

Genetic characterization of herbicide resistance and investigation of physiological basis of herbicide efficacy under temperature stress in grain sorghum (*Sorghum bicolor* (L.) Moench)

by

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Abstract

Grain sorghum (*Sorghum bicolor* (L.) Moench) is a multipurpose crop valued for food, feed, and fuel. Post-emergence (POST) herbicide applications are essential for effective weed control in grain sorghum to improve yields. Hydroxyphenyl pyruvate dioxygenase (HPPD) inhibitors (e.g., mesotrione and tembotrione) are widely used to control a broad spectrum of weeds. While HPPD-inhibiting herbicides are effective in corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.), their use in sorghum is restricted due to crop injury. Previous research identified and characterized HPPD-inhibitor resistant genotypes of sorghum. Rising global temperatures due to climate change and climate variability can alter herbicide efficacy and increase crop sensitivity to herbicide injury. Although the crop responses to herbicides under high-temperature (HT) stress have been reported, the physiological mechanisms underlying such responses remain poorly understood. The objectives of this thesis were to: 1) evaluate and characterize HPPD-resistant sorghum parental lines and their F₁ progeny for resistance to mesotrione and tembotrione; 2) evaluate the physiological basis of sorghum injury to HPPD-inhibitors (e.g., mesotrione) and synthetic auxinic herbicides (e.g. 2,4-D) under optimum (OT) and HT conditions. Experiments were conducted either in the greenhouse or in controlled environmental growth chambers. For Objective 1, mesotrione-resistant (G-10) and tembotrione-resistant (G-200, G-350) sorghum genotypes, along with the susceptible line S-1, were evaluated in the greenhouse. F₁ hybrids were produced via reciprocal crosses using the two resistant lines and various genotypes as parental lines. Mesotrione (0–8×; 1× = 105 g ai ha⁻¹) and tembotrione (0–4×; 1× = 92 g ai ha⁻¹) were applied on parental and hybrid progeny at the 4–5 leaf stage. G-10 and its F₁ hybrid with G-200 (G-200 × G-10) showed significantly higher mesotrione resistance, exhibiting 8.8-fold and 11.2-fold greater resistance, respectively, compared to the sensitive

genotype S-1. Visual injury scores and SPAD readings (chlorophyll index) confirmed better recovery and chlorophyll retention in resistant lines, supporting their use in breeding mesotrione-resistant cultivars. The genotype G-200 and its hybrid with G-10 ($G-10 \times G-200$) exhibited the highest resistance to tembotrione, showing 3.42-fold and 3.55-fold greater resistance, respectively, compared to the susceptible control. This was followed by G-350, which showed 2.42-fold greater resistance and also demonstrated higher biomass and chlorophyll content than the susceptible lines. Other genotypes and hybrids showed intermediate resistance. Overall, F_1 hybrids $G-200 \times G-10$ and $G-10 \times G-200$ exhibited the highest resistance to mesotrione and tembotrione, respectively, suggesting additive effects and potential for stacking resistance in sorghum breeding programs. For objective 2, sorghum genotypes RTx430 and P84G62 were grown under two temperature regimes: OT: (32/22°C day/night) and HT: (40/30°C day/night). A mixture of radiolabeled [^{14}C]- and unlabeled mesotrione or 2,4-D was applied to the fourth youngest leaf, and foliar uptake and translocation were assessed at 6 and 24 hours after treatment (HAT). A malathion (a known inhibitor of cytochrome P450 enzyme activity) pre-treatment assay was also used to evaluate the role of cytochrome P450 enzymes in herbicide detoxification under temperature stress. Results indicate that herbicide uptake increased under HT for both herbicides, however, most of the absorbed radioactivity for mesotrione remained in the treated leaf at HT, likely due to reduced metabolism, leading to greater injury. In contrast, under OT, a greater portion of the absorbed radioactive 2,4-D remained in the treated leaf, likely due to reduced metabolism associated with lower cytochrome P450 (CYP) activity, resulting in increased injury. The results of objective 1, provides a foundation for developing herbicide-resistant sorghum hybrids via introgression, which can help effective, POST weed management; and results from objective 2 suggests that temperature stress alters herbicide metabolism and

distribution differently depending on the herbicide. Under HT, reduced translocation of mesotrione likely due to impaired metabolism led to greater injury, whereas under OT, reduced translocation of 2,4-D from the treated leaf potentially due to lower cytochrome P450 (CYP) activity and metabolism also resulted in increased injury. Overall, the results of this research will help in effective weed management in grain sorghum.

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Chapter 1 - Introduction and Literature Review

1.1 Grain Sorghum and its Importance

Grain sorghum (*Sorghum bicolor* L. Moench) ranks as the fifth most widely produced cereal crop globally, valued for its exceptional resilience under harsh environmental conditions and its contribution to food and nutrition security, especially in arid and semi-arid regions (Sinha and Thomas, 2025). Its strong adaptability to drought, elevated temperatures, and poor soils makes it increasingly important in the face of changing climates and extreme environmental conditions (Eltahir et al., 2024). While it serves as a staple food for millions in regions of Africa and Asia, grain sorghum is also gaining attention for its nutritional and functional benefits, including high levels of dietary fiber, protein, antioxidants, and its gluten-free nature (da Silva et al., 2023).

Grain sorghum is primarily cultivated for animal feed and biofuel purposes in the United States (U.S.) (Mundia et al., 2019). By 2024, global grain sorghum production reached approximately 62.34 million metric tons, with the U.S. contributing about 8.73 million metric tons roughly 14% of the global supply, making it the leading producer worldwide (USDA FAS, 2024a). Domestically, Kansas and Texas are the top producing states, collectively accounting for over 80% of the U.S. sorghum production. Specifically, Kansas represents about 57% of national production, followed by Texas at 25% (USDA FAS, 2024b; Statista, 2025).

1.1.1 Challenges in Grain Sorghum Production

Although grain sorghum is well-adapted to drought and low-input environments, achieving optimal yields remains a challenge. One of the primary limitations is weed competition, particularly during the early stages of crop development, when slow seedling growth and limited early vigor reduce sorghum's competitiveness (Rizzardi et al., 2004;

Peerzada et al., 2017). Grass weeds from genera such as *Urochloa* (e.g., *Urochloa platyphylla* – broadleaf signalgrass), *Echinochloa* (e.g., *Echinochloa crusgalli* – barnyard grass), *Panicum* (e.g., *Panicum dichotomiflorum* – fall panicum), *Digitaria* (e.g., *Digitaria sanguinalis* – large crabgrass), and *Sorghum* (e.g., *Sorghum halepense* – Johnsongrass, *Sorghum bicolor* – shattercane) are especially troublesome in U.S. production systems, along with aggressive broadleaf weeds like Palmer amaranth (*Amaranthus palmeri*) and waterhemp (*Amaranthus tuberculatus*) (Van Wyche, 2020; Werle et al., 2016).

Notable problematic species include grass weeds such as foxtail (*Setaria* spp.), longspine sandbur (*Cenchrus longispinus*), prairie cupgrass (*Eriochloa contracta*), and witchgrass (*Panicum capillare*). Similarly, common broadleaf weeds include kochia (*Bassia scoparia*), common lambsquarters (*Chenopodium album* L.), Russian thistle (*Salsola kali*), redroot pigweed (*Amaranthus retroflexus*), tumble pigweed (*Amaranthus albus*), Venice mallow (*Hibiscus trionum*), velvetleaf (*Abutilon theophrasti*), common cocklebur (*Xanthium strumarium*), morningglory (*Ipomoea* spp.), devil's claw (*Proboscidea louisianica*), and common sunflower (*Helianthus annuus*) (Dille et al., 2020b; Thompson et al., 2019). Without any weed control measures, annual yield losses in U.S. grain sorghum production are estimated at 47%, translating to approximately \$953 million in economic losses (Dille et al., 2020).

Herbicide options for grain sorghum, both pre-emergence (PRE) and post-emergence (POST), are far more limited than in other major row crops. Group 15 PRE herbicides, such as acetochlor, S-metolachlor, and dimethenamid, are commonly used for grass weed suppression; however, their efficacy relies heavily on adequate rainfall or irrigation, and their residual activity may not extend throughout the growing season (K-State Research and Extension, 2025b). In

many sorghum-growing regions, dry soil conditions limit the activation of PRE herbicides, reducing their effectiveness (Henning et al., 2010).

Postemergence herbicide options are primarily limited to broadleaf control, leaving few effective chemical tools for managing grass weeds after crop emergence (Werle et al., 2016). This challenge is further exacerbated by the high cost of labeled herbicides, increasing cases of herbicide resistance, and the lack of grass-selective POST herbicides suitable for sorghum (Gholami et al., 2013; Mishra et al., 2015). Most commercial hybrids are sensitive to POST herbicides such as HPPD-inhibitors (e.g., mesotrione, tembotrione) and ACCase-inhibitors (e.g., quizalofop), often resulting in significant crop injury (Thompson et al., 2019; Griekspoor, 2023). Consequently, POST options for grass weed control in grain sorghum remain limited. However, herbicides like atrazine and 2,4-D are still widely used postemergence for weed control, particularly when tank-mixed to broaden the spectrum of control (Smith et al., 2010; Thompson et al., 2019). In sorghum, Texas panicum (annual grass weed) was effectively managed with atrazine when tank-mixed with pendimethalin or trifluralin, resulting in $\geq 97\%$ control with early postemergence (EPOST) and 76–100% with late postemergence (LPOST) applications (Grichar et al., 2005). Atrazine also enhances the efficacy of many herbicides by controlling broadleaf weeds and suppressing grass species like foxtails. However, its continued use raises concerns due to the emergence of resistant weed populations and potential environmental impacts (Thompson et al., 2019).

Due to its slow growth during the early stages, sorghum is particularly vulnerable to weed competition soon after emergence (Barber et al., 2015). On average, yield declines by 3.6% for each week that weeds are not controlled, and severe infestations within the first two weeks can reduce yields by as much as 20% (Barber et al., 2015; Smith et al., 1990). This highlights the

importance of maintaining a weed-free environment during a critical early-season period to avoid substantial yield losses. Enhancing sorghum's tolerance to early post-emergence herbicide applications, particularly those targeting grass weeds, could support a more effective weed management strategy (Bagavathiannan et al., 2018).

1.1.2 Hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitors as Post-Emergence Option for Weed Control in Grain Sorghum

Hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicides represent a relatively modern class of weed control chemistry, first introduced during the 1980s (Lee et al., 1997; van Almsick, 2009). HPPD is a key enzyme in the biosynthesis of plastoquinone and tocopherols, both of which are essential for carotenoid production in plants (Siefermann-Harms, 1987). Based on their site of action, HPPD inhibitors are classified as Group 27 herbicides by the Weed Science Society of America (WSSA) and the Herbicide Resistance Action Committee (HRAC) (Mallory-Smith & Retzinger, 2017). These herbicides fall into several chemical families, including: isoxazoles (e.g., isoxaflutole), pyrazolones (e.g., pyrasulfotole, tolypyralate, topramezone), triketones (e.g., bicyclopyrone, mesotrione, tembotrione), and isoxazolidinones (e.g., clomazone) (Lee et al., 1997).

Among these, mesotrione is extensively used in corn production for both pre- and post-emergence weed control due to inherent ability of corn to rapidly detoxify the herbicide through cytochrome P450-mediated metabolism (Mitchell et al., 2001; Ahrens et al., 2013). In contrast, grain sorghum exhibits high sensitivity to post-emergence applications of HPPD inhibitors, often resulting in visible bleaching symptoms and necrosis (Abit et al., 2009). Consequently, mesotrione is only labeled for pre-emergence (PRE) use in crops like sorghum and sugarcane

(Jhala et al., 2023). Similarly, tembotrione is used both PRE and POST in corn for broadleaf and grass weed control; however, it is not registered in sorghum due to crop injury.

Despite being in use for over two decades, the evolution of herbicide-resistant weed species to HPPD inhibitors has occurred more slowly compared to resistance development against other herbicide modes of action (Jhala et al., 2014; Kaundun, 2021). As of 2025, resistance to HPPD-inhibiting herbicides has been documented in only a few weed species globally—three broadleaf weeds (*Palmer amaranth*, *waterhemp*, and *wild radish*) and one grass weed (*Chinese sprangletop*) (Heap, 2025).

1.1.3 Herbicide-Resistant Sorghum: Need and Potential

Sorghum is increasingly recognized as a key crop for the future due to its high adaptability, efficient water use, and diverse end-uses including animal feed, human consumption, and biofuel production (Taylor et al., 2006; McCuistion et al., 2019; Stamenković et al., 2020). Despite its climate resilience, herbicide options, particularly for post-emergence (POST) weed control remain limited in grain sorghum (Sreeram et al., 2016; Tanya et al., 2020). Grass weeds pose a major challenge as few POST herbicides are registered due to the risk of crop injury (Thompson et al, 2019). In this context, the development of herbicide-resistant sorghum offers a promising solution for effective weed control.

However, grain sorghum's smaller global market share has led to less investment by agrochemical and seed companies in developing herbicide-resistant traits compared to major crops like corn and soybean (*Glycine max* L. Merril). Moreover, sorghum has not been a common target for genetically modified (GM) herbicide traits (Pandian et al., 2021). Another concern is the potential gene flow from herbicide-resistant sorghum to wild relatives such as shattercane and johnsongrass. Although recent studies suggest the risk is minimal (Hodnett et al.,

2019; Werle et al., 2017), effective stewardship practices are essential to prevent and manage any unintended trait transfer

To overcome the limited availability of POST herbicide options in sorghum, extensive breeding efforts have led to the development of herbicide-resistant hybrids. Key advances include traits conferring resistance to acetolactate synthase (ALS)-inhibiting herbicides (such as imidazolinone and sulfonylurea) and acetyl-CoA carboxylase (ACCase)-inhibitors, resulting in commercial technologies such as Inzen™, igrowth™, and Double Team™.

The first herbicide-resistant grain sorghum was developed at Kansas State University by transferring a modified ALS gene from an ALS-inhibitor-resistant shattercane biotype through conventional breeding (Tuinstra & Al-Khatib, 2011). This trait, originally branded by DuPont as *Inzen*™ and paired with *Zest*™ (nicosulfuron, an ALS-inhibitor), is now held by Corteva AgroSciences, though commercial hybrids are not yet available in the market. *igrowth*™, developed by Advanta Inc., via EMS-induced altered ALS gene conferring tolerance to imidazolinone (IMI) herbicides. It is already commercialized in Argentina and is expected to be launched in the U.S. soon. Meanwhile, Double Team™, an ACCase-inhibitor-tolerant sorghum, is being developed by Advanta in partnership with the United Sorghum Checkoff Program and marketed by S&W Seed Company (Pandian et al., 2021).

1.1.4 Mechanism of Herbicide Resistance

According to the WSSA (1998), herbicide resistance is “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type”. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis”. HRAC (2015) also defined herbicide resistance as “naturally occurring inheritable ability of some weed

biotypes within a given weed population to survive a herbicide treatment that should, under normal use conditions, effectively control that weed population”. The evolution of herbicide resistance in weeds remains a significant challenge in agriculture; meanwhile, resistance or tolerance traits in crops are widely utilized in modern farming to enable effective herbicide to use for weed control without harming the crop (Beckie et al., 2019). Herbicide resistance mechanisms are generally classified into two main types: target-site resistance (TSR) and non-target-site resistance (NTSR) (Dekker & Duke, 1995).

Target-site resistance (TSR) occurs when mutations in the herbicide’s target gene lead to amino acid substitutions that alter the protein’s structure, thereby reducing herbicide binding affinity. This mechanism may also involve gene amplification or overexpression, increase the abundance of the target enzyme and reduce herbicide efficacy. Typically, mutations in the gene encoding the enzyme, leading to structural changes in the protein. Such changes can hinder the herbicide’s ability to bind effectively to its intended site, thereby reducing or nullifying its herbicidal effect (Hanson et al., 2013). In short, target-site resistance arises either from structural modifications at the herbicide’s binding site typically due to point mutations or from overexpression of the target enzyme because of gene amplification, both of which reduce the herbicide’s efficacy (Délye et al., 2013). Target-site resistance is the most frequently observed resistance mechanism in weeds and is particularly common for herbicides that inhibit acetolactate synthase (ALS), acetyl-CoA carboxylase (ACCase), protoporphyrinogen oxidase (PPO), and photosystem II (PSII) (Gaines et al., 2020). A point mutation conferring ACCase-inhibitor resistance has been identified in *Setaria viridis*, where the substitution of isoleucine with leucine at position 1781 in the chloroplastic ACCase enzyme imparts resistance to the cyclohexanedione (CHD) herbicide sethoxydim (Yu et al., 2007). Another form of target-site

resistance involves gene overexpression or duplication, as seen in glyphosate-resistant *Lolium rigidum* and *Amaranthus palmeri*, where increased *EPSPS* gene copies confer resistance (Baerson et al., 2002; Gaines et al., 2010).

Non-target-site resistance (NTSR) involves physiological modifications that reduce the herbicide dose reaching its target site, typically through reduced uptake, impaired translocation, or enhanced sequestration and metabolism (Im et al., 2016; Powles & Yu, 2010). Among these, metabolic detoxification is the most widespread NTSR mechanism. It occurs through a four-phase detoxification pathway: Phase I – activation via oxidation by cytochrome P450 monooxygenases; Phase II – conjugation with hydrophilic molecules such as glutathione or sugars; Phase III – compartmentalization by ATP-binding cassette (ABC) transporters; and Phase IV – degradation of conjugated products in vacuoles or the apoplast (Yuan et al., 2006; Oh et al., 2016). Metabolism-based resistance has been documented in numerous weed species against various herbicides. One of the earliest discoveries of non-target-site resistance involving enhanced metabolism through cytochrome P450 enzymes was reported in *Lolium rigidum* biotypes in Australia and phenyl urea-resistant *Phalaris minor* populations in Asia (Werck-Reichhart et al., 2000). Likewise, Palmer amaranth exhibited rapid mesotrione metabolism, degrading over 90% of the herbicide within 24 hours post-application compared to susceptible plants (Nakka et al., 2017). Additionally, accelerated metabolism of tembotrione was identified as the primary mechanism of resistance in a Palmer amaranth population from Nebraska (Küpper et al., 2018). This mechanism is predominant in crop species that exhibit natural herbicide tolerance. Crops such as wheat, corn, and soybean display inherent tolerance to various ALS-inhibiting herbicides, largely due to their ability to metabolize these compounds through cytochrome P450 enzymes (Koeppel et al., 1997; Hinz & Owen, 1996; Brown et al., 1990; Yu &

Powles, 2014). Cytochrome P450s play a significant role in metabolizing herbicides like nicosulfuron, mesotrione, and tembotrione in corn (Liu et al., 2019), trifloxysulfuron-sodium in cotton (Thyssen et al., 2018), and chlorsulfuron in wheat (Xiang et al., 2006), among others.

1.2 Sources for Herbicide Resistance

1.2.1 Natural Variation

Sorghum possesses rich genetic diversity across five botanical races shaped by domestication and adaptation (Harlan & De Wet, 1972). Germplasm collections such as the USDA National Plant Germplasm System preserve over 41,000 accessions (Pandian, 2020), many containing underutilized traits related to herbicide resistance (Cuevas & Prom, 2020). This genetic diversity present in grain sorghum can be harnessed to identify resistance to herbicides like HPPD-inhibitors, which offer broad-spectrum weed control when applied post-emergence. Screening a diverse collection of cultivated grain sorghum hybrids revealed varying levels of resistance to HPPD-inhibitors such as mesotrione and tembotrione, indicating that natural variation for herbicide resistance exists within the sorghum germplasm (Abit & Al-Khatib, 2009; Cunha et al, 2016). Also, screening a wide of sorghum germplasm, Pandian et al. (2020) identified four sorghum genotypes with tolerance to HPPD-inhibiting herbicides mesotrione and tembotrione. Further characterization of these genotypes revealed that mesotrione tolerance is controlled by a dominant monogenic trait, while tembotrione tolerance is polygenic, involving several QTLs (Quantitative Trait Loci). These findings provide a strong foundation for characterizing genetic control of HPPD resistance and guiding its introgression into elite lines. The objectives of Chapter 2 and 3 of this thesis include evaluation of the response of mesotrione- and tembotrione-resistant parental lines and their crosses with U.S. genotypes to assess the transfer of HPPD-inhibitor resistance trait in different genetic backgrounds.

1.2.2 Wild Relatives as a Source of Resistance Allele

Wild relatives of cultivated crops often retain beneficial alleles lost during domestication, offering valuable traits for improving stress tolerance, adaptation, and yield (Castañeda-Álvarez et al., 2016; Harlan, 1976). In sorghum, weedy species like shattercane and johnsongrass commonly co-exist with cultivated fields and have evolved resistance to ALS- and ACCase-inhibiting herbicides due to repeated exposure in corn and soybean systems (Werle, 2016). These resistant traits have been successfully transferred into cultivated sorghum via conventional breeding. For example, a Kansas shattercane population carrying a Trp-574-Leu mutation in the ALS gene conferring resistance to sulfonylurea and imidazolinone herbicides was used to develop resistant grain sorghum hybrids (Tuinstra & Al-Khatib, 2011). Similarly, a Sudangrass biotype from Bolivia with a Trp-2027-Cys substitution in the ACCase gene provided resistance to APP herbicides like fluazifop-P and quizalofop-P (Kershner et al., 2012). These traits were introgressed into commercial hybrids (Tuinstra & Al-Khatib, 2017).

1.2.3 Induced Mutagenesis

Induced mutagenesis is a widely used approach to increase genetic diversity in crops that have experienced bottlenecks due to domestication and intensive selection, such as grain sorghum (Mace et al., 2013). Both chemical agents, like ethyl methanesulfonate (EMS), and physical mutagens, such as gamma irradiation, are employed to create random genetic variation, which can be harnessed for crop improvement (Jankowicz-Cieslak et al., 2017; USDA AMS, 2023). These techniques have led to the development of new varieties with valuable agronomic traits, including herbicide resistance, in several major crops. For example, Clearfield® rice and CoAxiom® wheat were developed using EMS mutagenesis to introduce resistance to ALS- and ACCase-inhibiting herbicides, respectively (Avila et al., 2005; Chhapekar et al., 2019). In

legumes, EMS-induced mutagenesis has produced lentil mutants induced genetic variation through gamma radiation (30 kR) tolerant to imazethapyr and metribuzin (Sharma et al, 2017). Similarly, large-scale EMS mutagenesis in rice has resulted in lines with robust tolerance to imazapic, demonstrating the scalability and effectiveness of induced mutagenesis for breeding herbicide-resistant crops (Oladosu et al., 2016). Herbicide-resistant grain sorghum lines have been developed using EMS as a chemical mutagen. Notably, mutations providing strong resistance to ACCase-inhibitors (Clement, 2013) and previously to ALS-inhibitors (Uriarte et al., 2019) have been patented and incorporated into the breeding of resistant sorghum hybrids. Tang et al. (2025) used EMS mutagenesis and large-scale screening to identify sorghum mutants with specific point mutations in the SbALS gene that confer strong resistance to imidazolinone herbicides. Their work demonstrated that these herbicide-resistant sorghum lines enable effective weed control and facilitate successful intercropping with similarly resistant soybean varieties.

1.2.4 Gene Editing

Advancements in gene-editing technologies have enabled precise modification of target genes. Among available tools like TALENs and ZFNs, CRISPR-Cas9 stands out as a faster, more efficient, and cost-effective method. Herbicide-resistant crop variants have been developed by modifying herbicide target sites. In contrast, screening large germplasm or mutant populations is time-consuming and may take up to 10 years to develop a commercial product (Ahmar et al, 2020). Gene editing, supported by genomic resources, allows precise modifications in the genome to develop herbicide-resistant traits in a short period of time. It enables targeted changes in herbicide-binding sites or promoter regions to boost expression of detoxifying enzymes like cytochrome P450s and GSTs, enhancing herbicide metabolism (Werck et al., 2000). Gene editing has successfully introduced target-site resistance in model plants like Arabidopsis and

major crops such as rice, maize, and soybean. For example, a Ser-653-Asn mutation in the ALS gene of *Arabidopsis* conferred resistance to imidazolinone herbicides (Dong et al., 2020). Similarly, CRISPR-Cas9 was used to introduce a Pro-171-Phe substitution in rice, resulting in the commercial herbicide-resistant variety Nangeng 46 (Kuang et al., 2020). Similarly, a modified, insensitive variant of the corn HPPD gene was introduced into soybean, providing tolerance to HPPD-inhibiting herbicides (Siehl et al., 2014). These advances demonstrate the potential of CRISPR-Cas9 and other technologies for developing herbicide-resistant sorghum lines.

1.2.5 Transgenic Approaches

Recently, herbicide resistance to HPPD inhibitors has been successfully achieved in both soybean and cotton through transgenic approaches. This was accomplished by introducing a modified bacterial HPPD gene (hppdPFW336) derived from *Pseudomonas fluorescens*, a non-pathogenic soil bacterium. The gene carries a single-point mutation (Gly336Trp) that enhances resistance to HPPD-inhibiting herbicides such as isoxaflutole. In soybean, this trait was incorporated into the FG72 event using biolistic (particle bombardment) transformation, whereas in cotton, the GHB811 event was developed via *Agrobacterium*-mediated transformation. Expression of the HPPD W336 protein in these crops allows for postemergence application of HPPD inhibitors without causing crop injury, thereby expanding weed management options. These traits are often combined with tolerance to other herbicides, including glyphosate, glufosinate, and dicamba, enabling the development of multiple herbicide-resistant cultivars (Hawkes et al., 2012; Dreesen et al., 2018; Joyner et al., 2022).

1.3 Genetic Mapping and Identification of Herbicide Resistance in Sorghum

The development of herbicide-tolerant grain sorghum is crucial for effective post-emergence weed control and long-term crop sustainability. In cases where resistance is governed by qualitative inheritance, selecting surviving plants after herbicide application is straightforward due to the clear trait expression and minimal environmental influence. However, when resistance is quantitatively inherited, more precise phenotyping tools such as visual injury assessments and SPAD chlorophyll (chlorophyll index) readings are necessary to capture subtle variations in herbicide response (Daniel et al., 2023). Advanced genomic tools such as bulk segregant analysis (BSA), especially when coupled with RNA sequencing (BSR-Seq), enable rapid identification of resistance loci by comparing pooled DNA or RNA from resistant and susceptible plants. This approach has been effective in corn, where CYP81A9 was identified as a resistance gene, offering greater speed and accuracy than conventional marker-assisted or GWAS (genome-wide association studies) methods (Liu et al., 2019).

Bi-parental QTL mapping using F₂ and recombinant inbred line populations continues to be very important in locating resistance genes. In sorghum, QTLs associated with tolerance to mesotrione and tembotrione as well as other abiotic and biotic stressors have been mapped successfully (Pandian et al., 2020; Sanchez et al., 2002; Tao et al., 2003; Punhuri et al., 2013). For instance, Bogner et al. (2018) identified a QTL linked to mesotrione and/or isoxazole resistance in soybean, while six distinct QTLs for tembotrione tolerance were reported in sorghum via linkage mapping (Pandian et al., 2020b). These findings highlight the polygenic nature of herbicide resistance in many crop species. Adhikari et al. (2020) performed a GWAS for fomesafen tolerance in sorghum and discovered significant SNPs (single nucleotide polymorphisms) near the *PPXI* gene. Despite the lack of coding region variation, their results

suggested that non-target site resistance mechanisms such as enhanced metabolic detoxification are likely at play, driven by multiple genes.

In Chapter 2 and 3 of this thesis, sorghum genotypes G-10 (mesotrione-resistant) and G-200 and G-350 (tembotrione-resistant), as parents and their crosses with US lines were evaluated for phenotypic data such as visual injury, chlorophyll content and biomass accumulation upon treatment with various doses of these herbicides. In future, molecular markers associated with mesotrione and tembotrione resistance will be developed and validated through Kompetitive Allele Specific PCR (Polymerase Chain Reactions) (KASP) assays, following the precedent set by Michelmore et al. (1991), who used RAPD (Random Amplified Polymorphic DNA) markers to map disease resistance in lettuce.

1.4 Marker-Assisted Introgression of Herbicide Resistance into Elite Sorghum Lines

Introgression is the stable incorporation of a gene or genomic region from a donor into a recurrent parent through repeated backcrossing. Selection is a key method for introducing valuable traits such as herbicide, drought, or disease resistance into elite crop varieties. When integrated with molecular marker tools, this strategy enables precise and accelerated trait transfer while maintaining desired agronomic characteristics. KASP genotyping has emerged as a cost-effective, high-throughput platform for SNP detection and is widely used in marker-assisted selection (Semagn et al., 2014). Its reliability and efficiency have been demonstrated across various crops. For example, Burow et al. (2019) applied KASP markers to enhance selection of *Striga* resistance in sorghum. In chickpea (*Cicer arietinum* L.), Kumar et al. (2017) used KASP assays to track ALS-inhibitor herbicide resistance, while Iquebal et al. (2022) validated herbicide

resistance markers in soybean. Similarly, in wheat, Vikram et al. (2016) utilized KASP to select drought-tolerant lines using validated QTLs.

Building on these advances, the future course of current research aims to use KASP assays to validate SNP markers linked to mesotrione and tembotrione resistance. These markers will then guide the introgression of herbicide resistance into elite sorghum R-lines through a marker-assisted backcrossing program. After multiple generations of selection and genotyping, homozygous BC₁F₂ plants will be evaluated in the field to confirm herbicide resistance and agronomic performance. This approach offers a practical alternative to transgenic breeding, which is constrained by high development costs and regulatory hurdles (Lassoued et al., 2019), as well as ecological concerns over gene flow to wild sorghum relatives like johnsongrass and shattercane (Ohadi et al., 2018). By leveraging marker-assisted selection and QTL-guided breeding, this thesis aims to develop herbicide-resistant hybrids that are environmentally sustainable and commercially viable

1.5 Climate Change and Impact on Agriculture

Climate change, characterized by long-term shifts in temperature and precipitation (WMO, 1992), is one of the most critical global challenges today. Since the Industrial revolution, rising concentrations of greenhouse gases, such as CO₂ (carbon dioxide), CH₄ (methane), and N₂O (nitrous oxide) have significantly contributed to climate change, climate variability and extreme events. These gases have increased by 40%, 150%, and 20%, respectively, since 1750 (IPCC, 2014). According to the IPCC sixth assessment report, human activities are the primary drivers of this warming, with global surface temperatures rising by approximately 0.8°C to 1.3°C between 1850 and 2019 (IPCC, 2023).

Agricultural production, which is fundamental to human development and survival, is increasingly impacted by the effects of rapid climate change. Climate change contributes to more frequent extreme weather events such as droughts, floods, erratic precipitation, extreme temperatures which in turn reduce crop yields and destabilize ecosystems (Karki & Mackey, 2020; Annie et al., 2023; Wu et al., 2023). Moreover, these climatic shifts complicate pest and disease management in crops, heightening the vulnerability of food systems (Monteleone et al., 2022; Yang et al., 2023). These also influences weed control, weed management and shifts of weed species in different crops. Both crop productivity and effective weed control can be significantly impacted by shifts in abiotic factors, primarily driven by climate change and variability (Dukes et al., 2009; Singer et al., 2013).

Rising temperatures can interfere with key developmental stages in crops, including vegetative development, time to flowering, and the timing of harvest (Baldocchi & Wong, 2008). Since each crop has a specific temperature threshold for successful reproduction, extreme heat can lead to reproductive failure (Prasad et al., 2017). For instance, corn struggles to reproduce above 32°C, while grain sorghum suffers from reduced pollen viability, germination, and seed set at temperatures exceeding 38°C (Djanaguiraman et al., 2014).

In addition to directly affecting crops, rising temperatures and different soil moisture and humidity levels can also influence weed proliferation and alter herbicide efficacy, ultimately impacting their effectiveness in weed control (Poorter & Navas, 2003; Dukes et al., 2009). Weeds typically adapt better to environmental stress than crops due to their greater genetic diversity and lack of selective breeding for uniform traits (Dukes & Mooney, 1999).

1.5.1 Impact of Rising Temperature on Corn and Grain Sorghum

Rising temperatures driven by climate change pose substantial threats to the productivity and physiological performance of two major cereal crops i.e., corn and grain sorghum. In corn, high temperatures during critical reproductive stages such as anthesis, silking, and grain filling significantly impair yield by reducing pollen viability and silk receptivity, often leading to kernel abortion and yield losses of up to 25-35% in some regions (Tiwari & Yadav, 2019). These stresses disrupt physiological processes, including photosynthesis and respiration, and trigger oxidative stress through elevated reactive oxygen species (ROS), necessitating enhanced antioxidant defenses (Tiwari & Yadav, 2019). Ultra-high temperatures, such as brief exposures to 48°C, can cause rapid declines in transpiration and photosynthetic activity, resulting in visible tissue dehydration (Ellwanger et al., 1973). Cytochrome P450 enzymes are affected in corn's adaptive responses to combined heat and biotic stresses (Li et al., 2023). Furthermore, with the projected rise in global temperatures, physiological optimum of corn may shift, with reproductive processes becoming increasingly vulnerable above 32-35°C (Korres et al., 2016).

Grain sorghum, although generally more resilient due to its C4 photosynthetic efficiency, is not immune to heat stress. Temperatures above the optimal reproductive range of 25-28°C can drastically reduce floret fertility and grain weight, with complete sterility observed at around 37°C (Prasad et al., 2015). The most heat-sensitive phases occur from 10 days before to 5 days after anthesis, during which elevated temperatures impair pollen viability, anther dehiscence, pollen tube growth, and pistil development (Djanaguiraman et al., 2018; Prasad et al., 2008). Even brief periods of heat stress can shorten the grain-filling phase and accelerate senescence, further compromising yield (Prasad et al., 2015; 2017). Climate projections suggest a 7-17% reduction in sorghum yields across arid and semi-arid regions by mid-century due to temperature

stress, underscoring the urgency for breeding heat-resilient cultivars adapted to changing climatic conditions (Prasad et al., 2015).

In crops, weed pressure remains a major constraint to productivity, with herbicides being the primary tool for weed management. However, herbicide efficacy is highly influenced by temperature extremes, with several reports indicating reduced performance under high-temperature stress. Moreover, elevated temperatures can increase crop injury due to a lower adaptive physiological capacity in crops compared to weeds. Thus, understanding the complex interplay between temperature stress, herbicide efficacy, and crop physiological responses is critical for developing sustainable weed management strategies under a changing climate.

1.5.2 Effect of Temperature on Herbicide Efficacy

Herbicides play a significant role in modern weed management due to their cost-effectiveness and reliability compared to other control strategies. However, climate change, particularly rising temperatures may affect herbicide performance by altering their behavior and enhancing weed competitiveness (Bailey, 2003). Environmental conditions at the time of herbicide application critically influence efficacy, as they affect herbicide absorption, translocation, metabolism, and interactions at the target site within susceptible weed species (Varanasi et al., 2016). The presence of detoxifying enzymes or antioxidant defense mechanisms within plants may also reduce herbicidal activity, thereby compromising weed control outcomes. These physiological and biochemical factors, acting independently or in combination, can enhance or diminish herbicide performance (Matzenbacher et al., 2014).

Temperature stress, in particular, can influence the effectiveness of foliar-applied herbicides by altering their uptake, internal movement, and breakdown within the plant (Jursík et al., 2023). Elevated temperatures may either enhance herbicide absorption and translocation, thus

improving efficacy or accelerate metabolic degradation, reducing herbicide persistence and activity (Johnson & Young, 2002). For example, Rudell et al. (2023) found that high temperatures reduced the efficacy of 2,4-D in Palmer amaranth by increasing its metabolic breakdown. Conversely, temperature elevation can improve the effectiveness of certain herbicide classes, such as PROTOX (protoporphyrinogen oxidase) inhibitors (Li et al., 2000; Fausey & Renner, 2001; Price et al., 2004; Hatterman-Valenti et al., 2011).

Greenhouse studies have shown that herbicides like flumiclorac and fluthiacet were significantly more effective at 40°C compared to 10°C in controlling common lambsquarters and redroot pigweeds, with fluthiacet demonstrating up to a threefold increase in activity (Fausey & Renner, 2001). In contrast, the efficacy of mesotrione, in Palmer amaranth under elevated temperatures (40/30°C), reduced primarily due to enhanced metabolic detoxification (Godar et al., 2015). While crops like corn and grain sorghum are generally tolerant to mesotrione through reduced uptake and P450-mediated metabolism (Hawkes et al., 2001; Mitchell et al., 2001; Abit & Al-Khatib, 2009; Pandian et al., 2023), however recent studies show that they may still suffer increased injury when grown under high-temperature conditions (Adari, 2024).

Although numerous studies have examined the effect of temperature on herbicide efficacy in weed species, fewer have explored its influence on crop responses to herbicides. This thesis investigates the physiological mechanisms underlying increased sensitivity grain sorghum to mesotrione and 2,4-D under elevated temperature stress. The following section delves further into the specific nature of crop injury caused by these herbicides at high temperatures.

1.5.3 Impact of Temperature on Herbicide Selectivity and Crop Injury

Herbicide metabolism in plants is primarily governed by endogenous enzymes, particularly cytochrome P450 monooxygenases (P450s), whose composition and activity vary

among species. These differences influence how rapidly herbicides are detoxified, forming the biochemical basis for species-specific sensitivity and crop-weed selectivity (Dimaano & Iwakami, 2020). In corn, for instance, herbicide selectivity is closely tied to its ability to rapidly metabolize certain herbicides via cytochrome P450s. Mesotrione is detoxified through P450-mediated ring hydroxylation, followed by conjugation into non-phytotoxic compounds (Hawkes et al., 2001). Similarly, 2,4-D is metabolized through oxidative processes and sugar conjugation, reducing its phytotoxic potential (Fang & Butts, 1954).

Although elevated temperatures alter herbicide efficacy (increase or decrease) on weeds, they may also compromise crop safety by reducing selectivity. For example, flumioxazin showed greater phytotoxicity to peanut (*Arachis hypogaea*) at 25°C compared to 15°C (Price et al., 2004), and sulfentrazone caused a marked reduction in soybean height as temperatures increased from 15°C to 34°C (Li et al., 2000). Similarly, greater injury was observed in corn treated with mesotrione, tembotrione, or 2,4-D under high temperature conditions (40/35°C day/night) compared to optimal conditions (30/25°C day/night) (Adari, 2024). These observations suggest that temperature-sensitive physiological responses, such as altered absorption, translocation, or metabolic detoxification may intensify herbicide injury in crops.

However, the specific mechanisms by which temperature affects crop-herbicide interactions remain insufficiently characterized. Understanding the physiological basis for this increased herbicide sensitivity under high temperature stress is crucial for developing temperature-resilient crop management practices. This forms the central focus of Chapter 4 of this thesis.

1.6 Summary and Objectives of the Research

Grain sorghum is recognized as one of the most climate-resilient crops, with applications ranging from food and feed to biofuel production. However, effective post-emergence control of grass weeds remains a significant challenge due to the limited availability of POST herbicide options. Although HPPD-inhibiting herbicides such as mesotrione and tembotrione are commonly utilized for both pre- and post-emergence weed control in corn, their post-emergence use in sorghum is restricted due to the risk of crop injury. Developing herbicide-resistant grain sorghum offers a promising solution to improve weed management in this crop. In a recent study, Pandian et al. (2020) identified four genotypes from a diversity panel, two exhibiting resistance to mesotrione and two to tembotrione when compared to a susceptible genotype. Evaluating these resistant lines and their F₁ hybrids generated with elite U.S. sorghum genotypes can facilitate the development of an effective introgression breeding program. Elevated temperatures significantly influence herbicide efficacy. The high genetic variability among weed species enables them to adapt more efficiently to combined temperature and herbicide stress, often gaining a competitive advantage over crops (Dukes & Mooney, 1999). This adaptability may stem from reduced herbicide uptake or accelerated metabolic detoxification. Research conducted in our laboratory has demonstrated that crops such as corn and sorghum exhibit increased sensitivity to herbicides like mesotrione and 2,4-D when subjected to high temperature stress. Despite these findings, limited research has been conducted to elucidate the underlying mechanisms responsible for this heightened sensitivity.

The overall goal of this thesis was to a) assess transfer of the herbicide resistance of previously identified grain sorghum genotypes into multiple genetic backgrounds. and b)

investigate the physiological mechanisms underlying mesotrione and 2,4-D injury in grain sorghum under HT. Thesis is organized into the following main Chapters.

Chapter 2: Genotypic characterization and evaluation of mesotrione-resistant sorghum genotypes and their F₁ progeny with diverse genetic backgrounds.

Chapter 3: Characterize the inheritance of tembotrione resistance in grain sorghum.

Chapter 4: Investigate the physiological basis of temperature stress on mesotrione and 2,4-D efficacy in grain sorghum.

Chapter 2 - Genotypic Characterization and Evaluation of Mesotrione Resistance in Grain Sorghum Parental and Hybrid Lines

Abstract

Post-emergent (POST) weed control options in grain sorghum are limited due to the crop's sensitivity to grass-selective herbicides. Mesotrione provides effective post-emergence control of a wide range of weeds, including grasses, but it is not registered for use in grain sorghum due to crop injury. This study aimed to assess the response of mesotrione resistant parent line (G-10) and F₁ progeny with diverse genetic backgrounds for mesotrione resistance for use in breeding herbicide-tolerant hybrids. A greenhouse experiment was conducted using sorghum genotypes with known differences in mesotrione sensitivity, including G-10 (resistant), S-1 (susceptible), and their F₁ hybrids with tembotrione-resistant lines (G-200, G-350). Plants were treated with mesotrione doses (0 to 840 g ai ha⁻¹, 1x=105 g ai ha⁻¹), and responses were assessed based on visual injury, chlorophyll index (SPAD readings), and biomass accumulation. The results indicated a wide range of genotypic variation in mesotrione response. The susceptible genotype S-1 showed a GR₅₀ of 38 g ai ha⁻¹, while the resistant line G-10 and its F₁ hybrid with G-200 exhibited significantly higher resistance (GR₅₀ = 337 and 427 g ai ha⁻¹, respectively) supporting mesotrione resistance controlled by single dominant gene. Visual injury and SPAD readings further confirmed that tolerant genotypes experienced less bleaching and maintained higher chlorophyll levels, with a delay in symptom development and faster recovery. The findings demonstrate the potential for using mesotrione-resistant genotypes, especially G-10 and its hybrids, in breeding programs to develop POST herbicide-resistant sorghum cultivars. This

study provides foundational insights into phenotypic variation for mesotrione resistance and supports ongoing genetic improvement efforts targeting weed control in sorghum.

2.1 Introduction

Grain sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop globally and the fourth-largest grain crop in the United States by acreage, following corn (*Zea mays* L.), soybean (*Glycine max* L. Merrill), and wheat (*Triticum aestivum* L.) (Hao et al., 2021; Ciampitti & Prasad, 2019; Dille et al., 2020). Grain sorghum is a highly versatile crop cultivated for various purposes, including food, livestock feed, and biofuel production (Ciampitti et al., 2019). Despite being a climate resilient crop, weed infestation particularly the control of grass weeds has been one of the major challenges in its production and can cause up to 60% of yield loss if left uncontrolled (Thompson et al., 2019; Dille et al., 2020). Although several POST herbicides effectively control broadleaf weeds in sorghum, options for grass weed control are limited due to the crop's sensitivity to grass-selective herbicides (Thompson et al., 2019).

The 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibitors (e.g., mesotrione and tembotrione) are predominantly utilized for selective preemergence (PRE) and postemergence (POST) weed control, mainly in crops such as corn, barley, oat, rice, sorghum, sugarcane, and wheat (Jhala et al., 2023). HPPD-inhibiting herbicides are commonly used for weed control in corn including grasses due to the crop's natural tolerance, which is primarily attributed to rapid oxidative metabolism via cytochrome P450 monooxygenases and lower herbicide absorption (Grossmann & Ehrhardt, 2007; Mitchell et al., 2001; Pataky et al., 2008). However, these herbicides are not registered as POST in grain sorghum due to crop injury. HPPD-inhibiting herbicides induce tissue bleaching by disrupting carotenoid biosynthesis, which leads to lipid peroxidation of cell membranes, resulting in tissue necrosis and ultimately plant death.

(Beaudegnies et al., 2009; Lee et al., 1998; Van Almsick, 2009). Although widely used, herbicide resistance to HPPD inhibitors has so far been confirmed only in common waterhemp (*Amaranthus tuberculatus*), Palmer amaranth (*Amaranthus palmeri*), wild radish (*Raphanus raphanistrum* L), and one grass weed, Chinese sprangletop (*Leptochloa chinensis* (L.) Nees.) (Nakka et al., 2017; Heap, 2025). Due to sorghum's susceptibility to POST-applied grass herbicides, grass weed control is currently dependent on pre-emergence (PRE) options. Given the low likelihood of discovering new herbicide modes of action selective to sorghum, developing herbicide-resistant hybrids such as those tolerant to ALS-, ACCase-, or HPPD-inhibitors offers a practical solution for enabling POST grass weed control (Thompson et al., 2019; Pandian et al., 2021).

Mesotrione is a synthetic herbicide from the triketone family, chemically known as 2-[4-(methylsulfonyl)-2-nitrobenzoyl]-1,3-cyclohexanedione. It acts by inhibiting the enzyme HPPD, which plays a vital role in tyrosine catabolism and the biosynthesis of plastoquinones, tocopherols, and carotenoids (Beaudegnies et al., 2009). Previous studies have demonstrated that some sorghum hybrids exhibit reduced injury from POST-applied mesotrione compared to more sensitive hybrids (Abit & Al-Khatib, 2009). Similarly, Pandian et al. (2023) identified two genotypes, G-1 and G-10, with a high level of resistance to mesotrione relative to a sensitive genotype, S-1. This resistance was associated with more rapid herbicide metabolism, suggesting the involvement of cytochrome P450 enzymes. Further inheritance studies indicated that mesotrione resistance in these genotypes is controlled by a single dominant gene.

Building on these findings, we hypothesize that hybrids from mesotrione-resistant genotypes (G-1 and G-10) crossed with U.S. sorghum lines will demonstrate enhanced resistance to mesotrione. The specific objectives of this study were to evaluate the response of parental

resistant genotypes and their hybrids to mesotrione application and quantify visual injury, chlorophyll content, and biomass accumulation.

2.2 Materials and Methods

2.2.1 Plant Material and Growth Conditions

This study included a range of sorghum genotypes with known differences in mesotrione sensitivity. The resistant genotype G-10 and the susceptible genotype S-1 were used along with their hybrids derived from crosses with tembotrione-resistant genotypes G-200 and G-350. Additional U.S. sorghum lines and hybrids, including Pioneer 84G62, RTx430, and DKS 37-07, were also evaluated. In all hybrid combinations, the first genotype listed was used as the female parent and the second as the male parent (e.g., G-200/G-10 indicates G-200 as the female and G-10 as the male). Seeds of all genotypes were individually planted in square pots ($6 \times 6 \times 6$ cm) filled with a commercial potting mix (ProMix Ultimate; Premier Tech Horticulture, Mississauga, Ontario, Canada) and grown in a greenhouse under controlled environmental conditions. The greenhouse was maintained at 25/20°C (day/night), with a 15/9 h photoperiod, $60 \pm 10\%$ relative humidity, and a light intensity of $750 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by both natural and supplemental lighting. Plants were watered as needed and fertilized weekly with MiracleGro (Scotts MiracleGro Products, Inc., Marysville, OH).

2.2.2 Whole Plant Dose-Response Assay

At 4-5 leaf stage, all the genotypes were treated with mesotrione (Callisto, Syngenta Group, Basel, Switzerland) along with 1% crop oil concentrate (COC) and 2.5% ammonium sulfate using a bench-top track spray chamber (Generation III, De Vries Manufacturing, Hollandale, MN). The chamber was fitted with a single flat-fan nozzle (80015LP TeeJet tip; Spraying Systems, Wheaton, IL, USA) operated at a speed of 2.96 mph, delivering a spray

volume of 187 L ha⁻¹ at a pressure of 36.5 psi. For the dose–response assay, sorghum seedlings were treated with mesotrione at rates of 0, 26.25, 52.5, 105 (field-recommended), 210, 420, and 840, g ai ha⁻¹. Aboveground biomass was collected at 3 WAT, dried in a hot air oven at 60°C for 72 hours, and then weighed. Each herbicide treatment included eight to ten replicates and was repeated once following the same protocol as described by Pandian et al (2023).

2.2.3 Visual Injury and Chlorophyll Index Measurement

Herbicide injury was evaluated at 1, 2, and 3 weeks after treatment (WAT) by visually scoring plants relative to non-treated controls, using a scale from 0% (no injury) to 100% (complete plant death). Chlorophyll index were measured on the upper surface of the fully opened fourth leaf with a collar. The measurements were taken 1, 2, and 3 weeks after treatment using a chlorophyll meter (SPAD 502 Plus Chlorophyll Meter, Spectrum Technologies, Inc., Aurora, IL, USA).

2.2.4 Statistical Analysis

The data from both runs of experiments were pooled as there was no significant difference between the runs from Lavene’s test ($P>0.05$). All the experiments were conducted in a completely randomized design and were repeated in time. The dry biomass data collected from each experiment was converted to relative dry biomass by using the following

Equation-1:

$$\text{Relative dry biomass (\% of non-treated)} = \frac{\text{dry biomass of individual treated plants}}{\text{average dry biomass of all the non-treated plants}} \times 100$$

The relative dry biomass calculated was subjected to non-linear regression analysis using a three-parameter log-logistic model using a ‘drc’ package (Knezevic et al., 2007; Ritz et al., 2015) available in R software (version 4.4.0). It uses the following equation.

Equation-2:

$$Y = \{d / 1 + \exp[b(\log - \log e)]\}$$

In the equation above Y is the response, and b is the dose, where d is the upper limit, b is the slope of the curve, and e is GR₅₀ which is the herbicide dose required for 50% reduction in plant biomass. To assess the fit of data to the regression model, a Lack-of-fit test was performed using the model fit function of 'drc.'

2.3 Results

2.3.1 Mesotrione Dose-response Assay

The analyses of dose-response results suggest considerable variation of sorghum genotypes to various doses of mesotrione (Figure 2.1 A and Figure 2.1 B). The susceptible line S-1 showed the lowest resistance, with a GR₅₀ of 38 g ai ha⁻¹, while the resistant parental line G-10 exhibited significantly higher resistance, with a GR₅₀ of 337 g ai ha⁻¹, consistent with the findings of Pandian et al. (2023). The highest level of resistance was observed in the F₁ progeny of G-200 x G-10 (G-200 as the female and G-10 as the male parent), with a GR₅₀ of 427 g ai ha⁻¹, representing an 11.2-fold increase in resistance compared to S-1 (Table 2.1).

Based on the visual injury and biomass accumulation, sorghum genotypes showed varied response. From highly sensitive, moderately sensitive, intermediate and resistant as shown in Table 4.1. Besides, S-1, RTx430, also had pronounced injury and biomass reduction with low GR₅₀ of 73 g ai ha⁻¹. Genotypes G-350, DKS37-07 and the F₁ hybrid of G-10 x G-350 showed moderate sensitivity to the mesotrione (Figure 2.1 A and Table 2.1). While as the genotypes G-200 and P84G62 showed intermediate response to mesotrione requiring approximately 4.1 and 4.9, fold higher mesotrione doses than S-1, respectively, to achieve 50% growth reduction (Table 2.1). Results were promising with genotypes involving mesotrione resistant parent G-10 and its

F₁ progeny hybrids G-10 x G-200 and G-200xG-10 showing high level of resistance to mesotrione with GR₅₀ of 337, 230, and 427 g ai ha⁻¹, respectively (Figure 2.1 A, Figure 2.1 B, and Table 2.1). These findings are consistent with Pandian et al. (2023) who found high level of resistance in G-10 and F₁ progeny of S-1 x G-10 with a GR₅₀ of 313 and 1336 g ai ha⁻¹, respectively under greenhouse conditions, primarily governed by a single dominant gene.

Overall, the dose-response results confirmed that the resistant genotypes maintained higher relative dry weight across increasing mesotrione doses, while susceptible genotypes exhibited a sharp decline. These findings demonstrate significant genotypic variation in mesotrione resistance and underscore the potential for utilizing resistant lines such as G-10 in breeding programs aimed at developing HPPD-inhibitor resistant sorghum varieties.

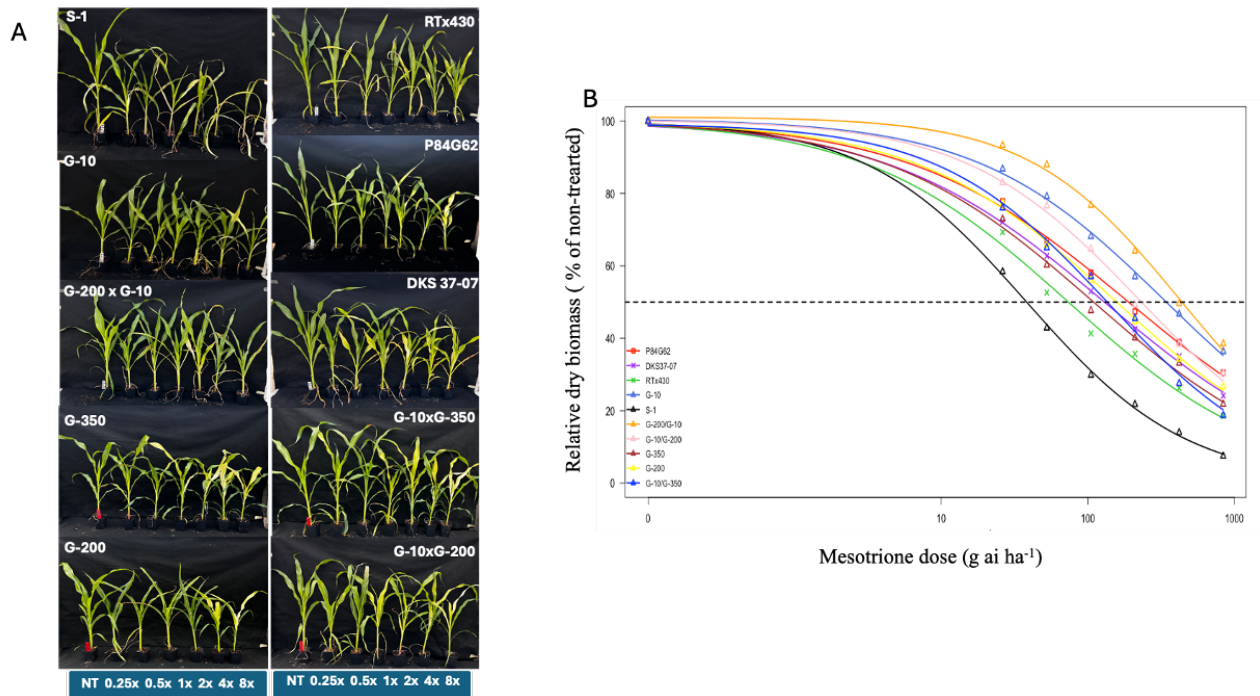


Figure 2.1 A) Response of parents S-1 (susceptible), G-10 (mesotrione-resistant), G-200 and G-350 (tembotrione-resistant), F₁ (G-200 x G-10), F₁ (G-10 x G-200), F₁ (G-10 x G-350), P84G62, DKS37-07, RTx430 (commercial hybrids/lines) to different doses of mesotrione. B) Mesotrione dose-response curves representing the above ground dry biomass S-1 (susceptible), G-10 (mesotrione-resistant), G-200 and G-350 (tembotrione-resistant), F₁ (G-200 x G-10), F₁ (G-10 x G-200), F₁ (G-10 x G-350), and commercial hybrids/lines viz., P84G62, DKS37-07, using the three-parameter log-logistic model.

Table 2.1 Regression parameters describing the response of sorghum genotypes and their F₁ progeny to mesotrione under greenhouse conditions (GR₅₀: dose required for 50% growth reduction; SE: standard error; RI: Resistance index R/S; Resistant/Susceptible)

Genotype	GR₅₀ (SE)	RI (R/S)
S-1	38 (5)	1
RTx430	73 (15)	1.9
G-350	108 (23)	2.8
DKS37-07	129 (21)	3.3
G-10 x G-350	138 (26)	3.6
G-200	157 (33)	4.1
P84G62	187 (30)	4.9
G-10 x G-200	230 (43)	6
G-10	337 (48)	8.8
G-200 x G-10	427 (71)	11.2

2.3.2 Visual Injury

Mesotrione injury symptoms (leaf chlorosis, bleaching, and subsequent necrosis) began to appear one WAT and became pronounced by 2 WAT. Thereafter, injury symptoms began to subside in intermediate and resistant genotypes as plants showed signs of recovery (Figure 2.1 A). Visual injury assessments following mesotrione application revealed substantial genotypic variation among the evaluated sorghum genotypes (Figure 2.2). The susceptible genotype S-1 exhibited > 50% of visual injury ($ED_{50} = 91 \text{ g ai ha}^{-1}$) with significant bleaching and biomass reduction at field recommended dose and increased with the increasing dose. In contrast, the resistant parental line G-10 displayed significantly reduced injury < 10% at 1x dose, with an ED_{50} of 296 g ai ha^{-1} and an SI of 3.2 (SI is the sensitivity index based on the ratio of ED_{50} value of resistant to susceptible, R/S); , indicating a nearly threefold increase in resistance relative to S-1. The highest level of mesotrione resistance was observed in the F_1 hybrid G-200 $\times$ G-10 (visual injury < 10% at 1x) with the requirement of $\sim$ five times more mesotrione required to cause 50% of visual injury than S-1.

Other genotypes also showed moderate to high levels of resistance. Commercial hybrid Pioneer 84G62 and the tembotrione-resistant parental line G-200 and the F_1 progeny of G-10/G-200 showed intermediate response with ED_{50} values of 210, 192, and 233 g ai ha^{-1} with < 30% of visual injury at 1x, respectively. Additionally, RTx430, DKS 37-07, G-10 $\times$ G-350, and G-350 >30% visual injury at 1x dose of mesotrione and displayed relatively moderate sensitivity, with ED_{50} values of 106, 116, 125, 133 g ai ha^{-1} , respectively. This shift in the visual injury trend could be linked to their genetic background; however, despite exhibiting more bleaching, these genotypes were still able to produce new growth by 3 WAT. These results were consistent with the visual trends found in dose-response assay (Figure 2.1 and Table 2.1), where more resistant

genotypes showed delayed onset and reduced magnitude of injury symptoms compared to susceptible lines.

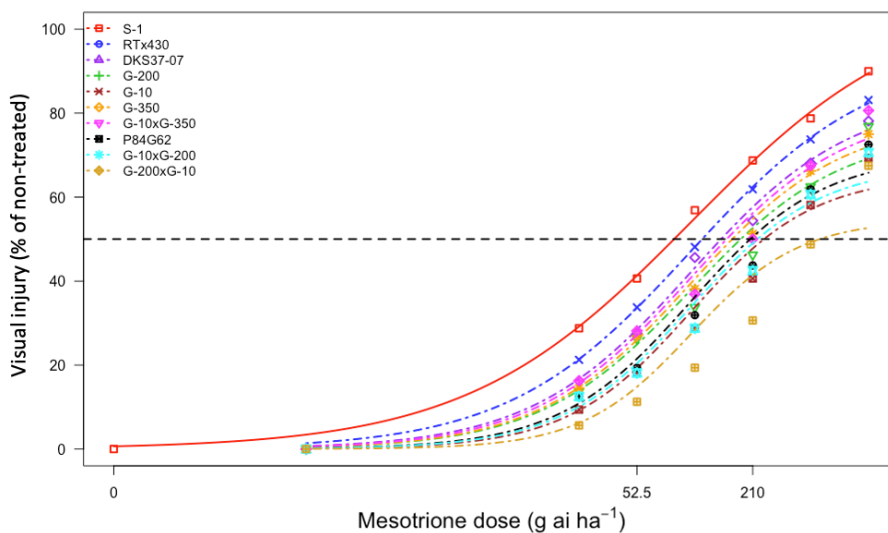


Figure 2.2 Mesotrione dose-response curves representing the visual injury of S-1 (susceptible), G-10 (mesotrione-resistant), G-200 and G-350 (tembotrione-resistant), F₁ (G-200 x G-10), F₁ (G-10 x G-200), F₁ (G-10 x G-350), and commercial hybrids/lines viz., P84G62, DKS37-07, using the three-parameter log-logistic model.

Table 2.2 Regression parameters describing the response of sorghum genotypes and their F₁ progeny to mesotrione under greenhouse conditions (ED₅₀: dose required for 50% visual injury; SE: standard error; SI: Sensitivity index R/S; Resistant/Susceptible)

Genotype	ED₅₀ (SE)	SI (R/S)
S-1	91 (18)	1
RTx430	106 (25)	1.1
G-350	133 (42)	1.7
DKS37-07	116 (27)	1.2
G-10 x G-350	125 (43)	1.6
G-200	192 (63)	2.1
P84G62	210 (55)	2.3
G-10 x G-200	233 (54)	2.5
G-10	296 (62)	3.2
G-200 x G-10	451 (89)	4.9

2.3.3 Chlorophyll Retention Response (SPAD Readings/Chlorophyll Index)

Mesotrione application causes characteristic bleaching symptoms by inhibiting carotenoid biosynthesis, resulting in oxidative degradation of chlorophyll and impaired photosynthesis (Beaudegnies et al., 2009; van Almsick, 2009; McCurdy et al., 2008).

Chlorophyll index, measured through SPAD readings, declined in a dose-dependent manner across all sorghum genotypes (Figure 2.1A and Figure 2.3). Among all genotypes, S-1 exhibited the highest sensitivity, with a sharp reduction in SPAD index even at low doses of mesotrione. The chlorophyll index dropped below 30% of the untreated control at the 0.5x rate, with an ED₅₀ of 78 g ai ha⁻¹, confirming its susceptibility to mesotrione-induced bleaching.

In contrast, the resistant parental line G-10 and its F₁ hybrid G-200 × G-10 maintained higher chlorophyll index under increasing mesotrione doses. These genotypes exhibited ED₅₀ values of 231 and 296 g ai ha⁻¹, respectively, and the highest sensitivity index values (2.96 and 3.79), indicating enhanced chlorophyll retention and improved physiological tolerance to oxidative stress. Intermediate chlorophyll retention was observed in genotypes like G-10 × G-200 (139 g ai ha⁻¹), P84G62 (130 g ai ha⁻¹), and G-200 (124 g ai ha⁻¹), with SI values ranging from 1.58 to 1.78. The hybrids G-10 × G-350 and DKS37-07 also demonstrated moderate resistance with ED₅₀s of 111 and 106 g ai ha⁻¹, respectively. Genotypes such as RTx430 (102 g ai ha⁻¹) and G-350 (98 g ai ha⁻¹) showed slightly increased resistance compared to S-1 but still belonged to the more sensitive group. While their ED₅₀ values were marginally higher, they still experienced considerable bleaching, particularly noticeable even at 3 WAT.

Despite the narrower range of ED₅₀ values observed for chlorophyll index compared to visual injury or biomass reduction, the trend remained consistent: more resistant genotypes

retained chlorophyll more effectively, while susceptible types like S-1 and RTx430 experienced rapid and sustained bleaching after mesotrione exposure.

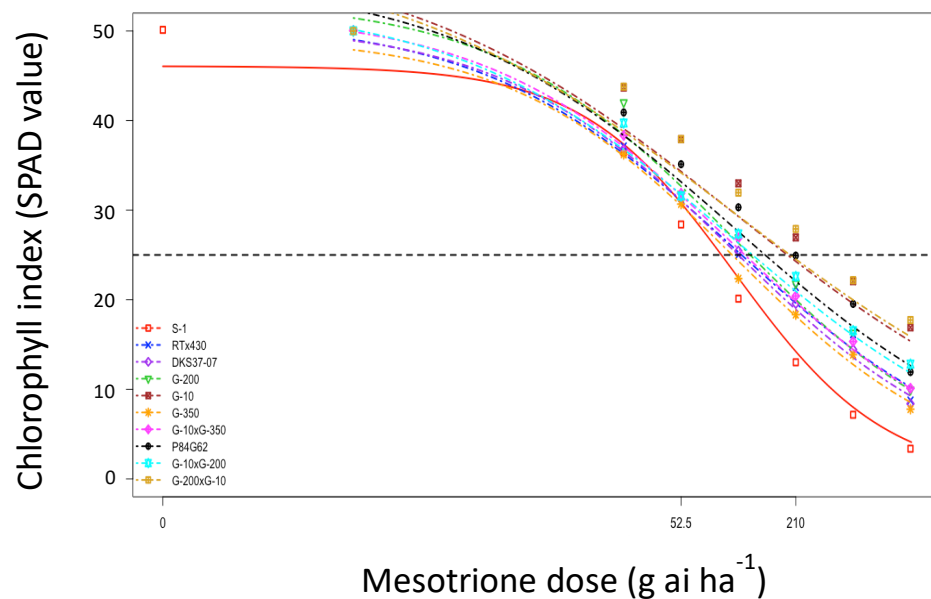


Figure 2.3 Mesotrione dose-response curves representing leaf chlorophyll index (% of non-treated, NT) of S-1 (susceptible), G-10 (resistant), G-200 and G-350 (tembotrione tolerant), F₁ (G-200 x G-10), F₁ (G-10 x G-200), F₁ (G-10 x G-350), and commercial hybrids/lines viz., P84G62, DKS37-07 at 3 WAT.

Table 2.3 Regression parameters describing the response of sorghum genotypes and their F₁ progeny to mesotrione under greenhouse conditions (ED₅₀: dose required for 50% of chlorophyll reduction; SE: standard error; SI: Sensitivity index R/S; Resistant/Susceptible)

Genotype	ED₅₀ (SE)	SI (R/S)
S-1	78 (26)	1
RTx430	102 (36)	1.3
G-350	98 (29)	1.25
DKS37-07	106 (39)	1.35
G-10 x G-350	111 (32)	1.42
G-200	124 (31)	1.58
P84G62	130 (43)	1.66
G-10 x G-200	139 (32)	1.78
G-10	231 (54)	2.96
G-200 x G-10	296 (53)	3.79

2.4 Discussion

Our findings demonstrated a consistent increase in mesotrione resistance the genotype G-10 and its F_1 progeny $G-200 \times G-10$ (Table 2.1). These results align with the observations of Pandian et al. (2023), who reported elevated resistance levels in G-10 and its F_1 progeny with S-1 ($S-1 \times G-10$), exhibiting GR_{50} values of 848 and 1336 g ai ha⁻¹, respectively much higher than the 70 g ai ha⁻¹ observed for S-1. Similarly, the visual injury symptoms recorded in this study followed trends consistent with earlier findings in grain sorghum exposed to mesotrione. Abit et al. (2009) screened 85 commercial hybrids and identified DKS35-70 as the most resistant, with a GR_{50} of 209 g ai ha⁻¹ under greenhouse conditions. In this research, although foliar application of mesotrione caused visible injury in all genotypes by 1 WAT, the resistant genotypes exhibited rapid recovery and had less than 10% injury by 3 WAT, compared to the persistent symptoms seen in sensitive and moderately sensitive lines. This variation in response is expected due to the diverse genetic backgrounds among sorghum hybrids, which can influence cuticle structure, herbicide absorption, translocation, and metabolism (Bunting et al., 2004; Armel et al., 2005).

The F_1 progeny of $G-200 \times G-10$ and its reciprocal cross exhibited ~11- and ~6-fold increases in mesotrione resistance relative to S-1, supporting the dominant inheritance of mesotrione resistance in G-10 findings consistent with those of Pandian et al. (2023). The reduced visual injury and higher chlorophyll index in resistant genotypes can be attributed to their superior ability to metabolize mesotrione, thereby minimizing oxidative stress. These results are further supported by earlier studies on sweet corn, where differential resistance to POST-applied mesotrione was linked to differences in metabolic rates. Tolerant cultivars metabolized mesotrione more rapidly than sensitive ones (O'Sullivan et al., 2002; Wichert et al.,

1999). Importantly, certain herbicide resistance in grain sorghum and other species has been shown to be metabolism-based rather than target-site mediated, primarily involving cytochrome P450 enzymes (Lu et al., 2020; Pandian et al., 2023). Mesotrione metabolism in sorghum hybrids has been previously confirmed (Abit and Al-Khatib, 2009), with cytochrome P450 enzymes converting mesotrione into the non-toxic metabolite 4-hydroxy-mesotrione (Alferness & Wiebe, 2002). This detoxification pathway is also observed in corn (Williams et al., 2008), Palmer amaranth (*Amaranthus palmeri*; Nakka et al., 2017), and common waterhemp (*Amaranthus tuberculatus*; Ma et al., 2013; Kaundun et al., 2017).

The rising challenge of herbicide-resistant weeds in grain sorghum particularly grass weeds has led to increasing interest in developing herbicide-resistant sorghum technologies. Pandian et al. (2023) reported that mesotrione resistance in grain sorghum is governed by a single dominant gene with minor-effect alleles, making it feasible to map and transfer this trait into elite U.S. sorghum backgrounds. Advanced molecular tools like bulk segregant analysis (BSA), genome-wide association studies (GWAS), single nucleotide polymorphism (SNP) discovery, and Kompetitive Allele-Specific PCR (KASP) genotyping have proven vital for identifying, mapping, and introgression herbicide resistance traits in crops and weeds.

In common waterhemp, BSA mapped HPPD-inhibitor resistance to two major QTLs. SSR markers such as *Amaranthus*_SSR345 enabled introgression of a 15.2-Mb resistance haplotype into susceptible biotypes through controlled crosses, achieving 92% transmission efficiency (Murphy et al., 2021). In rice, GWAS across diverse cultivars identified key resistance loci like RGlu6 and RGly8, with SNP markers (e.g., Chr6:12,784,501) facilitating the pyramiding of glufosinate, glyphosate, and mesotrione tolerance into elite lines (Xia et al., 2024). In sunflower (*Helianthus annuus* L.), marker-assisted backcrossing (MABC) enabled rapid

introgression of *AhasII* alleles, conferring resistance through systems like IMISUN (Imazethapyr Sunflower) and SURES (Sulfonylurea-Resistant Sunflower). Foreground selection with flanking SNP markers and background selection using genome-wide SSRs minimized linkage drag and reduced breeding cycles by 40% (Bulos et al., 2013). These approaches supported the development of hybrids like IMISUN, SURES, and CLPlus (Clearfield Plus). KASP assays have further streamlined breeding pipelines. In *Brassica napus*, high-throughput KASP screening for ALS mutations (e.g., *Pro197Ser*) replaced phenotypic selection, allowing genotyping of 5,000 BC₁F₁ plants per week and facilitating the efficient introgression of sulfonylurea resistance (Guo et al., 2023). Extensive field evaluation of mesotrione resistant parents and hybrids along with susceptible checks could provide a steppingstone in moving ahead for the development of herbicide resistant sorghum technology. Further research should focus on the identification of specific QTLs followed by marker identification and validation and finally the introgression of the trait of interest.

2.5 Conclusion

In conclusion, the sorghum genotype G-10 and its F₁ progeny, G-200 × G-10 and G-10 × G-200, exhibited a high level of resistance to mesotrione, characterized by minimal visual injury, higher chlorophyll index, and greater biomass accumulation, findings that align with previous studies. The monogenic inheritance of mesotrione resistance in G-10, as reported by Pandian et al. (2023), offers a valuable opportunity for precise introgression of this trait into elite U.S. sorghum lines through marker-assisted breeding. Overall, these results lay a strong groundwork for future genetic mapping and molecular studies aimed at advancing effective weed management strategies in grain sorghum.

Chapter 3 - Genotypic Characterization and Evaluation of Tembotrione Resistance in Grain Sorghum

Abstract

Post-emergence (POST) grass weed control in grain sorghum is limited due to crop sensitivity to grass-selective herbicides. Tembotrione, a 4-hydroxyphenylpyruvate dioxygenase (HPPD)-inhibiting herbicide, is effective against a broad spectrum of weeds but is not currently registered for use in grain sorghum due to crop injury concerns. This Chapter evaluated the response of tembotrione-resistant parental lines (G-200, G-350), a susceptible genotype (S-1), and their F₁ hybrids with different genetic backgrounds for tembotrione resistance to support breeding of herbicide-resistant sorghum cultivars. A greenhouse dose-response experiment was conducted using 22 sorghum genotypes treated with tembotrione (0 to 368 g ai ha⁻¹, 1x = 92 g ai ha⁻¹), and response was assessed based on visual injury, chlorophyll index (SPAD readings), and biomass accumulation. The results revealed considerable genotypic variation in response to tembotrione. The susceptible genotype S-1 showed a GR₅₀ of 49 g ai ha⁻¹, while the resistant parental line G-200 and its F₁ hybrid with G-10 showed much higher resistance (GR₅₀ = 186 and 174 g ai ha⁻¹, respectively), with the F₁ hybrid exhibiting the highest resistance index (RI = 3.55). Visual injury and chlorophyll index measurements aligned with biomass responses, showing less bleaching and faster recovery in tolerant genotypes. The enhanced tolerance of G-10 × G-200 hybrids suggest additive or stacked resistance mechanisms, likely due to polygenic inheritance in G-200 (previously reported) and potential complementary resistance traits from G-10. These findings demonstrate the potential of combining resistant genotypes to develop POST herbicide-tolerant sorghum hybrids and support the use of tembotrione-resistant lines in sorghum improvement programs.

3.1 Introduction

Grain sorghum (*Sorghum bicolor* (L.) Moench) is considered a key crop for both current and future agricultural systems, owing to its exceptional adaptability, higher water use efficiency, and wide range of applications (Taylor et al., 2006; Stamenković et al., 2020; McCuiston et al., 2019). Despite its resilience to various biotic and abiotic factors, grain sorghum yields are limited mostly by challenges like weed competition. A major decline in U.S. sorghum acreage over the past two decades is largely attributed to the limited availability of POST grass weed control herbicides (Sreeram et al., 2016; Tanya et al., 2020; Pandian et al., 2021).

The 4-hydroxyphenylpyruvate dioxygenase (HPPD) class of herbicides is widely used for controlling both preemergence (PRE) and postemergence (POST) broadleaf and grass weeds in crops like corn (*Zea mays* L.) and wheat (*Triticum aestivum*), which exhibit natural tolerance (Sutton et al., 2002; Wang et al., 2020). However, their use as POST herbicides in grain sorghum is limited due to the risk of crop injury. These herbicides function by inhibiting the HPPD enzyme, which plays a crucial role in converting 4-hydroxyphenyl pyruvate to homogentisate, an intermediate involved in the biosynthesis of plastoquinones and tocopherols in plants (Lee et al., 1998). Plastoquinone is vital for carotenoid production, which protects chlorophyll by capturing excited electrons generated during photosynthesis. When carotenoid synthesis is disrupted, chlorophyll becomes vulnerable to photo-oxidation, leading to tissue bleaching, necrosis, and ultimately plant death (Dankov et al., 2009).

Tembotrione, a triketone-class HPPD-inhibiting herbicide, is used for selective POST control of both grass and broadleaf weeds in corn (Stephenson et al., 2015). It ranks as the second most widely used HPPD-inhibiting herbicide in the United States, with reported annual

usage exceeding 200,000 kg (Jhala et al., 2023; USDA-NASS, 2018). Additionally, tembotrione has shown greater effectiveness against grass weeds compared to other triketone herbicides (Ahrens et al., 2013). It is worth noting that tembotrione provides more effective weed control compared to atrazine (Santos et al., 2025; Williams et al., 2011), making it a valuable addition to weed management programs that traditionally rely on atrazine in sorghum cultivation. While mesotrione, a related triketone, is approved for PRE application in sorghum but not for POST use, tembotrione remains unregistered for both PRE and POST applications in sorghum. The increasing prevalence of herbicide-resistant grass weeds, coupled with limited control options and the slow pace of new herbicide discovery, underscores the need for alternative strategies. Developing herbicide-resistant sorghum hybrids offers a promising solution to enhance POST grass weed control in sorghum (Thompson et al., 2019). Pandian et al. (2020) identified two sorghum genotypes, G-200 and G-350, with resistance to tembotrione compared to a susceptible line (S-1), following the screening of 317 lines from the Sorghum Association Panel (SAP). Genetic analyses of F₁ and F₂ generations indicated that resistance to tembotrione is governed by a partially dominant polygenic trait, potentially involving rapid detoxification through cytochrome P450 activity. QTL mapping using genotype-by-sequencing further revealed genomic regions linked to this resistance in both genotypes. Building on these findings, we hypothesize that hybrids resulting from crosses between the tolerant genotypes (G-200 and G-350) and U.S. sorghum lines will exhibit improved resistance to tembotrione. The specific objectives of this study were to evaluate the response of parental resistant genotypes and their hybrids to tembotrione application and quantify visual injury, chlorophyll index, and biomass accumulation.

3.2 Materials and Methods

3.2.1 Plant Material and Growth Conditions

This study included 22 sorghum genotypes, including tembotrione-resistant lines (G-200, G-350), the susceptible line S-1, and their crosses with the mesotrione-resistant parent G-10. Commercial hybrids, public inbreds, and their crosses with G-200 and G-350 were also evaluated.

The seeds of all grain sorghum genotypes were placed individually in square pots (6×6×6 cm) filled with potting mixture (ProMix Ultimate, Premier Tech Horticulture, Mississauga, Ontario, Canada) in a greenhouse. The greenhouse was maintained at 25/20°C, 15/9 h day/night photoperiod, 60 ± 10% relative humidity, and 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of natural and supplemental light. Plants were watered as needed and fertilized weekly with MiracleGro (Scotts MiracleGro Products, Inc., Marysville, OH).

3.2.2 Whole Plant Dose-response Assay

At 4-5 leaf stage, all the genotypes were treated with tembotrione (Laudis; Bayer Crop Science, St. Louis, MO, United States) with 0.25% methylated soy oil (Destiny; WinField United) as described by Pandian et al (2020) using a bench-top track spray chamber (Generation III, De Vries Manufacturing, Hollandale, MN). The chamber was fitted with a single flat-fan nozzle (80015LP TeeJet tip; Spraying Systems, Wheaton, IL, USA) operated at a speed of 2.96 mph, delivering a spray volume of 187 L ha⁻¹ at a pressure of 36.5 psi. For the dose-response assay, sorghum seedlings were treated with tembotrione at rates of 0, 23, 46, 92 (field-recommended), 184, and 368, g ai ha⁻¹. Aboveground biomass was collected at 3 WAT, dried in a hot air oven at 60°C for 72 hours, and then weighed. Each herbicide treatment included eight to ten replicates, and all experiments and was repeated once.

3.2.3 Visual Injury and Chlorophyll Index Measurement

Herbicide injury was evaluated at 1, 2, and 3 weeks after treatment (WAT) by visually scoring plants relative to non-treated controls, using a scale from 0% (no injury) to 100% (complete plant death). Chlorophyll index was recorded on the upper surface of the fully expanded fourth leaf, identified by the presence of a collar. Measurements were taken at 1, 2, and 3 weeks after treatment using a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies, Inc., Aurora, IL, USA).

3.2.4 Statistical Analysis

Data from both experimental runs were combined, as Lavene's test indicated no significant differences between runs ($P > 0.05$). All experiments followed a completely randomized design and were repeated over time. Dry biomass data from each experiment were converted to relative dry biomass using the following approach.

Equation-1:

Relative dry biomass (% of non-treated) =

dry biomass of individual treated plants/ average dry biomass of all the non-treated plants $\times 100$

The relative dry biomass calculated was subjected to non-linear regression analysis using a three-parameter log-logistic model (Equation-2) using a 'drc' package (Knezevic et al., 2007; Ritz et al., 2015) available in R software (version 4.4.0).

Equation-2:

$$Y = \{d / 1 + \exp[b(\log - \log e)]\}$$

In the equation above Y is the response, and b is the dose, where d is the upper limit, b is

the slope of the curve, and e is GR₅₀ which is the herbicide dose required for 50% reduction in plant biomass. To assess the fit of data to the regression model, a Lack-of-fit test was performed using the model fit function of ‘drc’.

3.3. Results

3.3.1 Whole Plant Dose-response Assay

Tembotrione dose-response data analysis revealed substantial genotypic variation among the evaluated sorghum lines and hybrids (Figure 3.1 A and B, Figure 3.2 A and B, and Figure 3.3 A and B). The susceptible check S-1 showed the lowest tolerance (GR₅₀ = 49 g ai ha⁻¹) and was used as the baseline for calculating resistance indices (RI). In contrast, G-10 × G-200 exhibited the highest resistance among F₁ progeny, with a GR₅₀ of 174 g ai ha⁻¹ representing a 3.55-fold increase over S-1 (Table 3.1).

Among the parental lines, G-200 showed highest resistance (GR₅₀ = 186 g ai ha⁻¹, RI = 3.79), while G-10, previously confirmed as resistant to mesotrione, exhibited moderate resistance to tembotrione (GR₅₀ = 85 g ai ha⁻¹, RI = 1.73). The F₁ hybrid G-10 × G-200 exceeded parental GR₅₀ values, suggesting stacking of resistance alleles. Given that resistance in G-200 is known to follow a partially dominant and polygenic inheritance pattern (Pandian et al., 2020), the enhanced tolerance in the hybrid indicates additive effects from both parents. Moreover, the prior confirmation of G-10’s mesotrione resistance (Pandian et al., 2023) supports the idea that it may contribute minor alleles or complementary mechanisms (e.g., metabolism or reduced translocation) that extend resistance across HPPD inhibitors.

Based on GR₅₀ values and visual injury responses, genotypes were classified into susceptible, moderate, intermediate, and resistant groups (Table 3.1). Along with S-1, RTx430 (GR₅₀ = 62 g ai ha⁻¹) showed high sensitivity. Moderate resistance was seen in Tx7000, DKS 37-

07, G-10, BTx399, and Tx7000 $\times$ G-200 (GR_{50} = 78–94 g ai ha⁻¹). Genotypes such as P84G62, R. LBK1, BTx623, BTx3197, BTx398, and RTx430 $\times$ G-200 (Figure 3.1 and Figure 3.2) showed intermediate resistance, requiring more than twice the GR_{50} of S-1. Hybrids such as G-10 $\times$ G-350, R. LBK1 $\times$ G-350, BTx398 $\times$ G-200, G-200 $\times$ BTx3197, and BTx623 $\times$ G-200 also exhibited intermediate resistance, with GR_{50} values between 99 and 140 g ai ha⁻¹ (Table 3.3). Among the most resistant entries were G-350 (140 g ai ha⁻¹), G-200 $\times$ G-10 (125 g ai ha⁻¹), G-200 (168 g ai ha⁻¹), and G-10 $\times$ G-200 (174 g ai ha⁻¹).

Overall, genotypes with higher GR_{50} values maintained greater relative biomass and exhibited less visual injury under increasing tembotrione doses. These findings highlighted polygenic and partially dominant nature of resistance in G-200, establishing G-10 $\times$ G-200 as a strong candidate for breeding HPPD-inhibitor-resistant sorghum cultivar.

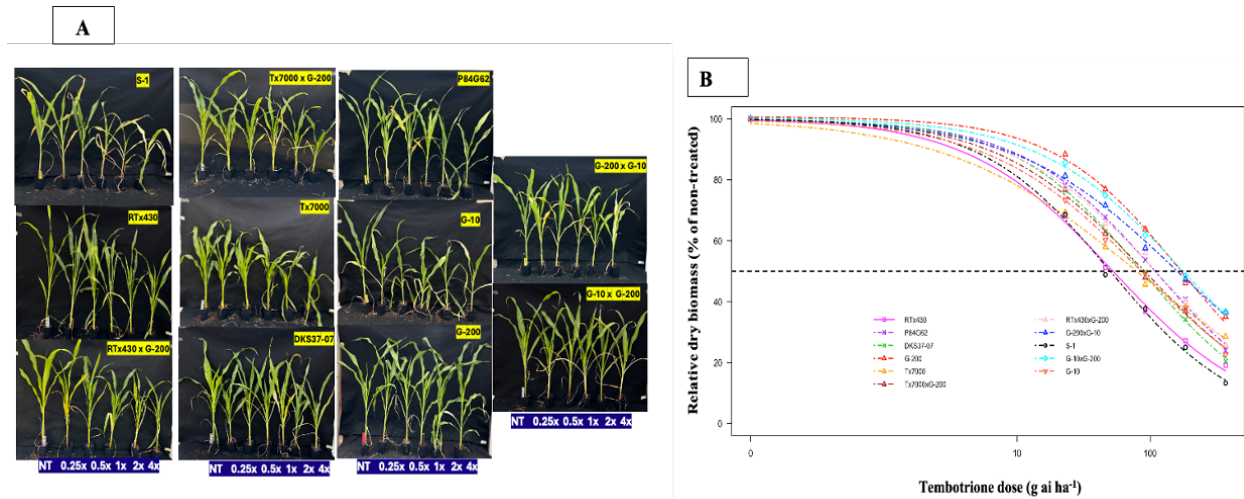


Figure 3.1A) Response of parents S-1 (susceptible), G-10, G-200 (tembotrione resistant), F₁ (G-200 x G-10), F₁ (G-10 x G-200), F₁ (Tx7000 x G-200), F₁ (RTx430 x G-200), and P84G62, DKS37-07, RTx430, Tx7000 (commercial hybrids and public inbreds) to different doses of tembotrione. B) Tembotrione dose-response curves representing the above ground dry biomass S-1 (susceptible), G-10, G-200 (tembotrione resistant), F₁ (G-200 x G-10), F₁ (G-10 x G-200), F₁ (Tx7000 x G-200), F₁ (RTx430 x G-200), and P84G62, DKS37-07, RTx430, Tx7000 (commercial hybrids/lines), using the three-parameter log-logistic model.

Table 3.1 Regression parameters describing the response of sorghum genotypes and their F₁ progeny to tembotrione under greenhouse conditions (GR₅₀: dose required for 50% growth reduction; SE: standard error; RI: Resistance index R/S; Resistant/Susceptible)

Genotype	GR50 (SE)	RI (R/S)
S-1	49 (6.9)	1
RTx430	62 (8)	1.26
Tx7000	78 (14)	1.59
G-10	85 (19)	1.73
DKS37-07	87 (12)	1.77
Tx7000 x G-200	89 (14)	1.81
P84G62	96 (16)	1.95
RTx430 x G-200	110 (16)	2.24
G-200 x G-10	125 (27)	2.55
G-200	186 (23)	3.79
G-10 x G-200	174 (38)	3.55

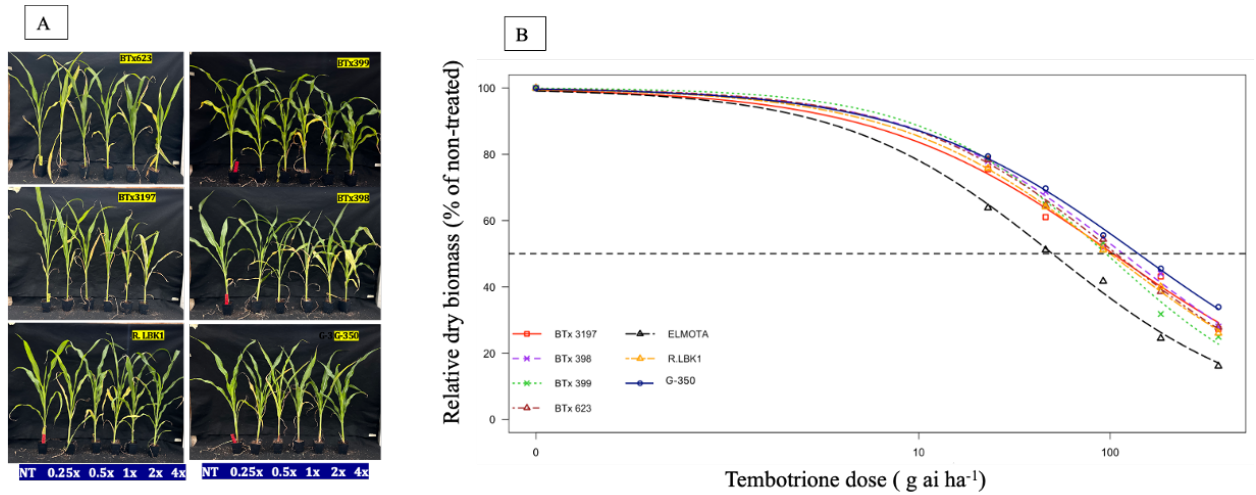


Figure 3.2 A) Response of sorghum genotypes, S-1 (susceptible, shown in Figure 5.1 A), BTx623, BTx399, BTx398, BTx3197, R. LBK1, and G-350 (tembotrione resistant), to different doses of tembotrione. B) Tembotrione dose-response curves representing the above ground dry biomass S-1 (susceptible), BTx623, BTx399, BTx398, BTx3197, R. LBK1, and G-350, using the four-parameter log-logistic model.

Table 3.2 Regression parameters describing the response of sorghum genotypes to tembotrione under greenhouse conditions (GR₅₀: dose required for 50% growth reduction; SE: standard error; RI: Resistance index R/S; Resistant/Susceptible)

Genotype	GR₅₀ (SE)	RI (R/S)
S-1	49 (6.9)	1
BTx399	94 (15)	1.91
BTx623	107 (14)	2.18
R.LBK1	100 (18)	2.04
BTx3197	103 (20)	2.1
BTx398	116 (21)	2.36
G-350	140 (41)	2.85

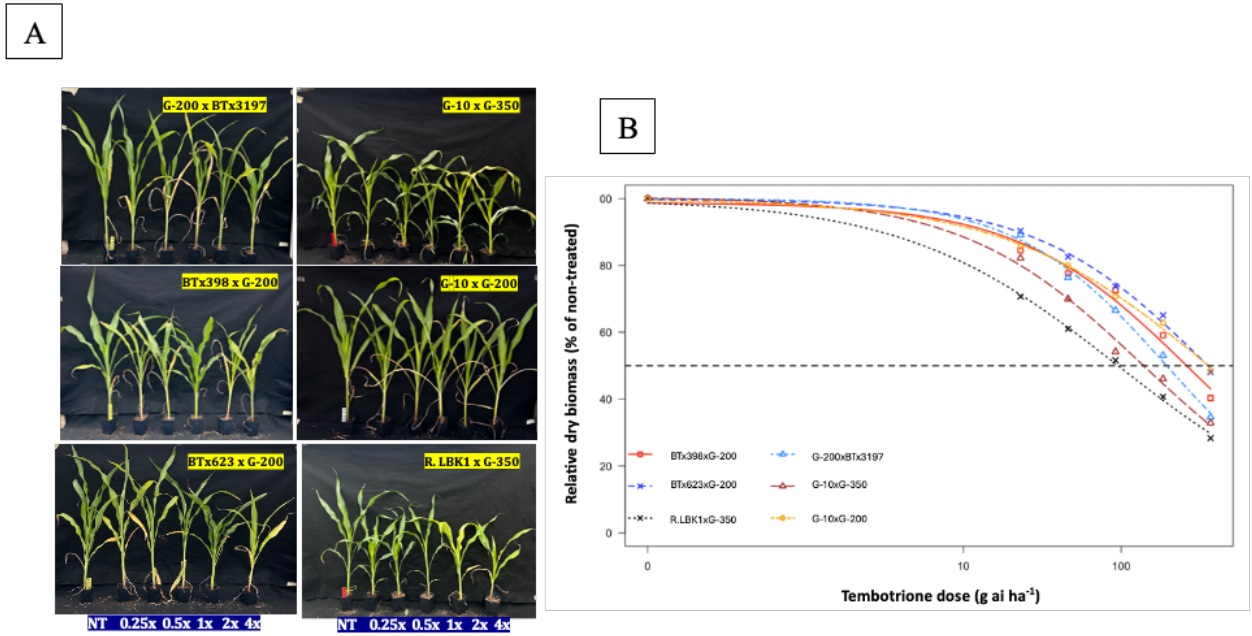


Figure 3.3 A) Response of sorghum F₁ hybrids, BTx623 x G-200, BTx398 x G-200, R. LBK1 x G-200, G-200 x BTx3197, G-10 x G-200, and G-10 x G-350, to different doses of tembotrione. B) Tembotrione dose-response curves representing the above ground dry biomass of F₁ hybrids, BTx623 x G-200, BTx398 x G-200, R. LBK1 x G-200, G-200 x BTx3197, G-10 x G-200, and G-10 x G-350, using the three-parameter log-logistic model.

Table 3.3 Regression parameters describing the response of sorghum genotypes and their F₁ progeny to tembotrione under greenhouse conditions (GR₅₀: dose required for 50% growth reduction; SE: standard error; RI: Resistance index R/S; Resistant/Susceptible)

Genotype	GR₅₀ (SE)	RI (R/S)
S-1	49 (6.9)	1
R.LBK1 x G-350	99 (18)	2.02
G-10 x G-350	105 (29)	2.14
BTx398 x G-200	132 (60)	2.69
G-200 x BTx3197	109 (37)	2.22
BTx623 x G-200	125 (44)	2.55
G-10 x G-200	174 (38)	3.55

3.3.2 Visual Injury Response of Sorghum Genotypes to Tembotrione

Tembotrione-induced injury symptoms, including chlorosis, bleaching, and necrosis, appeared within one week of application and peaked at 2 WAT. Injury began to subside by 3 WAT in intermediate and resistant genotypes, which displayed noticeable recovery through new leaf growth (Figure 3.1 A, Figure 3.2 A, and Figure 3.3 A). Visual assessments revealed clear genotypic differences in response to tembotrione, confirming trends observed in the GR₅₀-based biomass analysis.

The susceptible check S-1 exhibited >50% injury at the labeled field rate, with an ED₅₀ of 60 g ai ha⁻¹, while the F₁ hybrid G-10 × G-200 showed the highest resistance, requiring 215 g ai ha⁻¹ to achieve 50% visual injury and displaying <15% injury at the 1x (92 g ai ha⁻¹, field recommended rate) (Figure 3.1 A and Table 3.4). The resistant parent G-200, known to possess polygenic and partially dominant resistance, also showed strong resistance (ED₅₀ = 189 g ai ha⁻¹, SI = 3.15). Notably, G-10, previously confirmed to be resistant to mesotrione, exhibited moderate resistance to tembotrione with an ED₅₀ of 79 g ai ha⁻¹ (SI = 1.31), suggesting it may contribute minor alleles or complementary resistance mechanisms in F₁ crosses.

F₁ hybrids involving resistant parents consistently showed enhanced resistance. G-200 × G-10 (ED₅₀ = 129 g ai ha⁻¹), BTx398 × G-200 (ED₅₀ = 97 g ai ha⁻¹), BTx623 × G-200 (ED₅₀ = 113 g ai ha⁻¹), G-200 × BTx3197 (ED₅₀ = 104 g ai ha⁻¹), and G-10 × G-350 (ED₅₀ = 83 g ai ha⁻¹) demonstrated reduced injury compared to susceptible and moderately resistant lines, with most showing <30% injury at 1x rates (Table 3.4, Table 3.5 and Table 3.6).

Intermediate to moderately resistant responses were observed in DKS 37-07 (ED₅₀ = 97), BTx398 (ED₅₀ = 104 g ai ha⁻¹), R. LBK1 (ED₅₀ = 108 g ai ha⁻¹), BTx3197 (ED₅₀ = 99),

P84G62 ($ED_{50} = 101 \text{ g ai ha}^{-1}$), and R. LBK1 $\times$ G-350 ($ED_{50} = 74 \text{ g ai ha}^{-1}$). These genotypes displayed 25–40% injury at the field rate, suggesting partial resistance.

Genotypes such as BTx623 ($ED_{50} = 67 \text{ g ai ha}^{-1}$), Tx7000 ($ED_{50} = 88 \text{ g ai ha}^{-1}$), BTx399 ($ED_{50} = 82 \text{ g ai ha}^{-1}$), and RTx430 ($ED_{50} = 71 \text{ g ai ha}^{-1}$) exhibited moderate sensitivity, with 30–50% injury under field application rates. Crosses involving these genotypes and resistant parents (e.g., Tx7000 $\times$ G-200, RTx430 $\times$ G-200) showed slight improvements, but remained within the moderate category.

Overall, visual injury data aligned well with GR_{50} trends, where resistant genotypes maintained less injury and faster recovery under increasing herbicide stress. The high resistance observed in G-10 $\times$ G-200, exceeding both parental ED_{50} values, further supports the additive or stacking effect of resistance alleles. These findings validate the use of G-10 and G-200 as complementary resistance donors and underscore the genetic complexity governing HPPD-inhibitor resistance in sorghum.

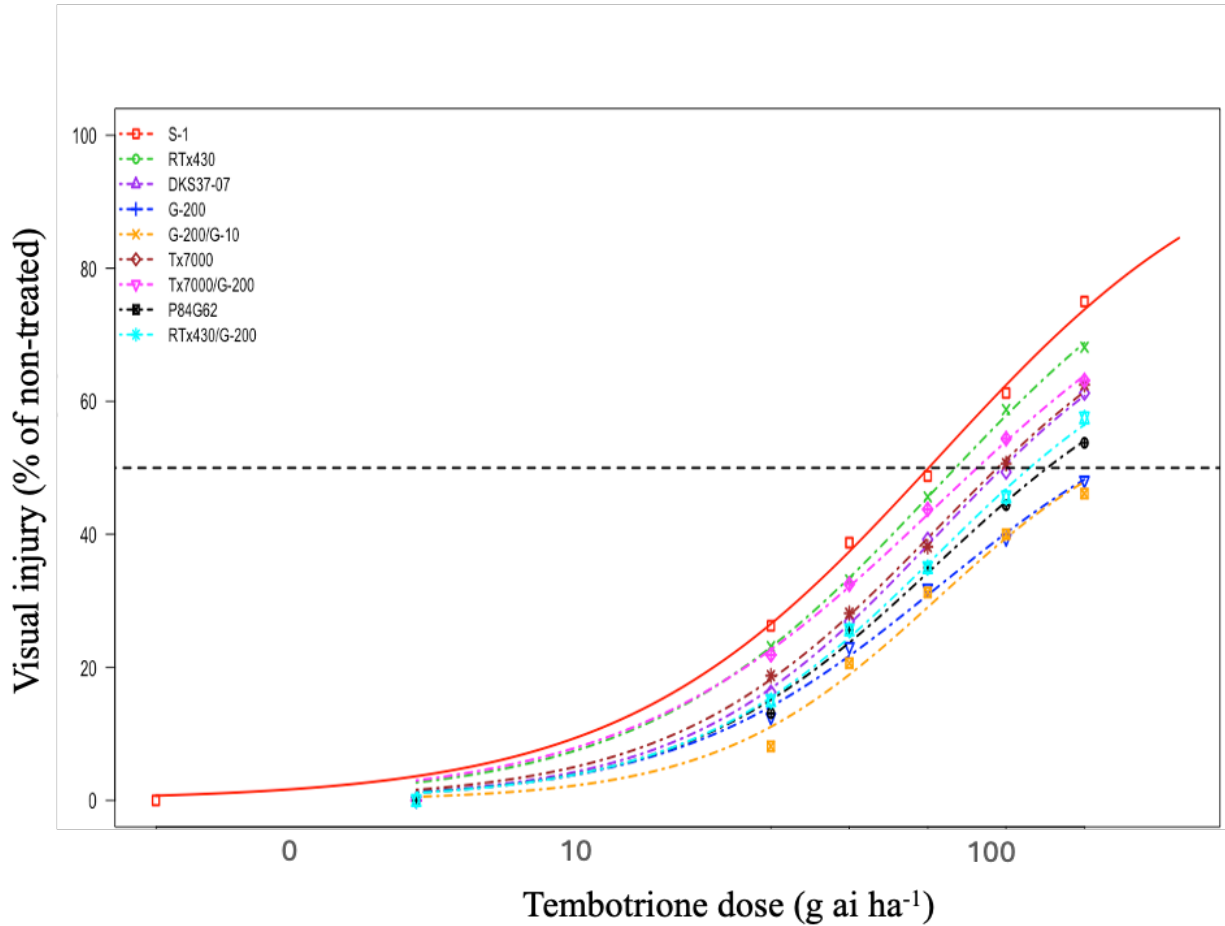


Figure 3.4 Tembotrione dose-response curves representing visual injury (% of non-treated, NT) S-1 (susceptible), G-10, G-200 (tembotrione resistant), F₁ (G-200 x G-10), F₁ (G-10 x G-200), F₁ (Tx7000 x G-200), F₁ (RTx430 x G-200), and P84G62, DKS37-07, RTx430, Tx7000 (commercial hybrids/lines), using the three-parameter log-logistic model.

Table 3.4 Regression parameters describing the response of sorghum genotypes to tembotrione under greenhouse conditions (ED₅₀: dose required for 50% visual injury; SE: standard error; RI: Sensitivity index R/S; Resistant/Susceptible)

Genotype	ED₅₀ (SE)	SI (R/S)
S-1	60 (8.8)	1
RTx430	71 (24)	1.18
Tx7000	88 (31)	1.46
G-10	79 (19)	1.31
DKS37-07	97 (35)	1.61
Tx7000 x G-200	70 (14)	1.16
P84G62	101 (16)	1.68
RTx430 x G-200	110 (31)	1.83
G-200 x G-10	129 (44)	2.15
G-200	189 (56)	3.15
G-10 x G-200	215 (64)	3.58

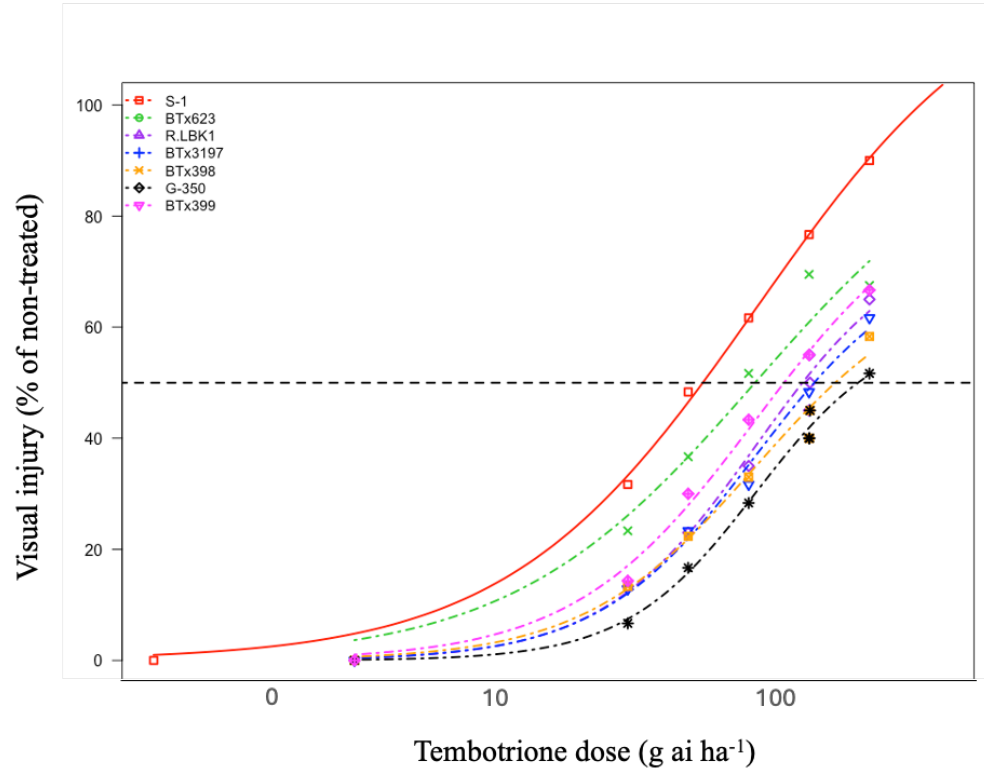


Figure 3.5 Tembotrione dose-response curves representing visual injury (% of non-treated, NT) of S-1 (susceptible), BTx623, BTx399, BTx398, BTx3197, R. LBK1, and G-350, using the three-parameter log-logistic model.

Table 3.5 Regression parameters describing the response of sorghum genotypes to tembotrione under greenhouse conditions (ED₅₀: dose required for 50% visual injury ; SE: standard error; RI: Sensitivity index R/S; Resistant/Susceptible)

Genotype	ED₅₀ (SE)	SI (R/S)
S-1	60 (8.8)	1
BTx399	82 (18)	1.36
BTx623	67 (21)	1.11
R.LBK1	108 (29)	1.8
BTx3197	99 (25)	1.65
BTx398	104 (29)	1.73
G-350	132 (38)	2.2

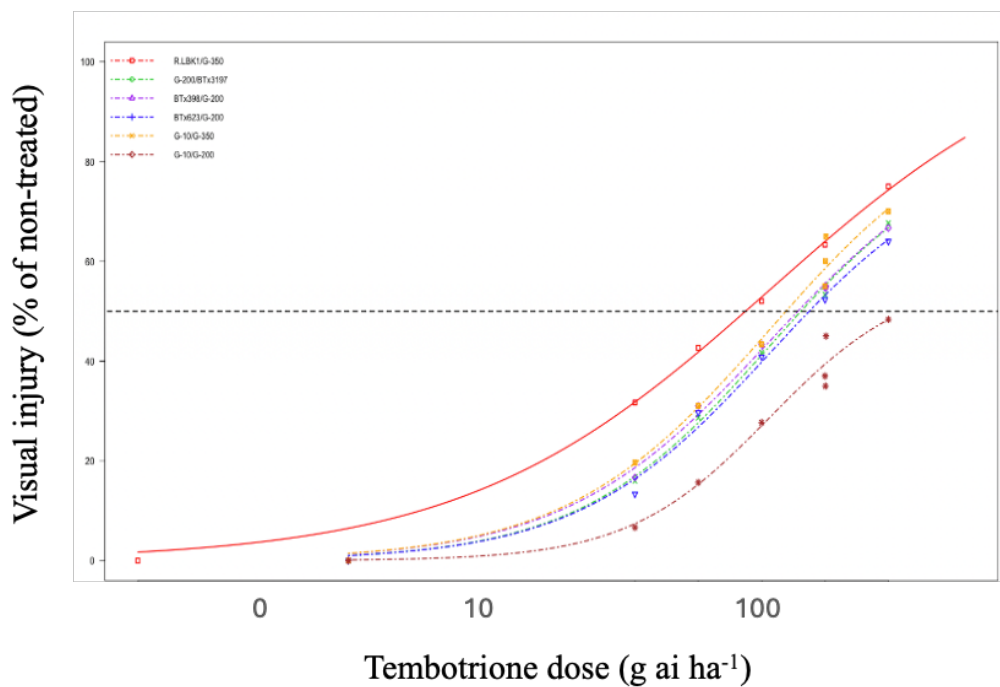


Figure 3.6 Tembotrione dose-response curves representing visual injury (% of non-treated, NT) of F₁ hybrids, BTx623 x G-200, BTx398 x G-200, R. LBK1 x G-200, G-200 x BTx3197, G-10 x G-200, and G-10 x G-350, using the three-parameter log-logistic model.

Table 3.6 Regression parameters describing the response of sorghum genotypes and their F₁ progeny to tembotrione under greenhouse conditions (ED₅₀: dose required for 50% visual injury;

SE: standard error; RI: Sensitivity index R/S; Resistant/Susceptible)

Genotype	ED₅₀ (SE)	SI (R/S)
S-1	60 (8.8)	1
R. LBK1 x G-350	74 (17)	1.23
G-10 x G-350	83 (25)	1.38
BTx398 x G-200	97 (34)	1.61
G-200 x BTx3197	104 (46)	1.73
BTx623 x G-200	113 (42)	1.88
G-10 x G-200	215 (64)	3.58

3.3.3 Chlorophyll Index (SPAD value)

The chlorophyll index (SPAD value) response, represented as ED₅₀ (the effective dose causing a 50% reduction in chlorophyll content), indicates substantial genotypic variation among sorghum lines and hybrids treated with tembotrione the susceptible control S-1 exhibited the lowest ED₅₀ of 41 g ai ha⁻¹, serving as the baseline (SI = 1). In contrast, genotypes such as G-200 (148 g ai ha⁻¹) and G-10 × G-200 (145 g ai ha⁻¹) showed the highest resistance, indicating over a threefold increase in resistance compared to S-1 (Figure 3.7). Other parental genotypes like RTx430 (68 g ai ha⁻¹), Tx7000 (81 g ai ha⁻¹), and G-10 (85 g ai ha⁻¹) exhibited moderate resistance, while their hybrids with G-200 (e.g., RTx430 × G-200, Tx7000 × G-200) consistently demonstrated enhanced ED₅₀ values ranging from 75 to 93 g ai ha⁻¹.

In the second set, G-350 (131 g ai ha⁻¹) and BTx3197 (88 g ai ha⁻¹) also displayed high resistance, while lines such as BTx398 (71 g ai ha⁻¹), BTx399 (82 g ai ha⁻¹), and BTx623 (74 g ai ha⁻¹) showed moderate levels. The hybrid R. LBK1 × G-350, however, had a lower ED₅₀ (61 g ai ha⁻¹) relative to G-350 (Figure 3.9). In the third set, G-10 × G-200 again emerged as the most tolerant hybrid (145 g ai ha⁻¹), followed by crosses like BTx623 × G-200 (98 g ai ha⁻¹) and G-200 × BTx3197 (91 g ai ha⁻¹), which had improved resistance compared to their susceptible parents. Overall, these findings emphasize the role of G-200 and G-350 as valuable donors of tembotrione resistance and support the use of these lines in breeding programs aiming to develop herbicide-resistant sorghum cultivars suitable for POST applications, particularly under high-temperature stress.

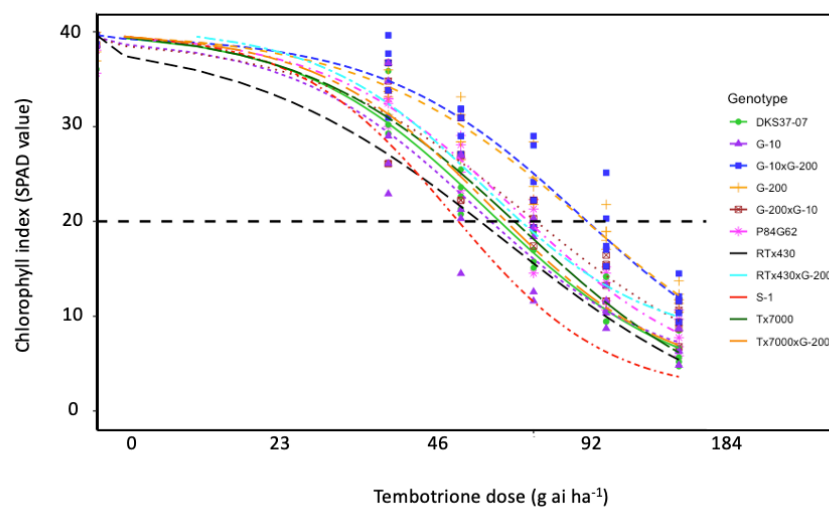


Figure 3.7 Tembotrione dose-response curves representing leaf chlorophyll index (% of non-treated, NT) S-1 (susceptible), G-10, G-200 (tembotrione resistant), F₁ (G-200 x G-10), F₁ (G-10 x G-200), F₁ (Tx7000 x G-200), F₁ (RTx430 x G-200), and P84G62, DKS37-07, RTx430, Tx7000 (commercial hybrids/lines), using the three-parameter log-logistic model.

Table 3.7 Regression parameters describing the response of sorghum genotypes and their F₁ progeny to tembotrione under greenhouse conditions (ED₅₀: dose required for 50% of chlorophyll index reduction; SE: standard error; RI: Sensitivity index SI; Resistant/Susceptible)

Genotype	ED₅₀ (SE)	SI (R/S)
S-1	41 (6)	1
RTx430	68 (26)	1.65
Tx7000	81 (30)	1.97
G-10	85 (27)	2.07
DKS37-07	72 (18)	1.75
Tx7000 x G-200	75 (23)	1.82
P84G62	83 (19)	2.02
RTx430 x G-200	93 (34)	2.26
G-200 x G-10	96 (39)	2.34
G-200	148 (31)	3.6
G-10 x G-200	145 (38)	3.53

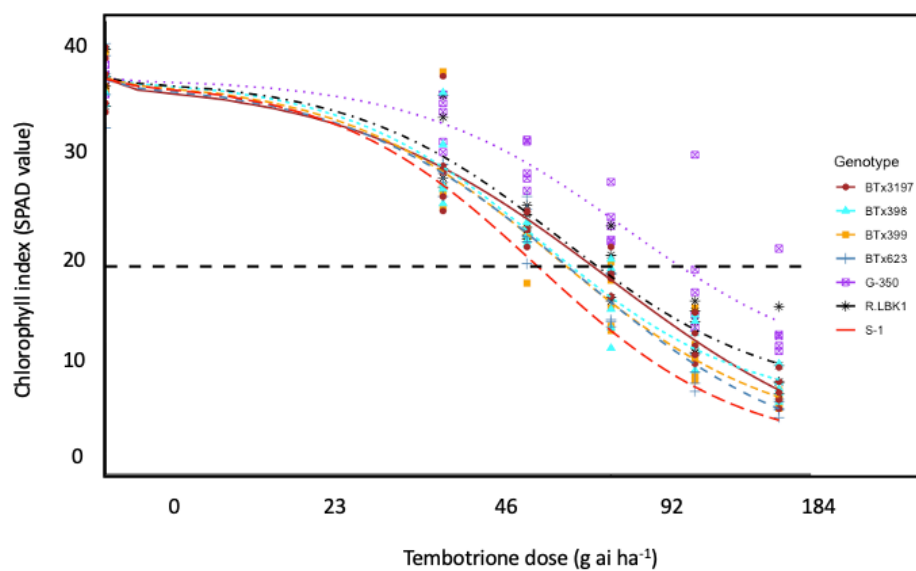


Figure 3.8 Tembotrione dose-response curves representing leaf chlorophyll index (% of non-treated, NT) of S-1 (susceptible), BTx623, BTx399, BTx398, BTx3197, R. LBK1, and G-350, using the three-parameter log-logistic model.

Table 3.8 Regression parameters describing the response of sorghum to tembotrione under greenhouse conditions (ED₅₀: dose required for 50% of chlorophyll index reduction; SE: standard error; RI: Sensitivity index SI; Resistant/Susceptible)

Genotype	ED₅₀ (SE)	SI (R/S)
S-1	41 (6)	1
BTx399	82 (27)	2
BTx623	74 (20)	1.8
R.LBK1	83 (31)	2.02
BTx3197	88 (25)	2.14
BTx398	71 (19)	1.73
G-350	131 (38)	3.19

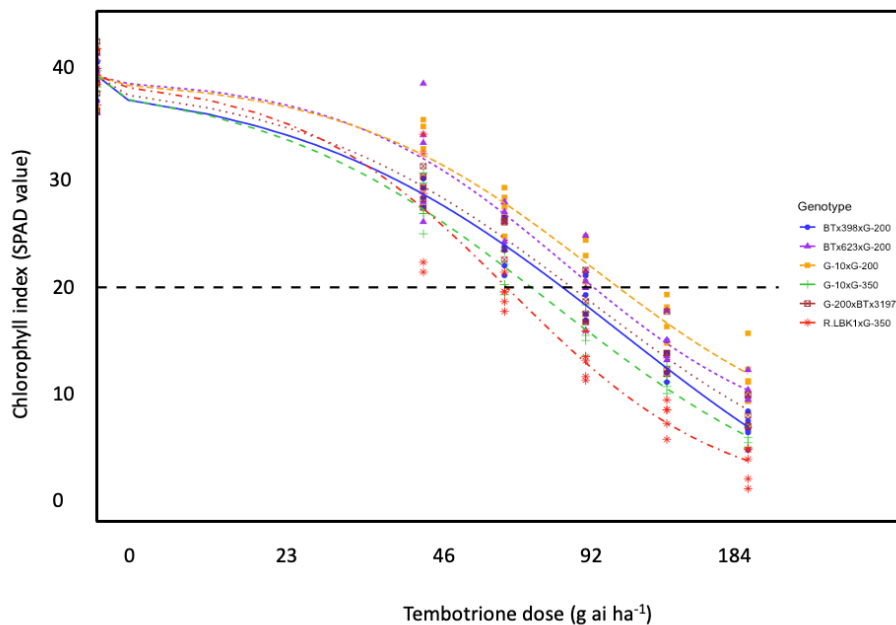


Figure 3.9 Tembotrione dose-response curves representing leaf chlorophyll index (% of non-treated, NT) of F₁ hybrids, BTx623 x G-200, BTx398 x G-200, R. LBK1 x G-200, G-200 x BTx3197, G-10 x G-200, and G-10 x G-350, using the three-parameter log-logistic model.

Table 3.9 Regression parameters describing the response of sorghum genotypes and their F₁ progeny to tembotrione under greenhouse conditions (ED₅₀: dose required for 50% of chlorophyll index reduction; SE: standard error; RI: Sensitivity index R/S; Resistant/Susceptible)

Genotype	ED₅₀ (SE)	SI (R/S)
S-1	41 (6)	1
R.LBK1 x G-350	61 (13)	1.48
G-10 x G-350	84 (25)	2.04
BTx398 x G-200	81 (20)	1.97
G-200 x BTx3197	91 (17)	2.21
BTx623 x G-200	98 (22)	2.39
G-10 x G-200	145 (38)	3.53

3.4 Discussion

Tembotrione, a HPPD-inhibiting herbicide, is commonly applied POST in corn for controlling grassy and broadleaf weeds, but it is not registered for use in sorghum due to crop injury (Damalas et al., 2018). In this study, several F_1 progeny such as G-10 $\times$ G-200, G-10 $\times$ G-350, RTx430 $\times$ G-200, and Tx7000 $\times$ G-200 developed from crosses between resistant (G-200, G-350) and relatively sensitive genotypes exhibited GR_{50} values comparable to or slightly higher than their resistant parents (Table 3.1–3.3), suggesting that tembotrione resistance in sorghum is a partially dominant trait. This aligns with Pandian et al. (2020), who reported intermediate GR_{50} values (104 and 117 g ai ha⁻¹) in F_1 progeny from S-1 $\times$ G-200 and S-1 $\times$ G-350, relative to their resistant parents G-200 (218 g ai ha⁻¹) and G-350 (172 g ai ha⁻¹).

Sorghum response to tembotrione is known to vary by genotype, application timing, dose, and environment. Early applications, especially before the five-leaf stage, increase the risk of crop injury, manifesting as foliar bleaching, stunted growth, and yield loss (Dan et al., 2012). Though commercial sorghum hybrids are generally susceptible, prior studies have documented variable responses among sorghum germplasm (Cunha et al., 2016), with susceptible genotypes experiencing severe injury including leaf necrosis and plant death (Galon et al., 2016), and yield reductions ranging from 25.9% to 61.9% (Santos et al., 2025). Our results further support this, showing broad variation in visual injury and chlorophyll retention among genotypes. Resistant genotypes (G-200, G-350) and their F_1 progeny exhibited the least visual injury and retained the most chlorophyll index at 3 WAT, while susceptible lines like S-1 and RTx430 showed greater injury (Figures 3.1–3.3).

HPPD-inhibitor resistance in sorghum is metabolism-based, not due to alterations in the HPPD target site, as confirmed by the absence of sequence differences between resistant (G-200, G-350) and susceptible (S-1) genotypes (Pandian et al., 2020). This mirrors findings in weed species and corn, where resistance to HPPD-inhibitors like tembotrione, mesotrione, and topramezone is conferred by enhanced metabolism via cytochrome P450 enzymes (Ma et al., 2013; Nakka et al., 2017; Küpper et al., 2018a; Elmore et al., 2015). In Palmer amaranth, CYP-mediated hydroxylation and glycosylation, along with overexpression of *CYP72A219* and *CYP81E8*, have been linked to resistance (Küpper, 2018b). In previous study, sorghum genotypes G-200 and G-350 exhibited significant biomass reduction when pre-treated with CYP inhibitors such as malathion or PBO before tembotrione application, supporting a CYP-mediated detoxification mechanism (Pandian et al., 2020).

Previous research suggested that in mesotrione-resistant waterhemp populations, resistance is inherited as a partially dominant, polygenic trait (Huffman et al., 2015; Kohlhas et al., 2018; Oliveira et al., 2018). In contrast, sweet corn sensitivity to tembotrione is governed by a single recessive allele (Williams & Pataky, 2008). Three QTLs associated with resistance were identified on chromosomes 2, 4, and 8, explaining 9–44% of the phenotypic variance for traits like visual injury and chlorophyll retention marking the first such report in grain sorghum (Pandian et al., 2020). Notably, the QTL on chromosome 8 showed the strongest effect and is a promising candidate for marker-assisted selection. Co-localization of these QTLs with previously reported loci related to chlorophyll fluorescence and photochemical quenching suggests possible pleiotropy (Fiedler et al., 2014; Ortiz et al., 2017). Use of genotype-by-sequencing and dense SNP markers enhanced mapping resolution, consistent with efforts in *Amaranthus tuberculatus* and rice (Murphy et al., 2021; Xia et al., 2024).

Interestingly, the F₁ hybrid G-10 × G-200 displayed even higher GR₅₀ (174 g ai ha⁻¹) and ED₅₀ (215 g ai ha⁻¹) than either of the parents, suggesting additive gene action or allele stacking. Though G-10 is less resistant to tembotrione than G-200, its known mesotrione resistance likely contributes minor alleles or alternative pathways that, when combined with G-200's major loci, enhance resistance. This strategy echoes previous allele stacking efforts in sorghum for anthracnose resistance, where ARG1, ARG2, and ARG3 were pyramided to achieve broad-spectrum immunity (Bayable et al., 2020; Muleta et al., 2022). Additionally, key pleiotropic loci (e.g., Dw1, Dw2) have been stacked to improve both disease resistance and agronomic traits such as plant height and biomass (Muleta et al., 2025; Zhao et al., 2016).

Altogether, these findings underscore the potential for improving tembotrione resistance by crossing G-200 and G-350 with elite commercial sorghum lines. Similar strategies have successfully enhanced traits like drought tolerance, stay-green, cold tolerance, and yield under diverse environments (Reddy et al., 2009; Subudhi et al., 2000; Knoll & Ejeta, 2008; Nagaraja-Reddy et al., 2013). The integration of high-density SNPs, linkage maps, and KASP assays has further streamlined breeding pipelines by allowing efficient tracking of resistance loci (Guo et al., 2023), offering a promising path toward the development of robust tembotrione-resistant sorghum cultivars.

3.5 Conclusion

In conclusion, we have identified F₁ hybrids derived from crosses between tembotrione-resistant genotypes (G-200, G-350) and moderately sensitive lines from diverse genetic backgrounds with elevated GR₅₀ and ED₅₀ values. These findings support the inheritance of tembotrione resistance as a partially dominant trait consistent with the previous findings by Pandian et al. (2020). Resistant genotypes and their hybrids also showed reduced visual injury

and greater chlorophyll index retention, providing a strong basis for introgression this trait into elite sorghum cultivars. Future research using segregating populations, such as F₂ progeny, along with the development of refined genetic maps and QTL analysis, will aid in identifying markers closely linked to tembotrione resistance. Validating these QTLs and advancing marker-assisted selection will be crucial for the development of triketone-herbicide-tolerant sorghum varieties

Chapter 4 -Physiological Response of Grain Sorghum (*Sorghum bicolor* (L.) Moench) to Mesotrione and 2,4-D under High-Temperature Stress.

Abstract

Effective post-emergence (POST) weed control is essential for maximizing grain sorghum productivity; however, high-temperature (HT) stress can adversely affect crop tolerance to herbicides. Previous studies have suggested that HT stress increases mesotrione injury in sorghum, whereas 2,4-D causes more injury under optimum temperature (OT) conditions. This study aimed to investigate the physiological basis of sorghum (P84G62 and RTx430) injury from mesotrione under high-temperature (HT) stress and increased sensitivity to 2,4-D under optimum temperature (OT) conditions. Grain sorghum genotypes were grown in two separate growth chambers maintained at either optimum temperature (OT) of 32/22°C or high temperature (HT) of 40/30°C conditions. At 4-5 leaf stage, a mixture of radiolabeled [^{14}C]- and unlabeled mesotrione or 2,4-D was applied separately to the fourth youngest leaf of each plant. Foliar uptake and translocation were quantified at 6 and 24 hours after treatment (HAT), and cytochrome P450 (CYP450)-mediated metabolism was assessed using malathion as an inhibitor. Results showed that herbicide uptake increased under HT for both herbicides, however, the most part of absorbed radioactive mesotrione remained in the treated leaf at HT, likely due to reduced metabolism, leading to greater injury. In contrast, under OT, a greater portion of the absorbed radioactive 2,4-D remained in the treated leaf, likely due to reduced metabolism associated with lower cytochrome P450 (CYP) activity, resulting in increased injury. The CYP450 inhibitor

assay further confirmed that mesotrione metabolism was suppressed under HT, indicated by significant biomass reduction when mesotrione was applied in combination with malathion. For 2,4-D, greater biomass reduction with malathion was observed under OT, likely due to reduced detoxification or CYP450 enzyme activity under these conditions. Overall, HT enhanced herbicide uptake but differentially affected the metabolism and distribution of mesotrione and 2,4-D, thereby influencing the extent of crop injury. These findings demonstrate that mesotrione and 2,4-D respond differently to temperature stress in terms of uptake, metabolism, and resulting crop injury. Therefore, selecting the appropriate herbicide and sorghum genotype is critical for minimizing injury and maintaining weed control efficacy, particularly under projected increases in global temperature.

4.1 Introduction

Grain sorghum (*Sorghum bicolor* L. Moench) is an important staple crop widely cultivated for food and fodder in the arid and semi-arid regions of Sub-Saharan Africa (SSA) and South Asia (SA). However, its productivity is significantly constrained by abiotic stresses, particularly drought and high temperature (HT), which are prevalent in these regions and contribute to ongoing food and nutritional insecurity (Prasad et al., 2021). The optimal temperature range for sorghum growth is 26-34°C during the vegetative stage (Hammer et al., 1993) and 25-28°C during the reproductive stage (Prasad et al., 2006). Temperatures exceeding 32°C beyond the physiological optimum are considered HT stress conditions as they disrupt normal growth and development. Climate models project that global air temperatures may rise by 3.7-4.8°C by the end of the 21st century, largely due to elevated carbon dioxide and other greenhouse gases (Pachauri & Meyer, 2014; Frank et al., 2015). In sorghum, HT stress is known to reduce chlorophyll content and photosynthetic activity while increasing membrane damage

(Djanaguiraman et al., 2010). Additionally, HT stress adversely affects a range of phenological, growth, physiological, and yield traits including plant height, leaf area, leaf development, pollen germination, seed set, and seed size, all of which contribute to substantial yield losses (Nguyen et al., 2013; Prasad et al., 2008; Prasad et al., 2021; Singh et al., 2016).

Weed interference poses another major challenge to grain sorghum production, resulting in considerable yield losses, reduced harvest efficiency, and limited weed control options (Burnside et al., 1969; Moore et al., 2004; Smith et al., 1990; Stahlman & Wicks, 2000; Thompson et al., 2019). The most widely adopted strategy for weed control in sorghum is the use of herbicides. However, post-emergence (POST) herbicide options are limited due to the risk of crop injury. POST herbicides such as 2,4-D, dicamba, prosulfuron, and bromoxynil are commonly used in sorghum for broad-spectrum weed control. In contrast, HPPD-inhibiting herbicides, which are effectively used in corn (*Zea mays* L.) due to its natural tolerance through rapid oxidative metabolism via cytochrome P450 monooxygenases and reduced herbicide absorption (Grossmann & Ehrhardt, 2007; Mitchell et al., 2001; Pataky et al., 2008), are not registered for POST use in sorghum because of the risk of crop damage. Nevertheless, some studies have reported reduced mesotrione-induced injury in certain grain sorghum genotypes when the herbicide is applied POST (Abit et al., 2009; Pandian et al., 2023).

2,4-D is a synthetic auxin herbicide that mimics the natural plant hormone auxin. Application of synthetic auxins like 2,4-D disrupts normal hormonal balance, leading to abnormal growth patterns such as twisting, curling, and ultimately the death of sensitive broadleaf species (Grossmann, 2000, 2003). Due to its broad-spectrum efficacy and affordability, 2,4-D is frequently applied post-emergence in sorghum. However, sorghum sensitivity to 2,4-D varies by genotype, with reported yield losses ranging from 12% to 27% (Burnside & Wicks,

1972). Crop injury is more pronounced with low-volatile ester formulations (Phillips, 1960), and recent studies show that yield and lodging in sorghum are influenced by both the application rate and timing of 2,4-D (Dan et al., 2010; Petter et al., 2011; Rosales-Robles et al., 2014).

Importantly, the efficacy and safety of herbicides are strongly influenced by environmental conditions.

Climate change, through increased carbon dioxide levels and altered temperature and precipitation patterns, is expected to impact multiple facets of weed management, including weed biology, establishment, competitiveness, distribution, and the effectiveness of control strategies (Bradley et al., 2010; Clements et al., 2004; Peters et al., 2014; Thuiller et al., 2008; Valerio et al., 2011; Waryszak et al., 2018; Ziska & Dukes, 2011). Among these factors, temperature plays a pivotal role in herbicide efficacy. The impact of temperature varies based on weed species and the herbicide's mode of action (Shyam et al., 2019). Elevated temperatures may alter herbicide uptake, translocation, and metabolism, and can induce pleiotropic effects (Dyer, 2018; Rudell et al., 2023). While much of the existing literature focuses on weed species, there is a lack of information about how crop species respond to herbicides under elevated temperatures. For instance, studies on key sorghum weeds such as Palmer amaranth and kochia have shown that HT stress reduces the efficacy of herbicides like 2,4-D, glyphosate, dicamba, and mesotrione (Ou et al., 2018; Ganie et al., 2017; Godar et al., 2015; McCurdy et al., 2008; Rudell et al., 2023; Shyam et al., 2019a). Specifically, in Palmer amaranth, HT reduced the effectiveness of 2,4-D and mesotrione (Godar et al., 2015; Rudell et al., 2023).

In contrast to weeds, crops may experience enhanced herbicide activity and resultant injury under HT stress. For example, in glyphosate-resistant soybean, an increase in temperature from 15°C to 35°C enhanced glyphosate efficacy, leading to crop injury (Pline et al., 1999).

Similarly, grain sorghum has shown increased sensitivity to MCPA, 2,4-D, bromoxynil, and dicamba under high temperature conditions, possibly due to changes in herbicide absorption, translocation, and metabolism (Hatzios & Penner, 1982; Pannacci & Bartolini, 2018). In grain sorghum, tolerance to herbicides like synthetic auxins and HPPD-inhibitors is thought to result from detoxification mechanisms mediated by cytochrome P450 (P450) enzymes (Abit & Al-Khatib, 2009; Pandian et al., 2023; Peterson et al., 2016). The activity of P450 enzymes, however, is temperature-dependent and can influence herbicide metabolism and efficacy (Mahan et al., 2004). Recent research by Adari (2024) observed that grain sorghum genotypes P84G62 and RTx430 exhibited increased mesotrione injury under high temperature stress. Interestingly, the opposite trend was observed with 2,4-D, where greater injury occurred under optimum temperature conditions.

Based on these findings, we hypothesize that under high temperature stress, the metabolism of mesotrione is reduced, leading to increased crop injury. Conversely, for 2,4-D, we hypothesize that decreased metabolism at optimum temperature results in more pronounced crop injury. Therefore, the objectives of this study are: (1) To investigate the physiological basis (uptake and translocation) of 2,4-D and mesotrione injury in grain sorghum under optimum and HT stress; and (2) To evaluate the metabolism of these herbicides through a cytochrome P450 inhibitor assay.

4.2. Materials and Methods

4.2.1 Materials

Two grain sorghum genotypes, Pioneer 84G62 (P84G62) and RTx430, were chosen for this study. RTx430 is a widely utilized inbred line that serves as a foundation for numerous grain

sorghum breeding programs (Miller, 1984). In contrast, P84G62 is a well-known, high-yielding commercial hybrid commonly grown in the United States (Maulana & Tesso, 2013).

4.2.2 Plant Growth Conditions

Seeds of the grain sorghum genotypes P84G62 and RTx430 were individually sown in square pots (6×6×6 cm) filled with a commercial potting mix (ProMix Ultimate, Premier Tech Horticulture, Mississauga, Ontario, Canada). For the mesotrione experiment, seedlings were initially germinated and grown in a greenhouse under controlled conditions of 32/22°C (day/night), with a 15/9 h photoperiod, a photosynthetic photon flux density of 750 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and relative humidity maintained at $60 \pm 10\%$. When plants reached the 3–4 leaf stage two days before herbicide application, they were transferred to two separate growth chambers for acclimatization under either optimum temperature (OT; 32/22°C, day/night) or high temperature (HT; 40/30°C, day/night) regimes. Both chambers maintained identical conditions: $60 \pm 5\%$ relative humidity and a 12-hour photoperiod with a photon flux density of 330 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the canopy level, provided by a combination of incandescent, fluorescent, and LED lights.

For the 2,4-D experiment, seeds of both genotypes were germinated and grown in growth chambers set to OT conditions. At the 3–4 leaf stage, two days prior to herbicide treatment, a subset of plants was transferred from the OT chamber to the HT chamber. Throughout both experiments, all plants were watered regularly as needed.

4.2.3 Absorption and Translocation of [^{14}C]-Mesotrione and 2,4-D

At the 4–5 leaf stage, sorghum plants (P84G62 and RTx430) grown under both temperature regimes, optimum temperature (OT) and high temperature (HT) were treated with radiolabeled herbicides to evaluate herbicide uptake and translocation. For the mesotrione treatment, each plant received ten 1- μL droplets (8000 dpm per droplet) of [phenyl- ^{14}C]-

mesotrione (total activity: 9.15 kBq; specific activity: 3290 MBq g⁻¹). To achieve a final concentration equivalent to rate of 210 g ai ha⁻¹, unlabeled mesotrione (Callisto®, Syngenta Crop Protection) was added to the radioactive solution. For improving droplet retention and herbicide absorption, crop oil concentrate (COC; Agridex®, Helena Chemical Company, USA) was included at 1% v/v, and ammonium sulfate (AMS; Liquid N-PAK®, Agrilience, USA) was added at 0.85% w/v.

For the 2,4-D treatment, a stock solution was prepared by mixing ¹⁴C-radiolabeled 2,4-D (American Radiolabeled Chemicals, St. Louis, MO; specific activity: 50 mCi mmol⁻¹) with a commercial 2,4-D amine formulation (2,4-D Amine 4L, Winfield United Solutions, Arden Hills, MN) to achieve an application rate of 1120 g ai ha⁻¹ (1x= 560 g ai ha⁻¹). Both herbicide solutions were prepared in a carrier volume of 187 L ha⁻¹ and applied to the adaxial (upper) surface of the fourth youngest fully expanded leaf.

After a 30-minute drying period, plants were returned to their respective growth chambers. Harvesting was conducted at 6 and 24 hours after treatment (HAT), and each plant was divided into the treated leaf (TL), tissues above the treated leaf (ATL), and below the treated leaf (BTL). The treated leaves were then washed with 5 ml of wash solution (containing 10% ethanol and 0.05% Tween 20) for 1 minute in a 20 ml scintillation vial to remove unabsorbed herbicide. The radioactivity was measured using liquid scintillation spectrometry (LSS; Beckman Coulter LS6500 Liquid Scintillation Counter, Beckman Coulter Inc., Fullerton, CT, USA). The three plant sections - treated leaf (TL), above treated leaf (ATL), and below treated leaf (BTL) were individually wrapped in tissue paper and oven-dried at 60°C for 2 to 3 days. To quantify absorbed radioactivity, the dried plant tissues were combusted using a biological oxidizer (OX-501; RJ Harvey Instrument, Tappan, NY, USA), and the resulting radioactivity

was measured again by LSS. Each treatment consisted of four biological replicates, and the experiment was repeated once.

The total absorbed [^{14}C]-herbicide was determined as follows:

$$\% \text{ absorption of } ^{14}\text{C herbicide} = (\text{total R applied} - \text{R of rinsate}) \times 100 / \text{total R applied};$$

where R = radioactivity.

The percent ^{14}C herbicide translocated (T) was determined using the following equation:

$\%T = 100 - [^{14}\text{C al} / (^{14}\text{C al} + ^{14}\text{C ot}) \times 100]$, where $^{14}\text{C al}$ is the amount of ^{14}C measured in the treated leaf, and $^{14}\text{C ot}$ is the amount of ^{14}C detected in other untreated tissues of the plant.

4.2.4 Cytochrome P450 - inhibitor Assay

To assess cytochrome P450 enzyme activity under optimum and HT conditions in grain sorghum, experiments were conducted using malathion, a known P450 inhibitor's, role in mesotrione and 2,4-D metabolism. Sorghum plants were grown in controlled growth chambers as previously described. Malathion (Spectracide Malathion Insect Spray Concentrate; Spectrum Brands) was applied at rates of 0, 2000 (M1), and 4000 (M2) g ai ha⁻¹, along with 0.25% non-ionic surfactant (NIS). One hour following the malathion treatment, mesotrione (1x= 105 g ai ha⁻¹) and 2,4-D (1x= 560 g ai ha⁻¹) were applied at a 2x dose than the field recommended dose with the appropriate adjuvants. A bench-top track spray chamber (Generation III; De Vries Manufacturing, Hollandale, MN, US) equipped with a single flat-fan nozzle (80015LP TeeJet tip; Spraying Systems, Wheaton, IL, US) delivering 187 L ha⁻¹ was used for malathion and herbicide application. In accordance with established protocols from our laboratory (Shyam et al., 2021a; Pandian et al., 2020b), a booster dose of 5 mM malathion was applied via soil drench 24 hours after the initial application, except in the foliar-only malathion treatment. Biomass was

harvested three weeks after treatment (WAT), oven-dried at 60 °C for 2–3 days, and then weighed to assess herbicide impact.

4.2.5 Statistical Analysis

The experiment followed a completely randomized factorial design with two main factors: growth temperature (optimum and high) and sampling time (6 and 24 hours after treatment). Each treatment combination was replicated four times. There was no interaction between the experimental runs and the treatments, hence, the data from each independent experiment were combined before subjecting to statistical analysis. Data for these experiments were analyzed by two-way ANOVA ($P < 0.05$). Data for all the experiments were significant for the main effects (growth temperatures), hence, post-hoc Tukey-HSD pairwise comparisons were used to test which simple effects were significantly different ($P < 0.05$) and was analyzed using R (version 4.4.0, R Core Team, 2024).

4.3 Results

4.3.1 Mesotrione Uptake and Translocation

Growth temperature significantly influenced the uptake and translocation of [^{14}C]-mesotrione in grain sorghum genotypes P84G62 and RTx430 (Figures 4.1 and 4.2). At 6 HAT, plants exposed to high temperature (HT) conditions absorbed more mesotrione compared to those grown under optimum temperature (OT). Specifically, at 6 HAT, P84G62 and RTx430 absorbed approximately 50% and 60% of the applied [^{14}C]-mesotrione, respectively, whereas uptake under OT conditions was reduced to about 35% and 40%, respectively (Figure 4.1 A). By 24 HAT, mesotrione absorption further increased under HT, with P84G62 and RTx430 showing uptake levels of ~75% and ~80%, respectively, compared to ~56% and ~58% under OT (Figure 4.1 B). This trend of elevated absorption under HT conditions aligns with previous findings

suggesting that heat-induced modifications in cuticle permeability or leaf surface characteristics can enhance herbicide entry (Johnson and Young, 2002).

Temperature also had a pronounced effect on mesotrione translocation. At 6 HAT, a larger proportion of the absorbed herbicide remained in the treated leaf (TL) under HT compared to OT. Under HT, ~42% (P84G62) and ~50% (RTx430) of the absorbed mesotrione was retained in the TL, whereas under OT, retention dropped to ~20% and ~27%, respectively (Figure 4.2 A). A similar pattern was observed at 24 HAT: HT-grown P84G62 and RTx430 retained ~32% and ~36% of mesotrione in the TL, respectively, while plants under OT conditions retained only ~12% and ~15% (Figure 4.2 B). These observations indicate that while high temperature enhances mesotrione uptake, it restricts its translocation away from the treated leaf, likely due to reduced metabolic degradation. A similar temperature-mediated increase in herbicide activity was reported by Pline et al. (1999), where glyphosate translocation to meristematic tissues in Roundup Ready® soybean increased significantly at 35°C compared to 15°C, leading to greater crop injury. Although mesotrione translocation was restricted under HT in the present study, the increased uptake combined with reduced metabolism parallels the broader pattern of heightened herbicide activity and injury risk under elevated temperatures. These findings are consistent with previous research by Adari (2024), who reported increased herbicide injury in both genotypes under HT stress, with RTx430 exhibiting greater sensitivity than P84G62.

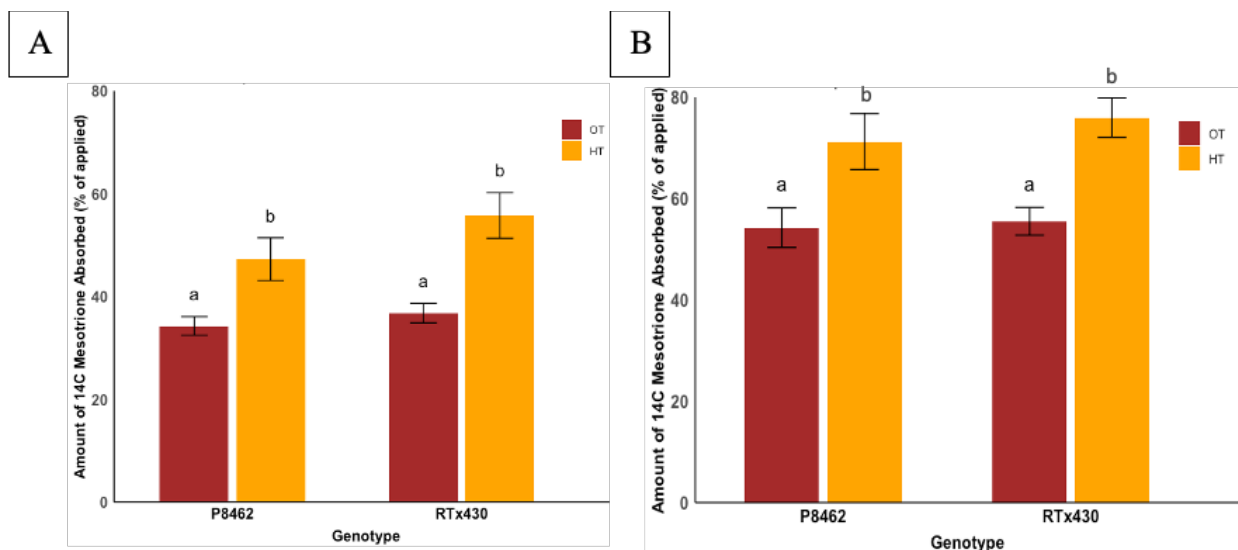


Figure 4.1 Absorption of [^{14}C] mesotrione in sorghum plants as percent applied at 6 (A) and 24 (B) HAT (hours after treatment)

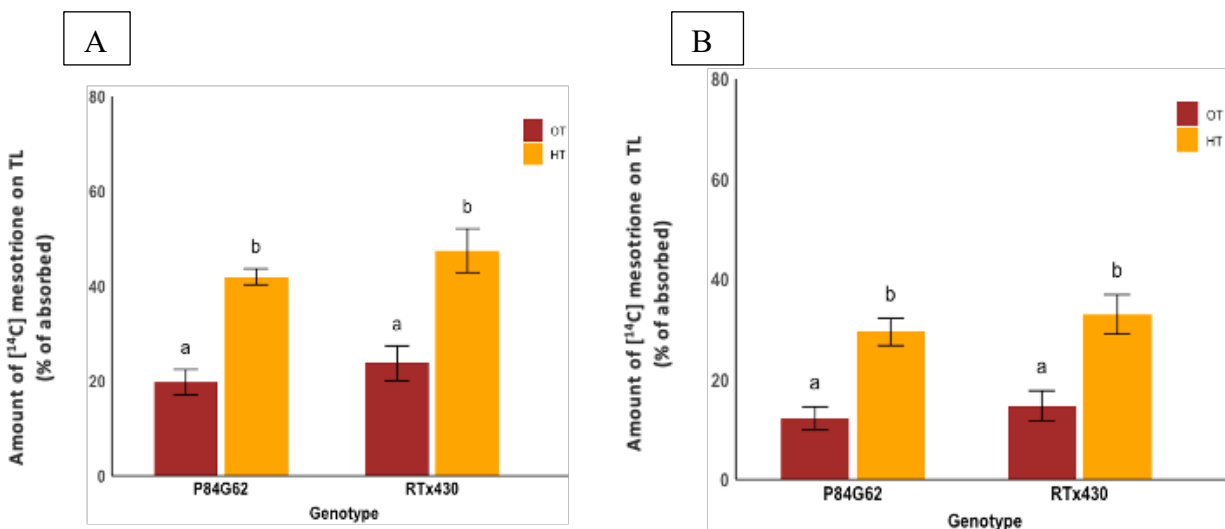


Figure 4.2 [^{14}C] mesotrione in treated leaf at 6 (A) and 24 (B) HAT (hours after treatment)

4.3.2 2,4-D Uptake and Translocation

Temperature exerted a notable influence on the uptake and movement of [^{14}C]-2,4-D in the grain sorghum genotypes P84G62 and RTx430 (Figures 4.3 and 4.4). At 6 HAT, plants exposed to HT conditions exhibited significantly greater absorption of 2,4-D compared to those grown under OT. Specifically, at 6 HAT under HT, P84G62 and RTx430 absorbed approximately 42% and 50% of the applied herbicide, respectively, whereas the corresponding absorption under OT was about 31% and 35% (Figure 4.3 A). By 24 HAT, absorption (% of applied) further increased in HT-grown plants, reaching approximately 58% for P84G62 and 65% for RTx430, compared to 43% and 48% under OT, respectively (Figure 4.3 B). These findings suggest that elevated temperature may enhance herbicide absorption, potentially due to increased cuticle permeability or temperature-induced changes in the leaf surface characteristics.

However, despite the higher uptake under HT, the translocation of 2,4-D away from the treated leaf (TL) was considerably reduced, likely due to rapid herbicide metabolism. At 6 HAT, OT-grown plants had ~ 37% (P84G62) and 40% (RTx430) of the absorbed 2,4-D in the TL, whereas under HT, translocation was limited to about 20% in both genotypes (Figure 4.4 A). This trend continued at 24 HAT, where OT-grown plants showed greater movement of the herbicide (~25% in P84G62 and ~30% in RTx430), while only ~10% of the absorbed 2,4-D was detected in the TL in both genotypes under HT (Figure 4.4 B). These results indicate that although high temperature increases initial 2,4-D absorption, it simultaneously limits systemic movement, likely due to accelerated metabolism.

The effect of temperature on herbicide uptake and efficacy is both species-specific and dependent on the herbicide's mode of action. Although HT enhanced 2,4-D absorption in sorghum, its rapid metabolism under these conditions could reduce the extent of crop injury

compared to OT. This pattern is supported by the findings of the previous study from our lab, which observed greater herbicide injury under OT conditions for both the genotypes. Similarly, previous research has reported reduced efficacy of 2,4-D at higher temperatures in weed species such as Palmer amaranth and common waterhemp (Rudell et al., 2023; Shyam et al., 2019).

In summary, high temperature conditions increased 2,4-D uptake in grain sorghum but restricted its translocation, likely due to rapid metabolism mediated by cytochrome P450 enzymes. This altered physiological response may explain the greater injury observed under OT conditions, corroborating earlier injury assessments and preliminary findings by Adari (2024).

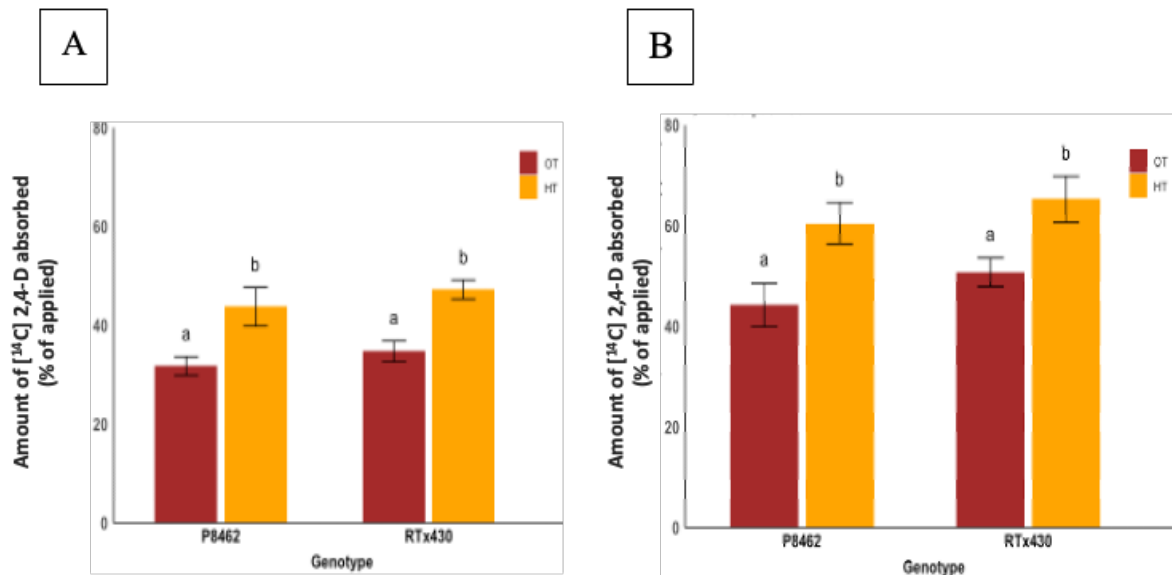


Figure 4.3 Absorption of [^{14}C] 2, 4-D in sorghum plants as percent applied at 6 (A) and 24 (B) HAT (hours after treatment)

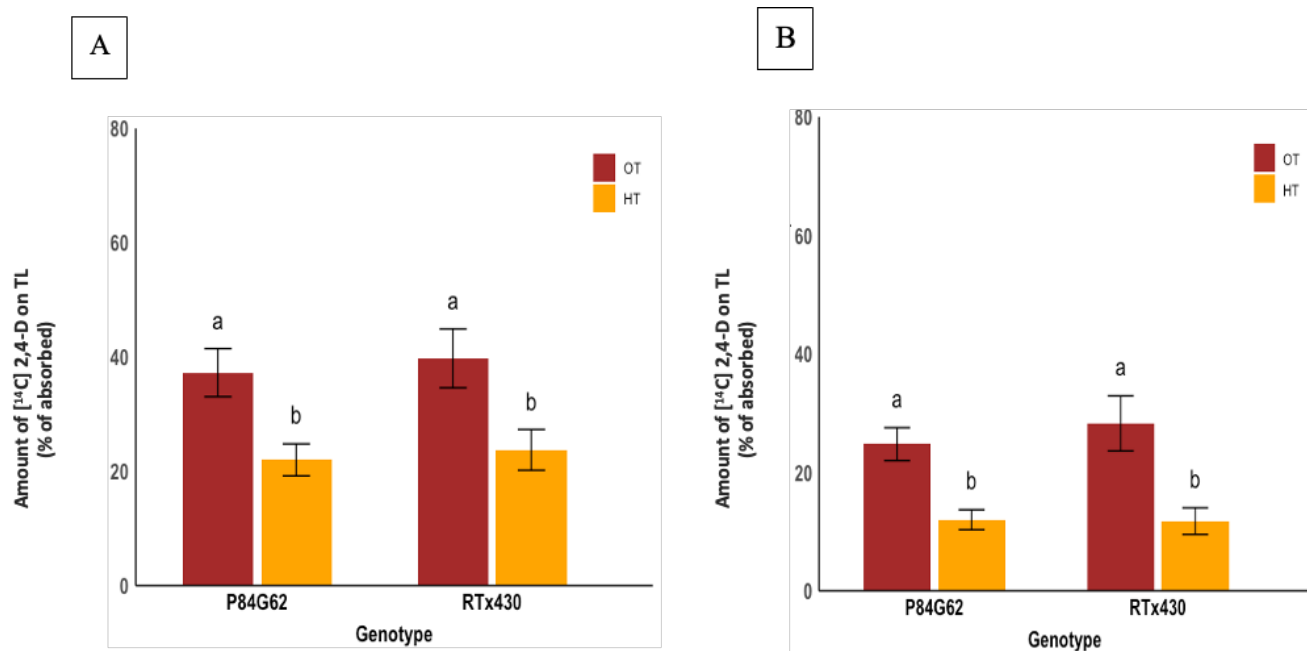


Figure 4.4 [^{14}C] 2,4-D in treated leaf at 6 (A) and 24 (B) HAT (hours after treatment)

4.3.3 CYP450 Inhibitor Assay for Mesotrione

The cytochrome P450 (CYP450) inhibitor assay demonstrated a strong interaction between mesotrione treatment, temperature, and metabolic inhibition in both grain sorghum genotypes, P84G62 and RTx430. Plants treated with a 2x dose of mesotrione ($1x = 105 \text{ g ai ha}^{-1}$) exhibited significant reductions in biomass under high temperature (HT) compared to optimum temperature (OT) conditions (Figures 4.5 and 4.6). Specifically, biomass of P84G62 plants under OT remained $\sim 75\%$ of the non-treated, but declined to $\sim 55\%$ under HT. A similar pattern was observed in RTx430, where biomass decreased from $\sim 70\%$ under OT to $\sim 48\%$ under HT (Figure 4.6 A and Figure 4.6 B).

When mesotrione was combined with the cytochrome P450 inhibitor malathion either at $2000 \text{ g ai ha}^{-1}$ (M1) or $4000 \text{ g ai ha}^{-1}$ (M2), the reduction in biomass was even more pronounced. In P84G62, the 2x+M1 biomass accumulation was $\sim 65\%$ under OT and reduced $\sim 30\%$ under HT. The 2x+M2 treatment further decreased biomass to $\sim 15\%$ under HT and while as biomass remained $\sim 60\%$ of non-treated under OT (Figure 4.6 A). In RTx430, biomass following 2x+M1 reduced to $\sim 20\%$ under HT while as its accumulation was still $\sim 60\%$ under OT (Figure 4.6 B). In response to mesotrione 2x+M2 treatment, a sharp decline to in biomass accumulation of RTx430, followed by complete death of plants was seen in plants grown under HT than at OT. Biomass remained $\sim 50\%$ under OT whereas it reduced $\sim 10\%$ under HT (Figure 4.5 and Figure 4.6 B). The significant biomass loss under HT in the presence of malathion suggests a strong inhibitory effect on mesotrione detoxification pathways likely due to the reduced activity of cytochrome P450.

These findings support the hypothesis that high temperature suppresses cytochrome P450-mediated metabolism of mesotrione in sorghum, leading to greater herbicide injury. The more drastic biomass reduction observed under HT, particularly in the 2x+M2 treatments, highlights the synergistic impact of temperature stress and P450 inhibition.

While both genotypes were sensitive to this combined stress, RTx430 appeared more susceptible to biomass loss under these conditions, consistent with prior injury assessments.

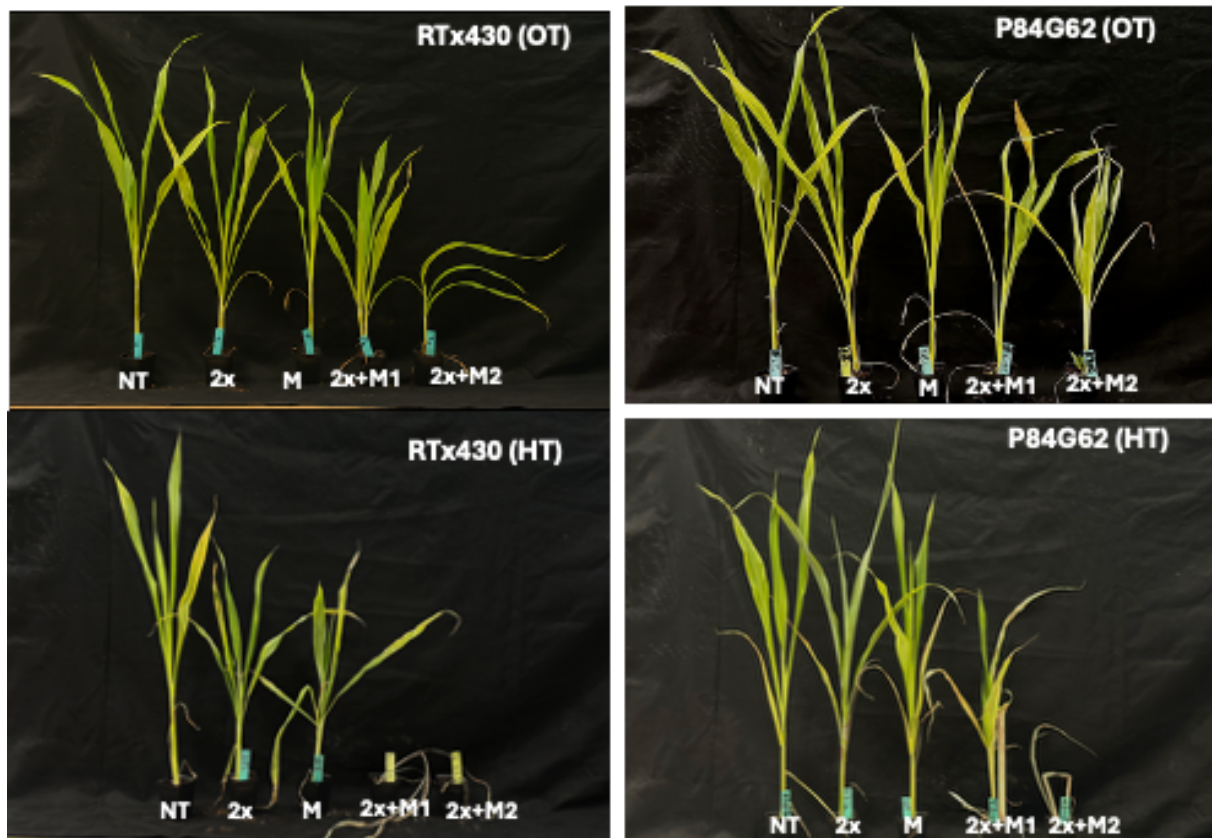


Figure 4.5 The response of sorghum plants at OT and HT to 2x dose of mesotrione (1x= 105 g ai ha⁻¹) or malathion treatments (M or M1: 2000 g ai ha⁻¹; M2: 4000 g ai ha⁻¹) followed by 2x dose of mesotrione; NT: Non-treated.

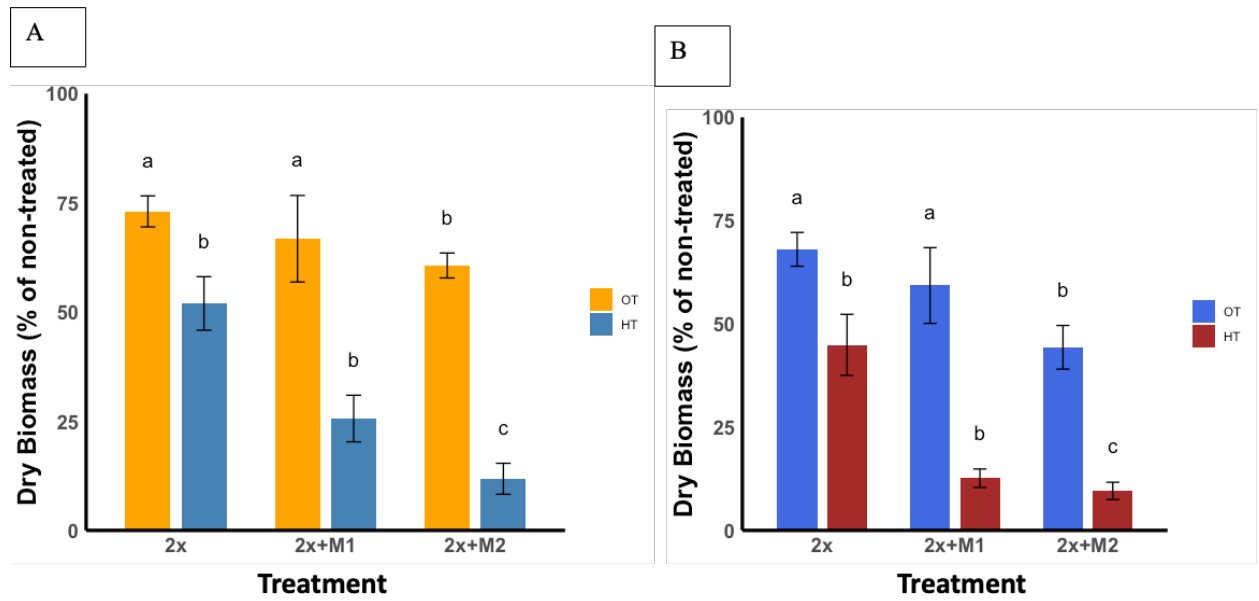


Figure 4.6 Relative dry weight of above ground biomass as percent of non-treated of sorghum A) P84G62 and B) RTx430 plants at OT and HT when treated with mesotrione alone or a combination of malathion and mesotrione at 3 WAT (weeks after treatment)

4.3.4 CYP450 Inhibitor Assay for 2,4-D

The cytochrome P450 (CYP450) inhibitor assay revealed a contrasting trend for 2,4-D compared to mesotrione, particularly in how the grain sorghum genotypes P84G62 and RTx430 responded under high temperature (HT) versus optimum temperature (OT) conditions. When treated with a 2x dose of 2,4-D (1120 g ai ha⁻¹), plants accumulated greater biomass under HT than under OT (Figures 4.7 and Figure 4.8). For P84G62, biomass under HT was approximately 70% of the non-treated control, compared to ~50% under OT. In RTx430, this difference was more pronounced, with biomass at ~70% under HT and ~45% under OT ((Figure 4.7 and Figure 4.8).

When 2,4-D was applied in combination with malathion at 2000 g ai ha⁻¹ (M1), the contrast remained evident. In P84G62, biomass under HT was ~65%, whereas it dropped to ~30% under OT. Similarly, RTx430 retained ~63% biomass under HT but showed a steep reduction to ~15% under OT. The highest malathion dose (4000 g ai ha⁻¹; M2) further increased the biomass reduction with P84G62 having ~10% under OT but remained at ~60% under HT. In RTx430, biomass fell to ~8 -10% under OT while still reaching ~40% under HT.

These results indicate that unlike mesotrione, 2,4-D injury in sorghum was more severe under OT than HT, suggesting reduced herbicide detoxification at lower temperatures. The higher biomass retention under HT, even in the presence of CYP450 inhibition, points to enhanced 2,4-D metabolism at elevated temperatures, potentially mitigating herbicide toxicity. RTx430 showed greater sensitivity under OT conditions compared to P84G62, consistent with previous visual injury assessments and uptake-translocation patterns.

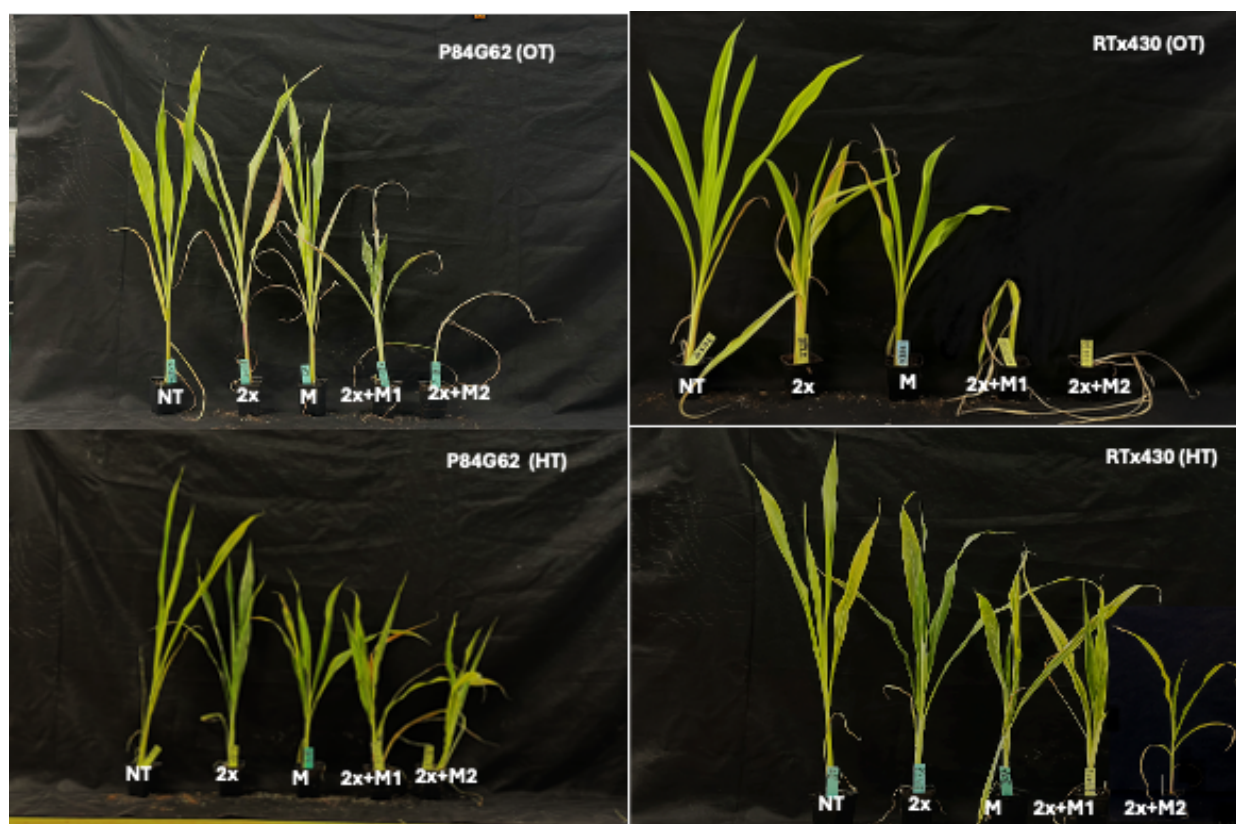


Figure 4.7 The response of sorghum plants at OT and HT to 2x dose of 2,4-D ($1x = 560 \text{ g ai ha}^{-1}$) or malathion treatments (M or M1: $2000 \text{ g ai ha}^{-1}$; M2: $4000 \text{ g ai ha}^{-1}$) followed by 2x dose of 2,4-D; NT: Non-treated.

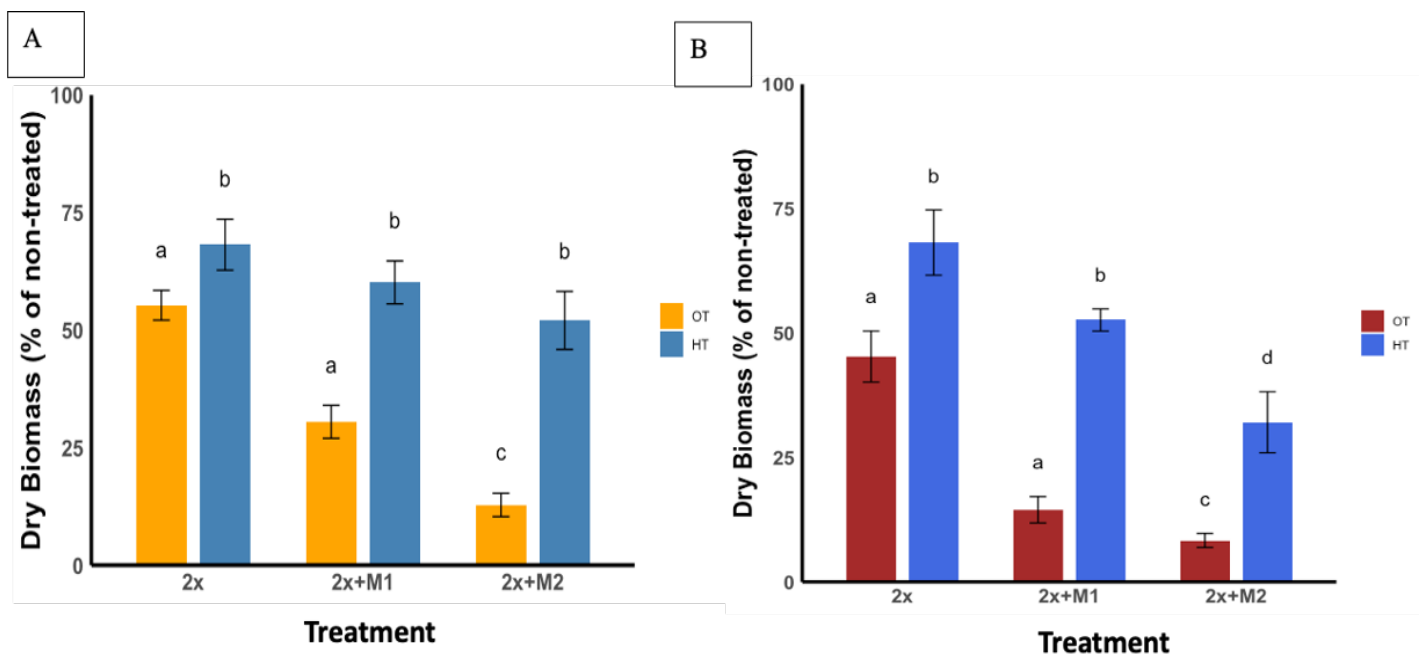


Figure 4.8 Relative dry weight of above ground biomass as percent of non-treated of sorghum A) P84G62 and B) RTx430 plants at OT and HT when treated with 2,4-D alone or a combination of malathion and 2,4-D at 3 WAT (weeks after treatment)

4.4 Discussion

Weed management in grain sorghum primarily relies on herbicides such as atrazine, S-metolachlor, dimethenamid-P, and alachlor for preemergence control, and 2,4-D, dicamba, prosulfuron, and bromoxynil for postemergence applications (Smith and Scott, 2010). However, sorghum is inherently sensitive to several commonly used herbicides, including glyphosate, glufosinate, and imazethapyr, which limits the options for effective weed control (Al-Khatib et al., 2003). Rising temperatures further complicate this challenge by altering herbicide efficacy and increasing the risk of crop injury. High-temperature (HT) stress is known to impact plant growth and may enhance herbicide-induced damage (Bagavathiannan et al., 2017). Visual injury symptoms serve as early indicators of herbicide impact and are often inversely correlated with crop yield. However, certain genotypes may exhibit resilience, recovering from initial injury with minimal yield loss (Carvalho et al., 2009).

In our study, sorghum genotypes P84G62 and RTx430 showed greater absorption and translocation of [^{14}C]-mesotrione under HT compared to OT, likely contributing to increased injury due to heat-induced alterations in the leaf cuticular surface or possibly due to reduced metabolic degradation (Figure 4.1 and Figure 4.2). Prior studies have also reported varied tolerance among grain sorghum genotypes to mesotrione under both field and greenhouse conditions, with some genotypes showing reduced injury (Abit et al., 2009; Pandian et al., 2023). The selectivity of crops to herbicides like mesotrione is often attributed to their capacity for rapid metabolism via cytochrome P450 enzymes. However, altered activity either increased or decreased of specific CYP genes in corn, such as *nsfl* or *ben1* located on chromosome 5, can enhance sensitivity to several P450-metabolized herbicides (Williams et al., 2006).

In this research, biomass data clearly indicated that the combination of mesotrione with increasing doses of malathion led to a substantial reduction in biomass in both sorghum genotypes under HT conditions compared to OT. This outcome suggests that elevated temperatures likely inhibit cytochrome P450 activity, thereby reducing the detoxification capacity for mesotrione and intensifying herbicide injury (Figure 4.5 and Figure 4.6). Although both genotypes exhibited increased sensitivity under HT stress, RTx430 showed a greater amount of [^{14}C]-mesotrione in the treated leaf at both 6 and 24 hours after treatment (HAT), likely due to slower metabolism, resulting in more pronounced injury compared to P84G62 (Figure 4.1 and Figure 4.2). While numerous studies have explored the effects of elevated temperature on herbicide efficacy in weeds, there remains limited literature investigating the combined impact of high temperature and herbicide stress on crop performance and physiological response.

Previous report suggests, the efficacy of glufosinate in glufosinate-susceptible cotton was highest at 27°C and reduced at 18 and 40°C (Manhal et al., 2006). Atrazine injury increased with higher temperature in barley after PRE application (Mulder & Nalewaja, 1978) and in common bean and redroot pigweed after POST application, likely due to enhanced foliar uptake and target-site binding (Al-Khatib et al., 1992). Previous studies show that mesotrione efficacy under high temperature varies by species. For example, Palmer amaranth exhibited reduced sensitivity at 40°C (Godar et al., 2015), while no difference was observed in perennial ryegrass and large crabgrass across 18–34°C (McCurdy et al., 2008, 2009). In contrast, common cocklebur and velvetleaf showed increased susceptibility at 32°C, whereas common waterhemp and large crabgrass were more sensitive at lower temperatures (Johnson & Young, 2002). These findings indicate that mesotrione response under HT is species dependent.

2,4-D is widely used postemergence in sorghum due to its broad-spectrum weed control and low cost. However, sorghum tolerance can vary with genotype, environment, and herbicide formulation, with ester forms causing more injury (Phillips, 1960). Recent studies also indicate that sorghum yield and lodging are influenced by both application timing and 2,4-D rate (Dan et al., 2010; Petter et al., 2011; Rosales-Robles et al., 2014). Temperature influences herbicide uptake and movement by affecting photosynthesis, enzyme activity, and cuticle properties. Warmer temperatures can enhance photosynthesis, enzyme function, and phloem loading, potentially increasing herbicide translocation. Additionally, changes in cuticle structure and stomatal opening may boost uptake. For 2,4-D, uptake tends to increase at higher temperatures, while translocation remains largely unaffected (Kirkwood, 1999; Pallas, 1960; Schultz & Burnside, 1980; Sharma & Vandenberg, 1970). Our results have shown that, despite greater absorption under HT, there was reduced translocation from the TL, likely due to the rapid metabolism of the herbicide (Figure 4.3 and Figure 4.4).

HT can enhance herbicide diffusion by reducing the viscosity of cuticular waxes and altering spray solution properties (Price, 1983). This leads to increased cuticle permeability and herbicide uptake (Fausey & Renner, 2001). Additionally, temperature-induced changes in phloem translocation, respiration, and cellular activity may further impact herbicide performance. In Palmer amaranth, 2,4-D and dicamba were more effective at lower temperatures (31/20°C) compared to higher temperatures (41/30°C) (Johnston et al., 2019). Conversely, the effectiveness of 2,4-D and glyphosate increased in common ragweed (*Ambrosia artemisiifolia*) and giant ragweed (*Ambrosia trifida*) under higher temperatures. Similarly, in our study, we found that sorghum grown at OT had reduced translocation of [¹⁴C]-2,4-D away from the treated leaf, likely due to reduced metabolism (Figure 4.4). High-temperature (HT) stress is known to

diminish herbicide efficacy by accelerating metabolic breakdown, mainly through enhanced cytochrome P450 enzyme activity (Johnson & Young, 2002; Hatzios & Penner, 1982; Frear et al., 1969; Frear & Swanson, 1996).

Our CYP inhibitor assay also showed that, under HT, increased activity of cytochromes in response to 2,4-D increased the metabolism in comparison to OT (Figure 4.7 and Figure 4.8). Light et al. (1999, 2001) observed that herbicides relying on enzyme inhibition may show reduced efficacy at higher temperatures due to thermal effects on enzyme–herbicide interactions. This temperature-dependent or kinetic tolerance can lead to diminished weed control if expressed in weeds, or improved crop safety if observed in the crop itself.

Variations in grain sorghum response to herbicides under temperature stress can be linked to differences in herbicide mode of action. Both HPPD and synthetic auxins affect the photosynthetic and physiological pathway but in distinct ways: HPPD inhibitors block plastoquinone synthesis, impacting carotenoid production (Amesz, 1973; Lee et al., 1997). In contrast, synthetic auxins like 2,4-D act by binding to Aux/IAA proteins, altering auxin-regulated gene expression and inducing ethylene and ABA production (Mithila et al., 2011; Peterson et al., 2016). Unlike HPPD, 2,4-D broadly suppresses multiple genes tied to photosynthesis (Gaines, 2020). This highlights the complexity of herbicide-induced injury under high temperature, as each herbicide class disrupts different plant functions and pathways. Therefore, future studies should focus on evaluating the effect of high or low temperature on the differential expression of CYPs responsible for herbicide metabolism.

4.5 Conclusion

This study highlights the contrasting responses of grain sorghum genotypes to mesotrione and 2,4-D under high-temperature (HT) stress, emphasizing the significant role of temperature in

affecting the herbicide absorption, translocation, and metabolism. High temperatures increased mesotrione uptake while reducing its translocation, likely due to impaired cytochrome P450-mediated detoxification. This was especially evident in RTx430, which showed greater injury and higher herbicide retention. In contrast, for 2,4-D, HT enhanced uptake but also increased metabolic breakdown, resulting in less translocation and reduced injury compared to OT, where metabolism was slower.

The cytochrome P450 inhibitor assay confirmed that temperature influences the activity of detoxification pathways differently for each herbicide. Rising temperatures and increasing herbicide resistance give weeds a competitive advantage over crops, particularly in sorghum, where POST herbicide options are limited and crop sensitivity is heightened under heat stress. Moreover, high temperatures can accelerate the evolution of non-target-site resistance (NTSR) in weeds by enhancing detoxification and altering herbicide efficacy (Reffati et al., 2019).

Therefore, the significance of this work lies in highlighting the importance of considering environmental conditions particularly temperature when applying herbicides, to ensure effective weed control while minimizing crop injury. These findings support strategic decision-making for herbicide timing; for example, applying mesotrione during cooler parts of the day or under moderate temperatures may reduce crop injury, whereas 2,4-D could be safer during warmer conditions due to enhanced metabolic detoxification. Such temperature-based recommendations can improve weed control efficacy while reducing the risk of herbicide-induced crop damage. Besides temperature, factors such as humidity, light, and rainfall also affect herbicide performance and crop response. Thus, further research on how these abiotic and plant-related variables influence herbicide efficacy and sorghum injury is essential for effective weed management.

Additionally, the observed genotypic differences in herbicide response under temperature stress suggest that breeding for improved herbicide tolerance is a viable approach. Future research should focus on examining the expression patterns of specific CYP450 genes across temperature regimes to better understand their role in herbicide metabolism and crop resilience.

Chapter 5 - General Discussion, Conclusions, Implications and Future Directions

Problems Addressed

Grain sorghum is recognized for its resilience; however, controlling grass weeds postemergence (POST) remains a significant challenge due to limited herbicide options. Although herbicides for preemergence (PRE) and POST control of broadleaf weeds are available, most commercial sorghum hybrids are highly sensitive to POST grass herbicides, including HPPD-, ALS-, and ACCase-inhibitors (Smith et al., 2010; Thompson et al., 2019). Consequently, grass weed management in sorghum primarily depends on PRE herbicide applications. Developing herbicide-resistant sorghum hybrids represents a promising and economical strategy for enabling broad-spectrum POST weed control. Prior studies have reported natural resistance to mesotrione (Abit et al., 2009) and tembotrione (Dan et al., 2010) in certain sorghum genotypes. Further screening of a sorghum diversity panel by Pandian et al. (2020, 2023) identified four key genotypes: G-1 and G-10 resistant to mesotrione, and G-200 and G-350 resistant to tembotrione alongside a susceptible genotype, S-1. Genetic analysis revealed that mesotrione resistance is governed by a single dominant gene, while tembotrione resistance appears to be a partially dominant polygenic trait.

Despite widespread use of HPPD-inhibiting herbicides in corn and other cereals, mesotrione and tembotrione (HPPD herbicides) are not currently registered for POST use in grain sorghum due to the risk of crop injury. However, the rich genetic diversity inherent in sorghum (Glaszmann et al., 2010; Harlan and De Wet, 1972) offers significant potential for breeding herbicide-tolerant varieties adapted to diverse agroecosystems. Chapters 2 and 3 of this thesis explored the response of mesotrione-resistant (G-10) and tembotrione-resistant (G-200

and G-350) parental lines, their reciprocal crosses, and F₁ progeny with diverse genetic backgrounds. Evaluations based on visual injury, biomass accumulation, and chlorophyll content demonstrated enhanced tolerance in several F₁ lines. These findings confirm the successful transfer of herbicide resistance traits and support the dominant and polygenic inheritance patterns of mesotrione and tembotrione resistance. This work contributes to the development of HPPD-inhibitor-resistant sorghum, offering a valuable tool for addressing the dual challenges of weed resistance and limited POST weed control options.

Rising temperatures due to climate change pose a significant threat to crop productivity. According to the IPCC (2022), future climate is projected to increase temperatures by 2–5 °C by the end of this century. These altered abiotic conditions particularly changes in temperature and climate variability can severely impact crop production and agronomic practices, including weed and pest management (Dukes et al., 2009; Singer et al., 2013). Herbicides remain a critical tool for weed control, offering growers a cost-effective and reliable method compared to other strategies. However, herbicide performance is also sensitive to environmental factors such as temperature, which can influence both herbicide behavior and plant response. Climate change may further exacerbate weed resistance due to enhanced weed vigor and altered herbicide efficacy (Varanasi et al., 2016).

In particular, high temperatures can negatively affect herbicide efficacy in crops like corn and grain sorghum by increasing herbicide-induced injury. While the physiological mechanisms underlying temperature effects on herbicide efficacy are well documented in some weed species, studies in major crops such as corn and grain sorghum remain limited. For instance, Plaine et al. (1999) reported greater translocation of ¹⁴C-glyphosate to meristematic tissues in Roundup Ready® soybean at 35 °C than at 15°C, indicating increased injury risk at elevated temperatures.

High temperatures can also accelerate herbicide metabolism or induce antioxidant enzyme activity that reduces herbicide efficacy. In a study by Godar et al. (2015), the efficacy of the HPPD-inhibitor mesotrione was significantly reduced in Palmer amaranth when exposed to high day/night temperatures (40/30°C), despite increased absorption. Also, there was a reduced translocation of mesotrione to growing tissues compared to plants grown at optimum (32.5/22.5°C) or low (25/15°C) temperatures. Recent research has also shown increased injury to corn and grain sorghum from POST-applied mesotrione and 2,4-D under high temperature conditions (Adari, 2024). This variability presents a challenge for weed management and underscores the need to investigate the physiological mechanisms behind crop injury under elevated temperatures. Chapters 4 of this thesis addresses this critical issue by exploring the physiological basis of mesotrione and 2,4-D-induced injury in grain sorghum under high temperature stress.

Key Findings

Chapter 2:

In this chapter, the results suggest a genotypic variation in response to mesotrione. The susceptible genotype S-1 exhibited the greatest sensitivity, with sharp reductions in biomass, severe bleaching symptoms, and a low GR₅₀ (38 g ai ha⁻¹) (Figure 2.1 and Table 2.1) and SPAD ED₅₀ (78 g ai ha⁻¹) (Figure 2.3 and Table 2.3). RTx430 showed similar sensitivity, though slightly more tolerant than S-1 (Figure 2.1). In contrast, the resistant parent G-10 and its F₁ hybrid G-200 × G-10 demonstrated strong resistance across all parameters, maintaining higher biomass and SPAD values with minimal visual injury (Figure 2.1, Figure 2.2 and Figure 2.3). The GR₅₀ and SPAD (chlorophyll index) ED₅₀ values (337 and 231 g ai ha⁻¹ for G-10; 427 and

296 g ai ha⁻¹ for G-200 × G-10, respectively) of these genotypes reflected consistent resistance to mesotrione (Table 2.1). Intermediate responses were observed in G-200, P84G62, and G-10 × G-200, which maintained moderate growth and chlorophyll index under mesotrione treatment (Figure 2.1 and Table 2.3). The alignment of biomass, visual injury, and SPAD trends confirm the previously reported (Pandian et al., 2023) dominant nature of inheritance of mesotrione resistance of G-10 and signifies its value for breeding herbicide-tolerant sorghum hybrids.

Chapter 3:

This chapter evaluated the performance of two tembotrione- resistant parental lines, G-200 and G-350, and their hybrids across diverse sorghum genetic backgrounds. Dose-response results revealed that G-200 showed a GR₅₀ of 186 g ai ha⁻¹, while G-350 also performed well (GR₅₀ = 140 g), placing both in the resistant category (Table 3.1 and Table. 3.2). Their hybrids exhibited varying levels of resistance. For example, G-10 × G-200 showed the highest GR₅₀ of 174, followed by hybrids like BTx398 × G-200, BTx623 × G-200, and G-200 × BTx3197, which showed intermediate GR₅₀ values ranging from 97 to 140 g ai ha⁻¹ (Table 3.2). Similarly, hybrids involving G-350, such as G-10 × G-350 and R. LBK1 × G-350 (Table 3.3), demonstrated moderate resistance, supporting the contribution of these parents to enhanced resistance to mesotrione in F₁ crosses.

Visual injury and chlorophyll index data aligned with GR₅₀ findings. Injury symptoms peaked at 2 weeks after treatment (WAT), with recovery observed in more resistant lines by 3 WAT. The hybrid G-10 × G-200 showed the greatest ED₅₀ of 215 g ai ha⁻¹, with <15% injury at the field rate. Parent G-200 also showed strong ED₅₀ of 189 g ai ha⁻¹, while G-350 hybrids had moderate ED₅₀ of 74–83 g ai ha⁻¹ (Table 3.4 and Table 3.5). In contrast, susceptible genotypes like S-1 and RTx430 showed >50% injury at the labeled rate. These results confirm that G-200

and G-350 can effectively transfer resistance traits when crossed with diverse sorghum lines, supporting their use as donor parents for breeding HPPD-inhibitor-resistant cultivars.

Overall, the results of Chapter 2 and 3 highlight the significant genotypic variation in sorghum response to HPPD-inhibiting herbicides mesotrione and tembotrione. The results suggest that the resistance traits, particularly from G-10, G-200, and G-350, can be effectively transferred to hybrids, with evidence of dominant or polygenic inheritance contributing to improved tolerance. These findings provide a strong foundation for developing herbicide-resistant sorghum cultivars, offering new opportunities for effective postemergence weed control.

Chapter 4:

This chapter examined the physiological responses of two grain sorghum genotypes, P84G62 and RTx430, to the herbicides mesotrione and 2,4-D under high (HT; 40/30°C day/night) and optimum temperature (OT) conditions. High temperature significantly increased the uptake of both herbicides in both genotypes. For mesotrione, however, a greater portion remained in the treated leaf at HT (Figure 4.2), indicating limited translocation likely due to slower metabolic breakdown (Johnson & Young, 2002). This effect was more pronounced in RTx430, which showed higher retention and greater herbicide injury (Figure 4.2), consistent with earlier findings (Adari, 2024). In contrast, under OT, a greater portion of the absorbed radioactive 2,4-D remained in the treated leaf, likely due to reduced metabolism associated with lower cytochrome P450 (CYP) activity, resulting in increased injury (Figure 4.4). Interestingly, greater injury occurred under OT, suggesting that 2,4-D detoxification is more efficient at higher temperatures a trend supported by studies in other species such as Palmer amaranth and waterhemp (Rudell et al., 2023; Shyam et al., 2019).

These patterns were further confirmed through CYP450 inhibitor assays. When mesotrione was applied with malathion, biomass decreased sharply under HT, with the most severe injury observed in the 2x+M2 treatment, particularly in RTx430 (Figure 4.5 and Figure 4.6). This indicates that high temperature, combined with reduced cytochrome P450 activity, enhances mesotrione toxicity. Conversely, 2,4-D-treated plants retained more biomass under HT than OT even with malathion, indicating that elevated temperature promotes 2,4-D metabolism and reduces injury. RTx430 again showed higher sensitivity than P84G62 under OT (Figure 4.7 and Figure 4.8). These results show that high temperature enhances mesotrione injury by increasing uptake but limiting its movement and metabolism, while 2,4-D is more damaging under optimum temperature, likely due to slower detoxification.

Future Prospects

At the genetic level, precise mapping of resistance loci remains essential. For mesotrione, future work should focus on identifying key genomic regions using genotyping-by-sequencing (GBS), while integrating complementary methods such as genome-wide association studies (GWAS) and linkage analysis to enhance resolution. In the case of tembotrione, previously identified QTLs (Pandian et al., 2020) must be validated using larger F₂ populations across multiple environments to improve the accuracy of resistance detection. Once the resistance loci are well defined, tightly linked molecular markers can be developed to facilitate marker-assisted selection (MAS) for introgression herbicide tolerance into elite sorghum breeding lines. These improved lines will serve as the foundation for developing high-yielding hybrids with robust tolerance to HPPD-inhibitor herbicides. To further expedite breeding efforts, advanced tools such as genomic selection, doubled haploid technology, and speed breeding should be adopted to shorten generation cycles and rapidly fix desirable traits. Ultimately, integrating molecular

genetics, stress physiology, and accelerated breeding approaches will enable the development of sorghum hybrids with stacked resistance to mesotrione or tembotrione.

Climate change, driven by a steady increase in atmospheric temperatures, poses a significant threat to crop productivity by altering the effectiveness of herbicide-based weed control strategies. Temperature stress can influence key physiological processes such as herbicide absorption, translocation, and metabolism, often resulting in variable herbicide efficacy and increased crop injury. Our findings demonstrate that high temperatures reduced mesotrione metabolism in both corn and sorghum, leading to greater injury, whereas 2,4-D caused less injury under the same conditions likely due to enhanced detoxification mechanisms. These contrasting responses highlight the need to further investigate herbicide behavior under temperature extremes and to better understand genotype-specific tolerance mechanisms.

Future research should prioritize evaluating widely grown corn and sorghum hybrids, rather than just parental lines, to assess herbicide tolerance under temperature stress. In addition to temperature, other environmental variables such as elevated carbon dioxide and increased humidity also need to be studied, as they may further influence herbicide performance and crop responses. A particular emphasis should be placed on studying the expression of temperature-responsive genes, including cytochrome P450s, to unravel the molecular basis of herbicide metabolism under stress conditions. These innovations will enhance the adaptability of sorghum to evolving climate conditions and contribute to more sustainable weed management solutions.

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