PHYSIOLOGY OF GLYPHOSATE-RESISTANT JOHNSONGRASS AND IMPLICATIONS FOR MANAGEMENT

A Dissertation Presented to

the Faculty of the Graduate School

University of Missouri-Columbia

In Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

---

by

SARAH E. DIXON

Dr. Reid J. Smeda, Supervising Professor

DECEMBER 2022

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

PHYSIOLOGY OF GLYPHOSATE-RESISTANT JOHNSONGRASS AND IMPLICATIONS FOR MANAGEMENT

presented by Sarah E. Dixon

a candidate for the degree of Doctor of Philosophy

and hereby certify that in their opinion it is worthy of acceptance.

Dr. Reid J. Smeda
Professor, University of Missouri

Dr. J. Andy Kendig
Adjunct Professor,
University of Missouri

Dr. Kevin Bradley
Professor, University of Missouri

Dr. Harley Naumann
Associate Teaching Professor,
University of Missouri

Dr. Lauren Sullivan
Assistant Professor,
Michigan State University

DEDICATION

This dissertation work is dedicated to my parents Bruce & Shirley Dixon, who survived raising me, and to my grandmother Adeline, who never had a limit to the number of books she would read aloud.

ACKNOWLEDGEMENTS

First and foremost, I thank Dr. Reid Smeda for his willingness to take me on as a graduate student in his research program. I must also thank Reid for entrusting me with many aspects of experimentation, both planned and unplanned. I am grateful for patience, encouragement, and feedback along the way. I would also like to thank Kevin Bradley and Harley Naumann for serving as instructors in my courses as well as serving on my committee. I owe thanks to Lauren Sullivan for allowing me to join her ecology seminar, and for the creative insights I gained from conversations with her lab members. I appreciate the concepts and philosophies I gained from Kevin, Harley, and Lauren's scholarship. I owe many thanks to Dr. Andy Kendig, who participated in my Ph.D. committee with aplomb, and offered valuable feedback along the way and in the refinement of the dissertation that follows.

Other faculty played a more indirect, but no less valuable role in my research and graduate education. I owe many thanks to Drs. Dean Volenberg and Jim Schoelz for sharing lab space with me, and for their feedback along the way. Drs. Zhiwei Fang and Murugesan Raju were indispensable resources for the microbiological aspects of this work. Drs. Christine Spinka and Mark Ellersieck both supported and challenged my growth in statistical reasoning. Drs. Mithila Jugulam and Chandrima Shyam at Kansas State University were incredibly gracious hosts and a pleasure to work with.

I owe a significant debt of gratitude to my graduate colleagues Matt Fleetwood, Emma Gaither, and Timmy Rice- especially Emma and Tim, who were willing to stay late on Fridays counting grass weeds, and always approached my projects as a team

responsibility. I am especially grateful for Shaun Billman, who kept us all on track despite the many unpredictable aspects of juggling research priorities. I must also thank Leanna Reichl and Brogan Eyre, who were among some of the best undergraduate research assistants one could hope to find. Andrew Biggs and Josh Reinbott are the unsung heroes of field research at Bradford, and I am grateful for all the ways they have accommodated and supported our research projects over the years. Finally, I would like to thank Justin Pollard, Greg Elmore, and Neha Rana for their sincere interest and investment in this research.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER II

CHAPTER III

CHAPTER IV

LIST OF FIGURES

CHAPTER II

CHAPTER III

CHAPTER IV

LIST OF APPENDIX FIGURES

ABSTRACT

Glyphosate, as the formulated commercial herbicide Roundup®, was introduced by Monsanto in 1974. The broad-spectrum activity of glyphosate made it an excellent candidate for engineering herbicide-resistant crops. Following the introduction and widespread adoption of glyphosate resistant crops, the amount of glyphosate applied to agricultural land increased dramatically. In 2016, a producer reported a failure of glyphosate to control johnsongrass within an agronomic field in Buchanan County, MO to the herbicide manufacturer. In response to the reported failure of glyphosate to control johnsongrass, plant material was collected, and the objectives of the following research were to 1) determine if glyphosate resistance is present in the Buchanan County, MO population and 2) determine the mechanism(s) imparting resistance, if found; and 3) evaluate prospective management practices for this johnsongrass population to mitigate the spread of herbicide resistance. Johnsongrass plants were subjected to a greenhouse dose-response assay to assess the potential for glyphosate resistance, which was confirmed when the dose required to reduce aboveground biomass by 50% (GR_{50}) was 1,073 g ae ha^{-1} for the putative glyphosate-resistant (gly-R) population versus 230 g for a comparative glyphosate-susceptible (gly-S) population. Potential non-target site resistance was assessed with a growth chamber study utilizing ^{14}C-glyphosate applied to potted plants to determine potential differences in herbicide absorption and translocation. Following the application of a solution containing ^{14}C-glyphosate to gly-R and gly-S plants in a growth chamber experiment, glyphosate absorption was not different between the two populations. However, at 96 hours after treatment (HAT), the gly-R population had translocated 36% of the absorbed herbicide out of the treated leaf versus 62% for the

gly-S population, and 42% less ^{14}C-glyphosate than the gly-S population to the root system. Potential target site mechanisms of glyphosate resistance were examined by sequencing fragments of the gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*). *EPSPS* expression, and expression of the glyphosate efflux transporter *EcABCC8* were evaluated by quantitative PCR (qPCR). Results of *EPSPS* sequencing identified no point mutations. One gly-R individual had 4.5-fold *EPSPS* expression relative to three gly-S plants. No evidence for *EPSPS* amplification was observed in the remaining four gly-R plants examined. Two plants were identified that overexpressed *EcABCC8* 2.2-fold compared to gly-S. Differential translocation of glyphosate is the primary mechanism of resistance to glyphosate in johnsongrass isolated from Buchanan County, MO. In addition, overexpression of *EPSPS* and *EcABCC8* may also contribute to glyphosate resistance within this population. In field experiments with soybean conducted in Buchanan County, Missouri in 2020 and 2021, weed density and biomass data confirmed that grass weed control from glufosinate consistently outperformed glyphosate at both study locations. While some treatments resulted in excellent control of gly-R johnsongrass, POST only programs place significant pressure on single herbicide modes of action, and the consequences of such an approach are discussed. Additional field studies examined the sensitivity of roadside johnsongrass populations to glyphosate. The GR_{50} of roadside populations was plotted against the Euclidean distance of each population from the gly-R population in distance-decay models. The distance of roadside johnsongrass from the original gly-R population predicted the GR_{50} of roadside populations within 173 g ha^{-1} of glyphosate, and an increase in sensitivity to glyphosate was predicted when populations were collected at further distances from the gly-R

source. A simulated roadside management study assessed the response of mature (1.5 m height) gly-R johnsongrass to fall-applied chemical weed control options suitable for roadside applications. When herbicides were applied to mature plants in the fall, results indicated that all treatments reduced the number of shoots from rhizome in the spring. Rhizomes from plants treated with glyphosate alone at 1,736 or 3,473 g ai ha^{-1} produced biomass reduced only 27 to 39% compared to non-treated controls. While effective chemical control options for johnsongrass were identified in both crop and non-crop field studies, a combination of chemical, mechanical, and cultural practices is likely to be the most successful practice to limit the tolerance of johnsongrass populations to glyphosate.

CHAPTER I

Literature Review

Development, preeminence, and impact of glyphosate. The commercialization of 2,4-D in the years following World War II initiated the transformation of agricultural weed management from primarily mechanical to chemical strategies. Agriculture, especially large-scale crop production systems, have relied on herbicides for weed management for the past 70+ years. However, only a few decades of that time were highly productive years for herbicide discovery. From 1980 to 2001, the rate of new herbicides commercialized was ~5.5 active ingredients (a.i.) per year. Following 2001, the rate of new a.i. commercialization for herbicides dropped to only ~2 per year (Duke, 2012). More importantly, in the decades that followed the herbicide discovery boon of the 1980's and 1990's, no novel herbicide modes of action were introduced until 2018 (Umetsu & Shirai, 2020). The restraints on herbicide discovery have largely resulted from the shifting economic landscape following the introduction of genetically modified crops such as glyphosate-resistant (gly-R) soybean (*Glycine max*) in 1996, followed two years later by the introduction of gly-R maize (*Zea mays*) (Duke, 2012).

Glyphosate was originally discovered by the Swiss chemist Dr. Henri Martin during a search for new compounds with pharmaceutical applications (Dill et al., 2010). Glyphosate failed to exhibit any pharmaceutical purpose, and no reports were made on its biological activity. Monsanto tested several aminomethylphosphinic acid (AMPA) compounds that showed weak herbicidal activity. The chemist Dr. John Franz was charged with the development of analogs with more significant herbicidal activity (Dill et al., 2010). Dr. Franz designed and synthesized the compound in 1970, and glyphosate as

the formulated commercial herbicide Roundup® was introduced by Monsanto in 1974 (Benbrook, 2016). The broad-spectrum activity of glyphosate rendered initial uses to instances where total vegetation control was desired, such as between orchard rows, prior to crop planting, or in non-crop areas such as roadside right-of-ways. Glyphosate targets the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), which is naturally occurring in plants, bacteria, and fungi but absent from mammalian systems (Dill et al., 2010).

In the shikimate pathway, glyphosate competes with the native substrate phosphoenol pyruvate (PEP) for binding near the active site in *EPSPS*. *EPSPS* transfers the enolpyruvl moiety from PEP to shikimate-3-phosphate (S3P), forming 5-enolpyruvlshikimate-3-phosphate (EPSP) and releasing a single inorganic phosphate (Schönbrunn et al., 2001). Glyphosate is noncompetitive with S3P, which is required for glyphosate binding in the "open" position of *EPSPS*. The open position exposes the active site of the enzyme and permits binding of glyphosate-S3P or PEP-S3P. In the closed position, *EPSPS* is temporarily inhibited when occupied by PEP-S3P, but gradually reverses. When *EPSPS* is bound by the glyphosate-S3P complex, however, the resulting nonfunctional enzyme conformation is quite stable (Alibhai & Stallings, 2001). Glyphosate is unique in that it is not known to bind to or inhibit any other enzyme, even those that utilize PEP as a substrate (Alibhai & Stallings, 2001).

An experiment to examine binding properties of both glyphosate and the native substrate PEP, when S3P was absent from reaction conditions, revealed that glyphosate's affinity for the free enzyme was weaker than PEP (Ream et al., 1992). Binding constants (Kd) for PEP in the absence of S3P were Kd = 0.39 mM versus Kd = 12 mM for

glyphosate (Ream et al., 1992). However, in the presence of S3P, as is the case in all natural plant conditions, glyphosate affinity for *EPSPS* increases 75,000 times over that of the free enzyme, whereas the native substrate's affinity only increases about 20-fold (Ream et al., 1992).

Once bound, the glyphosate-S3P-*EPSPS* complex is nonfunctional. The aromatic amino acids phenylalanine, tyrosine, and tryptophan result from the shikimic acid pathway. With *EPSPS* inhibited, their production ceases and levels are depleted, resulting in cessation of protein synthesis. Exogenous application of these amino acids reverses the growth inhibition observed after glyphosate application but fails to reverse glyphosate phytotoxicity (Lee, 1980). Visual symptoms of glyphosate toxicity in plants take 4 to 14 days to appear, and include stunted growth, chlorosis, wilting, leaf wrinkling or malformation, and necrosis (Franz et al., 1997). Varied evidence has been put forth for the mode of action (MOA) resulting from glyphosate toxicity, with the most promising of those indicating that the dysfunctional shikimate pathway may act as a carbon sink depriving other biosynthetic pathways of critical inputs. Despite its global status as an herbicide that transformed agriculture, the precise sequence of events resulting in death in sensitive plants remains unknown (Duke & Powles, 2008).

In the soil, the phosphonic acid moiety of glyphosate adsorbs to clay mineral and organic matter fractions, rendering glyphosate unavailable for root uptake (Sprankle et al., 1975). Rapid degradation by soil microbes further limits glyphosate's potential soil activity (Sprankle et al., 1975). However, when applied as foliar spray, glyphosate is absorbed across the leaf cuticle and moves readily in the apoplast and symplast (Jachetta et al., 1986; MacIsaac et al., 1991). Upon passive phloem loading, glyphosate

translocates readily to meristematic phloem “sinks”, contributing to its ability to control perennial plants by killing underground reproductive structures (Shaner, 2014). Evidence suggests that glyphosate relies on a transport mechanism to cross the plasma membrane, as glyphosate concentration in the cytosol increases in an ATP-dependent manner at rates contingent upon the extracellular concentration gradient (Denis & Delrot, 1993; Ge et al. 2013). In earlier studies on vacuolar glyphosate concentrations, evidence for only a unidirectional movement of the herbicide across the plasma membrane and tonoplast was presented (Ge et al., 2014). However, a recent study identified an ABC-type transporter that effluxes glyphosate from the cytosol to the apoplast, providing some of the first evidence for transporter-mediated bidirectional movement of glyphosate (Pan et al., 2021; Sammons & Gaines, 2014).

The broad-spectrum activity of glyphosate made it an excellent candidate for engineering herbicide-resistant crops; however, the effort to engineer glyphosate resistance in crop plants was a significant undertaking. Glyphosate resistance in crop plants results from the insertion of a gene for an insensitive *EPSPS* isolated from *Agrobacterium* spp. (Dill, 2005). Prior to the genetic engineering that permitted expression of a glyphosate insensitive *EPSPS*, two alternative strategies were attempted: overexpression of a sensitive *EPSPS*, and detoxification of the herbicidal molecule. Briefly, overexpression of a glyphosate-sensitive (gly-S) *EPSPS* failed due to the inability to generate many whole plants from cell culture lines in addition to the fitness penalties observed when whole petunia (*Petunia hybrida*) plants were generated (Shah et al., 1986). Expression of a glyphosate oxidase enzyme meant to detoxify the glyphosate molecule was insufficient to impart sufficient glyphosate resistance necessary for crop

varieties (Dill, 2005). Single point mutations generated enzyme mutants whose kinetics did not meet theoretical ideals, and thus were insufficient to impart the required level of glyphosate resistance in crop plants. These single point mutations were glycine for alanine at position 101 ($Gly_{101}Ala$) and serine for proline at 106 ($Pro_{106}Ser$), using the numbering system beginning at the start of the mature enzyme for *Arabidopsis thaliana*. In maize, a double $Thr_{102}Ile$ and $Pro_{106}Ser$ (TIPS) mutation resulted in the only other insensitive *EPSPS* used to generate glyphosate resistant crop varieties (Dill, 2005).

GR crops simplified weed management and provided superior control at costs comparable to or less than conventional herbicide programs. Patent protection for glyphosate expired in 2000, reducing the cost of glyphosate applications by introducing generic formulations (Benbrook, 2016). Anticipating durable competition from gly-R crops, agrochemical companies shifted research and discovery priorities at a time when company consolidation was dramatically reducing the diversity and competitiveness of the agricultural market (Dayan, 2019). Simultaneously, the cost of discovering, developing, and bringing a synthetic pesticide to commercialization rose from $184 million in 2000 to ~$286 million in 2016 (Dayan, 2019).

Changes in herbicide use patterns following the introduction of gly-R crops have frequently been blamed for the evolution of herbicide-resistant (HR) weed species. The most significant change in herbicide use following the introduction and widespread adoption of gly-R crops has been the dramatic increase in the amount of glyphosate applied to agricultural land. In 1995, prior to the introduction of gly-R crops, global glyphosate use was estimated at 51 million kg ai (Benbrook, 2016). By 2014, this number had risen to 747 million kg, a nearly 15-fold increase (Benbrook, 2016). In the United

States by 2014, gly-R soybeans alone accounted for 49% of the country's glyphosate use, with gly-R maize and cotton (*Gossypium hirsutum*) accounting for an additional 35% of the total ~90 million kg of glyphosate applied (Benbrook, 2016).

The evolution of HR weed accessions is a consequence of a lack of herbicide diversity. The use of a single herbicide MOA in the same place over time imposes a selection pressure for HR weeds (Beckie & Reboud, 2009). A study investigating the impacts of gly-R crops on herbicide diversity determined that the introduction of gly-R maize did not change the diversity of herbicide MOAs used in production (Kniss, 2018). With gly-R soybeans and cotton, however, increased use of glyphosate was associated with a concomitant decrease in herbicide diversity (Kniss, 2018). By 2014, gly-R soybeans and cotton alone were responsible for 77% of the total amount of glyphosate applied to agricultural land in the United States (Benbrook, 2016). Currently, 343 cases of glyphosate resistance have been reported in 56 weed species across 30 countries (Heap, 2022).

Herbicide resistance. While increased use of glyphosate combined with a decrease in herbicide diversity certainly represents a significant selection pressure for glyphosate-resistant weeds, previous research determined that weeds seem to have less capacity to evolve resistance to glyphosate compared to other herbicide MOAs (Beckie, 2006; Gustafson, 2008; Kniss, 2018). The evolution of weeds with target site resistance (TSR) to glyphosate was once thought to be a near-impossibility, owing in part to the highly conserved active site in *EPSPS* (Funke et al., 2006). However, glyphosate resistance has independently evolved in most of the 56 species reported, including mechanisms of resistance that are unique to glyphosate (Gaines et al., 2019).

Target site resistance typically refers to mutations in the genes encoding for the protein or enzyme that an herbicide targets. With glyphosate, however, over-expression of *EPSPS* or gene amplification has also been demonstrated to impart glyphosate resistance. In goosegrass (*Eleusine indica*) accessions that evolved resistance to glyphosate, constitutive expression of *EPSPS* at 9 to 30 times higher than that of the sensitive population imparted resistance 7.2 to 9.4 times greater than the sensitive population (Chen et al., 2020). Similarly, with windmill grass (*Chloris verticillata*), gene duplication in the gly-R population resulted in up to 48 times the number of *EPSPS* gene copies compared to the sensitive population, imparting glyphosate resistance up to 8.7 times that of the sensitive population (Ngo et al., 2018).

Mutations in the genes encoding target sites in herbicide-resistant weed populations are typically correlated with very high levels of resistance, as the herbicide is unable to bind to the modified protein that results. There are several known point mutations predicting amino acid changes in *EPSPS* that result in glyphosate resistance. It is interesting to note that, particularly with glyphosate, these point mutations typically do not predict amino acid substitutions within the active site where glyphosate binds, but instead modify enzyme conformation around the active site such that native substrate interactions can continue, but glyphosate can no longer bind (Powles & Yu, 2010). The first report of a mutation in *EPSPS* conferring resistance to glyphosate occurred with goosegrass (Lee & Ngim, 2000). To date, single, double, and triple mutation in *EPSPS* have been shown to impart glyphosate resistance in weed populations under glyphosate selection (Gaines et al., 2020).

For single nucleotide polymorphisms (SNPs) resulting in *EPSPS* mutants, most are located at the proline amino acid residue at position 106. A SNP at the initial base of the codon for Pro_{106} resulting in the substitution of alanine, serine, threonine or histadine has been reported in the gly-R grass weed species *Lolium rigidum*, *Eleusine indica*, *Echinochloa colona*, *Digitaria sanguinalis* and *Chloris virgata* (Yanniccari et al., 2022). The TIPS double mutation (Pro_{106}Ser mutation in conjunction with Thr_{102}Ile) once utilized to impart crop tolerance to glyphosate occurred naturally under glyphosate selection in *Eleusine indica,* and *Bidens pilosa* (Alcántara de la Cruz et al., 2016; Yu et al., 2015). A triple mutation was recently observed in *Amaranthus hybridus*, in which the TIPS double mutation was confirmed in addition to a Ala_{103}Val substitution (Perotti et al., 2019).

Non-target site resistance (NTSR) mechanisms in weeds arise from physiological processes that ultimately function to reduce amount of active herbicide reaching the target site. With glyphosate, the most observed NTSR mechanism is reduced translocation of the herbicide (Powles & Yu, 2010). Reduced translocation through the plant restricts the amount of absorbed glyphosate reaching meristematic tissues, or restricts glyphosate entry into the chloroplast, where *EPSPS* binding is required for phototoxicity (Jugulam & Shyam, 2019). There has been evidence for reduced glyphosate uptake in both *Lolium* spp. and johnsongrass (*Sorghum halepense* [L.] Pers.) (Powles & Yu, 2010). However, the role of reduced uptake or herbicide absorption in limiting the amount of glyphosate reaching the cytoplasm appears to be relatively minor in comparison to reduced translocation. The exact mechanism underlying reduced translocation of glyphosate remains to be elucidated. Previous authors have suggested the

possibility of modified chloroplast- or plasma membrane-bound transporter activity, in addition to vacuolar sequestration (Preston & Wakelin, 2008; Powles, 2008).

Recently, the identification of an upregulated ABC transporter in gly-R *Echinochloa colona* plants appears to be one of the first NTSR mechanisms whose genetic basis is known. *EcABCC8* is believed to efflux glyphosate into the apoplast. This mechanism reduces cytoplasmic concentrations of glyphosate and thus glyphosate toxicity, and overexpression of this gene in transgenic rice (*Oryza sativa*), maize, and soybean was sufficient to impart glyphosate resistance (Pan et al., 2021). Resistance indices (RI) from the ratio of the gly-R GR_{50} to the gly-S GR_{50} were 14, 13.8, and 17 for rice, maize, and soybean, respectively (Pan et al., 2021). *E. colona* with field-evolved glyphosate resistance expressed *EcABCC8* at rates 10-fold higher than the sensitive population (Pan et al., 2021). For this population, herbicide uptake and translocation patterns were similar to gly-S *E. colona* (Goh et al., 2018).

The last option for NSTR mechanisms is metabolism of the herbicide. Herbicide metabolism typically involves activity of cytochrome P450 monooxygenases (P450s) or glutathione *S*-transferases (GSTs). P450s reduce the phytotoxicity of a herbicide molecule via hydroxylation or dealkylation, and the resulting product may be further detoxified via conjugation to a sugar molecule and vacuolar sequestration of the metabolite-sugar complex (Powles & Yu, 2010). GSTs perform similar detoxification mechanisms via conjugation to glutathione. For glyphosate, however, there has long been insufficient evidence for a metabolic pathway in plants, although the microbial enzyme glyphosate oxidase (GOX) has been known for some time to be involved in glyphosate metabolism (Dill, 2005). For this reason, an early report of gly-R *Digitaria insularis* with

enhanced metabolism of glyphosate was met with skepticism (de Carvalho et al., 2011). However, in 2019, the first aldo-keto reductase involved in glyphosate metabolism was identified that resulted in the glyphosate metabolites AMPA and glyoxylate (Pan et al., 2019). Furthermore, when transcripts found in gly-R *Echinochloa colona* were overexpressed in transgenic rice seedlings, 8.5-fold overexpression of *EcAKR4-1* was sufficient to impart glyphosate resistance at levels 2 to 4 times greater than the susceptible (Pan et al., 2019).

Both TSR and NTSR mechanisms observed with gly-R species have been found to occur together, particularly with grass weed species. With gly-R goosegrass collected from citrus groves in Mexico, a $Pro_{106}Ser$ mutation was found occurring alongside overexpression of *EPSPS* (Gherekhloo et al., 2017). Likewise, a gly-R goosegrass population collected from a tea plantation in China exhibited a $Pro_{106}Ala$ mutation alongside increased gene copy number (Chen et al., 2020). Gly-R populations of *Lolium perenne* were shown to have differential herbicide absorption and translocation patterns in addition to a $Pro_{106}Ser$ mutation (Fernández-Moreno et al., 2017). The same mechanisms were found in *Chloris barbata* populations from Mexico (Bracamonte et al., 2016). Similarly, with *L. multiflorum*, a gly-R accession showed reduced herbicide absorption and translocation in addition to 21-fold greater expression of *EPSPS* (Fernández-Moreno et al., 2017). With gly-R *Poa annua* from California, a $Pro_{106}Leu$ mutation was identified alongside a 7-fold increase in gene copy number (Brunharo et al., 2019). The recent increase in the number of simultaneous glyphosate resistance mechanisms may partially reflect the expanding scope of investigations; however, it is

just as likely that the increase reflects the rapid adaptation of weedy species under continued glyphosate selection (Gaines et al., 2020).

Johnsongrass biology. Johnsongrass is a vigorous perennial grass species that likely originated approximately 2 million years ago during a hybridization event between *Sorghum bicolor* and *Sorghum propinquum* (Klein & Smith, 2021). An erect grass reaching up to 3 m in height, leaves are alternate with a prominent white midvein and ribbed sheaths, reaching 20 to 60 cm in length (McWhorter, 1989). The open, purplish panicle during flowering and creeping rhizomes are other distinguishing characteristics of the species. Johnsongrass can now be found on nearly every continent, owing to its significant investment in carbon assimilation and reproductive strategies.

The C4 photosynthetic pathway permits net photosynthesis rates ranging up to 48 $\mu mol \cdot CO_2 \cdot m^{-2} \cdot s^{-1}$, resulting in highly efficient carbon assimilation (Gulías et al., 2018; Stuart et al., 1985). In a study comparing johnsongrass to three other functionally similar C4 grasses: switchgrass (*Panicum virgatum*), little bluestem (*Schizachyrium scoparium*) and big bluestem (*Andropogon gerardii*), johnsongrass establishing from seed had higher relative growth rates, biomass accumulation, and specific leaf area (Reichmann et al., 2016). The photosynthetic nitrogen use efficiency (PNUE) of johnsongrass offers a distinct advantage for establishment in poorer quality soils. Under low nitrogen, phosphorus, and potassium, the PNUE of johnsongrass was approximately twice that of similar C4 grasses (Reichmann et al., 2016).

Johnsongrass is capable of both gametic reproduction by seeds and clonal (vegetative) reproduction by rhizomes. There appears to be a genetic linkage between the two reproductive strategies, as rhizome growth increases significantly during flowering

(McWhorter, 1989). Flowering may begin as early as 46 days after emergence (Klein & Smith, 2021). Although johnsongrass is capable of both self- and cross-pollination, detailed studies of flowering morphology suggest that most seed production results from xenogamy or cross-pollination (Kaur & Soodan, 2017). Stigmas emerge prior to pollen shed, and plants produce substantial amounts of pollen with viability above 90% at anther dehiscence. As well, bagged inflorescence experiments revealed that 78% more seed set occurred under open-pollinated conditions (Kaur & Soodan, 2017). Johnsongrass may cross-pollinate with other individuals or hybridize with feral or grain sorghum (*Sorghum bicolor*) (Klein & Smith, 2021).

Several authors report flowering occurring throughout the season. In field experiments with johnsongrass in competition with maize, one main period of seed production was observed in early summer, contributing 60 to 90% of the total season-long seed production observed (Ghersa et al., 1985; Scopel et al., 1988). In some cases, a second and lengthier flowering period was observed from late summer to early fall (Ghersa et al., 1985). These observed seed production peaks are likely in response to photoperiod, as johnsongrass flowering occurs under daylength ≤12 hours, with 13+ hour photoperiods apparently inhibitory to flowering (Knight & Bennett, 1953).

Johnsongrass seed production appears responsive to both inter- and intraspecific competition. The effects of interspecific competition were observed with field research in which johnsongrass competing with maize produced more than 32,000 $seeds^{-1} \cdot m^{-1}$, while plants grown on fallow land produced only 5,000 $seeds^{-1} \cdot m^{-1}$ (Ghersa et al., 1985). Similarly, both inter- and intra-specific competitive effects appear to impact johnsongrass in maize, where seed production was estimated at 2,350 when johnsongrass density was

17 plants per m^2 but dropped to only 87 seeds per plant when johnsongrass density was 63 plants per m^2 (Barroso et al., 2012). However, impacts of intraspecific competition appear minimal compared to those from competing species. When johnsongrass cultivated from a single plant was kept free from interspecies competition, seed production was estimated at 28,000 per plant when johnsongrass density was 190 stems·m^2 (Horowitz, 1973). Johnsongrass seedling recruitment may be sensitive to competition from parent plants. Inside a johnsongrass patch, the number of seedlings recruited was about 1% of the total number of seeds shed, whereas in tilled plots farther than 1.25 m from the patch, seedling recruitment was nearly equal to the number of seeds shed (Ghersa et al., 1993).

Estimates of seed dormancy range considerably for johnsongrass. The variability observed with johnsongrass seed dormancy is likely due to genetic factors underlying different ecotypes. When seeds from 44 ecotypes were exposed to temperatures alternating between 20 and 35°C, ~ 50% had less than 10% germinated seed by 14 days after sowing, and another ~30% had less than 20% germination (Taylorson & McWhorter, 1969). The remaining ~20% had seed germination ranging from 20 to 60% (Taylorson & McWhorter, 1969). In a longevity study, 37% of johnsongrass seed germinated after burial in soil for 5.5 years (Egley & Chandler, 1983). Dormancy appears due to the mechanical restriction of water imbibition by the hard seed coat (Bagavathiannan & Norsworthy, 2016; Taylorson & McWhorter, 1969).

The prolific seed production observed in johnsongrass is accompanied by rather rudimentary strategies for seed dispersal. The caryopsis (seed) contains simple bracts and rachilla that are likely insufficient for epizoochory (Kaur & Soodan, 2017). Johnsongrass

seed dispersal appears to occur via (in order of importance) seed rain, wind dispersal, and endozoochory. Seed rain was well described in field studies wherein 99% of the seed produced by a 10 by 10 m johnsongrass patch was contained within the patch, with the remaining 1% found within 2 m of the patch (Ghersa et al., 1993). A separate study found that 98% of seeds fell with a 5 m radius from experimental johnsongrass patches (Barroso et al., 2012). In both studies, the authors concluded that prevailing wind directions determined significant differences in the direction of seed dispersal (Barroso et al., 2012; Ghersa et al., 1993). When agronomic practices were incorporated in studies of seed dispersal, separate authors found that a maize combine dispersed johnsongrass seed up to 22 and 50 m from the seed source (Barroso et al., 2012; Ghersa et al., 1993). As well, the amount of johnsongrass seed dispersed by the combine was roughly equal to the amount dispersed under natural conditions (seed rain). Johnsongrass likely benefits from both strategies, as seed rain near the parent plant and vector-facilitated seed dispersal function to spatially separate risks to seedling establishment (Barroso et al., 2012).

The fate of dispersed seeds is often inauspicious. Seed predation by granivores and decay are two of the primary processes responsible for seed losses, and johnsongrass seed appears subject to significant predation. In field studies, 64 to 75% of johnsongrass seeds were lost from predator feeding trays (Bagavathiannan & Norsworthy, 2013). Similarly, Ghersa et al. (1985) found a 75% reduction in the number of johnsongrass seed present from fall to winter of the next year. After 1 year under field conditions, the viability of exposed johnsongrass seed was 48% (Bagavathiannan & Norsworthy, 2013). While the latter study aimed to attribute the loss of seed viability to environmental aging, the results for johnsongrass likely reflect the advantages of seed dormancy more than the perils of

post-dispersal exposure (Bagavathiannan & Norsworthy, 2013; Taylorson & McWhorter, 1969). In models for johnsongrass population expansion by seed reproduction, when seed losses were accounted for, one study concluded that reproduction by seed alone was insufficient to maintain the population (Scopel et al., 1988).

Vegetative reproduction plays a significant role in maintaining johnsongrass population growth. Rhizomes function to store excess carbohydrates and provide axillary reproductive structures from which vegetative propagation is possible (Monaghan, 1979). Rhizome growth initiates within 3 to 4 weeks of emergence, but remains relatively slow until flowering, at which point rhizome growth increases rapidly, with up to 70 m of rhizomes produced in a single season (Monaghan, 1979). Johnsongrass rhizomes also contribute to its perennial growth habit in climates where freezing winters kill aboveground plant tissue (Habyarimana et al., 2020). Johnsongrass requires some protection from cold climates to complete a perennial life cycle, as many biotypes possess rhizomes that can be rendered nonviable with freezing temperatures lower than -3 °C (Monaghan, 1979). In the spring, culms begin to emerge from rhizome around 15 °C, and typically possess a greater competitive ability due to early emergence and faster growth rates (Peerzada et al., 2017; Reichmann et al., 2016). Where rhizomes do not overwinter, johnsongrass acts as an annual, where plants are re-established from seed the following year. Seeds germinate in response to light and temperatures ranging from 25 to 30 °C (Peerzada et al., 2017).

Computer modeling to predict johnsongrass habitat found that suitable climate niches for johnsongrass exist on every continent except Antarctica (Barney & DiTomaso, 2011). For the United States and North America, 80% and 62% of land areas, respectively,

represent suitable habitat for johnsongrass establishment and growth (Klein & Smith, 2021). Currently, johnsongrass is widely naturalized across the United States as well as globally, occupying agricultural areas of the globe between latitude 55° N and 45° S (Warwick & Black, 1983).

Herbicide-resistant johnsongrass. Prior to the advent of grass-selective herbicides, johnsongrass control was primarily achieved through mechanical means (Klein & Smith, 2021). Grass-selective herbicides were developed in the 1970's and 1980's that permitted farmers to selectively manage grass weeds in dicot crops POST (Smeda et al., 1997). The graminicides are acetyl CoA carboxylase (ACCase)-inhibiting herbicides. The ACCase enzyme catalyzes the first step in fatty acid synthesis, and inhibition by these herbicides results in a depletion of phospholipids required for cellular membranes (Shaner, 2014). Repeated use of these chemistries led to the first report of resistance to ACCase-inhibiting herbicides in 1991 (Heap, 2022). The first report to describe graminicide resistance in two johnsongrass populations found that the dose required to reduce aboveground biomass by 50% (GR_{50}; I_{50}) was more than 388 times that of the reference susceptible population (Smeda et al., 1997). ACCase-inhibiting herbicide resistance in additional grass species confirmed the evolution of target site resistance conferring high levels of resistance when a mutation in the target enzyme rendered ACCase insensitive (Smeda et al., 1997). Less than 20 years later, a johnsongrass population with resistance to both ACCase inhibitors and glyphosate was reported in Argentina (Heap, 2022).

Similarly, acetolactate or acetohydroxyacid synthase (ALS or AHAS)-inhibiting herbicides have been widely available for use both preemergence (PRE) and postemergence (POST) in many crop species for more than 40 years (Powles & Yu,

2010). A number of these compounds control johnsongrass POST, such as nicosulfuron, foramsulfuron, and imazethapyr. The repeated use of these herbicides in maize led to the first report of an ALS-resistant johnsongrass population from Texas in 2000 (Heap, 2022). Later reports of separate ALS-resistant johnsongrass populations identified at least two target site mutations conferring resistance (Hernández et al., 2015). As with previous cases of ACCase-resistant johnsongrass, less than two decades elapsed before the first report of a population with resistance to both ALS and ACCase- inhibiting herbicides occurred (Heap, 2022).

The first report of a glyphosate-resistant johnsongrass population emerged from Argentina in 2005, where adoption of glyphosate-tolerant soybean was estimated to be greater than 95% (Vila-Aiub et al., 2007). Concomitantly, glyphosate use in Argentina in 2006 had increased to 12.5 times the amount used the decade prior (Vila-Aiub et al., 2007). The GR_{50} values for the glyphosate-resistant johnsongrass populations were 3.7 to 10.5 times higher rates of glyphosate than the susceptible (R/S ratio) (Vila-Aiub et al., 2007). Unlike the ACCase- and ALS-resistant johnsongrass populations, later work identified reduced herbicide uptake (10 to 25% less than S) and translocation (36% less) as the primary mechanism conferring glyphosate resistance (Vila-Aiub et al., 2012). No target site mutations were identified in this population, although the authors did not examine the possibility of *EPSPS* overexpression.

With johnsongrass, where mechanisms of glyphosate resistance have been investigated, independent populations have shown remarkable consistency. Since 2005, there have been seven new reports of evolved glyphosate resistance (gly-R) in johnsongrass populations. There is significant evidence in the literature to support the

theory that these cases of glyphosate resistance are independently evolved, not the result of vegetative spread or resistance inherited from a single individual. The widespread distribution of gly-R johnsongrass in Argentina enabled a population genetics study that determined the evolution of glyphosate resistance was not due to a single gly-R parent, as gly-R johnsongrass from different geographical regions were more closely related to their neighboring glyphosate sensitive (gly-S) conspecifics than they were to each other (Fernández et al., 2013). Of the seven reports made at the time of this writing, no one johnsongrass population has been subjected to research into all possible mechanisms of resistance to glyphosate. However, among those that have been the subject of research into mechanisms of resistance, three have concluded that reduced translocation was the primary mechanism underlying glyphosate resistance (Riar et al., 2011; Vázquez-Garcia et al., 2020; Vila-Aiub et al., 2007).

Justification. In 2016, a producer reported a failure of glyphosate to control johnsongrass within an agronomic field in Buchanan County, MO to the herbicide manufacturer. Over half of Buchanan County, MO is crop land in agricultural production. As of 2017, cultivation of more than 59,000 hectares produced in excess of $66,000,000 USD in total commodity sales (USDA, 2017). As well, johnsongrass is prolifically distributed across Buchanan County, occurring in both agricultural and non-agricultural areas, particularly along roadsides and in right-of-ways. Buchanan County producers report facing persistent johnsongrass pressure in agronomic fields (Wayne Flannery, personal communication) and the potential impacts of glyphosate-resistant johnsongrass in this area are significant.

In response to the reported failure of glyphosate to control johnsongrass, plant material was collected, and the objectives of the following research were to 1) determine if glyphosate resistance is present in the Buchanan County, MO population and 2) determine the mechanism(s) imparting resistance, if found; and 3) evaluate prospective management practices for this johnsongrass population to mitigate the spread of herbicide resistance.

LITERATURE CITED

Alcántara-de la Cruz, R, PT Fernández-Moreno, CV Ozuna, AM Rojano-Delgado, HE Cruz-Hipolito, JA Domínguez-Valenzuela, F Barro and R De Prado. 2016. Target and non-target site mechanisms developed by glyphosate-resistant hairy beggarticks (*Bidens pilosa* L.) populations from Mexico. Front. Plant Sci. 7: 1492.

Alibhai, MF and WC Stallings. 2001. Closing down on glyphosate inhibition—with a new structure for drug discovery. PNAS. 98(6): 2944-2946.

Bagavathiannan, MV and JK Norsworthy. 2016. Multiple-herbicide resistance is widespread in roadside Palmer amaranth populations. PLoS One. 11(4): e0148748.

Barney, JN and JM DiTomaso. 2011. Global climate niche estimates for bioenergy crops and invasive species of agronomic origin: potential problems and opportunities. PLoS One. 6(3): e17222.

Barroso, J, D Andújar, C San Martín, C Fernández-Quintanilla and J Dorado. 2012. Johnsongrass (*Sorghum halepense*) seed dispersal in corn crops under Mediterranean conditions. Weed Sci. 60(1): 34-41.

Beckie, HJ. 2006. Herbicide-resistant weeds: management tactics and practices. Weed Technol. 20(3): 793-814.

Beckie, HJ and X Reboud. 2009. Selecting for weed resistance: herbicide rotation and mixture. Weed Technol. 23(3): 363-370.

Benbrook, CM. 2016. Trends in glyphosate herbicide use in the United States and globally. Environ. Sci. Eur. 28(1): 1-15.

Bracamonte, E, PT Fernandez-Moreno, F Barro and R De Prado. 2016. Glyphosate-resistant *Parthenium hysterophorus* in the Caribbean Islands: non target site resistance and target site resistance in relation to resistance levels. Front. Plant Sci. 7: 1845.

Brunharo, CACG, S Morran, K Martin, ML Moretti and BD Hanson. 2019. *EPSPS* duplication and mutation involved in glyphosate resistance in the allotetraploid weed species *Poa annua* L. Pest Manag. Sci. 75(6): 1663-1670.

Chen, J, H Huang, S Wei, H Cui, X Li and C Zhang. 2020. Glyphosate resistance in *Eleusine indica*: *EPSPS* overexpression and P106A mutation evolved in the same individuals. Pestic. Biochem. Physiol. 164: 203-208.

Dayan, FE. 2019. Current status and future prospects in herbicide discovery. Plants. 8(9): 341.

de Carvalho, LB, PL Da Costa Aguiar Alves, F Gonzalez-Torralva, HE Cruz-Hipolito, AM Rojano-Delgado, R De Prado, J Gil-Humanes, F Barro and MD Luque de Castro. 2012. Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. J. Agric. Food Chem. 60(2): 615-622.

Denis, MH and S Delrot. 1993. Carrier-mediated uptake of glyphosate in broad bean (*Vicia faba*) via a phosphate transporter. Physiol. Plant. 87(4): 569-575.

Dill, GM. 2005. Glyphosate-resistant crops: history, status and future. Pest Manag. Sci. 61(3): 219-224.

Dill, GM, RD Sammons, PC Feng, F Kohn, K Kretzmer, A Mehrsheikh, M Bleeke, JL Honegger, D Farmer, D Wright and EA Haupfear. 2010. Glyphosate: discovery, development, applications, and properties. In: VK Nandula (Ed.). Glyphosate resistance in crops and weeds: history, development, and management. Hoboken, New Jersey, USA: Wiley.

Duke, SO and SB Powles. 2008. Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci. 64(4): 319-325.

Duke, SO. 2012. Why have no new herbicide modes of action appeared in recent years? Pest Manag. Sci. 68(4): 505-512.

Egley, GH and JM Chandler. 1983. Longevity of weed seeds after 5.5 years in the Stoneville 50-year buried-seed study. Weed Sci. 31(2): 264-270.

Fernández, L, LA De Haro, AJ Distefano, MC Martinez, V Lía, JC Papa, I Olea, D Tosto and HE Hopp. 2013. Population genetics structure of glyphosate-resistant johnsongrass (*Sorghum halepense* L. Pers) does not support a single origin of the resistance. Ecol. Evol. 3(10): 3388-3400.

Fernández-Moreno, PT, R Alcántara-de la Cruz, RJ Smeda and R De Prado. 2017. Differential resistance mechanisms to glyphosate result in fitness cost for *Lolium perenne* and L. *multiflorum*. Front. Plant Sci. 8: 1796.

Franz, JE, MK Mao and JA Sikorski. 1997. Glyphosate: A unique and global herbicide. Washington, USA: American Chemical Society.

Funke, T, H Han, ML Healy-Fried, M Fischer and E Schönbrunn. 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. PNAS. 103(35): 13010-13015.

Gaines, TA, SO Duke, S Morran, CAG Rigon, PJ Tranel, A Küpper and FE Dayan. 2020. Mechanisms of evolved herbicide resistance. J. Biol. Chem. 295(30): 10307-10330.

Gaines, TA, EL Patterson and P Neve. 2019. Molecular mechanisms of adaptive evolution revealed by global selection for glyphosate resistance. New Phytol. 223(4): 1770-1775.

Ge, X, DA d'Avignon, JJ Ackerman and RD Sammons. 2014. *In vivo* ^{31}P-nuclear magnetic resonance studies of glyphosate uptake, vacuolar sequestration, and tonoplast pump activity in glyphosate-resistant horseweed. Plant Physiol. 166(3): 1255-1268.

Gherekhloo, J, PT Fernández-Moreno, R Alcántara de la Cruz, E Sánchez-González, HE Cruz-Hipolito, JA Domínguez-Valenzuela and R De Prado. 2017. Pro-106-Ser mutation and *EPSPS* overexpression acting together simultaneously in glyphosate-resistant goosegrass (*Eleusine indica*). Sci. Rep. 7(1): 1-10.

Ghersa, CM, MA Martinez-Ghersa, EH Satorre, ML Van Esso and G Chichotky. 1993. Seed dispersal, distribution and recruitment of seedlings of *Sorghum halepense* (L.) Pers. Weed Res. 33: 79–88.

Ghersa, CM, EH Satorre and ML Van Esso. 1985. Seasonal patterns of johnsongrass seed production in different agricultural systems. Isr. J. Plant Sci. 34(1): 24-30.

Goh, SS, Q Yu, H Han, MM Vila-Aiub, R Busi and SB Powles. 2018. Non-target-site glyphosate resistance in *Echinochloa colona* from Western Australia. Crop Prot. 112: 257-263.

Gulías, J, R Melis, D Scordia, J Cifre, G Testa, SL Cosentino and C Porqueddu. 2018. Exploring the potential of wild perennial grasses as a biomass source in semi-arid Mediterranean environments. Ital. J. Agron. 13(2): 103-111.

Gustafson, DI. 2008. Sustainable use of glyphosate in North American cropping systems. Pest Manag. Sci. 64(4): 409-416.

Habyarimana, E, P De Franceschi, S Ercisli, FS Baloch and M Dall'Agata. 2020. Genome-wide association study for biomass related traits in a panel of *Sorghum bicolor* and S. *bicolor* × S. *halepense* populations. Front. Plant Sci. 11: 551305.

Heap, I. 2022. The international herbicide-resistant weed database. Available <www.weedscience.org>

Hernández, MJ, R León, AJ Fischer, M Gebauer, R Galdames and R Figueroa. 2015. Target-site resistance to nicosulfuron in Johnsongrass (*Sorghum halepense*) from Chilean corn fields. Weed Sci. 63(3): 631-640.

Horowitz, M. 1973. Spatial growth of *Sorghum halepense* (L.) Pers. Weed Res. 13(2): 200-208.

Jachetta, JJ, Appleby and L Boersma. 1986. Apoplastic and symplastic pathways of atrazine and glyphosate transport in shoots of seedling sunflower. Plant Physiol. 82(4): 1000-1007.

Jugulam, M and C Shyam. 2019. Non-target-site resistance to herbicides: recent developments. Plants. 8(10): 417-433.

Kaur, R and AS Soodan. 2017. Reproductive biology of *Sorghum halepense* (L.) Pers. (Poaceae; Panicoideae; Andropogoneae) in relation to invasibility. Flora. 229: 32-49.

Klein, P and CM Smith. 2021. Invasive johnsongrass, a threat to native grasslands and agriculture. Biologia. 76(2): 413-420.

Knight, WE and HW Bennett. 1953. Preliminary report of the effect of photoperiod and temperature on the flowering and growth of several southern grasses 1. Agron. J. 45(6): 268-269.

Kniss, AR. 2018. Genetically engineered herbicide-resistant crops and herbicide-resistant weed evolution in the United States. Weed Sci. 66(2): 260-273.

Lee, LJ and J Ngim. 2000. A first report of glyphosate-resistant goosegrass (*Eleusine indica* [L.] Gaertn) in Malaysia. Pest Manag. Sci. 56(4): 336-339.

Lee, TT. 1980. Characteristics of glyphosate inhibition of growth in soybean and tobacco callus cultures. Weed Res. 20(6): 365-369.

MacIsaac, SA, RN Paul and MD Devine. 1991. A scanning electron microscope study of glyphosate deposits in relation to foliar uptake. Pest. Sci. 31(1): 53-64.

McWhorter, CG. 1989. History, biology, and control of johnsongrass. In: CL Foy (Ed.). Reviews of weed science (4). Champaign, IL, USA: Weed Science Society of America.

Monaghan, N. 1979. The biology of johnson grass (*Sorghum halepense*). Weed Res. 19(4): 261-267.

Ngo, TD, JM Malone, P Boutsalis, G Gill and C Preston. 2018. *EPSPS* gene amplification conferring resistance to glyphosate in windmill grass (*Chloris truncata*) in Australia. Pest Manag. Sci. 74(5): 1101-1108.

Pan, L, Q Yu, H Han, L Mao, A Nyporko, L Fan, L Bai and SB Powles. 2019. Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinochloa colona*. Plant Physiol. 181(4): 1519-1534.

Pan, L, Q Yu, J Wang, H Han, L Mao, A Nyporko, A Maguza, L Fan, L Bai and SB Powles. 2021. An ABCC-type transporter endowing glyphosate resistance in plants. PNAS. 118(16): e2100136118.

Peerzada, AM, HH Ali, Z Hanif, AA Bajwa, L Kebaso, D Frimpong, N Iqbal, H Namubiru, S Hashim, G Rasool and S Manalil. 2017. Eco-biology, impact, and management of *Sorghum halepense* (L.) Pers. Biol. Invasions. pp. 1-19.

Perotti, VE, AS Larran, VE Palmieri, AK Martinatto, CE Alvarez, D Tuesca and HR Permingeat. 2019. A novel triple amino acid substitution in the *EPSPS* found in a high-level glyphosate resistant *Amaranthus hybridus* population from Argentina. Pest Manag. Sci. 75(5): 1242-1251.

Powles, SB and Q Yu. 2010. Evolution in action: plants resistant to herbicides. Annu. Rev. Plant Biol. 61: 317-347.

Powles, SB. 2008. Evolved glyphosate-resistant weeds around the world: lessons to be learnt. Pest Manag. Sci. 64(4): 360-365.

Preston, C and AM Wakelin. 2008. Resistance to glyphosate from altered herbicide translocation patterns. Pest Manag. Sci. 64(4): 372-376.

Ream, JE, HK Yuen, RB Frazier and JA Sikorski. 1992. *EPSPS*: binding studies using isothermal titration microcalorimetry and equilibrium dialysis and their implications for ligand recognition and kinetic mechanism. Biochem. 31(24): 5528-5534.

Reichmann, LG, S Schwinning, HW Polley and PA Fay. 2016. Traits of an invasive grass conferring an early growth advantage over native grasses. J. Plant Ecol. 9(6): 672-681.

Riar, DS, JK Norsworthy, DB Johnson, RC Scott and M Bagavathiannan. 2011. Glyphosate resistance in a johnsongrass (*Sorghum halepense*) biotype from Arkansas. Weed Sci. 59(3): 299-304.

Sammons, RD and TA Gaines. 2014. Glyphosate resistance: state of knowledge. Pest Manag. Sci. 70(9): 1367-1377.

Schönbrunn, E, S Eschenburg, WA Shuttleworth, JV Schloss, N Amrhein, JNS Evans and W Kabsch. 2001. Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. PNAS. 98(4): 1376-1380.

Scopel, AL, CL Ballare and CM Ghersa. 1988. Role of seed reproduction in the population ecology of *Sorghum halepense* in maize crops. J. Appl. Ecol. 25(3): 951-962.

Shah, DM, RB Horsch, HJ Klee, GM Kishore, JA Winter, NE Tumer, CM Hironaka, PR Sanders, CS Gasser, S Aykent and NR Siegel. 1986. Engineering herbicide tolerance in transgenic plants. Science. 233(4762): 478-481.

Shaner, DL. 2014. Herbicide handbook. Lawrence, KS, USA: Weed Science Society of America.

Smeda, RJ, CE Snipes and WL Barrentine. 1997. Identification of graminicide-resistant johnsongrass (*Sorghum halepense*). Weed Sci. 45(1): 132-137.

Sprankle, P, WF Meggitt and D Penner. 1975. Adsorption, mobility, and microbial degradation of glyphosate in the soil. Weed Sci. 23(3): 229-234.

Stuart, BL, DR Krieg and JR Abernathy. 1985. Photosynthesis and stomatal-conductance responses of johnsongrass (*Sorghum halepense*) to water stress. Weed Sci. 33(5): 635-639.

Taylorson, RB and CG McWhorter. 1969. Seed dormancy and germination in ecotypes of johnsongrass. Weed Sci. 17(3): 359-361.

Umetsu, N and Y Shirai. 2020. Development of novel pesticides in the 21st century. J. Pest. Sci 45(2): 54-74.

USDA National Agricultural Statistics Service. 2017 Census of Agriculture. Available <www.nass.usda.gov/AgCensus>.

Vázquez-Garcia, JG, C Palma-Bautista, AM Rojano-Delgado, R De Prado and J Menendez. 2020. The first case of glyphosate resistance in johnsongrass (*Sorghum halepense* [L.] Pers.) in Europe. Plants. 9(3): 313.

Vila-Aiub, MM, MC Balbi, AJ Distéfano, L Fernández, E Hopp, Q Yu and SB Powles. 2012. Glyphosate resistance in perennial *Sorghum halepense* (Johnsongrass), endowed by reduced glyphosate translocation and leaf uptake. Pest Manag. Sci. 68(3): 430-436.

Vila-Aiub, MM, MC Balbi, PE Gundel, CM Ghersa and SB Powles. 2007. Evolution of glyphosate-resistant johnsongrass (*Sorghum halepense*) in glyphosate-resistant soybean. Weed Sci. 55(6): 566-571.

Warwick, SI and LD Black. 1983. The biology of Canadian weeds: 61. *Sorghum halepense* (L.) Pers. Can. J. Plant Sci. 63(4): 997-1014.

Yanniccari, M, JG Vázquez-García, R Gigón, C Palma-Bautista, M Vila-Aiub and R De Prado. 2022. A novel *EPSPS* Pro-106-His mutation confers the first case of glyphosate resistance in *Digitaria sanguinalis*. Pest Manag. Sci. 78(7): 3135-3143.

Yu, Q, A Jalaludin, H Han, M Chen, RD Sammons and SB Powles. 2015. Evolution of a double amino acid substitution in the 5-enolpyruvylshikimate-3-phosphate synthase in *Eleusine indica* conferring high-level glyphosate resistance. Plant Physiol. 167(4): 1440-1447.

CHAPTER II

Glyphosate-Resistant Johnsongrass: Mechanism(s) of Resistance

Sarah E. Dixon, Mithila Jugulam, Murugesan Raju, Reid Smeda

ABSTRACT

Following a reported failure of glyphosate to control johnsongrass (*Sorghum halepense* [L.] Pers.) in an agronomic field in Buchanan County, MO, plants were subjected to a greenhouse dose-response assay to assess the potential for glyphosate resistance. The dose required to reduce aboveground biomass by 50% (GR_{50}) was 1,073 g ae ha^{-1} for the putative glyphosate-resistant (gly-R) population and 230 g for a comparative glyphosate-susceptible (gly-S) population, yielding a resistance index of 4.7. Of 14 gly-R plants, three survived an application of 6,323 g ha^{-1}. Potential target site mechanisms of glyphosate resistance were examined by sequencing fragments of the gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*); a shikimic acid assay was used to confirm these results. *EPSPS* expression and expression of the glyphosate efflux transporter *EcABCC8* were evaluated by quantitative PCR (qPCR). Non-target site resistance was assessed with a growth chamber study utilizing ^{14}C-glyphosate applied to potted plants to determine potential differences in herbicide absorption and translocation. Results of *EPSPS* sequencing identified no point mutations predicting amino acid changes at positions Gly_{101}, Thr_{102}, Ala_{103}, or Pro_{106} for 19 sequenced plants. For eight additional gly-R plants, no mutations were observed at Gly_{144} or Ala_{192}. In a leaf disc assay, the accumulation of shikimic acid was not significantly different between the gly-R and gly-S populations when glyphosate concentrations were >100 µM, which reinforced conclusions that glyphosate resistance was not due to an

altered target site. With acetolactate (*ALS*) as a housekeeping gene for *EPSPS* expression, one gly-R individual had 4.5-fold *EPSPS* expression relative to three gly-S plants. No evidence for *EPSPS* amplification was observed in the remaining four gly-R plants examined. Two plants were identified that overexpressed *EcABCC8* 2.2-fold compared to gly-S when *ARI8* was utilized as a housekeeping gene. Following the application of a solution containing ^{14}C-glyphosate to gly-R and gly-S plants at the three- to four-true leaf stage in a growth chamber experiment, glyphosate absorption was not different between the two populations. Absorption increased from 12 to 96 hours after treatment (HAT) to 56% of the total radioactivity recovered for both populations. At 96 HAT, the gly-R population had translocated 36% of the absorbed herbicide out of the treated leaf versus 62% for the gly-S population. Translocation to plant roots was most variable, with the gly-R population translocating 42% less ^{14}C-glyphosate than the gly-S population to the root system. The amount of herbicide recovered in aerial plant tissue outside of the treated leaf was similar between the two populations. Differential translocation of glyphosate is the primary mechanism of resistance to glyphosate in johnsongrass isolated from Buchanan County, MO. In addition, overexpression of *EPSPS* and *EcABCC8* may also contribute to glyphosate resistance within this population.

INTRODUCTION

Johnsongrass (*Sorghum halepense* [L.] Pers.) is a perennial species believed to have originated from west Asia in a hybridization event between *Sorghum bicolor* and *Sorghum propinquum* 1-2 million years ago (Klein & Smith, 2021; Paterson et al., 2020). In the post-Columbian era, human activities facilitated johnsongrass distribution across Asia, Africa, Europe, North and South America, and Australia. While widely regarded as

an invasive species, johnsongrass was intentionally introduced to the southern United States prior to 1830 as a forage crop and had dispersed into at least 21 states by 1985 (McWhorter, 1971). Currently, accessions have been confirmed in at least 47 states (Ceseski et al., 2017). Conservative modeling predicts that up to 80% of the continental United States will serve as suitable habitat for johnsongrass in the near future as mean global temperatures rise, even in the absence of regular precipitation (Barney & DiTomasio, 2011).

The potential for crop interference by johnsongrass is significant. Yield losses in row crops subjected to season-long competition with johnsongrass have reached 100% in maize, 88% in soybean, and 70% in cotton (Bridges & Chandler, 1987; Mitskas et al., 2003; Williams & Hayes, 1984). Previously, postemergence (POST) herbicides that controlled johnsongrass included organic arsenicals (e.g., methanearsonic acid [MSMA]; cacodylic acid) and plant growth regulators (e.g., mefluidide; metriflufen; dalapon); these have since become unavailable due to negative environmental impacts (Hamilton, 1966; Millhollon, 1985). Today, the current herbicides exhibiting selective control of johnsongrass POST are largely represented by the acetyl CoA carboxylase (ACCase)- and acetolactate synthase (ALS)- inhibiting herbicides, as well as the nonselective herbicides glufosinate and glyphosate.

Over-reliance on the ACCase- and ALS-inhibiting modes of action resulted in the first report of johnsongrass with resistance to ACCase-inhibiting herbicides in 1991, with an ALS-resistant biotype reported by 2000 (Heap, 2022; Smeda et al., 1997). Following the commercialization of genetically engineered glyphosate-resistant (gly-R) crops in 1996, the use of glyphosate as the primary herbicide for POST weed control was a

frequent occurrence (Johnson et al., 2014). The selection pressure imposed by widespread use of glyphosate POST resulted in the evolution of over 50 different glyphosate-resistant weed species globally by 2022 (Binimelis et al., 2009; Heap, 2022).

Glyphosate's unique mode of action is inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), the sixth enzyme in the shikimic acid pathway for biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Herrmann & Weaver, 1999). *EPSPS* performs the tandem conversion of substrates phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) to form 5-enolpyruvylshikimate-3-phosphate (EPSP) (Herrmann & Weaver, 1999). Glyphosate competes with PEP for binding but is noncompetitive with respect to S3P, as glyphosate binds specifically to the S3P-*EPSPS* complex in the enzyme's active site (Schönbrunn et al., 2001). Once bound, the resulting glyphosate-S3P-*EPSPS* complex is nonfunctional and quite stable (Ream et al., 1992). The resulting deregulation of the shikimic acid pathway results in an increase in shikimic acid. Shikimic acid increases as a result of the dephosphorylation of S3P to shikimate, as well as the lack of other metabolic pathways with significant capacity to metabolize excess shikimate (Herrmann & Weaver, 1999). The direction of carbon towards the now-dysfunctional pathway deprives other metabolic processes of critical photoassimilates, as the main branch of the shikimic acid pathway interacts with many other metabolic pathways (de Maria et al., 2006). While not completely understood, *EPSPS* inhibition by glyphosate disrupts metabolic functioning and ultimately leads to death in susceptible plants.

Weed resistance to glyphosate was once thought to be a near-impossibility, owing to the specificity of the herbicide-enzyme interaction as well as fitness penalties

associated with mutations in *EPSPS* (Binimelis et al., 2009). Single-point mutations near the enzyme's active site most frequently reduce affinity for the native substrate(s) in addition to glyphosate, representing a zero-sum game for enzymatic functioning (Dill, 2005). However, point mutations that result in amino acid substitutions in the enzyme typically impart very high levels of herbicide resistance, known as target site resistance (TSR). The most frequently reported of these is a substitution for proline at position 106 in *EPSPS*, where amino acid substitutions of alanine, cysteine, glycine, isoleucine, or threonine for proline at position 106 impart glyphosate resistance (numbered according to *Arabidopsis thaliana*) (Alibhai et al., 2010; Klee et al., 1987; Sammons & Gaines, 2014). In particular, the $Pro_{106}Gly$, $Pro_{106}Ala$ and $Pro_{106}Ser$ substitutions only slightly reduce affinity for PEP, but significantly reduce glyphosate affinity (Healy-Fried et al., 2007). As well, the double mutation $Thr_{102}Ile$ and $Pro_{106}Ser$ (TIPS) has been reported in both *Eleusine indica* and *Bidens pilosa,* imparting glyphosate resistance indices (RIs) of 180 and 20, respectively (Alcántara de la Cruz et al., 2016a; Yu et al., 2015). In 2018, with *Amaranthus hybridus*, a triple mutation (TAP-IVS) was reported in which a $Ala_{103}Val$ mutation was observed alongside $Thr_{102}Ile$ and $Pro_{106}Ser$ imparting >300-fold glyphosate resistance (Perotti et al., 2019). The remaining amino acid positions (101, 144, and 192) where mutations have been demonstrated to impart resistance in crop plants have yet to be reported with glyphosate resistance occurring in weedy species, but that should not preclude the possibility (Alibhai et al., 2010; Eichholtz et al., 2001; Sammons & Gaines, 2014).

A second TSR mechanism frequently observed in herbicide resistant weeds is overexpression of *EPSPS*, which is often correlated with increased genomic copy

number, as well as the magnitude of glyphosate resistance (Gaines et al., 2010). Gene amplification and/or duplication as a mechanism of resistance to glyphosate has been observed in several grass species, including *Bromus deandrus*, *Lolium perenne* ssp. *multiflorum,* and *Eleusine indica* (Gherekhloo et al., 2017; Malone et al., 2016; Salas et al., 2012).

Non-target site resistance (NTSR) mechanisms to glyphosate include reduced foliar absorption and translocation of the herbicide. Reduced herbicide uptake and/or translocation is the most reported NSTR mechanism of resistance to glyphosate (Jugulam & Shyam, 2019). Where the mechanism underlying reduced herbicide absorption has been explored, previous authors attributed the phenomenon to the density of epicuticular waxes (Alcántara de la Cruz et al., 2016b; Rojano-Delgado et al., 2012). Reduced translocation of glyphosate has often been attributed to vacuolar sequestration of the herbicide, which has been observed in *Conyza canadensis* and *Lolium* spp. (Ge et al., 2010; Ge et al., 2012). Vacuolar sequestration is suspected to result from modified ABC transporter activity, but the specific transporter or gene sub-family has yet to be identified (Sammons & Gaines, 2014).

Previously, gly-R johnsongrass accessions have been confirmed in the United States in Arkansas, Louisiana, and Mississippi, in addition to Argentina and Spain. Of these, three accessions were the subject of research into the mechanism of glyphosate resistance; in all three cases, reduced translocation was identified as the primary mechanism of resistance (Vila-Aiub et al., 2012; Riar et al., 2011; Vázquez-García et al., 2020). Within one population, differential herbicide absorption was also observed, with the gly-R population absorbing up to 20% less herbicide than the gly-S population (Vila-

Aiub et al., 2012). In 2016, following a report of an application of glyphosate that failed to control johnsongrass in Missouri, seeds of the putative gly-R biotype were collected for further investigation. The objectives of this research were to determine if glyphosate resistance was present with the *S. halepense* accession and, if confirmed, evaluate potential mechanisms underlying glyphosate resistance.

MATERIALS AND METHODS

Whole plant dose-response assay. When johnsongrass plants grown from seed collected from an agronomic field in Buchanan County, MO (39°34'51.3"N, 94°43'36.3"W) were initially subjected to a dose-response study to confirm resistance, a broad range of sensitivity was observed. Dose-response models did not describe the data well. However, field applications of up to 2,525 g ae ha^{-1} appeared to only temporarily stunt plant growth. Therefore, approximately 50 L of rhizomes were collected across the field in at least six sampling points on 23 Dec. 2019. Rhizomes were planted under greenhouse conditions in fiberglass trays filled with an 80:20 mix of peat-based potting media (Premier Tech Horticulture; Rivière-du-Loup, Quebec, Canada) and Mexico silt loam topsoil (fine, smectitic, mesic Vertic Epiaqualf). When emerged shoots reached 15 to 20 cm in height, 1,736 g ha^{-1} of glyphosate as the potassium salt (Bayer CropScience, MO, USA) was applied with a CO_2-pressurized backpack sprayer calibrated to deliver 140 L ha^{-1}. All treated shoots appeared to survive this application, with only plant stunting and temporary chlorosis and/or purpling observed in response to glyphosate at 1,736 g ha^{-1}. Surviving plants were allowed to grow in the greenhouse and develop rhizomes. Rhizomes were harvested on 2 Jan. 2020 for use in a greenhouse dose-response assay (Fig. 2.1). Rhizomes for the reference, susceptible population were dug from an

undeveloped urban area in Boone County, MO (38°54'38.9"N, 92°17'43.6"W) with no known history of herbicide use.

Rhizomes were cut into 3 to 7 cm fragments containing 2 to 3 nodes per piece and started in 15 cm pots in the same potting media as previously described. When plants reached 10 to 15 cm and had developed at least three true leaves, glyphosate was applied using a greenhouse track sprayer calibrated to deliver 140 L ha^{-1} at 8 doses ranging from 0 to 6,634 g ha^{-1} for the putative resistant biotype and 0 to 1,457 g ha^{-1} for the susceptible biotype. To avoid surfactant interference from the commercial formulation, when glyphosate was applied at doses above 1,457 g ha^{-1}, a custom mixture of glyphosate without surfactant (MON 78623) and the current commercial surfactant (MON 58121) supplied by the manufacturer was utilized (Bayer CropScience; St. Louis, MO, USA). At 21 days after treatment (DAT), plants were harvested at the soil line and dried at 50ºC for 72 hours, and plant dry weight was measured.

***EPSPS* gene sequencing.** Tissue from developing leaves of gly-R and gly-S individuals maintained in the greenhouse was collected and immediately frozen on dry ice. Total RNA was isolated from leaf tissue samples using RNeasy Plant Mini Kit (Qiagen; Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's instructions. Synthesis of cDNA was conducted using 3 µg of total RNA in combination with SuperScript IV Reverse Transcriptase and random hexamers (Invitrogen; Waltham, MA, USA). Following cDNA synthesis, samples were treated with 2 U of *E. coli* ribonuclease H to prohibit RNA carryover. To amplify a segment of *EPSPS* gene, primers were designed based on sequences listed under GenBank accessions KC914621.1, HQ436353.1, HQ436354.1, HQ436351.1, and HQ436352.1, resulting in

forward: 5’ TGGGGCCTTGAACACTCTTG 3’; reverse 5’ACATCCCCAAGAGCC-AAAGG 3’. These amplify a segment of the *EPSPS* gene encompassing the Thr_{102} and Pro_{106} positions previously described to confer glyphosate resistance in *Eleusine indica*, *Digitaria insularis,* and *Lolium* spp. (Sammons & Gaines, 2014). Amplification of cDNA by polymerase chain reaction (PCR) was conducted using 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM each primer, and 2 U Platinum Taq DNA polymerase (Invitrogen; Waltham, MA, USA). PCR cycling conditions were 94 °C for 2 min, followed by 39 cycles of 94 °C for 30 s, 61 °C for 45 s, and 72 °C for 30 s with a final extension at 72 °C for 10 min. For a subset of plants (*n*=8), primers previously designed by Fernández et al. (2013) and Vila-Aiub et al. (2012) were used (forward: 5’ TGAGGATGTTCACT-ACATGC 3’; reverse: 5’ CACATCACCCTGCAAACTGG 3’) to generate longer sequence reads that cover positions Gly_{144} and Ala_{192}. PCR cycling conditions for these were 94 °C for 2 min, followed by 39 cycles of 94 °C for 30 s, 56.5 °C for 45 s, and 72 °C for 30 s with a final extension at 72 °C for 10 min. PCR products were loaded in 1.5% agarose gel and bands were isolated using QIAquick Gel Extraction Kit (Qiagen; Hilden, North Rhine-Westphalia, Germany) according to the manufacturer’s instructions. PCR products were sequenced in duplicate using Sanger sequencing with Applied Biosystems BigDye Terminator v3.1 cycle sequencing chemistry (Thermo Fisher Scientific; Waltham, MA, USA). The resulting forward- and reverse-generated nucleotide sequences were constructed and submitted to GenBank under the accession numbers listed in Fig. 2.2. Sequences were searched in the GenBank database using the basic local alignment search tool. Sequences used for comparison were those obtained from gly-S leaf samples in

addition to gly-R (HQ436351.1; HQ436352.1) and gly-S (MK492471.1; HQ436354.1; HQ436353.1) johnsongrass accessions listed in GenBank.

Quantitative PCR. First-strand cDNA from gly-R and gly-S plants was synthesized from 2 µg total RNA as previously described. For *EPSPS* gene expression, efficiency curves were constructed by subjecting cDNA to 0, 1/5, 1/25, and 1/125 dilutions. cDNA was loaded into 96-well plates for quantitative PCR (qPCR) using the QuantStudio 3 real-time qPCR system (Thermo Fisher Scientific; Waltham, MA, USA).

Four replications for each primer + cDNA concentration were amplified in 25 µL reaction volumes containing 1 µL aliquots of cDNA, 0.2 µM each forward and reverse primers, and SYBR Green PCR Master Mix (Thermo Fisher Scientific; Waltham, MA, USA) diluted to 1x concentration. Reaction conditions were 50°C for 2 min followed by 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curves were constructed by holding the samples at 95°C for 1 min, then reducing the temperature to 60° C for 1 min, followed by increasing the temperature by 0.15°C every 5 s to 95°C. Only one plant sample resulted in multiple peaks by melt-curve analysis; this sample was not included in the final data for analysis.

Three primer pairs targeting the protein coding sequence for acetolactate synthase (*ALS*) were used as comparative controls, and three primer pairs targeting the protein coding sequences of *EPSPS* were used in qPCR. The primer pairs targeting *ALS* were forward: 5’ CAGTCCGTGTGACAA-AGAAGA 3’ and reverse: 5’ GCGGGACGA-TTATATCCAAGAG 3’; forward: 5’ CGCTCCATCACCAAA-CATAAC 3’ and reverse: 5’ CAGAAGAGGCGAGG-AAGAAA; forward: 5’ ACGTGTGACAGGGAA-GATTG 3’ and reverse: 5’ CATGTG-GCTGCTTGTTC-TTG 3’. Primer pairs targeting

the *EPSPS* sequence were forward: 5’ GATGGAGCCAGAGAGCTTAAC 3’ and reverse: 5’ GTGCGGACGTTGATTGTTTC 3’; forward: 5’ TGAGGTGTTTCCTCC-CAAATG 3’ and reverse: 5’ GTTTGCTGAG-GTACTGGAGATG 3’; forward: 5’ ATCTCCAGTACC-TCAGCAAAC 3’ and reverse: 5’ GCCTCAAGTGCAAGCTAT-TTC 3’. Initially, the six primer pairs were employed to confirm the absence of primer-dimers as well as primer efficiency. However, neither primer-dimers nor nonspecific amplification were observed for any primer pair. When data quality was confirmed according to Yuan et al. (2006), amplicons from each were included in data analysis.

The plasma membrane-bound ABC transporter *EcABCC8,* shown to efflux glyphosate into the apoplast and reduce cytoplasmic levels of the herbicide, was included in gene expression analysis (Pan et al., 2021). For *EcABCC8*, qPCR followed the same protocol as described above, with a few exceptions. Plants were treated with glyphosate at 1,446 g ha^{-1} and tissue was harvested at 6 hours after treatment. Tissue was collected from gly-R plants surviving the dose-response assay and four additional plants grown from seed produced by a gly-R individual (F1). Due to the induction of glyphosate stress, the housekeeping gene for these qPCR studies was *ARI8*, utilizing primers designed by Ulrich et al. (2021). EcABCC8 primers (forward: 5’ AAAGGATTCAGCTGGCAAGA 3’; reverse: 5’ TTCAACTTGGTGGGTCACAA 3’) were designed by referencing GenBank accession number MT249005 (Pan et al., 2021). Reverse-transcribed cDNA was subjected to 1/2, 1/4, and 1/8 dilutions, and reaction conditions were the same as described above. Samples labeled F1 denote plants grown from seed collected from putative gly-R plants.

Shikimic acid assay. An enzyme inhibition assay was conducted following the methods described in Amaro-Blanco et al. (2018) using living leaf tissue harvested from gly-R and gly-S johnsongrass. Leaf discs approximately 7 mm in diameter were removed from 8 plants per population. Discs were taken from blade of the youngest, fully expanded leaf when plants were at the three- to five-leaf stage.

Prior to experimentation, glyphosate as the potassium salt was added to 1 mM ammonium dihydrogen phosphate ($NH_4H_2PO_4$) (MilliporeSigma; Burlington, MA, USA) at pH 4.4 resulting in concentrations of 0, 1, 10, 50, 100, 250, 500, and 1000 µM. After weighing, leaf discs were transferred to 1.5 mL Eppendorf tubes containing 1 mL of 1 mM of $NH_4H_2PO_4$ with five discs per tube. Tubes were incubated in a growth chamber for 24 h under a constant photoperiod of 900 µmol m^2 s^{-1} at 30°C, with ambient humidity (uncontrolled) ranging from 27 to 35%. For each glyphosate concentration and population, six replicates were used. Standards were analyzed within each experimental run for quality control.

At 24 h, tubes were stored at −20°C until further analysis. For analysis, tubes were thawed at room temperature. Thereafter, 250 µL of 1.25 N HCl (MilliporeSigma; Burlington, MA, USA) was added and tubes were incubated at 50 °C for 20 min. A 125 µL aliquot from each Eppendorf tube was pipetted into a new 1.5 mL Eppendorf tube, and 500 µL of 0.25% w/v periodic acid and sodium metaperiodate (MilliporeSigma; Burlington, MA, USA) was added. Following incubation at room temperature for 90 min, 500 µL of 0.6 N sodium hydroxide (Midwest Scientific; St. Louis MO, USA) and 0.22 M sodium sulfite (MilliporeSigma; Burlington, MA, USA) was added and immediately transferred to cuvettes. Within 20 min, light absorption at 380 nm was measured using an

Evolution 300 UV-Vis spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA).

^{14}C-glyphosate absorption and translocation. Differential absorption and translocation were examined using ^{14}C-glyphosate as the isopropylamine salt (American Radiolabeled Chemicals; St. Louis, MO, USA) in repeated growth chamber experiments. The experimental design was a randomized complete block with five replications. Time, in units of hours after treatment (HAT) and population (gly-R versus gly-S) were main factors. The experimental unit was a single, potted johnsongrass plant started from rhizome pieces at the three- to five-leaf stage measuring 25 to 35 cm in height. Plants were treated with 315,000 dpm as two, 5 µL droplets of a solution containing ^{14}C-glyphosate and commercially formulated glyphosate (Bayer CropScience; St. Louis, MO, USA) at a rate of 868 g ae ha^{-1} with the addition of non-ionic surfactant (NIS) at 1% v/v (MFA Incorporated; Columbia, MO, USA). Plants were harvested at 12, 24, 48, 72, and 96 HAT and partitioned into parts consisting of the treated leaf, all aerial plant tissue above treated leaf, all aerial plant tissue below treated leaf, and root material.

When plants were harvested, the treated leaf was cut at the collar and placed into 15 mL conical centrifuge tubes containing 10 mL of leaf wash solution (0.25% NIS; 10% methanol; 89.75% $_{d}H_2O$) and vortexed for 60 s. This leaf wash solution was decanted and collected in a scintillation vial. The procedure was repeated, and following the second leaf wash, the treated leaf was placed in a paper envelope and dried at 50°C for 4 d. Radioactivity from leaf wash solutions was determined by liquid scintillation spectrometry using a Beckman LS 6500 Scintillation System (Beckman Coulter; Brea, CA, USA). Aerial plant tissue above and below the treated leaf were placed in envelopes

inside paper bags and oven-dried at 50°C for 4 d. Roots were allowed to air-dry for 24 h after harvest of the aerial tissues, and potting media was gently shaken from roots. Root material was placed in envelopes and dried as previously described. Following combustion in an OX-501 biological material oxidizer (RJ Harvey Instrument Corporation; Tappan, NY, USA), the gaseous combustion products were captured in liquid scintillation cocktail (Z Scientific; New City, NY, USA) and quantified by liquid scintillation spectrometry.

For each plant, the total amount of radiation recovered was determined as the sum of all plant parts as well as leaf wash solution, which averaged 98.3%. The amount of activity absorbed was calculated as a percent by dividing the sum of all plant parts by the total amount recovered (Olson et al., 1999). Herbicide translocation was also expressed as a percent, determined by the amount of radioactivity within a given plant part divided by the amount absorbed.

Statistical analysis. The whole-plant dose-response assay was a completely randomized design, repeated. In SAS Enterprise Guide 7.1 (SAS Institute; Cary, NC, USA) data were subjected to a mixed model using PROC GLM to confirm treatment effect. Results are means pooled from 14 replications over 2 runs, when no interaction was observed between runs and herbicide dose or between replication and dose. Plant dry biomass data were transformed as a percent of the non-treated control within each replication and subjected to non-linear regression (Fig. 2.1) using a four-parameter, log-logistic model (Seefeldt et al., 1995) (Eq. 2.1) in GraphPad Prism 7 (GraphPad Software; San Diego, CA, USA) according to Larran et al. (2017).

$$y = c + \frac{(d - c)}{1 + x / gly\text{-}R_{50}{}^{b}} \qquad \text{(Eq. 2.1)}$$

With qPCR, a data quality check was performed using linear regression for each sample + gene combination using SAS Enterprise Guide 8.3. Data quality was analyzed with the model $Ct = \beta_0 + \beta_{conc}X_{lconc} + \varepsilon$, where X_{lconc} is the log (base 2) transformed concentration of cDNA template, and β_0 and β_{conc} represent the intercept and slope of the regression line, respectively. Gly-R individuals were compared to the combined data of all three gly-S individuals. For each individual, the Ct number was plotted against the log-transformed concentration of cDNA template. Data quality was confirmed when the predicted slopes of lines were -1 (with $p < 0.0001$) (Yuan et al., 2006).

Similarly, a multiple linear regression (MLR) model (Eq. 2.2) including effects of population, gene, and cDNA concentration was utilized to test for differences between gene and population using a single degree of freedom contrast statement.

(Eq. 2.2)

$$Ct = \beta_0 + \beta_{conc}X_{iconc} + \beta X_{ipopulation} + \beta_{gene}X_{igene} + \beta_{conc*population}X_{iconc}X_{ipopulation} + \beta_{conc*gene}X_{iconc}X_{igene} + \beta_{gene*population}X_{igene}X_{ipopulation} + \beta_{conc*gene*population}X_{icocn}X_{\text{-}\ ipopulation}X_{igene} + \varepsilon$$

To estimate gene expression, the means of all three gly-S individuals were utilized to derive $\Delta\Delta Ct$ values for both gly-R and gly-S individuals.

The shikimic acid assay was arranged as a randomized complete block design with six replicates, repeated twice. A standard curve was constructed using known concentrations of shikimic acid (MilliporeSigma; Burlington, MA, USA) in assays without leaf discs. All data were corrected for background absorbance at 380 nm and shikimate concentrations determined by the standard curve were transformed from µg shikimate μL^{-1} solution to µg shikimate per gram fresh leaf weight. Data were combined

across experiments when no significant interactions were observed using PROC GLM in SAS Enterprise Guide 8.3 after assessing normality & homoscedasticity using PROC UNIVARIATE. Shikimic acid accumulation data were modeled using Eq. 2.1 with GraphPad Prism 7 software.

For the absorption and translocation of radiolabeled glyphosate, percent data were combined across both experiments when no significant interactions were observed using PROC GLM in SAS. Data normality & homoscedasticity were evaluated using PROC UNIVARIATE, and natural log transformed to meet the assumption of normality. Differences between the gly-R and gly-S populations were determined by ANOVA with significant means separated using Tukey's Honestly Significant Difference (HSD) at each harvest interval. For those intervals where significant differences between the two populations were observed, ANOVA was repeated using percent data and subjected to means separation using Tukey's HSD.

RESULTS

Whole plant dose-response assay. Data were able to be combined from experiments, and the log-logistic response model parameters showed the suspected gly-R population to be less susceptible to glyphosate than the susceptible control plants (p>0.0001 with R^2 of 0.99 for the independent models) (Table 2.1). The GR_{50} values for the gly-R and gly-S populations were 1,073 and 230 g ha^{-1}, respectively, yielding a resistance index of 4.7. The range of glyphosate doses utilized to determine the GR_{50} for the gly-R population (0 to 6,323 g ha^{-1}) was insufficient to determine the LD_{50} (lethal dose; or dose required for 50% population mortality). However, based on visual injury estimations, eight plants of

14 survived the application of 5,059 g ha^{-1}, and three plants survived 6,323 g ha^{-1} (data not shown).

***EPSPS* gene sequencing and quantitative PCR.** No mutations in the gene sequences were observed. On the longer sequence reads, single nucleotide polymorphisms (SNPs) were apparent between individuals, but none of these predicted significant amino acid substitutions. Furthermore, johnsongrass is tetraploid, and paralogous genes arising from duplication events can introduce false SNPs (Paterson et al., 2020). Given the absence of SNPs predicting amino acid substitutions at known positions, these polymorphisms were not considered further. Sequencing results ruled out the possibility of an insensitive *EPSPS* in this population.

EPSPS gene expression for the combined gly-S individuals was estimated at 2 ± 0.4 relative to *ALS*. For four of the five gly-R individuals, expression of *EPSPS* was with the range observed with susceptible plants (Fig. 2.3a). However, one gly-R individual showed increased expression of *EPSPS* (4.5 ± 1.1; $p < 0.0001$) relative to *ALS*.

The level of glyphosate sensitivity in the F1 individuals tested for expression of *EcABCC8* was not known. Here, the mean of three technical replicates for the gly-S individual was utilized to derive ΔΔCt values for the gly-S, gly-R and F1 individuals (Fig. 2.3b). In response to glyphosate stress, the gly-R individual overexpressed the plasma membrane-bound glyphosate transporter ($p < 0.0001$) with an estimated expression of 2.2 ± 0.4 relative to *ARI8*. Similarly, *EcABCC8* expression by one F1 plant was higher than the gly-S ($p < 0.0001$) with estimated expression levels at 2.3 ± 0.4.

Shikimic acid assay. For gly-S plants, shikimic acid accumulation occurred rapidly between 1 and 100 µM glyphosate (Fig. 2.4). Enzyme saturation (near-total inhibition)

appeared to occur between 250 and 500 µM. Shikimate was slower to accumulate in gly-R plants, with 50 µM glyphosate discriminating between the two populations (p= 0.0038). Gly-S plants yielded a mean of 71 µg shikimate per gram fresh leaf weight, whereas gly-R plants yielded only 27 µg. Above 100 µM glyphosate, differences were no longer observed, and gly-R plants accumulated similar levels of shikimate as gly-S plants at 1,000 µM glyphosate. It was unclear whether enzyme saturation occurred at 1,000 µM for gly-R plants.

^{14}C-glyphosate absorption and translocation. Absorption of ^{14}C-glyphosate was low but similar for both gly-R and gly-S plants, with a mean of 16.7 and 17.1% of the applied activity absorbed at 96 HAT, respectively (Fig. 2.5a). On treated leaves, the two, 5 µL droplets of ^{14}C-glyphosate solution resulted in copper-colored lesions on the leaf surface after the solution dried, which appeared suggestive of tissue necrosis. However, herbicide absorption did increase over time for both gly-R and gly-S plants, indicating that potential leaf burning (perhaps associated with the use of surfactant) did not completely inhibit absorption. No significant differences in herbicide absorption were observed at any interval after application (Fig. 2.5a).

Differences in herbicide translocation were apparent between gly-R and gly-S plants as early as 12 HAT (Fig. 2.5b). At 12 HAT, the gly-R population had translocated 64 and 30% less ^{14}C-glyphosate above the treated leaf and to the roots, respectively, than the gly-S population. By 24 HAT, the gly-R population had translocated 38% less herbicide out of the treated leaf to other plant tissues than the gly-S population. By 96 HAT, no differences were observed between gly-R and gly-S plants in the amount of herbicide translocated to aerial plant parts outside of the treated leaf (Fig. 2.5d). However, 42%

more herbicide remained in the treated leaf of gly-R plants at 96 HAT. In addition, significantly less herbicide was recovered from the roots of gly-R plants, with gly-S translocating up 46% more ^{14}C-glyphosate to the root system than gly-R plants.

DISCUSSION

The resistance index resulting from dose-response studies is consistent with the level of glyphosate resistance observed in previous gly-R johnsongrass, where RIs ranged from 3.5 to 10.5 (Riar et al., 2011; Vila-Aiub et al., 2012). A recent report of glyphosate resistance evolved within a johnsongrass population from Spain reports similar results, with RIs of 4.2 to 9 (Vázquez-García et al., 2020).

Where gly-R johnsongrass has previously been reported, only three accessions have been the subject of research into the mechanism of resistance. In two cases where potential TSR was examined, the authors found no evidence for mutations in *EPSPS* (Vázquez-García et al., 2020; Vila-Aiub et al., 2012). The results of sequencing 19 individuals confirm those of previous studies. As well, with *EPSPS* enzyme assays, insensitive target sites yield differential responses to glyphosate *in vivo* and are detectable with the type of leaf disc assay performed in this study (Gaines et al., 2010; Ngo et al., 2016; Ngo et al., 2018). Shikimic acid ($C_7H_{10}O_5$) accumulates with *EPSPS* inhibition due to dephosphorylation of shikimate-3-phosphate to shikimate (Herrmann & Weaver, 1999). In the case of an insensitive target site, when glyphosate does not bind readily with *EPSPS*, shikimic acid may not accumulate.

The results of the shikimic acid assay are consistent with the absence of a target site mutation mechanism. While *EPSPS* inhibition in gly-R plants required higher

concentrations of glyphosate, the similar shikimate accumulation in both gly-R and gly-S plants at doses above 100 μM is indicative of a glyphosate-sensitive *EPSPS*. This also supports results of the dose-response study, in that glyphosate could still be lethal for gly-R plants, albeit at significantly higher doses than gly-S plants. Finally, the results of the shikimic acid assay confirm those of Vázquez-García et al. (2020), in which the gly-R johnsongrass accession from Spain showed no differences in *EPSPS* sensitivity to glyphosate.

To our knowledge, no currently known gly-R johnsongrass populations have been subjected to analysis of *EPSPS* expression. However, there is some basis for comparison with the grass weeds *Echinochloa colona*, *Eleusine indica*, and *Lolium multiflorum* with field-evolved resistance to glyphosate. With glyphosate-resistant goosegrass (*Eleusine indica*), target site resistance was examined for four populations collected from a tea plantation in China. Two populations overexpressed *EPSPS* by 9.3- and 5.4-fold compared to the glyphosate susceptible population (Chen et al., 2020). This overexpression correlated to RIs of 7.2 and 4.9, respectively (Chen et al., 2020). The goosegrass population with an RI of 4.9 and overexpression at 5.4-fold is similar to the results described here, although both the level of resistance and *EPSPS* overexpression observed in the johnsongrass population were both lower, at 4.7 and 4.5, respectively.

Higher levels of gene duplication or amplification are expected to correlate with higher levels of glyphosate resistance. Compared to a few other gly-R weed species with *EPSPS* overexpression, gene amplification in the johnsongrass individual revealed here was low. For example, a goosegrass population with an RI of 9.3 overexpressed *EPSPS* by 30-fold, and with *Amaranthus palmeri*, genomic copy numbers from 5 to >160 were

observed in populations with RIs of 6.2 to 8 (Chen et al., 2020; Culpepper et al., 2006; Gaines et al., 2010; Gaines et al., 2012). However, not all gly-R weed populations overexpressing *EPSPS* have followed this trend. With gly-R *Lolium multiflorum*, a 21-fold increase in *EPSPS* expression correlated to RIs of only 2.3 (GR_{50}) or 4.3 (LD_{50}) (Fernández-Moreno et al., 2017). In more recent studies with gly-R goosegrass, *EPSPS* was overexpressed in one population by 3-fold (Zhang et al., 2021). However, when glyphosate stress was induced, this overexpression rose to 8-fold, which may partially explain why low levels of gene duplication or amplification observed without glyphosate stress could contribute more substantially to glyphosate resistance (Zhang et al., 2021). While not true for all plants tested, for the gly-R johnsongrass population from Missouri, *EPSPS* amplification is one potential mechanism contributing to glyphosate resistance.

Overexpression of *EcABCC8* is another possible mechanism contributing to glyphosate resistance in the gly-R johnsongrass population. Both TSR and NTSR mechanisms were explored for a population of junglerice (*Echinochloa colona*) with field-evolved resistance to glyphosate (RI= 5.6) (Goh et al., 2018). *EPSPS* expression was similar between the R and S biotypes, and when *EPSPS* expression failed to respond to induction via glyphosate treatment, genomic copy number was not evaluated (Goh et al., 2018). In 2021, this same population was found to overexpress the ABC transporter *EcABB8* by ten-fold relative to the S population (Pan et al., 2021). While this level of expression is nearly five times higher than that observed with johnsongrass (2.2), given the evidence for additional resistance mechanisms in the gly-R johnsongrass population, it cannot be ruled out.

For the three gly-R johnsongrass accessions in which glyphosate resistance was the subject of mechanism of resistance research, reduced translocation was identified as the primary mechanism of resistance for all (Riar et al., 2011; Vázquez-García et al., 2020; Vila-Aiub et al., 2012). Results of the ^{14}C-glyphosate absorption and translocation study agree with these. The first reported gly-R johnsongrass population retained 57% more glyphosate in the treated leaf than the susceptible population, resulting in RIs ranging from 3.5 to 10.5 (Vila-Aiub et al., 2007; Vila-Aiub et al., 2012). The second reported case, occurring in the United States, identified 28% greater glyphosate retention in the treated leaf of gly-R plants, imparting RIs of 5 to 7 (Riar et al., 2011). Recently in Spain, gly-R plants retained 47 – 57% more herbicide in the treated leaf, and RIs were estimated at 4.2 to 9 (Vázquez-García et al., 2020). For the gly-R johnsongrass population described here, 42% more glyphosate was retained in the treated leaf than the gly-S population, which is consistent with previously described data. These results suggest that reduced translocation of the herbicide is the primary mechanism underlying glyphosate resistance in this population.

The evolution of glyphosate resistance was confirmed in this population of johnsongrass originating from Buchanan County, MO. The level of glyphosate resistance is within the range previously reported for johnsongrass, and as with other gly-R johnsongrass populations, reduced translocation of the herbicide to the crown and roots is one mechanism imparting resistance. As with two previous reports, no mutations in *EPSPS* were discovered within this population. Expression of *EPSPS* has not been previously reported on with gly-R johnsongrass, nor has expression of *EcABCC8*, a transporter that effluxes glyphosate into the apoplast. In this research, some

overexpression of *EPSPS* was observed in one individual, which may be enhanced under glyphosate stress. When plants were challenged with glyphosate, overexpression of the *EcABCC8* transporter was observed in both parent and offspring individuals, suggesting a supporting role for epigenetic mechanisms to enhance glyphosate tolerance. This is the first study to report on gene expression of both *EPSPS* and *EcABCC8* in a population of johnsongrass with field-evolved resistance to glyphosate.

LITERATURE CITED

Alcántara-de la Cruz, R, PT Fernández-Moreno, CV Ozuna, AM Rojano-Delgado, HE Cruz-Hipolito, JA Domínguez-Valenzuela, F Barro and R De Prado. 2016a. Target and non-target site mechanisms developed by glyphosate-resistant hairy beggarticks (*Bidens pilosa* L.) populations from Mexico. Front. Plant Sci. 7: 1492.

Alcántara de la Cruz, R, F Barro, JA Domínguez-Valenzuela and R De Prado. 2016b. Physiological, morphological and biochemical studies of glyphosate tolerance in Mexican cologania (*Cologania broussonetii* (Balb.) DC.). Plant Physiol. Biochem. 98: 72-80.

Alibhai, MF C Cajacob, PCC Feng, GR. Heck, Y Qi, S Flasinski and WC Stallings. 2010. Glyphosate resistant class I 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*). U.S. Patent 7,723,575 issued May 25, 2010.

Amaro-Blanco, I, PT Fernández-Moreno, MD Osuna-Ruiz, F Bastida and R De Prado. 2018. Mechanisms of glyphosate resistance and response to alternative herbicide-based management in populations of the three *Conyza* species introduced in southern Spain. Pest Manag. Sci. 74(8): 1925-1937.

Barney, JN and JM DiTomaso. 2011. Global climate niche estimates for bioenergy crops and invasive species of agronomic origin: potential problems and opportunities. PLoS One. 6(3): e17222.

Binimelis, R, W Pengue and I Monterroso. 2009. "Transgenic treadmill": responses to the emergence and spread of glyphosate-resistant johnsongrass in Argentina. Geoforum. 40(4): 623-633.

Bridges, DC and JM Chandler. 1987. Influence of johnsongrass (*Sorghum halepense*) density and period of competition on cotton yield. Weed Sci. 35(1): 63-67.

Ceseski, AR, K Al-Khatib and JA Dahlberg. 2017. Distinguishing johnsongrass and young summer grass weeds. Available <https://escholarship.org/uc/item/0pw0r288>

Chen, J, H Huang, S Wei, H Cui, X Li and C Zhang. 2020. Glyphosate resistance in *Eleusine indica*: *EPSPS* overexpression and P106A mutation evolved in the same individuals. Pestic. Biochem. Physiol. 164: 203-208.

Culpepper, AS, TL Grey, WK Vencill, JM Kichler, TM Webster, SM Brown, AC York, JW Davis and WW Hanna. 2006. Glyphosate-resistant Palmer amaranth (*Amaranthus palmeri*) confirmed in Georgia. Weed Sci. 54(4): 620-626.

de María, N, JM Becerril, JI García-Plazaola, A Hernández, MR De Felipe and M Fernández-Pascual. 2006. New insights on glyphosate mode of action in nodular metabolism: role of shikimate accumulation. J Agric. Food Chem. 54(7): 2621-2628.

Dill, GM. 2005. Glyphosate-resistant crops: history, status and future. Pest Manag. Sci. 61(3): 219-224.

Eichholtz, DA, CS Gasser and GM Kishore. 2001. Modified gene encoding glyphosate-tolerant 5-enolpruvyl-3-phosphoshikimate synthase. U.S. Patent No. 6,225,114.

Fernández, L, LA De Haro, AJ Distefano, MC Martinez, V Lía, JC Papa, I Olea, D Tosto and HE Hopp. 2013. Population genetics structure of glyphosate-resistant johnsongrass (*Sorghum halepense* L. Pers) does not support a single origin of the resistance. Ecol. Evol. 3(10): 3388-3400.

Fernández-Moreno, PT, R Alcántara-de la Cruz, RJ Smeda and R De Prado. 2017. Differential resistance mechanisms to glyphosate result in fitness cost for *Lolium perenne* and L. *multiflorum*. Front. Plant Sci. 8: 1796.

Gaines, TA, W Zhang, D Wang, B Bukun, ST Chisholm, DL Shaner, SJ Nissen, WL Patzoldt, PJ Tranel, AS Culpepper and TL Grey. 2010. Gene amplification confers glyphosate resistance in *Amaranthus palmeri*. PNAS. 107(3): 1029-1034.

Gaines, TA, A Cripps and SB Powles. 2012. Evolved resistance to glyphosate in junglerice (*Echinochloa colona*) from the tropical Ord River region in Australia. Weed Technol. 26(3): 480-484.

Ge, X, DA d'Avignon, JJH Ackerman and RD Sammons. 2010. Rapid vacuolar sequestration: the horseweed glyphosate resistance mechanism. Pest Manag. Sci. 66(4): 345-348.

Ge, X, DA d'Avignon, JJH Ackerman, A Collavo, M Sattin, EL Ostrander, EL Hall, RD Sammons and C Preston. 2012. Vacuolar glyphosate-sequestration correlates with glyphosate resistance in ryegrass (*Lolium* spp.) from Australia, South America, and Europe: a ^{31}P-NMR investigation. J. Agric. Food Chem. 60(5): 1243-1250.

Gherekhloo, J, PT Fernández-Moreno, R Alcántara de la Cruz, E Sánchez-González, HE Cruz-Hipolito, JA Domínguez-Valenzuela and R De Prado. 2017. Pro-106-Ser mutation and *EPSPS* overexpression acting together simultaneously in glyphosate-resistant goosegrass (*Eleusine indica*). Sci. Rep. 7(1): 1-10.

Goh, SS, Q Yu, H Han, MM Vila-Aiub, R Busi and SB Powles. 2018. Non-target-site glyphosate resistance in *Echinochloa colona* from Western Australia. Crop Prot. 112: 257-263.

Hamilton, KC. 1966. Response of johnsongrass to herbicides. J. Prog. Ag. Arizona. 18(5): 19.

Healy-Fried, ML, T Funke, MA Priestman, H Han and E Schönbrunn. 2007. Structural basis of glyphosate tolerance resulting from mutations of Pro101 in *Escherichia coli* 5-enolpyruvylshikimate-3-phosphate synthase. J. Biol. Chem. 282(45): 32949-32955.

Heap, I. 2022. The international herbicide-resistant weed database. Available <www.weedscience.org>

Herrmann, KM and KH Weaver. 1999. The shikimate pathway. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 473-503.

Johnson, DB, JK Norsworthy and RC Scott. 2014. Herbicide programs for controlling glyphosate-resistant johnsongrass (*Sorghum halepense*) in glufosinate-resistant soybean. Weed Technol. 28(1): 10-18.

Jugulam, M and C Shyam. 2019. Non-target-site resistance to herbicides: recent developments. Plants. 8(10): 417-433.

Klee, HJ, YM Muskopf and CS Gasser. 1987. Cloning of an Arabidopsis thaliana gene encoding 5-enolpyruvylshikimate-3-phosphate synthase: sequence analysis and manipulation to obtain glyphosate-tolerant plants. Mol. Gen. Genetics. 210(3): 437-442.

Klein, P and CM Smith. 2021. Invasive johnsongrass, a threat to native grasslands and agriculture. Biologia. 76(2): 413-420.

Larran, AS, VE Palmieri, VE Perotti, L Lieber, F Tuesca and HR Permingeat. 2017. Target-site resistance to acetolactate synthase (ALS)-inhibiting herbicides in *Amaranthus palmeri* from Argentina. Pest Manag. Sci. 73(12): 2578-2584.

Malone, JM, S Morran, N Shirley, P Boutsalis and C Preston. 2016. *EPSPS* gene amplification in glyphosate resistant *Bromus diandrus*. Pest Manag. Sci. 72(1): 81-88.

McWhorter, CG. 1971. Introduction and spread of johnsongrass in the United States. Weed Sci. 19(5): 496-500.

Millhollon, RW. 1985. Progressive kill of rhizomatous johnsongrass (*Sorghum halepense*) from repeated treatment with dalapon, MSMA, or asulam. Weed Sci. 33(2): 216-221.

Mitskas, MB, CE Tsolis, IG Eleftherohorinos and CA Damalas. 2003. Interference between corn and johnsongrass (*Sorghum halepense*) from seed or rhizomes. Weed Sci. 51(4): 540-545.

Ngo, TD, M Krishnan, P Boutsalis, G Gill and C Preston. 2016. Target-site mutations conferring resistance to glyphosate in feathertop Rhodes grass (*Chloris virgata*) populations in Australia. Pest Manag. Sci. 74(5): 1094-1100.

Ngo, TD, JM Malone, P Boutsalis, G Gill and C Preston. 2018. *EPSPS* gene amplification conferring resistance to glyphosate in windmill grass (*Chloris truncata*) in Australia. Pest Manag. Sci. 74(5): 1101-1108.

Olson, BLS, K Al-Khatib, P Stahlman, S Parrish and S Moran. 1999. Absorption and translocation of MON 37500 in wheat and other grass species. Weed Sci. 47(1): 37-40.

Pan, Lang, Q Yu, J Wang, H Han, L Mao, A Nyporko, A Maguza, L Fan, L Bai and SB Powles. 2021. An ABCC-type transporter endowing glyphosate resistance in plants. PNAS. 118(16): e2100136118.

Paterson, AH, W Kong, RM Johnston, P Nabukalu, G Wu, WL Poehlman, VH Goff, K Isaacs, TH Lee, H Guo and D Zhang. 2020. The evolution of an invasive plant, *Sorghum halepense* L. ('Johnsongrass'). Front. Genet. 11: 317.

Perotti, VE, AS Larran, VE Palmieri, AK Martinatto, CE Alvarez, D Tuesca and HR Permingeat. 2019. A novel triple amino acid substitution in the *EPSPS* found in a high-level glyphosate resistant *Amaranthus hybridus* population from Argentina. Pest Manag. Sci. 75(5): 1242-1251.

Pfaffl, MW. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29(9): e45-e45.

Ream, JE, HK Yuen, RB Frazier and JA Sikorski. 1992. *EPSPS*: binding studies using isothermal titration microcalorimetry and equilibrium dialysis and their implications for ligand recognition and kinetic mechanism. Biochem. 31(24): 5528-5534.

Riar, DS, JK Norsworthy, DB Johnson, RC Scott and M Bagavathiannan. 2011. Glyphosate resistance in a johnsongrass (*Sorghum halepense*) biotype from Arkansas. Weed Sci. 59(3): 299-304.

Rojano-Delgado, AM, H Cruz-Hipolito, R De Prado, MD Luque de Castro and A Rodríguez Franco. 2012. Limited uptake, translocation and enhanced metabolic degradation contribute to glyphosate tolerance in *Mucuna pruriens* var. *utilis* plants. Phytochemistry. 73: 34-41.

Salas, RA, FE Dayan, Z Pan, SB Watson, JW Dickson, RC Scott and NR Burgos. 2012. *EPSPS* gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* ssp. *multiflorum*) from Arkansas. Pest Manag. Sci. 68(9): 1223-1230.

Sammons, RD and TA Gaines. 2014. Glyphosate resistance: state of knowledge. Pest Manag. Sci. 70(9): 1367-1377.

Schönbrunn, E, S Eschenburg, WA Shuttleworth, JV Schloss, N Amrhein, JNS Evans and W Kabsch. 2001. Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. PNAS. 98(4): 1376-1380.

Seefeldt, SS, JE Jensen and EP Fuerst. 1995. Log-logistic analysis of herbicide dose-response relationships. Weed Technol. 9(2): 218-227.

Smeda, RJ, CE Snipes and WL Barrentine. 1997. Identification of graminicide-resistant johnsongrass (*Sorghum halepense*). Weed Sci. 45(1): 132-137.

Ulrich, MN, E Muñiz-Padilla, A Corach, E Hopp and D Tosto. 2021. Validation of reference genes for quantitative PCR in johnsongrass (*Sorghum halepense* L.) under glyphosate stress. Plants. 10(8): 1555.

Vázquez-Garcia, JG, C Palma-Bautista, AM Rojano-Delgado, R De Prado and J Menendez. 2020. The first case of glyphosate resistance in johnsongrass (*Sorghum halepense* [L.] Pers.) in Europe. Plants. 9(3): 313.

Vila-Aiub, MM, MC Balbi, PE Gundel, CM Ghersa and Stephen B. Powles. 2007. Evolution of glyphosate-resistant johnsongrass (*Sorghum halepense*) in glyphosate-resistant soybean. Weed Sci. 55(6): 566-571.

Vila-Aiub, MM, MC Balbi, AJ Distéfano, L Fernández, E Hopp, Q Yu and SB Powles. 2012. Glyphosate resistance in perennial *Sorghum halepense* (Johnsongrass), endowed by reduced glyphosate translocation and leaf uptake. Pest Manag. Sci. 68(3): 430-436.

Williams, CS and RM Hayes. 1984. Johnsongrass (*Sorghum halepense*) competition in soybeans (*Glycine max*). Weed Sci. 32(4): 498-501.

Yu, Q, A Jalaludin, H Han, M Chen, RD Sammons and SB Powles. 2015. Evolution of a double amino acid substitution in the 5-enolpyruvylshikimate-3-phosphate synthase in *Eleusine indica* conferring high-level glyphosate resistance. Plant Physiol. 167(4): 1440-1447.

Yuan, JS, A Reed, F Chen and CN Stewart. 2006. Statistical analysis of real-time PCR data. BMC Bioinform. 7(1): 1-12.

Zhang, C, CJ Yu, Q Yu, WL Guo, TJ Zhang and XS Tian. 2021. Evolution of multiple target-site resistance mechanisms in individual plants of glyphosate-resistant *Eleusine indica* from China. Pest Manag. Sci. 77(10): 4810-4817.

Table 2.1. Glyphosate dose-response model parameters constructed from experimentation with glyphosate-resistant (gly-R) and glyphosate-susceptible (gly-S) johnsongrass accessions. Both models are $Y=C+(D-C) / 1+(X/GR50\text{^}b)$. *Y* is the dry biomass of plants harvested at 3 weeks after treatment and *X* is glyphosate dose as g ae ha^{-1}. *C* = lower limit of plant response and *D* = upper limit or non-treated plant response; both refer to the data transformed to a percent of control within replication. GR_{50} = the dose yielding 50% growth reduction. *b* describes the slope of the curve around the GR_{50}. R^2 values describe the goodness-of-fit of both models for the means of 14 replicates plus the standard error of the mean.

	Glyphosate dose-response	
	Johnsongrass accession	
Model parameter	Susceptible	Resistant
C	9.8	17
D	92.5	103
b	-3.06	-3.84
R^2	0.99	0.99
GR_{50}	230	1,073

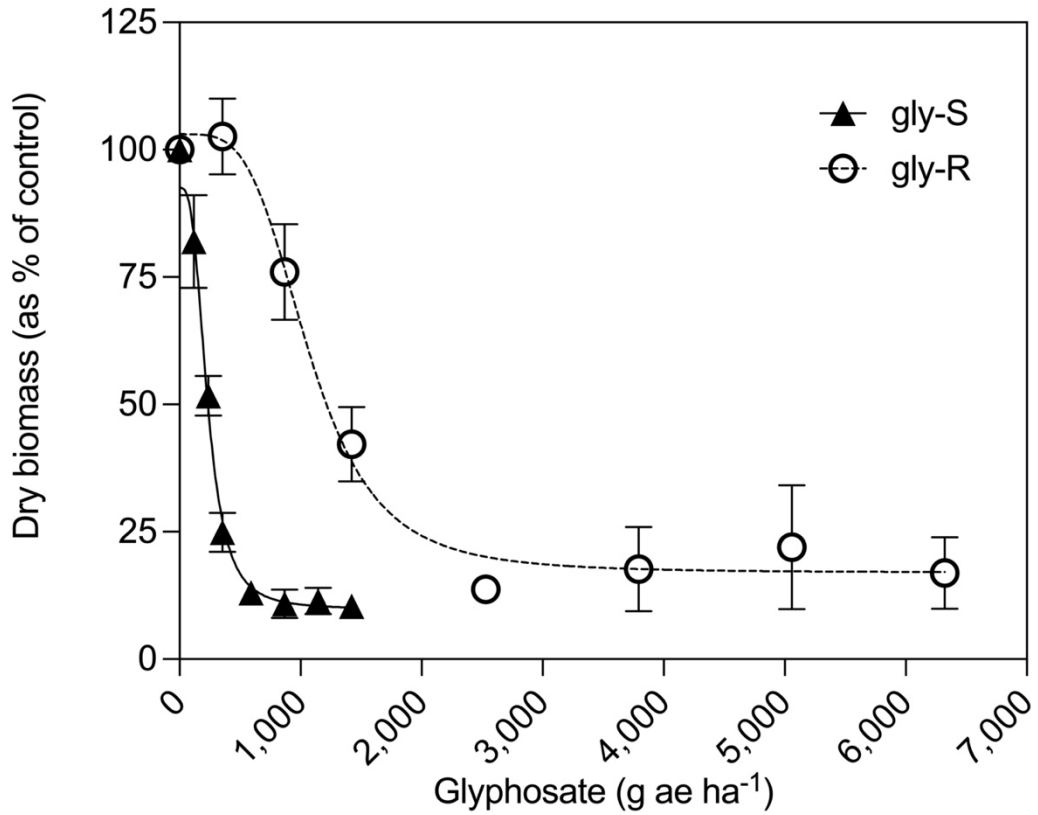


Fig. 2.1. Graph of glyphosate dose-response experiments with suspected glyphosate-resistant (gly-R) and glyphosate-susceptible (gly-S) johnsongrass accessions using the model $Y=C+(D-C) / 1+(X/GR_{50}\text{^}b)$. *Y* is the dry biomass of plants harvested at 3 weeks after treatment, transformed to a percent of control within replication, and *X* is glyphosate dose as g ae ha^{-1}. Lines are the response curves predicted from nonlinear regression with R^2 values of 0.99 for both the gly-R and gly-S biotypes. Symbols (▲ and ◯) represent the mean of 14 replicates. Vertical bars represent the standard error of the mean. Error bars not plotted are shorter than the symbol for the mean.

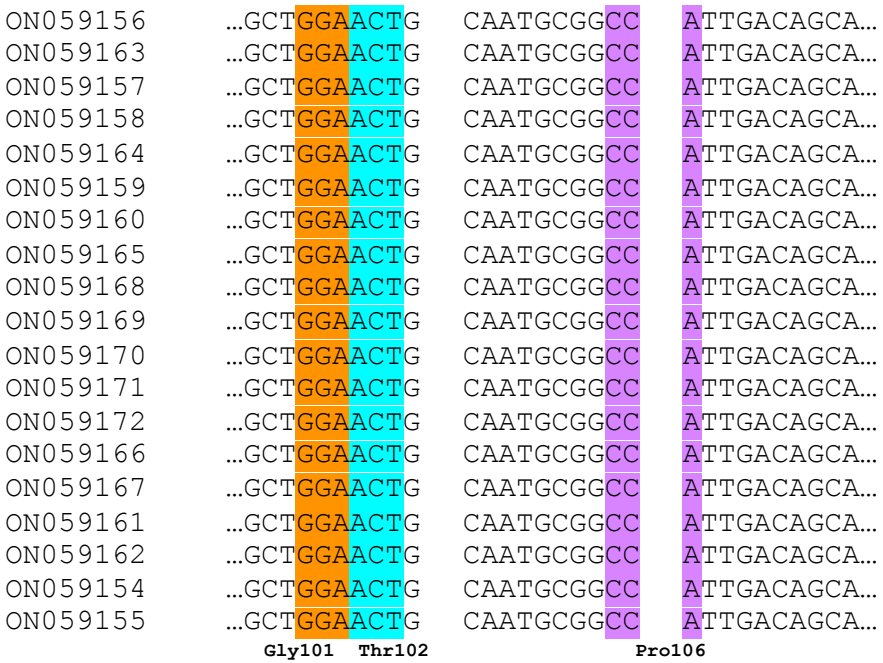


Fig. 2.2. Aligned nucleotide sequences of *EPSPS* gene fragments from glyphosate-resistant (gly-R) accessions of johnsongrass collected from Buchanan County, MO. Partial sequences shown in this figure cover positions at 101, 102, and 106 where point mutations have been reported to confer glyphosate resistance. Amino acid numbering is according to the numbering system for *Arabidopsis thaliana* (Klee et al., 1987). The entire gene fragment sequences are listed in the GenBank database available at <https://www.ncbi.nlm.nih.gov/genbank> under the accession numbers listed in the left-hand column.

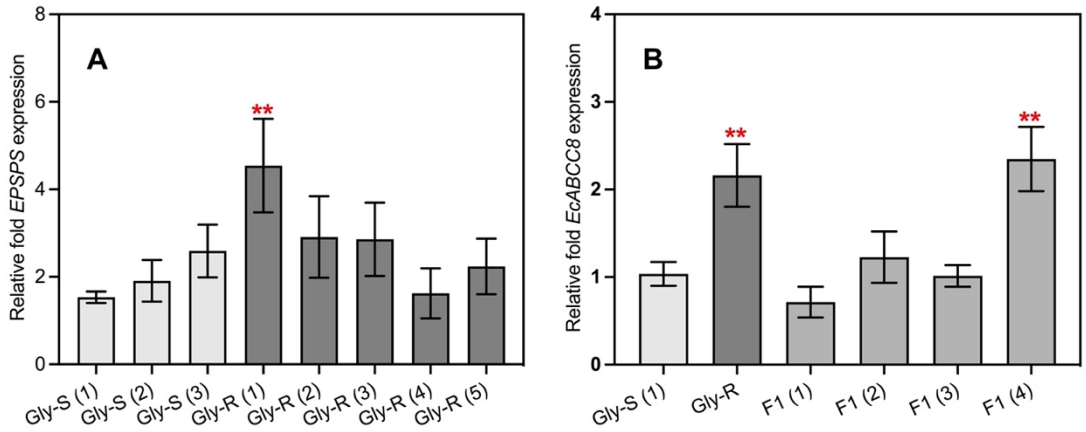


Fig. 2.3. Estimated fold change in gene expression for glyphosate-sensitive (gly-S) and glyphosate-resistant (gly-R) johnsongrass estimated using $2^{-\Delta\Delta Ct}$ (Pfaffl, 2001). Total RNA was extracted from plants and reverse-transcribed to cDNA, which served as the template for qPCR. (**A**) Gene expression was estimated for 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) and (**B**) the ABC transporter *EcABCC8.* Data were normalized relative to ALS and ARI8, respectively (Ulrich et al., 2021). Tissue was harvested 6 hours after treatment with glyphosate at 1,447 g ae ha^{-1} for *EcABCC8* expression only. F1 designates samples from plants grown from seed collected from putative gly-R plants. Vertical bars indicate the standard error of the means.

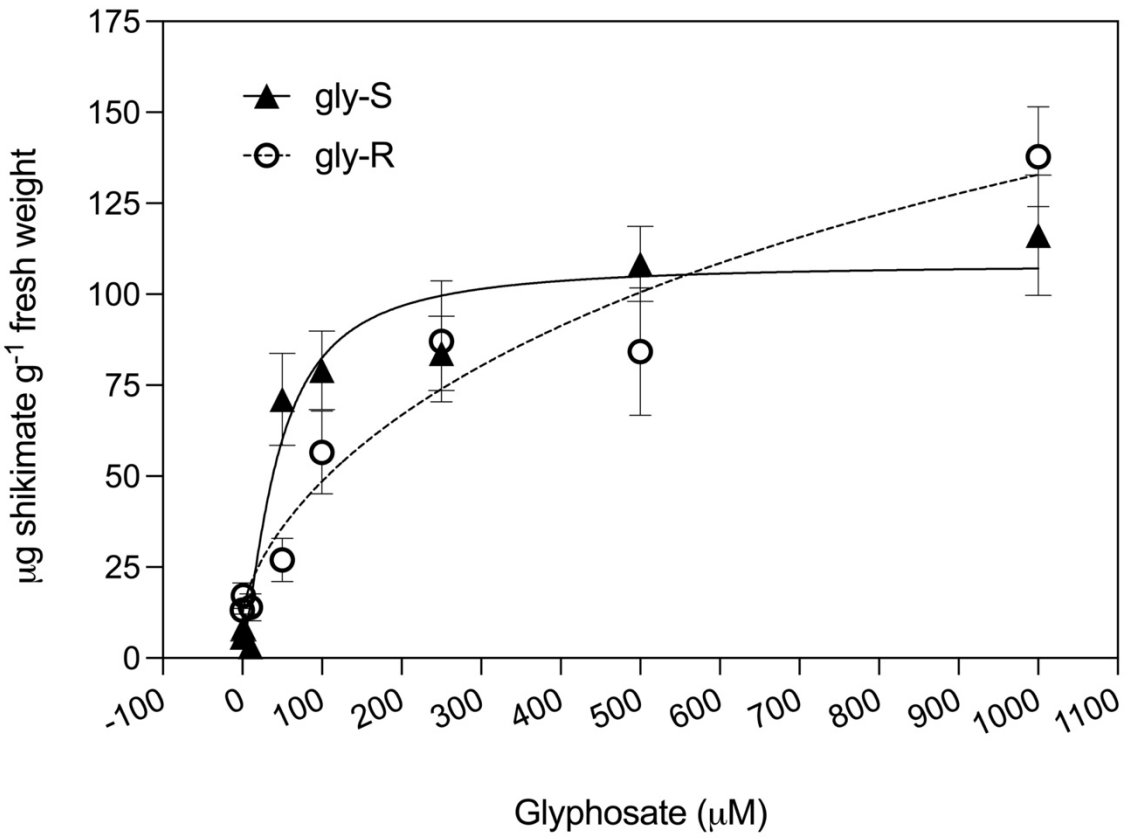


Fig. 2.4. Shikimic acid accumulation of leaf discs from glyphosate-resistant (gly-R) and glyphosate-susceptible (gly-S) johnsongrass accessions. The response curve for the gly-R accession is *Y*=3.215+(105.285)/1+([*X*/44.66]^1.385) with an R^2 value of 0.95. The response curve for the gly-S accession is *Y*=11.57+(310.73) / 1+([X/1933]^0.6755) with an R^2 value of 0.96. *Y* is the shikimic acid accumulation (extrapolated from a standard curve) per gram fresh weight of leaf discs incubated in solution with varying glyphosate concentrations (*X*) for 24 h. Symbols (▲ and ◯) represent the mean of 18 replicates pooled across three experiments. Vertical bars represent the standard error of the mean.

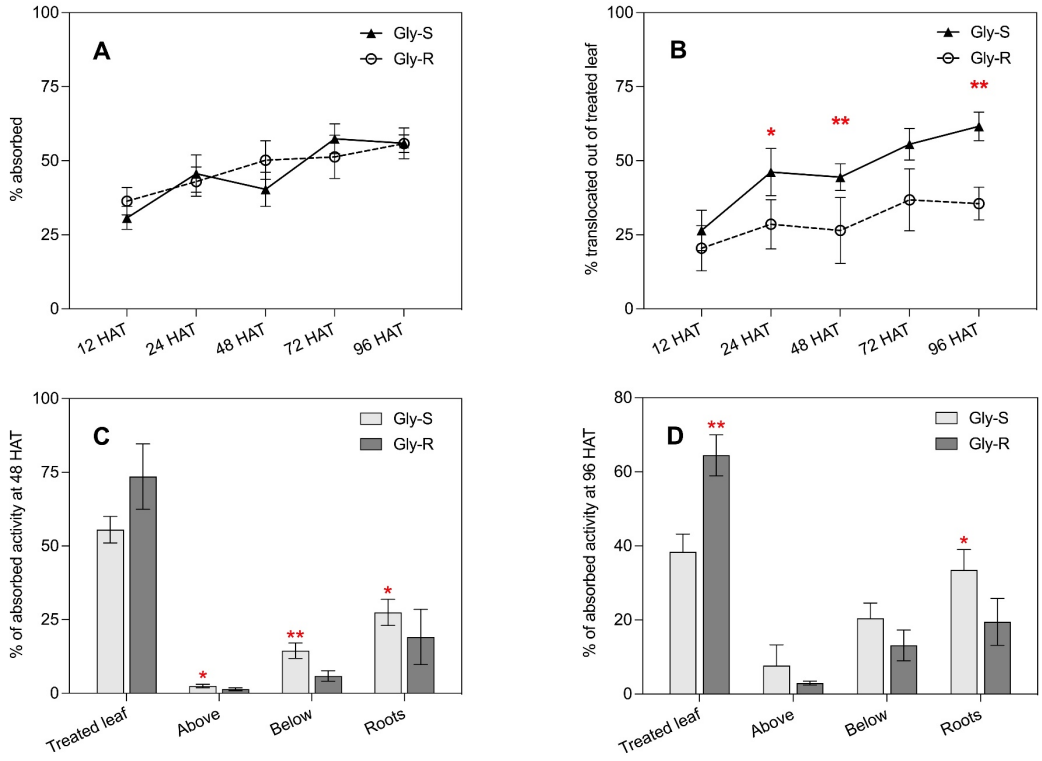


Fig. 2.5. ^{14}C-glyphosate absorption and distribution in glyphosate-resistant (gly-R) and glyphosate-susceptible (gly-S) johnsongrass plants during growth chamber experimentation. Droplets of a solution containing ^{14}C-glyphosate were applied to plants from both populations. Plants were maintained in a growth chamber until harvest and partitioning occurring at 12, 24, 48, 72, or 96 hours after treatment (HAT). (**A**) Amount of radioactivity absorbed by the treated leaf, expressed as a percent of the total radioactivity recovered. (**B**) Amount of radioactivity in plant parts outside of the treated leaf, expressed as a percent of the total radioactivity absorbed. (**C**) Amount of radioactivity in plant parts when harvested at 48 HAT, as a percent of activity absorbed. (**D**) Amount of radioactivity in plant parts when harvested at 96 HAT, as a percent of activity absorbed. Asterisks denote statistically significant differences using Tukey's Honestly Significant Difference, where *= significant at α=0.05 and **= significant at α=0.01. Vertical bars represent the standard errors of the means of 10 replicates pooled across two experiments.

CHAPTER III

Control of Glyphosate-resistant Johnsongrass (*Sorghum halepense* [L.] Pers.) in Soybean with Tolerance to Dicamba, Glufosinate, and Glyphosate

ABSTRACT

Field experiments were conducted at two sites in Buchanan County, Missouri in 2020 and 2021 to evaluate postemergence (POST) control of suspected glyphosate-resistant (gly-R) johnsongrass (*Sorghum halepense* [L.] Pers.) in soybean (*Glycine max*) with tolerance to dicamba, glufosinate, and glyphosate. POST programs included tank-mixed encapsulated acetochlor (1,259 g ai ha^{-1}), dicamba (563 g ai ha^{-1}), and glyphosate (1,263 g ai ha^{-1}) with clethodim (140 g ai ha^{-1}) or fluazifop-P-butyl (210 g ai ha^{-1}) as single-pass treatments. Two-pass POST treatments included encapsulated acetochlor (1,259 g), dicamba (563 g), and glyphosate (1,263 g) at the early POST application and glufosinate alone (655 g ai ha^{-1}), glufosinate (655 g) plus glyphosate (1,263 g), glyphosate alone (1,263 g), glyphosate (1,263 g) plus clethodim (140 g), and fluazifop-P-butyl alone (210 g) or fluazifop-P-butyl (85 g) with fenoxaprop-P-butyl (24 g ai ha^{-1}) at the mid-POST application. To confirm the presence of gly-R johnsongrass, rhizomes collected from both sites were utilized in greenhouse assays to assess herbicide resistance. Vegetatively propagated plants were subjected to a glyphosate dose-response study as well as a screening for multiple herbicide resistance. From dose-response models, the GR_{50} of johnsongrass collected from one site at 15.9 times that of the susceptible; for johnsongrass collected from the second site, the GR_{50} was 2.3 times higher. Based on visual estimates of grass weed control on a scale of 0 (no injury) to 100 (complete mortality) a single early POST application resulted in insufficient control of

johnsongrass, and annual grass weed species (*Panicum dichotomiflorum* and *Setaria* spp.) in three of four site years. At one site, the timing of applications in relation to the growth stage of rhizomatous johnsongrass was a critical factor in determining control. With significant gly-R johnsongrass pressure, two-pass treatments resulted in 53 to 96% control at two weeks after treatment (WAT) in one year, with only 20 to 72% control in the second year. Where pressure from gly-R johnsongrass was lower, treatments performed more consistently, with 94 to 99% control in one year at 2 WAT, and 95 to 99% in the second year. Weed density and biomass data confirmed that grass weed control from glufosinate consistently outperformed glyphosate at both study locations. While some treatments resulted in excellent control of gly-R johnsongrass, POST only programs place significant pressure on single herbicide modes of action. Consequences of such an approach are discussed. In the multiple resistance assay, no evidence was found for resistance to another herbicide mode of action in gly-R johnsongrass accessions.

INTRODUCTION

Johnsongrass is a vigorous perennial grass species that can be found on nearly every continent on the planet, where its occupation of agricultural areas requires significant investment in weed control strategy. For producers, the cost of controlling johnsongrass with herbicides can exceed $50 USD per hectacre (Klein & Smith, 2021).

A high level of johnsongrass control is required to avoid penalties at harvest in both the quantity and quality of soybean yield. Uncontrolled johnsongrass competing with soybean for 6 weeks at densities of approximately 3 culms m^{-2} reduced yield up to 38% (Williams & Hayes, 1984). At seven weeks, yield reductions reached 69%. Similarly,

when soybean cultivars were evaluated to determine competitiveness with johnsongrass, yield reductions ranged between 23 and 42% over a three-year period (McWhorter & Hartwig, 1972). A separate study determined that at least 70% johnsongrass control was required to avoid yield reductions in soybean at harvest, and nearly 100% johnsongrass control was required to avoid penalties in soybean quality at harvest (McWhorter & Anderson, 1981).

With conventional soybean cultivars, previous studies have identified several programs for johnsongrass control with varying degrees of success. Culpepper et al. (2000) achieved ≥ 95% control for nine separate weed species with imazaquin plus dimethenamid preemergence (PRE) followed by chlorimuron postemergence (POST); however, rhizomatous johnsongrass control from the same program did not exceed 73%. In a separate study, when ACCase-inhibiting herbicides were applied in single-pass or split applications (two-pass) POST to conventional soybeans, johnsongrass control at soybean harvest was improved up to 67% by two-pass POST applications of ACCase-inhibiting herbicides compared to a single pass (Bendixen, 1988). Furthermore, when the row spacing of soybeans was decreased from 76 cm to 25 cm, control from the two-pass POST system increased up to 89% (Bendixen, 1988).

Effective control of established johnsongrass benefits from POST herbicides with systemic translocation such as glyphosate that can suppress regrowth of the plant from rhizomes (Johnson et al., 2003). Glyphosate inhibits the enzyme 5-enolpyruvyl shikimate-3-phosphate synthase (*EPSPS*) in the biosynthetic pathway that produces the aromatic amino acids phenylalanine, tyrosine, and tryptophan (Heap & Duke, 2018). In soybean in particular, widespread adoption of genetically engineered (GE) soybean

cultivars with resistance to glyphosate reduced the diversity of herbicides utilized between 1990 and 2015 (Kniss, 2018). Concomitantly, the use of glyphosate in the United States increased across multiple cropping systems, with approximately 1.1 billion kg of glyphosate as the active ingredient applied between 2004 and 2014 (Benbrook, 2016).

Herbicide diversity, or the application of more than one mode of action, has been shown to reduce the likelihood of field-evolved herbicide resistance in weed species (Beckie & Reboud, 2009). However, as of 2014, GE soybeans and cotton with resistance to glyphosate were responsible for 77% of the total amount of glyphosate applied to agricultural land in the United States (Kniss, 2018). Currently, 343 cases of glyphosate resistance have been reported in 56 weed species across 30 countries (Heap, 2022). Currently, of the 31 reported cases of herbicide resistance in johnsongrass, 7 of these accessions involve resistance to glyphosate (Heap, 2022). Overreliance on acetyl-CoA (ACCase)- and acetolactate synthase (ALS)-inhibiting herbicides for johnsongrass control prior to widespread adoption of glyphosate resistant soybean cultivars provides the basis for most other herbicide resistant accessions (Klein & Smith, 2021).

In GE soybean cultivars with herbicide resistance, additional herbicide modes of action are available for selective control of glyphosate resistant (gly-R) johnsongrass. Glufosinate been the target of research into control of gly-R johnsongrass in glufosinate-resistant soybean. In Arkansas, two POST applications of glufosinate at 450 g ai ha^{-1} to gly-R johnsongrass achieved up 83% visual control by 10 weeks after soybean emergence (Johnson et al., 2014). However, glufosinate efficacy declined rapidly when johnsongrass plants reach ≥ 30 cm in height (Johnson et al., 2014). Increasingly, GE

herbicide resistant traits in soybean have been combined to permit the application of multiple modes of action POST in response to pressure from herbicide-resistant weeds (Nandula, 2019). Soybean cultivars are now available with resistance to glyphosate and glufosinate in addition to dicamba or 2,4-D (USDA, 2014; USDA, 2015). With glyphosate sensitive (gly-S) johnsongrass, both dicamba and 2,4-D have been demonstrated to reduce glyphosate leaf uptake up to 24%, and subsequent translocation to roots 33 to 45% (Flint & Barrett, 1989).

Recent confirmation of glyphosate resistance in a johnsongrass population in northwest Missouri poses a novel management challenge for producers. Selective control of gly-R johnsongrass in soybeans has been reported on previously, but the recent introduction of new GE soybean cultivars with resistance to glyphosate, glufosinate, and dicamba warranted a review of management practices that could be employed to control gly-R johnsongrass in soybean. At two field sites in Missouri in 2020 and 2021, suspected gly-R johnsongrass established in agronomic fields was a target weed species for evaluation of POST control in soybean with tolerance to dicamba, glufosinate, and glyphosate. In addition, greenhouse studies with field collected rhizomes from both study sites were conducted to assess herbicide resistance.

MATERIALS AND METHODS

Field study. Two field experiments within agronomic fields in corn-soybean rotation were conducted in Buchanan County, Missouri in 2020 and 2021. At both sites, a dicamba, glufosinate, and glyphosate resistant XtendFlex® soybean cultivar '4020RXF' (Bayer CropScience; St. Louis, MO, USA) was planted with a four-row research plot

planter at 346,000 seeds ha^{-1} in 76 cm rows on 19 May 2020 and 4 June 2021. Both sites were planted on the same day in each year. At both sites, individual plots measured 3 m wide by 6 m long. The first site, designated Belcher Branch (39°34'47.7"N, 94°44'17.6"W) was within an agronomic field under conventional tillage practices, and thus was tilled with a field cultivator prior to planting. The second site, hereafter referred to as Lake East (39°34'43.4"N, 94°43'32.5"W) was within an agronomic field under no-till management, and was planted without tillage. The soil type at Belcher Branch was a Judson silt loam (fine-silty, mixed, superactive, mesic Cumulic Hapludoll) with 3% organic matter (OM) and pH 6.4. At Lake East, the soil was a Knox silt loam (fine-silty, mixed, superactive, mesic Mollic Hapludalf) with 2.7% OM and pH 7.2.

Lake East was adjacent to the field in which the population of gly-R johnsongrass was first identified but separated by a forested buffer strip approximately 50 m wide. Both sites were selected based upon a history of difficult-to-control johnsongrass infestation (J. Pollard, personal communication). Following the first year of the study, johnsongrass rhizomes were dug from random locations across both fields and later subjected to a dose-response assay to determine if gly-R plants were present within the field study populations.

POST applications were made at one or both of two timings: early POST (EPOST), and mid-POST (MPOST) following the EPOST application when johnsongrass regrowth was observed. In 2020 at EPOST, grasses emerging from seed were 10 to 15 cm in height, while johnsongrass emerging from rhizome was approximately 45 to 60 cm in height. In 2021, both soybean planting as well as the EPOST application were delayed by wet field conditions. Annual grasses were 15 to 20 cm at the EPOST application, with

johnsongrass from rhizome ranging from 40 to 90 cm in height. EPOST applications (Table 3.1) were made on 8 July 2020 and 14 July 2021 using a CO_2-pressurized backpack sprayer equipped with TTI11002 air-induction flat fan nozzles (TeeJet; Springfield, IL, USA) calibrated to deliver 140 L ha^{-1}. MPOST applications (Table 3.1) were made on 23 July 2020 and 22 July 2021 as previously described, except with the use of XR8002 flat fan nozzles.

Data collected included weekly estimates of visual grass weed control as well as crop injury (on a scale of 0-100%, with 0 indicating no herbicide injury and 100 indicating complete mortality) from one week after treatment (WAT) with the EPOST application to 3 WAT with the MPOST application. Surviving grasses were collected weekly in two, 0.25 m^2 quadrats within a plot. Survival was defined as plants with <80% herbicide injury at the time of collection, and these were cut at the soil line and individual stems were counted to obtain grass weed density. Grass density data were extrapolated to 1 m^2. After obtaining weed density, grass weed biomass was combined across species to obtain biomass per plot after oven-drying at 50°C for 3 d. As the study was initiated prior to commercialization of this soybean cultivar, soybean yield was not recorded, and plants were destroyed following study completion.

Whole-plant greenhouse assays. Rhizomes collected from both field sites were compared to rhizomes from a known susceptible population dug from an undeveloped urban area (38°54'38.9"N, 92°17'43.6"W) approximately 230 km away, with no known history of herbicide use. Rhizomes were planted in 20 by 30 cm plastic trays filled with an 80:20 mix of peat-based potting media (Hummert International; Earth City, MO, USA) and Mexico silt loam topsoil (fine, smectitic, mesic Vertic Epiaqualf). When plants

emerged from rhizome, three from each population were randomly selected and transplanted into 60 L polyethylene tubs. These were allowed to grow in the greenhouse to produce new rhizomes for the dose-response assay. Harvested rhizomes were cut into fragments containing 2-3 nodes per piece and planted in 15 cm pots in potting mix as previously described. When plants reached 10 to 15 cm and had developed at least three true leaves, glyphosate was applied using a greenhouse track sprayer calibrated to deliver 140 L ha^{-1} at doses ranging from 0 to 7,235 g ha^{-1} for the putative resistant biotype and 0 to 1,457 g ha^{-1} for the susceptible biotype. At 21 days after treatment (DAT), plants were harvested at the soil line and oven-dried at 50ºC for 3 d. The weight of dry plant biomass was converted to a percent of the non-treated control within replication.

Following the first year of the field study, a separate greenhouse assay was conducted to assess the potential for multiple resistance. Johnsongrass plants from both gly-R and gly-S populations were started from rhizome as previously described and treated with a single herbicide mode of action at the three to four-leaf stage using a greenhouse track sprayer. Herbicides included clethodim (102 g ai ha^{-1}), fluazifop (350 g ai ha^{-1}), glufosinate (656 g ai ha^{-1}), glyphosate (1734 g ai ha^{-1}), imazapic (210 g ai ha^{-1}), imazethapyr (105 g ai ha^{-1}) or nicosulfuron (15 g ai ha^{-1}) plus a non-treated control. At 3 WAT, plants were harvested at the soil line and oven-dried at 50ºC for 3 d. The weight of dry plant biomass was converted to a percent of the non-treated control within replication, and the experiment was repeated.

Statistical analysis. Both field studies were arranged in a randomized complete block design with four replications. Field study data were subjected to a mixed linear model in SAS Enterprise Guide 8.3 (SAS Institute; Cary, NC, USA) using PROC GLIMMIX with

herbicide treatment as a fixed factor and replication as a random factor. All data were examined for significant interactions between treatments and site years. Where data were not normally distributed, a rank transformation was applied (Conover & Iman, 1981). Significant means were separated using Tukey's Honestly Significant Difference (HSD) at α=0.05. For the dose-response assay, biomass data were transformed as a percent of control within replication and subjected to nonlinear regression using a four-parameter, log-logistic model (Seefeldt et al., 1995) (Eq. 3.1) in GraphPad Prism 7 (GraphPad Software; San Diego, CA, USA) according to Larran et al. (2017). A lack-of-fit *F* test was performed to test the hypothesis that the curves have the same slope; i.e., if a more complex model (nonparallel curves) can be reduced to a simpler model (parallel curves).

$$y = c + \frac{(d - c)}{1 + x / gly\text{-}R_{50}{}^{b}} \qquad \text{(Eq. 3.1)}$$

For the multiple resistance assay, treatments were arranged in a randomized complete block design with six replications in the first study and five replications in the second. Data were subjected to a mixed linear model as previously described with herbicide treatment and population as fixed factors and replication as a random factor. Data were combined across experimental runs when no interaction was observed between run, population, and treatment.

RESULTS

Crop injury was minimal in 2020 and 2021 and was related to encapsulated acetochlor at the EPOST application, with drawstring and/or crinkled leaves (Appendix A.1). At the time of treatment, soybeans were V3-V4 stage in 2020 but had only reached V1-V2 at the EPOST application in 2021.

At Belcher Branch, the dominant grass weeds were johnsongrass, giant foxtail (*Setaria faberi*) and green foxtail (*Setaria viridis*), and to a lesser extent, fall panicum (*Panicum dichotomiflorum*) (Fig. 3.1). At the Lake East site, johnsongrass was well-established across the field, with many plants emerging from rhizome. In both 2020 and 2021, johnsongrass emerging from rhizome was further advanced in growth compared to grasses emerging from seed, and this seasonal growth pattern represented a significant challenge for weed management with regard to timing of POST applications.

In 2020, treatment differences for johnsongrass control were observed at both sites (Table 3.2). As expected, all two-pass POST treatments yielded greater control than a single early POST application. Fluazifop, fluazifop plus fenoxaprop, and clethodim plus glyphosate resulted in johnsongrass control ranging from 92 to 99% across both sites. When glyphosate, glufosinate, or glyphosate plus glufosinate were applied at MPOST, site-specific treatment differences emerged by 2 WAT. Glyphosate alone yielded 53% control at Lake East and 95% at Belcher Branch. Putative resistant biotypes of johnsongrass were suspected at both sites; however, results of glyphosate alone suggested that a gly-R biotype was more prevalent at Lake East than Belcher Branch (Table 3.2). Glufosinate alone resulted in johnsongrass control ranging from 79% at Lake East to 94% at Belcher Branch. At both sites, glyphosate plus glufosinate resulted in johnsongrass control estimates comparable to glufosinate applied alone, with a mean of 80% versus 79% control at Lake East for glyphosate plus glufosinate and glufosinate alone, respectively. For Belcher Branch, these estimates were 98 and 94% for glyphosate plus glufosinate and glufosinate alone, respectively.

No treatment differences were observed at Belcher Branch in 2021 (Table 3.3). Johnsongrass control was estimated at 90% for both single-pass treatments by 2 WAT with the MPOST application. Control ranged from 95 to 97% for all two-pass treatments at 2 WAT. At Lake East, applications made in 2021 to larger johnsongrass plants reduced the efficacy of all treatments compared to 2020. However, loss of efficacy at Lake East in 2021 was greater for glyphosate and glufosinate at MPOST than applications that included clethodim, fluazifop, or fluazifop plus fenoxaprop. By 2 WAT with the MPOST application, single passes with either clethodim or fluazifop tank-mixed with encapsulated acetochlor, dicamba, and glyphosate yielded greater visual control (59 and 48% for clethodim and fluazifop, respectively) than two-pass systems with either glufosinate alone (52%) or glufosinate plus glyphosate (45%) at the second application. When the second POST application included fluazifop, fluazifop plus fenoxaprop, or clethodim plus glyphosate, johnsongrass control ranged from 66 to 72% by 2 WAT.

Effective control of annual grass species at Belcher Branch was achieved in both years by multiple treatments (Table 3.3). In 2020, annual grass weed control means of 62 to 75% were achieved with a single-pass POST by 2 WAT with the MPOST application. Clethodim plus acetochlor, dicamba, and glyphosate resulted in 93% control; the tank-mix with fluazifop yielded 89% control (Table 3.3). While nearly all treatments at the MPOST application resulted in poorer control in 2021 compared to 2020, this may be due to later canopy closure of soybeans in 2021 compared to 2020. In 2020, canopy closure occurred around 3 WAT with the MPOST application. In 2021, canopy closure did not occur until approximately 5 WAT with the MPOST application. Effective annual grass weed control resulting from the EPOST application in 2021 (>90% for all treatments)

permitted continued emergence of grass weeds following the MPOST application. Regardless of the additional emergence of grass weeds, control from a single-pass treatment was acceptable (90%) by 2 weeks after MPOST applications were made. For two-pass treatments, control of annual grass weeds was excellent (>95%) by 2 WAT with the MPOST application.

Weed density. In 2020, no treatment differences were observed at either site for johnsongrass density by two weeks after the MPOST application (Table 3.4). At Belcher Branch, single-pass treatments yielded higher johnsongrass density than non-treated control plots by 2 WAT with the MPOST application. This probably resulted from tillage employed at Belcher Branch, which facilitates johnsongrass seedling recruitment, in combination with the first POST application reducing competition from annual grasses (Table 3.5). Similarly, a two-pass treatment with glufosinate at the MPOST application timing resulted in higher johnsongrass density than non-treated control plots. At Lake East, with plants emerging from both seed and rhizome, a comparable trend was observed; single-pass treatments yielded johnsongrass density roughly equal to that of the non-treated control (Table 3.4). However, the two-pass treatment with glufosinate at the MPOST application timing at Lake East appeared much more effective than the application at Belcher Branch, with a mean of 5 culms m^{-2} for glufosinate versus 23 to 26 for single pass treatments by two weeks after treatment.

Significant treatment differences in johnsongrass density were observed at both sites in 2021. At Lake East in 2021, clethodim plus glyphosate at the MPOST application resulted in a mean johnsongrass density of 28 culms m^{-2} versus non-treated control plots at 108 culms m^{-2} by two weeks after treatment. Glyphosate alone at MPOST however,

yielded a mean of 84 culms m^{-2} by two weeks after treatment. For Belcher Branch, the trend was similar, with reduced differences. Clethodim plus glyphosate at MPOST yielded a mean of 4 culms m^{-2} versus 6 for glyphosate alone. The increase in mean johnsongrass density at Belcher Branch within control plots from 2020 to 2021 appeared to be due to the number of plants emerging from seed, in conjunction with later soybean canopy closure in 2021.

Weed biomass. Trends in grass weed biomass generally followed those observed by visual estimates of control, but identified a few treatment differences that visual estimation did not. In 2020, when glyphosate alone was applied at MPOST, mean grass weed biomass was reduced by 80% from non-treated control plots at Belcher Branch and 67% at Lake East by 2 WAT (Table 3.6). When glufosinate was included with glyphosate at MPOST, biomass was reduced from control plots by 100% and 87% at Belcher Branch and Lake East, respectively. As with visual estimations, grass weed control at two weeks after treatment with fluazifop, fluazifop plus fenoxaprop, and clethodim plus glyphosate at the MPOST application timing was excellent, with surviving grass weed biomass reduced by 100% from non-treated control plots at Belcher Branch and 93 to 96% at Lake East.

In 2021, treatment differences were only observed at Lake East (Table 3.6). Glyphosate plus glufosinate applied at MPOST resulted in the greatest control at 2 WAT, with biomass reduced by 73% from non-treated controls. Two-pass treatments with either fluazifop, fluazifop plus fenoxaprop, or clethodim plus glyphosate were comparable, with biomass reduced by 52 to 62% compared to control plots. However, the application of all treatments to larger plants in 2021 compared to 2020 resulted in unacceptable

johnsongrass control. At Belcher Branch in 2021, no treatment differences were observed (Table 3.6). Grass weed biomass was reduced from controls by ≥95% for all treatments, except the two-pass treatment with glyphosate alone at the MPOST application. At Belcher Branch, glyphosate alone at the MPOST application reduced grass weed biomass by 84% at 2 WAT.

Dose-response assay. Johnsongrass sensitivity to glyphosate varied dramatically between the two field study locations (Fig. 3.2). Three models were fit to the dose-response data from susceptible population, Belcher Branch, and Lake East (p <0.0001; with R^2= 0.97, 0.96, and 0.99, respectively). Based on the GR_{50} values generated from dose-response models, the resistance indices (R:S ratio) were 2.3 for biotypes originating from Belcher Branch and 15.9 for those from Lake East.

The model derived from biotypes collected at Belcher Branch is consistent with field observations. Johnsongrass response to glyphosate alone at 2 WAT with the MPOST application was more varied than responses observed at Lake East, which was suggestive of either the presence of both gly-R and gly-S biotypes, or the type of low-level glyphosate resistance that was observed in the dose-response model. The model from Lake East was surprising, as the GR_{50} obtained from Lake East biotypes exceeded that of the original population used to confirm glyphosate resistance. The level of glyphosate resistance observed at Lake East is consistent with field observations of plant response to glyphosate alone at the MPOST application. The level of resistance was assumed to be higher than Belcher Branch, as glyphosate alone appeared to only temporarily stunt plant growth.

Multiple resistance screening. Johnsongrass response to ALS- and ACCase-inhibiting herbicides was similar for both gly-R and gly-S populations when these herbicides were applied to potted plants (Fig. 3.3). Visual injury data (*not shown*) confirmed the expected response to ALS inhibitors, in that injury symptoms were slower to develop than those of clethodim or fluazifop. The slower development of herbicide injury appeared to translate into higher biomass at harvest, although treatments were not significantly different between ALS and ACCase inhibitors. As well, imazapic, imazethapyr, and nicosulfuron generally did not completely control plants from either population. Injury symptoms from clethodim and fluazifop were faster to develop and resulted in lower plant biomass at 3 WAT. The greatest control of the seven herbicides examined resulted from glyphosate applied to gly-S plants, with biomass reduced 79% from controls, and no plants surviving an application of 1,734 g ha^{-1}. These results illustrate the significance of losing glyphosate efficacy as a tool for johnsongrass control. All plants from the gly-R population survived the 1,734 g application.

The response of gly-R plants to ACCase inhibitors is consistent with field observations at both sites in 2020 and 2021. Though not statistically significant, a 13% difference in plant response to glufosinate was observed. Glufosinate reduced gly-S plant biomass by 41% compared to non-treated control plants, whereas gly-R plant biomass was reduced by 28%. However, the activity of glufosinate is sensitive to environmental differences such as temperature and humidity (Anderson et al., 1993) and this trend was not observed in field studies with glufosinate alone at the MPOST application. In field studies with gly-R johnsongrass populations, glufosinate alone at the MPOST application reduced grass weed biomass to a greater extent than glyphosate alone in all site-years.

DISCUSSION

According to one manufacturer, the optimal growth stage for the application of encapsulated acetochlor POST to soybeans is V3-V4 (Bayer, 2020). Less injury was observed in 2020 when soybeans were at V3-V4 during the EPOST application than in 2021 when soybeans were V1-V2 at the EPOST application. Crop injury observed in 2020 and 2021 was consistent with previous reports of soybean injury in response to tank-mixes including acetochlor. With glufosinate plus acetochlor applied POST at 594 and 1,690 g ha^{-1}, respectively, two separate field studies reported less than 10% injury to glufosinate tolerant soybeans (Jhala et al., 2017; Kaur et al., 2014). In addition, with glyphosate tolerant soybean, Jhala et al. (2015) reported less than 10% injury in response to encapsulated acetochlor plus glyphosate at 1,680 and 870 g ha^{-1}.

Rhizomatous johnsongrass presents a challenge to the timing of herbicide applications. At Lake East, johnsongrass establishment across the field was ubiquitous. At Belcher Branch, johnsongrass was much more patchily established, with some plots containing rhizomatous johnsongrass and some appearing to only contain johnsongrass emerging from seed. Rhizomatous dispersal has been shown to result in johnsongrass patches with mean area of 17 m^2 from one plant in approximately two years (Horowitz, 1973). Furthermore, studies in maize (*Zea mays*) fields demonstrated that seedling recruitment of johnsongrass was dependent on tillage (Barroso et al., 2012). In the studies described here, where tillage was employed (Belcher Branch), perennial plants were more spatially isolated but seedling recruitment within plots appeared continuous until canopy closure. This pattern is reflected in our data in that higher johnsongrass densities were found within non-treated control plots at Belcher Branch vs. Lake East, but grass

weed biomass at Lake East was consistently higher. Highly effective treatments at Belcher Branch were appropriately timed to soybean growth stage in 2020, with canopy closure occurring approximately 3 WAT with the MPOST application. In 2021, however, the efficacy of many treatments may prove to be problematic under normal production practices, as canopy closure did not occur until approximately 5 WAT with the MPOST application. Based on observations, continued emergence of grass weeds would have necessitated a third POST application prior to soybean canopy closure.

In the greenhouse screening for multiple resistance in the gly-R johnsongrass population, the most effective herbicide tested was glyphosate applied to sensitive plants at 1,734 g ha^{-1}. These results illustrate the significance of losing glyphosate efficacy for johnsongrass control. The range of glyphosate resistance identified in dose-response studies with plants collected from field study sites is consistent with previous reports of gly-R johnsongrass. With other gly-R johnsongrass populations, resistance indices (RIs) ranged from 3.5 to 10.5 (Riar et al., 2011; Vila-Aiub et al., 2007). A recent report of glyphosate resistance evolved within a johnsongrass population from Spain reported a more conservative range, with RIs of 4.2 to 9 (Vázquez-García et al., 2020). Based on observations at Lake East, the RI of johnsongrass was expected to be higher than that at Belcher Branch, but the GR_{50} of this population exceeded expectations. Based on observations at Belcher Branch, the johnsongrass population occurring there appeared to more diverse, as responses to glyphosate were less consistent than those observed at Lake East. The range of glyphosate resistance reported for johnsongrass across both sites in this study should preclude the use of glyphosate for management.

Of the 31 unique cases of herbicide resistance in johnsongrass, only two have confirmed multiple resistance to more than one herbicide mode of action (Heap, 2022). However, it is important to note that overreliance on POST programs contributes to the evolution of herbicide-resistant weeds (Beckie et al., 2019; Vencill et al., 2012). Although ACCase-inhibiting herbicides improved control of gly-R johnsongrass in these field studies, this places significant selection pressure on these herbicides when applied alone. With resistance to glyphosate in the johnsongrass populations occurring in the field study sites, the application of POST treatments such as clethodim plus glyphosate would not relieve selection pressure for ACCase resistance. With the greenhouse study, the efficacy of ACCase- or ALS-inhibiting herbicides should only be considered as a single tool to manage johnsongrass, not form the basis of a long-term strategy.

In the greenhouse screening, the gly-R and gly-S populations differed in their response to glufosinate, but no statistical significance was observed. Other grass weed species with resistance to glyphosate been found to also demonstrate resistance to glufosinate. Glyphosate resistant *Lolium perenne*, *Lolium multiflorum*, and *Eleusine indica* have demonstrated levels of resistance to glufosinate ranging from 2.4 to 14-fold (Avila-Garcia & Mallory-Smith, 2011; Ghanizadeh et al., 2015; Jalaludin et al., 2015). However, the efficacy of glufosinate in field conditions with the same gly-R population provides sufficient evidence against the potential for multiple resistance in this population. Glufosinate is sensitive to environmental fluctuations such as temperature, humidity, and light intensity, and this is likely sufficient to explain the differential response observed between existing field populations and potted plants maintained in the greenhouse (Anderson et al., 1993; Petersen & Hurle, 2001).

Glufosinate exhibits activity on johnsongrass, but efficacy depends on the size of plants at treatment. In a previous field study, when glufosinate was applied at 400 g ha^{-1} to rhizomatous johnsongrass plants under 30 cm, visual control was estimated at 93% by 8 WAT (Culpepper et al., 2000). In a separate study, the combined mean of rhizomatous johnsongrass control from single (420 g ha^{-1}) or split (300 followed by 300 g ha^{-1}) applications of glufosinate was 85% by 6 WAT (Johnson et al., 2003). In this study, the application of 655 g ha^{-1} to plants under 30 cm achieved control up to 98% at Belcher Branch. These data agree with previous field studies, including one with gly-R johnsongrass, in which a two-pass POST system with glufosinate alone resulted in 97% visual control (Johnson et al., 2014). As plants mature, glufosinate efficacy on johnsongrass is reduced. With foliar-applied herbicides, large plants receive less herbicide per unit biomass than smaller plants when leaf surface area is considered (Wauchope et al., 1997). In a separate field study, the application of glufosinate at 590 g ha^{-1} to 60 cm plants resulted in only 69% control (Johnson et al., 2014). In our study, when glufosinate at 655 g ha^{-1} was applied to plants ranging from 60 to 90 cm, control was even less, with a mean of 52%. The use of glufosinate to control rhizomatous johnsongrass above 60 cm is not recommended. In the case of Lake East in 2021, both glufosinate at 655 and glyphosate at 1,263 g ha^{-1} were sublethal rates when applied to putative gly-R plants above 60 cm. The use of sublethal rates of herbicide has been demonstrated to contribute to the development of herbicide-resistant weeds (Doyle & Stypa, 2004; Markus et al., 2018; Vieira et al., 2019; Vila-Aiub et al., 2005). In the presence of glyphosate resistance, glufosinate control of gly-R johnsongrass is a valuable tool for preventing the development of individuals with multiple resistance. Glufosinate

provides a unique mode of action that can be employed in rotation with other herbicides, such as ALS and ACCase inhibitors. The use of glufosinate to control johnsongrass must be judicious to prevent the loss of another effective herbicide mode of action. In these studies, glufosinate at 655 g ha^{-1} was highly effective in controlling rhizomatous johnsongrass under 30 cm.

Johnsongrass emerging from rhizome typically possesses a greater competitive ability due to earlier emergence and faster growth rates than plants emerging from seed (Peerzada et al., 2017; Reichmann et al., 2016). The presence of rhizomatous johnsongrass in the field in addition to the glyphosate resistance observed in this population presents a significant challenge for one- or two-pass POST systems. As well, under this study design, the progression of annual weed emergence, soybean growth, and canopy closure all added complexity to the timing of herbicide application. Results of the data presented here clearly indicate that additional management strategies must be utilized to reduce pressure on POST herbicides.

Where tillage may be employed, as with the field study location at Belcher Branch, post-harvest fall cultivation can be an effective strategy to reduce populations, as tillage exposes rhizomes to both freezing and desiccation (McWhorter, 1972). While conventional preplant tillage contributes to johnsongrass seedling recruitment (Barroso et al., 2012), earlier studies revealed that intensive preplant tillage (≥ 6 diskings) provided johnsongrass control in soybeans comparable to the use of herbicides (McWhorter & Hartwig, 1965). In addition, PRE herbicides controlling johnsongrass emerging from seed can effectively complement the use of tillage for johnsongrass control. In the first POST applications made in these studies, the residual activity of acetochlor was expected

to suppress but not control johnsongrass emerging from seed (Bayer, 2020). The use of an effective PRE herbicide in conjunction with POST programs, in addition to alternating herbicide mode of action is strongly recommended (Norsworthy et al., 2012; Powles et al., 1996).

While many producers have moved away from preplant incorporated (PPI) herbicides, trifluralin has been shown to have good activity on seedling johnsongrass (Pike et al., 1991; McWhorter, 1974). The use of trifluralin plus metribuzin PPI in soybeans peaked in popularity in the 1980's, prior to the shift towards POST herbicide weed management (Pike et al., 1991). However, the combination of trifluralin plus metribuzin offers two unique modes of action with activity on seedling johnsongrass (Richard, 1998; McWhorter, 1974). In addition to metribuzin, several PRE herbicides that do not require soil incorporation are available with activity on johnsongrass, including dimethenamid, imazaquin, pendimethalin, and flumioxazin (Arnold & Hurst, 1982; Culpepper et al., 2000; Johnson et al., 1991; Johnson et al., 2014; Somerville et al., 2017). These herbicides represent five unique modes of action that can be utilized in soybean to reduce selection pressure on both PRE and POST herbicides (Beckie & Harker, 2017; Shaner, 2014).

The results of no-till field studies at Lake East over two years with POST only programs provide strong evidence for diversified weed management. No-till systems may face reduced pressure from seedling johnsongrass, but vegetative reproduction plays a significant role in maintaining johnsongrass populations (Scopel et al., 1988). Management programs should emphasize cultural practices such as the prevention of johnsongrass seed production prior to crop harvest, and preventing seed movement

(Barroso et al., 2012; Ghersa et al., 1993). Several PRE herbicides with activity on johnsongrass may be surface applied prior to planting (Shaner, 2014). Particularly for no-till, crop rotation is an important tool that can enable new herbicide modes of action for POST control of johnsongrass, such as nicosulfuron or tembotrione in maize (Camacho et al., 1991; Damalas et al., 2018). Mechanical, cultural, and diversified chemical weed management strategies, when utilized judiciously, can significantly reduce the selective pressure imposed on weed populations from overreliance on single herbicide modes of action.

LITERATURE CITED

Anderson, DM, CJ Swanton, JC Hall and BG Mersey. 1993. The influence of temperature and relative humidity on the efficacy of glufosinate-ammonium. Weed Res. 33(2): 139-147.

Arnold, BL and HR Hurst. 1982. Herbicides for controlling a mixed population of johnsongrass and common cocklebur in soybeans. Mississippi Agric. For. Exp. Sta. Bull. 906. 12 pp.

Avila-Garcia, WV and C Mallory-Smith. 2011. Glyphosate-resistant Italian ryegrass (*Lolium perenne*) populations also exhibit resistance to glufosinate. Weed Sci. 59(3): 305-309.

Barroso, J, D Andújar, C San Martín, C Fernández-Quintanilla and J Dorado. 2012. Johnsongrass (*Sorghum halepense*) seed dispersal in corn crops under Mediterranean conditions. Weed Sci. 60(1): 34-41.

Bayer CropScience. 2020. Warrant herbicide label. EPA Reg. No. 524-591. St. Louis, Missouri, USA.

Beckie, HJ and KN Harker. 2017. Our top 10 herbicide-resistant weed management practices. Pest Manag. Sci. 73(6): 1045-1052.

Beckie, HJ and X Reboud. 2009. Selecting for weed resistance: herbicide rotation and mixture. Weed Technol. 23(3): 363-370.

Beckie, HJ, MB Ashworth and KC Flower. 2019. Herbicide resistance management: Recent developments and trends. Plants. 8(6): 161.

Benbrook, CM. 2016. Trends in glyphosate herbicide use in the United States and globally. Environ. Sci. Eur. 28(1): 1-15.

Bendixen, LE. 1988. Soybean (*Glycine max*) competition helps herbicides control johnsongrass (*Sorghum halepense*). Weed Technol. 2(1): 46-48.

Camacho, RF, LJ Moshier, DW Morishita and DL Devlin. 1991. Rhizome johnsongrass (*Sorghum halepense*) control in corn (*Zea mays*) with primisulfuron and nicosulfuron. Weed Technol. 5(4): 789-794.

Conover, WJ and RL Iman. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. Am. Stat. 35(3): 124-129.

Culpepper, AS, AC York, RB Batts and KM Jennings. 2000. Weed management in glufosinate-and glyphosate-resistant soybean (*Glycine max*). Weed Technol. 14(1): 77-88.

Damalas, CA, TK Gitsopoulos, SD Koutroubas, C Alexoudis and I Georgoulas. 2018. Weed control and selectivity in maize (*Zea mays* L.) with tembotrione mixtures. Int. J. Pest Manag. 64(1): 11-18.

Doyle, P and M Stypa. 2004. Reduced herbicide rates—a Canadian perspective. Weed Technol. 18(4): 1157-1165.

Flint, JL and M Barrett. 1989. Antagonism of glyphosate toxicity to johnsongrass (*Sorghum halepense*) by 2, 4-D and dicamba. Weed Sci. 37(5): 700-705.

Ghanizadeh, H, KC Harrington and TK James. 2015. Glyphosate-resistant *Lolium multiflorum* and *Lolium perenne* populations from New Zealand are also resistant to glufosinate and amitrole. Crop Prot. 78: 1-4.

Ghersa, CM et al. 1993. Seed dispersal, distribution and recruitment of seedlings of *Sorghum halepense* (L.) Pers. Weed Res. 33:79–88.

Heap, I and SO Duke. 2018. Overview of glyphosate-resistant weeds worldwide. Pest Manag. Sci. 74(5): 1040-1049.

Heap, I. 2022. The international herbicide-resistant weed database. Available <www.weedscience.org>

Horowitz, M. 1973. Spatial growth of *Sorghum halepense* (L.) Pers. Weed Res. 13(2): 200-208.

Jalaludin, A., Q Yu, and SB Powles. 2015. Multiple resistance across glufosinate, glyphosate, paraquat and ACC ase-inhibiting herbicides in an *Eleusine indica* population. Weed Res. 55(1): 82-89.

Jhala, AJ, LD Sandell, D Sarangi, GR Kruger and SZ Knezevic. 2017. Control of glyphosate-resistant common waterhemp (*Amaranthus rudis*) in glufosinate-resistant soybean. Weed Technol. 31(1): 32-45.

Jhala, AJ, MS Malik and JB Willis. 2015. Weed control and crop tolerance of micro-encapsulated acetochlor applied sequentially in glyphosate-resistant soybean. Can. J. Plant Sci. 95(5): 973-981.

Johnson, WG, RE Frans and LD Parsch. 1991. Economics of johnsongrass (*Sorghum halepense*) control in soybeans (*Glycine max*). Weed Technol. 5(4): 765-770.

Johnson, WG, J Li, and JD Wait. 2003. Johnsongrass control, total nonstructural carbohydrates in rhizomes, and regrowth after application of herbicides used in herbicide-resistant corn (*Zea mays*). Weed Technol. 17(1): 36-41.

Johnson, DB, JK Norsworthy and RC Scott. 2014. Herbicide programs for controlling glyphosate-resistant johnsongrass (*Sorghum halepense*) in glufosinate-resistant soybean. Weed Technol. 28(1): 10-18.

Kaur, S, LD Sandell, JL Lindquist and AJ Jhala. 2014. Glyphosate-resistant giant ragweed (*Ambrosia trifida*) control in glufosinate-resistant soybean. Weed Technol. 28(4): 569-577.

Klein, P and CM Smith. 2021. Invasive johnsongrass, a threat to native grasslands and agriculture. Biologia. 76(2): 413-420.

Kniss, AR. 2018. Genetically engineered herbicide-resistant crops and herbicide-resistant weed evolution in the United States. Weed Sci. 66(2):260-273.

Larran, AS, VE Palmieri, VE Perotti, L Lieber, F Tuesca and HR Permingeat. 2017. Target-site resistance to acetolactate synthase (ALS)-inhibiting herbicides in *Amaranthus palmeri* from Argentina. Pest Manag. Sci. 73(12): 2578-2584.

Markus, C, A Pecinka, R Karan, JN Barney and A Merotto Jr. 2018. Epigenetic regulation–contribution to herbicide resistance in weeds?. Pest Manag. Sci. 74(2): 275-281.

McWhorter, CG and EE Hartwig. 1965. Effectiveness of preplanting tillage in relation to herbicides in controlling johnsongrass for soybean production 1. J Agron. 57(4):385-389.

McWhorter, CG and EE Hartwig. 1972. Competition of johnsongrass and cocklebur with six soybean varieties. Weed Sci. 20(1): 56-59.

McWhorter, CG and JM Anderson. 1981. The technical and economic effects of johnsongrass (*Sorghum halepense*) control in soybeans (*Glycine max*). Weed Sci. 29(3): 245-253.

McWhorter, CG. 1972. Factors affecting johnsongrass rhizome production and germination. Weed Sci. 20(1): 41-45.

McWhorter, CG. 1974. Johnsongrass control in soybeans by trifluralin and nitralin. Weed Sci. 22(2): 111-115.

Nandula, VK. 2019. Herbicide resistance traits in maize and soybean: current status and future outlook. Plants. 8(9): 337.

Norsworthy, JK, SM Ward, DR Shaw, RS Llewellyn, RL Nichols, TM Webster, KW Bradley, G Frisvold, SB Powles, NR Burgosand and WW Witt. 2012. Reducing the risks of herbicide resistance: best management practices and recommendations. Weed Sci. 60(SP1): 31-62.

Peerzada, AM, HH Ali, Z Hanif, AA Bajwa, L Kebaso, D Frimpong, N Iqbal, H Namubiru, S Hashim, G Rasool and S Manalil. 2017. Eco-biology, impact, and management of *Sorghum halepense* (L.) Pers. Biol. Invasions. pp. 1-19.

Petersen, J and K Hurle. 2001. Influence of climatic conditions and plant physiology on glufosinate-ammonium efficacy. Weed Res. 41(1):31-39.

Pike, DR, MD McGlamery and EL Knake. 1991. A case study of herbicide use. Weed Technol. 5(3): 639-646.

Powles, SB, C Preston, IB Bryan and AR Jutsum. 1996. Herbicide resistance: impact and management. Adv. Agron. 58: 57-93.

Reichmann, LG, S Schwinning, HW Polley and PA Fay. 2016. Traits of an invasive grass conferring an early growth advantage over native grasses. J. Plant Ecol. 9(6): 672-681.

Riar, DS, JK Norsworthy, DB Johnson, RC Scott and M Bagavathiannan. 2011. Glyphosate resistance in a johnsongrass (*Sorghum halepense*) biotype from Arkansas. Weed Sci. 59(3): 299-304.

Richard, EP. 1998. Control of perennated bermudagrass (*Cynodon dactylon*) and johnsongrass (*Sorghum halepense*) in sugarcane (*Saccharum* spp. hybrids). Weed Technol. 12(1): 128.

Scopel, AL, CL Ballare and CM Ghersa. 1988. Role of seed reproduction in the population ecology of *Sorghum halepense* in maize crops. J. Appl. Ecol. 25:951-962.

Seefeldt, SS, JE Jensen and EP Fuerst. 1995. Log-logistic analysis of herbicide dose-response relationships. Weed Technol. 9(2): 218-227.

Shaner, DL. 2014. Herbicide handbook. Lawrence, KS, USA: Weed Science Society of America.

Somerville, GJ, SB Powles, MJ Walsh and M Renton. 2017. Why was resistance to shorter-acting pre-emergence herbicides slower to evolve?. Pest Manag. Sci. 73(5): 844-851.

United States Department of Agriculture–Animal and Plant Health Inspection Service, USDA-APHIS. Dow AgroSciences LLC; determination of nonregulated status of herbicide resistant corn and soybeans. 22 Sept. 2014. 79 FR: 56555-56557.

United States Department of Agriculture–Animal and Plant Health Inspection Service, USDA-APHIS. Monsanto Co.; determination of nonregulated status of herbicide resistant soybean and cotton. 20 Jan. 2015. 80 FR: 2675-2676.

Vázquez-Garcia, JG, C Palma-Bautista, AM Rojano-Delgado, R De Prado and J Menendez. 2020. The first case of glyphosate resistance in johnsongrass (*Sorghum halepense* (L.) Pers.) in Europe. Plants 9(3): 313.

Vencill, WK, RL Nichols, TM Webster, JK Soteres, C Mallory-Smith, NR Burgos, WG Johnson and MR McClelland. 2012. Herbicide resistance: toward an understanding of resistance development and the impact of herbicide-resistant crops. Weed Sci. 60(SP1): 2-30.

Vieira, BC, JD Luck, KL Amundsen, TA Gaines, R Werle and GR Kruger. 2019. Response of *Amaranthus* spp. following exposure to sublethal herbicide rates via spray particle drift. PloS One. 14(7): e0220014.

Vila-Aiub, MM and CM Ghersa. 2005. Building up resistance by recurrently exposing target plants to sublethal doses of herbicide. Eur. J Agron. 22(2): 195-207.

Vila-Aiub, MM, MC Balbi, PE Gundel, CM Ghersa, and SB Powles. 2007. Evolution of glyphosate-resistant johnsongrass (*Sorghum halepense*) in glyphosate-resistant soybean. Weed Sci. 55(6): 566-571.

Wauchope, RD, HR Sumner and CC Dowler. 1997. A measurement of the total mass of spray and irrigation mixtures intercepted by small whole plants. Weed Technol. 11(3): 466-472.

Williams, CS and RM Hayes. 1984. Johnsongrass (*Sorghum halepense*) competition in soybeans (*Glycine max*). Weed Sci. 32(4): 498-501.

Table 3.1. List of herbicide treatments in soybean field studies with glyphosate-resistant johnsongrass in Buchanan County, Missouri in 2020 and 2021. Early POST treatments targeted 10 to 20 cm grass weeds and mid-POST treatments were applied one to two weeks later. [1]Abbrev.= treatment abbreviations utilized in following tables and figures. [2,4]Manufactured by WinField United. [3]Manufactured by Bayer CropScience. fb= followed by.

Herbicide programs POST targeting grass weeds in soybean					
[1]Treatment Abbrev.	Active ingredient	Rate (g ai ha^{-1})	Application timing	Adjuvant(s)	Adjuvant rate
NTC	Nontreated control				
ADG fb Gly	Acetochlor	1,259	Early POST		
	Dicamba	563	Early POST		
	Glyphosate	1,263	Early POST		
	Glyphosate	1,263	Mid-POST	Ammonium sulfate	0.5 g L^{-1}
ADG fb Gly+C	Acetochlor	1,259	Early POST		
	Dicamba	563	Early POST		
	Glyphosate	1,263	Early POST		
	Clethodim	140	Mid-POST	[2]Destiny® HC	0.5% v/v
	Glyphosate	1,263	Mid-POST		
ADG+C	Acetochlor	1,259	Early POST	[3]Intact™	0.5% v/v
	Clethodim	140	Early POST		
	Dicamba	563	Early POST		
	Glyphosate	1,263	Early POST		
ADG fb F	Acetochlor	1,259	Early POST	Intact™	0.5% v/v
	Dicamba	563	Early POST		
	Glyphosate	1,263	Early POST		
	Fluazifop-P-butyl	210	Mid-POST	[4]Class Act® Ridion	1% v/v
	Acetochlor	1,259	Early POST	Intact™	0.5% v/v
ADG+F	Dicamba	563	Early POST		
	Fluazifop-P-butyl	105	Early POST		
	Glyphosate	1,263	Early POST		
ADG fb F+F	Acetochlor	1,259	Early POST	Intact™	0.5% v/v
	Dicamba	563	Early POST		
	Glyphosate	1,263	Early POST		
	Fluazifop-P-butyl	85	Mid-POST	Class Act® Ridion	1% v/v
	Fenoxaprop-P-ethyl	24	Mid-POST		
ADG fb Glu+Gly	Acetochlor	1,259	Early POST	Intact™	0.5% v/v
	Dicamba	563	Early POST		
	Glyphosate	1,263	Early POST		
	Glufosinate	655	Mid-POST	Ammonium sulfate	0.5 g L^{-1}
	Glyphosate	1,263	Mid-POST		
ADG fb Glu	Acetochlor	1,259	Early POST	Intact™	0.5% v/v
	Dicamba	563	Early POST		
	Glyphosate	1,263	Early POST		
	Glufosinate	655	Mid-POST	Ammonium sulfate	0.5 g L^{-1}

Table 3.2. Johnsongrass control data from field studies targeting glyphosate-resistant johnsongrass in soybean conducted in Buchanan County, Missouri in 2020 and 2021. Early POST treatments (EPOST) targeted 10 to 20 cm grass weeds and mid-POST treatments (MPOST) were applied one to two weeks later. Visual estimations of weed control are based on a scale of 0 (no control) to 100% (complete plant mortality). No data are available for annual grass weed control at Lake East, as annual grass weeds were only present at Belcher Branch. Treatment means followed by the same letter are not significantly different at α=0.05 using Tukey's Honestly Significant Difference. [1]Treatment abbreviations listed in Table 3.1. [a] WAT= weeks after treatment. [b-m]= adjuvants listed below table.

	Application timing				Visual estimation of johnsongrass control (0-100%)			
					Belcher Branch		Lake East	
	EPOST		MPOST		2020	2021	2020	2021
nent abbrev.	Herbicides	Rates (g ai ha^{-1})	Herbicides	Rates (g ai ha^{-1})	2 WAT (MPOST)[a]	2 WAT (MPOST)	2 WAT (MPOST)	2 WAT (MPOST)
NTC	———	———	———	———	0 c	0 b	0 e	0 e
ADG fb Gly	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Glyphosate[b]	1,263	95 ab	96 a	53 d	20 d
ADG fb Gly+C	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Clethodim + glyphosate[c]	140 + 1,263	99 a	95 a	96 a	72 a
ADG+C	Acetochlor + dicamba + glyphosate + clethodim[d]	1,259 + 563 + 1,263 + 140	———	———	62 bc	90 a	17 de	48 bc
ADG fb F	Acetochlor + dicamba + glyphosate[e]	1,259 + 563 + 1,263	Fluazifop-P-butyl[f]	210	95 ab	97 a	92 ab	66 a
ADG+F	Acetochlor + dicamba + glyphosate + fluazifop-P-butyl[g]	1,259 + 563 + 1,263 + 105	———	———	75 bc	90 a	53 d	59 ab
ADG fb F+F	Acetochlor + dicamba + glyphosate[h]	1,259 + 563 + 1,263	Fenoxaprop-P-ethyl + fluazifop-P-butyl[i]	24+85	98 a	96 a	95 a	71 a
ADG fb Glu+Gly	Acetochlor + dicamba + glyphosate[j]	1,259 + 563 + 1,263	Glufosinate + glyphosate[k]	655 + 1,263	98 a	96 a	80 bc	45 c
ADG fb Glu	Acetochlor + dicamba + glyphosate[l]	1,259 + 563 + 1,263	Glufosinate[m]	655	94 ab	96 a	79 c	52 bc

[b, k, l, m]= included ammonium sulfate at 0.5 g L^{-1}

[d, e, g, h, j, l]= included Intact™ at 0.5% v/v

[f, i]= included Class Act Ridion® at 1% v/v

[c]= included Destiny HC® at 0.5% v/v

Table 3.3. Annual grass spp. weed control data from field studies targeting glyphosate-resistant johnsongrass in soybean conducted in Buchanan County, Missouri in 2020 and 2021. Early POST treatments (EPOST) targeted 10 to 20 cm grass weeds and mid-POST treatments (MPOST) were applied one to two weeks later. Visual estimations of weed control are based on a scale of 0 (no control) to 100% (complete plant mortality). No data are available for annual grass weed control at Lake East, as annual grass weeds were only present at Belcher Branch. Treatment means followed by the same letter are not significantly different at α=0.05 using Tukey's Honestly Significant Difference. [1]Treatment abbreviations listed in Table 3.1. [a] WAT= weeks after treatment. [b-m]= adjuvants listed below table.

	Application timing				Visual estimation of annual grass spp. control (0-100%) Belcher Branch	
	EPOST		MPOST		2020	2021
Treatment abbrev.	Herbicides	Rates (g ai ha^{-1})	Herbicides	Rates (g ai ha^{-1})	2 WAT (MPOST)[a]	2 WAT (MPOST)
NTC	——	——	——	——	0 d	0 b
ADG fb Gly	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Glyphosate[b]	1,263	99 a	96 a
ADG fb Gly+C	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Clethodim + glyphosate[c]	140 + 1,263	99 a	95 a
ADG+C	Acetochlor + dicamba + glyphosate + clethodim[d]	1,259 + 563 + 1,263 + 140	——	——	93 b	90 a
ADG fb F	Acetochlor + dicamba + glyphosate[e]	1,259 + 563 + 1,263	Fluazifop-P-butyl[f]	210	98 a	97 a
ADG+F	Acetochlor + dicamba + glyphosate + fluazifop-P-butyl[g]	1,259 + 563 + 1,263 + 105	——	——	89 c	90 a
ADG fb F+F	Acetochlor + dicamba + glyphosate[h]	1,259 + 563 + 1,263	Fenoxaprop-P-ethyl + fluazifop-P-butyl[i]	24+85	98 a	97 a
ADG fb Glu+Gly	Acetochlor + dicamba + glyphosate[j]	1,259 + 563 + 1,263	Glufosinate + glyphosate[k]	655 + 1,263	99 a	96 a
ADG fb Glu	Acetochlor + dicamba + glyphosate[l]	1,259 + 563 + 1,263	Glufosinate[m]	655	99 a	96 a

[b, k, l, m]= included ammonium sulfate at 0.5 g L^{-1}
[d, e, g, h, j, l]= included Intact™ at 0.5% v/v
[f, i]= included Class Act Ridion® at 1% v/v
[c]= included Destiny HC® at 0.5% v/v

Table 3.4. Johnsongrass density data from field studies targeting glyphosate-resistant johnsongrass in soybean conducted in Buchanan County, Missouri in 2020 and 2021. Only weeds with herbicide injury at 80% or less (based on a scale of 0 [no control] to 100% [complete plant mortality]) were considered as surviving herbicide treatment. Treatment means followed by the same letter are not significantly different at α=0.05 using Tukey's Honestly Significant Difference. Treatment abbreviations are listed in Table 3.1. [a]2 WAT (MPOST)= two weeks after treatment (WAT) with mid-POST application. [b-m]= adjuvants listed below table.

	Application timing				Johnsongrass density			
					Belcher Branch		Lake East	
	EPOST		MPOST		2020	2021	2020	2021
Treatment abbrev.	Herbicides	Rates (g ai ha^{-1})	Herbicides	Rates (g ai ha^{-1})	2 WAT (MPOST)[a]	2 WAT (MPOST)	2 WAT (MPOST)	2 WAT (MPOST)
NTC	———	———	———	———	74	191 b	49	107 b
ADG fb Gly	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Glyphosate[b]	1,263	72	11 ab	65	83 ab
ADG fb Gly+C	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Clethodim + glyphosate[c]	140 + 1,263	14	3 a	2	28 a
ADG+C	Acetochlor + dicamba + glyphosate + clethodim[d]	1,259 + 563 + 1,263 + 140	———	———	100	3 a	35	101 ab
ADG fb F	Acetochlor + dicamba + glyphosate[e]	1,259 + 563 + 1,263	Fluazifop-P-butyl[f]	210	60	2 a	4	53 ab
ADG+F	Acetochlor + dicamba + glyphosate + fluazifop-P-butyl[g]	1,259 + 563 + 1,263 + 105	———	———	91	6 a	53	82 ab
ADG fb F+F	Acetochlor + dicamba + glyphosate[h]	1,259 + 563 + 1,263	Fenoxaprop-P-ethyl + fluazifop-P-butyl[i]	24+85	54	3 a	45	51 ab
ADG fb Glu+Gly	Acetochlor + dicamba + glyphosate[j]	1,259 + 563 + 1,263	Glufosinate + glyphosate[k]	655 + 1,263	62	0 a	49	46 ab
ADG fb Glu	Acetochlor + dicamba + glyphosate[l]	1,259 + 563 + 1,263	Glufosinate[m]	655	89	0 a	4	47 ab

[b, k, l, m]= included ammonium sulfate at 0.5 g L^{-1}

[d, e, g, h, j, l]= included Intact™ at 0.5% v/v

[f, i]= included Class Act Ridion® at 1% v/v

[c]= included Destiny HC® at 0.5% v/v

Table 3.5. Annual grass weed density data from field studies targeting glyphosate-resistant johnsongrass in soybean conducted in Buchanan County, Missouri in 2020 and 2021. Early POST treatments (EPOST) targeted 15 to 20 cm grass weeds and mid-POST treatments (MPOST) were applied one to two weeks later. Grass densities were derived from two, 0.25 m^2 sampling areas within plots. Only weeds with herbicide injury at 80% or less (based on a scale of 0 [no control] to 100% [complete plant mortality]) were counted as surviving herbicide treatment. Treatment means followed by the same letter are not significantly different at α=0.05 using Tukey's Honestly Significant Difference. Treatment abbreviations are listed in Table 3.1. [a]2 WAT (MPOST)= two weeks after treatment (WAT) with mid-POST application. [b-m]= adjuvants listed below table.

	Application timing				Annual grass spp. density	
					Belcher Branch	
	EPOST		MPOST		2020	2021
Treatment abbrev.	Herbicides	Rates (g ai ha^{-1})	Herbicides	Rates (g ai ha^{-1})	2 WAT (MPOST)[a]	2 WAT (MPOST)
NTC	———	———	———	———	754 c	494 b
ADG fb Gly	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Glyphosate[b]	1,263	7 a	9 a
ADG fb Gly+C	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Clethodim + glyphosate[c]	140 + 1,263	15 ab	19 a
ADG+C	Acetochlor + dicamba + glyphosate + clethodim[d]	1,259 + 563 + 1,263 + 140	———	———	50 ab	26 a
ADG fb F	Acetochlor + dicamba + glyphosate[e]	1,259 + 563 + 1,263	Fluazifop-P-butyl[f]	210	31 ab	42 a
ADG+F	Acetochlor + dicamba + glyphosate + fluazifop-P-butyl[g]	1,259 + 563 + 1,263 + 105	———	———	137 b	15 a
ADG fb F+F	Acetochlor + dicamba + glyphosate[h]	1,259 + 563 + 1,263	Fenoxaprop-P-ethyl + fluazifop-P-butyl[i]	24+85	18 ab	14 a
ADG fb Glu+Gly	Acetochlor + dicamba + glyphosate[j]	1,259 + 563 + 1,263	Glufosinate + glyphosate[k]	655 + 1,263	14 ab	12 a
ADG fb Glu	Acetochlor + dicamba + glyphosate[l]	1,259 + 563 + 1,263	Glufosinate[m]	655	20 ab	27 a

[b, k, l, m]= included ammonium sulfate at 0.5 g L^{-1}

[d, e, g, h, j, l]= included Intact™ at 0.5% v/v

[f, i]= included Class Act Ridion® at 1% v/v

[c]= included Destiny HC® at 0.5% v/v

Table 3.6. Grass weed biomass data from field studies targeting glyphosate-resistant johnsongrass in soybean conducted in Buchanan County, Missouri in 2020 and 2021. Early POST treatments (EPOST) targeted 15 to 20 cm grass weeds and mid-POST treatments (MPOST) were applied one to two weeks later. Only weeds with herbicide injury at 80% or less (based on a scale of 0 [no control] to 100% [complete plant mortality]) were counted as surviving herbicide treatment. Treatment means followed by the same letter are not significantly different at α=0.05 using Tukey's Honestly Significant Difference. Treatment abbreviations are listed in Table 3.1. [a]WAT= weeks after treatment. [b-m]= adjuvants listed below table.

	Application timing				Grass weed biomass (% of nontreated control)			
	EPOST		MPOST		Belcher Branch		Lake East	
					2020	2021	2020	2021
Treatment abbrev.	Herbicides	Rates (g ai ha^{-1})	Herbicides	Rates (g ai ha^{-1})	2 WAT (MPOST)[a]	2 WAT (MPOST)	2 WAT (MPOST)	2 WAT (MPOST)
NTC	———	———	———	———	100 d	100	100 d	100 b
ADG fb Gly	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Glyphosate[b]	1,263	20 cd	16	33 bc	53 ab
ADG fb Gly+C	Acetochlor + dicamba + glyphosate	1,259 + 563 + 1,263	Clethodim + glyphosate[c]	140 + 1,263	0 a	5	3 a	38 ab
ADG+C	Acetochlor + dicamba + glyphosate + clethodim[d]	1,259 + 563 + 1,263 + 140	———	———	17 cd	2	59 cd	39 ab
ADG fb F	Acetochlor + dicamba + glyphosate[e]	1,259 + 563 + 1,263	Fluazifop-P-butyl[f]	210	0 a	1	7 a	48 ab
ADG+F	Acetochlor + dicamba + glyphosate + fluazifop-P-butyl[g]	1,259 + 563 + 1,263 + 105	———	———	11 bcd	1	41 cd	44 ab
ADG fb F+F	Acetochlor + dicamba + glyphosate[h]	1,259 + 563 + 1,263	Fenoxaprop-P-ethyl + fluazifop-P-butyl[i]	24+85	0 a	3	3 a	42 ab
ADG fb Glu+Gly	Acetochlor + dicamba + glyphosate[j]	1,259 + 563 + 1,263	Glufosinate + glyphosate[k]	655 + 1,263	0 a	4	13 ab	27 a
ADG fb Glu	Acetochlor + dicamba + glyphosate[l]	1,259 + 563 + 1,263	Glufosinate[m]	655	3 abc	2	13 ab	39 ab

[b, k, l, m]= included ammonium sulfate at 0.5 g L^{-1}

[d, e, g, h, j, l]= included Intact™ at 0.5% v/v

[f, i]= included Class Act Ridion® at 1% v/v

[c]= included Destiny HC® at 0.5% v/v

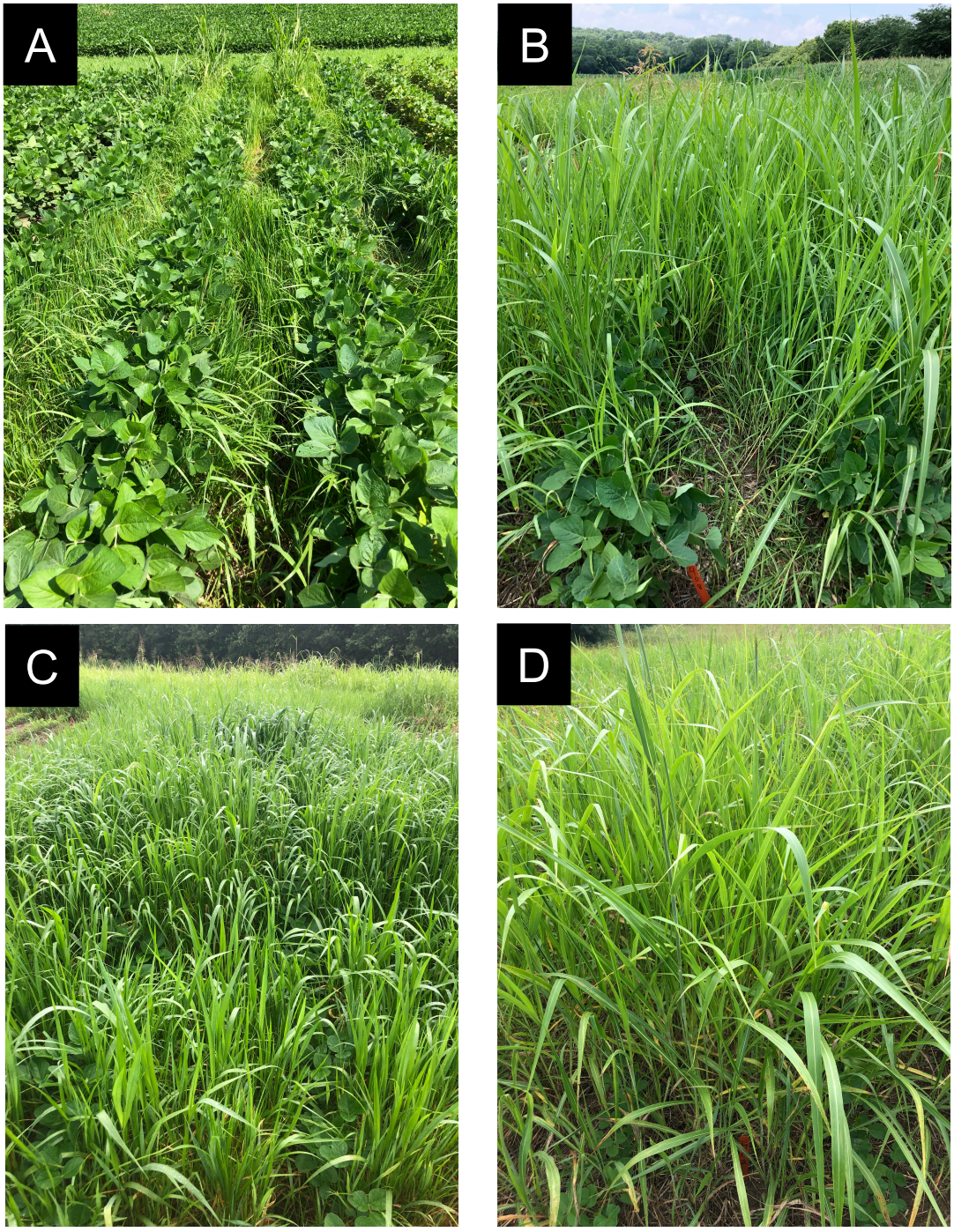


Fig. 3.1. Representative photos of non-treated control plots from field studies targeting glyphosate-resistant johnsongrass in soybean conducted in Buchanan County, Missouri in 2020 and 2021. A, C= Belcher Branch site in 2020 and 2021, at 48 and 32 days after planting (DAP), respectively. B, D= Lake East site in 2020 and 2021, at 48 and 32 DAP. Timing corresponds to 2 weeks after the treatment with B application.

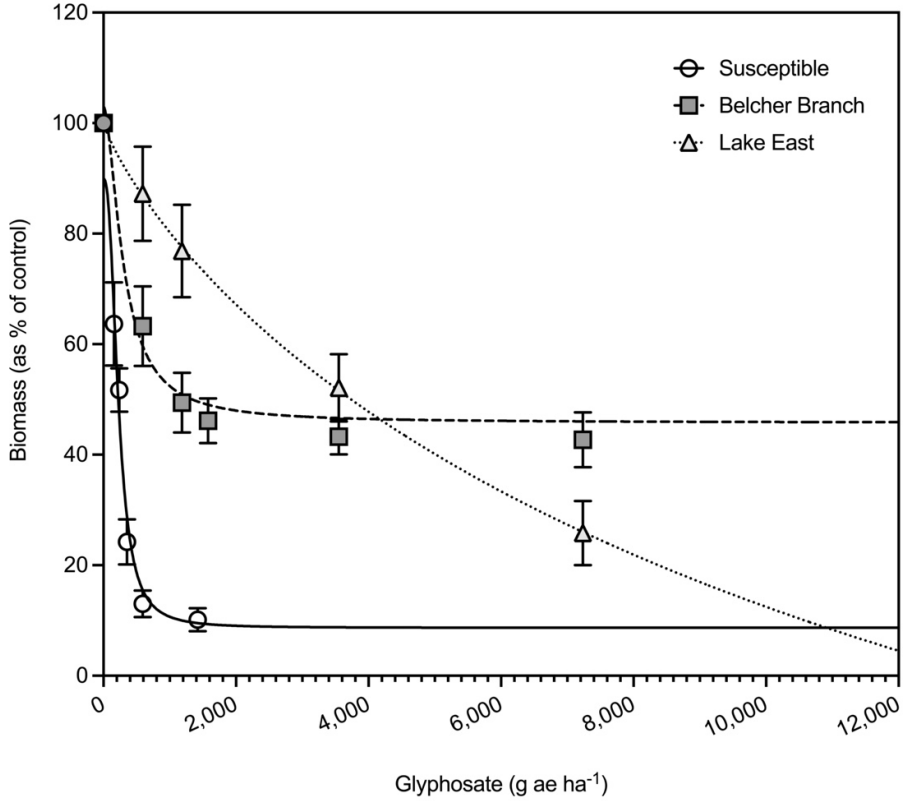


Fig. 3.2. Dose-response of johnsongrass biotypes to glyphosate. Biotypes were obtained from rhizomes collected from field study sites under investigation for glyphosate-resistant johnsongrass control in soybean conducted in Buchanan County, Missouri in 2020 and 2021. Plants were vegetatively propagated and maintained in a greenhouse prior to harvest at three weeks after treatment. Dry biomass data were transformed as a percent of non-treated control within replication and modeled using f(x)=C+(D-C)/(1+(x/[GR_{50}])^b). Curved lines are the models predicted from nonlinear regression with R^2 values of 0.82, 0.63, and 0.32 for susceptible, Belcher Branch, and Lake East, respectively. Vertical lines indicate the standard error of the mean. Symbols represent the mean of 14 replicates. Error bars not plotted are shorter than the symbol for the mean.

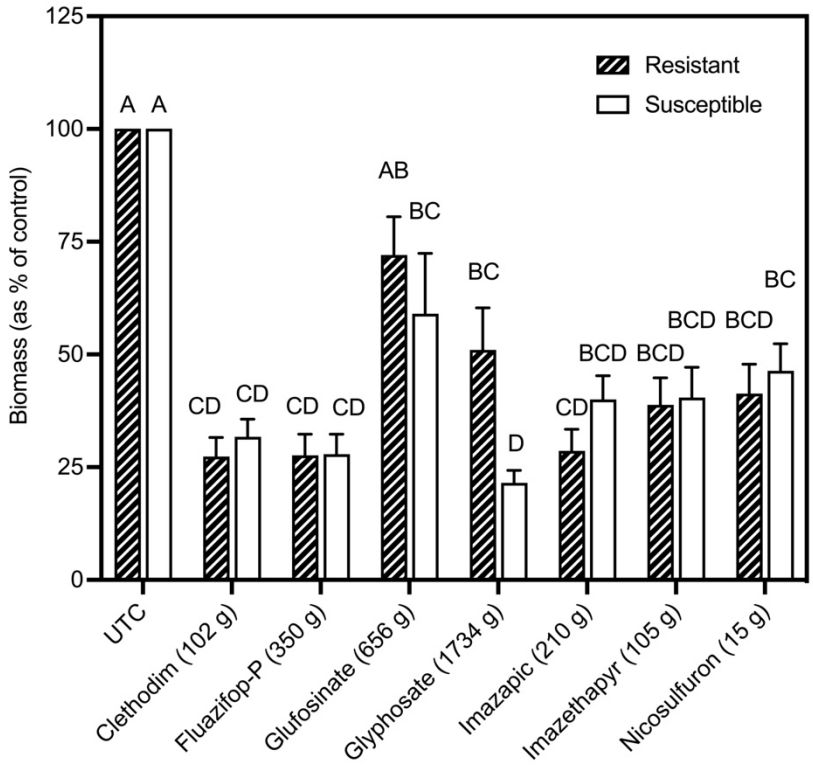


Fig. 3.3. Response of glyphosate-resistant (gly-R) and glyphosate-susceptible (gly-S) johnsongrass biotypes to different herbicide modes of action. Rates for herbicides are shown in parentheses as g ai ha^{-1}. Rhizomes of the gly-R biotype were obtained from a field in Buchanan County, Missouri where glyphosate resistance was first confirmed in johnsongrass. Rhizomes for the gly-S population were dug from an undeveloped urban area. The experiment was repeated, and data from both runs were combined to obtain the means of 11 replicates. Means followed by the same letter are not significantly different at α=0.05 using Tukey's Honestly Significant Difference. Vertical bars indicate the standard error of the means.

CHAPTER IV

Management of Roadside Johnsongrass (Sorghum halepense [L.] Pers.) Populations and Glyphosate Resistance

ABSTRACT

Roadways serve as vectors for plant dispersal, and johnsongrass (*Sorghum halepense* [L.] Pers.) in particular has expanded its habitat range utilizing non-crop areas such as roadsides. Following confirmation of glyphosate resistance in a population of johnsongrass isolated from an agronomic field in Buchanan County, MO, a study was conducted to determine the level of glyphosate sensitivity in roadside johnsongrass. Rhizomes were collected from roadside johnsongrass populations in a 30 km range around the glyphosate-resistant (gly-R) population and vegetatively propagated for use in whole-plant dose-response assays to estimate each population's GR_{50} (glyphosate dose required to reduce aboveground biomass by 50%). The GR_{50} of roadside populations was plotted against the Euclidean distance of each population from the gly-R population in distance-decay models. In a separate field study, vegetatively propagated plants of the gly-R biotype were utilized to assess the response of mature (1.5 m height) gly-R johnsongrass to fall-applied chemical weed control options suitable for roadside applications. Rhizomes were collected in the spring and subjected to a greenhouse assay to assess viability in addition to total nonstructural carbohydrate (NSC) content analysis. Glyphosate sensitivity in roadside populations ranged from GR_{50} values of 106 to 819 g ae ha^{-1}. The distance of roadside johnsongrass from the gly-R population predicted the GR_{50} of roadside populations within 173 g ha^{-1} of glyphosate. An increase in sensitivity to glyphosate was predicted when populations were collected at further distances from the

gly-R source. Results from a simulated roadside management study with the gly-R biotype in 2020 and 2021 indicated that all herbicide treatments reduced the number of shoots from rhizome in the spring. In the greenhouse assay, rhizomes sampled in early spring from plants treated with imazapic at 210 g ai ha^{-1} or imazapyr (841 g ai ha^{-1}) plus glyphosate (868 g ai ha^{-1}) did not produce any shoots. Rhizomes from plants treated with imazapic (210 g) plus glyphosate (868 g) produced only a single shoot across both study years. Consistent with the trait for glyphosate resistance, rhizomes from plants treated with glyphosate alone at 1,736 or 3,473 g ai ha^{-1} produced up to 15 shoots. Rhizomes from plants treated with glyphosate alone at 1,736 or 3,473 g ai ha^{-1} produced biomass reduced only 27 to 39% compared to non-treated controls. With rhizomes from plants treated with clethodim (272 g ai ha^{-1}), aminocyclopyrachlor (1,401 g ai ha^{-1}) plus imazapyr (423 g ai ha^{-1}) plus indaziflam (50 g ai ha^{-1}), foramsulfuron (101 g ai ha^{-1}) plus iodosulfuron (10 g ai ha^{-1}) plus thiencarbazone (42 g ai ha^{-1}), or sulfosulfuron (105 g ai ha^{-1}), shoot biomass was reduced 78 to 94% compared to non-treated controls. In the spring, rhizome NSC content ranged from 13 to 23%, and did not appear related to rhizome viability when estimated by greenhouse assay. With a significant range of glyphosate sensitivity observed in roadside populations, the results of a simulated roadside management field study indicate potential options that can be employed to limit the tolerance of roadside johnsongrass to glyphosate.

INTRODUCTION

Transportation corridors serve as vectors for plant dispersal, including non-native and weedy species (Gade, 2013; Vilà & Pujadas, 2001). Johnsongrass (*Sorghum halepense* (L.) Pers.), a C4, warm-season perennial grass with a broad habitat range, is

one such weed species. Johnsongrass invades both agricultural and non-agricultural areas, reducing native plant diversity. In agricultural fields, johnsongrass reduces productivity by competing with the crop and harboring viral diseases and their vectors (Klein & Smith, 2021). The spread of johnsongrass in the United States over the past ~200 years has been facilitated by habitat expansion into non-agricultural areas. In 2016, an investigation of more than 500 *S. halepense* individuals revealed that most plant genotypes (76%) collected from non-agricultural areas were those of recent evolution (Sezen et al., 2016).

In crop fields, management of established johnsongrass requires a significant investment in weed control strategy. For producers, the cost of controlling johnsongrass with herbicides can exceed $50 USD per hectacre (Klein & Smith, 2021). Uncontrolled johnsongrass competing with soybean (*Glycine max*) for seven weeks at densities of approximately 3 culms m^{-2} reduced yield up to 69% (Williams and Hayes, 1984). In corn (*Zea mays*), season-long competition from johnsongrass led to ≥ 80% yield loss compared to corn kept weed-free (Mitskas et al. 2003).

While conservation ecologists have debated the concept of weed ingress from unmanaged field edges, a number of studies have demonstrated movement of weedy species from field margins to cropland. McDonnell & Stiles (1983) documented bird-disseminated seed deposition along crop field edges that ranged up to 70 seeds m^{-2}. Wilson & Aebischer (1995) examined spatial patterns of dicotyledonous weeds in cereal crops. In one year, 23 weed species decreased in density from the field edge to the interior, while in the second year, 17 weed species followed the same pattern. In a separate study that considered the soil seedbank, the annual grass weeds barren brome

(*Bromus sterilis*) and quackgrass (*Elymus repens*) occurring in crop fields appeared to have originated from field margin populations (Marshall, 1989). The perception that weedy species along field margins invade agricultural fields is still common, despite the body of evidence against this phenomenon (Marshall, 2004).

In the United States, early perspectives on roadside vegetation held that management should be practiced for the purposes of human safety and aesthetic appearance (Disque, 1959). However, modern perspectives on roadside landscapes are far broader in scope, encompassing public concern regarding native ecosystems, invasive and endangered species, insect pollinators, and more. In recent years, roadside land management has come under increased public scrutiny for its impact on local ecosystems (NCHRP, 2022). The roadsides adjacent to the national highway system (rights-of-way) comprise over 1,375,000 hectares of land area, many of which require some degree of vegetation management (NCHRP, 2022).

Roadside vegetation management is frequently accomplished by mowing and chemical control via herbicide applications (Martin et al., 1996). Selective herbicides- those with activity on some plant species, but not all- are useful in controlling undesirable species while preserving others for erosion control (Montgomery et al., 2006). Nonselective herbicides such as glyphosate can be a cost-effective means of total vegetation control, and the use of glyphosate has been frequently recommended for roadside vegetation management (Johnson et al., 2010; McCullough & Shilling, 2020; Meyer et al., 1995; Miller & Middlebrooks, 1987). Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) in the biosynthetic pathway for the production of aromatic amino acids phenylalanine, tyrosine, and tryptophan (Heap &

Duke, 2018). The development of a genetically engineered glyphosate-resistant soybean (*Glycine max*) cultivar in 1996 led to widespread use of glyphosate postemergence (POST), particularly in soybean (Kniss, 2018). In the years that followed, the use of glyphosate in the United States increased across a number of crop systems, with approximately 1.1 billion kg of glyphosate applied between 2004 and 2014 (Benbrook, 2016). The selection pressure imposed by widespread use of glyphosate POST resulted in the evolution of over 50 different glyphosate-resistant (gly-R) weed species by 2022 (Heap, 2022).

In 2019, a population of johnsongrass isolated from an agronomic field in Buchanan County, Missouri was confirmed resistant to glyphosate. In 2020 and 2021, due to the frequent occurrence of johnsongrass on roadsides and within agricultural fields in this area, the following studies were conducted to ascertain the level of glyphosate sensitivity in roadside populations of johnsongrass, as well as assess the response of the gly-R biotype to chemical weed control utilized in non-crop areas.

MATERIALS AND METHODS

Sensitivity of roadside populations to glyphosate. To identify the levels of glyphosate sensitivity in roadside populations of johnsongrass, a series of dose-response studies were conducted on johnsongrass collected from roadsides in Buchanan County, MO. In October of 2020, rhizomes were collected from 18 established roadside stands of johnsongrass in a ~6 km search radius from the agricultural field in which the gly-R population was first identified. During an initial screening, a subsample of rhizome material was propagated in the greenhouse and treated with glyphosate at 1,736 g ae ha^{-1} when plants reached 20 to 30 cm in height. The results of this screening were used to

inform the search radius in 2021. Based on the number of plants surviving the initial dose of 1,736 g ha^{-1}, in October 2021, rhizomes were collected from another 18 roadside stands of johnsongrass at least 6 km from the site where the gly-R biotype was first identified and up to 29 km away. In total, rhizomes from 36 roadside populations were collected. Plants generated from rhizome material were transplanted into 60 L polyethylene tubs filled with a 50:50 mixture of peat-based potting media (Hummert International; Earth City, MO, USA) and Mexico silt loam topsoil (fine, smectitic, mesic Vertic Epiaqualf). Plants were allowed to mature in the greenhouse for a minimum of 3 months. Rhizomes were harvested from tubs, cut into 2 to 3 node pieces, and planted in 15 cm pots filled with a 70:30 mixture of potting media as previously described. A reference, glyphosate-susceptible population collected from Boone County, MO was vegetatively propagated in the same manner and utilized as an internal control within dose-response studies. At 15 to 30 cm in height, plants were treated with varied rates of glyphosate using a greenhouse track sprayer calibrated to deliver 140 L ha^{-1}. At 3 weeks after treatment (WAT), plants were harvested at the soil line and oven-dried at 55°C for 4 d.

Simulated roadside management study with glyphosate-resistant johnsongrass. Rhizomes of gly-R johnsongrass were collected from soybean fields in Buchanan County, MO in 2019. These were propagated in the greenhouse and treated with glyphosate at 1,736 g ha^{-1} when plants reached 20 to 30 cm in height. Surviving plants were transplanted into two adjacent fields formerly under row crop production in Boone County, MO in rows on 7 June 2020 and 10 June 2021 as single plants. The soil type for both fields was a Mexico silt loam (fine, smectitic, mesic Vertic Epiaqualf) with

2.8% organic matter (OM) and pH 7.2 for the field utilized in 2020; the field utilized in 2021 had 2.9% OM and pH 6.7. Approximately 2 months after plants established, rows were demarcated into 10 m^2 plots with two johnsongrass stands each. Once flowering initiated, stands were trimmed to 1.5 m height weekly to minimize pollen spread and seed production (Fig. 4.1).

On 18 Sept. 2020 and 20 Sept. 2021, treatments (Table 4.1) were applied via backpack sprayer calibrated to deliver 374 L ha^{-1} with the addition of 1% v/v methylated seed oil. Percent visual injury (with 0=no injury and 100=plant death) was recorded at 2 and 4 weeks after treatment (WAT). Prior to herbicide application, the number of tillers for each of the two johnsongrass bunches were recorded per plot to estimate plant size (Appendix A.4).

In the spring, the rhizomes of a single johnsongrass stand were dug from each plot and rhizome fresh weight was recorded (data not shown). Rhizomes were washed, and a random subsample was cut into 10, 5-node pieces. Five pieces of this subsample were oven-dried at 55°C for 7 d for non-structural carbohydrate (NSC) analysis. The remaining five were planted into 30 by 30 cm trays filled with 70:30 potting media as previously described and maintained in the greenhouse. Shoot emergence was counted biweekly until six weeks after planting (WAP), at which point shoots were harvested at the soil line and oven-dried at 55°C for 4 d.

Dried rhizome pieces were ground to pass a 40-mesh screen and ball-milled to a fine powder prior to partitioning into ~30 mg subsamples. Non-structural carbohydrates were evaluated following the protocol described in Landhäusser et al. (2018). Briefly, soluble sugars were extracted in ethanol by heating at 90ºC for 10 min and quantified

spectrophotometrically using a phenol-sulfuric acid method. A parallel assay without phenol was utilized to correct for potentially interfering substances. Starch was digested to glucose using α-amylase and amyloglucosidase, and the resulting glucose hydrolysate was quantified spectrophotometrically with a peroxidase-glucose oxidase preparation.

Statistical analysis. Dose-response studies on roadside johnsongrass populations were arranged in a randomized complete block design with six replications. For 10 populations, vegetative propagation yielded insufficient material for six replications, and these were eliminated from the final data set. Biomass data were transformed as a percent of control within replication and subjected to a mixed model using PROC GLM in SAS Enterprise Guide 8.3 (SAS Institute; Cary, NC, USA) to confirm population & treatment effects. Biomass data were then subjected to nonlinear regression using a four-parameter, log-logistic model (Seefeldt et al., 1995) (Eq. 3.1) in GraphPad Prism 7 (GraphPad Software; San Diego, CA, USA) according to Larran et al. (2017). A lack-of-fit *F* test was performed to test the hypothesis that the curves have the same slope; i.e., if a more complex model (nonparallel curves) can be reduced to a simpler model (parallel curves).

$$y = c + \frac{(d - c)}{1 + x / gly\text{-}R_{50}{}^{b}}, \qquad \text{(Eq. 4.1)}$$

where *y*= biomass data transformed as a percent of control within replication; *c*= mean response at highest dose; *d*=mean response at lowest dose; *x*= glyphosate as the isopropylamine salt, given in g ae ha^{-1}; and GR_{50} = growth reduction, or 50% reduction in aboveground biomass; and *b*= slope of the line around the GR_{50}.

A new dataset was constructed for roadside populations, using GR_{50} values estimated from dose-response models as the response variable and the Euclidean distance

of each roadside population from the confirmed gly-R population as the independent variable. Euclidean distance was calculated using the distance matrix function in QGIS version 3.26.2 (QGIS Development Team 2021; QGIS Geographic Information System, Open-Source Geospatial Foundation Project). A single outlier (population 13; Table 4.2) was identified using the ROUT method in GraphPad Prism 9 (Motulsky & Brown, 2006). A distance-decay function (Eq. 4.2) was fit to both the data set as a whole (n=27) as well as the dataset with the outlier removed in SigmaPlot 14.5 (IBM; Armonk, NY, USA). The exponential distance-decay function

$$f = y0 + [a \times (e^{(-b \times x)})], \quad \text{(Eq. 4.2)}$$

where y= GR_{50}, given in g ae ha^{-1}; x= distance of the roadside population from the confirmed gly-R population given in meters; $y0$= lower asymptote, or theoretical minimum GR_{50}; a= midpoint, or distance at midpoint (y) and b= slope of the curve around a.

The simulated roadside management study with gly-R johnsongrass was arranged as a randomized complete block design with four replications in 2020, repeated in 2021. Data were combined across years where no significant interaction was observed between herbicide treatment and study year. The number of shoots emerged from rhizome nodes was subjected to logistic regression using PROC LOGISTIC in SAS Enterprise Guide 8.3. Single degree of freedom contrast statements were utilized to detect differences in the estimates for the probability of a rhizome node producing a shoot. Visual injury, the biomass of shoots produced from rhizomes, and rhizome NSC data were subjected to ANOVA using PROC GLM in SAS Enterprise Guide 8.3 after assessing normality & homoscedasticity using PROC UNIVARIATE. Shoot biomass data were rank

transformed (Conover & Iman, 1981) and for all data, significant means were separated using Tukey's Honestly Significant Difference (HSD).

RESULTS

Sensitivity of roadside populations to glyphosate. A generalized linear model confirmed treatment effect for all populations. In nonlinear regression, the dose-response models described the data well; R^2 values ranged from 0.7314 to 0.9958 (Table 4.2). When plants were treated with the lowest dose of glyphosate (590 g ha^{-1}), biomass responses ranged from 73 to 109% of non-treated controls. At the highest rate utilized (3,550 g ha^{-1}), biomass responses ranged from 11 to 53% of the non-treated control. A broad range of glyphosate sensitivity was observed across 26 roadside populations, with GR_{50} values ranging from 106 to 819 g ha^{-1} (Table 4.2). Populations 1, 4, and 13 were less susceptible to glyphosate (p <0.0001) than the reference susceptible population that was utilized as an internal control. This reference population had a GR_{50} value of 227 g ha^{-1}, which fell within the lower range of glyphosate sensitivity observed in the roadside populations. However, nine of the roadside populations demonstrated greater sensitivity to glyphosate than the reference population.

A single johnsongrass population was identified as an outlier when GR_{50} values were compared to the distance from the agricultural field in which the gly-R population was first isolated (Table 4.2). This population (13) was collected 6.3 km from the confirmed gly-R population, along the outer road of an interstate highway. Given its proximity to a high-volume traffic corridor, the GR_{50} estimate is not likely due to error, as movement may be facilitated by vehicle traffic. Models for both the full population (n=27) & partial (n=26; population 13 removed) are presented for comparison (Fig. 4.3).

In both cases, the distance of each roadside population from the agricultural field in which gly-R johnsongrass predicted the GR_{50} estimate better than the null model, with p= 0.0012 and p<0.0001 for the full and partial population models, respectively.

For the full population model, distance from the gly-R biotype predicted the GR_{50} within an average of ± 173 g ha^{-1} (Fig. 4.3). The full population model predicted influence from the gly-R biotype up to 458 m away (b= -0.03; unitless), and a minimum threshold GR_{50} of 328 g ha^{-1} (asymptote). When population 13 was removed from the dataset, the model predicted the population GR_{50} within an average of ± 125 g ha^{-1} (Fig. 4.3). The model for the partial population model predicted influence from the gly-R biotype up to 13,825 m away (b= -0.01) and the estimated minimum GR_{50} was reduced to 264 g ha^{-1}.

Simulated roadside management study with glyphosate-resistant johnsongrass. With visual injury responses, the primary symptom of herbicide injury observed was leaf reddening and/or purpling. Minor chlorosis was observed in response to glyphosate. In both years, visual injury was generally poor, and no plant death was observed prior to the first killing frost (Fig. 4.4). A highly significant interaction was observed between herbicide treatment and year of application (p < 0.0001). In 2020, across all treatments, mean visual injury did not exceed 15%. In 2020, 28 days elapsed between the herbicide application and the first killing frost.

In 2021, 43 days elapsed between the herbicide application and the first killing frost (Fig. 4.4). Injury symptoms progressed more rapidly, and overall injury responses were greater in 2021 than in 2020. The greatest plant injury was observed in response to aminocyclopyrachlor plus imazapyr plus indaziflam, with a mean of 53% injury. Injury

responses to sulfosulfuron, and foramsulfuron plus iodosulfuron plus theincarbazone were 43 and 36%, respectively. Treatments including clethodim, glyphosate or imazapic yielded no more than 30% injury prior to the first killing frost, although chlorosis in response to glyphosate was more prominent in 2021 than in 2020.

When spring rhizome viability was examined in the greenhouse, no year by treatment interaction was observed (p= 0.9526) for the number of shoots produced by rhizomes, and data were combined across years (Fig. 4.5). While herbicide treatment was significant (p= 0.0450), no differences were detected between treatments for the probability of a rhizome producing shoots. Rhizomes sampled from plants treated with imazapic or glyphosate plus imazapyr did not produce any shoots in 2020 or 2021. Similarly, rhizomes from plants treated with glyphosate plus imazapic produced only a single shoot across both years, indicating that treatments with imazapic or imazapyr were effective in reducing rhizome viability. The treatments clethodim, sulfosulfuron, foramsulfuron plus iodosulfuron plus thiencarbazone, and aminocyclopyrachlor plus imazapyr plus indaziflam performed comparably well, producing only 3 to 6 shoots across both years. The glyphosate resistance trait was apparent in that plants treated with glyphosate alone at 1,736 or 3,473 g ha^{-1} produced a total of 15 and 12 shoots, respectively. Rhizomes sampled from non-treated control plots produced 32 shoots in total.

No interaction was observed between the amount of biomass produced by rhizome and study year (p= 0.6409; Fig. 4.6). As rhizomes from plants treated with imazapic or glyphosate plus imazapyr did not produce shoots, these treatments were significantly different than non-treated controls, where mean biomass was 11.4 g. Shoot

biomass from plants treated with glyphosate at 1,736 or 3,473 g ha^{-1} had a mean of 7.0 and 8.4 g, respectively. While not rising to the level of statistical significance, greater differences were observed between the treatments clethodim, sulfosulfuron, foramsulfuron plus iodosulfuron plus thiencarbazone, and aminocyclopyrachlor plus imazapyr plus indaziflam. When rhizomes were sampled from plants treated with sulfosulfuron, mean shoot biomass was 0.7 g, whereas mean biomass from clethodim, foramsulfuron plus iodosulfuron plus thiencarbazone, or aminocyclopyrachlor plus imazapyr plus indaziflam ranged from 1.6 to 2.5 g.

For total rhizome NSC, the effects of plant tillers, study year, and herbicide treatment were all nonsignificant, with p= 0.3070 for herbicide treatment (Fig. 4.6). Across both years, rhizomes from non-treated controls had a mean of 17.0% NSC content. Only rhizomes sampled from plants treated with imazapic or imazapyr plus glyphosate had less NSC content than controls, with means of 15.7 and 13.3% for imazapic plus glyphosate and imazapyr plus glyphosate. Rhizomes sampled from plants treated with glyphosate at either 1,736 or 3,473 g ha^{-1} were comparable to those from non-treated controls, with means of 17.5 and 17.1%, respectively. Rhizomes from plants not treated with glyphosate had consistently higher NSC content, ranging from 17.9 to 23.3% NSC content.

DISCUSSION

Sensitivity of roadside populations to glyphosate. The distance-decay models for the full and reduced populations may illustrate the signficance of vector-assisted dispersal. Vehicles on the roadway can themselves be vectors of seed dispersal, as can

equipment transported along roadways or utilized in roadside mowing (Clifford, 1959; McCanny & Cavers, 1988; Strykstra et al., 1997). In Australia, human activities assisted with the dispersal of 204 of 233 noxious weed species examined (Panetta & Scanlan, 1995). Although the full population sample size was small, a single johnsongrass population collected from a high traffic volume interstate accounted for 28% of error in the model (S=172.6 g ha^{-1}). However, when this population was removed from the dataset, a suprising element of the resulting model was the significant decrease in slope that estimated influence from the gly-R population up to 13.8 km away (Fig. 4.4). Studies of plant dispersal at this scale are limited. Johnsongrass patches expanding via vegetation reproduction from rhizomes has been estimated at a rate of only 1-2 m per year (Horowitz, 1973). Seed dispersal occurs primarily through seed rain, and field studies have found that the overwhelming majority of seeds (≥98%) fall within 10 m of the parent plant. However, when seed movement by tillage equipment was included in field studies of seed dispersal, researchers documented the transport of seeds up to 1.8 km from the source (Mayer et al., 2002). In this study, the prediction of influence from the gly-R population up to 13.8 km away likely results from a lack of model precision (S= 125.1), but could also arise from movement of the trait for glyphosate resistance.

From 1990 to 2014, in the United States alone, over 1.1 billion kg of glyphosate as the active ingredient was applied (Benbrook, 2016). During this time, agriculture's share of glyphosate use far exceeded that which was utilized in non-agricultural settings. Agricultural use accounted for 87.8% of the total amount of glyphosate applied between 1990 and 2014, with soybean production alone responsible for 31.5% (Benbrook, 2016).

From this perspective, one must conclude that the selection pressure imposed by glyphosate on weed species has largely occurred within cropland.

Still, non-crop applicators are not entirely inculpable. Since the beginning of glyphosate manufacturing in 1974, over 63.5 million kg of glyphosate has been applied in non-agricultural settings (Benbrook, 2016). While it is difficult to know what portion of that use was applied to roadsides, an abundance of publications directing roadside management practices can be found proposing the use of glyphosate (Johnson et al., 2010; McCullough & Shilling, 2020; Meyer et al., 1995; Miller & Middlebrooks, 1987).

Several criteria must be met in order to confirm the evolution of herbicide resistance in a new species or biotype, perhaps the most critical of these being that a biotype meets the definition of herbicide resistance. A scientific definition, in combination with an agricultural field definition, are the parameters that both the Weed Science Society of America and the Global Herbicide Resistance Action Committee utilize to define herbicide resistance (Heap, 2022). The scientific definition of herbicide resistance is given as "*a genetically inherited statistical difference in herbicide response between two weed populations of the same species*", while the agricultural field definition requires that the species or biotype "*must survive the recommended rate of herbicide under normal field conditions*" (Heap, 2022). The methods utilized in this study to characterize the sensitivity of roadside populations of johnsongrass to glyphosate are the same as those that can be utilized to confirm the evolution of herbicide resistance. However, in order to confirm glyphosate resistance in roadside populations of johnsongrass, especially at the levels observed in more tolerant populations, we would advocate for additional data generated from repeated experiments. Alternatively, the

inclusion of an alternate method, such as dose-response study on plants propagated from seed, or quantification of population survival, would better support a claim of evolved resistance (Beckie et al., 2000; Seefeldt et al., 1995).

Heritability of herbicide resistance is another important criterion utilized in determining when a population has evolved resistance (Heap, 2022). Plants arising from vegetative propagules are genetically identical to the parent. With sexual reproduction, inheritance of the trait for herbicide resistance has been demonstrated in johnsongrass. In 2022, a biotype with resistance to acetolactate synthase (ALS)-inhibiting herbicides was transplanted to serve as a pollen donor in a field study with ALS-sensitive johnsongrass (Maity et al., 2022). In this instance, ALS resistance was due to a single point mutation ($Trp_{574}Leu$) that could be confirmed in hybrid seedlings resulting from cross-pollination of the ALS resistant and sensitive biotypes. The authors found rates of pollen-mediated gene flow (PMGF) ranging from 0.8 to 8.7% at 50 m from the pollen source, when PMGF was evaluated as the number of hybrid offspring surviving a discriminate herbicide dose (Maity et al., 2022).

Quantifying the segregation of a glyphosate resistance trait dispersing in johnsongrass populations would be complicated by several factors. Foremost, the current absence of a single genetic marker for glyphosate resistance in johnsongrass. While mutations in the target site (gene encoding *EPSPS*) that confer resistance to glyphosate have been observed in other weedy grass species, they have yet to be identified in johnsongrass (Alarcón-Reverte et al., 2014; de Carvalho et al., 2012; Ngo et al., 2016; Riar et al., 2011; Vázquez-Garcia et al., 2020; Vila-Aiub et al., 2012; Yu et al., 2015).

The genetic basis of other non-target site resistance (NTSR) mechanisms to glyphosate are not due to point mutations; rather, they have been shown to result from gene amplification and/or duplication (Malone et al., 2016; Pan et al., 2019; Pan et al., 2021; Salas et al., 2012). While johnsongrass is capable of both self- and cross-pollination, its reproductive biology favors outcrossing (Kaur & Soodan, 2017). Genetic mapping of johnsongrass affirms this theory, as broad introgression in the johnsongrass genome resulting from crossing with both wild and cultivated *Sorghum bicolor* has been identified (Paterson et al., 2020). Further complicating matters, the occurrence of more than one glyphosate resistance mechanism has frequently been documented. Within a gly-R population of *Digitaria insularis*, for example, differential herbicide translocation, enhanced glyphosate metabolism, and point mutations in the target site were all identified as mechanisms contributing to glyphosate resistance (de Carvalho et al., 2012). Regardless of how the trait for glyphosate resistance may move with roadside populations, the range of glyphosate sensitivity determined here provides sufficient evidence to inform management decisions.

Simulated roadside management study. Perennial grasses transport photoassimilates to roots in the fall, thus making fall-applied systemic herbicides particularly effective. Many herbicides translocate readily in the phloem, and herbicide movement to meristems in perennial root organs can prevent reemergence in the spring (Fick & Moser 1978; Singh & Coleman 1974; Sosebee & Wiebe 1973). However, seasonal patterns of plant senescence and dormancy may mask apparent injury to mature plants, leading to erroneous conclusions regarding herbicide efficacy. For johnsongrass, **e**stimates of spring rhizome viability likely represent the most accurate assessment of

herbicide efficacy (Anderson et al., 1960). The greenhouse assay provides the most direct estimation of potential spring regrowth, and was chosen to ensure no encroachment of rhizomes from neighboring plots.

The response of johnsongrass rhizomes to treatments containing imazapic or imazapyr suggests that multiple resistance to both glyphosate and ALS-inhibiting herbicides was not present in the gly-R biotype(s), although three cases of multiple herbicide resistance in johnsongrass have been reported (Heap, 2022; Malidža & Rajković, 2018). Similarly, there was insufficient evidence for multiple resistance to acetyl CoA carboxylase (ACCase)-inhibiting herbicides. While rhizomes sampled from plants treated with clethodim produced more shoots than treatments with ALS-inhibiting herbicides, the total number of shoots produced was 56% less than rhizomes from plants treated with glyphosate.

The individual herbicides imazapic and sulfosulfuron have been shown to result in johnsongrass control in field experiments previously, though data on fall applications to mature stands is limited. Gricher et al. (2008) reported ≥ 90% johnsongrass control from 140 g ai ha^{-1} of imazapic when this rate was applied to johnsongrass ≤ 25 cm in height. In a greenhouse study, Ferrell et al. (2003) determined that regrowth from rhizomatous johnsongrass was reduced 99% from controls when imazapic was applied at 70 g ai ha^{-1} to plants ≤ 40 cm in height. Despite differences in plant size, maturity, and herbicide rate, the results of the rhizome viability assay for plants treated with imazapic agree with the findings of these studies. With sulfosulfuron, when 70 g ai ha^{-1} was applied to johnsongrass 0.5 to 1 m in height, Wright (2006) reported up to 74% johnsongrass control, though regrowth was not examined. Only four shoots were

produced by rhizomes sampled from plants treated with 105 g ai ha^{-1} of sulfosulron in this experiment, which is consistent with good, albeit incomplete control.

The herbicide combinations foramsulfuron plus iodosulfuron plus theincarbazone and aminocyclopyrachlor plus imazapyr plus indaziflam are pre-mixed and marketed as Derigo® and Plainview™ SC, respectively. In this study, the maximum labeled rate of Derigo® was applied to 1.5 m johnsongrass stands (Bayer, 2014). Comparison studies are limited, but in a field experiment with maize (*Zea mays*), the application of foramsulfuron (45 g) and iodosulfuron (1.5 g) plus isoxadifen-ethyl (2 kg) to johnsongrass 25 to 30 cm in height provided 95% control up to 5 WAT (Torma et al., 2006). With aminocyclopyrachlor plus imazapyr plus indaziflam, only imazapyr has appreciable activity on johnsongrass (Shaner, 2014). Rhizomes from plants treated with imazapyr at 424 g in combination with aminocyclopyrachlor plus indaziflam produced a total of 5 shoots over 2 years. Compared to the zero shoots produced from rhizomes sampled from plants treated with imazapyr at 841 g in combination with glyphosate at 868 g, rhizome viability appeared quite responsive to imazapyr at 424 g when in combination with aminocyclopyrachlor plus indaziflam.

After shoots emerged from rhizome, the development of aerial biomass was no longer strictly a function of rhizome carbohydrate reserves. When plants emerging from rhizome are grown in the dark, etiolated shoots are positively correlated with total NSC (Dovrat et al., 1971; Mitchell et al., 1998). While this approach may have resulted in a stronger correlation between shoot biomass and rhizome NSC data in this experiment, light-grown shoots better approximate expected outcomes in field conditions.

An earlier study also examined the response of johnsongrass rhizome NSC to ALS-inhibiting herbicides such as sulfosulfuron, imazapic, and imazapyr. In a greenhouse study, Johnson et al. (2003) found that rhizomes harvested 3 WAT had total NSC content ranging from 10 to 28% w/w, a range consistent with that observed in this study. Also similar to the results obtained from this field study, Johnson et al. (2003) found inconsistent responses of rhizome NSC in relation to shoot regrowth potential for ALS-inhibiting herbicides. At 3 WAT, total NSC in rhizomes from plants treated with primisulfuron at 40 g ha^{-1} were reduced 32% from the means of the non-treated control. However, rhizomes from plants treated with primisulfuron had the greatest regrowth potential when estimated as shoot biomass 3 weeks after cutting. Herbicide mechanism of action, rather than individual treatments, may best explain the response of total rhizome NSC.

The ALS-inhibiting herbicides interrupt the synthesis of branched-chain amino acids, lower the total free amino acid pool, and interfere with protein metabolism (Zhou et al., 2007). Downstream effects include slowed cell division, the induction of fermentative respiration, and reduced translocation of photoassimilates (Zhou et al., 2007). By supplying *Thlaspi arvense* (L.) seedlings with $^{14}CO_2$, Bestman et al. (1990) found that the application of chlorsulfuron did not reduce net carbon assimilation, but did reduce translocation out of source leaves, resulting in leaf sucrose concentrations at nearly 2.5 times that of controls. Similarly, Royuela et al. (2000) found that sucrose and starch accumulated in the roots of pea plants (*Pisum sativum* L.) in a dose-dependent manner when plants were supplied with imazethapyr. While increased NSC content in

plant organs appears indicative of treatment with ALS-inhibiting herbicides, it likely does accurately reflect toxicity of the herbicide.

Another mode of action employed in this field study is the inhibition of ACCase. ACCase inhibitors such as clethodim target lipid synthesis in plants (Shaner, 2014). In sensitive plants, fatty acid synthesis is inhibited by ≥ 90% (Burton et al., 1989). Reductions in lipid and protein content have been observed in response to ACCase inhibitors (Abdel-Wahab et al., 2021). While pinoxaden is selective for grass weeds in wheat (*Triticum aestivum*), a recent study revealed that the carbohydrate content of wheat plants increased up to 4% following herbicide application (Abdel-Wahab et al., 2021). In this study, the NSC content of rhizomes from sensitive plants treated with clethodim increased 14%. However, the number of shoots produced from rhizome following treatment with clethodim was reduced 81% compared to non-treated controls. Similar to the ALS-inhibiting herbicides, the NSC content of rhizomes following treatment with clethodim does not appear to reflect toxicity of the herbicide.

Dysregulation of the shikimic acid pathway resulting from treatment with glyphosate interrupts normal carbon assimilation processes. With this biotype of gly-R johnsongrass, the response of total rhizome NSC suggests that *EPSPS* may still inhibited to some extent, as the only two treatments to reduce total rhizome NSC from controls both contained glyphosate. However, this was not reflected in biomass production by rhizomes sampled from plants treated with glyphosate. Biomass from plants treated with glyphosate was reduced only 27 to 39% from non-treated controls, indicating that the NSC content of gly-R rhizomes was an unreliable predictor of vegetatively-propagated plant vigor.

The two treatments containing glyphosate in this study were employed to demonstrate its lack of efficacy on this johnsongrass population, as the use of glyphosate to control glyphosate-resistant weeds is not recommended. Similarly, caution should be exercised in utilizing ALS-inhibiting herbicides alone to control gly-R johnsongrass, as the number of ALS resistant johnsongrass populations is nearly twice that of glyphosate resistant accessions (Heap, 2022). If chemical control is desired, roadside vegetation managers should recall that the application of more than one herbicide mode of action has been shown to reduce the likelihood of field-evolved herbicide resistance in weed species (Beckie & Reboud, 2009). In addition, seeding pasture grasses can reduce johnsongrass pressure (McWhorter, 1989). Repeated mowing reduces rhizome growth, and properly timed mowing can prevent seed set and dispersal (Warwick & Black, 1983). A combination of chemical, mechanical, and cultural practices is likely to be the most successful practice to limit the tolerance of roadside johnsongrass to glyphosate.

LITERATURE CITED

Alarcón-Reverte, R, A García, SB Watson, I Abdallah, S Sabaté, MJ Hernández, FE Dayan and AJ Fischer. 2014. Concerted action of target-site mutations and high *EPSPS* activity in glyphosate-resistant junglerice (*Echinocloa colona*) from California. Pest Manag. Sci. 71: 996–1007.

Abdel-Wahab, SIZ, AAA Aioub, REME Salem and AEA El-Sobki. 2021. Do the herbicides pinoxaden, tribenuron-methyl, and pyroxsulam influence wheat (*Triticum aestivum* L.) physiological parameters?. Environ. Sci. Pollut. Res. 28 (37): 51961-51970.

Anderson, LE, AP Appleby and JW Weseloh. 1960. Characteristics of johnsongrass rhizomes. Weeds. 8(3): 402-406.

Bayer CropScience. 2014. Derigo herbicide label. EPA Reg. No. 432-1533. St. Louis, Missouri, USA.

Beckie, HJ, IM Heap, RJ Smeda and LM Hall. 2000. Screening for herbicide resistance in weeds. Weed Technol. 14(2): 428-445.

Beckie, HJ and X Reboud. 2009. Selecting for weed resistance: herbicide rotation and mixture. Weed Technol. 23(3): 363-370.

Benbrook, CM. 2016. Trends in glyphosate herbicide use in the United States and globally. Environ. Sci. Eur. 28(1): 1-15.

Bestman, HD, MD Devine and WH Vanden Born. 1990. Herbicide chlorsulfuron decreases assimilate transport out of treated leaves of field pennycress (*Thlaspi arvense* L.) seedlings. Plant Physiol. 93(4): 1441-1448.

Burton, JD, JW Gronwald, DA Somers, BG Gengenbach and DL Wyse. 1989. Inhibition of corn acetyl-CoA carboxylase by cyclohexanedione and aryloxyphenoxy-propionate herbicides. Pestic. Biochem. Physiol. 34(1): 76-85.

Clifford, HT. 1959. Seed dispersal by motor vehicles. J. Ecol. 47(2): 311-315.

Conover, WJ and RL Iman. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. Am. Stat. 35(3): 124-129.

de Carvalho, LB, PL Da Costa Aguiar Alves, F Gonzalez-Torralva, HE Cruz-Hipolito, AM Rojano-Delgado, R De Prado, J Gil-Humanes, F Barro, and MD Luque de Castro. 2012. Pool of resistance mechanisms to glyphosate in *Digitaria insularis*. J. Agric. Food Chem. 60(2): 615-622.

Disque, EA. 1959. Selective cutting of roadside vegetation for improved highway safety, appearance and use. Highway Research Board. 43:42. Available <https://trid.trb.org/view/1178546>

Dovrat, A, GP Dirven and B Dienum. 1971. The influence of defoliation and nitrogen on the regrowth of Rhodes grass (*Chloris gayana* Kunth). 1. Dry matter production and tillering. Neth. J. Agri. Sci. 19(2): 94-101.

Duke, SO and SB Powles. 2008. Glyphosate: a once-in-a-century herbicide. Pest Manag. Sci. 64(4): 319-325.

Ferrell, JA, HJ Earl and WK Vencill. 2003. The effect of selected herbicides on CO_2 assimilation, chlorophyll fluorescence, and stomatal conductance in johnsongrass (*Sorghum halepense* L). Weed Sci. 51 (1): 28-31.

Fick, WH and LE Moser. 1978. ^{14}Carbon translocation in three warm-season grasses as affected by stage of development. J. Range Manag. 50.

Gade, KJ. 2013. Freeways as corridors for plant dispersal: a case study from central Arizona. Proc. 2013 ICOET. Scottsdale, Arizona, USA.

Ghersa, CM, MA Martinez-Ghersa, EH Satorre, ML Van Esso and G Chichotky. 1993. Seed dispersal, distribution and recruitment of seedlings of *Sorghum halepense* (L.) Pers. Weed Res. 33: 79–88.

Grichar, WJ, PA Baumann, TA Baughman and JD Nerada. 2008. Weed control and bermudagrass tolerance to imazapic plus 2, 4-D. Weed Technol. 22(1): 97-100.

Heap, I and SO Duke. 2018. Overview of glyphosate-resistant weeds worldwide. Pest Manag. Sci. 74(5):1040-1049.

Heap, I. 2022. The international herbicide-resistant weed database. Available <www.weedscience.org>

Horowitz, M. 1973. Spatial growth of *Sorghum halepense* (L.) Pers. Weed Res. 13(2): 200-208.

Johnson, WG, J Li and JD Wait. 2003. Johnsongrass control, total nonstructural carbohydrates in rhizomes, and regrowth after application of herbicides used in herbicide-resistant corn (*Zea mays*). Weed Technol. 17(1): 36-41.

Johnson, JM, KL Lloyd, JC Sellmer and AE Gover. 2010. Roadside vegetation management research - 2010 report. Pennsylvania Department of Transportation. <https://trid.trb.org/view/924829>

Kaur, R and AS Soodan. 2017. Reproductive biology of *Sorghum halepense* (L.) Pers. (Poaceae; Panicoideae; Andropogoneae) in relation to invasibility. Flora. 229: 32-49.

Klein, P and CM Smith. 2021. Invasive johnsongrass, a threat to native grasslands and agriculture. Biologia. 76(2): 413-420.

Kniss AR. 2018. Genetically engineered herbicide-resistant crops and herbicide-resistant weed evolution in the United States. Weed Sci. 66(2): 260-273.

Kong, W, C Kim, VH Goff, D Zhang and AH Paterson. 2015. Genetic analysis of rhizomatousness and its relationship with vegetative branching of *Sorghum bicolor* × S. *propinquum* recombinant inbred lines. Am. J. Bot. 102: 718–724.

Landhäusser, SM, PS Chow, LT Dickman, ME Furze, I Kuhlman, S Schmid, J Wiesenbauer, B Wild, G Gleixner, H Hartmann and G Hoch. 2018. Standardized protocols and procedures can precisely and accurately quantify non-structural carbohydrates. Tree Physiol. 38(12): 1764-1778.

Maity, A, B Young, N Subramanian and M Bagavathiannan. 2022. Pollen-mediated transfer of herbicide resistance between johnsongrass (*Sorghum halepense*) biotypes. Sci. Rep. 12(1): 1-13.

Malidža, G and M Rajković. 2018. First case of multiple resistance of Johnsongrass (*Sorghum halepense* (L.) Pers.) to ALS- and ACCase-inhibiting herbicides in Serbia. Proc. Perspect. Chall. Weed Control Weed Resist. Herbicide Eur. 29–30 Nov.

Malone, JM, S Morran, N Shirley, P Boutsalis and C Preston. 2016. *EPSPS* gene amplification in glyphosate resistant *Bromus diandrus*. Pest Manag. Sci. 72: 81-88.

Marshall, EJP. 1989. Distribution patterns of plants associated with arable field edges. J. Appl. Ecol. (1): 247-257.

Marshall, EJP. 2004. Agricultural landscapes: field margin habitats and their interaction with crop production. J. Crop Improv. 12(1): 365-404.

Martin, DL, LM Cargill and DP Montgomery. 1996. Roadside vegetation management. Oklahoma Department of Transportation. Available <https://trid.trb.org/view/478526>

Mayer, F, H Albrecht and J Pfadenhauer. 2002. Secondary dispersal of seeds in the soil seed bank by cultivation. JDPD. 18: 551-560.

McCanny, SJ and PB Cavers. 1988. Spread of proso millet (*Panicum miliaceum* L.) in Ontario, Canada. II. Dispersal by combines. Weed Res. 28(2): 67-72.

McCullough, P and D Shilling. 2020. Enhancing extension recommendations for improving herbicide resistance management on Georgia roadsides. Georgia Department of Transportation. Available <https://trid.trb.org/view/1722500>

McWhorter, CG. 1989. History, biology, and control of johnsongrass. In: CL Foy (Ed.). Reviews of weed science (4). Champaign, IL, USA: Weed Science Society of America.

Meyer, RE, CL Benner and WG McCully. 1995. Management of vegetation on the pavement edge and adjacent shoulder. Interim report. Texas Department of Transportation. Available <https://trid.trb.org/view/447692>

Miller, JF and PB Middlebrooks. 1987. Herbicides for highway use. Final report – phase III. Georgia Department of Transportation. Available <https://trid.trb.org/view/285744>

Mitchell, AR, EA Rechel and RL Dovel. 1998. Three methods for determining storage carbohydrate concentration in peppermint (*Mentha piperita*) rhizomes. HortSci. 33(4): 754-756.

Mitskas, MB, CE Tsolis, IG Eleftherohorinos and CA Damalas. 2003. Interference between corn and johnsongrass (*Sorghum halepense*) from seed or rhizomes. Weed Sci. 51(4): 540-545.

Monaghan, N. 1979. The biology of Johnson grass (*Sorghum halepense*). Weed Res. 19(4): 261-267.

Montgomery, DP, CC Evans, and DL Martin. 2006. Roadside vegetation management guidelines. Oklahoma Department of Transportation. 50 p.

Motulsky, HJ and RE Brown. 2006. Detecting outliers when fitting data with nonlinear regression–a new method based on robust nonlinear regression and the false discovery rate. BMC Bioinform. 7(1): 1-20.

National Cooperative Highway Research Program (NCHRP), American Association of State Highway and Transportation Officials. Tools and technology for roadside landscape asset management. Available <https://trid.trb.org/view/1854196>

Ngo, TD, M Krishnan, P Boutsalis, G Gill and C Preston. 2016. Target-site mutations conferring resistance to glyphosate in feathertop Rhodes grass (*Chloris virgata*) populations in Australia. Pest Manag. Sci. 74(5): 1094-1100.

Pan, Lang, Q Yu, H Han, L Mao, A Nyporko, L Fan, L Bai and SB Powles. 2019. Aldo-keto reductase metabolizes glyphosate and confers glyphosate resistance in *Echinochloa colona*. Plant Physiol. 181(4): 1519-1534.

Pan, Lang, Q Yu, J Wang, H Han, L Mao, A Nyporko, A Maguza, L Fan, L Bai and SB Powles. 2021. An ABCC-type transporter endowing glyphosate resistance in plants. PNAS. 118(16): e2100136118.

Panetta, FD and JC Scanlan. 1995. Human involvement in the spread of noxious weeds: what plants should be declared and when should control be enforced? Plant Prot. Q. 10: 69.

Paterson, AH, W Kong, RM Johnston, P Nabukalu, G Wu, WL Poehlman, VH Goff, K Isaacs, TH Lee, H Guo and D Zhang. 2020. The evolution of an invasive plant, *Sorghum halepense* L. ('Johnsongrass'). Front. Genet. 11: 317.

Pratley, J et al. 1999. Resistance to glyphosate in *Lolium rigidum*. I. Bioevaluation. Weed Sci. 47: 405–411.

Riar, DS, JK Norsworthy, DB Johnson, RC Scott and M Bagavathiannan. 2011. Glyphosate resistance in a johnsongrass (*Sorghum halepense*) biotype from Arkansas. Weed Sci. 59(3): 299-304.

Royuela, M, A Gonzalez, EM Gonzalez, C Arrese-Igor, PM Aparicio-Tejo and C Gonzalez-Murua. 2000. Physiological consequences of continuous, sublethal imazethapyr supply to pea plants. J. Plant Physiol. 157(3): 345-354.

Salas, RA, FE Dayan, Z Pan, SB Watson, JW Dickson, RC Scott and NR Burgos. 2012. *EPSPS* gene amplification in glyphosate-resistant Italian ryegrass (*Lolium perenne* spp. *multiflorum*) from Arkansas. Pest Manag. Sci. 68: 1223-1230.

Seefeldt, SS, JE Jensen and EP Fuerst. 1995. Log-logistic analysis of herbicide dose-response relationships. Weed Technol. 9(2): 218-227.

Sezen, UU, JN Barney, DZ Atwater, GA Pederson, JF Pederson, JM Chandler, TS Cox, S Cox, P Dotray, D Kopec and SE Smith. 2016. Multi-phase US spread and habitat expansion of a post-Columbian invasive, *Sorghum halepense*. PLoS One. 11: e01644584.

Shaner, DL. 2014. Herbicide handbook. Lawrence, KS, USA: Weed Science Society of America.

Singh, JS and DC Coleman. 1974. Distribution of photo-assimilated carbon in the root system of a shortgrass prairie. J. Ecol. (1): 359-365.

Sosebee, RE and HH Wiebe. 1973. Effect of phenological development on radiophosphorus translocation from leaves in crested wheatgrass. Oecologia. 13(2): 103-112.

Strykstra, RJ, GL Verweij and JP Bakker. 1997. Seed dispersal by mowing machinery in a Dutch brook valley system. Acta Bot. Neerl. 46(4): 387-401.

Tang, HV and GH Liang. 1988. The genomic relationship between cultivated sorghum [*Sorghum bicolor* (L.) Moench] and Johnsongrass [S. *halepense* (L.) Pers.]: a re-evaluation. Theoret. Appl. Genetics. 76: 277–284.

Torma, M, G Kazinczi and L Hódi. 2006. Postemergence herbicide treatments in maize against difficult to control weeds in Hungary. J. Plant Dis. Prot. (20): 781.

Vázquez-Garcia, JG, C Palma-Bautista, AM Rojano-Delgado, R De Prado and J Menendez. 2020. The first case of glyphosate resistance in johnsongrass (*Sorghum halepense* [L.] Pers.) in Europe. Plants. 9(3): 313.

Vila-Aiub, MM, MC Balbi, AJ Distéfano, L Fernández, E Hopp, Q Yu and SB Powles. 2012. Glyphosate resistance in perennial *Sorghum halepense* (Johnsongrass), endowed by reduced glyphosate translocation and leaf uptake. Pest Manag. Sci. 68(3): 430-436.

Vilà, M and J Pujadas. 2001. Land-use and socio-economic correlates of plant invasions in European and North African countries. Biol. Conserv. 100(3): 397-401.

Warwick, SI and LD Black. 1983. The biology of Canadian weeds: 61. *Sorghum halepense* (L.) Pers. Can. J. Plant Sci. 63(4): 997-1014.

Williams, CS and RM Hayes. 1984. Johnsongrass (*Sorghum halepense*) competition in soybeans (*Glycine max*). Weed Sci. 32(4): 498-501.

Wilson, PJ and NJ Aebischer. 1995. The distribution of dicotyledonous arable weeds in relation to distance from the field edge. J. Appl. Ecol. (1): 295-310.

Wright, RS. 2006. Determining factors affecting antagonism of sulfosulfuron tank-mixed with glyphosate for control of johnsongrass [Master's thesis, Mississippi State University]. ProQuest Dissertations Publishing.

Yu, Q, A Jalaludin, H Han, M Chen, RD Sammons and SB Powles. 2015. Evolution of a double amino acid substitution in the 5-enolpyruvylshikimate-3-phosphate synthase in *Eleusine indica* conferring high-level glyphosate resistance. Plant Physiol. 167(4): 1440-1447.

Zhou, Q, W Liu, Y Zhang and KK Liu. 2007. Action mechanisms of acetolactate synthase-inhibiting herbicides. Pestic. Biochem. Phys. 89(2): 89-96.

Table 4.1. Herbicide treatments applied in a simulated roadside johnsongrass management study conducted in the fall of 2020 and 2021 in Boone County, MO. Herbicides were applied to johnsongrass stands approximately 1.5 m in height on 18 Sept. 2020 and 20 Sept. 2021. The experiment was a randomized complete block design, repeated. Herbicides were applied by a CO_2-pressurized backpack sprayer calibrated to deliver 374 L ha^{-1} through four TeeJet 8004 flat-fan nozzles. Abbreviations are utilized in following figures to identify herbicide treatments.

Herbicide treatments applied to glyphosate-resistant johnsongrass

Treatment	Active ingredient(s)	Rate (g ai ha^{-1})	Adjuvant(s)	Adjuvant rate
NTC	Untreated control	-	-	-
AMINO+IMAZAPYR+INDAZ	Aminocyclopyrachlor	140	MSO	1% v/v
	Imazapyr	424		
	Indaziflam	50		
CLETH	Clethodim	272	MSO	1% v/v
FORAM+IODO+THIEN	Foramsulfuron	101	MSO	1% v/v
	Iodosulfuron-methyl	10		
	Thiencarbazone-methyl	42		
GLY (1736 g)	Glyphosate	1,736	AMS	20 g L^{-1}
			MSO	1% v/v
GLY (3473 g)	Glyphosate	3,473	AMS	
			MSO	1% v/v
GLY+IMAZAPIC	Glyphosate	868	AMS	20 g L^{-1}
	Imazapic	210	MSO	1% v/v
GLY+IMAZAPYR	Glyphosate	868	AMS	20 g L^{-1}
	Imazapyr	841	MSO	1% v/v
IMAZAPIC	Imazapic	210	MSO	1% v/v
SULFO	Sulfosulfuron	105	MSO	1% v/v

Table 4.2. Glyphosate dose-response model parameters for roadside johnsongrass populations. Single johnsongrass plants vegetatively propagated from rhizomes collected on roadsides in Buchanan County, MO were treated with glyphosate using a greenhouse track sprayer calibrated to deliver 140 L ha^{-1} in a single experiment with a randomized complete block design. Parameters for the model Y=C+(D-C) / 1+(X/GR_{50}^b) (where y= biomass data transformed as a percent of control within replication; C= mean response at highest dose; D=mean response at lowest dose, x= glyphosate as g ae ha^{-1}; and GR_{50} = growth reduction, or 50% reduction in the amount of aboveground biomass; and b describing the slope of the line around the GR_{50}) were estimated from the means of a minimum of six replications using GraphPad Prism 9.

Glyphosate dose-response studies on roadside johnsongrass collections					
	Model parameters				Model fit
Population	Bottom *[% of control]*	Top *[% of control]*	Slope *[unitless]*	GR_{50} *[g ae ha^{-1}]*	R^2
1	48.1	84.3	-2.13	555	0.934
2	41.2	109.0	-1.01	408	0.912
3	48.1	90.8	-1.05	265	0.883
4	41.9	104.6	-1.96	480	0.951
5	23.9	73.2	-1.07	186	0.823
6	37.9	90.3	-0.97	288	0.900
7	53.3	109.2	-2.45	698	0.902
8	30.9	99.6	-1.83	339	0.996
9	26.2	82.8	-1.15	211	0.902
10	51.1	91.0	-1.09	296	0.919
11	41.3	86.9	-1.01	242	0.830
12	33.6	106.3	-1.47	203	0.987
13	36.6	104.0	-1.25	819	0.935
14	32.9	102.6	-1.90	173	0.973
15	41.0	89.9	-1.00	309	0.731
16	22.8	84.6	-1.26	311	0.938
17	43.2	101.7	-1.67	169	0.977
18	49.0	107.4	-2.01	106	0.868
19	29.4	82.6	-1.15	202	0.891
20	48.1	100.3	-1.84	249	0.985
21	34.1	100.6	-1.81	251	0.990
22	30.2	80.4	-3.54	505	0.875
23	30.1	90.1	-1.72	283	0.956
24	31.2	85.1	-1.10	224	0.905
25	11.6	87.9	-0.76	443	0.959
26	32.9	85.2	-1.08	220	0.877

Fig. 4.1. Photo of glyphosate-resistant johnsongrass plots in a simulated roadside management study conducted in Boone County, MO in 2020 and 2021. Herbicides were applied to johnsongrass stands approximately 1.5 m in height on 18 Sept. 2020 and 20 Sept. 2021. The experiment was a randomized complete block design, repeated. Photo taken on 9 Nov. 2021.

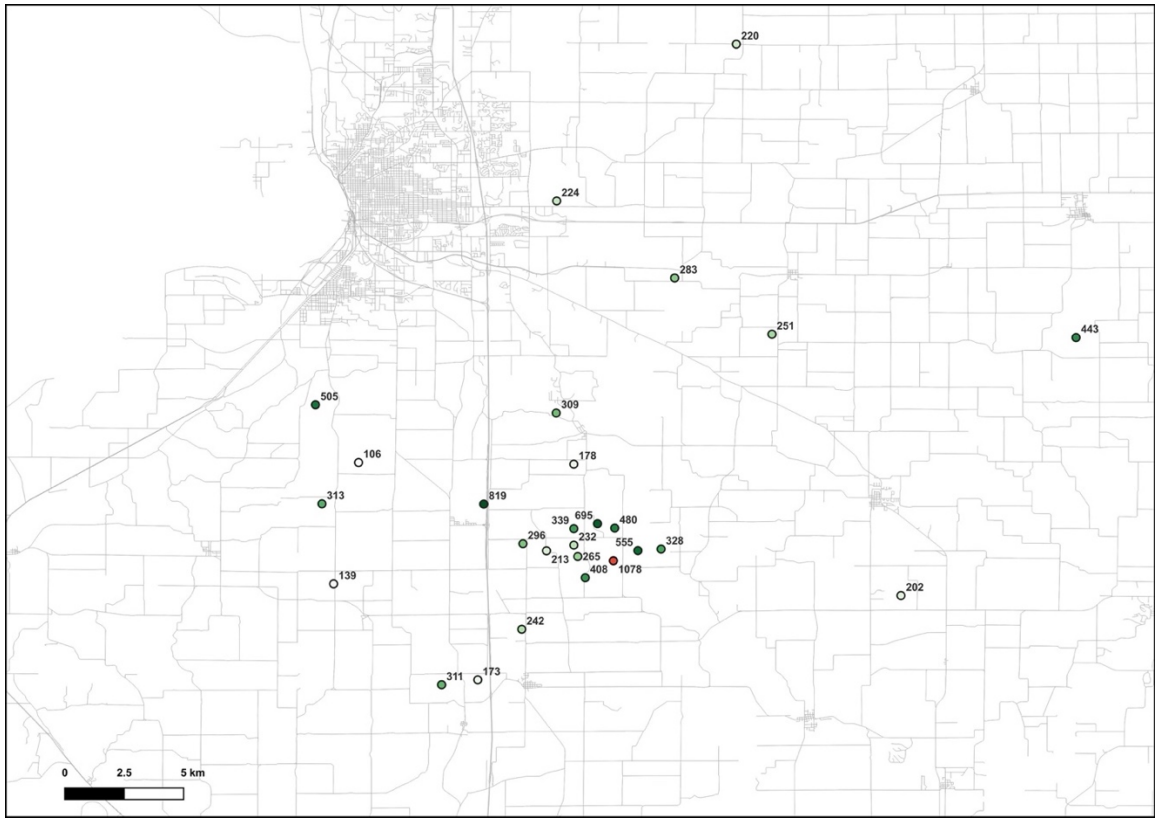


Fig. 4.2. Map of roadside johnsongrass populations collected from Buchanan County, MO in October of 2020 and 2021. Johnsongrass populations were vegetatively propagated and subjected to glyphosate dose-response studies to estimate sensitivity to glyphosate. Pale- to dark-green circles indicate the location of rhizomes collected from roadsides recorded by handheld GPS device (±10 m error). The red-orange circle indicates the confirmed glyphosate-resistant johnsongrass population collected from an agronomic field in 2019. Data labels are GR_{50} values, or glyphosate dose required to reduce aboveground biomass by 50%, given as grams acid equivalent per hectare. Gray lines indicate public roadways as described by the Missouri Department of Transportation (data obtained from Missouri Spatial Data Information Service, May 2022).

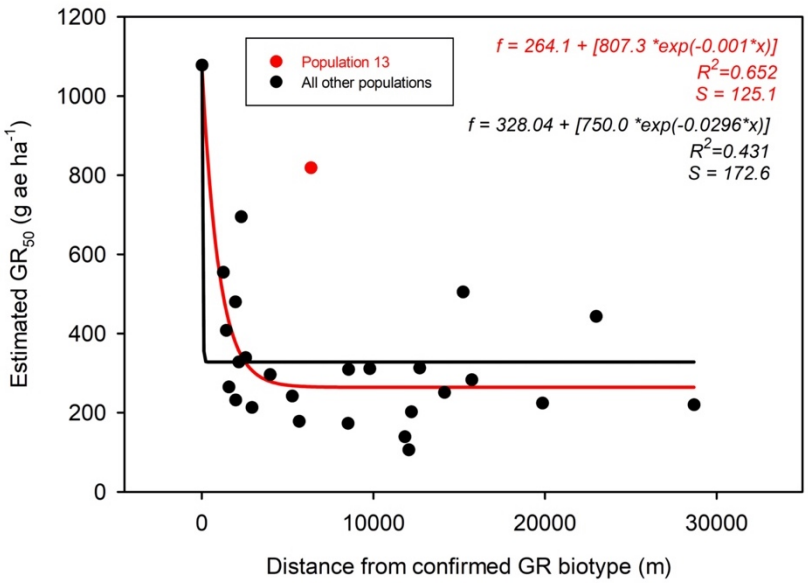


Fig. 4.3. Distance-decay models applied to data on the sensitivity of roadside johnsongrass populations in Buchanan County, MO to glyphosate and the distance of each population from a confirmed glyphosate-resistant biotype. Sensitivity to glyphosate was estimated from dose-response studies as the GR_{50}, or glyphosate dose required to reduce aboveground biomass by 50%, given as grams acid equivalent per hectare on the y-axis. Distance on the x-axis is given in meters and refers to the distance of each roadside population from a confirmed glyphosate-resistant biotype collected from an agronomic field in 2019. Distance was determined by the distance matrix function in QGIS version 3.26.2. A distance-decay function was fit to all data (symbols in black) as well as data with a single outlier removed (symbols in red).

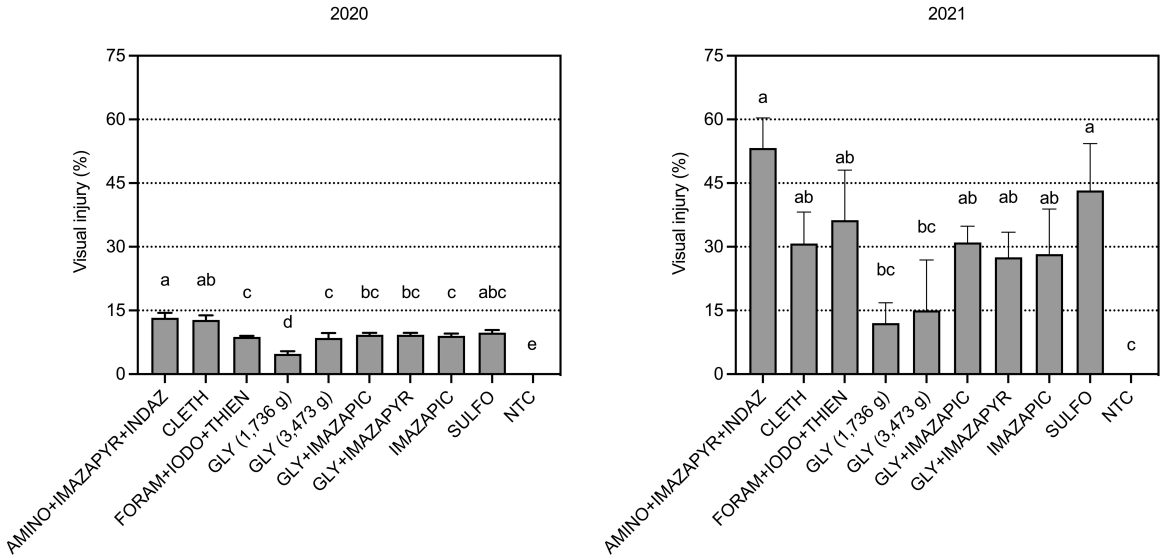


Fig. 4.4. Visual injury responses of glyphosate-resistant johnsongrass plots at 4 weeks after treatment with fall-applied herbicide treatments applied in a simulated roadside johnsongrass management study conducted in the fall of 2020 and 2021 in Boone County, MO. The experiment was a randomized complete block design, repeated. Herbicides were applied to johnsongrass stands approximately 1.5 m in height on 18 Sept. 2020 and 20 Sept. 2021. Visual injury was estimated on a scale of 0 to 100 with 0=no injury and 100=complete plant mortality. Data represent the means of four replications. Vertical bars indicate the standard error of the means. Treatment abbreviations on the x-axis are listed in Table 4.1.

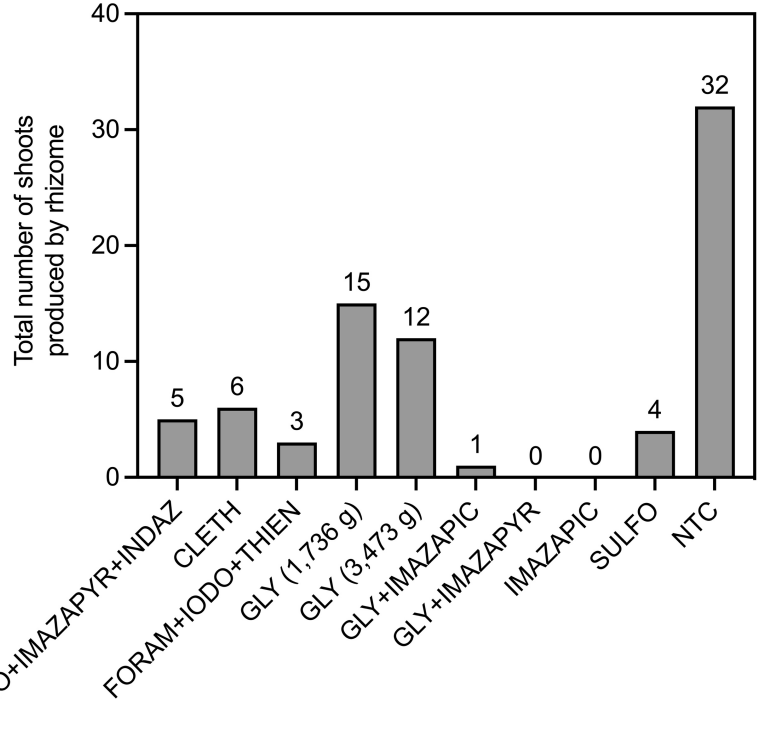


Fig. 4.5. Total number of shoots produced from rhizomes of glyphosate-resistant johnsongrass treated with fall-applied herbicides applied in a simulated roadside management study conducted in the fall of 2020 and 2021 in Boone County, MO. The experiment was a randomized complete block design, repeated. Herbicides were applied to johnsongrass stands approximately 1.5 m in height on 18 Sept. 2020 and 20 Sept. 2021. Rhizomes were harvested from plots on 21 May 2021 and 23 May 2022, cut into five, 5-node pieces and planted in the greenhouse to estimate viability. Data represent the total of eight replications from 2020-2021 and 2021-2022 at 6 weeks after planting. Treatment abbreviations on the x-axis are listed in Table 4.1.

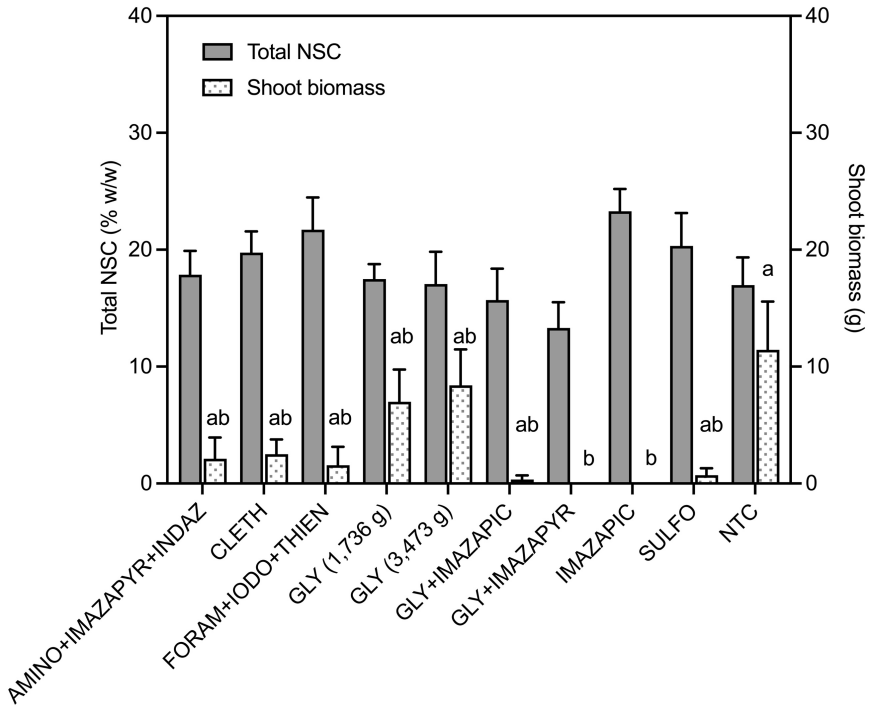


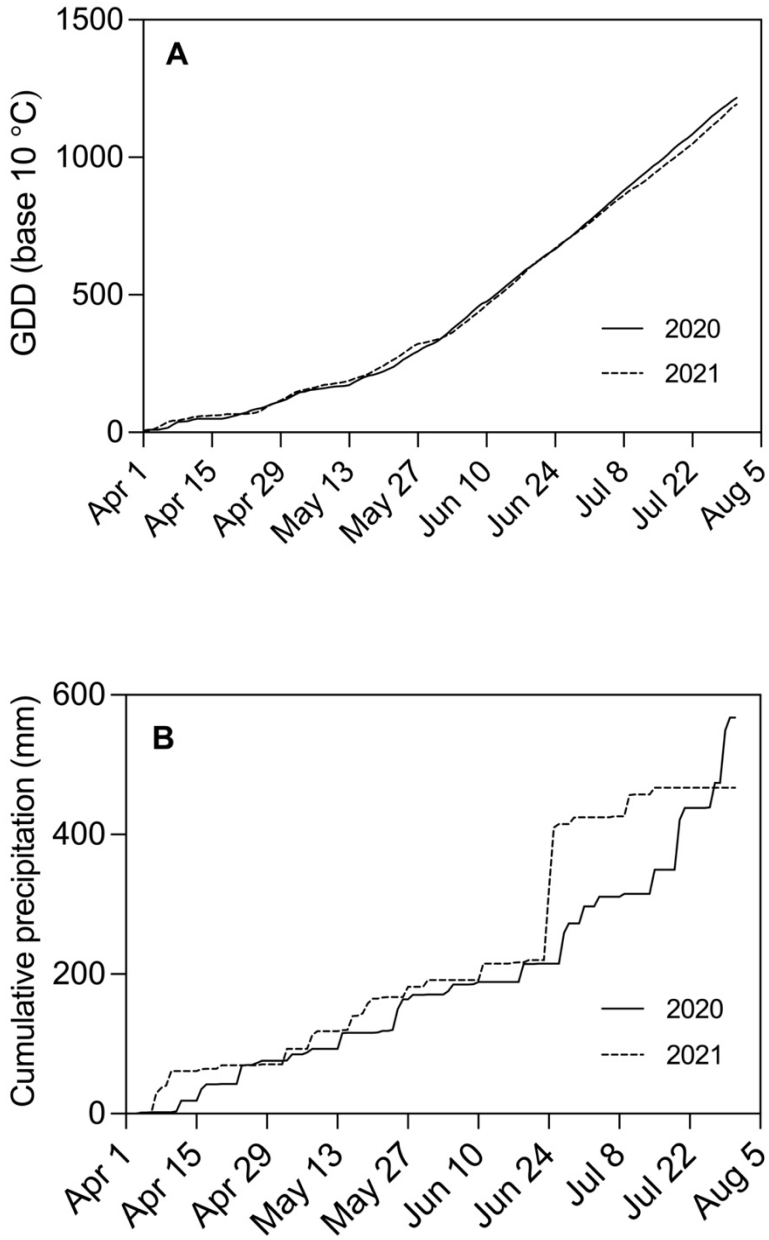
Fig. 4.6. Estimated viability of johnsongrass rhizomes by non-structural carbohydrate analysis (NSC) and a greenhouse assay. Herbicides were applied to glyphosate-resistant johnsongrass in a simulated roadside management study conducted in the fall of 2020 and 2021 in Boone County, MO. Rhizome viability was estimated in a greenhouse assay where shoots produced from rhizome were harvested at 6 weeks after planting. Dry biomass of shoots is presented on the right y-axis. Total NSC of dried rhizomes is presented on the left y-axis as a % sugar weight (starch converted to glucose hydrolysate) to % weight of rhizome matter. NSC content was estimated following the method described in Landhäusser et al. (2018). Data are the means of four replications. Vertical bars indicate the standard error of the means. Treatment abbreviations on the x-axis are listed in Table 4.1.

APPENDIX

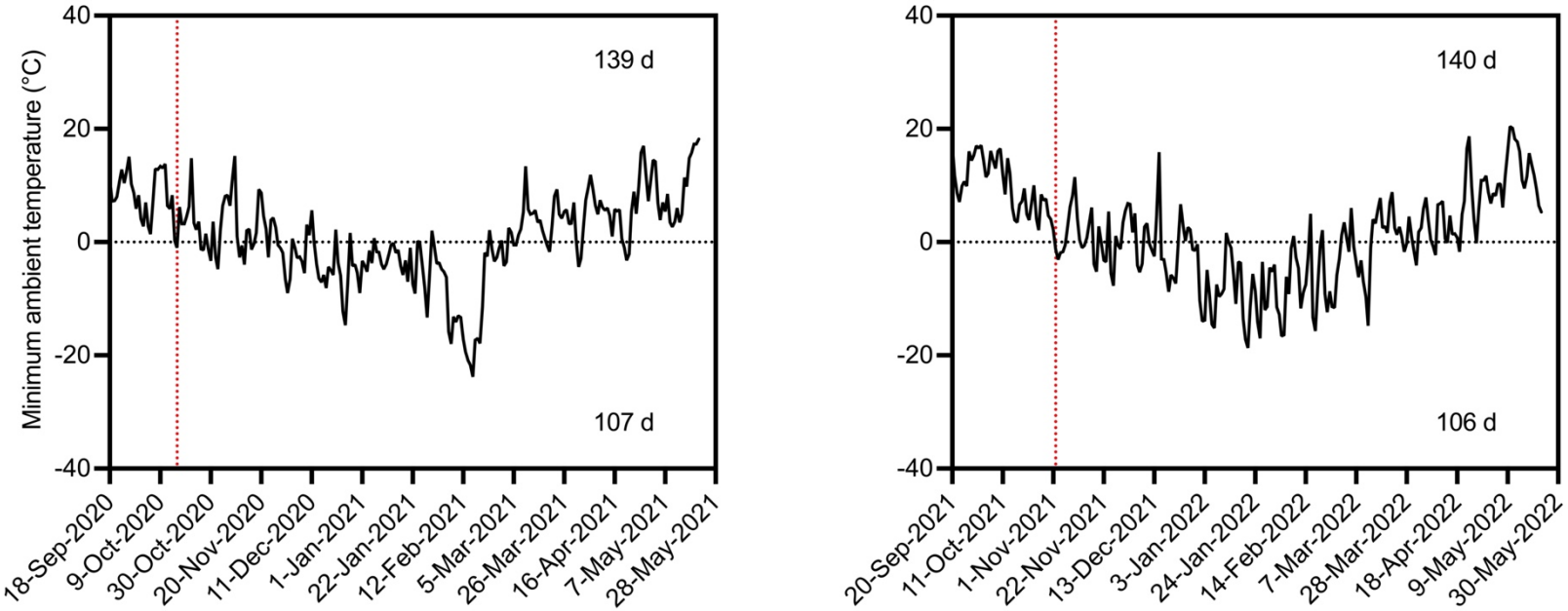
A.1 Soybean injury data from field studies with glyphosate-resistant johnsongrass conducted in Buchanan County, Missouri in 2020 and 2021. Early POST treatments (EPOST) targeted 10 to 20 cm grass weeds and mid-POST treatments (MPOST) were applied one to two weeks later. Visual estimations of crop injury are based on a scale of 0 (no herbicide injury) to 100% (complete plant mortality). Treatment means followed by the same letter are not significantly different at α=0.05 using Tukey's Honestly Significant Difference. [1]Treatment abbreviations are listed in Table 3.1. [2] 2 WAT (A)= two weeks after treatment (WAT) with A application. 2 WAT (B)= two weeks after treatment (WAT) with B application.

	Crop injury (0-100%)			
	2020		2021	
	Crop Injury	Crop Injury	Crop Injury	Crop Injury
	2 WAT (EPOST)[2]	2 WAT (MPOST)	2 WAT (EPOST)	2 WAT (MPOST)
Treatment (Site)[1]	Mean %	Mean %	Mean %	Mean %
Belcher Branch				
NTC	0 b	0	0 b	0 b
ADG fb Gly	4 a	0	11 a	11 a
ADG fb Gly+C	4 a	0	10 a	10 a
ADG+C	3 ab	0	11 a	11 a
ADG fb F	4 a	0	13 a	13 a
ADG+F	0 b	0	12 a	12 a
ADG fb F+F	4 a	0	11 a	11 a
ADG fb Glu+Gly	4 a	0	12 a	12 a
ADG fb Glu	0 b	0	13 a	13 a
Lake East				
NTC	0 c	0 d	0 b	0 b
ADG fb Gly	10 a	1 cd	9 a	9 a
ADG fb Gly+C	8 ab	5 ab	10 a	10 a
ADG+C	7 abc	0 d	9 a	9 a
ADG fb F	11 a	6 a	10 a	10 a
ADG+F	2 bc	0 d	11 a	11 a
ADG fb F+F	9 ab	5 ab	11 a	11 a
ADG fb Glu+Gly	9 ab	3 ab	8 a	8 a
ADG fb Glu	9 ab	2 bc	10 a	10 a

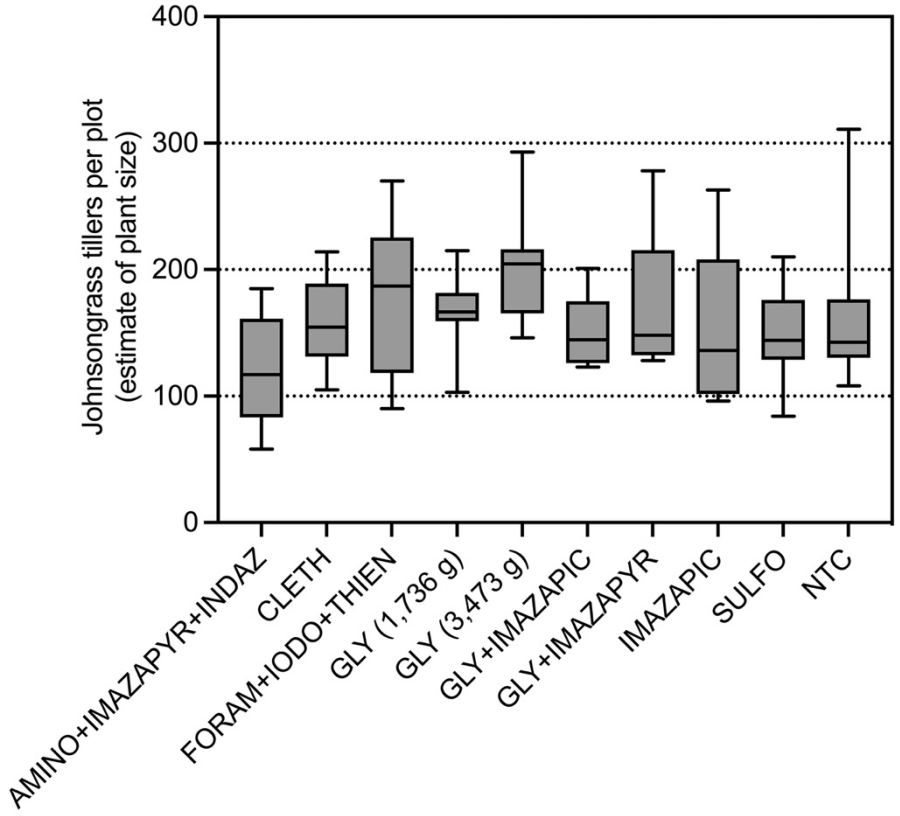
A.2. Summary of weather conditions at Buchanan County, Missouri field research sites during 1 Apr. through 1 Aug. in 2020 and 2021. (A) Cumulative growing degree days [GDD (base 10 °C)] and (B) cumulative daily precipitation.



A.3. Lowest ambient temperatures at the research site in Boone County, MO where a study was conducted simulating roadside management of glyphosate-resistant johnsongrass with fall-applied herbicides in 2020 and 2021. Herbicides were applied to johnsongrass stands approximately 1.5 m in height on 18 Sept. 2020 and 20 Sept. 2021 in separate studies. Rhizomes were harvested from plots on 21 May 2021 and 23 May 2022 for estimates of viability after overwintering. Vertical dashed lines indicate dates of the first killing frost (16 Oct. 2020 and 2 Nov. 2021) in 2020 and 2021.



A.4. Plant size of glyphosate-resistant johnsongrass, as estimated by tiller counts, prior to herbicide applications in a simulated roadside management study conducted in the fall of 2020 in Boone County, MO. The experiment was a randomized complete block design repeated in 2021. Box and whisker plots represent the data distribution of plant size estimated by the sum of tillers in each plot assigned to treatments (x-axis) in 2020 and 2021. Gray boxes extend from the first to the third quartile of the data distribution. Vertical lines extend to the minimum and maximum values. Horizontal black lines across the middle of boxes represent the mean.



VITA

Sarah Dixon was born on August 1, 1985 in Columbia, Missouri to Bruce Arden & Shirley Ann Dixon. She graduated from David H. Hickman High School in 2004 and the University of Missouri in 2009 with a Bachelor of Arts in Sociology. She continued her education at the University of Missouri, completing her Master of Science degree in Plant, Insect and Microbial Sciences in 2019. In December of 2022, she will receive her Doctorate of Philosophy in Plant, Insect and Microbial Sciences.