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RESEARCH ARTICLE

New insights into the mode of action of thiamethoxam on seed germination physiology

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ABSTRACT

In order to contribute to understanding the mode of action of the thiamethoxam in plants, we have carried out a virtual screening and in silico molecular docking study with TIA. The pharmacophoric search for protein ligands with three-dimensional structures similar to TIA resulted in several protein ligands with Tanimoto score equal to or greater tha 0.5, but only the ligand isoniazid (ISZ) was bound to a protein from plant origin, which was an ascorbate peroxidase (APX). Molecular docking of ISZ and TIA to several APX's revealed that TIA binds more strongly to these enzymes than ISZ, suggesting that TIA acts on plants through inhibition of this enzyme. When seedlings obtained from wheat and soybean seeds treated with different concentrations ot TIA, the activity of APX decreased with increasing TIA concentration. Therefore, both in silico and experimental results suggest that TIA can promotes plant growth through inhibition of the enzyme APX.

Keywords: Antioxidant; Docking; Metabolism; Pesticide; Plant

INTRODUCTION

Thiamethoxam (TIA; 3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-*N*-nitro-1,3,5-oxadiazinan-4-imine) is a broad spectrum insecticide of the neonicotinoid group, used either as a foliar spray or as a seed treatment (Maienfisch et al., 2002; Nauen et al., 2003; Mourtzinis et al., 2019). Substances in this group are agonists of the post-synaptic nicotinic acetylcholine receptors (*nAChRs*), which makes them efficient insecticides (Jeschke and Nauen, 2008; Estrada Atehortúa et al., 2016).

In addition to affecting insects, such substances can also have beneficial effects on plants (Wulff et al., 2019). Specifically, in the case of TIA, preliminary studies conducted by our research group or international researchers showed that this insecticide could improve soybean seeds germination (Cataneo et al., 2010; Junior et al., 2019); enhance wheat root development (Macedo and Castro, 2012); increase root growth and nitrogen uptake, resulting in gains of grass quality (Macedo et al., 2013); and increase seedling vigor in the presence of weed competition in corn (Afifi et al., 2015). Furthermore, neonicotinoids can also modify stress tolerance in plants. For example, they can improve drought tolerance in soybean (Cataneo et al. 2010) and cold tolerance in wheat (Larsen and Falk, 2013).

Currently, some research appointed that for regular seed germination and initial plantlet growth is necessary to have regulated concentrations of ROS because these compounds are considered an essential signal for seed germination (Bailly et al., 2008; El-Maarouf-Bouteau and Bailly, 2008), whereas in the presence of ROS donors occur the stimulation of seed germination, and in the presence of ROS scavengers the seed germination is inhibited (Leymarie et al., 2012).

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Deciphering the mechanism of action of substances in general is of great scientific and economic importance, as it allows, for example, the development of new biologically more efficient chemical structures than their predecessors (Schenone et al., 2013). For this purpose, a tool that has proved to be of great importance is bioinformatics, which can roughly be defined as an interdisciplinary field of science that develops computational methods and tools for the manipulation and understanding of biological data. In other words, bioinformatics is used for *in silico* analysis of biological issues, employing mathematical and statistical techniques (Lesk, 2013). Furthermore, it allows fast, low-cost manipulation of genes, proteins, and organic substances. The speeds with which various calculations can be performed *in silico* are very high, which allows the handling of a number of parameters much higher than those that can be investigated experimentally. As a result, there is usually a great acceleration of work development with the use of bioinformatics (Leelananda and Lindert; 2016). It has been successfully employed for the development of new products for agronomic use (Elanchezhian, 2012; Gong et al., 2013; Yang et al., 2018). For example, bioinformatics has been used as a tool in the development of heterocyclic derivatives with insecticidal activity (Fahmy et al., 2018). It is also possible to mention the use of bioinformatics to identify G-protein coupled receptors as attractive targets for the development of new insecticides (Ngai and McDowell, 2017).

This study aims to understand the biostimulant activity of thiamethoxam on seed vigor, in this study, we used computational tools to identify the enzymatic target. Later bioassays were performed on soybean and wheat seeds to elucidate the results found *in silico*.

MATERIALS AND METHODS

In silico identification of thiamethoxam enzymatic target in plants

Conformational search

Using the software ChemSketch 12.01 (https://www.acdlabs.com/), the chemical structure of thiamethoxam (TIA) was drawn with the nitro group in the same side (TIA-Z) and in the opposite side (TIA-E) in relation to the group (2-chloro-1,3-thiazol-5-yl)methyl (Fig. 1). The chemical structure of isoniazid (ISZ) was also drawn using the same software. The three drawn structures were converted to three-dimensional structures with the same software, to be submitted to conformational searches with the software Open3Dalign 2.3 (Tosco et al., 2011). To this end, 1000 molecular dynamics simulations were performed at 300 °C, with 1000 steps of 1 fs in each, which means that 1,000,000 conformations were generated. The Merck

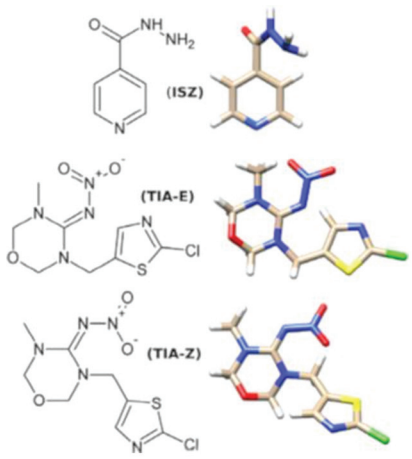

Fig 1. Two- and three-dimensional structures of thiamethoxam (TIA-E and TIA-Z) and isoniazid (ISZ) that were employed in the conformational search.

Molecular Force Field (MMFF94) was used, considering the solvent (water) through the Generalized Born and Surface Area (GBSA) method. The most stable conformation for each structure, as well as all those up to 10 kcal/mol less stable, were submitted to optimization with the computer program Mopac2016 17,270L (Stewart, 2016). In this case the solvent (water) was considered through the Conductor-like Screening Model (COSMO). The conformations with the lowest energies were considered the most stable.

Pharmacophore search

Initially, the three-dimensional chemical structures of ligands from proteins in the databank of Ligand Expo (Feng et al., 2004) were downloaded (http://ligand-expo.rcsb.org/) and submitted to hydrogen atoms addition using the software OpenBabel 2.3.2 (O'Boyle et al., 2011), to be aligned to the most stable conformations of TIA-E and TIA-Z through the software Align_it 1.0.4 (Taminau et al., 2008), which used standard values for all parameters. Only proteins produced by plants, with ligands that presented Tanimoto scores equal to or above 0.5 were selected for the next step.

Ascorbate peroxidase

48 three-dimensional structures and amino acids sequences of the enzyme ascorbate peroxidase (APX), produced by Soybean (*Glycine max* (L.) Merr.), pea (*Psium sativum* L.), tobaco (*Nicotiana tabacum* L.) and Arabdopsis (*Arabidopsis thaliana* (L.) Heynh), were downloaded (http://www.rcsb.org/) from the RCSB Protein Data Bank (Berman et al., 2000). The amino acids sequences were aligned through the software Ugene 1.22.1 (Okonechnikov et al., 2012), which employed the algorithm Clustal Omega 1.2.1 (Sievers et al., 2011) with 10 iterations. The pdb files of the enzymes 2vcn, 2vcs and 2vcf (Metcalfe et al., 2008), as well as all those ones with similarities equal

to or greater than 95% in relation to 2vcn, 2vcs and 2vcf, were submitted to the Python *script* MakeMultimer (http://watcut.uwaterloo.ca/tools/makemultimer/index) to generate the corresponding monomeric three-dimensional structures. Their three-dimensional structures were then aligned with the softwre Lovoalign 1.1.0 (Martínez et al., 2007). The enzymes 2vcs, 2vcn and 2vcf, as well as all those ones with root mean square deviation (RMSD) equal to or less than 2.0 Å in relation to 2vcs, 2vcn or 2vcf, were selected. Then, those enzymes with mutation or missing residues in the 2vcf binding region of ISZ were discarded.

Docking

The pdb file of each selected APX was converted to the pdbqt format using the software AutodockTools 1.5.7rc1 (Morris et al., 2009). Analogously, the pdb files of the most stable conformations of TIA-E, TIA-Z and ISA were converted to the pdbqt format using the same software. Using the software Autogrid 4.2.6 (Morris et al., 2009), atomic affinities were calculated in a region of 22 x 22 x 22 (x, y, z) $Å^3$, which was centered in the region of ISZ binding to 2vcf. The software Autodock 4.2.6 (Morris et al., 2009) used such affinities to dock TIA-E, TIA-Z and ISZ to all the selected APX's. Except for the ga_num_evals parameter, which was raised from 2500000 to 5000000, the other parameters were kept with the program default values.

Effect of thiamethoxam on soybean and wheat

For bioassay the commercial insecticide Cruiser 350F® (Syngenta Crop Protection, Paulínia, SP, Brazil), whose active ingredient is TIA (35 g/L), was applied on wheat (*Triticum aestivum* L. 'BRS264') and soybean (*Glycine max* (L.) Merrill 'Monsoy 6210') was evaluated. The seed germination test was performed as recommended by the Rules for Seed Analysis (BRASIL, 2009). For two minutes, the seeds were disinfected by immersion in a 2% (g/mL) sodium hypochlorite solution. Then washed them in running water until the complete removal of sodium hypochlorite.

According to the recommendation by the manufacturer of the mentioned insecticide, in the case of wheat the commercial product (Cruiser 350F®) was used at doses of 0, 50, 100 and 200 mL per 100 kg of seeds, while for soybean, 0, 100, 200 and 400 mL of Cruiser 350F® were used per 100 kg of seeds. For both plans the mixtures of seeds and the pesticide were vigorously shaked in a plastic bag for 2 min. Then, seeds were distributed on previously sterilized 28 x 38 cm *Germitest* papers (material free of debris, impurities, fungi and bacteria), previously the *Germitest* papers foils were moistened with distillate water for seed sowing, which were rolled up and placed inside a Biochemical Oxygen Demand (B.O.D.) germinator regulated to 20 ºC (± 5 ºC), in a 24-hour cycle, with a 12h photoperiod. Four repetitions (*Germitest* papers) were used per treatment/concentration/seed, and in each paper were placed 50 seeds. After nine days, fresh seedlings (200 mg) from each *Germitest* paper were crushed in a mortar containing a solution (1.5 mL) made up from: 750 µL of 200 mM potassium phosphate buffer (pH 7.8); 15 µl 10 mM EDTA; 150 µL 200 mM ascorbic acid; and 585 µL ultra-pure water. The resulting mixtures were centrifugated at 12000 g to afford plant extracts, in which ascorbate peroxidase activity was measured according to the method described in the literature (Nakano and Asada, 1981). Briefly, a sample (37.5 µL) of each plant extract was added to 3000 µL of a solution containing: 1500 µL of potassium phosphate buffer pH 7.0 (200 mM), 150 µL of ascorbic acid (10 mM), 1200 µL of ultra pure H_2O and 150 µL of H_2O_2 (2 mM). The activity was determined every ten seconds, for 60 seconds, through the measure of absorbance at 290 nm. The results were expressed in µmol min^{-1} mg^{-1} of protein.

Ascorbate peroxidase activity underwent regression analysis, with the best-fitted mathematical models selected when the analysis of the *F* test for the regression was significant ($P<0.05$) and the determination coefficient (R^2) showed more accuracy, electing, thus, the linear polynomial ($\hat{y} = a_0 + a_1x + a_2x^2$) or the quadratic polynomial ($\hat{y} = a_0 + a_1x + a_2x^2 + a_3x^3$) for all variables that showed significance. Furthermore, to improve the regression model estimation we adopted the residue regression decomposition in "pure error" and "lack of fit". R^2 values were found by dividing the sum of squares regression by the sum of squares of treatment. Analyses were conducted in SISVAR Software (Ferreira, 2011).

RESULTS AND DISCUSSION

This research proposed to understand the chemical induction of TIA on physiological seed responses. This pesticide molecule induces changes in plant metabolism, and the imbalance in ROS compounds can promote benefic effects, such as seed germination and plantlet improved growth.

In silico identification of thiamethoxam enzymatic target in plants

As the computer program used did not allow the interconversion of the TIA-E and TIA-Z structures (Fig. 1), it was important to use both to represent TIA in the performed calculations. After the conformational search, eight conformations were obtained for each of these chemical structures, whose populations, according to the Boltzmann distribution, were all greater than or equal to 4% of the total. The RMSD values between the conformations of each initial structure ranged

from 0.6 to 1.9 Å. Consequently, they were all used in the pharmacophoric search, as the Aling_it program makes rigid overlaps. That is, it does not rotate around chemical bonds in order to find more appropriate conformations. This procedure allowed the selection of several three-dimensional protein structures whose ligands had Tanimoto scores equal to or above 0.5: KatG catalase peroxidase, actinorhodin polyketide ketoreductase, androgen receptor ligand-binding domain, arylamine N-acetyltransferase, lactoferrin, lactoperoxidase, cyclophilin A, cytochrome C peroxidase, ascorbate peroxidase (APX), flavoprotein pentaerythritol tetranitrate reductase, carbonic anhydrase II, CinD nitroreductase and RadB recombinase. Among these proteins, the only ones that had plants origin were APX's 2vcn, 2vcs and 2vcf (Metcalfe et al., 2008). The ligand (isoniazid - ISZ) of such enzymes had a Tanimoto score of 0.51 with conformations of the TIA-E and TIA-Z structures (Fig. 2), suggesting that TIA may also bind to APX's, affecting their enzymatic activities.

Reactive oxygen species can damage important components of plant cells, such as DNA, proteins and lipids. In addition, such species are also used in lignin formation, leaf abscission, fruit ripening, and flowering. Therefore, it is extremely important for plant to maintain a balance of these species. To this end, one of the most important enzymes is APX, which catalyzes the reduction of H_2O_2, which is done at the expense of ascorbic acid (Dabrowska et al., 2007), which is oxidized in the process, and which has various functions in plant cells (Zhang, 2013). Consequently, inhibition of APX's can significantly affect plant development.

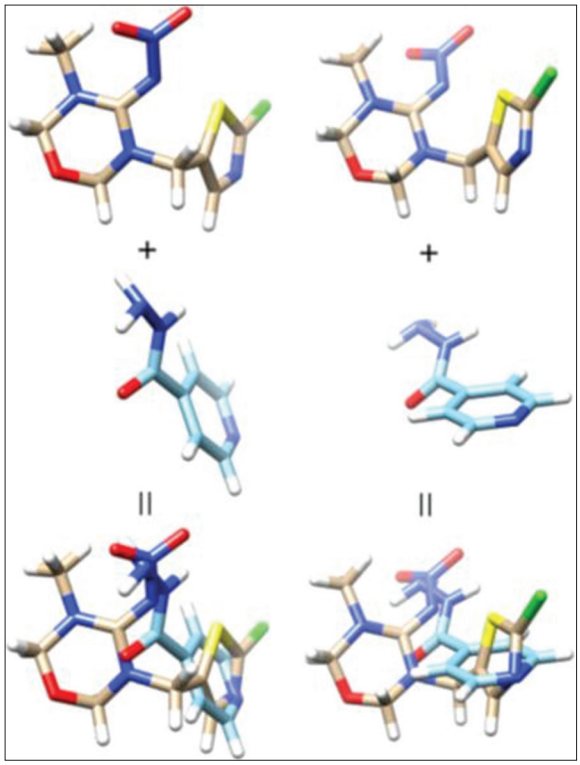

Fig 2. Examples of overlaps of thiamethoxam (TIA) with isoniazid (ISZ) in the conformation observed in its complex with ascorbate peroxidase 2vcs (Metcalfe et al., 2008)(Metcalfe et al., 2008). Image was generated in the software UCSF Chimera 1.10.1 (Pettersen et al., 2004).

Given the potential observed for the inhibition of APX's by TIA, we sought to deepen the work *in silico* to investigate the affinity of such substance for these enzymes. Although there are 48 three-dimensional plant-produced APX structures in the RCSB Protein Data Bank (http://www.pdb.org;), several of them had amino acid sequences with similarities of less than 95% to 2vcn, 2vcs and 2vcf (Metcalfe et al., 2008). Consequently, they were discarded. Several of them were also discarded because they are mutants. In addition, in some three-dimensional structures there were missing amino acid residues at or near the ISZ binding site. Consequently, such enzymes were also discarded. This left the following APX's to be used in the *in silico* study: 1apx (Patterson and Poulos, 1995), 1oag (Sharp et al., 2003), 1v0h (Sharp et al., 2004), 2ghh (Badyal et al., 2006), 2ghk (Badyal et al., 2006), 2vcf (Metcalfe et al., 2008), 2xi6 (Gumiero et al., 2010), 2xif (Gumiero et al., 2010), 2xih (Gumiero et al., 2010), 5jpr (Kwon et al., 2016) and 5jqr (Kwon et al., 2016). After aligning the three-dimensional structures of such enzymes, the RMSD values for 2vcf were all equal to or less than 0.6 Å (Fig. 3). It is worth mentioning that the RMSD between 2vcf and 2vcn or 2vcs (both mutants) was only 0.2 Å.

It was observed that the RMSD value between the docked and experimentally complexed ISZ structures was only 0.2 Å, making it evident that the *in silico* docking process is capable of reproducing the experimental result for ISZ. When all ISZ dockings in ascorbate peroxidases were compared to the dockings performed with TIA, it was

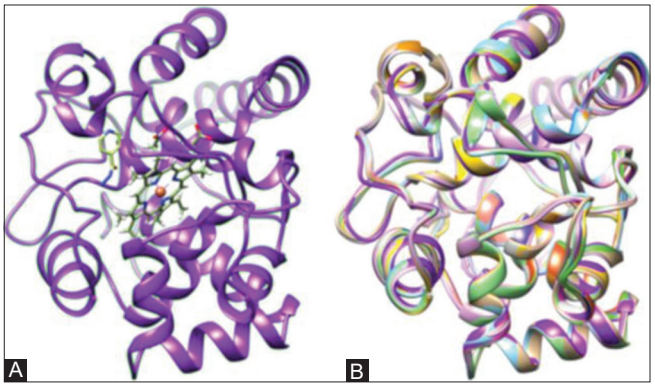

Fig 3. A) Three-dimensional structure of ascorbate peroxidase 2vcf, in which the presence of isoniazid (ISZ) and Fe-containing protoporphyrin IX (HEM) was made explicit. B) Three-dimensional structures of ascorbate peroxidases 1apx, 1oag, 1v0h, 2ghh, 2ghk, 2xi6, 2xif, 2xih, 5jpr and 5jqr, aligned with the 2vcf structure. The image was generated with the computer program UCSF Chimera 1.10.1 (Pettersen, et al., 2004).

clearly observed that this insecticide had more affinity for APX's than ISZ (Fig. 4).

The docked TIA and ISZ structures presented good overlaps between them (Fig. 5), though this was slightly different from that observed during the pharmacophoric search (Fig. 2).

Looking more closely at the interactions of ISZ with APX, one realizes that there is a hydrogen bond between one of the hydrogen atoms of the ISZ hydrazine group and the nitrogen atom of amino acid residue ALA134 of the enzyme. Analogous interaction is not observed between the TIA six-membered ring oxygen atom and the same amino acid residue (Fig. 6). However, since hydrogen bonds between nitrogen atoms are among the weakest of this kind (Abraham et al., 1989; Laurence and Berthelot, 2000), such hydrogen bond interaction was not enough to make ISZ have more affinity for the enzyme than TIA. Specifically for ascorbate peroxidase 2vcf, the affinity of ISZ was -4.9 kcal/mol, while that of TIA was -9.2 kcal/mol.

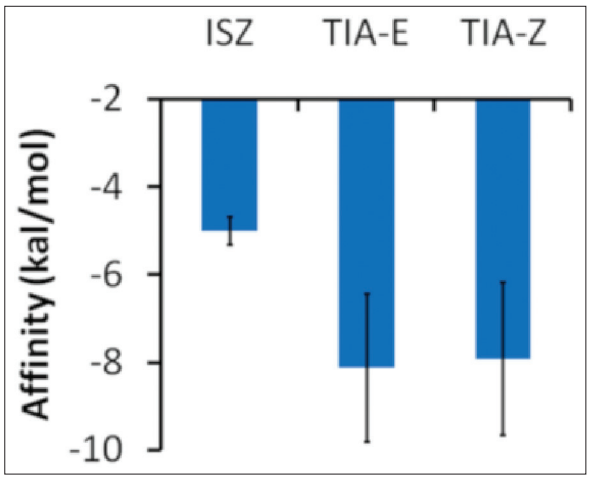

Fig 4. Affinities of izoniazid (ISZ) and the two structures used for thiamethoxam (TIA-E and TIA-Z) by the ascorbate peroxidases of plant origin. Error bars correspond to standard deviations.

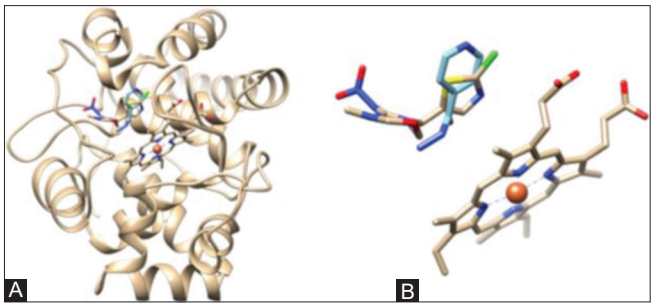

Fig 5. A) Structure of the ascorbate peroxidase 2vcf containing protoporphyrin IX with Fe (HEM) and the anchored structures of thiamethoxam (TIA) and isoniazid (ISZ). B) Amplification of HEM, ISZ and TIA, without enzyme structure and without hydrogen atoms. The image was generated using the computer program UCSF Chimera 1.10.1 (Pettersen et al., 2004).

It is interesting to note that the three-dimensional structure of APX 2vcf is derived from the ISZ-containing enzyme crystal at its active site, which probably shaped such a site to maximize the attractive energies between the ligand and the enzyme. However, for other enzymes, without ISZ or any other ligand in their active sites, in the experimentally obtained crystalline structures, slightly different interactions were observed between the docked ligands and the enzyme. For example, for 1apx enzyme (Patterson and Poulos, 1995), hydrogen bonds are observed between ISZ and amino acid residues HIS42 and TRP41. For TIA only hydrogen bonding with HIS42 is observed (Fig. 7). However, even so TIA has affinity of -9.0 kcal/mol, while ISZ has affinity of -5.1 kcal/mol. Thus, all results suggest that TIA is a better binder for APX's than ISZ. Consequently, TIA is likely to act on plant development by inhibiting its APX.

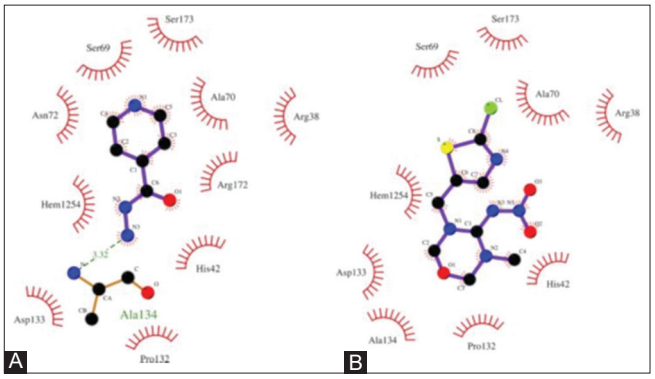

Fig 6. A) Two-dimensional representation of isoniazid (ISZ) interactions with protoporphyrin IX with Fe (HEM) and amino acid residues of ascorbate peroxidase 2vcf. B) Two-dimensional representation of the interactions of thiamethoxam (TIA) with HEM and amino acid residues of 2vcf. The image was generated using the computer program LigPlot + 1.4.5 (Wallace et al., 1995).

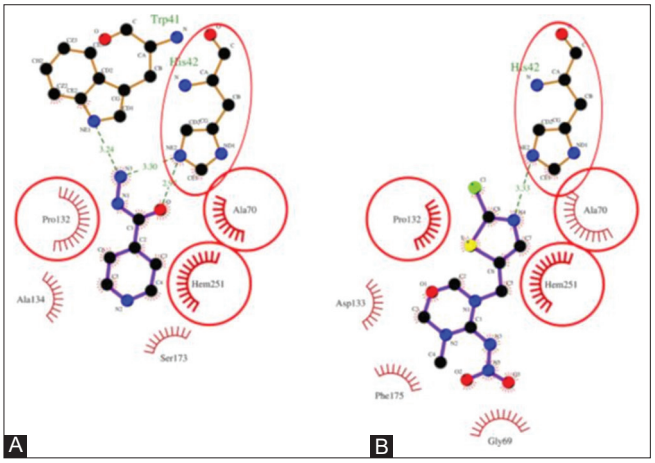

Fig 7. A) Two-dimensional representation of isoniazid (ISZ) interactions with protoporphyrin IX with Fe (HEM) and amino acid residues of ascorbate perdoxidase 1apx. B) Two-dimensional representation of thiamethoxam (TIA) interactions with HEM and amino acid residues of 1apx (Patterson and Poulos 1995). The image was generated using the computer program LigPlot + 1.4.5 (Wallace et al. 1995).

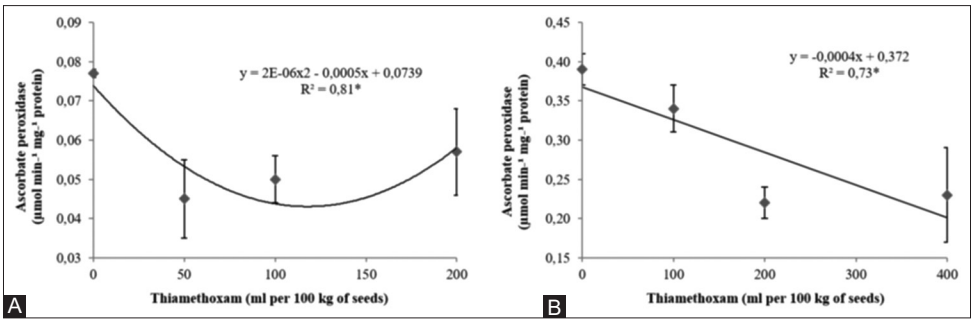

Fig 8. Ascorbate peroxidase activity in seedlings obtained from seeds treated with different doses of thiamethoxam: A) wheat; B) soybean.

Effect of thiamethoxam on soybean and wheat ascorbate peroxidase

The treatment of seeds of a monocotyledonous species (*T. aestivum*) and an eudicotyledonous species (*G. max*) with TIA causes considerable reductions in APX activities, with a quadratic fit in the regression for wheat (Fig. 8A) and linear fit in the regression for soybean (Fig. 8B).

These data corroborate the results of the computational calculations, since the increase of TIA concentration in seed treatment promoted a significant decrease in APX activity. As this decrease is accompanied by plant growth, perhaps the inhibitory action of TIA on this enzyme is inducing a refined control of oxidative activity on cell membranes to allow cell expansion (Dietz, 2003; Foyer and Noctor, 2013). In addition, the change in this enzyme activity, reported to occur during cellular differentiation, could also contribute to regulate wall plasticity (Dietz, 2003; Foyer and Noctor, 2013). These possibilities are in line with the fact that peroxidases are a growth-limiting factors in the outer epidermal wall of the coleoptile according to histochemical assays (Schöpfer, 1996). Thus, when peroxidase inhibitors are used under *in vitro* condition, wall-stiffening through reaction with H_20_2 is suppressed (Schöpfer, 1996). Furthermore, hydrogen peroxide can act in plant redox homeostasis to negatively regulate abscisic acid (ABA) and zeatin riboside (ZR) content in pea plantlets tissue (Barba-Espin et al., 2010).

At the end of this study, it was possible to identify a potential pathway of regulation/signaling of the pesticide TIA, which regulates the balance of peroxidase in plant tissues and allows a fine adjustment in ROS production its action on plant cell expansion. These results suggest that this mechanism is involved in the biostimulant response observed by Macedo and Castro (2011); Junior *et al.* (2019)) of TIA when applied to treat soybean and wheat seeds. These plant responses can promote a real rentability to farms.

CONCLUSIONS

Virtual screening through a pharmacophoric search, followed by *in silico* molecular docking, suggests that thiamethoxam binds more strongly to ascorbate peroxidase than isoniazid. This result is in line with the experimental quantification of ascorbate peroxidase activity in seedlings obtained from seeds of soybean and wheat that were treated with thiamethoxam. As the concentration of thiamethoxan increased, the activity of ascorbate peroxidase decreased, corroborating the result of the *in silico* study, which suggested that thiamethoxam caused plant growth by inhibiting the enzyme ascorbate peroxidase.

Authors' Contributions

Iandra Rocha Barbosa conducted the experiment and performed the statistical analysis; Danúbia Aparecida Costa Nobre assisted the greenhouse and laboratorial analysis, and performed the statistical analysis; Denilson Ferreira Oliveira carried out the bioinformatic analysis and assisted wrote the manuscript; Geraldo Humberto Silva designed the experiment, wrote the manuscript and supervised the assays; Willian Rodrigues Macedo designed the experiment, wrote the manuscript and supervised the assays. All authors commented on previous versions of this manuscript and agree with it publication.

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