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ISOCITRATE LYASE AS A MOLECULAR TARGET FOR WEED SUPPRESSION: *IN SILICO*, *IN VITRO* AND *IN VIVO* APPROACHES

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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 doutor em Ciências Biológicas.

Orientadora: Prof[a]. Dr[a]. Emy Luiza Ishii-Iwamoto
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BIOGRAFIA

Paulo Vinicius Moreira da Costa Menezes nasceu em 20 de janeiro de 1990, em Vilhena, Rondônia. Possui graduação em Ciências Biológicas (licenciatura) pela Universidade Estadual de Maringá (2015). Em 2018, recebeu o título de mestre em Ciências Biológicas nesta mesma Instituição, onde investigou as alterações bioquímicas, anatômicas e morfológicas causadas pela fração butanólica da palhada de *Urochloa ruziziensis* e da saponina esteroidal protodioscina sobre as plantas daninhas *Ipomoea grandifolia* e *Digitaria insularis*, estudo este posteriormente publicado no periódico Plant Physiology and Biochemistry (2021). Em 2020, iniciou seus estudos no curso de graduação em Estatística (bacharel) nesta mesma Instituição no intuito de aprimorar seus conhecimentos nas Ciências Exatas, e em 2021 tornou-se sócio da SBPC (Sociedade Brasileira para o Progresso da Ciência). Atualmente está concluindo o doutorado (2018-2022) no Laboratório de Oxidações Biológicas (Departamento de Bioquímica) da Universidade Estadual de Maringá pelo mesmo Programa de Pós-Graduação, onde trabalha com bioquímica de plantas sob a supervisão da Prof[a]. Dr[a]. Emy Luiza Ishii-Iwamoto. Ao longo de sua trajetória acadêmica desenvolveu pesquisa nas áreas de bioquímica e fisiologia vegetal envolvendo os seguintes tópicos: plantas daninhas, alelopatia, metabolismo secundário de plantas, metabolismo energético e oxidativo, compostos naturais com potencial biológico e bioquímica computacional. Ainda, é entusiasta de estudos científicos aplicados no campo da etnobotânica.

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Deixo aqui o meu tributo.

"We wish to pursue the truth no matter where it leads — but to find the truth, we need imagination and skepticism both. We will not be afraid to speculate, but we will be careful to distinguish speculation from fact. The cosmos is full beyond measure of elegant truths; of exquisite interrelationships; of the awesome machinery of nature. The surface of the Earth is the shore of the cosmic ocean. On this shore we've learned most of what we know. Recently we've waded a little way out, maybe ankle deep, and the water seems inviting. Some part of our being knows this is where we came from. We long to return. And we can. Because the cosmos is also within us. We're made of star-stuff. We are a way for the cosmos to know itself."

Carl Sagan (1934 – 1996)

"…I stand at the seashore, alone, and start to think.

There are the rushing waves, mountains of molecules
Each stupidly minding its own business
Trillions apart, yet forming white surf in unison

Ages on ages, before any eyes could see
Year after year, thunderously pounding the shore as now
For whom, for what?
On a dead planet, with no life to entertain

Never at rest, tortured by energy
Wasted prodigiously by the sun, poured into space
A mite makes the sea roar

Deep in the sea, all molecules repeat the patterns
Of one another till complex new ones are formed
They make others like themselves
And a new dance starts

Growing in size and complexity
Living things, masses of atoms, DNA, protein
Dancing a pattern ever more intricate

Out of the cradle onto the dry land
Here it is standing
Atoms with consciousness, matter with curiosity
Stands at the sea, wonders at wondering

I, a universe of atoms
An atom in the universe"

Richard P. Feynman (1918-1988)

ÍNDICE

APRESENTAÇÃO

Esta tese é composta por dois capítulos: o capítulo 1 compreende um artigo sobre a modelagem da enzima Isocitrato liase de *Arabidopsis thaliana*, e a prospecção *in silico* de possíveis inibidores para esta enzima no intuito de analisá-la como um possível alvo molecular para a descoberta de um novo mecanismo de ação herbicida para plantas daninhas. Este artigo foi intitulado "**Isocitrate lyase as a molecular target for weed supression: *in sílico, in vivo* and *in vitro* studies**". O capítulo 2, por sua vez, é um aprofundamento do estudo do primeiro capítulo, sendo constituído por um artigo que investiga os modos de ação do ácido itacônico sobre o metabolismo energético e oxidativo da planta daninha *Euphorbia heterophylla* durante o processo de germinação, intitulado: "**The modes of action of itaconic acid on the initial development of *Euphorbia heterophylla***". Conforme as resoluções do Programa de Pós-Graduação em Ciências Biológicas (PBC), os artigos foram redigidos seguindo as normas das revistas as quais serão submetidas, conforme descrito abaixo:

Capítulo 1 – Menezes, P.V.M.C.; Henrique, I.F.; Kajihara, L.A.; Stulp, G.F.; Wakida, E.; Contesoto, I.C.; Wagner, A.L.S.; Mantovanelli, G.C.; Silva, R.C.; Oliveira, H.C.; Mito, M.S.; Constantin, R.P.; Ferrarese-Filho, O.; Seixas, F.A.V.; Ishii-Iwamoto, E.L. Isocitrate lyase as a molecular target for weed supression: *in sílico*, *in vivo* and *in vitro* studies. Será submetido ao periódico ***Pesticide Biochemistry and Physiology*** (fator de impacto em agosto de 2022: 4,966. https://www.sciencedirect.com/journal/pesticide-biochemistry-and-physiology).

Capítulo 2 – Menezes, P.V.M.C.; Henrique, I.F.; Kajihara, L.A.; Anunciação, A.R.P.; Salomão, J.M.; Stulp, G.F.; Wakida, E.; Contesoto, I.C.; Wagner, A.L.S.; Mantovanelli, G.C.; Mito, M.S.; Constantin, R.P.; Ferrarese-Filho, O.; Seixas, F.A.V.; Ishii-Iwamoto, E.L. The modes of action of itaconic acid on the initial development of *Euphorbia heterophylla*. Será submetido ao periódico ***Physiologia Plantarum*** (fator de impacto em agosto de 2022: 5,081. https://physiologiaplantarum.org).

RESUMO GERAL

A Isocitrato liase (ICL, EC: 4.1.3.1) é uma enzima-chave do ciclo do glioxilato expressa em sementes oleaginosas durante o processo de germinação, que juntamente com outras vias metabólicas (β-oxidação e a gliconeogênese), permite a conversão de ácidos graxos em moléculas de glicose, que são fundamentais para o embrião em desenvolvimento. Além de desempenhar este papel central na conversão de reservas lipídicas em carboidratos por meio de sua função anaplerótica, esta enzima não é expressa em mamíferos e outros vertebrados, o que a torna uma candidata promissora como alvo molecular herbicida. Desta maneira, o objetivo do presente estudo foi o de modelar a estrutura tridimensional da ICL de *Arabidopsis thaliana* por meio de ferramentas de bioquímica computacional no intuito de utilizá-la como alvo em estudos de virtual *screening* (*in silico*), e posteriormente analisá-la como alvo molecular herbicida por meio de análises *in vitro* e *in vivo* com os ligantes prospectados sobre diversas espécies de plantas daninhas, com a posterior análise dos seus modos de ação. Para isso, a sequência de aminoácidos da ICL de *Arabidopsis thaliana* (Uniprot id: P28297) foi utilizada para pesquisar padrões estruturais por meio de um BLASTp contra o banco de dados do Protein Data Bank. Após as etapas de modelagem e minimização, a estrutura quartenária final da ICL de *A. thaliana* (ArThaICL) ligada ao seu substrato glioxilato e ao cofator magnésio foi então utilizada em simulações de *redocking* por meio dos programas Vina rodado na interface gráfica PyRx e Molegro-6.1. A média de rmsd obtida para a reprodução da pose do isocitrato pelos dois algoritmos foi de 0,571 Å, o que validou o modelo desenvolvido para a etapa de *docking* molecular. As bibliotecas de compostos analisados foram constituídas pelos inibidores conhecidos da ICL descritos no Brenda Database e pela biblioteca da SigmaAldrich obtida em 2020. Dentre os ligantes encontrados no virtual *screening*, o itaconato e o tartarato obtiveram bons *scores* e foram selecionados para testes *in vitro* e *in vivo* sobre espécies com diferentes graus de reservas lipídicas, sendo elas: *Arabidopsis thaliana*, *Sesamum indicum*, *Amaranthus hybridus*, *Bidens pilosa*, *Euphorbia heterophylla*, *Ipomoea grandifolia* e *Urochloa decumbens*. Embora o tartarato não tenha causado efeitos sobre nenhum dos parâmetros analisados, o itaconato, inicialmente pesquisado como antiparasitário em modelo animal, causou alterações diferenciais significativas sobre os parâmetros de crescimento inicial, com uma notável correlação entre inibição no comprimento das plântulas e dependência de reservas lipídicas. As espécies *A. thaliana* e *S. indicum* foram as mais afetadas pelo itaconato, com inibições de 81,4 e 68,7%, respectivamente, nos seus comprimentos radiculares, na concentração de 1 mM. As espécies *E. heterophylla* e *B. pilosa* apresentaram graus intermediários de inibição com reduções de 27,5 e 38,1%, respectivamente. As espécies restantes, menos dependentes de reservas lipídicas, não apresentaram inibições nos parâmetros de crescimento inicial, o que é um forte indício da atuação deste composto sobre o ciclo do glioxilato. A avaliação dos efeitos do itaconato sobre as atividades *in vitro* das ICL extraídas dos cotilédones de *S. indicum* e *E. heterophylla* demostrou os efeitos diretos deste composto por meio de inibições dose-dependentes, com valores de IC_{25} (concentração de inibição) e IC_{50} de 23,49 e 81,74 µM, para a ICL de *S. indicum*, respectivamente, e de 23,42 e 67,19 µM para a ICL de *E. heterophylla*, respectivamente. Posteriormente, a espécie *E. heterophylla* foi escolhida para análises adicionais, onde buscou-se compreender os modos de ação do itaconato sobre parâmetros oxidativos e energéticos do seu metabolismo. Inicialmente, as atividades das ICL extraídas dos cotilédones de *E. heterophylla*, incubados na presença de itaconato 1 mM, foram avaliadas em intervalos

de 24 horas até o período final de 120 horas, onde constatou-se inibição da enzima em todos os intervalos testados, o que indica que mesmo sob condições *in vivo* este tratamento possui a capacidade de atuar sobre o seu alvo molecular em seu compartimento. Posteriormente, foram avaliadas as atividades de enzimas da via glicolítica, da via das pentoses fosfato e do ciclo do ácido cítrico extraídas de cotilédones e raízes de plântulas crescidas na presença de itaconato 1 mM. Foram observadas inibições de 56,5 e 56,7%, para a glicoquinase (GK) de cotilédones e raízes, respectivamente, 66,3%, para a glicose-6-fosfato desidrogenase (G6PDH) de cotilédones, 32,5% para a piruvato quinase (PK) de raízes, e 35,7% para a malato desidrogenase (MDH) de cotilédones. Ainda, a atividade da enzima fosfoenolpiruvato desidrogenase (PEPCK) de cotilédones também apresentou a notável inibição de 60,6%, quando tratadas com itaconato 1 mM nestas mesmas condições. Por outro lado, enzimas do sistema de defesa antioxidante extraídas dos cotilédones de *E. heterophylla*, testadas sob a mesmas condições, não apresentaram nenhuma alteração significativa, embora uma redução de 21,2% no conteúdo de ROS neste mesmo tecido tenha sido observada. Finalmente, a respiração de ápices radiculares foi inibida de maneira dose-dependente (concentrações de itaconato: 0.25 – 1 mM), com inibições de 36,9 e 48,5% das respirações total e KCN-sensível, respectivamente, pela concentração de itaconato 1 mM, e valores de IC_{25} de 251,1 e 243,3 µM, respectivamente. Assim, as alterações metabólicas causadas pelo itaconato foram consequências de sua atuação primária sobre o ciclo do glioxilato, que gerou escassez de substratos para a gliconeogênese, inibindo-a nos cotilédones, o que consequentemente reduziu a mobilização de açúcares (na forma de sacarose) para os meristemas radiculares. A escassez de carboidratos reduziu também a eficiência da respiração celular, gerando assim um *deficit* energético e escassez de precursores importantes para os processos biossintéticos, com a consequente inibição sobre o desenvolvimento das plântulas de *E. heterophylla*. O conjunto dos resultados obtidos permite concluir que a ICL desempenha um papel central durante a mobilização das reservas lipídicas e a inibição dessa enzima reduz drasticamente o crescimento inicial das plântulas, por meio de uma deficiência de glicose em um momento crítico do seu desenvolvimento. Esta inibição no desenvolvimento inicial pode reduzir consideravelmente a capacidade das plantas daninhas, dependentes de reservas lipídicas de competirem com as culturas de interesse.

Palavras-chave: Ciclo do glioxilato, plantas daninhas, germinação, modos de ação

GENERAL ABSTRACT

Isocitrate lyase (ICL, EC: 4.1.3.1) is a key enzyme of the glyoxylate cycle expressed in oilseeds during the germination process, which, together with other metabolic pathways (β-oxidation and gluconeogenesis), allows the conversion of fatty acids into glucose molecules, which are critical for the developing embryo. In addition to playing this central role in the conversion of lipid stores into carbohydrates through its anaplerotic function, this enzyme is not expressed in mammals and other vertebrates, which makes it a promising candidate as an herbicide molecular target. Thus, the aim of the present study was to model the three-dimensional structure of the ICL of *Arabidopsis thaliana* using computational biochemistry tools in order to use it as a target in virtual screening studies (*in silico*), and later analyze it as an herbicide molecular target through *in vitro* and *in vivo* analyzes with the prospected ligands on several weed species, with the subsequent analysis of their modes of action. For this, the amino acid sequence of the *Arabidopsis thaliana* ICL (Uniprot id: P28297) was used to search for structural patterns through a BLASTp against the Protein Data Bank database. After the modeling and minimization steps, the final quaternary structure of the *A. thaliana* ICL (ArThaICL) linked to its glyoxylate substrate and to the magnesium cofactor was then used in redocking simulations using the Vina programs running on the PyRx and Molegro- 6.1. The average rmsd obtained for the reproduction of the isocitrate pose by the two algorithms was 0.571 Å, which validated the model developed for the molecular docking step. The libraries of compounds analyzed were constituted by the known inhibitors of ICL described in the Brenda Database and by the SigmaAldrich library obtained in 2020. Among the ligands found in the virtual screening, itaconate and tartrate obtained good scores and were selected for *in vitro* and *in vivo* tests on species with different degrees of lipid reserves, namely: *Arabidopsis thaliana*, *Sesamum indicum*, *Amaranthus hybridus*, *Bidens pilosa*, *Euphorbia heterophylla*, *Ipomoea grandifolia* and *Urochloa decumbens*. Although tartrate had no effect on any of the parameters analyzed, itaconate, initially researched as an antiparasitic in an animal model, caused significant differential changes on initial growth parameters, with a remarkable correlation between inhibition in seedling length and dependence of lipid reserves. The species *A. thaliana* and *S. indicum* were the most affected by itaconate, with inhibitions of 81.4 and 68.7%, respectively, in their root lengths, at a concentration of 1 mM. The species *E. heterophylla* and *B. pilosa* showed intermediate degrees of inhibition with reductions of 27.5 and 38.1%, respectively. The remaining species, less dependent on lipid reserves, did not show inhibition in the initial growth parameters, which is a strong indication of the action of this compound on the glyoxylate cycle. The evaluation of the effects of itaconate on the *in vitro* activities of the ICL extracted from the cotyledons of *S. indicum* and *E. heterophylla* demonstrated the direct effects of this compound through dose-dependent inhibitions, with values of IC_{25} (inhibition concentration) and IC_{50} of 23.49 and 81.74 µM, for the ICL of *S. indicum*, respectively, and 23.42 and 67.19 µM for the ICL of *E. heterophylla*, respectively. Subsequently, the species *E. heterophylla* was chosen for further analysis, which sought to understand the modes of action of itaconate on oxidative and energetic parameters of its metabolism. Initially, the activities of the ICL extracted from the cotyledons of *E. heterophylla*, incubated in the presence of 1 mM itaconate, were evaluated at 24-hour intervals until the final period of 120 hours, when enzyme inhibition was observed at all tested intervals, which indicates that even under *in vivo* conditions this treatment has the ability to act on its molecular target in its compartment. Subsequently, the activities of

enzymes of the glycolytic pathway, the pentose phosphate pathway and the citric acid cycle extracted from cotyledons and roots of seedlings grown in the presence of 1 mM itaconate were evaluated. Inhibitions of 56.5 and 56.7% were observed for glucokinase (GK) from cotyledons and roots, respectively, 66.3%, for glucose-6-phosphate dehydrogenase (G6PDH) from cotyledons, 32.5% for pyruvate kinase (PK) from roots, and 35.7% for malate dehydrogenase (MDH) from cotyledons. Furthermore, the activity of the enzyme phosphoenolpyruvate dehydrogenase (PEPCK) of cotyledons also showed a remarkable inhibition of 60.6%, when treated with 1 mM itaconate under these same conditions. On the other hand, enzymes of the antioxidant defense system extracted from the cotyledons of *E. heterophylla*, tested under the same conditions, did not show any significant change, although a reduction of 21.2% in the ROS content in this same tissue was observed. Finally, root apexes respiration was inhibited in a dose-dependent manner (itaconate concentrations: 0.25 – 1 mM), with inhibitions of 36.9 and 48.5% of total and KCN-sensitive respirations, respectively, by the concentration of 1 mM itaconate, and IC_{25} values of 251.1 and 243.3 µM, respectively. Thus, the metabolic alterations caused by itaconate were consequences of its primary action on the glyoxylate cycle, which generated a shortage of substrates for gluconeogenesis, inhibiting it in the cotyledons, which consequently reduced the mobilization of sugars (in the form of sucrose) to the root meristems. The scarcity of carbohydrates also reduced the efficiency of cellular respiration, thus generating an energy deficit and scarcity of important precursors for biosynthetic processes, with the consequent inhibition of the development of *E. heterophylla* seedlings. The set of results obtained allows us to conclude that ICL plays a central role during the mobilization of lipid reserves and the inhibition of this enzyme drastically reduces the initial growth of seedlings, through a glucose deficiency at a critical moment in their development. This inhibition of early development can considerably reduce the ability of weeds, dependent on lipid reserves, to compete with the crops.

Keywords: Glyoxylate cycle, weeds, germination, modes of action

CHAPTER 1

Isocitrate lyase as a molecular target for weed suppression: *in silico*, *in vivo* and *in vitro* studies

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Abbreviations

ABA	Abscisic acid	**G6PDH**	Glucose-6-phosphate dehydrogenase
ACCase	Acetyl-coenzyme A carboxylase	**GA**	Gibberellic acid
ALS	Acetolactase	**GK**	Glucokinase
ArThaICL	*Arabidopsis thaliana* ICL model	**HDS**	Honestly Significant Difference
CG	Conjugate gradient	**ICL**	Isocitrate lyase
DENs	Phenylpyrazolin	**MDH**	Malate dehydrogenase
DIMs	Cyclohexanedione	**MOA**	Mechanism of action
EPSP	5-enolpyruvyl-shikimate-3-phosphate	**PEPCK**	Phosphoenolpyruvate carboxykinase
FOPs	Aryloxyphenoxypropionate	**PK**	Pyruvate kinase

Abstract

Traditional herbicides suppress weeds through various mechanisms of action (MOA). Since 1980, no new MOA has been introduced to the market, and the growing number of resistant weed biotypes emphasizes the importance of finding new herbicides with different molecular targets. Isocitrate lyase (ICL) is a key enzyme in the glyoxylate cycle that plays a central role in the mobilization of seed lipid reserves, allowing the conversion of fatty acids into sugars, essential for the embryo during its initial development. The present study aimed to investigate ICL as a potential herbicide target through *in silico* and biological approaches. A three-dimensional model of ICL was developed and some potential ligands were screened by molecular docking simulations. Among them, itaconate and tartrate were selected for biological assays, evaluating their effects in a concentration range of 50-1000 µM on the ICL activity and on germination and growth of *Arabidopsis thaliana*, *Bidens pilosa*, *Sesamum indicum*, *Ipomoea grandifolia*, *Amaranthus hybridus*, *Euphorbia heterophylla* and *Urochloa decumbens*. Itaconate had a species-specific effect, inhibiting the initial growth of seedlings that are more dependent on lipid reserves. The activity of ICL extracted from *E. heterophylla* and *S. indicum* cotyledons was inhibited by itaconate, but not by tartrate. Our results supported the modeling of a selected enzyme structure and the virtual screening of ligands through bioinformatics approaches as a promising tool in the search for new molecular targets and compounds as alternatives to traditional herbicides. In addition, the choice of ICL as a molecular target seems to be very promising, since inhibitors of this enzyme can reduce the initial development, fitness and the characteristic aggressiveness of weeds during seedling establishment with selectivity for species not dependent on lipid reserves.

Keywords: Glyoxylate cycle, bioinformatics, herbicides, itaconate

1. Introduction

Weeds are known for their ability to negatively impact the yield of crops due to competition for natural resources in the environment, such as nutrients, moisture and light, which can cause an estimated loss of productivity till 34% (Oerke, 2006). Herbicides provide effective and economical weed control and are the primary method in agronomic crops, replacing manual labor, animals and mechanical weed control (Heap, 2014).

Traditional herbicides are able to suppress weeds through several known mechanisms of action (MOA), generally inhibiting vital processes in the plant metabolism, including amino acids and lipid biosynthesis (Duke, 1990). Within the class of amino acids inhibitors, there are broad-spectrum herbicides such as glyphosate, an inhibitor of 5-enolpyruvyl-shikimate-3-phospate synthase (EPSP synthase), a key enzyme of the shikimate pathway, responsible for the aromatic amino acids biosynthesis (Duke, 1990). Still, another very important class of herbicides are the inhibitors of acetolactate synthase (ALS), a key enzyme of the branched amino acids biosynthesis pathway (Duke, 1990). In the case of herbicides that inhibit lipid synthesis, the molecular target is acetyl-coenzyme A carboxylase (ACCase), an important enzyme involved in fatty acid biosynthesis process (Devine, 1997), and among the inhibitors that exert their effects through this MOA are the aryloxyphenoxypropionate (FOPs), cyclohexanedione (DIMs), and phenylpyrazolin (DENs) (Yu et al., 2010). The ACCase inhibitors are used selectively in dicotyledonous (dicot) crops species to control grass (monocot) weeds species in the fields: this selectivity is based on the different sensitivity of ACCase from monocot or dicot species to these herbicides (Burton et al., 1989; Konishi and Sasaki, 1994).

Currently, a problem that has been worrying agronomists and researchers worldwide is the staggering increase in the prevalence of weed biotypes resistant to traditional herbicides, including those used in transgenic crops such as the glyphosate (Baek et al., 2021; Westwood et al., 2018). This has leading to great efforts in research and studies to find substances with different MOA (Westwood et al., 2018), however, since 1980, no new herbicide with different MOA has been introduced into the market (Duke, 2012) and the growing trend of new herbicide-resistant weed biotypes threatens to make almost all existing herbicides unusable by 2050. In addition to this difficulty in

the control of weeds, the concomitant increase in the world population, which is expected to reach 9 billion people on that date, means that the world demand for food is needed to increase significantly (Westwood et al., 2018).

In fact, the weed science discipline is experiencing right now one of its most critical and challenging moments. Resolutions in computing power and automation, along with the integration of old and new weed management technologies, could provide integrated weed management and resistance management strategies that will be more sustainable than the technologies that are now failing (Westwood et al., 2018). Within this perspective, the use of substances with new molecular targets, capable of precisely controlling specific weed species, can contribute to solutions to the challenges imposed in the coming years.

The discovery of inhibitors of key enzymes of fundamental metabolic pathways, such as carbon assimilation, amino acid biosynthesis, sulfur assimilation, lignification and germination can be accomplished by modeling of the three-dimensional structure of selected enzyme and prospection of inhibitors through bioinformatics tools. Among the candidate enzymes that act in the germination process, isocitrate lyase (ICL) stands out, a key enzyme in the glyoxylate cycle that plays a central role in the mobilization of lipid reserves during the germination of oil seeds, allowing the conversion of fatty acids into sugars, essential for the embryo during its initial development (Graham, 2008). In addition to playing an important role during a critical stage of the life cycle of weed species dependent on lipid reserves, this enzyme has some desirable characteristics, such as not being expressed by mammals, which drastically reduces the chances of inhibitors of this enzyme being toxic to humans (Graham, 2008). In order to obtain inhibitory substances for this enzyme, in the present work *in silico* approaches were carried out through the development of three-dimensional modeling of ICL structure and molecular docking analysis. *In vitro* and *in vivo* assays were also performed to evaluate the effects of selected inhibitors on the germination and initial development of several weed species with different degrees of dependence on lipid reserves.

2. Material and Methods

2.1. Reagents and plant material

DL-isocitrate, ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF) and itaconic acid were purchased commercially from Sigma Chemical Co. (St. Louis, USA). The other reagents purchased were of the highest purity available. Seeds of *B. pilosa*, *I. grandifolia*, *A. hybridus*, *U. decumbens* and *E. heterophylla* were purchased from Cosmos Agrícola Produtos e Serviços Rurais LTDA-Brazil. Seeds of *A. thaliana* and *S. indicum* were obtained by donation.

2.2. Modeling the structure of Arabidopsis thaliana ICL bound to isocitrate and Mg^{2+} cofactor

The amino acid sequence of isocitrate lyase (ICL) from *Arabidopsis thaliana* (Uniprot id: P28297) was used for the present study. For the structure modeling process, the sequence was used to search for structural templates through a BLASTp against the Protein Data Bank database. The structure of the *Aspergillus nidulans* ICL (pdb id: 1dqu) resolved at 2.80 Å resolution (Britton et al., 2000) was used as the main template of the amino acid sequence, and the structure of the *Magnaporthe oryzae* ICL (pdb id: 5e9g) resolved at 2.10 Å resolution (Park et al., 2016) was used as a secondary template for the ligands glyoxylate, glycerol, Mg^{2+}, structural waters, and also to cover gaps in the alignment of the main template sequence. A total of 1,000 models of the quaternary structure of the *Arabidopsis thaliana* ICL (ArThaICL) bonded to the glyoxylate ligand and the magnesium cofactor were generated using the Modeller program (Webb and Sali, 2016), where the best model was chosen based on the stereochemical evaluation by the Procheck program (Collaborative Computational Project N 4, 1994). The final modeled structure was used in docking simulation, using the Vina program (Trott and Olson, 2010) to choose the best pose of the isocitrate substrate, using the program's default settings.

The final modeled structure had the glyoxylate and glycerol ligands replaced by the best pose of the isocitrate ligand (CID 5459771) and then the entire complex was subjected to energy minimization simulations to maximize the interactions between protein residues with the isocitrate ligand. For this, the NAMD/VMD program package (Humphrey et al., 1996; Phillips et al., 2005) and the Chamm36 force field (Mackerell et al., 2004) were used. The isocitrate ligand force field was generated by the SwissParam server (Zoete et al., 2011), in the same format, where the minimization

occurred in stages. Initially the entire system was virtually immersed in a periodic box with TIP3 water whose margins were at least 10 Å away from the outermost surface of the molecule. Sufficient amounts of Na^+ ions were added to neutralize system charges. In the first step of minimization, the isocitrate ligand was kept fixed in space and the coordinates of the protein, water, salts and cofactor were submitted to 20,000 steps of minimization by conjugate gradient (CG). In the second step, all atoms were minimized by another 10,000 steps of CG. The complex structure resulting from this second stage was used in the redocking and virtual screening simulations.

2.3. Virtual screening

The final minimized structure of ArThaICL bounded to isocitrate substrate and Mg^{2+} cofactor was used as a target for virtual screening simulations. The choosing of programs that were used in the screening occurred by the isocitrate substrate redocking in the minimized complex. The Vina programs (Trott and Olson, 2010) running on the PyRx (Dallakyan and Olson, 2015) graphical interface and Molegro-6.1 (Thomsen and Christensen, 2006) were both able to reproduce the isocitrate pose, being considered validated for the virtual screening. The Vina program used the standard search and ranking algorithm with a search box with dimensions of 10, 10 and 10 Å in the x, y and z axes, centered on the ligand. The Molegro program used the algorithms Iterated Simplex (Ant colony Optimization) for search and PLATS Score for ranking, with a search radius of 7.0 Å centered on the isocitrate ligand. Screening scores were ordered by best Rerank score. The library of compounds analyzed was formed by the known ICL inhibitors described in the Brenda database (Schomburg et al., 2012), plus the library from SigmaAldrich building blocks obtained in January/2020. The screening was initially carried out with the Vina program and the ligands with the best scores in relation to isocitrate were grouped in a folder called best-results, which were used for four new screenings as a kind of validation. The best-results were also evaluated four times with the Molegro program where the ligands that were repeated in all eight simulations were visually inspected in the protein complex, selected and purchased for *in vitro* studies. Among the ligands found in the virtual screening, itaconate was chosen to be used as the ICL inhibitor in the later stages of this study.

2.4. Seed germination and seedling initial growth

Seeds of *A. thaliana*, *B. pilosa*, *S. indicum*, *I. grandifolia*, *A. hybridus*, *E. heterophylla* and *U. decumbens* had been previously selected for size and appearance. The seeds of *I. grandifolia* and *U. decumbens* were scarified with sulfuric acid for 45 and 15 minutes, respectively, to break seed dormancy. After washing with distilled water, seeds were placed in plastic boxes (Gerbox® 110 × 110 × 50 mm) containing 40 mL of semi-solid agar 0.8% (w/v) with itaconate at 0, 50, 100, 500 and 1000 μM concentrations and tartrate at 0 or 1000 μM concentrations. Itaconate and tartrate were dissolved in the agar medium before its solidification. Each treatment, including controls, was replicated five times, and each replicate consisted of 50 seeds, except in the case of *A. thaliana*, where each replicate consisted of 16 seeds. Boxes were randomly placed in a germination chamber (photon flux density of approximately 230 μmol m^{-2} s^{-1}). The *A. thaliana* seeds were maintained at a constant temperature of 20°C, with a 12-h photoperiod (light/dark); *S. indicum* and *E. heterophylla* seeds were maintained at a constant temperature of 25°C, with a 12-h photoperiod (light/dark); *B. pilosa*, *A. hybridus* and *U. decumbens* were maintained in photoperiod to 8/16 h (light/dark) at 30°C and 20°C, respectively; and *I. grandifolia* was maintained at a constant temperature of 30°C, with a 12-h photoperiod (light/dark). At the end of treatment (120 hours), roots and aerial parts of seedlings were excised for measurements of their lengths and fresh weights. Then, plant materials were placed into the oven for 48 h at 60°C, to determine their dry weight. The germinated seeds were counted daily until 120 h of treatment for calculations of mean germination time (*t*), speed of germination (*S*), and speed of accumulated germination (S_A) (Chiapusio et al., 1997; Labouriau and Osborn, 1984). The mean germination time was calculated by equation 1:

$$t = \sum ni\ ti \sum ni \quad (1)$$

t are the mean germination time, and *ni* is the number of germinated seeds between the times ti_{-1} and *ti*.

The speed of germination was calculated by equation 2:

$$S = (N1/T1) + (N2 - N1) \times 1/2 + (N3 - N2) \times 1/3 + \ldots (Nn - Nn_{-1}) \times 1/n \quad (2)$$

S represents the speed of germination and *N* represents the proportion of germinated seeds obtained in the first (*T1*), second (*T2*), third (*T3*), … , (n_{-1}) hours.
The speed of accumulated germination was calculated by equation 3:

$$S_A = (N1/T1) + (N2/T2) + (N3/T3) + ... (Nn/Tn) \quad (3)$$

S_A represents the speed of accumulated germination and *N* represents the proportion of germinated seeds at time (*T1*), time (*T2*), time (*T3*), ⋯(*Tn*), hours.

2.5. Extraction and determination of isocitrate lyase activity

The enzymatic extracts were obtained according to Khan and McFadden (1979), with modifications. For extraction, 25-100 mg of cotyledons or imbibed seeds when cotyledons were not easily accessed, were macerated in a cold mortar, with 1 mL of a medium containing 50 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF. Then, the material was centrifuged at 15,000 × *g* for 20 minutes, at 4°C. The supernatant was used as source of the enzymes. The ICL activity test was performed according to Roche et al. (1970), with modifications. The reaction was carried out by using 50 µl of extract and 700 µl of a reaction medium, containing 70 mM potassium phosphate buffer (pH 7.6), 5 mM $MgCl_2$, 1.8 mM DTT and 4.25 mM DL-isocitrate. The reaction was incubated for 15 minutes at 30°C. After 15 minutes, the reaction was stopped with 150 µl of 10% TCA and the proteins were precipitated with centrifugation at 5,000 × *g*, for 10 minutes at 4°C. A 500 µl aliquot of the supernatant was diluted in an identical volume of distilled water. In each tube, 25 µl of 5% phenylhydrazine hydrochloride was added, shaken vigorously and incubated for 1 minute in a water bath at 100ºC. Then, 500 µl of 2 N HCl were added to the tubes, and after 5 minutes, 25 µl aliquots of 25% potassium ferricyanide were added. The tubes were again vigorously shaken for complete homogenization and, after 15 minutes, the concentration of the glyoxylate formed was read at 520 nm in a spectrophotometer (ε = 5.3 10^4 M^{-1} cm^{-1}). The enzyme activity was expressed in nmols $minute^{-1}$ mg^{-1} of protein.

2.6. Determination of the activity of isocitrate lyase during the germination process, and evaluation of ICL inhibitors on the enzyme activity

The activity of isocitrate lyase was measured in extracts of untreated seedlings, grown at 24 hours intervals until the end of 120 hours, to determine the time period in which the maximum (peak) activity of the enzyme was reached in each of the species tested. Once the peak time was determined, this time was selected to obtain the enzymatic extracts that were used for evaluating of the direct effects of itaconate and tartrate in the concentration range of 10, 25, 50, 100, 500 and 1000 µM. Inhibitors were added to reaction medium before measurements of enzyme activity. The assays followed the same methodology described above, and the enzyme activity was expressed in nmols $minute^{-1}$ mg^{-1} of protein.

Statistical analysis

The data presented in the graphs were expressed as mean ± standard errors (S.E.) of independent preparations; these were analyzed by analysis of variance (ANOVA), with significant differences between the means identified by Tukey's Honestly Significant Difference (HSD) test ($p \leq 0.05$) using RStudio software. The IC_{50} and IC_{25} concentrations were calculated by numerical interpolation using the point-to-point function of GraphPad Prism 5 software.

3. Results and discussion

3.1. Structural model of ArThaICL validation and in silico results

The identity between the sequences of the ICL (target protein) of *A. thaliana* with that of the template protein of *Aspergillus nidulans* was 52.8%, considered sufficient for modeling by homology (Fig. S1). The best model presented 99% of the residues in the allowed regions of the Ramachandran plot (Fig. S2), presenting a stereochemical quality that allowed its use in virtual screening studies. Figures 1A and 1B shows the final structure of the minimized complex, whose one chain (Fig. 1C) was

used in the virtual screening studies, as well as the main interactions of the active site residues with the isocitrate substrate (Fig. 2).

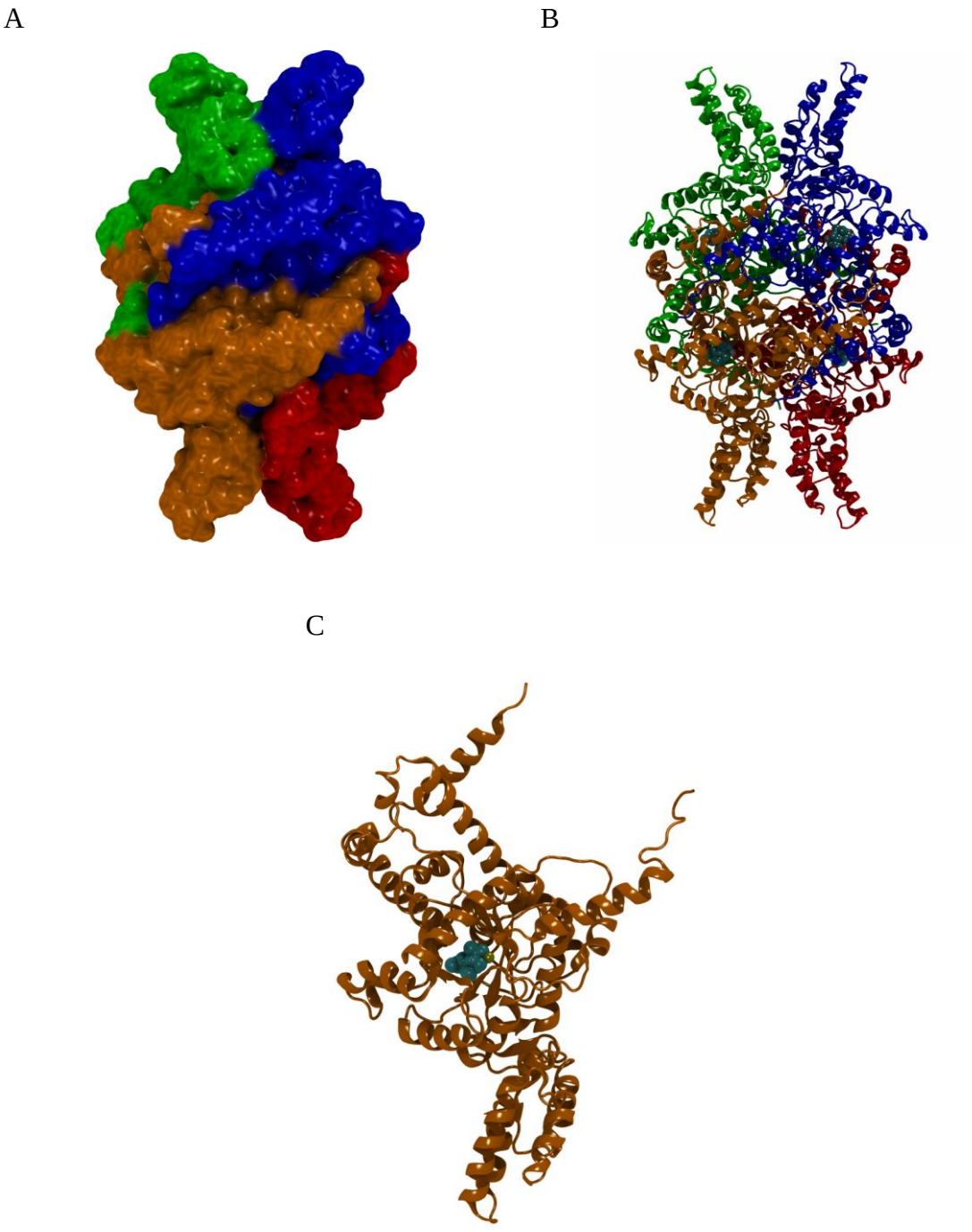


Figure 1 - Representations of the ArThaICL structure. Representation highlighting the quaternary arrangement of the four chains (A). Quaternary ribbon structure highlighting the four chains and the isocitrate (cyan) and magnesium cofactor (yellow) ligands (B). Single chain ribbon structure used in the docking simulations, showing the isocitrate in cyan and the magnesium cofactor in yellow (C).

The selection of the most suitable programs to perform the virtual screening simulations was made by redocking the isocitrate ligand in the modeled complex. The Vina and Molegro programs were able to reproduce the modeled pose of isocitrate with a rmsd average of 0.571 Å, being then selected for the virtual screening step (Fig. 3A, B). The scores found for isocitrate (substrate) (Fig. 4A) with the programs used in redocking are shown in Table 1. For the virtual screening, two libraries were used. The first was formed by known ICL inhibitors available in the Brenda Enzymes database (Schomburg et al., 2012), consisting of 94 compounds. The second library called Sigma-Aldrich Building Blocks was obtained from the Zinc15 database (Sterling and Irwin, 2015) and contained 78,000 molecules. The scores of some ligands found in the simulations were highlighted in table 1: homoisocitrate, tartrate and itaconate, respectively (Fig. 4C, E, G). It is important to note that all these ligands have very similar structures to isocitrate (Fig. 4A), having basically the same chemical groups, such as carboxylic acids, carbonyls, methylenes and hydroxyls, and small and flexible carbon skeletons having between 4 to 7 carbons atoms, which may explain the similar interactions that these organic acids undergo by interacting with the catalytic groups at the ArThaICL site, as well as the original substrate (Fig. 2).

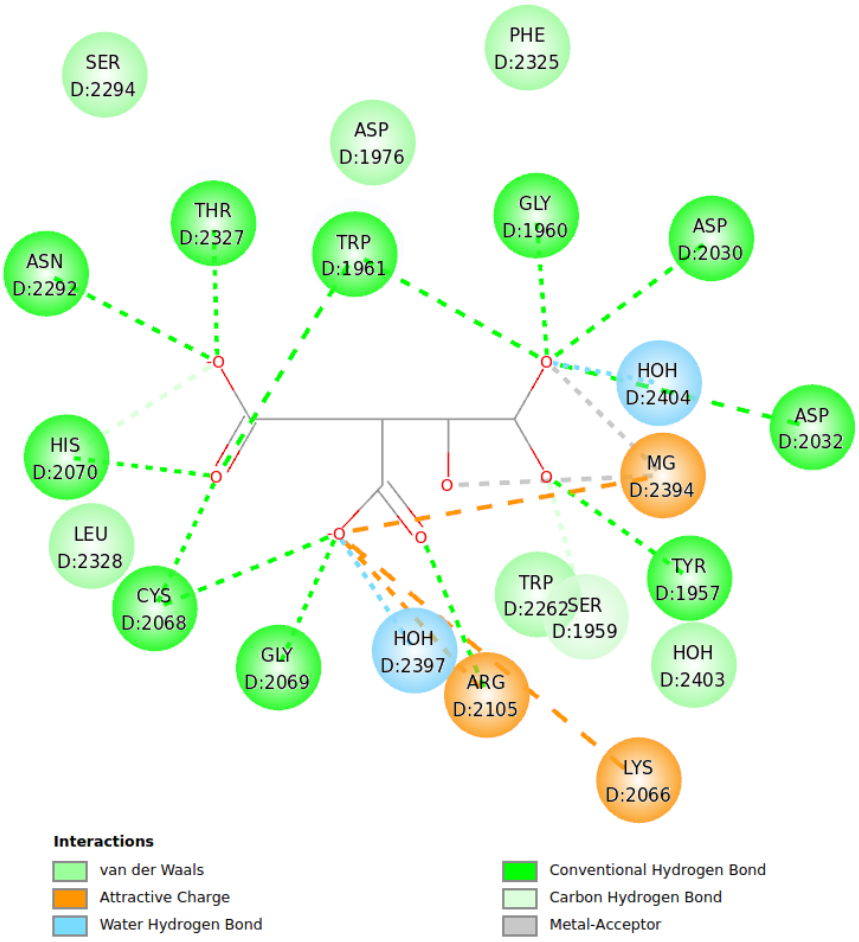


Figure 2 – Map of interactions between isocitrate substrate and ArThaICL residues in the active site. Generated by Discovery Studio program (Biovia DS, 2020)

A B

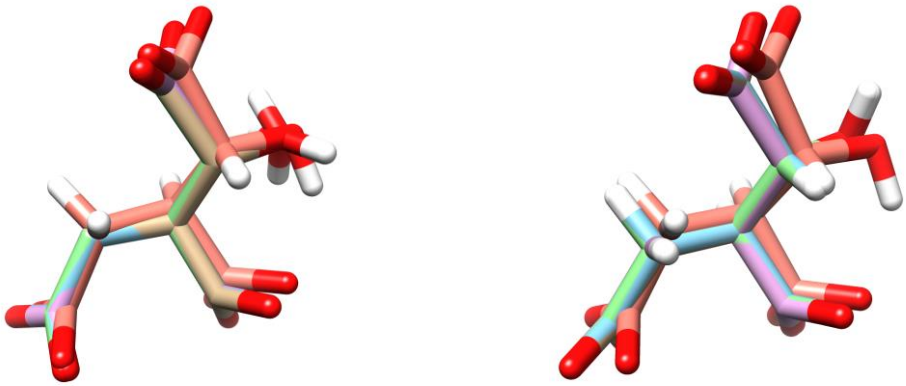


Figure 3 – Superposition of the four poses found with the redocking of the isocitrate ligand compared to the pose of the modeled (reference) ligand in dark pink. Poses found with the Vina program (A) and poses found with the Molegro program (B).

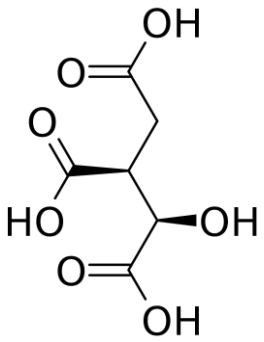
Table 1 – Average scores found in the redocking of the reference ligand isocitrate with the two programs selected in this study.

	Vina program	Molegro program
Isocitrate	-7.50 ± 0.00	-106.5 ± 0.50
Homoisocitrate	-7.10 ± 0.00	-108.43 ± 0.08
Tartrate	-7.00 ± 0.00	-80.83 ± 1.79
Itaconate	-6.15 ± 0.05	-79.32 ± 0.23

Values are means of score ± SE (n=4 for isocitrate, n=2 for homoisocitrate, tartrate and itaconate).

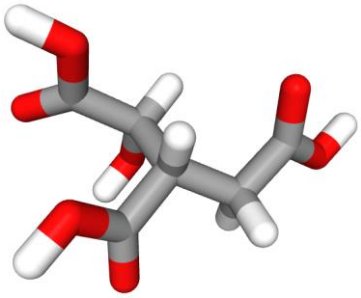
Despite having obtained the lowest score among the 3 ligands, itaconate has been previously described as a potent ICL inhibitor (Khan and McFadden, 1979; McFadden and Purohit, 1977; Runquist and Kruger, 1999; Vincenzini et al., 1986). Furthermore, the fact that it has desirable characteristics such as low toxicity, low cost, proven efficacy and high solubility were taken into account to select it in the present study to investigate whether ICL enzyme as a molecular target is effective to inhibit the initial development of weeds. Therefore, most of the subsequent assays of this study were carried out using itaconate, and some experiments were performed with tartrate.

A

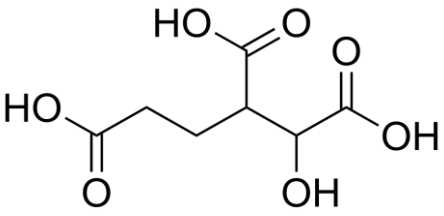


Isocitric acid

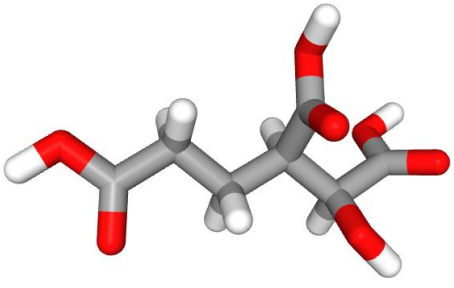
B



C

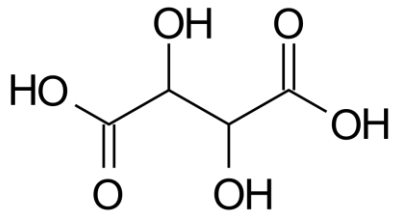


D

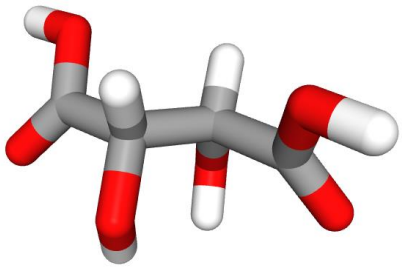


Homoisocitric acid

E

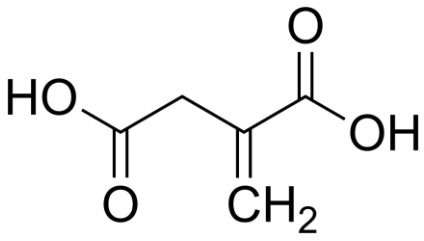


F

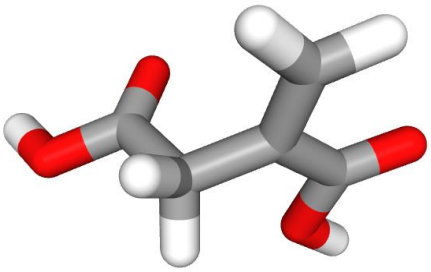


Tartaric acid

G



H



Itaconic acid

Figure 4 – Representation of the 2D and 3D sticks chemical structures of isocitric acid (A, B), homoisocitric acid (C, D), tartaric acid (E, F) and itaconic acid (G, H). At physiological pH there is a predominance of its conjugated base, isocitrate, homoisocitrate, tartrate and itaconate, respectively.

3.2. Effects of itaconate on the germination and initial growth of A. thaliana, B. pilosa, S. indicum, I. grandifolia, A. hybridus, E. heterophylla and U. decumbens

The following species were selected for *in vivo* and *in vitro* studies: *A. thaliana*, considering that the ICL amino acid sequence of this species was used for the virtual screening of inhibitors; *Sesamum indicum* for being a culture known for the high lipid content of its seeds; the eudicots weeds species *A. hybridus*, *B. pilosa*, *E. heterophylla* and *I. grandifolia*, and the monocot weed species *U. decumbens.*

Table 2 shows that itaconate in the concentration range from 50 to 1000 µM does not affect the germination of the different species tested, since none of the germination indices was significantly modified. However, the growth of resulting seedlings was altered (Figures 5 and 6) with changes in the lengths and weights of the

seedlings that varied from inhibition or stimulus, in addition to no alterations in some species.

Table 2 – Germination percentage (G%), speed of germination (*S*), speed of accumulated germination (S_A), and mean germination time (*t*) of *A. thaliana*, *S. indicum*, *A. hybridus*, *B. pilosa*, *E. heterophylla*, I. *grandifolia* and *U. decumbens* grown for 24–120 hours, treated with 0 (control), 50, 100, 500 and 1000 µM of itaconate.

Weed species	Itaconate (µM)	(G%)	($S^{¥}$)	($S_A^{¥}$)	(*t*)
A. thaliana	0	88.75 ± 5.00 a	0.059 ± 0.004 a	0.52 ± 0.03 a	70.4 ± 1.50 a
	50	87.5 ± 1.97 a	0.063 ± 0.002 a	0.50 ± 0.03 a	69.8 ± 2.26 a
	100	83.75 ± 1.53 a	0.057 ± 0.02 a	0.49 ± 0.02 a	69.6 ± 0.35 a
	500	87.50 ± 1.98 a	0.055 ± 0.001 a	0.48 ± 0.01 a	71.3 ± 0.84 a
	1000	83.75 ± 4.68 a	0.051 ± 0.004 a	0.47 ± 0.03 a	71.9 ± 2.45 a
S. indicum	0	87.6 ± 2.71 a	0.91 ± 0.02 a	3.84 ± 0.10 a	26.9 ± 0.61 a
	50	89.2 ± 1.20 a	0.89 ± 0.02 a	3.77 ± 0.05 a	26.9 ± 0.93 a
	100	89.2 ± 1.50 a	0.87 ± 0.02 a	3.70 ± 0.08 a	27.5 ± 0.81 a
	500	91.2 ± 1.50 a	0.87 ± 0.01 a	3.72 ± 0.05 a	27.0 ± 0.44 a
	1000	92.8 ± 2.50 a	0.90 ± 0.02 a	3.86 ± 0.09 a	27.0 ± 0.58 a
A. hybridus	0	82.4 ± 5.31 a	0.39 ± 0.04 a	2.54 ± 0.18 a	46.0 ± 1.12 a
	50	82.0 ± 0.89 a	0.32 ± 0.03 a	2.26 ± 0.14 a	48.9 ± 1.00 a
	100	85.2 ± 4.08 a	0.42 ± 0.01 a	2.77 ± 0.04 a	44.9 ± 0.67 a
	500	85.6 ± 3.65 a	0.36 ± 0.03 a	2.43 ± 0.17 a	45.7 ± 1.10 a
	1000	84.8 ± 3.44 a	0.42 ± 0.03 a	2.69 ± 0.11 a	45.6 ± 1.02 a
B. pilosa	0	67.6 ± 2.04 a	0.25 ± 0.01 a	1.46 ± 0.05 a	53.0 ± 1.07 a
	50	61.6 ± 2.79 a	0.24 ± 0.01 a	1.39 ± 0.05 a	52.9 ± 1.08 a
	100	63.6 ± 2.79 a	0.24 ± 0.02 a	1.39 ± 0.05 a	55.2 ± 1.46 a
	500	67.6 ± 3.54 a	0.22 ± 0.03 a	1.25 ± 0.12 a	52.7 ± 2.35 a
	1000	62.8 ± 1.36 a	0.20 ± 0.01 a	1.18 ± 0.05 a	55.2 ± 0.62 a
E. heterophylla	0	49.4 ± 2.41 a	0.40 ± 0.03 a	1.89 ± 0.14 a	35.8 ± 1.31 a
	50	56.0 ± 2.97 a	0.42 ± 0.03 a	2.06 ± 0.16 a	36.9 ± 0.73 a
	100	52.8 ± 1.96 a	0.39 ± 0.01 a	1.80 ± 0.08 a	38.1 ± 1.96 a
	500	48.0 ± 3.03 a	0.33 ± 0.02 a	1.61 ± 0.12 a	41.2 ± 1.89 a
	1000	51.2 ± 3.44 a	0.35 ± 0.02 a	1.63 ± 0.07 a	39.3 ± 1.94 a
I. grandifolia	0	74.8 ± 3.38 a	0.86 ± 0.02 a	3.60 ± 0.08 a	24.7 ± 0.34 a

	50	81.6 ± 3.19 a	0.86 ± 0.03 a	3.59 ± 0.12 a	24.6 ± 0.25 a
	100	77.6 ± 2.79 a	0.91 ± 0.01 a	3.78 ± 0.05 a	24.6 ± 0.26 a
	500	79.6 ± 2.56 a	0.87 ± 0.01 a	3.66 ± 0.06 a	24.3 ± 0.22 a
	1000	82.8 ± 1.86 a	0.88 ± 0.02 a	3.67 ± 0.08 a	24.9 ± 0.23 a
U. decumbens	0	63.6 ± 4.40 a	0.20 ± 0.01 a	1.28 ± 0.08 a	55.1 ± 0.81 a
	50	61.2 ± 2.50 a	0.19 ± 0.01 a	1.22 ± 0.04 a	56.0 ± 0.78 a
	100	64.0 ± 3.52 a	0.19 ± 0.01 a	1.27 ± 0.06 a	55.1 ± 1.18 a
	500	60.4 ± 2.71 a	0.19 ± 0.01 a	1.22 ± 0.06 a	56.2 ± 0.49 a
	1000	61.6 ± 5.20 a	0.18 ± 0.01 a	1.20 ± 0.09 a	58.9 ± 1.29 a

[¥]Seeds germinated per hour**.** Values are means ± SE (n=5). Different letters indicate means that differ significantly, according to Tukey's HSD test at $p \leq 0.05$

In *A. thaliana*, itaconate induced a dose-dependent inhibition in the length of roots with IC_{25} and IC_{50} of 46.36 and 131.7 μM, respectively (Fig. 5A and Table 3). The length of stem was reduced only by the maximal concentration assayed of 1000 μM (-29.89%). At the same concentration, the length of roots was 81.39% reduced compared with untreated seedlings. The fresh weight biomass of seedlings was not significantly altered, and due to the very low values the dry weight biomass cannot be precisely evaluated.

Itaconate also caused a dose-dependent inhibition in the development of *S. indicum* with reduction in the length and in the fresh and dry weights of seedlings (Figs. 5C, D). The degrees of inhibition caused by 1000 μM itaconate were similar to that observed in *A. thaliana*: 68.7% and 45.8% in the length of roots and stems, respectively (Figs. 5C). The IC_{25} for root length reduction was 53.91 μM (Table 3). In the case of biomass, the concentration of 1000 μM also caused notable inhibitions, with a reduction of 60.6% and 53.3% in fresh and dry biomass weights, respectively (Figs. 5D).

In *A. hybridus*, itaconate induced distinct effects from those found in *A. thaliana* and *S. indicum*: the root growth was not altered (Fig. 5E) and a dose-dependent stimulus occurred in the stem, with an increase of 44.6% and 89.2% in the length and fresh biomass weight, respectively, at the maximal concentration assayed (1000 μM) (Fig. 5E).

Inhibition in the seedling growth was the main effect of itaconate in *B. pilosa*, but the sensitivity of this species was lower in relation to *A. thaliana* and *S. indicum*

(Fig. 5G). The IC_{25} for root length was 439.10 μM, a value approximately one order of magnitude greater than that of *A. thaliana* (Table 3). A reduction of 38.1% in the root length was found at 1000 μM concentration. Itaconate also reduced stem length and the fresh and dry biomass weights of *B. pilosa*: at a concentration of 1000 μM, these parameters were reduced in 28.5%, 59.1% and 30.9%, respectively, when compared with the untreated seedlings (Figs. 5H).

Table 3 – Concentrations of itaconate needed for 25% and 50% of roots length and ICL activity inhibition (IC_{25} and IC_{50}).

Weed species	Root length		ICL activity	
	IC_{25} (μM)	IC_{50} (μM)	IC_{25} (μM)	IC_{50} (μM)
A. thaliana	46.36 ± 9.169 a	131.7 ± 40.09 a	-	-
S. indicum	53.91 ± 16.91 a	314.1 ± 77.38 a	23.49 ± 1.727 a	81.74 ± 6.011 a
B. pilosa	439.10 ± 79.61 b	-	-	-
E. heterophylla	423.90 ± 146.5 b	-	23.42 ± 4.114 a	67.19 ± 8.998 a

Values are means ± SE (n = 5 or n = 6). Different letters indicate means that differ significantly, according to Student's T-test at $p \leq 0.05$

In *E. heterophylla*, itaconate also inhibited the seedlings growth: a dose-dependent inhibition on root length was observed, with a maximal inhibition of 27.5% (1000 μM concentration) (Fig. 6A) and a IC_{25} value of 423.9 μM, a value that was significantly higher than that of *A. thaliana* and *S. indicum*, and similar to that of *B. pilosa* (Table 3). Unlike most of other species assayed, stem length was not altered by itaconate (Fig. 6A), but the fresh and dry biomass weights were reduced by 14% and 79.9% respectively, at the maximum assayed concentrations of 1000 μM (Fig. 6B).

I. grandifolia was less sensitive to itaconate as was also observed in *A. hybridus*: the root length was not altered (Fig. 6C) and stem length was increased (+19.9%, by 1000 μM concentration) (Fig. 6C) with no change in the fresh and dry biomass weights (Figs. 6D).

Finally, treatments with itaconate did not change any of the parameters of initial growth of *U. decumbens* seedlings even at the highest concentration of 1000 μM (Figs. 6E, F).

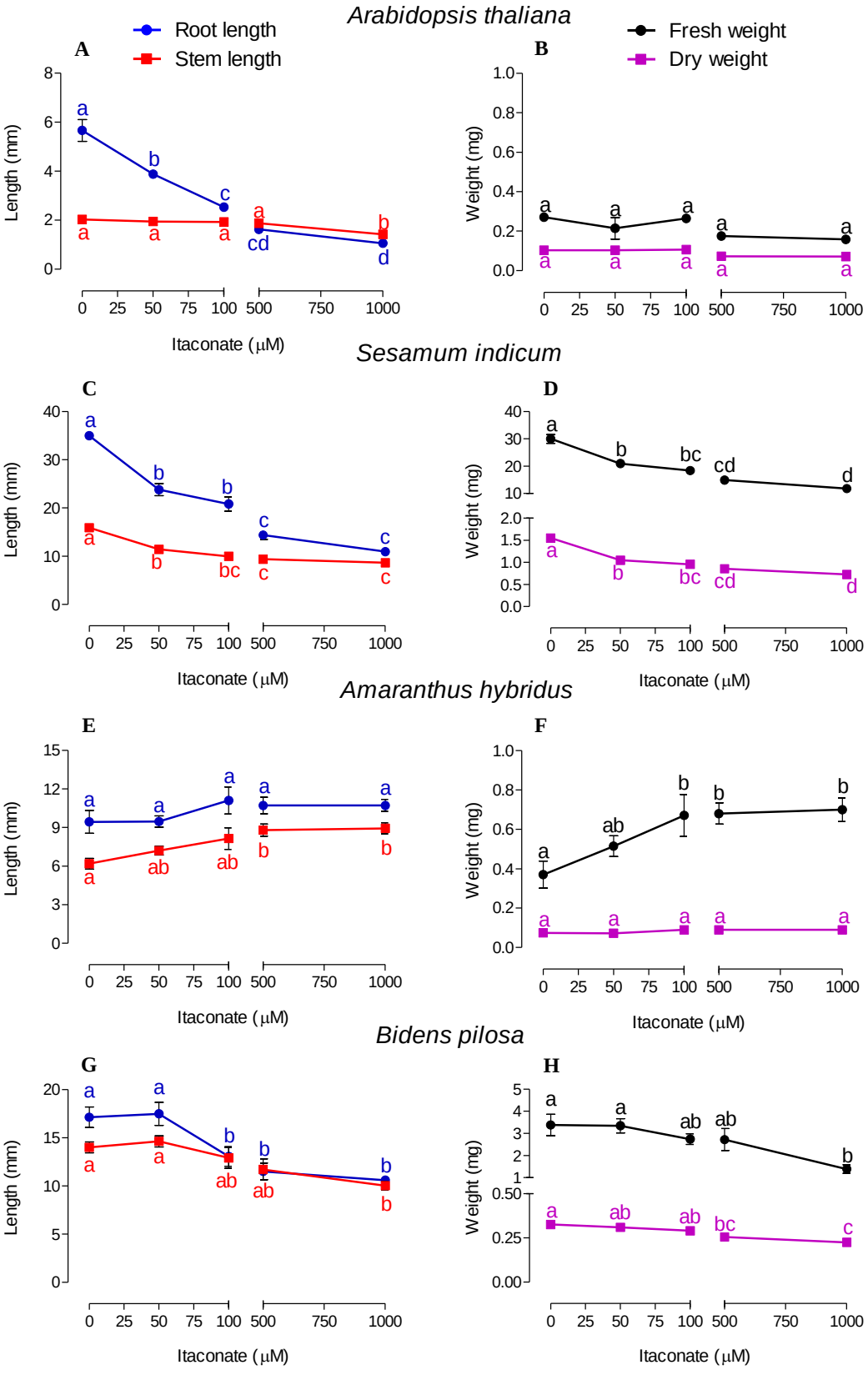


Figure 5 - Effects of itaconate on root lengths (blue lines), aerial parts lengths (red lines), fresh weights (black lines), and dry weights (purple lines) of the seedlings of *A. thaliana* (A, B), S. *indicum* (C, D), *A.*

hybridus (E, F) and *B. pilosa* (G, H), treated for 120 hours. Values are means ± SE (n=5). Different letters indicate that means differed significantly according to Tukey's HSD test at $p \leq 0.05$

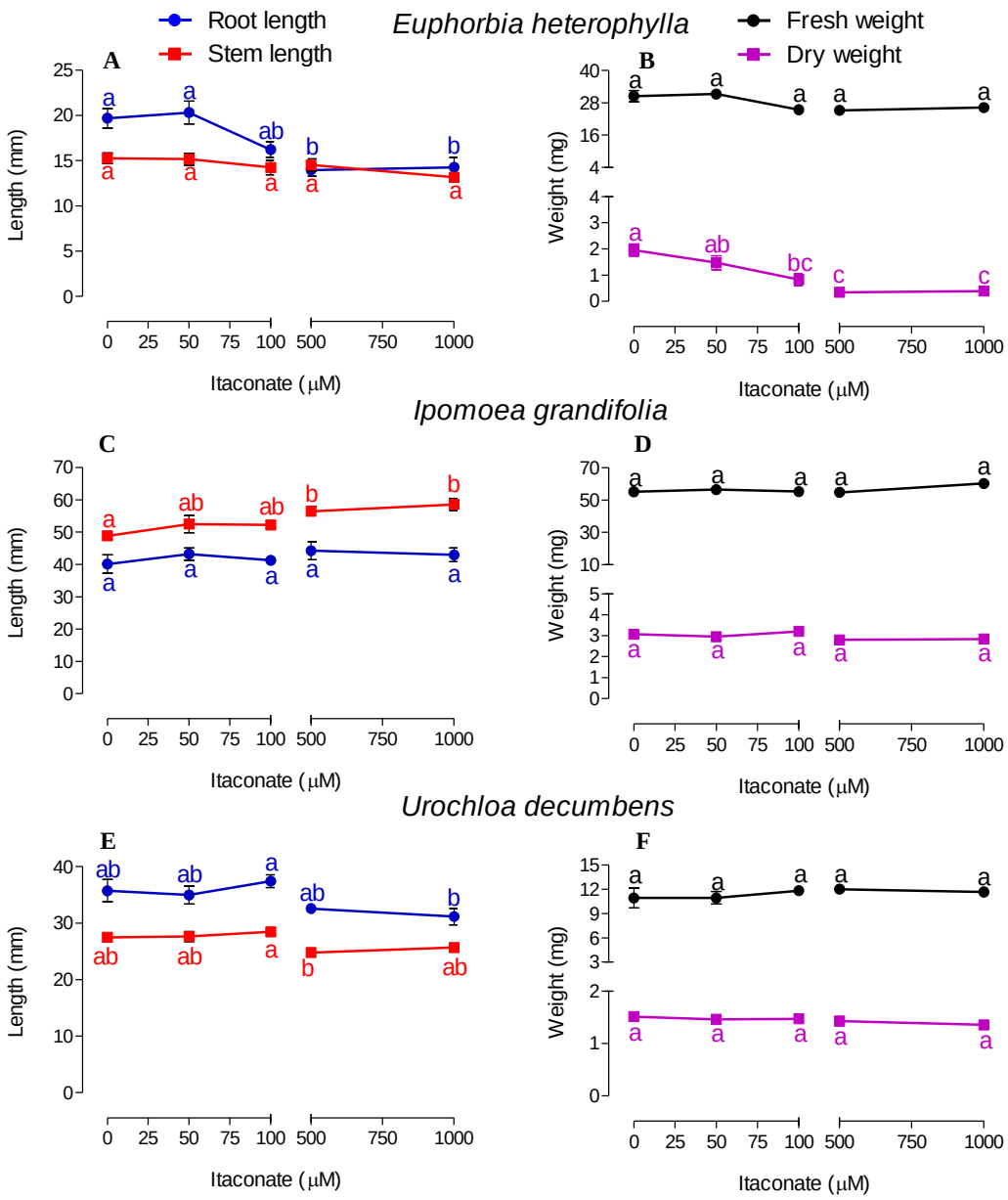


Figure 6 - Effects of itaconate on root lengths (blue lines), aerial parts lengths (red lines), fresh weights (black lines), and dry weights (purple lines) of the seedlings of *A. hybridus* (A, B), *E. heterophylla* (C, D) and *U. decumbens* (E, F), treated for 120 hours. Values are means ± SE (n=5). Different letters indicate that means differed significantly according to Tukey's HSD test at $p \leq 0.05$

3.3. The time course of isocitrate lyase activity during seedlings development and the effects of itaconate and tartrate on extracted enzymes

To determine the time course of the isocitrate lyase activity in each selected species during the germination process and initial development, the enzyme was extracted at 24-hour intervals until 120 hours after seed imbibition. As can be seen in figure 7, the species *S. indicum* and *E. heterophylla* exhibited higher enzyme activity compared to *A. hybridus, B. pilosa, I. grandifolia* and *U. decumbens.* The time course of enzymatic activity in *S. indicum* and *E. heterophylla* shows that ICL activity gradually increased, reaching a maximum value which was followed by a decrease to minimum values at 120 hours of growth. The time course curve of ICL activity in *E. heterophylla* was delayed and compressed in relation to that of *S. indicum,* so that the maximum activity (peak) was 48 hours in *S. indicum* and 96 hours in *E. heterophylla*. At its respective time peak, the ICL activity in *S. indicum* was 2.7-fold greater than that of *E. heterophylla*. ICL activity in other species *B. pilosa*, *I. grandifolia*, *A. hybridus* and *U. decumbens* was significantly lower at all time intervals (Fig. 7). The small amounts of tissues from *A. thaliana* at the initial growth of seedlings did not allow measurements of ICL activity.

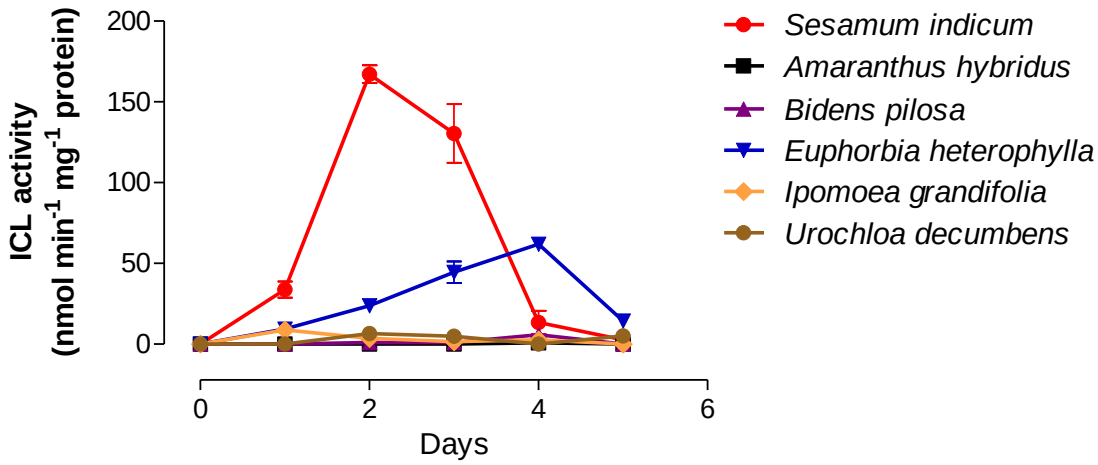


Figure 7 - Determination of the activity of the isocitrate lyase enzyme during the germination and initial development of *B. pilosa* (purple), *S. indicum* (red), *I. grandifolia* (cream), *A. hybridus* (black), *E. heterophylla* (blue) and *U. decumbens* (brown). Enzymes activity was evaluated in the extracts from cotyledons of seedlings or imbibed seeds grown at intervals of 24 hours until the end of 120 hours. Values are means ± SE (n=6).

To evaluate the *in vitro* effects of two selected inhibitors itaconate and tartrate on ICL activity, the enzyme was extracted from untreated seedlings of *S. indicum* and *E. heterophylla* grown at their peak times. Enzyme activity was measured in the presence

of itaconate or tartrate in a concentration range of 10-1000 µM. As can be seen in Figure 8, in the absence of inhibitors, ICL activity of *S. indicum* was higher than in *E. heterophylla*, and in both species a dose-dependent inhibition of ICL activity by itaconate was observed. At the highest concentration of 1000 µM, ICL activity was reduced by 88.1 % (*S. indicum*) and 91% (*E. heterophylla*) and the itaconate IC_{25} and IC_{50} were 23.49 and 81.74 µM for *S. indicum* and 23.42 and 67.19 µM for *E. heterophylla*, respectively (Fig. 8 and Table 3).

Different from itaconate, tartrate assayed at the same concentration range did not inhibit ICL activity of *S. indicum* or *E. heterophylla*. Furthermore, when tartrate at the concentration of 1000 µM was tested under *in vivo* conditions, no significant changes were observed in the development of *A. thaliana*, *S. indicum* and *E. heterophylla* seedlings (Fig. 9).

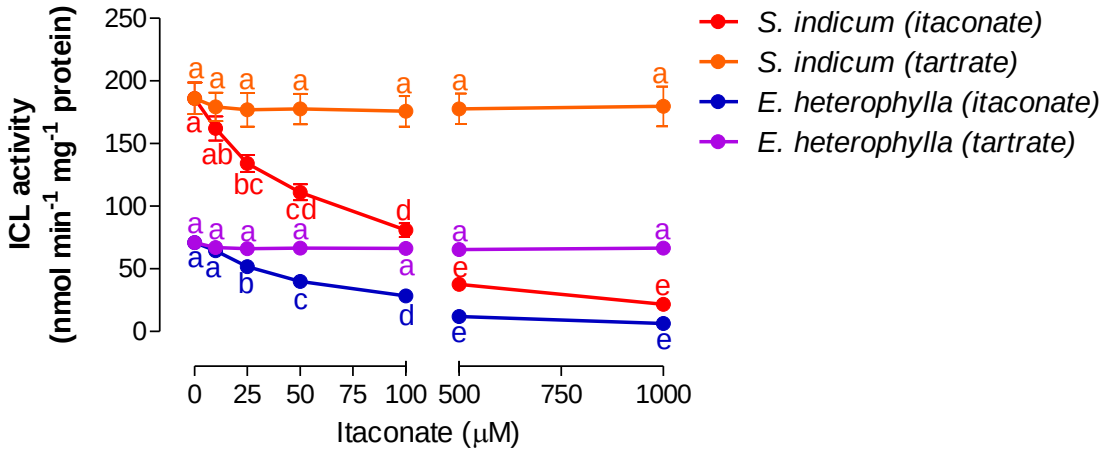


Figure 8 - Effects of itaconate (red and blue) and tartrate (orange and purple) on the enzyme isocitrate lyase extracted from cotyledons of *S. indicum* and *E. heterophylla*, grown for 48 and 96 hours (activity peak), respectively. The tested concentrations of itaconate and tartrate were 10, 25, 50, 100, 500 and 1000 µM. The compounds were incubated together with the substrate in the reaction medium. Each data point is the mean ± SE (n=5). Different letters indicate means that differ significantly, according to Tukey's HSD test at $p \leq 0.05$

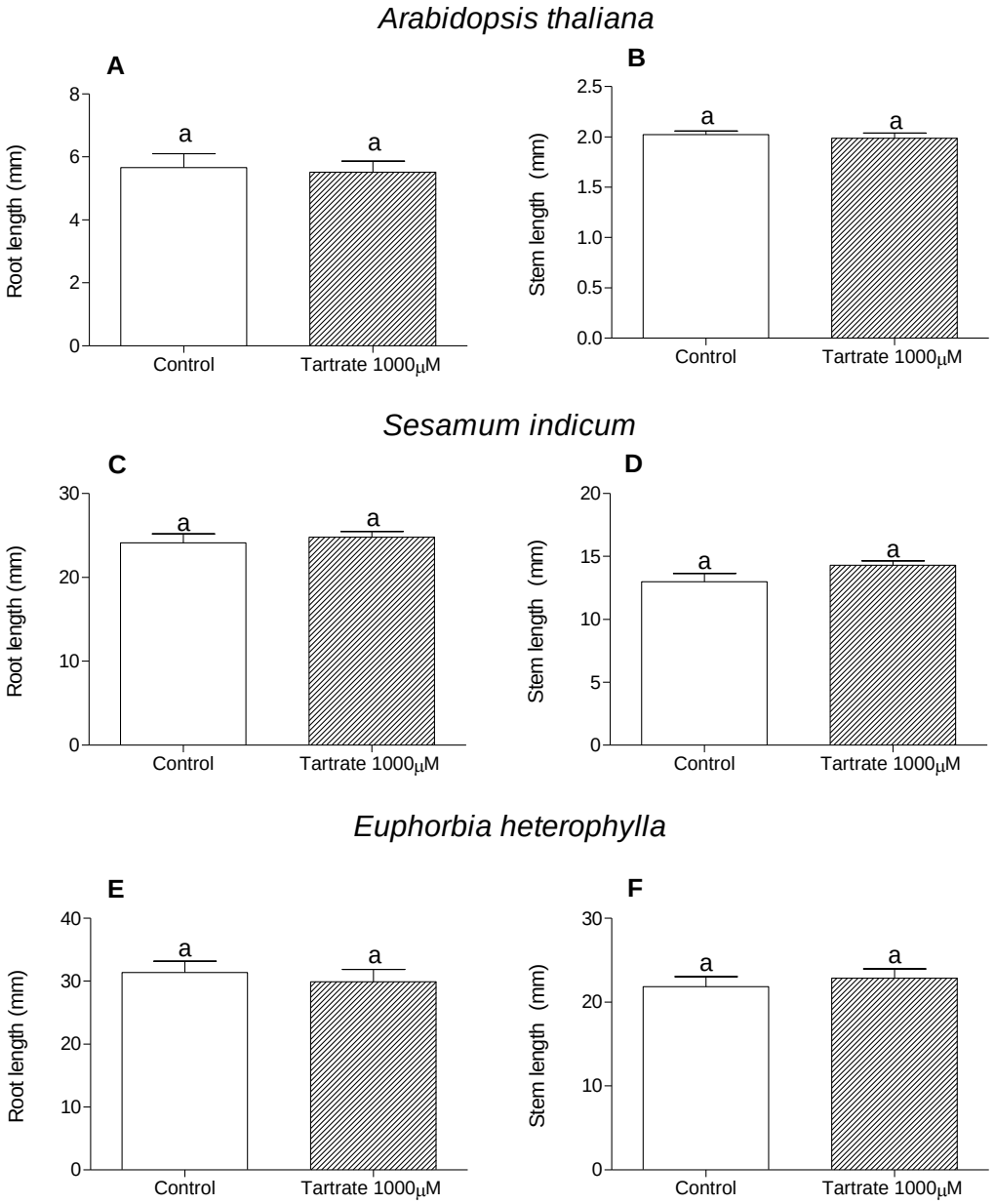


Figure 9 - Root and stem lengths of the seedlings of *A. thaliana* (A, B), S. *indicum* (C, D) and *E. heterophylla* (E, F) grown in the presence of tartrate 1000 μM for 120 hours. Values are means ± SE (n=5). Different letters indicate that means differed significantly according to Tukey's HSD test at $p \leq 0.05$

4. Discussion

The present study elaborated a structural model for isocitrate lyase (ArThaICL) that was built based on the amino acids sequence of *Arabidopsis thaliana*, a model oil plant, using *Aspergillus nidulans* and *Magnaporthe oryzae* ICL as templates for

homology modeling. After the model validation, an active site ligand screening was performed in order to find possible ICL inhibitors through molecular docking techniques. Among the highlighting compounds found in the virtual screening are homoisocitrate, tartrate and itaconate. Itaconate is a succinate structural analog, previously described as an inhibitor for this enzyme (Khan and McFadden, 1979; McFadden and Purohit, 1977; Runquist and Kruger, 1999; Vincenzini et al., 1986). Using itaconate as inhibitor, the present study evaluated the ICL as an herbicide molecular target by analyzing the consequences of the inhibition of glyoxylate cycle to germination and initial development of several weed species.

For the three-dimensional model elaboration, the best model obtained for homology modeling presented 99% of the amino acid residues in the allowed regions of the Ramachandran plot (Fig. S1), thus presenting the necessary stereochemical quality for the virtual screening studies. The decision to model the structure of ArThaICL in the presence of isocitrate was due to this compound being the real substrate of the enzyme. Furthermore, the crystallographic template structure has the ligands glyoxylate (product) and glycerol in the active site, which could leave the enzyme in a catalytically unfavorable conformation. This is very important when prospecting an inhibitor for the active form of the protein, therefore, after insertion of the isocitrate through docking, the structure of the complex was minimized to avoid stereochemical collisions of the protein residues with the new ligand, as well as to bring it into a catalytically more favorable conformation.

The selection of the most suitable programs to perform the virtual screening simulations was made by redocking the isocitrate ligand in the modeled complex. If the program is able to reproduce the modeled/crystallographic pose with a rmsd less than 1.0 Å and with reproducibility, it means that the program is able to "understand" the interactions that the protein makes with the ligand and is validated for use in library screening simulations with unknown binders. In addition, the score provided with redocking serves as a cutoff point for selecting other ligands that are more likely to bind, as well as the modeled pose serves as a structural guide for selecting the best pose of an unknown ligand, through the positioning of the pharmacophoric groups that interact with the protein (Fig. 2). The Vina and Molegro programs were able to reproduce the modeled pose of isocitrate with an average rmsd of 0.571 Å, which demonstrates the quality of the model developed in the present work.

Among the ligands selected by molecular docking**,** itaconate and tartrate were commercially available to conduct *in vivo* and *in vitro* assays in weed species. Expected effects of an ICL/glyoxylate cycle inhibitor were obtained with itaconate. Among the assayed species *A. thaliana* – the model plant – and *S. indicum* – the positive control – were the most sensitive ones relative to inhibition on root seedling growth. The weed species tested exhibited differential sensitivity to itaconate: *B. pilosa* and *E. heterophylla* were also inhibited, but to a lesser extent compared to *A. thaliana* and *S. indicum*; *A. hybridus and I. grandifolia* were not inhibited, on the contrary, a small stimulus was found, and *U. decumbens* was practically insensitive.

During the initial stages of development, embryos are heterotrophic organisms, depending on the reserves stored in the seeds. For species with great proportion of lipid reserves, dependence on the glyoxylate cycle is vital until seedlings begin to develop the first green leaves, producing sugars through photosynthesis (Cornah and Smith, 2002; Eastmond and Graham, 2001). During seed imbibition the expression of the ICL gene as well as its synthesis occurs de novo (Martins et al., 2000), processes that are regulated by the hormones gibberellic acid (GA) and abscisic acid (ABA), among other factors (Cornah and Smith, 2002). As ICL synthesis occurs, it begins to direct the carbon flow from the breakdown of fatty acids to gluconeogenesis through the synthesis of carbon skeletons with 3 or more carbon atoms, which can serve as precursors for this pathway (Graham, 2008). The time required for ICL expression and synthesis may vary from species to species, which is illustrated by the results obtained with *S. indicum* and *E. heterophylla* revealing that the ICL of *S. indicum* has the maximum activity (peak) after 48 hours of seed imbibition and in *E. heterophylla* after a period of 96 hours.

In general, a good correlation was found between the *in vivo* sensitivity of the tested species and the ICL activity measured in their cotyledons during germination and early development. *S. indicum* that had higher sensitivity to itaconate presented higher ICL activity, as expected due to its high lipid reserve content (Nzikou et al., 2009). The sensitive weed species *E. heterophylla* showed considerable ICL activity. Low or negligible activity was found in cotyledons of *A. hybridus, I. grandifolia, U. decumbes*, species insensitive to *in vivo* inhibition by itaconate. The exception was *B. pilosa* which exhibited low ICL activity, but the growth of seedling was reduced in a similar extent to *E. heterophylla*. Further evidence that ICL inhibition contributed to *in vivo* effect of

itaconate on sensitive species was the *in vitro* assay revealing a direct inhibition of itaconate on ICL extracted from cotyledons of *S. indicum* and *E. heterophylla*.

The strong relationship between inhibition by itaconate on seedling growth and in ICL activity with the proportion of lipid in the seeds is another evidence that ICL was the main molecular target of itaconate under *in vivo* conditions. *A. thaliana* and *S. indicum*, the most sensitive species, have a high proportion of oil on their seed reserves: an average of 40.3 and 54.0%, respectively (Tashiro et al., 1990). *E. heterophylla* and *B. pilosa* that were inhibited in a lower extent compared with *A. thaliana* and *S. indicum* have minor proportion of oils: around 20% of seed dry mass in *E. heterophylla* and 12.8% in *B. pilosa* (Santos et al., 2002). The insensitive weed *A. hybridus* has a small amount of lipids (4.66%) (Szabóová et al., 2020). There is no information relative to proportion of oil in seeds of *I. grandifolia*, but in species of the same genus values from 1.83% (*I. carnea*) to 14.09% (*I. hederacea*) were described (Abiodun et al., 2017; Zia-Ul-Haq et al., 2012). *U. decumbes* is a grass from Poacea family characterized for having starch as the main seed nutrient and low content of lipids, as for example *Sorghum bicolor* (4.3%) and *Secale cereale* (2.65%) (Beloshapka et al., 2016; Nyström et al., 2008). Our data are in agreement with those found in *Ricinus communis* demonstrating that the lack of ICL can be lethal to species dependent on lipid reserves (Cornah and Smith, 2002).

Although itaconate inhibited similarly the ICL activity of cotyledons of *S. indicum* and *E. heterophylla*, as indicated by their IC_{25} and IC_{50} values, concentrations nearly tenfold higher were required to inhibit the root growth of *E. heterophylla* compared with *S. indicum.* This finding is in agreement with the lower dependence of the glyoxylate cycle/lipids to sustain the development of *E. heterophylla* seedlings. In this way, the species which do not depend exclusively on the ICL activity for sugars availability were less affected by itaconate under *in vivo* condition.

It seems unequivocal that ICL is a molecular target of itaconate under *in vivo* conditions, but the effects found in *B. pilosa* and *A. hybridus* suggested the existence of additional mechanisms. The growth of *B. pilosa* seedlings was inhibited by itaconate in a similar degree compared with *E. heterophylla*, as indicated by their values of IC_{25}, but its seeds have lower content of lipids (Santos et al., 2002) and also lower ICL activity in its cotyledons suggesting lower dependence on glyoxylate cycle. On contrary, in *I. grandifolia* and *A. hybridus* itaconate induced, instead of inhibition a stimulus in

seedling growth, particularly on stem growth. It remains to be studied which metabolic processes not directly related to glyoxylate cycle were altered by itaconate in these three weed species.

From the results obtained *in vivo*, it is possible to verify that although the effects of itaconate caused considerable inhibition on the initial development of several weed species, the germination parameters were not affected by this compound. Such phenomenon is quite common, since the latter tends to be less sensitive (Ferreira and Borghetti, 2004). Contrary to seed embryos, seedlings have direct contact with compounds dissolved in the medium. The glyoxylate cycle is important for the initial development of the oilseed embryo, but it has been suggested that the cycle is not essential for germination to occur, playing a much more important role in the initial vigor and in the competitive capacity of the seedling in the environment than in the capacity of germinating (Eastmond and Graham, 2001; Graham, 2008; Penfield et al., 2005). In agreement, our data revealed that the ICL activity from cotyledons of *S. indicum* that is low at the first 24 hours after seed imbibition, increased rapidly after this time.

Tartrate, another ICL ligand revealed by molecular docking, did not exert effects on seedling growth and/or ICL activity in species sensitive to itaconate when assayed in the same concentration range. Lack or weak *in vitro* effects of a putative inhibitor revealed *in silico* studies was not uncommon, and several factors could contribute to this result, including the structural and conformational differences between the *in silico* ICL structure with the *in vivo* structure of enzyme of different species; the influence of the reaction medium used for *in vitro* enzyme measurements (pH, concentration of substrates and co-factors) that can change the conformational structure of enzyme and its interaction with the substrate; the type of enzyme inhibition, water solubility and others. In *Linum usitatissimum* L. itaconate is described as a non-competitive inhibitor of ICL (Khan and McFadden, 1979). In the present work, a saturating substrate concentration (4.25 mM D-isocitrate) was used in the reaction medium to measure ICL activity, a concentration that is higher than the maximum concentration of the tested inhibitors (1.0 mM). In this condition, different from non-competitive inhibitors, the effect of a competitive inhibitor would not be evidenced. Further studies performing classical enzymatic kinetic assays may clarify this question, a desirable approach for all ICL inhibitors revealed by molecular docking. Irrespective on this, the experimental

results obtained with itaconate were strong evidence that present *in vivo*/*vitro*/*silico* strategy for ICL inhibitor screening efficiently worked**.**

5. Conclusion

The fact that itaconate efficiently inhibited the growth of *A. thaliana* and also of other different species *S. indicum*, *E. heterophylla* and *B. pilosa* validated the ArThaICL model simulations and bioinformatics *in silico* performed in this work for the development of an herbicide based on ICL inhibition. ICL plays an important role during the mobilization of lipid reserves, and its inhibition by compounds such as itaconate has the ability to drastically reduce the initial growth of seedlings by causing a sugar deficiency at a crucial moment of their development, possible impacting other biochemical parameters. This drastic reduction in seedling development can considerably reduce the ability of these weeds species to compete with crops of interest in the fields. The present work also evidenced the importance of *in vivo* assays in different weed species, not only in model species. Itaconate specifically inhibited the weed species most dependent on lipid reserves, not harming the development of other species. Therefore, ICL inhibitors could be used selectively for non-oleaginous crops to control specific weeds, an approach that can contribute to more precise herbicide application. Although more studies are still needed for the development of a commercial herbicide based on ICL inhibition, the present work demonstrated that the *in silico* strategy to discover a selective inhibitor is a promising insight in the search for new compounds, mechanisms and modes of action of herbicides, thus helping to discover new alternatives for the control of herbicide-resistant weed species in a sustainable way.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Appendix A Supplementary data

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SP|P28298|ACEA_EMENI ----------MSYIEEEDQRYWDEVAAVKNWWKDSRWRYTKRPFTAEQIVAKRGNLKIEYP 51
SP|P28297|ACEA_ARATH MAA--SFSVPSMIMEEEGRFEAEVAEVQTWWSSERFKLTRRPYTARDVVALRGHLKQGYA 58
SP|P0CT06|ACEA_MAGO7 MASKNMVNPAVEPSMEDDLFAREVAEVKQWWSDPRWRYTKRPFTAEQIVSKRGNLKIEYP 60
                                  *:  :  *** *: **.. *:: *:**:**.::*: **:**  *

SP|P28298|ACEA_EMENI SNVQAKKLWGILERNFKNKEASFTYGCLDPTMVTQMAKYLDTVYVSGWQSSSTASSTDEP 111
SP|P28297|ACEA_ARATH SNEMAKKLWRTLKSHQANGTASRTFGALDPVQVTMMAKHLDTIYVSGWQCSSTHTSTNEP 118
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                     **  :****  *: .  :  ** *:*.*:*. ** ***:***:******.*** :*::**

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SP|P28297|ACEA_ARATH GPDLADYPYDTVPNKVEHLFFAQQYHDRKQREARMSMSREERTKTPFVDYLKPIIADGDT 178
SP|P0CT06|ACEA_MAGO7 GPDLADYPYTTVPNKVSHLFMAQLFHDRKQRHERLSAPKSERSKLQNIDYLRPIIADADT 180
                     .*******  ******.**::** :******. *::  :.:* *   :***:*****.**

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                     *.** **.:** ***:*:****:*****:  ************ :********* * *

SP|P28298|ACEA_EMENI DIMGTDLLAIARTDSEAATLITSTIDHRDHPFIIGSTNPDIQP--LNDLMVMAEQAGKNG 289
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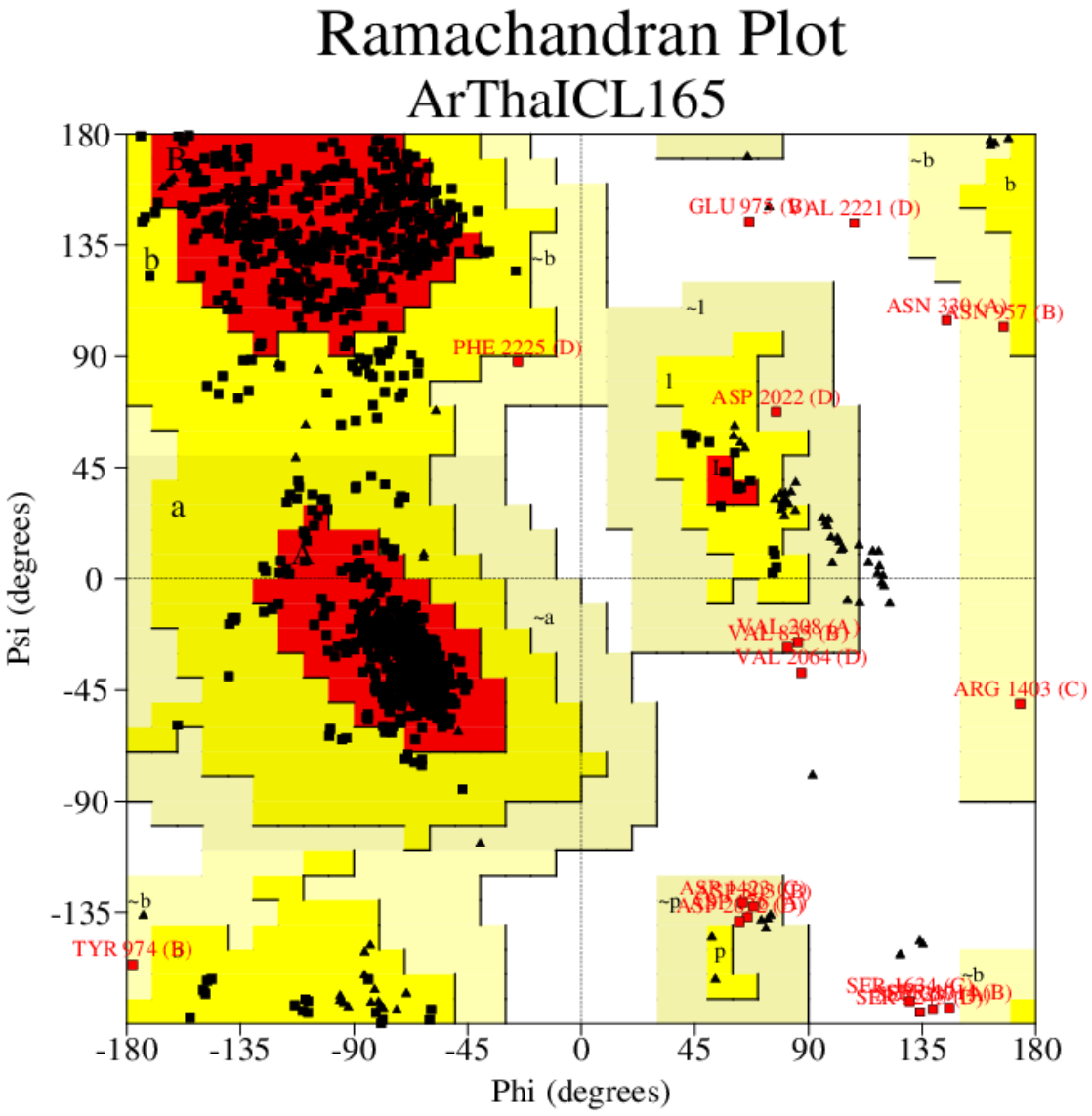
SP|P0CT06|ACEA_MAGO7 KEFAEGVHAVYPEQKLAYNLSPSFNWKTAMP-RDEQETYIRRLAGLGYCWQFITLAGLHT 473
                     .:**:*:::  **  ***********. :    ::   :* *:. ***.********:*:

SP|P28298|ACEA_EMENI TALISDTFAKAYAKQGMRAYGELVQEPEMANGVDVVTHQKWSGANYVDNMLKMITGGVSS 523
SP|P28297|ACEA_ARATH DALVVDTFAKDYARRGMLAYVERIQREERTHGVDTLAHQKWSGANYYDRYLKTVQGGISS 538
SP|P0CT06|ACEA_MAGO7 TALISDRFARAYSEVGMRAYGELVQEPEMELGVDVVKHQKWSGATYVDELQKMVTGGVSS 533
                      **: * **: *:. ** ** * :*. *   ***.: *******.* *.  * : **:**

SP|P28298|ACEA_EMENI TAAMGKGVTEDQFKS---------------------- 538
SP|P28297|ACEA_ARATH TAAMGKGVTEEQFKESWTRPGADGMGEGTSLVVAKSRM 576
SP|P0CT06|ACEA_MAGO7 TAAMGKGVTEDQFH------------------------ 547
                     **********:**:
```

Figure S1 – Clustal omega alignment between the amino acid sequences of the target protein of *Arabidopsis thaliana* (P28297) with templates of *Aspergillus nidulans* (P28298) and *Magnaporthe oryzae* (P0CT06). The identity between the three sequences was 47.4%. Yellow regions represent gaps in alignment with templates.



Plot statistics

Residues in most favoured regions [A,B,L]	1785	93.0%
Residues in additional allowed regions [a,b,l,p]	116	6.0%
Residues in generously allowed regions [~a,~b,~l,~p]	11	0.6%
Residues in disallowed regions	8	0.4%
	----	------
Number of non-glycine and non-proline residues	1920	100.0%
Number of end-residues (excl. Gly and Pro)	318	
Number of glycine residues (shown as triangles)	148	
Number of proline residues	64	

Total number of residues	2450	

Figure S2 – Ramachandran plot of the best ICL model of *Arabidopsis thaliana* generated by the Modeller program.

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CHAPTER 2

The modes of action of itaconic acid on the initial development of *Euphorbia heterophylla*

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Abstract

The study of new herbicide sites of action and a better understanding of the modes of action of natural compounds can contribute to the search for alternative solutions in weeds control in a healthier and more sustainable way. In a previous study, itaconate was found to exert significant inhibitions on the initial development of *Euphorbia heterophylla*, an aggressive weed species. Although it was clear that the main factor for these effects on *E. heterophylla* development was due to the inhibition of the enzyme Isocitrate lyase, the modes of action of this compound are still not well understood. The present study aimed to evaluate the modes of action of itaconate on *E. heterophylla* through the analysis of energetic and oxidative metabolism parameters. For this, the concentration of 1 mM of itaconate was tested on several different enzyme activities from cotyledons and roots of *E. heterophylla* incubated for 120 hours in the presence of the compound. Furthermore, the ROS contents of the cotyledons and the cellular respiration rate of the root apexes under this treatment were also evaluated. According to the obtained results, the metabolic alterations caused by itaconate in *E. heterophylla* were mainly consequences of its primary action in the glyoxylate cycle, which impaired gluconeogenesis in cotyledons, thus reducing the mobilization of sugars to the root's meristems. The lower apport of sugar in the roots led to a decrease in the efficiency of the mitochondrial respiratory chain and also the supply of metabolites to the biosynthetic processes, with consequent repercussion on the growth of the *E. heterophylla* seedlings.

Keywords: Respiration, gluconeogenesis, weeds

1-Introduction

The use of synthetic herbicides is the most efficient strategy to control weeds species in the fields, and although it is widely used, there is abundant evidence about its harmfulness to both the environment and human health (Gupta, 2018; Hasenbein et al., 2017; Heap, 2014; Rose et al., 2016). In addition, the control of weed biotypes resistant to traditional herbicides is one of the great challenges of modern agriculture (Farooq et al., 2020; Macías et al., 2019; Radhakrishnan et al., 2018). The study of new herbicide action sites and a better understanding of the modes of action of natural compounds can greatly contribute to the search for alternative solutions to traditional herbicides, thus enabling weed control in a healthier and more sustainable way (Fujii, 2001; Hiradate et al., 2010).

In a previous study (Menezes et al., 2022), the enzyme Isocitrate lyase (ICL) was evaluated as a possible new herbicide molecular target through the development of a computational model using bioinformatics tools, followed by molecular docking analyzes. Itaconic acid lies among the prospected molecules, an organic natural compound that is produced by mammalian macrophages through the decarboxylation of cis-aconitate in order to control the growth of pathogenic microorganisms, such as *Salmonella enterica* and *Mycobacterium tuberculosis*, which are dependent on the glyoxylate cycle under specific situations for their life cycle (Michelucci et al., 2013). Although itaconic acid exerts important immunological functions and is a strong ICL inhibitor of plants like *Linum usitatissimum* L. and microorganisms like *S. enterica* and *M. tuberculosis* (Khan & McFadden, 1979; Michelucci et al., 2013), studies exploring its effects on plants are still quite scarce. Menezes et al. (2022) tested the effects of itaconate on some crop and weed species, and a strong correlation was found between the inhibition of the initial development of the seedlings and the proportions of their lipid reserves (Menezes et al., 2022). Among the weeds studied was *E. heterophylla*, a weed well known for its ability to compete very successfully with the crop, especially in the early stages of establishment, due to its ability to grow very rapidly and form a dense canopy over the young crop plants (Wilson, 1981). In addition to having an aggressive behavior, this species already accumulates multiple resistances to several herbicides, among them glyphosate and acetolactate synthase (ALS) inhibitors (Trezzi et al., 2005; Vidal et al., 2007), which emphasizes the importance of finding new alternatives to control this weed species.

Itaconic acid has been shown to exert significant inhibitions on initial seedling development and ICL activity isolated from cotyledons of *E. heterophylla* (Menezes et al., 2022). Although ICL seems to be the primary site of itaconate in this weed, the metabolic consequences of this action, particularly on energy metabolism and antioxidant defense system

in both cotyledons and roots are not known. Such information is essential for understanding the modes of action of a potential new herbicide, and therefore, the aim of the present study was to investigate the effects of itaconate on oxidative stress parameters, antioxidant enzymes activities and respiratory activity in *E. heterophylla* seedlings.

2-Material and Methods

2.1-Reagents and plant material

DL-isocitrate, ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), 2′,7′,-dichlorofluorescin diacetate (DCFDA), and itaconic acid were purchased commercially from Sigma Chemical Co. (St. Louis, USA). The other reagents purchased were of the highest purity available. Seeds of *E. heterophylla* were purchased from Cosmos Agrícola Produtos e Serviços Rurais LTDA-Brazil.

2.2-Germination and growth of *E. heterophylla*

Seeds of *E. heterophylla* had been previously selected by size and appearance. After washing with distilled water, 50 seeds were placed in plastic boxes (Gerbox® 110 × 110 × 50 mm) containing 40 mL of semi-solid agar 0.8% (w/v) and itaconate at the concentrations of 0 (control) or 1 mM. Itaconate was dissolved in the agar medium before its solidification, and the boxes were randomly placed in a germination chamber (photon flux density of approximately 230 μmol m^{-2} s^{-1}). The *E. heterophylla* seeds were maintained at a constant temperature of 25°C, with a 12 hours photoperiod (light/dark) for a period of 120 hours until use.

2.3-Evaluation of ICL activity extracted from cotyledons of *E. heterophylla* grown in the presence or absence of itaconate

The ICL activity was evaluated at intervals of 24 hours after seeds imbibition until the end of 120 hours of seedling grown in the presence or absence of 1 mM itaconate. The enzymatic extracts were obtained according to Khan & McFadden (1979), with modifications. For enzyme extraction, 50 mg of cotyledons were macerated in a cold mortar, with 1 mL of a medium containing 50 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF. Then, the material was centrifuged at 15,000 × *g* for 20 minutes, at 4°C. The supernatant was used as the source of the enzyme (crude enzymatic extract). The ICL activity

test was performed according to Roche et al. (1970), with modifications. The reaction was carried out by using 50 µl of extract (0.03-0.1 mg de protein) and 700 µl of a reaction medium, containing 70 mM potassium phosphate buffer (pH 7.6), 5 mM MgCl, 1.8 mM DTT and 4.25 mM DL-isocitrate. The reaction was incubated for 15 minutes at 30°C, and after 15 minutes the reaction was stopped with 150 µl of 10% TCA and the proteins were precipitated with centrifugation at 5,000 × *g*, for 10 minutes at 4°C. A 500 µl aliquot of the supernatant was diluted in an identical volume of distilled water. In each tube, 25 µl of 5% phenylhydrazine hydrochloride was added, shaken vigorously and incubated for 1 minute in a water bath at 100ºC. Then, 500 µl of 2 N HCl were added to the tubes, and after 5 minutes, 25 µl aliquots of 25% potassium ferricyanide were added. The tubes were again vigorously shaken for complete homogenization and, after 15 minutes, the concentration of the glyoxylate formed was read at 520 nm in a spectrophotometer ($\varepsilon = 5.3\ 10^4\ M^{-1}\ cm^{-1}$). The enzyme activity was expressed in nmols $minute^{-1}$ mg^{-1} of protein.

2.4-Measurements of the activities of enzymes related to glucose metabolism in cotyledons and roots of *E. heterophylla*

For the extraction of Glucokinase (GK), Glucose-6-phosphate dehydrogenase (G6PDH), Pyruvate kinase (PK) and Malate dehydrogenase (MDH), approximately 50 mg of cotyledons or roots of *E. heterophylla* incubated for 120 hours in the presence or absence of itaconate 1 mM were triturated with extraction buffer containing 50 mM HEPES-KOH (pH 7.4), 0.25 M sucrose, 70 mM KCl, 10 mM $MgCl_2$, 1 mM Na-EDTA, 10 mM 2-mercaptoethanol and 1 mM PMSF, and centrifuged for 20 minutes at 4°C at 10,500 × *g*. For the extraction of Phosphoenolpyruvate carboxykinase (PEPCK), approximately 100 mg of cotyledons of *E. heterophylla* incubated in the same conditions were triturated with extraction buffer containing 0.2 M Bicine-KOH (pH 9.8) and 50 mM DTT, and centrifuged for 30 minutes at 4ºC at 20,000 × *g*. In both cases, the supernatants were used as source of enzymes. GK (EC 2.7.1.1) activity was measured by monitoring at 25ºC the reduction of $NADP^+$ (λ = 340 nm) in a medium containing 30-100 µl of enzymatic extract (0.01-0.125 mg of protein), 42 mM triethanolamine-HCl (pH 7.6), 2.22 mM glucose, 6.7 mM $MgCl_2$, 2.7 mM Na-ATP, 0.73 mM $NADP^+$ and 5 units mL^{-1} G6PDH (EC 1.1.1.49) ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). G6PDH (EC 1.1.1.49) activity was measured by monitoring at 25ºC the reduction of $NADP^+$ (λ = 340 nm) in a medium containing 30-100 µl of enzymatic extract (0.01-0.125 mg of protein), 86 mM triethanolamine-HCl (pH 7.6), 1.2 mM glucose-6-phosphate, 6.7 mM $MgCl_2$ and 0.37 mM $NADP^+$ ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). PK (EC 2.7.1.40) activity was determined by monitoring at 25ºC the rate of NADH oxidation (λ = 340 nm) in a medium containing 30-100 µl of enzymatic extract (0.01-0.15 mg of protein), 86

mM triethanolamine-HCl (pH 7.6), 0.2 mM NADH, 2.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM K-PEP, 4.7 mM K-ADP and 5 units mL^{-1} Lactate dehydrogenase (LDH) (EC 1.1.1.27) (ε = 6.22 mM^{-1} cm^{-1}). MDH (EC 1.1.1.37) activity was measured by monitoring at 25ºC the NADH oxidation oxidation (λ = 340 nm) in a medium containing 30-50 µl of enzymatic extract (0.075-0.125 mg of protein), 94 mM triethanolamine-HCl (pH 7.5), 0.2 mM NADH and 0.5 mM oxaloacetate (ε = 6.22 mM^{-1} cm^{-1}). Finally, the carboxylation activity of PEPCK (EC 4.1.1.32) was measured in a continuous assay at 25ºC by monitoring the NADH oxidation (λ = 340 nm) in a medium containing 30 µl of enzymatic extract (0.075-0.15 mg of protein), 100 mM HEPES (pH 7.0), 100 mM KCl, 90 mM $KHCO_3$, 0.5 mM PEP, 1.0 mM ADP, 5 µM $MnCl_2$, 4 mM $MgCl_2$, 0.14 mM NADH and 6 units mL^{-1} of MDH (ε = 6.22 mM^{-1} cm^{-1}). The enzymes activity was expressed as nmols min^{-1} mg^{-1} protein.

2.5-Measurement of the antioxidant defense enzymes in cotyledons of *E. heterophylla*

Approximately 50 mg of *E. heterophylla* cotyledons treated or not with 1 mM itaconate for 120 hours were transferred to a cold mortar and macerated with 1 mL of a medium containing 0.1 M phosphate buffer (pH 6.8) and 0.1% polyvinylpyrrolidone (PVP). The extracts were then centrifuged at 4ºC, for 30 min, at 7,400 × *g*. The supernatant was used as the source of the enzymes. All the operations were carried out at 4ºC. Catalase (CAT; EC 1.11.1.6) activity was measured in a medium containing 50 mM phosphate buffer (pH 6.8), 5.8 mM H_2O_2, and the enzyme extract (0.01–0.05 mg protein). The H_2O_2 consumption was measured by a spectrophotometer at 240 nm (ε = 43.6 M^{-1} cm^{-1}) (Aebi, 1984). Peroxidase activity (POD; EC 1.11.1.7) was measured in a medium containing 50 mM phosphate buffer (pH 6.8), 5.8 mM H_2O_2, 0.15 mM pyrogallol, and the enzyme extract (0.01–0.05 mg of protein). The POD activity was calculated by determining the amount of purpurogallin formed by a spectrophotometer at 420 nm (ε = 2.64 mM^{-1} cm^{-1}) (Pütter, 1974). The activity of Ascorbate peroxidase (APX; EC 1.11.1.11) was measured in a medium containing 50 mM phosphate buffer (pH 6.8), 0.5 mM ascorbate, 15 mM H_2O_2, and the enzyme extract (0.01–0.05 mg protein). The ascorbate oxidation was measured by a spectrophotometer at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) (Nakano & Asada, 1981). Glutathione reductase (GR; EC 1.8.1.7) activity was measured in a medium containing 50 mM phosphate buffer (pH 7.5), 1 mM GSSG, 0.1 mM NADPH, and the enzyme extract (0.01–0.05 mg of protein). The NADPH consumption was measured by a spectrophotometer, at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) (Foyer & Halliwell, 1976).

2.6-Determination of the reactive oxygen species (ROS) content in cotyledons of *E. heterophylla*

The determination of ROS was carried out according to the procedure of Jambunathan (2010), with some modifications. Approximately 50 mg of *E. heterophylla* cotyledons treated or not with 1 mM itaconate were pulverized with liquid nitrogen using a mortar and pestle. Then, the material was transferred to a plastic tube, mixed with 2 mL of 10 mM Tris-HCl buffer (pH 7.2), and centrifuged at 12,000 × *g* for 20 min and 4ºC. The supernatants were transferred to glass tubes and diluted 1:20 with the Tris-HCl buffer. To start the fluorescence reaction with ROS, 10 µL of 2',7'-dichlorofluorescin diacetate (DCFDA 1 mM prepared in dimethyl sulfoxide DMSO) was pipetted into each tube that was rapidly homogenized with a vortex and kept in the dark for approximately 15 minutes. The wavelengths (λ) used were 504 nm and 526 nm for excitation and emission, respectively. The ROS contents were expressed in fluorescence units per microgram of fresh weight (FW).

2.7-Respiratory activity measurements in primary apexes of *E. heterophylla*

The respiratory activity of the root apexes of *E. heterophylla*, treated or not with 1 mM itaconate for 120 hours was measured at 25ºC using a Clark type electrode that was inserted into an acrylic chamber and connected to a polarograph (Ishii-Iwamoto et al., 2006). Approximately 20 mg of the root apexes were cut into segments with lengths of 2–3 mm, and they were immediately placed into the oxygen electrode vessel with 2 mL of a nutrient solution (pH 5.8) containing 2 mM $Ca(NO_3)_2$, 2 mM KNO_3, 27 µM $FeCl_3$, 0.43 mM NH_4Cl, 0.75 mM $MgSO_4$ and 20 µM NaH_2PO_4. The rate of oxygen consumption was expressed in nanomols of oxygen consumed per minute per amount of tissue (milligrams), assuming the initial concentration of dissolved oxygen of 240 µM at 25ºC. To estimate the contribution of mitochondrial ferrocytochrome-*c*:oxygenoxidoreductase (COX; EC 1.9.3.1) to the overall O_2 uptake, 200 µM KCN was added to the reaction medium approximately 5 minutes after the addition of the apexes (KCN-sensitive respiration). The residual oxygen consumption was defined as KCN-insensitive respiration.

2.8-Determination of proteins

The protein content of the primary root extracts was determined according to Bradford (1976), using bovine serum albumin as standard. The range of concentrations in the standard

curve was 0.5–20 mg% with an r^2 of 0.934. Each plant extract was diluted to reach a protein concentration of less than 15 mg%.

2.9-Statistical analysis

The data presented in the graphs were expressed as mean ± standard errors (S.E.) of independent preparations; these were analyzed by analysis of variance (ANOVA), with significant differences between the means identified by Tukey's Honestly Significant Difference (HSD) test ($p \leq 0.05$) using RStudio software.

3-Results

3.1-Effects of itaconate on the ICL activity of *E. heterophylla* during the germination and initial development

The presence of 1 mM itaconate in the agar medium where *E. heterophylla* seeds were incubated was sufficient to cause significant inhibitions in the ICL activity *in vivo* at all tested intervals. As shown in Figure 1, in the absence of itaconate (control) the ICL activity in the cotyledons increased during the first 4 days, reaching the peak of activity of 62.0 nmols min^{-1} mg^{-1} protein at 96 hours after the incubation. In the presence of itaconate the ICL activity was reduced by 63.46%, 43.05%, 54.88% and 70.95% in the intervals of 24, 48, 72 and 96 hours, respectively, when compared with the corresponding values in control seedlings. At the terminus of experimental period (120 hours) when the enzyme activity in control seedlings decreased abruptly relative to peak activity, further reduction was found in the treatment with itaconate (64.70%) (Fig. 1).

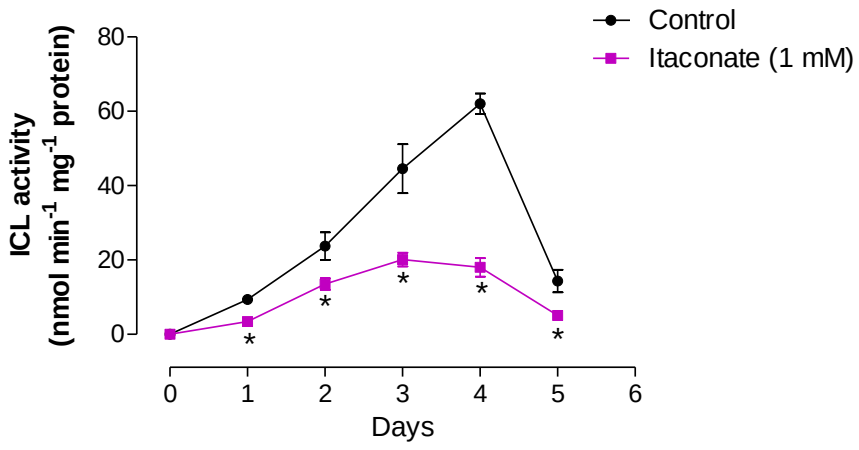

Figure 1 – Effects of itaconate on the enzymatic activity of ICL during the germination and initial development of *E. heterophylla*. *E. heterophylla* was grown in the absence or presence of 1.0 mM itaconate for 120 hours. Cotyledons were removed from seedlings at intervals of 24 hours until the end of 120 hours to obtain the crude extract of ICL enzyme. Values of ICL activity are means ± SE (n=6). The symbol (*) indicates that means between the control and the correspondent treatment differed significantly according to Tukey's HSD test at $p \leq 0.05$

3.2-Effects of itaconate on the activity of enzymes related to carbohydrate metabolism in cotyledons and roots of *E. heterophylla*

Figure 2 show the activities of some enzymes of glucose metabolism in cotyledons and roots of *E. heterophylla*, grown in the absence or presence of 1.0 mM itaconate. The significant activity of Phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme of gluconeogenesis pathway was detected only in extracts of cotyledons. The activities of enzymes related to glucose catabolism, Glucokinase (GK), Glucose-6-phosphate dehydrogenase (G6PDH) and Pyruvate kinase (PK) were higher in extracts of roots than in cotyledons. Roots and cotyledons had similar activities of Malate dehydrogenase (MDH). When seedlings were grown in the presence of 1.0 mM itaconate, a remarkable 60.62% inhibition of PEPCK activity was observed in cotyledons (Fig. 2A). Itaconate also reduced GK activity in both cotyledons (-56.47%) and roots (-56.72%) compared to the corresponding controls (Fig. 2B). G6PDH extracted from cotyledons was reduced by 66.25%, while no significant changes were induced by itaconate in G6PDH extracted from roots (Fig. 2C). Differently from the activities of GK and G6PDH, the treatment with itaconate caused a stimulus of 85.48% in the PK activity of the cotyledons, contrasting with an inhibition of 32.46% in the enzyme extracted from the roots when compared to their respective controls (Fig. 2D). In turn, the MDH activity of cotyledon and root extracts was inhibited by 35.73% and 10.24%, respectively, by the treatment with itaconate (Fig. 2 E).

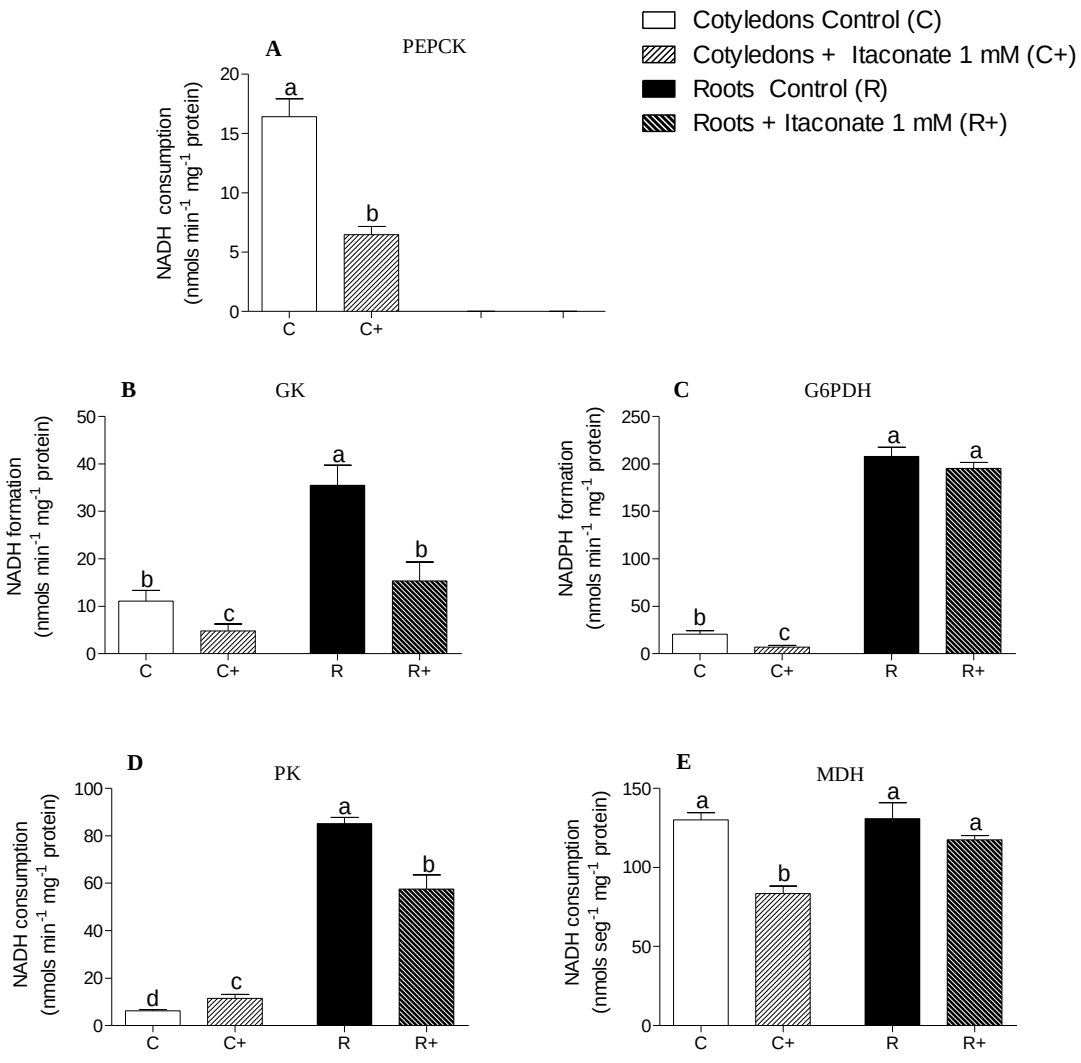

Figure 2 – Effects of itaconate on the activities of enzymes related to glucose metabolism in cotyledons (C) and roots (R) of *E. heterophylla*. Treatment with itaconate 1 mM is represented by the signal (+). (A) Phosphoenolpyruvate carboxykinase (PEPCK), (B) Glucokinase (GK), (C) Glucose-6-phosphate dehydrogenase (G6PDH), (D) Pyruvate kinase (PK) and (E) Malate dehydrogenase (MDH). Itaconate was dissolved in the agar medium at the concentration of 1 mM, and the seedlings were grown in the absence or presence of this compound for 120 hours. Cotyledons and roots were excised for isolation of crude extract of enzymes and ICL activity was measured as described in material and methods. Values are means ± SE (n=8). Different letters indicate that means differed significantly according to Tukey's HSD test at $p \leq 0.05$

3.3-Effects of itaconate on the antioxidant enzymes and on the ROS contents in the cotyledons of *E. heterophylla*

The activities of antioxidant enzymes extracted from the cotyledons of *E. heterophylla*, Catalase (CAT), Peroxidase (POD), Ascorbate peroxidase (APx) and Glutathione reductase (GR) were not significantly altered by 1.0 mM itaconate treatments as shown in Figure 3.

Itaconate reduced ROS content in the cotyledons of *E. heterophylla* seedlings by 21.19% relative to values found in untreated seedlings (Fig. 4).

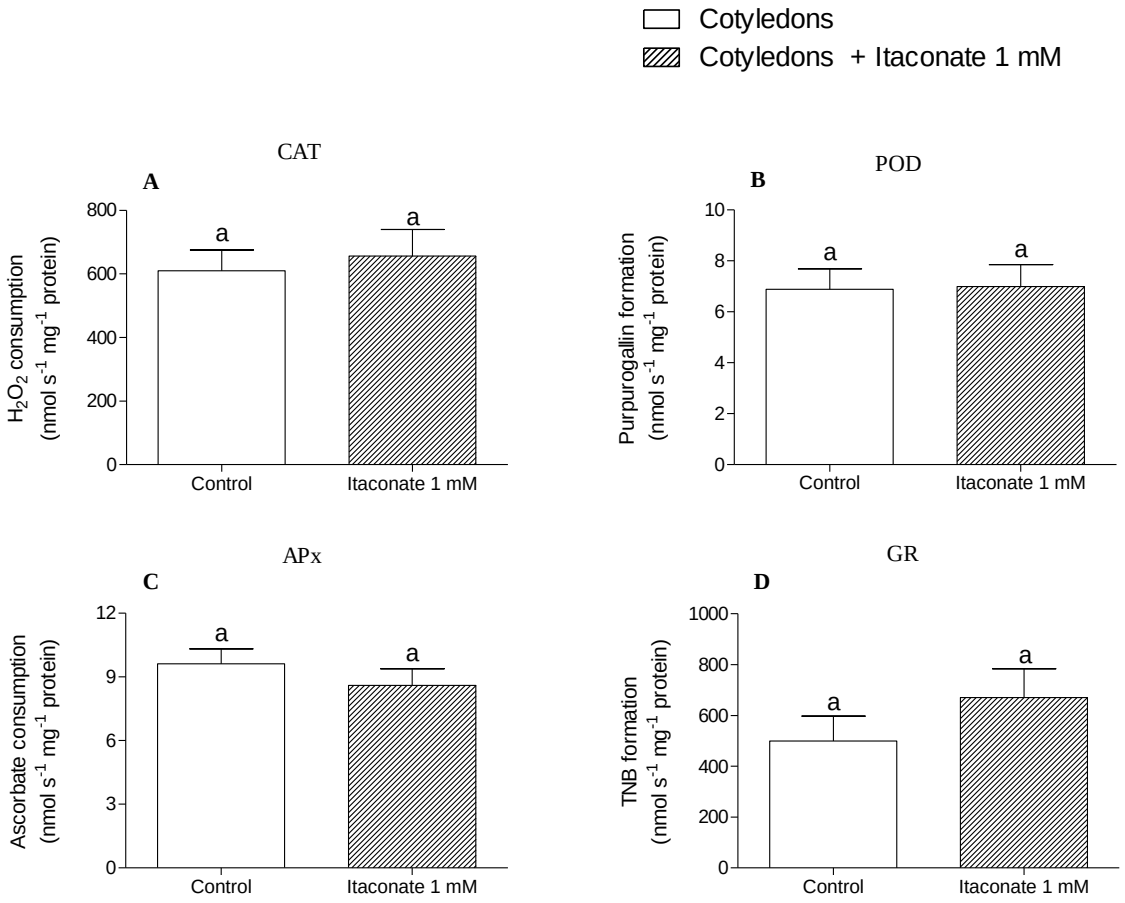

Figure 3 – Effects of itaconate on the activities of Catalase (CAT), (B) Peroxidase (POD), (C) Ascorbate peroxidase (APx) and (D) Glutathione reductase (GR). The enzymes were extracted from cotyledons of *E. heterophylla* grown in the absence or presence of 1.0 mM itaconate for 120 hours. Values are means ± SE (n=6). Different letters indicate that means differed significantly according to Tukey's HSD test at $p \leq 0.05$

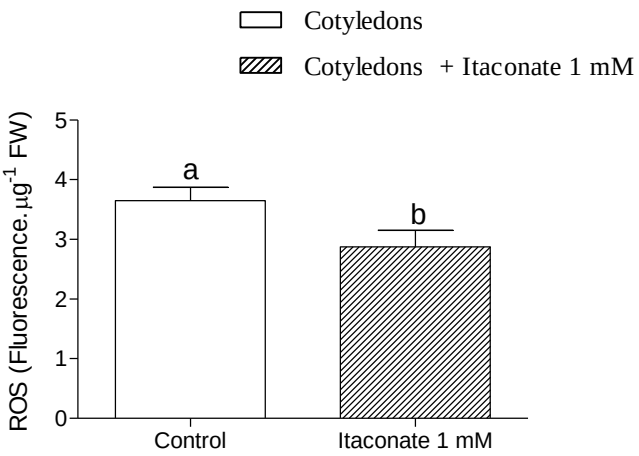

Figure 4 – Effects of itaconate on the ROS contents in the cotyledons of *E. heterophylla*. ROS was measured in cotyledons of *E. heterophylla* grown in the absence or presence of 1.0 mM itaconate for 120 hours. Values are means ± SE (n=8). Different letters indicate that means differed significantly according to Tukey's HSD test at $p \leq 0.05$

3.4-Effects of itaconate on the respiratory activity of the root apexes of *E. heterophylla* seedlings

The respiratory activity of root apexes of *E. heterophylla* grown in the presence of itaconate at concentrations of 0.25, 0.5 and 1.0 mM is shown in Figure 5. Control seedlings had a total respiratory rate of approximately 740.7 nmols min^{-1} g^{-1} FW, a value resulting from the sum of KCN-sensitive and KCN-insensitive respiration (Fig. 5). Itaconate induced a dose-dependent inhibition of total respiration with a decrease of 25.07%, 37.60% and 36.95% at concentrations of 0.25, 0.5 and 1.0 mM, respectively, when compared to the untreated seedlings (Fig. 5). This inhibition was mainly due to the decrease in KCN-sensitive respiration, which was 48.89% and 48.52% reduced at concentrations of 0.5 and 1 mM itaconate, respectively (Fig. 5). KCN-insensitive respiration was not significantly altered by itaconate.

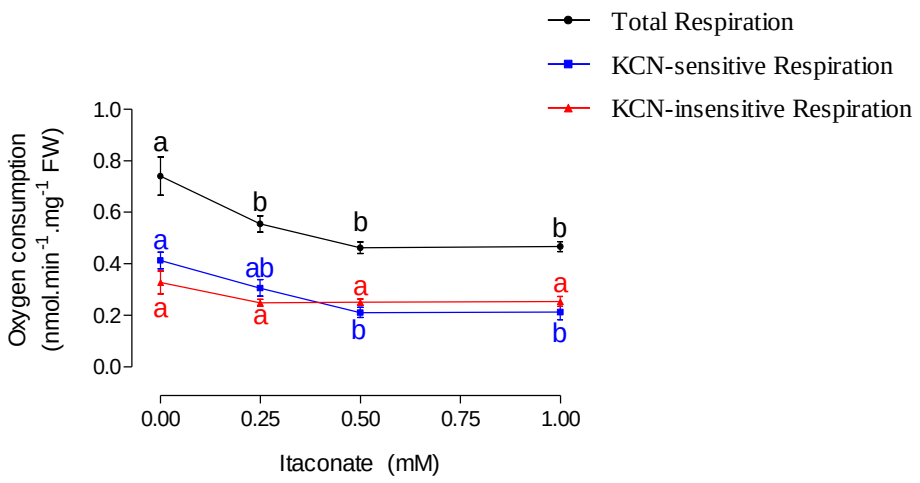

Figure 5 – Respiratory activity of roots apexes from primary roots of *E. heterophylla* grown in the absence (0) or presence of itaconate at concentrations of 0.25, 0.5 and 1 mM. The oxygen consumption of the root apexes was measured in the presence or absence of the inhibitor KCN (200 µM): total respiration (black line), KCN-sensitive (blue line) and KCN-insensitive (red line). Each data point is the means ± SE (n=6). Different letters indicate that means differed significantly according to Tukey's HSD test at $p \leq 0.05$

4-Discussion

In a previous study we have demonstrated that itaconic acid inhibits the initial development of *E. heterophylla* seedlings and also exerts a direct inhibition on ICL activity extracted from their cotyledons (Menezes et al., 2022). The present study revealed details of the metabolic changes induced by itaconic acid, most of them consequence of an impairment of glyoxylate cycle, as expected, but additional modes of actions were also revealed.

Corroborating the direct *in vitro* inhibition caused by itaconate in the activity of ICL extracted from cotyledons (Menezes et al., 2022), when *E. heterophylla* was cultivated in the presence of 1.0 mM of itaconate, the time curve of ICL activity, from seed imbibition to 120 h of treatment, was substantially depressed in relation to that found in untreated seedlings (Fig. 1). The peak of ICL activity that was at 96 hours after seeds imbibition in untreated seedlings, under treatment with 1 mM itaconate, became at 72 hours, with an activity rate 67.6% smaller (Fig. 1). The concentration that reduced *in vitro* 50% of ICL activity (IC_{50}) has demonstrated to be 67.19 µM (Menezes et al., 2022), a concentration well below that used to this experimental series (1.0 mM). Different factors may contribute to this difference: under *in vivo* conditions, the inhibitor must first be absorbed by the seedlings, diffuse through the tissues, so it can finally reach the compartment (glyoxysomes) where the glyoxylate cycle occurs. Furthermore, it is important to take into account that in some species, the seedlings could eventually metabolize the itaconate molecules, degrading it or converting into another non-active compound before it

reaches the ICL, or even compartmentalize it in a segregated location, so that only a small fraction of the availably itaconate will succeed to reach its molecular target (Coleman et al., 1997; Gani et al., 2021; Zhang & Yang, 2021). Even with the addition of these variables imposed by the *in vivo* conditions, the results demonstrate the effectiveness of itaconate in inhibiting the ICL activity in cotyledons of *E. heterophylla*, interfering with glyoxylate cycle and the metabolic pathways related to conversion of lipids to sugar during the germination and initial development of this species.

Glyoxylate cycle has an anaplerotic role, allowing the synthesis *de novo* of carbon skeletons, such as succinate and oxaloacetate, and these molecules are used as precursors for gluconeogenesis (Beevers, 1980; Runquist & Kruger, 1999). Therefore, it was expected that the inhibition caused by itaconate on the ICL activity reduces the carbon flow through this pathway, decreasing the production of glucose. Accordingly, in cotyledons of seedlings grown in the presence of 1.0 mM itaconate it was found a considerable reduction (-60.6%) in the activity of Phosphoenolpyruvate carboxykinase (PEPCK), a key-enzyme that acts at the beginning of the gluconeogenesis pathway. This reduction in PEPCK activity may reflects changes at the enzymatic regulation level or a repression of the gene expression in the cotyledons of *E. heterophylla* due to reduction in the supply of enzyme substrate.

The glucose produced in the cotyledons by the glyoxylate cycle together with gluconeogenesis is later exported to other tissues in the form of sucrose (Graham, 2008; Runquist & Kruger, 1999). During the germination process, tissues such as root meristems are in intense metabolic activity and depend on the arrival of carbohydrates from this reserve tissue to keep active (Desvoyes et al., 2021; Perilli et al., 2012). Root meristem is continuously undergoing mitosis, thus requiring both energy and reduction power (ATP and NADPH) that can be supplied by glycolysis and pentose phosphate pathways, also requiring carbon skeletons as precursors and building blocks for the tissues that are being synthetized. In agreement, a considerably higher activity of enzymes related to glycolysis and pentose phosphate pathways, GK, PK and G6PDH were found in the roots when compared to their respective isoenzymes in cotyledons (Fig. 2B, C, D).

When the seedlings were grown in the presence of 1.0 mM itaconate, the activity of the glycolytic enzymes GK and PK in the roots was decreased, an effect probably secondary to the shortage of substrates provided by cotyledons (Fig. 2B, D). Different responses were found in cotyledons, where the activity of G6PDH and MDH enzymes that were not altered by itaconate in the roots, were both significantly reduced in the cotyledons, indicating that processes required to glucose synthesis such as the pentose phosphate pathway, the tricarboxylic acid cycle (TCA), or even the malate aspartate shuttle may be impaired in this tissue (Fig. 2C, E). On the other hand, the behavior of glycolytic enzymes in this tissue diverged, whereas the activity of GK was

56.47% reduced the activity of PK was 85.48% stimulated (Fig. 2B, D). These changes may represent a compensatory response to the reduced supply of citric acid cycle intermediates by the inhibited glyoxylate cycle, in order to allow alternative anaplerotic pathways, such as that involving the pyruvate carboxylase (PC, EC number: 6.4.1.1) (Wurtele & Nikolau, 1990).

By evaluating the enzymes of the antioxidant defense system in the cotyledons, Catalase (CAT), Peroxidase (POD), Ascorbate peroxidase (APx) and Glutathione reductase (GR) it was found that none of these enzymes was significantly altered by itaconate (Fig. 3). In addition, a reduction in reactive oxygen species (ROS) contents was observed in the cotyledons (Fig. 4), which emphasizes the absence of severe oxidative damage.

The mobilization of the lipid bodies in cotyledons releases fatty acids and glycerol, the fatty acids enter the glyoxysomes, where they are broken down into 2-carbon fragments by β-oxidation enzymes. In this pathway hydrogen peroxide (H_2O_2) is generated by the enzyme Acyl-coenzyme A oxidase (ACX, EC: 1.3.3.6) (Baker et al., 2006; Graham, 2008). The inhibition of the ICL/glyoxylate cycle in the cotyledons by itaconate tends to decrease the carbon flow through the β-oxidation pathway, thus decreasing the ROS production by ACX. It is important to mention that under conditions where the glyoxylate cycle is inhibited, the acetyl-CoA molecules produced by β-oxidation that cannot be used as precursors for gluconeogenesis may be completely oxidized in the TCA to ATP synthesis in the mitochondrial cellular respiration (Cornah & Smith, 2002).

The respiratory activity of *E. heterophylla* roots apexes were reduced in both the total and the KCN-sensitive respirations. KCN sensitive respiration represents the oxygen consumption through mitochondrial cytochrome oxidase pathways, that is linked to ADP phosphorylation (Abrahim et al., 2003). Itaconate seems to induce, thus, an energy deficit on the roots, particularly in the meristems, with consequent impairment on seedling growth. In accordance to this assumption, the concentrations that caused 25% of inhibition on the root apexes total and KCN-sensitive respirations (IC_{25}) was 251.12 and 243.32 µM, respectively, values lower, but in the same order of magnitude from the values obtained in inhibition of roots growth (IC_{25} = 423.90 µM) (Menezes et al., 2022). A deficiency of sugars that are not being produced and exported from the cotyledons to roots can explain the reduction in the oxygen consumption by mitochondrial respiration. Furthermore, another possibility may be also considered: itaconate is a structural analogue of succinate, which has been demonstrated to compete for the active site of Succinate dehydrogenase (SDH, EC: 1.3.5.1) in macrophages (Lampropoulou et al., 2016). This enzyme is located in the complex II of respiratory chain (Abrahim et al., 2003) and in this way, if this enzyme in *E. heterophylla* is also sensitive to itaconate, an interference on the electron supply from complex II could be another mechanism of itaconate action on the root respiratory activity.

5-Conclusion

The present study allowed us to conclude that the metabolic alterations caused by itaconate in *E. heterophylla* were mainly consequences of its primary action in the glyoxylate cycle, which impaired gluconeogenesis in cotyledons, thus reducing the mobilization of sugars to the root's meristems. The lower apport of sugar in the roots led to a decrease in the efficiency of the mitochondrial respiratory chain and also the supply of metabolites to the biosynthetic processes, with consequent repercussion on the growth of the *E. heterophylla* seedlings.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Author contributions

Osvaldo Ferrarese-Filho, Emy Luiza Ishii-Iwamoto and Flavio Augusto Vicente Seixas conceived the research. Paulo Vinicius Moreira da Costa Menezes conducted the experiments. Emy Luiza Ishii-Iwamoto, Flavio Augusto Vicente Seixas and Paulo Vinicius Moreira da Costa Menezes participated in methodological tasks and data analysis. Paulo Vinicius Moreira da Costa Menezes and Emy Luiza Ishii-Iwamoto wrote the manuscript with suggestions of all authors.

Data availability statement

The data that support the findings of this study are available in the supplementary material of this article.

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