University of São Paulo
"Luiz de Queiroz" College of Agriculture

Interaction of PPO-inhibitor herbicide mixtures and mechanistically studies thereof

Ana Paula Meirelles Menzani

Thesis presented to obtain the degree of Doctor in Science. Area: Crop Science

Piracicaba
2017

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Menzani, Ana Paula Meirelles

Interaction of PPO-inhibitor herbicide mixtures and mechanistically studies thereof / Ana Paula Meirelles Menzani. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2017.

110 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

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Acknowledgements

I thank the University of Sao Paulo (USP), College of Agriculture “Luiz de Queiroz” (ESALQ) for the valuable contribution in my professional development.

I wish to thank my advisor Prof. Dr. Pedro Jacob Christoffoleti, in particular, for mentoring me during my project; his encouragement, technical assistance, and patience, that made this possible.

I would like to express my sincere gratitude to BASF for enabling me to conclude this important step in my personal and professional development. I am grateful for the opportunity to work on such a dynamic project that has provided me the opportunity to develop the tools necessary to succeed as a scientist conducting research within the discipline of Weed Science.

I thank Richard Evans from BASF Corporation for his support, guidance, corrections and insights, attention to detail, and expertise throughout the course of my degree that made this possible. I thank him for remaining confident in my abilities. I thank the committee members of each step of this thesis: Dr. Raphael Aponte, Dr. Stefan Tresch, Dr. Iwona Stephan, Dr. Klaus Kreuz, Michael Knapp and Anette Griebel, from global research crop protection in BASF SE and also, Troy Klingaman from BASF Corporation, for all their scientific and valuable insights and advice.

No doctoral work would have been possible or successful without the support of Ademar De-Geroni, Luiz José, Walter Dias and the herbicides team of Valdomiro Donizete and Sabrina Bandeira. I would like to thank as many people as possible who helped me along my journey, and in particular I would like to thank Eliana Rodante Piffer for all support in English language learning and Bianca Martins for helping in the statistical analysis in Chapter III.

I would like to thank my family, especially my mother Meli Mazzi, for her tremendous support, guidance, and unconditional love, which she has unselfishly given throughout my entire life. The successes in my life would not have been possible without her. To Vanair and Valdemir, I thank you both for your wonderful friendships, support and guidance.

Finally, I would like to thank my lovely husband Paulo Cesar Menzani (and our family), for his support and tremendous love. I’m very grateful that there’s a wonderful man supporting me.

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RESUMO

Interação de herbicidas inibidores da PPO e estudos fisiologicamente relacionados

Agricultura é responsável por fornecer alimento e fibras necessárias para sustentar a população mundial. Controle de plantas daninhas é essencial para obter uma boa produtividade. O uso intensivo de herbicidas que age no mesmo sitio de ação ou são detoxificados por processos similares pelas plantas daninhas resulta geralmente no desenvolvimento de plantas daninhas resistentes a um herbicida específico ou à uma classe de herbicidas. A resistência de plantas daninhas devido ao uso de culturas tolerantes à glifosato tem se tornado um dos mais sérios problemas na agricultura. Inibidores da PPO pode ser uma ferramenta para mitigar o desenvolvimento de plantas daninhas resistentes. Há poucos relatos de plantas daninhas resistentes a este mecanismo de ação. Quando aplicados na dose recomendada, apresentam perfil toxicológico favorável e além disso, a maioria dos herbicidas deste grupo são compatíveis com plantio direto. No entanto, são mais eficientes em dicotiledôneas do que em monocotiledôneas. O objetivo dessa tese foi fornecer informações em relação a associação binária de herbicidas inibidores da PPO no controle de plantas daninhas. As associações mostraram efeito sinérgico no controle de *Echinochloa crus-galli* e milho voluntário, além do controle de dicotiledôneas. Avaliou-se também a atividade de alguns inibidores da PPO na inibição de 50% da enzima PPO2 e observou-se que para inibir 50% da PPO2 necessitou de menor quantidade de trifludimoxazin e flumioxazin em todas as plantas testadas, enquanto que sulfentrazone e saflufenacil, que associados apresentaram a melhor eficácia nos ensaios de campo, mostraram que precisar de maiores concentrações para inibir a PPO2 comparada aos outros produtos. A absorção e translocação destes produtos, isolados ou em mistura, mostraram que a absorção foi mais lenta em milho do que em *E. crus-galli*. Os herbicidas apresentaram comportamentos similares, sendo absorvidos quase 95% até 72 horas após aplicação, com exceção do trifludimoxazin, que foi significativamente mais lento que os outros. Saflufenacil foi o herbicida que apresentou melhor translocação na folha aplicada, enquanto trifludimoxazin não apresentou nenhuma translocação. Em relação às associações, os produtos mostraram diferenças na absorção e translocação, variando conforme as plantas daninhas estudadas. Alguns eventos tolerantes a inibidores da PPO foram avaliados em *Arabidopsis thaliana* e indicaram como potenciais eventos para ser desenvolvidos nas culturas de interesse.

Palavras-chave: Inibidores da Protox; Associação de herbicidas; IC_{50}; Absorção e translocação; Tolerância à herbicidas

ABSTRACT

Interaction of PPO-inhibitor herbicide mixtures and mechanistically studies thereof

Crop production provides the food and fiber necessary to sustain the world's population. Effective weed management is critical to maintaining agricultural productivity. Intensive or continuous use of herbicides that act on the same target site, or are detoxified by similar processes within crops and target weeds frequently results in the development of weeds resistant to a specific herbicide or class of herbicides. Weed resistance due to the extensive use of glyphosate in glyphosate tolerant crop systems has become one of the most serious issues facing agriculture today. Thus PPO-inhibitor herbicides are an alternative mechanism of action that have the potential to mitigate the development of resistant weeds in weed control systems where crop tolerance is sufficient to allow them to be effectively deployed. While there are few reports of weed resistance to PPO-inhibitor herbicides it has not developed to the extent that it is a commercial problem for growers. When used at recommended doses, they typically have favorable regulatory profiles. Furthermore, most of them are highly compatible with no-tillage agriculture. However, PPO-inhibitor herbicides are typically more active on dicots than monocots. This thesis provides information with regards to effectiveness of certain binary mixtures of PPO herbicides as weed control agents. The mixtures showed synergistic effects and could control monocots as *Echinochloa crus-galli* and volunteer corn besides control the dicots species. Regarding the compounds studied, trifludimoxazin and flumioxazin required less amount of compound to inhibit 50% of PPO2 activity in all plants species tested, while sulfentrazone and saflufenacil, one of the best mixtures in the field, showed that needed bigger concentrations to inhibit 50% of the PPO2 compared to other compounds evaluated.The absorption and translocation of these compounds individually or in mixtures, showed that as single compounds, the absorption was slower in maize than *E. crus-galli* and there was no difference among the herbicides except trifludimoxazin, that was significantly lower than the other compounds. Saflufenacil was the herbicide that showed the best translocation out of treated leaf point, while trifludimoxazin has not shown any translocation out of leaf treated. In mixtures, the compounds showed some differences in absorption and translocation, which it was variable according to species studied. Some traits of PPO-tolerant were also evaluated in *Arabidopsis thaliana* which indicates potential traits to be developed in crops of interest.

Keywords: PPO-inhibitors; Binary mixtures; IC_{50}; Absorption and translocation; Herbicide-resistant crops

1 INTRODUCTION

For more than 10000 years, plants have been cultivated to provide food and fiber to sustain the world's population. The need to cultivate plants for the purpose of feeding and clothing humanity inspired the development of organized agriculture. Threats to agricultural productivity may have serious consequences for humanity.

Effective weed management is critical to maintaining agricultural productivity. Weeds reduce crop yield and quality by competing with crops for light, water, and nutrients. Weed competition results in billions of dollars in global crop losses annually. Herbicides are an important tool used by growers to manage weeds for the purpose of improving agricultural productivity and preserving crop yields and quality.

The introduction of herbicide resistant crops technology, such as glyphosate-resistant, declined the use of other herbicides options and less investment by industry to discover new herbicide active ingredients. Also, this technology enabled a weed control practice that is effective, easy-to-use, economical, and safe, resulting in a great change in the strategy of managing weeds. As a consequence, a single mechanism of action has been used to manage weeds, resulting in a big issue to resistant weed management.

However, growers usually do not recognize the weed potential to evolve resistance to glyphosate, until the biotypes appear in their fields and, unfortunately, for long time the chemical industry has not commercialized herbicides with new mechanism of action. Part of the reason that there have not been new mechanism of action or target site is because it is not easy and also regulator thresholds for suppressing innovation, banning actives and reducing options.

Furthermore, among the reason for the low discovery of new herbicides is the great number of chemicals that must be tested to discover a new herbicide sharply increase and the investment, besides be very high, glyphosate-resistant crops, in particular Roundup Ready soybeans, or Clearfield-crops had reduced the market opportunities.

Growers have to diversify the herbicides use to mitigate the spread of resistant weeds because almost all commercially available herbicides mechanisms of action have documented cases of herbicide-resistant weeds. It is known that tank-mixtures of different compound is highly used by growers and also could be a tool for weed resistance management. The mixtures of herbicides can be resulted in additive, synergic or antagonistic effect and need to be evaluated before applied.

Furthermore, one strategy to sustain the weed control is to develop additional trait that provides resistance to herbicides with alternative mechanisms of action. After three decades and billions of dollars invested in research and development, only few transgenic herbicide traits are commercially available.

One particular herbicidal class, known as the protoporphyrinogen oxidase inhibitor herbicides, for short called PPO or Protox, has been reported only 13 weeds with resistance to this herbicide class. *Amaranthus tuberculatus* and *A. palmeri* were the first weeds species documented with PPO-resistance, which was due to a single codon deletion Gly210 in a dual-targeting gene known as PPX2L.

The few resistant cases reported may due to the relatively short-lived selection pressure of these fast-acting foliar herbicides applied. However, the development of more persistent soil-active PPO-inhibitors might increase the selection pressure and consequently may raise the likelihood of resistance development.

PPO-inhibitors are a very important herbicide target that has been under utilized due to crop tolerance restrictions. Thus PPO herbicide tolerant would enable an effective tool to be deployed more broadly in weed management systems.

The PPO enzyme catalyzes the conversion of Protoporphyrinogen-IX (Protogen IX) to Protoporphyrin-IX, which is the last common step in the biosynthesis of heme and chlorophyll molecules. In plants, two PPO isoforms are encoded by two different PPO nuclear genes, PPO1 and PPO2, which are located in plastids and mitochondria respectively, however, PPO2 isoforms are dual targeted to both organelles.

The herbicide action occurs by enzyme competition between PPO herbicide and Protoporphyrinogen-IX. Since the PPO family of herbicides has more affinity for the enzyme, Protoporphyrinogen-IX accumulates in the chloroplast or mitochondria and diffuses into the cytoplasm which subsequently is converted to Protoporphyrin-IX by plasma membrane peroxidases and enzymatic oxidation. Exposure to light causes formation of singlet oxygen and other oxidative species, resulting in membrane disruption and subsequent cell death.

PPO-inhibitors control broadleaf weed selectively. The symptoms observed on the foliage as leaf cupping, crinkling, bronzing, and necrosis may be observed after two days of application in post emergence conditions.

The PPO enzyme is inhibited by several herbicides chemical class such as diphenyl ether, phenyl imides, triazolinones, pyrazoles and pyrimidinedione. There

are many advantageous characteristics of PPO-inhibitors that include have low mammalian toxicity, low effective doses, rapid onset of action and long residual activity on some herbicides.

For those reasons, the main objectives of this thesis were: 1) identifying key potential binary mixtures of PPO-inhibitors for weed control, mainly monocots, since it is known that PPO herbicides are generally more active on dicots than on monocots, although the enzyme target appears to be equally sensitive to the herbicides; 2) measuring the effectiveness of PPO-inhibitor herbicides in inhibiting PPO1 and PPO2 enzyme by IC_{50} (half maximal inhibitory concentration) from *Amaranthus tuberculatus, Setaria viridis* and *Alopecurus myosuroides*; 3) better understanding about the mechanism of absorption and translocation of those compounds either single or mixtures; and finally, 4) finding a good trait to develop new herbicide-resistant crops, by evaluating the activity of PPO compounds in transgenic *Arabidopsis thaliana* with different PPO isoforms. Furthermore, since two traits were based on natural mutations enabling discuss resistance management and finally herbicide tolerant traits discussion.

1.2 Literature Review

Protoporphyrinogen oxidase, also called PPO or Protox, is a key enzyme in the synthesis of chlorophyll and heme. Protox-inhibitor herbicides inhibit this enzyme which catalyzes the six-electron oxidation of Protoporphyrinogen-IX to Protoporphyrin-IX. They also are referred to as tetrapyrrole-biosynthesis inhibitors or Protoporphyrin-IX synthesis inhibitors (DAILEY et al., 1995; SMITH et al., 1993).

It is the last common pathway in the production of heme and chlorophyll. While the production of chlorophyll, a light-harvesting pigment, is an essential process for all green photosynthetic organisms, heme is an essential cofactor in cytochromes, hemoglobin, oxygenases, peroxidases and catalases, which are important in stress reduction due to the ability to inactivate free radicals (CHAUDIÈRE & FERRARI-ILIOU, 1999). This characteristic makes PPO an excellent enzyme target for herbicide development (LEHNEN et al., 1990; JACOBS et al., 1991; DAYAN & WATSON, 2011).

The herbicide action occurs by enzyme competition between PPO herbicide and Protoporphyrinogen-IX. Since the PPO family of herbicides has more affinity for

the enzyme, Protoporphyrinogen-IX accumulates in the chloroplast or mitochondria and diffuses into the cytoplasm and is converted through enzymatic oxidation by the plasma membrane peroxidases to Protoporphyrin-IX (JACOBS et al., 1991; DAILEY et al., 1995). Once in the cytoplasm, Protoporphyrin-IX cannot return to the chloroplast because it is highly lipophilic (LEHNEN et al., 1990).

Exposure to light causes formation of singlet oxygen and other oxidative species, resulting in membrane disruption and subsequent cell death. PPO inhibitor herbicides have characteristically a very rapid contact action, causing leaf burning, desiccation and growth inhibition resulting in complete death of plants (JACOBS et al., 1991; MORI & SCHROEDER, 2004; DAYAN & WATSON, 2011).

Duke et al. (1991) reported that the damage resulting from the peroxidation of lipids is the initial degradation of the plasma lemma and tonoplast membranes, followed by inhibition of photosynthesis and evolution of ethylene. However, at the primary site of action which is the direct interaction of light and herbicide. Furthermore, the events that cause tissue damage and associated necrosis, are not associated with the primary target and are always due to membrane damage caused by lipid peroxidation of polyunsaturated fatty acids (HESS, 2000).

PPO-inhibiting herbicides are mainly applied in post emergence in the initial growth stages of the weeds. These compounds have much higher activity in post emergence than in pre-emergence, or only a small residual activity in the soils, except sulfentrazone, oxyfluorfen and flumioxazin. (RODRIGUES & ALMEIDA, 2011; DAYAN & DUKE, 2010).

Many PPO-inhibitors provide selective control of broadleaf weeds. The symptoms observed on the foliage are leaf cupping, crinkling, bronzing, and necrosis may be observed two days following post emergence application. The metabolization of Diphenyl Ether Protoporphyrinogen (DPE) Oxidase Inhibitors are the most important mechanism of tolerance by crops. In soybean, for instance, occurs the breaking of ether linkage between the phenyl groups, producing metabolites without herbicidal activity (DAYAN & DUKE, 2010).

In pre-emergence applications, tissue necrosis is initiated when plants emerge above the soil surface and are exposed to light. Crop injury also can happen if heavy rains occur when the plants are emerging through the soil surface. The splashing, from rainfall, causes high concentrations of the herbicide to make contact with the

hypocotyls, cotyledons, and growing points causing tissue necrosis (HARTZLER, 2004).

Protox-inhibitor herbicides show differences in absorption across plants species, however translocation is commonly limited. Nevertheless, slight differences in absorption and translocation can occur, which might explain the tolerance differences of these herbicides. Most of the DPE herbicides showed a higher level of tolerance in soybean due to a lower dose of absorption, limited translocation and metabolization of herbicides in the species (RITTER & COBLE, 1981).

These products cause some symptoms in the leaves of crops, mainly soybean, however these crops recovered rapidly, and the yield is not affected (VIDRINE et al., 1993). Thus, the basis for selectivity of this chemical group can also be attributed to minimum absorption and translocation of herbicide, herbicide sequestration or enhanced mitochondrial PPO enzyme concentration, which serves as a reductant for excess of cytoplasm Protoporphyrinogen (HIGGINS et al., 1988; MATSUMOTO et al., 1999; WARABI et al., 2001).

Root uptake of foliar active compounds is generally poor. Most of DPE herbicides are not translocated beyond the point of absorption. However, some of them are translocated by xylem. Some studies showed that absorption and translocation of DPE herbicides may be affected by temperature and humidity (RITTER & COBLE, 1981).

Sulfentrazone, as a soil active compound, after root uptake and leave translocation is rapidly metabolized without any harm to plant (DAYAN & DUKE, 1997). In soybean cells, resistance to oxyfluorfen was selected because increasing the concentration of enzyme in PROTOX mitochondria, which enabled higher ability to use excess Protoporphyrin-IX present in the cytoplasm (WARABI et al., 2001).

The flumiclorac soybean tolerance is due to reduced absorption and translocation, and high detoxification of this herbicide, while corn tolerance is due to reduced leaf retention and its high metabolization (FAUSEY & RENNER, 2000).

When these herbicides are applied in pre-emergence conditions, they cause the death of plant at the time which they come in contact with the layer treated of soil. Sub lethal doses may produce symptoms of bronzing in young leaves, while the drift of small drops cause white spots and necrosis in young leave (AHRENS, 1994).

The shoot absorption is influenced by characteristics of the leaf surface, such as composition, thickness and arrangement of the cuticle, the environmental conditions and the physicochemical characteristics of the herbicide (VIDAL, 2002).

While the absorption in pre-emergence herbicides is influenced primarily by anatomical and physiological barriers to the pathway of herbicides and physicochemical characteristics thereof. In some species, the herbicides coming to ground are preferably absorbed by the root system of the plants, while in others they are absorbed mainly by the shoots of emerging parts, such as the hypocotyl, epicotyl and coleoptile (ESHEL & PREDEVILLE, 1967).

Dayan & Duke (2010) highlighted that these compounds are effective at very low application doses and have generally good ecotoxicology and human toxicology profiles at recommended application doses. Most of them are highly compatible with the no-tillage agriculture.

The classification of herbicides based on mechanism of action has undergone changes over time, both due to the discovery of new herbicides and the elucidation of the sites of actions in plants. Herbicide Resistance Action Committee (HRAC) system for classifying herbicides by mechanism of action and chemical classes is currently the internationally accepted standard (Table 1).

Trifludimoxazin is a new herbicide, also a PPO-inhibitor, and belongs to Triazinone chemical class (not shown in the table). This herbicide has been developed by BASF SE, but it is still under registration process in some countries such as Canada, Australia, United States and Argentina.

Table 1 - Main PPO-inhibitors herbicides distributed by chemical class, common name, structure and their main crops recommended and the application time, respectively. Table adapted from Dayan & Duke, 2010.

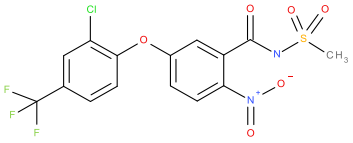
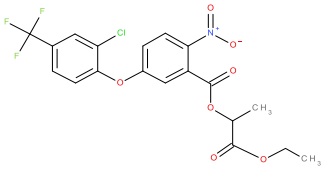
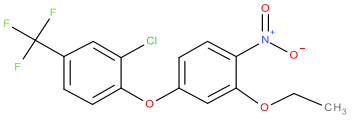
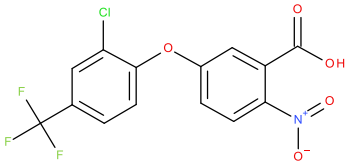
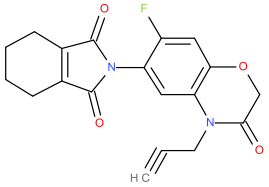
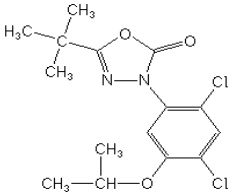
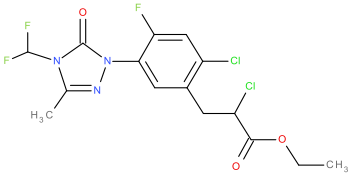
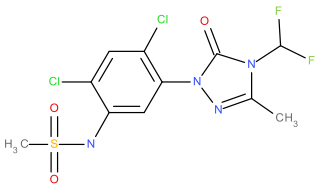
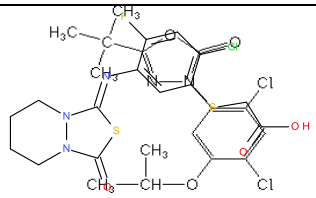
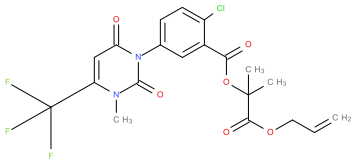
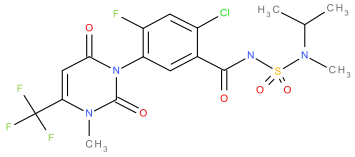
Chemical Class	Common Name	Structure	Main Crop	Application
Diphenyl ether (DPE)	Fomesafen		Soybean	Post emergence
Diphenyl ether (DPE)	Lactofen		Soybean	Post emergence
Diphenyl ether (DPE)	Oxyfluorfen		Vegetable crops	Pre and Post emergence
Diphenyl ether (DPE)	Acifluorfen		Soybean, peanut, rice	Post emergence
N-Phenyl-phthalimides	Flumioxazin		Soybean, peanut	Pre emergence
Oxadiazoles	Oxadiazon		Grasses, ornamentals and vegetable crops	Pre and Post emergence
Triazolinones	Carfentrazone		Cereal crops	Post emergence
Triazolinones	Sulfentrazone		Soybean, sugarcane, tobacco	Pre emergence

Table 1 - [Continuation] Main PPO-inhibitors herbicides distributed by chemical class, common name, structure and their main crops recommended and the application time, respectively. Table adapted from Dayan & Duke, 2010.

Chemical Class	Common Name	Structure	Main Crop	Application
Thiadiazoles	Fluthiacet		Soybean and corn	Post emergence
Pyrimidinedione	Butafenacil		Cotton defoliant	Post emergence
Pyrimidinedione	Saflufenacil		Soybean, sugarcane, desiccation	Post emergence

1.2.1 Protoporphyrinogen oxidase enzyme (Protox or PPO)

Two isoforms of PPO, namely PPO1 (targeted to the chloroplast and encoded by the gene PPX1) and PPO2 (mitochondrial PPO, encoded by the gene PPX2), have been found in plants (LERMONTOVA et al., 1997; POWLES & YU, 2010). According to Dayan & Duke (2010), plant PPO1 is compartmentalized in the thylakoid and in the envelope membranes of chloroplasts, whereas the mitochondrial isoform PPO2 is localized on the outer surface of the inner mitochondrial membrane. In the chloroplast, the porphyrin pathway leads to both chlorophyll and heme, whereas it leads exclusively to heme in the mitochondrion (DAYAN & DUKE, 1997).

Two Protox isoenzymes have been described in tobacco, a plastidic and a mitochondrial form. Protox genes or cDNAs have been cloned from *Escherichia coli*, *Bacillus subtilis*, human, cow, mouse, and yeast. The molecular masses of these Protox gene products range from 50 to 60 kDa, except for the 21-kDa *E. coli.* The N terminus is most widely conserved, and the deduced amino acid sequences of PPO1 and PPO2 are only 27.3% (WATANABE et al., 2001).

The authors still explain that the translation product of PPO1 cDNA translocates to chloroplasts, whereas PPO2 are targeted to mitochondria, suggesting

that tobacco Protox exists in chloroplasts and mitochondria as isoenzymes. Since Protox is the final common enzyme in the chlorophyll and heme biosynthetic pathways in plants, Protox should play a role in distributing Proto IX to both pathways.

Analysis using electron microscopy has demonstrated that spinach PPO1 preferentially associates with the stromal side of the thylakoid membrane, while a small fraction of PPO1 is located on the stromal side of the inner envelope membrane (CHE et al., 2000).

There have been no investigations into the precise suborganellar location of PPO mitochondrial. Little is known about the transport mechanism of mitochondria PPO1, which in general, proteins transported into the mitochondria have an N-terminal targeting peptide that is processed after transport is complete (GLASER et al., 1998).

While tobacco PPO2 and other homologs do not process the typical mitochondrial targeting sequence at their N termini. Trials conducted *in vitro* by Lermontova et al. (1997) showed that tobacco PPO2 is transported to mitochondria without any size reduction. The mechanism by which this occurs is not yet understood.

1.2.2 Chemical Group: Pyrimidinediones

Saflufenacil [N' - [2 - cloro - 4 - fluoro - 5 - (3 - metil - 2,6 - dioxo - 4 - (trifluorometil) -3,6 -di-hidro - 1 (2H) pirimidinil) benzoil-N - isopropil-N - metilsulfamida] is a new herbicide developed by BASF for pre-plant burndown and pre-emergent broadleaf weed control in corn, soybean, cotton, wheat and sorghum (BOWE et al., 2008; GROSSMANN et al, 2010; LIEBL et al., 2008; GEIER et al., 2009; SOLTANI et al., 2010; LYON & KNISS, 2010).

Grossman et al. (2010) and Menalled (2011) confirmed that saflufenacil has the same physiological changes in plants that are caused by other herbicides that are PPO inhibitor.

Saflufenacil is translocated mainly in xylem and has limited mobility in the phloem (LIEBL et al., 2008; ASHIGH & HALL, 2010). Grossmann et al. (2010) reported that saflufenacil is a weak acid, which ionizes in solution and provide mobility in xylem and phloem distributing systemically in the plant.

Injury symptoms to susceptible species appear within a few hours, and these plants die in 1 to 3 days (FRIHAUF et al., 2010a; LIEBL et al., 2008). Most of selectivity studies have been conducted in corn, which is able to restrict the translocation of herbicide from the leaves to other parts of plant due to the fast metabolism that occurs in its early metabolites.

It demonstrates pre-emergence selectivity based on physical positioning and rapid metabolism in tolerant crops (BOWE et al., 2008). Several studies have approached saflufenacil efficacy in pre and post emergence. Researchers compared the application of saflufenacil in pre-versus post emergence. Saflufenacil is easily absorbed by the foliar tissue of the plant rather than root tissue.

Studies suggest that the plants are 100 times more sensitive for foliar application saflufenacil than the roots (GROSSMAN et al., 2011). It can explain why saflufenacil has better performance in post emergence application.

Geier at al. (2009) mentioned that saflufenacil applied in post emergence at 6 to 30 g.ha-1 reduced population density of 5 broadleaf weeds (*Chorispora tenella, Descurainia sophia, Amaranthus palmeri, A. retroflexus e A. albus*) by 63 to 93% in a dose response study.

Waggoner et al. (2011) observed that saflufenacil at 25g.ha^{-1} provided more than 95% of control of *Conyza canadensis* and it was an optimal dose for tank mixtures with glyphosate, glufosinate and paraquat. Whereas Owen et al. (2011) reported that saflufenacil at 25 and 50 g.ha^{-1} also controlled *C. canadensis* and showed a residual effect of 51 days in cotton. In the spring, 50 g.ha^{-1} of saflufenacil provided 8 weeks of residual control in *C. canadensis* (DAVIS et al., 2010) showing in addition of burndown control, it also provided efficient residual.

Saflufenacil is broadleaf weed herbicide and needs to be tank mixed with other herbicides to increase weed control spectrum (JHALA et al., 2013). The mixtures of two products can result in additive, synergic or antagonistic effect. When the result is greater than expected, the mixture is called synergistic, when the result is less than expected, it is considered antagonistic and when the result is similar to expected, it is called of additive. There are several methods to calculate synergistic or antagonistic effects between herbicides. Colby (1967) method is a classic methodology valid only for cases where the combination components exhibit similar non-action (TREZZI et al., 2007).

Several studies have been developed in Brazil in order to evaluate synergistic or antagonistic effect between glyphosate and saflufenacil. Valente et al. (2010) reported that the addition of 50 and 70 g.ha^{-1} of saflufenacil in 1080 g.ha^{-1} of glyphosate provided control above 90% of *Conyza* sp. at 24 days after treatment. Dalazen (2012) observed that this mixture avoids regrowth of weeds.

The same authors compared the efficacy of saflufenacil alone and in tank mixture with Glyphosate (1061 g.ha^{-1}) and noticed that all tank mixture treatments controlled *C. canadensis* seven days after application regardless the dose of saflufenacil used (6,3; 12,5; 25 e 50 g a.i. ha^{-1}). Saflufenacil alone did not show good efficacy in this study.

According to Anónymous (2008), the application of both saflufenacil and glyphosate in burndown pre-planting improve the control of weeds compared to glyphosate alone, and provide good residual control.

The tank mixture of saflufenacil and glyphosate, respectively in the doses of 24,5 + 1188; 35,0 + 1188 and 49,0 + 1188 g of active ingredient per hectare, provided excellent control of *Sida rhombifolia, Bidens pilosa* and *Brachiaria decumbens* in the citrus trial according to Foloni et al. (2010).

Saflufenacil can be considered complementary to glyphosate, being recommended this mixture to control tough weeds, such as *Conyza* due to saflufenacil allows glyphosate mobility, resulting in a possible synergistic interaction (BOWE et al., 2008). Frihauf et al. (2010b) reported that wheat crops absorbed from 2.8 to 3.5 times more Saflufenacil when applied in tank mixture with 2.4-D amine compared to saflufenacil alone.

According to Diesel et al. (2012a), the mixtures of saflufenacil and metribuzin improved the control and reduced the dry matter of *Alternanthera tenella* showing a synergistic effect. However, in another study conducted by these authors, clomazone applied alone did not provide good efficacy of *A. tenella*, while the mixture with saflufenacil showed the same results of saflufenacil alone (DIESEL et al., 2012b).

Adjuvants are typically mixed with herbicides to improve efficacy or modify certain properties of the solution, facilitating the application or minimize potential problems by improving herbicide activity.

Several laboratories studies were conducted to measure the absorption and translocation of saflufenacil in plants. Frihauf et al. (2010b) reported that adjuvants

improved the absorption of saflufenacil from less than 20% to 80% in foliar tissue of winter wheat.

Furthermore, the saflufenacil absorption has been increased when in tank mix with glyphosate formulation, that includes its own surfactant. This increased absorption has been attributed to the high surfactant loading. Saflufenacil in tank mixture with glyphosate pure compound and a surfactant resulted in a similar absorption of saflufenacil solo plus adjuvants (ASHIGH & HALL, 2010).

The authors also reported that, in cabbage crops these mixtures resulted in reduced translocation of this herbicide and it may due to the adjuvant presence, improving the quick contact action, and resulting in less translocation of glyphosate. They concluded that the absorption and translocation of saflufenacil may be influenced by the tank mix with other herbicides or even by the adjuvants addition.

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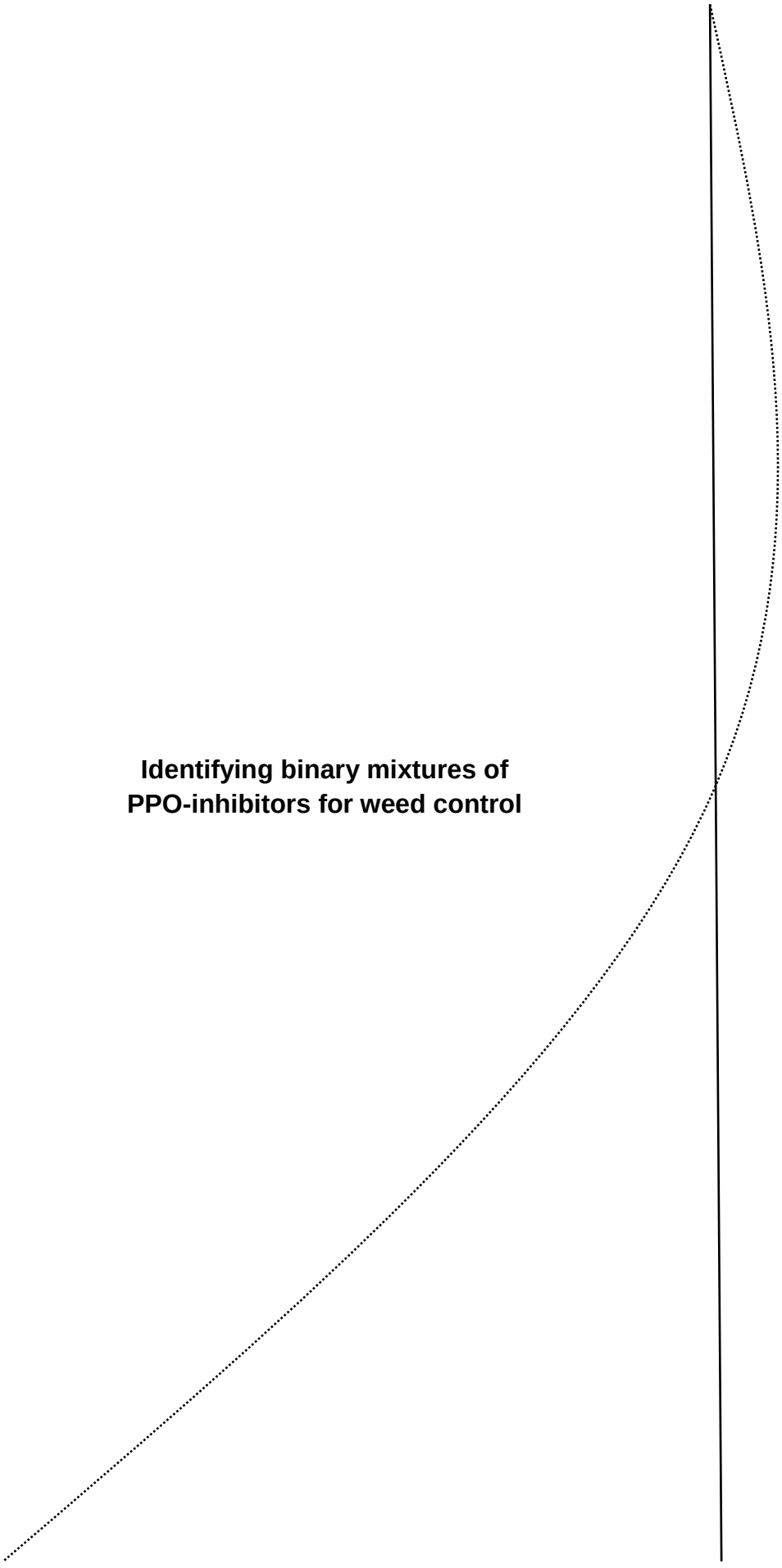
CHAPTER I

Identifying binary mixtures of PPO-inhibitors for weed control



Abstract

Identifying key binary mixtures of PPO-inhibitors for weed control

Identifying herbicides with broad spectrum of weeds would be useful to avoid and manage the spread of resistant weeds in the agriculture. Among known herbicide chemistries and mechanisms of action, PPO inhibitors would be a valuable tool for future management of weeds resistant to glyphosate, ALS herbicides, PSII herbicides, HPPD herbicides, and other mechanisms of action because due to relatively lower selection pressure PPO resistance has not had a major impact on the utility of PPO herbicides. Additionally, the accessibility of the target, and structural diversity of the chemistry classes that there are many options within PPO herbicides to provide effective control of weeds and address the challenges related to weed resistance. For this reason, the main objective of this research was identifying binary mixtures of PPO-inhibitors that would effectively control monocot weeds. The studies were conducted in Santo Antonio de Posse/Brazil and Seymour/United Stated with two weeds and two volunteer crops: *Amaranthus* sp. and *Echinochloa crus-galli, Glycina max* and *Zea mays*. Saflufenacil and carfentrazone-ethyl were chosen due to a good post emergence efficacy, while trifludimoxazin, flumioxazin and sulfentrazone were selected due to their residuality. The best mixtures to improve the spectrum of control were saflufenacil with flumioxazin, sulfentrazone, or trifludimoxazin. These binary mixtures controlled *E. crus-galli* and volunteer corn as well all dicots species evaluated. In addition, it is likely that this tank mix treatment would control volunteer glyphosate-resistant crops.

Keywords: Monocots control; Saflufenacil; Tank-mix; Synergism

2.1 Introduction

In Brazil, soybean (*Glycine max* L.) and maize are cultivated on 33.9 and17.4 million hectares, respectively. Area planted to soybean accounts for over 65% of the total area cultivated in Brazil (CONAB, 2017). In the United States of America, the estimated area planted with corn for all purposes is 36.8 ha for 2017, down 3 percent from last year, and soybean was 36.2 million hectares, up 7 percent from last year (USDA, 2017).

Weed control is one of the major activities associated with crop production. If not controlled, weeds may compete with the crop for nutrition, water, and light, and may also increase pest problems (SINGH, 2011).

A large number of herbicides families are directly or indirectly influenced by photochemical reactions. These herbicides inhibit electron flow in photosystem II in the photosynthetic light reaction; capture electrons in photosystem I in the

photosynthetic light reaction; inhibit glutamine synthetize in the nitrogen assimilation pathway; directly or indirectly inhibit carotenoid biosynthesis; or inhibit protoporphyrinogen oxidase during chlorophyll biosynthesis (HESS, 2000).

PPO-inhibiting herbicides were commercialized in the 1960's and their market share reached about 10% of all herbicide value in the late 1990s. The site of action of this chemical group only was known in 1989 (MATRINGE et al., 1989).

The widespread adoption of glyphosate-resistant crops has caused a significant reduction of the field application of PPO inhibitors, and these herbicides accounted for only 1.3% of total value herbicide output in the United States in 2006 (DAYAN & DUKE, 2010).

The introduction and extensive utilization of glyphosate-resistant soybean cultivars and corn hybrids led to weeds shifts and selection of glyphosate-resistant weeds in the United States and Brazil led to an increased need for alternative herbicide programs including tank mixes with herbicides having a different mechanism of action (BECKIE, 2006; DAYAN & DUKE, 2010).

Therefore, a strategy is required to avoid glyphosate-resistant weeds by identifying a new herbicide chemistry with a different mechanism of action (SINGH, 2011) and including non-glyphosate-resistant crops in the rotation (WILSON et al., 2007).

It is also important to highlight that the glyphosate-resistant volunteer maize and soybean has become a problem in the agriculture system, mainly when maize succeeds soybean in crop protection and vice versa, and both are glyphosate-resistant crops (DEEN et al., 2006; SOLTANI et al., 2006). And besides that, no major new site-of-action for herbicides has been introduced into the marketplace for about 20 year (BECKIE & TARDIF, 2012).

Currently, only 13 Protox inhibitors-resistant weeds have been reported in the world, according to Heap (2017). Dayan & Duke (2010) mentioned that the market niche for Protox inhibitors was beginning to expand to weed control in monocot crops.

It is important to highlight that among known herbicide chemistries and mechanisms of action, PPO inhibitors would be a valuable tool for future management of weeds resistant to glyphosate, ALS herbicides, PSII herbicides, HPPD herbicides, and other mechanisms of action because due to relatively lower

selection pressure PPO resistance has not had a major impact on the utility of PPO herbicides.

Additionally, the accessibility of the target, and structural diversity of the chemistry classes that there are many options within PPO herbicides to provide effective control of weeds and address the challenges related to weed resistance, enabling the development of new compounds by other companies.

The main objective of this phase was identifying key mixtures of PPO-inhibitors in order to improve the spectrum of the compounds alone, controlling monocots and dicot weeds. For this was evaluate efficacy and potential synergism of binary mixtures of PPO-inhibitors in the control of *Glycina max*, *Zea mays*, *Amaranthus* sp. and *Echinochloa crus-galli* in two different regions. Saflufenacil and carfentrazone-ethyl were chosen due to a good efficacy in burndown application related in the literature.

Already for residual control, trifludimoxazin, flumioxazin and sulfentrazone were chosen also based on literature (DAYAN & DUKE, 2010, EVANS in personal contact). The mixtures mentioned in the project were chosen based on intern previous work.

Trifludimoxazin is a new PPO-inhibitor herbicide, and belongs to Triazinone chemical class. This herbicide has been developed by BASF SE, but it is still under registration process in some countries such as Australia, Canada, United States and Argentina.

2.2 Material and Methods

The trials were conducted in two different regions both important to soybean and maize production. The first trial was conducted in Midwest Research Farm located in Seymour, Illinois, United States (Latitude: 40.4º, Longitude: 88.4º and Altitude: 244 m) from May to June, 2014. The area was previously used for maize.

The soil was classified as Clay Silty, which chemical and particle size analyses are presented in the Tables 2.1 and 2.2. The area was prepared without herbicide application and then one row of maize (variety Channel 213-52) and one row of soybean (variety ASGROW 3832) were sowed on May, 30th 2014. Maize and soybean were used as indicators of monocots and dicots as well as volunteer crops. *Amaranthus palmeri* and *Echinochloa crus-galli* (natural infestation) also were evaluated as well.

The second and third trial were conducted at the Agricultural Research Station located in Santo Antônio de Posse, São Paulo, Brazil (Latitude: 22,6ºS, Longitude: 46.9ºW and Altitude: 609 m) from March to May, 2015 and after, February to March, 2016. The soil was classified as Clay Sandy, which chemical and particle size analyses are presented in the Tables 2.1 and 2.2.

The weeds were seeded since the area did not show good infestation of the species in study. As *Amaranthus palmeri* is an invasive weed in Brazil, *Amaranthus viridis* and *Amaranthus hybridus* were seeded instead. The same occurred with the varieties of maize and soybean and the most meaningful varieties were seeded then.

The seeds of weeds were collected at the Research Station in the season 2013-2014 and stored at 5ºC until used in this study. The area was prepared and sowed one row of each plant, maize (variety DK 390), soybean (variety BMX Potência), *Amaranthus viridis, A. hybridus* and *Echinochloa crus-galli*.

Table 2.1 – Soil chemical analysis of Seymour, USA and Santo Antônio de Posse, BR respectively.

Area	pH	M.O.	P	K	Ca	Mg	B
	$CaCl_2$	%	$mg.dm^{-3}$		$mmol.dm^{-3}$		$mg.dm^{-3}$
Seymour, USA	6.1	3.2	38.5	0.48	134.7	224.5	0.29
Sto. Antônio de Posse, BR (2015)	5.2	2.3	71	2.6	17	5	0.25
Sto. Antônio de Posse, BR (2016)	5.4	1.6	33	2.9	18	7	0.71

Table 2.2 – Soil particle size analysis of Seymour, USA and Santo Antônio de Posse, BR respectively.

Area	Sand	Silt	Clay	Soil Texture
		%		
Seymour, USA	20	52	28	Clay Silty
Sto. Antônio de Posse, BR (2015)	48.6	11.1	40.3	Clay Sandy
Sto. Antônio de Posse, BR (2016)	46.1	8.5	45.4	Clay Sandy

The treatments are mentioned on Table 2.3, which the doses of products applied isolated were combined in tank mixes according to low, intermediate and high doses. Herbicide treatments were applied in late post emergence, when the weeds were 6 to 8 leaves of dicots or 3 to 4 tillers to monocots. In the untreated was evaluated the cover percentage of weeds.

Table 2.3 – Treatments: herbicides, products concentration/formulation and doses (g a.i.ha^{-1}) applied in the trials.

Herbicides	Product Concentration (g kg^{-1})/Formulation	Doses (g a.i.ha^{-1})
Untreated		
Saflufenacil*	700 WG	12.5
		25.0
		50.0
Trifludimoxazin*	500 SC	12.5
		25.0
		50.0
Flumyoxazin*	500 WP	35.0
		70.0
		140.0
Sulfentrazone*	500 SC	105.0
		210.0
		420.0
Carfentrazone-ethyl*	400 EC	8.75
		17.5
		35.0
Saflufenacil + Flumyoxazin*	700 WG + 500 WP	12.5 + 35.0
		25.0 + 70.0
		50.0 + 140.0
Saflufenacil + Trifludimoxazin*	700 WG + 500 SC	12.5 + 12.5
		25.0 + 25.0
		50.0 + 50.0
Saflufenacil + Sulfentrazone*	700 WG + 500 SC	12.5 + 105.0
		25.0 + 210.0
		50.0 + 420.0
Trifludimoxazin + Carfentrazone-ethyl*	500 SC + 400 EC	12.5 + 8.75
		25.0 + 17.5
		50.0 + 35.0

*All herbicide treatments included DASH 0.5% v/v.

The meteorological data of Seymour and Santo Antônio de Posse during the period of development of trials are presented in the Figure 2.1 and the precipitation and irrigation are presented in the Figure 2.2, respectively.

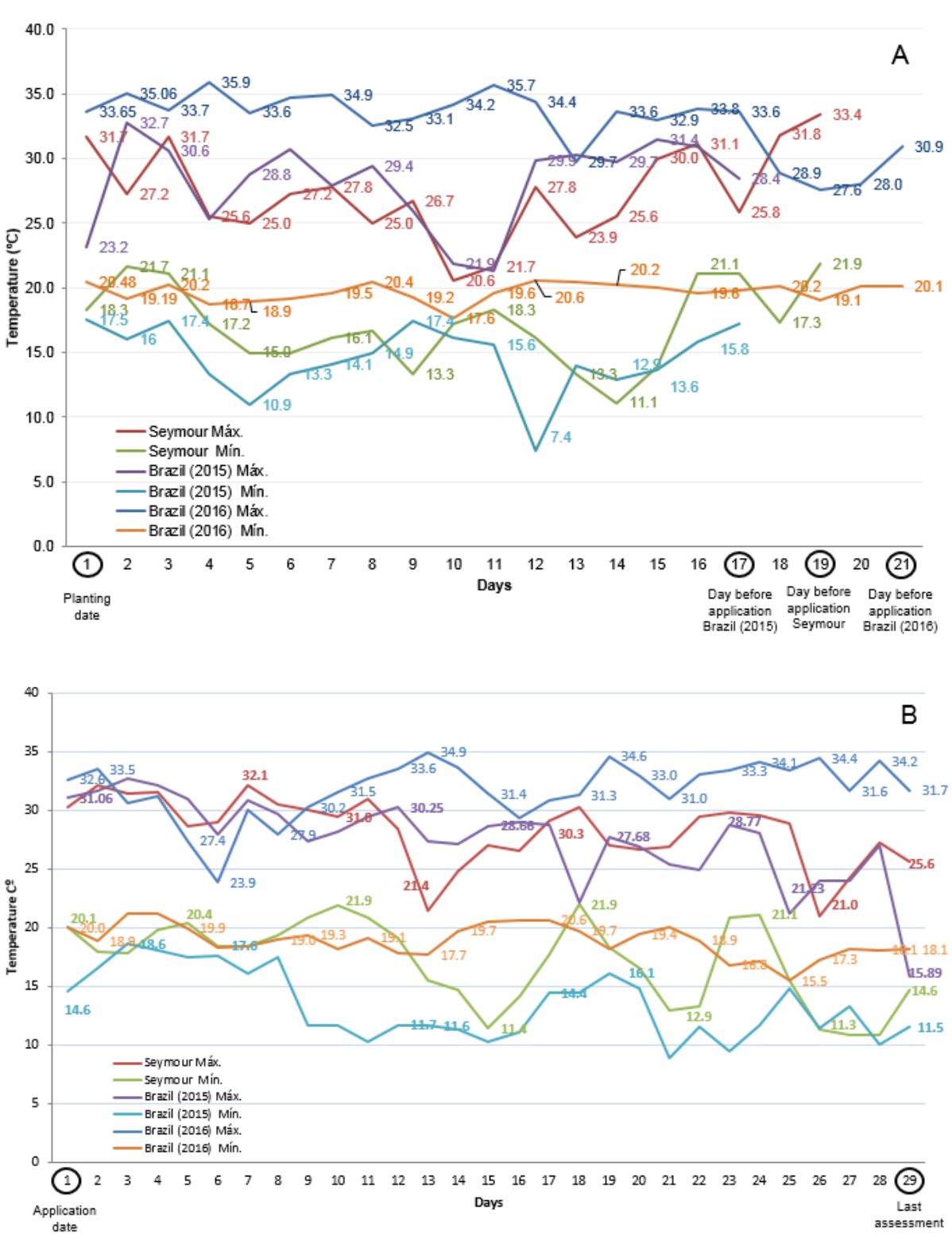


Figure 2.1 – Maximum and Minimum temperatures (ºC) in **A** from planting to one day before applying the treatments and in **B**, from application to last day of assessments in the both trials.

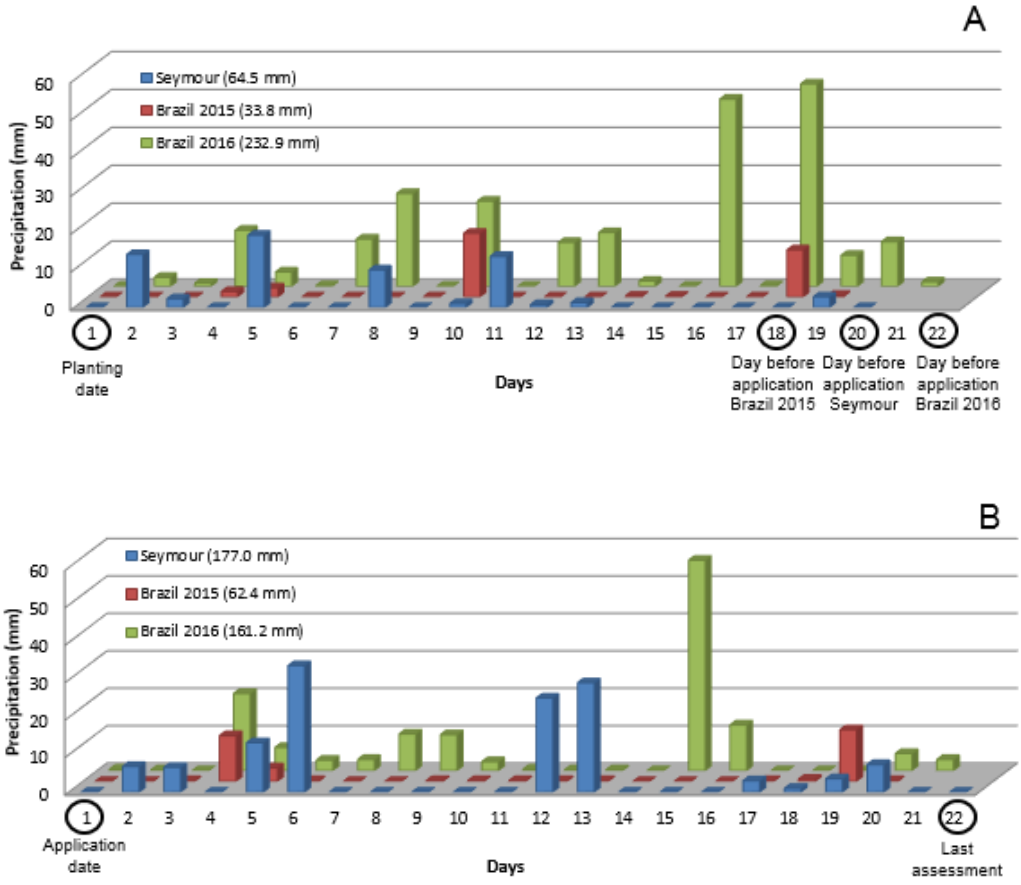


Figure 2.2 – Precipitations + Irrigations (mm) in **A** from planting to one day before apply the treatments and in **B**, from application to last day of assessments in the both trials.

The herbicides treatments were manually applied with a knapsack sprayer pressurized with compressed air at fitted with 80.01 nozzle (TeeJet, Spraying Systems Co.) calibrated to deliver 200 $L.ha^{-1}$ at 2 bar. At the both sites, the plot size was 2m by 5 m or 10 m^2. The information from the moment of application is described in the Table 2.4.

Weed control was visually evaluated at 7, 14, 21 and 28 days after application (DAA) on a scale of 0 to 100%, where 0% being no control and 100% being complete control of weeds at the time of observation compared with non-treated control, according to methodology proposed by Velini (1995).

Table 2.4 – Information about application date, start time and end time of application, nozzles type, pressure, water volume, air temperature, air humidity, wind speed, cloudy cover in both locations.

Application/Weather	Seymour	Sto Antonio de Posse	
		1st rep	2nd rep
Application date	6/20/2014	4/7/2015	2/25/2016
Hour from	15:02	8:50	10:20
Hour to	16:05	10:00	11:40
Nozzles type	XR 80.02	XR 80.01	XR 80.01
Pressure (bar)	3.3	2	2
Vol (L/ha)	200	200	200
Air Temp (ºC)	30.3	27	31.2
RH (%)	61	58	55
Wind Speed (km/h)	4.8	1	1
Could cover (%)	60	0	88

It was measured the percentage of phytotoxicity effects means, percentage reduction in volume of the weeds, in comparison with the untreated plot plus percentage degree of damage to the remaining weed canopy.

The experiments were conducted in a randomized complete block design with three repetitions. The data from each field experiment was analyzed separately. Data were subjected to analysis of variance (ANOVA) using the statistical analysis software version 9.2 (SAS Institute Inc. Cary, NC), SAS, 2002.

Normality, homogeneity of variance, and interactions of treatments in greenhouse repeat experiments and field experiments were tested. Where the ANOVA indicated treatment effects were significant, means were separated at Scott Knott ($\alpha = 0.05$). The data of percent weed control were arcsine transformed before analysis; however, non-transformed percentages are presented with mean separation based on transformed data.

2.3 Results and Discussion

Maize, soybean, *Echinochloa crus-galli* and *Amaranthus* spp. control were evaluated at 7, 14, 21 and 28 days after application (DAA). However, since the assessments with 14 and 28 DAA were the most representative, these data are shown in the Table 2.5 and 2.6.

The efficacy of PPO herbicides on dicots is known and it could be confirmed by the trials conducted in Seymour and Santo Antônio de Posse. According to Vidal (1997), PPO inhibitors control broadleaf weeds selectivity and the metabolism is the most important mechanism to provide tolerance in the crops.

PPO-inhibitor herbicides as single compounds or mixtures were effective for controlling broadleaf weeds at every dose tested and at every evaluation date, except for carfentrazone-ethyl. Carfentrazone-ethyl at the lowest dose tested did not effectively control broadleaf weeds at either location.

As mentioned before, soybean was used as indicator of dicots as well as volunteer crops. This group of herbicides cause temporary injury to the foliage of treated crops, however crops normally recover rapidly and yields are not affected (GRAHAM, 2005; VIDRINE et al., 1996). However, the mixtures and saflufenacil as single compound to Brazil, and every PPO-inhibitor herbicide in the USA were effective in the volunteer soybean.

It is because these both references probably refer to herbicides like diphenyl ether (1996) and carfentrazone-ethyl, flumioxazin and sulfrentrazone (2005), not referring to herbicides such as saflufenacil or trifludimoxazin that are a new chemical class.

Furthermore, soybean control was higher in USA than in Brazil. Saflufenacil, trifludimoxazin and sulfentrazone showed similar control (> 90%) to the binary mixtures. Flumioxazin and carfentrazone-ethyl showed lower control than the other single compounds.

Whereas in Brazil, only saflufenacil did not differ significantly of the mixtures showing control above 90%. Regarding the mixtures, only carfentrazone-ethyl + trifludimoxazin in the lowest dose showed less control (< 80%) at 14 and 28 days after application.

Studies conducted in California confirmed that saflufenacil is a strong tool to control several annual broadleaf weeds, including glyphosate-resistant weeds such as *Conyza bonariensis* and *C. canadensis* (JHALA et al., 2013).

Waggoner et al. (2011) reported that glyphosate tank mixed with saflufenacil reduced density of glyphosate resistant *C. canadensis* to less than three plants/meter. Therefore, as *C. canadensis* is glyphosate resistant, only saflufenacil was controlling effectively the weed and perhaps glyphosate was helping saflufenacil to translocate inside the plant.

Saflufenacil is translocated mainly in the xylem with limited or no mobility in the phloem (LIEBL et al., 2008; ASHIGH & HALL, 2010). Grossmann et al. (2010) reported that saflufenacil is weak acid, which ionizes in solution and provide is mobile in xylem and phloem distributing systemically in the plant.

Duke & Powles (2008) reported that tank mixtures of herbicides are an important aspect of a glyphosate stewardship program, and Jhala, et al. (2013) added that herbicides with different mechanism of action will reduce the selection pressure and occurrence of glyphosate-resistant weeds. Thus there are very important tools to facilitate the rotation of herbicides as well as other resistant technologies in the future.

The trials showed that the herbicides performed better on dicots than in monocots. This might be due to the mechanism of action as it is primarily a dicot herbicide; therefore, it is not much effective on monocrop species (BASF Corp., 2010). It is probably because of mostly to present lack of phloem mobility since the target is sensitive.

The mixtures provided a higher level of monocot control in the USA as compared to Brazil. Treatments of single active ingredients showed poor control of monocots, except sulfentrazone that showed the best monocots control in both locations and saflufenacil in the highest dose to *E. crus-galli* control in the USA (Table 4.1).

Dayan & Duke (1996) related that Protox-inhibitors herbicides control both monocotyledonous and dicotyledonous weeds. However, most of monocots could metabolize the PPO inhibitors showing some initial necrosis that did not affect the plant development (MORAN et al., 2011).

For example, experiments conducted to evaluate winter wheat (*Triticum aestivum*) response to post emergence application of saflufenacil suggested some necrosis at 10 to 20 DAA, but it was not evident at 30 DAA (FRIHAUF et al., 2010).

According to Singh et al. (2011), the application of saflufenacil alone was not effective for controlling monocot weeds, however it provided similar broadleaf weed control compared to glyphosate applied alone.

Nevertheless, the addition of one more PPO inhibitor herbicide provided significantly better control, achieving almost 100% control of monocots in some mixtures (Table 2.5 and 2.6) compared with the herbicides applied alone at 7, 14, 21 and 28 DAA. Mixtures showed better control than the individual active ingredients in both experiments (Figure 2.1 and 2.2), except to *Amaranthus* spp. which did not show significant difference herbicides tested.

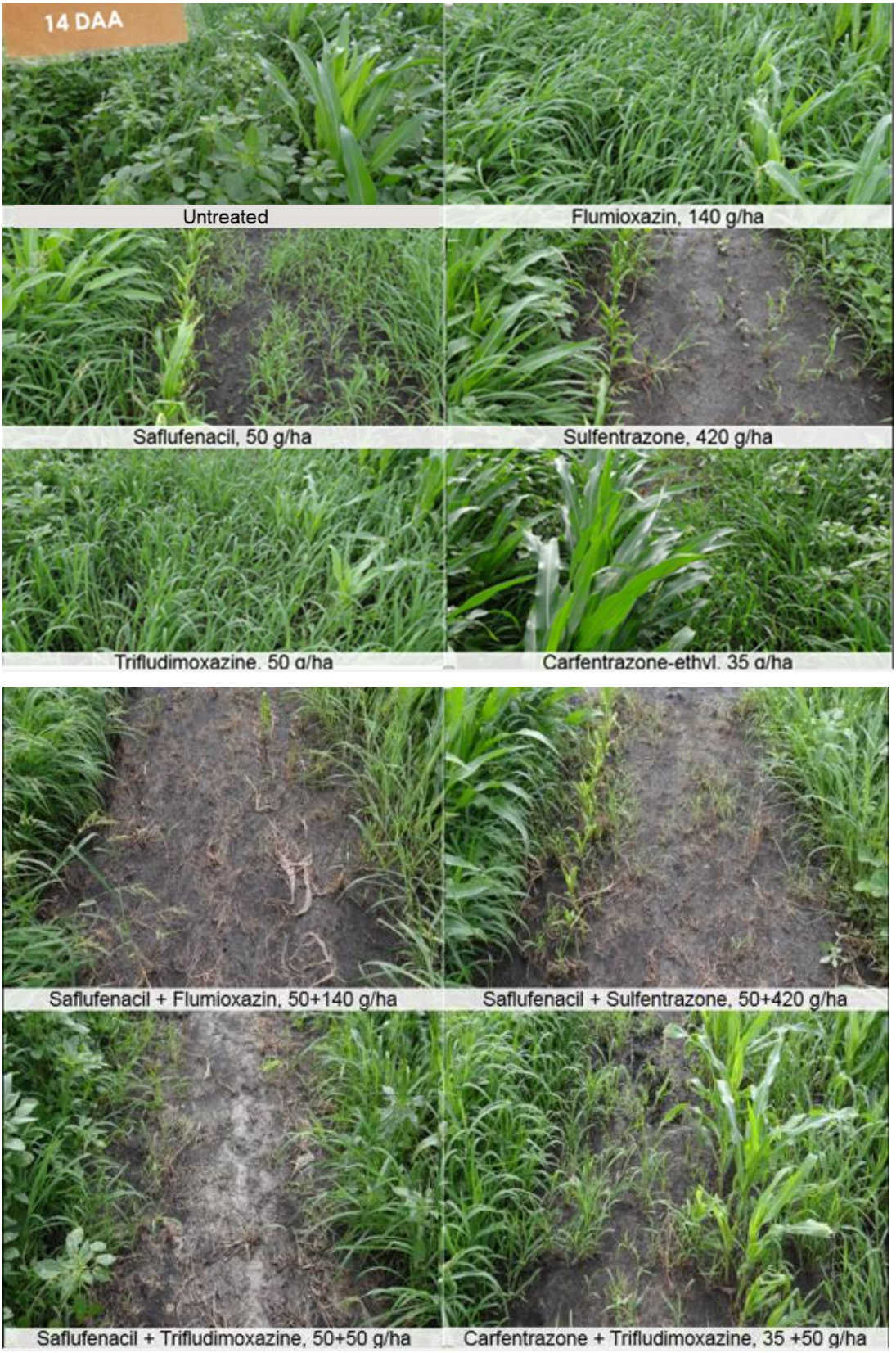


Figure 2.1 – Pictures of maize (ZEAMD) – one row, soybean (GLXMA) – one row, *Echinochloa crus-galli* (ECHCG) and *Amaranthus palmeri* (AMAPA) – natural infestation, at 14 days after application (DAA). Seymour, USA, 2014.

Figure 2.2 – Pictures of maize (ZEAMD), soybean (GLXMA), *Echinochloa crus-galli* (ECHCG)**,** *Amaranthus viridis* (AMAVI) and *A. hybridus* (AMACH) – one row of each, at 7 days after application (DAA). Santo Antônio de Posse, Brazil, 2015.

In the USA, the superior mixture concepts were saflufenacil with flumioxazin, sulfentrazone, or trifludimoxazin.

Synergism, that is cooperative action of two components of a mixture, such that the total effect is greater or more prolonged than the sum of effects of the two taken independently, Colby formula was used in order to calculate the possible synergism among the mixtures. Colby (1967) method is a classic methodology valid only for cases where the combination components exhibit similar non-action (TREZZI et al., 2007).

Saflufenacil + flumioxazin, saflufenacil + trifludimoxazin and saflufenacil + sulfentrazone showed synergism for control of both maize and *E. crus-galli* in each location. In general, carfentrazone-ethyl + trifludimoxazin showed synergism only on *E. crus-galli* and in Brazil this mixture showed synergism also on soybeans.

Valente et al. (2010) reported that the addition of 50 and 70 g.ha^{-1} of saflufenacil in 1080 g.ha^{-1} of glyphosate provided above 90% control of *Conyza* sp. at 24 days after treatment. Dalazen (2012) observed that this mixture reduces regrowth of weeds.

The same authors compared the efficacy of saflufenacil alone and in tank mixture with glyphosate (1061 g.ha^{-1}) and noticed that all tank mixture treatments controlled *C. canadensis* seven days after application regardless the dose of saflufenacil used (6,3; 12,5; 25 e 50 g i.a. ha^{-1}). Saflufenacil alone did not show good efficacy in this study.

According to Anónymous (2008), the application of both saflufenacil and glyphosate in burndown pre-planting improve the control of weeds compared to glyphosate alone, and provide good residual control.

Although the monocot control was lower in Brazil than in the USA, the best mixture was saflufenacil + sulfentrazone which improved the control of maize and *E. cruz*-galli, 84.2% and 85% at 28 DAA, respectively at the highest dose. The mixture of trifludimoxazin + carfentrazone-ethyl showed the worst control of monocots in both locations.

It was known that while PPO herbicides are generally more active on dicots than on monocots, but the enzyme target appears to be equally sensitive to the herbicides (LI & NICHOLL, 2005).

There are two isoforms of PPO in plants, the plastidic PPO1 and the mitochondrial PPO2 (LERMONTOVA et al., 1997); being PPO1 located in the

thylakoid and in the envelope membranes of chloroplasts and PPO2 located on the outer surface of the inner mitochondrial membrane (FERREIRA et al., 1988).

Dayan et al. (2010) mentioned that in the presence of inhibitors, in plants with PPO2-resistant in the mitochondria, plant have enabled expressing the PPO-resistant in the chloroplast to survive.

It may explain the difference the efficacy when apply only one compound and the tank mixture of two compounds. Likely, one can have more affinity with one PPO and the second, with another PPO, being a synergistic effect. This and other questions await future investigation.

It has known the glyphosate-resistant volunteer maize and soybean has become a problem in agriculture system, mainly when maize succeed soybean in crop protection and vice versa, and both are glyphosate-resistant crops (DEEN et al., 2006; SOLTANI et al., 2006).

Grossmann et al. (2011) reported that maize has shown natural tolerance to saflufenacil as a consequence of more rapid metabolism of the herbicide in the shoot and root tissue, compared to broadleaf weeds. Besides that, low translocation of root-absorbed herbicide into the shoot tissue also favours maize tolerance to saflufenacil.

Gazziero (2015) reported that many growers used to mixture two to five products in the same tank in order to control, besides other pests and fungi, volunteer crops. It is worth to highlight that in *E. crus-galli* control, sulfentrazone solo showed similar control of the mixtures, however on the other hand to maize control, the mixtures showed significant improvement, proving that mixtures present broad spectrum of weeds control.

Probably, maize is able to restrict the translocation of herbicide to other parts of plant from leaves, due to a fast metabolism that occur in the initial metabolites which it demonstrated selectivity studies of saflufenacil (FRIHAUF et al., 2010).

Experiments conducted to determine the response of maize to post emergence application of saflufenacil suggested that addition of an adjuvant to saflufenacil caused 99% injury at three-leaf stage and reduced the yield when compared with saflufenacil without adjuvant (SOLTANI et al., 2009).

Overall, it was determined that saflufenacil, flumioxazin, sulfentrazone, trifludimoxazin or carfentrazone-ethyl applied alone were not as effective as applied in tank mixes for dicots and monocot control.

Moreover, tank mixing saflufenacil with flumioxazin, sulfentrazone, or with trifludimoxazin were usually comparable. The residual provided by sulfentrazone, flumioxazin and trifludimoxazin make mixtures including these herbicides more interesting.

Singh et al. (2011) reported that application of herbicides as a tank mixture is a popular method adopted by growers due to improve the broad spectrum weed control in a single application that reduces labor and fuel costs.

Therefore, more research is required to understand why PPO herbicides interact to improve the spectrum of weeds controlled. The effect could be attributed to differences in absorption, translocation, and metabolism in different species or also could be due to the differences to PPO1 and PPO2 sensitivity.

Table 2.5 – Efficacy in percentage of control of maize (ZEAMD), soybean (GLXMA), *Echinochloa crus-galli* (ECHCG) and *Amaranthus palmeri* (AMAPA) at 14 and 28 days after application (DAA). The values in the check are percentage of cover. Seymour, USA, 2014.

	Treatments	FO	(g i.a/ha)	GLXMA				AMAPA				ZEAMD				ECHCG			
				14 DAA		28 DAA		14 DAA		28 DAA		14 DAA		28 DAA		14 DAA		28 DAA	
1	Untreated			[25.0]		[25.0]		[51.7]		[51.7]		[30.0]		[30.0]		[13.3]		[13.3]	
2	Saflufenacil *	WG	12.5	86.7	b	93.3	a	96.7	a	97.3	a	13.3	e	10.0	d	28.3	d	38.3	c
3	Saflufenacil *	WG	25	90.0	b	91.7	a	95.0	a	91.7	a	25.0	e	21.7	d	46.7	c	61.7	b
4	Saflufenacil *	WG	50	96.3	a	98.7	a	100.0	a	100.0	a	65.0	c	56.7	c	71.7	b	78.3	b
5	Trifludimoxazine *	SC	12.5	78.3	c	81.7	b	98.3	a	100.0	a	41.7	d	36.7	c	20.0	d	28.3	d
6	Trifludimoxazine *	SC	25	95.0	a	97.7	a	100.0	a	100.0	a	50.0	d	46.7	c	15.0	d	30.0	d
7	Trifludimoxazine *	SC	50	90.7	b	95.0	a	100.0	a	100.0	a	51.7	d	51.7	c	21.7	d	35.0	d
8	Flumioxazin *	WG	35	66.7	d	70.0	c	95.0	a	100.0	a	35.0	d	28.3	d	13.3	d	25.0	d
9	Flumioxazin *	WG	70	66.7	d	75.0	c	98.3	a	100.0	a	53.3	d	33.3	c	16.7	d	21.7	d
10	Flumioxazin *	WG	140	85.0	b	93.3	a	100.0	a	100.0	a	61.7	c	51.7	c	33.3	d	48.3	c
11	Sulfentrazone *	SC	105	86.7	b	83.3	b	98.3	a	100.0	a	63.3	c	60.0	b	63.3	b	73.3	b
12	Sulfentrazone *	SC	210	88.3	b	92.3	a	96.7	a	98.3	a	63.3	c	51.7	c	73.3	b	75.7	b
13	Sulfentrazone *	SC	420	89.0	b	91.0	a	98.3	a	99.0	a	71.7	c	60.0	b	84.0	a	89.0	a
14	Carfentrazone *	EC	8.75	88.3	b	86.7	b	90.0	b	90.0	a	21.7	e	10.0	d	30.0	d	48.3	c
15	Carfentrazone *	EC	17.5	85.0	b	86.7	b	90.0	b	93.3	a	18.3	e	10.0	d	26.7	d	31.7	d
16	Carfentrazone *	EC	35	81.7	c	86.7	b	82.0	c	76.7	b	15.0	e	10.0	d	45.0	c	61.7	b
17	Saflufenacil + Flumioxazin *	WG+WG	12.5 + 35	91.0	b	94.0	a	98.5	a	100.0	a	45.0	d	50.0	c	65.0	b	72.5	b
18	Saflufenacil + Flumioxazin *	WG+WG	25 + 70	97.3	a	98.0	a	100.0	a	100.0	a	78.3	b	85.0	a	90.0	a	91.3	a
19	Saflufenacil + Flumioxazin *	WG+WG	50 + 140	99.7	a	99.0	a	100.0	a	100.0	a	98.7	a	98.7	a	97.0	a	97.7	a
20	Saflufenacil + Trifludimoxazine *	WG+SC	12.5 + 12.5	96.7	a	97.7	a	98.0	a	99.0	a	46.7	d	43.3	c	70.0	b	67.3	b
21	Saflufenacil + Trifludimoxazine *	WG+SC	25 + 25	99.7	a	98.7	a	100.0	a	100.0	a	70.0	c	63.3	b	91.0	a	92.3	a
22	Saflufenacil + Trifludimoxazine *	WG+SC	50 + 50	100.0	a	100.0	a	100.0	a	100.0	a	99.7	a	99.0	a	98.3	a	97.7	a
23	Saflufenacil + Sulfentrazone *	WG+SC	12.5 + 105	95.0	a	96.0	a	97.7	a	96.7	a	83.3	b	81.7	a	83.3	a	88.3	a
24	Saflufenacil + Sulfentrazone *	WG+SC	25 + 210	95.0	a	95.0	a	99.3	a	98.3	a	78.3	b	81.7	a	87.0	a	89.7	a
25	Saflufenacil + Sulfentrazone *	WG+SC	50 + 420	99.0	a	99.3	a	100.0	a	100.0	a	83.3	b	89.0	a	93.0	a	96.0	a
26	Carfentrazone + Trifludimoxazine *	EC+SC	8.75 + 12.5	91.7	b	97.0	a	98.7	a	99.0	a	45.0	d	36.7	c	55.0	c	65.0	b
27	Carfentrazone + Trifludimoxazine *	EC+SC	17.5 + 25	93.3	a	97.0	a	100.0	a	100.0	a	50.0	d	55.0	c	46.7	c	58.3	b
28	Carfentrazone + Trifludimoxazine *	EC+SC	35 + 50	96.7	a	98.7	a	99.7	a	100.0	a	53.3	d	48.3	c	73.3	b	75.0	b
	CV (%)			7.6		5.7		4.9		6.0		13.5		20.2		20.3		15.6	

*All herbicide treatments included DASH 0.5% v/v.
Means (n=3) within columns with no common letter are significantly different according to test group average Scott_Knott (α=0.05).

Table 2.6 – Efficacy in percentage of control of maize (ZEAMD), soybean (GLXMA), *Echinochloa crus-galli* (ECHCG)**,** *Amaranthus viridis* (AMAVI) and *A. hybridus* (AMACH) at 14 and 28 days after application (DAA). The values in the check are percentage of cover. Santo Antônio de Posse, Brazil, 2015 e 2016.

Treatments	FO	(g i.a/ha)	GLXMA		AMAVI		AMACH		ZEAMD		ECHCG	
			14 DAA	28 DAA	14 DAA	28 DAA	14 DAA	28 DAA	14 DAA	28 DAA	14 DAA	28 DAA
1 Untreated			[19.0]	[22.8]	[5.5]	[6.3]	[1.5]	[2.5]	[38.0]	[48.0]	[6.8]	[9.2]
2 Saflufenacil *	WG	12.5	70.8 c	75.0 b	99.0 a	99.7 a	99.3 a	99.8 a	7.2 i	5.3 g	35.0 f	12.5 e
3 Saflufenacil *	WG	25	93.7 a	94.3 a	100.0 a	100.0 a	100.0 a	100.0 a	9.5 i	5.7 g	45.0 e	9.8 e
4 Saflufenacil *	WG	50	97.2 a	96.2 a	100.0 a	100.0 a	100.0 a	100.0 a	14.5 h	14.3 g	69.2 c	15.8 e
5 Trifludimoxazine *	SC	12.5	53.3 e	53.3 d	99.3 a	99.3 a	100.0 a	100.0 a	18.3 h	20.8 f	23.3 g	37.5 d
6 Trifludimoxazine *	SC	25	69.2 c	77.2 b	99.5 a	100.0 a	100.0 a	100.0 a	29.2 g	32.5 e	29.2 g	16.7 e
7 Trifludimoxazine *	SC	50	80.8 b	82.8 b	100.0 a	100.0 a	100.0 a	100.0 a	38.3 f	34.2 e	35.8 f	17.5 e
8 Flumioxazin *	WG	35	35.8 f	30.8 e	100.0 a	100.0 a	100.0 a	100.0 a	25.0 g	22.5 f	22.5 g	35.8 d
9 Flumioxazin *	WG	70	53.3 e	50.0 d	100.0 a	100.0 a	100.0 a	100.0 a	31.7 g	30.0 e	32.5 f	21.7 e
10 Flumioxazin *	WG	140	59.2 e	53.3 d	100.0 a	100.0 a	100.0 a	100.0 a	46.7 e	38.3 e	44.2 e	13.3 e
11 Sulfentrazone *	SC	105	50.8 e	50.8 d	100.0 a	100.0 a	100.0 a	100.0 a	25.0 g	34.2 e	44.2 e	25.8 e
12 Sulfentrazone *	SC	210	65.8 d	60.8 c	100.0 a	100.0 a	100.0 a	100.0 a	35.8 f	48.3 d	60.0 d	29.2 d
13 Sulfentrazone *	SC	420	81.7 b	80.0 b	100.0 a	100.0 a	100.0 a	100.0 a	74.2 b	71.7 b	88.3 a	84.2 a
14 Carfentrazone *	EC	8.75	23.3 g	17.5 f	70.5 c	95.0 b	78.3 b	96.0 c	7.0 i	6.5 g	10.0 h	44.2 d
15 Carfentrazone *	EC	17.5	62.5 d	65.8 c	93.3 b	95.5 b	97.8 a	97.7 b	8.3 i	5.0 g	22.5 g	16.7 e
16 Carfentrazone *	EC	35	70.8 c	74.2 b	96.8 b	98.5 a	99.0 a	98.3 b	11.7 i	8.7 g	23.3 g	21.7 e
17 Saflufenacil + Flumioxazin *	WG+WG	12.5 + 35	92.0 a	94.5 a	100.0 a	100.0 a	100.0 a	100.0 a	39.2 f	29.2 e	57.5 d	50.0 c
18 Saflufenacil + Flumioxazin *	WG+WG	25 + 70	92.2 a	95.8 a	100.0 a	100.0 a	100.0 a	100.0 a	50.0 d	41.7 d	81.7 b	53.3 c
19 Saflufenacil + Flumioxazin *	WG+WG	50 + 140	98.0 a	94.8 a	100.0 a	100.0 a	100.0 a	100.0 a	72.5 b	59.2 c	91.7 a	34.2 d
20 Saflufenacil + Trifludimoxazine *	WG+SC	12.5 + 12.5	96.7 a	94.2 a	100.0 a	100.0 a	100.0 a	100.0 a	37.5 f	33.3 e	59.2 d	60.0 c
21 Saflufenacil + Trifludimoxazine *	WG+SC	25 + 25	99.3 a	99.0 a	100.0 a	100.0 a	100.0 a	100.0 a	44.2 e	44.2 d	75.8 b	96.3 a
22 Saflufenacil + Trifludimoxazine *	WG+SC	50 + 50	99.3 a	99.7 a	100.0 a	100.0 a	100.0 a	100.0 a	61.7 c	50.8 d	86.8 a	66.7 b
23 Saflufenacil + Sulfentrazone *	WG+SC	12.5 + 105	87.5 b	87.5 a	100.0 a	100.0 a	100.0 a	100.0 a	55.0 d	47.5 d	81.3 b	72.5 b
24 Saflufenacil + Sulfentrazone *	WG+SC	25 + 210	95.3 a	93.3 a	100.0 a	100.0 a	100.0 a	100.0 a	84.7 a	73.3 b	90.2 a	57.5 c
25 Saflufenacil + Sulfentrazone *	WG+SC	50 + 420	97.2 a	97.0 a	100.0 a	100.0 a	100.0 a	100.0 a	88.3 a	84.2 a	97.0 a	85.0 a
26 Carfentrazone + Trifludimoxazine *	EC+SC	8.75 + 12.5	74.2 c	79.2 b	100.0 a	100.0 a	100.0 a	100.0 a	25.8 g	26.7 f	32.5 f	35.0 d
27 Carfentrazone + Trifludimoxazine *	EC+SC	17.5 + 25	93.5 a	94.5 a	100.0 a	100.0 a	100.0 a	100.0 a	40.8 f	35.8 e	50.0 e	28.3 d
28 Carfentrazone + Trifludimoxazine *	EC+SC	35 + 50	98.0 a	98.3 a	100.0 a	100.0 a	100.0 a	100.0 a	67.5 c	57.5 c	76.7 b	61.7 c
CV (%)			5.2	5.54	2.1	1.0	1.3	0.5	9.6	12.8	9.6	17.2

*All herbicide treatments included DASH 0.5% v/v.
Means (n=3) within columns with no common letter are significantly different according to test group average Scott_Knott (α=0.05).

2.4 Conclusion

The best mixtures to improve the spectrum of control were saflufenacil with flumioxazin, sulfentrazone, or trifludimoxazin. Those mixtures also have shown synergism effects by Colby formula. The less efficacious mixtures was carfentrazone-ethyl + trifludimoxazin.

The aforementioned mixtures controlled *E. crus-galli* and volunteer maize as well as dicots species evaluated. In addition, the tank mix treatment could also be effective for volunteer glyphosate-resistant crops control.

The dose of each compound in the mixtures depends on the species and region. However, at the highest all mixtures provided effective control of the weed species evaluated.

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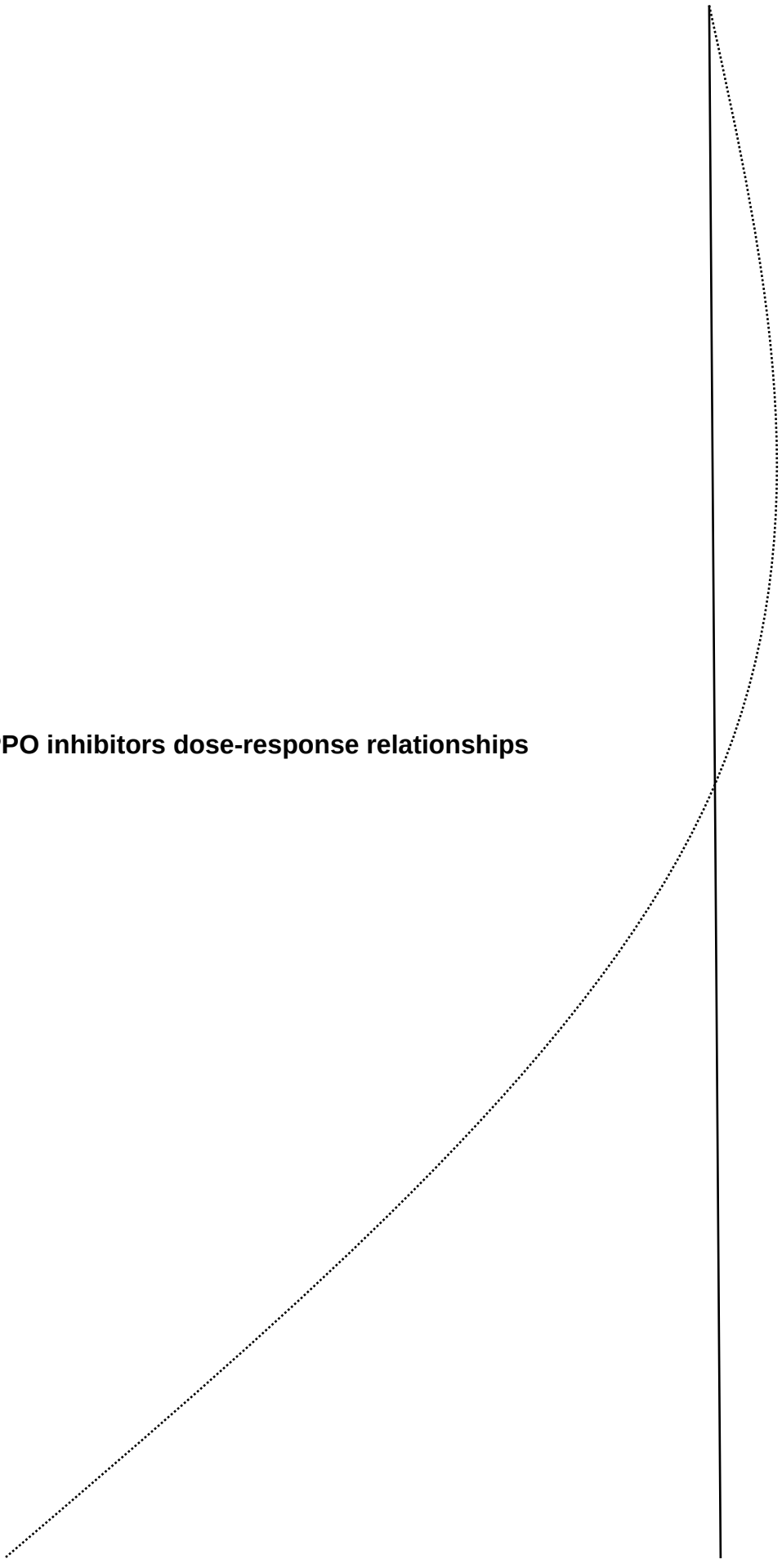
CHAPTER II

PPO inhibitors dose-response relationships



Abstract

PPO inhibitors dose-response relationships

Protoporphyrinogen oxidase is a key enzyme in the chlorophyll or heme synthesis. In plants, two isoforms of PPO, namely PPO1 (targeted to the chloroplast) and PPO2 (mitochondrial PPO) have been identified. Studies were conducted to evaluate the effect of selected PPO-inhibitor herbicides in inhibiting PPO1 and PPO2 enzyme by IC_{50} (half maximal inhibitory concentration) from *Amaranthus tuberculatus, Setaria viridis* and *Alopecurus myosuroides*. Unfortunately, PPO1 could not be expressed in the test system so no results were for this enzyme. However, mitochondrial PPO enzyme from *A. tuberculatus, A. myosuroides* and *S. italica* was sensitive to inhibition by saflufenacil, trifludimoxazin, flumioxazin, carfentrazone-ethyl and sulfentrazone. Trifludimoxazin and flumioxazin required less amount of compound to inhibit 50% of PPO2 activity in all plants species tested, while sulfentrazone and saflufenacil, showed that the lowest unit activity needed bigger concentrations to inhibit 50% of the PPO2 compared to other compounds evaluated. In order to reduce the risk of resistance management, trifludimoxazin was the most active herbicide on the PPO resistant enzymes with the Dg210 and R128L mutations.

Keywords: PPO-inhibitors herbicides; IC_{50}; Natural mutations

3.1 Introduction

Protoporphyrinogen oxidase, also called PPO or Protox, is a key enzyme in the chlorophyll or heme synthesis. Herbicides from this group inhibit this enzyme which catalyzes the six-electron oxidation of Protoporphyrinogen-IX to Protoporphyrin-IX. They also are referred to as tetrapyrrole-biosynthesis inhibitors or Protoporphyrin-IX synthesis inhibitors (DAILEY et al., 1995; SMITH et al., 1993).

It is the last common pathway in the production of heme and chlorophyll. While the production of chlorophyll, a light-harvesting pigment, is an essential process for all green photosynthetic organisms, heme is an essential cofactor in cytochromes, hemoglobin, oxygenases, peroxidases and catalases, which are important in stress reduction due to the ability to inactivate free radicals (CHAUDIÈRE & FERRARI-ILIOU, 1999). This characteristic makes PPO an excellent enzyme target for herbicide development (LEHNEN et al., 1990; JACOBS et al., 1991; DAYAN & WATSON, 2011).

The herbicide action occurs by enzyme competition between Protox-herbicides and Protoporphyrinogen-IX. Since the PPO family of herbicides has more affinity for the enzyme, Protoporphyrinogen-IX accumulates in the chloroplast or mitochondria and diffuses into the cytoplasm which subsequently is converted to Protoporphyrin-IX by plasma membrane peroxidases, enzymatic oxidation (JACOBS

et al., 1991; DAILEY et al., 1995). Once in the cytoplasm, Protoporphyrin-IX cannot to return to the chloroplast because it is highly lipophilic (LEHNEN et al., 1990).

Exposure to light causes formation of singlet oxygen and other oxidative species, resulting in membrane disruption and subsequent cell death. PPO-inhibitors herbicides have characteristically a very rapid contact action, causing leaf burning, desiccation and growth inhibition resulting in complete death of plants (JACOBS et al., 1991; MORI & SCHROEDER, 2004; DAYAN & WATSON, 2011).

Duke et al. (1991) reported that the damage resulting from the peroxidation of lipids is the initial degradation of the plasmalemma and tonoplast membranes, followed by inhibition of photosynthesis and evolution of ethylene. In all instances the involvement of light in herbicide action is not a direct interaction of light and herbicide, but are the primary site of action. Furthermore, the events that cause tissue damage that are necrosis, are not associated with the primary target and are always due to membrane damage caused by lipid peroxidation of polyunsaturated fatty acids (HESS, 2000).

In plants, two isoforms of PPO, namely PPO1 (targeted to the chloroplast and encoded by the gene PPX1) and PPO2 (mitochondrial PPO, encoded by the gene PPX2), have been found (LERMONTOVA et al., 1997; POWLES & YU, 2010).

According to Dayan & Duke (2010), plant PPO1 is compartmentalized in the thylakoid and in the envelope membranes of chloroplasts, whereas the mitochondrial isoform PPO2 is localized on the outer surface of the inner mitochondrial membrane. In the chloroplast, the porphyrin pathway leads to both chlorophyll and heme, whereas it leads exclusively to heme in the mitochondrion (DAYAN & DUKE, 1997).

Two PPO isoenzymes have been described in tobacco, a plastidic and a mitochondrial form. PPO genes or cDNAs have been cloned from *Escherichia coli*, *Bacillus subtilis*, human, cow, mouse, and yeast. The molecular masses of these Protox gene products range from 50 to 60 kDa, except for the 21-kDa *E. coli.* The N terminus is most widely conserved, and the deduced amino acid sequences of PPO1 and PPO2 are only 27.3% similar (WATANABE et al., 2001).

The authors still explain that the translation product of PPX-I cDNA translocates to chloroplasts, whereas PPX-II is targeted to mitochondria, suggesting that tobacco Protox exists in chloroplasts and mitochondria as isoenzymes. Since Protox is the final common enzyme in the chlorophyll and heme biosynthetic pathways in plants, Protox should play a role in distributing Proto IX to both

pathways. However, the knowledge of plant mitochondrial PPO is poor in comparison with plastidal PPO.

There have no investigations into the precise suborganellar location of PPO mitochondrial. Little is known about the transport mechanism of mitochondria PPO1, which in general, proteins transported into the mitochondria have an N-terminal targeting peptide that is processed after transport is complete (GLASER et al., 1998).

Some analysis using electron microscopy has demonstrated that spinach PPO 1 preferentially associates with the stromal side of the thylakoid membrane, while a small fraction of PPO1 is located on the stromal side of the inner envelope membrane (CHE et al., 2000).

While tobacco PPO2 and other homologs do not process the typical mitochondrial targeting sequence at their N termini. Trials conducted *in vitro* by Lermontova et al. (1997) showed that tobacco PPO2 is transported to mitochondria without any size reduction. The mechanism by which this occurs is not yet understood.

According to Seefeldt (1995), the relationship between herbicide dose and plant response is of fundamental importance in understanding herbicide efficacy and mechanism of action. However, although the molecular site of PPO-inhibitors has been established, the interaction of PPO-inhibitors and their effects in the PPO1 and PPO2 enzymes remain unresolved.

The main propose of this work was to measure of the effectiveness of PPO-inhibitor herbicides in inhibiting PPO1 and PPO2 enzyme by IC_{50} (half maximal inhibitory concentration) from *Amaranthus tuberculatus, Setaria viridis* and *Alopecurus myosuroides*. Furthermore, to evaluate the efficacy of those herbicides in the activity of *A. tuberculatus* PPO2 with two different mutations (one substitution of Arginine per Leucine in the position of 128 (R128L) and one deletion of Glycine in the position of 210 (dG210).

3.2 Material and Methods

This trial was conducted in the Herbicides Molecular Biology Laboratory located in Limburgerhof, Germany in August, 2014.

3.2.1 Cloning

Protoporphyrinogen Oxidase 1 and 2 (PPO1 and PPO2) wild type sequences and variants thereof from *Arabidopsis thaliana* and *Amaranthus tuberculatus* were synthesized and cloned by Geneart (Geneart AG, Regensburg, Germany).

Plasmids were isolated from *E. coli* TOP10 by performing a plasmid mini preparation. Subcloning of PPO1 genes was performed by digesting with NcoI and XhoI and ligating in-frame into the pET24d N-His vectors with various fusion proteins. The table 3.1 describes the vectors used with the given fusion protein.

Table 3.1 – Vector library information used with fusion protein and their sizes (kDa). Limburgehof, Germany, 2014.

Vector + Fusion Protein	Fusion Protein Name	Size (kDa)
pET24d N-His GB1	GB1: Fragment from *Streptococcus*; B1 Ig binding domain Solubility enhancement tag.	8.9
pET24d N-His MBP	MBP: Maltose-binding protein from *E. coli*. Increases expression and solubility. Note: Can creates soluble inclusion bodies	43.1
pET24d N-His trx	trx: Thioredoxin A from *E. coli*. Solubility enhancement tag, and can also increases target protein expression.	14.3
pET24d N-His zz	zz: IgG binding domain of Protein A from *Staphylococcal aureus*. Codon optimized double Z-domain repeat. Solubility enhancement tag, and can also increases target protein expression.	16.9
pET24d N-His GST	GST: Glutathione S-transferase from *Schistosoma japonicum* Classical affinity tag, not the best solubility-tag exists as dimer, careful with dimerfolding target proteins	28.1
pET24d N-His nusA	nusA: Transcription elongation factor from *E. coli*, prevents transcription termination. Solubility enhancement tag, and can also increases target protein expression.	57.4
pET24d N-His mGFP	mGFP: monomeric GFP. Monitor protein expression trials	29.7
pET24d N-His dsbAin	dsbAin: Disulfide Bond Isomerase A from *E. coli*. DsbA catalyses disulfide bridge formation. Intracellular variant, preferentially to be used in Origami expression strain.	23.9
pET24d N-His dsbCin	dsbCin: Disulfide Bond Isomerase C from *E. coli*. DsbC catalyses disulfide bridge formation. Intracellular variant, preferentially to be used in Origami expression strain.	26.3

Transformants were grown on Luria-Bertani (LB) media containing 50 $mg.mL^{-1}$ kanamycin and grown at 37ºC over night.

3.2.2 Expression and purification of PPO1

There is no data available for expression and purification of PPO1 in the literature. This was tentatively based on PPO2 methods. Clones in pET24d N-His were transformed into NiCo21 and Arctic Express DE3 RIL strain of *E. coli.* Cells were grown in 100 mL of ZY-Autoinduction -media with kanamycin, with overnight shaking at 37 ºC. It was taken 100µL of a pre-culture (with the antibiotics Kanamycin 50µg/ml plus Gentamycin 20µg/ml) from the Arctic Express strain were diluted in 100 mL of LB without antibiotics and grown at 30ºC shaking for 3 hours, induced with 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and grown at 12 ºC shaking for 24 more hours.

The arctic express culture did not grow successfully. NiCo21cultures were diluted in 100 mL of ZY (N-Z amine yeast) autoinduction-media with antibiotic, 100 µL of $MgSO_4$ (1M) and grown at 37ºC shaking for 5 hours and grown at 25ºC shaking for an additional 21 hours. Cultures were harvested by centrifugation at 6000xg, and stored as pellets at -80 ºC until further use. Cells were lysed using a Q-Sonica Sonicator.

The protein purification was made using a Macherey–Nagel Protino Ni–IDA Kit. The purification was made in a 4°C cool area to protect the protein as good as possible. After Gel analysis, no soluble PPO 1 combined with the fusion proteins was found.

3.2.3 PPO Activity and Inhibition Assay

In order to obtain the dose-response (IC_{50} values), PPO2 from *Amaranthus tuberculatus*, *Alopecurus myosuroides* and *Setaria italica* were expressed and purified as described above. Protogen was prepared in an external Laboratory as a 216µM stock (1mM of KOH,105vV; ETOH, 125mMTrisHCl; PH8,5,2,5mM; EDTA,2,5mM GSH) for enzymatic protoporphyrinogen oxidation, a late step in heme synthesis, Enzyme 28 (1982) 206–219).

In an Assaymix was added 100 mM of Tris HCl pH 7.3; 1 mM of EDTA pH 8.0; 5 mMof Dithiothreitol (DTT); 0.0085% of Tween 80; 125 ng of PPO2 enzyme from *Setaria* sp.; and finally, was added 3.24 µM of Protoporphyrinogen (PPO-substrate) to start the reaction. Is was distributed in the roboter Biomek 4000 with 4% of DMSO. In this equipment was also added 60 µL of water, 10 µL of compound solution in

DMSO 80%, mixed and incubated for 30 min in order to allow the compound reach perfect contact with the enzyme and finally added 3 µL of substrate.

Dose-response curves with the PPO inhibitors saflufenacil, trifludimoxazin, flumioxazin, carfentrazone-ethyl, sulfentrazone were obtained in the presence of 3,24 µM Protogen. The compounds were not measured in mixtures as done in Chapter I, III and IV since the herbicides act in the same site of action and only one could binding with the enzyme. Dose-response was measured between the inhibitor concentration range of $1{,}0x10^{-05}$ to $1{,}0x10^{-12}$ M. The excitation and emission bandwidths were set at 1.5 and 30 nm, respectively.

All assays were made in duplicates or triplicates and measured using a POLARstar 30 Optima / Galaxy (BMG) with excitation at 405 nm and emission monitored at 630 nm. Molar concentrations of compound required for 50% enzyme inhibition (IC_{50} values) were calculated by fitting the values to the dose-response equation using non-linear regression analysis. IC_{50} were calculated with linear slopes of fluorescence units/min during a 30min measurement time and dose response was calculated by SAS (SAS Circle, Box 8000, Cary, NC 27512-80).

3.3 Results and Discussion

3.3.1 Recombinant Expression and Purification of PPO1 with Fusion Proteins

Every fusion protein (Table 3.1) was cloned with PPO1 gene in the N terminus of a protein fusion. These nine proteins were expressed however in insoluble protein. The only two expression pattern were PPO1 fused with trx or dsbAin proteins. Due to the gel electrophoresis is limited by narrowness visual bands, the nine proteins were evaluated in activity assay. However, the nine proteins were found without any activity.

3.3.2 PPO 2 assay

The enzyme PPO2 from *A. tuberculatus, A. myosuroides* and *S. italica* was sensitive to inhibition by saflufenacil, trifludimoxazin, flumioxazin, carfentrazone-ethyl and sulfentrazone (Table 3.2). According to Dayan & Duke (2010), the inhibition of PPO is proportional to the ability of each compound to bind to that particular site on PPO.

The IC_{50} concentrations obtained for PPO inhibition were around 0.78 nM saflufenacil in *A. tuberculatus,* 4.1 nM in *S. italica*, being less sensitive to PPO from

A. myosuroides (211 nM). Grossmann et al. (2010) reported IC_{50} concentrations for PPO inhibition were around 4.0 nM saflufenacil in *Zea mays*, *Abutilon theophrasti, Solanum nigrum*.

Table 3.2 – Effects of PPO-inhibitors herbicides on PPO2 activity extracted and assayed from *Amaranthus tuberculatus* (AMATU) wild type and natural mutations (dG210 and R128L), *Alopecurus myosuroides* (ALOMY) and *Setaria italica* (SETIT) in nanoMolar (nM). Limburgehof, Germany, 2014.

Chemical Class	Common name	Concentrations required for 50% inhibition IC50 (nM)				
		AMATU			ALOMY	SETIT
		wild type	dG210	R128L		
Pyrimidinedione	Saflufenacil	0.780	1,600.000	116.000	211.000	4.100
Triazinone	Trifludimoxazine	0.060	2.116	0.075	0.172	0.135
N-Phenyl-phthalimides	Flumioxazin	0.096	124.046	91.900	0.144	0.183
Triazolinones	Sulfentrazone	1.030	14,285.714	11,627.906	89.200	49.800
Triazolinones	Carfentrazone	0.560	775.640	0.630	1.470	4.273

Saflufenacil in PPO2 from *A. tuberculatus* was 5 to 270 times less sensitive than in *S. italica* and *A. myosuroides,* respectively. The same tendency was observed to carfentrazone-ethyl and sulfentrazone, may because both species are monocots from Poaceae family.

PPO enzyme activity *in vitro* was inhibited by saflufenacil, 50% inhibition in a concentration range from 0.2 to 2.0 nM, with no clear differences between corn and broadleaf weed species (GROSSMAN et al., 2011). These authors also reported that concentration of flumioxazin was lower to inhibit 50% of PPO than saflufenacil.

The IC_{50} value for sulfentrazone was 1.2 and 1 µM in etioplast preparations from two soybean varieties (DAYAN et al., 1997). Acifluorfen-methyl was strongly inhibitory to protoporphyrinogen oxidase activities showing the IC_{50} of 4 nM for the corn etioplast enzyme (PPO1) and the results proposed that protoporphyrinogen oxidase is a cellular target for diphenyl ether herbicides according to MATRINGE et al. (1989).

Trifludimoxazin and flumioxazin required less amount of compound to inhibit 50% of PPO2 activity from all species tested and there was not increased in monocots genes, while sulfentrazone and saflufenacil, one of the best mixtures in the field (Chapter I), showed that needed bigger concentrations to inhibit 50% of the PPO2 and even high when PPO2 came from monocots.

Grossmann et al. (2011) also reported saflufenacil inhibited PPO enzyme activity with lower intensity than flumioxazin, which was 11 to 100-fold lower to different weeds species tested. However, this low intensity lead to slightly delayed

injury of vascular tissues after foliar absorption, which may be enable long-distance transport of the herbicide within the plant.

It was needed lower concentration of carfentrazone-ethyl than sulfentrazone in order to inhibit 50% of PPO2 enzyme, both at the same chemical class. The same has been seen in the studied conducted by Thompson & Nissen (2000) which has shown that carfentrazone-ethyl was 50 to 110 times stronger inhibitors of PPO, based on IC_{50} values than sulfentrazone.

Dayan and Duke (1997) reported that although PPO-inhibitors appear to have similar broad structural characteristics which compete for the binding site by mimicking the conformation of half the biological substrate, the nature of the binding site remains a mystery.

The IC_{50} obtained for a herbicide on a species may not reflect the species susceptibility. Differential susceptibility to acifluorfen on mustard, cucumber and *Ipomoea* spp. showed up to be due to differences in Proto IX accumulation in response to the herbicide. In some cases, differences in Proto IX accumulation appear to be due to differences in activity of the porphyrin pathway (SHERMAN et al, 1991).

In addition, inhibition of absorption or sequestration of the herbicide should be considered in the field trials. Perhaps, sulfentrazone and saflufenacil was reaching the enzymes easily than trifludimoxazin and flumioxazin in the field (Chapter I).

For instance, uptake reduction of oxyfluorfen was determined to be one of the factor contributing to natural resistance of rice, and there is some evidence that it might contribute to natural resistance to other PPO-inhibitors in other species (DAYAN & DUKE, 1997).

Since the IC_{50} from PPO1 there was not available, it was not possible to affirm if the double mixtures of PPO herbicides seen in the field (Chapter I) have more affinity with PPO1 or PPO2.

These isoforms share little sequence identity, that is 25%, but they are located in different subcellular targeting. While PPO1 are located in plastids, PPO 2 are located in the mitochondria although there are some isoforms that are located in both organelles (LERMONTOVA et al., 1997; WATANABE, et al., 2001; SALAS et al., 2016).

Hao et al. (2009) reported that in plants not exposed to inhibitors, the enzyme's catalytic efficiency might be partially compensated by the native

chloroplastic PPO. However, Li & Nicholl (2005) assumed that despite of PPO herbicides is generally more active on dicots than on monocots, the enzyme target appears to be equally sensitive to the herbicides, and perhaps the difference between dicots and monocots might be in absorption, translocations and metabolism of PPO inhibitors.

Concentration of compounds sharply increased to inhibit 50 percent of resistant PPO enzyme mutants (dG210 and R128L) for all herbicides tested. Gly210 deletion in PPO was around 14000 times less sensitive than the wild-type *A. tuberculatus* to sulfentrazone, 2000 times less to saflufenacil, 1300 times less to flumioxazin and carfentrazone-ethyl and only, 35 times sensitive than the wild type to trifludimoxazin.

Dayan et al. (2010) observed PPO-resistant was 100 to 500 times less sensitive than the wild-type *A. tuberculatus* PPO-sensitive to the diphenyl ether inhibitors. In all case studies by the authors, the regression curves suggest that the Gly210 deletion has altered the architecture of the *A. tuberculatus* PPO substrate binding domain, enabling the PPO-resistant inhibitor to still bind the enzymes, however with lower affinities than the wild type enzymes.

The mechanism of PPO-inhibitor resistance is a unique target-site amino acid deletion, that involves the loss of a glycine at position 210 in the mitochondrial isoform of PPO enzyme. According to Riggins & Tranel (2012), loss this amino acid is considered to have occurred via a slippage-like mechanism within a trinucleotide, that alters the binding domain of the enzyme without negatively affecting substrate affinity, reducing by at least 100-fold PPO-inhibiting herbicides sensitivity.

The same tendency was observed either to dG210 or R128L mutation. The latter one was required only 0.075 nM of trifludimoxazin to inhibit 50% of PPO2 from *A. tuberculatus* mutant.

In the case of sulfentrazone, the concentration increased about 14000 and 12000-fold more to inhibit dG210 and R128L in PPO, respectively, while carfentrazone-ethyl, the same chemical class of sulfentrazone, required almost 1400 times more to inhibit dG210 and only 1.125 to inhibit the substitution R128L. Saflufenacil was about 150-fold less sensitive in the PPO-mutation (R128L) than the wild type whereas flumioxazin was about 1000-fold less sensitive.

Jung et al. (2010) have worked with transgenic rice line M4, transformed with *Myxococcus xanthus* PPO gene, that was about 200-fold more resistant to

oxyfluorfen than wildtype rice in whole-plant bioassays and cross-resistance to other PPO-herbicides such as carfentrazone-ethyl. M4 line was more resistant to oxyfluorfen (more than 200-fold), followed by acifluorfen (15-fold) and carfentrazone-ethyl (12-fold).

To reduce the risk of resistance management, trifludimoxazin showed the most active compound to control these species in both natural mutations dG210 and R128L. In the Chapter IV was observed that flumioxazin also showed excellent tool to manage those PPO-resistant natural mutations.

Although there were not many cases of PPO-resistant cases reported in the field, while resistance to these other herbicides has evolved relatively rapidly, PPO-inhibitors have a single site of action at highly potent as inhibitors at the molecular level, implying that the Protogen binding site is promiscuous to evolve resistance (DAYAN & DUKE, 1997).

Dayan &Duke (1997) and Dayan et al. (2010) reported that there were few cases of PPO-resistant weeds reported might due to the relatively short-lived selection pressure of these fast-acting foliar herbicides applied. Nevertheless, if the present methods and doses of use these herbicides are continues, resistance might be evolved, as it has been for paraquat, another fast-acting herbicide. Furthermore, the development of more persistent soil-active PPO-inhibitors might increase the selection pressure and consequently raises likely the resistance development.

It indicates that more diverse array of other mechanism of action herbicides, mechanical, and cultural practices should be applied together in order to control the resistant species and provide more sustainability for this technology (GREEN & OWEN, 2011; POWELS, 2008).

3.4 Conclusion

Unfortunately, the only two soluble proteins expression pattern to PPO1 was fused with trx or dsbAin and were found without any activity.

However, mitochondrial PPO enzyme from *A. tuberculatus, A. myosuroides* and *S. italica* was sensitive to inhibition by saflufenacil, trifludimoxazin, flumioxazin, carfentrazone-ethyl and sulfentrazone.

Trifludimoxazin and flumioxazin required less amount of compound to inhibit 50% of PPO2 activity in all plants species tested, while sulfentrazone and

saflufenacil, one of the best mixtures in the field, showed that needed bigger concentrations to inhibit 50% of the PPO2 compared to other compounds evaluated.

In order to reduce the risk of resistance management, trifludimoxazin showed the most active compound to inhibit 50% of the PPO2 enzyme from these species even in the both natural mutations dG210 and R128L.

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CHAPTER III

Foliar absorption and plant translocation of PPO-inhibitors herbicides in *Echinochloa crus-galli* and *Zea mays*

Abstract

Foliar absorption and plant translocation of PPO-inhibitors herbicides in *Echinochloa crus-galli* and *Zea mays*

Inhibition of PPO is herbicide mechanism of action that has successfully been used for weed management in agriculture since 1960s. The widespread adoption of glyphosate-resistant crops has caused reduction of the field application of PPO inhibitors, which controls broadleaf weed selectively and show differences in absorption among plants species, while the translocation be commonly limited. Since it is known that the binary mixtures of PPO-inhibitors herbicides improved the control of monocots plants, the main propose of this work was toinvestigate the absorption and translocation of saflufenacil, sulfentrazone, trifludimoxazin and cafentrazone as well as the mixtures saflufenacil + trifludimoxazin, saflufenacil + sulfentrazone and trifludimoxazin + carfentrazone-ethyl in *Echinochloa crus-galli* and maize (*Zea mays*). Plants with the application of one single droplet were individually dissected into the treated leaf, the rest of the aerial part, and the root and the evaluations were done by UPLC-MS/MS (ultra-high-performance liquid chromatography coupled to tandem mass spectrometry). As single compounds, the absorption was slower in maize than *Echinochloa crus-galli*. There was no difference among the herbicides overtime except trifludimoxazin, which was significantly lower, which indicates that the absorption of trifludimoxazin may be slower than the other compounds. Saflufenacil was the herbicide that showed more translocation out of the treated, while trifludimoxazin showed no translocation out of leaf treated. The translocation of saflufenacil decreased considerably when in mixture with trifludimoxazin or sulfentrazone, while there was no difference in foliar absorption. On the other hand, trifludimoxazin + carfentrazone-ethyl showed the highest absorption in *E. crus-galli* and maize at 6 HAA. Carfentrazone-ethyl solo was better translocated than the mixture with trifludimoxazin in maize and *E. crus-galli,* which indicated that absorption and translocation is dependent on species and perhaps other mechanisms such as metabolism needed to be studied in other to explain better the synergy of binary mixtures of PPO-inhibitors.

Keywords: Absorption; Binary mixtures; Saflufenacil; Monocots

4.1 Introduction

Inhibition of PPO is a herbicide mechanism of action that has successfully been used for weed management in agriculture since the introduction of the first chemical group, diphenyl ethers, in the 1960s (MATRINGE et al., 1993; DAYAN & DUKE, 2010). The widespread adoption of glyphosate-resistant crops has caused reduction of the field application of PPO inhibitors (DUKE & POWLES, 2008).

Protoporphyrinogen oxidase, also called PPO or Protox, is a key enzyme in the chlorophyll or heme synthesis. Protox-inhibitor herbicides inhibit this enzyme which catalyzes the six-electron oxidation of Protoporphyrinogen-IX to Protoporphyrin-IX. They also are referred to as tetrapyrrole-biosynthesis inhibitors or Protoporphyrin-IX synthesis inhibitors (DAILEY et al., 1995; SMITH et al., 1993).

It is the last common pathway in the production of heme and chlorophyll. While the production of chlorophyll, a light-harvesting pigment, is an essential process for all green photosynthetic organisms, heme is an essential cofactor in cytochromes, oxygenases, peroxidases and catalases, which are important in stress reduction due to the ability to inactivate free radicals (CHAUDIÈRE & FERRARI-ILIOU, 1999). This characteristic makes PPO an excellent enzyme target for herbicide development (LEHNEN et al., 1990; JACOBS et al., 1991; DAYAN & WATSON, 2011).

There are many advantageous characteristics of PPO-inhibitors that include a broad herbicidal spectrum, have low mammalian toxicity, low effective doses, rapid onset of action and long residual activity of some herbicides, as sulfentrazone, in this group (SALAS et al., 2016).

PPO-inhibitors herbicides are products not systemic that control broadleaf weeds selectively (MATZENBACHER et al., 2014). The symptoms observed on the foliage are leaf cupping, crinkling, bronzing, and necrosis, that may be observed after two days of application in post emergence application (DAYAN & DUKE, 2010).

Protox-inhibitor herbicides show differences in absorption among plants species, however the translocation is commonly limited. Nevertheless, slight differences in absorption and translocation can occur, which might explain the tolerance differences at these herbicides. Most of the Diphenyl Ether Protoporphyrinogen (DPE) Oxidase Inhibitors herbicides showed bigger tolerance in soybean due to lesser speed of absorption, translocation and metabolization of the herbicides in the species (RITTER & COBLE, 1981).

Furthermore, the selectivity base of this chemical group can also be attributed minimum absorption and translocation of the herbicide, herbicide sequestration or enhanced mitochondrial PPO enzyme concentration, which serves as a reductant for excess cytoplasm Protoporphyrinogen (HIGGINS et al., 1988; MATSUMOTO et al., 1999; WARABI et al., 2001).

The flumiclorac soybean tolerance is due to reduced absorption and translocation, and high detoxification of this herbicide, while corn tolerance is due to reduced leaf retention and its high metabolization (FAUSEY & RENNER, 2000).

Root uptake of foliar active compounds is generally poor. Most of DPE herbicides are not translocated beyond the point of absorption. However, some of them are translocated by xylem. Some studies showed that absorption and translocation of DPE herbicides may be affected by temperature and humidity (RITTER & COBLE, 1981).

Sulfentrazone, as a soil active compound, after root uptake and leave translocation is rapidly metabolized without any harm to plant (DAYAN & DUKE, 1997). In soybean cells, resistance to oxyfluorfen was selected because of increasing the concentration of the PROTOX in the mitochondria, which enabled higher ability to use excess Protoporphyrin-IX present in the cytoplasm (WARABI et al., 2001).

The shoot absorption is influenced by characteristics of the leaf surface, such as composition, thickness and arrangement of the cuticle, the environmental conditions and the physicochemical characteristics of the herbicide (VIDAL, 2002).

While the absorption of pre-emergence herbicides is influenced primarily by anatomical and physiological barriers to the pathway of herbicides and physicochemical characteristics thereof. In some species, the herbicides coming to ground are preferably absorbed by the root system of the plants, while in others they are absorbed mainly by the shoot emerging parts, such as hypocotyl, epicotyl and coleoptile (ESHEL & PREDEVILLE, 1967).

Dayan & Duke (2010) highlighted that these compounds are effective at very low application doses and have generally good ecotoxicology and human toxicology profiles at recommended application doses. Most of them are highly compatible with the no-tillage agriculture.

As shown there are many information about absorption and translocation of DPE chemical class. It was known that while PPO herbicides are generally more active on dicots than on monocots, but the enzyme target appears to be equally sensitive to the herbicides (LI & NICHOLL, 2005).

As glyphosate-resistant weeds is sharply increasing after the herbicide-tolerant crops adoption, it is essential measure the absorption and translocation of binary mixture of PPO-inhibitor herbicides in some monocots plants in order to evaluate the behavior of these molecules in Poaceae, family of plants.

Since it has known that the binary mixtures of PPO-inhibitors herbicides improved the control of monocots plants, the main propose of this work was investigate the absorption and translocation of saflufenacil, sulfentrazone, trifludimoxazin and cafentrazone as well as the mixtures saflufenacil + trifludimoxazin, saflufenacil + sulfentrazone and trifludimoxazin + carfentrazone-ethyl in *Echinochloa crus-galli* and maize (*Zea mays*).

4.2 Material and Methods

Greenhouse-grown corn (*Zea mays*) and *Echinochloa crus-galli* were used at the two-three leaf stage. This trial was conducted in ADME Laboratory of BASF located in Limburgerhof, Germany from August to September, 2015.

Saflufenacil, sulfentrazone, trifludimoxazin and cafentrazone as well as the mixtures: saflufenacil + trifludimoxazin, saflufenacil + sulfentrazone, and trifludimoxazin + carfentrazone-ethyl were also used in the field trials in Brazil and as mentioned in the Chapter I. Based on field results the binary mixtures, saflufenacil + trifludimoxazin, saflufenacil + sulfentrazone, and trifludimoxazin + carfentrazone-ethyl, the two best and worst mixtures, respectively were selected for these studies, as well as the herbicides alone, in order to provide better understanding of the field results.

Saflufenacil and carfentrazone-ethyl were chosen due to a good efficacy in burndown application related in the literature, while for residual control, trifludimoxazin and sulfentrazone were chosen also based on literature (DAYAN & DUKE, 2010, EVANS in personal contact). The mixtures mentioned in the project were chosen based on intern previous work.

The application consisted of 2.5 $\mu g.mL^{-1}$ test compound alone (saflufenacil, sulfentrazone, trifludimoxazin and cafentrazone) dissolved in 0.25% acetone, 0.025% DMSO, and 0.2% Tween 20 (v/v). One 5 µL single-droplet of the treatments, corresponding to 0.0125 µg of test compound, was applied to the adaxial surface of the second leaf of those plants (Figure 4.1).

The ratio used to apply the mixture of PPO compounds was the same used in field trials (Chapter I). The application mixtures consisted of 5.0 $\mu g.mL^{-1}$ test compound dissolved in 0.5% aceton, 0.05% DMSO, and 0.2% Tween 20 (v/v). The mixtures were saflufenacil + trifludimoxazin (M1), saflufenacil + sulfentrazone (M2), and trifludimoxazin + carfentrazone-ethyl (M3). One 5-µl single-droplet of the

treatments also was applied to the adaxial surface and the corresponding of active ingredient is shown in the Table 4.1.

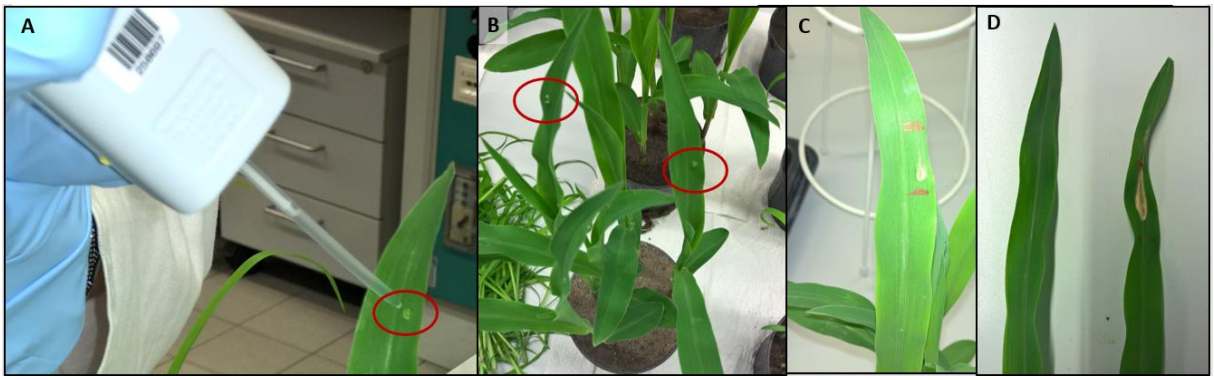

Figure 4.1 – PPO-inhibitor herbicides was applied with on single-droplet in the adaxial surface of the second leaf in maize plants (A e B), the herbicides symptoms at 24 hours of application(C) and 3 days after application (D) of different compounds.

Table 4.1 – Concentration of active ingredient of mixtures compounds found in one 5 µL single-droplet.

Mixtures	a.i/single-droplet (µg)
Saflufenacil + Trifludimoxazin (M1)	0.0125 + 0.0125
Saflufenacil + Sulfentrazone (M2)	0.0025 + 0.0225
Trifludimoxazin + Carfentrazone-ethyl (M3)	0.015 + 0.010

Plants were incubated in a plant growth chamber at 24/22°C (day/night), 65% relative humidity, 18 h day^{-1} light at 5400 Lux (fluorescent lighting), with constant irrigation. Application mixtures were also spotted onto glass slides and incubated in the plant growth chamber to assess possible non-biological depletion of test compounds.

One day and 3 days after application (DAA) each plant was individually dissected into the treated leaf, the rest of the aerial part, and the root. The additional evaluation was made with 6 hours after application, which each plant was individually dissected into the treated leaf. The treated leaf was immersed in acetonitrile-water (1:1, v/v) for 20 s with gentle agitation to remove the non-absorbed deposit of test compound from the leaf surface. All plant sections were extracted with acetonitrile-water (1:1) using a tissue homogenizer. The test compounds applied onto glass slides were recovered by vigorous rinsing with acetonitrile-water (1:1).

Additional plant treatments were conducted in parallel and harvested immediately after application to determine the total compound recovery at time zero.

These results will provide quantitative data on the foliar absorption, distribution into different plant parts (i.e. translocation), and total recovery of active ingredient (i.e. metabolic stability).

The evaluations were done by UPLC-MS/MS (ultra-high performance liquid chromatography coupled to tandem mass spectrometry). The MS/MS equipment was an AB Sciex API4000 or API5500 triple-quadrupole instrument operated in multiple-reaction monitoring mode. This state-of-the-art instrument and methodology allows for very high sensitivity and selectivity in quantitative target analyte determination.

The analysis was based on HATAMI et al. (2016). The effects of maize and *E. crus-galli* and the time, as well as the interaction with PPO-inhibitor herbicides Absorption and translocation were subjected to ANOVA. Both species *Zea mays* and *E. crus-galli* were considered as a fixed factor while the time was considered as a random factor.

The means and standard errors (average) of PPO-inhibitor herbicides absorption and translocation were calculated for all parts of the plants, and the means were analyzed by different groups. For each analysis, assumptions such as equal variance and normal distribution were evaluated. When required, the Tukey test at 5% probability was used for mean separation. Statistical analyses were performed using the SAS Institute Inc. Cary, NC software (version 9.2), SAS, 2002.

4.3 Results and Discussion

PPO-inhibitors herbicides absorption was determined by the difference between the amount of the molecules applied and the amount recovered in the leaf wash. The majority of herbicide was recovered from the treated leaf.

Recovery of saflufenacil, sulfentrazone, trifludimoxazin and carfentrazone-ethyl in the leaf wash was greater than 90% for *E. crus-galli* and *Z. mays* at the zero-harvest interval (data not shown).

The absorption of trifludimoxazin was significantly lower as compared to the other individual herbicides in *E. crus-galli* at 24 hours after application (HAA). However, there were no differences among the herbicides overtime except trifludimoxazin, which indicates that the absorption of trifludimoxazin was slower than the other compounds evaluated (Table 4.2).

Whereas in maize, the absorption was slower as compared to *E. crus-galli* for all herbicides applied at 6 hours after treatment, which increased overtime (Table

4.2). However, carfentrazone-ethyl and sulfentrazone showed greater absorption at 24 hours after application, followed by saflufenacil and trifludimoxazin, which also showed less absorption at 72 hours after application.

Grossmann et al. (2011) reported that maize has shown natural tolerance to saflufenacil as a consequence of more rapid metabolism of the herbicide in the shoot and root tissue, compared to broadleaf weeds. Besides that, low translocation of root-absorbed herbicide into the shoot tissue also favors maize tolerance to saflufenacil.

As a single compound, saflufenacil was the herbicide that showed most translocation out of the treated leaf, reaching about 5 percent in *E. crus-galli* and only 1 percent in *Z. mays*. This compound showed the best translocation at 24 HAA. Saflufenacil and sulfentrazone showed greater translocation than carfentrazone-ethyl, which showed the least translocation, at 72 HAA (Table 4.3). Whereas, sulfrentrazone showed greater translocation in maize than in *E. crus-galli*, the opposite of saflufenacil.

Trifludimoxazin has not shown any translocation out of the treated leaf in *E. crus-galli* and maize and for this reason, this compound was excluded from the statistical analysis.

According to Reis et al. (2015), effective control of weeds species is influenced by initial absorption and subsequent translocation of sufficient herbicide to the site of action where the herbicide is phytotoxic. They reported that aminocyclopyrachlor + metsulfuron-methyl was absorbed only 20% by leaves and the translocation did not exceed 5%, but that this amount was enough to achieve the target site and control *Tecoma stans*.

Most post emergence-applied PPO-inhibitors are promptly absorbed through the leaves. However, some chemical class like diphenyl ether herbicides are not translocated beyond the point of absorption and on the other hand others can be readily translocated by the xylem (DAYAN & DUKE, 2010).

Saflufenacil is translocated mainly in xylem and has limited mobility in the phloem (LIEBL et al., 2008; ASHIGH & HALL, 2010). Grossmann et al. (2010) reported that saflufenacil is a weak acid, which ionizes in solution and provide mobility in xylem and phloem distributing systemically in the plant.

According to Kleier's prediction model, the physical properties of saflufenacil, in terms of ionization constant in aqueous solution value (pKa = 4.4) and

octanol/water partitioning coefficient (LogKow = 2.6) are nearly ideal for phloem systemicity (KLEIER et al., 1998).

About 80% of the applied saflufenacil radioactivity was taken up within 16 hours after treatment in maize, *Solanum nigrum* and *Ipomoea purpurea*. During this time, saflufenacil was able to move systemically within the plant, from the treated leaf in phloem vascular tissue beyond to areas of meristematic growth and storage in residual shoot parts and the root (GROSSMANN et al., 2011).

Those authors also reported that PPO-inhibitors have limited symplastic phloem movement, such as flumioxazin and butafenacil, causing only contact action with tissue necrosis on the treated leaf. Despite saflufenacil and butafenacil have pyrimidinedione core structure, only saflufenacil have a side-chain carrying an acidic proton that confirms the importance of the weak acid moiety at the pyrimidinedione structure.

Table 4.2 – Foliar absorption (%) of the herbicides as single compounds applied at 6, 24 and 72 hours after application (HAA) in *Echinochloa crus-galli* and maize (*Zea mays*). Limburgerhof, Germany, 2015.

		Absorption (%)	
Herbicides	**HAA**	***E. cruz-galli***	***Zea mays***
Saflufenacil	6	45.42 BC	22.30 E
Saflufenacil	24	89.98 A	60.46 DC
Saflufenacil	72	97.78 A	92.46 AB
Trifludimoxazin	6	22.05 C	17.62 E
Trifludimoxazin	24	29.22 C	45.07 D
Trifludimoxazin	72	70.98 AB	77.30 BC
Sulfentrazone	6	51.74 BC	8.77 E
Sulfentrazone	24	82.52 A	94.54 A
Sulfentrazone	72	86.30 A	97.42 A
Carfentrazone	6	27.21 C	6.86 E
Carfentrazone	24	87.99 A	91.68 AB
Carfentrazone	72	94.66 A	99.30 A

Means (n=3) within columns with no common letter are significantly different according to test group average Tukey (α=0.05).

There was no significant difference in the absorption of *E. crus-galli* and maize when saflufenacil was applied solo or in mixture with trifludimoxazin or sulfentrazone during the time. However, it was observed that the absorption sharply increased after 6 HAA for those treatments (Table 4.4).

Table 4.3 – Translocation (%) of the herbicides as single compounds applied at 24 and 72 hours after application (HAA) in *Echinochloa crus-galli* and maize (*Zea mays*). Limburgerhof, Germany, 2015.

		Translocation (%) to treated leaf	
Herbicides	**HAA**	***E. cruz-galli***	***Zea mays***
Saflufenacil	**24**	3.833 A	0.766 A
Saflufenacil	**72**	2.200 B	0.533 AB
Sulfentrazone	**24**	0.016 C	0.246 B
Sulfentrazone	**72**	0.020 C	0.243 BC
Carfentrazone	**24**	0.005 C	0.011 C
Carfentrazone	**72**	0.007 C	0.009 C

Means (n=3) within columns with no common letter are significantly different according to test group average Tukey (α=0.05).

The absorption was higher in *E. crus-galli* than in maize at 6 and 24 HAA, which demonstrated that either saflufenacil, alone or in mixtures, showed slower absorption in maize, that equaled at 72 HAA due to increase significantly overtime. The slow absorption of maize might explain the results from the field (Chapter I), which the mixtures in the lowest doses reached better control in *E. crus-galli* than in maize.

Translocation of saflufenacil decreased considerably when in mixture with trifludimoxazin or sulfentrazone (Table 4.5). Although glyphosate does not belong to the same mechanism of action, similar results have been seen by Ashigh & Hall (2010), that reported the addition of glyphosate reduced the translocation of saflufenacil radiolabeled in glyphosate-susceptible plants, while translocation was not affected in glyphosate-resistant canola, which indicated that it is dependent of the species.

Furthermore, the phytotoxicity of saflufenacil reduced the activity of glyphosate, which might reduce its translocation in all plant species studied. Increased absorption of saflufenacil by the addition of glyphosate plus adjuvant appears to increase its contact activity.

Regarding sulfentrazone, there were no difference between the application as single compound or in mixture with saflufenacil in both *E. crus-galli* and *Z. mays* and it was less than 0.02% of translocation (data not shown).

Starke & Oliver (1996) reported that sulfentrazone plus glyphosate tank mixtures were antagonistic at all dose combinations for *E. crus-galli* and *Amaranthus palmeri*, indicating that these herbicides are not complementary in tank mixtures.

Fadayomi & Warren (1977) reported that there was limited movement of nitrofen and oxyfluorfen, both diphenyl-ether herbicides, from the point of application on leaves. While the extent of movement occurred in the same way to soybean and greenbean, apparently, there was more absorption of both herbicides by soybean, and probably mechanism besides absorption and translocation could be responsible for this difference, since soybean is more tolerant to those herbicides.

Table 4.4 – Foliar absorption (%) of saflufenacil, as single compounds and in mixtures, applied at 6, 24 and 72 hours after application (HAA) in *Echinochloa crus-galli* and maize (*Zea mays*). Limburgerhof, Germany, 2015.

		Absorption (%)	
Herbicides	**HAA**	***E. cruz-galli***	***Zea mays***
Saflufenacil	**6**	45.42 B	22.30 C
Saflufenacil	**24**	89.98 A	60.46 B
Saflufenacil	**72**	97.78 A	92.46 A
Saflufenacil+Trifludimoxazin	**6**	41.72 B	15.69 C
Saflufenacil+Trifludimoxazin	**24**	84.79 A	65.18 B
Saflufenacil+Trifludimoxazin	**72**	96.35 A	85.67 A
Saflufenacil+Sulfentrazone	**6**	39.61 B	24.30 C
Saflufenacil+Sulfentrazone	**24**	93.67 A	62.74 B
Saflufenacil+Sulfentrazone	**72**	93.60 A	95.03 A

Means (n=3) within columns with no common letter are significantly different according to test group average Tukey (α=0.05).

Table 4.5 – Translocation (%) of saflufenacil, as single compounds and in mixtures, applied at 24 and 72 hours after application (HAA) in *Echinochloa crus-galli* and maize (*Zea mays*). Limburgerhof, Germany, 2015.

		Translocation (%) to treated leaf	
Herbicides	**HAA**	***E. cruz-galli***	***Zea mays***
Saflufenacil	**24**	3.833 A	0.767 A
Saflufenacil	**72**	2.200 B	0.533 A
Saflufenacil+Trifludimoxazin	**24**	0.024 C	0.005 B
Saflufenacil+Trifludimoxazin	**72**	0.028 C	0.007 B
Saflufenacil+Sulfentrazone	**24**	0.035 C	0.024 B
Saflufenacil+Sulfentrazone	**72**	0.015 C	0.039 B

Means (n=3) within columns with no common letter are significantly different according to test group average Tukey (α=0.05).

Different from saflufenacil and mixtures, that was observed that the absorption sharply increased after 6 HAA, trifludimoxazin + carfentrazone-ethyl showed the highest absorption in *E. crus-galli* and maize at 6 HAA and this mixture also showed the worst control in the field for both targets (Table 4.6 and Chapter I).

Whereas the absorption of trifludimoxazin, alone or in mixture with saflufenacil, followed the same pattern of other compounds, that is increased overtime. Trifludimoxazin + saflufenacil was one of the mixtures that has shown synergistic

effects in the field, while the mixture of trifludimoxazin and carfentrazone-ethyl has not shown it (Chapter I).

Nevertheless, trifludimoxazin has shown any translocation in *E. crus-galli* and *Z. mays*. For this reason, this herbicide was not evaluated in the statistical analysis. Also, in mixtures with carfentrazone-ethyl or saflufenacil, the translocation was too low reaching only 0.2% in the mixture of trifludimoxazin + carfentrazone-ethyl (data not shown).

Table 4.6 – Foliar absorption (%) of trifludimoxazin, as single compounds and in mixtures, applied at 6, 24 and 72 hours after application (HAA) in *Echinochloa crus-galli* and maize (*Zea mays*). Limburgerhof, Germany, 2015.

		Absorption (%)	
Herbicides	**HAA**	***E. cruz-galli***	***Zea mays***
Trifludimoxazin	**6**	22.05 C	17.62 C
Trifludimoxazin	**24**	29.22 C	45.07 B
Trifludimoxazin	**72**	70.98 AB	77.30 A
Trifludimoxazin+Saflufenacil	**6**	15.57 C	9.50 C
Trifludimoxazin+Saflufenacil	**24**	39.76 BC	43.20 B
Trifludimoxazin+Saflufenacil	**72**	80.63 A	79.03 A
Trifludimoxazin+Carfentrazone	**6**	73.62 A	79.60 A
Trifludimoxazin+Carfentrazone	**24**	84.76 A	81.93 A
Trifludimoxazin+Carfentrazone	**72**	81.61 A	89.40 A

Means (n=3) within columns with no common letter are significantly different according to test group average Tukey (α=0.05).

Likewise the trifludimoxazin results, carfentrazone-ethyl also was not much absorbed (27.21% in *E. crus-galli* and 4.57% in *Z.* mays) at 6 HAA, and also increased overtime, reaching 94.66 and 99.30%, respectively. However, in mixture with trifludimoxazin, the absorption of carfentrazone-ethyl was significantly high at 6 HAA (83.84% in *E. crus-galli* and 62.46% in *Z.* mays), showing also a flat absorption overtime (Table 4.7).

The opposite was seen in the translocation, where carfentrazone-ethyl solo was better translocated than the mixture with trifludimoxazin in maize and *E. crus-galli* (Table 4.8).

Studies conducted by Thompson & Nissen (2000) showed that the absorption of carfentrazone-ethyl was rapid in *Abutilon theophrasti*, that is 70% within 2 hours after treatment, while soybean has absorbed greater than 90% of carfentrazone-ethyl in the first 2 hours after treatment. They also reported that carfentrazone-ethyl absorption in corn, that is a tolerant specie, was similar to absorption by *A.*

theophrasti in the presence of adjuvants and that the absorption is limited without the use of adjuvants.

Corroborating with the results obtain, those authors reported that the translocation is species dependent, and overall less than 1 to 5% of carfentrazone-ethyl was translocated out of the treated leaf at 24 hours after application.

Carfentrazone-ethyl also was less absorbed by maize than *E. crus-galli* following the same tendency of other compounds, despite both reached more than 90% of absorption at 72 HAA.

It was observed that in weed resistance with non-target-site mechanisms, foliar absorption and translocation out of treated sections could be the responsible for significantly less absorption of the compounds causing the resistance. However, the results obtained by Hatami et al. (2016) demonstrated that the absorption and translocation of the herbicide were not the cause of ALS inhibitor herbicides developed by *Rapistrum rugosum*.

Table 4.7 – Absorption (%) of carfentrazone-ethyl, as single compounds and in mixture with trifludimoxazin, applied at 6, 24 and 72 hours after application (HAA) in *Echinochloa crus-galli* and maize (*Zea mays*). Limburgerhof, Germany, 2015.

		Absorption (%)	
Herbicides	**HAA**	***E. cruz-galli***	***Zea mays***
Carfentrazone	**6**	27.21 B	4.57 C
Carfentrazone	**24**	87.99 A	91.68 A
Carfentrazone	**72**	94.66 A	99.30 A
Carfentrazone+Trifludimoxazin	**6**	83.84 A	62.46 B
Carfentrazone+Trifludimoxazin	**24**	93.49 A	94.02 A
Carfentrazone+Trifludimoxazin	**72**	90.56 A	98.57 A

Means (n=3) within columns with no common letter are significantly different according to test group average Tukey (α=0.05).

Table 4.8 – Translocation to treated leaf (%) of carfentrazone-ethyl, as single compounds and in mixture with trifludimoxazin, applied at 24 and 72 hours after application (HAA) in *Echinochloa crus-galli* and maize (*Zea mays*). Limburgerhof, Germany, 2015.

		Translocation (%) to treated leaf	
Herbicides	**HAA**	***E. cruz-galli***	***Zea mays***
Carfentrazone	**24**	0.005 AB	0.011 A
Carfentrazone	**72**	0.007 A	0.013 A
Carfentrazone+Trifludimoxazin	**24**	0.001 B	0.005 AB
Carfentrazone+Trifludimoxazin	**72**	0.003 B	0.003 B

Means (n=3) within columns with no common letter are significantly different according to test group average Tukey (α=0.05).

Thompson & Nissen (2000) still complemented that selectivity of carfentrazone-ethyl cannot be explained by absorption, although the rapid absorption

of carfentrazone-ethyl in soybean may be a factor contributing to limited soybean tolerance. Overall, these studies have shown that absorption and translocation is dependent of species and perhaps other mechanism are involved such as metabolism that could explain better those differences.

Lewis et al. (2013) observed the same to synthetic auxin herbicides, that absorption, translocation and also metabolism can vary depending on plant species and compounds.

It's also important to highlight that light intensity, humidity and temperature can increase or decrease the absorption and translocation of the herbicides. For example, Grossmann & Ehrhardt (2007) studying topramezone tolerant mechanism in corn, observed that the foliar absorption decreased 50% in dark conditions, and 50% in temperatures below of 8°C, consequently reducing the translocation in 75%.

High temperatures provide better efficacy of PPO-inhibitors herbicides due to favor the absorption as a result of changes in the composition and the permeability of the cuticle, and high dose of chemical reaction. It is important to keep in mind that each plant species has an optimum temperature for tissue development (MATZENBACHER et al, 2014).

Those authors also reported that high temperature associated with high values of relative humidity may cause strong hydration of the cuticle, which also favors the absorption and the efficacy of PPO-inhibitors.

4.4 Conclusion

As single compounds, the absorption was slower in maize than *Echinochloa crus-galli*. There were no differences across herbicides overtime except trifludimoxazin, that was significantly lower than the other compounds.

Saflufenacil was the herbicide that showed the best translocation out of treated leaf, reaching about 5.0 percent in *E. crus-galli* and only 1 percent in *Z. mays*, while trifludimoxazin has not shown any translocation out of leaf treated.

The translocation of saflufenacil decreased considerably when in mixture with trifludimoxazin or sulfentrazone, while there was not difference in foliar absorption. Saflufenacil, alone or in mixtures, showed slower absorption in maize in the first 6 HAA, while trifludimoxazin + carfentrazone-ethyl showed the highest absorption in *E. crus-galli* and maize already in the first 6 HAA.

Carfentrazone-ethyl showed the same tendency of other compounds in foliar absorption, low at the beginning and increased overtime. However, the opposite was seen in the translocation, where carfentrazone-ethyl solo was better translocated than the mixture with trifludimoxazin in maize and *E. cruz-galli,* which indicated that it's dependent of species and perhaps other mechanism are involved such as metabolism.

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CHAPTER IV

Herbicide tolerant model system: *Arabidopsis thaliana* transgenic lines with insensitive PPO1 and PPO2 isoforms

Abstract

Herbicide tolerant model system: *Arabidopsis thaliana* transgenic lines with insensitive PPO1 and PPO2 isoforms

The introduction of herbicide resistant crops has changed the strategy to manage weeds, since it is based on only a single mechanism of action, resulting in a big issue to resistant weeds management. One strategy to sustain the weed control is to develop additional trait that provides resistant crops to herbicides with alternative mechanisms of action. PPO tolerant crops that enable PPO-inhibitors herbicides to be used selectively in crop is an alternative approach to control weeds. Transgenic *Arabidopsis thaliana* with different PPO isoforms: PPO2 overexpressed from *Amaranthus tuberculatus;* PPO1 overexpressed from *A. thaliana;* PPO1 Acuron mutant from *A. thaliana;* PPO2 with two different mutations: L397Q + F420M and R128A + F420M; PPO2 with two natural mutation: deletion of Glycine in the 210 position and the substitution of R128L both in *A. tuberculatus* comparing with the *A. thaliana* wild type have been evaluated under the application of several PPO-inhibitors herbicides. *A. thaliana* with gene of PPO2 mutations from *A. tuberculatus* with the both substitutions L397Q + F420M and R128A + F420M showed the best performance in terms of tolerance, followed by natural mutations (dG210 and R128L) that were slightly better than PPO1 Acuron mutation. Regarding PPO-herbicides, sulfentrazone and carfentrazone-ethyl, followed by saflufenacil as well as the mixtures saflufenacil + sulfentrazone or carfentrazone-ethyl + trifludimoxazin were the treatments more selective to those traits.

Keywords: PPO-inhibitors; Herbicide resistant crops; Resistant weed management

5.1 Introduction

Herbicide-resistant crops, mainly glyphosate-resistant crops, have transformed the strategic of growers to managing weeds since 1996. This technology enabled a new herbicide system, that was effective, easy-to-use, economical, and safe. However, only a single mechanism of action to control weeds resulted in a big issue to manage resistant weeds (GREEN & OWEN, 2011; MORTENSEN et al., 2012; RIGGINS & TRANEL, 2012; WRIGHT et al., 2010).

Wright et al. (2010) reported that the use of additional weed control mechanisms which complement glyphosate-resistant crops are strongly needed. One strategy to sustain the weed control is to develop additional trait that provides resistance to herbicides with alternative mechanisms of action. After three decades and billions of dollars invested in research and development, only a few transgenic herbicide traits are commercially available (GREEN & OWEN, 2011).

Protoporphyrinogen oxidase, called PPO or Protox, has been used for many years to broadleaf weeds control. However, during the late 1990s had a slowly decline in use due to the widespread adoption of glyphosate-resistant crop varieties. However, as glyphosate resistance weeds continues to increase, growers are once again relying on PPO-inhibiting herbicides as an alternative approach to control weeds (RIGGINS & TRANEL, 2012).

PPO is a key enzyme in the chlorophyll or heme synthesis. Protox-inhibitor herbicides inhibit this enzyme, which catalyzes the six-electron oxidation of Protoporphyrinogen-IX to Protoporphyrin-IX. They also are referred to as tetrapyrrole-biosynthesis inhibitors or Protoporphyrin-IX synthesis inhibitors (DAILEY et al., 1995; SMITH et al., 1993).

It is the last common pathway in the production of heme and chlorophyll. While the production of chlorophyll, a light-harvesting pigment, is an essential process for all green photosynthetic organisms, heme is an essential cofactor in cytochromes, hemoglobin, oxygenases, peroxidases and catalases, which are important in stress reduction due to the ability to inactivate free radicals (CHAUDIÈRE & FERRARI-ILIOU, 1999). This characteristic makes PPO an excellent enzyme target for herbicide development (LEHNEN et al., 1990; JACOBS et al., 1991; DAYAN & WATSON, 2011).

The herbicide action occurs by enzyme competition between Protox herbicide and Protoporphyrinogen-IX. Since the PPO family of herbicides has more affinity for the enzyme, Protoporphyrinogen-IX accumulates in the chloroplast or mitochondria and diffuses into the cytoplasm which subsequently is converted to Protoporphyrin-IX by plasma membrane peroxidases, enzymatic oxidation (JACOBS et al., 1991; DAILEY et al., 1995). Once in the cytoplasm, Protoporphyrin-IX cannot return to the chloroplast because it is highly lipophilic (LEHNEN et al., 1990).

Exposure to light causes formation of singlet oxygen and other oxidative species, resulting in membrane disruption and subsequent cell death. PPO inhibitor herbicides have characteristically a very rapid contact action, causing leaf burning, desiccation and growth inhibition, resulting in complete death of plants (JACOBS et al., 1991; MORI & SCHROEDER, 2004; DAYAN & WATSON, 2011).

In plants, two isoforms of PPO, namely PPO1 (targeted to the chloroplast and encoded by the gene PPX1) and PPO2 (mitochondrial PPO, encoded by the gene PPX2), have been found (LERMONTOVA et al., 1997; POWLES & YU, 2010).

According to Dayan & Duke (2010), plant PPO1 is compartmentalized in the thylakoid and in the envelope membranes of chloroplasts, whereas the mitochondrial isoform PPO2 is localized on the outer surface of the inner mitochondrial membrane. In the chloroplast, the porphyrin pathway leads to both chlorophyll and heme, whereas it leads exclusively to heme in the mitochondrion (DAYAN & DUKE, 1997).

Two Protox isoenzymes have been described in tobacco, a plastidic and a mitochondrial form. Protox genes or cDNAs have been cloned from *Escherichia coli*, *Bacillus subtilis*, human, cow, mouse, and yeast. The molecular masses of these Protox gene products range from 50 to 60 kDa, except for the 21-kDa *E. coli.* The N terminus is most widely conserved, and the deduced amino acid sequences of PPO1 and PPO2 are only 27.3% similar (WATANABE et al., 2001).

PPO enzyme mutations tend to reduce the enzymatic activity, that might explain the relatively slow evolution of resistant weeds to this 40-year-old herbicide class. Some companies continue to synthesize analogues and commercialize new PPO-inhibiting herbicides, that is the example of saflufenacil, launched in 2010 and labeled for wide variety of crops (GREEN & OWEN, 2011).

Resistance to these herbicides has been slow to develop in the field (HEAP, 2017). The figure 5.1 shown a global list of herbicide-resistant weeds that compare PPO-inhibitor herbicides to major herbicide mechanisms of action (adapted from POWLES & YU, 2010).

Dayan &Duke (1997) and Dayan et al. (2010) reported that this could be due to the relatively short-lived selection pressure of these fast-acting foliar herbicides applied. However, the development of more persistent soil-active PPO-inhibitors might increase the selection pressure and consequently raises likely the resistance development.

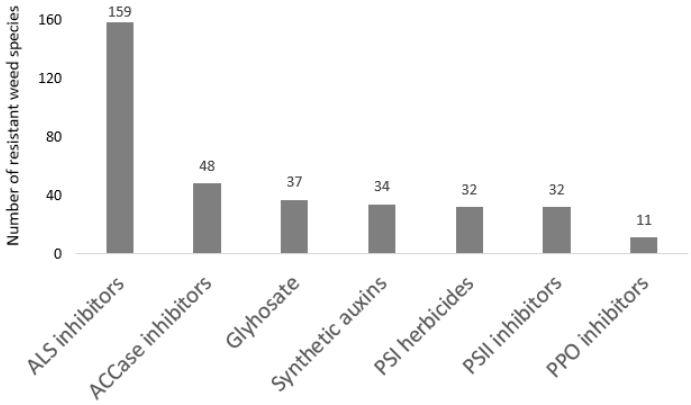

Figure 5.1 - Number of weeds species that have evolved resistance to major herbicides mechanisms of action (HEAP, 2017; adapted from POWELS et al., 2010).

Besides that, Dayan & Duke (2010) highlighted that these compounds are effective at very low application doses and have generally good ecotoxicology and human toxicology profiles at recommended application doses. Most of them are highly compatible with the no-tillage agriculture.

The first weed to evolve resistance to PPO herbicides was *Amaranthus tuberculatus* in 2001. This resistance was attributed to target-site mutation in the PPX2 gene. A unique target-site amino acid deletion (Gly210) confer resistance in this specie (SALAS et al., 2016)

PPO herbicide-resistant maize plants have been reported by isolating PPO genes and herbicide-resistant mutants. At the same time, PPO inhibitor-resistant rice was developed by expression of the *Bacillus subtilis* PPO gene via targeting the gene into either chloroplast or cytoplasm. Also, other ways have been reported such as conventional tissue culture methods, expression of modified co-factors of the protoporphyrin IX binding subunit proteins, over-expression of wild-type plant PPO gene, and engineering of P-450 monooxygenases to degrade the PPO inhibitor (LI & NICHOLL, 2005).

Also, was reported by Ha et al. (2003) that rice PPO-resistance was strongly related to increase the amount of PPO production, which reduced the metabolic inhibiting effect of the herbicide. Jung et al. (2009) has confirmed that the transgenic rice line (M4) was about 200-fold more resistant to oxyfluorfen, acifluorfen, carfentrazone-ethyl that the wild type (WT) in transplanted and direct-seeded rice.

Transgenic crops resistant to PPO-inhibiting herbicides have been developed and the technology even received the trade name Acuron. The first PPO-resistant

corn used a double mutant PPO, PPO1 from *A. thaliana*. The broad-spectrum weed control and soil residual activity of PPO herbicides could be useful in corn, soybean, and cotton, but the resistance management should be considered (GREEN & OWEN, 2011).

Dayan & Duke (1997) reported that crop resistance to PPO-inhibitors could be manipulated by alteration of the enzymes that degrade the herbicide, that is PPO, the enzymes that degrade Protogen and/or Proto IX, and the herbicide-resistant, that is peroxidase-like enzyme that generates Proto IX in herbicide-treated plants.

The main proposal of this work was to evaluate the activity of PPO compounds in transgenic *Arabidopsis thaliana* with different PPO isoforms: PPO2 overexpressed from *A. tuberculatus;* PPO1 overexpressed from *A. thaliana;* PPO1 Acuron mutant from *A. thaliana;* PPO2 with two different mutations: L397Q + F420M and R128A + F420M; PPO2 with two natural mutation: deletion of Glycine in the 210 position and the substitution of R128L both in *A. tuberculatus* comparing with the *A. thaliana* wild type. Furthermore, since two traits were based on natural mutations enabling discuss resistance management and finally herbicide selectivity traits discussion.

5.2 Material and Methods

Seven traits were chosen, some of them were represented with two events, and a broad range of PPO compounds as single compounds followed by different mixtures were applied in order to reach the answers. These compounds were also used in the field trials in Brazil and as mentioned in the Chapter I, saflufenacil and carfentrazone-ethyl were chosen due to a good efficacy in burndown application related in the literature. Already for residual control, trifludimoxazin, flumioxazin and sulfentrazone were chosen also based on literature (DAYAN & DUKE, 2010, EVANS in personal contact). The mixtures mentioned in the project were chosen based on intern previous work.

This trial was conducted in Herbicides Greenhouse at the Agricultural Research Station located in Limburgerhof, Germany from August to September, 2015.

Transgenic *A. thaliana* with PPO respectively insensitive PPO1 and PPO2 isoforms (Table 5.1), overexpressed different variations of the PPO genes under control of strong constitutive promotor PcUbi and Imazamox as a selective marker (AHAS gene) also including mutations avoiding binding of the PPO inhibitors to the

target peptide. The transformed seeds (F2) were obtained from Metanomix® with null, homo and heterozygotes. The best 15 events were chosen previously by germination assay and molecular analysis. The germination assessments were made in dose-response curves with imazamox doses and then, was made the ranking with the best ones. By molecular analysis was given preference to the simple insertion events to prevent possible additional effects.

The wild type plants, that is non-genetic plants that occurs in nature, was used as reference in order to distinguish from mutant forms. *A. thaliana* mutant forms were grown on substrate on 14-August-2015 (16-22 TºC and 16 h of light) and treated with imazamox (40g/ha) + 1% Dash. This pre-application was made to select only genetic plants, avoiding mixtures with wild type. After 18 days, these transgenic lines were transplanted each one in single pots and maintained in the chamber (16-22 TºC and 16 h light) for seven more days in order to acclimatize the plants to prevent possible stressed plants during the application (Figure 5.2).

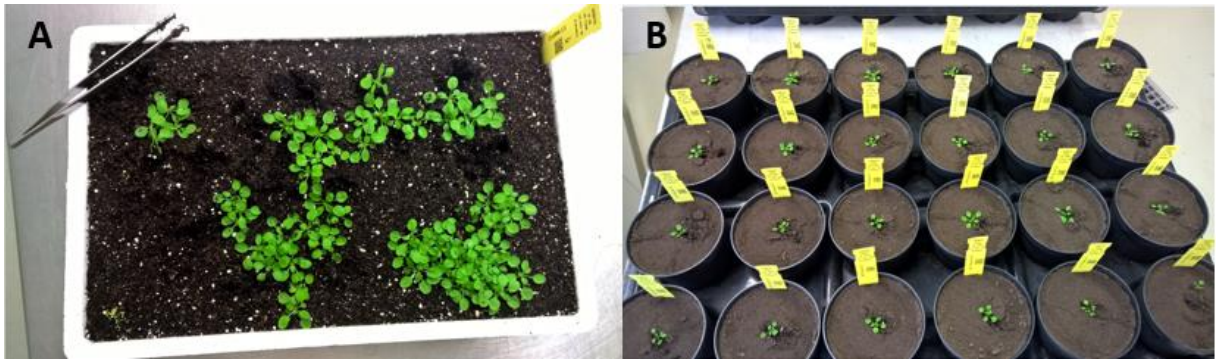

Figure 5.2 – Plants selected by Imazamox application (A) and transplanted in single pots (B).

The plants were transferred to greenhouse with 16h of darkness for the purpose of avoid flowering and obtain the leaves stronger. Four weeks after sowed, the plants were treated in a small rosette stage with PPO inhibitor compounds solo (saflufenacil, trifludimoxazin, flumioxazine, sulfentrazone, carfentrazone-ethyl) and the respective mixtures (Table 5.2).

Trifludimoxazin is a new PPO-inhibitor herbicide, and belongs to Triazinone chemical class. This herbicide has been developed by BASF SE, but it is still under registration process in some countries such as Australia, Canada, United States and Argentina.

The application doses were based on the doses used previously in the field (Chapter I). The highest dose corresponds to two fold of the highest dose used in the field and the wide ratio among treatments is due to include all trait effects. In case of the mixtures candidates, the doses were chosen based on the half of the single concentration of each compound to mixtures may have the same activity like single compounds.

Table 5.1 - Transgenic *Arabidopsis thaliana* with PPO inhibitor insensitive PPO 1 and PPO 2 isoforms. Limburgerhof, Germany, 2015.

Treatments	Constructs	Events	Gene	Observation
1	1	ET 0443 E	AMATU_PPO2_wt	PPO2 overexpressed without mutations
2	1	ET 0443 G	AMATU_PPO2_wt	PPO2 overexpressed without mutations
3	2	ET 0770 A	AMATU_PPO2_L397Q_F420M	PPO2 mutations
4	3	ET 0469 Q	AMATU_PPO2_R128A_F420M	PPO2 mutations
5	4	ET 0448 F	AMATU_PPO2_dG210	PPO2 natural mutations
6	5	ET 0442 M	AMATU_PPO2_R128L	PPO2 natural mutations
7	6	ET 0446 C	At_PPO1_wt	PPO1 overexpressed without mutations
8	6	ET 0446 L	At_PPO1_wt	PPO1 overexpressed without mutations
9	7	ET 0445 D	At_PPO1_Ac	PPO1 from Acuron mutation
10	7	ET 0445 E	At_PPO1_Ac	PPO1 from Acuron mutation
11	8	ET 0737	MC24 (WT)	Wild type

The herbicides treatments were applied with a robot sprayer machine pressurized with compressed air, fitted with XR 110.015 nozzle (TeeJet, Spraying Systems Co.) calibrated to deliver 375 $L.ha^{-1}$ at 2.1 bar.

Phytotoxicity assessments were conducted at 7 and 14 days after application, where 0% was assigned to no phytotoxic effects of the herbicide, and 100% when plants were completely controlled, according to the methodology proposed by Velini (1995). The percentage of phytotoxicity effects means percentage reduction in volume of the weeds in comparison with the untreated plot plus percentage degree of damage to the remaining plant biomass.

Table 5.2 – Treatments: herbicides, products concentration/formulation and doses (grams of active ingredient per hectare – g a.i.ha^{-1}) applied in the trials.

Herbicides	Product Concentration (g kg^{-1})/Formulation	Doses (g a.i.ha^{-1})
Untreated		
Saflufenacil *	342 SC	2,5
		25
		100
Trifludimoxazine *	500 SC	2,5
		25
		100
Flumyoxazin *	510 WG	7
		70
		280
Sulfentrazone *	480 SC	21
		210
		840
Carfentrazone *	240 EC	1,75
		17,5
		70
Saflufenacil + Flumyoxazin *	342 SC + 510 WG	1,25 + 3,5
		12,5 + 35
		50 + 140
Saflufenacil + Trifludimoxazine *	342 SC + 500 SC	1,25 + 1,25
		12,5 + 12,5
		50 + 50
Saflufenacil + Sulfentrazone *	342 SC + 480 SC	1,25 + 10,5
		12,5 + 105
		50 + 420
Trifludimoxazine + Carfentrazone *	500 SC + 240 EC	1,25 + 0,88
		12,5 + 8,75
		50 + 35

*All herbicides treatments included DASH 0.5% v/v.

The experiments were conducted in a randomized complete block design with two repetitions. The data from each field experiment was analyzed separately. Data were subjected to analysis of variance (ANOVA) using the statistical analysis software version 9.2 (SAS Institute Inc. Cary, NC). Normality, homogeneity of variance, and interactions of treatments in greenhouse were tested. Where the ANOVA indicated treatment effects were significant, means were separated at Scott Knott ($\alpha = 0.05$). The data of percent weed control were arcsine transformed before analysis; however, non-transformed percentages are presented with mean separation based on transformed data.

5.3 Results and Discussion

PPO-inhibitors as single compounds or in binary mixtures had similar activity on susceptible wild type *A. thaliana,* reaching greater than 95% of control at the lowest doses tested (Table 5.3).

The overexpression of wild type PPO1 and overexpression of wild type PPO2 are represented by two events also in the Table 5.3. Based on these results, PPO compounds both alone or in mixture had activity in both PPO isoforms (Figure 5.3).

However, flumioxazin and the mixture contained saflufenacil showed the less selectivity to those transgenic *A. thaliana*, followed by saflufenacil and trifludimoxazin that showed similar performance, being slight more selective to wild type PPO2 overexpressed.

Li & Nicholl (2005) related that over-expressing native PPO genes in plants has been used to improve plant resistant to PPO-inhibitory herbicides. Transgenic tobacco plants over-expressing the wild type *A. thaliana* PPO enzyme had 5 to 7 times more enzyme activity and were more resistant to the diphenyl ether herbicide.

The resistance to this chemical class was conferred by over-expression of the plastidic PPO gene isoform, that neutralized herbicidal action. It prevented the accumulation of the substrate protoporphyrinogen IX, and abolished the light-dependent phytotoxicity caused by PPO-herbicides (LI & NICHOLL, 2005).

Sulfentrazone and carfentrazone-ethyl showed the best selectivity among the tested compounds, however while sulfentrazone showed better activity in PPO2 overexpressed and consequently less selectivity, carfentrazone-ethyl provided better control in PPO1 overexpressed. It could be an indication that sulfentrazone has better activity on PPO2 enzyme while carfentrazone-ethyl on PPO1 enzyme, although in order to have clear answer it is recommended compare the same species. In this case, the overexpression of PPO1 came from *A. thaliana* while PPO2 overexpressed came from *Amaranthus tuberculatus*.

Figure 5.3 – Pictures of transgenic *A. thaliana* (1): overexpressed the wildtype PPO1 enzyme from *A. thaliana* and (2) overexpressed the wildtype PPO2 enzyme from *A. tuberculatus.* From left to right, there is the untreated, highest, medium and lowest dose of (A) Saflufenacil, (B) Trifludimoxazin, (C) Flumioxazin, (D) Sulfentrazone, (E) Carfentrazone-ethyl, (F) Saflufenacil + Flumioxazin, (G) Saflufenacil + Trifludimoxazin, (H) Saflufenacil + Sulfentrazone and (I) Carfentrazone-ethyl + Trifludimoxazin, at 14 days after application. Limburgerhof, Germany, 2015.

Nevertheless, when sulfentrazone and carfentrazone-ethyl were in mixture with another PPO inhibitor showed there was similar activity on both PPO isoforms, decreasing the selectivity like the other mixtures in the Table 5.3.

Singh et al. (2011) reported that application of herbicides as a tank mixture is a popular method adopted by growers due to improved broad spectrum weed control from a single application that reduces labor and fuel costs.

PPO1 Acuron mutation performed better than overexpression of wild type PPO1 (Table 5.4). In this case, flumioxazin and the mixture of flumioxazin+ saflufenacil was not selective to transgenic *A. thaliana*. Regarding single compounds, trifludimoxazin was not selective at the medium and highest dose. Besides that, trifludimoxazin in mixture with saflufenacil or carfentrazone-ethyl followed the same tendency, being not selective at the highest doses. The better mixture to be used with this technology was saflufenacil and sulfentrazone which in the field provided the best control of monocots and broadleaf weeds (Chapter I).

According to Green & Owen (2011), transgenic crops resistant to PPO-inhibiting herbicides received the trade name Acuron and the first PPO-resistant corn used a double mutant PPO, that was PPO1 from *A. thaliana*. While to confer resistance to PPO-resistant rice was used overexpression of the naturally resistant *Bacillus subtilis* PPO gene and also to select for overexpression of wild type PPO genes, have been used an increasing gene copy number and tissue culture.

A. thaliana with gene of PPO2 mutations from *A. tuberculatus* with the both substitutions L397Q + F420M and R128A + F420M showed the best performance in terms of selectivity (Table 5.4), indicating that both could be a good trait and future method for controlling undesired vegetation at crop cultivation site.

Even Flumioxazin that showed outstanding control of the other events, showed slight less activity for these PPO2 mutations. In that case, only the mixture of flumioxazin with saflufenacil did not show good selectivity, the other ones showed good tolerance for those traits. The most selective single compounds were saflufenacil, sulfentrazone and carfentrazone-ethyl.

Table 5.5 shown PPO2 from two natural mutations, being one substitution of Arginine per Leucine in the position of 128 (R128L) and one deletion of Glycine in the position of 210 (dG210). Both traits showed similar tolerance towards the treatments with broad range of PPO compounds, except to flumioxazin, followed by the mixture of flumioxazin + saflufenacil and trifludimoxazin (Figure 5.4).

Table 5.3 – Control (in percentage) of transgenic *Arabidopsis thaliana* with PPO1 overexpressed in *Arabisopsis thaliana* (At_PPO1_wt in two different events) and PPO2 isoforms overexpressed without mutation from *Amaranthus tuberculatus* (AMATU_PPO2_wt in two different mutation), at 7 and 14 days after application (DAA). Limburgerhof, Germany, 2015.

	Treatments	FO	(g i.a/ha)	Wild type		At_PPO1_wt (C)		At_PPO1_wt (L)		AMATU_PPO2_wt (E)		AMATU_PPO2_wt (G)	
				7 DAA	14 DAA	7 DAA	14 DAA	7 DAA	14 DAA	7 DAA	14 DAA	7 DAA	14 DAA
1	Untreated			--	--	--	--	--	--	--	--	--	--
2	Saflufenacil *	WG	12.5	100.0 a	100.0 a	62.5 d	30.0 d	84.0 c	65.0 b	72.5 c	55.0 c	75.0 e	62.5 d
3	Saflufenacil *	WG	25	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
4	Saflufenacil *	WG	50	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
5	Trifludimoxazine *	SC	12.5	99.0 a	100.0 a	50.0 d	32.5 d	72.5 e	50.0 b	65.0 d	62.5 c	67.5 e	42.5 e
6	Trifludimoxazine *	SC	25	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
7	Trifludimoxazine *	SC	50	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
8	Flumioxazin *	WG	35	95.0 b	100.0 a	90.0 b	100.0 a	90.0 c	99.0 a	85.0 c	99.0 a	90.0 c	98.0 b
9	Flumioxazin *	WG	70	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
10	Flumioxazin *	WG	140	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	98.0 b	100.0 a
11	Sulfentrazone *	SC	105	96.5 b	92.5 b	57.5 d	57.5 c	65.0 e	60.0 b	55.0 d	50.0 c	65.0 e	52.5 e
12	Sulfentrazone *	SC	210	100.0 a	100.0 a	82.5 c	82.5 b	77.5 d	75.0 b	94.0 b	92.5 b	87.5 c	92.5 b
13	Sulfentrazone *	SC	420	100.0 a	100.0 a	90.0 b	95.0 b	95.0 b	95.0 a	100.0 a	100.0 a	99.0 b	100.0 a
14	Carfentrazone *	EC	8.75	100.0 a	100.0 a	52.5 d	57.5 c	65.0 e	57.5 b	37.5 e	37.5 c	37.5 f	37.5 e
15	Carfentrazone *	EC	17.5	100.0 a	100.0 a	98.0 a	97.5 a	100.0 a	100.0 a	77.5 c	72.5 c	80.0 d	62.5 d
16	Carfentrazone *	EC	35	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	95.0 b	90.0 b	90.0 c	82.5 c
17	Saflufenacil + Flumioxazin *	WG+WG	12.5 + 35	100.0 a	100.0 a	99.0 a	100.0 a	98.0 b	100.0 a	98.0 b	100.0 a	98.0 b	100.0 a
18	Saflufenacil + Flumioxazin *	WG+WG	25 + 70	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	99.0 a	100.0 a	100.0 a	100.0 a
19	Saflufenacil + Flumioxazin *	WG+WG	50 + 140	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
20	Saflufenacil + Trifludimoxazine *	WG+SC	12.5 + 12.5	100.0 a	100.0 a	90.0 b	85.0 b	94.0 b	91.5 a	84.0 c	82.5 b	87.5 c	75.0 c
21	Saflufenacil + Trifludimoxazine *	WG+SC	25 + 25	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
22	Saflufenacil + Trifludimoxazine *	WG+SC	50 + 50	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
23	Saflufenacil + Sulfentrazone *	WG+SC	12.5 + 105	100.0 a	100.0 a	75.0 c	60.0 c	55.0 e	20.0 c	62.5 d	55.0 c	70.0 e	62.5 d
24	Saflufenacil + Sulfentrazone *	WG+SC	25 + 210	100.0 a	100.0 a	99.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
25	Saflufenacil + Sulfentrazone *	WG+SC	50 + 420	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
26	Carfentrazone + Trifludimoxazine *	EC+SC	8.75 + 12.5	100.0 a	100.0 a	37.5 d	37.5 d	67.5 e	60.0 b	60.0 d	55.0 c	42.5 f	40.0 e
27	Carfentrazone + Trifludimoxazine *	EC+SC	17.5 + 25	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
28	Carfentrazone + Trifludimoxazine *	EC+SC	35 + 50	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a
	CV (%)			1.4	3.5	6.6	6.7	4.6	9.2	6.2	8.9	3.2	5.5

*All herbicide treatments included DASH 0.5% v/v.
Means (n=3) within columns with no common letter are significantly different according to test group average Scott_Knott (α=0.05).

Table 5.4 – Control (in percentage) of transgenic *Arabidopsis thaliana* with PPO1 from Acuron mutation (At_PPO1_Acuron in two different events) and PPO2 from two different mutations (L397Q+F420M and R128L+F420M) at 7 and 14 days after application (DAA). Limburgerhof, Germany, 2015.

Treatments	FO	(g i.a/ha)	Wild type		At_PPO1_Acuron (D)		At_PPO1_Acuron (E)		AMATU_PPO2_L397Q_F420M		AMATU_PPO2_R128A_F420M	
			7 DAA	14 DAA	7 DAA	14 DAA	7 DAA	14 DAA	7 DAA	14 DAA	7 DAA	14 DAA
1 Untreated			--	--	--	--	--	--	--	--	--	--
2 Saflufenacil *	WG	12.5	100.0 a	100.0 a	12.5 e	0.0 e	7.5 c	2.5 c	17.5 b	10.0 e	0.0 d	2.5 e
3 Saflufenacil *	WG	25	100.0 a	100.0 a	62.5 c	45.0 c	37.5 b	25.0 b	25.0 b	10.0 e	2.5 d	2.5 e
4 Saflufenacil *	WG	50	100.0 a	100.0 a	82.5 b	77.5 b	72.5 a	65.0 a	42.5 b	35.0 d	7.5 d	10.0 d
5 Trifludimoxazine *	SC	12.5	99.0 a	100.0 a	50.0 c	27.5 d	12.5 c	7.5 c	10.0 b	5.0 e	7.5 d	0.0 e
6 Trifludimoxazine *	SC	25	100.0 a	100.0 a	99.0 a	99.0 a	92.5 a	92.5 a	70.0 a	62.5 c	62.5 a	45.0 b
7 Trifludimoxazine *	SC	50	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	85.0 a	84.0 b	70.0 a	55.0 b
8 Flumioxazin *	WG	35	95.0 b	100.0 a	90.0 b	99.0 a	90.0 a	99.0 a	85.0 a	99.0 a	91.5 a	99.0 a
9 Flumioxazin *	WG	70	100.0 a	100.0 a	98.0 a	100.0 a	98.0 a	100.0 a	90.0 a	100.0 a	85.0 a	100.0 a
10 Flumioxazin *	WG	140	100.0 a	100.0 a	99.0 a	100.0 a	99.0 a	100.0 a	90.0 a	100.0 a	90.0 a	100.0 a
11 Sulfentrazone *	SC	105	96.5 b	92.5 b	5.0 e	2.5 e	7.5 c	2.5 c	17.5 b	10.0 e	5.0 d	0.0 e
12 Sulfentrazone *	SC	210	100.0 a	100.0 a	30.0 d	30.0 d	37.5 b	25.0 b	25.0 b	17.5 e	12.5 c	2.5 e
13 Sulfentrazone *	SC	420	100.0 a	100.0 a	75.0 b	67.5 b	50.0 a	40.0 b	40.0 b	42.5 d	32.5 b	5.0 d
14 Carfentrazone *	EC	8.75	100.0 a	100.0 a	17.5 d	17.5 d	15.0 c	7.5 c	22.5 b	15.0 e	5.0 d	0.0 e
15 Carfentrazone *	EC	17.5	100.0 a	100.0 a	72.5 b	62.5 b	70.0 a	65.0 a	20.0 b	15.0 e	15.0 c	2.5 e
16 Carfentrazone *	EC	35	100.0 a	100.0 a	87.5 b	80.0 b	82.5 a	77.5 a	20.0 b	12.5 e	17.5 c	2.5 e
17 Saflufenacil + Flumioxazin *	WG+WG	12.5 + 35	100.0 a	100.0 a	98.0 a	100.0 a	96.5 a	100.0 a	85.0 a	100.0 a	85.0 a	98.0 a
18 Saflufenacil + Flumioxazin *	WG+WG	25 + 70	100.0 a	100.0 a	98.0 a	100.0 a	98.0 a	100.0 a	85.0 a	100.0 a	85.0 a	99.0 a
19 Saflufenacil + Flumioxazin *	WG+WG	50 + 140	100.0 a	100.0 a	98.0 a	100.0 a	98.0 a	100.0 a	85.0 a	100.0 a	92.5 a	100.0 a
20 Saflufenacil + Trifludimoxazine *	WG+SC	12.5 + 12.5	100.0 a	100.0 a	72.5 b	55.0 b	62.5 a	52.5 a	20.0 b	27.5 e	7.5 d	7.5 d
21 Saflufenacil + Trifludimoxazine *	WG+SC	25 + 25	100.0 a	100.0 a	95.0 a	90.0 a	91.5 a	85.0 a	37.5 b	40.0 d	27.5 c	25.0 c
22 Saflufenacil + Trifludimoxazine *	WG+SC	50 + 50	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	100.0 a	67.5 a	62.5 c	65.0 a	45.0 b
23 Saflufenacil + Sulfentrazone *	WG+SC	12.5 + 105	100.0 a	100.0 a	20.0 d	17.5 d	5.0 c	7.5 c	7.5 b	10.0 e	5.0 d	5.0 d
24 Saflufenacil + Sulfentrazone *	WG+SC	25 + 210	100.0 a	100.0 a	62.5 c	45.0 c	42.5 b	35.0 b	20.0 b	17.5 e	5.0 d	5.0 d
25 Saflufenacil + Sulfentrazone *	WG+SC	50 + 420	100.0 a	100.0 a	82.5 b	70.0 b	65.0 a	57.5 a	17.5 b	17.5 e	27.5 c	7.5 d
26 Carfentrazone + Trifludimoxazine *	EC+SC	8.75 + 12.5	100.0 a	100.0 a	20.0 d	30.0 d	22.5 b	15.0 b	12.5 b	7.5 e	12.5 d	12.5 d
27 Carfentrazone + Trifludimoxazine *	EC+SC	17.5 + 25	100.0 a	100.0 a	100.0 a	100.0 a	91.5 a	85.0 a	25.0 b	17.5 e	40.0 b	30.0 b
28 Carfentrazone + Trifludimoxazine *	EC+SC	35 + 50	100.0 a	100.0 a	100.0 a	100.0 a	99.0 a	100.0 a	70.0 a	65.0 c	60.0 a	45.0 b
CV (%)			1.4	3.5	8.2	9.0	14.8	16.7	12.5	15.6	21.0	14.7

*All herbicide treatments included DASH 0.5% v/v.
Means (n=3) within columns with no common letter are significantly different according to test group average Scott_Knott (α=0.05).

Table 5.5 – Control (in percentage) of transgenic *Arabidopsis thaliana* with PPO2 from two different natural mutations (dG210 and R128L) at 7 and 14 days after application (DAA). Limburgerhof, Germany, 2015.

	Treatments	FO	(g i.a/ha)	Wild type				AMATU_PPO2_dG210				AMATU_PPO2_R128L			
				7 DAA		14 DAA		7 DAA		14 DAA		7 DAA		14 DAA	
1	Untreated			--		--		--		--		--		--	
2	Saflufenacil *	WG	12.5	100.0	a	100.0	a	10.0	f	0.0	e	7.5	g	0.0	e
3	Saflufenacil *	WG	25	100.0	a	100.0	a	67.5	c	62.5	b	62.5	d	47.5	b
4	Saflufenacil *	WG	50	100.0	a	100.0	a	80.0	b	82.5	a	72.5	c	67.5	b
5	Trifludimoxazine *	SC	12.5	99.0	a	100.0	a	27.5	e	2.5	e	22.5	f	12.5	d
6	Trifludimoxazine *	SC	25	100.0	a	100.0	a	95.0	a	90.0	a	80.0	c	70.0	b
7	Trifludimoxazine *	SC	50	100.0	a	100.0	a	99.0	a	100.0	a	94.0	a	90.0	a
8	Flumioxazin *	WG	35	95.0	b	100.0	a	90.0	b	100.0	a	90.0	b	99.0	a
9	Flumioxazin *	WG	70	100.0	a	100.0	a	96.5	a	100.0	a	98.0	a	100.0	a
10	Flumioxazin *	WG	140	100.0	a	100.0	a	98.0	a	100.0	a	98.0	a	100.0	a
11	Sulfentrazone *	SC	105	96.5	b	92.5	b	30.0	e	7.5	e	10.0	g	0.0	e
12	Sulfentrazone *	SC	210	100.0	a	100.0	a	47.5	d	37.5	c	20.0	f	0.0	e
13	Sulfentrazone *	SC	420	100.0	a	100.0	a	60.0	c	55.0	b	40.0	e	10.0	d
14	Carfentrazone *	EC	8.75	100.0	a	100.0	a	25.0	e	15.0	d	20.0	f	10.0	d
15	Carfentrazone *	EC	17.5	100.0	a	100.0	a	42.5	d	32.5	c	57.5	d	32.5	b
16	Carfentrazone *	EC	35	100.0	a	100.0	a	65.0	c	55.0	b	75.0	c	62.5	b
17	Saflufenacil + Flumioxazin *	WG+WG	12.5 + 35	100.0	a	100.0	a	94.0	a	100.0	a	96.5	a	100.0	a
18	Saflufenacil + Flumioxazin *	WG+WG	25 + 70	100.0	a	100.0	a	98.0	a	100.0	a	98.0	a	100.0	a
19	Saflufenacil + Flumioxazin *	WG+WG	50 + 140	100.0	a	100.0	a	98.0	a	100.0	a	98.0	a	100.0	a
20	Saflufenacil + Trifludimoxazine *	WG+SC	12.5 + 12.5	100.0	a	100.0	a	55.0	c	25.0	d	22.5	f	22.5	c
21	Saflufenacil + Trifludimoxazine *	WG+SC	25 + 25	100.0	a	100.0	a	85.0	b	67.5	b	77.5	c	62.5	b
22	Saflufenacil + Trifludimoxazine *	WG+SC	50 + 50	100.0	a	100.0	a	96.5	a	95.0	a	87.5	b	75.0	a
23	Saflufenacil + Sulfentrazone *	WG+SC	12.5 + 105	100.0	a	100.0	a	25.0	e	5.0	e	10.0	g	2.5	e
24	Saflufenacil + Sulfentrazone *	WG+SC	25 + 210	100.0	a	100.0	a	60.0	c	42.5	c	35.0	e	27.5	c
25	Saflufenacil + Sulfentrazone *	WG+SC	50 + 420	100.0	a	100.0	a	77.5	b	77.5	b	65.0	d	60.0	b
26	Carfentrazone + Trifludimoxazine *	EC+SC	8.75 + 12.5	100.0	a	100.0	a	40.0	d	20.0	d	15.0	f	37.5	b
27	Carfentrazone + Trifludimoxazine *	EC+SC	17.5 + 25	100.0	a	100.0	a	87.5	b	77.5	b	70.0	d	57.5	b
28	Carfentrazone + Trifludimoxazine *	EC+SC	35 + 50	100.0	a	100.0	a	96.5	a	92.5	a	82.5	c	75.0	a
	CV (%)			1.4		3.5		8.5		11.1		9.0		16.1	

*All herbicide treatments included DASH 0.5% v/v.
Means (n=3) within columns with no common letter are significantly different according to test group average Scott_Knott (α=0.05).

Figure 5.4 – Pictures of transgenic *A. thaliana* with two natural mutations (1) dG210 and (2) R128L from *A. tuberculatus.* From left to right, there is the untreated, highest, medium and lowest dose of (A) Saflufenacil, (B) Trifludimoxazin, (C) Flumioxazin, (D) Sulfentrazone, (E) Carfentrazone-ethyl, (F) Saflufenacil + Flumioxazin, (G) Saflufenacil + Trifludimoxazin, (H) Saflufenacil + Sulfentrazone and (I) Carfentrazone-ethyl + Trifludimoxazin, at 14 days after application. Limburgerhof, Germany, 2015.

Those mutations could also be a good option for future trait, being sulfentrazone and carfentrazone-ethyl, followed by saflufenacil as well as the mixtures saflufenacil + sulfentrazone or carfentrazone-ethyl + trifludimoxazin were the treatments more selective to these traits.

Flumioxazin and trifludimoxazin showed the best activity in those natural mutations and in this case, could be recommended to resistant management in weeds with these mutations.

The mechanism of PPO-inhibitor resistance is a unique target-site amino acid deletion in the PPX2 gene (mitochondrial PPO2 is encoded by the PPX2), that involves the loss of a glycine at position 210 in the mitochondrial isoform of PPO enzyme.

According to Riggins & Tranel (2012), loss this amino acid is considered to have occurred via a slippage-like mechanism within a trinucleotide, that alters the binding domain of the enzyme without negatively affecting substrate affinity, reducing by at least 100-fold PPO-inhibiting herbicides sensitivity.

In resistant *A. tuberculatus* for the PXX2L gene, that encodes both chloroplastic and mitochondrial PPO and the requirement for simultaneous loss of three nucleotides in the coding sequence of the target gene, should limit the evolution of this deletion, although further four resistant *A. tuberculatus* populations have been reported (POWLES & YU, 2010).

Actually, this mutation conferring resistance is unusual that it involves an amino acid deletion rather than a substitution and these authors confirmed that dG210 is the predominant mechanism found in *A. tuberculatus* populations PPO-herbicides resistant (TRINGLUM et al., 2011). Powles & Yu (2010) mentioned whether Gly210 substitution occurred rather than deletions, that the substitutions of Gly210 would provide either little or no resistance, or greatly reduce PPO functionality.

This deletion did not affect the affinity of protoporphyrinogen IX nor the FAD content, but decreased the catalytic efficiency of the enzyme, incurring tenfold-lower PPO activity than does the wild type. The deletion of Gly210 of the mitochondrial PPO imparts herbicide resistance to this dual-target protein without severely affecting its normal physiological function (DAYAN et al, 2010).

Besides that, the mixtures of flumioxazin + saflufenacil even in the lowest doses as well as trifludimoxazin + saflufenacil or carfentrazone-ethyl in the medium to highest dose showed good control of *A. thaliana* with those natural mutations. In the opposite that seen in the field, saflufenacil + sulfentrazone showed the lowest activity in these transgenic *A. thaliana* and in order to have more comparable results

it should be conducted in the field with natural weed species whether these mutations were developed in the field.

According to Li & Nicholl (2005), PPO resistance technology is not dependent on any single herbicide or mutant PPO gene. Ward & Volrath (2001) demonstrated that PPO mutants confer resistance to one PPO inhibitor also confer resistance to a variety of PPO-inhibitor compounds, being commercial or experimental. Most PPO mutations occur at sites where gene homology is very high at the DNA sequence level.

Dayan & Duke (1997) reported that the insertion of a gene into the crop that codes for herbicide-degrading enzyme might seem simple. However, the action of this enzyme would have to be fast because PPO-inhibitors work faster than any other herbicides.

Despite of flumioxazin shows good control of all events tested and the interaction of binary mixtures of PPO herbicides only provided faintly better control of PPO-resistant species, it indicates that more diverse array of other mechanism of action herbicides, mechanical, and cultural practices should be applied together in order to control the resistant species and provide more sustainability for this technology (GREEN & OWEN, 2011; POWELS, 2008).

To reduce weed populations and selection pressures that drive the evolution of resistant weeds, Mortensen et al. (2012) recommended crop rotation, cover crops, competitive crop cultivars, use of tillage and finally, targeted herbicide application.

Natural resistance to PPO-inhibitors has been slow to evolve (POWELS et al., 2010). Only thirteen PPO-resistant weeds were reported, being *A. palmeri* and *A. tuberculatus*, reported in the United States, the most important ones (HEAP, 2017).

However, if with the introduction of glyphosate, in the mid-1996s, scientists believed that evolution of glyphosate-resistant weeds and the levels of resistance would be very slow, it's was not recommended underestimate the potential for weeds become resistant (DUKE & POWLES, 2008).

Losing PPO-inhibiting herbicides as an effective chemical class would be a problem for farmers because this chemical class have long been the go-to option to control glyphosate-resistant. Overreliance on this group of herbicides, as in the others, allows weeds to select for resistance to herbicide's mechanism of action, like what happened with glyphosate and ALS-inhibiting herbicides (BECKIE & TARDIF, 2012; HOPKINS, 2017).

According to Green & Owen (2011), new herbicides-resistant crops technologies will provide more solutions for growers to manage resistant weeds, but will not replace the long-term need to diversify weed management strategic and discover herbicides with new mechanisms of action.

5.4 Conclusion

Based on the results, the overexpression of PPO1 and PPO2 isoforms in *A. thaliana* was not a good option for a trait in the future, because even some compounds showed some tolerance for the transgenic *A. thaliana,* it was not enough for crop selectivity.

However, *A. thaliana* with gene of PPO2 mutations from *A. tuberculatus* with the both substitutions L397Q + F420M and R128A + F420M showed the best performance in terms of selectivity, followed by natural mutations (dG210 and R128L) that were faintly better than PPO1 Acuron mutation.

Sulfentrazone and carfentrazone-ethyl, followed by saflufenacil as well as the mixtures saflufenacil + sulfentrazone or carfentrazone-ethyl + trifludimoxazin were the treatments more selective to these traits.

Regarding weed resistance management, flumioxazin and trifludimoxazin showed the best activity in those natural mutations. Nevertheless, in order to provide more sustainability for this technology, other mechanism of action herbicides, mechanical, and cultural practices should be applied together.

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CHAPTER V

Concluding remarks

6.1 Final considerations

Effective weed management is critical to maintaining agricultural productivity, that is responsible to sustain more than 7 billion people. Inhibition of PPO is herbicide mechanism action that has successfully been used for weed management in agriculture since 1960s.

The widespread adoption of glyphosate-resistant crops has caused reduction of the field application of PPO inhibitors, which controls broadleaf weed selectively. New herbicides-resistant crops technologies will provide more solutions for growers to manage resistant weeds, but will not replace the long-term need to diversify weed management strategic and discover herbicides with new mechanisms of action.

It’s known that chemical industry has not commercialized herbicides with a new mechanism of action for a long time. This partly because the number of chemical that must be tested to discover a new herbicide has increased considerably, besides of the high resources that needed to be invested and also reduced market opportunity in the past due to glyphosate-resistant crop.

The idea of this thesis was work with potential synergic binary mixtures of PPO-inhibitors for weed control, mainly monocots and investigate what factor might cause this synergy, since it is known that PPO herbicides are generally more active on dicots than on monocots, although the enzyme target appears to be equally sensitive to the herbicides.

Also, it was important understand the mechanistically studies thereof through of measurement of the effectiveness of PPO-inhibitor herbicides in inhibiting PPO1 and PPO2 enzyme by IC_{50}; absorption and translocation of these mixtures and finally, finding a good trait to develop new herbicide-resistant crops, also enabling discuss resistance management and finally herbicide tolerant traits discussion.

Controlling of both broadleaf and monocot weed is necessary for successful development and growth of crops. Since saflufenacil is a broadleaf herbicide; therefore, for a broad-spectrum weeds control, it needs to be tank mixed with other herbicides.

In this studied, the best mixtures to improve spectrum control were saflufenacil with flumioxazin, or sulfentrazone or trifludimoxazin. Those mixtures also have shown synergism effects.

They could control monocots as *E. cruz galli* and volunteer maize besides control the dicots species. In addition, it is likely that these tank mix treatment could be effective for control of volunteer glyphosate-resistant crops.

The doses of each compound in the mixtures depends on the species and region. However, all the highest doses tested can be recommend in tank mixtures. The worst mixture was carfentrazone-ethyl + trifludimoxazin.

To better understand the interaction of those synergism, PPO1 and PPO2 enzyme was evaluated by $IC_{50.}$ However, PPO1 could not be expressed to be evaluated the IC_{50} using the same steps of PPO2 enzyme and there is not any reported in the literature how express this enzyme.

However, mitochondrial PPO enzyme from *Amaranthus tuberculatus, Alopecurus myosuroides* and *Setaria italica* was sensitive to inhibition by saflufenacil, trifludimoxazin, flumioxazin, carfentrazone-ethyl and sulfentrazone. Trifludimoxazin and flumioxazin required less amount of compound to inhibit 50% of PPO2 activity in all plants species tested, while sulfentrazone and saflufenacil, showed that needed bigger concentrations to inhibit 50% of the PPO2 compared to other compounds evaluated.

In order to deeply investigated those synergism, studies of absorption and translocation were conducted. However, only saflufenacil, trifludimoxazin, carfentrazone-ethyl and sulfentrazone was chosen and their respective mixtures, due to the size of trial and resources to conduct it. As single compounds, the absorption was slower in maize than *Echinochloa crus-galli*. There was no difference among the herbicides overtime except trifludimoxazin, that was significantly lower, which indicates that the absorption of trifludimoxazin may be slower than the other compounds.

Saflufenacil was the herbicide that showed more translocation out of treated leaf, reaching about 5 percent in *E. crus-galli* and only 1 percent in *Z. mays*. This compound showed the best translocation at 24 HAA, while trifludimoxazin have shown any translocation out of leaf treated.

Surprisingly, there was not difference in foliar absorption of saflufenacil when in mixture with trifludimoxazin or sulfentrazone, while the translocation decreased considerably. Saflufenacil, alone or in mixtures, showed slower absorption in maize in the first 6 HAA, which might explain the results from the field, where the mixtures in the lowest doses reached better control in *E. crus-galli* than in maize.

On the other hand, trifludimoxazin + carfentrazone-ethyl showed the highest absorption in *E. crus-galli* and maize already in the first 6 HAA and this mixture also showed the worst control in the field for both targets.

Carfentrazone-ethyl showed the same tendency of other compounds in foliar absorption, low at the beginning and increased overtime. However, the opposite was seen in the translocation, where carfentrazone-ethyl solo was better translocated than the mixture with trifludimoxazin in maize and *E. cruz-galli,* which indicated that it's dependent of species and perhaps other mechanism are involved such as metabolism and could explain better those synergisms.

Already studies conducted in *Arabidopsis thaliana* in order to find some options to future PPO-inhibitors traits, *A. thaliana* with gene of PPO2 mutations from *A. tuberculatus* with the both substitutions L397Q + F420M and R128A + F420M showed the best performance in terms of selectivity, followed by natural mutations (dG210 and R128L) that were faintly better than PPO1 Acuron mutation.

Overexpression of PPO1 and PPO2 isoforms in *A. thaliana* was not a good option for a trait in the future, because even some compounds showed some selectivity for the transgenic *A. thaliana,* it was not enough for crop selectivity.

Regarding weed resistance management, flumioxazin and trifludimoxazin as single compounds, and saflufenacil + trifludimoxazin showed the best activity in those natural mutations. Also, trifludimoxazin showed the most active compound to inhibit 50% of the PPO2 enzyme from *A. tuberculatus*, even in the both natural mutations dG210 and R128L, *S. viridis* and *Alopecurus myosuroides* in the IC_{50} studies.

This thesis showed that PPO-inhibitor herbicides is an excellent tool to manage monocot and dicot weeds, and to develop a new PPO inhibitor-resistant crops in order to provide more solutions to growers and improve their crop production in the field. Nevertheless, in order to provide more sustainability for this technology, it's important to highlight that other mechanism of action herbicides, mechanical, and cultural practices should be applied together.