzymes, a "classic" bifunctional enzyme (*EcDQD/SDH1*) and an NAD⁺/NADH-dependent quinate dehydrogenase (*EcDQD/SDH4a*) were also found. Thus, the DHQD/SDH intermediates may diverge into other pathways, such as chorismate biosynthesis (shikimate pathway) and gallic and quinic acid biosynthesis (Figs. 2h, i).

From a collection of 5500 compounds, 24 were identified as *Pseudomonas putida* shikimate dehydrogenase (AroE) inhibitors with polyphenolic structures. Two compounds were highly inhibitors, epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) (Table 1), in addition to reducing the growth of *P. putida* (Peek et al. 2014b). The *Toxoplasma gondii* AROM complex (*TgSDH*) SDH was isolated and characterized, and EGCG partially inhibited *TgSDH* (Peek et al. 2014a). EGCG and ECG are also suitable inhibitors of DHQD–SDH in *A. thaliana* (Peek et al. 2014b).

1,6-Dihydroxy-2-oxoisonicotinic acid derivatives (Table 1) were synthesized by Baillie et al. (1972). *In vitro*, reversible and irreversible inhibitions were observed on pea SDH, but no herbicidal effect was noted *in vivo*. Additionally, *in vitro*, a triazolothiadiazole derivative, IMB-SD62 (Table 1), inhibited SDH of *M. tuberculosis*, revealing an anti-tuberculosis action. For *in vivo* conditions, a possible reduction of the acute infection in rats caused by the bacteria has been suggested (Deng et al. 2018).

Shikimate kinase (SK)

Shikimate kinase (SK) is an Mg²⁺-dependent enzyme that catalyzes the fifth reaction of the pathway. It phosphorylates the C3 hydroxyl group of shikimate to form shikimate-3-phosphate by using ATP as a cosubstrate (Figs. 1 and 2f).

Shikimate is produced in the shoots and green stems of etiolated plants of *S. bicolor*, and SK was partially characterized in these tissues (Bowen and Kosuge 1977). The stoichiometry

reaction for the formation of shikimate-3-phosphate and ADP revealed equal amounts of ATP and shikimate, and the highest activity was obtained at pH 9 with Mg^{2+} and Mn^{2+} . Alone or together, aromatic amino acids did not interfere with enzyme activity, but *p*-coumarate and caffeate inhibited it (Bowen and Kosuge 1979). A preference for divalent cations, especially Mg^{2+} , and optimal activity at pH 9.5 were also observed in spinach (*Spinacia oleracea*) chloroplasts (Schmidt et al. 1990). The enzyme had a single polypeptide (30 kDa). It was unstable after purification, requiring the addition of bovine serum albumin and thioredoxins to improve its stability. In addition, a slight inhibition by ADP was observed, suggesting that the enzyme is associated with pathway regulation according to energy status.

In tomato, a cDNA fragment referring to the SK gene has been isolated; its N-terminal end has a chloroplast transit peptide, and the molecular mass is 33 kDa. Only one gene for SK has been found (Schmidt et al. 1991). In rice (*Oryza sativa*), three genes for SK have been found (*OsSK1*, *OsSK2*, *OsSK3*). All proteins are imported into the chloroplast but are expressed differently; *OsSK1* and *OsSK2* after treatment with an elicitor and *OsSK1* and *OsSK3* during the panicle development stage (Kasai et al. 2005). Two SKA genes (*AtSK1* and *AtSK2*) and two SK-like genes (*AtSKL1* and *AtSKL2*) were found in *A. thaliana* (Fucile et al. 2008). Both genes are differentially expressed in tissues. *AtSK1* is related to genes involved in stress responses, while *AtSK2* engages in the shikimate pathway and secondary metabolism genes. *AtSKL1* and *AtSKL2* are similarly expressed but lack SK activity (Fucile et al. 2011). *AtSKL1* is involved in chloroplast development (Xu et al. 2018). *Cassia obtusifolia* SK (*CoSK*) has been analyzed *in silico* and cloned (Liu et al. 2015). Got by homology modeling, the 3D structure of *CoSK* is α - β - α -folded with parallel β -sheets flanked by α -helices and four domains (CORE, NB, ESB, and LID).

Even with information about the three-dimensional structure of SK in plants, the search for molecules with inhibitory action on this enzyme has been focused on microorganisms, primarily *M. tuberculosis* (*MtSK*), as described in Table 1 (Vianna and De Azevedo 2012; Rajput et al. 2016; Pandey et al. 2016; Prado et al. 2016; Masoko et al. 2016; Simithy et al. 2018b, a; Rahul Reddy et al. 2020). As an example, ilimaquinone (Simithy et al. 2018b) and 6-cyclohexamidomanzamine A (Simithy et al. 2018a) inhibited *MtSK* irreversibly and in combination, respectively (Table 1). However, ilimaquinone inhibited other enzymes, such as lactate dehydrogenase. Additionally, rottlerin inhibited *MtSK* (*M. tuberculosis*), *MsSK* (*M. smegmatis*), and mycobacteria (Pandey et al. 2016).

Bioinformatics has been a helpful tool to prospect *MtSK* inhibitors (Vianna and De Azevedo 2012; Rajput et al. 2016; Rahul Reddy et al. 2020), and several libraries can be screened to provide a specific number of molecules with anti-tuberculosis actions. For example, this reduces cost and time with *in vitro* and *in vivo* analyses. In addition to tuberculosis, virtual screening has been used for SK of other microorganisms, such as *Shigella sonnei* (*SsSK*), and two compounds (Zinc2135897 and CB4275815; Table 1) were good inhibitors (Ahmad et al. 2018). Selected *N,N*-dibenzyl-5-aminoshikimate acid analogs competitively inhibited SK from *M. tuberculosis* and *H. pylori* (Table 1). The *N,N*-di(3-bromo-1H-indol-5-ylmethyl)amine derivative (compound 4d) was the most efficient inhibitor (K_i of 8.2 μM) of *MtSK*, while the *N,N*-di(naphth-2-ylmethyl)amine derivative (compound 4b) (K_i of 300 nM) inhibited *HpSK* (Prado et al. 2016). In another study also involving *MtSk* and *HpSK*, shikimic acid derivatives (Table 1) were analyzed, showing different potentialities for *M. tuberculosis* and *H. pylori* as a result of the different conformations caused by the binding of the compound in each of the enzymes (Pernas et al. 2019).

Applying another approach, Masoko et al. (2016) evaluated the effect of *Sutherlandia frutescens* extract on *MtSK* activity. This plant was chosen because it is used as an herbal agent against diseases, including tuberculosis. The dichloromethane:methanol (1:1) extract showed the best IC_{50} (0.1 $\mu\text{g/mL}$); the most active subfraction against *MtSK* had only γ -linolenic acid, with an IC_{50} of 3.7 $\mu\text{g/mL}$ (Table 1). Hexanol extract of *Alpinia galangal* (L.) Willd also inhibited the activity of *MtSK*, in which seven compounds (genistein, pinocembrin, kaempferide, naringenin, 1,7-diphenyl-4-hepten-3-one, acacetin, and cirsimaritin) present in this extract were identified, which may interact synergistically leading to such inhibition (Patrick et al. 2021). A similar study was performed with compounds from the therapeutic herb mango ginger (*Curcuma amada*) against *H. pylori* SK (*HpSK*), and gentisic acid was suggested to be a promising *in vitro* inhibitor (Divyashri et al. 2021).

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS)

EPSPS catalyzes the sixth reaction by the transfer of enolpyruvyl from phosphoenolpyruvate (PEP) to the 5'-oxygen atom of shikimate-3-phosphate (S3P), forming EPSP and releasing inorganic phosphate (Figs. 1 and 2f). EPSPS is classified into class I (in plants and most bacteria) and class II (in bacteria such as *Agrobacterium sp.*).

EPSPS is a well-studied enzyme of the shikimate pathway because it is the glyphosate target and the most widely used herbicide worldwide. Acting on different weeds, glyphosate [*N*-(phosphonomethyl)-glycine] (Table 1) is a nonselective herbicide that is postemergent and has good translocation within plants but is relatively slow to act (Duke and Powles 2008; Sherwani et al. 2015). Purified EPSPS has a molar mass of 51 kDa from pea, spinach, lettuce (*Lactuca sativa*), and maize (*Zea mays*). Glyphosate inhibited dicot and monocot plants, confirming their nonselectivity (Mousdale and Coggins 1986a). A study with purified EPSPS of *Nicotiana silvestres* reinforced the action of glyphosate as a competitive inhibitor for PEP and uncompetitive for S3P. From 0.12 to 1.0 mM, glyphosate inhibited plant growth and accumulated shikimate (Rubin et al. 1984).

A glyphosate-tolerant *Petunia hybrida* plant was 20 times more resistant than the nontolerant plant; it showed high EPSPS activity (Steinrücken et al. 1986). Similar behavior was noted in cultured cells of *Corydalis sempervirens* under glyphosate action (Holländer-Czytko and Amrhein 1987). Already, in glyphosate-resistant carrot (*Daucus carota*), some clones had only one copy of the EPSPS gene with a 13 kb insertion. In contrast, others had a 16 kb insertion and two EPSPS copies in reverse (Suh et al. 1993).

In sorghum shoots, inhibition by glyphosate is independent of EPSPS isozymes (Ream et al. 1988). Two maize EPSPS isoforms were slightly stimulated by the aromatic amino acids chorismate and anthranilate. Glyphosate inhibited both isozymes, but form II was more catalytically efficient (Forlani 1997). In petunia and tomato, cDNA encoding EPSPS was isolated and sequenced, and the similarity between the amino acid sequences was 93% (Gasser et al. 1988).

The structure of *O. sativa* EPSPS (*OsEPSPS*) was obtained by homology modeling and comparison with other EPSPS. *OsEPSPS* exhibits α -helix and β -leaves in higher proportions (Yaqoob et al. 2016). EPSPS from *E. coli* (*EcEPSPS*) and *M. tuberculosis* (*MtEPSPS*) have two globular, hemispherical domains with three repeated folding units of $\beta\alpha\beta\alpha\beta\beta$ and two conformations, "open" or "closed". The primary sequence of *OsEPSPS* has 515 amino acid residues, with conservation in most species evaluated and showing potential involvement in catalysis (Stallings et al. 1991; Pereira et al. 2003). Cocrystallization of *EcEPSPS* with glyphosate and S3P or with S3P alone proved that S3P closes the domains with a screw motion and the active site located in the interdomain cleft. Glyphosate binding is close to S3P but does not alter the EPSPS–S3P complex (Schonbrunn et al. 2001). Unlike *OsEPSPS*, obtaining the three-dimensional structure of EPSPS from *Arabidopsis thaliana* (*AtEPSPS*)

occurred through crystallization of the protein, which was in the open conformation of the enzyme, the way the substrate binds, and shares 45% sequence identity with the enzyme *Ec*EPSPS. *At*EPSPS is a monomeric protein with two distinct domains. Precisely in the interdomain hinge, five compounds showed good binding energies through *in silico* docking, pointed out as a promising site for the search for new inhibitors, which can interfere with the closing of the enzyme and consequently in its activity (Ruszkowski and Forlani 2022). Through site-directed mutagenesis projection, for example, it was found that by replacing proline 101 with serine, *E. coli* EPSPS (*Ec*EPSPS) was tolerant to glyphosate (Stallings et al. 1991). Thus, some studies focus on finding mutations related to glyphosate resistance and the emergence of weeds resistant to it (Powles and Preston 2006; Takano et al. 2019; Franci et al. 2020). Other studies have sought to identify molecules similar to glyphosate, although none have been as effective as this herbicide. For example, Pansegrau et al. (1991) synthesized two competitive inhibitors for shikimate 3-phosphate: mono-deoxy-shikimate 3-phosphate and 5-amino-shikimate 3-phosphate (Table 1). In another work, *N*-amino-glyphosate proved to be an excellent competitive inhibitor of *E. coli* EPSPS, but it was decomposed at room temperature (Knowles et al. 1993). Phosphoenolpyruvate derivatives inhibit *M. tuberculosis* EPSPS (de Oliveira et al. 2020). Two phosphonate diastereoisomers competitively inhibited the *Petunia hybrid* EPSPS; the 2R isomer was the most potent (Alberg and Bartlett 1989). Substitution of the 3-phosphate group with 3-malonate or 3-hydroximalonate ether revealed competitive inhibition of S3P in *E. coli* EPSPS, suggesting that exchange of this group can generate molecules with inhibitory potential (Miller et al. 1995; Shah et al. 1997). Finally, fluorinated analogs or stable phosphonates of the tetrahedral reaction intermediate (Fig. 2f) were evaluated on glyphosate-sensitive (form I) and glyphosate-tolerant (form II) EPSPS. All analogs competitively inhibited both forms, but EPSPS II was more tolerant. Thus, the intermediate tetrahedral state between the two enzyme forms may change conformation and energy (Funke et al. 2007).

Chorismate synthase (CS)

Chorismate synthase (CS) catalyzes the dephosphorylation of EPSP to form chorismate, and it requires reduced flavin mononucleotide phosphate (FMN) as a cofactor (Fig. 2g). In plants and bacteria, CS is monofunctional, and FMN comes from exogenous medium. In contrast, CS is bifunctional and NADPH-dependent in fungi and protozoa (Macheroux et al. 1999;

Ehammer et al. 2007; Khera et al. 2019). Chorismate synthase was first found in pea chloroplast fractions (Mousdale and Coggins 1986b). Subsequently, CS was purified from *C. sempervirens*, which preferred FMN; FAD and riboflavin reduced its activity (Schaller et al. 1990).

The CS cDNA from *C. sempervirens* and *O. sativa* encode proteins with 447-438 amino acid residues and 48% homology to CS from *E. coli*. The N-terminal region corresponds to the chloroplast transit peptide, and only one gene was found (Schaller et al. 1991; Pagolu et al. 2016). Two CS genes (*LeCS1* and *LeCS2*) were identified in the haploid tomato genome. These genes had 88% similarity in the amino acid sequence and, like *C. sempervirens*, had a single transit peptide. Both genes are more highly expressed in flowers and roots; *LeCS1* is more highly expressed than *LeCS2* (Gorlach et al. 1993). However, because of alternative splicing in one gene (*LeCS2*), three isoenzymes can be found in tomatoes (CS1, CS2, and CS2 Δ). When expression in *E. coli* was performed, only CS1 and CS2 were enzymatically active, showing tetramer forms, while CS2 Δ was unstable (Braun et al. 1996). Expression of rice CS (*OsCS*) in *E. coli* *aroC* mutants in the absence of tryptophan allowed these bacteria to grow, suggesting a functional complement. In addition, bathophenanthroline inhibited *OsCS* (Table 1) (Pagolu et al. 2016).

Unlike what we have seen thus far, signs targeting the peroxisomes of *P. hybrida* (*PhCS*) were found in addition to the plastid transit peptide. With flowering, *PhCS* activities were noted in chloroplasts and peroxisomes. Silencing the enzyme reduced its catalytic efficiency, plant growth, and the contents of carotenoids, anthocyanin, and chlorophylls *a* and *b*. The levels of aromatic amino acids were not altered, but the folate contents were reduced in leaves and sepals (Zhong et al. 2020).

From *in silico* molecular modeling, it was possible to obtain the three-dimensional structure of *Plasmodium falciparum* CS (*PfCS*) and find EPSP analogs with inhibitory actions. The compound 6,7-dihydroxy-2-[1-(4-morpholin-4-yl-phenyl)-meth-(*E*)-ylidene]-benzofuran-3-one (Table 1) was the most potent (Tapas et al. 2011). Using homology modeling and virtual screening, it was possible to identify inhibitors of *Paracoccidioides brasiliensis* CS (*PbCS*) (Rodrigues-Vendramini et al. 2018; Bueno et al. 2019). *In vivo* and *in vitro* assays with (1*S*,2*S*,3*aS*,4*S*,9*bR*)-1-chloro-6-nitro-2-(2-nitrophenyl)sulfanyl-2,3,3*a*,4,5,9*b*-hexahydro-1*H*-cyclopenta[*c*]quinoline-4-carboxylic acid (Table 1) reduced fungal infection in the lungs of mice infected with *P. brasiliensis* (Rodrigues-Vendramini et al. 2018). Additionally, prospected by virtual screening, phenaphthazine (Table 1) showed

synergistic antifungal activity when combined with amphotericin B. It inhibited *in vitro* PbCS activity without any cytotoxic effect on mammalian cells (Bueno et al. 2019). Still following this line of comparative modeling, it was possible to obtain the three-dimensional structure of the CS of *Toxoplasma gondii* and by virtual screening to find four compounds with potential inhibitory action, two benzofuran analogs, and two EPSP analogs (Table 1) (Singh and Qureshi 2022).

Crude extracts of *Zingiber officinale* Roscoe inhibited the growth of *Plasmodium falciparum* *in vitro*. From the isolation, the compound 6-paradol (1-(4-hydroxy-3-methoxyphenyl)decan-3-one) (Table 1) was discovered, which *in silico* proved to be a potent inhibitor of PfCS *in vitro* and showed inhibition in the nanomolar range (Awasthi et al. 2022).

Bottlenecks and outlook

As described here, the shikimate pathway is predominant in microorganisms related to human health and plants to a lesser but no less necessary extent. Obtaining enzyme inhibitors of the metabolic pathway has proven crucial in disease control in humans and weeds. However, resistance to current bacterial agents has become a severe problem affecting healthcare systems with damaging reflections on society (Chambers and DeLeo 2009; Khawbung et al. 2021). Allied with these problems, there are few studies aimed at inhibiting the enzymes of this pathway in plants. Thus, the bottlenecks present in the health field extend to agriculture because of the increasing number of resistant weeds and the limited availability of herbicides with specific mechanisms of action (Heap 2021). For example, the first report of glyphosate-resistant weeds was from 1996 in *Lolium rigidum*. With GMOs and indiscriminate herbicide use, the number of resistant weeds has increased exponentially; to date, there are over 50 species of resistant weeds (Heap 2021). As weeds compete with crop plants for light, water, and nutrients, productivity is primarily affected (Lu et al. 2016; Ramella et al. 2020). Thus, diversifying weed management practices and discovering new molecules and/or mechanisms of action are significant challenges.

The shikimate pathway is well defined in microorganisms with relevant information on reaction, function, localization, and regulation. Although plants have a fair amount of information, new gaps and questions will arise. We know very little about what is yet to be revealed. Among these points is the location of the enzymes, presented as exclusively chloroplastic, since the characterized genes encode enzymes with a sequence referring to

plasmatic transit (Maeda and Dudareva 2012). However, studies point to diversification in their localizations. For example, a second cytosolic DHQD–SDH was reported in tobacco (Ding et al. 2007), while in petunia, a CS was described in peroxisomes (Zhong et al. 2020). The discovery of over one DHQD–SDH is an excellent case in point because, in addition to the enzyme with canonical activity, DHQD–SDH affects the biosynthesis of gallic acid and possibly quinic acid (Huang et al. 2019; Tahara et al. 2021). These pieces of evidence are in line with the “dual pathway hypothesis”, considering the existence of a plastid and extraplastid shikimate pathway occurring concomitantly; Lynch (2022) recently reviewed this. Given this complexity, added to the pressure exerted in the search for new agents, *in silico* tools allow the testing of thousands of molecules on specific targets, reducing time and costs (Isa and Malik 2017; Isa et al. 2018). The discovery of molecules with herbicidal actions will not be miraculous despite all this. The joint application of eco-friendly management technologies and chemical and biological practices points to a long way to go in the coming years. The challenge is on!

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Figures

Fig. 1. The shikimate pathway. Phosphoenolpyruvate (PEP); erythrose 4-phosphate (E4P); 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP); DAHP synthase (DAHPS) of *Arabidopsis thaliana* (Bhattacharya and Kumar 2012); 3-dehydroquinate; inorganic phosphate (Pi); 3-dehydroquinate synthase (DHQS) dimer from *Actinidia chinensis* (Mittelstädt et al. 2013); 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQD–SDH) of *A. thaliana* (ID PDB: 2O7Q_1) (Singh and Christendat 2007); shikimate kinase (SK) from *A. thaliana* (ID PDB: 3NWJ) (Fucile et al. 2011); 5-enolpyruvylshikimate 3-phosphate (EPSP); EPSP synthase (EPSPS) of *Oryza sativa* (Yaqoob et al. 2016); chorismate synthase (CS) tetramer from *M. tuberculosis* (ID PDB: 2G85) (Dias et al. 2006); tyrosine (Tyr); phenylalanine (Phe); tryptophan (Trp).

Fig. 2. Reaction mechanisms of the shikimate pathway. **a)** reaction catalyzed by DAHPS; **b)** reaction steps catalyzed by DHQS; **c)** reactions catalyzed by DHQ type I and II; **d)** reaction catalyzed by SDH; **e)** reaction catalyzed by SK; **f)** reaction catalyzed by EPSP synthase; **g)** monofunctional and bifunctional reaction catalyzed by CS; **h)** quinate biosynthesis by quinate dehydrogenase (QDH); **i)** gallate biosynthesis by dehydroshikimate dehydrogenase (DSDG).

Figure 1

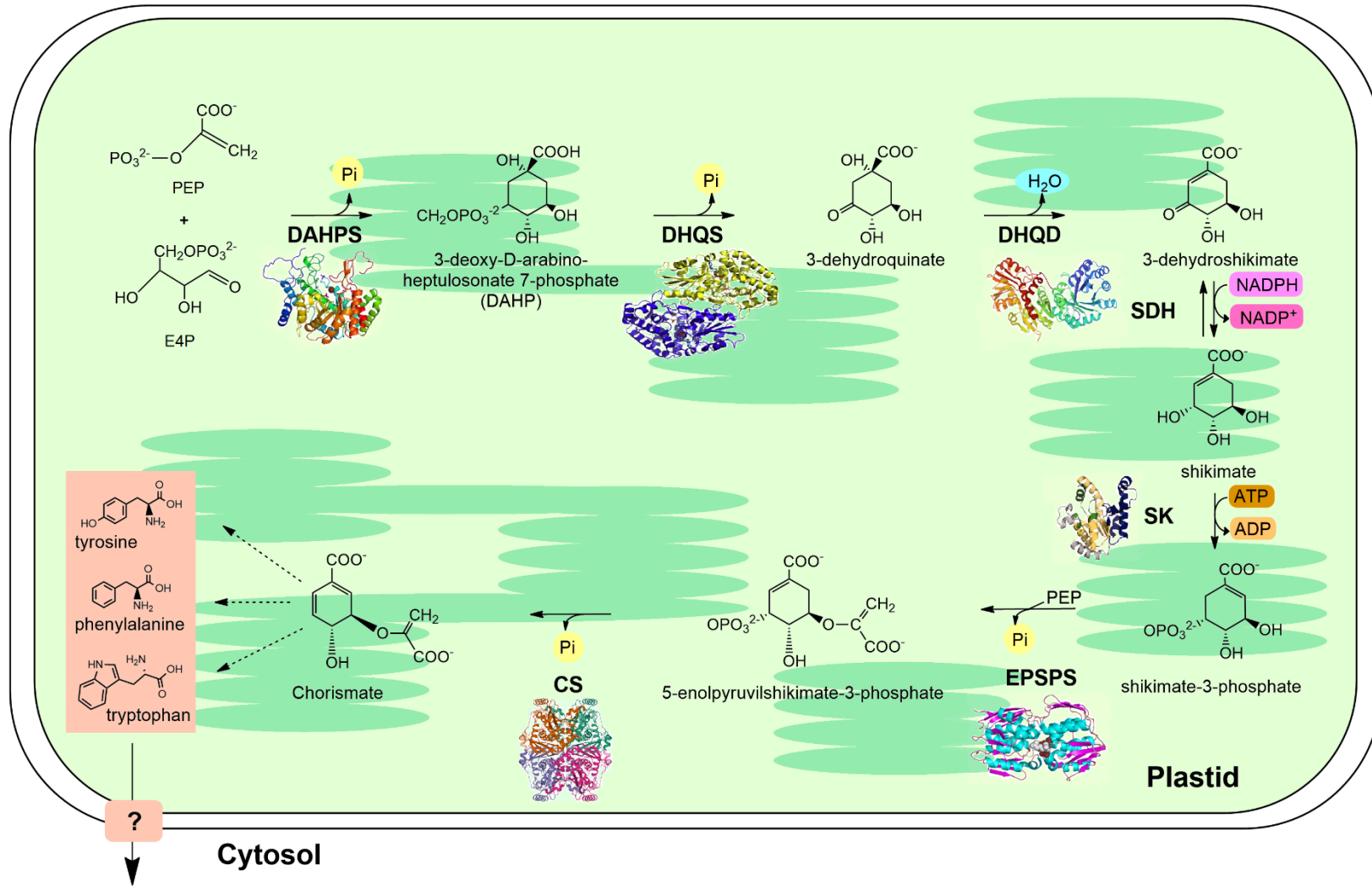


Figure 2

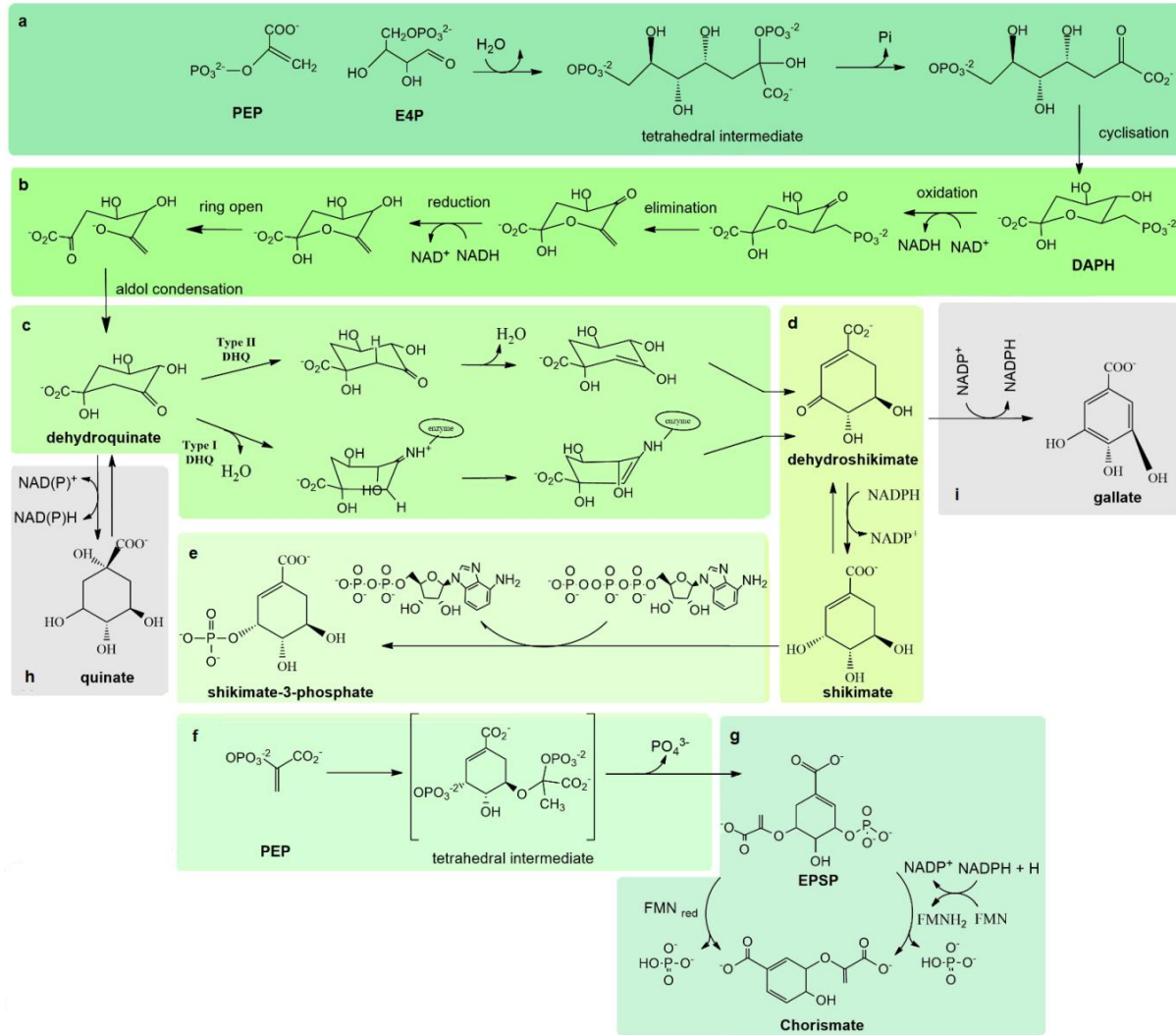
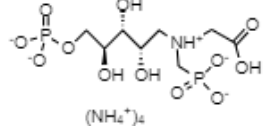
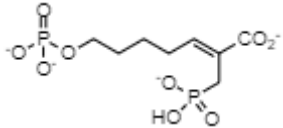
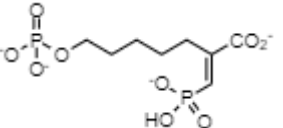
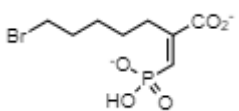
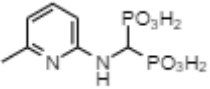
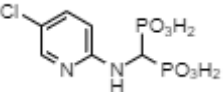
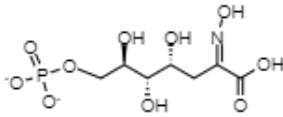
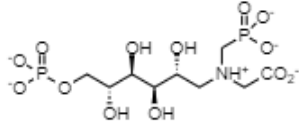
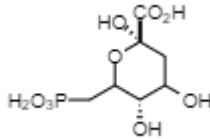
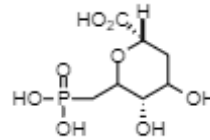
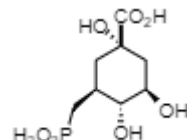
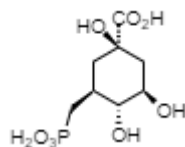


Table 1 - Enzymes of the shikimate pathway and related inhibitors

Enzyme	Compound	Chemical structure	<i>in silico</i>	<i>in vitro</i>	<i>in vivo</i>	References
DAHPS	Tetraammonium (((carboxymethyl)[(2 <i>S</i> ,3 <i>R</i> ,4 <i>S</i>)-2,3,4-trihydroxy-5-(phosphonatoxy)pentyl]amino)methyl)phosphonate				<i>E. coli</i>	(Walker and Parker 2006)
	Potassium 2-phosphonylmethyl-7-phosphorylhept-2-enoate		<i>E. coli</i>	<i>E. coli</i>		(Walker et al. 2011)
	(<i>E,Z</i>)-potassium 7-(phosphoryl)-2-(phosphonomethenyl)heptanoate		<i>E. coli</i>	<i>E. coli</i>		(Walker et al. 2011)
	[(1 <i>E</i>)-7-bromo-2-carboxyhept-1-en-1-yl]phosphonate		<i>E. coli</i>	<i>E. coli</i>		(Walker et al. 2011)
	<i>N</i> -2-(6-methyl-pyridyl)- amino-methylene bisphosphonic acid				<i>N. plumbaginifolia</i>	(Forlani et al. 1996)
	<i>N</i> -2-(5-chloro-pyridyl)-amino-methylene bisphosphonic acid				<i>N. plumbaginifolia</i>	(Forlani et al. 1996)

	DAHP oxime		<i>C. glutamicum</i>	<i>E. coli</i>	(Balachandran et al. 2016; de Oliveira et al. 2020)
	2-[(phosphonomethyl)[(2 <i>R</i> ,3 <i>R</i> ,4 <i>R</i> ,5 <i>R</i>)-2,3,4,5-tetrahydroxy-6-(phosphonatooxy)hexyl]azaniumyl]acetate		<i>C. glutamicum</i>		(de Oliveira et al. 2020)
DHQS	3-deoxy-D-arabino-heptulosonic acid 7-phosphonate		<i>E. coli</i>	<i>P. sativum</i>	(Myrvold et al. 1989; Pompliano et al. 1989)
			<i>P. sativum</i>	<i>E. crusgalli</i>	
				<i>S. viridis</i>	
				<i>S. halepense</i>	
				<i>A. fatua</i>	
	α -(2,6-anhydro-3-deoxy-D-arabino-heptulopyranosid)onate 7-phosphonate		<i>E. coli</i>		(Myrvold et al. 1989;
			<i>P. sativum</i>		Pompliano et al. 1989)
	Carbaphosphonate			<i>P. sativum</i>	(Montchamp et al. 1992)
				<i>E. crusgalli</i>	
				<i>S. viridis</i>	
				<i>S. halepense</i>	
				<i>A. fatua</i>	

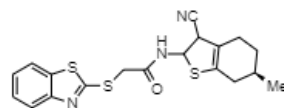
Epimeric carbaphosphonate



P. sativum
E. crusgalli
S. viridis
S. halepense
A. fatua

(Montchamp et al. 1992)

HTS11955

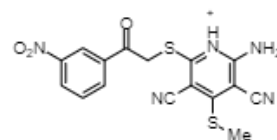


H. pylori

H. pylori

(Liu et al. 2008)

RH00573

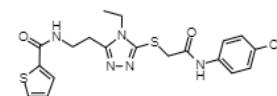


H. pylori

H. pylori

(Liu et al. 2008)

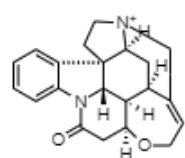
ZincC633887



M. tuberculosis

(Isa et al. 2018)

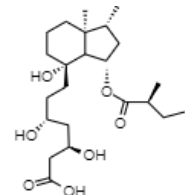
PubChem73393



M. tuberculosis

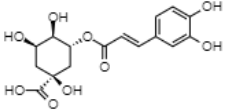
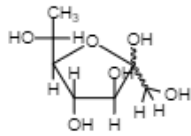
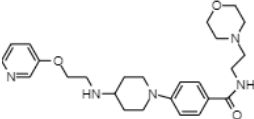
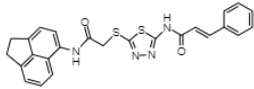
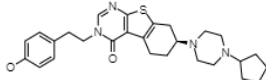
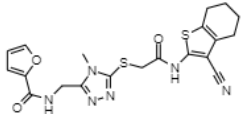
(Isa et al. 2018)

Lead 1

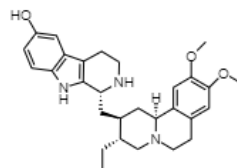


M. tuberculosis
(AroB)

(Sivaranjani et al. 2019)

	Chlorogenic acid		<i>P. alcalifaciens</i>	<i>P. alcalifaciens</i>	<i>P. alcalifaciens</i> <i>M. catarrhalis</i> <i>S. aureus</i> <i>E. coli</i>	(Neetu et al. 2020)
	7-deoxy-sedoheptulose (7dSh)				<i>A. variabilis</i> <i>S. cerevisiae</i> <i>A. thaliana</i>	(Brilisauer et al. 2019)
DHQD	Zinc14981770		<i>M. tuberculosis</i>	-		(Isa and Malik 2017)
	Zinc01147665		<i>M. tuberculosis</i>			(Isa and Malik 2017)
	Zinc22910025		<i>M. tuberculosis</i>			(Isa and Malik 2017)
	Zinc8442077		<i>M. tuberculosis</i>			(Isa and Malik 2017)

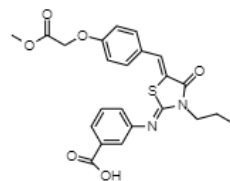
PubChem72341



M. tuberculosis

(Isa and Malik
2017)

Lead 1

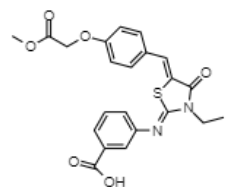


M. tuberculosis

M. tuberculosis

(Petersen et al.
2015)

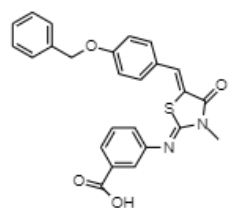
Lead 1a



M. tuberculosis

(Petersen et al.
2015)

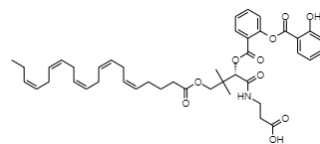
Lead 1b



M. tuberculosis

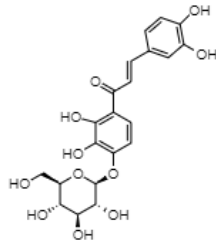
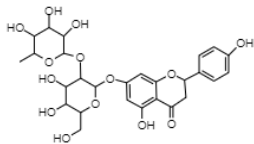
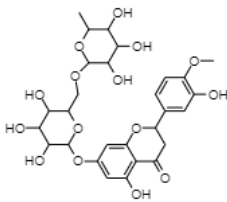
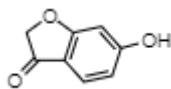
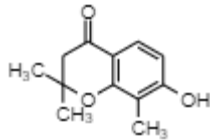
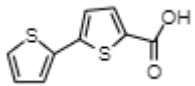
(Petersen et al.
2015)

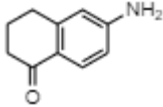
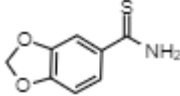
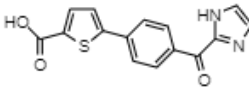
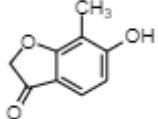
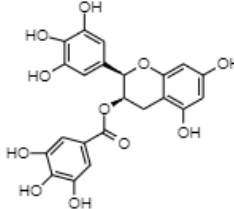
MOL19424



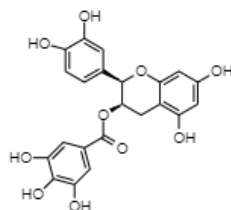
M. tuberculosis

(Souza et al.
2022)

Marein		<i>M. tuberculosis</i>		(Cheung et al. 2014)	
Naringin		<i>M. tuberculosis</i>	<i>M. tuberculosis</i>	(Lone et al. 2017)	
Hesperidin		<i>M. tuberculosis</i>	<i>M. tuberculosis</i>	(Lone et al. 2017)	
SDH	6-hydroxy-2,3-dihydrobenzo[b]furan-3-one (compound 894)		<i>S. aureus</i>	<i>S. aureus</i>	(Avitia-Domínguez et al. 2014)
	7-hydroxy-2,2,8-trimethyl-2,3-dihydro-4H-chromen-4-one (compound 766)		<i>S. aureus</i>	<i>S. aureus</i>	(Avitia-Domínguez et al. 2014)
	2,2'-bithiophene-5-carboxylic acid (compound 238)		<i>S. aureus</i>	<i>S. aureus</i>	(Avitia-Domínguez et

6-amino-1,2,3,4-tetrahydronaphthalen-1-one (compound 1)		<i>S. aureus</i>	<i>S. aureus</i>	al. 2014) (Enríquez-Mendiola et al. 2019)
1,3-benzodioxole-5-carbothioamide (compound 2)		<i>S. aureus</i>	<i>S. aureus</i>	(Enríquez-Mendiola et al. 2019)
5-[4-(1H-imidazol-2-ylcarbonyl)phenyl]thiophene-2-carboxylic acid (compound 3)		<i>S. aureus</i>	<i>S. aureus</i>	(Enríquez-Mendiola et al. 2019)
6-hydroxy-7-methyl-1-benzofuran-3(2H)-one (compound 4)		<i>S. aureus</i>	<i>S. aureus</i>	(Enríquez-Mendiola et al. 2019)
Epigallocatechin gallate (EGCG)			<i>T. gondii</i> <i>P. putida</i> <i>A. thaliana</i> (<i>DHQ/SDH</i>)	(Peek et al. 2014b, a)

Epicatechin gallate (ECG)

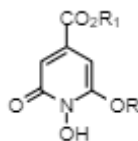


P. putida
A. thaliana
(*DHQ/SDH*)

(Peek et al.
2014b)

1,6-dihydroxy-2-oxoisonicotinic acid derivatives

R=CH₂C₆H₅, R₁=H; R= Ph, R₁=H; R=C₆H₄NHCOCH₂I, R₁=H;
R=C₆H₄NHCOCH₂I, R₁=C₂H₅

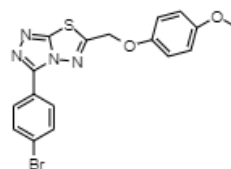


P. sativum

No herbicide effect
(*Pisum sativum*,
Sinapis alba, *Linum*
usitatissimum,
Lolium sp., *Hordeum*
vulgare, *Zea mays*,
Beta vulgaris)

(Baillie et al.
1972)

IMB-SD62



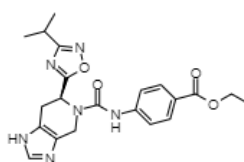
M. tuberculosis

Mouse infected with
M. tuberculosis
H37Rv

(Deng et al.
2018)

SK

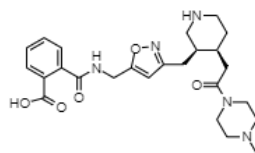
Zinc15707201



M. tuberculosis

(Vianna and De
Azevedo 2012)

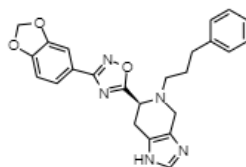
Zinc20462780



M. tuberculosis

(Vianna and De Azevedo 2012)

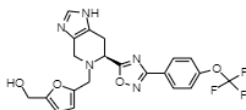
Zinc15707234



M. tuberculosis

(Vianna and De Azevedo 2012)

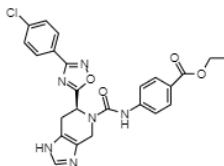
Zinc15675581



M. tuberculosis

(Vianna and De Azevedo 2012)

Zinc15707188



M. tuberculosis

(Vianna and De Azevedo 2012)

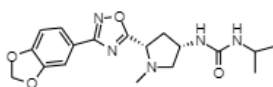
Zinc22936889

-

M. tuberculosis

(Vianna and De Azevedo 2012)

Zinc20464408



M. tuberculosis

(Vianna and De Azevedo 2012)

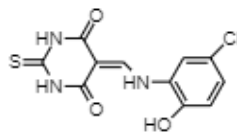
Zinc22936937

-

M. tuberculosis

(Vianna and De Azevedo 2012)

5631296



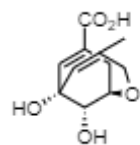
M. tuberculosis

M. tuberculosis

M. tuberculosis

(Rajput et al. 2016)

(1R,6S,10S)-6,10-Dihydroxy-4-methyl-2-oxabicyclo[4.3.1]deca-4(Z),7-diene-8-carboxylic acid

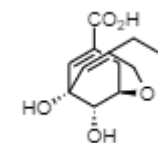


M. tuberculosis
and *H. pylori*

M. tuberculosis
and *H. pylori*

(Pernas et al. 2019)

(1R,6S,10S)-4-Ethyl-6,10-dihydroxy-2-oxabicyclo[4.3.1]deca-4(Z),7-diene-8-carboxylic acid

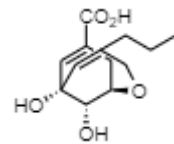


M. tuberculosis
and *H. pylori*

M. tuberculosis
and *H. pylori*

(Pernas et al. 2019)

(1R,6S,10S)-6,10-Dihydroxy-4-propyl-2-oxabicyclo[4.3.1]deca-4(Z),7-diene-8-carboxylic acid

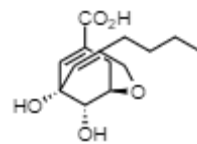


M. tuberculosis
and *H. pylori*

M. tuberculosis
and *H. pylori*

(Pernas et al. 2019)

(1R,6S,10S)-4-Butyl-6,10-dihydroxy-2-oxabicyclo[4.3.1]deca-4(Z),7-diene-8-carboxylic acid

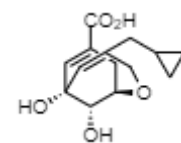


M. tuberculosis
and *H. pylori*

M. tuberculosis
and *H. pylori*

(Pernas et al. 2019)

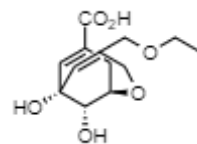
(1R,6S,10S)-4-Cyclopropylmethyl-6,10-dihydroxy-2-oxabicyclo[4.3.1]deca-4(Z),7-diene-8-carboxylic acid



M. tuberculosis
and *H. pylori*

(Pernas et al. 2019)

(1R,6S,10S)-4-Ethoxymethyl-6,10-dihydroxy-2-oxabicyclo[4.3.1]deca-4(Z),7-diene-8-carboxylic acid

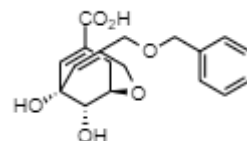


M. tuberculosis
and *H. pylori*

M. tuberculosis
and *H. pylori*

(Pernas et al.
2019)

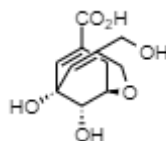
(1R,6S,10S)-4-Benzoyloxymethyl-6,10-dihydroxy-2-oxabicyclo[4.3.1]deca-4(Z),7-diene-8-carboxylic acid



M. tuberculosis
and *H. pylori*

(Pernas et al.
2019)

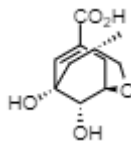
(1R,6S,10S)-4-Hydroxymethyl-6,10-dihydroxy-2-oxabicyclo[4.3.1]deca-4(Z),7-diene-8-carboxylic acid



M. tuberculosis
and *H. pylori*

(Pernas et al.
2019)

(1R,4S,6S,10S)-6,10-dihydroxy-4-methyl-2-oxabicyclo[4.3.1]dec-7-ene-8-carboxylic acid

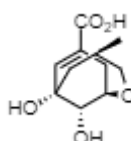


M. tuberculosis
and *H. pylori*

M. tuberculosis
and *H. pylori*

(Pernas et al.
2019)

(1R,4R,6S,10S)-6,10-dihydroxy-4-methyl-2-oxabicyclo[4.3.1]dec-7-ene-8-carboxylic acid



M. tuberculosis
and *H. pylori*

M. tuberculosis
and *H. pylori*

(Pernas et al.
2019)

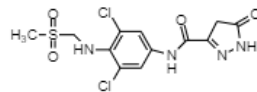
α -linolenic acid from *Sutherlandia frutescens*



M. tuberculosis

(Masoko et al.
2016)

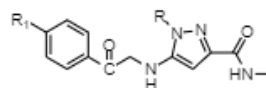
Compound 14



M. tuberculosis

(Rahul Reddy et al. 2020)

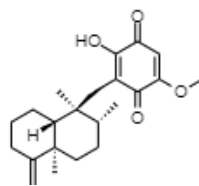
Compound 19 R: -H, R₁: -CH₃; 23 R: -C₆H₅, R₁: -CH₃; 31 R: -CH₃, R₁: -CH₃; 33 R: -CH₃, R₁: -F; 21 R: -H, R₁: -F; 22 R: -H, R₁: -CF₃; 24 R: -C₆H₅, R₁: -NO₂; 25 R: -C₆H₅, R₁: -F; 26 R: -C₆H₅, R₁: -CF₃; 27 R: -2,4-diNO₂C₆H₃, R₁: -CH₃; 30 R: -2,4-diNO₂C₆H₃, R₁: -CF₃; 32 R: -CH₃, R₁: -NO₂; 34 R: -CH₃, R₁: -CF₃



M. tuberculosis

(Rahul Reddy et al. 2020)

Ilimaquinone

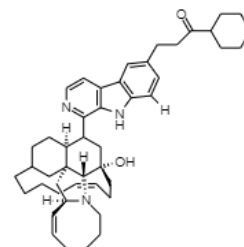


M. tuberculosis

M. tuberculosis

(Simithy et al. 2018b)

6-cyclohexamidomanzamine A

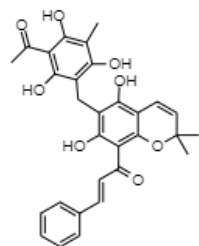


M. tuberculosis

M. tuberculosis

(Simithy et al. 2018a)

Rottlerin



M. smegmatis

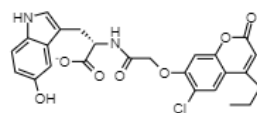
M. tuberculosis

Mycobacteria

M. tuberculosis

(Pandey et al. 2016)

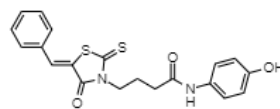
Zinc2135897



S. sonnei

(Ahmad et al. 2018)

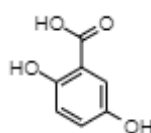
CB4275815



S. sonnei

(Ahmad et al. 2018)

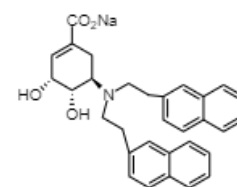
Gentisic acid



H. pylori

(Divyashri et al. 2021)

Sodium (3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(naphth-2-yl)methylaminocyclohex-1-ene-1-carboxylate) (compound 4b)

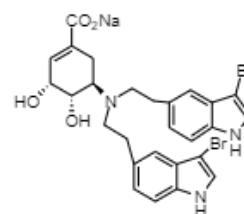


H. pylori

H. pylori

(Prado et al. 2016)

Sodium (3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(3-bromo)indol-5-yl)methylaminocyclohex-1-ene-1-carboxylate) (compound 4d)



M. tuberculosis

M. tuberculosis

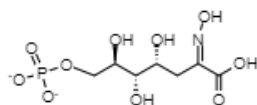
(Prado et al. 2016)

Hexanol extract of *Alpinia galangal* (L.) Willd

M. tuberculosis

(Patrick et al. 2021)

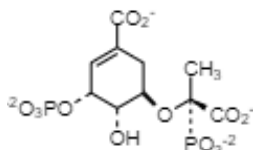
DAHP oxime



M. tuberculosis

(de Oliveira et al. 2020)

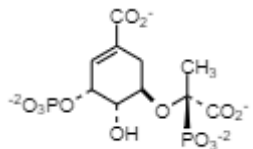
Shikimate 3-phosphate-5-carboxymethyl-(2*R*)-phosphonate



P. hybrida

(Alberg and Bartlett 1989)

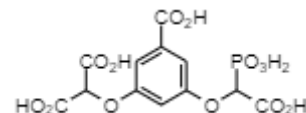
Shikimate 3-phosphate-5-carboxymethyl-(2*S*)-phosphonate



P. hybrida

(Alberg and Bartlett 1989)

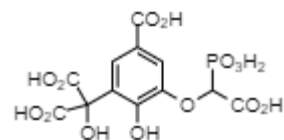
2(3-carboxy-5-(carboxy(phosphono)methoxy)phenoxy)malonic acid



E. coli

(Miller et al. 1995)

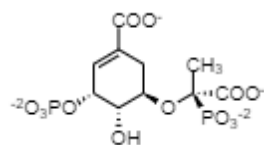
2-(5-carboxy-3-(carboxy(phosphono)methoxy)-2-hydroxyphenyl)-2-hydroxymalonic acid



E. coli

(Shah et al. 1997)

(3*R*,4*S*,5*R*)-4-hydroxy-5-[(2*S*)-1-hydroxy-1-oxo-2-phosphono-propan-2-yl]oxy-3-phosphonoxy-cyclohexene-1-carboxylic acid

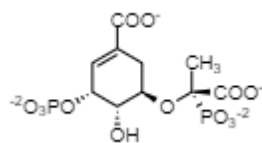


E. coli

EPSPS of *S. aureus*,
Agrobacterium sp. CP4, and *E. coli*

(Funke et al. 2007)

(3*R*,4*S*,5*R*)-4-hydroxy-5-[(2*R*)-1-hydroxy-1-oxo-2-phosphono-propan-2-yl]oxy-3-phosphonoxy-cyclohexene-1-carboxylic acid

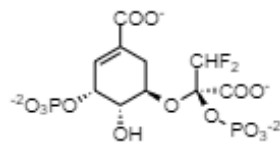


E. coli and
Agrobacterium
sp. CP4 EPSPS

EPSPS of *S. aureus*,
Agrobacterium
sp. CP4, and *E. coli*

(Funke et al. 2007)

(3*R*,4*S*,5*R*)-5-[(2*R*)-1,1-difluoro-3-hydroxy-3-oxo-2-phosphonoxy-propan-2-yl]oxy-4-hydroxy-3-phosphonoxy-cyclohexene-1-carboxylic acid

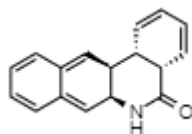


E. coli and
Agrobacterium
sp. CP4 EPSPS

EPSPS of *S. aureus*,
Agrobacterium
sp. CP4, and *E. coli*

(Funke et al. 2007)

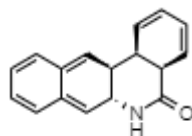
Zinc393031



A. thaliana

(Ruszkowski and Forlani 2022)

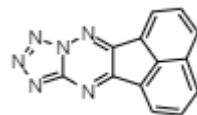
Zinc4532574



A. thaliana

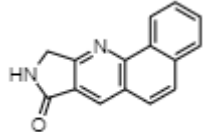
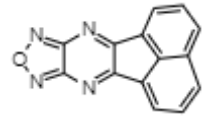
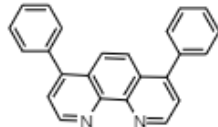
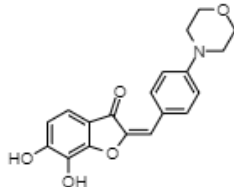
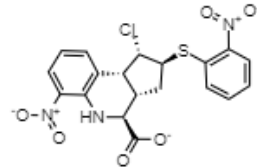
(Ruszkowski and Forlani 2022)

Zinc75286738

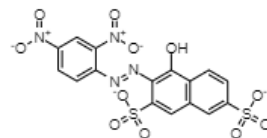


A. thaliana

(Ruszkowski and Forlani 2022)

	Zinc96319279		<i>A. thaliana</i>			(Ruszkowski and Forlani 2022)
	Zinc1508817		<i>A. thaliana</i>			(Ruszkowski and Forlani 2022)
CS	Bathophenanthroline			<i>E. coli</i>		(Pagolu et al. 2016)
	6,7-dihydroxy-2-[1-(4-morpholin-4-yl-phenyl)-meth-(E)-ylidene]-benzofuran-3-one		<i>P. falciparum</i>			(Tapas et al. 2011)
	(1 <i>S</i> ,2 <i>S</i> ,3 <i>aS</i> ,4 <i>S</i> ,9 <i>bR</i>)-1-chloro-6-nitro-2-(2-nitrophenyl)sulfanyl-2,3,3 <i>a</i> ,4,5,9 <i>b</i> -hexahydro-1 <i>H</i> -cyclopenta[<i>c</i>]quinoline-4-carboxylic acid		<i>P. brasiliensis</i>	<i>P. brasiliensis</i>	Mice infected with <i>P. brasiliensis</i>	(Rodrigues-Vendramini et al. 2018)

Phenaphthazine

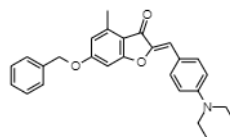


C. albicans

P. brasiliensis

(Bueno et al. 2019)

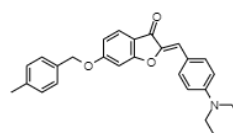
(2Z)-2-[[4-(diethylamino)phenyl]methylidene]-4-methyl-6-phenylmethoxy-1-benzofuran-3-one



T. gondii

(Singh and Qureshi 2022)

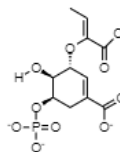
(2Z)-2-[[4-(diethylamino)phenyl]methylidene]-6-[(4-methylphenyl)methoxy]-1-benzofuran-3-one



T. gondii

(Singh and Qureshi 2022)

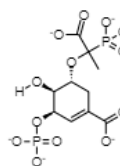
(3R,4S,5R)-3-[(Z)-1-carboxylatoprop-1-enoyl]-4-hydroxy-5-phosphonatooxycyclohexene-1-carboxylate



T. gondii

(Singh and Qureshi 2022)

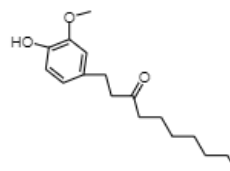
(3R,4S,5R)-5-[(1R)-1-carboxylato-1-phosphonatoethoxy]-4-hydroxy-3-phosphonatooxycyclohexene-1-carboxylate



T. gondii

(Singh and Qureshi 2022)

6-paradol (1-(4-hydroxy-3-methoxyphenyl)decan-3-one)



P. falciparum

P. falciparum

(Awasthi et al. 2022)

CAPÍTULO II

ARTIGO

6-Nitroquinazoline-2,4-diol um inibidor da chiquimato desidrogenase. Ensaio *in vitro* e *in vivo*.

Este artigo será submetido ao Periódico *Plant Physiology and Biochemistry*.

***6-Nitroquinazoline-2,4-diol* um inibidor da chiquimato desidrogenase.
Ensaio *in vitro* e *in vivo*.**

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Resumo

A via do chiquimato é responsável pelo fornecimento do precursor para a biossíntese dos aminoácidos aromáticos, sendo de extrema importância não só para a síntese de proteínas, mas na geração de metabólitos secundários nas plantas. Esta via está ausente em animais, servindo assim como alvo para o desenvolvimento de medicamentos e herbicidas. O objetivo deste trabalho foi prospectar inibidores para a chiquimato desidrogenase, uma enzima chave da via. Para isso, varredura virtual e *docking* molecular foram realizadas no sítio ativo da chiquimato desidrogenase de *Arabidopsis thaliana* (AtSDH), sendo o composto 6-nitroquinazoline-2,4-diol (NQD) selecionado. *In vitro* na concentração de 250 μM de NQD houve a inibição da atividade enzimática, sendo constatada redução da V_{max} sem alteração de K_m . *In vivo* sob cultivo hidropônico, a 500 μM de NQD o comprimento radicular de plântulas de soja e milho foi reduzido, teores de proteínas totais e de alguns aminoácidos aumentaram, mas sem alterações no teor de lignina. Em milho, NQD ocasionou em um acúmulo expressivo de ácido gálico, o qual pode ser sintetizado a partir do desidrochiquimato. Tratamentos de NQD juntamente com glifosato confirmaram que a ação do composto selecionado antecede a reação catalisada pela 5-enolpiruvilchiquimato-3-fosfato sintase, ou seja, sobre a SDH. Entretanto, há uma diferença de sensibilidade para a enzima de soja e de milho, sendo esta última mais sensível a ação do composto. No conjunto, os dados revelaram que NQD tem ação inibitória sobre a chiquimato desidrogenase, tanto *in vitro* como *in vivo*, apresentando uma maior potencialidade para SDH de milho.

Palavra-chave: Via do chiquimato; Chiquimato desidrogenase; Virtual screening; *Docking molecular*.

1. Introdução

Nas plantas, 30% do carbono fixado é direcionado para a via do chiquimato gerando os aminoácidos aromáticos (fenilalanina, tirosina e triptofano), os quais são importantes para a síntese proteica e como precursores de compostos primordiais para o crescimento e desenvolvimento. Além disso, eles estão envolvidos em respostas das plantas às alterações ambientais (Coruzzi et al., 2015; Maeda e Dudareva, 2012; Santos-Sánchez et al., 2019; Tzin e Galili, 2010).

A via do chiquimato é composta por sete reações, iniciando com derivados do metabolismo central do carbono, fosfoenolpiruvato e eritrose-4-fosfato, provenientes respectivamente da glicólise e da via das pentoses fosfato, a fim de gerar corismato como precursor dos aminoácidos aromáticos (Tzin e Galili, 2010) (Figura 1). A via está presente em plantas, bactérias, fungos e alguns parasitas apicomplexos, diferindo quanto ao modo de regulação, organização e localização celular das enzimas (Gientka e Duszkievicz-Reinhard, 2009; Mousdale e Coggins, 1985; Roberts et al., 1998).

As enzimas envolvidas na biossíntese de aminoácidos (aromáticos, cadeia ramificada e de glutamina) são pontos de atuação de alguns herbicidas disponíveis no mercado. Entretanto, a ocorrência e o aumento do número de plantas daninhas resistentes aos herbicidas vêm atenuando suas eficiências e aplicabilidades (Hall et al., 2020; Heap, 2021).

Plantas daninhas acarretam prejuízos aos produtores uma vez que competem por fatores primordiais para o crescimento e desenvolvimento das culturas, reduzindo a produtividade (Gazziero et al., 2012; Lu et al., 2016; Ramella et al., 2020). Com o advento dos herbicidas sintéticos, o controle de plantas daninhas passou a ser realizado de forma mais eficiente. Todavia, o uso excessivo e indiscriminado, sem a utilização de

outras práticas de manejo resultou em plantas daninhas resistentes, sendo agravado com o aparecimento de casos de resistência múltipla ou cruzada (Heap, 2021; Vargas e Roman, 2006). Desse modo, a busca por novas moléculas e/ou mecanismo de ação torna-se imprescindível para contornar este agravante problema. Pelo fato da via do chiquimato estar ausente em animais, e com a ocorrência de microrganismos e plantas daninhas resistentes aos produtos disponíveis comercialmente, as enzimas desta via são potenciais alvos para o desenvolvimento de novas drogas (Coracini e Azevedo, 2014; Derrer et al., 2013; Enríquez-Mendiola et al., 2019).

Nas plantas, a terceira e quarta reações da via do chiquimato são catalisadas por uma enzima bifuncional, a 3-desidroquinato desidratase-chiquimato desidrogenase (DHQD/SDH); nas bactérias são monofuncionais, e nos fungos fazem parte do complexo pentafuncional AROM (segunda à quinta reações) (Figura 1) (Coruzzi et al., 2015; Maeda e Dudareva, 2012; Singh e Christendat, 2006). A supressão da DHQD/SDH em plantas de tabaco por RNA de interferência (RNAi) reduziu a atividade da DHQD/SDH, o crescimento e a biomassa; em alguns casos as plantas transgênicas morreram após alguns dias (Ding et al., 2007), indicando um potencial alvo de estudo. A SDH ou chiquimato:NADP⁺ oxidoreductase (EC 1.1.1.25), catalisa a redução dependente de NADPH do desidrochiquimato a chiquimato (Figura 1) (Maeda e Dudareva, 2012).

A SDH vem sendo explorada visando uso na área médica contra microrganismos como *Mycobacterium tuberculosis* (Deng et al., 2018), *Staphylococcus aureus* (Avitia-Domínguez et al., 2014; Enríquez-Mendiola et al., 2019) e *Toxoplasma gondii* (Peek et al., 2014a). A avaliação da SDH em plantas como um novo sítio de ação herbicida foi avaliada por Baillie et al. (1972). Entretanto, os resultados obtidos *in vitro* com derivados do ácido 1,6-dihidroxi-2-oxoisonicotínico não se estenderam para um

potencial herbicida *in vivo*. Para DHQD/SDH de *A. thaliana*, os compostos epigalocatequina galato e epicatequina galato foram bons inibidores *in vitro* (Peek et al., 2014b); contudo não foram avaliados *in vivo*.

Neste contexto, o objetivo do presente trabalho foi prospectar, por meio de ferramentas *in silico*, inibidores candidatos para chiquimato desidrogenase (SDH). Posteriormente, foi avaliado o potencial inibitório *in vitro* para DHQD/SDH de *A. thaliana*, e *in vivo* por meio de cultivo hidropônico com plântulas de soja e milho.

2. Material e Métodos

2.1 Estrutura tridimensional da DHQD/SDH de *Arabidopsis thaliana*

Os procedimentos *in silico* para a obtenção do modelo da estrutura tridimensional da DHQD/SDH de *Arabidopsis thaliana* foram realizados no laboratório de Bioquímica Estrutural da Universidade Estadual de Maringá, Campus Umuarama.

A estrutura cristalográfica da DHQD/SDH cloroplastidial de *Arabidopsis thaliana* (*Athaliana*-DHQD/SDH; EC 4.2.1.10), na presença do cofator NADPH e do ligante 3-desidrochiquimato (DHK) (Singh e Christendat, 2007) foi obtida na base de dados Protein Data Bank (PDB ID: 2O7S). Um grupo -OH foi adicionado a um dos ligantes DHK (posição 9241) para que este se tornasse o ligante DHQ (desidroquinato), o qual é o substrato do domínio DHQD. As águas estruturais foram mantidas e os demais íons oriundos da solução de cristalização foram removidos. Os *gaps* da estrutura cristalográfica, decorrentes de falta de densidade eletrônica entre os resíduos 436 a 454 e 510 a 511, foram modelados por meio do programa Modeller (Webb e Sali, 2016) com base na sequência de aminoácidos (Uniprot ID: Q9SQT8). Os heteroátomos de S-hidroxicisteína foram substituídos por cisteínas e as conformações alternativas das

cadeias laterais foram removidas por meio do programa PDSet (Collaborative Computational Project, 1994).

A estrutura modelada da *A. thaliana*-DHQD/SDH foi submetida aos procedimentos de dinâmica molecular para minimização de energia da estrutura, por meio do programa NAMD2 (Phillips et al., 2005) a fim de maximizar a interação da proteína com os ligantes. O campo de força utilizado para a proteína e o cofator foi o Charmm (Mackerell et al., 2004) e para os ligantes os campos de força foram gerados pelo servidor SwissParam (Zoete et al., 2011) no mesmo formato. A minimização ocorreu em ciclos. No primeiro ciclo, os átomos da proteína, águas e sais foram submetidos a 20.000 passos de minimização por gradiente conjugado (CG), enquanto os átomos dos ligantes e cofator foram mantidos fixos no espaço. No segundo ciclo, todos os átomos do sistema foram submetidos a mais 10.000 passos de CG. As coordenadas espaciais da *Athaliana*-DHQD/SDH originárias deste segundo ciclo foram utilizadas nas simulações de *docking*.

Os protocolos de *docking molecular* foram estabelecidos independentemente para cada domínio da proteína, por meio de *redocking* do ligante cristalográfico usando os programas AutoDock versão 4.2.3 (Morris et al., 2009) e Molegro versão 6.0 (Thomsen e Christensen, 2006). Os protocolos foram considerados validados quando o programa foi capaz de reproduzir a pose do ligante cristalográfico em todas as repetições e com raiz quadrada média da distância (rmsd) menor que 0,5 Å.

2.2 Simulações de *docking* e varredura virtual

Para o domínio SDH o programa AutoDock implementado na interface gráfica Pyrx (Wolf, 2009) utilizou o algoritmo padrão de busca e ranqueamento com restrições de busca de dimensões 40, 40 e 40 nos eixos x, y e z e *grid* de 0,375 Å, centrado no ligante

DHK. O programa Molegro utilizou a função *PLANTS Score* para ranqueamento e a função *Iterated Simplex (Ant Colony Optimization)* para busca, com x, y e z de 37,34, 42,42 e 84,16 Å, respectivamente e raio de busca de 8 Å centrado no ligante. A estrutura do ligante (ZINC103687345) no formato *.sdf também foi utilizada nas simulações de docking, onde este foi denominado ligante exógeno.

As simulações de varredura virtual foram realizadas na biblioteca da Sigma (Sigma-Aldrich, MO, EUA), o DHK foi incluído na biblioteca, a fim de que as moléculas mais bem classificadas em relação ao substrato fossem consideradas. As moléculas selecionadas foram baixadas no formato *.sdf. Após a primeira triagem, elas foram submetidas a mais três triagens no programa AutoDock e mais 4 novas triagens no programa Molegro, visando a eliminação de falso-positivos e garantir a reprodutibilidade.

2.3 Expressão e purificação da proteína DHQD/SDH

O plasmídeo utilizado para expressar a proteína DHQD/SDH de *A. thaliana* foi gentilmente cedido pelo grupo de pesquisa da Universidade de Toronto (Singh e Christendat, 2006). Esta proteína foi expressa a partir da construção plasmidial $\Delta 88$ DHQ-SDH em pET28 modificado que expressa os resíduos 89-603 desta enzima. Os sítios de restrição utilizados foram *NdeI* e *BamHI* e a cauda de histidina se encontra na extremidade C-terminal da proteína (Singh e Christendat, 2006).

A expressão da proteína $\Delta 88$ DHQ-SDH foi realizada de acordo com Singh e Christendat (2006), com modificações. A cepa de bactérias *E. coli* utilizadas foi a BL21 (DE3), crescida em meio líquido Luria-Bertani (LB) contendo o antibiótico canamicina ($50 \mu\text{g mL}^{-1}$). Primeiramente foi realizada uma pré-cultura incubada a 37°C sob agitação durante a noite. Em seguida, $500 \mu\text{L}$ desta pré-cultura foram adicionados em 50 mL de

meio líquido LB contendo o antibiótico. Após alcançar $OD_{600\text{ nm}}$ entre 0,6-0,8, as culturas foram induzidas com 0,4 mM de β -D-tiogalactopiranosídeo (IPTG) durante 20 horas a 18°C, sob agitação. Após a indução, as células foram separadas por centrifugação (15 minutos, 5000 g, 4°C) e armazenadas para a etapa de purificação.

A proteína $\Delta 88\text{DHQ-SDH}$ foi purificada por meio de uma coluna de cromatografia por afinidade ao níquel (GE Healthcare, Uppsala, Sweden). Durante a purificação e a diálise foi utilizado o tampão Tris HCl 10 mM (pH 7,5) contendo 500 mM NaCl. Este mesmo tampão contendo 40, 150 e 250 mM de imidazol foi usado para eluir a proteína purificada da coluna. Após a diálise, foi realizada a dosagem de proteína pela metodologia de Bradford (1976) e análise de espectrometria de massas (Material Suplementar).

2.4 Atividade enzimática *in vitro*

A atividade enzimática da SDH foi realizada pelo monitoramento da produção de NADPH na presença de chiquimato, a 340 nm ($\epsilon = 6,22\text{ mM}^{-1}\text{ cm}^{-1}$). Para a reação foram utilizados: tampão 0,1 M Tris-HCl (pH 8,8), 1 nM da enzima $\Delta 88\text{DHQ-SDH}$, 0,1 a 2 mM de chiquimato em tampão 0,1 M Tris-HCl (pH 8,8) e 0 ou 250 μM de 6-nitroquinazoline-2,4-diol (NQD) em tampão 0,1M Tris-HCl (pH 8,8). Para iniciar a reação 2 mM de NADP^+ foram adicionados, e a redução de NADP^+ foi monitorada por 5 minutos a 23°C (Marchiosi et al., 2009; Singh e Christendat, 2006). Os valores de velocidade máxima ($V_{\text{máx}}$) e constante de Michaelis-Menten (K_m) foram obtidos por meio do ajuste a equação de Michaelis-Menten as velocidades iniciais (com ou sem NQD), com método de ajuste de regressão de mínimos quadrados (Prism versão 8.0.1, Graph Pad Software Inc., EUA).

2.5 Germinação e crescimento das plântulas em hidroponia

As sementes de soja (*Glycine max* L. Merrill cv. BRS 232) e milho (*Zea mays* L. cv. IPR-164) foram desinfetadas em solução hipoclorito de sódio (NaClO) 2% durante 5 minutos e lavadas exaustivamente com água deionizada. A seguir, as sementes foram depositadas entre folhas de papel Germitest[®], previamente umedecidas, as quais foram enroladas e acondicionadas em tubos de germinação. A germinação ocorreu em câmara escura, a 25°C, durante 3 dias. Após este período, as plântulas foram transferidas para sistemas de hidroponia contendo solução nutritiva (Dong et al., 2006) (pH 6,0), com ou sem o composto selecionado (500 µM de NQD). Os recipientes foram acondicionados em bancada de cultivo durante 24 a 96 horas (25°C ± 2, fotoperíodo de 12/12 h). Para incubação de 96 horas, no tempo de 48 horas foi adicionado solução nutritiva (1/6 de força) (Dong et al., 2006) nos sistemas. Para análise de comprimento, as raízes foram medidas antes e após o período de incubação, sendo expresso em centímetros. Em seguida, as raízes e os caules foram separados com auxílio de um estilete.

2.6 Consumo de solução nutritiva e estudo da absorção de NQD pelas plântulas.

A depleção do NQD, a partir da solução nutritiva, foi realizada por cromatografia líquida de alta eficiência (HPLC). Para isso, alíquotas de 1 mL foram coletadas a cada 24 h até o quarto dia de cultivo. Nos tempos, 24, 48, 72 e 96 h, e antes de se proceder a coleta o volume de solução nutritiva dos sistemas de cultivo foi aferido com água deionizada para o volume inicial de incubação. As alíquotas permaneceram em freezer (-20°C) até a realização das análises. As amostras foram filtradas em membranas de 0,45 µm e analisadas (20 µL) em HPLC Prominence (Shimadzu[®], Tóquio, Japão) com detector UV-VIS (SPD-10A). A detecção do NQD ocorreu por meio de uma corrida

isocrática, fase móvel ácido acético 4%: metanol 70% (50:50), fluxo de 0,8 mL min⁻¹, comprimento de onda de 310 nm, coluna C18 (250 x 4,6 mm, 5 µm; Supelco Discovery[®]), empregando uma curva de calibração para a quantificação do NQD. A captação de solução nutritiva foi monitorada mensurando-se o volume de solução consumida a cada 24 h.

Para determinar a quantidade de NQD absorvida, 0,2 g de raízes frescas foram maceradas com 2,5 mL de metanol 65%, posteriormente foram centrifugadas a 3200 g, por 15 minutos a 4°C. O sobrenadante foi filtrado em membrana de 0,45 µm e analisado (20 µL) em HPLC Prominence (Shimadzu[®], Tóquio, Japão), detector UV-VIS (SPD-10A), corrida isocrática com fase móvel ácido acético 4%: metanol 70% (50:50), fluxo de 0,8 mL min⁻¹, comprimento de onda de 310 nm, coluna C18 (250 x 4,6 mm, 5 µm; Supelco Discovery[®]). O rendimento dessa extração foi estimado (ver em Material Suplementar).

2.7 Dosagem de proteína

Para dosagem de proteínas 0,5 g de material fresco foi macerado com nitrogênio líquido, PVP e tampão fosfato (100 mM de tampão fosfato de potássio, 1 mM EDTA, 3 mM DTT, pH 7,5) (Azevedo et al., 1998). Posteriormente foi transferido para eppendorf e centrifugado a 13000 g por 30 minutos a 4°C. O sobrenadante foi empregado para a realização da dosagem pelo método de Bradford (1976), usando como padrão albumina do soro bovino.

2.8 Determinação do conteúdo de aminoácidos

A determinação de aminoácidos foi realizada segundo Astarita et al. (2003) e Pieruzzi (2009) com modificações. As biomassas frescas armazenadas a -80 °C (0,2 g)

foram maceradas com etanol 80% (v/v) e posteriormente rotaevaporadas a fim de eliminar o etanol. À amostra foram adicionados 2 mL de água MiliQ e centrifugada a 10.000 g por 10 minutos. O sobrenadante foi utilizado para as análises, por meio de derivatização com OPA-borato (Benson e Hare, 1975) e analisado em HPLC. Os aminoácidos foram separados com auxílio de uma coluna C18 (4,6 x 250 mm, 5 μ m; Shimadzu® Shim-pack GIST) equipada com pré-coluna (C18, 4 x 3.0 mm; Phenomenex® SecurityGuard). Para eluição foram utilizadas as fases “A” (acetato de sódio 50 mM (pH 7,2) contendo metanol (2%), tetrahydrofurano (2%) e ácido acético (0,7%)) e “B” (metanol 65%) com o seguinte gradiente: 0 min A 80% e B 20%, 5 min A 72% e B 28%, 39 min A 42% e B 58%, 40 min A 25% e B 75%, 56 min A 5% e B 95%, 59 min A 4% e B 96% e 61 min B 100%, com fluxo de 0,8 mL min⁻¹. O detector de fluorescência Shimadzu (modelo RF-20A) foi ajustado para excitação em 265 nm e emissão em 480 nm. Os tempos de retenção e as concentrações dos aminoácidos foram determinados por comparação com um padrão Sigma AAS-18 (contendo Asp, Glu, Ser, Arg, Gly, Thr, Ala, Tyr, Met, Val, Phe, Ile, Leu, Lys) acrescido de Asn e Gln. A partir deste padrão foram preparadas diferentes soluções (0,5 a 31,25 μ mol L⁻¹) utilizadas para a construção de curvas de calibração (Jarrett et al., 1986; Marur et al., 1994).

2.9 Determinação dos ácidos fenólicos

A quantificação dos ácidos fenólicos foi realizada em espectrofotômetro (Herrig et al., 2002) e por HPLC (Marchiosi et al., 2009). Para isso, raízes secas foram homogeneizadas com HCl 2N, posteriormente foram transferidos para vidros com tampa rosqueáveis e colocados à fervura por 30 minutos. Logo após, as amostras foram resfriadas e filtradas com papel filtro. Para a análise por espectrofotômetro as amostras foram misturadas com Na₂CO₃ e o reagente de Folin-Ciocalteu, e permaneceram no

escuro, a temperatura ambiente por uma hora, após esse tempo as amostras foram lidas em 750 nm. Para a análise em HPLC, as amostras foram diluídas, filtradas em membranas de 0,45 µm e analisadas (20 µL) em HPLC Prominence (Shimadzu[®], Tóquio, Japão) com detector UV-VIS (SPD-10A). A fase móvel foi metanol:ácido acético 4% (30:70) de modo isocrático, com fluxo de 0,8 mL min⁻¹. A separação foi realizada utilizando uma coluna C18 (250 x 4,6 mm, 5 µm; Supelco Discovery[®]). Os compostos foram identificados em 254 nm pela comparação com os tempos de retenção dos padrões.

2.10 Teste com glifosato e dosagem de chiquimato

As sementes de soja e milho foram desinfetadas em solução hipoclorito de sódio (NaClO) 2% durante 5 minutos, e a seguir lavadas exaustivamente com água deionizada. As sementes foram depositadas entre folhas de papel Germitest[®], previamente umedecidas, as quais foram enroladas e acondicionadas em tubos de germinação. A germinação ocorreu em câmara escura, a 25°C, durante 3 dias. Após este período, as plântulas foram transferidas para sistemas de hidroponia contendo solução nutritiva (Dong et al., 2006) (pH 6,0), para soja foram testados: glifosato (100 µM), glifosato mais NQD (100 e 500 µM, respectivamente) e controle (sem glifosato ou NQD). Para milho foi testado glifosato (25 µM), glifosato mais NQD (25 e 500 µM, respectivamente) e controle (sem glifosato ou NQD). Os recipientes contendo as plântulas de soja foram incubados por 48 horas e as plântulas de milho por 96 horas (25°C ± 2, fotoperíodo de 12/12 h). As análises de comprimento e biomassa frescas foram realizadas da mesma forma que no item 2.5. As biomassas frescas (0,5 g) obtidas foram homogeneizadas com HCL 0,25 N, posteriormente foram centrifugadas (1200 g, 10 min), o sobrenadante foi usado para a dosagem de chiquimato. As amostras foram

diluídas, filtradas em membranas de 0,45 μm e analisadas (20 μL) em HPLC Prominence (Shimadzu[®], Tóquio, Japão) com detector UV-VIS (SPD-10A), coluna C18 (250 x 4,6 mm, 5 μm ; Shim-pack CLC-ODS(M)[®] Shimadzu), fluxo de 0,8 mL min⁻¹, fase móvel ácido fosfórico 3.5 mM de forma isocrática. O chiquimato foi detectado pela comparação por meio do tempo de retenção do padrão (50 μM) (Bonini et al., 2009).

2.11 Determinação do conteúdo de lignina

O conteúdo de lignina foi determinado pelo método de brometo de acetila (Moreira-Vilar et al., 2014). Parede celular isenta de proteínas (10 mg) foram incubadas com uma solução de brometo de acetila 25% em ácido acético (250 μL) a 70°C por 30 minutos. Após, a reação foi colocada em banho de gelo, e adicionado NaOH 2M (400 μL), 7,5M de hidroxilamina (50 μL) e ácido acético glacial (2 mL). Após centrifugação (10000 g, 5 min), foi realizada a leitura do sobrenadante a 280 nm.

2.12 Análise estatística

Para análise estatística o software *Graph Pad Prism*[®] (versão 8 Graph Pad Software Inc., EUA) foi empregado. Os dados foram expressos como a média de experimentos independentes \pm erro padrão das médias. As diferenças entre os parâmetros foram analisadas pelo teste *t* não pareado, comparação múltipla de Dunnett ou Tukey, valores de $p \leq 0,05$ foram considerados estatisticamente significativos.

3. Resultados

3.1 Estrutura tridimensional da DHQD/SDH de *Arabidopsis thaliana*

As simulações de *redocking* são importantes para identificar quais programas e algoritmos compreendem as interações que a proteína realiza com cada ligante

crystalográfico ou modelado e, assim, identificar quais programas são capazes de aplicar estas mesmas interações a ligantes desconhecidos. Quando um programa efetua o *redocking* do ligante cristalográfico, o qual chamamos de endógeno, considera-o como tendo a pose ideal e parte desta para buscar a pose ideal, o qual seria uma tarefa fácil. Quando utilizamos este mesmo ligante partindo de uma pose aleatória, o qual chamamos de ligante exógeno, é uma tarefa mais difícil para encontrar a pose ideal. Portanto, é necessário validar os programas e algoritmos de *docking* com os ligantes endógenos (cristalográficos) e exógenos (de uma base de dados externa). A Tabela 1 mostra os escores e rmsd médios obtidos com o *redocking* dos ligantes DHQ e DHK.

Os valores médios dos escores dos ligantes de referência DHQ e DHK mostrados na Tabela 1, serviram como nota de corte na busca de outros ligantes por meio de simulações de varredura virtual usando os programas validados.

3.2 Simulações de docking e varredura virtual

Iniciamos a varredura virtual com uma biblioteca contendo 214.000 moléculas, da primeira varredura para o domínio SDH foram selecionados 1042 compostos. Após a repetição dos protocolos foram selecionados 8 compostos, destes o composto Zinc330539, denominado *6-nitroquinazoline-2,4-diol* (NQD) foi o que apresentou o melhor escore (Figura 3). O NQD foi adquirido junto a BLD Pharmatech Ltd. e empregado nas análises *in vitro* e *in vivo*.

3.3 Atividade enzimática *in vitro* da SDH

Para avaliar o efeito do NQD sobre a DHQD/SDH de *Arabidopsis thaliana* (Figura 4 e Tabela Suplementar 2) foi testada a concentração de 250 μM de NQD na presença de 10 a 2000 μM de chiquimato e concentração saturante de NADP^+ (2 mM). Na

concentração de 250 μM , NQD inibiu em 33% a atividade da SDH sob meia saturação de chiquimato. Os valores de V_{max} e K_m demonstram que o NQD reduziu em 48% a V_{max} sem alterar o K_m da reação (Tabela 2 e Figura 5).

3.4 Crescimento das plântulas em hidroponia

Após 24 e 48 horas de incubação, 500 μM de NQD reduziu em 30% e 50%, respectivamente, os comprimentos radiculares das plântulas de soja. Após 96 horas não se observaram alterações significativas (Figura 6A). A biomassa fresca foi reduzida em 23%, após 48 horas de incubação (dados não mostrados).

Em milho, 500 μM de NQD reduziu o comprimento da raiz em 25%, após 48 horas. Após 96 horas, a redução foi de 29% (Figura 6B). Já, a biomassa fresca foi determinada apenas em 96 horas, mostrando uma redução de 19% em relação ao controle (dados não mostrados).

3.6 Consumo de solução nutritiva e estudo da absorção de NQD pelas plântulas.

O consumo de solução nutritiva apresentou similaridade em ambas às espécies de plantas ocorrendo redução na captação de solução a partir de 48 horas (Figura 7). Na soja, as reduções foram de 38% e 32% (Figura 7A) e no milho foram de 40% e 30% (Figura 7B), respectivamente, após 48 e 96 horas.

Em soja, a depleção (12%) de NQD se iniciou após 24 horas de incubação. Nos tempos subsequentes, ao final das 96 horas, 41% de NQD foi depletado. Em contrapartida, no milho, a depleção (5%) foi mais acentuada a partir das 72 horas; ao final das 96 horas a depleção foi de 14% (Figura 8). Sistemas controle de NQD, ou seja,

incubados sem plantas, demonstraram baixa degradação do composto nos tempos avaliados (Figura Suplementar 1).

As raízes de soja e milho tiveram diferenças na proporção de NQD absorvida no período de 96 horas, a partir da metodologia empregada foi possível extrair 83,61 e 52,22 μg de NQD g^{-1} de biomassa fresca, respectivamente (Figura 9). Onde o rendimento da extração foi de 87 a 77% para raízes de soja e milho, reciprocamente (Tabela Suplementar 1).

3.7 Dosagem de proteínas

A exposição à 500 μM de NQD acarretou em aumentos de 39% e 36% no teor de proteínas totais, em raízes de soja e milho, respectivamente (Tabela 3).

3.8 Determinação do conteúdo de aminoácidos

A partir da análise do conteúdo de aminoácidos por HPLC foi possível verificar aumentos significativos de asparagina, alanina, valina e lisina (48%, 101%, 96% e 44%, respectivamente) em soja (Figura 10A). Em milho, aumentos significativos foram observados para aspartato (52%), histidina (145%), arginina (150%), treonina (55%), alanina (48%), tirosina (48%), valina (81%) e fenilalanina (102%) (Figura 10B).

3.9 Determinação dos ácidos fenólicos

Em soja, o teor de ácidos fenólicos totais aumentou 21% após exposição por 48 horas com NQD (Figura 11A). Não foram observadas alterações nos teores dos ácidos gálico (GA), *p*-hidroxibenzaldeído (*p*HB), protocatecuico (PRO), vanílico (VA), *p*-cumárico (*p*-CA) e ferúlico (FA) quando comparado com o controle (Figura 12A). Em contrapartida, no milho, embora o teor de ácidos fenólicos totais não tenha sido alterado

(Figura 11B), observou-se um aumento expressivo (4000 %) de ácido gálico (Figura 12B).

3.10 Teste com glifosato e dosagem de chiquimato

Em plântulas de soja submetidas a 100 μM de glifosato ou 100 μM de glifosato + 500 μM de NQD os comprimentos radiculares reduziram 45% e 57% em comparação com o controle (Tabela Suplementar 3). Em milho, na concentração de 25 μM de glifosato ou 25 μM de glifosato + 500 μM de NQD, não houve alterações estatisticamente significativas no comprimento radicular (Tabela Suplementar 4).

Em soja, o tratamento com glifosato acumulou 1,3 mg de chiquimato g^{-1} de biomassa fresca, enquanto o tratamento com glifosato + NQD gerou 0,6 mg de chiquimato g^{-1} de biomassa fresca, ou seja, uma redução de 54% no teor de chiquimato (Figura 13A). Em milho, a incubação com 25 μM de glifosato acumulou 0,7 mg de chiquimato g^{-1} de biomassa fresca, todavia, quando as plântulas foram incubadas com glifosato (25 μM) + NQD (500 μM) não foi observado acúmulo de chiquimato (Figura 13C).

3.11 Determinação do conteúdo de lignina

Os teores de lignina não foram alterados quando as plântulas foram submetidas a 500 μM de NQD por 48 horas (soja) e 96 horas (milho) (Tabela 4).

4. Discussão

A estrutura da DHQD/SDH de *Arabidopsis thaliana* apresenta uma forma côncava e com dois domínios funcionais distintos. A porção SDH tem um domínio de ligação ao nucleotídeo na região C-terminal e ao substrato em N-terminal. Um aspecto interessante é que os sítios ativos da DHQD e SDH estão face a face, garantindo desta forma, um

acurado direcionamento dos intermediários para a via do chiquimato, minimizando assim a perda para vias concorrentes (Singh e Christendat, 2007; 2006). Neste trabalho, o domínio SDH serviu como escopo para a varredura virtual por meio de *docking molecular*.

A varredura *in silico* em bibliotecas de compostos químicos vem sendo largamente empregada na busca por novos medicamentos, por se tratar de um método de baixo custo, alto rendimento na identificação de moléculas em pouco tempo (Avitia-Domínguez et al., 2014; Isa et al., 2018; Petersen et al., 2015; Rodrigues-Vendramini et al., 2018). Estas ferramentas podem ser expandidas na busca por moléculas inibidoras de enzimas vegetais, com aplicabilidade como herbicidas (Foletto-Felipe, 2021; Fu et al., 2019), o que foi explorado neste trabalho. A partir das varreduras virtuais, dois compostos foram encontrados como bons candidatos e o ZINC 330539 (6-nitroquinazoline-2,4-diol; NQD) foi adquirido para realização dos ensaios. Derivados de *quinazoline-2,4-dione* apresentam várias atividades, como antifúngico (Noureldin et al., 2018), antitumoral (Gangjee et al., 1998) e atividade herbicida com sítio de atuação para 4-hidroxifenilpiruvato dioxigenase (HPPD) (Wang et al., 2015).

Para a determinação da atividade *in vitro* empregamos a enzima $\Delta 88\text{DHQ-SDH}$ de *Arabidopsis thaliana* (Singh e Christendat, 2007), a qual foi expressa e purificada. A atividade da SDH na presença de 250 μM de NQD foi reduzida em cerca de 33% sob meia saturação, demonstrando que o composto apresenta ação inibitória *in vitro*. Tanto a varredura virtual como as simulações de *docking* foram para o sítio ativo do desidrochiquimato, buscando desta forma, um inibidor competitivo para este substrato. Todavia, os valores de $V_{\text{máx}}$ e K_{m} obtidos sugerem uma inibição do tipo não competitiva com o desidrochiquimato (Nelson. David L. e Cox, 2018).

Os testes *in vivo* com 500 μM de NQD causaram reduções nos comprimentos radiculares de plântulas de soja e milho seguido de reduções nas biomassas frescas. Além disso, nós constatamos que plantas submetidas ao NQD absorveram um volume menor de solução nutritiva em comparação com o controle, o que pode estar associado com o menor crescimento radicular. Além disso, constatamos que a absorção via radicular do composto foi 37% maior em plântulas de soja do que em milho, o que pode estar relacionado com a maior restrição no crescimento radicular nestas plântulas. Reduções no crescimento e de biomassa foram relatadas após a supressão da DHQD/SDH em tabaco, acompanhado de diminuição no teor dos aminoácidos aromáticos (Ding et al., 2007). Entretanto, isso não foi observado em nosso estudo uma vez que NQD aumentou os teores de aminoácidos aromáticos em raízes de milho (tirosina e fenilalanina) e de outros aminoácidos nas raízes de ambas as espécies avaliadas, como os aminoácidos não polares e alifáticos (alanina e valina), carregados positivamente (lisina, histidina, arginina) e negativamente (aspartato), e os polares não carregados (asparagina, treonina). A inibição da 3-desidroquinato sintase em cultura de *A. variabilis* aumentou aminoácidos não-aromáticos (leucina, isoleucina, valina e arginina). Entretanto, ocorreu redução dos aminoácidos aromáticos (Brilisauer et al., 2019), em oposição ao que observamos aqui. Desse modo, os aumentos de alguns aminoácidos e dos teores de proteínas totais podem estar relacionados a uma resposta de estresse, haja vista que modificações do metabolismo de aminoácidos vêm sendo relatados como um gatilho à essa resposta (Batista-Silva et al., 2019; Coruzzi et al., 2015; Hildebrandt, 2018; Tzin e Galili, 2010).

Ácidos fenólicos são metabólitos secundários que desempenham funções importantes nas plantas, sendo alguns deles provenientes de intermediários da via do chiquimato (Santos-Sánchez et al., 2019). O tratamento com NQD em soja aumentou o conteúdo

dos ácidos fenólicos totais, mas não alterou o conteúdo de nenhum ácido fenólico simples especificamente. Em milho, nós observamos um resultado diferente, não sendo constatada modificação nos teores de ácidos fenólicos totais, mas notamos um aumento expressivo no teor de ácido gálico. Aumentos de derivados de benzoatos, *p*-hidroxibenzoato e vanilina foram observados em plântulas de soja suscetíveis ao glifosato, sendo também constatado acúmulo de chiquimato, o qual poderia estar sendo convertido em desidrochiquimato → protocatecuato → *p*-hidroxibenzoato → vanilina (Marchiosi et al., 2009). Alguns estudos demonstraram a ocorrência de mais de uma DHQD/SDH em determinadas plantas, estando relacionadas não apenas com a formação de chiquimato, mas envolvidas na síntese de galato e, muito provavelmente, de quinato. Isso foi relatado em *Nicotiana tabacum* (Ding et al., 2007), *Populus trichocarpa* (Guo et al., 2014), *Betula pubescens* (Ossipov et al., 2003), *Vitis vinífera* (Bontpart et al., 2016), *Camellia sinensis* (Huang et al., 2019) e, mais recentemente, em *Eucalyptus camaldulensis* tolerante ao alumínio (Tahara et al., 2021). Dessa maneira, não pode ser descartada a presença de mais de uma DHQD/SDH em plantas de milho.

Para evidenciar a atuação do NQD *in vivo* foram realizadas aplicações de NQD mais glifosato. O glifosato [*N*-(fosfonometil)-glicina] é um herbicida não seletivo, pós-emergente, de boa translocação e que atua sobre a EPSP sintase, o que resulta no acúmulo de chiquimato (Duke e Powles, 2008; Steinrücken e Amrhein, 1980). Como esperado, experimentos com glifosato em soja reduziram o comprimento radicular e a biomassa fresca. Por outro lado, o tratamento com 100 µM glifosato + 500 µM NQD reduziu mais expressivamente o comprimento radicular ao passo que a redução da biomassa fresca foi similar ao tratamento só com 100 µM glifosato. Em contrapartida, o teor de chiquimato após o tratamento com 100 µM glifosato + 500 µM NQD foi reduzido em 54% em relação ao tratamento só com glifosato, indicando que a atividade

da SDH não foi completamente inibida, mas parcialmente, permitindo o fluxo através da via.

Em milho, não houve um comprometimento do comprimento radicular, seja somente com 25 μM glifosato ou em conjunto com 500 μM NQD, todavia, não foi detectado chiquimato nas plântulas incubadas com 25 μM glifosato + 500 μM NQD, demonstrando que *in vivo* a SDH de milho é mais fortemente inibida que a SDH de soja. Estes resultados, associados com o acúmulo de ácido gálico observado nas plântulas de milho, apontam para um mecanismo de transbordamento da via, onde o desidrochiquimato acumulado é desviado para a síntese de ácido gálico, similar ao observado no balanço na síntese de sacarose e amido (Zeeman, 2015). Além disso, não se pode desconsiderar que este ácido gálico gerado esteja sendo empregado em um processo de desintoxicação, como já relatado em plantas *E. camaldulensis*. Nesse caso, identificou-se uma SDH responsável pela produção de ácido gálico, o qual era precursor de um tanino hidrolisável empregado no processo de desintoxicação de alumínio (Tahara et al., 2021).

As vias do chiquimato e dos fenilpropanoides, a qual é a responsável pela biossíntese de lignina, estão interligadas. A lignina é um heteropolímero fenólico e a sua formação se inicia com fenilalanina e/ou tirosina, as quais sofrerão várias reações de hidroxilações e metilações para gerar os monômeros que a constituem, isto é, *p*-hidroxifenil (H), guaiacil (G) e siringil (S) (Marchiosi et al., 2020). Além dos aminoácidos aromáticos serem importantes na etapa inicial, o intermediário chiquimato tem uma atuação central na via de formação de lignina. O chiquimato está envolvido na reação de transesterificação do *p*-cumaroil-CoA em *p*-cumaroil-chiquimato pela ação da hidroxicinamoil-CoA:chiquimato/quinato hidroxicinamoiltransferase (HCT). O produto desta reação é hidroxilado pela *p*-coumaroil-chiquimato/quinato 3-hidroxilase (C3H)

gerando o cafeoil chiquimato, o qual pode sofrer novamente uma transesterificação pela HCT ou clivagem pela cafeoil-chiquimato esterase (CSE), originando o cafeoil-CoA ou cafeato, respectivamente (Hoffmann et al., 2003; Schoch et al., 2006; Vanholme et al., 2013). Os produtos formados seguem subsequentes reações em direção aos álcoois coniferílico e sinapil, enquanto o chiquimato liberado permanece disponível para novas reações com HCT.

Regulações negativas na expressão destas enzimas resultaram, na maioria dos casos, em reduções no teor do conteúdo de lignina, alterações na composição monomérica, com característico aumento de H (Martarello et al., 2021). Em outro estudo, visando reduzir a disponibilidade de chiquimato citosólico, uma chiquimato cinase bacteriana foi introduzida em álamo híbrido, e algumas linhagens apresentaram redução do crescimento, alteração da composição monomérica da lignina (aumento de *p*-hidroxifenil e redução de guaiacil e siringil), mas sem alteração do teor de lignina em comparação ao tipo selvagem (Hu et al., 2022). Em nosso trabalho, observamos que em milho o NQD aumentou os aminoácidos aromáticos (tirosina e fenilalanina), mas não alterou o conteúdo de lignina, sendo isso observado nas duas espécies de plantas estudadas. Desta forma, não podemos descartar que alterações na composição monomérica da lignina possam ter ocorrido.

5. Conclusões

A partir de ferramentas de bioinformática obtivemos um inibidor para SDH de *Arabidopsis thaliana*, o composto *6-nitroquinazoline-2,4-diol* (NQD) o qual demonstrou atividade inibitória *in vitro* para DHQD/SDH de *A. thaliana*, na concentração de 250 μ M. *In vivo* sua ação inibitória também foi observada, na

concentração de 500 μ M de NQD, ocasionando reduções no comprimento radicular de plântulas de soja e milho. A incubação de plântulas de soja e milho com NQD conjuntamente com glifosato possibilitou confirmar que o ponto de atuação do composto precede a reação da EPSP sintase, ou seja, ocorre sobre a SDH. Em suma, NQD *in vivo* aparenta ser um potente inibidor para SDH de milho, enquanto a SDH de soja apresentou uma menor sensibilidade à ação de tal composto. A presença expressiva de ácido gálico em plântulas de milho associado com a maior inibição *in vivo* após exposição ao NQD corrobora para sua ação sobre a SDH desta, e indica um modo diferencial de atuação para as espécies de plântulas estudadas, necessitando desta forma, de mais experimentos para identificar estes modos de ação.

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Tabelas

Tabela 1. Escores médios obtidos com o *redocking* nos programas Autodock e Molegro, para os ligantes cristalográficos e os ligantes obtidos da base de dados Zinc15.

	Ligante Cristalográfico				Ligante do banco de dados Zinc	
	Autodock		Molegro		Autodock	Molegro
	Média score	Rmsd (Å)	Média score	Rmsd (Å)	Média score	Média score
DHQ	-7.98 ± 0.09	0.32 ± 0.02	-81.38 ± 0.02	0.27 ± 0.00	-7.88 ± 0.10	-79.69 ± 0.24
DHK	-8.25 ± 0.02	0.22 ± 0.03	-67.35 ± 0.01	0.21 ± 0.01	-7.26 ± 0.21	-62.63 ± 0.00

Tabela 2. Parâmetros cinéticos obtidos pelo ajuste da equação de Michaelis-Menten dos resultados experimentais para enzima chiquimato-desidrogenase.

NQD (μM)	$V_{\text{máx}}$ ($\mu\text{mol h}^{-1} \text{ g de proteínas}^{-1}$)	K_M (μM)
0	475,7	282,9
250	248,3	258,1

Tabela 3. Teor de proteínas totais (mg% de proteínas) em plântulas de soja (48 horas) e milho (96 horas) incubadas com 0 ou 500 μ M NQD.

Plântula	Controle	500 μM NQD	Diferença (%)
Soja	334 \pm 16,52	465,6 \pm 19,51*	39%
Milho	222,9 \pm 7,91	303,0 \pm 27,20*	36%

Valores médios \pm erro padrão da média (n = 4), significativamente diferente do controle é marcado com asterisco (*), ($p \leq 0,05$, teste t não pareado).

Tabela 4. Teor de lignina em plântulas de soja incubadas por 48 horas e de milho incubadas por 96 horas com 0 ou 500 μM NQD.

Amostra	Soja	Milho
0	68,73 + 2,436	144,3 + 3,036
500 μM NQD	66,56 + 1,401	149,9 + 2,819

Valores médios \pm erro padrão da média (n=5), significativamente diferentes do controle são marcados com asterisco (*), ($p \leq 0,05$, teste t não pareado).

Figuras

Fig. 1. Via do chiquimato

Fig. 2. Estrutura tridimensional da DHQD/SDH de *Arabidopsis thaliana* empregada nas simulações de *docking molecular*. Domínio DHQD (magenta) contendo desidroquinato, domínio SDH (verde) contendo desidrochiquimato e NADPH. Identificação de cada átomo: oxigênio (vermelho), carbono (cinza claro), hidrogênio (cinza escuro), nitrogênio (azul escuro) e fosforo (laranja).

Fig. 3. Composto selecionado por *virtual screening* 6-nitroquinazoline-2,4-diol (-8,52 kcal mol⁻¹) apresentou o melhor score em relação ao substrato 3-desidrochiquimato (-7,51 kcal mol⁻¹). Identificação de cada átomo: oxigênio (vermelho), carbono (cinza), nitrogênio (azul escuro) e hidrogênio (azul claro).

Fig. 4. Gel de poliacrilamida (SDS-PAGE) 12 % com amostras da purificação da proteína $\Delta 88$ DHQ-SDH a partir da cromatografia de afinidade ao níquel. kDa = quilodáton; MM = massa molecular padrão; Extrato bruto = sobrenadante após a lise das bactérias; FT = do inglês *flow through*, ou seja, o que eluiu diretamente pela coluna; lavagem = injeção de tampão (10 mM Tris HCl, 500 mM NaCl, pH 7,5) para remoção das impurezas que interagiram fracamente com a resina; 40 mM, 150 mM ou 250 mM = injeção de tampão contendo imidazol nas concentrações de 40, 150 ou 250 mM. Seta: indicação da proteína $\Delta 88$ DHQ-SDH com cerca de 58 kDa.

Fig. 5. Gráfico de Michaelis-Menten de $\Delta 88$ DHQ-SDH de *Arabidopsis thaliana*. Reação contendo 0,1 M Tris-HCl (pH 8.8), 1 nM da enzima, 0,1 a 2 mM de ácido chiquímico, 2 mM de NADP⁺, 0 μ M (●) e 250 μ M (■) de NQD.

Fig. 6. Comprimento das raízes de soja em 24, 48 e 96 horas com 0 ou 500 μM de NQD (A) (24 h, n = 4; 48 h, n = 8; 96 h, n = 3). Comprimento das raízes de milho em 24, 48 e 96 horas com 0 ou 500 μM de NQD (B) (n = 4). Valores médios \pm erro padrão da média, significativamente diferentes do controle são marcados com asterisco (*), teste *t* não pareado ($p \leq 0,05$).

Fig. 7. Consumo de solução nutritiva em plantas de soja (A) e milho (B) até 96 horas de incubação com 0 (●) ou 500 μM de NQD (■). Valores médios \pm erro padrão da média (A, n = 3; B, n = 4), significativamente diferentes do controle são marcados com asterisco (*), teste *t* não pareado ($p \leq 0,05$).

Fig. 8. Depleção de 500 μM de NQD em plântulas de soja e milho até 96 horas de incubação. Valores médios \pm erro padrão da média (500 μM NQD, soja, n = 3 (▲); 500 μM NQD, milho, n = 4 (■)), significativamente diferentes do tempo 0 são marcados com asterisco (*), teste de Dunnett (*One-way ANOVA*) ($p \leq 0,05$).

Fig. 9. Absorção radicular em 96 horas de incubação com 500 μM de NQD em plântulas de soja e milho. Valores máximos e mínimos, n=4 ($p \leq 0,05$, teste *t* não pareado).

Fig. 10. Conteúdo de aminoácidos em plântulas de soja (A) e milho (B), incubadas por 48 e 96 horas, respectivamente, com 0 ou 500 μM . Asp, aspartato; glu, glutamato; asn, asparagina; ser, serina; gln, glutamina; his, histidina; arg, arginina; gly, glicina; thr, treonina; ala, alanina; tyr, tirosina; met, metionina; val, valina; phe, fenilalanina; ile, isoleucina; leu, leucina; lys, lisina. Valores médios \pm erro padrão da média (n=4), significativamente diferentes do controle são marcados com asterisco (*), ($p \leq 0,05$, teste *t* não pareado).

Fig. 11. Ácidos fenólicos totais em plântulas de soja incubadas por 48 horas com 0 ou 500 μM de NQD (A). Ácidos fenólicos totais em plântulas de milho incubadas por 96 horas com 0 ou 500 μM de NQD (B). Valores médios \pm erro padrão da média ($n = 4$), significativamente diferentes do controle são marcados com asterisco (*), ($p \leq 0,05$, teste t não pareado).

Fig. 12. Ácidos fenólicos em plântulas de soja incubadas por 48 horas com 0 ou 500 μM de NQD (A). Ácidos fenólicos em plântulas de milho incubadas por 96 horas com 0 ou 500 μM de NQD (B). GA, ácido gálico; $p\text{HB}$, p -hidroxibenzaldeído; PRO, ácido protocatecuico; VA, ácido vanílico; CA, ácido cafeico; $p\text{-CA}$ – ácido p -cumárico; FA, ácido ferúlico e nd, não detectado. Valores médios \pm erro padrão da média ($n = 4$), significativamente diferentes do controle são marcados com asterisco (*), ($p \leq 0,05$, teste t não pareado).

Fig. 13. Teor de chiquimato em raízes de soja (A) incubadas por 48 horas com 0 (controle), 100 μM de glifosato, 100 μM de glifosato + 500 μM de NQD; milho (B) incubado por 96 horas com 0 (controle), 25 μM de glifosato, 25 μM de glifosato + 500 μM de NQD. nd = não detectado. Valores médios ($n = 3$) \pm erro padrão da média, seguido por letras diferentes (a, b, c) são significativamente diferentes (teste de Tukey, *two-way* ANOVA. $p \leq 0,05$).

Figura 1

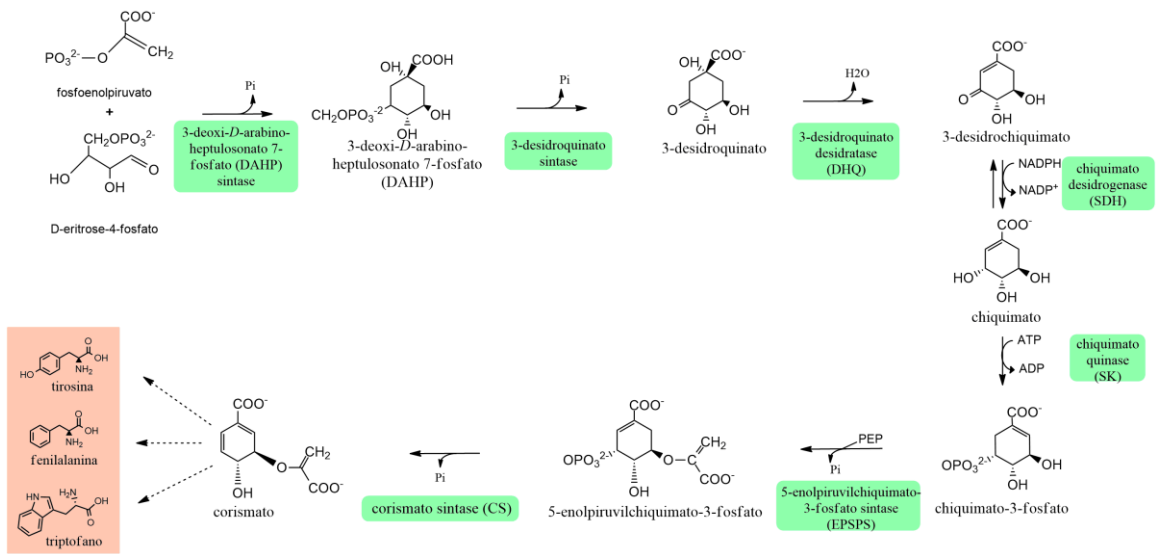


Figura 2

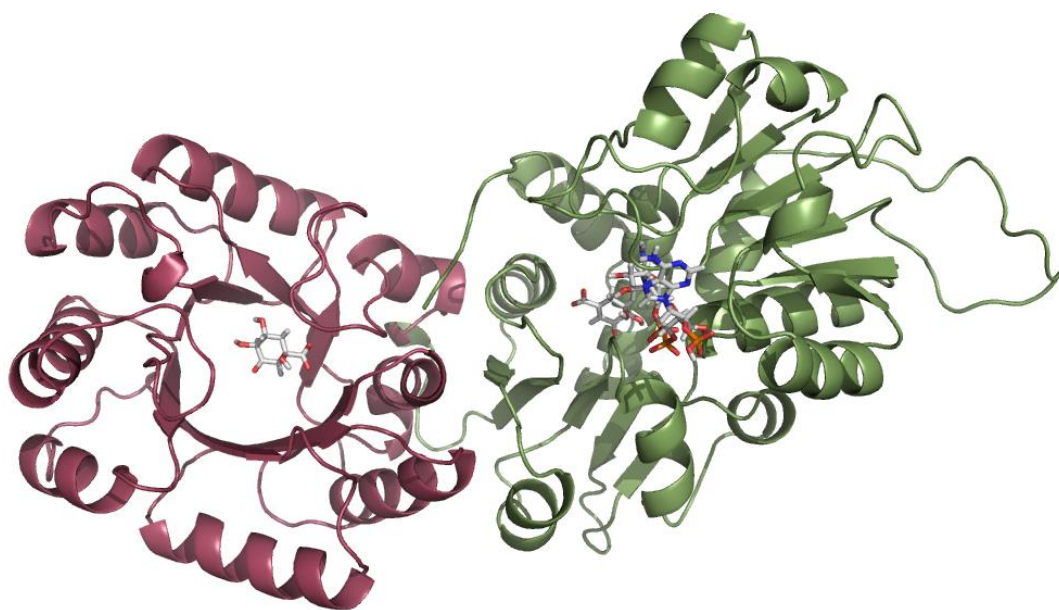


Figura 3

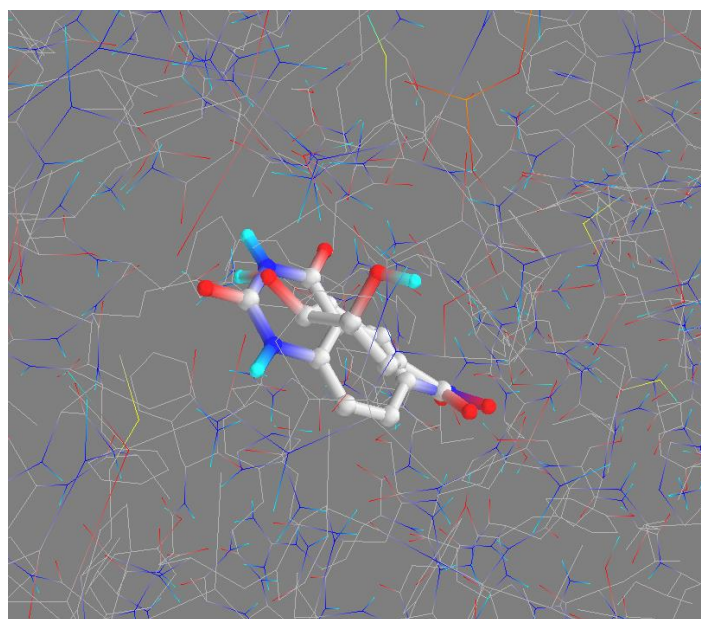


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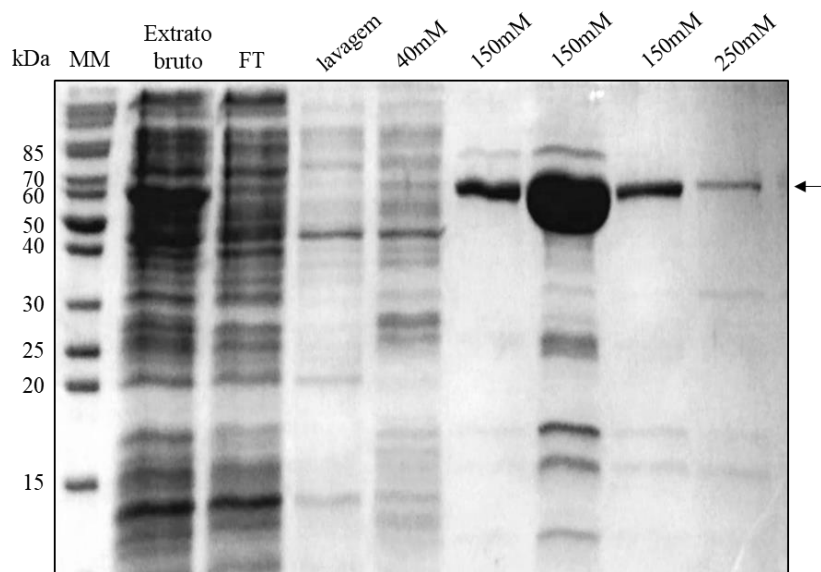


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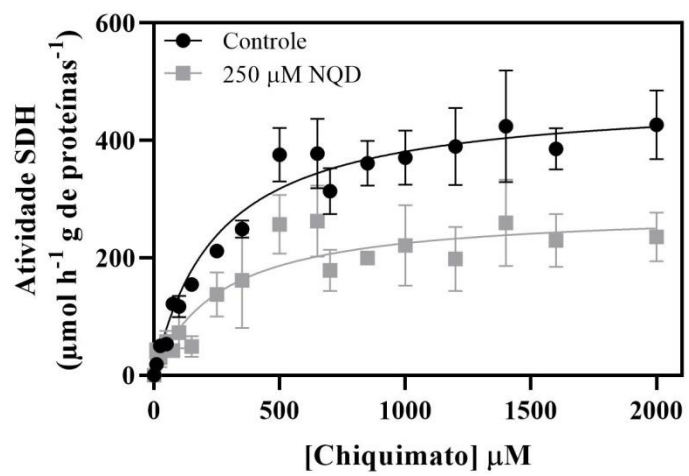


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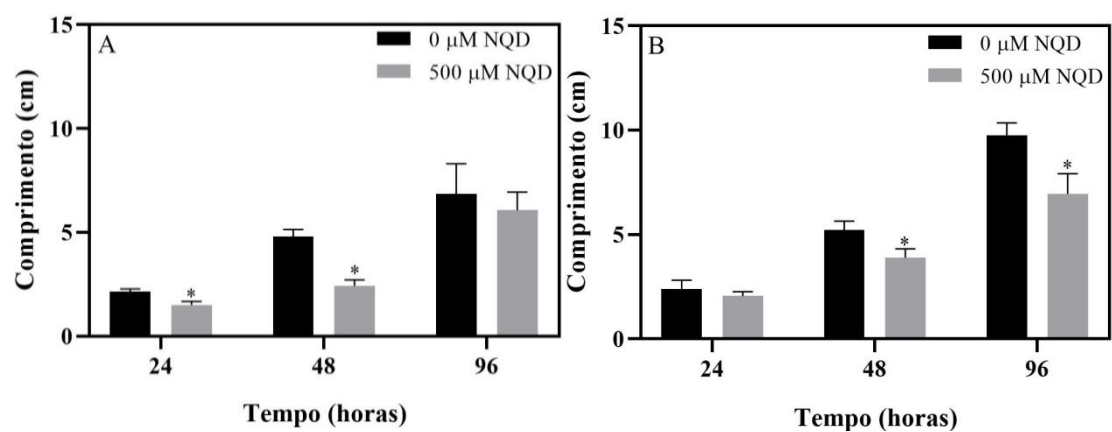


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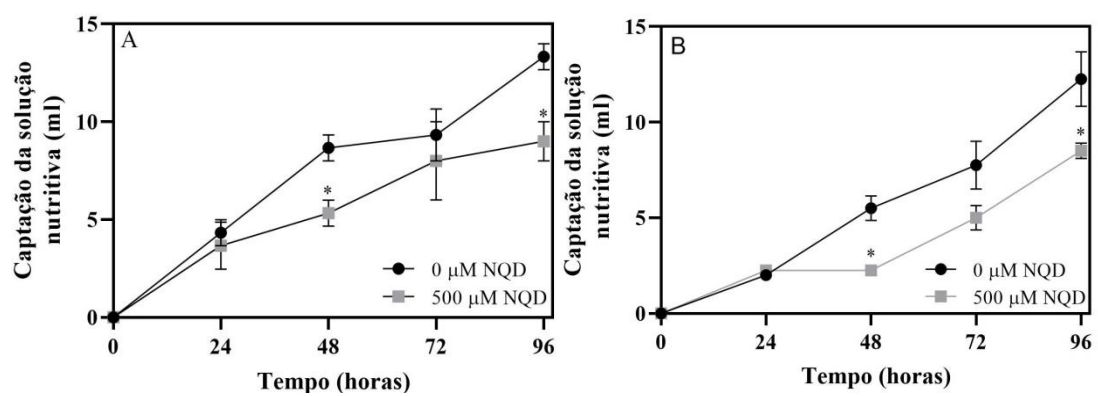


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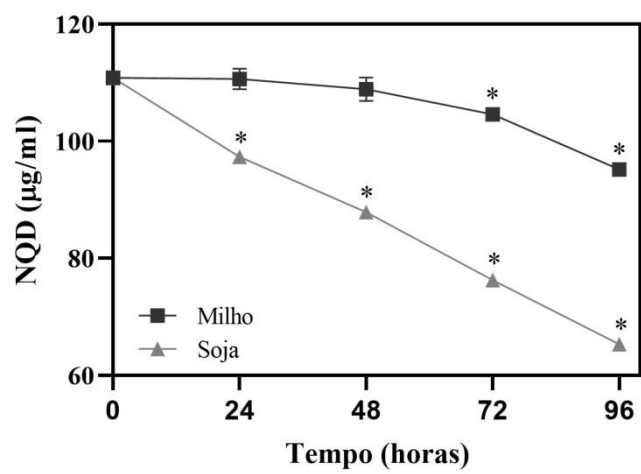


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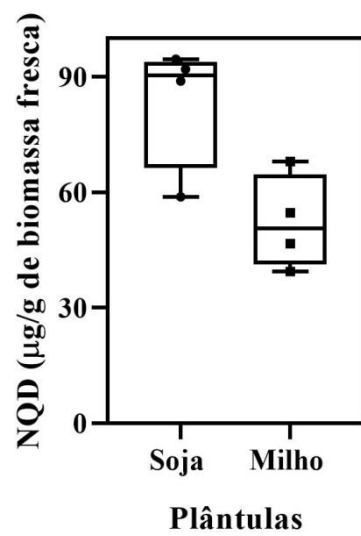


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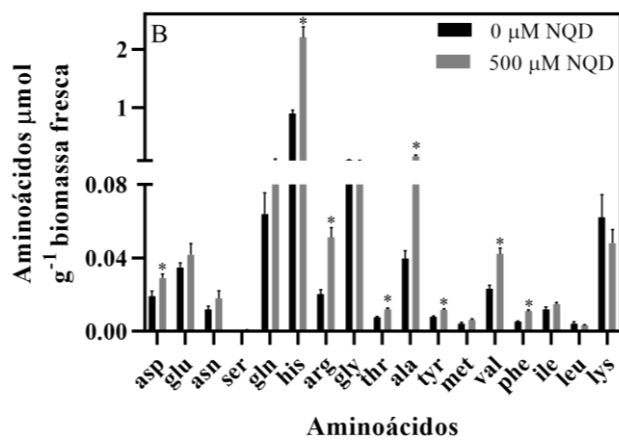
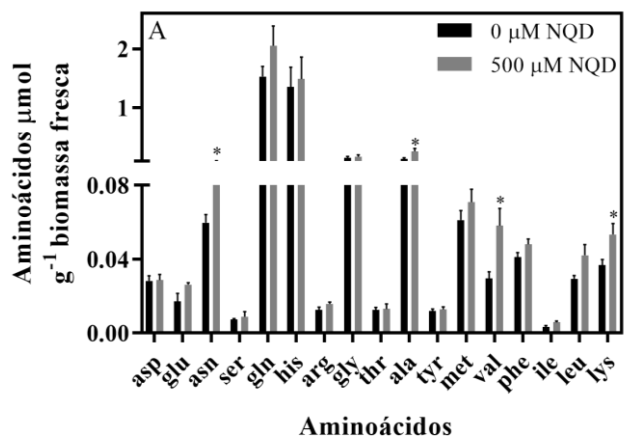


Figura 11

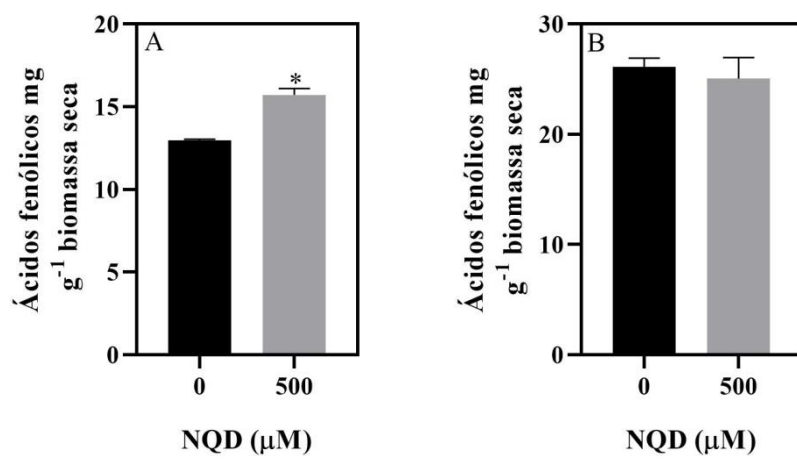


Figura 12

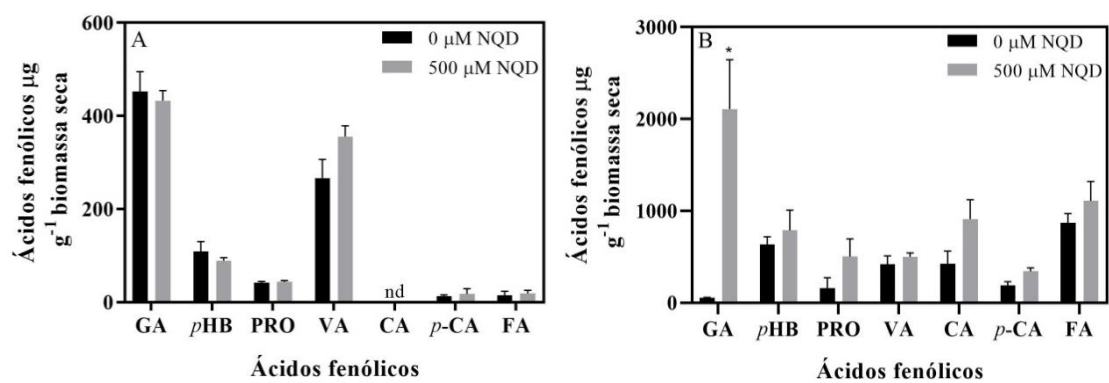
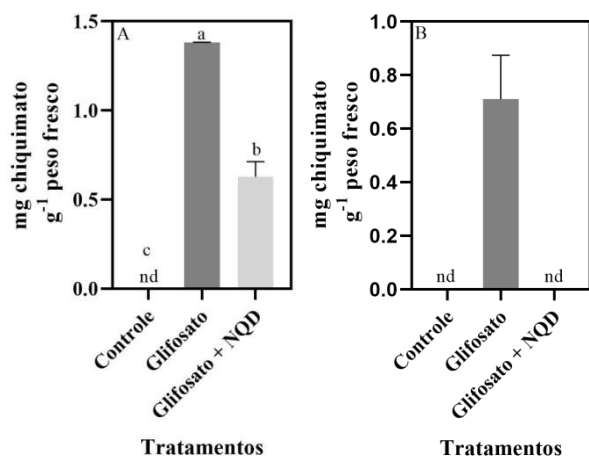


Figura 13



Material Suplementar

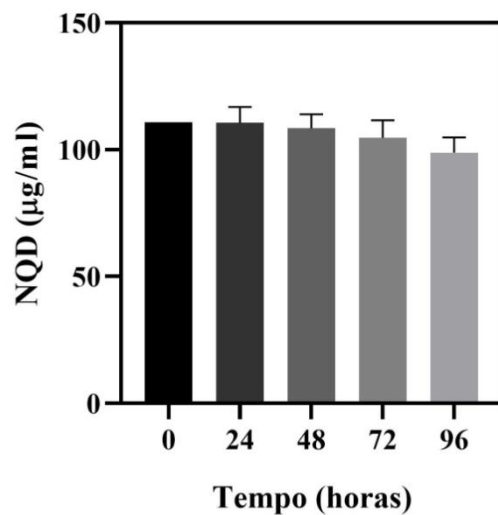


Figura Suplementar 1. Depleção de 500 µM de NQD em sistema sem plântulas até 96 horas de incubação. Valores médios \pm erro padrão da média, teste de Dunnett (*One-way ANOVA*) ($p \leq 0,05$).

Rendimento da extração de absorção do NQD

As sementes de soja (*Glycine max* L. Merrill cv. BRS 232) e milho (*Zea mays* L. cv. IPR-164) foram desinfetadas em NaClO 2% por 5 minutos, depois foram lavadas exaustivamente com água deionizada. A seguir, as sementes foram depositadas entre folhas de papel Germitest[®], previamente umedecidas, as quais foram enroladas e acondicionadas em tubos de germinação, onde permaneceram em câmara escura, a 25°C, durante 3 dias. Após este período, as plântulas foram transferidas para sistemas de hidroponia contendo solução nutritiva (Dong et al., 2006) (pH 6,0), sendo cultivadas por 96 horas (25°C ± 2, fotoperíodo de 12/12 h), sendo que em 48 horas foi adicionado solução nutritiva (1/6 de força). Após, este período 0,2 g de raízes foram maceradas com 2,5 mL de metanol 65% contendo 125 µM de NQD, posteriormente as amostras foram centrifugadas a 3200 rpm, 4°C por 15 min. O sobrenadante foi diluído (5x) em fase móvel, ácido acético 4%: metanol 70% (50:50), filtrado em membrana de 0,45 µm e analisado (20 µL) em HPLC Prominence (Shimadzu[®], Tóquio, Japão), detector UV-VIS (SPD-10A), corrida isocrática, fluxo de 0,8 mL min⁻¹, comprimento de onda de 310 nm, coluna C18 (250 x 4,6 mm, 5 µm; Supelco Discovery[®]). As áreas das amostras foram comparadas com a área obtida do padrão de NQD 25 µM.

Tabela Suplementar 1. Rendimento da extração do NQD em raízes de soja e milho incubadas por 96 horas, valores médios (n = 3).

Amostra	Área (mAU)	Rendimento (%)
Padrão 125 µM	1853996	
Soja	1624525	87%
Milho	1442945	77%

Digestão da $\Delta 88\text{DHQ-SDH}$ e análise em Espectrometria de Massas

A enzima $\Delta 88\text{DHQ-SDH}$ foi digerida segundo protocolo estabelecido pelo Laboratório Nacional de Biociências (LNBio, 2008), com modificações. A uma alíquota (50 μL) obtida da purificação foi adicionado ureia 8M (1:1), posteriormente foi adicionado 5 mM de DTT, sendo mantidos a 56°C por 25 min. A solução foi resfriada a temperatura ambiente antes da adição de 14 mM de IAA, permanecendo em repouso por 30 min protegido da luz. Após este período foi adicionado 5mM de DTT, permanecendo em repouso por mais 15 min em temperatura ambiente protegido da luz. Para reduzir a concentração da ureia a 0,8 M foram adicionados 400 μL de 50 mM de bicarbonato de amônio. Seguido de adição de 1mM de CaCl_2 . A amostra foi digerida a 37°C por 16-20 h, com a adição de 20 μL de tripsina (1:50). A reação foi interrompida com a adição de 10 μL de ácido fórmico. A amostra foi centrifugada a 3000 g, por 15 min a 4°C, e o sobrenadante foi reservado em eppendorf, permanecendo no gelo até a injeção no NanoUPLC-MS^E, a qual foi realizada de acordo com Foletto-Felipe (2021).

Tabela Suplementar 2. Identificação de proteínas por espectrometria de massas.

Código do	Descrição	Massa molecular	Score	Cobertura
Uniprot		(Da)		(%)
Q9SQT8	Bifuncional 3-desidroquinato desidratase/chiquimato desidrogenase de <i>Arabidopsis</i> <i>thaliana</i>	66138	13717	75.79
A0A119LPF9	Proteína transmembrana <i>Arabidopsis thaliana</i>	12109	5708	43.65

Tabela Suplementar 3. Parâmetros biométricos de soja incubada por 48 horas, com 100 µM de glifosato, 100 µM de glifosato + 500 µM de NQD ou na ausência de ambos (controle). Valores médios (n = 3) ± erro padrão da média, seguido por letras diferentes (a, b) são significativamente diferentes, teste de Tukey (*One-way ANOVA*) ($p \leq 0,05$).

Soja	Comprimento 48 h	Biomassa fresca 48 h
Controle	4,943 ± 0,4296 ^a	1,110 ± 0,0479 ^a
Glifosato	2,620 ± 0,3612 ^b	0,829 ± 0,0551 ^b
Glifosato + NQD	2,097 ± 0,1260 ^b	0,814 ± 0,0474 ^b

Tabela Suplementar 4. Parâmetros biométricos de milho incubado por 96 horas, com 25 µM de glifosato, 25 µM de glifosato + 500 µM de NQD ou na ausência de ambos (controle). Valores médios (n = 3) ± erro padrão da média, seguido por letras diferentes (a, b) são significativamente diferentes, teste de Tukey (*One-way ANOVA*) ($p \leq 0,05$).

Milho	Comprimento 96 h	Biomassa fresca 96 h
Controle	6.917 ± 1,309 ^a	0.9877 ± 0,1871 ^a
Glifosato	3,793 ± 0,5359 ^a	0,7395 ± 0,0716 ^a
Glifosato + NQD	6,230 ± 0,3522 ^a	0,8785 ± 0,0311 ^a