THESIS

THE NEED FOR NEW INHIBITORS OF PHOTOSYNTHESIS IN AGRICULTURAL SETTINGS, AND THE NOVEL HERBICIDAL COMPOUND AS9057

Submitted by

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ABSTRACT

THE NEED FOR NEW INHIBITORS OF PHOTOSYNTHESIS IN AGRICULTURAL SETTINGS, AND THE NOVEL HERBICIDAL COMPOUND AS9057

Due to increased food demand, the need for use of herbicides is both necessary and on the rise. Several herbicide classes target photosynthetic electron transport: HRAC Groups 5, 6, and 22. These herbicides are used in large amounts in many different cropping systems to control several species of broadleaf and grass weeds. The first chapter provides a comprehensive review of what these photosynthesis inhibitors are, how they are used and their mode of action. Presently, commercial herbicides only inhibit electron flow at two different sites (PSII and PSI). Those which inhibit electron flow at PSII block the movement of electrons down the electron transport chain, while those which inhibit at PSI accept electrons. Necrosis developing on the leaves of plants treated with PSII and PSI inhibitors is due to the accumulation of reactive oxygen species. Evolution of resistance, toxicity concerns, and other limitations of these herbicides call for the exploration of new chemistries that can be used to target this pathway.

One of these new chemistries has been identified as AS9057. AS9057 is a natural product identified as a novel herbicide with a potentially new mode of action using AI4AI, an AI platform for herbicide discovery developed by Agrematch. Greenhouse trials demonstrated that the herbicidal activity of AS9057 was light-dependent. The rapid burndown symptoms-developing on treated plants, combined with its chemical structure, suggested that AS9057 may target photosystem II. Measurements of photosynthetic electron transport rates in treated plants alongside data from oxygen evolution assays did not support this hypothesis. Further experiments suggested the AS9057 may instead act as an electron diverter. Oxygen consumption assays in isolated thylakoid membranes using a variety of electron transport inhibitors revealed that AS9057 likely acts on photosystem I in a similar manner to paraquat, but

at a potentially new step between P700 and $NADP^+$. This is consistent with other reports that AS9057 can act as an electron acceptor for flavoproteins. Ferredoxin-$NAPD^+$ reductase is a flavoprotein with a redox potential similar to that of AS9057. Thus, it is currently hypothesized that AS9057 acts as an electron acceptor at or near the ferredoxin to form a radical and generate reactive oxygen species which causes the light-dependent herbicidal effect which is observed in treated plants from greenhouse trials.

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INTRODUCTION

Many issues are faced by growers as they continue to meet global food demands. With these demands rising and the surrounding climate beginning to change at a rapid pace, the effects of these issues are amplified. One of the largest of these issues that growers must combat is weeds (Gianessi, 2013). Weeds threaten agricultural crops by monopolizing resources such as light, water, space, and available nutrients. The most efficient and cost effective way to manage these weeds is with herbicides (Gianessi & Reigner, 2017). One of the major pathways which can be targeted by herbicides is the light reaction of photosynthesis, and there are several groups of herbicides which inhibit the light reaction of photosynthesis at different sites along the pathway. Currently, only two sites of the light reaction are targeted by commercial herbicides: photosystem II (PSII) and photosystem I (PSI). These herbicides reside in HRAC groups 5, 6, and 22 which combined consist of over 80 unique chemistries (Heap, 2023). Despite the large number of herbicides which make up these groups, only 22 are commercially available in the Unites States. The limited number of herbicides which are commercially available can be explained by the limited spectra of activity which many of these herbicides have (Martin, 1987).

Even those herbicides which are available have their drawbacks. Many of the Group 5 and 6 compounds are soluble in water and translocate in soil easily, meaning they can leach into bodies of water and persist in the environment for extended periods of time. They can be particularly harmful to aquatic ecosystems (Bottoni et al., 1996; Muller et al., 2008; Rodgers, 1968). Though it is contested that the repeated exposure to these compounds is suspected to cause disruption of the endocrine system (Moore & Waring, 1998). Alternatively, compounds in Group 22 are often subject to restricted or limited use due to their high relative toxicity to mammals (Tsai, 2013). These compounds are also soluble in water and quite mobile with long half-lives (Donaher & Van den Hurk, 2023). Additionally, many species have been reported to exhibit resistance to these compounds due to their repeated use (Rigon et al., 2020). Over 100 species have been reported to be resistant to one or more compounds from Groups 5, 6, or 22 (Hawkes, 2014; Heap, 2023).

Because of the previously mentioned challenges faced with the available commercial herbicides, the discovery of novel herbicides which are safer and more effective is more important than ever before. Being one of the most well understood biological processes, the light reaction of photosynthesis is a great place to begin searching for novel herbicides which fit that criteria (Draber et al., 1991). AS9057 has been identified as a novel herbicidal compound. Its activity is strongly light dependent, suggesting that it may act somewhere along the photosynthetic electron transport chain. This study aims to confirm this herbicidal activity and identify the precise mode of action of AS9057 through a series of biochemical assays. Because AS9057 is a natural product which has been used in the human diet for a long time (Duval et al., 2016; E. M. Malik & C. E. Müller, 2016), it is speculated that AS9057 will be a safer alternative to herbicides which are currently used commercially.

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CHAPTER 1: IS THERE A PLACE FOR NEW HERBICIDES WHICH TARGET PHOTOSYNTHETIC ELECTRON TRANSPORT?

INTRODUCTION

As global food demand continues to rise, there is an increased need for new agricultural practices that maintain and sustain the current levels of food production and trade. Among practices pivotal to these current production levels is the use of herbicides (Gianessi & Reigner, 2017). Conventional agriculture employs a variety of herbicides with different modes of action to control weeds which threaten the yield of agricultural crops (Gianessi, 2013). However, the management of weeds with the herbicides that are currently available is facing some challenges due to several factors such as the evolution of resistance, environmental risks associated with certain herbicides, and mammalian toxicity. Currently, there are over 500 cases of unique herbicide resistance that have been reported globally (Heap, 2023). Additionally, many herbicides have restricted-use labels or have been banned in various places around the world due to their perceived risks (Kniss, 2017).

Several herbicide classes inhibit photosynthesis, the core physiological process that all plants rely on to generate their own chemical energy in the form of carbohydrates using light energy, CO_2, and H_2O (Youvan & Marrs, 1987). Photosynthesis is one of the most studied and well-understood biological processes, and herbicides have been instrumental in dissecting this complex pathway (Dayan et al., 2010; Draber et al., 1991). However, the current commercial herbicides which target photosynthesis pose issues due to many weed species evolving resistance to them, limited use due to the toxic nature of some of these compounds, and their lasting impacts on the environment (Gianessi, 2013). To assess the need for new photosynthetic inhibitors for agricultural use, it is important to understand their current relevance to food production, how these herbicides work, how resistance evolves, and the factors limiting their use.

WHAT ARE PHOTOSYNTHETIC INHIBITORS?

Most photosynthetic inhibitors cause necrosis in plant tissue by disrupting the photosynthetic electron transport chain. Currently, there are two modes of action for commercial herbicides targeting photosynthesis, namely blocking electron transport at the D1 protein of photosystem II (PSII) and electron diversion from photosystem I (PSI) (Fuerst & Norman, 1991). Despite there being multiple other possible targets in the light reactions of photosynthesis, only these two modes of action, PSII, and PSI, are used in agriculture as herbicides targets. Over 80 commercial herbicides targeting PSII have been classified in either Group 5 or 6 by the Herbicide Resistance Action Committee (HRAC) (Figure 1.1). While both groups block electron transport in the photosynthetic electron transport chain by competing for the binding site of plastoquinone on the D1 protein, sensitivity to group 5 PSII-inhibiting herbicides is reduced by mutations at Ser264, whereas sensitivity to group 6 herbicides is reduced by mutations in the D1 protein at His215 (Fuerst & Norman, 1991) . Group 5 consists of 8 different chemical classes while Group 6 consists of 3 chemical classes (Figure 1.1). PSI herbicides are classified by the HRAC as Group 22 herbicides otherwise known as pyridiniums (Figure 1.1). The two most common pyridinium herbicides are paraquat and diquat (Fuerst & Norman, 1991). These herbicides divert electrons from PSI and generate free radical intermediates that produce large amounts of reactive oxygen species (ROS) to cause phytotoxicity (Krieger-Liszkay et al., 2011).

Photosynthetic inhibitors mainly control broadleaf weeds but can also be used to manage some varieties of grass weeds. Group 5 contains the most numbers of chemical classes (e.g., triazines, triazolinones, triazinones, uracils, amides, ureas, pyridazinones, and phenyl carbamates) (Figure 1.1) (Heap, 2023). The two largest classes within Group 5 are the triazines and ureas, with 27 individual herbicides in each (Figure 1.2). Group 6 consists of the nitriles, phenyl-pyridazines, and benzothiadiazinones chemical classes. Group 22 is limited to four pyridiniums (Figures 1.1 and 1.2), however, only two of them are used as commercial products. It is important to note that only 22 of the over 80 registered herbicides in Groups 5, 6, and 22 are commercially available in the United States. This

is because many of the group 5 and 6 herbicides have limited spectra of activity and only a few of them are used for their broad spectrum of activity (Martin, 1987). The remaining chemical classes utilize only 1 or 2 unique compounds for commercial agriculture (Figure 1.2).

USE OF HERBICIDES WHICH TARGET THE LIGHT REACTION OF PHOTOSYNTHESIS

Group 5 and 6 herbicides are widely used for the control of a variety of weed species. The triazine atrazine is by far the most used Group 5 herbicide and is applied primarily on corn, with more than 32,000 metric tons applied in per year in the United States (Figure 1.3). Outside of atrazine (and simazine), the other relevant Group 5 herbicides are used in a variety of cropping systems. For example, the triazinone metribuzin is used in soybean cropping systems, whereas the urea herbicide diuron is used primarily in cotton systems and the amide propanil is used almost exclusively in rice systems (Figure 1.3). Group 6 herbicides are generally used either in soybean or wheat systems (Figure 1.3). Paraquat is the most used Group 22 herbicide, with almost 7× the amount applied in comparison to diquat. Paraquat is used as a nonselective herbicide or defoliant in a variety of cropping systems including soybean, corn, cotton, orchards, and wheat, with the most metric tons applied to soybean (Figure 1.3).

With the dominance of atrazine, most of the photosynthetic inhibiting herbicides are applied in the corn belt region of the Unites States. As of 2018, the states with the highest use are Kansas and Illinois which each use over 5 million tons of these herbicides, closely followed by Nebraska and Iowa which each use approximately 3-5 million tons. Other states which use notable amounts of these photosynthetic inhibitors are Texas, Arkansas, Missouri, Indiana, and Ohio, each using about 2-3 million tons per state (Figure 1.4).

Group 5 herbicides have a market value of nearly 2.2 billion USD/year (Figure 1.5). This is primarily accounted for by atrazine (800 million USD/year), as the most used photosynthesis inhibiting herbicide in the United States by a large margin. Group 22 herbicides amount to 935 million USD /year, consisting mostly of paraquat at 560 million USD /year, which is consistent with its lower use in metric tons (Figure

1.5). Bentazon leads the market value for the Group 6 herbicides and accounts for 217 million USD/year out of a total 227 million USD/year, amounting to the lowest market value of all three herbicide groups targeting photosynthesis (Figure 1.5). Nonetheless, the market trends between 2017 and 2022 reflect a 17% increase in market value for group 5 herbicides, an 8% increase for group 6, and a 23% increase for herbicides in Group 22.

OVERVIEW OF THE LIGHT REACTION OF PHOTOSYNTHESIS

The process of photosynthesis in plants is split into two phases; the light or Hill reactions and the dark reactions or Calvin cycle (Niyogi et al., 2015). For this review, we will only briefly describe the light reactions. The light reactions of photosynthesis are localized within the thylakoid membranes residing in chloroplasts, the specialized photosynthetic organelles that arose from an ancient endosymbiosis event (Niyogi et al., 2015). Light, CO_2, and water are the three components required to synthesize sugar in plants, while O_2 is a by-product of these reactions (Whitmarsh & Govindjee, 1999). First, water is split into O_2, protons and electrons at the water splitting complex (Figure 1.6). These electrons enter the photosynthetic electron transport chain, while the protons accumulate within the lumen and drive photophosphorylation of ADP into ATP via the chloroplast ATP synthase (Youvan & Marrs, 1987). The electrons enter the photosynthetic electron transport chain on the lumen side of PSII following the oxidation of P680, the first checkpoint in the photosynthetic electron transport chain. These electrons are excited by light energy from a redox potential of c. +100 mV to a redox potential of c. -500 mV. Once on the stroma side of PSII, these electrons exit PSII and are transferred to plastoquinone (PQ) (Whitmarsh & Govindjee, 1999). PQ accepts two electrons from PSII and two protons from the stroma to form PQH_2 and transfers these electrons with a redox potential of c. +200 mV to cytochrome b6/f complex (Figure 1.6) (Herbert, 1975). The cytochrome b6/f complex then shuttles these electrons to the blue copper protein plastocyanin inside the lumen which has a redox potential of c. +400 mV(Gross, 1993). Plastocyanin then transfers these electrons to P700 in PSI where light energy excites them from the redox potential of c. +400 mV to c. -500 mV. From here, the redox potential of these electrons slowly decreases until they are

used by ferredoxin/FNR to convert $NADP^+$ to NADPH (Dai et al., 2004). The protons from the water-splitting reaction travel to ATP synthase where they are used in the photophosphorylation of ADP to form ATP.

HOW COMMERCIAL HERBICIDES TARGETING PHOTOSYNTHESIS WORK?

All group 5 and 6 herbicides are selective. They control the desired weed species without injuring the crops on which they are used. The main mechanism of selectivity relies on the differential metabolism of the herbicides. For example, atrazine is very safe for corn because it rapidly detoxifies this herbicide via the action of glutathione-S transferases. Sensitive weeds are not able to metabolize atrazine fast enough (Davis et al., 1964; Shimabukuro, 1967; Shimabukuro et al., 1971). On the other hand, group 22 herbicides are not considered selective because they are not rapidly metabolized by any plants (Kim & Kim, 2020; Sagar, 1987).

With respect to their mechanism of action, PSII herbicides inhibit photosynthesis by binding at the D1 protein on the reducing side of PSII. Here, they competitively bind to the plastoquinone binding site and halt electron flow after they have been initially excited by light energy in the P680 reaction center and have traveled through PSII (Shipman, 1981; Trebst et al., 1983). These herbicides bind competitively at the Q_B site due to their higher affinity to the site than that of the plastoquinone itself (Figure 1.7) (Oettmeier, 1999). Because electron transport is inhibited at this point, electrons can then no longer be shuttled through the rest of the photosynthetic electron transport system and photosynthesis is subsequently halted. Thus, treated plants are no longer able to produce ATP or NADPH which are both required for sustaining plant life (Droppa et al., 1981). Because the H bonding of Group 5 herbicides interacts with Ser264 and Group 6 herbicides interact with His215, they are sensitive to mutation in these respective residues in which they bind (Figure 1.7B). These sites are where the plastoquinone in PSII binds, and, thus, when herbicidal compounds bind there, they block the photosynthetic electron transfer process (Amesz, 1973). This blockage of electrons causes the production of singlet oxygen, a type of

ROS, and other free radicals. Chlorotic damage which is observed in treated plants is a result of the photo-oxidative damage caused by these ROS (Hess, 2000; Pallett & Dodge, 1980; Rutherford & Krieger-Liszkay, 2001; Traxler et al., 2023). In plants which exhibit resistance to PSII inhibitors, the active ingredient of the herbicides are broken down into mobile, nontoxic metabolites and, thus, become no longer phytotoxic (Rigon et al., 2020). Alternatively, there are no known cases of metabolism-based resistance to PSI inhibitors.

As an aside, it should be noted that PSII is a highly promiscuous target, meaning that thousands of molecules have been identified to inhibit PSII in high throughput screens carried by the Ag Chem industry. On the other hand, PSI inhibitors are much less common.

Group 22 herbicides such as paraquat and diquat disrupt the photosynthetic electron transport chain at PSI (Dodge & Harris, 1970). With redox potentials c. -450 mV, these herbicides act as electron acceptors as they emerge from PSI and form reactive radical intermediates which react with free oxygen to generate ROS, causing lipid peroxidation and rapid necrosis of photosynthetically active tissue (Krieger-Liszkay et al., 2011). These compounds cause the accumulation of both hydrogen peroxide and superoxide in levels higher than those that can be quenched by the plants and therefor cause tissue necrosis. This accumulation of ROS occurs via a series of reactions known as the Fenton reaction and the Haber-Weiss Reaction. The Fenton reaction yields hydroxide (OH^-) and hydroxyl radical ($OH^\bullet$) with the reactants Iron (II) and hydrogen peroxide (H_2O_2). Hydroxyl radicals produced by the Fenton reaction are highly unstable and the most toxic of the reactive oxygen species as the initiator of lipid peroxidation (Traxler et al., 2023). The hydroxyl radical can also be produced through the Haber-Weiss reaction, in which hydrogen peroxide and superoxide radicals are catalyzed by Iron (II). This reaction yields not only the hydroxyl radical but hydroxide as well (Traxler et al., 2023). In plants, the overproduction of ROS may cause DNA, protein, and lipid damage which ultimately causes cell death (Plaza et al., 2021). Additionally, this inhibits the production of NADPH because electron transport is diverted at a point likely before the ferredoxin and before it is used to produce NADPH.

PSI-inhibiting compounds are contact herbicides, meaning that they act in contact with plant tissue exposed to light and do not readily translocate in side of the plant (Funderburk & Lawrence, 1964). This also means that plants are mostly unable to metabolize PSI herbicides because they kill tissues so rapidly. Because these herbicides do not bind to a specific site and the compound itself causes the phytotoxic effect, it has been difficult for plants to evolve resistance to Group 22 herbicides (discussed below).

RESISTANCE TO INHIBITORS OF PHOTOSYNTHESIS

Repeated use of the same herbicide has driven evolution of resistance in weeds (Rigon et al., 2020). Resistance can result from target-site mutations and/or overexpression (TSR), or via other nontarget-site (NTSR) mechanisms that alter the way plants detoxify, move, or compartmentalize the herbicides (Gaines et al., 2020). Because inhibitors of photosynthesis have been widely used in agriculture for many years, many weed species have evolved resistance to herbicides targeting the photosynthetic electron transport chain (Gronwald, 1997). Resistance to inhibitors of PSII has evolved in 92 weed species such as common ragweed, redroot pigweed, bluegrass, and knotweed, and has been reported widely across the globe, but primarily in the United States, Canada, and France (Figures 1.8 and 1.9) (Heap, 2023; Pfister & Arntzen, 1979). Resistance to Group 5 herbicides is reported in 87 species while resistance to Group 6 herbicides is only reported in 5 species as of 2023 (Heap, 2023). Many species have evolved resistance to triazines, a Group 5 class of herbicides. Since reduced sensitivity to triazines is linked to mutations at Ser264 on the D1 protein of the PSII complex, most of the triazine resistance cases involve mutations at that site (Battaglino et al., 2021; Gronwald, 1997; Oettmeier, 1999; Pfister & Arntzen, 1979). In Group 5 herbicides, a Ser264 to Gly mutation is responsible for resistance to triazines in the D1 protein (Funderburk & Lawrence, 1964; Oettmeier, 1999). This Ser264 to Gly mutation has been found in over 50 independent species across more than 20 different nations (Oettmeier, 1999). However, biotypes that exhibit resistance to triazines due to this mutation are not resistant to urea-type herbicides which are chemically distinct (Powels & Preston). Several varieties of double and triple mutants also exist with mutations at two or three different sites respectively. These mutations cause resistance to more than one

herbicide in the same plant (cross-resistance). Some resistance to PSII herbicides has been found which does not involve TSR mutations. Instead, these plants evolved metabolism-based NTSR that detoxify the Group 5 herbicides via the activity of glutathione S-transferases (Oettmeier, 1999).

In some instances, species exhibit negative cross-resistance. Negative cross resistance is described as a mutation that causes resistance at one site and hypersensitivity at another site. Some weeds present resistance to triazines, while still sensitive to ureas. Weeds which are resistant to triazines have greater sensitivity to herbicides from Groups 1, 30, and 6 (Fuerst et al., 1986; Gadamski et al., 2000).

Though it is less common, PSI resistance has been confirmed. However, it should be noted that resistance to PSI herbicides has evolved more recently than the resistance to PSII-inhibiting herbicides, which is likely attributed to the higher rates and higher frequency of use of PSII herbicides. Paraquat resistance has been reported in 28 weed species across 14 different countries (Hawkes, 2014). Like PSII inhibitors, both monocot (*Eleusine indica* and *Lolium rigidum*) and dicot (*Conyza* spp. and others) weed species have evolved resistance to PSI inhibitors, which seems more difficult to evolve because these herbicides do not bind to a specific protein. The lack of ligand/protein interaction in this type of inhibition is not likely to result in a target site mutation imparting resistance to PSI inhibitors. Additionally, PSI herbicides are fast-acting, so plants have difficulty evolving mechanisms of NTSR to them. The compounds themselves accept electrons to generate ROS and cause oxidative stress. It is currently hypothesized that plants resistant to ROS-generating herbicides may have an increased antioxidant system and so rapidly quench the excessive superoxide and hydrogen peroxide (Amorim et al., 2022). Plants overexpressing two or more enzymes in the Halliwell-Asada cycle (i.e. CuZnSOD, MnSOD, stromal ascorbate peroxidase, or dehydroascorbate reductase) have higher tolerance to PSI herbicide application. Overexpressing these enzymes in plants causes the rapid inactivation of peroxide to reduce ROS and its effects (Hawkes, 2014). Similar effects are also seen in plants which overexpress catalase. Some studies also suggest that resistance to PSI herbicides can be caused by mutations that cause the sequestration of these herbicides into plant cell vacuoles (Hawkes, 2014).

LIMITATIONS OF INHIBITORS OF PHOTOSYNTHESIS

Despite their effectiveness, herbicides targeting photosynthesis have certain drawbacks. PSII inhibitors, particularly triazines, persist in ecosystems for extended amounts of time. They may damage those ecosystems which exist in natural bodies of water (Muller et al., 2008). Additionally, these compounds are highly mobile in soil, allowing for high levels of soil translocation and leaching into water bodies. This leaching is caused by the relatively high polarity of such compounds (Bottoni et al., 1996; Rodgers, 1968). These herbicides are also suspected to be endocrine disruptors, meaning that exposure to these compounds causes interference with the endocrine system, the system in animals which is responsible for the synthesis and regulation of hormones (Moore & Waring, 1998). Effects of endocrine disruption include increased risk of certain cancers, interference with reproduction and development, and defects in other bodily systems such as the nervous or immune systems (Casals-Casas & Desvergne, 2011). In other certain species, this can cause hermaphroditic individuals (Evans, 2022). In humans, the primary means of exposure to such compounds is through the drinking water which is generally sourced from the ground water in which these herbicides leach into. Due to these health and environmental concerns, atrazine has been banned in Uruguay (Avila et al., 2020).

The primary limitation of the use of PSI inhibitors like paraquat and diquat is their high relative toxicity. Paraquat is the most abundantly used PSI herbicide with over 8,000 metric tons per year applied in the United States. This pyridinium is considered a dangerous substance. Paraquat can be fatal in even small amounts when swallowed, inhaled, or even when in contact with skin. with oral and dermal LD_{50}'s in rat of about 80 mg/kg. Prolonged and repeated exposure to paraquat can cause extreme organ damage, primarily to its target organs which is the respiratory system (EPA, 2024). Paraquat disrupts mitochondrial electron transport and also causes heart, kidney, and liver failure. Paraquat exposure has also been linked to Parkinson's disease (Tangamornsuksan et al., 2019). Additionally, there is no known cure or antidote for paraquat poisoning.

Like PSII herbicides, PSI herbicides may also be toxic to aquatic ecosystems, having large toxicity effects on algae, bacteria, fish, and other aquatic invertebrates (Tsai, 2013). Paraquat is water soluble and can move quickly in these aquatic environments, though it is not mobile in soils. Paraquat persists in the environment for extended periods of time and can have a half-life of over 6 years (Donaher & Van den Hurk, 2023).

Though paraquat continues to be used in the United States, its use is highly regulated and even banned in several other parts of the world including several South American Countries (Avila et al., 2020). In Uruguay, paraquat requires a professional prescription to be used and is the only herbicide of which this is required. In 2020, paraquat was permanently banned in Brazil. In Colombia, only two prohibitions of herbicides are in effect, one of which being the aerial application of paraquat (Avila et al., 2020). In each of these nations and several others, paraquat is frequently put under review time and time again due to its known human health and environmental effects.

Additionally, a limitation which is presented by the herbicides which we currently use commercially is the lack of sites which are targeted. While Groups 5, 6 and 22 target either PSII or PSI, other compounds can target other aspects of photosynthesis. For example, 2,5-dibromo-6-isopropyl-3-methyl-1,4- benzoquinone (DBMIB) blocks the photosynthetic electron transport chain at the cytochrome b6/f complex (Bauer & Wijnands, 1974; Trebst et al., 1970). Though the mode of action of DBMIB was identified before the 1980s and its relative toxicity is low, DBMIB is not used as a commercial herbicide likely because it can inhibit the respiratory process when handled or used incorrectly. Furthermore, glutaraldehyde inhibits electron transport at the level of plastocyanin (Hardt & Kok, 1977), but has similar toxicity effects as those of paraquat. However, glutaraldehyde is a very toxic reagent used to stabilize proteins in microscopy. Consequently, this compound has never been developed as a herbicide targeting this site of action.

IS THERE A NEED FOR NEW HERBICIDES TARGETING PHOTOSYNTHESIS?

Herbicides targeting photosynthesis have been used in agriculture since the 1960s (Ross & Kreiger, 1980). Even though there are many cases of herbicide resistance (Gronwald, 1997; Rigon et al., 2020) and some concerns over their environmental toxicity, these compounds remain popular. Herbicide discovery is a long and difficult process, but an important one to pursue to address current pressing issues facing farmers. Despite the 21.1% increase in the cost of herbicide development since 1995 (Agbioinvestor, 2024), photosynthesis is one of the best understood biological processes, and it has been a successful target for the development of valuable herbicides. Consequently, research on new herbicides inhibiting photosynthesis aiming to identify molecules with modes of action outside of known PSII and PSI targets, or that target known modes of action but with novel interactions within these sites should be pursued. However, while the combined market value of herbicides targeting photosynthesis is 3.4 billion USD/year, nearly 50% of that value rests in one active ingredient from each group (atrazine for Group 5, bentazon for Group 6, and paraquat for Group 22). Therefore, yes, the need for new herbicides which target this pathway is prevalent. However, potential new herbicides targeting photosynthesis will have to perform better, cost less, overcome resistance, and have better environmental and toxicological profiles than the three leading active ingredients.

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FIGURES

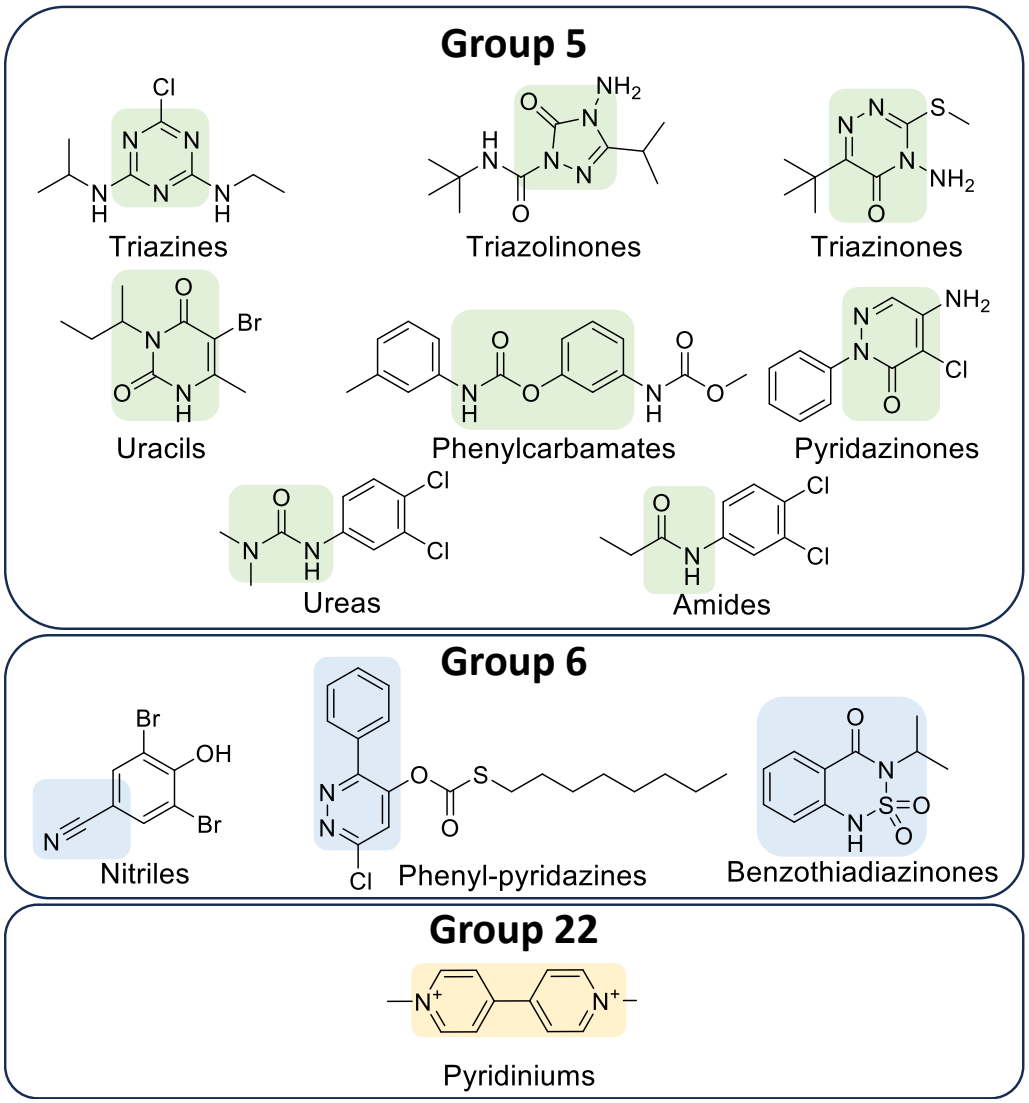

Figure 1.1. Structural characteristics of commercial herbicides targeting photosynthesis. Examples of Group 5 herbicides: Atrazine (triazines), amicarbazone (triazolinones), metribuzin (triazinones), bromacil (uracils), phenmedipham (phenylcarbamates), chloridazon (pyridazinones), and propanil (amides). Examples of Group 6 herbicides: Bromoxynil (nitriles), pyridate (phenyl-pyridazines) and bentazon (benzothiadiazinones). Example of Group 22 herbicides: Paraquat (pyridinium)

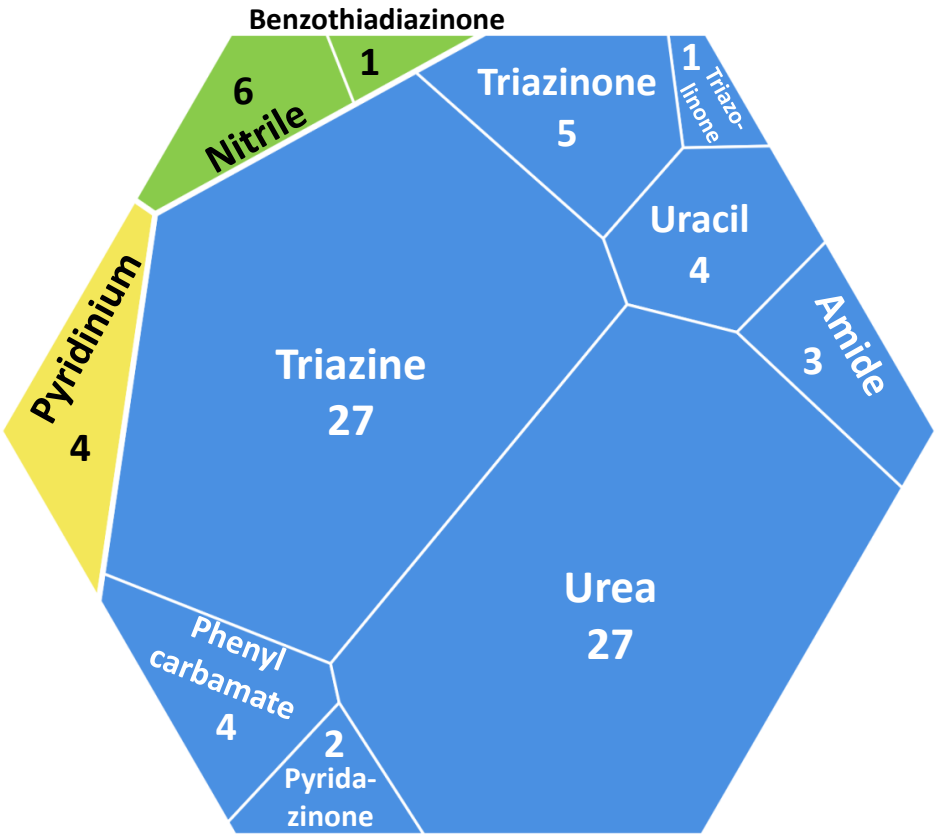

Figure 1.2. Relative size of each chemical class within HRAC Groups 5 (blue), 6 (green) and 22 (yellow) herbicides. Of the 81 registered herbicides used to generate this figure, only 5 triazines, 2 triazinones, 2 uracils, 2 phenylcarbamates, 1 pyridazinone, 5 ureas, 1 amide, 1 nitrile, 1 benzothiadiazinone and 2 pyridiniums were used in the United States in 2018. Data from USGS Pesticide National Synthesis Project (USGS, 2024).

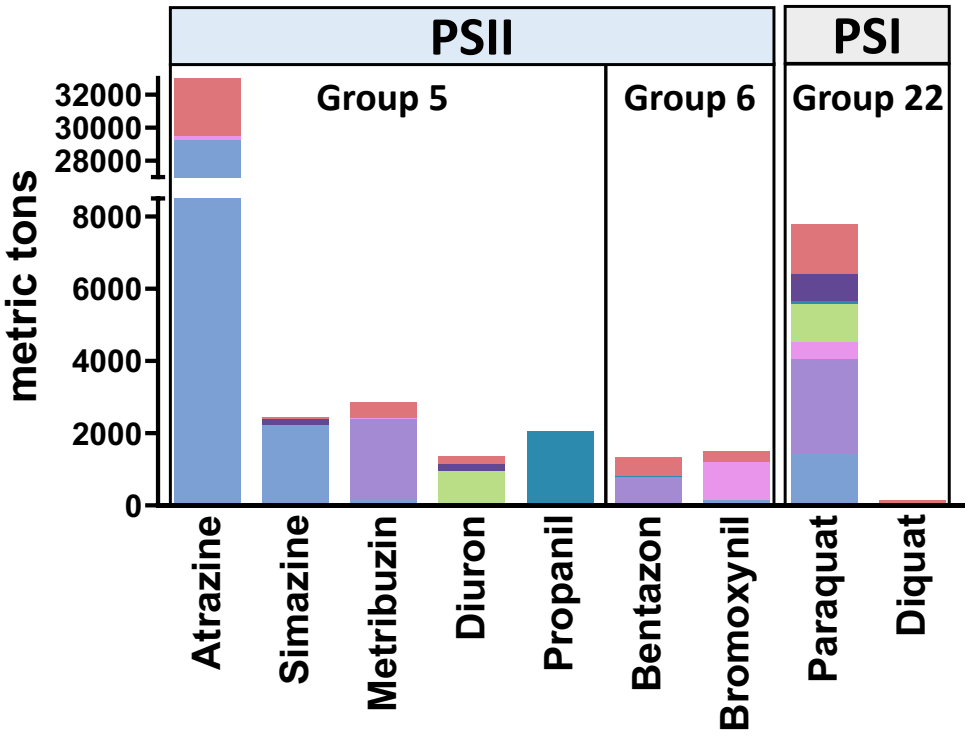

Figure 1.3. Most used herbicides targeting photosynthesis in the United States (in metric tons). Herbicides are organized by HRAC Groups 5 and 6 targeting photosystem II (PSII) and HRAC Group 22 targeting photosystem I (PSI). Atrazine and simazine are triazines, metribuzin is a triazinone, diuron is a urea, and propanil is an amide. Bentazon is a benzothiadiazinone and bromoxynil is a nitrile. Paraquat and diquat are pyridiniums. =corn; =soybean; =wheat; =cotton; =rice; =orchards and grapes; =other crops. Most recent complete data available is for 2018, obtained from USGS Pesticide National Synthesis Project (USGS, 2024).

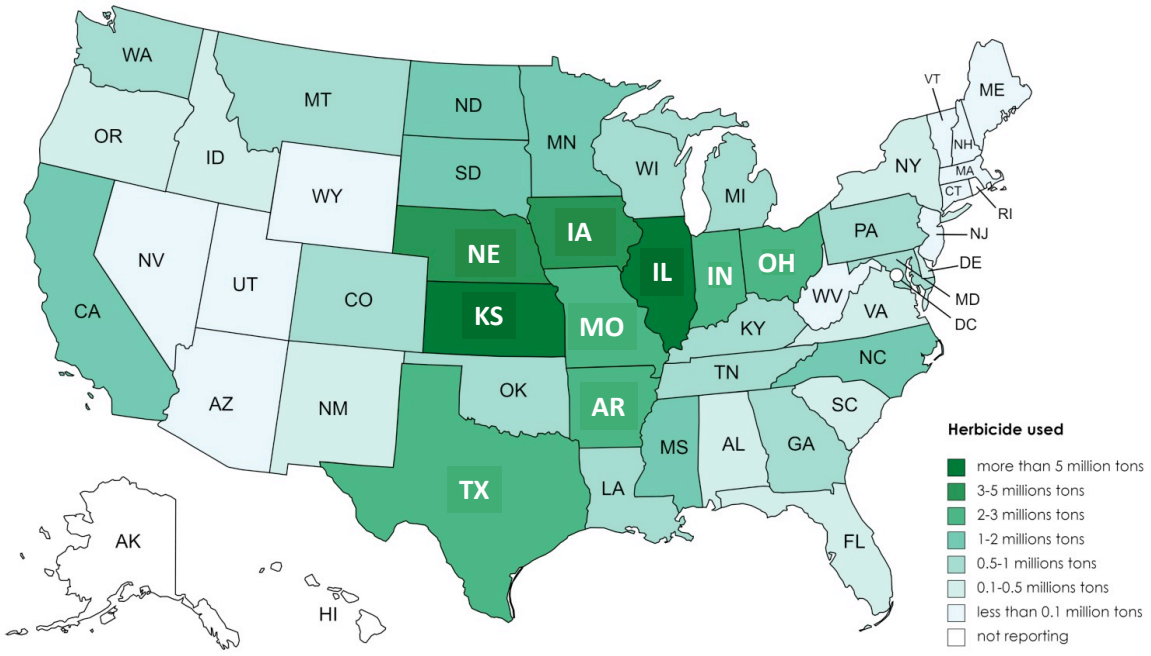

Figure 1.4. The total amount of HRAC Groups 5, 6, and 22 herbicides used in each state in 2018. Data from USGS Pesticide National Synthesis Project (USGS, 2024). The map was generated with MapChart (https://www.mapchart.net/usa.html).

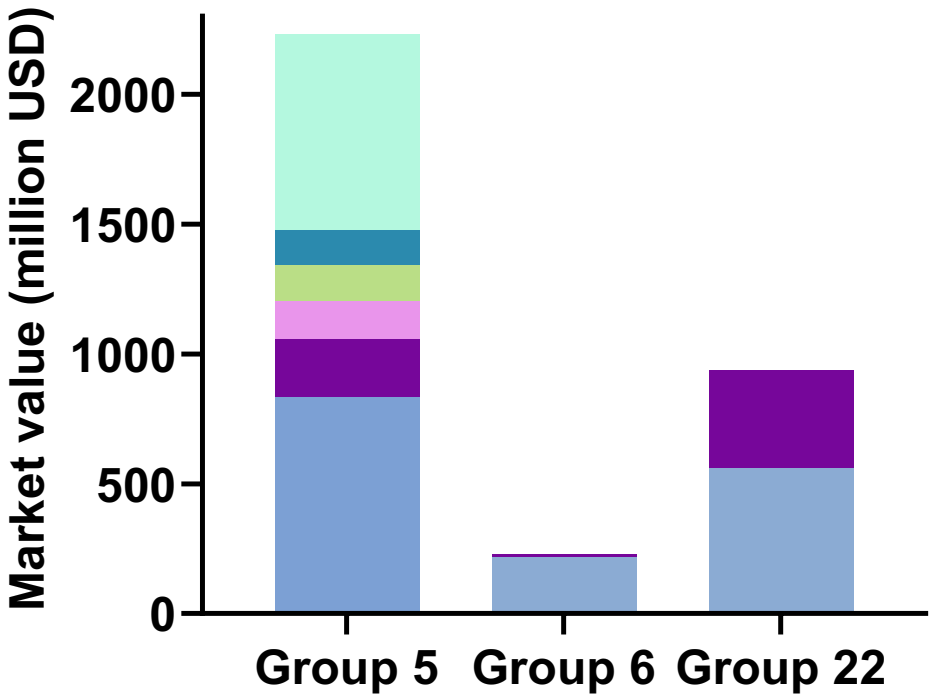

Figure 1.5. 2022 Worldwide market value of herbicides targeting photosynthesis. Group 5 herbicides included =atrazine; =metribuzin; =diuron; =tebuthiuron; =metamitron; =19 other herbicides. Group 6 herbicides included =bentazon; =pyridate. Group 22 herbicides included =paraquat; =diquat. Data kindly provided by AgbioInvestor.com.

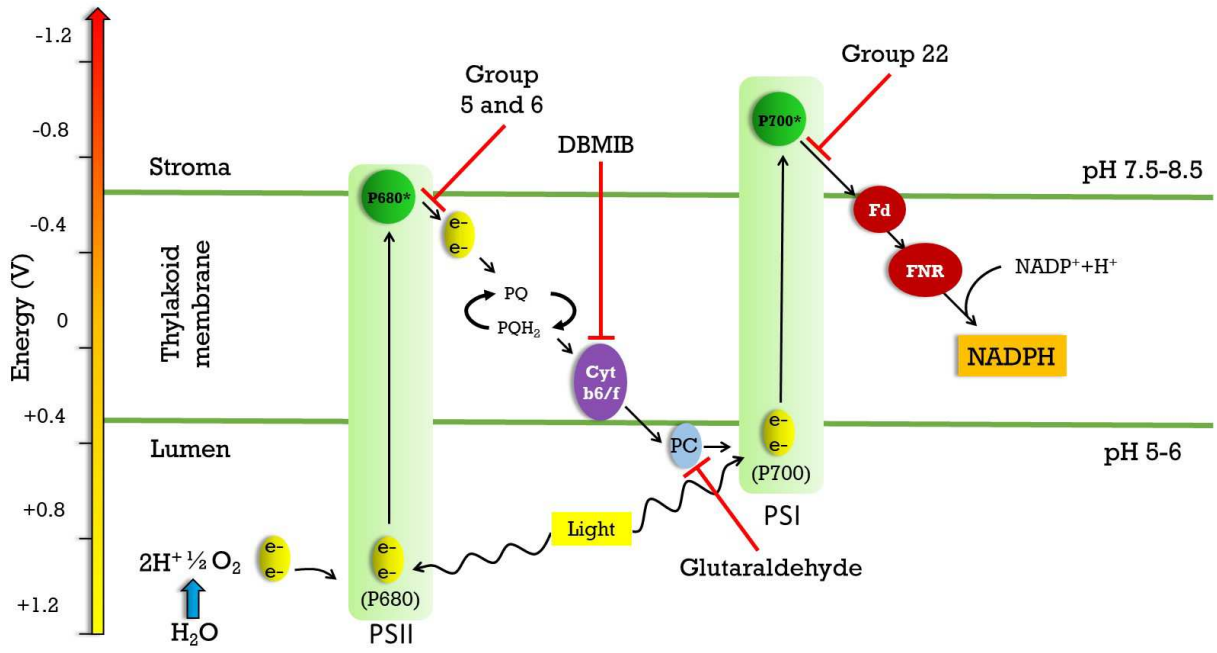

Figure 1.6. Z-scheme of photosynthetic electron transport chain that points where inhibitors act along the chain. Photosystem II (PSII), photosystem I (PSI), plastoquinone (PQ), plastoquinol (PQH_2), 2,5-dibromo-6-isopropyl-3-methyl-1,4- benzoquinone (DBMIB), plastocyanin (PC), ferredoxin (Fd), ferredoxin/NADP reductase (FNR).

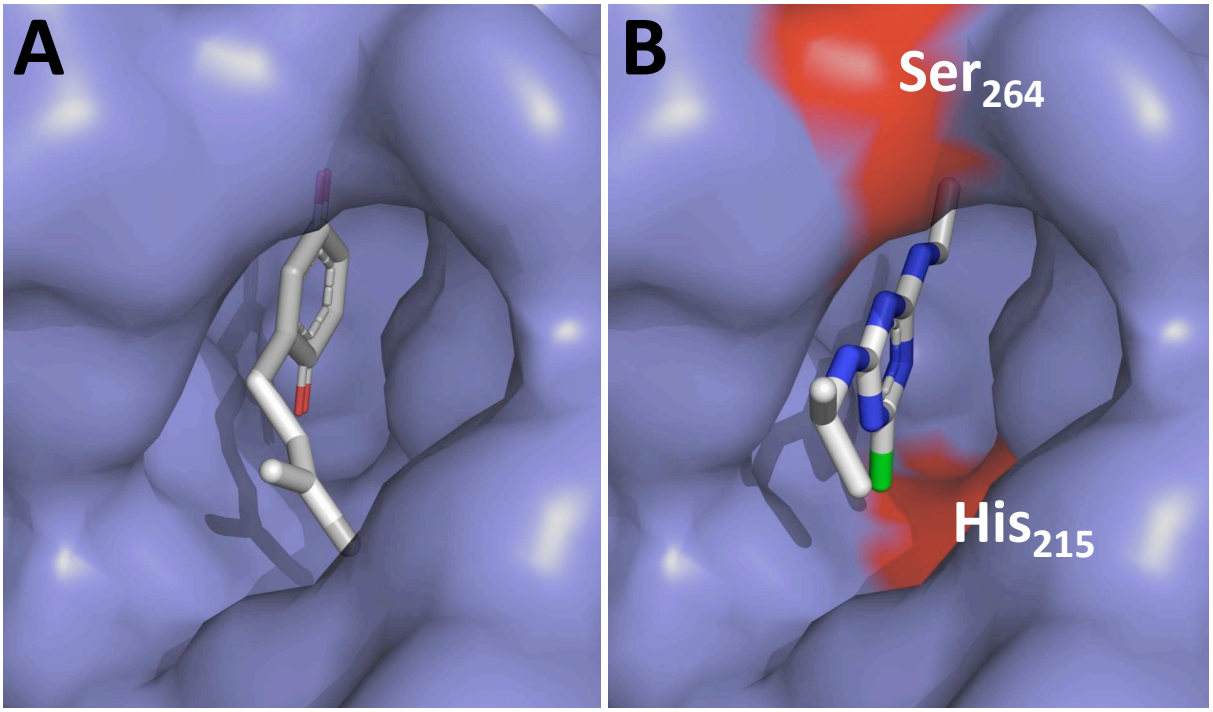

Figure 1.7. Crystal structure of the D1 protein of *Arabidopsis thaliana* (PDB: 7OUI) showing the plastoquinone binding pocket with either A) pentyl benzoquinone (analog of plastoquinone) or B) atrazine. Reduced sensitivity to Group 5 herbicides is caused by mutations in Ser264 whereas reduced sensitivity to Group 6 herbicides is caused by mutations in His215 (shown in red in panel B). The coordinates of atrazine binding were obtained from the crystal structure of *Rhodopseudomonas viridis* reaction center (PDB: 5PRC).

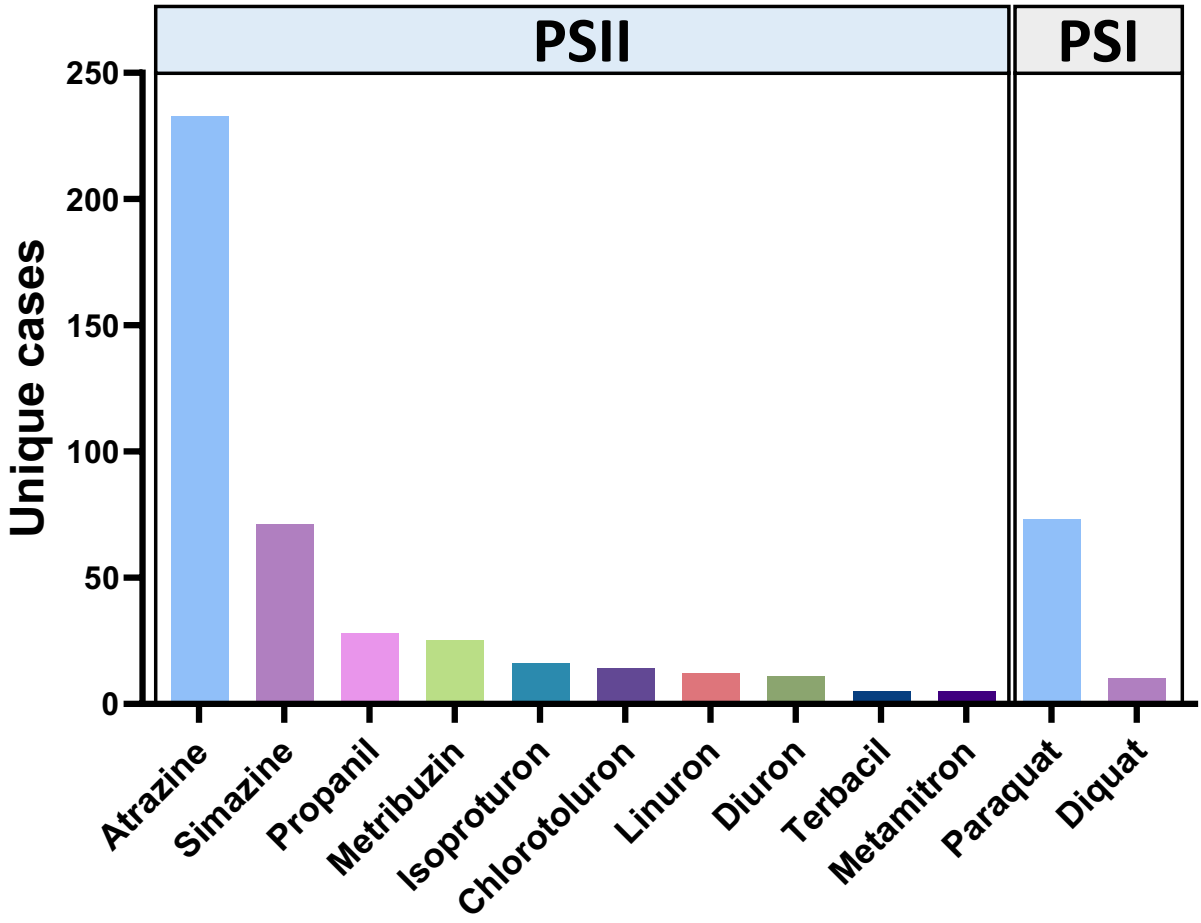

Figure 1.8. Unique cases of herbicide resistance in photosynthetic inhibitors grouped by active compound.

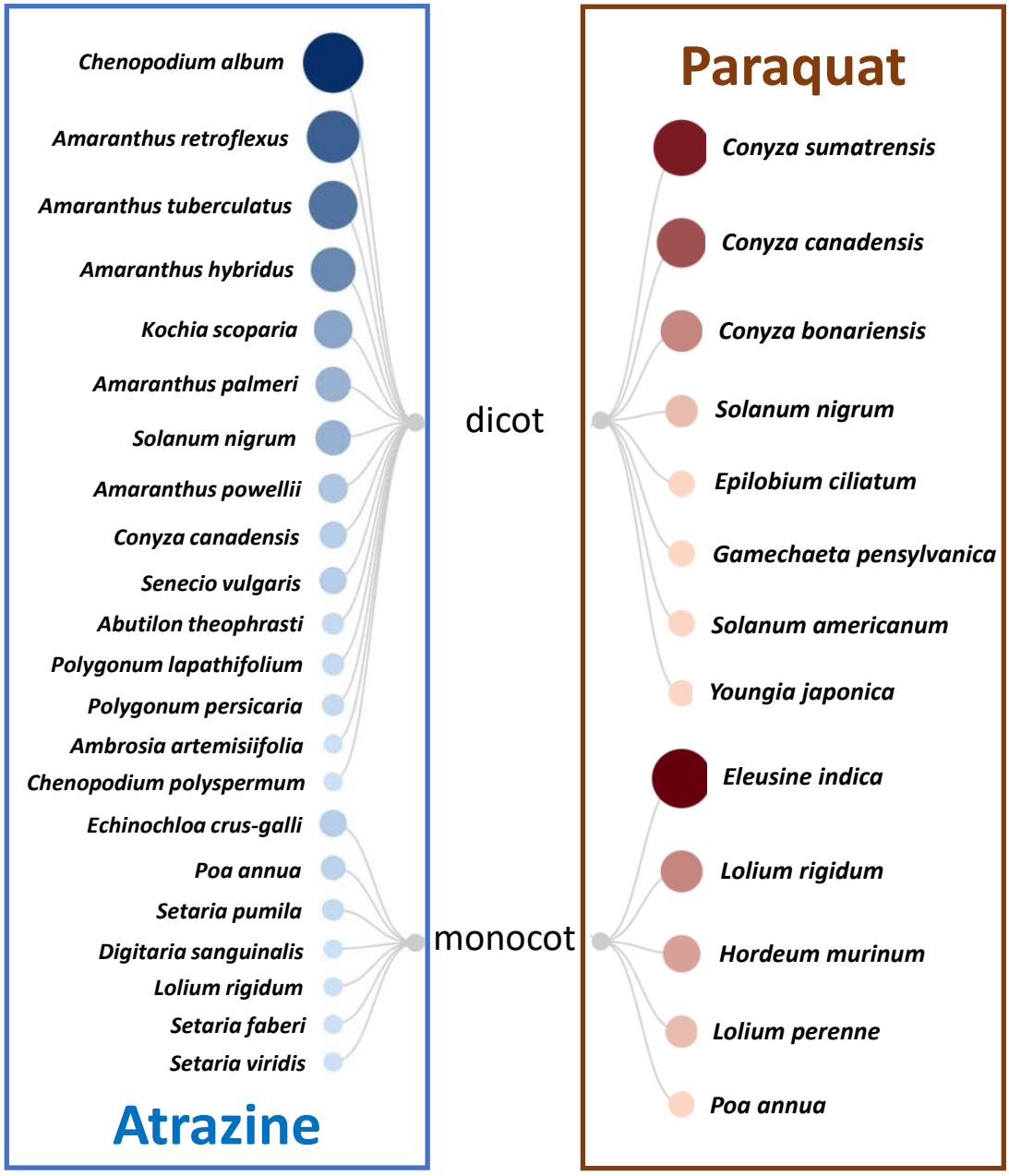

Figure 1.9. Most common monocot and dicot weed species with resistance to the PSII herbicide atrazine (blue) and PSI herbicide paraquat (brown).

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CHAPTER 2: THE NATURAL HERBICIDE AS9057 TARGETS PHOTOSYSTEM I

INTRODUCTION

Increases in the world population continue to put pressure on global food production. Farmers face many challenges endeavoring to meet the ever-increasing demand, including the need for new weed management tools (Dayan, 2019). Herbicides remain the most effective and cost-efficient way to manage weeds in agroecosystems where they compete for the light, nutrients, and space needed to grow crops (Gianessi & Reigner, 2017). However, after decades of agricultural herbicide use, many weeds have evolved resistance to several groups of herbicides (Duke, 2012; Gaines et al., 2020). New modes of action must be discovered to address this problem. Despite this growing need for new modes of action and many scientific efforts to discover them, there have been few new modes of action discoveries in nearly 20 years (Dayan, 2019; Duke, 2012). As a part of these modes of action discovery efforts, Agrematch has developed a proprietary Artificial Intelligence platform, AI4AI^{TM}, which identifies compounds with predicted new modes of action (Barker AL, 2023).

AS9057 (4,5-dihydroxyanthraquinone-2-carboxylic acid) (Figure 2.1), also known as rhein, is a natural anthraquinone present in numerous plant species, including rhubarb (*Rheum* spp. L) (Duval et al., 2016). Anthraquinones are highly pigmented molecules that form the largest group of natural dyes (Dulo et al., 2021). This class of compounds has had multiple industrial (Duval et al., 2016) and medicinal applications (Chien et al., 2015; Enas M. Malik & Christa E. Müller, 2016).

The biological activities of AS9057 include antifungal, anti-inflammatory, and antioxidant properties and has several medical applications (Duval et al., 2016). A study on the chemical properties of AS9057 also suggested that its redox potential would make it a suitable electron acceptor from some flavoproteins and electron transport systems (Egerer et al., 1982).

Though AS9057 has been investigated for a variety of biological activities or industrial uses, nothing was known about its herbicidal activity. AS9057 is toxic to hepatocytes by accumulating in the mitochondria, redox cycling, and generating oxygen free radicals causing loss of mitochondrial

membrane integrity and subsequently halting the production of ATP (Bironaite & Öllinger, 1997). Though not many studies have been done surrounding the effects of AS9057 on plants, the herbicidal properties of quinones have been studied extensively, including benzoquinones (Dayan et al., 2009; González-Ibarra et al., 2005; Nain-Perez et al., 2016; Uddin et al., 2013), naphthoquinones (Durán et al., 2019; Jewess et al., 2002) and anthraquinone (Oettmeier et al., 1988; Schrader et al., 2003). One study showed that similar quinones inhibit chloroplastic ATPase activity. Another reported inhibition of the photosynthetic electron transport chain at some location between PSII and PSI, most likely by competing with plastoquinone at the Q_B binding site as do the group 5 and 6 herbicides (i.e., triazine, urea and nitrile herbicides) (Strotmann et al., 1982; Trebst et al., 1970). Additionally, many quinones are present as allelochemicals in soils released by plants fungi, or other microorganisms, and are hypothesized to disrupt electron transport by forming semiquinone radical intermediates (Uchimiya & Stone, 2009).

AS9057 was predicted by the AI4AI algorithm to have a new mode of action and was selected as a herbicidal lead. Preliminary studies confirmed the herbicidal activity of AS9057, and its action is strongly light-dependent, suggesting that it interferes with photosynthesis. While the structural features of AS9057 suggest that it might act as an inhibitor of photosystem II (Oettmeier et al., 1988), we report that this natural anthraquinone targets a different step in photosynthesis and its contact activity involves the generation of high amounts of reactive oxygen species leading to lipid peroxidation and desiccation of the leaves.

MATERIALS AND METHODS

Herbicidal activity and Dose Response

For Growth chamber herbicidal activity assays, *Amaranthus palmeri, Chenopodium quinoa, Solanum nigrum, Setaria viridis* and *Gossypium hirsutum* were planted in 9×9 cm pots filled with potting mix. Growth conditions were 16 h light / 8 h dark with 30°C and 25°C, respectively. All plants were watered as needed.

AS-9057 powder was mixed with a solution of 2% (v/v) Monoisopropanolamine (obtained from

Merck, Sigma-Aldrich® 471291), 2% (v/v) Dash® HC (obtained from BASF) and 0.25% (v/v) Genapol® X-080 (obtained from Merck, Sigma-Aldrich® 48750). Plants were sprayed 7-15 days after sowing at the 1-3 true leaf stage using a VL-SET Paasche Airbrush at a 1,000 L ha-1 application volume. Each treatment contained 2-3 pots as repeats. Herbicidal activity was visually assessed and scored 20 days after application.

For dose-response assays, Palmer amaranth (*Amaranthus palmeri*) was planted in pots with clay soil and grown in a Net-house. Conditions were approximately 12 h light / 12 h dark with 29°C on average and minimum and maximum temperature of 19°C and 46°C respectively. Average relative humidity was 67%. All plants were watered as needed. Scythe® herbicide (Gowan) was diluted with water. AS-9057 powder was mixed with a solution of 2% (v/v) Monoisopropanolamine (obtained from Merck, Sigma-Aldrich® 471291), 2% (v/v) Dash® HC (obtained from BASF) and 0.25% (v/v) Genapol® X-080 (obtained from Merck, Sigma-Aldrich® 48750). Weeds were sprayed 10 days after sowing at the 4-leaf stage using a DeVries Generation 4 Research Track Sprayer with a TEE JET nozzle 80015EVS. The spray speed was 2 KMH, spray pressure was 40 PSI and total spraying volume was 400 L/ha. Each pot contained 6 plants and each treatment contained 5 pots as repeats. The herbicidal activity was assessed and scored 14 days after application by cutting and weighing the aerial portion of the plants in each pot.

For Growth chamber herbicidal activity assays, *Amaranthus palmeri, Chenopodium quinoa, Solanum nigrum, Setaria viridis* and *Gossypium hirsutum* were planted in 9×9 cm pots filled with potting mix. Growth conditions were 16 h light / 8 h dark with 30°C and 25°C, respectively. All plants were watered as needed.

AS-9057 solution was prepared as previously described. Plants were sprayed 7-15 days after sowing at the 1-3 true leaf stage using a VL-SET Paasche Airbrush at a 1,000 L ha-1 application volume. Each treatment contained 2-3 pots as repeats. Herbicidal activity was visually assessed and scored 20 days after application.

Electrolyte Leakage Assay

Ten cucumber cotyledon discs were floated in a bathing medium (5% sucrose, 2 mM MES pH 6) in petri dishes (Dayan et al., 2008). AS9057 was added to each of these Petri dishes to the final concentrations of 0, 1.5, 3, 6, and 12 μM. Three replicate dishes of each concentration were made. These dishes were incubated in the dark overnight and then placed under high light intensity (1,000 μmol/m^2/s). Data for electrolyte leakage was then collected using a conductivity meter with a FiveEasy Plus FP30 conductivity meter connected to an InLab 751-4 mm microprobe (Mettler Toledo, Columbus, OH 43240) (Dayan & Watson, 2011).

Reactive Oxygen Species (ROS) Accumulation Assay

Cucumber cotyledon discs were prepared in AS9057 solutions as described above and incubated under high light intensity for 5 h. Hydrogen peroxide was quantified using a diaminobenzidine (DAB) stain. The DAB stain solution consists of 300 mg DAB dissolved in 275 mL of deionized water. The pH was adjusted to 3.8 using HCl. Then 150 μL of Tween 20 (Millipore Sigma, St Louis, MO) was added to the solution to act as a surfactant followed by15 mL of 200 mM Na_2HPO_4 was then added to neutralize the solution (Takano et al., 2019).

Leaf discs were then transferred into tubes with 2-5 mL of DAB solution, and kept under low vacuum (15 mm Hg) for 1 h with shaking at 50-100 rpm. Samples were then heated at 75°C for 30 min. DAB solution was removed and replaced by 2-5 mL of destaining solution consisting of ethanol:acetic acid:glycerol (3:1:1 by volume). Destaining was repeated to remove all pigments. Samples were transferred to white background blotting paper and scanned at the highest resolution. H_2O_2 accumulation as indicated by the intensity of the color in the leaf discs was then quantified using imaging software (Photoshop CS3, Adobe, San Jose, CA) by converting images to grey scale (Takano et al., 2019). Background levels of untreated discs were subtracted from the treated discs.

Photosynthetic Electron Transport Assay

Ten leaf discs of six-leaf stage wild type Palmer amaranth were prepared and floated in Petri dishes

on 10 mL of bathing medium (5% sucrose, 2 mM MES pH 6). AS9057 was dissolved in the previously described surfactant solution at a concentration of 10 mg/mL and diuron, a known inhibitor of PSII, was dissolved in dimethyl sulfoxide (DMSO) (Battaglino et al., 2021). Water and DMSO were used as controls. 100 µL of the tested herbicidal compounds stock solutions and controls were added to the leaf discs. Three replicates of each Petri dish were made. Dishes containing leaf discs were incubated in the dark overnight and then placed under high light intensity (1,000 µmol/m^2/s). Photosynthetic electron transport rates were then collected using a leaf fluorometer (Opti-Sciences Y(II), Light adapted meter, Hudson, NH USA) (Dayan et al., 2009).

Extraction of Thylakoids

Thylakoids were extracted from fresh organic spinach leaves purchased at a local store according to (Dayan et al., 2017). Fifty grams of leaves are collected and homogenized with 250 mL of extraction buffer consisting of 1,650 mM sorbitol, 50 mM HEPES, 25 mM cysteine, 5 mM $MgCl_2$, and 5 mM EDTA. The homogenized leaves were filtered through one layer of Miracloth (Millipore Sigma, St Louis, MO) lined inside two layers of cheesecloth, and centrifuged in 50 mL aliquots in disposable tubes (Falcon, Fisher Scientific, Waltham, WA) at 3,000 g for 30 min. The supernatant was discarded, and the chloroplast pellets were resuspended in resuspension buffer solution containing 1,650 mM sorbitol, 50 mM HEPES, 5 mM dithiothreitol, 5 mM $MgCl_2$, and 5 mM EDTA. Thylakoids prep was transferred to microfuges in 500 µL aliquots, frozen and stored at -80°C until use.

Photosystem II Oxygen Evolution Assay

The oxygen evolution assay is conducted in a Clark electrode (Oxytherm+ System, Hansatech Instrument, Norfolk, UK). This experiment is conducted at 25°C. 50 µL of the thylakoid prep and 30 µL of the test compounds (from 100 × stock solutions) were added to 3 ml of the assay solution (800 mM sucrose, 50 mM MES-NaOH, 15 mM $CaCl_2$, and 1 mM ferricyanide equilibrated at 25°C) in the chamber (Dayan et al., 2017). The stir bar is turned on and the thylakoids were allowed to mix with buffer for 1

min, the light is turned on to initiate photosynthesis for an additional minute prior to recording oxygen evolution. Then, the compound of interest (either AS9057 or paraquat) is added and initial slope and changes in the slope are recorded. In this assay, AS9057 and diuron were used to test their effect on photosystem II activity.

Photosystem I Oxygen Uptake Assays

This assay uses the same buffer as for the PSII assay, except that the electron acceptor ferricyanide is not added to the solution. As with the standard oxygen evolution assay described above, 3 mL of this buffer and 50 µL of extracted thylakoid are added into the chamber and allowed to mix for 1 min before turning the light on (Dayan et al., 2017). Oxygen uptake was monitored for 1 min. After this additional minute, 30 µL of the compound of interest is added and slopes and changes in slopes are recorded. Compounds of interest in this case are 1 mM ferricyanide which is used as a terminal electron acceptor for photosystem II, 1 mM paraquat which is a PSI electron diverter, 0.33 mM AS9057, and the surfactant solution ensures there is no effect caused by the components of this solution.

Additional oxygen uptake assays were performed in the same manner as all previous assays using a buffer that consisted of 0.6 M sorbitol, 5 mM $MgCl_2$, 25 mM HEPES, 0.5 mM NH_4Cl, and 20 µM diuron or 5 µM DBMIB (2, 5-dibromo-3-methyl-6-isopropylbenzoquinone, Millipore Sigma, St Louis, MO) with 50 µL of extracted thylakoid membrane. These two components are allowed to mix in the chamber for 30 s, the light is then turned on and the contents mix for an additional 30 s. Then 30 µL of each test compound was added to the assay and a measurement took place for two minutes. In this assay, diuron blocks the source of electron at PSII and in a separate assay DBMIB blocks the electron at the cytochrome b6/f complex (Malkin, 1981).

Ab Initio Calculations

The structure of AS9057 was obtained from the x-ray analysis of a cocrystal salt solvate of AS9057 and berberine (Yang et al., 2022) deposited in The Cambridge Crystallographic Data Centre (CCDC) (Groom et

al., 2016). Spartan20 (Version 1.1.4, Wavefunction, Inc. Irvine, CA 92612) was used to remove berberine and solvent. The bond angles and length were corrected by submitting the structure to geometric minimization and energy optimization using density function theory calculations (wB97X-D 6-31*).

Statistical Analysis

Experiments comparing means were analyzed with a one-way ANOVA test followed by a Duncan multiple range analysis test ($p < 0.05$). The data were analyzed using RStudio 2023.06.2 Build 561R with R statistical package (Release 4.3.1) and module agricolae.

RESULTS

Dose-Response Curves

Dose-response curves were conducted to assess the efficacy of AS9057. AS9057 was herbicidal to a variety of weed species (Table 1), reducing fresh weight, and its mode of action was light-dependent, causing rapid desiccation of the foliage of the plants treated (Figure 2.2). When compared with Scythe (pelargonic acid), an herbicidal soap that is the current leading natural product used as an herbicide (Coleman & Penner, 2008), AS9057 is 54 times more potent. AS9057 GR_{50}=71±25 g/ha, Scythe GR_{50}=3,834±480 g/ha and are statistically different at $p < 0.05$ (Figure 2.3).

Effect of AS9057 on ROS Accumulation and Electrolyte Leakage

The presence of reactive oxygen species (ROS), such as H_2O_2, in treated plants or leaf discs, is indicative of oxidative stress. AS9057 caused a rapid and dose-dependent accumulation of H_2O_2 in leaf discs which had been incubated overnight in the dark and then exposed to high light intensity (Figure 2.4A). The ROS generated by AS9057 resulted in a loss of cell membrane integrity in treated plants. As the dose of AS9057 which plants were treated with increased, the amount of H_2O_2 which accumulated in these leaf discs also increased (Figure 2.4B). This means that the herbicidal mechanism of action by which AS9057 acts is likely through the membrane leakage caused by lipid peroxidation from the over-

abundance of ROS.

Effect of AS9057 on Photosystem II

There is no statistical difference in photosynthetic electron transport activity in plants exposed to doses of AS9057 ranging from 0 to 1,000 g/ha (Figure 2.5A). Since low uptake and/or rapid translocation may have been contributing factors to the lack of effect *in planta*, electron transport rates were also measured in cucumber cotyledon discs exposed to AS9057 or diuron, compared to water and DMSO as controls. While diuron completely inhibited electron transport, AS9057 did not have any effect, with electron transport rates similar to the blank formulation control (Figure 2.5B). The oxygen evolution assay, which measures levels of molecular oxygen produced by photosynthesis during the assay, can be used to detect PSII inhibitors *in vitro*. Oxygen evolution is observed in the presence of AS9057, further suggesting this natural herbicide does not inhibit PSII (Figure 2.5C).

Effect of AS9057 on Photosystem I

The oxygen consumption assay was performed to assess the effect of AS9057 on PSI. In this assay, oxygen evolution is measured in isolated thylakoid membranes exposed to either paraquat, a known PSI inhibitor, or AS9057. The dependence on electron transport from PSII was assessed using diuron. Both paraquat and AS9057 caused oxygen consumption by accepting electrons from PSI and reducing molecular oxygen to ROS (Figure 2.6A). Additionally, this process was stopped when electron transport is blocked at PSII by diuron. This suggests that both herbicides require electrons from PSII but act as electron diverters at the level of PSI. Rose bengal, a photosensitizing herbicidal compound (Knox & Dodge, 1984), generated ROS whether or not PSII electron transport was blocked. These data provide further evidence that AS9057 acts as an electron diverter such as the dipyridinium herbicide paraquat (Figure 2.6A).

To further investigate where AS9057 accepts an electron in the photosynthetic electron transport chain, another assay was performed using DBMIB instead of diuron. This substituted benzoquinone

blocks electron transport at the cytochrome b6/f complex rather than PSII (Chain & Malkin, 1979; Rich et al., 1991). In this assay, AS9057 once again mirrored the behavior of paraquat and is unable to produce ROS and cause oxygen consumption in the presence of DBMIB. Thus, AS9057 accepts the electron at a point in the chain after the cytochrome b6/f complex.

Chemical properties of AS9057

AS90576 is a 9,10-anthraquinone whose chemical properties match the average properties of postemergence herbicides identified by Tice (Table 2) (Tice, 2001). The chemical properties and biological activities of 9,10-anthraquinones are pH-dependent (Bardagi et al., 2018; Campos-Martin et al., 2006; Trung et al., 2021). The effect of pH on AS9057 is illustrated by a distinct inflection at λ=470 nm between acidic and alkaline environments (Figure 2.6A). There is a red shift (bathochromic shift) at pH>8, where AS9057 exists predominantly as its radical ion with an absorption maximum at λ=510 nm. The blue shift (hypsochromic shift) to an absorption maximum at λ=430 nm at pH<8 is due to a chemical change to the predominantly enolate ion form of AS9057 (Bardagi et al., 2018). It should be noted that absorbance of rose bengal has an absorption maximum at λ=550 nm (Knox & Dodge, 1984), but this molecule acts as a photosensitizer rather than targeting photosynthetic electron transport (Figure 2.6A).

AS9057 is a highly conjugated 9,10-anthraquinone and *ab initio* calculations identified carbon 9 as the atom to accept one electron from PSI to initiate the formation of an AS9057 radical involved in ROS and lipid peroxidation (Figure 2.7B). Previous work reported that the redox potential of AS9057 was pH-dependent, with values of -280 and -335 mV at pH 7 and pH 9, respectively(Egerer et al., 1982).

Herbicidal Activity of Related Products

An activity screen was performed using 12 products related to AS9057 either structurally, in their herbicidal activity or in their color. This screen included herbicidal activity, and assays assessing the effect on PSII and PSI (as described above). Studying the mode of action of products which bear similarities to AS9057 can help better develop the understanding of AS9057's mode of action. Lawson

methyl ether (a naphthoquinone) was the only compound to have a similar activity in the oxygen consumption assay as AS9057 (Supplemental Table 1). Interestingly, naphthoquinones are normally associated with inhibition of PSII (Durán et al., 2019; Jewess et al., 2002). Pyridazocidin, is only previously described natural product to act as an electron diverter on the reducing end of PSI (Gerwick et al., 1997).

DISCUSSION

Initial tests confirmed the herbicidal activity of AS9057 on several plant species (Table 1). The contact activity of AS9057 was strongly light-dependent and caused rapid burndown of the foliage. As a natural herbicide, AS9057 is more than 50 times more active on Palmer amaranth than the herbicidal soap Scythe (pelargonic acid) (Figure 2.3), indicating that its mechanism may involve photosynthesis, rather than some other mechanical disruption of the cuticle. In the presence of light, AS9057 induced a dramatic accumulation of ROS and concomitant disruption of the plasma membrane integrity (Figure 2.4) (Dayan et al., 2017).

AI4AI predicted that AS9057 had a new mode of action. 9,10-Anthraquinones are photoreactive and thus might act as an inhibitor of PSII (Oettmeier et al., 1988; Schrader et al., 2003). However, AS9057 did not inhibit photosynthetic electron transport *in planta* or in cotyledon disks, nor inhibit oxygen evolution in isolated thylakoid membranes (Figure 2.5). Therefore, the possibility that the contact activity of AS9057 relies on another light-dependent herbicidal mechanism of action that involve ROS accumulation and membrane destruction was tested and eliminated (i.e., inhibition of protoporphyrinogen oxidase (group 14) or glutamine synthetase (group 10)) (Supplemental Figure 1) (Barker et al., 2023; Takano et al., 2019).

Consequently, the possibility that AS9057 targeted a site in photosynthesis other than PSII led us to test whether AS9057 acted as a photosynthetic electron diverter in a manner similar to paraquat, a group 22 herbicide targeting PSI (Krieger-Liszkay et al., 2011). Rapid oxygen consumption caused by AS9057 in isolated thylakoid membranes was identical to that observed with paraquat (Figure 2.6). Furthermore,

this process required photosynthetic electron transport and was affected by both diuron and DBMIB, which block electron transport either at PSII or cytochrome b6/f complex, respectively (Figure 2.6) (Battaglino et al., 2021; Chain & Malkin, 1979; Rich et al., 1991). A summary of the experiments pointing to PSI as the target of AS9057 is described in Figure 2.8.

Blocking electron transports with both diuron and DBMIB highlights that AS9057 relies on these electrons for its mechanism of action, but that it acts at a point past PSII (Figure 2.8). The experiment using diuron confirmed electron transport is inhibited past PSII while the experiment done with DBMIB confirms that AS9057 acts at a point past the cytochrome b6/f complex and pointing to PSI as the likely site of action of AS9057 (Figure 2.8) (Malkin, 1981). Though the exact site of action of paraquat is not known, it is near photosystem I (Krieger-Liszkay et al., 2011). Because the activity of AS9057 is like that of paraquat, it is proposed that the site of action is also near PSI, and this generates AS9057 radicals resulting in ROS formation (Figure 2.8).

Since AS9057 acts within the thylakoid membrane where the pH ranges from 6.5-8.5 (Figure 2.9), its redox potential will be between -280 to -335 mV (Egerer et al., 1982). The redox potentials of ferredoxin and FNR are approximately -400 mV and -300 mV, respectively (Corrado et al., 1996; Doelle, 1975). Thus, due to the redox potentials and pH within the thylakoid membrane, it is inferred that the precise site of action of AS9057 is between ferredoxin and FNR in the photosynthetic electron transport system. Egerer et al., 1982 reported that AS9057 acts as an electron acceptor, and has an affinity for flavoproteins (Egerer et al., 1982). Since FNR is a flavoprotein (Corrado et al., 1996), we are postulating that AS9057 diverts electrons from the photosynthetic electron transport system from FNR, forms a radical, and initiates a ROS cascade resulting in its herbicidal contact activity.

The pH-dependent reactivity of AS9057 (Figure 2.7B) has important implications for its herbicidal activity, where the pH-dependence determines which ion of AS9057 is involved in ROS formation (Figure 2.9B). During photosynthesis, the pH of the stroma where AS9057 diverts electrons from PSI is alkaline, whereas the pH of the lumen, where most of the O_2 is produced by the water-splitting complex, is acidic. Consequently, AS9057 will exist predominantly as its radical ion in the stroma and as its enolate

ion in the lumen (Figure 2.9).

Accordingly, the putative mechanism of radical formation involving AS9057 occurs in three steps. In the initiation step at > pH 8, AS9057 complexes with biological Fe^{2+} through an α-ketol group by an associative mechanism resulting in an octahedral, O_h, radical anionic complex ($AS9057^{\bullet-}$) (Figure 2.7B). While the existence of an octahedral complex remains elusive, it is thought to react with O_2 to yield superoxide and the oxidized complex Fe^{3+}·AS9057 thus initiating the catalytic cycle shown in equation 1.

$$Fe^{2+}.AS9057^{\bullet-} + O_2 \rightarrow Fe^{3+}.AS9057 + O_2^{\bullet-} \quad \text{(eq. 1)}$$

Transfer of the electron through the chain oxidizes Fe^{2+} to Fe^{3+} releasing superoxide (equation 1). Superoxide is a weak Brønsted base that can abstract a proton from weakly acidic organic compounds such as lipids (Sawyer et al., 1978) and induces the propagation of this radical-driven lipid peroxidation cycle. The intermediate oxygen evolved in equations 2 and 3 reenters equation 1 to propagate this reaction, resulting in increased ROS concentration.

Under basic conditions in the stroma, hydroperoxyl radical (shown in brackets) decomposes to hydroxide ion (equation 2), which recycles to equation 1 and generates more superoxide. In this reaction, hydroperoxyl radical decomposes so rapidly that it does not participate directly in the lipid peroxidation cycle (Behar et al., 1970).

$$O_2^{\bullet-} \rightarrow 2O_2^- + 2H^+ \rightarrow [2HO_2^{\bullet}] \rightarrow OH^- \quad pH > 8 \qquad \text{(eq. 2)}$$

In the acidic environment of the lumen, hydroperoxyl radical produced as an intermediate decomposes to dioxygen and hydrogen peroxide (equation 3). These reactions all proceed with second order, bimolecular, kinetics (Bardagi et al., 2018).

$$O_2^{\bullet-} \rightarrow 2O_2^- + 2H^+ \rightarrow [2HO_2^{\bullet}] \rightarrow O_2 + H_2O_2 \quad pH < 8 \qquad \text{(eq. 3)}$$

Therefore, the herbicidal activity of AS9057 is associated with the light-dependent generation of ROS (namely superoxide, hydroxide ion and hydrogen peroxide as described in equations 1, 2 and 3) that causes lipid peroxidation and ultimately results the death of photosynthetically active tissues.

CONCLUSION

Increased instances of resistance and harmful effects of herbicides which are currently used commercially drive the need for new chemical tools to manage weeds (Zhang et al., 2023). AS9057 was identified by the AI4AI algorithm as a herbicidal lead with a potential new mode of action. The herbicidal activity was confirmed on several plant species. This investigation on its mode of action points to PSI as the target of AS9057, form a radical by accepting an electron, and drives a ROS cascade. This is significant because paraquat and diquat are the only known compounds which act in this way, and both are toxic and highly dangerous synthetic compounds which persist in the environment for extended periods of time (Tsai, 2013). Many natural products have been used as the basis for discovery of these synthetic products and evidence suggests that these natural products may be more effective and less harmful than their synthetic counterparts (Gerwick & Sparks, 2014). Because AS9057 acts in the same way and is a natural product, it offers a safer alternative to the PSI inhibitors which are currently used in commercial agriculture.

ACKNOWLEDGMENTS

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TABLES

Table 1. Percentage control of AS9057 of different weed species when sprayed at 50 g/ha. Plant injury was assessed 20 days after herbicide application.

Species	**Control (%)**[a]
Amaranthus palmeri	100
Chenopodium quinoa	100
Solanum nigrum	98±3
Setaria viridis	60±40
Gossypium hirsutum	3±6

[a]0% control means no injury at all and 100% control means complete death of the plants.

Table 2. The chemical properties of AS9057

Properties	AS9057	Tice rules[a]
Molecular weight	284.22	186-491
mlog P	2.2	0-4.6
H-bond donor	3	0-2
H-bond acceptor	2	1-7

[a]Tice rules from Tice, 2001(Tice, 2001)

FIGURES

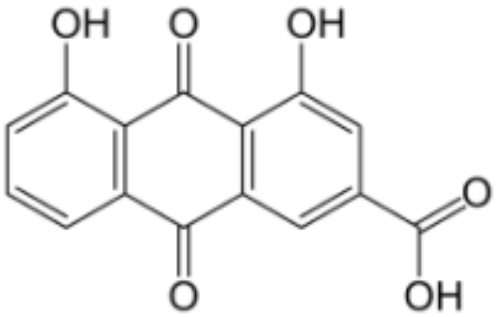

Figure 2.1. Chemical structure of AS9057

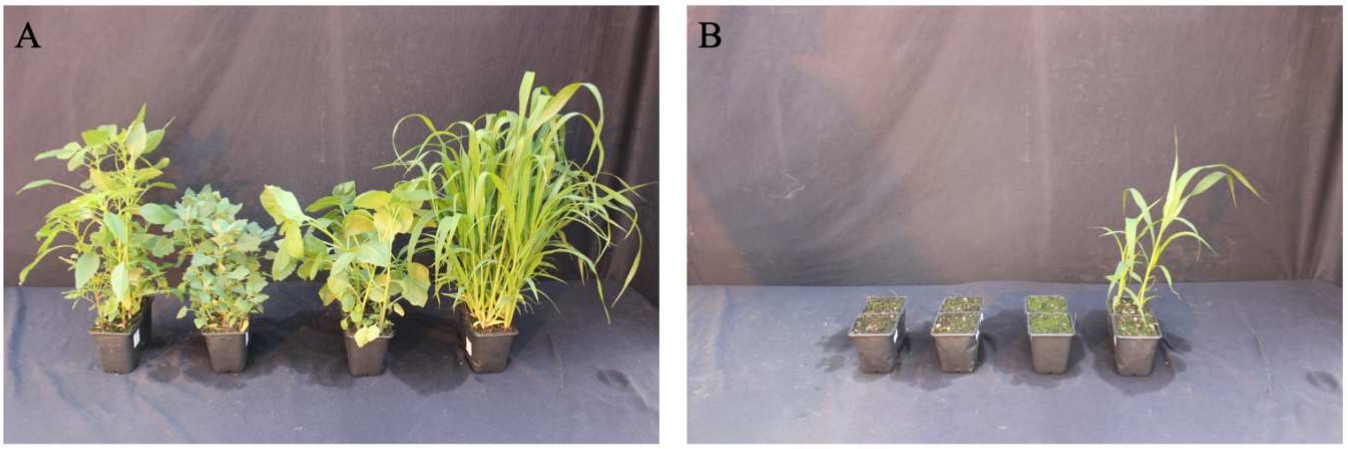

Figure 2.2. Control (A) and treated (B) side by side comparison of *A. palmeri*, *C. quinoa*, *S. nigrum*, and *S. viridis* (left to right) 20 DAT. Treated plants were treated at 50 g/ha.

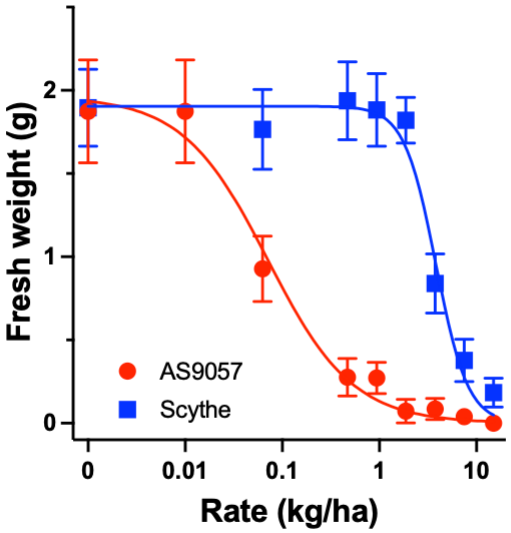

Figure 2.3. Dose response curve and linear regression of AS9057 (●) and Scythe or pelargonic acid (■) sprayed on Palmer amaranth at 0, 0.06, 0.47, 0.94, 1.9, 3.8, 7.5, and 15 kg/ha, n=5, error bars = standard error of the means.

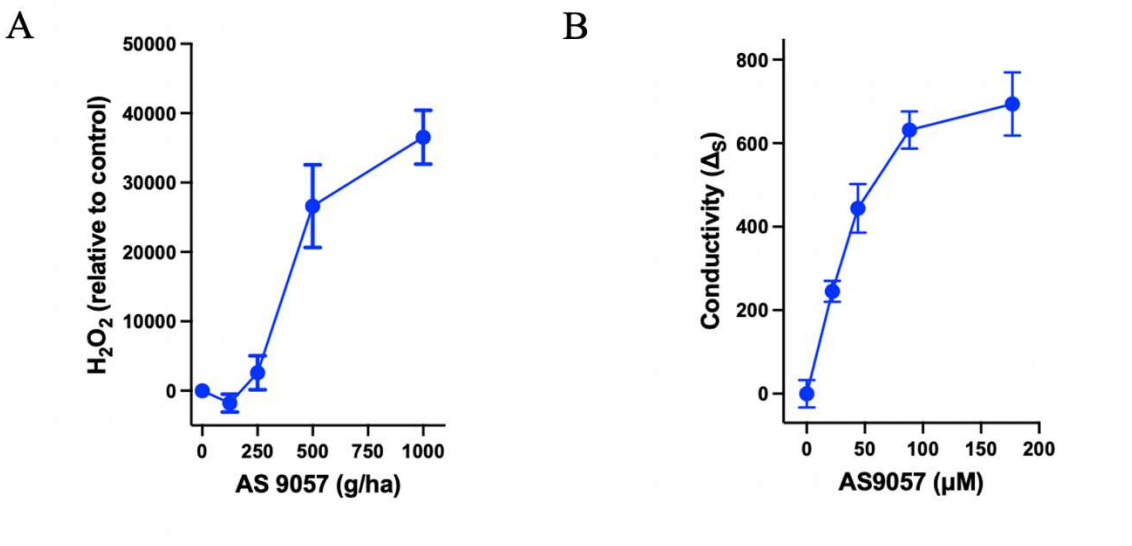

Figure 2.4. Effect of AS9057 on A) hydrogen peroxide accumulation (n=10, error bars = standard error), and B) electrolyte leakage from leaf discs exposed to AS9057, as measured as conductivity (n=4, error bars = standard error).

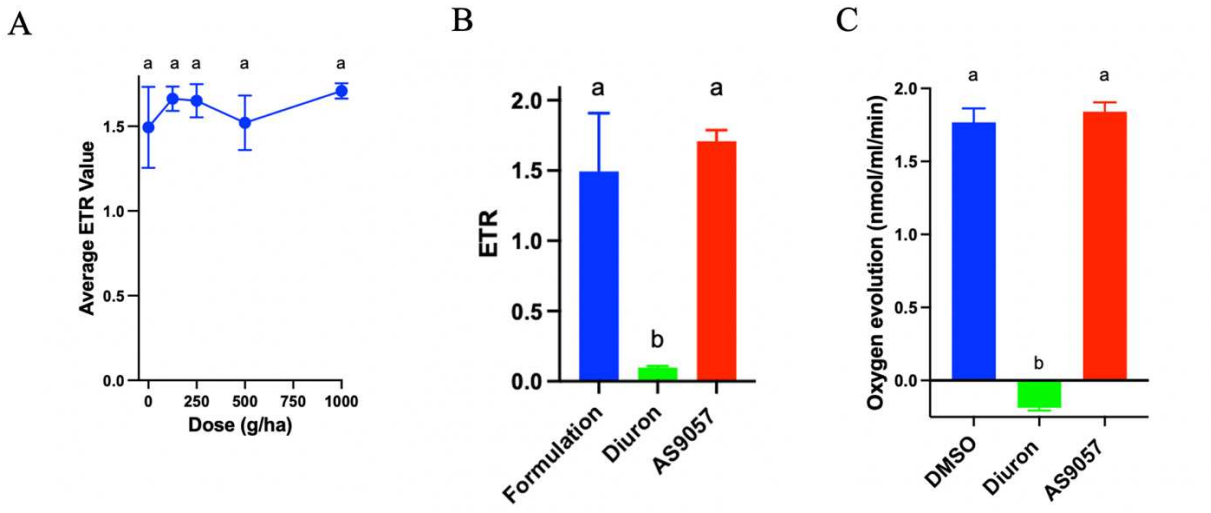

Figure 2.5. A) Dose-response of AS9057 on ETR in leaves of plants sprayed in the greenhouse, error bars = standard error, n=3, ETR was not statistically different, p =0.479. B) Electron transport rates in leaf discs which were exposed to 177 µM of AS9057 relative to a negative control containing only the formulation which AS9057 is dissolved in or a positive control of diuron (100 µM). Error bars = standard error, n=3. C) Oxygen evolution in isolated chloroplast. DMSO (control), 200 µM AS9057, and 100 µM diuron, n=3, error bars = standard error. Bars with the same letter are not statistically different according to Duncan multiple range analysis at $p < 0.05$.

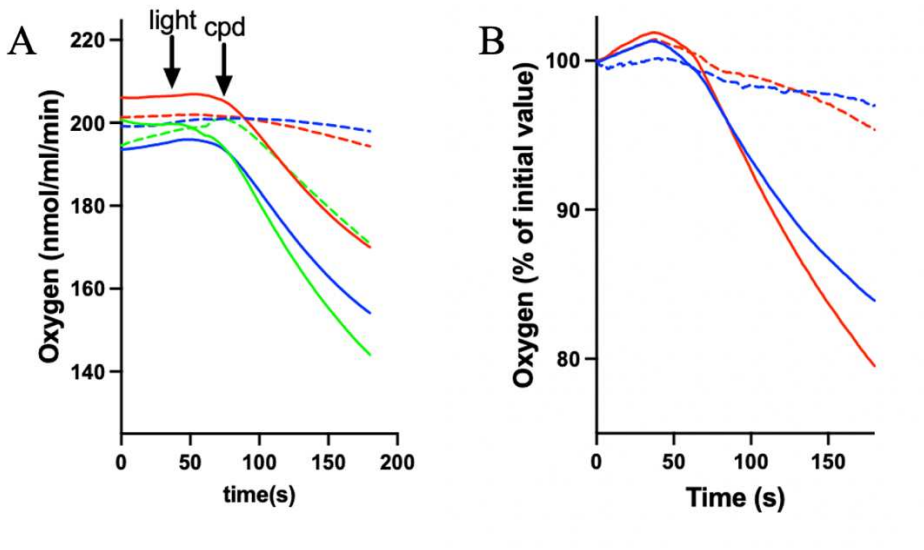

Figure 2.6. Requirement of photosynthetic electron transport on the mode of action of AS9057. Diuron and DBMIB were used to block electron transport at PSII or cytochrome b6/f complex, respectively. A) Photosystem I oxygen consumption by 0.01 mM paraquat (●), 0.01 mM AS9057 (●) or 0.01 mM rose bengal (a photosensitizer) (●) with (dotted lines) and without (solid lines) 0.01 mM diuron. B) Photosystem I oxygen consumption by 0.01 mM paraquat (●) and 0.01 mM AS9057 (●) with (dotted lines) or without (solid lines) DBMIB.

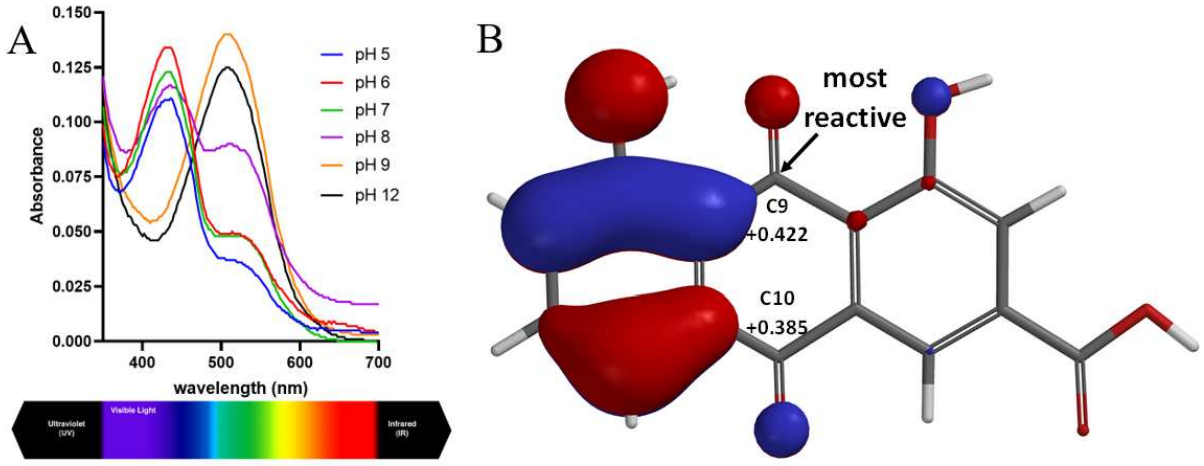

Figure 2.7. A) Absorption spectra of AS9057 in aqueous environments ranging from pH 5 to 12. B) Structure of AS9057 following *ab initio* energy calculation. Red and blue surfaces represent the highest occupied molecular orbitals (HOMO). The carbons C9 and C10 are indicated with their respective Mulliken atomic charge following *ab initio* energy calculation. Red and blue surfaces represent the highest occupied molecular orbitals (HOMO).

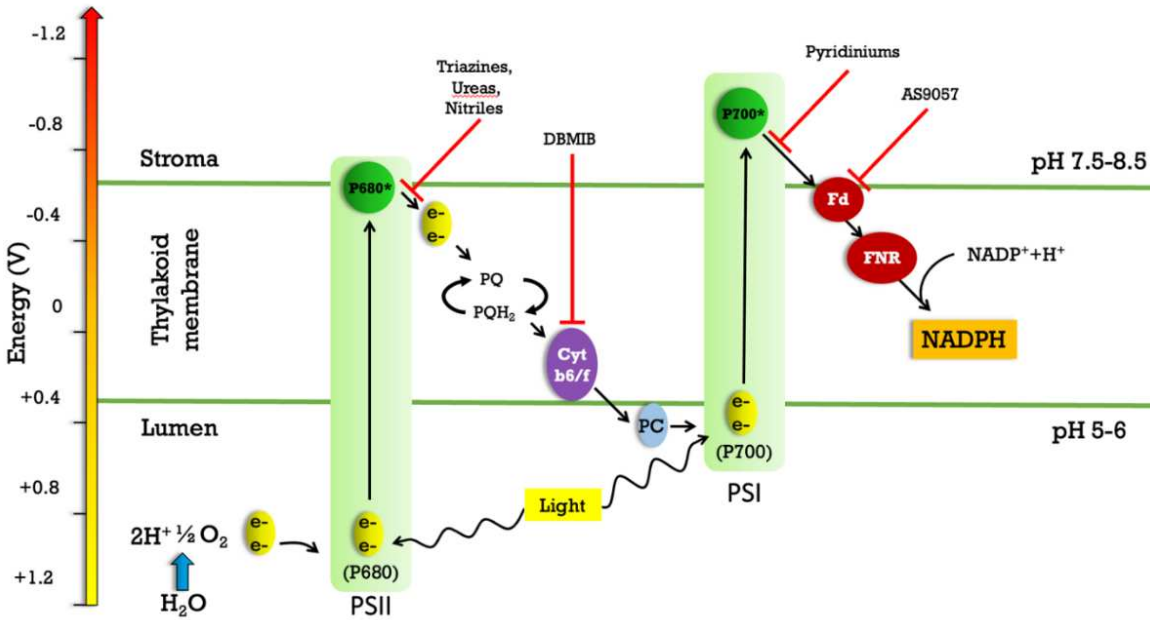

Figure 2.8. Z-scheme of photosynthetic electron transport system, showing sites of action of AS9057and other herbicide classes which were used to aid in the discovery of AS9057's inhibition of PSI.

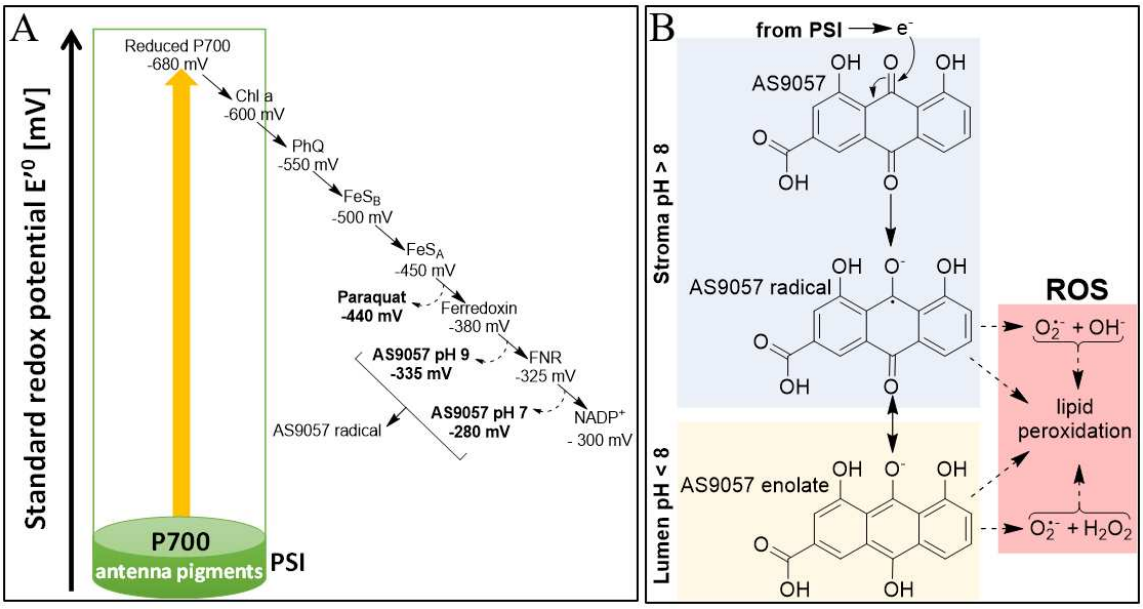

Figure 2.9. A) Current hypothesis of where AS9057 diverts electrons between photosystem I and the formation of NADPH by ferredoxin-$NADP^+$ reductase (FNR). The vertical scale illustrates the redox potential of the various steps between photosystem I and the reduction of $NADP^+$ to NADPH. The redox potential of paraquat and AS9057 are sufficiently different to suggest that they divert electrons at different points within this electron transport chain. Chl a = chlorophyll a; PhQ = phylloquinone; FeSA = iron-sulfur cluster A; FeSB = iron-sulfur cluster B. B) Putative mechanism of ROS formation by AS9057 reaction intermediates as affected by pH of chloroplast localization. Under high light intensity, the pH of the stroma is >8 and the pH of lumen is <8.

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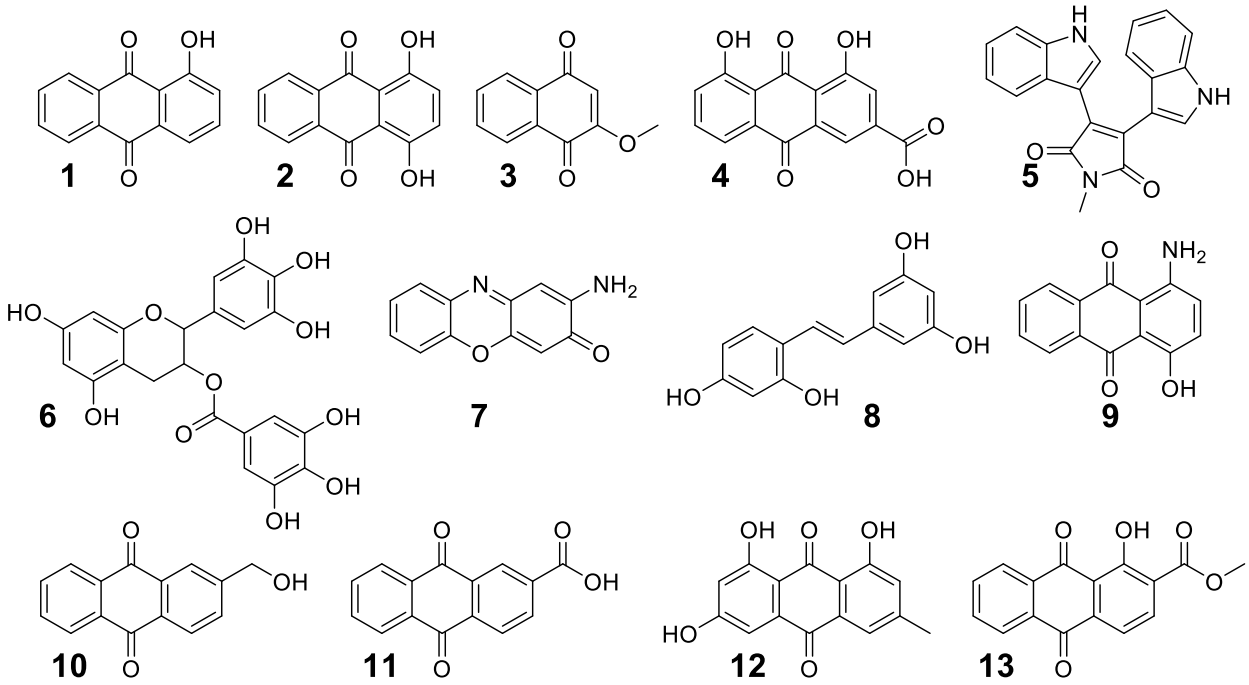
APPENDIX

Supplemental Table 1. Structures, description, and activity for compounds tested in this study.



Cpd	CAS	Name	GH[a]	PET[b]	PSII[c]	PSI[d]
1[e]	129-43-1	1-Hydroxyanthraquinone	Yes	No	No	No
2[e]	81-64-1	Quinizarin	Yes	No	No	No
3[e]	2348-82-5	Lawsone methyl ether	Yes	No	No	Yes
4[e]	478-43-3	AS9057	Yes	No	No	Yes
5[e]	113963-68-1	Bisindolylmaleimide V	No	No	No	No
6[e]	989-51-5	Epigallocatechin gallate	No	No	No	No
7[e]	1916-59-2	Questiomycin A	No	No	No	No
8[e]	29700-22-9	Oxyresveratrol	No	No	No	No
9[e]	116-85-8	1-Amino-4-hydroxyanthraquinone	No	No	No	No
10	17241-59-7	Anthraquinone-2-methanol	Yes	No	No	No

11	117-78-2	Anthraquinone-2-carboxylic acid	Yes	No	No	No
12	518-82-1	Emodin	Yes	No	No	No
13	332102-66-6	Rubiawallin C	Yes	No	No	No

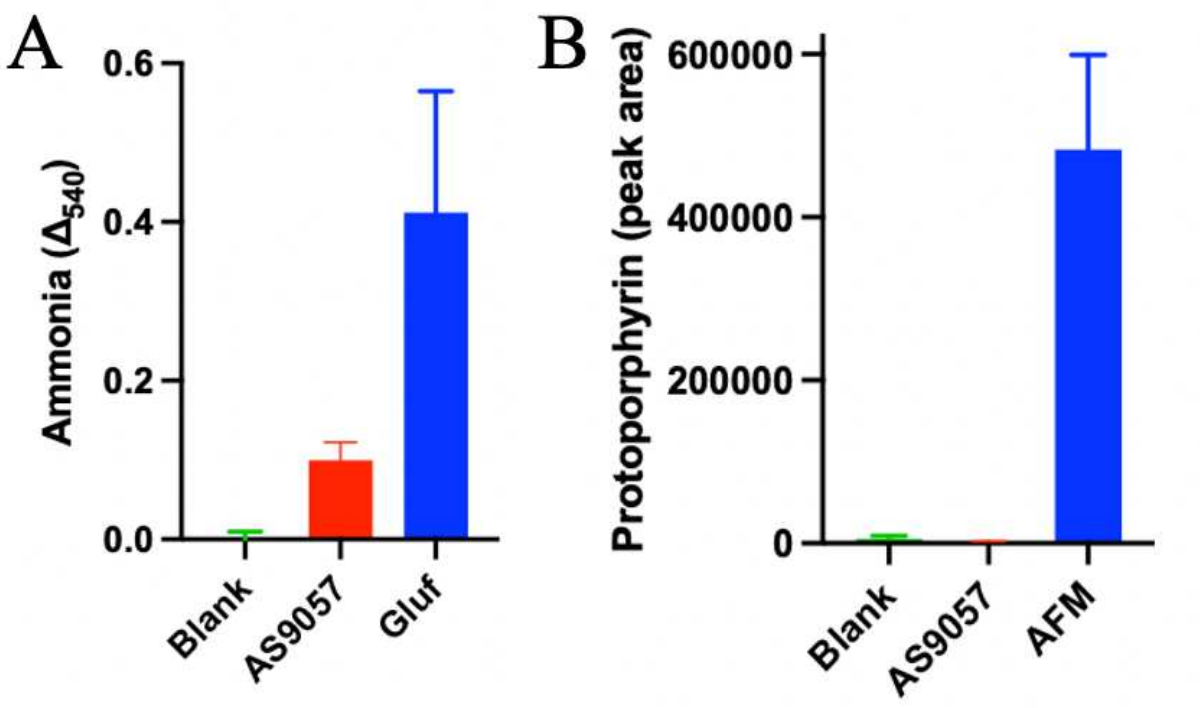
[a] Denotes herbicidal activity of the compounds.

[b] Denotes inhibition of photosynthetic electron transport in leaf discs.

[c] Denotes inhibition of photosystem II oxygen evolution in isolated thylakoid membranes.

[d] Denotes diversion of electrons from photosystem I in isolated thylakoid membranes.

[e] Denotes compounds that are red pigments.



Supplemental figure 1. A) NH_3 accumulation in plants treated with AS9057 (177 μM) compared to glufosinate (100 μM). B) Proto accumulation levels in plants treated with AS9057 (177 μM) compared to AFM (100 μM)