DISSERTATION

# QUIZALOFOP-RESISTANT WHEAT: BIOCHEMICAL CHARACTERIZATION OF THE AXIGEN™ TRAIT AND CORRESPONDING METABOLISM

Submitted by

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ABSTRACT

QUIZALOFOP-RESISTANT WHEAT: BIOCHEMICAL CHARACTERIZATION OF THE AXIGEN™ TRAIT AND CORRESPONDING METABOLISM

A new weed management tool in wheat, the CoAXium™ Wheat Production System, incorporates quizalofop-resistant wheat, a specialized formulation of quizalofop (Aggressor™), and a stewardship management program for effective management of annual grasses with otherwise limited control options. The AXigen™ trait confers resistance primarily through a single-point mutation in *ACC1.* The mutation causes an alanine to valine substitution at position 2004 in wheat acetyl-CoA carboxylase (ACCase) relative to the *Alopecurus myosuriodes* reference. Through greenhouse and biochemical studies paired with protein homology modelling and simulations, the research presented herein provides strong evidence that a conformational change imparted by the amino acid substitution results in quizalofop-resistant ACCase. Conversely, the mutation conveys negative cross-resistance to haloxyfop, a similar herbicide to quizalofop with a smaller molecular volume. The remaining research objectives focus on quizalofop metabolism in CoAXium™ wheat. Liquid chromatography-mass spectrometry measurements of quizalofop content over time from liquid demonstrate cooler temperature conditions (4.5°C) delay quizalofop metabolism by 4 times compared to warmer temperature conditions (19°C). Reduced temperatures also delay quizalofop metabolism to the same extent in the following annual grass weed species: *Aegilops cylindrica, Bromus tectorum*, and *Secale cereale.* Further, additional studies suggest herbicide metabolism mechanisms

enhance overall CoAXium™ wheat quizalofop resistance. Despite similar ACCase resistance, resistant winter and spring wheat varieties convey varying degrees of whole-plant resistance. In winter wheat but not spring wheat, increased resistance corresponds to a shorter quizalofop half-life, implying faster metabolism boosts overall resistance. Treatment of resistant spring wheat varieties with cloquintocet, a metabolism-boosting safener, increases overall resistance. Follow-up differential expression analysis of cloquintocet-treated plants may support differential metabolism findings and lead to identification of putative candidate genes associated with upregulated herbicide metabolism, such as cytochrome P450 monooxygenases, glutathione-*S*-transferases, and glycosyltransferases.

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**Other Contributors**

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# DEDICATION

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# CHAPTER 1: THE COAXIUM™ WHEAT PRODUCTION SYSTEM: A NEW HERBICIDE-RESISTANT SYSTEM FOR ANNUAL GRASS WEED CONTROL AND INTEGRATED WEED MANAGEMENT


## Summary

This review highlights the importance of wheat as a global food source and describes a novel multi-institutional, public-private partnership between Colorado State University, the Colorado Wheat Research Foundation, and private chemical and seed companies that resulted in the development of a new herbicide-resistant wheat production system.


## Introduction

Wheat (*Triticum aestivum* L.) is a key crop for global food security because the world population consumes approximately one-fifth of calories and proteins as wheat products (Pena-Bautista et al. 2017). Wheat is a staple food due to its high yield potential, efficient harvestability, extensive genetic diversity for development of locally adapted varieties, and a biochemical grain profile that forms a malleable, multipurpose dough (Shewry 2009). Another feature enhancing food security is wheat's long-term storability, which buffers against unpredictable weather or price fluctuations.

The genetic diversity of wheat enables global production across a spectrum of environments that includes low to high altitude areas and tropical to temperate climates. The global harvest total for 2020 was forecast at 763 million metric tons (MMT), which is similar to the 2019 harvest estimate of 762 MMT but greater than the 2016 to 2018 harvest average of 752 MMT (FAO 2020). In 2019, more than 50% of the global harvest originated in the People's Republic of China, the Republic of India, the Russian Federation, the United States of America

(USA), and France (Figure 1.1) (FAO 2021). A significant share of the world food economy involves wheat trade, as evidenced by the 2019 global wheat export market value of approximately $44.1 billion US dollars (Simoes and Hidalgo 2020). For that year, the top five exporting countries (the Russian Federation, USA, Canada, France, and Ukraine) accounted for about 57.6% of the export market value, equivalent to $25.4 billion US dollars for about 115 MMT of wheat (FAO 2021). Wheat is a major source of income for small-scale farmers worldwide (Pena-Bautista et al. 2017).

Countries with developing economies consumed 77% of the global wheat production over the past decade and accounted for 90% of imports (Enghiad et al. 2017). Annual average global wheat consumption in 2017 and 2018 was 67.4 kilograms per capita, however consumption was nearly three-fold greater for the top five consuming countries of Tunisia, Turkey, Algeria, Morocco, and Egypt (FAO 2020). The top five importers in 2019 were Indonesia (11.0 MMT), Egypt (10.4 MMT), Turkey (10.0 MMT), Italy (7.5 MMT), and the Philippines (7.2 MMT) (FAO 2021). Except for Italy, the top five countries by wheat consumption and imports are considered to be developing economies. The world population is expected to reach 9.7 billion by 2050, with most growth predicted to be in the least developed countries (UN 2019). Wheat will play a critical role in feeding the world population, as it is a versatile yet affordable food source.

To feed an expanding global population, wheat productivity needs to improve or at least be sustained, which may be attained by continual improvements of pathogen and insect resistance coupled with reductions in production costs. Weeds are responsible for the largest percent crop yield loss of all the pests in wheat, causing an average of 7.7% yield losses (but can

result in up to 23% loss in productivity) (Oerke 2006). Improving weed control is a practical strategy for maintaining wheat productivity and reducing production costs. Annual grasses are the most problematic weeds in wheat as they thrive under similar growing conditions. Current management of these weeds centers on herbicides, though options are limited during the growing season due to their negative impacts on wheat and the growing number of herbicide resistance cases. To ensure sustainable wheat production despite herbicide-resistant weeds, integrated weed management (IWM) should be bolstered by diversifying herbicide programs and utilizing more cultural and mechanical weed control methods. A new technology that can contribute to IWM in wheat is the CoAXium™ Wheat Production System (http://www.coaxiumwps.com). The system facilitates excellent, cost-effective control of winter annual grass weed species by enabling a novel use of quizalofop in a winter wheat production system (Figure 1.2). Further, the system provides a new opportunity to increase adoption of integrated weed management through promoting non-herbicide weed control via a unique stewardship agreement.

**Winter Annual Grass Weed Management in Winter Wheat**

*General*

Weed control in wheat relies predominately on herbicides where growers commonly implement mode of action rotations and herbicide tank mixtures to minimize the selection of herbicide-resistant weeds. Winter annual grass control in wheat involves several herbicide modes of action including inhibition of acetyl-CoA carboxylase (ACCase), acetolactate synthase (ALS), microtubule assembly, photosynthesis at photosystem II, enolpyruvyl shikimate phosphate synthase (EPSPS), protoporphyrinogen oxidase, and very long-chain fatty acid

synthesis (Table 1.1). During the winter wheat growing season specifically, producers primarily use herbicides that inhibit ACCase, ALS, and photosynthesis. Three distinct chemistries comprise ACCase-inhibitors, including aryloxyphenoxypropionates (FOPs), cyclohexanediones (DIMs), and a phenylpyrazoline (pinoxaden). Post-emergent use of ACCase-inhibitors is limited to some FOPs and pinoxaden in wheat due to a general lack of selectivity of the DIMs. Producers apply the ALS-inhibitor, imazamox, post-emergence by taking advantage of Clearfield™ Production Systems that feature imazamox-resistant wheat varieties (Nakka et al. 2019).

Wheat growers utilize IWM practices that supplement herbicide programs with cultural, mechanical, or preventative weed control tactics. The most regularly employed non-herbicide methods are using competitive wheat varieties, strategic fertilization, crop rotation, intercropping, fallow, and using weed-free certified seed and farm equipment sanitation. There are several less employed but promising methods that also fall under the IWM umbrella. Methods of weed control conducted prior to planting include cover cropping, residue mulching, stale seedbed preparation, or conventional tillage (Chhokar and Sharma 2012; Jabran et al. 2017). Seeding tactics that minimize weed infestations are using weed-free seed stock, early sowing, increased seed density, decreased row spacing, and sowing in an east to west orientation. During early wheat growth, harrowing can decrease weed biomass with little no impact on wheat yield (Rueda-Ayala et al. 2011). To reduce weed seedbank populations significantly at harvest, Australian researchers have developed several techniques such as a chaff mill implement for combines or specialized combines with integrated chaff mills that grind and destroy annual grass weed seeds (Walsh et al. 2018). Lastly, an effective non-herbicide

weed control method between growing seasons includes residue retention (Mesbah et al. 2019).

*Major Weeds in Wheat*

Common winter annual grass species in wheat production around the globe are oat (*Avena* sp.), foxtail grasses (*Alopecurus* sp., including blackgrass), brome (*Bromus* sp.), ryegrass (*Lolium* sp.), and canarygrass (*Phalaris* sp.), although there are additional problematic weed species across growing regions (Figure 1.3). Reliance on herbicides to control these weeds has led to dominance of a few, highly competitive species with the genetic diversity necessary to evolve herbicide resistance. Resistance to a single herbicide mode of action with cross-resistance is not unusual and some species also exhibit multiple resistance to two or more modes of action (Figure 1.3). The most problematic species globally in terms of herbicide resistance are blackgrass (*Alopecurus myosuroides*), wild oat (*Avena fatua*), Italian ryegrass (*Lolium perenne* ssp. *multiflorum*), and rigid or annual ryegrass (*Lolium rigidum*) (Figure 1.3).

**The CoAXium™ Wheat Production System**

*The System*

The CoAXium™ Wheat Production system comprises 1) wheat varieties with the AXigen™ trait for quizalofop resistance (referred to as “tolerance” or “tolerant” by industry), 2) a specialized herbicide formulation of quizalofop-p-ethyl under the tradename Aggressor™, and 3) a grower stewardship agreement developed through a private-public collaboration among the Colorado Wheat Research Foundation, Inc. (CWRF); Albaugh, LLC; and Vilmorin and Company (parent company of Limagrain Cereal Seeds, LLC). Before all components of the system were brought together, CWRF partnered with Colorado State University (CSU) to

develop a post-emergence tool in addition to Clearfield™ Production Systems to control crop-debilitating annual grass weed species in winter wheat and reduce selection for imazamox resistance. Quizalofop resistance was prioritized as a breeding target due to quizalofop exhibiting a different mode of action, lower production cost, and increased control of feral rye than imazamox, as well as minimal toxicity and limited soil residual activity. Researchers at CSU identified a novel quizalofop resistance trait from a mutagenized wheat (Ostlie et al. 2015) population and then introgressed the trait into elite varieties. Albaugh, LLC formulated and optimized Aggressor™ herbicide for specific use with the resistant varieties. Varieties featuring the AXigen™ trait are now commercially available through PlainsGold™ (CWRF); Limagrain Cereal Seeds, LLC; and other third-party licensees. In the United States, wheat breeders continue to develop locally adapted varieties with the AXigen™ trait.

*Trait Development*

Plant growth requires the enzyme ACCase because it carries out the first step of fatty acid biosynthesis. Two forms of ACCase exist in plants, one of which is inhibited by quizalofop while the other is unaffected. Broadleaf plant species typically produce both forms of ACCase and tolerate the herbicide, whereas grass species only make the susceptible form and are damaged or killed following inhibition. Wheat, a grass species, is very sensitive to quizalofop, but CoAXium™ wheat varieties tolerate rates of the herbicide that provide effective in crop control of problematic weeds because of the presence of the AXigen™ trait.

With support from CWRF, researchers at CSU initiated the AXigen™ trait discovery by applying the chemical mutagen ethyl methanesulfonate to seeds to induce random DNA mutations in Hatcher winter wheat (Ostlie et al. 2015). This method is not considered to be

genetic modification in the context of genetically modified organisms (GMOs) as it does not involve recombinant DNA technology or transgenesis. These mutagenized seeds were planted in the field and young plants were treated with quizalofop. Resistance was confirmed in surviving plants through additional quizalofop treatments. Wheat is a hexaploid species, meaning it has seven pairs of chromosomes from each of three ancestral species (designated as genomes A, B, and D), thus three copies of every gene. The researchers found that quizalofop resistance was associated with identical mutation in the ACCase gene from any one of the three wheat genomes. The mutation, which occurs at position 2004 in the amino acid reference sequence of blackgrass, causes an amino acid substitution of a smaller alanine for a larger valine in the ACCase enzyme structure. The substitution prevents quizalofop from binding to and inhibiting ACCase (Figure 1.4).

To develop quizalofop-resistant wheat varieties for commercial production, the Wheat Breeding and Genetics Program at CSU first crossed two wheat plants, one with a copy of the AXigen™ trait in genome A and the other in genome D, with the elite winter wheat variety Byrd. Copies of the trait in each of the formerly mentioned genomes bestow more resistance than a copy in genome B (Ostlie et al. 2015). Progeny from each of the crosses were then crossed together to generate wheat with two homozygous copies of the AXigen™ trait. The combination of two copies of the trait in one wheat variety imparts resistance to labeled rates of quizalofop. Initial CoAXium™ varieties, which were named LCS Fusion AX and Incline AX, were selected from progeny of the last cross.

*Benefits*

An advantage of the CoAXium™ Wheat Production System is the ability to use quizalofop, which is affordable and exhibits low environmental toxicity due to soil degradation within a few days of application (Mantzos et al. 2017). A more significant advantage is that the system can be alternated with Clearfield™ Production Systems to minimize post-emergence reliance on imazamox, thereby reducing selection for imazamox-resistant weeds. Before the introduction of the CoAXium™ Wheat Production System, growers in certain wheat regions in the United States were limited to imazamox to control problematic annual grass species such as feral rye (*Secale cereale*) and jointed goatgrass (*Aegilops cylindrica*) during the growing season. Aggressor™ controls both weeds at label rates (77 to 91 g ai $ha^{-1}$). In field trials across Oklahoma, Kansas, and Colorado in the United States, label rates imparted 92-100% season-long control of feral rye (Kumar et al. 2020) (Figure 1.2). The CoAXium™ Wheat Production System enables control of other weeds as well. At 62 g ai $ha^{-1}$ of a quizalofop formulation other than Aggressor™, a greenhouse study demonstrated a significant biomass reduction of downy brome (*Bromus tectorum*) and Japanese brome (*Bromus japonicus*) (Metier et al. 2019), whereas canola (*Brassica napus*) field trials showed a decrease in percent by weight of Italian ryegrass in most field sites at the same rate (Bushong et al. 2017).

To delay herbicide resistance evolution in weeds and prolong the utility of the CoAXium™ Wheat Production System, growers must adhere to a stewardship agreement that follows integrated weed management principles. Guidelines mirror those described in the *Winter Annual Grass Weed Management in Winter Wheat* section in this review, from diversifying herbicide mode of action to incorporating cultural and mechanical weed control

methods. Aggressor™ application rates should not deviate from label recommendations for specific grass species. The incorporation of spring crops in rotations is encouraged and CoAXium™ wheat should not be grown for more than two years in a row. Weed control with mechanical methods and herbicides other than ACCase-inhibitors are advised before harvest, after harvest, and during fallow to minimize the weed seed bank. Planting weed-free certified seed, practicing zero-tolerance for weed escapes, and sanitizing equipment will further diminish the weed seed bank. Using weed-free seed additionally minimizes the chance of resistant gene flow to weed species.

*Drawbacks*

Though the CoAXium™ Wheat Production System provides many advantages, there is still the possibility of selecting for quizalofop-resistance in grass weed species. The Herbicide-Resistance Weed Database (Heap 2021) lists several cases of quizalofop resistance around the globe. Summer annual grasses represent more resistance cases than winter annual grasses since farmers use quizalofop more often in spring or summer crops. The handful of quizalofop resistance cases in winter annual grasses supports the potential selection of additional cases, especially as the CoAXium™ Wheat Production System extends large-scale quizalofop use to the winter growing season. In China, quizalofop-resistant American sloughgrass (*Beckmannia syzigachne*) has been discovered in a cropping rotation that includes wheat (Zhu et al. 2020). Most recently, Chinese scientists identified quizalofop-resistant Asia Minor bluegrass with a 50% growth reduction rate ($GR_{50}$) lower than Aggressor label rates (Chen et al. 2020). Researchers have also described quizalofop-resistant Italian ryegrass in both Italy (Collavo et al. 2017) and the United States (Rauch et al. 2017), and some of the latter were confirmed to be

cross-resistant to other ACCase-inhibiting herbicides. Several instances of quizalofop-resistant smooth barley (*Hordeum murinum* L. ssp. *glaucum* (Steud.) Tzvelev) have also been found in Australia, with $GR_{50}$ values ranging from 90 to 231 g ai $ha^{-1}$ (Shergill et al. 2017).

In addition to weed resistance to a single herbicide, cross-resistance may occur between herbicides in different chemical families within the same mode of action. Point mutations that confer resistance to ACCase-inhibitors have been known to provide cross-resistance (Jang et al. 2013). Biotypes of blackgrass and Italian ryegrass resistant to FOPs such as diclofop and fenoxaprop occur globally, and the CoAXium™ Wheat Production System will not be practical in production regions with these weed populations because the biotypes are likely cross-resistant to quizalofop. A final potential disadvantage of the CoAXium™ Wheat Production System could be reduced efficacy due to climate change. Generally, ACCase inhibitor metabolism increases at higher temperatures, which may reduce efficacy in certain species as global temperature rises, particularly coupled to higher atmospheric $CO_2$ that increases metabolism in some instances (Varanasi et al. 2016).

**Conclusions**

Wheat is a globally significant crop. Further adoption of IWM is critical to achieve yields required to feed a growing population while overcoming persistent challenges such as herbicide-resistant weeds and climate change. The CoAXium™ Wheat Production System affords a practical means of promoting IWM through a stewardship agreement and the ability to incorporate an additional mode of action into herbicide management programs. This new tool is a welcome opportunity for growers who otherwise have limited annual grass weed control options during the growing season. The unique stewardship agreement of the system

promotes the longevity of this novel tool. Because the AXigen™ trait does not originate from transgenic modification, CoAXium™ wheat will be more acceptable to global consumers as production increases.

**Table**

Table 1.1. Herbicide modes of action and active ingredients used for cool-season annual pre- or post-emergence grass control in winter wheat.

| HRAC Group[1] | Mode of Action | Active Ingredients | Application Timing |
|---|---|---|---|
| 1 | Acetyl-CoA carboxylase | clodinafop-propargyl, diclofop-methyl, fenoxaprop-P-ethyl, *pinoxaden, t*ralkoxydim | Post |
| 2 | Acetolactate synthase | chlorsulfuron, flupyrsulfuron-methyl-sodium, imazamethabenz-methyl, imazamox, iodosulfuron-methyl-sodium, mesosulfuron-methyl, metsulfuron-methyl, propoxycarbazone-sodium, pyroxsulam, sulfosulfuron, thifensulfuron-methyl, tribenuron-methyl | Pre, Post |
| 3 | Microtubule assembly | pendimethalin | Pre |
| 5 | Photosystem II: serine-264 binders | chlorotoluron, isoproturon, metribuzin | Post |
| 6 | Photosystem II: histidine-215 binders | bromoxynil | Post |
| 9 | Enolpyruvyl shikimate phosphate synthase | glyphosate[2] | Pre |
| 14 | Protoporphyrinogen oxidase | carfentrazone-ethyl | Post |
| 15 | Very long-chain fatty acid synthases | flufenacet, pyroxasulfone, tri-allate | Pre, Post |
| 0 | Unknown | difenzoquat, flamprop-methyl | Post |

[1]Herbicide Resistance Action Committee (http://www.hracglobal.com)
[2]Glyphosate is used only in fallow and not in wheat

**Figures**

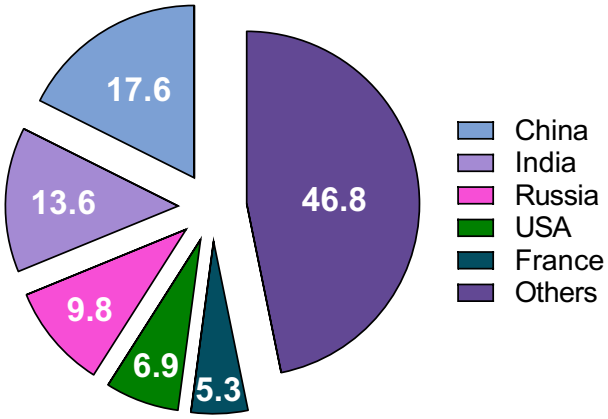



Figure 1.1. Major wheat producing nations. Values represent percent of global world production (FAO 2021)

Figure 1.2. Colorado CoAXium™ wheat trial plots: (Left) treated with 93 g ai $ha^{-1}$ (12 fl oz $ac^{-1}$) Aggressor™ + 1% MSO and (right) untreated infested with feral rye (Photo by Eric P. Westra)

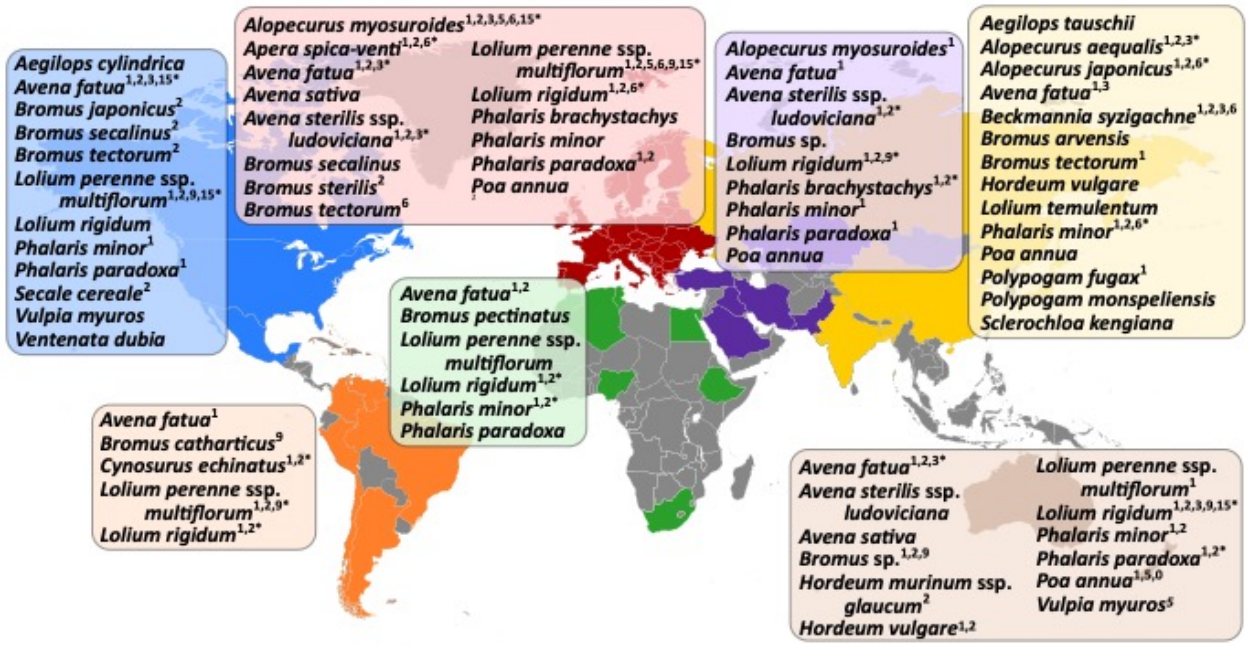



Figure 1.3. Winter annual grass weeds and herbicide resistance in wheat cropping systems around the world

[#]Numbers next to weed species indicate known cases of resistance to HRAC herbicide groups listed in Table 1.1 (Heap 2021).
[*]An asterisk next to a weed species denotes one or more cases of multiple resistance (Heap 2021).

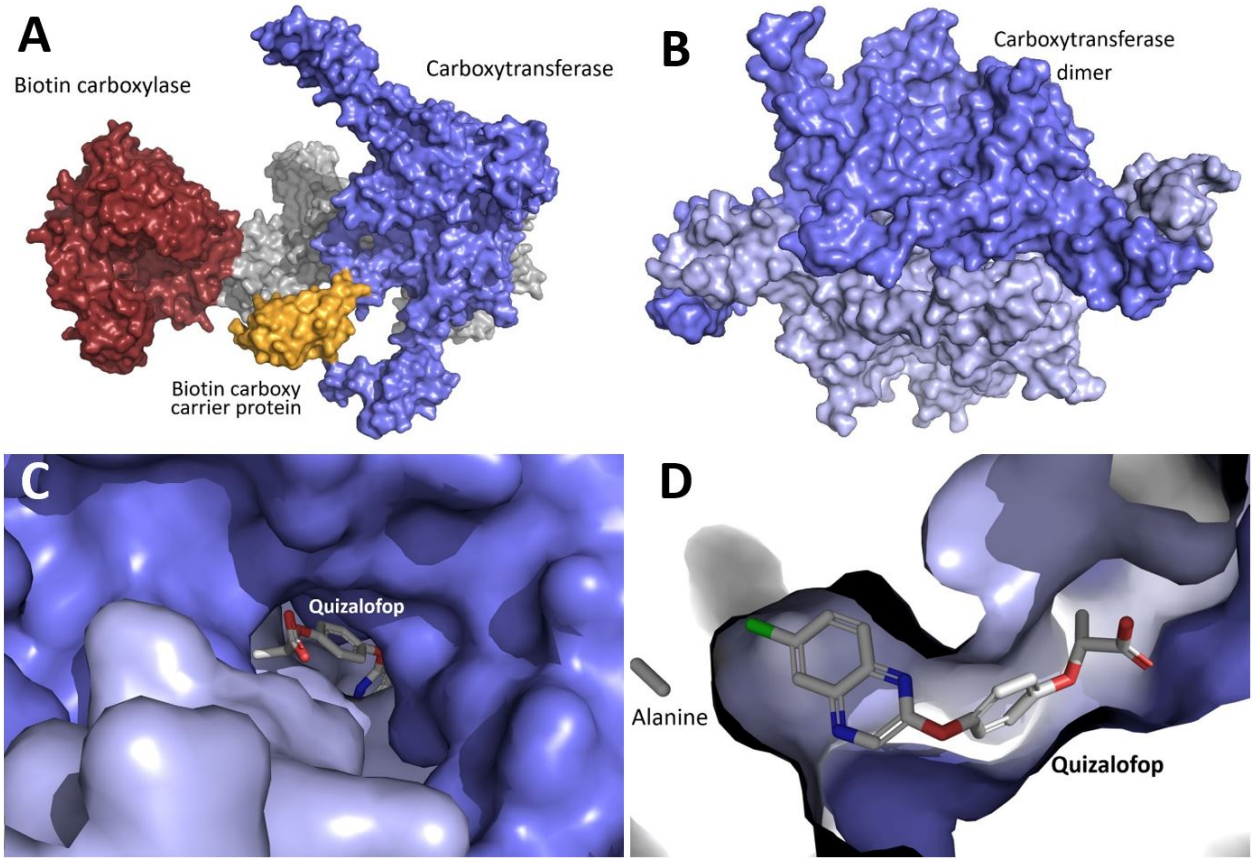



Figure 1.4. Herbicide binding pocket on wheat ACCase and location of the alanine to valine substitution at position 2004 imparting quizalofop resistance. A) Wheat ACCase is a large multifunctional protein that has biotin carboxylase and carboxytransferase activities as well as a biotin carboxy carrier protein. B) The functional enzyme consists of a homodimer. This image shows the dimerization of the carboxytransferase part of the enzyme in purple and light blue. C) ACCase inhibitors like quizalofop bind in a pocket formed at the interface of the carboxytransferase dimer. D) Alanine 2004 in involved in forming the back end of the pocket where quizalofop binds. A substitution to a larger valine reduces the size of the pocket and quizalofop no longer fits in the pocket.

**References**

Bushong J, Peeper T, Boyles M, Stone A. 2017. Italian ryegrass (*Lolium perenne*), feral cereal rye (*Secale cereale*), and volunteer wheat (*Triticum aestivum*) control in winter canola. Weed Technology. 25(3):344-349.

Chen W, Wu L, Wang J, Yu Q, Bai L, Pan L. 2020. Quizalofop-p-ethyl resistance in *Polypogon fugax* involves glutathione *S*-transferases. Pest Management Science. 76(11):3800-3805.

Chhokar RS, Sharma I. 2012. Weed management strategies in wheat: A review. Journal of Wheat Research. 4(2):1-21.

Collavo A, Panozzo S, Allegri A, Sattin M. 2017. A "stressed" alfalfa-based cropping system leads to the selection of quizalofop-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*). Weed Science. 64(4):683-694.

Enghiad A, Ufer D, Countryman AM, Thilmany DD. 2017. An overview of global wheat market fundamentals in an era of climate concerns. International Journal of Agronomy. 2017:1-15.

FAO. 2020. Food outlook- Biannual report on global food markets.

FAOSTAT Statistical Database. 2021. Rome; [accessed 3 April 2021].

Heap, I. The International Herbicide-Resistance Weed Database. 2021. [accessed 3 April 2021].

Jabran K, Mahmood K, Melander B, Bajwa AA, Kudsk P. 2017. Weed dynamics and management in wheat. Advances in Agronomy. p. 97-166.

Jang S, Marjanovic J, Gornicki P. 2013. Resistance to herbicides caused by single amino acid mutations in acetyl-CoA carboxylase in resistant populations of grassy weeds. New Phytologist. 197(4):1110-1116.

Kumar V, Liu R, Manuchehri MR, Westra EP, Gaines TA, Shelton CW. 2020. Feral rye control in quizalofop-resistant wheat in central great plains. Agronomy Journal. 113(1):407-418.

Mantzos N, Antonopoulou M, Katsoulakou S, Hela D, Konstantinou I. 2017. Soil degradation of metazachlor and quizalofop-p-ethyl herbicides on tlc plates under natural solar light and dark conditions. International Journal of Environmental Analytical Chemistry. 97(7):606-622.

Mesbah A, Nilahyane A, Ghimire B, Beck L, Ghimire R. 2019. Efficacy of cover crops on weed suppression, wheat yield, and water conservation in winter wheat–sorghum–fallow. Crop Science. 59(4):1745-1752.

Metier EP, Lehnhoff EA, Mangold J, Rinella MJ, Rew LJ. 2019. Control of downy brome (*Bromus tectorum*) and japanese brome (*Bromus japonicus*) using glyphosate and four graminicides: Effects of herbicide rate, plant size, species, and accession. Weed Technology. 34(2):284-291.

Nakka S, Jugulam M, Peterson D, Asif M. 2019. Herbicide resistance: Development of wheat production systems and current status of resistant weeds in wheat cropping systems. The Crop Journal. 7(6):750-760.

Oerke EC. 2006. Crop losses to pests. The Journal of Agricultural Science. 144(1):31-43.

Ostlie M, Haley SD, Anderson V, Shaner D, Manmathan H, Beil C, Westra P. 2015. Development and characterization of mutant winter wheat (*Triticum aestivum* L.) accessions resistant to the herbicide quizalofop. Theoretical and Applied Genetics. 128(2):343-351.

Pena-Bautista RJ, Hernandez-Espinosa N, Jones JM, Guzman C, Braun HJ. 2017. Wheat-based foods: Their global and regional importance in the food supply, nutrition, and health.

The wheat and nutrition series: A compilation of studies on wheat and health. Mexico City, Mexico: CIMMYT. p. 148-166.

Rauch TA, Thill DC, Gersdorf SA, Price WJ. 2017. Widespread occurrence of herbicide-resistant Italian ryegrass (*Lolium multiflorum*) in northern Idaho and eastern Washington. Weed Technology. 24(3):281-288.

Rueda-Ayala VP, Rasmussen J, Gerhards R, Fournaise NE. 2011. The influence of post-emergence weed harrowing on selectivity, crop recovery and crop yield in different growth stages of winter wheat. Weed Research. 51(5):478-488.

Shergill LS, Malone J, Boutsalis P, Preston C, Gill G. 2017. Basis of ACCase and ALS inhibitor resistance in *Hordeum glaucum* Steud. Pest Management Science. 73(8):1638-1647.

Shewry PR. 2009. Wheat. Journal of Experimental Botany. 60(6):1537-1553.

Wheat. 2020. [accessed 3 April 2021]. http://oec.world.

UN. 2019. World population prospects 2019: Highlights (st/esa/ser.A/423). In: Department of Economic and Social Affairs PD, editor.

Varanasi A, Prasad PVV, Jugulam M. 2016. Impact of climate change factors on weeds and herbicide efficacy. Advances in Agronomy. p. 107-146.

Walsh MJ, Broster JC, Schwartz-Lazaro LM, Norsworthy JK, Davis AS, Tidemann BD, Beckie HJ, Lyon DJ, Soni N, Neve P et al. 2018. Opportunities and challenges for harvest weed seed control in global cropping systems. Pest Management Science. 74(10):2235-2245.

Zhu J, Wang J, DiTommaso A, Zhang C, Zheng G, Liang W, Islam F, Yang C, Chen X, Zhou W. 2020. Weed research status, challenges, and opportunities in china. Crop Protection. 134.

# CHAPTER 2: BIOCHEMICAL AND STRUCTURAL CHARACTERIZATION OF QUIZALOFOP-RESISTANT WHEAT ACETYL-COA CARBOXYLASE

**Summary**

A novel nucleotide mutation in *ACC1* resulting in an alanine to valine amino acid substitution in acetyl-CoA carboxylase (ACCase) at position 2004 of the *Alopecurus myosuroides* reference sequence (A2004V) imparts quizalofop resistance in wheat. Genotypes endowed with one or two homozygous mutant *ACC1* homoeologs are 7- and 68-fold more resistant to quizalofop than a wildtype variety in greenhouse experiments, respectively. *In vitro* assays of ACCase activities in protein extracts from these varieties demonstrate a 3.8- and 39.4-fold increase in resistance to quizalofop in the single and double-mutants relative to the wildtype. The A2004V mutation does not alter the specific activity of wheat ACCase, suggesting that ACCase mutants retain their normal catalytic functions. Modeling of wildtype and quizalofop-resistant wheat ACCase demonstrates that the A2004V amino acid substitution causes a reduction in the volume of the binding pocket that hinders quizalofop's interaction with ACCase. Docking studies confirm that the mutation reduces the binding affinity of quizalofop. Interestingly, the models suggest that the A2004V mutation does not affect haloxyfop binding. Follow up *in vivo* and *in vitro* experiments reveal that the mutation, in fact, imparts negative cross-resistance to haloxyfop, with quizalofop-resistant varieties exhibiting more sensitivity to haloxyfop than the wildtype variety.

**Introduction**

Acetyl-CoA carboxylase (ACCase or EC 6.4.1.2) catalyzes the first committed and rate-limiting step of fatty acid biosynthesis, converting acetyl-CoA to malonyl-CoA (Numa et al. 1965). The three primary functional components of ACCase are biotin carboxylase, biotin carboxyl carrier protein, and carboxyl transferase (see Figure 1 in (Takano et al. 2021). Mechanistically, biotin carboxylase first reduces a molecule of ATP to transfer a carboxyl group from bicarbonate to the prosthetic biotin arm of the biotin carboxyl carrier protein domain. The biotin arm then moves the newly acquired carboxyl group to the carboxyl transferase domain, which carboxylates acetyl-CoA to form malonyl-CoA. Most plants have two forms of ACCase, a eukaryotic multifunctional homomeric form located in the cytosol and a prokaryotic multi-subunit form localized to plastids (Sasaki and Nagano 2004). Grasses differ from most plants by having only the eukaryotic form of the enzyme in both the cytosol and plastids (Konishi et al. 1996). Group 1 herbicides specifically target the eukaryotic form of ACCase by binding to the carboxyl transferase domain and inhibiting the second catalytic step (Nikolskaya et al. 1999), whereas these herbicides have little activity on the prokaryotic form. Consequently, group 1 herbicides are excellent graminicides (Dayan et al. 2019).

The recently commercialized CoAXium™ Wheat Production System consists of a specialized quizalofop formulation under the tradename Aggressor™ for use on wheat varieties with the AXigen™ quizalofop resistance trait coupled with a stewardship program aiming to delay resistance in weeds. Quizalofop, applied as the proherbicide form quizalofop-p-ethyl, controls grasses postemergence. Following cuticle penetration, the proherbicide is bioactivated to the active form quizalofop acid *in planta*. Plants transport the active quizalofop to

meristematic tissue, which contains a large concentration of plastid-bound ACCase. Plastid-bound wheat ACCase encoded by the gene *ACC1* occurs as a homodimer with three functional domains per monomer (Gornicki and Haselkorn 1993). Though biochemical characterization of quizalofop's binding to ACCase has not been studied, work on other aryloxyphenoxy-propionate herbicides demonstrates that these are reversible, noncompetitive inhibitors of grass ACCase (Burton et al. 1991; Rendina et al. 1988).

The AXigen™ trait is a mutant quizalofop-resistant wheat ACCase. Researchers discovered the trait via ethyl methanesulfonate (EMS) mutagenesis and subsequent screening for resistance to quizalofop (Ostlie et al. 2015). The nucleotide substitution in *ACC1* imparting quizalofop resistance in CoAXium™ wheat corresponds to position 2004 in the ACCase amino acid sequence from the *Alopecurus myosuroides* reference sequence (Ostlie et al. 2015). This mutation causes an amino acid substitution of an alanine for a slightly larger valine residue in ACCase (A2004V). Initially, the researchers identified three accessions with heterozygous resistance-conferring mutations for each of three *ACC1* gene homoeologs in hexaploid wheat (2n=6x=42) on chromosome 2 and later developed a line with a homozygous mutation for each homoeolog. Not all homoeologs contribute equally to quizalofop resistance, with the A to V mutation on the D homoeolog providing the greatest level of resistance, followed by the same mutation in the A and B homoeologs. The Wheat Breeding and Genetics Program at Colorado State University introgressed the mutations from the A and D *ACC1* homoeologs into an elite genetic background and developed the initial CoAXium™ varieties featuring the mutation in both homoeologs.

Our objectives were to determine if the amino acid substitution affects the specific activity of wheat ACCase and to describe how the substitution imparts resistance at biochemical and structural levels. We further quantified fold-differences in whole-plant and enzymatic resistance between wheat with wildtype ACCase and wheat with one or two mutant ACCase homoeologs.

We first determined whole-plant quizalofop resistance in wheat with one or two mutant ACCase homoeologs by comparison to wildtype, susceptible wheat and contrasted these experiments to the effect of quizalofop in *in vitro* assays of wheat ACCase. We then modeled the changes in the ACCase protein structure and its interaction with the herbicide using homology modelling, molecular dynamic simulations, and docking.

**Materials and Methods**

*Plant Material and Growing Conditions*

Four wheat genotypes were used to compare whole-plant resistance of lines with wildtype and mutant ACCase as well as specific activity and resistance of extracted ACCase. The AF10 and Hatcher lines were provided by Dr. Scott Haley from the Department of Soil and Crop Sciences, and the varieties Byrd, and Incline AX were obtained from the Colorado Wheat Research Foundation. All experiments carried out on these wheat varieties were done with their permission and complied with local and national regulations. Byrd is a quizalofop-susceptible variety that was the recurrent parent for Incline AX, a CoAXium™ variety, whereas Hatcher is the original mutagenized variety. AF10 is one of the initial quizalofop-resistant lines, which contains a homozygous mutant *ACC1* homoeolog in sub-genome D. Incline AX exhibits two mutant homoeologs in sub-genomes A and D. Byrd was the wildtype, non-mutant in all

physical experiments except for the quizalofop plant growth dose-response experiment, where Hatcher was substituted. AF10 and Incline AX were consistently used as single- and double-gene mutants, respectively, in the same experiments.

Plants for the whole-plant dose responses and ACCase extractions were grown from seed in soilless media. Plants were maintained in a greenhouse environment with temperatures from 20 to 25°C, relative humidity between 50 to 70%, and a 14-hour daylength with natural lighting supplemented by sodium halide lamps.

*Herbicide Dose Effect on Plant Growth*

Prior to reaching the jointing stage, wheat seedlings were treated with herbicide. Untreated seedlings of each line were also retained to normalize data. Eleven rates of herbicide were used in each dose-response experiment. Quizalofop herbicide doses for the susceptible line ranged from 3.85 and 108 g ae $ha^{-1}$, whereas doses for resistant lines ranged from 15.4 to 493 g ae $ha^{-1}$. Haloxyfop doses ranged from 15.4 to 616 g ae $ha^{-1}$ for all wheat lines. Non-ionic surfactant was added to each dose (0.25% v/v). All treatments were applied in a single pass at 40 cm above the plant canopy using a spray chamber. Each treatment was triplicated, with four plants comprising a replicate. Fresh leaf biomass was harvested three weeks after treatment. Biomass was adjusted in some instances to account for germination of fewer than four plants per replicate.

*Enzyme Extraction*

Approximately 5.0 g of crown tissue was harvested from each genotype at the two-leaf stage, flash frozen in liquid nitrogen, and stored at -80 °C. Enzyme extraction and activity assay were modified from previously published methods (Dayan et al. 2017; Seefeldt et al. 1996; Yu

et al. 2004). Frozen wheat tissue was powdered in liquid nitrogen. All subsequent extraction steps were carried out on ice or in a cold room to maintain an extract temperature of 4 °C. Ground tissue was transferred to 14.85 mL extraction buffer (100 mM Trizma, 20 mM DTT, 2 mM L-ascorbic acid, 1 mM EDTA, 0.5% w/v PVP-40, 0.5% w/v insoluble PVP-20, and 10% v/v glycerol; pH 8.0) and 150 µL of 100 mM PMSF (dissolved in 100% ethanol). The tissue was homogenized at max speed for 30 s with a homogenizer probe. Homogenate was filtered by hand with Miracloth, followed by centrifugation for 30 min at a speed of 25,000 x g. The resulting supernatant was slowly brought to 66% saturation with solid ammonium sulfate and stirred for an hour.

Ammonium sulfate solutions were centrifuged using the same parameters as the previous centrifugation step to precipitate ACCase and similar proteins. After discarding the supernatant, the protein pellets were resuspended with 2.5 mL elution buffer (50 mM tricine, 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mM DTT; pH = 8.0). The protein extracts were desalted using a gravity protocol with conditioned PD-10 columns. Desalted protein extracts were eluted with 3.5 mL of elution buffer and mixed with 25% v/v glycerol.

*Enzyme Specific Activity Assay*

To determine ACCase specific activity, four 40 µL aliquots of fresh protein extract per genotype were preincubated for 3 min in assay solution without acetyl-CoA. Acetyl-CoA was subsequently added to three aliquots to initiate enzyme activity, whereas water was added in lieu of acetyl-CoA for background readings. Preincubation and reaction steps were maintained at 32 °C with moderate shaking. Final concentrations of assay solution components for each 200 µL reaction (including enzyme aliquot) were as follows: 20 mM tricine (pH = 8.3), 10 mM

KCl, 10 mM $MgCl_2$, 5 mM ATP, 3.24 mM $NaHCO_3$, 2.5 mM DTT, 0.1% w/v BSA, 0.25 mM acetyl-CoA, and 0.25 mM $NaH^{14}CO_3$ (provides 18.5 kBq per reaction). After 10 min, enzyme reactions were quenched with 20 µL of 12 M hydrochloric acid. Solutions were transferred to 20 mL scintillation vials and left uncapped overnight to enable volatilization of unincorporated carbonate to $CO_2$. Reactions were carried out in a fume hood equipped with a $^{14}C$ filter.

The following day, 10 mL of scintillation cocktail was added to each vial. Vials were capped and vortexed for 20 s prior to analysis. Radioactivity was measured with a liquid scintillation analyzer in disintegrations per min (DPM).

*Herbicide Dose Effect on ACCase Activity*

Specific activity dose-responses were conducted using the same protocol as the specific activity assay but with the addition of herbicide immediately preceding the preincubation step. Dilution series were prepared with quizalofop acid and haloxyfop acid analytical standards dissolved in 100% acetonitrile. Triplicated dose treatments per genotype included background readings (water added in lieu of acetyl-CoA), null herbicide doses, and doses that provided 0.0100, 0.100, 1.00, 10.0, 100, and 1000 µM herbicide in the final assay solution.

*Homology Modeling of Wheat Carboxyl Transferase Domain*

The segments of wheat ACCase sequence where the A2004V substitution is located were aligned to the sequence of the CT domain of yeast ACCase using EMBOSS Needle (Li et al. 2015). This information was used to build a homology model of wheat ACCase based on the crystal coordinates of the carboxyl transferase (CT) domain of yeast ACCase (1UYS)(Zhang et al. 2004) available from the RCSB protein data bank. The homology modeling pipeline was similar to that reported previously to model the binding of glufosinate on resistant glutamine

synthetase from *Lolium perenne* (Brunharo et al. 2019). A preliminary model was obtained using Modeller 10.0 (Webb and Sali 2014; 2017). The model was assembled in its functional dimer configuration and refined using GROMACS (version 2018.3) (Abraham et al. 2015; Hess et al. 2008) on a workstation with two processors (96 threads) and video cards (10 GB GPU memory each). Steric clashes or inappropriate geometries were corrected through molecular dynamics simulation and evaluated using MolProbity (Chen et al. 2010; Davis et al. 2007) as described before.

Proteins and ligand interactions were visualized using PyMOL v.2.3.3 (Delano 2002). Additionally, the docking of quizalofop and haloxyfop to native and mutant carboxyl transferase dimers of wheat was measured using established protocols developed for Autodock (Goodsell et al. 2021; Morris et al. 2012). Briefly, the receptor was defined to encompass residues lining the herbicide binding domain. Amino acid charges were calculated using Gasteiger charges including all polar hydrogens (Gasteiger and Marsili 1980). The docking imposed a covalent map to involve the nitrogen hydrogen of ILE 222 in the docking of the herbicides within the gridbox. A total of 100 poses were generated.

*Statistical Analysis*

All statistical analysis was conducted with packages in RStudio v.1.2.5033 with R v.3.6.2 (R Core Team 2019). To model the effect of herbicide on plant growth, fresh weight data were first normalized to percent of control means. Data was transformed with a Box-Cox transformation. For quizalofop data, a two-parameter log-logistic regression was fit using the LL.4 function of package drc (Ritz et al. 2015):

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

where lower limit *c* is fixed at 0, upper limit *d* is fixed at 100, *m* is the slope, and *e* is the 50% growth reducing dose ($GR_{50}$). A four-parameter Brain-Cousens model was fit using the BC.4 function of package drc for haloxyfop data:

$$y = c + \frac{d-c+f}{1+\exp\left(m[\log(x)-\ddot{e}]\right)}, \qquad (2)$$

where lower limit *c* is fixed at 0, *d* is the upper limit, *f* is the hormesis effect, with *m* and *e* as additional parameters without direct interpretation. The $GR_{50}$ of the haloxyfop model was estimated through parameterization with the ED function of package drc. A resistance factor ($R:S_{50}$) was calculated by dividing $GR_{50}$'s of quizalofop-resistant wheat lines by the $GR_{50}$ of a wildtype, susceptible line with the EDcomp function of package drc. Student's t-tests ($\alpha$=0.05) of resistance factor ratios were used to identify significant fold differences in resistance between estimated $GR_{50}$ doses.

A linear calibration curve of absorbance versus BSA concentration was fit using the lm function of package stats (R Core Team 2019) to estimate wheat ACCase extract protein concentrations:

$$y = mx + b, \qquad (3)$$

where *m* and *b* constants and standard deviations were $2.76 \cdot 10^{-3} \pm 3.21 \cdot 10^{-4}$ and $1.40 \cdot 10^{-1} \pm 1.32 \cdot 10^{-3}$, respectively. The residual standard error of the calibration was $1.06 \cdot 10^{-3}$ with a multiple R-squared value of 0.987. Genotype protein concentrations were calculated from the calibration and adjusted for dilution.

Per genotype, the DPM measurement of a reaction to which water was added instead of acetyl-CoA was subtracted from replicate specific activity measurements as background activity. Mean DPM enzyme specific activity measurements were converted to $^{14}C$ pmol units

using manufacturer provided activity of $^{14}C$-labeled sodium bicarbonate. Specific activity was then weighted by protein concentration to yield units of $^{14}C$ pmol·mg$^{-1}$ protein. Standard errors reflect error propagation of DPM measurement replicates and protein concentration. Specific activity was compared between genotypes using ordinary one-way ANOVA F-protected Student's t-tests ($\alpha$=0.05, n=3) with anova of package stats and emmeans of package emmeans (Lenth 2020; R Core Team 2019).

For specific activity dose-response analysis, background activity was subtracted from DPM readings. Background corrected DPM readings were normalized to percent residual activity using control means for each genotype. Data was modelled using the same methods as for the effect of quizalofop on whole-plant growth, with the exception that *e* represents the 50% enzyme inhibition dose ($I_{50}$). Similar resistance factors (R:S) were calculated using $I_{50}$ estimates, followed by Student's t-tests ($\alpha$=0.05) of ratios to identify significant fold differences in resistance between estimated doses.

**Results**

*Herbicide Dose Effect on Plant Growth*

Three wheat genotypes were grown in the greenhouse to assess the contribution of a single or double homozygous *ACC1* mutation leading to whole-plant quizalofop resistance and to examine cross-resistance to haloxyfop. In general, whole-plant resistance to either quizalofop or haloxyfop varies by the number of mutant *ACC1* homoeologs present in a wheat genotype. The residual standard error of the 2-parameter log-logistic regression model with Box-Cox transformation of quizalofop doses is 11.5 with 97 degrees of freedom. For the

haloxyfop doses, the residual standard error of the 4-parameter Brain-Cousens model with Box-Cox transformation is 2.14 with 92 degrees of freedom.

The estimated quizalofop doses that reduce fresh biomass growth normalized as a percent of untreated controls by 50% ($GR_{50}$) range from 7.13 to 485 g ae·ha$^{-1}$, while haloxyfop $GR_{50}$ values range from 26.5 to 125 g ae·ha$^{-1}$ (Table 2.1 and Figure 2.1). Based on $GR_{50}$ values, single- and double-mutant genotypes are 7.00 and 68.0 times more resistant to quizalofop than the wildtype, susceptible genotype, respectively. Both quizalofop-resistant genotypes are more sensitive to haloxyfop than the wildtype. The single-mutant is about three-fold and the double-mutant is about five-fold more sensitive, respectively, than the susceptible genotype (Table 2.1). According to Student's t-tests, single- and double-mutant genotype resistance is significantly different from the susceptible genotype for both herbicides.

*Enzyme Specific Activity Assay*

Crude enzyme preparations containing ACCase activity, the target enzyme of quizalofop and other group 1 herbicides, were extracted from wheat to compare specific activity and to determine enzyme-level resistance of the same three genotypes used in the whole-plant resistance studies. Enzyme extract specific activity range from 2.66 to 3.91 nmol·mg protein$^{-1}$ (Figure 2.2). Values are not significantly different between genotypes ($F_{2,6} = 0.278$).

*Herbicide Dose Effect on ACCase Activity*

Enzyme-level resistance to either quizalofop or haloxyfop varies by the number of mutant ACC1 homoeologs present in a wheat genotype. The residual standard error of the 2-parameter log-logistic regression model with Box-Cox transformation of quizalofop doses is

0.435 with 48 degrees of freedom. A similar model of haloxyfop doses has a residual error of 0.940 with 55 degrees of freedom.

The estimated quizalofop doses that inhibit ACCase activity normalized as a percent of untreated controls to 50% ($I_{50}$) range 0.486 to 19.3 µM, while haloxyfop $I_{50}$ values range from 0.968 to 7.63 µM (Table 2.2 and Figure 2.3). ACCase extracts from single- and double-mutant genotypes are 3.8 and 39.4 times more resistant to quizalofop than the wildtype, susceptible genotype, respectively, based upon estimated $I_{50}$ values. Conversely, ACCase extracts from the same quizalofop-resistant genotypes are less resistant to haloxyfop than the susceptible genotype. The single- and double-mutants are between eight- and seven-fold more sensitive to haloxyfop than the wildtype (Table 2.2 and Figure 2.3).

While the resistance factor for the double-mutant genotype is statistically significant for quizalofop in Student's t-test, the resistance factors for the single-mutant genotype is not statistically significant though the p-value is close to the significance threshold. Resistance factors for the single- and double-mutant genotypes are significantly different from the susceptible genotype for haloxyfop.

Enzyme-level resistance factors are nearly equal to those calculated from the whole-plant dose-response trial despite a lack of significance for the single- and double-mutants compared to the susceptible. This disparity may be due to the smaller sample size used in the enzyme dose-responses, which causes larger confidence intervals for $I_{50}$ estimates across genotypes. Nonetheless, our results suggest that quizalofop resistance conferred by the AXigen™ trait is additive at both the whole-plant and enzyme-level. Estimated $GR_{50}$ values in the whole-plant dose response trials for the susceptible and single-mutant genotype were

within confidence intervals provided by Ostlie et al. (2015), yet our resistance factor estimates for the single-mutant genotype based on these values is nearly double.

The single- and double-mutants are more sensitive to haloxyfop relative to the susceptible genotype in both whole-plant and enzyme-level dose-response trials. These results suggest that while the ACCase conformational change induced by the A2004V amino acid substitution increases quizalofop-resistance, the change causes negative cross-resistance to haloxyfop (e.g., increases susceptibility to haloxyfop).

*Homology Modeling of Wheat Carboxyl Transferase Domain and Docking of Quizalofop and Haloxyfop*

Both quizalofop and haloxyfop are group 1 herbicides that target eukaryotic forms of ACCase. However, our biological studies in the greenhouse and biochemical characterization of ACCase reveal that the alanine to valine mutation imparts resistance to quizalofop both *in planta* and *in vivo*, while causing negative cross-resistance to haloxyfop. Though quizalofop and haloxyfop are structurally related (Figure 2.4A and B), quizalofop is a slightly larger molecule due to its heterobicyclic 6-chloro-quinoxalin-2-yl rings relative to the monocyclic 3-chloro-5-(trifluoromethyl)pyridin-2-yl ring of haloxyfop, with CPK volumes of 303.97 and 317.09 $A^3$, respectively (Table 2.3). Quizalofop has a smaller molecular weight than haloxyfop despite a larger volume. Both herbicides are lipophilic, though quizalofop is more so according to a greater partition coefficient (log*P*). Quizalofop has a slightly larger dipole moment than haloxyfop, indicating it is more polar. The herbicide molecules have a similar electrostatic potential (Figure 2.4C and D).

The docking energy of quizalofop increases from -6.070 to -5.700 kcal·mol$^{-1}$ from the wildtype to resistant form, whereas the docking score of haloxyfop decreases slightly from -5.074 to -5.211 kcal·mol$^{-1}$ (Table 2.4). The number of correct docking poses and root-mean-square deviation of atomic poses for all docking combinations are within acceptable limits. Clearly, both quizalofop and haloxyfop bind to the catalytic domain of the wildtype carboxyl transferase (Figures 2.5A and C). The A2004V amino acid substitution is located at the bottom left of the herbicide binding domain, where the methyl side chain of alanine occupies a smaller volume than that of the isopropyl side chain of valine. The asterisk in Figure 2.5B highlights the strong steric hindrance between the volume occupied by the valine side chain and the 6-chloro-quinoxalin-2-yl group of quizalofop. The same A2004V amino acid substitution does not prevent binding of haloxyfop (Figure 2.5D).

**Discussion**

Whole-plant quizalofop dose greenhouse experiments confirm resistance in quizalofop-resistant wheat genotypes (Table 2.1). Dose-response curves shift right with an increasing number of *ACC1* resistance homoeologs (Figure 2.1A), therefore the AXigen™ quizalofop resistance trait is additive and the double- is more resistant than the single-mutant genotype. The level of resistance to quizalofop at the enzyme-level parallels that observed in the greenhouse study (Table 2.2 and Figure 2.3A), establishing a strong relationship between the number of mutant resistant homoeologs with phenotypic and enzymatic responses.

Though the resistance factors are not significantly different for the single- and double-mutants compared to the susceptible in the enzyme-level dose-response trial with quizalofop, the values are nearly equal to the resistance factors calculated from the whole-plant dose-

response trial. The statistical disparity may be due to the smaller sample size used in the enzyme dose-responses, which causes larger confidence intervals for $I_{50}$ estimates across genotypes. Nonetheless, our results suggest that quizalofop resistance conferred by the AXigen™ trait is additive at both the whole-plant and enzyme-level. Estimated $GR_{50}$ values in the whole-plant dose response trials for the susceptible and single-mutant genotype were within confidence intervals provided by Ostlie et al. (2015), however, our resistance factor for the single-mutant genotype based on these values is nearly double.

Follow-up experiments with haloxyfop demonstrate that quizalofop-resistant genotypes are more sensitive to haloxyfop than the wildtype genotype in both whole-plant (Table 2.1) and enzyme-level (Table 2.2) dose-response trials. The leftward shift of whole-plant dose-response curves with an increasing number of mutant resisistant homoeologs indicates that the quizalofop resistance mutation imparts negative cross-resistance to haloxyfop (Figure 2.1B). Additionally, single- and double-mutant genotypes are more sensitive to haloxyfop than the susceptible genotype at the enzyme-level (Figure 2.3B), confirming the negative cross-resistance pattern observed in the greenhouse.

Computational experiments confirm aforementioned biological and biochemical findings. Protein modeling indicates that the location of the quizalofop resistance conferring A2004V substitution in wheat is within the binding domain of ACCase inhibitors, imparting a direct conformational shift (Figure 2.5). An increase in docking energy from the wildtype to quizalofop-resistant wheat ACCase model indicates reduced quizalofop binding affinity (Table 2.4) that is evident in 3D models (Figure 2.5A and B).

While the conformational change of the A2004V substitution reduces the volume of the binding pocket sufficiently to impact the binding of quizalofop because of a large heterobicyclic group (Figure 2.5A and B), it does not affect the binding of haloxyfop with a smaller monocyclic 3-chloro-5-(trifluoromethyl)pyridin-2-yl group (Figure 2.5C and D). This is further supported by haloxyfop docking energy calculations, which slightly decrease from wildtype to quizalofop-resistant ACCase (Table 2.4). These results suggest that while the conformational change associated with quizalofop-resistant ACCase increases quizalofop resistance, the change causes negative cross-resistance to haloxyfop (e.g. increases susceptibility to haloxyfop). A similar observation was reported in mutations imparting resistance to the phytoene desaturase inhibitor fluridone resulting in negative cross-resistance to some of the other phytoene desaturase-inhibiting herbicides (Arias et al. 2006).

An additional biochemical assay indicates that the conformational change associated with quizalofop-resistant ACCase does not affect specific activity. Enzyme extracts from genotypes with susceptible, wildtype *ACC1* and one or two resistant *ACC1* homoeologs lack significant differences in specific activity. Similar specific activity estimates imply that the conformational change induced by the A2004V amino acid substitution does not affect ACCase activity. This finding supports field trials that found comparable CoAXium™ wheat yields following quizalofop treatment to non-treated, quizalofop-susceptible wheat yields (data not shown).

Both quizalofop and haloxyfop are group 1 herbicides that target eukaryotic forms of ACCase, though quizalofop occupies more volume (Table 2.3 and Figure 2.4). Our biological studies in the greenhouse and biochemical characterization of ACCase reveal that the *ACC1*

mutation resulting in the A2004V amino acid substitution imparts resistance to quizalofop both *in planta* and *in vivo*, while causing negative cross-resistance to haloxyfop. The negative cross-resistance to haloxyfop is not concerning for the CoAXium™ Wheat Production System because the system is only available in the United States, where haloxyfop is not labeled for use in wheat.

A potential shortcoming of the outlined experiments is the variable genetic background of the genotypes. Genotypes were selected based on a similar genetic background of existing plant materials, however differences still exist that could affect experimental findings. Near-isogenic line development to compare copy numbers of quizalofop-resistant *ACC1* homoeologs would eliminate potential confounding effects of variable genetic background.

**Conclusions**

The AXigen™ trait, which confers quizalofop resistance in wheat, consists of a point mutation in *ACC1* resulting in an A2004V amino acid substitution in ACCase. We applied a combination of biological, biochemical, and computational methods in the study outlined herein to divulge the precise biochemical resistance mechanism imparted by the mutation. We found that the mutation induces a conformational change in ACCase that results in reduced binding affinity for quizalofop, therefore conferring quizalofop resistance at both the biochemical and whole-plant level. Simultaneously, the conformational change increases binding affinity for haloxyfop, leading to negative cross-resistance. Though negative cross-resistance is a rare phenomenon between herbicides with the same mode of action, the differential spatial size of quizalofop and haloxyfop molecules explain this finding. This study further demonstrates the utility of protein modelling and computational analysis for elucidating

biochemical mechanisms of resistance that are otherwise difficult to pinpoint through standard laboratory protocols.

**Tables**

Table 2.1. Relative herbicide dose effects on whole-plant growth by genotype[1] for 50% growth reduction ($GR_{50}$)[2]

| Herbicide | Mutant *ACC1* Homoeologs | $GR_{50}$ | R:S[3] | R:S p-value[4] |
|---|---|---|---|---|
| Quizalofop | 0 | 7.13 (1.0) | - | - |
| | 1 | 50.1(5.8) | 7.0 (1.31) | <0.0001 |
| | 2 | 485 (43.6) | 68.0 (11.6) | <0.0001 |
| haloxyfop | 0 | 125 (28.7) | - | - |
| | 1 | 40.4 (13.4) | 0.32 (0.130) | <0.0001 |
| | 2 | 26.5 (6.1) | 0.21 (0.07) | <0.0001 |

[1]Genotype refers to the number of mutant homozygous homoeologs in *ACC1* that result in a A2004V amino acid substitution and quizalofop-resistance, with up to three possible on group 2 chromosomes in hexaploid wheat (2n = 6x = 42).
[2]Leaf fresh weight values (g) normalized to percent untreated controls and transformed with Box-Cox transformation per genotype for each herbicide. Quizalofop dose effect modeled with a 2-parameter log-logistic function and haloxyfop dose effect modeled with a 4-parameter Brain-Cousens function to estimate $GR_{50}$ values.
[3]Resistance factor, calculated by dividing the $GR_{50}$ of a quizalofop-resistant genotype by the susceptible genotype $GR_{50}$.
[4]Student's t-test p-value ($\alpha$=0.05) of resistance factor ratios.

Table 2.2. Relative herbicide dose effects on ACCase activity by genotype[1] for 50% inhibition ($I_{50}$)[2]

| Herbicide | Mutant *ACC1* Homoeologs | $I_{50}$ | R:S[3] | R:S p-value[4] |
|---|---|---|---|---|
| Quizalofop | 0 | 0.49 (0.13) | - | - |
| | 1 | 1.84 (0.52) | 3.8 (1.5) | 0.0634 |
| | 2 | 19.30 (4.01) | 39.4 (13.3) | 0.00571 |
| Haloxyfop | 0 | 7.63 (1.08) | - | - |
| | 1 | 0.97 (0.21) | 0.13 (0.003) | <0.0001 |
| | 2 | 1.12 (0.25) | 0.15 (0.004) | <0.0001 |

[1]Genotype refers to the number of mutant homozygous homoeologs in *ACC1* that result in a A2004V amino acid substitution and quizalofop-resistance, with up to three possible on group 2 chromosomes in hexaploid wheat (2n = 6x = 42).
[2]Activity values (DPM) normalized to percent untreated controls and transformed with Box-Cox transformation per genotype for each herbicide. The dose effect of each herbicide is modeled with a 2-parameter log-logistic function to estimate $I_{50}$ values.
[3]Resistance factor, calculated by dividing the $I_{50}$ of a quizalofop-resistant genotype by the susceptible genotype $I_{50}$.
[4]Student's t-test p-value ($\alpha$=0.05) of resistance factor ratios.

Table 2.3. Physicochemical properties of quizalofop and haloxyfop

| Parameters | Quizalofop | Haloxyfop |
|---|---|---|
| Mw | 344.75 | 361.70 |
| log*P* | 1.89 | 0.65 |
| CPK volume $A^3$ | 317.09 | 303.97 |
| Energy (1000 kj·$mol^{-1}$) | -4,006.2 | -4,445.5 |
| Dipole | 3.51 | 3.08 |

Table 2.4**.** Docking parameters of quizalofop and haloxyfop on wheat ACCase

| | Quizalofop | Haloxyfop |
|---|---|---|
| | **wildtype wheat ACCase** | |
| Correct docking | 92/110 | 82/110 |
| Docking energy | -6.070±0.095 | -5.074±0.206 |
| RMSD | 0.273±0.021 | 0.245±0.033 |
| | **quizalofop-resistant wheat ACCase** | |
| Correct docking | 95/110 | 104/110 |
| Docking energy | -5.700±0.230 | -5.211± 0.221 |
| RMSD | 0.434±0.034 | 0.242± 0.035 |

## Figures

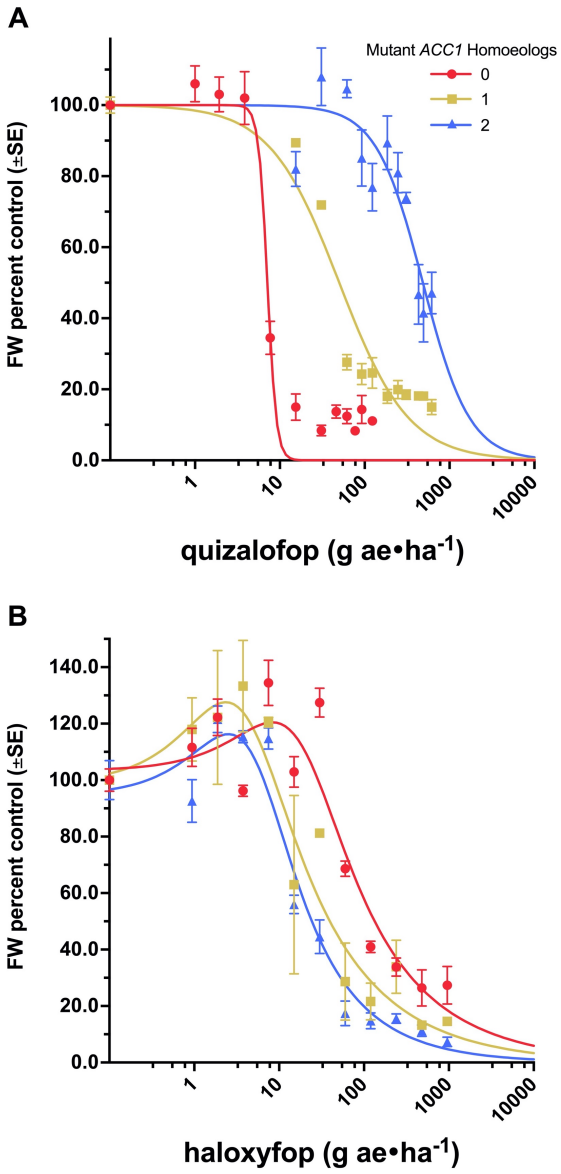



Figure 2.1. Models[1] of quizalofop (A) and haloxyfop (B) herbicide dose effects on whole-plant growth by genotype[2]

[1]Leaf fresh weight values (g) normalized to percent untreated controls and transformed with Box-Cox transformation per genotype for each herbicide. Quizalofop dose effect modeled with a 2-parameter log-logistic function (A) and haloxyfop dose effect modeled with a 4-parameter Brain-Cousens function (B).
[2]Genotype refers to the number of mutant homozygous homoeologs in *ACC1* that result in a A2004V amino acid substitution and quizalofop-resistance, with up to three possible on group 2 chromosomes in hexaploid wheat (2n = 6x = 42).

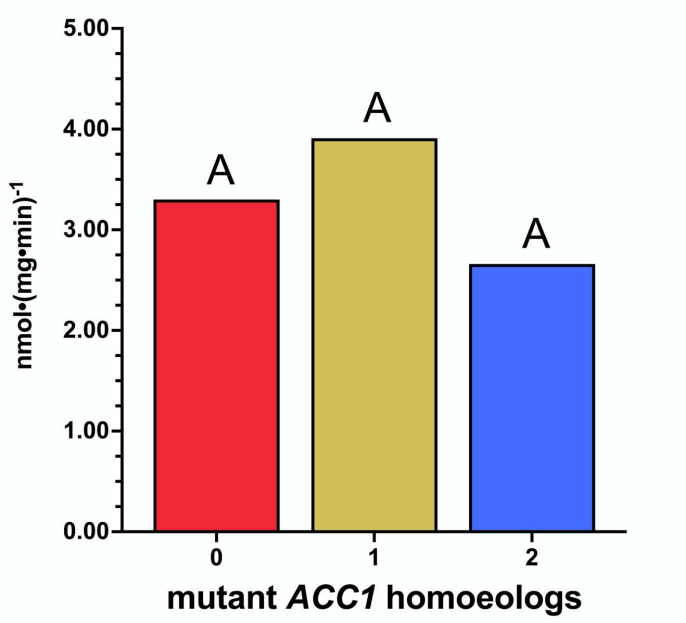



Figure 2.2. ACCase specific activity by genotype[1,2]

[1]Genotype refers to the number of mutant homozygous homoeologs in *ACC1* that result in a A2004V amino acid substitution and quizalofop-resistance, with up to three possible on group 2 chromosomes in hexaploid wheat (2n = 6x = 42).
[2]Genotype specific activities labeled with the same capital letter are not significantly different according to Student's t-tests (α=0.05).

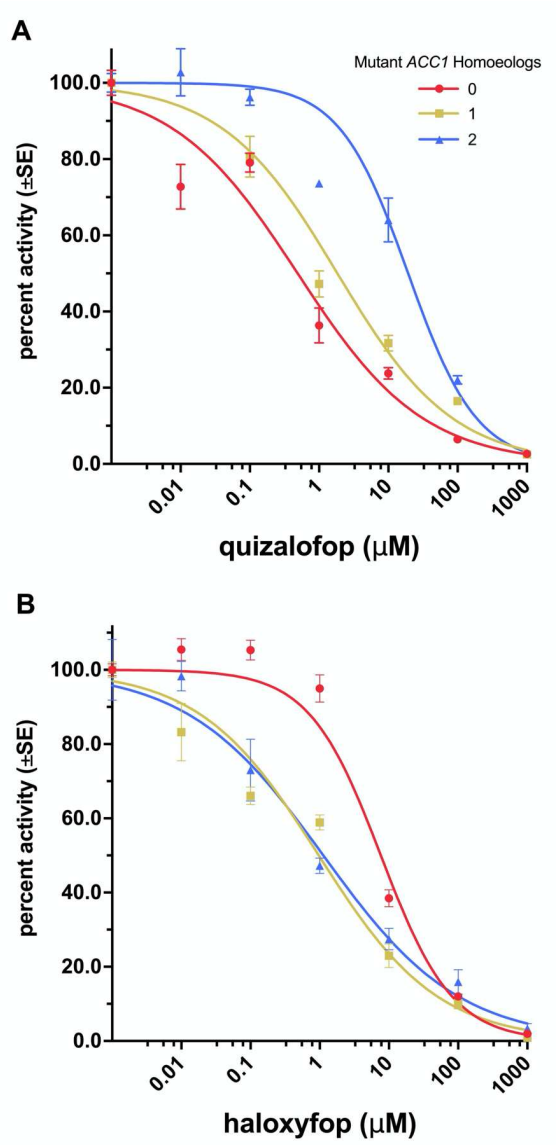



Figure 2.3. Models[1] of quizalofop (A) and haloxyfop (B) herbicide dose effects on ACCase activity by genotype[2]

[1]Activity values (DPM) normalized to percent untreated controls and transformed with Box-Cox transformation per genotype for each herbicide. The dose effect of each herbicide is modeled with a 2-parameter log-logistic function.
[2]Genotype refers to the number of mutant homozygous homoeologs in *ACC1* that result in a A2004V amino acid substitution and quizalofop-resistance, with up to three possible on group 2 chromosomes in hexaploid wheat (2n = 6x = 42).

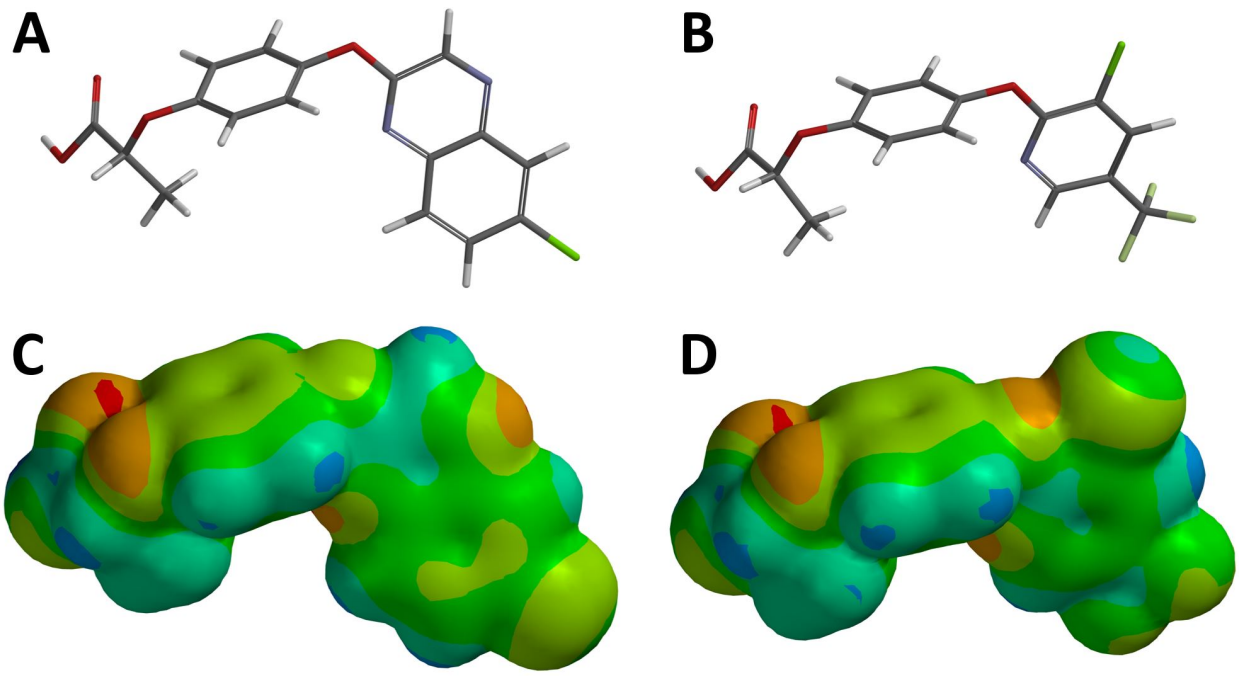



Figure 2.4**.** Structures of A) quizalofop and B) haloxyfop showing the carbons in gray, hydrogen in white, oxygen in red, nitrogen in blue, chlorine in green and fluorine in cyan on the left side. Electrostatic potential maps of C) quizalofop and D) haloxyfop. Colors on the volume represent range from electronegative (red) to electropositive (blue) partial charges.

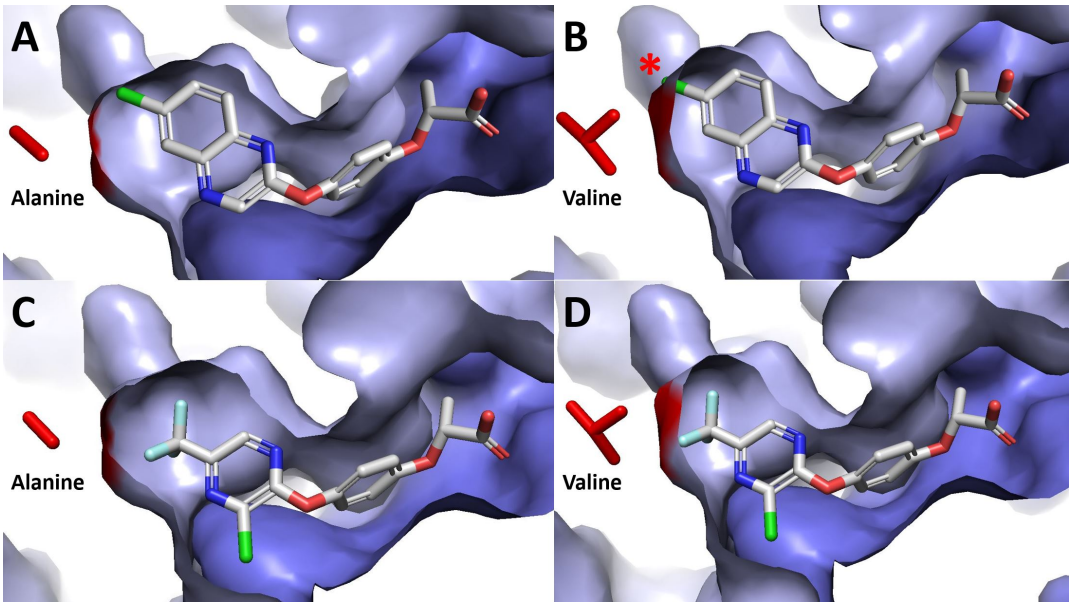



Figure 2.5. Comparison of the docking of quizalofop on A) wildtype carboxyl transferase dimer of wheat with alanine (in red) and B) mutated carboxyl transferase dimer of wheat with valine mutation (in red) to the docking of haloxyfop on C) wildtype carboxyl transferase dimer of wheat with alanine (in red) and D) mutated carboxyl transferase dimer of wheat with valine mutation (in red). Asterisk in panel B denotes the breach of the surface of the protein by quizalofop due to the increase volume of the valine side chain in the mutated ACCase.

**References**

Abraham MJ, Murtola T, Schulz R, Páll S, Smith JC, Hess B, Lindahl E. 2015. Gromacs: High performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX. 1-2:19-25.

Arias RS, Dayan FE, Michel A, Howell J, Scheffler BE. 2006. Characterization of a higher plant herbicide-resistant phytoene desaturase and its use as a selectable marker. Plant Biotechnology Journal. 4:263-273.

Brunharo CACG, Takano HK, Mallory-Smith CA, Dayan FE, Hanson BD. 2019. Role of glutamine synthetase isogenes and herbicide metabolism in the mechanism of resistance to glufosinate in *Lolium perenne* L. spp. *multiflorum* biotypes from Oregon. Journal of Agricultural Food Chemistry. 67(31):8431-8440.

Burton JD, Gronwald JW, Keith RA, Somers DA, Gengenbach BG, Wyse DL. 1991. Kinetics of inhibition of acetyl-coenzyme A carboxylase by sethoxydim and haloxyfop. Pesticide Biochemistry and Physiology. 39:100-109.

Chen VB, Arendall WB, 3rd, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC. 2010. Molprobity: All-atom structure validation for macromolecular crystallography. Acta Crystallographica Section D: Biology Crystallography. 66(Pt 1):12-21.

Davis IW, Leaver-Fay A, Chen VB, Block JN, Kapral GJ, Wang X, Murray LW, Arendall WB, 3rd, Snoeyink J, Richardson JS et al. 2007. Molprobity: All-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Research. 35(Web Server issue):W375-383.

Dayan FE, Barker A, Bough R, Ortiz M, Takano H, Duke SO. 2019. Herbicide mechanisms of action and resistance. In: Moo-Young M. Comprehensive biotechnology. Cambridge, MA: Academic Press. P. 36-48.

Dayan FE, Owens DK, Corniani N, Silva FML, Watson SB, Howell JL, Shaner DL. 2017. Biochemical markers and enzyme assays for herbicide mode of action and resistance studies. Weed Science. 63(SP1):23-63.

Delano WL. The PyMOL molecular graphics system. 2002. [accessed 2021 September 1]. http://www.pymol.org.

Gasteiger J, Marsili M. 1980. Iterative partial equalization of orbital electronegativity- a rapid access to atomic charges. Tetrahedron. 36(22):3219-3288.

Goodsell DS, Sanner MF, Olson AJ, Forli S. 2021. The autodock suite at 30. Protein Science. 30(1):31-43.

Gornicki P, Haselkorn R. 1993. Wheat acetyl-CoA carboxylase. Plant Molecular Biology. 22:547-552.

Hess B, Kutzner C, van der Spoel D, Lindahl E. 2008. Gromacs 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. Journal of Chemical Theory and Computation. 4(3):435-447.

Konishi T, Shinohara K, Yamada K, Sasaki Y. 1996. Acetyl-CoA carboxylase in higher plants: Most plants other than graminae have both the prokaryotic and the eukaryotic forms of this enzyme. Plant Cell Physiology. 37(2):117-122.

Lenth R. Emmeans: Estimated marginal means, aka least-squares means. 2020. [accessed]. https://CRAN.R-project.org/package=emmeans.

Li W, Cowley A, Uludag M, Gur T, McWilliam H, Squizzato S, Park YM, Buso N, Lopez R. 2015. The embl-ebi bioinformatics web and programmatic tools framework. Nucleic Acids Research. 43(W1):W580-584.

Morris G, Goodsell D, Pique M, Lindstrom W, Huey R, Forli S, Hart WE, Halliday S, Belew R, Olson AJ. Autodock version 4.2. 2012. [accessed 2021 September 1]. http://autodock.scripps.edu/faqs-help/manual/autodock-4-2-user-guide/AutoDock4.2.6_UserGuide.pdf.

Nikolskaya T, Zagnitko O, Tervzadze G, Haselkorn R, Gornicki P. 1999. Herbicide sensitivity determinant of wheat plastid acetyl-CoA carboxylase is located in a 400-amino acid fragment of the carboxyltransferase domain. Proceedings of the National Academy of Sciences USA. 96(25):14647-14651.

Numa S, Bortz WM, Lynen F. 1965. Regulation of fatty acid synthesis and the acetyl-CoA carboxylation step. Advances in Enzyme Regulation. 3:407-423.

Ostlie M, Haley SD, Anderson V, Shaner D, Manmathan H, Beil C, Westra P. 2015. Development and characterization of mutant winter wheat (*Triticum aestivum* L.) accessions resistant to the herbicide quizalofop. Theoretical and Applied Genetics. 128(2):343-351.

R Core Team. R: A language and environment for statistcal computing. 2019. Vienna, Austria: R Foundation for Statistical Computing; [accessed 2021 September 1]. http://www.R-project.org/.

Rendina AR, Felts JM, Beaudoin JD, Craig-Kennard AC, Look LL, Paraskos SL, Hagenah JA. 1988. Kinetic characterization, stereoselectivity, and species selectivity of the inhibition of

plant acetyl-CoA carboxylase by the aryloxyphenoxypropionic acid grass herbicides. Archives of Biochemistry and Biophysics. 265(1):219-225.

Ritz C, Baty F, Streibig JC, Gerhard D. 2015. Dose-response analysis using R. PLoS One. 10(12):e0146021.

Sasaki Y, Nagano Y. 2004. Plant acetyl-CoA carboxylase: Structure, biosynthesis, regulation, and gene manipulation for plant breeding. Bioscience, Biotechnology, and Biochemistry. 68(6):1175-1184.

Seefeldt SS, Fuerst EP, Gealy DR, Shukla A, Irzyk GP, Devine MD. 1996. Mechanisms of resistance to diclofop of two wild oat (*Avena fatua*) biotypes from the Willamette Valley of Oregon. Weed Science. 44(4):776-781.

Takano HK, Ovejero RFL, Belchior GG, Maymone GPL, Dayan FE. 2021. ACCase-inhibiting herbicides: Mechanism of action, resistance evolution and stewardship. Scientia Agricola. 78(1).

Webb B, Sali A. 2014. A comparative protein structure modeling using modeller. Current Protocols in Bioinformatics. 47:5.6.1-32.

Webb B, Sali A. 2017. Protein structure modeling with modeller. Functional Genomics: Methods in Molecular Biology. New York, NY: Humana Press.

Yu Q, Shane Friesen LJ, Zhang XQ, Powles SB. 2004. Tolerance to acetolactate synthase and acetyl-coenzyme A carboxylase inhibiting herbicides in *Vulpia bromoides* is conferred by two co-existing resistance mechanisms. Pesticide Biochemistry and Physiology. 78(1):21-30.

Zhang H, Tweel B, Tong L. 2004. Molecular basis for the inhibition of the carboxyltransferase domain of acetyl-coenzyme-a carboxylase by haloxyfop and diclofop. Proceedings of the National Academy of Sciences USA. 101(16):5910-5915.

# CHAPTER 3: LOW TEMPERATURE SLOWS METABOLIC DETOXIFICATION OF QUIZALOFOP IN RESISTANT WINTER WHEAT AND THREE ANNUAL GRASS WEED SPECIES


**Summary**

Quizalofop-resistant wheat is the core component of the recently commercialized CoAXium™ Wheat Production System. As with other herbicides, quizalofop provides better weed control at early growth stages. Application at these stages under ideal temperatures are sometimes proceeded by unpredicted, rapid temperature decreases. Temperature shifts may cause crop injury or impact weed control efficacy. In the following study, we examine the effect of reduced temperature on quizalofop content and metabolism in CoAXium™ winter wheat and three weed species. Temperature conditions are either 19°C or 4.5°C daytime temperatures with tissue sampling over 5 timepoints (1 to 16 or 18 days after treatment, DAT). Analysis features liquid chromatography coupled to mass spectrometry detection of the active form of quizalofop, quizalofop acid. Wheat reaches the greatest quizalofop content 2 DAT in the warmer temperature whereas the maximum amount occurs 8 DAT in the cooler temperature. There are differences in maximums for weed species as well, where downy brome (*Bromus tectorum* L.) and jointed goatgrass (*Aegilops cylindrica* Host) have a greater maximum content at the cooler temperature, relative to feral rye (*Secale cereale* L.). The presence of maximum content differences in the aforementioned weeds suggests greater absorption and/or de-esterification of quizalofop proherbicide to the active form at cooler temperatures. Quizalofop content trends over time reveal delayed, suppressed metabolism under cooler conditions for wheat and weeds. Based on trends across timepoints in the warmer temperatures for all

species, quizalofop content peaks within 1 to 2 DAT and consistently decreases thereafter. In contrast, content at cooler temperatures peak around 8 to 9 DAT and are generally followed by minimal decreases in content. Final dry shoot tissue biomass does not necessarily correspond to differences in metabolism, as final wheat biomass is not different between treatments. Weed biomass is only different for jointed goatgrass, which has a greater biomass in the cooler temperature.

**Introduction**

The CoAXium™ Wheat Production System centers on wheat with the AXigen™ trait for quizalofop resistance (Bough et al., 2021). The AXigen™ trait is an alanine to valine mutation at position 2004 of wheat acetyl-CoA carboxylase (ACCase) (Ostlie et al., 2015) causing an enzyme conformational change that imparts resistance to quizalofop acid (QZA) (submitted manuscript). The system provides effective post-emergence control of annual weedy grasses in winter wheat where control options are limited and is paired with a stewardship program to mitigate development of herbicide-resistant weeds. The original QZA-resistant winter wheat mutant accessions were discovered at Colorado State University. Homozygous resistance mutations in two of three wheat sub-genomes were introgressed into a susceptible elite genetic background. High-performing progeny were selected and released as initial CoAXium™ varieties in partnership with the Colorado Wheat Research Foundation, LLC.

The CoAXium™ Wheat Production System consists of a customized proherbicide quizalofop-p ethyl (QPE) formulation under the tradename Aggressor™. The proherbicide passively penetrates cuticle tissue and is subsequently bioactivated to the active QZA form by

carboxyesterases in wheat and other grass species (Haslam et al., 2001;Cummins and Edwards, 2004).

Though QPE application is more desirable at early growth stages in winter wheat (i.e. 4$^{th}$ leaf stage) to provide maximal weed control, environmental conditions may rapidly change from ideal to sub-optimal after treatment. Spring herbicide applications in High Plains winter wheat production are particularly risky due to unpredicted weather shifts that can cause sudden temperature plunges, as decreased temperatures proceeding herbicide application may result in unintended crop injury. For example, an investigation in Canadian winter wheat of the effect of application timing and temperature of several herbicides demonstrates that cooler temperatures cause crop injury after the application of 2,4-D alone or in combination with dichlorprop, as well as a mixture of dicamba, MCPA, and mecoprop (Robinson et al., 2015).

Plants that are acclimated to cold may be able to tolerate herbicides despite reduced temperatures, as is the case with rice (*Oryza sativa* L.) and bispyribac (Martini et al., 2021). Conversely, reduced temperatures generally suppress plant growth and slow metabolism, especially in plants that have been dehardened, or lost acclimation to cool temperatures as is the case for most plants in spring. One reason for this is that most enzymes have an optimal temperature range and deviations from the optimal range cause decreased enzyme activity. Optimal enzyme temperature ranges are typically above freezing. Further, herbicide absorption, translocation, accumulation, metabolism, and sequestration may be impeded at low temperatures (Kudsk and Kristensen, 1992;Ghanizadeh and Harrington, 2017), which may explain crop injury due to otherwise safe herbicides.

Low temperatures can also negatively affect herbicide efficacy. In Palmer amaranth (*Amaranthus palmeri* S. Wats.), pyrithiobac suppresses growth most at a moderate temperature (27°C) compared to low (18°C) or high (40°C) temperatures, where efficacy is reduced (Mahan et al., 2004). Efficacy is reduced below and above the moderate temperature because an enzyme involved in metabolism is more active at all temperatures besides the moderate temperature, resulting in resistance. Thermal-dependency has also been observed for glutathione reductase, an enzyme involved in herbicide metabolism, in spinach (*Spinacia oleraceae* L.), corn (*Zea mays* L.), and cucumber (*Cucumis sativus* L.) (Mahan et al., 1990). QPE activation in particular may be reduced by low temperatures in weed species, as both herbicide absorption and de-esterification are affected by temperature.

Climate change may impact the efficacy of current weed management strategies. It has been postulated that elevated temperatures and $CO_2$ levels can reduce the efficacy of many pesticides by altering metabolism and translocation of xenobiotics in target organisms (Matzrafi, 2019), and this applies to increase resistance to herbicides in weeds due to lower herbicide persistence in the soil and greater abilities to metabolize herbicides (Refatti et al., 2019;Korres and Dayan, 2020). This seems to be particularly true for postemergent gramicide (Neal et al., 1990;Cieslik et al., 2013). Typically, increased herbicidal activity of ACCase inhibitors at higher air humidity and temperature (up to a certain point) are associated with increased absorption and the translocation of the herbicides. Finally, low temperatures reduce the overall metabolic activity in the plant, which makes plants generally less susceptible to herbicides. However, metabolic degradation of ACCase inhibitors may also be reduced at lower temperatures, thus prolonging the duration of exposure to the active ingredient. Warmer

temperatures can increase the risk of herbicide-resistant weeds due to enhanced detoxification. For example, detoxification of pinoxaden was higher under elevated temperatures, resulting in poor herbicide performance (Matzrafi et al., 2016). Similarly, increasing the air temperature from 18 to 30°C reduced fluazifop efficacy on green foxtail [*Setaria viridis* (L.) P. Beauv.] at low herbicide rates but this did not affect Japanese millet [*Echinochloa esculenta* (A. Braun) H. Scholz] control (Smeda and Putnam, 1990). Similarly, the efficacy of haloxyfop-methyl was less injurious to green foxtail and proso millet (*Panicum milliaceum* L.) when under higher temperature and relative humidity (Shekoofa et al., 2020).

Studying the effect of low temperatures on wheat or weed metabolism is warranted due to the potential for unforeseen, temperature-dependent injury following QPE application to CoAXium™ winter wheat. The objective of our study was to investigate the effect of decreased temperature on QZA content and metabolism after herbicide application in CoAXium™ winter wheat and three weed species. We focus on herbicide metabolism because of the propensity of this mechanism to overcome injury and the existence of several known cases of hindered herbicide metabolism at low temperatures. Our metabolism measurements, which rely on liquid chromatography coupled to mass spectrometry (LC-MS/MS) to target and quantify QZA in plant tissues, may also serve as an indicator of proherbicide absorption and de-esterification.

## Material and Methods

### *Plant Material and Initial Growing Conditions*

Initially, the effect of temperature on QZA metabolism in two winter wheat varieties was investigated. The cultivars were CoAXium™ varieties LCS Fusion AX and Incline AX, both of

which were derived from EMS mutagenesis of Hatcher seed and are resistant to QZA through a double homozygous resistance mutation for the *ACC1* gene in sub-genomes A and D. The varieties share a pedigree of approximately 69% Byrd and 31% Hatcher.

Four wheat seeds were sown per each 10×10 cm container of peat-based soilless media and germinated in a greenhouse. After two weeks, seedlings had begun jointing and were moved to a cold room for six weeks of vernalization. The cold room was maintained at 4.5°C (40°F) and 60% relative humidity with 13.5 h of light provided per day.

The study was expanded to three weedy annual grass species in Colorado winter wheat cropping systems: downy brome (*Bromus tectorum* L.), feral rye (*Secale cereale* L.), and jointed goatgrass (*Aegilops cylindrica* Host). Field-collected weed seed was surface sterilized with 10% bleach for 15 min and rinsed five times with tap water prior to germination on wetted filter paper in petri dishes. Then, weed seedlings were transferred to the same sized containers as wheat with equivalent seedlings per container. Initial growth conditions mirrored those of wheat.

*Herbicide Application*

Following vernalization, wheat plants were moved to a greenhouse to de-harden. After a week and a half, QPE (Aggressor, Albaugh, Inc) was applied at a rate equivalent to 77.1 g ae $ha^{-1}$ (10.0 fl oz $acre^{-1}$) with 0.25% NIS in a single pass at 40 cm above the plant canopy using a research table sprayer (model Gen IV, Devries Manufacturing, Hollandale, MN). The same protocol was followed for weed species, with the exception of a reduced QPE rate equivalent to 15.0 g ae $ha^{-1}$ (1.95 fl oz $acre^{-1}$) in order to challenge the plants without killing them.

*Temperature Conditions*

Half of the plants of each wheat cultivar and weed species were moved to a controlled environment with a daytime temperature of 19°C whereas the other half were moved to a controlled environment with a daytime temperature of 4.5°C. Both environments featured a nighttime temperature of 4.5°C and 60% relative humidity. Temperature conditions reflect the average daytime temperature in northern Colorado in April (19°C ) as well as a below-average daytime temperature variation (4.5°C). Daylengths were 13.5 h of light providing a photon flux of 132 µmol $m^{-2}$ $sec^{-1}$. Pre- and post-herbicide application temperature conditions are summarized in Table 3.1.

*Metabolism Experimental Design and Harvest*

The overall design for metabolism experiments was a three-way factorial design of two wheat cultivars or three weed species by two temperature treatments across five discrete timepoints in two repeated trials. Plants of each wheat variety were harvested from both temperature regimes at 1, 2, 4, 8, and 18 d after treatment (DAT), whereas plants of each weed species were harvested at 1, 2, 4, 9, and 16 DAT. For wheat, each cultivar and timepoint combination was quintuplicated, with three to four plants comprising a replicate. In the first iteration of the weed metabolism experiment, species and timepoint combination was also quintuplicated, though three to four plants comprised a replicate for downy brome and feral rye and two plants for jointed goatgrass. A weed species replicate in the second trial included four plants. At harvest, wheat and weed shoot tissue was removed, weighed, and washed with 20% v/v acetone in water to remove herbicide residue. Shoots were stored at -20°C until processing.

*Herbicide Extraction, Detection, and Quantification*

QZA was extracted from approximately 2.5 g of plant tissue using a modified QuEChERS ("quick, easy, cheap, effective, rugged, safe") protocol. Samples were first homogenized in 10 mL distilled water with a homogenizer probe. An equivalent volume of acetonitrile with 0.1% formic acid was added each sample. Samples were subsequently vortexed, then mixed for 10 min at room temperature and 250 rpm. Each sample was immediately vortexed following the addition of a salt mixture (4 g $MgSO_4$ and 1 g NaCl). To induce biphasic separation, samples were centrifuged for 10 min at room temperature and 4,000×g. Supernatant from each sample was filtered with 0.2 µm PTFE for ultra-pure liquid chromatographic (UPLC) analysis.

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 time was 3.2 min.

Samples were grouped by timepoint and replicate number with a randomized treatment order for UPLC analysis in all trials. Samples were injected in 10 µL aliquots. Prior to, in the middle of, and after sample runs, serial dilutions of QZA analytical standard (≥95%, Sigma-Aldrich) in acetonitrile with 0.1% v/v formic acid were injected to quantify QZA peak counts. Several blank replicates were also included throughout sample runs to monitor method consistency and signal-to-noise ratio. In the first wheat trial, standard concentrations were 2.00, 6.00, 18.0, 54.0, 162, and 486 pg $\mu L^{-1}$ while concentrations in the second wheat trial were 2.06, 6.17, 18.5, 55.6, 167, and 500 pg $\mu L^{-1}$. Standard concentrations were 0, 0.690, 2.06, 6.17, 18.5, 55.6, 167, and 500 pg $\mu L^{-1}$ in the first and second weed trials.

*Biomass Experimental Design and Harvest*

The overall design for biomass experiments was a two-way factorial design of the same wheat cultivars and weed species from the metabolism experiments. Plants were propagated, treated with herbicide, and exposed to the environmental conditions as described in the first three method sections. Plants of each wheat variety and weed species from temperature regimes were harvested at 18 and 16 DAT, respectively. Every wheat variety or weed species by temperature treatment combination had five replicates comprised of four plants. At harvest, wheat and weed shoot tissue was removed, dried in a drying oven, and weighed for dry biomass.

*Statistical Analysis*

All statistical analysis was conducted with packages in RStudio v.1.2.5033 with R v.3.6.2. For each metabolism trial, a linear calibration curve of peak means weighted

by standard deviations versus standard concentration was fit using the stats function of package lm:

$$y = mx + b, \qquad (1)$$

where *y* is concentration and *x* is peak area, while *m* and *b* are constants. Sample peak areas were converted to concentration using linear calibration curves and were normalized by sample fresh mass before statistical analysis. Individual observations within temperature treatments by cultivar were screened for outliers using 1.5 interquartile range limits below or above the first or third quartile. Normality and variance diagnostics were used to identify additional outliers and to ensure ANOVA assumptions were fulfilled. Datasets were $\log_{10}$-transformed as necessary to approximate a normal distribution.

Homogeneity of wheat and weed metabolism data across trials was assessed using a Levene's test ($\alpha = 0.05$) to determine if trial data could be pooled. For each trial, a three-way ANOVA ($\alpha = 0.05$) was used to evaluate timepoint, temperature treatment, 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 temperature treatments per cultivar as well as content between cultivars per temperature treatments were contrasted using F-protected Student's t-tests ($\alpha = 0.05$). Further, temperature 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 temperature 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 temperature treatment, variety or species, and their interaction as significant predictors for biomass. When ANOVA had a significant F-test, biomass values were further contrasted between temperature treatments per variety or species using F-protected Student's t-tests ($\alpha = 0.05$). Pairwise comparisons were not conducted if the ANOVA F-test was not significant.

**Results**

*Statistical Overview of the Data*

The Levene's test for homogeneity of data by wheat variety and weed species between trials indicates data is heterogenous and therefore cannot be pooled. Efforts were made to account for confounding effects between trials, including germination, vernalization and environmental conditions (i.e. daylength, light quantity, temperatures, watering, and fertilization), yet the heterogeneity of the data implies an unidentified source of variation. Variation may be associated with systematic errors related to UPLC equipment as sample sets were analyzed at different moments in time. Nonetheless, overall trends are consistent between replications in both years for wheat and weeds. For brevity, only data from one of the trials is presented and discussed hereafter. Supporting biomass data was also recorded in the second but not first trial. Results of the duplicate trial are available as supplementary tables (S1 and 2) and figures (S1 and 2). Statistical analysis further reveals that the 4 DAT weed species timepoint in the second trial is an outlier, therefore subsequent analysis excludes this timepoint.

*Metabolism of QZA in Winter Wheat and Impact on Dry Biomass*

There are no significant differences in maximum QZA amounts between temperature conditions for both varieties individually or between maximum concentrations by variety at the same temperature (Table 3.2). A lack of maximum content differences suggests that wheat varieties absorb and de-esterify QPE proherbicide at similar rates. The wheat varieties are further unlikely to exhibit decreased herbicide absorption or de-esterification at cooler temperatures. Timing of maximum QZA content is also consistent, where maximum content occurs at 2 DAT at 19°C but is delayed until 8 DAT at 4.5°C for both varieties.

There are several significant differences in QZA content between temperature conditions by timepoint for both wheat varieties (Figure 3.1). Though content is greater in LCS Fusion AX at 19°C than at 4.5°C at 1 through 4 DAT, content is greater at 4.5°C at 8 through 18 DAT (Figure 3.1A). In Incline AX, content is greater under the 19°C versus 4.5°C conditions at 1 and 2 DAT, the same at 4 DAT, and smaller at 19°C by 8 and 18 DAT (Figure 3.1B).

Examining QZA content differences over time at each temperature by variety, there are significant difference in all treatment combinations (Figure 3.1). Within the 19°C treatment of LCS Fusion AX, QZA content is the same from 1 to 4 DAT, decreases by 8 DAT, then decreases further by 18 DAT (Figure 3.1A). In the 4.5°C treatment, content is mostly the same between 1 and 4 DAT, then increases to a peak at 8 DAT after which content plateaus and remains the same by 18 DAT. Trends are similar at both temperatures with Incline AX (Figure 3.1B).

Differences collectively imply that QZA content is initially greater at 19°C versus 4.5°C, but varieties can metabolize QZA and significantly reduce content by 4 DAT. In the 4.5°C treatment, QZA accumulates until 8 DAT but does not decrease significantly thereafter.

Metabolism of QZA most likely occurs at cooler temperatures but proceeds at a slower rate than captured within the duration of this study.

Despite potential metabolism differences for QZA between temperature treatments, dry biomass is not significantly different between varieties by temperature combinations (Figure 3.2). This implies that decreased metabolism at cooler temperatures does not results in greater crop injury.

*Metabolism of QZA in Weed Species and Impact on Dry Biomass*

There are significant differences in maximum QZA content between temperature treatments for downy brome and jointed goatgrass but not feral rye (Table 3.3). For the former two species, the maximum amount is greater at 4.5°C than at 19°C. Across species, content is greatest in jointed goatgrass for both temperature treatments. Downy brome and feral rye have a similar content at 19°C, though downy brome has a greater content than feral rye at 4.5°C. A greater content in any of the pairwise comparisons implies greater absorption and/or de-esterification of QPE. In this study, therefore, downy brome and jointed goatgrass absorb more proherbicide or convert more herbicide to the active form at cooler relative to warmer temperatures.

Moreover, peak QZA content timing varies between species. Maximum QZA content at 19°C occurs at 1 and 2 DAT in feral rye and jointed goatgrass, respectively, while maximum content for both species occurs at 9 DAT at 4.5°C. In downy brome, maximum QZA content appears at 1 DAT in both temperatures. The absence of a timing difference for maximum QZA content in downy brome suggests that metabolism is not as reduced at cooler temperatures relative to the other weed species.

There are several significant differences in QZA content between temperature treatments by timepoint per weed species (Figure 3.3). For downy brome, the only content difference is at 9 DAT, where content is greater at 4.5°C relative to 19°C (Figure 3.3A). QZA content in feral rye is greater at 19°C than 4.5°C at 1 DAT, the same at 2 DAT, and greater at 4.5°C at 9 and 16 DAT (Figure 3.3B). Finally, jointed goatgrass has a greater QZA content at 19°C than 4.5°C at 1 and 2 DAT, but a greater content reached at later timepoints under colder conditions (Figure 3.3C). Differences for feral rye and jointed goatgrass are similar to those of winter wheat. QZA content is initially greater at 19°C versus 4.5°C, but content is metabolized and reduced between 2 and 9 DAT.

Over time, there are also QZA content differences for most temperature treatments across weed species (Figure 3.3). Differences over timepoints for any given treatment implies metabolism is occurring. In the 19°C treatment of downy brome, QZA content peaks from 1 to 2 DAT and decreases by 9 DAT, after which content plateaus to 16 DAT (Figure 3.3A). The 4.5°C treatment of downy brome peaks later between 1 to 9 DAT and decreases by 16 DAT. Trends of both feral rye temperature treatments are the same as downy brome (Figure 3.3B). Jointed goatgrass trends at 19°C are slightly different than the other two weed species, where content decreases between 9 and 16 DAT rather than plateauing (Figure 3.3C). Content across the 4.5°C treatment of jointed goatgrass is not significantly different.

Despite potential metabolism differences for QZA between temperature treatments, dry biomass is only significantly different between temperature treatments for jointed goatgrass but no other weed species (Figure 3.4). Specifically, biomass is greater at 4.5°C than 19°C treatment. This implies that decreased metabolism at cooler temperatures does not affect

overall plant growth for downy brome or feral rye, but that jointed goatgrass potentially increases growth in response to the presence of herbicide at cooler temperatures. If metabolism is decreased in cool temperatures, increasing growth is an effective means of diluting herbicide content.

**Discussion**

*Metabolism of QZA in Winter Wheat and Impact on Dry Biomass*

In order to measure the rate of metabolic degradation of QZA, the recommended field rate of QPE (77.1 g ae $ha^{-1}$) was applied to the herbicide-resistant CoAXium™ wheat varieties. Overall, QZA metabolism is reduced in cooler temperatures relative to warmer temperatures. This is consistent with previous studies that indicate suppressed herbicide metabolism at cooler temperatures in other grass crops, such as bispyribac in rice (Martini et al., 2015) or metolachlor in corn (Viger et al., 1991). Despite the striking delayed metabolism of QZA with wheat grown at 4.5°C, relative to 19°C, there was no difference in dry biomass between wheat varieties (Figure 3.2). This implies that decreased metabolism at cooler temperatures does not result in greater crop injury, most likely because of the presence of the AXigen™ resistant trait in these varieties. A similar study mentions unaffected yield despite visible injury in wheat at 14 days following application of various herbicides at low temperatures (Robinson et al., 2015). While the metabolism curves in wheat differ as a result of temperature, the maximum QZA contents observed were the same, suggesting that overall absorption of QPE is the same regardless of temperature. One other factor impacted by low temperature may be the rate of bioactivation of QPE into QZA by native carboxyesterases (Table 3.2).

*Metabolism of QZA in Weed Species and Impact on Dry Biomass*

The rate of QZA degradation QZA in weeds was measured following application of lower rate of QPE (15.0 g ae $ha^{-1}$), relative to the dose used on CoAXium™ wheat, in order to challenge the plants without killing them. Both feral rye and jointed goatgrass have a greater maximum content at 4.5°C versus 19°C, suggesting increased rates of proherbicide absorption or activation. Other studies report decreased herbicide absorption at cooler temperatures in grass crops, including bisypribac in rice (Martini et al., 2015). Conversely, absorption is sometimes increased at cooler temperatures in crops, such as metolachlor in corn (Viger et al., 1991). Further experiments are needed to validate differential absorption or activation differences due to temperature. This may be achieved by measuring QPE in addition to quizalofop acid with UPLC-MS/MS or with conventional radiochemical absorption assays.

A greater content in any of the pairwise comparisons implies greater absorption and/or de-esterification of QPE. In this study, therefore, downy brome and jointed goatgrass absorb more proherbicide or convert more herbicide to the active form at cooler relative to warmer temperatures. Other studies confirm decreased herbicide absorption at cooler temperatures in some weeds, including nicosulfuron in quackgrass [*Elymus repens* (L.) Gould] and metribuzin in downy brome as well as jointed goatgrass (Buman et al., 1992;Bruce et al., 1996).

Differences on the whole imply that QZA content is initially greater at 19°C versus 4.5°C, but that all weed species can metabolize QZA and significantly reduce content by 4 DAT. QZA accumulates until 9 DAT at 4.5°C, however, subsequent metabolism is less certain due to timepoint limitations. QZA metabolism is delayed in cooler temperatures relative to warmer temperatures. Reduced temperatures may increase certain weed species' susceptibility to

herbicides and cause growth reductions at lower doses, as seen with the application of mesotrione to waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] and large crabgrass [*Digitaria sanguinalis* (L.) Scop.] under cool temperatures (Johnson and Young, 2002). Increased susceptibility may be attributed to suppressed metabolism.

**Conclusions**

The capacity of CoAXium™ wheat varieties to metabolize QZA is reduced by lower temperature but this is not sufficient to overcome the protection to this herbicide afforded by the AXigen™ resistance trait, with no impact on biomass accumulation. All weed species have the capacity to metabolize QZA under both temperature conditions except for jointed goatgrass at 4.5°C, but as with wheat, the rate of metabolic degradation is slower at the colder temperatures. The impact of temperature on metabolism of the herbicide does not affect its efficacy on the weed species except for jointed goatgrass, which is better controlled at 4.5°C.

**Tables**

Table 3.1. Overview of pre- and post-herbicide treatment durations and temperatures.

| | Description | Duration | Temperature (°C, Day/Night) |
|---|---|---|---|
| **Pre-treatment** | Seedling Growth | 2 weeks | 22/18 |
| | Vernalization | 6 weeks | 4.5/4.5 |
| | Dehardening | 10 days | 22/18 |
| **Post-treatment[1]** | 19°C | 16-18 days[2] | 19/4.5 |
| | 4.5°C | 16-18 days | 4.5/4.5 |

[1]Both post-treatments were held at 60% relative humidity and with a daylength of 13.5 h of light providing a photon flux of 132 µmol $m^{-2}$ $sec^{-1}$.
[2]Treatment duration was 16 days for weed species and 18 days for wheat varieties.

Table 3.2. Maximum quizalofop content differences between temperature treatments per winter wheat variety and by temperature treatment across varieties.

| | Temperature (°C) | Maximum Content ($log_{10}$(ng.g $FW^{-1}$) ± SE) | | DAT[1] |
|---|---|---|---|---|
| LCS Fusion AX | 19 | 1.22 ± 0.110 | a[2] | 2 |
| | 4.5 | 1.07 ± 0.0983 | a | 8 |
| Incline AX | 19 | 1.24 ± 0.0983 | a | 2 |
| | 4.5 | 1.13 ± 0.110 | a | 8 |

[1]Days after treatment.
[2]There were no significant differences between least square means of temperature treatments per variety (lowercase letters) according to F-protected Student's t-tests ($\alpha$=0.05).

Table 3.3. Maximum quizalofop content differences between temperature treatments per weed species and by temperature treatment across species.

| Species | Temperature (°C) | Maximum Content (ng.g $FW^{-1}$ ± SE) | | | | DAT[1] |
|---|---|---|---|---|---|---|
| Downy brome | 19 | 52.3 ± 4.02 | b[2] | B[3] | | 1 |
| | 4.5 | 57.3 ± 4.02 | a | | B | 1 |
| Feral rye | 19 | 54.4 ± 5.19 | a | B | | 1 |
| | 4.5 | 53.3 ± 4.02 | a | | C | 9 |
| Jointed goatgrass | 19 | 67.7 ± 4.02 | a | A | | 2 |
| | 4.5 | 57.2 ± 4.02 | b | | A | 9 |

[1]Days after treatment.
[2]For each species, different lowercase letters represent significantly different least square means between temperature treatments according to F-protected Student's t-tests ($\alpha$=0.05).
[3]Different capital letters represent significantly different least square means within a respective temperature treatment across varieties according to F-protected Student's t-tests ($\alpha$=0.05).

## Figures

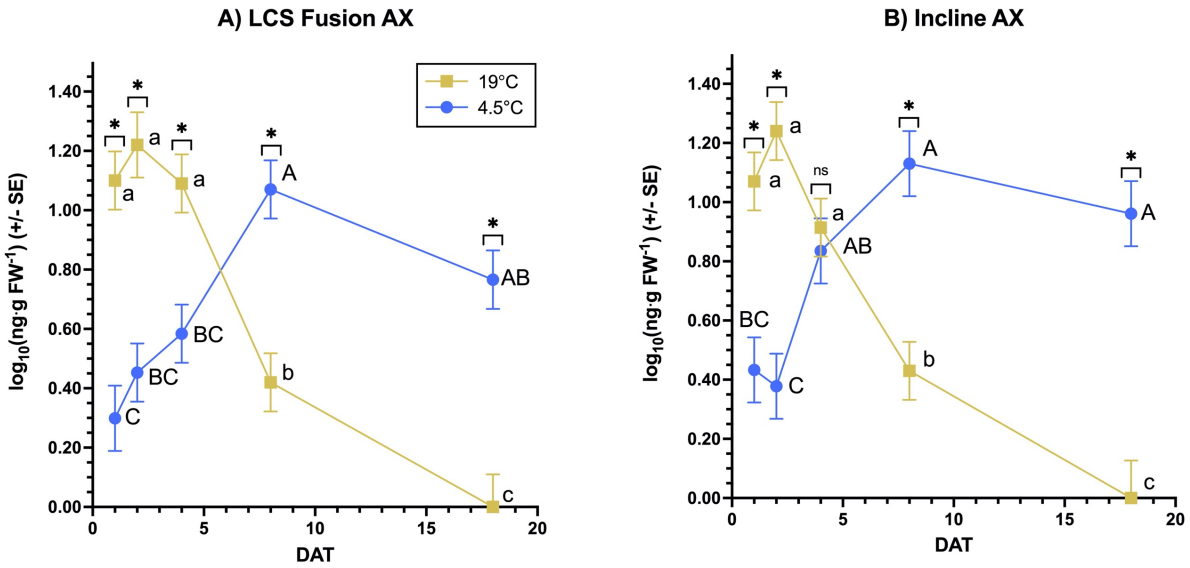



Figure 3.1. Quizalofop acid content over time by temperature treatment per winter wheat variety[1,2]

[1]For each variety (A-B), an asterisk specifies significantly different least square means between temperature treatments at a timepoint, while 'ns' indicates no significant differences according to F-protected Student's t-tests ($\alpha$=0.05).
[2]By variety, different lowercase letters and capital letters represent significantly different least square means between timepoints in 19°C and 4.5°C treatments, respectively, according to Student's t-tests with Tukey's HSD adjustments ($\alpha$=0.05).

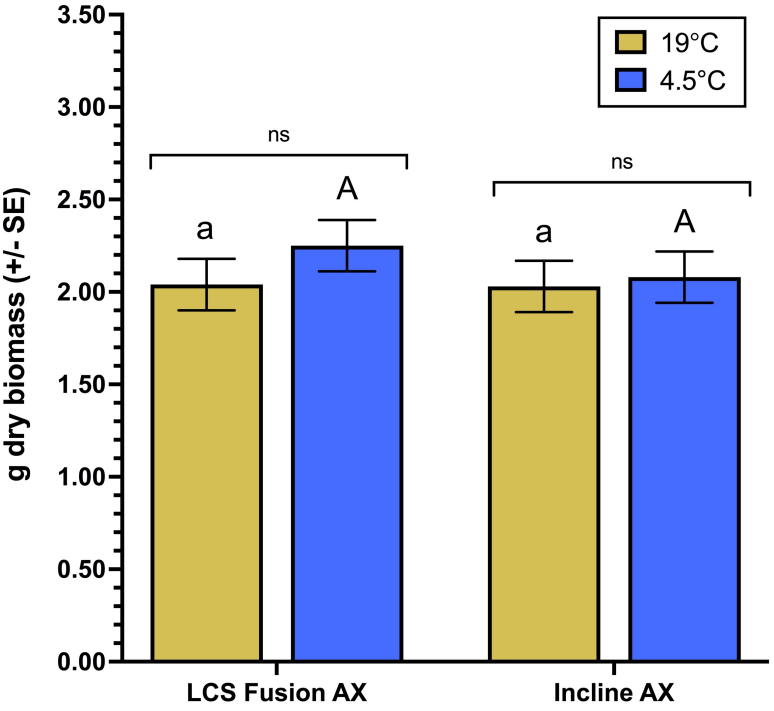



Figure 3.2. Final dry biomass by temperature treatment per winter wheat variety[1,2]

[1]The abbreviation 'ns' indicates no significant differences between least square means of temperature treatments per variety according to F-protected Student's t-tests ($\alpha$=0.05).

[2]Different lowercase letters and capital letters represent significantly different least square means across varieties between 19°C and 4.5°C treatments, respectively, according to F-protected Student's t-tests ($\alpha$=0.05).

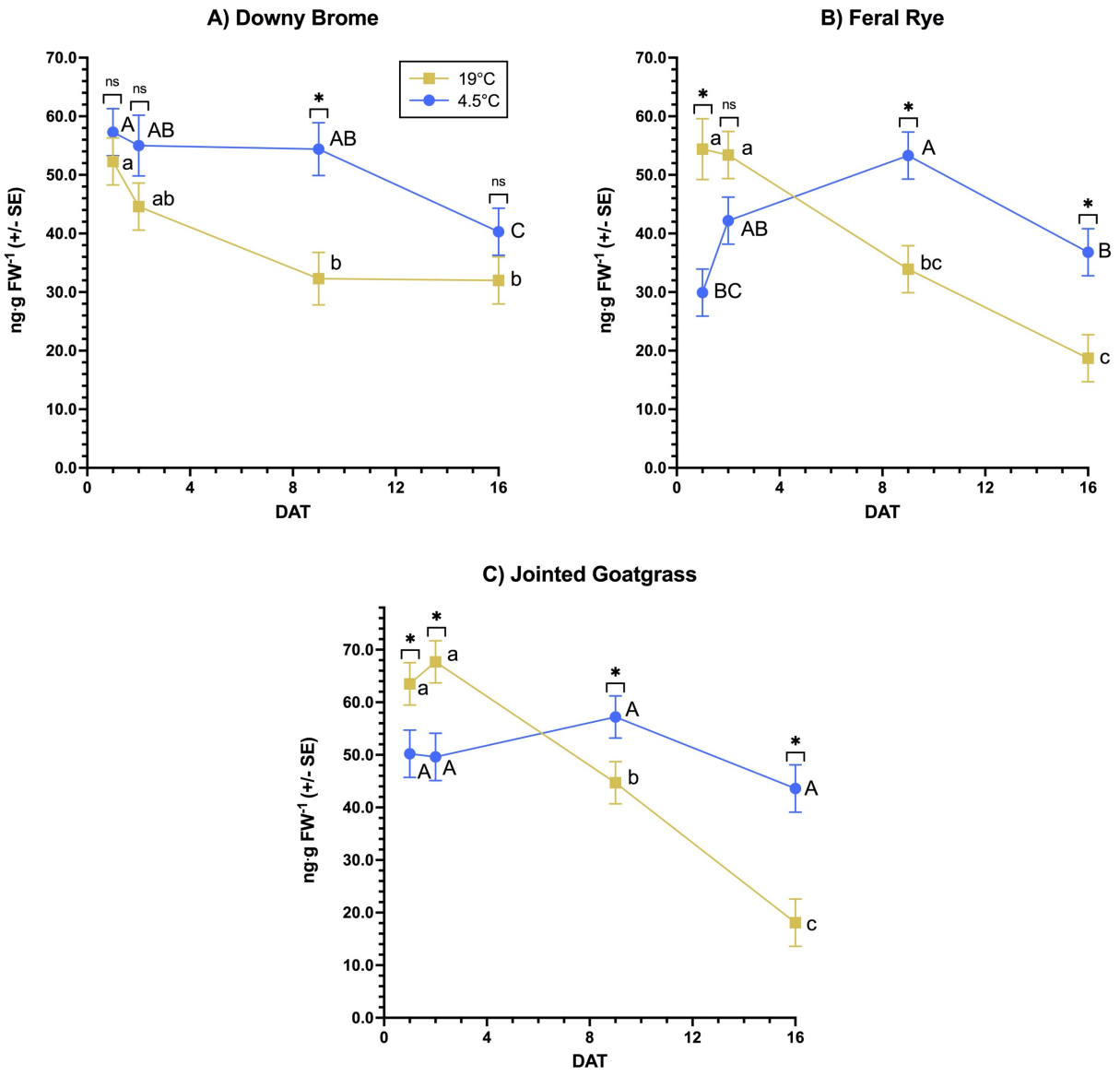



Figure 3.3. Quizalofop acid content over time by temperature treatment per weed species[1,2]

[1]For each species (A-C), an asterisk specifies significantly different least square means between temperature treatments at a timepoint, while 'ns' indicates no significant differences according to F-protected Student's t-tests ($\alpha$=0.05).

[2]By species, different lowercase letters and capital letters represent significantly different least square means between timepoints in 19°C and 4.5°C treatments, respectively, according to Student's t-tests with Tukey's HSD adjustments ($\alpha$=0.05).

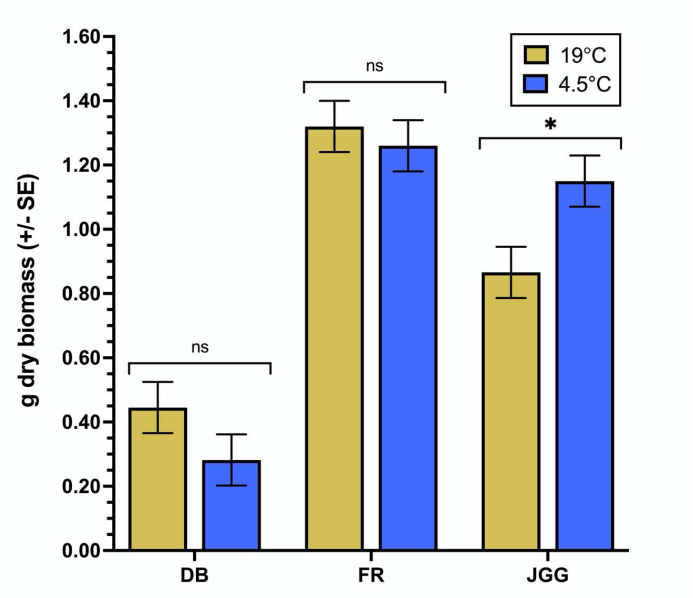



Figure 3.4. Final dry biomass by temperature treatment per weed species.[1,2]

[1]For each species, an asterisk specifies a significant difference between least square means of temperature treatments, while 'ns' indicates no significant differences according to F-protected Student's t-tests ($\alpha$=0.05).

**Supplementary Tables**

Supplemental Table S3.1. Maximum quizalofop content differences between temperature treatments per winter wheat variety and by temperature treatment across varieties.

| Variety | Temperature (°C) | Maximum Content ($log_{10}$(ng.g $FW^{-1}$) ± SE) | | | | DAT[1] |
|---|---|---|---|---|---|---|
| LCS Fusion AX | 19 | 1.29 ± 0.0862 | a[2] | A[3] | | 2 |
| | 4.5 | 1.03 ± 0.0771 | b | | B | 4 |
| Incline AX | 19 | 1.04 ± 0.0771 | b | B | | 2 |
| | 4.5 | 1.28 ± 0.0771 | a | | A | 1 |

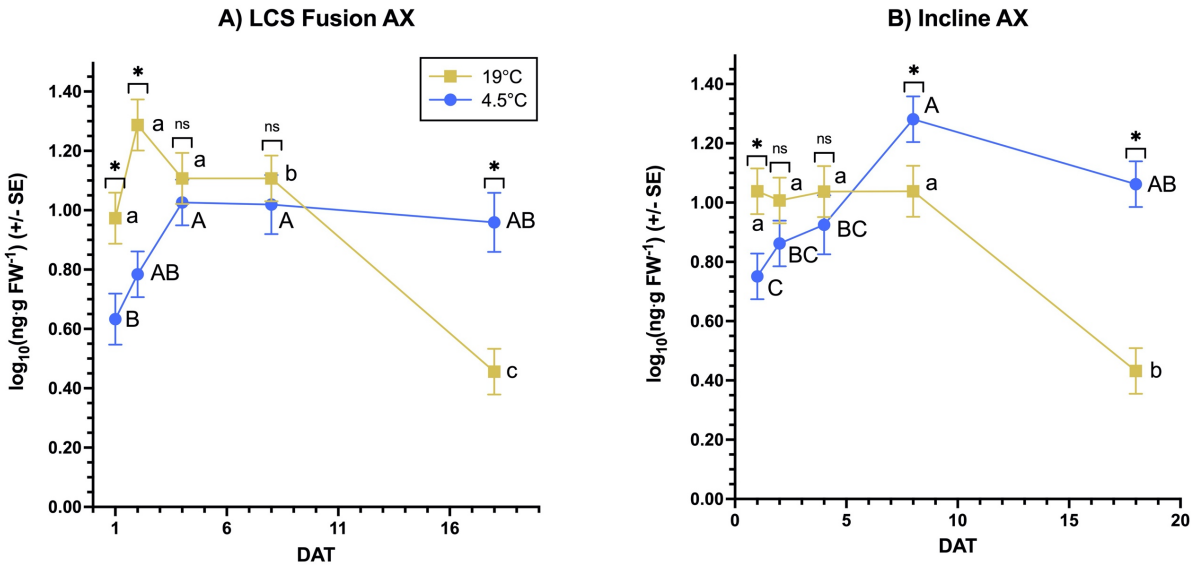
[1]Days after treatment.
[2]For each variety, different lowercase letters represent significantly different least square means between temperature treatments according to F-protected Student's t-tests ($\alpha$=0.05).
[3]Different capital letters represent significantly different least square means within a respective temperature treatment across varieties according to F-protected Student's t-tests ($\alpha$=0.05).

Supplemental Table S3.2. Maximum quizalofop content differences between temperature treatments per weed species and by temperature treatment across species.

| Species | Temperature (°C) | Maximum Content (ng.g $FW^{-1}$ ± SE) | | | | DAT[1] |
|---|---|---|---|---|---|---|
| Downy brome | 19 | 13.9 ± 1.82 | a[2] | A[3] | | 2 |
| | 4.5 | 18.5 ± 1.82 | a | | A | 1 |
| Feral rye | 19 | 17.1 ± 1.82 | a | A | | 1 |
| | 4.5 | 14.1 ± 1.82 | a | | A | 4 |
| Jointed goatgrass | 19 | 9.12 ± 2.35 | a | B | | 1 |
| | 4.5 | 6.71 ± 2.35 | a | | B | 2 |

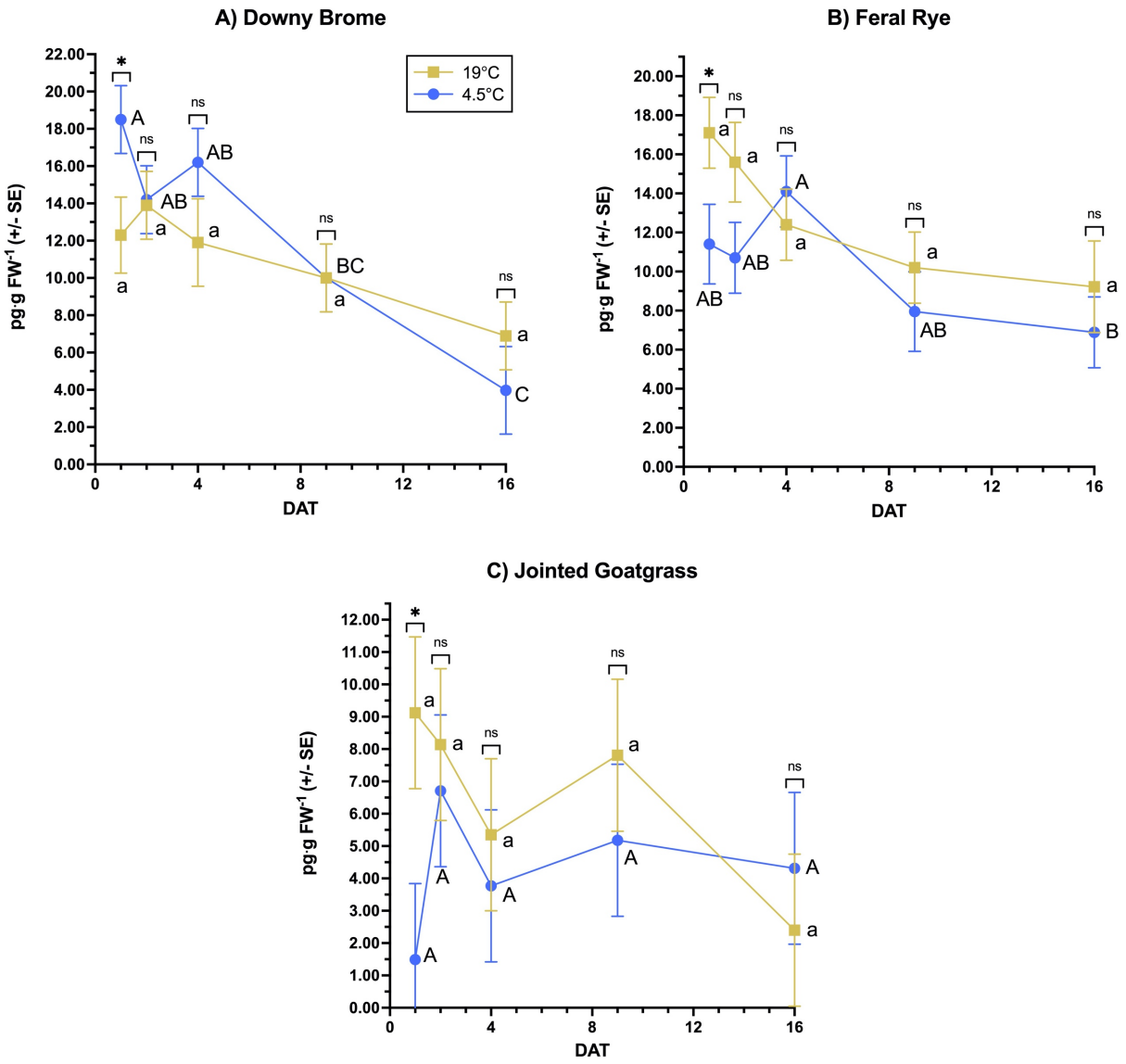
[1]Days after treatment.
[2]For each species, different lowercase letters represent significantly different least square means between temperature treatments according to F-protected Student's t-tests ($\alpha$=0.05).
[3]Different capital letters represent significantly different least square means within a respective temperature treatment across varieties according to F-protected Student's t-tests ($\alpha$=0.05).

## Supplementary Figures




Supplemental Figure S3.1. Quizalofop acid content over time by temperature treatment per winter wheat variety.[1,2]

[1]For each variety (A-B), a * specifies significantly different least square means between temperature treatments at a timepoint, while 'ns' indicates no significant differences according to F-protected Student's t-tests (a=0.05).
[2]By variety, different lowercase letters and capital letters represent significantly different least square means between timepoints in 19°C and 4.5°C treatments, respectively, according to Student's t-tests with Tukey's HSD adjustments ($\alpha$=0.05).




Supplemental Figure S3.2. Quizalofop acid content over time by temperature treatment per weed species.[1,2]

[1]For each species (A-C), a * specifies significantly different least square means between temperature treatments at a timepoint, while 'ns' indicates no significant differences according to F-protected Student's t-tests (a=0.05).
[2]By species, different lowercase letters and capital letters represent significantly different least square means between timepoints in 19°C and 4.5°C treatments, respectively, according to Student's t-tests with Tukey's HSD adjustments ($\alpha$=0.05).

**References**

Bough RA, Gaines TA, Westra EP, Haley S, Erker B, Shelton C, Reinheimer J, Dayan FE. 2021. The CoAXium™ Wheat Production System: A new herbicide-resistant system for annual grass weed control and integrated weed management. Outlooks on Pest Management. 32(4):151-157.

Bruce JA, Carey JB, Penner D, Kells JJ. 1996. Effect of growth stage and environment on foliar absorption, translocation, metabolism, and activity of nicosulfuron in quackgrass (*Elytrigia repens*). Weed Science. 44(3):447-454.

Buman RA, Gealy DR, Ogg AGJ. 1992. Effect of temperature on root absorption of metribuzin and its ethylthio analog by winter wheat (*Triticum aestivum*), jointed goatgrass (*Aegilops cylindrica*), and downy brome (*Bromus tectorum*). Weed Science. 40(4):517-521.

Cieslik LF, Vidal RA, Trezzi MM. 2013. Environmental factors affecting the efficacy of accase-inhibiting herbicides: A review. Planta Daninha. 31(2):483-489.

Cummins I, Edwards R. 2004. Purification and cloning of an esterase from the weed black-grass (*Alopecurus myosuroides*), which bioactivates aryloxyphenoxypropionate herbicides. Plant J. 39(6):894-904.

Ghanizadeh H, Harrington KC. 2017. Non-target site mechanisms of resistance to herbicides. Critical Reviews in Plant Sciences. 36(1):24-34.

Haslam R, Raveton M, Cole DJ, Pallett KE, Coleman JOD. 2001. The identification and properties of apoplastic carboxylesterases from wheat that catalyse deesterification of herbicides. Pesticide Biochemistry and Physiology. 71(3):178-189.

Johnson BC, Young BG. 2002. Influence of temperature and relative humidity on the foliar activity of mesotrione. Weed Science. 50:157-161.

Korres NE, Dayan FE. 2020. Effects of climate change on crops and weeds: The need for climate-smart adaptation paradigm. Outlooks on Pest Management. 31:210-215.

Kudsk P, Kristensen JL. 1992. Effect of environmental factors on herbicide performance. Proceedings of the First International Weed Control Congress; 1992; Melbourne, Australia. Melbourne, Australia: Weed Science Society of Victoria Inc. p. 173-186.

Mahan JR, Burke JJ, O'rzech KA. 1990. Thermal dependence of the apparent *km* of glutathione reductases from three plant species. Plant Physiology. 93:822-824.

Mahan JR, Dotray PA, Light GG. 2004. Thermal dependence of enzyme function and inhibition; implications for, herbicide efficacy and tolerance. Physiologia Plantarum. 120:187-195.

Martini LDF, Roma-Burgos N, Tseng TM, Fipke VM, Noldin AJ, de Avila, L.A. 2021. Acclimation to cold stress reduces injury from low temperature and bispyribac-sodium on rice. Pest Management Science. 77(9):4016-4025.

Martini LF, Burgos NR, Noldin JA, de Avila LA, Salas RA. 2015. Absorption, translocation and metabolism of bispyribac-sodium on rice seedlings under cold stress. Pest Management Science. 71(7):1021-1029.

Matzrafi M. 2019. Climate change exacerbates pest damage through reduced pesticide efficacy. Pest Management Science. 75(1):9-13.

Matzrafi M, Seiwert B, Reemtsma T, Rubin B, Peleg Z. 2016. Climate change increases the risk of herbicide-resistant weeds due to enhanced detoxification. Planta. 244(6):1217-1227.

Neal JC, Bhowmik PC, Senesac AF. 1990. Factors influencing fenoxaprop efficacy in cool-season turfgrass. Weed Technology. 4(2):272-278.

Ostlie M, Haley SD, Anderson V, Shaner D, Manmathan H, Beil C, Westra P. 2015. Development and characterization of mutant winter wheat (*Triticum aestivum* L.) accessions resistant to the herbicide quizalofop. Theoretical and Applied Genetics. 128(2):343-351.

Refatti JP, de Avila LA, Camargo ER, Ziska LH, Oliveira C, Salas-Perez R, Rouse CE, Roma-Burgos N. 2019. High [$co_2$] and temperature increase resistance to cyhalofop-butyl in multiple-resistant *Echinochloa colona*. Frontiers in Plant Science. 10(529).

Robinson MA, Letarte J, Cowbrough MJ, Sikkema PH, Tardif FJ. 2015. Winter wheat (triticum aestivum l.) response to herbicides as affected by application timing and temperature. Canadian Journal of Plant Science. 95(2):325-333.

Shekoofa A, Brosnan JT, Vargas JJ, Tuck DP, Elmore MT. 2020. Environmental effects on efficacy of herbicides for postemergence goosegrass (*Eleusine indica*) control. Scientific Reports. 10(1):20579.

Smeda RJ, Putnam AR. 1990. Influence of temperature, rainfall, grass species, and growth stage on efficacy of fluazifop. Weed Technology. 4(2):349-355.

Viger PR, Eberlein CV, Fuerst EP, Gronwald JW. 1991. Effects of CGA-154281 and temperature on metolachlor absorption and metabolism, glutathione content, and glutathione-*S*-transferase activity in corn (*Zea mays*). Weed Science. 39(3):324-328.

# CHAPTER 4: CONTRIBUTION OF METABOLISM TO OVERALL QUIZALOFOP RESISTANCE IN WHEAT VARIETIES WITH THE AXIGEN™ TRAIT

**Summary**

The CoAXium™ Wheat Production System, a new weed control tool, incorporates quizalofop-resistant wheat with a specialized formulation of quizalofop-p-ethyl (Aggressor™) and stewardship guidelines for system longevity. A homozygous mutation for a single-gene trait patented under the name AXigen™ confers quizalofop resistance in CoAXium™ varieties. Specifically, the trait encodes quizalofop-resistant acetyl-CoA carboxylase. Non-target site herbicide resistance associated with AXigen™ resistance has not been investigated, though these mechanisms may contribute to overall resistance. Knowledge of additional quantitative resistance has the potential to facilitate efficient breeding of regionally adapted varieties with quizalofop resistance and to further increase in-field quizalofop treatment safety margins. The following study features several biological experiments that suggest herbicide metabolism differences exist between varieties with the AXigen™ trait. Methods include enzyme and whole-plant quizalofop resistance quantification, plant tissue quizalofop content measurement over time, and assessment of the effect of cloquintocet-mexyl, a metabolism-boosting wheat safener, on quizalofop resistance. Despite similar enzyme-level resistance between resistant varieties, whole-plant resistance differs. Quizalofop content half-lives also differ between two CoAXium™ winter wheat varieties, where Incline AX has a shorter half-life than LCS Fusion AX. Moreover, the inclusion of cloquintocet with quizalofop enhances metabolism in experimental spring wheat lines with the AXigen™ trait, thereby compensating for reduced whole-plant

resistance compared to the CoAXium™ winter wheat varieties. A differential expression analysis of all varieties treated with cloquintocet may provide additional evidence of differential metabolism from biological methods. Parental genotype inclusion in the differential expression analysis may further reveal the hereditary source of metabolism differences.

**Introduction**

The CoAXium™ Wheat Production System features varieties with the AXigen™ trait for quizalofop resistance. The trait confers resistance through a point mutation that causes a single amino acid substitution in acetyl-CoA carboxylase (ACCase), the enzyme target of quizalofop and other aryloxyphenoxypropionate (FOP), cyclohexanedione (DIM), or phenylpyrazoline (DEN) group 1 herbicides. Though few highly effective post-emergent herbicides exist for weedy annual grasses in winter wheat (*Triticum aestivum* L.) such as feral rye (*Secale cereale* L.), the CoAXium™ System offers an additional tool that provides efficacious, affordable control (Kumar et al. 2020). The CoAXium™ System further incorporates a novel stewardship component by promoting management techniques that extend the field lifetime of quizalofop and delay the development of herbicide-resistant weeds.

Colorado State University developed initial CoAXium™ varieties in conjunction with the Colorado Wheat Research Foundation, LLC following discovery of the AXigen™ trait in a population mutagenized by ethyl methanesulfonate. Specifically, the trait involves a mutation in the *ACC1* gene causes an alanine to valine amino acid substitution in ACCase at position 2004 relative to the *Alopecurus myosuroides* protein sequence (Ostlie et al. 2015). Through conventional breeding, CoAXium™ varieties boast two homologous, homozygous resistance mutations in A and D wheat sub-genomes. Two copies of the AXigen™ trait sufficiently impart

resistance to label rates of Aggressor™ (Albaugh, LLC), the quizalofop-p-ethyl formulation used within the CoAXium™ system (Wheat Breeding and Genetics Program 2020; Limagrain Cereal Seeds 2021).

Target-site or single gene resistance constitutes the primary herbicide resistance mechanism in commercialized herbicide-resistant crops, including wheat (Nakka et al. 2019). Herbicide resistance in weeds can be conferred by similar target-site resistance, non-target site resistance, or a combination thereof. Regarding non-target site resistance specifically, the majority of enhanced herbicide metabolism mechanisms involve increased activity of cytochrome P450 monooxygenases (P450s), glutathione-S-transferases (GSTs), glycosyltransferases (GTs), esterases, or hydrolases (Delye et al. 2013).

Herbicide metabolism comprises four distinct phases (Carrvalho et al. 2009). In the first phase, herbicide molecules undergo chemical modifications such as oxidation, reduction, hydrolysis, oxygenation, or hydroxylation. The most prevalent enzymes associated with phase I are P450s. Phase II involves conjugation of herbicide metabolites from phase I with sugars, amino acids, or glutathione. Predominant enzymes that carry out this phase include GSTs and GTs. Phase III comprises further conjugation of herbicide metabolism or transport to vacuoles, the latter case often mediated by ATP binding cassette (ABC) promotors. Occasionally, herbicide metabolism includes phase IV, where metabolites are compartmentalized to vacuoles or associated with cell wall components. Over the course of metabolism, herbicide metabolite mobility and phytotoxicity decrease.

The genetic sequence encoding for ACCase is highly conserved across wheat homoeologs (Gornicki et al. 1997), likely owing to the fact that the enzyme is critical to fatty

acid synthesis and therefore plant growth (Sasaki and Nagano 2004). Transcription for all three wheat ACCase homoeologs occurs at similar rates (Chalupska et al. 2008). More recent analyses further indicate similar ACCase expression in comparable plant tissues (Borrill and Ramirez-Gonzalez 2016; Ramirez-Gonzalez et al. 2018). Despite these wheat ACCase characteristics, initial AXigen™ trait research revealed differences in overall quizalofop resistance among homoeologs (Ostlie et al. 2015). It is unknown whether additional metabolism mechanisms contribute to overall plant resistance, yet both mutagenesis and selective breeding may result in unintended carryover or exclusion of non-target traits. Knowledge of non-target site genes that enhance resistance conferred by target-site genes may augment introgression of the AXigen™ trait into locally adapted varieties and reduce the chances of crop injury in specific environmental conditions.

The purpose of this study was to investigate metabolism associated with quizalofop resistance in wheat in order to identify metabolism mechanisms that have the potential to boost overall resistance in CoAXium™ varieties and expedite AXigen™ trait introgression. First, enzyme-level quizalofop resistance was examined in ACCase extracted from wheat varieties or experimental lines with the AXigen™ trait as well as background parents. Whole-plant resistance was then quantified, quizalofop content was measured over time following quizalofop application, and the effect of cloquintocet-mexyl safener on quizalofop resistance was compared in select experimental lines.

## Materials and Methods

### *Plant Material and Growing Conditions*

Quizalofop metabolism in resistant wheat was investigated using seven winter and spring wheat varieties or experimental lines (Table 4.1). Three susceptible varieties were included for their background contribution to quizalofop-resistant genotypes, specifically Hatcher, Byrd, and Express. The remaining four genotypes were resistant, including winter wheat CoAXium™ varieties LCS Fusion AX (Limagrain Cereal Seeds, Inc) and Incline AX (PlainsGold, Inc) as well as experimental spring wheat lines COS14-D002 and COS14-D004 from the Wheat Breeding and Genetics Program at Colorado State University. All resistant genotypes contain two resistance-conferring mutant *ACC1* homoeologs (Ostlie et al. 2015) in sub-genomes A and D. The seven genotypes were used in each experiment, with the exception of the whole-plant dose-response experiment, where Byrd was omitted, as well as the cloquintocet dose-response experiment analysis, where only Express, COS14-D002, and COS14-D004 were used.

Plants from all experiments were grown from seed in soilless media. Plants were cultivated in a greenhouse environment with relative humidity between 50 to 70%, daytime temperatures from 20 to 25°C, and a 14-hour daylength with natural lighting supplemented by sodium halide lamps. Water, fertilizer, and pest management was provided as necessary.

### *Acetyl-CoA Carboxylase Activity Over Increasing Herbicide Doses*

Acetyl-CoA carboxylase was extracted from susceptible and resistant wheat varieties to determine enzyme-level quizalofop resistance. At the two-leaf growth stage, 5.0 g of crown tissue was harvested from each variety, flash frozen in liquid nitrogen, and stored at -80°C until extraction. Previously published protocols were adapted for the enzyme extraction and activity

assay methods outlined in this manuscript (Dayan et al. 2017; Seefeldt et al. 1996; Yu et al. 2004). To initiate extraction, frozen wheat tissue was ground using liquid nitrogen in a mortar and pestle. Subsequent extraction steps were performed on ice to maintain sample temperatures at 4°C. Tissue was immediately transferred to 14.85 mL extraction buffer (100 mM Trizma, 20 mM dithiothreitol (DTT), 2 mM L-ascorbic acid, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% w/v polyvinylpyrrolidone (PVP-40), 0.5% w/v insoluble PVP-20, and 10% v/v glycerol; pH 8.0) with 150 µL of 100 mM phenylmethylsulfonyl fluoride (PMSF) (dissolved in 100% ethanol) and mixed with a homogenizer probe at max speed for 30 s. Homogenate was filtered through Miracloth prior to centrifugation for 30 min at 25,000 x g.

Supernatants were transferred to beakers in a 4°C cold room, where remaining extraction steps were conducted. Each supernatant was brought to 66% saturation with solid ammonium sulfate and gently stirred. After an hour, ACCase and similarly sized proteins were precipitated via centrifugation using the same parameters as the previous centrifugation step. Supernatant was discarded and remaining protein pellets were resuspended with 2.5 mL elution buffer (50 mM tricine, 50 mM KCl, 2.5 mM $MgCl_2$, and 1 mM DTT; pH = 8.0). To improve extract stability, protein resuspensions were desalted using conditioned PD-10 columns and eluted with 3.5 mL of elution buffer. Extracts were diluted to 25% v/v with glycerol and stored at -80°C prior to activity dose-response assays.

A single assay was conducted per genotype and consisted of reactions with 40 µL of enzyme extract. Each assay consisted of 8 triplicated treatments for a total of 24 reactions. One treatment was background acetyl-CoA readings, another was a null herbicide treatment, and

the remaining six were herbicide dose treatments. Herbicide doses were prepared as a dilution series with quizalofop acid analytical standard (≥95%, Sigma-Aldrich) dissolved in 100% acetonitrile to provide 0.0100, 0.100, 1.00, 10.0, 100, and 1000 µM herbicide in final reaction solutions.

Enzyme extracts and assay solutions were combined in 1.5 mL microtubes prior to preincubation. Subsequently, herbicide doses were provided in 10 µL aliquots. Solutions were preincubated for 3 minutes at 32°C with moderate shaking. Following preincubation, enzyme activity was initiated using acetyl-CoA substrate. Water was used in place of acetyl-CoA for background readings. Reaction mixtures were maintained at the same temperature and shaking speed as preincubation. Concentrations of assay solution components for each 200 µL reaction excluding herbicide doses were as follows: 20 mM tricine (pH = 8.3), 10 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 3.24 mM $NaHCO_3$, 2.5 mM DTT, 0.1% w/v BSA, 0.25 mM acetyl-CoA, and 0.25 mM $NaH^{14}CO_3$ (provides 18.5 kBq per reaction). After 10 minutes, reactions were quenched with 20 µL of 12 M hydrochloric acid, transferred to 20 mL scintillation vials, and left uncapped overnight to enable volatilization of unincorporated carbonate to CO2. Assays were conducted in a fume hood equipped with a 14C filter.

A 10 mL volume of scintillation cocktail was added to each vial after overnight volatilization. Vials were capped and vigorously vortexed for 20 s. Sample radioactivity, proportional to ACCase activity, was measured in disintegrations per minute (DPM) with a liquid scintillation analyzer.

*Whole-Plant Injury Over Increasing Herbicide Doses*

Susceptible and resistant wheat genotypes were subjected to a range of herbicide doses to quantify plant-level quizalofop resistance. Following the two-leaf growth stage but before jointing, plants were retained as untreated controls or treated with a series of 11 quizalofop doses in triplicate, with four plants comprising a replicate. Herbicide doses for the susceptible varieties ranged from 1.00 to 123 g ae $ha^{-1}$, whereas doses for resistant genotypes ranged from 15.4 to 616 g ae $ha^{-1}$. Treatments were applied using singles passes in a spray chamber with the sprayer height at 40 cm above the plant canopy. Non-ionic surfactant (NIS) was added to each dose at 0.25% v/v. Three weeks after treatment, visual injury assessments were determined by comparing plants to untreated controls and assigning ratings on a scale from 0 to 100%, no injury to total death.

*Herbicide Content Over Time*

To model and compare quizalofop metabolism, herbicide content in susceptible and resistant wheat genotypes was measured over time using ultrapure liquid chromatography (UPLC) coupled to mass spectrometry (MS/MS). At the four-leaf growth stage, plants were treated with 1.30 g ae $ha^{-1}$ of quizalofop and 0.25% NIS using the same methods as the plant-level dose response. Leaf tissue was collected across timepoints following herbicide treatment, where each variety was triplicated with four plants per replicate. Tissue samples were harvested at 0.5, 1, 3, and 9 days after treatment (DAT) for all genotypes, whereas only resistant genotypes were sampled at 17 DAT. Samples were weighed (approximately 2.5 g of tissue per sample), washed with 20% v/v acetone in water, cut into pieces, frozen on ice, and stored at -20°C until herbicide extraction.

Quizalofop was extracted from tissue using a modified QuEChERS ("quick, easy, cheap, effective, rugged, safe") protocol. After thawing, samples were homogenized in 10 mL distilled water. An equal volume of acetonitrile with 0.1% formic acid was then added and samples were vortexed. Samples were shaken for 10 min at room temperature and 250 rpm. A salt mixture was added to each sample (4 g $MgSO_4$ and 1 g NaCl) and samples were immediately vortexed before centrifugation for 10 min at room temperature and 4500 rpm. About 1.5 mL of resulting supernatant from each sample was filtered with 0.2 µM PTFE.

Samples were injected to the instrument in 1 µL aliquots. Serial dilutions of quizalofop acid analytical standard (≥95%, Sigma-Aldrich) dissolved in acetonitrile with 0.1% v/v formic acid were dispersed throughout sample runs for quality assurance and to quantify quizalofop peak counts. Standard concentrations were 4.10, 10.2, 25.6, 64.0, 160, 400, and 1000 pg $\mu L^{-1}$. Blank replicates were also included in sample runs.

The UPLC-MS/MS instrument included 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 polar 100 x 4.6 mm column packed with 2.6 µm C-18 at a pore size of 100 Å (Phenomenex Kinetex) and heated to 40°C was used for reverse-phase binary gradient separation in conjunction with distilled water and acetonitrile solvents with 0.1% v/v formic acid. Solvent flow was held at a constant rate of 0.400 mL $min^{-1}$. Prior to detection, solvent was heated to 400°C, ionized through positive electrospray ionization, and desolvated at 250 °C. Nebulizing and drying He gas flow was maintained at 24 mL $min^{-1}$. Non-target ions were filtered by the triple quadrupole using multiple reaction 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 and daughter ions had a m/z of 345.00 and 299.00, respectively, with an average retention time of 4.9 min.

*Whole-Plant Regrowth Over Increasing Herbicide Doses in Combination with Cloquintocet*

Express, COS14-D002, and COS14-D002 were subject to a range of quizalofop doses with or without added cloquintocet to evaluate the effect of safener on quizalofop resistance between the genotypes. Plants were treated and replicated similarly to the whole-plant dose-response experiment. Cloquintocet was included as an additional treatment effect to a series of 6 quizalofop doses and a control. Quizalofop rates for Express, a susceptible variety, were equivalent to 0.308, 0.925, 2.85, 8.55, 25.7, and 77.1 g ae $ha^{-1}$, whereas rates for both resistant genotypes were equivalent to 24.1, 48.2, 96.3, 193, 385, and 771 g ae $ha^{-1}$. Cloquintocet was included at a rate of 8.0 g ae $ha^{-1}$ in safener treatments. All application mixtures included 0.25% v/v NIS. Three weeks after treatment, leaf tissue at a height greater than 5 cm above the soil surface was removed. Leaf biomass regrowth was then collected five-and-a-half weeks after treatment, dried, and weighed for final dry biomass.

*Statistical Analysis*

All statistical analysis was conducted with packages in RStudio v.1.2.5033 with R v.3.6.2 except for Student's t-tests, which were ran in GraphPad Prism v. 9.1.2 using extracted means and errors from R analyses. Before comparing enzyme-level quizalofop resistance between genotypes, background acetyl-CoA activity was subtracted from ACCase activity readings. Background-corrected readings were then normalized to percent residual activity using untreated control means as 100% for each variety. The activity of each variety was regressed

over quizalofop dose with a two-parameter log-logistic regression using the LL.4 function of package drc:

$$y = c + \frac{d-c}{1+\exp\left(m[\log(x)-\tilde{e}]\right)}, \quad (1)$$

where lower limit c is fixed at 0, upper limit d is fixed at 100, m is the slope, and e is the 50% enzyme inhibition dose ($I_{50}$). The $I_{50}$ estimates and errors were contrasted using F-protected Student's t-tests ($\alpha$=0.05).

Plant-level quizalofop resistance was determined and compared similarly to enzyme-level resistance. Prior to analysis, visual ratings were reversed so that untreated datapoints were equal to 100% rather than 0% for decreasing rather than increasing regressions. Visual ratings were regressed over quizalofop dose using the same two-parameter log-logistic regression as previously described, though e is the 50% visual injury dose ($VI_{50}$) rather than the $I_{50}$. The $VI_{50}$ estimates and errors were compared in the same manner as enzyme-level $I_{50}$ values.

Quizalofop metabolism was modeled by regressing quizalofop content over DAT. Peak counts were converted to concentration using a weighted linear calibration curve of analytical standard peak means fit using the stats function of package lm:

$$y = mx + b, \quad (2)$$

where y is concentration and x is peak area, while m and b are constants. Inverse standard deviations were used as the weighting factor. Quizalofop content per sample was estimated through the calibration, then normalized by fresh sample mass. Concentrations were regressed over time using the two-parameter exponential decay function, EXD.2 of package drc:

$$y = c + (d-c) \cdot \exp\left(\frac{-x}{e}\right), \quad (3)$$

where lower limit c is fixed at 0, d is the upper limit, and e is the decay slope. The quizalofop half-life ($t_{1/2}$) of each variety was estimated through parameterization with the ED function of package drc. All $t_{1/2}$ estimates and errors were compared using F-protected Student's t-tests ($\alpha$=0.05).

The effect of cloquintocet on quizalofop resistance was compared among spring wheat genotypes using methods comparable to that of the enzyme-level resistance analysis. Dry regrowth biomass was regressed over quizalofop doses using the same four-parameter log-logistic regression, yet lower limits c and d were not fixed and e is the 50% growth reduction dose ($GR_{50}$) rather than $I_{50}$. When possible, $GR_{50}$ estimates and errors were compared between treatments with or without cloquintocet per variety using F-protected Student's t-tests ($\alpha$=0.05).

**Results and Discussion**

Dose-response models of ACCase activity highlight enzyme-level quizalofop resistance differences between susceptible and resistant wheat genotypes. Enzyme extracts from resistant genotypes contain ACCase with a resistance-conferring amino acid substitution, which causes resistant variety dose-response curves to shift right relative to susceptible curves for both winter (Figure 4.1A) and spring wheat (Figure 4.1B). This rightward shift indicates increased herbicide resistance. Susceptible and resistant wheat genotypes exhibit similar regressions.

The comparison of $I_{50}$ estimates among genotypes supports dose-response regression trends. All resistant genotypes have a significantly greater $I_{50}$ than susceptible varieties (Figure 4.2), though no significant differences were observed across all susceptible or all resistant genotypes. Results suggest that quizalofop inhibits ACCase activity to a greater degree in

susceptible than resistant genotypes. Resistant winter wheat genotypes have slightly greater resistance than spring wheat genotypes, though values do not differ significantly. Other herbicide-resistant crops with enzyme-based resistance display similar enzyme inhibition trends between susceptible and resistant genotypes, including imazamox-susceptible and -resistant Clearfield™ wheat varieties (Rojano-Delgado et al. 2014).

Dose-response models of quizalofop injury accentuate plant-level resistance differences between susceptible and resistant wheat genotypes that may be attributable to mechanisms in addition to enzyme-level resistance. Similar to the ACCase activity dose-response regressions, resistant genotypes have greater quizalofop resistance than susceptible varieties in both winter (Figure 4.3A) and spring wheat (Figure 4.3B), specified by dose-response curves that shift right. However, unlike ACCase activity dose-response curves, regression trends between resistant genotypes within winter or spring wheat exhibit minor differences. Incline AX and COS14-D004 are slightly more resistant than LCS Fusion AX and COS14-D002, respectively, as shown by curves shifted to the right and significant differences according to Student's t-tests.

The $VI_{50}$ estimate comparisons between genotypes reflect plant injury dose-response regression trends. All resistant genotypes have a significantly greater $VI_{50}$ than susceptible varieties, where significant differences were observed among resistant genotypes but not between susceptible varieties (Figure 4.4). As illustrated by the regression curves, there are significant difference between genotypes for wheat and spring wheat. Incline AX is more resistant than LCS Fusion AX for winter wheat varieties, while COS14-D004 is more resistant than COS14-D002 for spring wheat experimental lines. Resistance differences between resistant

genotypes supports the hypothesis of differential herbicide metabolism despite equivalent enzyme resistance.

Regressions of quizalofop content over DAT expose some metabolism differences between wheat genotypes. Quizalofop metabolism is faster in resistant genotypes, whose curves tend to be left of susceptible varieties (Figure 4.5). In winter wheat specifically, metabolism is faster in Incline AX, followed by LCS Fusion AX, Byrd, and Hatcher. Metabolism is faster in COS14-D002 than the other spring wheat genotypes.

Susceptible variety $t_{1/2}$ estimates are similar, with some estimates significantly greater than that of certain resistant genotypes (Figure 4.6). Resistant variety $t_{1/2}$ estimates vary. Amongst resistant winter wheat varieties, Incline AX has a shorter half-life than LCS Fusion AX. Resistant spring wheat experimental lines do not have significantly different half-lives, though COS14-D002 has a relatively shorter half-life than COS14-D004. Metabolism differences provide further evidence of differential quizalofop metabolism among resistant genotypes, particularly for resistant winter wheat varieties. The increased resistance of Incline AX compared to LCS Fusion AX found in the whole-plant dose-response experiment may correspond to the faster rate of metabolism. In contrast, resistant spring wheat variety half-lives are not significantly different despite differences in the whole-plant dose-response experiment. As previously mentioned, overall herbicide resistance in plants may be due to both targeted and non-targeted resistance mechanisms.

Additional dose-response models of the combined effect of increasing quizalofop doses and cloquintocet demonstrates that augmented metabolism enhances resistance in spring wheat experimental lines. Dose-response curves shift right with the addition of cloquintocet to

quizalofop for all genotypes (Figure 4.7). The effect of safener is most pronounced in resistant genotypes (Figure 4.7B and C), where $GR_{50}$ values are beyond the range of quizalofop doses tested (Table 4.2). All $GR_{50}$ values for treatments without cloquintocet are consistent with the whole-plant dose-response experiment of winter and spring wheat genotypes.

Herbicide metabolism expression varies widely among crop and weed species, which contributes to increased herbicide selectivity in addition to single-gene resistance mechanisms (Dimaano and Iwakami 2021). Variable whole-plant resistance between wheat varieties with the AXigen™ trait may be attributable to differences in herbicide metabolism. A recent review of specific P450s implicated in herbicide metabolism directly associates five P450s with ACCase-inhibitors (Nandula et al. 2019). Compared to other plant families, grass species have the highest reported number of P450 genes associated with metabolism. Several herbicide metabolism studies with safener also reveal increased activity of metabolism mechanisms. In wheat, cloquintocet treatment increases GST gene upregulation (Theodoulou et al. 2003) and enzyme activity in foliage (Taylor et al. 2013), as well as GT activity (Edwards et al. 2005). A study in sorghum [Sorghum bicolor (L.) Moench) cites that safener increases expression of P450s, GSTs, GTs, and a few other herbicide metabolism mechanisms (Baek et al. 2019).

**Conclusions**

Results collectively provide evidence of differential herbicide metabolism mechanisms associated with overall resistance of quizalofop-resistant wheat genotypes. Though all genotypes contain the same qualitative trait for resistance and exhibit similar enzyme-level resistance, whole-plant resistance differs in both winter and spring wheat. Specifically, of the two resistant winter wheat varieties examined, Incline AX exhibits greater whole-plant

resistance than LCS Fusion AX, while resistant spring wheat variety COS14-D004 exhibits greater resistance than COS14-D002. Measurement and regression of quizalofop over time following treatment reveals a shorter quizalofop half-life in Incline AX versus LCS Fusion AX but not COS14-D004 versus COS14-D002, suggesting some whole-plant resistance differences may be due to differential metabolism. Treating spring wheat genotypes with cloquintocet increases overall resistance to quizalofop, implying that metabolism of the herbicide contributes to selectivity of this ACCase inhibitor. Further, the safener results in combination with chapter 3 results on the temperature-dependence of quizalofop metabolism highlight the dynamic nature of metabolism. Data collected for a differential expression analysis of winter and spring wheat genotypes treated with cloquintocet may reveal putative genes associated with differential metabolism. The background source of any upregulated herbicide metabolism genes may also be uncovered by including resistant spring wheat experimental lines as well as winter or spring susceptible donor parents in the analysis. After gene validation with quantitative PCR, molecular markers for improved quizalofop metabolism would be a practical means of enhancing quizalofop resistance in new CoAXium™ wheat variety development.

**Tables**

Table 4.1. Wheat genotype[1] overview

| Wheat Type | Genotype | Relation to Quizalofop |
|---|---|---|
| Winter | Byrd | Susceptible |
| | Hatcher | Susceptible |
| | LCS Fusion AX | Resistant |
| | Incline AX | Resistant |
| Spring | Express | Susceptible |
| | COS14-D002 | Resistant |
| | COS14-D004 | Resistant |

[1]Variety or experimental line.

Table 4.2. Comparison of plant regrowth $GR_{50}$ estimates[1] for quizalofop in combination with cloquintocet

| | Genotype | | | |
|---|---|---|---|---|
| | Express | | COS14-D002 | COS14-D004 |
| **Treatment** | $GR_{50}$ (g ae·ha$^{-1}$) | | | |
| Quizalofop | 4.83±0.602 | B | 124±15.2 | 309±73.9 |
| Quizalofop + Cloquintocet | 23.5±5.80 | A | >771 | >771 |

[1]Regrowth biomass was modeled with a 4-parameter log-logistic function to estimate $GR_{50}$ values where possible.
[2]Different capital letters represent significantly different $GR_{50}$ estimates between treatments for Express according to F-protected Student's t-tests ($\alpha$=0.05). Estimates for remaining genotypes could not be compared due to the limited range of the data and regressions.

**Figures**

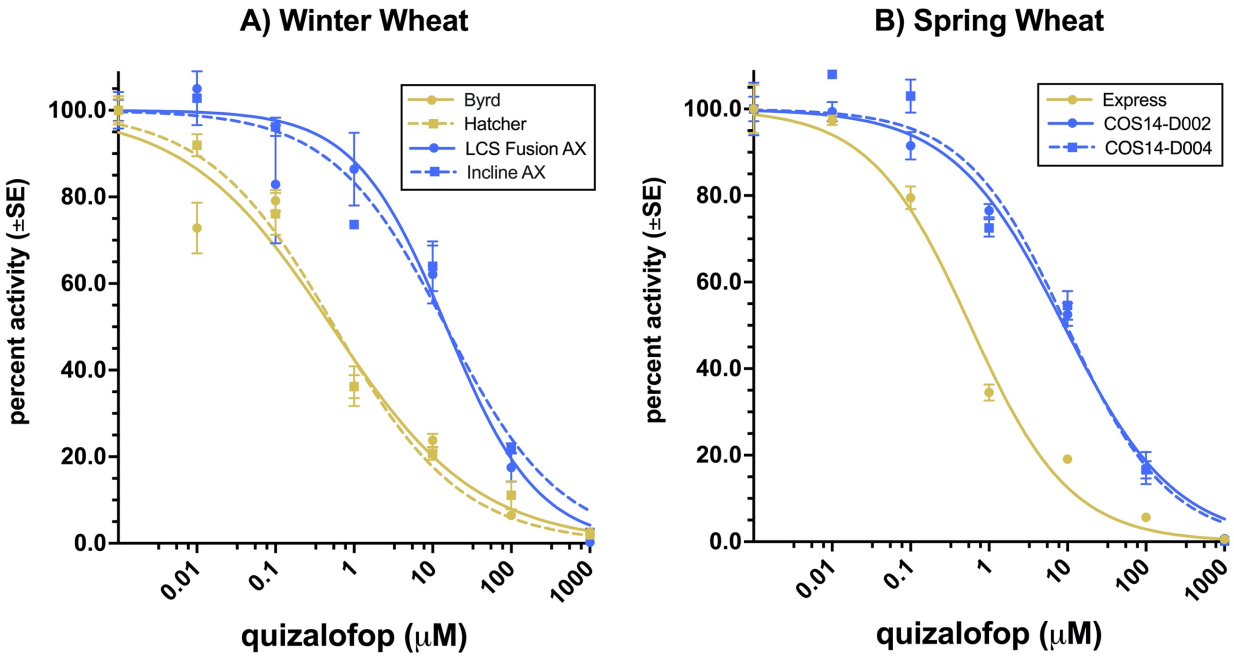



Figure 4.1. Dose-response curves of acetyl-CoA carboxylase activity[1] versus quizalofop dose by genotype[2]

[1]Activity values (DPM) normalized to percent untreated controls per variety, then dose effect was modeled with a 2-parameter log-logistic function.
[2]Susceptible genotypes are colored yellow, whereas resistant genotypes are colored blue.

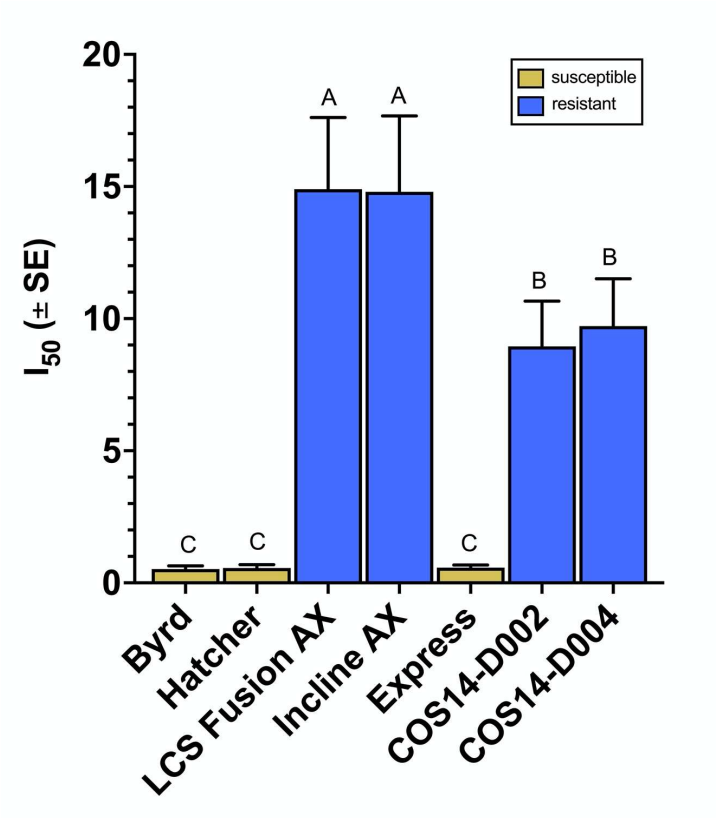



Figure 4.2. Comparison of acetyl-CoA carboxylase $I_{50}$ estimates[1] for quizalofop between genotypes[2]

[1]Activity values (DPM) normalized to percent untreated controls per variety, then dose effect was modeled with a 2-parameter log-logistic function to estimate $I_{50}$ values.
[2]Different capital letters represent significantly different $I_{50}$ estimates between genotypes according to F-protected Student's t-tests ($\alpha$=0.05).

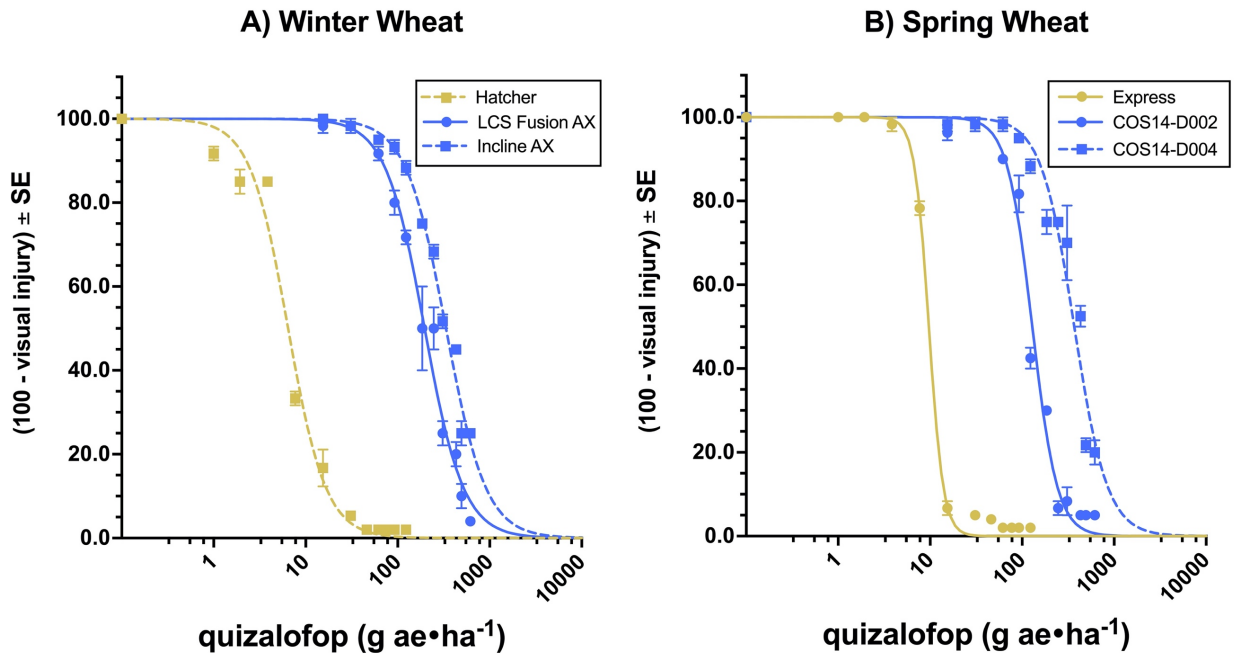



Figure 4.3. Dose-response curves of whole-plant visual injury[1] versus quizalofop dose by genotype[2]

[1]Visual ratings reversed, then dose effect was modeled with a 2-parameter log-logistic function.
[2]Susceptible genotypes are colored yellow, whereas resistant genotypes are colored blue.

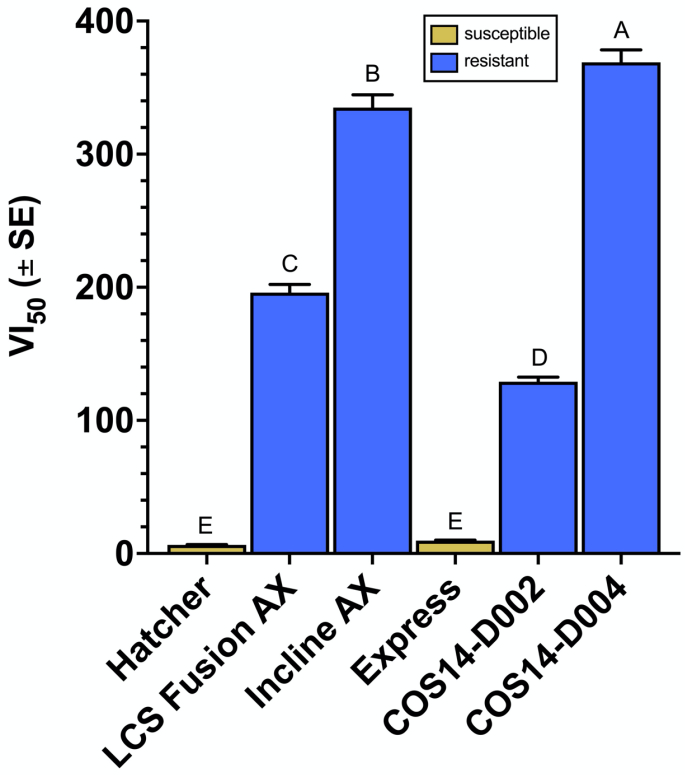



Figure 4.4. Comparison of whole-plant visual injury $VI_{50}$ estimates[1] for quizalofop between genotypes[2]

[1]Visual ratings reversed, then dose effect was modeled with a 2-parameter log-logistic function to estimate $VI_{50}$ values.
[2]Different capital letters represent significantly different $VI_{50}$ estimates between genotypes according to F-protected Student's t-tests ($\alpha$=0.05).

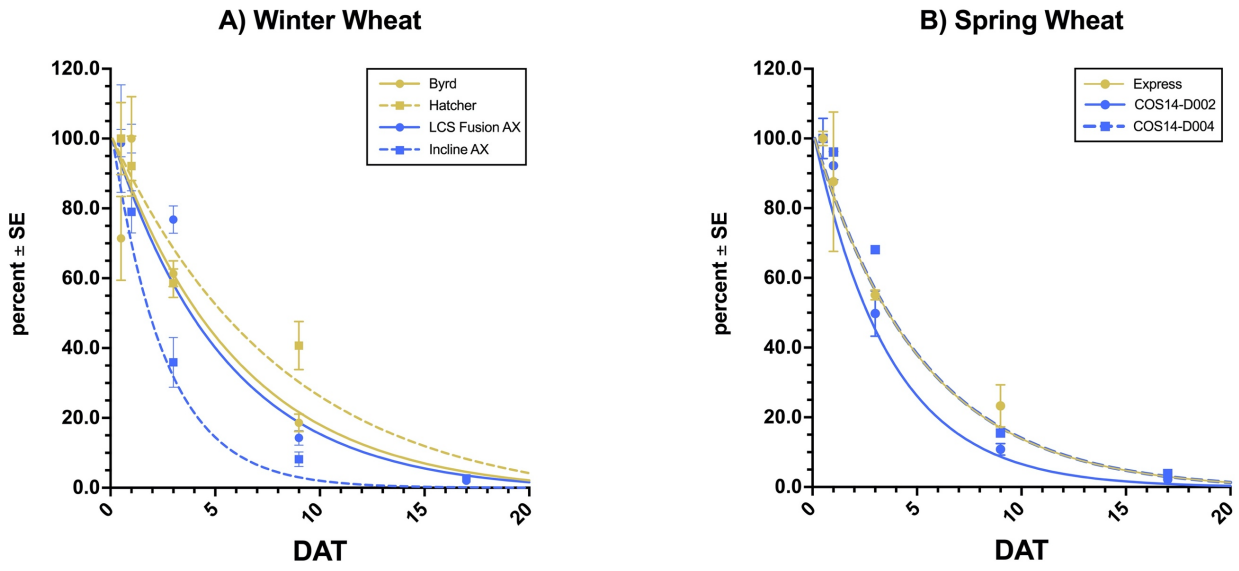



Figure 4.5. Exponential decay curves of quizalofop content[1] versus days after treatment (DAT) by genotype[2]

[1]Content estimated through calibration, normalized by fresh sample mass, then dose effect was modeled with a 2-parameter exponential decay function. For visualization purposes, content is further normalized to percent.
[2]Susceptible genotypes are colored yellow, whereas resistant genotypes are colored blue.

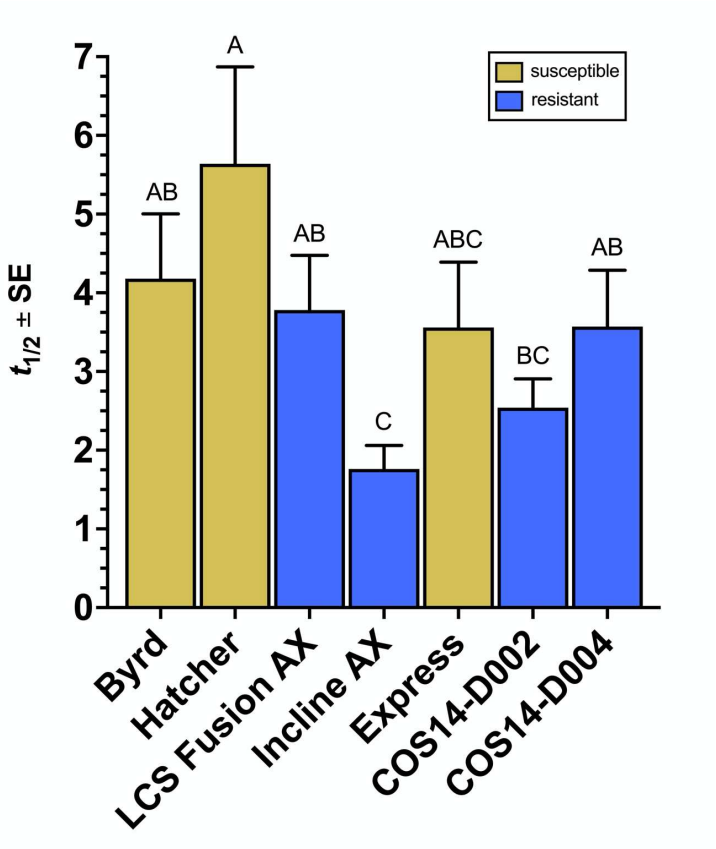



Figure 4.6. Comparison of quizalofop content $t_{1/2}$ estimates[1] between genotypes[2]

[1]Content estimated through calibration, normalized by fresh sample mass, then dose effect was modeled with a 2-parameter exponential decay function to estimate $t_{1/2}$ values.
[2]Different capital letters represent significantly different $t_{1/2}$ estimates between genotypes according to F-protected Student's t-tests ($\alpha$=0.05).

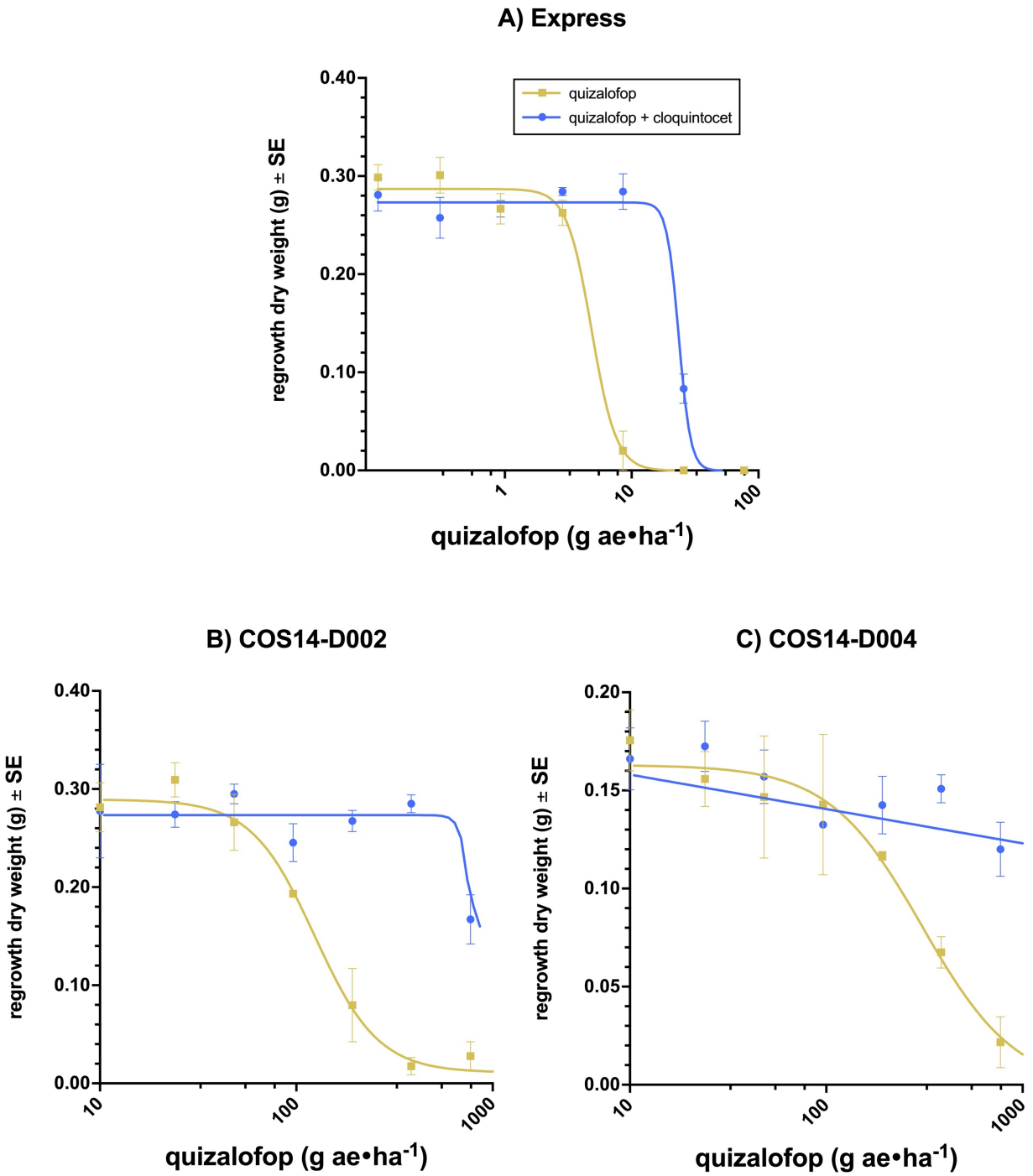



Figure 4.7. Dose-response curves of plant regrowth[1] versus quizalofop dose in combination with cloquintocet

[1]Regrowth biomass was modeled with a 4-parameter log-logistic function.

**References**

Baek YS, Goodrich LV, Brown PJ, James BT, Moose SP, Lambert KN, Riechers DE. 2019. Transcriptome profiling and genome-wide association studies reveal GSTs and other defense genes involved in multiple signaling pathways induced by herbicide safener in grain sorghum. Frontiers in Plant Science. 10:192.

Borrill P, Ramirez-Gonzalez R, Uauy C. 2016. Expvip: A customizable rna-seq data analysis and visualization platform. Plant Physiology. 170(4):2172-2186.

Carrvalho SJP, Nicolai M, Ferreira RR, Oliveira Figueira AV, Christoffoleti PJ. 2009. Herbicide selectivity by differential metabolism: Considerations for reducing crop damages. Scientia Agricola. 66(1):136-142.

Chalupska D, Lee HY, Faris JD, Evrard A, Chalhoub B, Haselkorn R, Gornicki P. 2008. *ACC* homoeoloci and the evolution of wheat genomes. Proceedings of the National Academy of Sciences USA. 105(28):9691-9696.

Dayan FE, Owens DK, Corniani N, Silva FML, Watson SB, Howell JL, Shaner DL. 2017. Biochemical markers and enzyme assays for herbicide mode of action and resistance studies. Weed Science. 63(SP1):23-63.

Delye C, Jasieniuk M, Le Corre V. 2013. Deciphering the evolution of herbicide resistance in weeds. Trends in Genetics. 29(11):649-658.

Dimaano NG, Iwakami S. 2021. Cytochrome P450-mediated herbicide metabolism in plants: Current understanding and prospects. Pest Management Science. 77(1):22-32.

Edwards R, Del Buono D, Fordham M, Skipsey M, Brazier, M., Dixon DP, Cummins I. 2005. Differential induction of glutathione transferases and glucosyltransferases in wheat, maize, and *Arabidopsis thaliana* by herbicide safeners. Z Naturforsch. 60c:307-316.

Gornicki P, Faris JD, King I, Podkowinski J, Gill B, Haselkorn R. 1997. Plastid-localized acetyl-CoA carboxylase of bread wheat is encoded by a single gene on each of the three ancestral chromosome sets. Proceedings of the National Academy of Sciences USA. 94:14179-14184.

Kumar V, Liu R, Manuchehri MR, Westra EP, Gaines TA, Shelton CW. 2020. Feral rye control in quizalofop-resistant wheat in central Great Plains. Agronomy Journal. 113(1):407-418.

Limagrain Cereal Seeds. LCS Fusion Ax. [accessed 2021 September 1st]. https://limagraincerealseeds.com/northern-plains/hard-red-winter-wheat-seed/lcs-fusion-ax/.

Nakka S, Jugulam M, Peterson D, Asif M. 2019. Herbicide resistance: Development of wheat production systems and current status of resistant weeds in wheat cropping systems. The Crop Journal. 7(6):750-760.

Nandula VK, Riechers DE, Ferhatoglu Y, Barrett M, Duke SO, Dayan FE, Goldberg-Cavalleri A, Tétard-Jones C, Wortley DJ, Onkokesung N et al. 2019. Herbicide metabolism: Crop selectivity, bioactivation, weed resistance, and regulation. Weed Science. 67(2):149-175.

Ostlie M, Haley SD, Anderson V, Shaner D, Manmathan H, Beil C, Westra P. 2015. Development and characterization of mutant winter wheat (*Triticum aestivum* l.) accessions resistant to the herbicide quizalofop. Theoretical and Applied Genetics. 128(2):343-351.

Ramirez-Gonzalez R, Borrill P, Lang D, Harrington SA, Brinton J, Venturini L, Davey M, Jacobs J, Van Ex F, Pasha A, et al. 2018. The transcriptional landscape of polyploid wheat. Science. 361(6403).

Rojano-Delgado AM, Priego-Capote F, Luque de Castro MD, De Prado R. 2014. Mechanism of imazamox resistance of the Clearfield® wheat cultivar for better weed control. Agronomy for Sustainable Development. 35(2):639-648.

Sasaki Y, Nagano Y. 2004. Plant acetyl-CoA carboxylase: Structure, biosynthesis, regulation, and gene manipulation for plant breeding. Bioscience, Biotechnology, and Biochemistry. 68(6):1175-1184.

Seefeldt SS, Fuerst EP, Gealy DR, Shukla A, Irzyk GP, Devine MD. 1996. Mechanisms of resistance to diclofop of two wild oat (*Avena fatua*) biotypes from the Willamette Valley of Oregon. Weed Science. 44(4):776-781.

Taylor VL, Cummins I, Brazier-Hicks M, Edwards R. 2013. Protective responses induced by herbicide safeners in wheat. Environmental and Experimental Botany. 88(C):93-99.

Theodoulou FL, Clark IM, He XL, Pallett KE, Cole DJ, Hallahan DL. 2003. Co-induction of glutathione-*S*-transferases and multidrug resistance associated protein by xenobiotics in wheat. Pest Management Science. 59(2):202-214.

Wheat Breeding and Genetics Program. Varieties. 2020. Colorado State University; [accessed 2021 September 1st]. wheat.agsci.colostate.edu/varieties/.

Yu Q, Shane Friesen LJ, Zhang XQ, Powles SB. 2004. Tolerance to acetolactate synthase and acetyl-coenzyme A carboxylase inhibiting herbicides in *Vulpia bromoides* is conferred by

two co-existing resistance mechanisms. Pesticide Biochemistry and Physiology. 78(1):21-30.

# APPENDIX I: RNA-SEQ DATA COLLECTION

The RNA-seq experiment was designed as a two-way factorial experiment of seven genotypes and a safener treatment (presence or absence), with four replicates per combination totaling 14 combinations and 56 individual samples. Plants were grown in containers with four separate inserts (6 cm wide x 6 cm long x 5 cm height) with one seed planted per insert. At the two-leaf stage (approximately 1 week following emergence), half of plants were treated with 8.09 g $ha^{-1}$ cloquintocet-mexyl safener with 0.25% v/v NIS using an overhead sprayer as described for the plant-level dose response experiment. The remaining half plants were untreated. Leaf tissue was harvested 12 hours after the safener treatment and flash frozen in liquid nitrogen. Tissue was stored at -80°C until processing.

Each tissue sample was homogenized in liquid nitrogen using a mortar and pestle. A Direct-zol RNA MiniPrep Plus Kit was used for RNA extraction (Zymo Research). The kit relies on TRIzol® for RNA extraction, as well as wash buffers and columns for sample purification. Final RNA samples were eluted in DNase/RNase-free water. RNA aliquots were frozen in liquid nitrogen and stored at -80°C. Samples were extracted in a randomized order to avoid batch effects. Extract quality was measured with a TapeStation 2200 (Agilent Technologies) to ensure quality guidelines of ≥28S/18S and ≥6.5 RIN. Samples were submitted to BGI Genomics for sequencing using the Illumina-seq system. Adapters were removed from sequence reads. Sequencing produced 43-49 million sequence reads per sample. Corresponding filename metadata is provided in Appendix Table A1.

Appendix Table A1. RNA-seq Metadata

| Genotype | Cloquintocet | Replication | Forward File | Reverse File |
|---|---|---|---|---|
| Byrd | untreated | 1 | 5_3_1.fq.gz | 5_3_2.fq.gz |
| Byrd | untreated | 2 | 3_3_1.fq.gz | 3_3_2.fq.gz |
| Byrd | untreated | 3 | 3_1_1.fq.gz | 3_1_2.fq.gz |
| Byrd | untreated | 4 | 1_4_1.fq.gz | 1_4_2.fq.gz |
| Byrd | treated | 1 | 4_8_1.fq.gz | 4_8_2.fq.gz |
| Byrd | treated | 2 | 6_2_1.fq.gz | 6_2_2.fq.gz |
| Byrd | treated | 3 | 2_7_1.fq.gz | 2_7_2.fq.gz |
| Byrd | treated | 4 | 1_2_1.fq.gz | 1_2_2.fq.gz |
| Express | untreated | 1 | 6_6_1.fq.gz | 6_6_2.fq.gz |
| Express | untreated | 2 | 5_2_1.fq.gz | 5_2_2.fq.gz |
| Express | untreated | 3 | 2_8_1.fq.gz | 2_8_2.fq.gz |
| Express | untreated | 4 | 1_1_1.fq.gz | 1_1_2.fq.gz |
| Express | treated | 1 | 5_5_1.fq.gz | 5_5_2.fq.gz |
| Express | treated | 2 | 7_1_1.fq.gz | 7_1_2.fq.gz |
| Express | treated | 3 | 2_5_1.fq.gz | 2_5_2.fq.gz |
| Express | treated | 4 | 3_7_1.fq.gz | 3_7_2.fq.gz |
| LCS Fusion AX | untreated | 1 | 6_7_1.fq.gz | 6_7_2.fq.gz |
| LCS Fusion AX | untreated | 2 | 6_8_1.fq.gz | 6_8_2.fq.gz |
| LCS Fusion AX | untreated | 3 | 7_7_1.fq.gz | 7_7_2.fq.gz |
| LCS Fusion AX | untreated | 4 | 1_3_1.fq.gz | 1_3_2.fq.gz |
| LCS Fusion AX | treated | 1 | 4_6_1.fq.gz | 4_6_2.fq.gz |
| LCS Fusion AX | treated | 2 | 7_8_1.fq.gz | 7_8_2.fq.gz |
| LCS Fusion AX | treated | 3 | 3_6_1.fq.gz | 3_6_2.fq.gz |
| LCS Fusion AX | treated | 4 | 3_4_1.fq.gz | 3_4_2.fq.gz |
| Hatcher | untreated | 1 | 7_4_1.fq.gz | 7_4_2.fq.gz |
| Hatcher | untreated | 2 | 5_1_1.fq.gz | 5_1_2.fq.gz |
| Hatcher | untreated | 3 | 2_6_1.fq.gz | 2_6_2.fq.gz |
| Hatcher | untreated | 4 | 1_8_1.fq.gz | 1_8_2.fq.gz |
| Hatcher | treated | 1 | 4_7_1.fq.gz | 4_7_2.fq.gz |
| Hatcher | treated | 2 | 7_5_1.fq.gz | 7_5_2.fq.gz |
| Hatcher | treated | 3 | 4_2_1.fq.gz | 4_2_2.fq.gz |
| Hatcher | treated | 4 | 3_2_1.fq.gz | 3_2_2.fq.gz |
| Incline AX | untreated | 1 | 9_1_1.fq.gz | 9_1_2.fq.gz |
| Incline AX | untreated | 2 | 6_5_1.fq.gz | 6_5_2.fq.gz |
| Incline AX | untreated | 3 | 7_2_1.fq.gz | 7_2_2.fq.gz |
| Incline AX | untreated | 4 | 2_2_1.fq.gz | 2_2_2.fq.gz |
| Incline AX | treated | 1 | 9_3_1.fq.gz | 9_3_2.fq.gz |

| | | | | |
|---|---|---|---|---|
| Incline AX | treated | 2 | 5_8_1.fq.gz | 5_8_2.fq.gz |
| Incline AX | treated | 3 | 3_5_1.fq.gz | 3_5_2.fq.gz |
| Incline AX | treated | 4 | 4_1_1.fq.gz | 4_1_2.fq.gz |
| COS14-D002 | untreated | 1 | 5_7_1.fq.gz | 5_7_2.fq.gz |
| COS14-D002 | untreated | 2 | 4_4_1.fq.gz | 4_4_2.fq.gz |
| COS14-D002 | untreated | 3 | 5_4_1.fq.gz | 5_4_2.fq.gz |
| COS14-D002 | untreated | 4 | 1_6_1.fq.gz | 1_6_2.fq.gz |
| COS14-D002 | treated | 1 | 4_3_1.fq.gz | 4_3_2.fq.gz |
| COS14-D002 | treated | 2 | 7_3_1.fq.gz | 7_3_2.fq.gz |
| COS14-D002 | treated | 3 | 3_8_1.fq.gz | 3_8_2.fq.gz |
| COS14-D002 | treated | 4 | 1_5_1.fq.gz | 1_5_2.fq.gz |
| COS14-D004 | untreated | 1 | 6_3_1.fq.gz | 6_3_2.fq.gz |
| COS14-D004 | untreated | 2 | 9_2_1.fq.gz | 9_2_2.fq.gz |
| COS14-D004 | untreated | 3 | 7_6_1.fq.gz | 7_6_2.fq.gz |
| COS14-D004 | untreated | 4 | 2_1_1.fq.gz | 2_1_2.fq.gz |
| COS14-D004 | treated | 1 | 9_4_1.fq.gz | 9_4_2.fq.gz |
| COS14-D004 | treated | 2 | 6_4_1.fq.gz | 6_4_2.fq.gz |
| COS14-D004 | treated | 3 | 2_4_1.fq.gz | 2_4_2.fq.gz |
| COS14-D004 | treated | 4 | 1_7_1.fq.gz | 1_7_2.fq.gz |