THESIS

FACTORS CONTRIBUTING TO HERBICIDE RESPONSE IN COAXIUM WHEAT

Submitted by

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ABSTRACT

FACTORS CONTRIBUTING TO HERBICIDE RESPONSE IN COAXIUM WHEAT

Compared to other pests, weed competition has the most significant negative impact on wheat grain yield. Understanding the contribution of metabolism in overall tolerance to herbicides can lead to new methods for controlling weeds in wheat. Glutathione S-transferase's (GSTs) role in the detoxification of herbicides has been studied since 1970. Previous literature reported increased resistance to herbicides with higher GST activity in black grass (*Alopecurus myosuroides*) and Asia minor bluegrass (*Polypogon fugax*). Resistance could be reversed by inhibiting GST activity. This research assesses the role of Phase 2 plant cell metabolism by testing (GST) inhibition to see if it influences the metabolism of quizalofop P-ethyl (QPE) in winter wheat (*Triticum aestivum*). We hypothesized that the addition of a safener would make the wheat more tolerant to the herbicide while the addition of a GST inhibitor would make the wheat more sensitive to QPE. Experiments were conducted analyzing the QPE effect on whole-plant biomass and an LC-MS/MS analysis of the amount of quizalofop acid (QZA) found in plant extracts. Safeners enhanced herbicide metabolism which increased CoAXium wheat tolerance to QPE. GST inhibitors, conversely, decreased herbicide metabolism causing CoAXium wheat to be more sensitive to QPE. Understanding the contribution of metabolism in overall resistance to herbicides can lead to breeding improvements for more herbicide-tolerant wheat varieties and new methods for controlling weeds in wheat.

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CHAPTER 1: GLUTATHIONE *S*-TRANSFERASE'S ROLE IN HERBICIDE METABOLISM: CROPS VS. WEEDS

SUMMARY

The authors briefly describe the discovery and structure of glutathione *S*-transferases (GST), and how these enzymes are involved in herbicide metabolism. GSTs contribute to both herbicide selectivity in crops and herbicide resistance in weeds. This review highlights examples from research on selected crops and weeds demonstrating how these enzymes can have both positive and negative impacts on the efficacy of herbicides.

INTRODUCTION

Glutathione *S*-transferases (GSTs) form a large and complex enzyme superfamily found in all organisms. Plants rely on GSTs for a range of essential functions, including primary and secondary metabolisms, stress tolerance, and cell signaling. The involvement of plant GSTs in xenobiotic detoxification was first reported in 1970 (Frear & Swanson 1970). We will survey the state of knowledge and speculate on future research that could enable us to harness the power of this enzyme superfamily to enhance herbicide selectivity and fight herbicide resistance.

REVIEW

1.1 Historical Perspective

Plant GSTs have been studied for over five decades, and a word cloud analysis based on the primary literature on these enzymes illustrates their roles in herbicide detoxification (Figure 1.1). A brief history review highlights important breakthroughs in GST research (Dixon & Edwards 2010). In 1970, GSTs were discovered to play a key role in herbicide selectivity by rapidly metabolizing atrazine in maize. This finding ushered in a great research interest in their role in herbicide detoxification. In the late 1980s and early 1990s, the role GSTs play in plant stress response from pathogens, oxidative stress, and heavy metal toxicity was studied extensively (Marrs 1996). Thus began a new era of classifying GST functions. In

1996, the first plant GST tertiary structure was elucidated in *Arabidopsis thaliana,* which shares about 60% protein sequence homology with its maize counterpart (Reinemer *et al.* 1996). This served as a model structure to conduct more studies on herbicide selectivity and resistance. In 2000, the release of the Arabidopsis genome (through advanced genomic tools) enabled deeper analysis of the GST superfamily. While there has been a plethora of research on crop GSTs, very little was known about GSTs' role in weeds. In 2019, the International Weed Genomics Consortium (IWGC) was formed to study the genomes of important weeds and create a collaborative place to study weed biology and management (https://www.weedgenomics.org/about/) (Montgomery *et al.* 2023). Access to weed genomes will allow detailed studies of weed GSTs which could lead to new weed management techniques.

The discovery of herbicides has revolutionized crop production around the world. Most conventional herbicides used for crop production (i.e., herbicides not requiring resistance traits in transgenic, gene-edited, or mutagenesis-derived crops) are selective herbicides that kill weeds without injury to the crops. Selective herbicides have greatly benefited growers for many years (Oerke 2006). In most cases, natural herbicide selectivity is based on differential rates of metabolic detoxification between the crops and the weeds and GSTs play an important role in contributing to the selectivity of herbicides.

GSTs are important because of their potential contribution to agronomic value. Understanding GST's role in xenobiotic detoxification in plants can be used to enhance herbicide selectivity on crops and/or inhibit GST activity to prevent weeds from metabolizing herbicides.

1.2 Plant GSTs Classification and Structure

Plant GSTs can be classified into eight distinct types (Table 1). The soluble dimeric classes, phi (F) and tau (U) are the most numerous in plants and are, incidentally, the most important GSTs involved in herbicide metabolism. The phi class detoxifies herbicides in corn and sorghum while the tau class is involved in detoxification of herbicides in wheat, soybeans, and rice (Edwards *et al.* 2005).

As a group, GSTs are globular soluble proteins with two main domains. The glutathione binding domain is relatively conserved across GSTs, whereas the xenobiotic binding domain is less conserved between members of the GST superfamily (shown in green and red in Figure 1.2A, respectively). Two

subgroups of cytosolic GSTs differ in how they interact with glutathione. The Y-GST group uses a tyrosine residue to activate glutathione, whereas the S/C-GST uses either serine or cysteine residues. The maize GST involved in atrazine metabolism, for example, is a S/C-GST-type enzyme since it relies on serine to activate glutathione (Figure 1.2B) (Prade *et al.* 1998).

The ability of GSTs to detoxify xenobiotics (e.g., herbicides) requires an abundant supply of reduced glutathione (GSH). Typically, GSTs attack specific carbon, sulfur, or nitrogen atoms of the molecules with which they are reacting. To do so, GSTs first bind both the herbicide (or other substrate) within a hydrophobic site and GSH at the adjacent more hydrophilic site (G-site). When the reactants are in close proximity, the thiol group of GSH is activated (shown as yellow bond in Figure 1.2B), which enables the conjugation of GSH to the other substrate.

1.3 A Herbicide Detoxification Primer

There are three phases of herbicide metabolism in the plant cells (Figure 1.3). When a herbicide enters the cell, detoxification can start in phase 1 or phase 2. Phase 1 metabolism takes place in the endoplasmic reticulum (ER) of the cell and is most often mediated by cytochrome P450 monooxygenases. Phase 2 metabolism occurs in the cytoplasm. Here the herbicide can be detoxified via conjugation by a variety of enzyme classes, including glutathione *S*-transferases (GST), glycosyl transferases (GT), amino acid transferases (AT), or malonyl transferases (MT) (Rigon *et al.* 2020). The herbicide metabolites are then sequestered in the vacuole via ATP-binding cassette (ABC) transporters or incorporated into the cell wall.

1.4 How GSTs Work

As mentioned above, GSTs detoxify a wide variety of xenobiotics across chemical classes, producing non-toxic conjugated metabolites (part of phase 2 metabolism). With respect to herbicides, GSTs can detoxify herbicides from many chemical classes inhibiting different target sites. GSTs can react with halogens such as chlorine (replacing it with glutathione), as exemplified by atrazine, a group 5 triazine herbicide targeting photosystem 2, chlorimuron-ethyl, a group 2 sulfonylurea herbicide targeting acetolactate synthase, and alachlor, a group 15 chloroaceanilide herbicide targeting very long-chain fatty acid elongases (Figure 1.4). Even though flufenacet is also a group 15 herbicide that possesses a halogen

group, GSTs react with this type of herbicide differently, breaking the molecule in two pieces, leaving a hydroxy metabolite (red OH) and a conjugated metabolite (Figure 1.4). GSTs can also detoxify the diphenyl ether herbicides fluorodifen, a protoporphyrinogen oxidase inhibitor (group 14), and fenoxaprop, an acetyl-CoA carboxylase inhibitor (group 1). The action of GST breaks these herbicides in two at the oxygen bridge, releasing a hydroxy metabolite and a conjugated metabolite (Figure 1.4).

1.5 Evolution of Herbicide Resistance

Repeated use of the same herbicide has led to the evolution of resistance in weeds (Rigon *et al.* 2020). This is the inherited ability of the weed population to survive exposure to a herbicide that previously would have killed it. Broadly speaking, resistance can result from mutations in the target site (TSR) of a herbicide, or via other non-target-site (NTSR) mechanisms that alter the way plants detoxify or compartmentalize the herbicides (Gaines *et al.* 2020). Regardless of the mechanisms of resistance involved, these herbicides have lost their selectivity. The struggle between selectivity and resistance is a difficult balancing act that must be maintained to prolong the usefulness of chemical weed management tools for our farmers.

1.6 Harnessing GSTs to Protect Crops from Herbicides

The GST conjugation system for herbicide detoxification has received a great deal of interest due to its potential application to enhance herbicide selectivity in crops (Cummins *et al.* 2011). Research on GSTs in maize flourished in the 1980s due to their contribution to herbicide selectivity and the economic impact of these herbicides on the world economy. In corn, the phi class of GST is predominantly responsible for herbicide selectivity (Table 1). Two GST isoenzymes, GST I (constitutively present in the plant) and GST II (induced by a safener) detoxify the herbicide alachlor in etiolated corn tissue (Mozer *et al.* 1983) but the number of GSTs expressed in safener-treated plants was two to three-fold greater than in untreated plants. Furthermore, GST levels can differ by genotype with herbicide-tolerant species having increased GST activity when exposed to herbicides (Sari-Gorla *et al.* 1993), confirming their important role in herbicide detoxification. As an enzyme superfamily, plants have many different GSTs, and the number of constitutive GSTs characterized in selected crop and weed species can vary greatly (Figure 1.5).

A study investigating differential herbicide tolerance in two different maize cultivars revealed that the rate of metolachlor detoxification was faster in cultivars with higher levels of GST activity (Li *et al.* 2017). Another important crop for GST research is wheat. This system differs from corn because wheat is hexaploid and the main GSTs involved in herbicide detoxification belong to the tau class. Edwards and Cole found that safeners, i.e. fenchlorazole-ethyl and dicholormid, induced the expression of some GSTs in wheat, thereby increasing the overall level of GST activity in these plants (Edwards & Cole 1996). Researchers characterized the first tau GST, *Ta*GSTU4-4, that metabolized fenoxaprop in safener-treated hexaploid wheat (Thom *et al.* 2002). A study in 2021 identified and characterized GST genes in three important wheat species (*Triticum aestivum*, *Triticum durum*, *Triticum urartu*) and a related grass species (*Aegilops tauschii*) (Hao *et al.* 2021). Wheat is an ideal model for polyploid research, and this study is a steppingstone to further research identifying GST markers to breed new wheat varieties with enhanced herbicide metabolism.

1.7 Repressing GSTs to Lower Herbicide Resistance in Weeds

NTSR is one of the greatest threats to weed management in cropping systems (Rigon *et al.* 2020). It can lead to multiple herbicide resistance (MHR) since the weeds have a central defense against multiple herbicides targeting any number of target sites in the plant (Cummins *et al.* 2013). However, GST inhibitors have been used in research studies to restore herbicidal activity, thus making the weeds susceptible to the herbicide, suggesting that GSTs can be involved in resistance to herbicides.

Alopecurus myosuroides (blackgrass) is a major weed in cereal crops in Europe that decreases crop yield and quality. In 1982, blackgrass biotypes evolved MHR (Cummins *et al.* 2013), thus causing a loss of chemical control on these crops. However, resistance could be reversed by spraying blackgrass with 4-chloro-7-nitrobenzoxadiazole (NBD-CL), a GST inhibitor, 48 h before spraying the herbicide chlorotoluron. Another study (Dücker *et al.* 2020) demonstrated that flufenacet metabolic degradation was reduced in four populations of blackgrass treated with the GST inhibitors tridiphane and ethacrynic acid.

A two-pronged approach has been used to test how GST activity could be mitigated on blackgrass – chemical and structural biology (Schwarz *et al.* 2021). They confirmed that NBD-Cl reversed MHR by

reducing GST activity. They subsequently discovered that natural flavonoid-based inhibitors could do the same. Their study revealed a connection between flavonoid binding and stress tolerance. This exciting discovery needs to be explored more to understand how this could be used in agriculture.

Polypogon fugax (Asia minor bluegrass) is a winter annual weed in Asian countries. It is an annual grass with a life cycle similar to wheat and other crops that greatly impacts cereal crop quality and yield. A dose-response study (Chen *et al.* 2020) on Asia minor bluegrass mirrored the results of the studies on blackgrass. They confirmed that resistance to the herbicide quizalofop-p-ethyl could be reversed by spraying this weed with the GST inhibitor NBD-Cl.

CONCLUSIONS

One of the most important functions of GSTs is their ability to detoxify herbicides through glutathione conjugation. As such, the relationship between GSTs and herbicides is a double-edged sword. On one hand, they are the basis of herbicide selectivity by rapidly metabolizing herbicides in crops, either through constitutive or induced GST expression. On the other hand, GSTs can be hijacked to impart herbicide resistance to weeds. Either way, recent advances in plant genome sequencing will provide new tools to identify specific GST markers that can be utilized in breeding programs to enhance herbicide selectivity in crops or can lead to specific GSTs involved in herbicide resistance in weeds. A better understanding of GSTs in plant systems will help design new tools for weed management that will benefit farmers.

TABLES

Table 1.1: Overview of classes of plant GSTs and their main functions (Dixon & Edwards 2010).

CLASSES	STRUCTURE	FUNCTION
Phi	dimer	herbicide detoxifying activity
Tau	dimer	herbicide detoxifying activity
Theta	dimer	glutathione-dependent peroxidase activity
Zeta	dimer	dehalogenation and isomerization activity
Lambda	monomer	oxidoreductase activity, not involved in conjugation reaction
DHAR[a]	monomer	oxidoreductase activity, not involved in conjugation reaction
TCHQD[b]	prokaryotic-like	unknown in higher plants
MAPEG[c]	trimer	unknown in higher plants

[a] Dehydroascorbate reductase
[b] Tetrachlorohydroquinone dehalogenase
[c] Membrane-associated proteins in eicosanoid and glutathione metabolism

FIGURES

Figure 1.1: Word cloud generated from 907 articles queried on the Web of Science for any article with herbicide and glutathione *S*-transferase in the title or abstract (figure created with www.wordart.com).

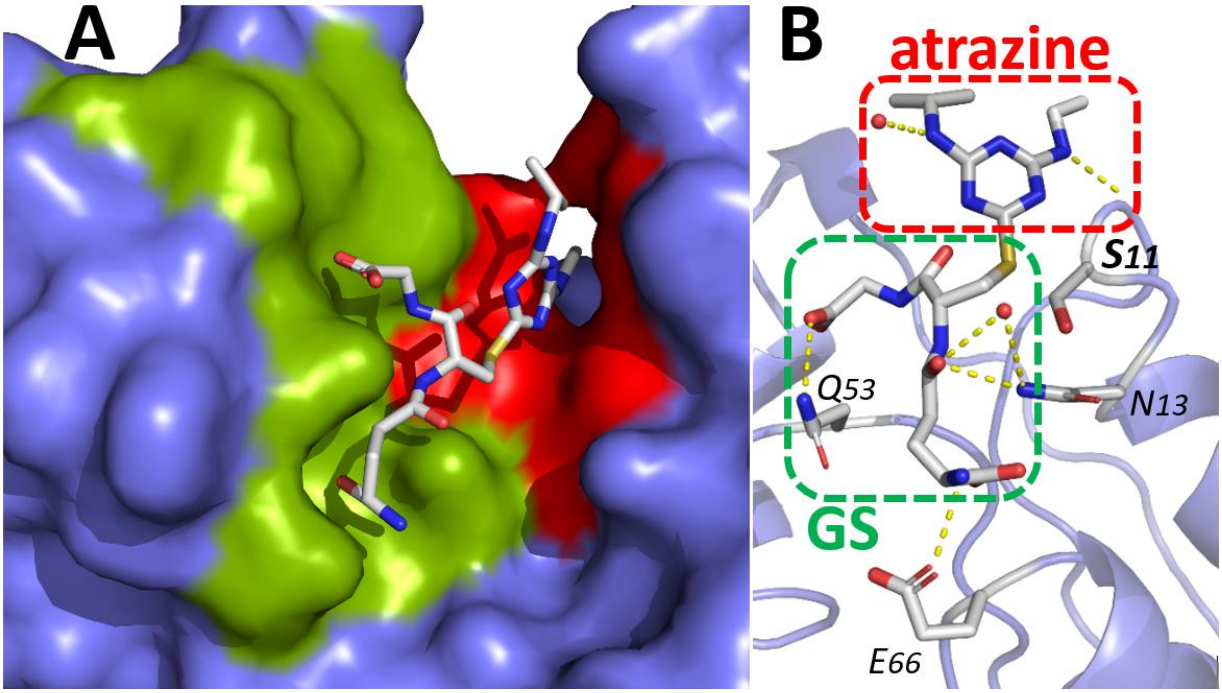

Figure 1.2: A) Maize glutathione S-transferase with atrazine-glutathione conjugate bound across the glutathione binding site (green surface) and xenobiotic binding site (red surface) from Prade et al. (1998). B) Close-up of the interactions between atrazine-glutathione conjugate and GST. Top red dotted oval identifies atrazine and bottom green dotted oval glutathione components of the conjugate, respectively. Q53 (glutamine53), N13 (asparagine13), and E66 (glutamate66) are amino acids involved in electrostatic interactions between the enzyme and the conjugate. The small red spheres are water molecules involved in the binding of the conjugate in the GST catalytic domain. The bold S11 (serine11) identifies the key amino acid involved in activating glutathione, making this a S/C-GST-type enzyme.

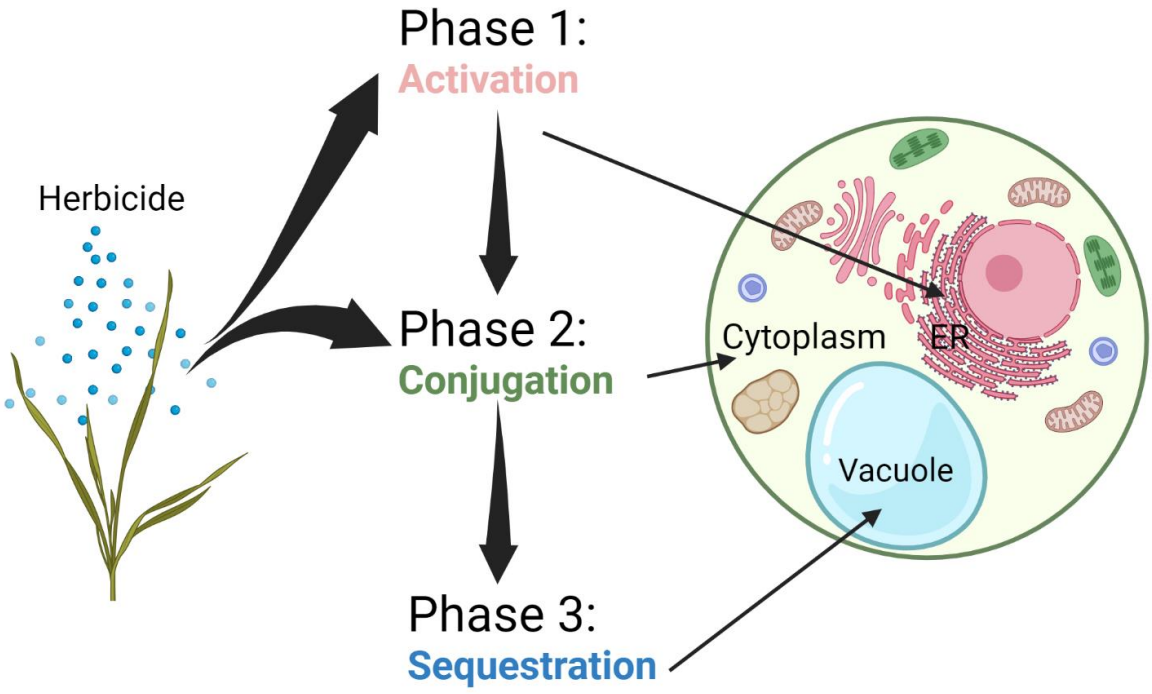

Figure 1.3. Phases of herbicide metabolism – function and location. ER=endoplasmic reticulum. (Figure created with BioRender).

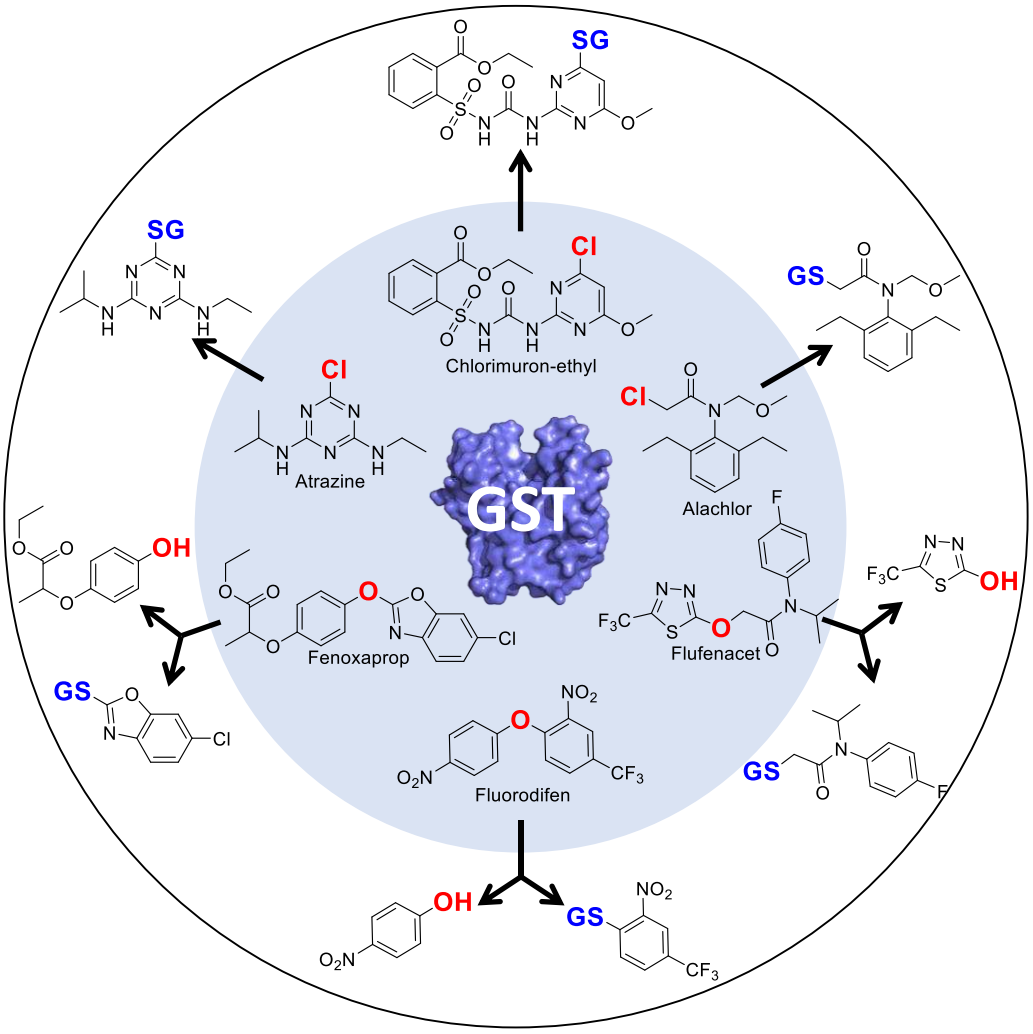

Figure 1.4. Examples of herbicide chemical classes detoxified by plant GSTs. Structures of herbicides are shown in the blue circle. The atom targeted by the GSTs are shown in bold red letters (Cl = chlorine and O = oxygen). The structures in the outside circle represent the metabolites resulting from GST activity. The position of the glutathione is shown as GS in bold blue.

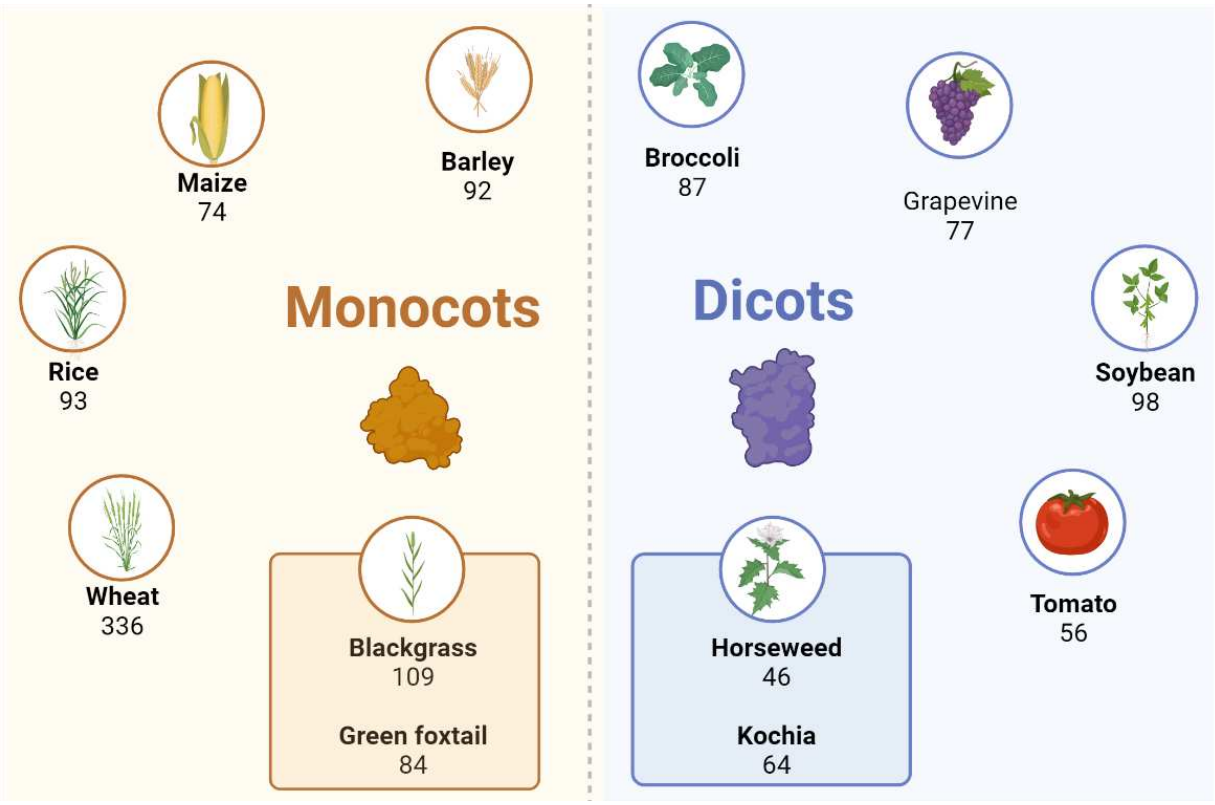

Figure 1.5. Numbers of plant GSTs in selected crops (Cunningham *et al.* 2021) and weeds [International Weed Genomics Consortium (https://www.weedgenomics.org/)]: the genomes were provided by the following papers: blackgrass (Kersten *et al.* 2023), green foxtail (Thielen *et al.* 2020), horseweed (Laforest *et al.* 2020), and kochia (Hall *et al.* 2023) (figure created with BioRender).

CHAPTER 2: ROLE OF METABOLISM IN SENSITIVITY TO QUIZALOFOP-P-ETHYL IN COAXIUM WHEAT

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important staple crops in the world providing carbohydrates, protein, vitamin B, energy, and dietary fibers for the human diet. In addition, wheat can be used for animal food, soaps, adhesives, and for many other purposes. Growing wheat requires less water than other staple crops and can be cultivated in a range of climates (Shewry & Hey 2015). Weed competition has the greatest negative impact on wheat grain yield, relative to other pests.

In 2008, farmers in Colorado were looking for a new winter wheat variety that would provide them with better control over tough annual winter grasses, such as, feral rye (*Secale cereale* L.), jointed goat grass (*Aegilops cylindrica* Host), and downy brome (*Bromus tectorum* L.). They were also looking for an alternative mode of action herbicide (the available Clearfield wheat system employs a group 2 herbicide, imazamox) to help delay herbicide resistance in these grass weeds that plague winter wheat. Through a partnership with CSU and Colorado Wheat Research Foundation (CWRF), a non-GMO AXigen™ trait was developed that provided tolerance to a specially formulated post-emergence Group 1 grass herbicide (quizalofop), Aggressor™ (https://www.coaxiumwps.com/about-us/). This new CoAXium™ wheat production system has better control over competing winter annual grass weeds while providing crop protection for the wheat from the herbicide.

In 2018, the CoAXium™ wheat production system was released to the public. Currently this wheat is planted on 1,900,000 acres in 13 different states. On average, about 70% of these acres are sprayed with Aggressor™ herbicide, depending on conditions such as drought, weed pressure, and winter kill.

To prolong the benefits of the CoAXium™ wheat production system and prevent weeds from developing resistance, all growers are required to sign a stewardship agreement. The growers are required to buy new certified seed from authorized dealers each year. They need to follow the Aggressor™ label

for rates and application timing. Best management practices must be implemented, such as crop rotation guidelines (CoAXium™ wheat should only be planted in 2 of 6 years), start with a clean seed bed, scout fields for weeds before and after application, control any weed escapes, and manage weeds on borders and during fallow seasons.

Farmers reported some unexpected injuries on their CoAXium™ wheat crops in certain environmental conditions in 2021. Looking at the effect of QPE on CoAXium™ winter wheat from Raven Bough's PhD research (figure 2.1 B) (Bough 2021), we notice that the two CoAXium™ seed lines show different levels of control. Since we know that all CoAXium™ lines have the AXigen™ trait which should protect them from QPE injury, we hypothesize that something must be going on in the metabolism. This project seeks to understand the contribution of metabolism in overall resistance to QPE in CoAXium™ winter wheat. Our research questions are: 1) How does metabolism affect overall tolerance to QPE in CoAXium™ winter wheat and 2) Which enzymes contribute to QPE metabolism in wheat.

Plants metabolize xenobiotics (e.g., herbicides) in three phases: Phase 1) activation of the herbicide occurs, most often involving cytochrome P450 monooxygenases, Phase 2) conjugation of herbicide (or its metabolites) to a hydrophilic building block (e.g., sugar, amino acid, glutathione) and Phase 3) sequestration and degradation (moves the conjugated metabolites to the vacuole or incorporates in the cell wall) (Rigon *et al.* 2020). First, we want to understand which phase is most involved in QPE metabolism. This can then lead us to discover which metabolites may be responsible for herbicide metabolism. Armed with this new information CSU breeders can develop improved CoAXium™ wheat varieties that have more tolerance to QPE.

MATERIALS AND METHODS

2.1 Plant Material and Growth

All experiments were conducted in the CSU Weed Lab greenhouse in Fort Collins, Colorado. This controlled environment maintained daytime temperatures of 20 – 25°C (shade cloths, fans and vents

activate as needed to maintain the selected temperature range), relative humidity about 75%, and 14-hour day length (sodium halide lamps turn on to supplement natural light when needed). Wheat plants were planted with four seeds in potting soil in pots (350 mL capacity). A tablespoon of Osmocote Plus (15-9-12) fertilizer was added to the pots at the time of planting for slow, continuous release. Four replicates were planted for each treatment. Pots were placed in trays and bottom watered until germination. The trays were randomly arranged on benches where they are manually watered as needed after germination.

2.2 Herbicide Application

Once the wheat plants reach the 3-4 leaf stage, following any pretreatments, they were sprayed with quizalofop-P-ethyl (QPE) at various doses with a single pass about 40 cm above the tops of the plants with a research table sprayer (model Gen IV, Devries Manufacturing, Hollandale, MN).

2.3 Biomass Experiments on Whole Plant

2.3.1 Safener Experiment

The effect of the safener, cloquintocet, was evaluated on two CoAXium™ winter wheat varieties, LCS Fusion AX (Limagrain Cereals, Inc.) and Incline AX (PlainsGold, Inc.) in controlled greenhouse conditions following planting and herbicide application protocols as described above. A dose-response experiment was conducted with QPE rates of 0, 24, 48, 96, 193, 385, and 771 g ae ha^{-1} with and without cloquintocet (Bough 2021). The cloquintocet rate was 8.0 g ae ha^{-1} in the safener-treated plants. All herbicide mixtures included 0.25% v/v of the adjuvant NIS. Three weeks after treatment, leaf tissue was cut above the meristematic region (approximately 5 cm above the soil surface) and discarded. At 5 ½ weeks after treatment, the regrowth was harvested, weighed, and dried in an oven at 65°C.

2.3.2 Metabolic Inhibitors Experiments

The effect of two cytochrome P450 monooxygenase inhibitors, malathion and phorate was examined on one wheat line, Fusion AX. Wheat seeds were planted in 64 pots using the planting procedures above. At the 3-4 leaf stage, 32 of the pots were sprayed with a dose of malathion (2000 g ae ha^{-1}). Twenty-four hours later all 64 pots were sprayed with a dose-response of QPE rates of 0, 8, 16, 24, 32, 40, 56, and 80 g ae ha^{-1}. Fresh weight was collected, weighed, and put in the oven at 65°C to dry 21

DAT. We repeated this experiment with a lower dose of malathion (1000 g ae ha^{-1}). We also conducted a dose-response experiment on Fusion AX winter wheat with a pretreatment of 1 g per pot of phorate at the 3-4 leaf stage followed by application of QPE rates of 0, 24.1, 48.2, 96.3, 193, 385, and 771 g ae ha^{-1} 48 h later. Fresh weight was harvested 21 DAT, weighed, and put in the oven at 65°C to dry.

The phase 2 metabolism effect of the GST inhibitor 4-chloro-7-nitrobenzoxadiazole (NBD) was evaluated on Incline AX wheat. The seeds were planted following the planting protocols described above. A pretreatment of NBD (216 mg dissolved in 15 mL DMSO and added to 135 mL water + 0.25% NIS) was sprayed on half of the plants at 270 g ae ha^{-1} at 187 L ha^{-1} at the 3-4 leaf stage. QPE + 0.25% NIS dose-response rates of 0, 24.1, 48.2, 96.3, 193, 385, and 771 g ae ha^{-1} was sprayed on wheat seedlings 48 h later. NBD was sprayed again on the plants with the NBD treatment at 4 and 12 DAT. At 21 DAT, fresh weight was harvested, weighed, and placed in an oven at 65°C for drying. Dry weights were measured after 3 d in the oven.

2.4 Herbicide Extraction Protocol

Quizalofop acid (QZA) was extracted following a modified QuEChERS crude pesticide extraction method (Bough *et al.* 2022). The plant tissue was homogenized in water, releasing plant tissue cell contents. Pesticides are somewhat purified from cell contents by partitioning into acetonitrile. The salt precipitates the proteins and nucleic acids. Quizalofop or other acidic pesticides were collected in the top organic layer for LC-MSMS analysis after centrifugation.

Plant tissue was rinsed with 20% acetone v/v in water by shaking in a jar for 1 min, then patted dry and stored at -20°C until ready to process. Frozen samples were ground to a powder with mortar and pestle with liquid nitrogen. Approximately 2.5 g of plant tissue was placed in a new tube with 10.0 mL distilled water and 10.0 mL acetonitrile + 0.1% formic acid and homogenized with a large homogenizer probe until it looked like a green smoothie (30 – 60 seconds). Next, the tubes were placed in a shaker for 20 min at room temperature and 250 rpm, followed by 10 min in a Sorvall Legend X1R centrifuge with a TX400 rotor at 3800 g. The supernatant was placed in a clean tube with one QuEChERS salt packet and immediately vigorously shaken to avoid clumping. Samples were vortexed for about 15 sec to mix well

and centrifuged again for 10 min at 3800 g. Finally, 1.5 mL of each supernatant was filtered through a 0.2 µM PTFE filter into an LC-MS vial, sealed with a cap, and stored at -20°C until ready to put in the LC-MS machine for analysis.

2.5 LC-MS/MS Protocol

The UPLC instrument consisted of a Nexera X2 UPLC with two LC-30 AD pumps, a SiL-30 ACMP autosampler, a DGU-20A5 Prominence Degasser, a CTO-30A column oven, and an 8040 triple quadrupole detector (Shimadzu, Kyoto, Japan). A 2.6 µm 100 × 4.6 mm reversed-phase C18 column packed with a pore size of 100 Å (Phenomenex Kinetex) was heated to 40°C for binary gradient separation. Solvent A was distilled water with 0.1% v/v formic acid and solvent B was acetonitrile with 0.1% v/v formic acid. A controller maintained the flow rate at 0.400 mL min^{-1}. Samples were heated to 400°C and ionized using positive electrospray ionization with a He gas nebulizing flow of 24 mL min^{-1}. Desolvation was carried out at 250 °C with a He gas drying flow of 24 mL min^{-1}. The triple quadrupole filtered non-target ions using an MRM mode with a Q1 pre-bias of -24.0 V, a Q2 pre-bias of -21.0 V, and a Q3 pre-bias of -21.0 V. Targeted parent ions had a m/z of 345.00 while daughter ions were selected at 299.00 m/z. The average retention times were 5.8 and 6.9 min for QZA and QPE, respectively (Supplemental Figure 1). Samples were injected in 10 µL volumes and both QPE and QZA were quantified using a pre-established calibration curve (Supplemental Figure 1).

2.6 Quizalofop Metabolism Experiments

Two metabolism experiments were conducted, one to analyze the effect of safener on QZE metabolism and the second to analyze the effect of repeated doses of NBD on QZE metabolism.

For the safener experiment, two winter wheat lines, LCS Fusion AX and Incline AX were planted using the planting protocol described above. At the 3-4 leaf stage, QPE (193 g ae ha^{-1}) + 0.25% NIS was sprayed on all plants. Plant tissue was collected at 4, 8, and 18 DAT, rinsed and placed in -80°C until

ready for processing. Once samples from all days had been collected, the herbicide extraction protocol described above was followed. Then, analysis of the amount of the QPE active ingredient, quizalofop acid (QZA) was conducted using the LC-MS protocol described above.

Incline AX wheat seeds were planted following the planting protocols described above. This time plants were culled from four to two in each pot. At the 3-4 leaf stage, NBD + 0.25% NIS was sprayed on half of the plants. After 48 h, QPE rate of 193 g ae ha^{-1} + 0.25% NIS was sprayed on all plants. Plant tissue was collected at 2, 4, 12, and 16 DAT, rinsed and placed in -80°C until ready for processing. Once samples from all days had been collected, the samples were processed and analyzed as described above.

2.7 Statistical Analysis

All statistical analysis was conducted with packages in RStudio v.2023.06.2 Build 561 with R v.4.3.1. For each metabolism trial, a linear calibration curve of peak means weighted by standard deviations vs. standard concentration was fit using the stats function of package lm. Sample peak areas were converted to quizalofop-P-ethyl and quizalofop-p using linear calibration curves and were normalized by sample fresh mass before statistical analysis.

For each trial, a three-way ANOVA ($\alpha = 0.05$) was used to evaluate timepoint, temperature regimen, variety or species, and all interactions as significant predictors for herbicide content prior to identification of specific mean differences. Maximum timepoint least square mean estimates of content between treatment per variety as well as content between varieties per temperature regimen were contrasted using F-protected Student's t-tests ($\alpha = 0.05$). Further, treatment least square mean estimates at each timepoint were contrasted using F-protected Student's t-tests ($\alpha = 0.05$). To determine presence of metabolism within each treatment, timepoint least square mean estimates were compared using Student's t-tests with Tukey's HSD multiple comparison adjustments ($\alpha = 0.05$).

Using dry weight data from the biomass experiments, a two-way ANOVA ($\alpha = 0.05$) was used to evaluate treatment and variety, and their interaction as significant predictors for biomass. When ANOVA

had a significant F-test, biomass values were further contrasted between treatment per variety using F-protected Student's t-tests ($\alpha = 0.05$). Pairwise comparisons were not conducted if the ANOVA F-test was not significant.

To model the effect of herbicide on plant growth or metabolism, fresh weight, dry weight or herbicide amount were used and analyzed with a three-parameter log-logistic regression LL.3 [drc] (Ritz & Streibig 2005) function:

$$y = \frac{d}{1+\exp\left(m(\log(x)-\log(e)\right)} \qquad (1)$$

where lower limit is fixed at 0, upper limit is d, m is the slope, and e is the 50% point in the curve.

RESULTS AND DISCUSSION

3.1 Safener Experiments

The comparison of CoAXium™ winter wheat plants treated with and without cloquintocet (safener) on whole plant biomass highlights the increased tolerance of the safener-treated plants to the herbicide QPE. At the highest dose of QPE, in both Fusion AX and Incline AX, the plants without cloquintocet were killed, while those treated with cloquintocet had almost the same dry-weight biomass as the control plants (Figure 2.2).

The metabolism experiment further proves the safening effect on wheat plants treated with cloquintocet. At 4 DAT, the safened plants have 53% less QZA (active ingredient of QPE) in the Fusion AX plants and 30% less QZA in the Incline AX plants (Figure 2.3). Comparison of $T_{1/2}$ (time for 50% metabolism of the herbicide) with and without cloquintocet (Table 2.2) show that it took 2.9 days less (InclineAX) and 4.1 days less (FusionAX) to metabolize QPE when the safener was added. These results demonstrate that the herbicide metabolism has increased in the plants treated with cloquintocet, thus protecting the plants from the herbicide.

Our research coincides with previous literature findings showing that plant tolerance to xenobiotics can be manipulated with safeners (Mozer *et al.* 1983) (Sari-Gorla *et al.* 1993). This use of safeners provides a great experimental tool to analyze the plant metabolism of herbicides (Edwards *et al.* 2005).

3.2 Phase 1 P450 Experiments

Previous literature had shown inhibition of cytochrome P450 monooxygenases with pretreatments of malathion (Ma *et al.* 2013) in corn and phorate (Busi *et al.* 2017) in *Lolium rigidum*. Dose-response experiments conducted on CoAXium™ winter wheat plants pretreated with phorate (Figure 2.4) and malathion (Supplemental Figure 2) did not show a change in tolerance to the herbicide QPE. A comparison of visual injury and dry-weight amounts showed no difference in the plants with or without the inhibitor pretreatments. GR_{50} (effective dose that reduces growth by 50%) results (Table 2.1 Phase 1) confirm that there is no difference between the control and plants treated with the phorate. Quizalofop-P-ethyl did not show a synergistic effect with the phorate that has occurred with other herbicides (Busi *et al.* 2017).

3.3 Phase 2 GST Inhibitor Experiments

The wheat plants with the repeated application of the GST inhibitor, NBD, showed more injury at all doses, as evidenced by the lower biomass fresh weight numbers (Figure 2.5A) of the NBD-treated plants. Further analysis from the LC-MS/MS looking at metabolism results revealed that plant tissue from all NBD-treated wheat had higher levels of QZA (Figure 2.5B) making the plants more sensitive to QPE. Statistical analysis confirms the difference between plants treated with repeated does of NBD. The GR_{50} results (Table 2.1 Phase 2) show the effective dose needed to reduce growth by 50% in the NBD-treated plants is almost half that of the control. In Table 2.2, the $T_{1/2}$ shows that the NBD-treated plants take 4.5 days longer to metabolize the herbicide active ingredient (QZA).

These results mirror previous GST inhibition studies conducted on *Alopercurus myosuroides* (Dücker *et al.* 2020) and *Polypogon fugax* (Chen *et al.* 2020). Phase 2 plant cell herbicide metabolism can be altered with GST inhibitors, thus affecting the overall tolerance of CoAXium™ winter wheat to QPE.

3.4 Conclusions

Manipulating GST numbers in phase two plant cell metabolism can affect herbicide tolerance levels in plants. Safeners can increase GST numbers to enhance herbicide metabolism resulting in an increase in CoAXium™ wheat tolerance to QPE. GST inhibitors, conversely, can decrease herbicide metabolism

causing CoAXium™ wheat to be more sensitive to QPE. Understanding the contribution of metabolism in overall resistance to herbicides can lead to breeding improvements for more herbicide-tolerant wheat varieties and new methods for controlling weeds in wheat. The next steps to build on this research are: 1) Conduct GSH and GST assays to analyze the differences in the amounts of GSTs between different CoAXium™ wheat varieties, 2) Identify the herbicide metabolite, 3) Identify specific genes and markers with RNAseq, and 4) Validate the activity of the target enzyme.

TABLES

Table 2.1: Effect of metabolic inhibitors on efficacy of QPE on CoAXium™ wheat InclineAX

Metabolism Phase	QPE		QPE+inhibitor	
	GR_{50} (g ae ha^{-1})[a]			
Phase 1[b]	377.3±105.6	A	363.0±87.6	A
Phase 2[c]	340.0±41.8	A	177.80±22.6	B

[a] Numbers in rows followed by the same letter are not different at $P < 0.05$ according to Duncan's multiple range test.
[b]Cytochrome P450 monoxygenases (Phorate)
[c]Conjugation – GSTs (NBD-Cl)

Table 2.2: Effect of the safener cloquintocet and the metabolic inhibitor NBD-Cl on rate of metabolic degradation of quizalofop acid in CoAXium™ wheat

Wheat variety	$T_{1/2}$ (days)[a,b]			
	QPE		QPE+cloquinocet	
Incline AX	7.7±0.4	A	4.8±0.2	B
Fusion AX	7.2±0.3	A	3.1±0.8	B
	QPE		QPE+NBD-Cl	
Incline AX	8.5±0.9	A	13.0±1.4	B

[a] Time for 50% metabolism of the herbicide
[b] Numbers in columns followed by the same letter are not different at P <0.05 according to Duncan's multiple range test.

FIGURES

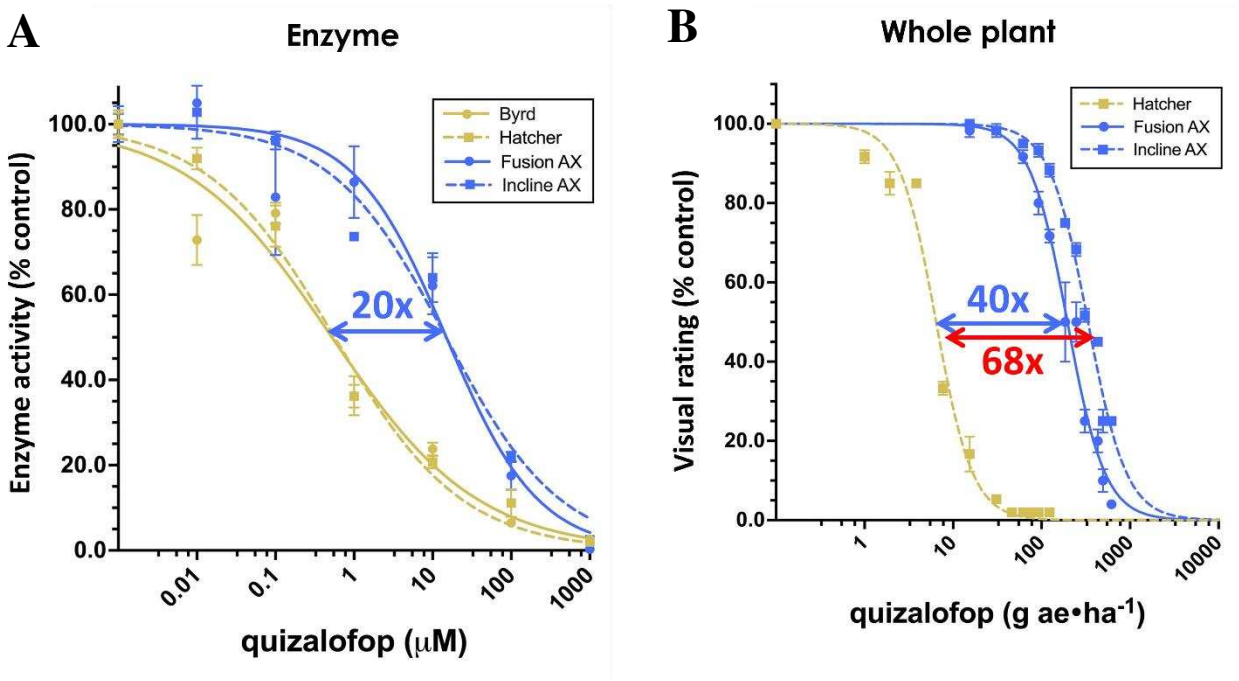

Figure 2.1: Effect of quizalofop-P-ethyl (QPE) on winter wheat, lines without the AXigen™ trait (Byrd and Hatcher) and lines with the AXigen™ trait (FusionAX and InclineAX).

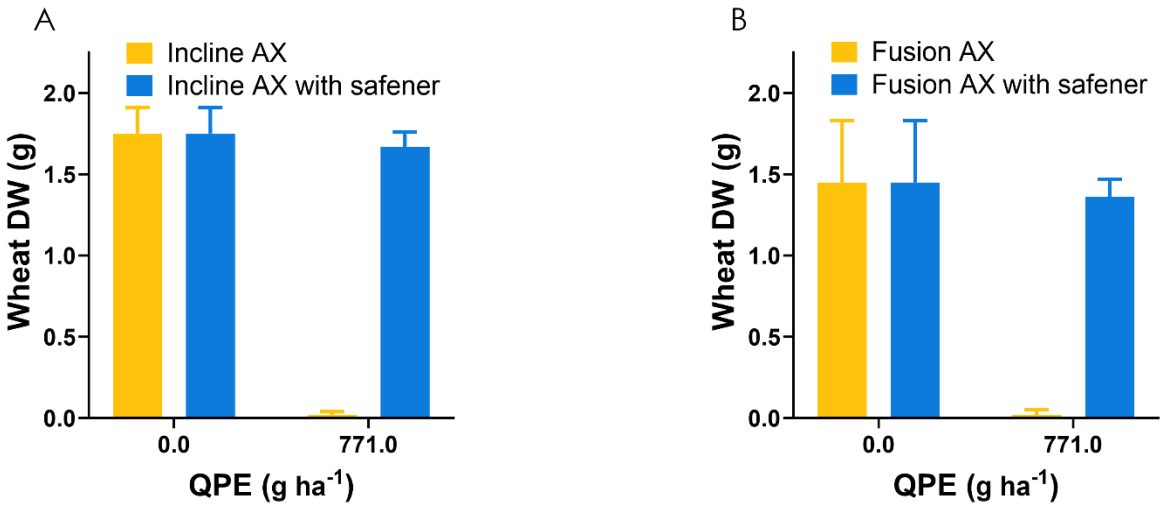

Figure 2.2: Effect of cloquintocet (safener) on whole plant wheat tolerance to quizalofop-p-ethyl (QPE). A) Incline AX and B) Fusion AX leaf regrowth dry weight (DW) in control plants and plants treated with the highest rate of QPE with and without safener.

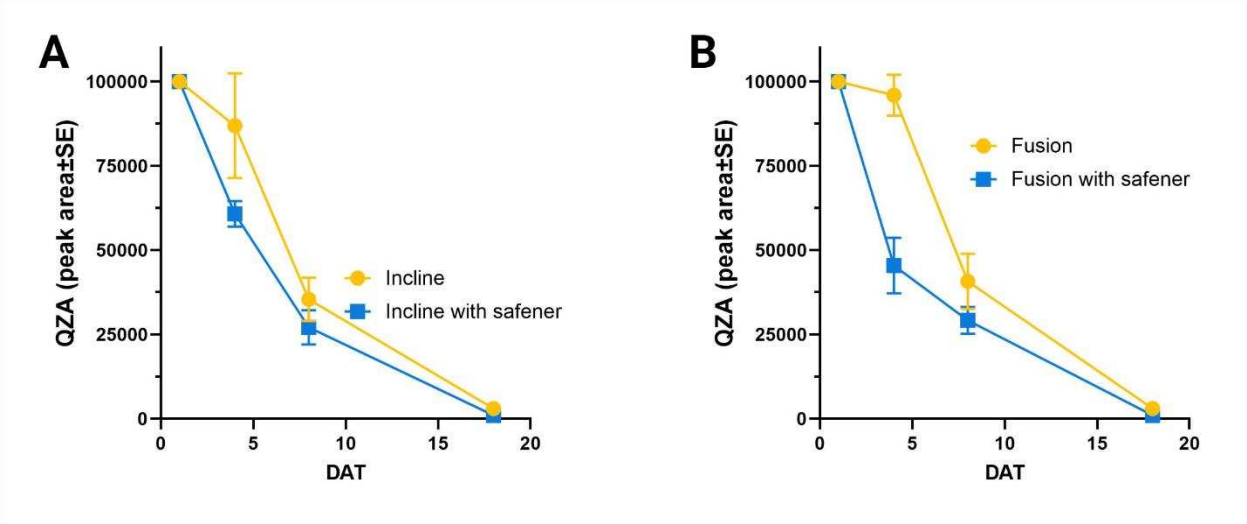

Figure 2.3: Effect of safener, cloquintocet, on herbicide metabolism of quizalofop-P-ethyl (QPE) in wheat plants. A) Incline AX and B) Fusion AX. The Y axis shows QPE active ingredient (QZA) amounts with and without safener. The X axis shows the number of days after treatment (DAT).

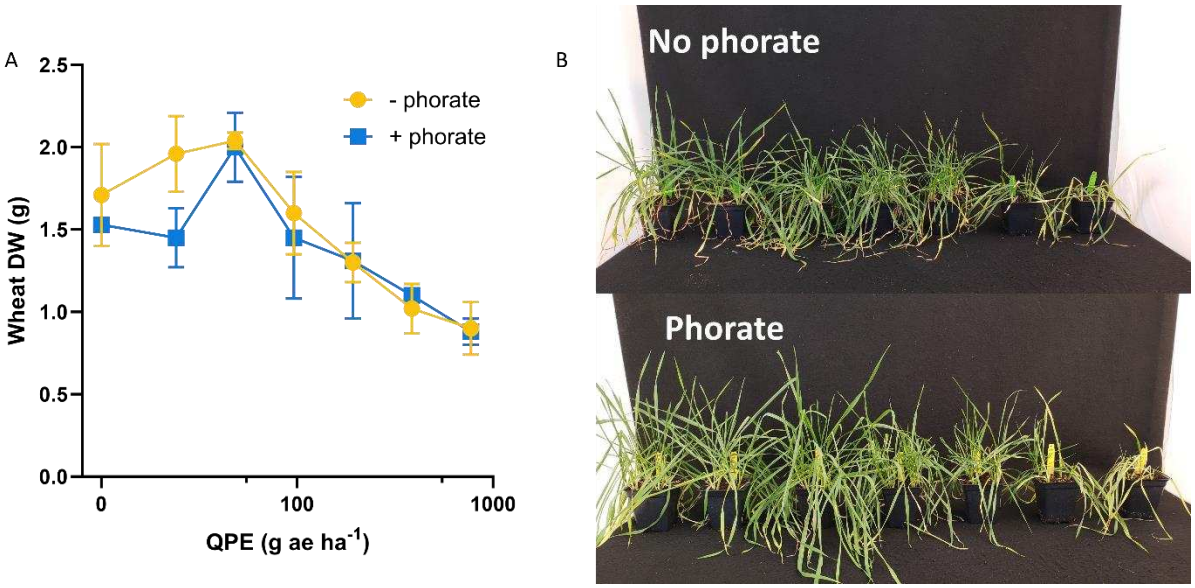

Figure 2.4: Effect of P450 inhibitor, phorate, on wheat's (InclineAX variety) sensitivity to quizalofop-P-ethyl (QPE). A) Wheat leaf dry weight (DW) in plants treated with increasing rates of QPE with or without phorate. B) visual injury of wheat treated with QPE with and without phorate at 21 DAT.

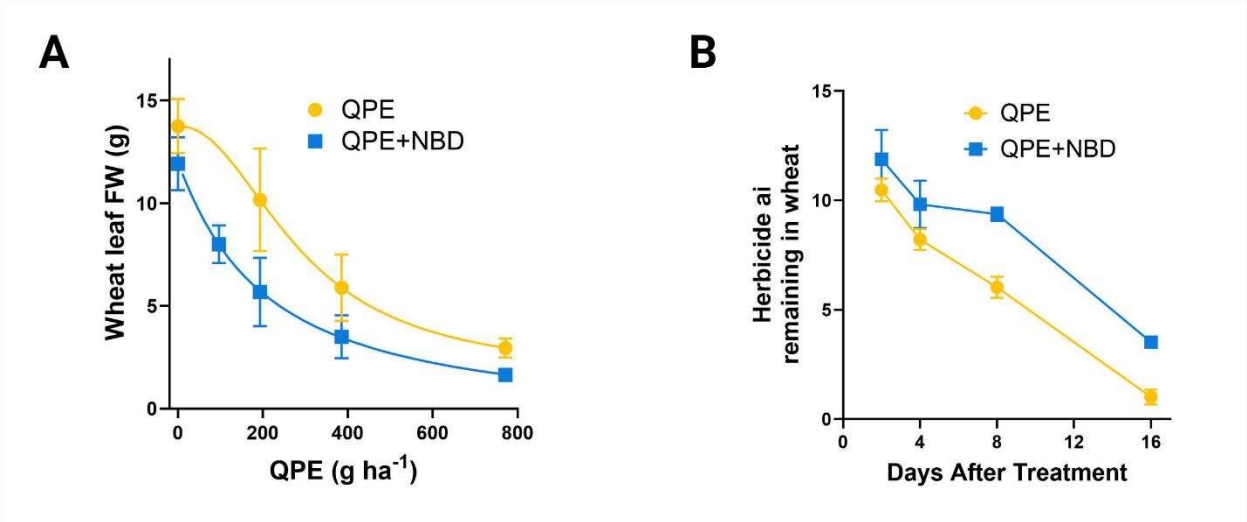

Figure 2.5: Effect of GST inhibitor, NBD, on wheat (InclineAX variety) tolerance to quizalofop-P-ethyl (QPE). A) Fresh weight (FW) of plants treated with increasing levels of QPE, with and without NBD. B) Amount of herbicide (QPE) active ingredient QZA in ug/g at 2, 4, 8, and 16 Days After Treatment.

CHAPTER 3: DEVELOPMENT OF A RAPID THROUGHPUT SCREEN BIOASSAY FOR QUIZALOFOP TOLERANCE TO EXPERIMENTAL COAXIUM™ WHEAT SEED LINES

INTRODUCTION

There is a continual need to develop and release new wheat varieties with improved yield, higher resistance to pests and diseases, and better performance in the current changing climatic conditions (Glenn *et al.* 2017). The process of creating new wheat varieties spans several years, starting from the initial crosses to developing new seed lines accessible to farmers.

Many steps are involved in the wheat breeding process (Rutkoski *et al.* 2022). First, breeders select parent plants that have desirable traits such as high yield, disease resistance, and good-quality grain. The selected parents are then crossed to create new hybrids (inbreeding). These new genetic varieties are grown and evaluated for their traits, and the best ones are selected to continue breeding. This process is repeated over several generations until a desired variety is created. The new variety is then tested in multiple seasons and geographies to ensure it has the desired traits and there are no unintended changes to the variety. Exceptional lines will be released to certified seed producers who will then sell to farmers for cultivation. The wheat breeding process involves extensive testing on thousands of plants and can take up to 12 or more years to complete (https://cropwatch.unl.edu/development-wheat-variety).

The Colorado State University (CSU) wheat breeding program in partnership with the Colorado wheat industry has been producing new varieties since 1963. Their goal is to develop and release new varieties that will thrive in Colorado's harsh winter conditions (https://agsci.colostate.edu/wheat/) and outcompete annual grass weeds. This involves years of testing in greenhouse conditions, at least three years of field trials, and end-use quality testing of the final product.

Weeds compete with crops for water, light, space, and nutrients. In winter wheat (*Triticum aestivum* L.) the weeds that have the highest impact on yield reduction are the winter annual grasses, primarily, feral rye (*Secale cereale* L.), jointed goat grass (*Aegilops cylindrica* Host), and downy brome (*Bromus tectorum* L.) (Kumar *et al.* 2021) (Hildebrandt *et al.* 2022). The primary method for controlling these

grass weeds in winter wheat is with post-emergent herbicides. Although wheat farmers employ some other non-chemical weed management strategies, the heavy reliance on herbicides leads to the development of herbicide-resistant weeds.

Colorado Wheat Research Foundation partnered with CSU researchers to develop a new winter wheat variety that would provide farmers with an additional winter wheat option with a new mode of action. The CoAXium™ Wheat Production system consists of three components: 1) elite wheat varieties that have the non-GMO AXigen™ trait which confers resistance to quizalofop-*P*-ethyl (QPE), 2) a patented group 1 herbicide formulation of QPE, Aggressor, and 3) a stewardship agreement that must be signed by all farmers to help preserve the trait and prevent weed resistance (Bough *et al.* 2021).

Every year CSU continues to develop improved CoAXium™ varieties to perform well in changing field conditions, out compete weeds, and provide better yields. A whole-plant greenhouse screen bioassay tool that would quickly provide data to breeders about new wheat seed line tolerance to herbicides would save time and money in the breeding process. Such bioassay tool was developed to generate whole-plant response curves of the new wheat seed lines exposed to QPE in about 30 d. The data provides insights on how wheat plants respond to the different doses of the herbicide and ranks seed lines in order of their tolerance to QPE and to help breeders select the better-performing varieties for the field trials.

MATERIALS AND METHODS

3.1 Plant Material and Growth

A dose-response screen bioassay under greenhouse conditions at Colorado State University, Fort Collins, Colorado was developed to be able to rapidly test new CoAXium™ wheat lines for their tolerance level to the herbicide quizalofop-P-ethyl (QPE) (Albaugh, LLC, Rosalia, WA). This controlled environment maintained daytime temperatures of 20 – 25°C (shade cloths, fans and vents activate as needed to maintain the selected temperature range), relative humidity about 75%, and 14-hour day length (sodium halide lamps turn on to supplement natural light when needed). Four wheat seeds were planted in pots (350 mL capacity) filled with potting soil and 14.75 g of Osmocote Plus (15-9-12) fertilizer. Extra

pots were planted to ensure that there were four replicates for each dose of each experimental seed line after germination. The pots were placed randomly in trays in the greenhouse and bottom watered to keep the soil evenly moist for germination. After germination, pots were watered as needed to keep roots from drying out.

3.2 Greenhouse Screen Assay Method 1

Eleven wheat lines (10 CoAXium™ lines and 1 non- CoAXium™ line) were planted using the above planting method for our first screen assay. Plants were sprayed with one of three QPE treatments (96, 193, 385 g ae ha^{-}) mixed with 1% v/v NIS adjuvant (Activator 90, Loveland Products, Greeley, CO) plus a non-treated control at the 3-4 leaf stage. All treatments were applied with a single pass about 40 cm above the tops of the plants with a research table sprayer (model Gen IV, Devries Manufacturing, Hollandale, MN) equipped with a TeeJet 2E 800 brass nozzle at a spray volume of 270 L/ha. Plant height measurements were taken every 7 d, and then fresh weight was measured at 21 DAT. After conducting several bioassays with this method, we refined the procedure to method 2 below.

3.3 Greenhouse Screen Assay Method 2

Forty-one experimental wheat lines, selected by the breeder, were planted using the same planting methods as above. At about three weeks (3-4 leaf stage) (Figures 3.1 and 3.2), the plants were sprayed in the research table sprayer with one of three QPE doses (48, 96, and 193 g ae ha^{-1}) mixed with an adjuvant (1% v/v NIS) plus a non-treated control. The rates were chosen that would provide injury but not kill the plants. At 3 d after spraying the QPE, the plant growth was cut just above the meristem (Figures 3.1 and 3.3). Seven d later the regrowth for each plant was cut and fresh weight was recorded (Supplemental Figures 4-6).

3.4 Statistical Analysis

All statistical analysis was conducted with packages in RStudio v.2022.12.0 Build 353 with R v.4.2.2. A few outliers were removed from the data but there were always at least three replicates for every dose and seed line. Since no significant experiment repetition was conducted, only the interaction of the

herbicide on the experimental seed lines is discussed. Using fresh weight data from the Method 2 experiments, a two-way ANOVA ($\alpha = 0.05$) was used to evaluate treatment and variety, and their interaction as significant predictors for biomass. Next, we looked at percent FW compared to control, created a dose-response curve in the DRC package in RStudio and estimated the rate required to reduce FW 50% (GR50).

RESULTS AND DISCUSSION

The response of the 41 experimental seed lines to the increasing herbicide rates on the amount of regrowth tissue in the wheat plants after herbicide spraying was consistent within each seed line: control showed the highest level of regrowth, and the 193 g ae ha^{-1} rate showed the lowest amount of regrowth (Table 3.1). The greatest differences were observed both visually and by fresh weight in the 48 and 96 g ae ha^{-1} rates which are presented in this paper (Figures 3.5 and 3.6). Looking at the fresh weight lines (1 and 2) had the least amount of regrowth at the 48 g ae ha^{-1} rate, indicating the lowest tolerance to QPE. The five lines with the most regrowth (30, 29, 17, 36, 32) show the highest tolerance to QPE at the 48 g ae ha^{-1} rate. The two-way ANOVA result (p-value < .05) validates that there was significant difference in the biomass of the different experimental lines in response to the herbicide doses (Table 3.2).

The FW regrowth results show both the vigor of the plant and their tolerance to the herbicide. A seed line that could show greater growth potential does not necessarily have a higher tolerance to the herbicide. Looking at the percent of fresh weight (FW) in relationship to control (treated/untreated) at the 48 g ae ha^{-1} rate shows 13 of the lines had average regrowth above 60% and only 3 lines were above 90% regrowth (Figure 3.7). The top five most tolerant seed lines, except for line 29, were different from the above FW results (33, 29, 38, 27, 35) When looking at these metrics we are normalizing growth and showing only the best tolerance to the herbicide.

The GR50 estimates (Table 3.3) for two of the top lines were, 33 (96.0±5.8) and 27 (69.6±12.1), consistent with the above percent of FW results. Four of the lines from the highest performing lines that

had at least 50% regrowth (Figure 3.8) when treated with 48 g ae ha^{-1} rate (33, 30, 32, and 39) were in the top seven results for all three metrics (FW, percent FW compared to control, and GR50 estimates).

The goals of this project were to create a quick whole-plant screen bioassay that would provide the CSU breeder of CoAXium™ wheat with an herbicide tolerance metric that would facilitate breeding decisions on new experimental wheat lines. The method 2 bioassay completes in half the time of method 1 and refined the herbicide doses to those that provide information on seed line tolerance to herbicide dose without killing the plants.

This experiment was a blind experiment as the researcher had no knowledge of the seed line traits. A blind experiment has the advantages of minimizing bias and enhancing objectivity in data interpretation since the researcher will not be influenced by any preconceived ideas about the seed lines. Blind experiments also contribute to scientific rigor, by reducing bias, the experiments are conducted in a methodologically sound manner increasing the validity of their results.

CONCLUSIONS

Usually, herbicide testing is conducted in the final stages (e.g., seed increase phases prior to commercial release) of breeding programs (Leon & Tillman 2015). The CoAXium™ wheat production system is effective because the wheat is bred to be resistant to a very specific herbicide - Aggresor™. It is critical that the wheat breeders know the level of tolerance to this herbicide early in the breeding process. This allows them to remove sensitive lines, design new crosses, or make recommendations for potential label rate changes.

Field screening is time-consuming, weather dependent, prone to diseases, and expensive. A rapid, dependable whole-plant screen bioassay as part of the yearly breeding activity (Leon & Tillman 2015) in a controlled greenhouse environment provides clear, observable results that provide information to wheat breeders for selection strategies. Based on these results our method two screen bioassay has proven to be a quick and effective tool that would benefit the CSU breeding program.

RECOMMENDATIONS

After refining the method for this screen bioassay (herbicide rates, timing of planting/spraying/regrowth) there are a couple of suggestions that may improve this tool and provide the CSU breeder with more valuable information. In future bioassay screens we would lower the NIS rate to 0.25%, as this is a better rate for the surfactant in a greenhouse study. The addition of a wheat line without the CoAXium™ trait and one of the top commercial CoAXium™ lines with each screen will show how the experimental lines compare under the same environmental conditions each time. This will also help to create a threshold amount of regrowth where any experimental lines below the threshold would not be considered for moving forward in the breeding process. Finally, asking the CSU breeder what metric would be most valuable to add to his trait spreadsheet. Perhaps a yes/no answer, ie. meets the accepted threshold for herbicide tolerance would be best. Or, maybe a quantitative answer, ie. GR_{50} or IR_{50}.

TABLES

Table 3.1: Amount of fresh weight for each of the herbicide doses (control, 48, 96, and 193 g ae ha^{-1}) for the 41 experimental lines.

Line	Control	48	96	193
1	2.57	0.15	0.00	0.00
2	2.46	0.13	0.00	0.00
3	2.50	0.95	0.12	0.00
4	2.69	1.33	0.40	0.00
5	2.40	1.03	0.16	0.00
6	2.92	1.19	0.23	0.06
7	2.51	1.02	0.20	0.00
8	2.82	1.63	0.09	0.00
9	2.44	0.74	0.20	0.00
10	2.52	0.90	0.19	0.00
11	2.44	2.00	0.35	0.00
12	3.03	0.62	0.18	0.00
13	2.82	0.95	0.00	0.00
14	3.78	1.79	0.37	0.07
15	2.80	0.72	0.00	0.00
16	4.00	0.87	0.09	0.00
17	3.05	1.70	0.31	0.00
18	2.22	1.45	0.30	0.00
19	2.27	0.96	0.14	0.00
20	2.21	1.07	0.21	0.00
21	2.51	0.71	0.24	0.00
22	2.20	0.86	0.00	0.00
23	1.47	0.75	0.29	0.00
24	2.50	1.16	0.18	0.00
25	1.89	1.05	0.27	0.00
26	1.65	0.96	0.41	0.00
27	1.83	1.49	0.39	0.00
28	2.50	1.54	0.43	0.00
29	2.91	2.30	1.18	0.00
30	3.71	2.75	0.83	0.00
31	3.06	1.49	0.90	0.00
32	2.88	2.12	0.65	0.00
33	1.88	1.85	0.94	0.00
34	2.24	1.39	1.01	0.00
35	2.84	1.89	0.26	0.00
36	3.12	2.19	1.44	0.00
37	2.20	1.12	0.18	0.00
38	1.53	1.29	0.32	0.00
39	2.57	1.87	0.52	0.00
40	2.12	1.23	0.13	0.00
41	1.59	0.76	0.03	0.00

Table 3.2: Two-way ANOVA results on dose 48 g data (built in RStudio).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Line	1	10.87	10.869	27.05	5.92e-07***
Residuals	162	65.1	0.402		
Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Table 3.3: Summary of highest performing lines that had more than 50% regrowth when treated at 48 g/ha quizalofop-P ethyl, relative to untreated control.

Wheat line	Regrowth FW (@48 g/ha)	Regrowth percent (@48 g/ha)	GR50 (g/ha)
8	1.63	57.85	51.0±4.4
11	2.00	81.99	54.8± 2.5
17	2.27	55.83	51.0±3.5
18	1.44	65.09	57.1±5.2
25	1.05	55.42	51.8±7.9
27	1.49	81.64	69.6±12.1
28	1.54	61.40	57.8±8.8
30	2.75	74.04	68.0±6.5
32	2.12	73.52	67.8±7.4
33	1.85	98.27	96.0±5.8
35	1.89	66.40	56.3±10.2
37	1.12	50.96	48.6±3.5
38	1.29	84.01	63.5±10.5
39	1.87	72.74	73.1±8.7
40	1.23	57.90	51.7±5.0

FIGURES

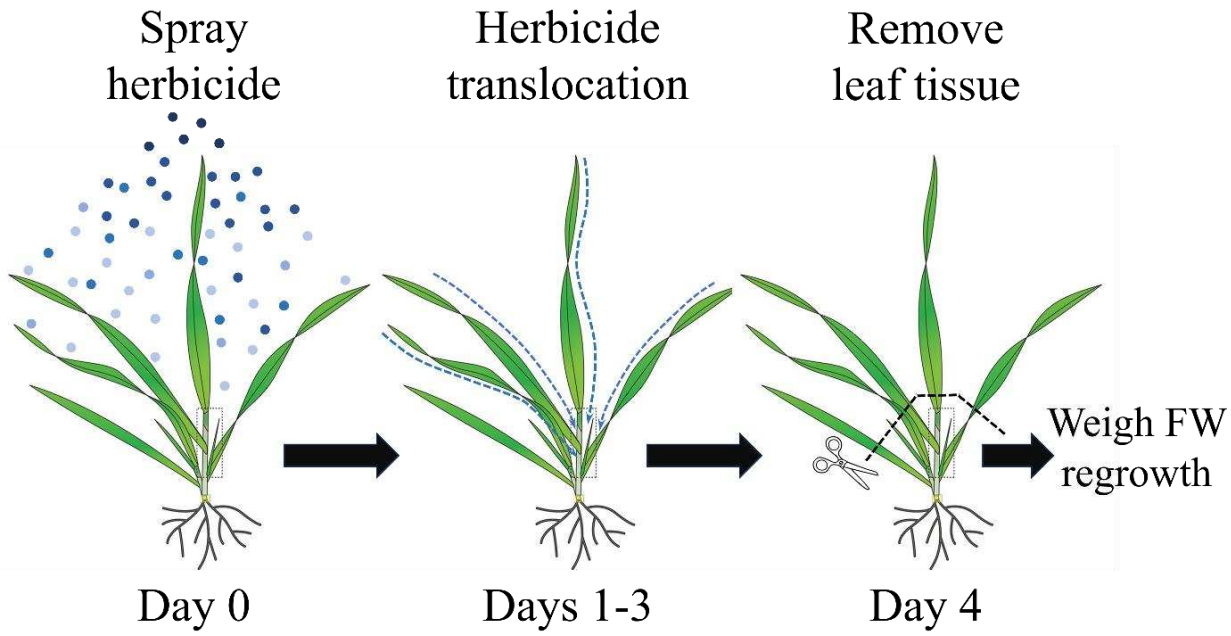

Figure 3.1: Illustration of the steps in the screen assay protocol. On day 0, wheat seedlings are 3-weeks-old and at their 3-4 leaf growth stage, when the herbicide is applied. The herbicide is allowed to translocate to the meristems for 3 d. Leaf tissue beyond the meristems is removed on day 4. Leaves are allowed to regrow for 7 d before being collected for biomass quantification.

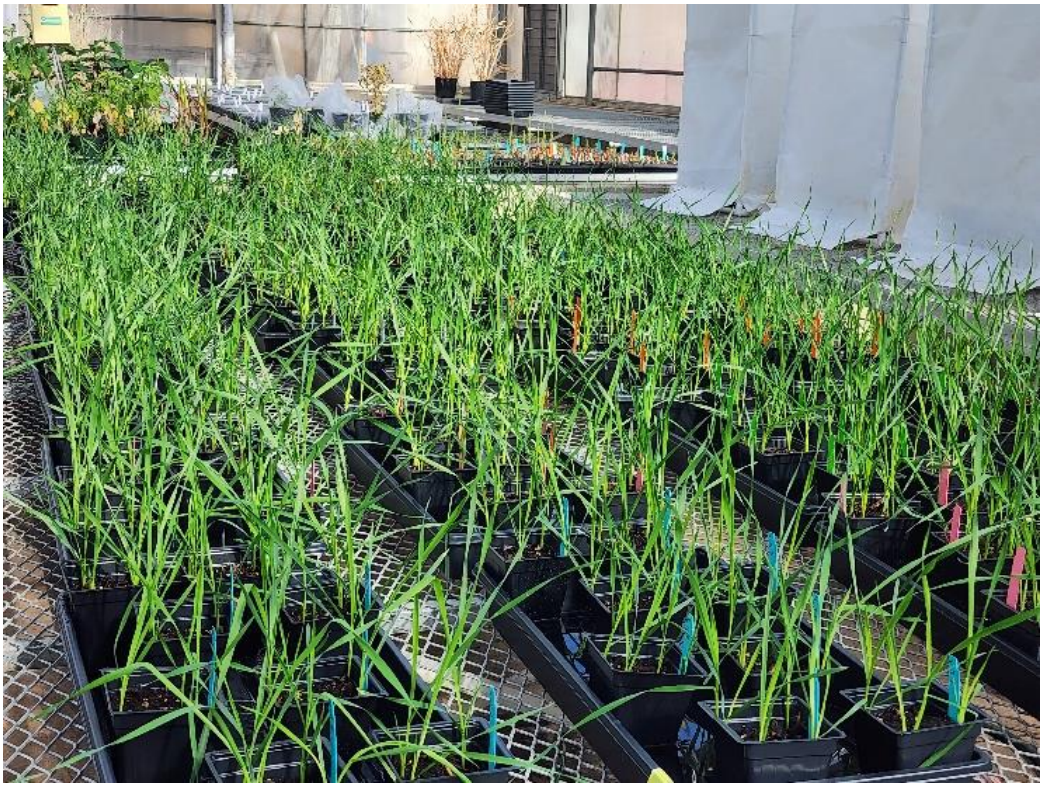

Figure 3.2: CoAXium™ wheat plants at three weeks of growth in the greenhouse. This corresponds to the 3-4 leaf stage when quizalofop-p-ethyl is applied.

Figure 3.4: CoAXium™ wheat plants 3 d after herbicide spray cut just above the meristem.

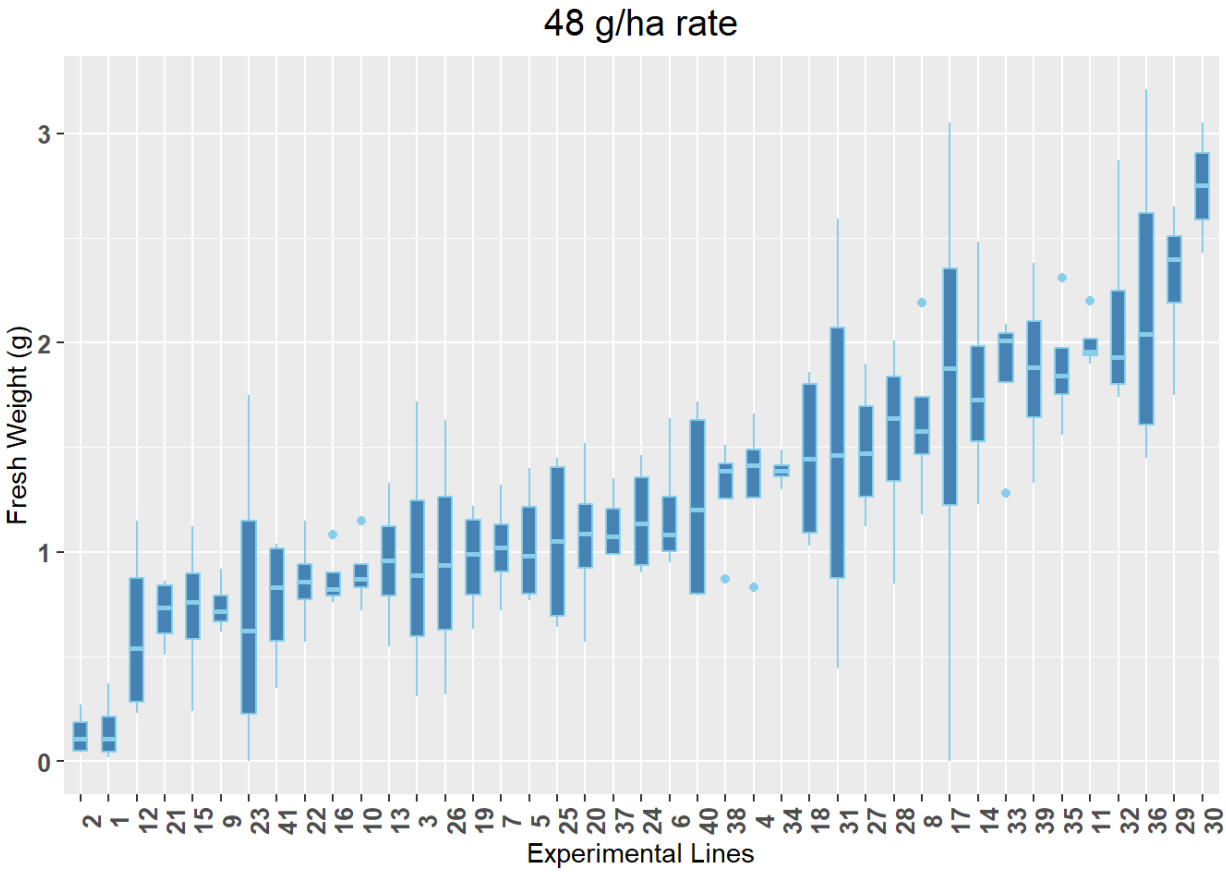

Figure 3.5: Wheat leaf regrowth fresh weight of 41 experimental seed lines exposed to 48 g ae ha^{-1} QPE. Lines were ranked from lowest to highest biomass 7 d after first cutting.

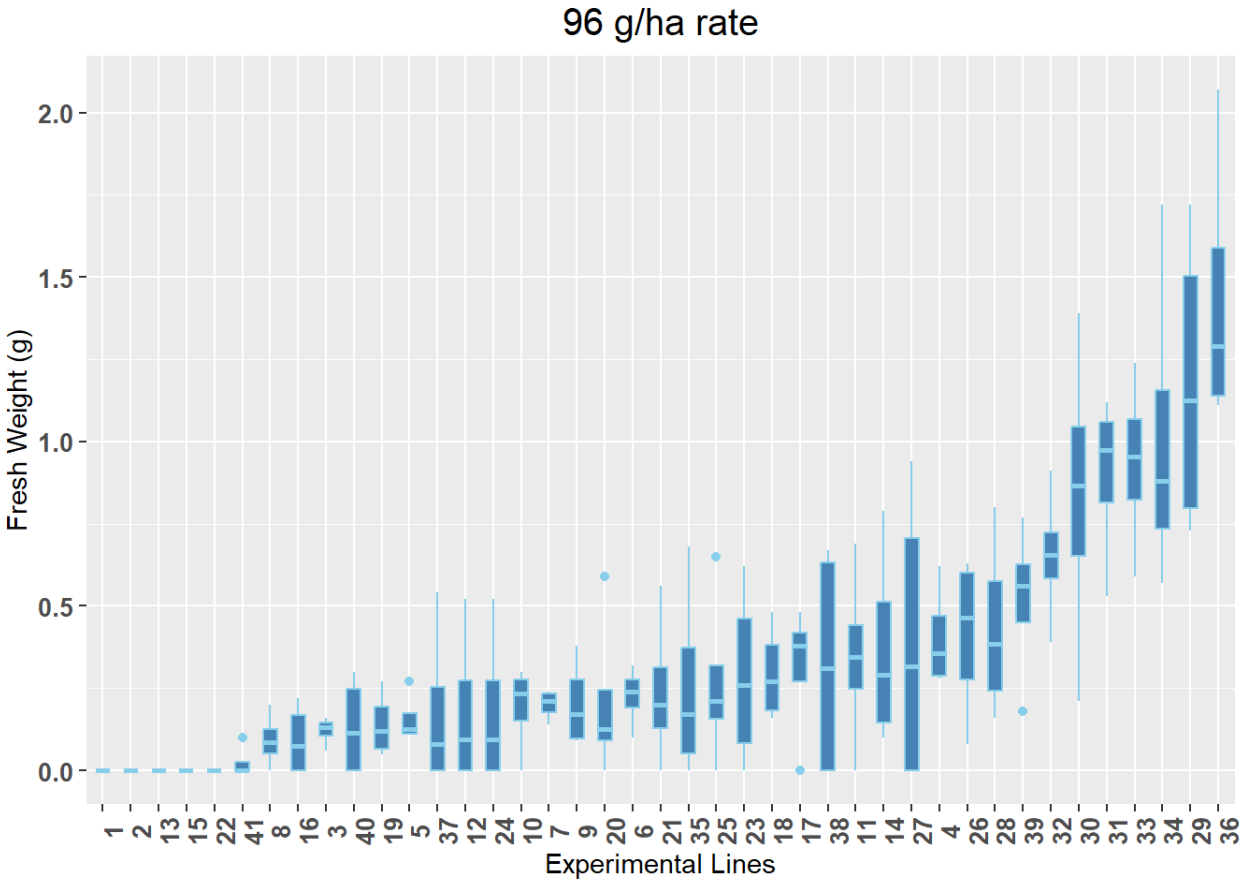

Figure 3.6: Wheat leaf regrowth fresh weight of 41 experimental seed lines exposed to 96 g ae ha^{-1} QPE. Lines were ranked from lowest to highest biomass 7 d after first cutting.

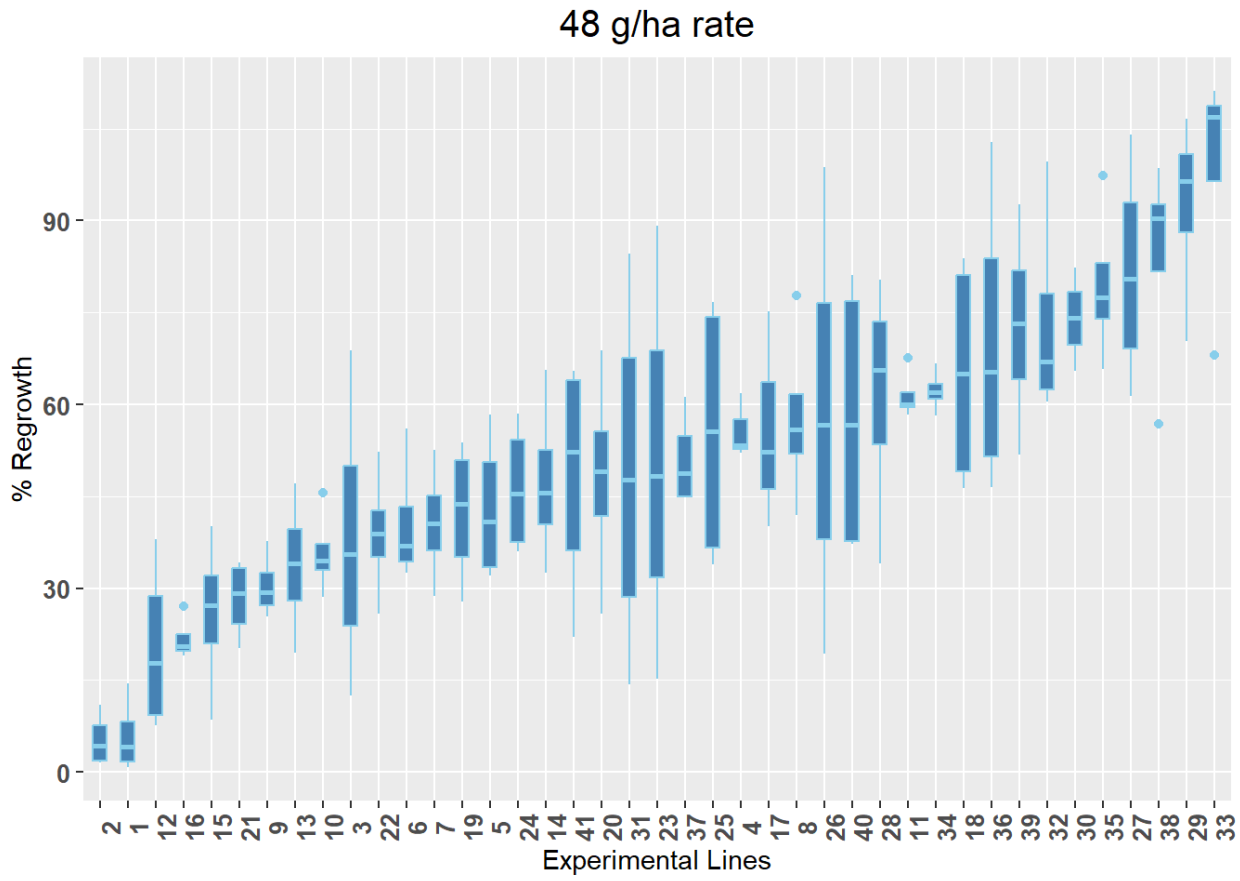

Figure 3.7. Percent regrowth of fresh weight (treated/untreated) at 48 g ae ha^{-1} rate. X axis shows the experimental seed line number. Y-axis represents the percent growth relative to untreated controls.

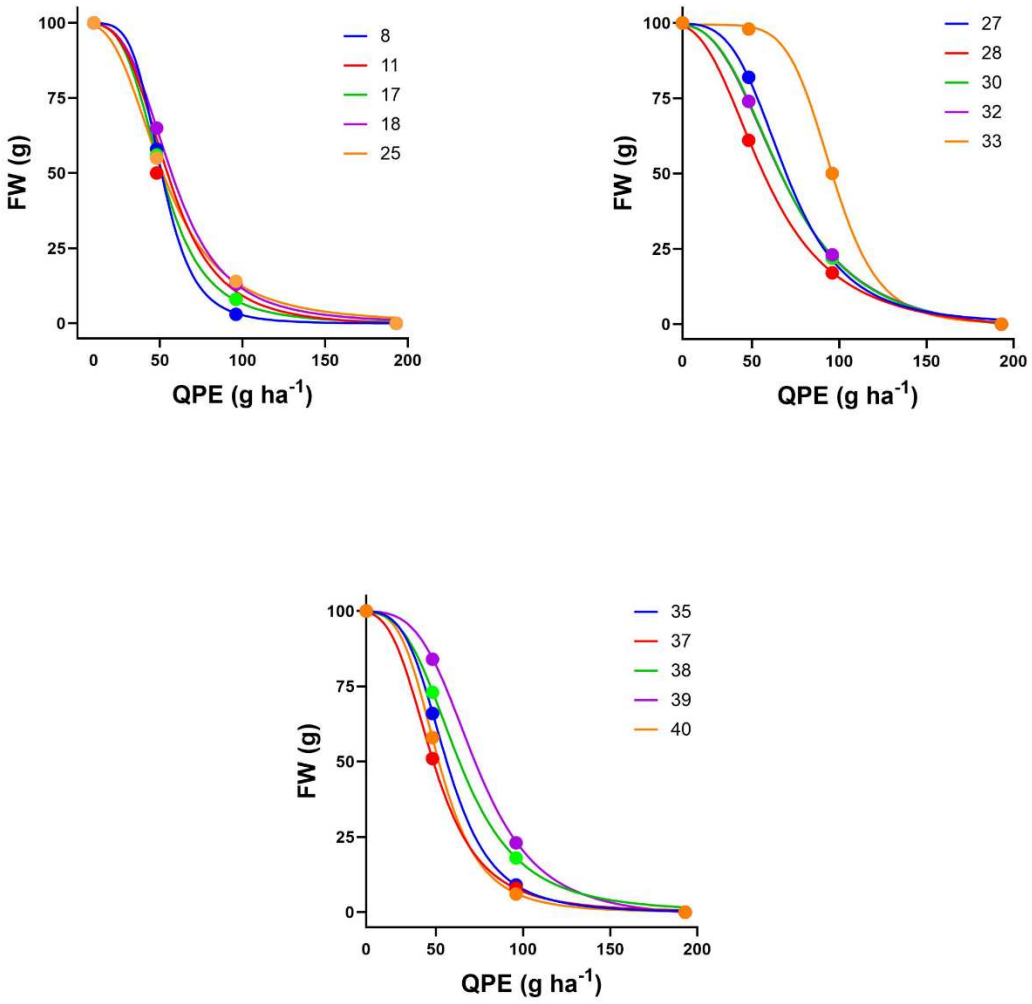

Figure 3.8. Dose response curves from percent growth of fresh weight (FW) from experimental lines with at least 50% regrowth conducted in RStudio DRC package.

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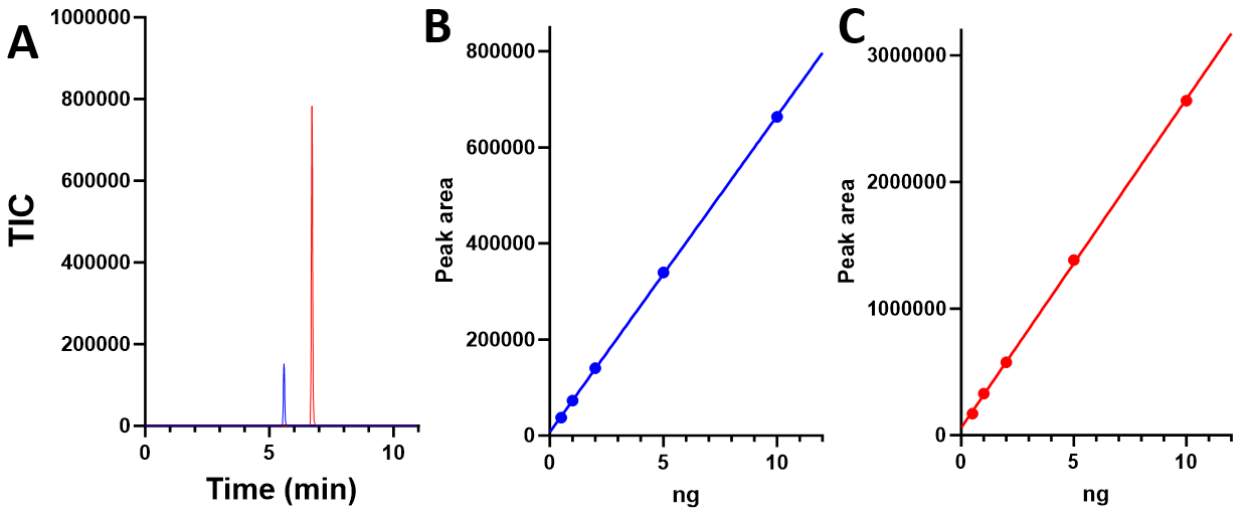
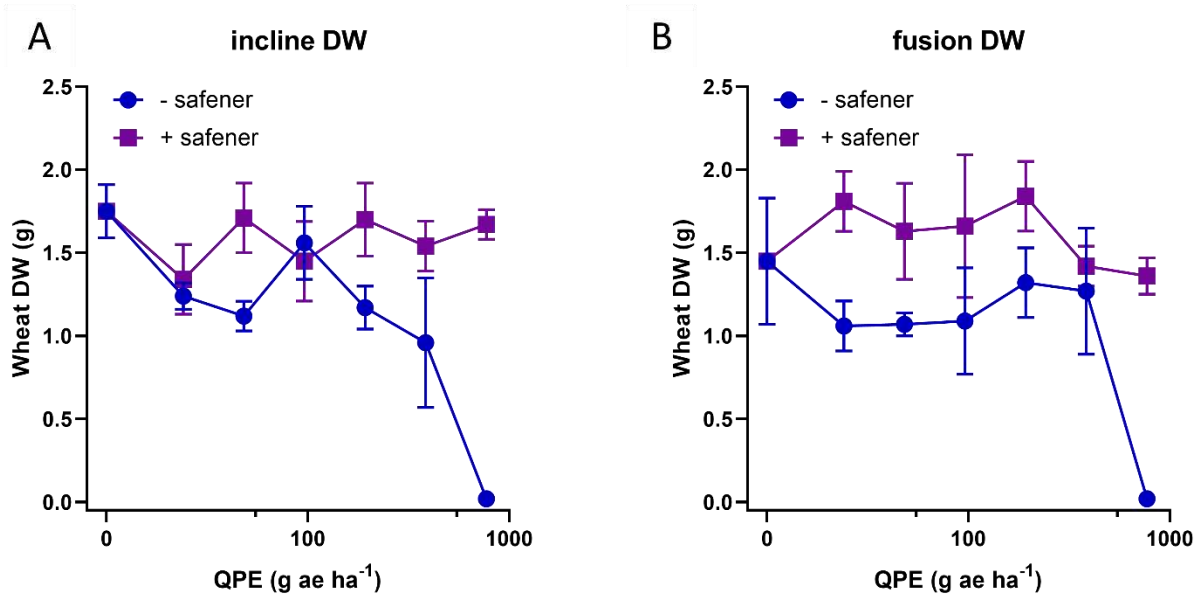
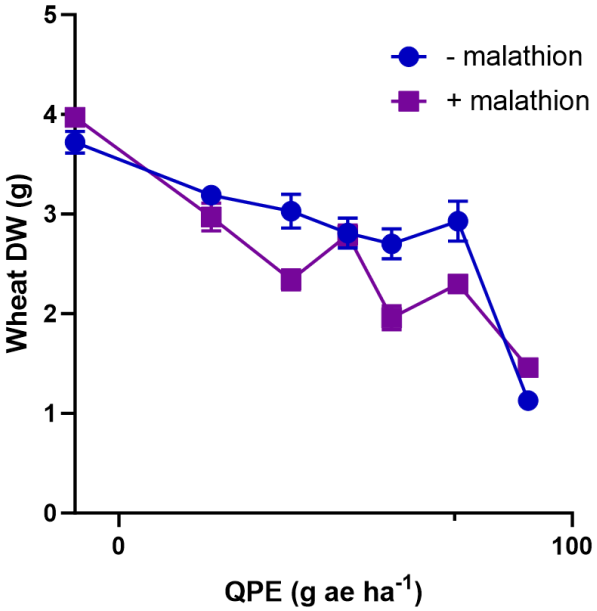
APPENDIX



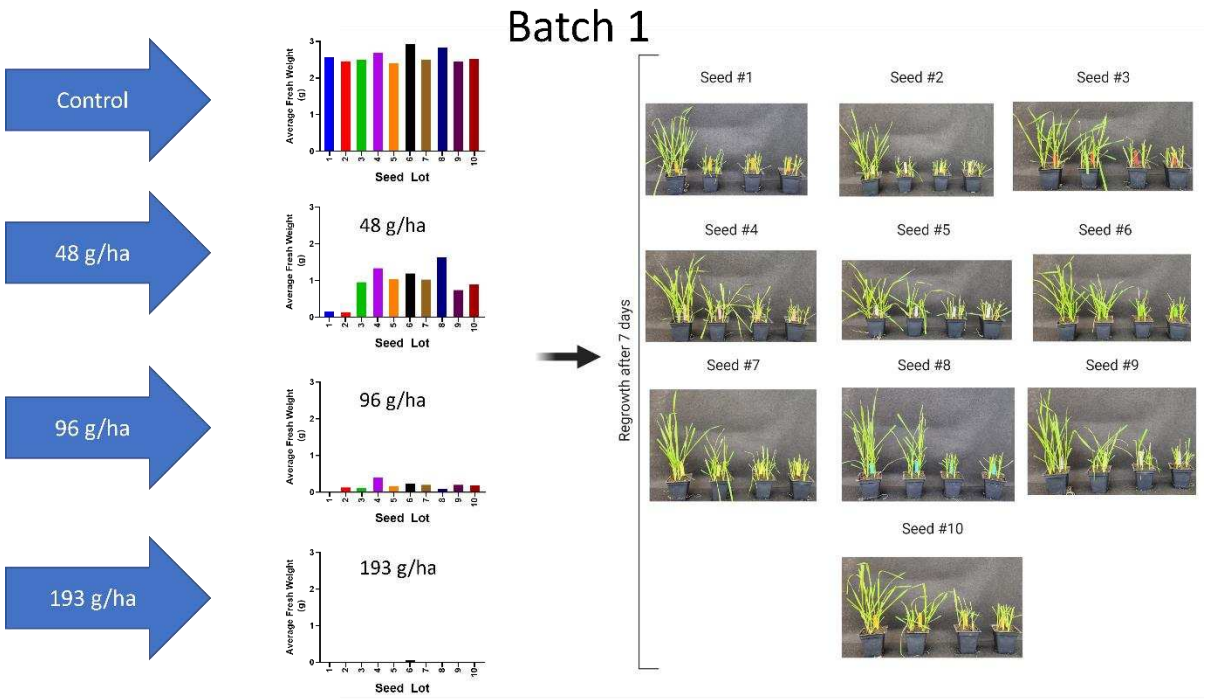
Supplemental Figure 1: Detection of quizalofop-p-ethyl (QPE) and quizalofop-p acid (QZA) by LC-MS/MS analysis. A) Representative chromatograms of QPE and QZA. B) Calibration curve of QPE: linear regression Y = 267996x and R^2=0.999. C) Calibration curve of QZA: linear regression Y = 66839.6x and R^2=0.999.



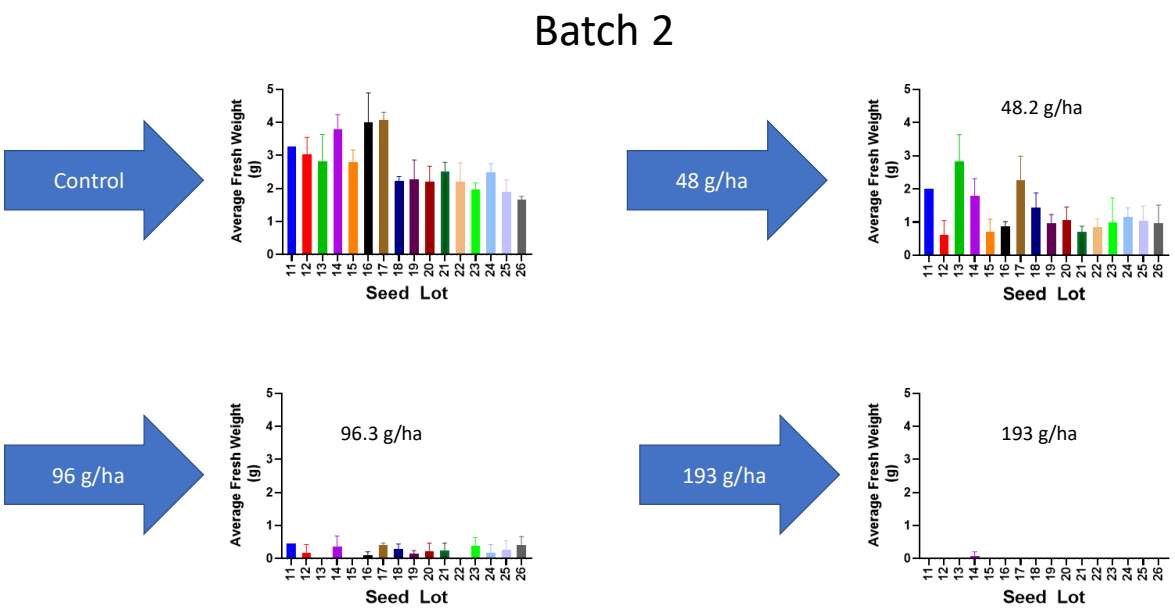
Supplemental Figure 2: Effect of the safener, cloquintocet, on wheat tolerance to quizalofop-p-ethyl. A) Incline AX and B) Fusion AX leaf regrowth dry weight (DW) in plants treated with increasing rates of quizalofop with and without safener.



Supplemental Figure 3: Effect of P450 inhibitor, malathion on wheat's sensitivity to QPE. Wheat leaf dry weight in plants treated with increasing rates of quizalofop with or without malathion.



Supplemental Figure 4: Example of fresh weight results for all four QPE doses in batch one of the screen bioassay.



Supplemental Figure 5: Example of fresh weight results for all four QPE doses in batch two of the screen bioassay.

Supplemental Figure 6: Example of regrowth results for all four QPE doses in batch two of the screen bioassay.