Improved herbicide selectivity in tomato by safening action of benoxacor, 2,4,6-T, melatonin, and fenclorim

Tabata Raissa de Oliveira

Mississippi State University, tabata.raissa@hotmail.com

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Improved herbicide selectivity in tomato by safening action of benoxacor, 2,4,6-T, melatonin, and fenclorim

By

Tabata Raissa de Oliveira

Approved by:

Te-Ming (Paul) Tseng (Major Professor)
Shaun R. Broderick (Co-Major Professor)
Taghi Bararpour
Dean E. Riechers
Michael Cox (Graduate Coordinator)
Scott T. Willard (Dean, College of Agriculture and Life Sciences)

A Thesis
Submitted to the Faculty of Mississippi State University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Plant & Soil Sciences with a concentration in Weed Science in the Department of Plant & Soil Sciences

Mississippi State, Mississippi

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This Master's Thesis successfully addressed the challenge of weed management in tomato production systems by enhancing the tomato plant's tolerance to potent herbicides using safeners. The primary objective of this study was to employ safeners to boost the tomato plant's resilience against herbicides known for their effectiveness in controlling troublesome weeds such as yellow and purple nutsedge, annual grasses, and pigweed species. Additionally, we investigated whether the use of safeners might lead to any antagonistic interactions with the tested herbicides.

The project's overarching tasks involved evaluating the safening effects of benoxacor, fenclorim, 2,4,6-trichlorophenoxyacetic acid, and melatonin on tomatoes when exposed to specific herbicides. These safeners, identified through our research, underwent testing in controlled environments, including greenhouses and laboratories, alongside standard herbicides that are commonly used and approved for tomato cultivation. We assessed factors such as injury levels, glutathione S-transferase (GST) activity, and the tomato plants' potential and degree of tolerance to the applied herbicides.
DEDICATION

This Master’s Thesis Project is dedicated to my family and God who provided essential support during my master’s degree.
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I am immensely grateful to numerous individuals and groups who helped me along the way. First, I sincerely appreciate my advisors, Dr. Tseng, Te-Ming Paul, and Dr. Shaun R. Broderick, for their invaluable time, guidance, and unwavering support throughout my journey toward this degree. They have taught and shaped me in ways that will help me throughout my career.

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CHAPTER I
INTRODUCTION

Tomatoes (*Solanum lycopersicum*) are a highly significant crop, ranking second only to potatoes (*Solanum tuberosum* L.) in terms of global production. More than 182.3 million tons of tomato fruits are cultivated annually across approximately 4.85 million hectares (Quinet et al. 2019). Tomato cultivation faces multiple challenges that contribute to reduced yields, both directly and indirectly. These challenges include issues such as using low-quality seeds, adverse climatic conditions, the presence of pests, and the effective management of weeds (Tolman et al. 2004; Singh et al. 2017; Clark et al. 1998).

Among the various pest classes, it is noteworthy that weeds emerge as the primary factor contributing to a significant decrease in tomato crop yields (Clark et al. 1998). Effective weed management is of paramount importance as it serves to prevent a decline in both product quality and overall productivity. Weeds, by interfering with the growth of crops, place a strain on essential natural resources such as soil, water, nutrients, sunlight, and more (Morales et al. 2003). In tomato production, various approaches can be employed to inhibit weed growth, including cultural, mechanical, biological, and chemical methods.

Herbicides are one of the most efficient methods for weed control in agricultural production (Gwatidzo et al. 2023). Selecting the appropriate herbicide to target specific weed species is a critical decision. Equally vital is considering external factors, such as temperature, wind speed, and moisture levels, which are imperative for optimizing herbicide effectiveness (Johnson &
Young, 2002) while simultaneously minimizing herbicide drift beyond its intended application area ( Richardson et al. 2019). According to the United States Environmental Protection Agency, drift is classified as dust or droplets being transported through the air during or immediately following pesticide application to locations different from where they were intended to target (EPA. 2022).

Tomato is very sensitive to many different herbicides. According to Medeiros et al. 2023, some herbicides such as 2,4-D and dicamba, are harmful to tomato production, and even small amounts caused by drift from other applications compromise the production. Additionally, the recommendation to apply pre-herbicides such as sulfentrazone, S-metolachlor, or fomesafen to control weeds in tomato production depends on the herbicide effectiveness and weed density (Flint M & Klonsky K, 1985) and for that reason to ensure maximum quality and high yield an effective weed management is necessary that can be associated with non-chemical control which including mechanical and cultural control.

To overcome the challenges posed by limited chemical control options in tomato production and to improve both the quality and yield for growers, it is imperative to explore supplementary weed control strategies. These strategies should effectively mitigate weed pressure around the crop while safeguarding fruit yield and quality. One promising avenue entails the utilization of safeners to enhance herbicide tolerance in tomatoes, thereby strengthening weed control strategies.

**Herbicides**

**2,4-D and Dicamba**

2,4-D (2,4-dichlorophenoxyacetic acid) is classified as a synthetic auxin herbicide and has been widely used to control broadleaf weed since the 1940s (Hamner & Tukey. 1944). It was
developed during World War II, but early research remained confidential until the war’s end (Peterson. 1967). Two different formulations of 2,4-D (salts and esters) are used commercially, and a third, 2,4-D choline, is introduced in the marketplace (Peterson et al. 2016). Their structural formulae are shown in Figure 1.

Reaction of 2,4-D acid with an amine form 2,4-D salt which is used to control broadleaf weeds (James et al. 2023). Some amine salt forms of 2,4-D include isopropylamine, triisopropanolamine, diethanolamine, and dimethylamine, which can be powder or liquid formulations (Peterson et al. 2016). When the acid of 2,4-D reacts with an alcohol form ester and combines with and alkyl chain of no more than four carbons, the chemical structure is more readily volatilized. This group includes methyl, isopropyl, and butyl esters (Marth & Mitchell, 1949; Peterson et al. 2016). However, to decrease the volatilization problem caused by 2,4-D since the 1980s, these formulations have been changed to include more than four carbons, thus forming low-volatile esters (Peterson et al. 2016). It is critical to know that there is a correlation between the length of the carbon chain and herbicide volatilization: the longer the carbon chain, the more significant the reduction in volatility. Recently introduced to the market, the formulation of choline is different from that of amine salts or esters. Even though it has the same effect as weed control, the new formulation of 2,4-D has different inherent volatility characteristics. According to (Barbieri et al. 2023; Peterson et al. 2016) 2,4-D choline is 96% less volatile than 2,4-D ester because the increased hydrogen bonds between the choline cation and the carboxylate anion of the herbicide molecule increase the intermolecular forces within the formulation, making it less likely for the herbicide volatilize (Magomadov & Malieva, 2023).

Another important synthetic auxin herbicide is dicamba (3,6-dichloro-2-methoxybenzoic acid), used to control broadleaf weeds (Müller & Applebyki, 2000) and is found in different crops.
Its structural formula is shown in figure 2. In 2016, Monsanto registered a new genetically engineered technology of soybean (Roundup Ready 2 Xtend®), which confers resistance to dicamba and glyphosate. This advancement improves weed control, even in glyphosate-resistant weeds (Bayer, 2021).

Dicamba and 2,4-D synthetic auxin herbicides induce cell elongation by increasing the activity of enzymes, and the most characteristic symptoms caused by these herbicides are uncontrolled cell division and growth, usually in meristematic regions that accumulate photosynthate assimilates and herbicides from the phloem (Klaus Grossmann. 2010; Figure 3). Both herbicides are volatile chemicals and easily promote a high risk of off-target movement that causes damage in non-resistant plants (Jones et al. 2019; Werle et al. 2022). However, according to Mueller et al. (2022), the dicamba formulation is more volatile than 2,4-D when applied in fresh plants, thus in 2020, the German agricultural company Bayer agreed to pay up to $400 million to farmers who suffered damage from dicamba sprayed on neighboring soybean or cotton fields from 2015 through 2020 (Bayer. 2020).

In 2012, 2,4-D was the fifth herbicide used in the United States, and in 2018, about 43% of U.S soybean acreage was planted with dicamba-tolerant seed (USDA. 2019 & Freisthler et al. 2022). These herbicides have been commonly used in the field to protect and decrease competition between weeds and grass or cereal crops (Monaco et al. 2002), which are often located close to different production, for instance, in tomato crops, which are not resistant to dicamba and 2,4-D. Thus, it is critically important to invest in technologies and alternatives to prevent injury and control damage caused by off-target herbicides in other crops that are non-resistant, such as tomato production.
Metribuzin and Sulfentrazone

According to a USDA report in 2021, tomatoes placed the top three vegetables in terms of harvested area and production in the United States. Due to its versatility in being grown either in field conditions or in the greenhouse, the tomato has become the world’s third-largest vegetable produced by the United States (Ma M et al. 2023). However, the management varies regarding local production, topography, soil type, cultivation objective (marketable or processed), variety selection, plant density, plant architecture, and so on (Logendra et al. 2001). An agricultural standard established to increase the yield of tomato production includes pest control (insects, diseases, and weeds; Reddy. 2012).

Diseases, insects, and weeds in tomato production can considerably reduce yield. Weeds alone have been reported to reduce tomato yields by 36-92% (Armelina. 1983; Samant & Prusty 2014) and reduce fruit quality and market value (Mennan et al. 2020), thus weed management is essential before and after the crop establishment (Yaacoby et al. 2023).

Herbicide use is more effective than non-chemical methods in that they target more weed species and provide better control of different weed species (Mohamed et al. 2023); however, their use in vegetable production on a large scale can promote adverse effects such as resistance, drift, and environmental impacts (Mennan et al. 2020). Sulfentrazone, S-metolachlor, fomesafen, and metribuzin (Met) have been approved for tomato production worldwide (Mohamed et al. 2023).

Metribuzin (4-amino-6-tert-butyl-3-methylsulfanyl-1,2,4-triazin-5-one) is a pre-emergent herbicide (Figure 4) approved for controlling broadleaf weeds and certain grasses in several crops, including tomatoes and potatoes production. Metribuzin inhibits photosystem II by blocking electron transport at the D1 protein, resulting in chlorosis and leaf tissue necrosis (Figure 5). Although this herbicide is one of the most used herbicides to control different weed species, its
use may cause injury to tomato and potato plants, especially under certain stress conditions (Chaudhari et al. 2017; Hatterman-Valenti et al. 1994).

Several weed species compete with tomatoes in production, but two of the most problematic species are often purple and yellow nutsedge (Cyperus rotundus spp; Cyperus esculentus spp). Morales et al. (2003) observed that yellow nutsedge reduced the total marketable tomato yield by 65%. One of the reasons yellow and purple nutsedge reduce yields so much is because they interfere with nitrogen absorption, an essential element for vegetative growth during tomato establishment (Morales et al. 1997). Herbicides such as sulfentrazone have been effective in controlling nutsedge and annual grasses (Niekamp. 2001)

Sulfentrazone (5-oxo-1,2,4-triazole; Figure 6) is an herbicide applied pre-emergence or at post-planting, depending on the weed species that acts on the inhibition of the protoporphyrinogen oxidase (PPO or Protox) enzyme. This herbicide interrupts the biosynthetic pathway responsible for chlorophyll production. The leaves become chlorotic once treated tissue is exposed to light, and necrosis develops as “bronzing” (Figure 7). Sulfentrazone is an herbicide recommended in soybeans and tobacco to control different weeds, which can be rotated with several crops, such as vegetables or grain crops. Although sulfentrazone was recently registered for use in tomato crops in Florida, it can potentially cause carryover injury to crops such as cotton, onion, snap bean, tomato, and watermelon. Sandhu et al. (2022) reported that sulfentrazone applied pre-emergence (PRE) to soybean caused < 18% injury in tomatoes at 840 g a.i./ha, which is the highest application rate for this herbicide (Pekarek et al. 2010; Rachuy et al. 2022).

To overcome limited chemical control options, supplemental weed control strategies are needed to effectively reduce the weed pressure around tomato crops while protecting the yield and
fruit quality. One of the promising weed control options is using safeners to improve herbicide tolerance in tomatoes, thereby enhancing weed control (Rosinger. 2014).

**Safeners**

Safeners are chemical compounds used to protect different crop plants against herbicide damage. Although there is evidence that safeners use various mechanisms to upregulate enzymes such as GST and P450, herbicide degradation and detoxification in plants are not well established. Safeners’ modes of action regarding the molecular and genetic mechanisms and their action pathways are poorly understood (Duhoux et al. 2017; Hu L et al. 2020).

Otto Hoffman first introduced the safener concept in the late 1940s from an accidental experiment he found in tomato plants treated with 2,4,6-T. They did not suffer damage after contact with 2,4-D vapor (Jablonkai et al. 2013). In 1971, these compounds were introduced as a new tool to the market, and the first commercial safener, 1,8-naphthalic anhydride, was used as a seed treatment in corn (Hoffman, 1969). Since then, safeners have created significant interest in the chemical industry due to their physiological and biochemical features, which activate important mechanisms in the plant without reducing activity in target weed species and contribute to reducing herbicide resistance (Duhoux et al. 2017).

For the past 15 years, research and development have contributed to finding new safeners and subsequent commercialization. These compounds represent diverse chemistries, as illustrated in Table 1 (Deng 2022).

**Fenclorim**

The safener fenclorim (4,6-dichloro-2-phenylpyrimidine), depicted in Figure 9, was initially discovered in 1991. Its primary purpose was to safeguard and enhance the tolerance of
rice (*Oryza sativa* L.) against pretilachlor, a chloroacetanilide herbicide, as well as other herbicides like acetochlor, *S*-metolachlor, dimethachlor, and metazachlor, which are commonly used for controlling annual grasses and broadleaved weeds. Fenclorim can be applied in combination with chloroacetanilide herbicides as a tank mix (Han & Hatzios 1991; Chen 2013).

In addition to its role in safeguarding rice against pretilachlor, fenclorim has recently been explored for its application in Arabidopsis thaliana cell culture (Jablonkai 2013). The mechanism through which fenclorim enhances its protective effects involves the upregulation of a crucial enzyme known as Glutathione S-transferases (GSTs), facilitating detoxification in fenclorim-safened rice or *Arabidopsis thaliana* (Jablonkai 2013; Brazier-Hicks, 2008; and Hu L et al. 2020). The intricate workings of the GST mechanism will be elaborated upon in the subsequent review.

**Benoxacor**

Benoxacor is a safener for dichloroacetamide herbicides, and its molecular structure is depicted in Figure 8. Hatzios & Cottingham (1991) published the first study regarding using benoxacor for safening plants. This safener was initially employed to safeguard maize crops from the potential harm caused by metolachlor, one of the most widely used herbicides in the Midwestern corn belt of the United States. This choice was prompted by the high concentration of metolachlor detected in groundwater, surpassing levels of other herbicides (Fuerst 1995; Simonsen et al. 2020).

In the protection of maize crops, benoxacor is typically applied in combination with metolachlor, often in formulations like Dual II Magnum by Syngenta, which is a widely used commercial product containing *S*-metolachlor (Simonsen et al. 2020). It is worth noting that benoxacor undergoes direct photolysis, leading to the formation of monochlorinated and dechlorinated in the presence of light. Additionally, benoxacor acts as a photosensitizer for
metolachlor. When exposed to sunlight, this combination of safener and herbicide generates reactive oxygen species (ROS) that have the potential to cause damage to cellular structures (McFadden. 2021). The biochemical mechanism of benoxacor which contribute to its protective effect is the detoxification of herbicide by conjugation with tripeptide Glutathione (GSH) (Narayanankutty et al. 2019) and is used as a cofactor to detoxify peroxides generated from oxygen radicals, reduce oxygen centers on DNA (Sies. 1999).

Most herbicide metabolism studies examining benoxacor have indicated that herbicide safeners facilitate the conjugation process in plant metabolism. This induction leads to enhancing GST isoenzymes specifically for chloroacetanilide herbicides (Cottingham et al. 1991).

2,4,6-T

2,4,6-Trichlorophenoxyacetic (2,4,6-T) (Figure 10) is a chemical substance that results from the breaking of an imidazole ring (Polese et al. 2006). 2,4,6-T is one of the first safeners discovered when, in 1947, Hoffman accidentally found that 2,4,6-T protected tomatoes against 2,4-D in a warm glasshouse (Hoffman. 1953).

This safener has been used to protect several crops. Crowdy and Wain (1951) used 2,4,6-trichlorophenoxyacetic to protect beans against Botrytis fabae Sardina. In another study, wheat treated with 2,4,6-T did not show phytotoxic symptoms from barbamate (Hoffman 1953). In 1952, Hoffman demonstrated the protective effect of 2,4,6-T in tomatoes. His findings revealed that plants treated with a safener did not exhibit the usual epinasty symptoms caused by 2,4-D herbicide vapors. Additionally, tomatoes exposed to 2,4,6-T showed signs of ripening stimulation (Hoffman, 1953). Although 2,4,6-T was one of the first safeners found, the concept of safener was not proposed until 1962, and since then, hundreds of potential safeners have been created to protect plants from multiple modes of herbicide action for various crops (Ahrens et al. 2013).
cases, safeners were created through a chemical structure that mimicked herbicides. Safeners act in many ways to neutralize herbicides. Principally, they work based on the structure-activity similarity, adopting a structure like the herbicide, as depicted in Figures 11A and 11B. As noted above, most of the 2,4,6-T studies have been reported in the late 1940s, and understanding the differential responses of various crops and weeds to safeners, especially among dicotyledonous plants with limited registered safeners, remains a complex challenge (Lanasa et al. 2022; Oloye et al. 2021). Hence, there is a need for further research to address the many unanswered questions regarding the use of 2,4,6-T to safeguard crops from the effects of 2,4-D or other herbicides.

Melatonin

Melatonin hormone is synthesized from tryptophan, and in humans, it is primarily released from the pineal gland in response to darkness. The pineal gland (glandula pinealis) was first described and named in Greece from the resemblance in shape and size of the stone pine (Pinus pinea) seeds by physician Galen (131 C.E.-201 C.E.). In France, the philosopher Rene Descartes (1596-1650) described the function of the gland as “the principal seat of the soul” (Kitay & Altschule. 1954; Hardeland et al. 2006). However, the secretory product melatonin (N-acetyl-5-methoxytryptamine) produced from the pineal gland was first characterized in 1958 by the North American dermatologist Aaron B. Lerner, who isolated the hormone from bovines (Lerner et al. 1958; Lerner. 1959). The relationship between melatonin synthesis and circadian rhythm in humans and mammals was described in 1975 by Lynch and colleagues. In the following decades, melatonin has been found to benefit animals, humans, and plants (Lynch et al. 1975).

Melatonin in humans and mammals has different actions that regulate many biological functions, such as immunity, sleep, reproduction, and circadian rhythm (Wang et al. 2022). Recent
studies have explored the use of melatonin to prevent various cancers (Li et al. 2017) due to its antioxidant activity and other signaling processes (Zhang & Zhang. 2014; Hacışevki & Bab. 2018).

In 1995, melatonin was found in many plants, such as bananas, tomatoes, cucumbers, and beetroot (Dubbels et al. 1995). Since then, new studies have used melatonin to mitigate abiotic and biotic stress and increase yields. Many studies report on the benefits of melatonin in cell enlargement, root development, delayed flowering, and improved fruit yield and quality (Arnao & Hernández-Ruiz. 2020). The protective role of melatonin in plants may also confer herbicide resistance to drift because it is a potent direct scavenger of free radicals (Hacışevki & Bab. 2018).

**Plant Metabolism of Herbicides**

Most plants undergo three distinct phases to facilitate the detoxification of xenobiotic compounds, such as herbicides and insecticides, ultimately converting them into intermediate substances with reduced phytotoxicity (Sandermann Jr. 1992). Phase I involves different reactions such as oxidation, reduction, and hydrolysis, followed by Phase II, which results in the primary conjugation with endogenous substrates such as glucose, amino acids, or glutathione. Reduced glutathione is widely distributed in plant tissues and protects cells from oxidative damage. Finally, Phase III consists of secondary conjugation and creates insoluble residues or sequestration of the herbicide into the vacuole (Davies & Caseley. 1999).

Using herbicide safener to protect plants against xenobiotic effects is known to go through glutathione conjugation during Phase II. Previous studies have shown that the conjugation of herbicides pretilachlor and acetochlor raised by fenclorim and other safeners can synthesize GSH, which leads to an induction of GST activity (Han & Hatzios. 1991; Riechers et al. 1997)
Glutathione-S-Transferase

The detoxification of xenobiotic and endobiotic compounds is catalyzed by glutathione S-transferase (GST), which is widely found in eukaryotes and prokaryotes cells and well-studied in plants and other organisms such as animals, fungi, and bacteria (Monticol et al. 2017).

Frear and Swanson (1970) state that the defense of *Zea mays* against herbicide s-triazine was involved by GST’s action, which led to other studies proving the importance of GST to herbicide tolerance in different crops such as tobacco, tomato, citrus, etc. Safeners are involved in increasing a plant’s herbicide tolerance. They primarily decrease the ability of herbicides to inhibit normal cellular function and reach their target sites. This is possible either by the relationship between herbicide molecule and safener chemical reaction promoting the reduction of herbicide uptake by plants or by reducing herbicide metabolites due to safener action. Based on different reports, one of the potential actions of GST activity is to protect cells from oxidative damage to DNA and lipid membrane peroxidation (Duhoux et al. 2017).

These studies hold significant promise in demonstrating the efficacy of safeners in enabling herbicide use for weed control while safeguarding crops against drift. However, further research is essential to deepen our understanding of safeners within agricultural contexts. Moreover, there is a need to develop more targeted approaches to enhance our comprehension of the relationship between herbicide-safeners and the detoxification of metabolism within plant cells.
Figures and Tables

Table 1  Commercial and naturally occurring safeners, as well as safener candidates enhance GST activity.

<table>
<thead>
<tr>
<th>Commercial Safeners</th>
<th>Commercial Safeners</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,8-naphthalic anhydride (NA)</td>
<td>dichlormid</td>
</tr>
<tr>
<td>R-28725</td>
<td>R-29148</td>
</tr>
<tr>
<td>furilazole</td>
<td>AD-67</td>
</tr>
<tr>
<td>benoxacor</td>
<td>MG-191</td>
</tr>
<tr>
<td>cyometrinil</td>
<td>oxabetrinil</td>
</tr>
<tr>
<td>fluoxfenim</td>
<td>acetamate</td>
</tr>
<tr>
<td>fenclorim</td>
<td>fenchlorazole-ethyl</td>
</tr>
<tr>
<td>isoxadifen-ethyl</td>
<td>mefenpyr-diethyl</td>
</tr>
<tr>
<td>cluquintocet-mexyl</td>
<td>cyprosulfamide</td>
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</table>

**Natural safeners**

<table>
<thead>
<tr>
<th>Natural safeners</th>
<th>Natural safeners</th>
</tr>
</thead>
<tbody>
<tr>
<td>melatonin</td>
<td>gibberellin</td>
</tr>
<tr>
<td>sanshools</td>
<td>isopimpinellin</td>
</tr>
<tr>
<td>5-methoxypsoralen</td>
<td>Z-ligustilide</td>
</tr>
<tr>
<td>brassinolide</td>
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</tr>
</tbody>
</table>

**Safener candidates**

<table>
<thead>
<tr>
<th>Safener candidates</th>
<th>Safener candidates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-4-(2-substituted hydrazinyl)-6-chloro-2-phenypyrimidines</td>
<td>N-alkyl amides</td>
</tr>
<tr>
<td>diazabicyclo derivatives</td>
<td>1,3-disubstituted imidazolidine or hexahydropyrimidine derivatives</td>
</tr>
<tr>
<td>phenyl isoxazole analogues</td>
<td>diazabicyclo derivatives</td>
</tr>
<tr>
<td>ester-substituted pyrazole derivatives</td>
<td>substituted phenyl oxazole derivatives</td>
</tr>
<tr>
<td>substituted dichloroacetylphenyl sulfonamide derivatives</td>
<td>quinoxaline derivatives</td>
</tr>
<tr>
<td>substituted oxazole isoxazole carboxamides</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1 2,4-dichlorophenoxyacetic acid molecule (2,4-D) is a phenoxyacetic acid herbicide used post-emergence for selective control of broadleaf weeds.
Figure 2  3,6-dichloro-2-methoxybenzoic acid molecule (Dicamba) is a methoxybenzoic acid used post-emergence for selective control of broadleaf weeds.
Figure 3  Effects of auxin herbicides on tomato plants.
Figure 4  4-amino-6-tert-butyl-3-methylsulfanyl-1,2,4-triazin-5-one (metribuzin) is an herbicide used both pre- and post-emergence in crops including soybean, potatoes, tomatoes, and others.
Figure 5  Effect of metribuzin herbicide on tomato leaves.
5-oxo-1,2,4-triazole (Sulfentrazone) is an herbicide applied pre-emergence or at post-planting, depending on the weed species that acts on the inhibition of the protoporphyrinogen oxidase (PPO or Protox) enzyme.
Figure 7  Effects of sulfentrazone herbicide on tomato plants.
Figure 8  2,2-dichloro-1-(3-methyl-2,3-dihydro-1,4-benzoazin-4-yl) ethenone (Benoxacor) molecule is an herbicide safener used in S-metalochlor.
4,6-dichloro-2-phenylpyrimidine (Fenclorim) molecule structure. It was first discovered in 1991 and used to protect rice seeds from herbicide application.
Figure 10  2,4,6-Trichlorophenoxyacetic acid (2,4,6-T) molecule structure was first discovered as a safener accidentally in 1947 by Hoffman.
Figure 11  Structural similarity of A) 2,4-D (herbicide) and B) 2,4,6-T (synthetic auxin herbicide used as a safener).
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CHAPTER II
ASSESSING THE PROTECTIVE EFFECTS OF BENOXACOR, FENCLORIM,
MELATONIN, AND 2,4,6-TRICHLOROPHENOXYPHENYLACETIC ACID
AGAINST HERBICIDE INJURY IN TOMATO

Portions of this chapter have been submitted for publication in Agrochemical Journal.

Abstract
Safeners protect crops by enhancing their ability to metabolize various compounds, including herbicides. They primarily work by increasing the crop's tolerance to herbicide damage, activating herbicide-metabolizing proteins, and aiding in their detoxification. This study aimed to investigate the chemical effects of safeners in tomato cultivation and focus on injury reduction and tissue protection. The experiment followed a randomized factorial design (5x4) with four replications repeated twice. We evaluated the effects of herbicides (dicamba, 2,4-D, metribuzin, and sulfentrazone at 1/100) and safeners (benoxacor, fenclorim, melatonin, 2,4,6-T, and an untreated control). Safeners were applied to the seeds before sowing, and herbicides were used as a foliar spray 25 days after sowing (DAS). Visual injury was evaluated 7, 14, and 21 days after application (DAA). Biomass measurements were taken 21 DAA. Results showed that preconditioning tomato seeds with 2,4,6-T, melatonin, and fenclorim 7 DAA significantly decreased injury by 25, 25, and 23%, respectively.

Moreover, applying melatonin, benoxacor, and 2,4,6-T 21 DAA led to significantly greater dry biomass, which increased by 1.5, 1.42, and 1.44 times, respectively, compared to the control.
This research provides valuable insights into the chemical effects of benoxacor, fenclorim, 2,4,6-T, and melatonin safeners in tomato cultivation. The findings demonstrate the potential for pre-conditional tomato plants with 2,4,6-T, melatonin, and fenclorim to reduce injury while applying melatonin, benoxacor, and 2,4,6-T can increase dry biomass. Understanding plant defense mechanisms and the protective effects of safeners against herbicide damage contributes to developing effective weed management strategies.

**Keywords:** Plant protection, safeners, herbicide drift, herbicide resistance, detoxification mechanisms.

**Introduction**

Tomatoes (*Solanum lycopersicum*) are a highly valued and widely cultivated crop, ranking second only to potatoes (*Solanum tuberosum* L.) in production. With over 182.3 million tons of tomato fruits grown on approximately 4.85 million hectares yearly (Quinet et al. 2019), tomatoes play a crucial role in global food production and supply. However, tomato production faces numerous challenges that directly and indirectly impact yields, including low-quality seeds, adverse climatic conditions, pest infestations, and weed interference (Tolman et al. 2004; Singh et al. 2017). Among these factors, weeds pose a significant threat to tomato crops. Weeds compete with tomatoes for essential resources such as water, nutrients, sunlight, and space, leading to reduced crop growth and productivity (Clark et al. 1998). Effective weed management is, therefore, vital to maintaining high-quality tomato yields and preventing economic losses in the agricultural industry (Yaacoby et al. 2023).

While herbicide application is a commonly employed method for weed control in agricultural production (Gwatidzo et al. 2023), selecting appropriate herbicides carefully based on the target weed species is essential. Additionally, environmental factors such as temperature, wind
speed, and moisture levels must be considered during herbicide application to ensure optimal
effectiveness and minimize unintended off-target effects (Johnson and Young 2002); (Richardson
et al. 2020). Tomato plants are known to be particularly sensitive to many herbicides. Some
commonly used herbicides, such as 2,4-D and dicamba, can be highly detrimental to tomato
production. Even small amounts of these herbicides, resulting from drift or contamination, can
severely compromise tomato crop growth and yield (Medeiros et al. 2023). Implementing effective
weed management strategies is crucial to achieve maximum quality and high yields in tomato
cultivation. A comprehensive approach may involve both chemical and non-chemical control
methods. Pre-herbicides, such as sulfentrazone, S-metolachlor, or fomesafen, may be
recommended based on their effectiveness against specific weed species and the density of weed
populations (Jablonkai 2013; Flint and Klonsky).

Furthermore, non-chemical controls, including mechanical and cultural practices, can
supplement herbicide use and help manage weeds effectively. Farmers and agricultural
practitioners can use various weed management approaches to minimize weed-related challenges,
reduce resource competition, and optimize tomato crop growth and productivity. Understanding
the specific weed dynamics and implementing appropriate weed management strategies are key
steps toward achieving successful tomato production while preserving the agricultural systems'
overall health and sustainability. One of the promising weed control options is to use safeners to
improve herbicide tolerance in tomato, thereby enhancing weed control (Rosinger, 2014). Otto
Hoffman introduced the safener concept in the late 1940s after discovering that tomato plants
treated with 2,4,6-T did not suffer damage after contact with 2,4-D vapor ((Jablonkai 2013)). In
1971, these compounds were introduced as a new tool to the market, and the first commercial
safener, 1,8-naphthalic anhydride, was used as a seed treatment in corn (Hoffman, 1969). Since
then, safeners have generated significant interest from the chemical industry due to their physiological and biochemical features to activate important mechanisms in plants without reducing herbicide efficacy in target weed species. Additionally, their use could help reduce or slow the development of herbicide-resistant weed populations (Duhoux and Délye 2017). The use of fenclorim (4,6-dichloro-2-phenylpyrimidine) as a safener occurred in 1991 to protect and increase the tolerance of rice (*Oryza sativa* L.) against pretilachlor (Han and Hatzios 1991; Chen et al. 2013).

Benoxacor was also discovered in the 1990s. (Cottingham and Hatzios 1991) reported that benoxacor was used as a safener in maize to protect it from metolachlor injury. Metolachlor is among the top five herbicides applied in the mid-western corn belt of the United States. It is so widely used that it can be found in high concentrations in groundwater, among other herbicides (Fuerst et al. 1995; Simonsen et al. 2020). More recent research on melatonin has discussed its benefits in plants through cell enlargement and root development, delayed flowering, and improved fruit yield, quality, and preserving the mineral balance in heat-stressed tomato plants (Arnao and Hernández-Ruiz 2020; Kaya et al. 2022).

Safener use may protect various crops against herbicide drift. Consequently, this investigation examined the absence and presence of different safeners with distinct modes of action to protect tomatoes against herbicide applications.

**Material and Methods**

**Tomato transplants, pre-conditioning, and herbicide treatments**

Experiments were conducted in 2022 and 2023 at the R.R. Foil Plant Science Research Center of Mississippi State University in Starkville, MS (lat. 32.46936111°N, long. 88.78333333°E). The study utilized a completely randomized design with four replications,
repeated twice, and arranged in a 4x5 factorial design. Factor A represented the herbicide treatments, including four herbicides, while factor B consisted of four safeners and a non-treated (safener control) group.

Tomato seeds were immersed in the solution with safener and methanol for 1 hour before planting. Each safener was dissolved in methanol at the following micromolar concentrations: 10 \( \mu M \) for benoxacor (TCI America\textsuperscript{TM}, >98.0%), 2.98 \( \mu M \) for fenclorim (TCI America\textsuperscript{TM}, >98.0%), 5,066 \( \mu M \) for 2,4,6-T (TCI America\textsuperscript{TM}, >98.0%), and 100 \( \mu M \) for melatonin (Alfa Aesar 99.0+%) \{Citation\}. The control treatment was treated with methanol only. Seedlings were generated by sowing the seeds into 72-cell plug trays filled with a soilless potting media (Pro-mix BX; Rivière-du-Loup, Quebec, Canada) and cultivated in a greenhouse. The tomato plugs were transplanted 21 days later into 0.815-L pots filled with the same soilless media. Herbicides were applied at 25 days after sowing (DAS) using a calibrated spray chamber delivering 187 L · ha\(^{-1}\) and equipped with AIXR 1102 nozzles (TeeJet Technologies, Wheaton, IL) at a spray pressure of 275.8 KPa. The specific herbicide applications are listed in Table 1.

Crop injury was visually evaluated at 7, 14, and 21 days after application (DAA) using a scale ranging from 0% to 100%, where 0% represented normal growth without herbicide symptoms and 100% indicated complete desiccation of shoot tissues. The criteria for scoring included various symptoms such as leaf chlorosis, cupping, rolling, stem twisting, stunting, necrosis, and epinasty.

**Shoot biomass**

At 21 DAA, the shoot tissues were harvested, stored in paper bags, oven-dried at 60 °C for 72 hours, and weighed to obtain the dry biomass weights.
Statistical Analysis

All data were fitted using a standard least-squares (LS) model in JMP Pro 16.1 (SAS Institute Inc., Cary, NC, USA). The main effects analyzed included the safeners (benoxacor, fenclorim, 2,4,6-T, and melatonin) and herbicides (sulfentrazone, metribuzin, dicamba, and 2,4-D) at 1% of the field rate (Table 1). Interactions between the main effects were also assessed in relation to dry biomass and injury. For data that met the assumptions of the ANOVA test, treatment means were separated using the LS Means Differences Tukey Honest Significant Difference (HSD) test, which was conducted at a significance level of $\alpha = 0.05$.

Results

Crop Injury

Significant differences in crop injury were observed in tomato leaves 7, 14, and 21 days after herbicide application (DAA) ($P < 0.05$). The herbicide applications exhibited varying injury levels, as indicated by the injury scale mentioned in the materials and methods section (Figure 5). At 7 DAA, fenclorim, 2,4,6-T, and melatonin treatments resulted in lower injury than the control, while benoxacor treatment did not show a significant difference. The highest injury levels were observed in the benoxacor and control treatments (33% and 32%, respectively), while the fenclorim treatment showed the lowest injury (23%), followed by melatonin and 2,4,6-T (25% each) (Figure 2).

At 14 DAA, significant differences in crop injury were observed in tomato leaves. The benoxacor and fenclorim treatments did not show a significant difference compared to the control, while 2,4,6-T and melatonin treatments resulted in significant differences (Figure 3). At 21 DAA, the 2,4,6-T and melatonin treatments effectively reduced herbicide-induced injury, showing significant differences from the fenclorim, benoxacor, and control treatments (Figure 4).
All herbicides caused more than 50% injury in tomato leaves and stems 21 DAA, except for fenclorim, which reduced injury from sulfentrazone by more than 50%, and 2,4,6-T, which reduced injury from dicamba, 2,4-D, and metribuzin. Melatonin also reduced 2,4-D injury by more than 50%; however, benoxacor did not reduce herbicide injury (Figure 5).

**Shoot Dry Biomass**

The shoot dry biomass of tomato plants 21 DAA showed significant differences ($P < 0.05$) among the treatments. The melatonin, 2,4,6-T, and benoxacor treatments had higher dry biomass accumulation post-herbicide application than the control. In contrast, the fenclorim treatment did not differ in accumulation from the treatments or the control (Figure 6). The pre-conditioning with melatonin accumulated 1.50 times more dry biomass than the control, while 2,4,6-T and benoxacor accumulated 1.44 and 1.42 times more, respectively. The fenclorim treatment dry biomass was not statistically different from the control. Still, the seed treated with this safener had 1.36 fold more tomato biomass compared to the control (Figure 6).

The dry biomass of tomatoes pre-conditioned with safeners was greater among the herbicide treatments than the control. The combination of herbicides and safeners had showed a significant difference compared to the control plants (Figure 5). Although dicamba did not show a significant difference compared to metribuzin and sulfentrazone, it resulted in the lowest increase in tomato biomass (1.10 times), while metribuzin and sulfentrazone treatments (1.21 and 1.33, respectively) allowed for more dry biomass accumulation (Figure 7). Conversely, 2,4-D treatment resulted in the greatest increase (1.76 times) in tomato biomass compared to the control.
Discussion

Pre-conditioning tomatoes with safeners melatonin, 2,4,6-T, and fenclorim reduced crop injury compared to the benoxacor and control treatments seven days after herbicide application (DAA). These findings align with previous studies, such as (Castro et al. 2020), which reported the protective effects of fenclorim on tomato seeds treated with different herbicides. The exact molecular mechanism by which safeners provide plant protection against herbicide injury remains unclear, and further research is needed to understand their modes of action in crops. For instance, 2,4,6-T has been shown to suppress epinasty induced by 2,4-D vapor and stimulate tomato ripening (Hoffmann 1953). The advantageous effects of melatonin in plants, including its influence on cell enlargement, root development, and stress mitigation, have been highlighted in numerous studies (Arnao and Hernández-Ruiz 2020). Melatonin's role as a potent scavenger of free radicals and its ability to detoxify various chemical contaminants make it a promising candidate for mitigating herbicide drift (Hacışevki & Baba, 2018). These observations are consistent with the results obtained in this study.

At 14 DAA, plants pre-conditioned with melatonin and 2,4,6-T exhibited the lowest injury percentages, while the control, fenclorim, and benoxacor treatments showed higher injury levels. The antioxidant capacity of melatonin allows it to scavenge reactive oxygen and nitrogen species, thereby protecting plants from environmental stress (Arnao and Hernández-Ruiz 2014). The protective effects of safeners have been documented in other plant species. For example, prior immersion in a solution with 2,4,6-T has inhibited stem curvature in peas exposed to 2,4-D (Hartman 1959).

Data from 21 DAA indicated that seeds treated with fenclorim, melatonin, and 2,4,6-T resulted in less tomato tissue injury compared to benoxacor and the control group. Furthermore,
all treatments showed a more significant dry biomass accumulation than the control. Fenclorim has been shown to promote antioxidant effects and protect rice plants from the impacts of pretilachlor (Hu et al. 2021). Similarly, pretreatment with melatonin reduced membrane damage and lipid oxidation and stimulated antioxidant enzyme activity in poplar leaves (Hacışevki & Baba, 2018). Although benoxacor did not result in less injury than the control treatment at 21 DAA (Figure 4), seeds pretreated with benoxacor had higher dry biomass accumulation post-herbicide application than the control (Figure 6). The biochemical mechanism of benoxacor involves the detoxification of herbicides through conjugation with the tripeptide glutathione (GSH), an antioxidant enzyme found in various organisms (Narayanankutty et al. 2019).

Despite the protective effects observed with melatonin, fenclorim, and 2,4,6-T, the toxicity of herbicides on tomato leaves increased as the number of days after application increased.

Tomatoes are susceptible to various herbicides, especially 2,4-D and dicamba, where even minimal, unintended drift risks the entire crop (Medeiros et al. 2023). The recommendation for pre-herbicide application in tomato production depends on herbicide efficacy and weed density (Flint and Klonsky). Therefore, effective weed management is crucial for ensuring optimum quality and high yield, which may involve non-chemical control methods such as mechanical and cultural practices.

**Conclusion**

This study demonstrated that pre-conditioning tomatoes with safeners significantly increased the tolerance of tomato plants to herbicide exposure. Fenclorim, 2,4,6-T, and melatonin exhibited notable levels of protection against injury on tomato tissues compared to the control. Moreover, 2,4,6-T, melatonin, and benoxacor resulted in more dry biomass accumulation than the control.
These findings emphasize the importance of utilizing safeners to enhance a crop's resilience to sulfentrazone, metribuzin, dicamba, and 2,4-D and mitigate their potential damage. It is crucial to recognize that herbicides from different families with varying modes of action, such as PPO, PSII, and auxin herbicides, can have detrimental effects on tomato plants due to their high sensitivity. Even minimal amounts of these herbicides can cause significant harm and compromise yields. Therefore, the agricultural industry and tomato growers must be cautious and take appropriate measures to prevent damage.

The use of specific safeners tailored to different crops and herbicide applications holds promise in improving herbicide formulations and ensuring adequate protection for tomato crops. Further research is warranted to deepen our understanding of the mechanisms underlying safeners' protective effects and their interaction with specific herbicides. Furthermore, exploring additional safeners and their efficacy in various crop-herbicide combinations would provide valuable insights for developing comprehensive weed management strategies and promoting sustainable agriculture practices.

Overall, this study highlights the potential of safeners as a valuable tool for protecting tomato crops from herbicide-induced injury. Implementing safener pre-conditioning in tomato cultivation practices can contribute to developing more effective herbicide formulations and help safeguard the productivity and quality of tomato yields.
Tables and Figures

Table 2  
List of herbicide treatments and their rates used in the greenhouse study at the R. R. Foil Plant Science Research Center at Mississippi State University, Starkville, MS

<table>
<thead>
<tr>
<th>Herbicide group</th>
<th>Herbicide</th>
<th>Rate</th>
<th>Trade names</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aryl triazinone</td>
<td>Sulfentrazone</td>
<td>0.22</td>
<td>Spartan</td>
<td>FMC</td>
</tr>
<tr>
<td>Triazinone</td>
<td>Metribuzin</td>
<td>0.84</td>
<td>Glory</td>
<td>Adama Agricultural Solutions</td>
</tr>
<tr>
<td>Benzoic acids</td>
<td>Dicamba</td>
<td>0.22</td>
<td>XtendiMax</td>
<td>Bayer</td>
</tr>
<tr>
<td>Phenoxy</td>
<td>2,4-D</td>
<td>0.2</td>
<td>Enlist One</td>
<td>Corteva</td>
</tr>
</tbody>
</table>
Table 3  Shoot dry biomass values of tomato plants with and without safeners compared under different herbicide applications 21 days after herbicide treatment. Means were separated within assessments using the Tukey test at P = 0.68. Means followed by the same letter do not differ.

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Year</th>
<th>Control</th>
<th>Fenclorim</th>
<th>Benoxacor</th>
<th>Melatonin</th>
<th>2,4,6-T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>g plant⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>2</td>
<td>0.96 c</td>
<td>1.51 b</td>
<td>1.48 b</td>
<td>1.80 ab</td>
<td>2 a</td>
</tr>
<tr>
<td>Dicamba</td>
<td>2</td>
<td>0.43 d</td>
<td>0.53 cd</td>
<td>0.52 cd</td>
<td>0.51 cd</td>
<td>0.34 d</td>
</tr>
<tr>
<td>Metribuzin</td>
<td>2</td>
<td>0.55 cd</td>
<td>0.61 cd</td>
<td>0.75 cd</td>
<td>0.73 cd</td>
<td>0.59 cd</td>
</tr>
<tr>
<td>Sulfentrazone</td>
<td>2</td>
<td>0.51 cd</td>
<td>0.69 cd</td>
<td>0.73 cd</td>
<td>0.72 cd</td>
<td>0.58 cd</td>
</tr>
</tbody>
</table>
Figure 12  Visual injury caused by (A) dicamba (XtendiMax), (B) sulfentrazone (Spartan), (C) 2,4-D (Enlist one), and (D) metribuzin (Glory) applications 25 days after sowing the tomato seeds pretreated with safeners.
Figure 13  Visible estimates of injury at 7 days after application of herbicides in tomato plants treated with safeners before sowing. Means were separated within assessments using the Tukey test at P < .0001. Means followed by the same letter do not differ.
Figure 14  Visible estimates of injury at 14 days after application of herbicides in tomato plants treated with safeners before sowing. Means were separated within assessments using the Tukey test at P < .0001. Means followed by the same letter do not differ.
Figure 15  Visible estimates of injury at 21 days after application of herbicides in tomato plants treated with safeners before sowing. Means were separated within assessments using the Tukey test at $P < .0001$. Means followed by the same letter do not differ.
Figure 16  Visible estimates of injury at 21 days after application of herbicides in tomato plants treated with safeners before sowing. Means were separated within assessments using the Tukey test at $P = 0.54$. Means followed by the same letter do not differ.
Figure 17  Shoot dry biomass of tomato plants at 21 days after application of herbicides treated with safeners before sowing. Means were separated within assessments using the student’s t-distribution test at $P = 0.24$. Means followed by the same letter do not differ.
Figure 18  Safener protection in tomato at 7 DAA of herbicides (A) Dicamba, (B) 2,4-D, (C) sulfentrazone, and (D) metribuzin. Herbicides were applied at 1:100 of the field rate. Tomatoes were pre-treated with 10 $\mu$M of safeners (1) fenclorim, 0.67gL$^{-1}$ of (2) benoxacor, 100 $\mu$M of (3) melatonin, 255.48 $\mu$M of (4) 2,4,6-T. (5) served as a control of no safeners. Visible estimates of injury were recorded for each treatment.
References


CHAPTER III
IDENTIFICATION OF ACTIVITY OF GLUTATHIONE S-TRANSFERASE FROM SOLANUM LYPERSICUM TREATED WITH BENOXACOR, FENCLORIM, MELATONIN, AND 2,4,6-TRICHLOROPHENOXYACETIC ACID TO PROTECT AGAINST HERBICIDE DRIFT.

Abstract
Pesticide residues, including herbicides and insecticides, frequently promote injury to vegetables such as tomatoes. Thus, developing scientific approaches to protect crops against herbicide injury is extremely important. One of the potential approaches is the use of herbicide safeners, which is a chemical compound that promotes herbicide detoxification. The safener detoxification is a complex process that involves various signaling pathways, such as phase II (chemical conjugation), which participate in the primary conjugation with endogenous substrates such as glutathione. The detoxification of xenobiotic and endobiotic compounds is catalyzed by glutathione S-transferase (GST). This study evaluates the GST activity of tomatoes treated with safeners after herbicide applications. The experiment was conducted at the Weed Physiology Laboratory in 2023. Treatments were arranged in a completely randomized design with four replications in a randomized factorial design (4x5). Factor A consisted of four herbicide treatments (dicamba, 2,4-D, sulfentrazone, and metribuzin), while factor B consisted of five safeners (melatonin, 2,4,6-T, fenclorim, benoxacor, non-safeners). Samples were collected at 0, 3, and 7 DAA. Enzymatic activities were evaluated by using a GST assay kit in which the 1-chloro-2,4-
dinitrobenzene was used as the enzymatic substrate. Benoxacor and fenclorim increased GST activities by 54 and 42%, respectively. The safeners likely induce GST isoenzymes. However, additional research is required to confirm and identify the specific GST isoforms induced by the safeners that also exhibit an affinity for the applied herbicides.

**Introduction**

Weed control methods have undergone a significant transformation over the past century. Initially, these methods relied on cultural and mechanical approaches. However, they have evolved to combine mechanical and chemical methods, with chemical compounds now playing a predominant role (Bagavathiannan et al. 2019). Some herbicides promote crop damage by effectively managing weed populations (Dhouib et al. 2016). Pesticide residues, including herbicides and insecticides, frequently promote injury to vegetables such as tomatoes (Greenland. 2003). Thus, developing scientific approaches to protect crops against herbicide injury is extremely important.

One potential approach is using herbicide safeners, which are chemical compounds employed to enhance the selectivity of herbicides between crops and weeds and promote herbicide detoxification (Giannakopoulos et al. 2020). They are frequently employed for weed control in large crops such as grasses, rice, sorghum, and corn (Giraldo et al. 2022). The safener detoxification is a complex process that involves various signaling pathways and mechanisms. Most plants undergo a three-phase process to facilitate the detoxification of xenobiotic compounds like herbicides and insecticides and convert them into intermediate substances with reduced phytotoxicity (Sandermann Jr. 1992).

In Phase I, chemical modification or activation occurs through oxidation, reduction, and hydrolysis reactions. This phase prepares xenobiotics for further processing. Phase II, known as
chemical conjugation, follows Phase I. Xenobiotics are primarily conjugated with endogenous substrates like glucose, amino acids, or glutathione. Glutathione, widely distributed in plant tissues, protects plant cells from oxidative damage during this phase. Phase III, referred to as transport/compartmentalization, is the final step. It involves the secondary conjugation of xenobiotics and is responsible for creating insoluble residues or sequestering them in vacuoles. This phase ensures the safe containment or elimination of the modified xenobiotics (Davies & Caseley. 1999).

Using herbicide safeners to protect plants against xenobiotic effects is known to go through glutathione S-transferase (GSTs) conjugation during Phase II, promoting detoxification. Acting as the primary phase II detoxification enzymes, GSTs safeguard living cells by facilitating the conjugation of reduced glutathione (GSH) to a diverse range of electrophilic molecules originating from internal and external sources (Edwards. 2011). Previous studies have shown that the conjugation of herbicides pretilachlor and acetochlor raised by fenclorim and other safeners can synthesize GSH, which leads to an induction of GST activity (Han & Hatzios. 1991; Riechers et al. 1997).

The detoxification of xenobiotic and endobiotic compounds is catalyzed by GST, which is widely found in eukaryotes and prokaryotic cells and is well-studied in plants and other organisms such as animals, fungi, and bacteria (Monticolo et al. 2017). Tests for GST induction using various safeners showed that cloquintocet-mexyl, fenchlorazole, and fluxofenim enhanced GST levels in wheat, protecting against butachlor. Additionally, benoxacor and fenchlorazole could increase the activity of GST in maize (Scarponi et al. 2006). In a separate study, Frear and Swanson (1970) highlighted the role of GST in the defense of Zea mays against s-triazine herbicides. Subsequent research further confirmed the significance of GST in herbicide tolerance across various crops,
including tobacco, tomato, citrus, and more (Karavangeli et al. 2005; Cicero et al. 2015; Csiszár et al. 2014). In plants, these compounds primarily act to reduce the effectiveness of herbicides in inhibiting their target sites. This reduction can occur through chemical reactions between the herbicide molecule and the safener, leading to decreased herbicide uptake by plants. Additionally, safeners can reduce herbicide metabolites. Notably, GST activity plays a pivotal role in safeguarding plant cells against oxidative DNA damage and lipid peroxidation in the membrane, as Duhoux et al. (2017) suggested.

While different studies have demonstrated the potential of safeners as a novel solution to numerous weed control challenges and have established their effectiveness in enhancing crop tolerance to herbicides, the ongoing advancement of improved crop protection through safeners hinges on the implementation of more targeted approaches and the deepening of our understanding. Moreover, we need to improve the knowledge of the correlation between herbicide-safeners and metabolism detoxification in plant cells. This study aims to identify, quantify, and establish a correlation between the activity of GST enzymes in tomatoes, which had been pre-treated with safeners, after herbicide application.

**Material and Methods**

**Plant Materials**

Studies were conducted at the R.R. Foil Plant Science Research Center of Mississippi State University and the Weed Physiology laboratory at the Department of Plant and Soil Science of Mississippi State University in Starkville, MS, in 2023. Treatments were arranged in a completely randomized design with four replications and arranged in a 4x5 factorial design.

Factor A consisted of four herbicide treatments and included metribuzin (Glory – Adama Ltd Agricultural Solutions) at 0.84 kg ai ha\(^{-1}\), sulfentrazone (Spartan – FMC Corporation) at 0.22
kg ai ha\(^{-1}\), dicamba (XtendiMax – Bayer) at 0.22 kg ai ha\(^{-1}\), and 2,4-D (Enlist One – Corteva\(^{TM}\)) while factor B consisted of five safeners (melatonin, 2,4,6-T, fenclorim, benoxacor, non-safeners) and included 10 µM for benoxacor (TCI America\(^{TM}\), > 98.0%), 0.67 gL \(^{-1}\) for fenclorim (TCI America\(^{TM}\), > 98.0%), 255.5 µM for 2,4,6-T (TCI America\(^{TM}\), > 98.0%), and 100 µM for melatonin (Alfa Aesar 99.0+ %), following previous studies (Fuerst et al. 1993; Shen et al. 2013; Hartman. 1959). The safener control seeds were treated with methanol only.

Herbicides were applied at 25 days after sowing (DAS) using a spray chamber calibrated to deliver 187 L ha\(^{-1}\) fitted with an AIXR 11002 nozzle (TeeJet Technologies, Wheaton, IL) and maintained at a spray pressure of 275.8 KPa.

**GST enzyme-essay**

Leaf samples were collected at 0, 3, and 7 days after herbicide application and frozen at -80°C until use. Enzymatic activities of the GST were determined by using a glutathione S-transferase assay kit (Sigma-Aldrich., MO, USA), in which the 1-chloro-2,4-dinitrobenzene (CDNB) was used as the enzymatic substrate. All assays were replicated four times. A solution containing 200 mM reduced L-glutathione and 100 mM (CDNB) in Dulbecco’s phosphate-buffered saline was prepared and used within 1 hour (solution A2). Tris-EDTA-polyvinylpyrrolidone (PUPP) buffer (100 mM Tris (Hydroxymethyl) aminomethane (C\(_4\)H\(_{11}\)NO\(_3\)), 1 mM EDTA [pH 7.8/7.5], and 7.5% w/v) was used to lyse, wash, and dissolve to obtain a clarified crude extract.

Five grams of frozen tomato tissue was added to 1.5-mL microcentrifuge tubes and homogenized using a Precellys\(^{®}\) Evolution Homogenizer at 7,200 RPM for 20 seconds using glass beads. After homogenization, 500 µL of Tris-EDTA-PUPP was added and centrifuged at 120 RPM for 20 minutes. The supernatant of the samples was transferred to another tube, and 10 µL of
solution A2 was added. GST activity was assayed spectrophotometrically at 340 nm. GST catalyzes the conjugation of L-GSH to CNDB through the thiol rate group of GSH.

\[
\text{GSH} + \text{CNDB} \xrightarrow{\text{GST}} \text{GS-DNB Conjugate} + \text{HCl} \quad (1)
\]

The reaction product, GS-DNB Conjugate, absorbs at 340 nm (A_{340\text{nm}}).

**GST- calculations**

The rate of increase in the absorption is directly proportional to the GST activity in the samples. The GSH conjugation was calculated from the increase in absorption from the reagent blank at 340 nm of the assay mixture. To calculate the change in absorbance $A_{340\text{nm}}$/minute, in the linear range of the plot, for the sample and the blank using the following equation:

\[
A_{340\text{nm}}/\text{min} = \frac{A_{340\text{nm}} (\text{final read}) - A_{340\text{nm}} (\text{initial read})}{\text{Reaction time (min.)}} \quad (2)
\]

Subtract the $A_{340\text{nm}}$/min of the blank from the $A_{340\text{nm}}$/min of the sample. It used this rate for the calculation of the GST-specific activity.
GST- specific activity

\[
\frac{A_{\text{satur/min}} \times V (ml) \times \text{dil}}{\mu\text{M/}\text{ml/min}} = \frac{\text{mol/\text{ml/min}}}{\varepsilon_{\mu\text{M}} \times V_{\text{enc}} (ml)}
\]  \hspace{1cm} (3)

Where:

dil - the dilution factor of the original sample

\(\varepsilon_{\mu\text{M}} \text{ (mM}^{-1} \text{ cm}^{-1})\) - the extinction coefficient for CDNB conjugate at 340 nm

\(V\) - The reaction volume (0.2 ml)

\(V_{\text{enc}}\) - The volume of the enzyme sample tested (0.05 ml)

**Statistical Analyzes**

All data were analyzed with LS-means in JMP Pro 16.1 (SAS Institute Inc., Cary, NC, USA). For data that met the assumptions of ANOVA, treatment means were separated using the LS Means Differences Tukey Honest Significant Difference (HSD) test, which was conducted at a significance level at \(\alpha = 0.05\). The main effects analyzed included the safeners (benoxacor, fenclorim, 2,4,6-T, and melatonin) and herbicides (sulfentrazone, metribuzin, dicamba, and 2,4-D) at 1% of the field rate. Interactions between the main effects were assessed in relation to GST activity.

**Results**

GST levels were determined in 484 samples treated with five different safener treatments at 0, 3, and 7 days after herbicide application (Figure 20). We hypothesized that safeners would increase GST activity over time after herbicide application. The herbicide and safener treatments significantly affected GST activity in tomato plants.
Safeners

Seeds treated with benoxacor and fenclorim significantly increased GST activity by 87 and 86%. Seeds treated with melatonin did not result in a significant difference in GST activity compared to the control; however, the treatment led to a 68% increase in GST activity compared to the control. Tomato seeds pretreated with 2,4,6-T did not result in a significant difference in GST activity and led to lower activity than the control and other safeners used (Figure 19).

Herbicides

The herbicide application had a significant effect on GST activity ($P < .0001$). Metribuzin and sulfentrazone increased GST activity compared to 2,4-D and dicamba herbicides. The application of sulfentrazone and metribuzin resulted in a more than 100% increase in GST activity compared to dicamba, as well as after the application of 2,4-D. Among all herbicide treatments, the highest enzyme activity was found with metribuzin and sulfentrazone (Table 4).

Safeners x herbicides

The GST activity of tomato leaves in this study showed a significant interaction ($P < .0001$) between safener and herbicide. Pretreatment with benoxacor resulted in a greater GST activity with metribuzin (4.2 times) and fenclorim (2.4 times) compared with plants without safeners (Table 5).

Discussion

The results demonstrated that the presence of safeners in the pre-treated tomato seeds significantly influenced the GST activity of benoxacor and fenclorim by 87 and 86%, respectively, compared to the control. Although melatonin did not result in a significant difference, seeds treated with this safener increased by 67% compared to the control treatment. Furthermore, the interaction
between safeners, herbicides, and timing of application impacted the GST results. GSTs have been associated with various stress responses, including biotic and abiotic attacks (Marrs, 1996). In crops like tomato, the expression of GSTs can be stimulated to protect cells from prooxidant-induced cell death (Kilili et al. 2004). Our results are consistent with those of Scarponi et al. (2006), who observed increases in glutathione S-transferase (GST) activity ranging from 64.8 to 30.5% in maize shoots treated with benoxacor due to induction of GST isoenzymes by the safener. Hu et al. (2021) found similar results in GST activity where Fen increased the activity of GST in rice. This result suggests that safeners enhance herbicide metabolism (Brazier et al. 2020) and increase the gene expression involved in herbicide detoxification (Riechers et al. 2010). There was no significant difference in GST activity in tomato leaves treated with melatonin; however, seeds treated with melatonin exhibited a 68% increase in enzymatic activity compared to the control treatment. In their study, Kanwar et al. (2020) elucidated the mechanism of detoxification by melatonin in tomatoes. They observed an enhancement in the activities of GSH genes, indicating a potential avenue for further optimization of these genes.

While herbicides are the primary means of weed control in commercial tomato production, tomatoes generally exhibit tolerance to most herbicides. Herbicides such as sulfentrazone, S-metolachlor, fomesafen, and metribuzin (Met) are compounds approved for tomato production worldwide (Mohamed et al. 2023). Meanwhile, 2,4-D and dicamba cause higher yield losses when sprayed at the early vegetative stage and are very sensitive to tomato production (Kruger et al. 2017; Soukupová & Koudela. 2023). In our study, when comparing the herbicides applied, the results indicate that the application of sulfentrazone led to an increase in GST activity of more than 100% compared to dicamba and 2,4-D. The application of metribuzin also resulted in a more than 100% increase in GST activity compared to dicamba and 2,4-D herbicides. According to Zhao et
al. (2023), several herbicides and safeners share common plant uptake sites. Therefore, some safeners may exert their detoxifying effects by influencing the absorption and translocation of herbicides within plants (Davies & Caseley. 1999). In addition, some herbicides are easily metabolically degraded by GST, such as triazines, which belong to the group of photosystem II inhibitors, and sulfentrazone, another herbicide (Cummins et al. 2011). Nevertheless, some studies have shown that safeners do not have a direct or indirect effect on herbicide translocation (Kocher. 2005; Scarponi et al. 2006), thus, the influence of safeners on herbicide uptake and movement is still controversial within crops. Furthermore, conclusive evidence is lacking to support the idea that safeners can directly disrupt the herbicide transport process within crops (Zhao et al. 2023).

The timing of herbicide application affected GST activity, with the highest levels observed at 7 days, followed by 0 and 3 days after herbicide application (Figure 20). The tests of GST activity showed an initial increase to $4 \times 10^{-4} \mu \text{mol/mL/min}$ at 0 DAA, followed by a decrease to $2.24 \times 10^{-4} \mu \text{mol/ml/min}$ at three days after herbicide application. Subsequently, there was a rise to levels ranging from $5.7 \times 10^{-4} \mu \text{mol/mL/min}$ 7 days after the application. In our study, benoxacor treatment significantly increased GST activity by 7 to $8 \times 10^{-4} \mu \text{mol/mL/min}$ at 0 and 7 days after treatment. Although not considerably higher, fenclorim resulted in $5, 4, \text{and } 7 \times 10^{-4} \mu \text{mol/mL/min}$ GST activity compared to untreated at 0, 3, and 7 days after herbicide treatment (Table 6). Earlier work by Scarponi et al. (2006) showed that wheat shoots treated with herbicide safeners benoxacor produced a 21.1% increase in enzyme activity at two days, while at 0 and 3 DAA, they found a 64.8 and 30.5% increase in enzyme activity compared to the control. Fenclorim resulted in a 39.1% increase in enzyme activity in maize at 2 DAA and a 48.8% increase in wheat at 3 DAA. However, the impact of different safeners on GST activity compared to untreated samples is not easy to explain based on the present data. One plausible explanation might involve the stress-inducing
nature of activated safeners following herbicide application on tomato plants. Additional research is needed to confirm this hypothesis.

Numerous studies have documented different organisms’ or safeners’ capacity to boost GST activity when exposed to various herbicides (Skipsey et al. 2005; Moreland et al. 1993; Dixon et al. 1997), but not including the combination between safeners and herbicides used on the present study. The current findings demonstrate an $8 \times 10^{-4} \mu mol/mL/min$ increase in GST activity when sulfentrazone is combined with fenclorim. Benoxacor enhances metribuzin-induced GST activity by $17 \times 10^{-4} \mu mol/mL/min$, while fenclorim and melatonin range from $10$ to $9 \times 10^{-4} \mu mol/mL/min$ in tomato leaves after treatment. Additionally, dicamba’s GST activity was enhanced when combined with fenclorim ($2.43 \times 10^{-4} \mu mol/mL/min$) and melatonin ($2.21 \times 10^{-4} \mu mol/mL/min$). In contrast, 2,4-D enhanced GST activity on tomato leaves treated with benoxacor and fenclorim (Table 5). Therefore, GST activity was found to be safener-induced in tomato leaves after herbicide applications with different MOAs. This hypothesis gains support from previous research. For instance, Hu L et al. (2021) observed a substantial $45.3\%$ increase in the expression of genes related to GST in rice when treated with fenclorim compared to the control group. Additionally, Scarponi et al. (2006) reported similar findings in maize treated with benoxacor, where GST enzyme levels significantly increased after exposure to terbuthylazine ($1.48 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein) and butachlor ($0.37 \text{ nmol min}^{-1} \text{ mg}^{-1}$ protein), as compared to the control group.

**Conclusion**

Our study demonstrated that safeners in pre-treated tomato seeds impacted GST activity. Notably, benoxacor, fenclorim, and melatonin showed a positive trend. In contrast, the 2,4,6-T safener did not yield the same increase in GST activity. Herbicide application also significantly influenced GST activity, with metribuzin and sulfentrazone leading to the highest increases.
compared to dicamba and 2,4-D. Furthermore, the timing of herbicide application affected GST activity, with the highest levels observed seven days after herbicide application. The interaction between safeners and herbicides demonstrated that benoxacor and fenclorim enhanced GST activity when combined with metribuzin. This suggests that safeners may enhance herbicide metabolism and gene expression related to herbicide detoxification in tomato crops. In summary, our findings highlight the potential of safeners to modulate GST activity and enhance herbicide tolerance in tomato plants. This study improves knowledge regarding the use of herbicide safeners in vegetables. Further research is needed to elucidate the underlying mechanisms of this interaction.
## Tables and Figures

**Table 4**

Means of GST activity (μmol/ml/min) under different herbicide applications. The data represent means of quadruplicate determinations. Means followed by the same letter are not significantly different ($P < .0001$) at the 5% level using the Tukey test.

<table>
<thead>
<tr>
<th>Herbicides</th>
<th>Rate (kg ai ha$^{-1}$)</th>
<th>GST activity (μmol/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metribuzin</td>
<td>0.84</td>
<td>9.03 a</td>
</tr>
<tr>
<td>Sulfentrazone</td>
<td>0.22</td>
<td>4.59 b</td>
</tr>
<tr>
<td>Dicamba</td>
<td>0.22</td>
<td>1.53 c</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.2</td>
<td>0.70 c</td>
</tr>
</tbody>
</table>
Table 5  GST activity (μmol/ ml/ min) of untreated and safener-treated tomato plants. The data represent means of quadruplicate determinations. Means followed by the same letter are not significantly different ($P < 0.05$) at the 5% level using the Tukey test.

GST ($10^{-4}$ μmol/ml/min)

<table>
<thead>
<tr>
<th>Herbicides</th>
<th>Control</th>
<th>Benoxacor</th>
<th>Fenclorim</th>
<th>Melatonin</th>
<th>2,4,6-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metribuzin</td>
<td>4.14 bcde</td>
<td>17.21 a</td>
<td>10.08 b</td>
<td>9.87 bc</td>
<td>cde</td>
</tr>
<tr>
<td>Sulfentrazone</td>
<td>5.93 bcde</td>
<td>2.12 de</td>
<td>7.91 bcd</td>
<td>5.51 bcde</td>
<td>1.48 e</td>
</tr>
<tr>
<td>Dicamba</td>
<td>0.98 e</td>
<td>0.82 e</td>
<td>2.43 e</td>
<td>2.29 de</td>
<td>0.89 e</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.45 e</td>
<td>1.09 e</td>
<td>0.89 e</td>
<td>0.78 e</td>
<td>0.24 e</td>
</tr>
</tbody>
</table>
Table 6  GST activity of untreated and safener-treated tomato plants. The data represent means of quadruplicate determinations * are significantly different from the untreated sample at the 5% level using the Tukey test.

GST (CDNB) activity ($10^{-4}$ μmol/ml/min)

<table>
<thead>
<tr>
<th>Safeners</th>
<th>Days after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Benoxacor</td>
<td>7*</td>
</tr>
<tr>
<td>Fenclorim</td>
<td>5</td>
</tr>
<tr>
<td>Melatonin</td>
<td>5</td>
</tr>
<tr>
<td>2,4,6-T</td>
<td>2</td>
</tr>
<tr>
<td>Untreated</td>
<td>2</td>
</tr>
</tbody>
</table>
Means of GST-specific activity ($10^{-4} \, \mu\text{mol/ ml/ min}$) calculated of untreated and safener-treated tomato plants. The data represent means of quadruplicate determinations. Means followed by the same letter are not significantly different ($P = 0.0001$) at the 5% level using the Tukey test.
Figure 20  Means of GST (μmol/ ml/ min) activity in response to different days after herbicide application. Means followed by the same letter are not significantly different ($P = 0.001$) at the 5% level using the Tukey test.
References


Cicero LL, Madesis P, Tsaftaris A, Piero AR. Tobacco plants over-expressing the sweet orange tau glutathione transferases (CsGSTUs) acquire tolerance to the diphenyl ether herbicide fluorodifen and to salt and drought stresses. Phytochemistry. 2015 Aug 1;116:69-77.


