Investigating the suitability of chlorophyll fluorescence imaging for determining the mode of action of herbicides, phytotoxicity, and the effect of adjuvants

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Abstract

Chlorophyll fluorescence is light re-emitted from a plant, to expel excess energy not being used for photochemistry. Chlorophyll fluorescence imaging (CFI) is a well-established method for the analysis of plant photosynthetic health and any perturbations. Herbicides, the most used type of pesticide, can have several possible modes of action (MOA), some of which affect the photosynthetic reactions of a plant, either directly or indirectly. As all plant processes ultimately depend on photosynthesis, any disruption to metabolisms will affect chlorophyll fluorescence. Therefore, the effects of herbicides should be detectable using CFI. With only a single new herbicidal MOA being introduced in the past 30-40 years, and with growing numbers of herbicidal resistant weeds and a need to increase food production for the growing global population, novel tools to screen for new MOAs are needed. The present research successfully showcased the potential utility of CFI as a tool in herbicidal research. Study 1 utilised *Lemna minor* to examine CFI as a high-throughput MOA screening tool. The results demonstrated that CFI was an extremely effective tool for detecting herbicides with a MOA targeting photosystem II (PSII), producing conclusive results within 24-hours. Study 2 utilised three important terrestrial species, to determine the efficacies of herbicides, and the effect of adjuvants, to gain a deeper understanding of the potential information that can be obtained from CFI. This study demonstrated that CFI can detect the effects of a range of MOAs on plants, not just those targeting PSII. Both studies found that CFI was able to detect the effects of adjuvants on the efficacies of herbicides. Future research should use CFI alongside traditional methods. The present work provides a key developmental step in producing a methods basis for the utilisation of CFI for MOA determination, with key benefits being rapid generation of results with minimal costs.

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Abbreviations

ADP	Adenosine diphosphate
AMARE	*Amaranthus retroflexus*
ATP	Adenosine triphosphate
BRSNN	*Brassica napus*
CF	Chlorophyll fluorescence
CFI	Chlorophyll fluorescence imaging
DMSO	Dimethyl sulfoxide
ECHCG	*Echinochloa crus-galli*
EEE	Excess excitation energy
F_m	Maximum fluorescence of dark adapted material
F_m'	Maximum fluorescence of light adapted material
F_o	Minimum fluorescence of dark adapted material
F_o'	Minimum fluorescence of light adapted material
F_q'/F_m'	Operating efficiency of PSII in light adapted material
F_q'/F_v'	PSII maximum efficiency factor
F_v	Variable fluorescence from dark adapted material
F_v/F_m	PSII maximum efficiency in dark adapted material
F_v'	Variable fluorescence from light adapted material
F_v'/F_m'	PSII maximum efficiency in light adapted material
HPLC	High-performance liquid chromatography
HRAC	Herbicide resistance action committee
MOA	Mode of action
$NADP^+$	Nicotinamide adenine dinucleotide phosphate

NADPH	Reduced form of $NADP^+$
NPQ	Non-photochemical quenching
pKa	Value to represent the strength of an acid
PSI	Photosystem I
PSII	Photosystem II
Q_A	Primary quinone electron acceptor of PSII
ROS	Reactive oxygen species
SLES	Sodium laureth sulfate

1. Introduction

1.1 Background

For the majority of human history (roughly 280,000 BCE - 10,000 BCE) hunting and gathering were the predominant methods for sustaining life, with the development of agriculture (i.e. cultivation of crops and domestication of animals) being a relatively modern invention, from approximately 10,000 BCE (Gowdy, 2021; Lewin, 2009). This shift from hunting and gathering to farming is known as the neolithic revolution (Weisdorf, 2005). The genesis of agriculture occurred independently in multiple areas of the globe, and marked a fundamental change in human lifestyle, and led to the rise of modern civilisations. The start of humans cultivating crops specifically for consumption and usage, coincided with the advent of managing invasive species, also known as weeds (Hay, 1974). Today, when averaged across all significant agricultural crops, weed competition results in yield losses of c.40% on a worldwide level, which is the highest cause of loss across all pests (Oerke, 2006).

There is a need for improving yields to feed the growing population - in 1999 the World Bank estimated that ~90% of the required increase in food production would need to come from increasing the yields of crop already grown on farmed areas, as opposed to increasing the amount of land used for growing food crops (Evans, 1999). This need for increasing food protection has only grown since this publication, with the global population having recently reached 8 billion (on 15th November 2022), and expected to hit almost 10 billion by 2050 (United Nations Department of Economic and Social Affairs, 2022). Whilst the number of undernourished people has decreased by around 50% since 1992, this number still stands at a more than substantial 820 million people, with numbers of those that are undernourished (both relatively and absolutely) rising since 2014 (FAO, 2018). There are various methods this could be done by;

however, one major way is by protecting the crops/yield, by controlling weeds and other pests. Weeds are unwanted species that reduce yields by competing for space, nutrients in soil, water, and light (Merfield, 2022; Renton and Chauhan, 2017).

For most of agricultural history, the removal of weeds has been a labour intensive manual and mechanical process, either using bare hands, primitive tools, livestock, or eventually mechanically/steam powered implements/assistance (Bell, 2015). The usage of biological methods of weed control had been established for centuries, however the method of control as we know it in modern times only first started at the turn of the 20th century, with the first major successes in the 1920s and ‘30s (Peng, 1983). However, it was not until the commercial development of 2,4-D in 1945, the first synthetic organic herbicide, that the usage of chemical weed control began to increase and become more commonplace (Peterson et al., 2016).

In more recent history, from the middle of the 20th Century until the 1980s, a novel herbicidal mode of action (MOA) was introduced frequently, with approximately a new MOA becoming commercially available every two to three years (Dayan, 2019). However, this rate of discovery slowed down towards the end of the century, and for the past 3 decades only one novel herbicidal MOA has been developed, with the plant dihydrogen phosphate dehydrogenase inhibitor Tetflupyrolimet being registered by FMC agricultural Solutions in 2019, and commercialisation being expected for 2024 (Dayan, 2019; Mylne and Stubbs, 2020; Sukhoverkov et al., 2021). Additionally, the fact that herbicides are some of the most regulated chemicals on the planet, and that the cost of registering a new herbicide has increased greatly, have made it very difficult to develop a new product (McDougal, 2018). The total registration and other related costs of developing a new active ingredient have reached approximately $100 million.

Herbicides, chemicals used to kill plants, are vitally important to modern agriculture, with the livelihoods and lives of millions of people relying on them, illustrated by the increasing usage of herbicides globally, partially as an attempt to increase crop yields (Gianessi, 2013; Holt, 2013). Approximately 60% of all pesticides used worldwide are herbicides, which equates to ~2 million tonnes of active ingredient being used per annum (Dayan, 2019; Sharma et al., 2019). There is an increase in lobbying against the use of herbicides due to the negative effects that they may have on unintended non-targets, for example Glyphosate, the most commonly used herbicide globally, likely has carcinogenic properties (Duke and Powles, 2008; Guyton et al., 2015; Tarazona et al., 2017). However, the banning of these herbicides due to these concerns could and would have massive consequences on both crop yield and farm income. For example, both the EU and Mexico plan to ban the use of Glyphosate by as soon as 2024 (Alcántara-de la Cruz *et al.*, 2021; Kudsk and Mathiassen, 2020). This would lead to predicted yield losses of up to 40% as well as massive economic losses, with UK farmers expected to see losses of almost £1 billion per annum (£940 million), if the UK government also applies this in interests of standardisation.

There has been a large amount of research into herbicides in recent years, with a portion of this work being focused on herbicidal resistance, as opposed to the development of novel herbicides and understanding herbicidal modes of action, due to growing amounts of resistant species and regulation (de Souza Barros et al., 2021; Jamaludheen et al., 2022). The usage of herbicide resistant crops is important and prevalent, as they mean that herbicides can be applied to a whole field without suffering losses to crop yield. For example, in 2012, 73%, 80%, and 93% of corn, cotton, and soybean planted in the United States respectively were Glyphosate resistant (Korres et al., 2019). The need for herbicides other than Glyphosate has decreased due to its dominance of the herbicidal market, yet an increasing number of species are becoming Glyphosate resistant (Duke, 2012; Duke and Dayan, 2022). With the principal tool for weed control in modern agriculture being herbicides, which provide an effective solution to the problem that most modern farms face, the rise of herbicide resistant species due to the vast overuse of herbicides is a growing concern, with 267 herbicide resistant weed species, accounting for 21 of the 31 known herbicidal MOAs (Harker and O'Donovan, 2013; Heap, 2022). To control the emergence of weed resistance to conventional herbicides, novel modes of action are urgently needed, and in some situations, farmers have run out of, or are running out of herbicide options. This situation creates opportunities for herbicide products with new MOAs to control these weeds and for new tools in herbicide resistance management. Although research into herbicide resistant crops is an important field, that is not the main focus of the present work.

1.2 Herbicides

Herbicides, defined as chemicals used to suppress the growth of, or kill, plants, are a necessary part of modern agriculture, as weeds can cause a reduction in crop quality, as well as increasing yield losses (Holt, 2013; Kudsk and Streibig, 2003; Oettmeier, 1999). Therefore, the use of herbicides has led to an improvement in both the amount, and quality of crops. Over the past 100 years there have been great improvements in the yields of agricultural crops (LeBaron *et al.*, 2008). In the US, tomato yields show the largest difference, with yields having increased by 811% from 1920 to 2004, but is also mirrored in the more major crops of corn and wheat, which have increased yields of 525% and 283% respectively (Warren, 1998). These commercially driven great increases in crop yields are due in great part to the usage of herbicides and adjuvants minimising competition from weeds. The necessity and importance of herbicides is well established, with the development and subsequent widespread usage of herbicides being described as one of the most important advances in agriculture, and that modern agriculture would not exist if not for the usage of herbicides (Blackshaw *et al.*, 2006; Oettmeier, 1999; Pike *et al.*, 1991). In the past 100 years since their first discovery, herbicides have become an integral and essential part of modern agriculture, with their use being so intrinsic that a total ban of their use would lead to a 20-90% decrease in farmer earnings (Anonymous, 1999; Kudsk and Streibig, 2003). In spite of their prevalence and intrinsic adoption by agricultural sectors, there is a perpetually increasing interest in minimising the amount of herbicides used, not only for economical commercial reasons, but also because of growing concerns surrounding their effects on human and environmental well-being – an interest which can be partially addressed by the use of adjuvants, and understanding the effects of adjuvants on the efficacies of herbicidal mixtures (Blackshaw *et al.*, 2006; Pacanoski, 2015). Herbicides are found in a wide variety of environments, from agricultural, aquatic, metropolitan, and forest locations, being used to cut back the density of undesired vegetation, whilst selective herbicides are safe to desired plants, not preventing their growth (Green, 2014; Holt, 2013). However, herbicides that are widely used around the world are often non-selective and broad spectrum, meaning they act on a wide range of species, limiting their use in agricultural regions/scenarios (Vats, 2015). Due to this, the application of herbicides can expedite the reduction of species diversity in an environment, leading to the natural selection of species adapted to the active ingredients.

Herbicides can fall into either one of two categories, depending on their method of application; either soil-applied or foliage-applied (Kudsk et al., 2017). As implied by their names, the two groups differ in the site of application and uptake on the target plant. Soil-applied herbicides are applied to sub-terranean parts, such as the roots, by being incorporated into the soil, whereas foliage-applied herbicides pertain to the above ground sections, for example the leaves or stem. Soil-applied herbicides can be used as a preventative measure, whereas foliage-applied herbicides can only be used after weed development (Qasem, 2011). Additionally, soil-applied herbicides can be grouped into two sub-types; pre-plant or pre-emergent (Vats, 2015). Pre-plant herbicides are applied to the soil before seeds are planted. This differs from pre-emergence herbicides which are added to the soil after the seed is planted, but before the seed has germinated. Post-emergence herbicides are applied to the foliage of plant after it has germinated and emerged from the soil (Vats, 2015). Herbicides can also be split into a further two categories: contact or systemic. Contact herbicides only effect the plant tissue which it comes into direct contact with, whereas systemic herbicides are absorbed into the plant, and translocated around it by either the xylem or phloem. Contact herbicides work faster, however systemic herbicides provide a longer lasting solution to weed problems (Vats, 2015).

Herbicides are not the only organic chemicals that are found in agricultural settings, with there also being a prevalence of antibiotics, hormones, and other toxic chemical compounds (such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins (PCDDs)), which persist in the environment, which can magnify and cause damage throughout food chains (Bernes, 1998; Jones and De Voogt, 1999; Zhang *et al.*, 2017). Like herbicides, these other pollutants such as hormones and antibiotics are also subjected to strict restrictions, due to their unpredictable negative effects on the environment, despite having otherwise beneficial properties/effects. The source of these organic pollutants are often other agricultural businesses (e.g. dairy farms) or waste from more metropolitan/industrial areas (Sarizadeh *et al.*, 2022; Sun *et al.*, 2015). The uptake of these pollutants, including herbicides, into flora, can be through either the roots or the leaves of the plants (Zhang *et al.*, 2017). The present research incidentally looks at the uptake of herbicides through both of these methods, by utilising various methods and species (specifically the aquatic species *Lemna minor* and other terrestrial species).

Two pathways for the transport of organic pollutants have been reported. For short distance transport, intracellular and intercellular mechanisms are used, and for long distance translocation, the conducting tissues of the xylem and phloem are utilised, depending on the size of the molecule and their ability to permeate cell membranes (Taiz and Zeiger, 2010). The apoplastic translocation of solubilised herbicides in the xylem is the main method of transport for soil-applied herbicides, due to the transport of water from the root to the shoot of the plant – with these herbicides tending to accumulate in mature leaves, as these leaves transpire the most amount of water (Nissen *et al.*, 2005). On the other hand, foliar-applied herbicides that have the necessary characteristics (e.g. appropriate size and pKa) can be translocated via the phloem, from the site of application (photosynthetically active leaves) to areas of growth (shoot and root meristems). The translocation of herbicides is not limited to either the phloem or the xylem, with many herbicides moving throughout plants via both systems. Molecules with a smaller size can permeate membranes more easily, and therefore are more likely to be able to be translocated via the xylem and phloem, whereas those that are of a larger size have a lower permeability in membranes, and hence cannot be transported as effectively (Zhang *et al.*, 2017). Along with these organic pollutants, the phloem is also used for the transportation of carbohydrates, amino acids, hormones, RNAs, and proteins to name just a few, with water being the most abundant substance in the phloem, and carbohydrates being the most concentrated solutes found (Turgeon and Wolf, 2009). Before the herbicides can be translocated, the chemicals need to be absorbed by the plant. One of the main factors affecting the uptake of chemicals (namely herbicides) into plants is the lipophilicity of the chemical, which is defined as the partition coefficient between octanol and water, P (usually given as logarithm partition coefficient; logP), as well as the partition coefficient between octanol and air (Krähmer *et al.*, 2021; Wang and Liu, 2007). Due to this, the lipid content of the roots is the biological factor that has the most effect on the uptake herbicides that are absorbed via the roots. In this work, herbicides with a wide range of partition coefficients were utilised, as this is an important factor in the uptake of herbicides and determining the efficacies of said herbicides.

Herbicides can have various modes of action (MOA), which covers an array of ways in which these chemicals influence plant physiology, biochemistry, and molecular mechanisms (Manuchehri, 2017; Shaffer, 2016). Knowledge of the MOA of a herbicide can help to understand the effect on plants and predict effectiveness, as well as long term use. It has previously been discussed that it is difficult to screen herbicides due to the many potential modes of action, as well as the potential for herbicides to have multiple tertiary MOAs (Duke, 1990). The first herbicidal MOA discovered were auxin herbicides, MCPA and 2,4-D, and some of the most widely used being triazines (such as atrazine) and phenylurea herbicides (such as Isoproturon), with these herbicides and MOAs all having different efficacies and toxicities (Kudsk and Streibig, 2003).

As of March 2020, the HRAC (Herbicide Resistance Action Committee) updated their mode of action classification, to capture necessary updates surrounding novel discoveries on herbicidal modes of action, as shown in table 1.1 (HRAC, 2020a). The revision saw the addition of five new modes of action, as well as the inclusion of 15 new active ingredients (HRAC, 2020b). Prior to the revision in 2020, the previous latest update was in 2010, showing that herbicidal research and development is still developing, even after a period with no major breakthroughs in commercialising new and safe modes of action. The HRAC predicted in 2020 that over the course of the following 10 years a further two to four herbicidal modes of action would be discovered (Ward, 2020).

As evidenced by the large amount of time in between updates from the HRAC, there are a number of challenges and difficulties that researchers and companies may face when developing new herbicides with novel modes of action (MOA). Some of these challenges include:

Finding new targets: Herbicides typically work by interfering with specific biological processes in plants, such as photosynthesis or cell division. However, many of the most important and well-understood targets have already been exploited by existing herbicides, so it may be difficult to find new targets that are both effective and specific to plants. These new targets are needed as farmers are running out of options for dealing with invasive weeds, due to growing populations of herbicide resistant weeds as well as bans on existing herbicides.

Table 1.1. A list of the known herbicidal modes of action that have resistant weeds, along with example herbicides of each class. Information obtained from the Herbicide Resistance Action Committee (HRAC, 2020a), and The International Herbicide Resistant Weed Database (Heap, n.d.).

Mode of Action	**Example Herbicide**
Inhibition of Acetyl CoA Carboxylase	Sethoxydim
Inhibition of Acetolactate Synthase	Chlorsulfuron
PSII Inhibitors - Serine 264 Binders	Chlorotoluron
PSII Inhibitors - Histidine 215 Binders	Bromoxynil
PSI Electron Diversion	Paraquat
Inhibition of Protoporphyrinogen Oxidase	Oxyfluorfen
Phytoene Desaturase inhibitors	Diflufenican
Inhibition of Hydroxyphenyl Pyruvate Dioxygenase	Isoxaflutole
Inhibition of Lycopene Cyclase	Amitrole
Inhibition of Microtubule Assembly	Clomazone
Inhibition of Enolpyruvyl Shikimate Phosphate Synthase	Glyphosate
Inhibition of Glutamine Synthetase	Glufosinate-ammonium
Inhibition of Microtubule Assembly 2	Trifluralin
Inhibition of Microtubule Organization	Propham
Very Long-Chain Fatty Acid Synthesis inhibitors	Butachlor
Inhibition of Cellulose Synthesis	Dichlobenil
Auxin Mimics	2,4-D
Antimicrotubule mitotic disrupter	Flamprop-methyl
Nucleic acid inhibitors	MSMA
Unknown	Endothall
Cell elongation inhibitors	Difenzoquat

Legislation, testing, and validation: Legislation plays a significant role in developing a new herbicide, requiring extensive testing to determine the efficacy, safety, and environmental impacts of the herbicide. This process can be time-consuming and expensive, and it may require access to specialized equipment and expertise, serving as a great barrier in the development and introduction of new herbicides. Additionally, new herbicides must be validated by regulatory agencies and laws, such as the Food Quality Protection Act (FQPA) in the US and Plant Protection Products Regulation (PPP) in the EU, which set standards for the use and residue levels of herbicides in food and the environment. This can add further delays and costs to the development process.

Resistance: Plants can evolve resistance to herbicides over time, which can reduce the effectiveness of the herbicide and require the development of new herbicides with different MOAs to combat the resistant plants. This can create a cycle of resistance and herbicide development, which can be costly and challenging to manage.

Environmental concerns: Herbicides can have unintended impacts on the environment, such as harming beneficial insects or contaminating soil and water. This can lead to public opposition to herbicide use, as well as regulatory hurdles and restrictions on herbicide development and use.

Overall, developing new herbicides with novel MOAs is complex and a challenging process that requires significant resources, expertise, and persistence. However, the need for effective herbicides to control weeds and protect crops remains critical, so researchers and companies must invest in these areas of research, to ensure suitable crop production for the future.

1.3 Herbicidal mode of action (MOA) affecting photosystem II

Some herbicides have a mode of action which affect photosystem II (PSII). These PSII herbicides act by competing with plastoquinone for binding at the D1 protein site (Muller et al., 2008). With plastoquinone being the charge carrier from PSII to cytochrome b6f, photosynthetic electron transfer is halted, so ATP and NADPH production is reduced and consequently the amount of excess excitation energy (EEE) increases, which must be dissipated or driven elsewhere. The decrease in photochemical efficiency causes an increase in dissipation elsewhere, and if not dissipated sufficiently, can cause damage to the photosystems, which is how PSII herbicides act.

Herbicides that target photosystem II, do so at the reducing side of the reaction centre protein complex in chloroplast thylakoid membranes (Oettmeier, 1999). The herbicides compete for binding of the Q_B niche of the D1 protein binding site with plastoquinone. The herbicidal molecules have a higher affinity for the said binding site than plastoquinone, and therefore stop plastoquinone from binding. The consequence of this is that the herbicides block electron transfer from PSII to the cytochrome b6f complex, via Q_A and Q_B. Blocking of electron transport promotes a reduction of nicotinamide-adenine dinucleotide phosphate (NADPH) being synthesised (Heap, n.d.). The binding of the herbicide molecule additionally halts the production of ATP, as well as the reduction of CO_2 due to the lack of NADPH, both of these products being necessary for plant growth. However, after exposure to a PSII herbicide, plant death occurs (in most cases) due to factors other than the onsetting “starvation” and is a result of the accumulation of highly reactive radicals, such as triplet state chlorophyll (Rutherford and Krieger-Liszkay, 2001; Zhu *et al.*, 2009). Triplet state chlorophyll, the formation of which is promoted by the plant unsuccessfully re-oxidizing

Q_A, can react with the diradical ground-state oxygen, a reactive oxygen species (Bayr, 2005). The interaction between these molecules forms a singlet oxygen molecule. Both singlet oxygen and triplet chlorophyll can remove a hydrogen from unsaturated lipids, creating a lipid radical, in a process known as lipid peroxidation (Fujita, 2002). A chain reaction of lipid peroxidation starts due to the reaction of the newly formed unstable lipid radicals with molecular oxygen, creating a peroxyl-fatty acid radical, which in turn can react with a non-radical lipid, perpetuating the cycle (Hogg and Kalyanaraman, 1999). Lipid peroxidation causes damage to the phospholipid bilayer of the thylakoid membrane via oxidisation, causing the cells and cell contents to dry, which ultimately comes to a loss of chlorophyll and carotenoids, and producing ROS (Sherwani *et al.*, 2015; Vladimirov *et al.*, 1980). A few PSII herbicides may also inhibit carotenoid biosynthesis, the products of which protect from photooxidative damage (Duke, 1990; Sandmann and Böger, 2020; Tracewell *et al.*, 2001). When a PSII herbicide is bound, electron transfer is halted, and there is an accumulation of excess excitation energy (EEE) (Battaglino *et al.*, 2021; Markwell and Namuth, 2003). This EEE must be dissipated sufficiently, and if not, the EEE can cause photoinhibition and damage to the photosystems of the plant due to the development of reactive oxygen species (ROS) (Pospíšil, 2016). EEE can be quenched via non-photochemical quenching (NPQ) (released as heat) and chlorophyll fluorescence (Muller *et al.*, 2001). A change in CF and quenching directly effects the efficiency of PSII, which is evidenced by the parameter F_v'/F_m', which provides an estimate of the maximum efficiency of PSII in light adapted material (Technologica). The change in CF emissions due to PSII herbicides binding is detectable utilising CFI, and can be used to infer the efficiency of other photochemical processes - this is how PSII inhibiting herbicides can be detected and identified using CFI (Juneau *et al.*, 2007; Legendre *et al.*, 2021; Park *et al.*, 2016).

Herbicides have been classified into various groups, which are subsets of the herbicides MOA (Sherwani *et al.*, 2015). Herbicides belonging to the same group all have the same MOA, however, may have different structures and belong to different chemical families. There are many classes of selective herbicides that have a MOA affecting PSII, with Triazines being one such group (Sherwani *et al.*, 2015; Shukla and Devine, 2008). Due to the effective mode of action, triazines, as well as other PSII MOA classes, are used to control a wide range of agricultural crops. However, the high efficacy and constant, repeated use has resulted in weeds with a resistance to these herbicides persisting due to natural selection. Weeds may be either completely resistant, or simply require a much higher dose of herbicides to be effective. Other classes of herbicides targeting photosystem II include ureas, amides, biscarbamates, benzothiadiazinones, phenylpyridazines, and cyanophenols (Oettmeier, 1999). On the D1 protein, there are two amino acid sites for herbicides to bind to photosystem II, depending on the herbicides preferred binding orientation in the Q_B niche: Serine 264 and Histidine 215, with this being a distinct classification by the HRAC due to no cross-resistance being found between the two groups (Battaglino *et al.*, 2021; Heap, n.d.; HRAC, 2020a). Additionally, Phenylalanine 265 also contributes to the binding of herbicides in PSII, with hydrogen bonds forming between the amino acid and the herbicide molecule (Fuerst and Norman, 1991). PSII inhibitors that bind to the locus Serine 264 include ureas and amides, and those that bind to Histidine 215 include nitriles and phenylpyridazines. Triazines, due to their efficacy, as well as compatibility with other herbicides, are used for broad-spectrum weed control and are essential for the production of many crops, such as corn (LeBaron *et al.*, 2008). Atrazine, one such herbicide of the Triazine class, was found to increase yields of corn by 9 bushels per acre (approximately 0.23 metric tons per acre), compared to other herbicides (according to the US Environmental Protection Agency (IRED, 2006)). Along with this, using other herbicides would come at an increased cost to the growers. These factors combined could result in a loss of approximately $28 per acre, when not using atrazine.

1.4 Adjuvants

Adjuvants are chemicals which can be added to herbicides to increase their efficacy, or modify action or physical properties, with particular aim to enhance performance (Hazen, 2000; Pacanoski, 2015). This is done by employing a myriad of mechanisms, such as improving mixing of a spray mixture with the herbicide active ingredient, changing how effective the plant is at taking up the herbicide, or even just reducing the amount of variance in performance caused by non-optimum conditions, subsequently reducing the amount of herbicide used (Kudsk, 2002). Different methods have a range of effectiveness, benefits, as well as practicality. The use of adjuvants has proved to be effective at decreasing the required amount of herbicide, for similar efficacies (Blackshaw *et al.*, 2006).

Adjuvants, along with any other ingredients in a herbicide mixture other than the key active ingredient, are usually considered biologically inactive (Kudsk and Streibig, 2003). Their presence is simply to increase or decrease the amount of active ingredient which reaches the site of action, by helping it to reach the site. However, some adjuvants themselves can be toxic to plants, as well as humans and other aspects of the environment (Mesnage and Antoniou, 2018). Unlike the active ingredients of herbicides, the phytotoxicity of adjuvants is often disregarded, and they do not face the same level of regulations and laws that herbicides themselves do, however they are still highly regulated depending on how they are used. This lack of assessment is indicated by the fact that there is no acceptable daily intake of adjuvants, and their uses in both agricultural and metropolitan settings could lead to high amounts of exposure and ingestion. This lack of daily intake guidelines is particularly egregious due to the fact that it has previously been shown that it is in fact relatively simple to assess the effect of adjuvants, and to use this information to determine dose recommendations (Streibig and Kudsk, 1993).

Adjuvants can have a variety of effects, from improving the uniformity of spray droplet size (Landim *et al.*, 2019), enhancing the mixing of the overall solution with the active ingredient of the herbicide, and increasing the efficacy of the herbicide (leading to lower concentrations of active ingredient being used and therefore lower costs due to less active ingredient being purchased) (Green and Hazen, 1998; Underwood, 2000). Additionally, adjuvants can help to minimise the leaching and persistence of the herbicidal active ingredient into the soil and environment (Alva and Singh, 1991). Despite there being numerous methods of action for adjuvants, they can generally be classified into two subgroups (and then even further into smaller subdivisions): special purpose adjuvants and activator adjuvants (Curran *et al.*, 1999).

Surfactants are one class of adjuvant, which work as a solvent, decreasing the surface tension of the herbicide mixture, allowing it to spread more, covering a larger surface area of the plant, and therefore increasing the rate of uptake (Song *et al.*, 2012). An example of a widely used surfactant is sodium laurel ether sulfate (SLES).

Previous research has shown that chlorophyll fluorescence imaging is an appropriate tool for determining the changes in efficacies of herbicides due to the addition of adjuvants, and the present study aims to corroborate and build upon these findings (Zhang *et al.*, 2019; Zhang *et al.*, 2022).

1.5 Chlorophyll fluorescence

Chlorophyll within plant leaves absorb light energy and use this to drive photochemistry and the production of ATP and NADPH (Maxwell and Johnson, 2000). There are also two other possible fates for the light energy absorbed; it can be dissipated as heat in a process called non-photochemical quenching (NPQ), or reemitted at a longer wavelength (approximately 400-700nm) known as chlorophyll fluorescence, which can be used to determine the light use efficiency of the plant (Kalaji *et al.*, 2017; Murchie and Lawson, 2013). These three fundamental processes are in a constant state of competition, with the efficiency of one increasing, causing a decrease in the efficiency of the other two. Due to this underlying relationship, CF can therefore be used to determine the photochemical yield of photosystem II (PSII), which is approximately proportionate to the operating efficiency of photosynthesis, as ~90% of chlorophyll fluorescence originates from photosystem II. The extent to which light energy is actively used in photochemical reactions determines plant photosynthetic efficiency. Chlorophyll fluorescence imaging (CFI) can be used to acquire information regarding photosynthetic efficiency and plant health because of the underlying link between chlorophyll fluorescence emissions and photochemistry, with an example CF trace being evidenced in Figure 1.1.

CFI is an ideal tool for screening herbicides and determining whether they have a mode of action affecting the photosystems, as both biotic and abiotic factors (such as the application of herbicides) can influence a plant’s photosynthetic efficiency and can cause spatial heterogeneities to occur, which can be detected using CFI. Additionally, conventional methods utilising HPLC (High-performance liquid chromatography) and mass spec (which have been used for assessing herbicide residues), have large overheads of technicality, price, and time (Park *et al.*, 2016). Whilst CFI cannot detect herbicide residues in leaf tissue, it can be used as a complimentary tool for biokinetic studies, and does not have the same limitations as those named previously.

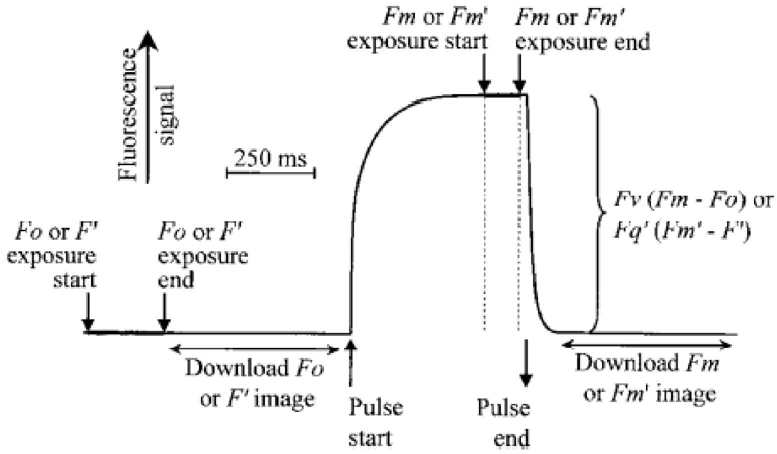


Figure 1.1. Example of a chlorophyll fluorescence trace, copied from Baker, Oxborough, Lawson, and Morison, 2001.

During photochemistry, the conversion of light energy to chemical energy occurs across a membrane, with many proteins and subunits and components involved (Andersson and Styring, 1991; Croce and van Amerongen, 2011; Utschig *et al.*, 2018; Wollman *et al.*, 1999). Photosystem II is a membrane protein complex made up of multiple subunits found in the thylakoid membrane of chloroplasts, and, despite its name, is the first protein complex in the light-dependent reactions of oxygenic photosynthesis (Barber, 2003). Photosystem IIs function is to oxidise water and reduce plastoquinone, using energy from light (Barber, 2003; Vass, 2012). The binding of plastoquinone occurs at the D1 protein, which PSII herbicides compete for, with there being two sites for binding, which distinguish between multiple classes of these herbicides with PSII targeting MOAs (triazine and urea herbicides). The enzymatic process of PSII catalysing water-splitting chemistry are so highly conserved to the point where it is the only case found across all life on earth (Vinyard *et al.*, 2013). The only two kinds of chlorophyll which are found in plants and green algae are chlorophyll a and b, both of which act as photoreceptors and are vital for performing photosynthesis, with chlorophyll b being only slightly structurally different to chlorophyll a, swapping a methyl group for an aldehyde group (Khaleghi *et al.*, 2012; Kimball, 2000). Despite there being numerous versions, chlorophyll a is the most widely utilised by PSII reaction centres, with chlorophyll b mainly acting as an accessory pigment, passing trapped light energy onto chlorophyll a, with the ratio between the two being an important factor for the light absorption efficiency of photosynthesis (Kume *et al.*, 2018; Sonobe *et al.*, 2020; Tanaka and Tanaka, 2000). In PSII, the process of splitting water molecules produces electrons, protons, and molecular oxygen (Barber, 2003; Renger, 2012; Rögner *et al.*, 1996). The electrons have two functions; to reduce $NADP^+$ to NADPH, and to create an electrochemical charge that provides the energy for pumping protons from the stroma of the chloroplast into the interior of the thylakoid (Cooper, 2000). On the other hand, the protons are essential in the conversion of ADP to ATP during photophosphorylation (Arnon *et al.*, 1954). As photosystem II is the initial main step in the performance of photosynthesis, PSII is also the main source of chlorophyll fluorescence, with approximately 90% of all CF originating from here (Coe *et al.*, 2015; Govindjee, 1995).

Parameter development for chlorophyll fluorescence originated with Kautsky and Hirsch (1931) publishing their seminal work “Neue versuche zur kohlensäureassimilation" (New experiments on carbon dioxide assimilation). Kautsky and Hirsch discovered the Kautsky effect, which evidences a decrease in chlorophyll fluorescence after the illumination of leaves that have been dark-adapted (Banks, 2017; Berry, 2018). The accepted model for the physiological basis of chlorophyll fluorescence is the Q_A model (with Q_A being the primary quinone electron acceptor of PSII) (Kalaji *et al.*, 2014; Schansker *et al.*, 2014; Stirbet and Govindjee, 2012). This model describes the process after a plants photosynthetic material is exposed to light after being dark-adapted, in which the fluorescence increases from minimum (F_o) to maximum (F_m) (Baker, 2008; Maxwell and Johnson, 2000; Murchie and Lawson, 2013). The Q_A model is based on the assumption that the increase in fluorescence observed from F_o to F_m is due to the reduction of Q_A, which is correlated with open PSII reaction centres becoming closed (Belgio *et al.*, 2014; Murchie and Lawson, 2013; Schansker *et al.*, 2014). Open reaction centres are those which are able to perform photochemistry (i.e. the reduction of Q_A by P680, a special chlorophyll which acts as the primary electron donor in PSII), and those that are closed are not able to (Q_A is fully reduced (Q_A^-), and P680 cannot pass on another electron until Q_A becomes oxidised by passing on an electron to Q_B) (Baker, 2008; Barber and Archer, 2001; Murchie and Lawson, 2013). F_o, minimum fluorescence, occurs when all reaction centres are open with Q_A being fully oxidised, and when all reaction centres are closed, Q_A is said to be fully reduced, with this being the point of F_m, maximum fluorescence. At F_m the reduction of Q_A occurs at a rate faster than the reoxidation of Q_A^-. The fluorescence transient is inversely correlated with the rate of CO_2 assimilation of the plant, with changes in chlorophyll fluorescence upon illumination of a dark-adapted leaf directly correlating with changes in CO_2 assimilation (Baker, 2008; McAlister and Myers, 1940).

When dark-adapted plants (NPQ is fully relaxed, Q_A is fully oxidised, and reaction centres are open) are exposed to dim light energy (less than 1 μmol m^{-2} s^{-1}), the chlorophyll fluorescence signal is the minimal level of fluorescence, F_o (Lawson *et al.*, 2002; Maxwell and Johnson, 2000; Murchie and Lawson, 2013). This is the level of fluorescence when all reaction centres of PSII are open, and therefore capable of photochemistry. After F_o has been recorded, a saturating pulse of bright actinic light (greater than 6000 μmol m^{-2} s^{-1}) can be used to obtain the maximum value of fluorescence, F_m, where all reaction centres are closed, Q_A is fully reduced, therefore stopping the possibility of photochemistry (Baker *et al.*, 2001; Barbagallo *et al.*, 2003; Lawson *et al.*, 2002). The increase of chlorophyll fluorescence emission from minimum (F_o) to maximum (F_m) reflects the reduction of Q_A. The difference between the minimal and maximal levels of fluorescence of dark-adapted material is the variable fluorescence of the material, F_v. This can also be measured for light-adapted material, using light adapted values for minimal, maximum, and variable fluorescence; F_o', F_m', and F_v' respectively (Baker, 2008). F_v/F_m and F_v'/F_m' estimate the maximum quantum efficiency of PSII, in the dark and light respectively. In between F_o' and F_m' is F', which is the fluorescence signal at any point between these two values (Lawson *et al.*, 2002; Technologica). F' is different to F_v and F_q', which are respectively the difference between F_o and F_m, and the difference between F' immediately before and at the peak of a saturating pulse. F', along with F_m', can be used to determine the operating efficiency of photosystem II in light adapted material (also termed F_q'/F_m') (Baker *et al.*, 2001; Technologica). The operating efficiency of photosystem II has been termed "the single most useful fluorescence parameter", and it can provide an estimate of the quantum efficiency of linear electron transport through photosystem II (Baker *et al.*, 2007; Baker *et al.*, 2001).

Environmental factors that cause damage via the impairment of PSII efficiency (often referred to as photoinhibition) cause a distinctive decline in the maximum efficiency of PSII (F_v/F_m), associated with the increase of F_o (Krause and Weis, 1991; Murchie and Lawson, 2013). F_v/F_m measurements of between 0.78 to the theoretical ideal value of 0.83 (in dark-adapted plants) are correlated with healthy and unstressed plant material (Björkman and Demmig, 1987; Murchie and Lawson, 2013; Percival, 2005). The ideal PSII maximum efficiency value of 0.83 relates to the photon yield of photosynthesis, which can be defined as the amount of CO_2 assimilated per mole of photons absorbed**,** and hence the fluorescence transients association with the rate of CO_2 assimilation of the plant (Baker, 2008; McAlister and Myers, 1940)**.**

In conclusion, chlorophyll fluorescence research has been a cornerstone in aiding our understanding of photosynthesis, and has become a key and universal method for researching the photosynthetic efficiency of plants in vivo (Nedbal and Whitmarsh, 2004).

1.6 Chlorophyll fluorescence imaging

Chlorophyll fluorescence imaging (CFI) is a non-invasive, rapid, and inexpensive method of measuring a plants photosynthetic efficiency, and provides a different way of looking at CF, providing spatial and temporal assessments (Gorbe and Calatayud, 2012; Maxwell and Johnson, 2000; Murchie and Lawson, 2013).

CFI has a great number of applications, including visualising differences in photosynthetic performance caused by both biotic and abiotic stress, screening populations of plants for mutants, as well as evidencing a diverse spectrum of plant properties that can cause photosynthetic heterogeneity, such as developmental stage, and nutritional state (Gorbe and Calatayud, 2012; Guidi *et al.*, 2019; Nedbal and Whitmarsh, 2004; Ogawa and Sonoike, 2021; Pérez-Bueno *et al.*, 2019). These various uses are possible due to a few useful characteristics of chlorophyll fluorescence, for example unique chlorophyll fluorescence emissions allowing chlorophyll fluorescence imaging to be used as a rapid method for screening plants (Varotto *et al.*, 2000), and it's extremely sensitive nature (Ning *et al.*, 1997).

Chlorophyll fluorescence imaging works by taking measurements of various parameters associated with the plants light-dependent reactions and processes, with both light and dark adapted values being required to do so (Lawson *et al.*, 2002). Chlorophyll fluorescence imaging devices have been adapted to fit a variety of uses, for example, at both microscopic and whole plant levels, as well as *in situ* laboratory experiments and mobile field experiments (Baker *et al.*, 2001; Bolhàr-Nordenkampf and Öquist, 1993; Lawson *et al.*, 2002; Logan *et al.*, 2007; López-Calcagno *et al.*, 2020; Nedbal and Whitmarsh, 2004; Pérez-Bueno *et al.*, 2019; Simkin *et al.*, 2020; Trampe *et al.*, 2011; Vialet-Chabrand *et al.*, 2017; Zarco-Tejada *et al.*, 2009). Chlorophyll fluorescence imaging is a tool designed to give an accurate depiction of plant stress in a rapid and visually intuitive manner, detailing the fluorescence emissions of cells, leaves, and up to whole plants or even ecosystems and areas, allowing the spatial and temporal assessment of plants (Frankenberg *et al.*, 2018; Gower, 2016; Gower *et al.*, 2004). CFI allows for the examination of the unique chlorophyll fluorescence signatures and their heterogeneity across leaf section and time (Nedbal and Whitmarsh, 2004). These differences can arise due to multiple factors that occur internally to the plant, which would not otherwise be noticeable through other measurements of chlorophyll fluorescence, such as DLE (delayed light emission) and thermal imaging, due to their restriction in giving single point measurements (Ellenson and Amundson, 1982; Ellenson and Raba, 1983; Hashimoto *et al.*, 1984; Omasa and Takayama, 2003). These spatial variations in photosynthesis are just one of many features of leaves revealed by chlorophyll fluorescence imaging, and can arise due to various reasons such as leaf morphology, developmental state, or effects of pesticides (Barbagallo *et al.*, 2003; Murchie and Lawson, 2013; Nedbal and Whitmarsh, 2004; Pérez-Bueno *et al.*, 2019).

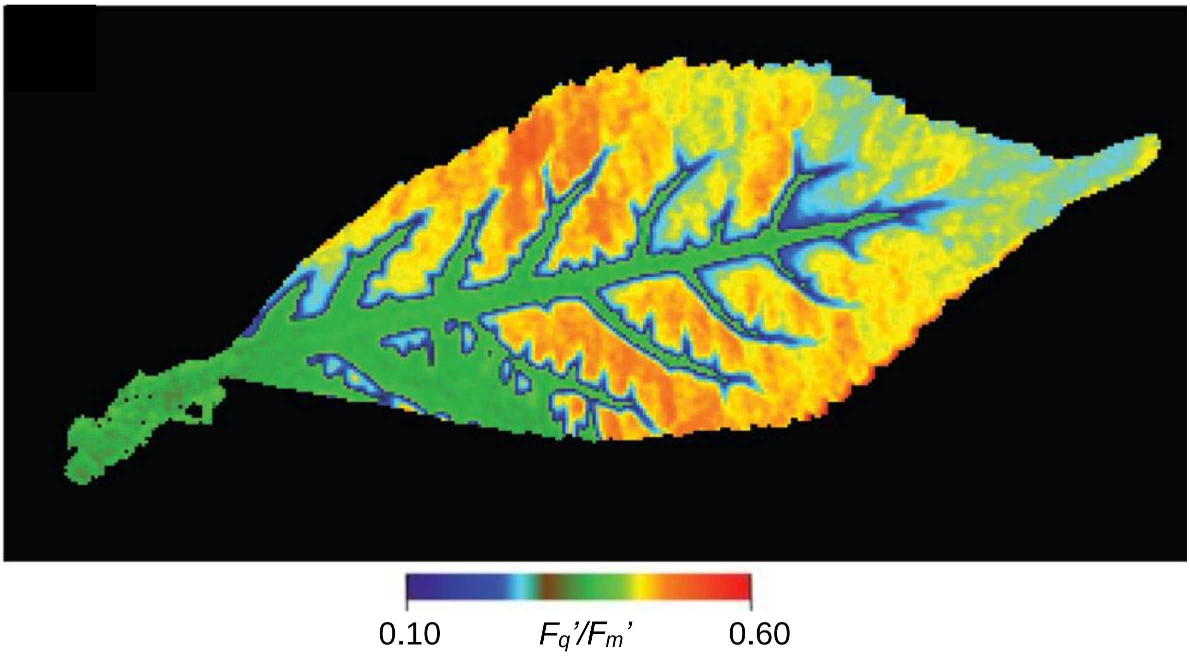


Figure 1.2. Example chlorophyll fluorescence image showing the decrease in photosynthetic efficiency (F_q'/F_m') of a cut elder leaf due to effect of the PSII herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, or Diuron) (Taken from Murchie and Lawson, 2013). The 'warmer' colours (yellows/reds) indicate healthy tissue, and the 'colder' colours (greens/blues) indicate stressed tissue.

Other than chlorophyll fluorescence imaging, different technologies, such as thermal imaging (Hashimoto *et al.*, 1984) and DLE imaging (Ellenson and Raba, 1983), were used to try and detect spatially differing responses of plants under various stressors, such as herbicides (Blaich *et al.*, 1982; Omasa *et al.*, 1987; Takayama and Omasa, 2005). Whilst somewhat effective, these methods do not indicate where in the photosynthetic apparatus the inhibition was occurring, only that it was occurring, which is a major factor for herbicide MOA determination. Chlorophyll fluorescence imaging differs from other conventional methods in that it allows the resolution of photosynthetic performance over the surface of a leaf, whereas others (such as gas exchange) are much more integrated (Nedbal and Whitmarsh, 2004). This allows greater attention to be paid to spatial differences across the leaf, which can differ greatly, and indicate specific areas of stress, which is valuable for herbicide MOA determination.

The specific herbicides with an MOA targeting PSII can be detected using chlorophyll fluorescence imaging. The decrease in efficiency causes an increase in dissipation elsewhere, and if not dissipated sufficiently, can cause damage to the photosystems. Hence when PSII is blocked by an herbicide, the chlorophyll fluorescence signal increases, and the effects can therefore be detected using chlorophyll fluorescence imaging. Other herbicidal modes of action which are visible by chlorophyll fluorescence imaging are those that affect photosystem I, and uncouplers, however only due to the ‘knock-on’ effects that these inhibitors have on PSII. Herbicides which have a mode of action that affects PSI act as electron acceptors, which consequently redirects the normal electron flow away from the photochemical process and generates reactive oxygen species by reacting with molecular oxygen (Juneau *et al.*, 2007; Liu *et al.*, 2013). This can be detected with CFI, as the process of photosynthesis has been disrupted. It is possible to differentiate between the PSI and PSII modes of action whilst using CFI, due to an important distinguishing factor between their inhibitory mechanisms. PSI inhibitors do not have any effect if plants are kept in the dark, as they require light to generate the superoxide radicals, whilst PSII inhibitors do have an effect without any light being present (Dayan and Zaccaro, 2012). Uncouplers act by dissociating electron transport from ATP synthesis, dissipating the energy state of the membrane of the thylakoid, before the energy can be used to perform phosphorylation of ADP – the electrons can cause lipid peroxidation, and subsequently the effects of the uncoupler can be detected using CFI (Dayan and Zaccaro, 2012; Moreland, 1980).

1.7 Summary

Herbicides, and the adjuvants used to aid their use, are a major and vital part of modern agriculture, with many people's livelihoods depending on their efficacy. The use of herbicides has been cause for concern, with the toxicity to humans and any collateral damage they cause being an area of legislation and research. Specifically, the classes of herbicides that affect photosystem II are very effective and therefore have come under large scrutiny – understanding a herbicides MOA is a major step in developing novel and safe products.

Chlorophyll fluorescence is one of the uses of light energy absorbed by a plant, and its intrinsic relationship with the photosynthetic efficiency of the plant makes it an ideal target of study. Research into the field of chlorophyll fluorescence is not modern, however it is with recent reviews and applications that CF and CFI have become an integral part of plant physiology research. Research into chlorophyll fluorescence, and specifically utilising CFI, has helped to develop an understanding of both a plant's underlying photosynthetic mechanisms, as well as the factors affecting it. Both biotic and abiotic factors can influence a plant's photosynthetic efficiency and can cause spatial heterogeneities to occur. These differences can be detected using chlorophyll fluorescence imaging.

There are multiple chlorophyll fluorescence parameters that can be used to assess a plants photosynthetic efficiency, which can be visualised using chlorophyll fluorescence imaging. With the majority of chlorophyll fluorescence emissions from photosystem II, understanding the maximum or operating efficiency of PSII by looking at the chlorophyll fluorescence emissions is vital to understanding the photosynthetic efficiency of the plant. Chlorophyll fluorescence imaging is an ideal tool for identifying herbicides which have a mode of action which affects the photosynthetic capabilities of a plant, namely PSII herbicides. The development of a method to screen a large number of novel herbicides to accurately identify those which act by binding to PSII, or affect the photosystems in other indirect ways, would be a great step forward in the process of efficient MOA diagnosis in the herbicide discovery pipeline. Chlorophyll fluorescence imaging gives a much better picture and more information about the specific mechanisms of the herbicidal modes of action than whole plant tests would be able to as it can provide both spatial and temporal data over whole leaves or plants, however whole plant visual assessments are eventually required to assess the biological activity of the herbicides (Fu *et al.*, 2022). In summary, research into chlorophyll fluorescence, and the technique of imaging its emission, have both been pivotally important steps in understanding a plants photosynthetic efficiency.

The aims of the present research are to determine whether chlorophyll fluorescence imaging is a suitable tool for herbicidal MOA and toxicity detection, and to determine how specific the methodology is at doing so, as well as what conditions are needed to detect them along with any other herbicides that affect the photosystems as either a secondary or tertiary mode of action. Subsequently and finally, the type and concentrations of adjuvants to be used that would increase the efficacy or applicability of various PSII herbicides will be assessed. This research aims to screen as many herbicidal standards as possible using chlorophyll fluorescence imaging, as whilst CFI is not a perfect solution, it is hypothesised that CFI will give reliable and rapid feedback on MOAs targeting PSII, showcasing any that directly or indirectly affect a plants photosynthetic efficiency.

2. Study 1 – Herbicidal MOA Screening Utilising *Lemna minor*

2.1 Introduction

Recently, research in herbicides has gained increasing attention due to proposed bans/replacements and the potential drastic effects this may have, leading to a need for new ways of developing and detecting herbicidal MOAs (Alcántara-de la Cruz *et al.*, 2021; Böcker *et al.*, 2019; Cashman *et al.*, 1981; Kudsk and Mathiassen, 2020; Stuart *et al.*, 2022; Walsh and Kingwell, 2021). The removal of certain valuable herbicides from commercial availability, as well as the increasing numbers of herbicide resistant species, only intensifies the need for the development and identification of novel herbicide modes of actions (MOA) (Heap, 2014). Herbicides are amongst the most regulated and controlled chemical substances in the world, due to their potential subsequent effects on the environment and human health (McDougal, 2018). The quantity of legislative rules and regulations, as well as increased registration costs, that crop protection companies have to go through for these novel chemicals, inhibit their development (Flynn and Naylor, 2002). Due to this, new methods are required for initial screening of many compounds, before more intensive investigation and development of a selected few which show promise. Ideally, such work needs to be high throughput, producing quick and reliable data, that can be extrapolated past the restraints of assays.

Chlorophyll fluorescence imaging is a rapid, inexpensive, adaptable, and non-invasive method for determining the photosynthetic performance of a plant (Murchie and Lawson, 2013). Results can be obtained in as little as 20 seconds, making it an ideal method for high throughput analysis. CFI can provide various measurements dependent on whether the plant is dark adapted, or light adapted – the following research only utilised dark adapted measurements, as this is a more rapid approach and provides plenty enough data for the work required. The use of rapid CFI is not exclusive to herbicidal research, and was not developed for this purpose, however this is an applied aspect of it, as there are many areas of plant biology where it is relevant and required and utilised in an increasing amount of research areas (Barbagallo *et al.*, 2003). Traditionally, greenhouse screening has entailed a thorough visual evaluation of herbicidal action, two to three weeks after treatment. Contrastingly, CFI can be used after a much shorter time frame – in the case of this experiment, after as little as 20 minutes. Previously, chlorophyll fluorescence imaging has been used in concurrence with multiple *Lemna* species, including *L. minor*, for determining toxic chemical effects on the photosynthetic capabilities of the plant (Dewez *et al.*, 2018; Frankart *et al.*, 2003; Park *et al.*, 2016).

Plants of *Lemna minor* were selected to be used for this assay for a number of reasons, aside from the fact that *Lemna minor* is easy to grow, it has previously been used in scientific studies to assess the efficacies of herbicides (Frankart *et al.*, 2003; Park *et al.*, 2016). As an aquatic plant *L. minor* is also relevant in the study of the application of herbicides due to the large amounts of run-off and leaching which can affect aquatic environments/ecosystems (Klöppel *et al.*, 1997; Prado *et al.*, 2009). Despite not being the main focus of the study, the methodology developed here could also be exceedingly applicable to further work in this area. *Lemna* plants have a simple structure, small size, ease and rate of growth, and high homogeneity. These traits make them the ideal species to be used for this research.

Adjuvants, in the context of pesticides, are defined as chemicals added to a herbicidal mixture to improve the efficacy of, affect the action of, or change a physical property of, the herbicide, with the specific aim of strengthening its performance -

therefore reducing amounts of active ingredient needed (Hazen, 2000; Pacanoski, 2015). The two adjuvants used for this research were Sodium Laureth Sulfate (SLES) and Tween 20 (polyoxyethylene sorbitan monolaurate). SLES and Tween 20 are commonly used adjuvants in the agricultural industry, and are known to be effective at increasing the efficacy of herbicides. Whilst the mode of action of these adjuvants are not fully elucidated, both SLES and Tween 20 act as surfactants, decreasing the surface tension of the herbicidal mixture, and along with a combination of other mechanisms, and improve rate of uptake. SLES has previously been shown to exhibit cellular toxicity itself, although still considerably lower in phytotoxic levels than other known severely toxic chemicals (Song *et al.*, 2012).

Tween 20 ($C_{26}H_{50}O_{10}$) (Fig. 6.1a), a non-ionic surfactant, is a widely used and effective component in many herbicidal mixtures, and has been a choice adjuvant for multiple decades (O'SULLIVAN and O'DONOVAN, 1980; Penfield *et al.*, 2015). SLES ($C_{14}H_{29}NaO_5S$) (Fig. 6.2b), an ethoxylated form of SLS, is more commonly known as a chemical frequently found in skin care and cleaning products and personal care products (e.g. toothpastes and shampoos), however it has also been utilised as an adjuvant in the agrochemical industry (TMR, 2020).

Previous studies have shown that chlorophyll fluorescence imaging is an ideal tool for observing the effects of pollutants on the photosynthetic processes of *Lemna*, as differences in the photosynthetic efficiencies of the plants are observed before any physical visual effects in the growth processes of the plant are seen(Juneau *et al.*, 2003). Additionally, *Lemna* has been shown to be a suitable organism for chlorophyll fluorescence research(Dewez *et al.*, 2018). This research aims to corroborate these findings.

The aim of this research was to study the effects of various herbicidal modes of action on plant photosynthetic efficiency (specifically *Lemna minor*), using chlorophyll fluorescence imaging and information on how CFI may be used as a high throughput screening tool for herbicides. Additionally, the effects of adjuvants on herbicidal efficacy were examined. The findings of this research aims to expand on previous work utilising *Lemna minor* and CFI for herbicidal screening, such as Park et al (2016) and Juneau et al (2003), and provide a basis for herbicidal screening techniques utilising chlorophyll fluorescence imaging in the future.

2.2 Materials and Methods

2.2.1 Plant material and growth conditions

For all experiments in this chapter, *Lemna minor* were obtained from the Canadian Phycological Culture Centre, University of Waterloo, strain number 490. Plants were grown in 500ml conical flasks containing MHE10MM (modified Hoagland's solution) inorganic media, and then grown in larger, 500mL plastic tubs containing the same media when the flasks became full. The plants were grown at 25°C and 50% humidity, on 16/8-hour day/night cycle, in a controlled environment growth room (Sanyo room, Weiss Technik control panel). The plants were transplanted into 24-well plates (1 plant per well), and further grown in 1.5 mL of media. The plants were grown in the same conditions after transplanting, for a mean of 7-10 days, until confluent and filling the wells.

2.2.2 Preliminary tests

Before the main adjuvant study could commence, some measures were taken, including a series of smaller experiments outlined below.

Herbicide concentration. Plants of *L. minor* were grown using the same methods as outlined above in section 2.2.1, and transplanted into, and further grown in, 24-well plates in an identical manner. 15µl of 10mg/ml test compound dissolved in DMSO were added to the top wells, with each subsequent well in the column being a 2-fold dilution of the well above, utilising DMSO as the solubilising agent. The columns therefore contained 4 concentrations of the test herbicides, with concentrations of 100ppm, 50ppm, 25ppm, and 12.5ppm. The 24-well plates also contained one column of the positive control Atrazine (with an initial concentration of 1mg/ml), and the negative control of DMSO (kept at 15µl per well throughout the column). After being treated with the test compounds, the plates were covered with a lid to avoid evaporation, and then imaged.

Chlorophyll Fluorescence Imaging for herbicide concentration determination. The CFImaging system (Technologica Ltd, Colchester, UK) was used to image the chlorophyll fluorescence of plants treated with different herbicide compounds. Plants were dark adapted for 20 min, in a room maintained at 21°C. For measurement collection, a plate was placed inside the imaging system and a set protocol within the Fluorimager software (Technologica Ltd, Colchester, UK) was used for image collection. In the protocol, plants were exposed to an actinic light intensity of 0µmol m^{-2} s^{-1} for 20 s and an image of minimal fluorescence (F_o) captured, after which a saturating pulse of 2000µmol m^{-2} s^{-1}, for 800ms, and an image of maximum fluorescence (F_m) was captured. After image capture and data exportation, the plates were removed from the imager and kept wrapped in tinfoil inside the dark box attached to the imager, to ensure they stay in the dark-adapted state. Images were collected at

T0, T1, T3, T5, and T24, with the numbers indicating the number of hours after the first image had been taken.

Adjuvant concentration. Adjuvant concentration phytotoxicity tests were carried out before the main experimental phase, to determine a 'safe' dosage of adjuvant to conduct the assays with. Concentrations of 0.2%, 0.1%, and 0.05% were tested, none of which showed large amounts of phytotoxicity, for either of the adjuvants. These concentrations were within the range of concentrations used in spray tanks, when products are combined for application in the field. Therefore, the assay could be performed using the higher 0.2% concentration, and all phytotoxicity detected would be due to the tested herbicides.

Light intensity of saturating pulse. The previous work assessing herbicide concentration had been conducted using a saturating pulse of 2000μmol m^{-2} s^{-1}. Comparison experiments were conducted to determine if the saturating pulse should instead be the maximum intensity of 6401 μmol m^{-2} s^{-1}. To perform the comparison, 24-well plates containing *L. minor* were dark adapted for 20 minutes, and then subjected to a light regime using various actinic light intensities (0, 100, and 250μmol m^{-2} s^{-1}) and the two saturating pulse intensities, 2000 and 6401 μmol m^{-2} s^{-1} used to obtain F_m. It was found that the higher intensity saturating pulse of 6401 μmol m^{-2} s^{-1} resulted in higher and more accurate F_v/F_m values than the lower intensity saturating pulse of 2000 μmol m^{-2} s^{-1}, as it ensured that all reaction centres were closed. Therefore, the higher intensity saturating pulse was used for the following adjuvant assay.

Assay length determination. To determine an appropriate length of assay, plants of *L. minor* were kept in the dark-adapted state for 48 hours, whilst being imaged at the time points T0, T1, T3, T5, T24, and T48, to determine if there was a decrease in the photosynthetic health of the plants over this sort of time frame. Due to this, all subsequent measurements were taken at a maximum of 24-hours after the initial image, as any effects on the photosynthetic efficiency could be attributed to the herbicide and/or adjuvant, as opposed to stress due to a lack of light. Additionally, one of the main desired outcomes of these assays was for rapid data collection, with the longer time frame of 48-hours achieving the opposite of that without any noticeable benefits.

From these preliminary tests, it was determined that the main assay assessing adjuvant efficacy would be conducted over a time course of 24-hours, maintaining the plants in the dark, utilising a herbicidal concentration of 100ppm, an adjuvant concentration of 0.2%, and a saturating pulse of 6401μmol m^{-2} s^{-1}.

2.2.3 Adjuvant assay layout

	1	**2**	**3**	**4**	**5**	**6**
A	H1	H2	H3	H4	H5	H6
B	H1+Tween	H2+Tween	H3+Tween	H4+Tween	H5+Tween	H6+Tween
C	H1+SLES	H2+SLES	H3+SLES	H4+SLES	H5+SLES	H6+SLES
D	Atrazine	Atrazine	Atrazine	DMSO	DMSO	DMSO

Figure 2.1. 24-well plate layout for adjuvant efficacy assay.

For adjuvant efficacy experiments, the 24-well plate used was set up as shown in Figure 2.1. Per 24-well plate of *L. minor*, six herbicides were tested three times: once on its own at a concentration of 100ppm (10mg/ml), once with the adjuvant Tween 20 (10mg/ml herbicide, 0.2% adjuvant concentration), and once with the adjuvant SLES (10mg/ml herbicide, 0.2% adjuvant concentration). As well as the test herbicides, the wells also contained three wells each of positive and negative controls, Atrazine (a PSII herbicide) and DMSO respectively. The positive control of Atrazine was at 1mg/ml (10ppm), and the DMSO concentration was 100ppm. It is important to note that for every subsequent replicate, the position of the adjuvants was swapped, to avoid any potential contamination from the wells containing the positive control, Atrazine.

2.2.4 Experimental procedure

Adjuvants, herbicides, and controls were added to the 24-well plates in the light, which were then immediately wrapped in tin foil, so as to dark adapt the plants, and left for 20 minutes. The plates of *L. minor* were then imaged using the CFImaging system (Technologica Ltd, Colchester, UK). For measurement collection, one plate was placed inside the imaging system at a time. A set protocol within the Fluorimager software (Technologica Ltd, Colchester, UK) was used for image collection. The plants were exposed to an actinic light intensity of 0 µmol m^{-2} s^{-1} for 20 seconds inside the imager, and then exposed to a saturating pulse of 6401 µmol m^{-2} s^{-1}, for 800ms. A saturating pulse of this strength ensures the closure of all reaction centres, as developed by the precursory tests. The parameters of F_o, F_m, and F_v/F_m were recorded (fluorescence minimum, fluorescence maximum, and maximum efficiency of PSII respectively).

After this initial image had been taken (T0), the plates were removed from the imager and re-wrapped in tinfoil to ensure the plants stay in the dark-adapted state. Subsequent images were taken, using the same protocol, 1, 3, 5, and 24 hours after the initial image. After the 24-hour image had been taken, the plates were disposed of following the proper practices.

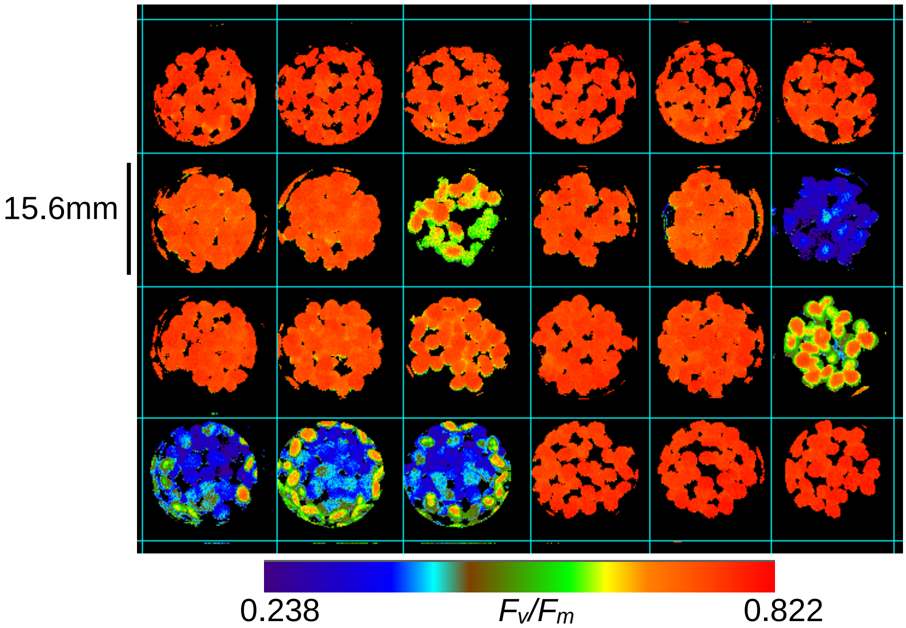


Figure 2.2. Example F_v/F_m image taken by the chlorophyll fluorescence imager of a 24-well plate containing *L. minor*, and treated with herbicides and adjuvants, 1 hour after application (T1). The image is using the same 24-well plate layout as shown in figure 2.

It can be seen in the example of Figure 4 that the controls of Atrazine and DMSO are working as intended, with the positive control Atrazine decreasing F_v/F_m by approximately 0.2-0.3. Meanwhile, the DMSO negative control plants are remaining healthy, with values of ~0.78. It can be seen that for the herbicides in the 3rd and 6th columns, the adjuvants are already having an effect on their efficacy.

2.2.5 Data analysis and statistics

All F_v/F_m values (for adjuvant studies) were normalised against the mean DMSO (negative control) F_v/F_m values of their respective plates, to account for any whole plate differences between the replicates. A normalised F_v/F_m value of 1 indicates a value equal to the DMSO value for the plate, a value of less than 1 indicates lower F_v/F_m values than the DMSO control, and a value of greater than 1 indicates higher F_v/F_m values than the DMSO control. This normalisation was performed to only show decreases in the plant's photosynthetic health due to the addition of the herbicides and adjuvants, as opposed to any differences seen due to the plants suffering from other abiotic factors, such as; being kept in the dark for extended periods of time, or being in a small amount of media, leading to decreased nutrients towards the end of the assay, as well biotic factors such as differences in stock plant health before the assay had begun. The DMSO controls showed F_v/F_m values of ~0.78 at T0, and ~0.72 after 24 hours.

After each image time point, the images were analysed and edited in the Fluorimager software, with subsequent numerical data being exported into Microsoft Excel and R studio for further analysis and statistical tests. All statistical analyses, namely analyses of variance (ANOVA) and post-hoc Tukey tests, were performed using the open-source package R (R Core Team, Vienna Austria 2019, version 3.6.3 for Windows), utilising R Studio (version 1.1.463).

2.3 Results

Initial screening of two replicates of 110 herbicide mode of action standards was carried out, and these data used to select 36 herbicides which showed 'interesting' results when utilising one or both of the adjuvants for further replicates. The herbicides selected as 'interesting' had either a large or unexpected effect, or were of potentially commercial relevance to Syngenta, and of these 36 herbicides, the herbicides which showed a difference of 0.25 normalised F_v/F_m or greater between treatments were then selected – 12 of the tested herbicides showed differences this great.

2.3.1 Effect of herbicides on photosystem II

After narrowing down from the initial 110 herbicides to the 36 most relevant, with further replicates performed, some of the herbicides were observed to affect the maximum photosynthetic efficiency of PSII in *L. minor*, whereas other herbicides were not seen to affect F_v/F_m any greater than the negative controls of DMSO (Fig 2.3a). Those that had differing modes of action were harder to be detected using CFI, especially within the constraints of the assays time frame. The results below show that chlorophyll fluorescence imaging is an appropriate tool for this work. An analysis of variance (ANOVA) showed that there was a significant difference between the effects of the herbicides on F_v/F_m, which can be attributed to some of the herbicides having MOAs that target PSII, and therefore directly affect the CF emissions of the plant (p<0.001).

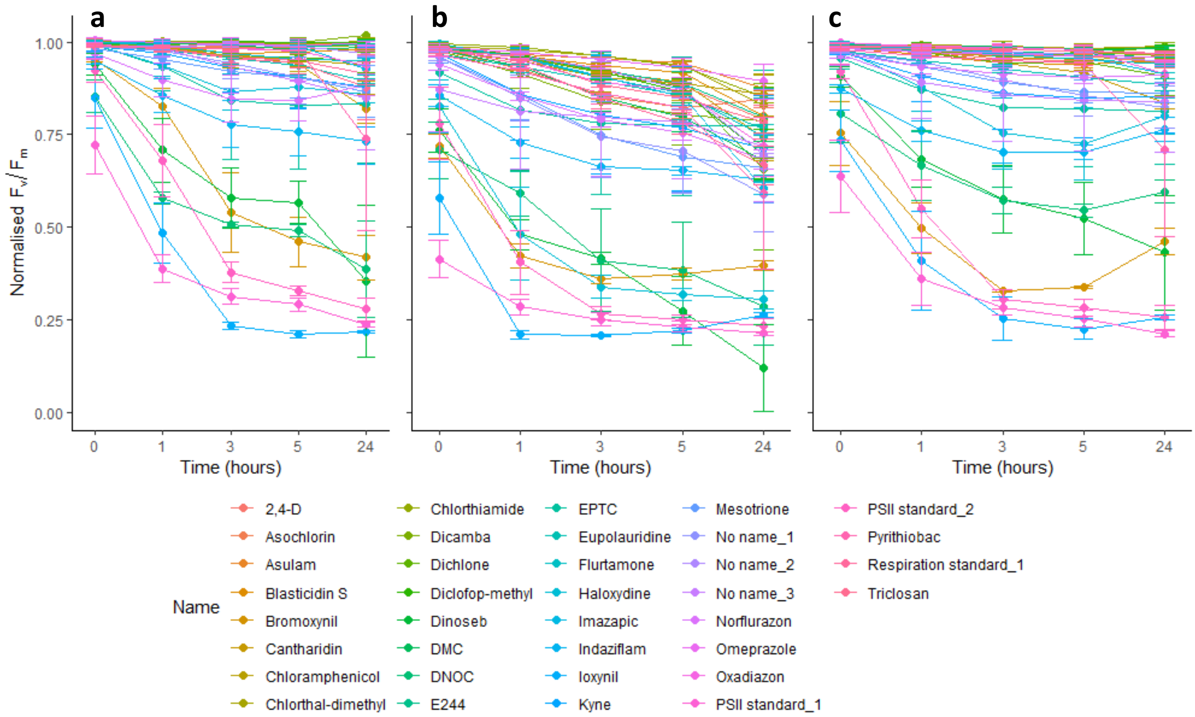


Figure 2.3. Effect of 36 herbicides on the maximum efficiency of PSII (normalised against negative control DMSO) over a time course of 24 hours. (**a**) Treatment utilising just the herbicide, (**b**) shows the treatment of the herbicide with the addition of SLES, and (**c**) shows results when Tween 20 was included in the herbicidal

mixture. Data shows the mean F_v/F_m values recorded, with error bars representing ± standard error (n=4).

2.3.2 Effect of adjuvant treatment on efficacy of herbicides

The figure below (Figure 2.4) shows results for 12 herbicides selected from above that had a difference of 0.25 or greater in the normalised F_v/F_m value when the herbicide was used with and without an adjuvant. A tabular summary of these results can be found in the appendix (Table 6.1).

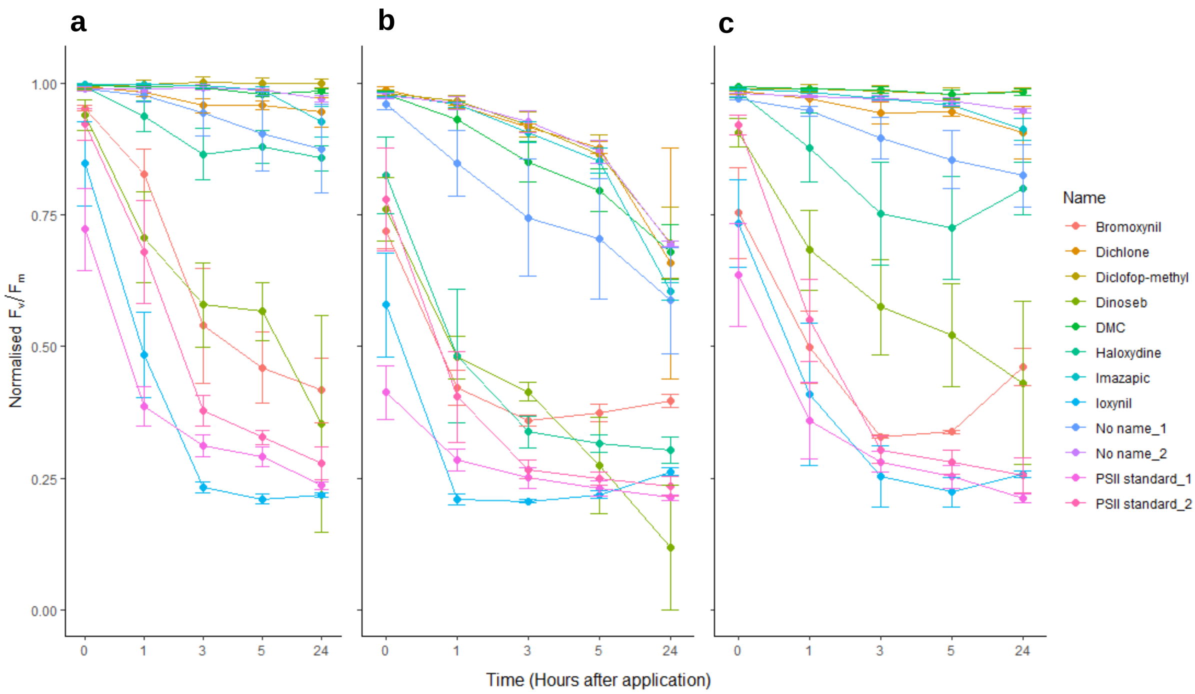


Figure 2.4. The effects of the 12 herbicides, most affected by the addition of an adjuvant, on the maximum photosynthetic efficiency of PSII over a time course of 24 hours, normalised against negative control values. The results are split into three graphs separating the treatments out, with (**a**) containing the herbicide treatment, (**b**) with the addition of SLES, and (**c**) with the inclusion of Tween 20. Data shows the mean F_v/F_m values recorded, with error bars representing ± standard error (n=4).

The treatments which included either of the adjuvants proved to have larger effects on the maximum photosynthetic capabilities of the plant (i.e. greater decreases in F_v/F_m) when compared to just the herbicide on its own (Fig 2.4) ($p<0.001$). The adjuvant SLES had the largest effect on herbicide efficiency, showing the greatest decrease in F_v/F_m (Fig 2.4b). The addition of the adjuvant Tween 20 increases the efficacies of herbicides in some cases, however overall these differences were not enough to be significant when averaged across the 12 herbicides (Fig 2.4c).

2.3.3 SLES vs Tween 20

There was a significant difference between the effects of Tween 20 and SLES ($p<0.001$), with the latter proving to have been a much more effective adjuvant, increasing herbicidal activity and decreasing F_v/F_m, specifically after 24 hours (Fig 2.5).

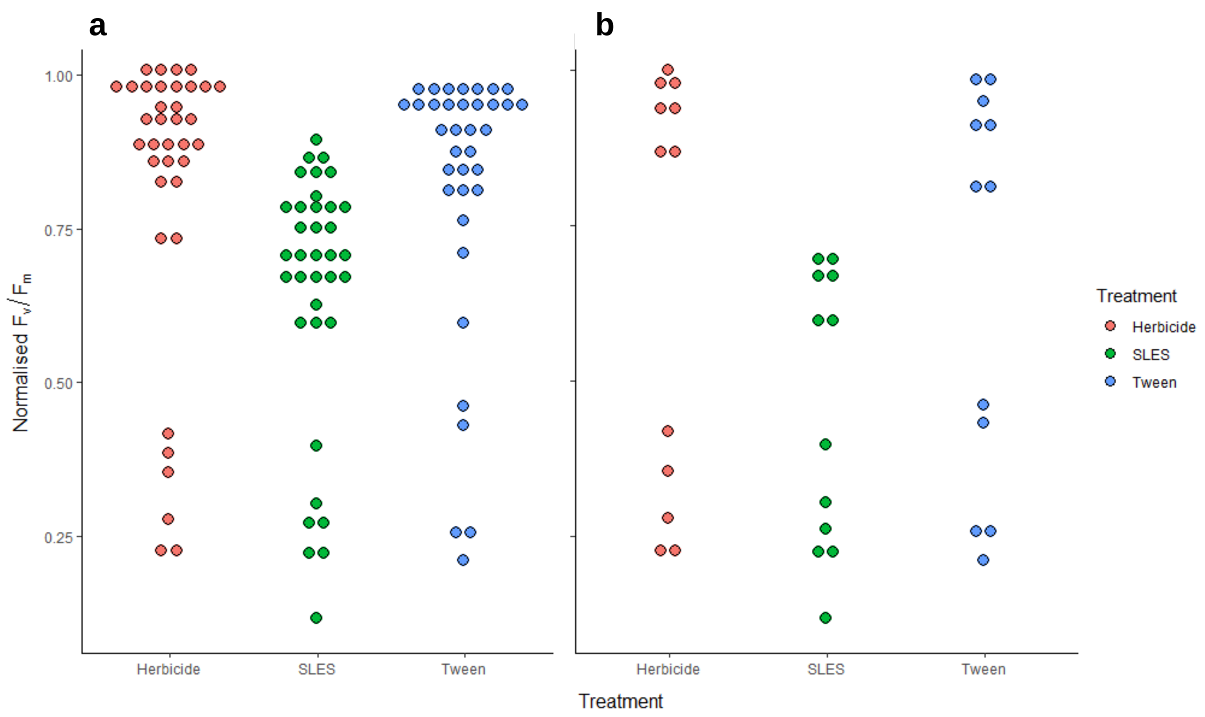


Figure 2.5. Differences in the effect of the two adjuvants SLES and Tween 20 in altering the efficacy of herbicides targeting PSII, seen after 24 hours. (**a**) shows the 36 herbicides selected after the initial 110, and (**b**) shows the 12 herbicides of the 36 that were most affected by the addition of an adjuvant. Most of the herbicides greatly affected by the addition of an adjuvant were affected by the addition of SLES, rather than Tween 20.

2.3.4 Change in efficacy of specific herbicides

Bromoxynil. Across the entire time series, there was a significant difference between the effects of the treatments ($p<0.001$), with the two adjuvant treatments showing lower normalised F_v/F_m than the herbicide treatment alone (around 0.75 compared to 1), and with immediate effect (SLES-H $p<0.001$; Tween-H $p<0.001$). However, the herbicide treatment does reach the same levels of effect after 24-hours, reducing the recorded F_v/F_m values to similar levels to the adjuvant treatments - the adjuvants increased the speed of effect of Bromoxynil, rather than increasing the magnitude of effect.

Dichlone. The application of Dichlone showed no significant differences over the time course in F_v/F_m , with the data values not being significantly different from the normalised negative controls of DMSO ($p=0.054$). However, the data for SLES, 24 h after application, clearly shows a large error bar, and so these results and the effect of Dichlone with the addition of adjuvants on F_v/F_m should be investigated further.

Diclofop-methyl. As with Dichlone, Diclofop-methyl alone and with Tween 20 shows little effect on F_v/F_m across the majority of the time series. However, there was a significant difference between the effects of the treatments on the normalised F_v/F_m of the *Lemna*, due to the effect observed after 24 h with the herbicide plus SLES (ANOVA $p<0.001$; Tukey SLES-H $p<0.001$; SLES-Tween $p<0.001$). With the addition of SLES, Diclofop-methyl reduced F_v/F_m to ~0.7 normalised F_v/F_m, whereas the herbicide alone and with Tween 20 remained around normalised F_v/F_m of 1.

Dinoseb. Dinoseb showed a steady decrease in the F_v/F_m across all treatments, with the SLES treatment consistently lower than both the Dinoseb on its own, and Dinoseb with the addition of Tween 20 (ANOVA p<0.001; Tukey SLES-H p<0.01; SLES-Tween p<0.001). No differences were observed in F_v/F_m between the Dinoseb on its own and with the addition of the adjuvant Tween 20 (p>0.05) (Fig 2.6d).

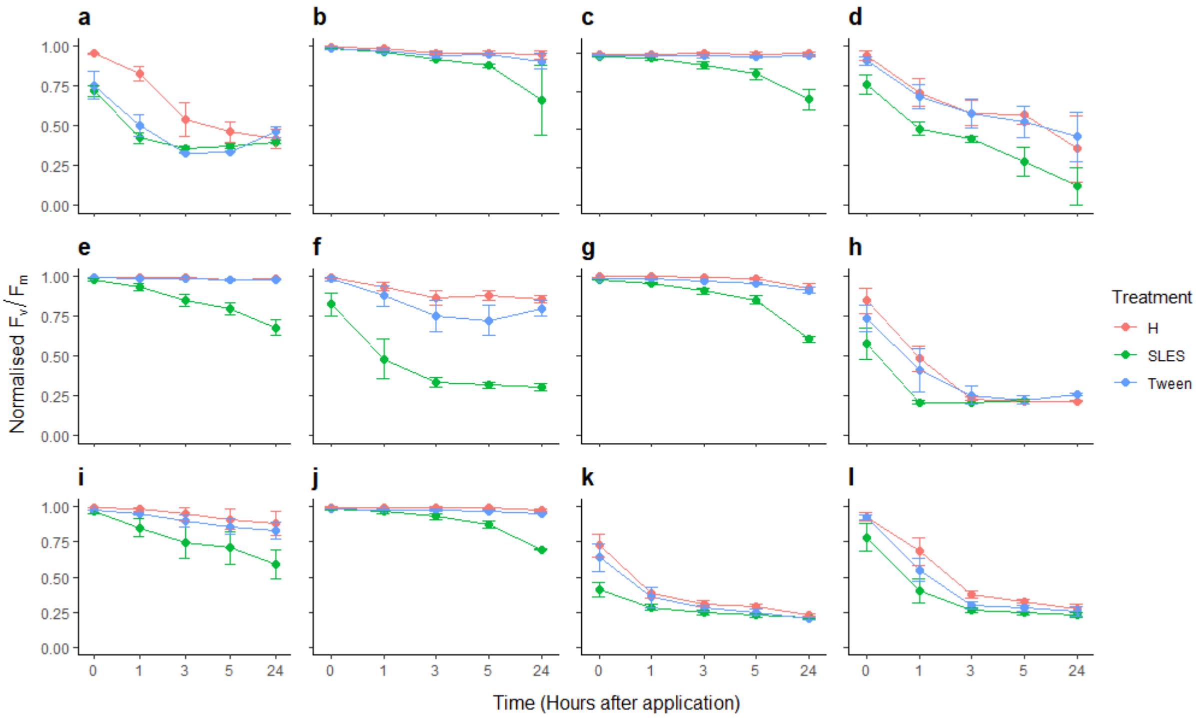


Figure 2.6. Effects of 12 selected herbicides on the maximum efficiency of PSII (F_v/F_m) of *Lemna minor*. The herbicides displayed are as follows: (**a**) Bromoxynil, (**b**) Dichlone, (**c**) Diclofop-methyl, (**d**) Dinoseb, (**e**) DMC, (**f**) Haloxydine, (**g**) Imazapic, (**h**) Ioxynil, (**i**) No name 1, (**j**) No name 2, (**k**) PSII standard 1, and (**l**) PSII standard 2. Graphs show three treatments: the herbicide on its own, with the addition of SLES, and with the addition of Tween 20. F_v/F_m values are normalised against the negative controls of the respective 24-well plates. Error bars represent ± standard error (n=4).

DMC. After 24 h exposure to DMC, F_v/F_m in *Lemna* plants was not significantly different to the negative control plants. However, with the addition of SLES to the herbicidal mixture, the normalised F_v/F_m of the plants decreased constantly over the 24 h time period. There was a significant difference between the SLES treatment and the other treatments over the time frame of 24 h ($p<0.001$). There was no significant difference between the treatment with the addition of Tween 20 and the herbicidal treatment alone ($p>0.05$).

Haloxydine. The efficacy of the herbicide Haloxydine was greatly affected by the addition of the adjuvant SLES ($p<0.001$), with F_v/F_m values much lower than the Tween and Herbicide treatments (SLES-H $p<0.001$; SLES-Tween $p<0.01$). However, Tween 20 did not prove to lower the F_v/F_m significantly more than using Haloxydine without an adjuvant ($p>0.05$).

Imazapic. No meaningful effects on F_v/F_m were observed with the addition of Imazapic, as well as Imazapic with Tween 20, when compared to the negative control – however with the introduction of SLES to the mixture, a significant effect was seen ($p<0.001$), with a decreasing in *Lemna* F_v/F_m by approximately 40% between 3 and 24 hours (SLES-H $p<0.001$; SLES-Tween 20 $p<0.001$).

Ioxynil. For all three treatments, a large decrease of PSII maximum efficiency was observed. Values decreased to ~0.25 normalised F_v/F_m (which equates to approximately 0.18 true F_v/F_m) within 1 hour post treatment application. This large effect was seen across all treatments and so no significant differences were observed between the treatments ($p>0.05$).

No Name 1. The herbicide No Name 1 showed a steady, although minor, decrease in the maximum photosynthetic efficiency across all treatments, with the

SLES treatment being consistently lower than the other two (SLES-H $p<0.001$; SLES-Tween 20 $p<0.01$). Both the herbicide alone and herbicide with Tween 20 treatments can be seen to have a noticeable effect on F_v/F_m after 24 h, and so it can be hypothesised that over a longer time series there would be a much larger effect on the F_v/F_m of the plants than is seen in the 24 h time frame. The Tween 20 and herbicide alone treatments could eventually affect the plants with the same magnitude as the SLES treatment.

No Name 2. A significant difference ($p<0.001$) was observed between treatments over the entire time course, with most of the differentiation occurring after 5 and 24 hours between the SLES treatment, the herbicide treatment alone, and the herbicide plus Tween 20 treatment (SLES-H $p<0.001$, Tween-SLES $p<0.001$).

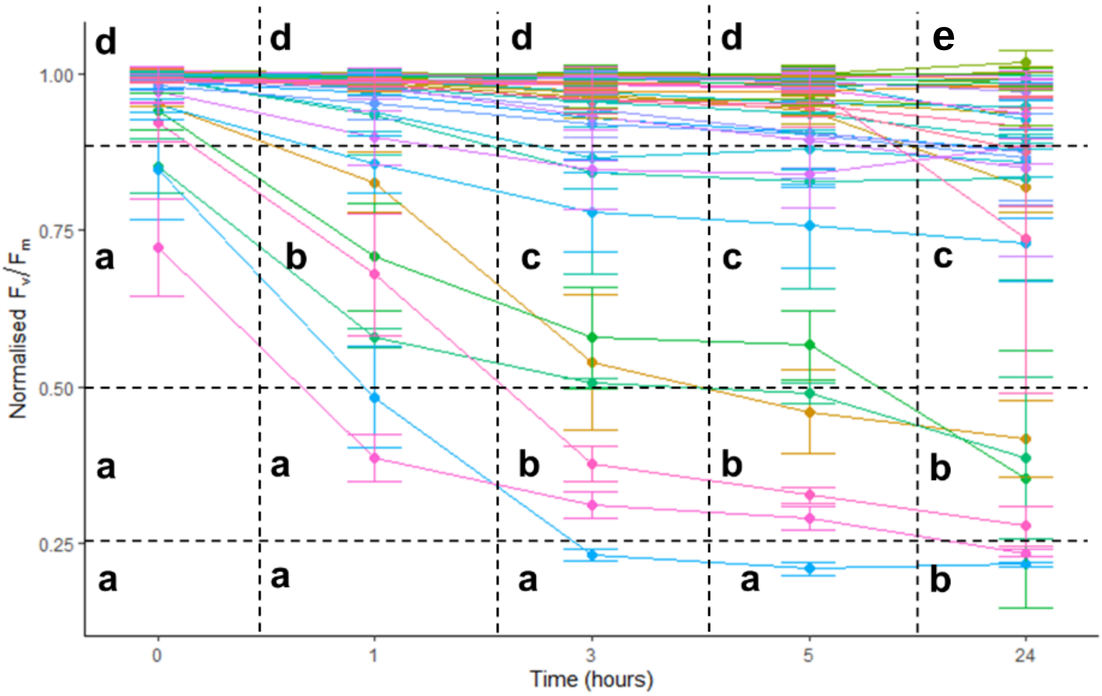
PSII Standard 1 & 2. As expected given the names, both PSII standard 1 and 2 had a large effect on the maximum efficiency of PSII over the 24-hour time frame, with effects being seen immediately (T0) compared to the negative controls. PSII standard 1 showed a significant difference in efficacy with the addition of adjuvants, with SLES treatment resulting in lower F_v/F_m than the herbicide alone, and the herbicide with the addition of Tween 20 ($p<0.01$). 20 mins post application, PSII standard I with SLES resulted in normalised F_v/F_m values of ~0.4 (~0.31 true F_v/F_m), with the herbicide alone and with the addition of Tween 20 resulted in normalised F_v/F_m values of ~0.75 (~0.585 true F_v/F_m). However, after three hours there was no discernible difference between the treatments, with all reducing F_v/F_m to minimal levels (~0.1 true F_v/F_m). A similar story was observed for PSII standard 2. A significant difference between the treatments was observed ($p<0.05$), specifically when comparing PSII standard 2 with SLES to the herbicide on its own or with the addition of Tween 20 (SLES-H $p<0.05$). However, the effect of PSII standard 2 plus SLES took a slightly longer amount of time to reach a similar level of effect when compared to PSII standard 1 plus SLES, with PSII standard 2 plus SLES decreasing true F_v/F_m to ~0.3 after 1 hour, whereas PSII standard 1 only took 20 mins to have the same effect.

2.3.5 Effect of time on damage to photosystems

As might be expected, there is a significant difference ($p<0.001$) between the amount of damage a herbicide has on the maximum photosynthetic efficiency (F_v/F_m) immediately after being applied compared to an extended period of time (3, 5, or 24 hours) (Fig 2.3a) (T0-T3 $p<0.001$; T0-T5 $p<0.001$; T0-T24 $p<0.001$).

2.3.6 PSII MOA prediction

Analysing the data as outlined in Figure 2.7, could be used to determine whether a herbicide has a mode of action affecting PSII, by assessing the decrease in the maximum efficiency of PSII, in relation to the amount of time passed since application. The boundaries in Figure 2.7 are arbitrary, given the limited data set and amount of replicates in this study, however with further work, a similar approach could be developed that would be more robust, and used as an early screen for modes of action. This approach could be developed further with the inclusion of light adapted CFI data.



Annotation	Likelihood of PSII MOA
a	Highly Likely
b	Likely
c	Possibly
d	PSII can't be ruled out
e	Unlikely

Figure 2.7. Effect of 36 herbicides on the maximum efficiency of photosystem II of *Lemna minor*, normalised against negative controls. Gridlines and letter notations overlayed indicate arbitrary likelihood that the herbicide has a mode of action affecting photosystem II, with a table legend explaining the annotations. Error bars represent ± standard error (n=4).

2.4 Discussion

The aim of this experiment was to assess the impact of herbicidal application to *Lemna minor* on the maximum photosynthetic efficiency of PSII (F_v/F_m) using chlorophyll fluorescence imaging. Significant differences between treatments (herbicide application alone, and with the addition of either SLES or Tween 20) were found, with these differences becoming more apparent after 24 hours, although some treatments proved to take effect almost immediately (after 20 minutes dark adaption, T0).

It can be seen from the results, due to the observed decreases in F_v/F_m, that some herbicides affect the maximum photosynthetic efficiency of a plant, by targeting the photosystems (specifically PSII), and that they can be detected utilising chlorophyll fluorescence imaging, confirming the hypothesis of this work (Juneau *et al.*, 2007; Park *et al.*, 2016). However, many did not, and therefore their effect cannot easily and definitively be detected using dark adapted data from chlorophyll fluorescence imaging over the time period used in this study. Herbicides whose mode of action do not directly target PSII can potentially be detected using CFI of F_v/F_m, however a longer time frame would need to be utilised, as the effects of these herbicides takes longer to have an effect on the photosystems than the 24 h time period used here. The number of herbicides that resulted in a decrease in the maximum efficiency of photosynthesis when an adjuvant was added increased in this study. The adjuvant SLES was found to be the more effective of the two adjuvants tested, as it increased the efficacy and/or the rate of effect of more herbicides than Tween 20, and to a greater degree. The herbicides which greatly affected the F_v/F_m (decreases of >0.5 normalised, to true values of less than ~0.4 F_v/F_m after 24 hours, Fig. 2.7) of *L. minor* in the given assay can be deduced to have a mode of action affecting photosystem II. Additionally, as the assay was performed using dark adapted data, herbicides directly affecting photosystem I would not initially have an effect on PSII and therefore CF emissions (Juneau *et al.*, 2007). Herbicides with an MOA targeting PSI would possibly take longer for an effect on the efficiency of PSII to be observed, which was not detectable in the 24 h time frame of this study. This methodology helps to ensure that those herbicides that did have a very large observed effect on F_v/F_m were specifically targeting PSII, as opposed to PSI. It can be deduced that some herbicide's affect PSII as a secondary or tertiary mode of action, and not as their primary mode of action, due to detectable changes in the CF emissions, for example, Imazapic (Fig 2.6g). Imazapic has a primary mode of action affecting the amino acid biosynthesis of the plant, however with the addition of the adjuvant SLES, Imazapic can be seen to have a large effect on the F_v/F_m, and so it could be determined that Imazapic also effects PSII as a secondary MOA (Sebastian *et al.*, 2016). Further evidence that employing CFI is a useful technique for this research comes from the low false negative rate of this assay, which shows that all known PSII herbicides from the standards list were easily detectable.

Preliminary testing showed that using the adjuvant SLES at a concentration of 0.2% had no phytotoxic effect on the plant, however the results shown here indicate that many of the herbicides had improved efficacy after 24-hours when the adjuvant was included (shown by a decrease in F_v/F_m). Future work should test the potential phytotoxic effects of the two adjuvants, across more samples, and at a variation of concentrations. Additional replicates could be performed of either the entire preliminary list of herbicides (110), or of the reduced list (36 herbicides), to minimise assay length.

The 12 herbicides which were most affected by the addition of an adjuvant were analysed separately. These herbicides highlighted the efficacy of the adjuvant SLES, with Tween 20 being seemingly less effective. The difference in timing for observing the effects can be noted down to differences in herbicidal modes of action (Juneau *et al.*, 2007). Without the addition of any adjuvants, the five herbicides Bromoxynil, Dinoseb, Haloxydine, PSII standard 1, and PSII standard 2, caused a large decrease in F_v/F_m after 24 hours (from approximately 0.78 true F_v/F_m to ~0.55-0.25 true F_v/F_m). However, with the addition of an adjuvant, mainly SLES, the effect of the herbicide on F_v/F_m was seen much quicker, after 0-3 hours rather than 24. Three of these five herbicides (Bromoxynil, PSII standard 1, and PSII standard 2) had a mode of action specifically targeting PSII, one, Dinoseb, acts as an uncoupler of electron transport for ATP synthesis, and the herbicide Haloxydine has a MOA inhibiting homogentisate solanesyltransferase (HRAC, 2020a; Qu *et al.*, 2021). These data suggest that Haloxydine could have a secondary mode of action targeting photosynthetic electron transport, specifically at photosystem II.

The data presented here demonstrate that CFI is an appropriate tool for higher throughput assessment of herbicidal mode of action. The methods here could also be modified for higher throughput, utilising 96 well plates or higher, using either *Lemna minor*, algal cultures, or even chloroplasts (Barbagallo *et al.*, 2003). If CFI was more widely adopted for higher throughput herbicidal assessment, it may help with the identification of new MOAs that are commercially relevant, with there being only a single new MOA being introduced in the past 40 years (Dayan, 2019; Sukhoverkov *et al.*, 2021). The necessity of high throughput screening has become increasingly evident in the agrochemical industry, due to being a method of improving the translation of novel products from concepts to being commercially viable (Ridley *et al.*, 1998). Combining the practicalities of both chlorophyll fluorescence imaging and using *Lemna minor* as a model test species is an effective higher throughput screening approach for the initial screening task of determining early-stage development ideas about the MOAs of various herbicides – whether it be affecting PSII (directly or indirectly) or otherwise.

This research has provided useful data for the assessment of screening herbicides for the mode of action, and the effect that two common adjuvants have on efficacy, utilising the technique of chlorophyll fluorescence imaging. The results of this assay highlight two ways in which the adjuvants affect the efficacy of PSII herbicides; those that increase the rate of effect, and those that increase the magnitude of effect. Future work into the understanding of herbicidal modes of action, as well as the development of novel herbicides, should use chlorophyll fluorescence imaging as a key component in the process, as it gives accurate and repeatable results, in a rapid and inexpensive manner. This assay has proved that CFI is a promising and valuable tool for this type of research, and that it is a valid method that could be used more extensively in the understanding of existing, and development of novel, herbicides.

3. Study 2 – Determination of Herbicidal and Adjuvant Efficacies and Phytotoxicities on Relevant Terrestrial Species

3.1 Introduction

3.1.1 Background

Weeds are one of the most important yield-determining factors in crops and can cause significant yield losses (Asaduzzaman *et al.*, 2020). This is especially prevalent and important in arable crops, such as Rapeseed (*Brassica napus*), given that it is the second most important source of vegetable oil worldwide, with approximate revenue losses of $54 million in Australia alone (Brennan and Bolland, 2007; Llewellyn *et al.*, 2016). The significant yield loss from weeds is due to the resulting competition for resources such as light, water, nutrients, as well as space in the field – the more space that is taken up by invasive species, the less space there is for the desired crop. For example, wild radish is a prevalent annual weed, and as little as 4 wild radish plants per square metre reduced *Brassica napus* yield by as much as 11%, with 64 wild radish plants per square metre reducing *Brassica napus* yield by up to 91% (Blackshaw *et al.*, 2002). The oil content contained in the *Brassica* seeds is additionally negatively impacted by the competing weeds, causing limited resources (McMullan *et al.*, 1994).

Herbicides are the main tool for weed management in the oil crop *Brassica napus*, which is more commonly known as canola or rapeseed (Harker and O'Donovan 2013). Initially, growers depended heavily on pre-plant-incorporated herbicides, but now there is much greater reliance on post-emergence herbicides for weed control. Pre-plant (or pre-emergent) herbicides are active in the soil, and kill the weed seed when it is germinating, however post-plant (or post-emergent) herbicides are applied after the plant has emerged from the soil and kills it at this point (Krähmer *et al.*, 2021). Pre-emergence herbicides are mainly taken up by the roots, whereas post-emergent herbicides are mainly taken up via leaves and stems. This trend has enabled the widespread adoption of reduced tillage, especially with the advent of herbicide-tolerant *Brassica napus* cultivars, including genetically modified (GM) *Brassica napus*, such as the events T45 ("Liberty Link canola"), MS8, RF3, ATR-409, and GT73*(COGEM, 2019; Kumar et al., 1998; Lemerle et al., 2011)*. The availability of the associated broad-spectrum action herbicides has aided the control of problematic weeds such as wild radish, shepherd's purse (*Capsella bursa-pastoris*) and wild garlic (*Allium vineale*), which all act as invasive species, and impact the yields of *Brassica napus* harvests(Grey *et al.*, 2006). Consequently, current rapeseed rotation and seeding techniques are highly dependent on herbicides, but the repetitive use of any one herbicide on large populations of genetically variable weed species selects for resistant weed biotypes (Heap 2018).

3.1.2 Herbicidal modes of action

A specific group of herbicides were chosen for this assay. They were chosen as to cover a wide range of known and common modes of action, from each major MOA class. All active ingredients selected were post-emergent and foliar applied, ands interact directly with the leaf. An additional interest was given to minimizing where possible the hazard and contamination risks potentially posed by the active ingredients, with the safest active ingredients being selected. Further information about the mechanistic properties and targeting sites of the herbicidal modes of action will be detailed below.

Table 3.1. The various herbicides used in the present research, and their respective modes of action and partition coefficients (PPDB, 2023).

Mode of Action	**Herbicide**	**logP**
Photosystem I	Paraquat	-4.50
Photosystem II	Bromoxynil	0.27
Amino Acid Biosynthesis	Glyphosate	-6.28
Fatty Acid Biosynthesis	Diclofop-methyl	4.80
NH_4 Assimilation/Photorespiration	Glufosinate	-3.96
Plastoquinone Biosynthesis	Mesotrione	0.11

Photosystem I & II. More herbicide classes have inhibitory effects on photosynthetic activity than any other physiological process (Duke, 1990). This experiment utilised the photosystem II inhibitor Bromoxynil, and the photosystem I inhibitor Paraquat.

Amino acid biosynthesis. Herbicides which act as amino acid biosynthesis inhibitors typically have a broad-spectrum of activity caused by the high levels of conservation of the amino acid biosynthesis pathways in plants (Hall *et al.*, 2020). Glyphosate is one such amino acid biosynthesis inhibitor, and competitively inhibits the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), the penultimate enzyme in the shikimate pathway (Duke, 1990). The shikimate pathway links the metabolism of carbohydrates to the biosynthesis of folates and aromatic amino acids via seven steps, with over 30% of all carbon fixed by plants traveling through this pathway (Herrmann and Weaver, 1999; Shaner, 2006). When glyphosate inhibits EPSP synthase, the pathway is deregulated, and an accumulation of shikimate and shikimate-3-phosphate occurs, having toxic effects, as well as deregulating carbon metabolism (de María *et al.*, 2006). Additionally, the reduced synthesis of aromatic amino acids leads to limited protein, cell wall, and secondary plant product synthesis (Velini *et al.*, 2009).

Lipid (fatty acid) biosynthesis. Diclofop-methyl targets the plasma membrane and acetyl-CoA carboxylase (Shimabukuro and Hoffer, 1994). Acetyl-CoA catalyses the production of malonyl-CoA, which is the first intermediate in fatty acid biosynthesis, and inhibiting the enzyme acetyl-CoA stops this synthesis (Konishi and Sasaki, 1994). Although herbicides affecting acetyl-CoA do not directly affect the photosystems, the photosynthetic health of the plant is affected due to the creation of reactive oxygen species (Kaiser *et al.*, 2013). The production of ROS causes multiple damaging reactions, which interrupt the electron transport from PSII to PSI, which consequently affects the chlorophyll fluorescence emissions. Due to this, herbicides which have a mode of action affecting fatty acid biosynthesis should be detectable via chlorophyll fluorescence imaging systems, although possibly not as rapidly as those herbicides which directly affect the photosystems.

NH_4 assimilation/photorespiration. The herbicidal effect of Glufosinate ammonium can be attributed to the accumulation of NH_4^+, caused by the inhibition of glutamine synthetase (Donthi and Kumar, 2022; Loux *et al.*, 2019b; Takano *et al.*, 2020; Takano and Dayan, 2020). The build-up of this phytotoxic ammonia disrupts cell membranes and inhibits photosynthesis, causing eventual plant death (PPDB, 2023).

Plastoquinone biosynthesis. Herbicides which inhibit plastoquinone, inhibit the processes of carotenoid biosynthesis as well as photosynthetic electron transport, and cause bleaching phytotoxicity due to a lack of photoprotection (see Figure 3.1) (Wakabayashi and Böger, 2002). As a plastoquinone biosynthesis inhibitor, Mesotrione interferes with the synthesis of homogentisate, which inhibits the enzyme p-hydroxyphenylpyruvate dioxygenase (HPPD) (Mitchell *et al.*, 2001). HPPD catalyses the synthesis of homogentisic acid, which is an antecedent of plastoquinone and vitamin E (Matringe *et al.*, 2005).

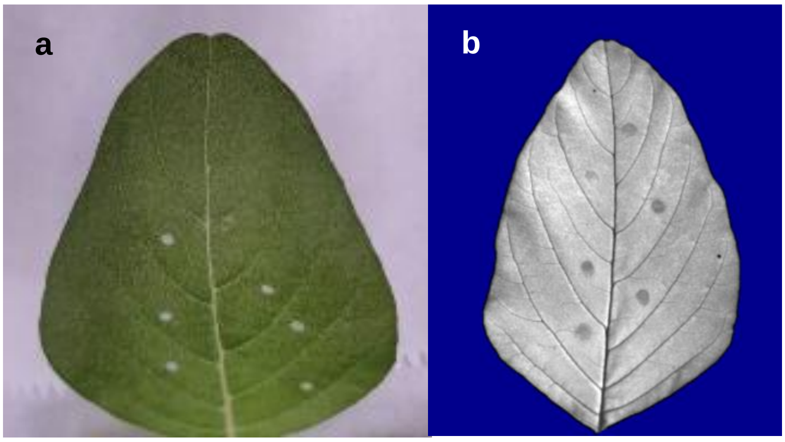


Figure 3.1. Bleaching effects caused by the plastoquinone biosynthesis inhibitor Mesotrione, as seen on a leaf of *Amaranthus retroflexus*, 3 hours after the application of the treatment. (**a**) Shows a photograph of the *Amaranthus* leaf, and (**b**) shows a CF image of the leaf.

3.1.3 Adjuvants

The aim of the usage of adjuvants is to reduce the amounts of active ingredients needed in a herbicidal mixture to attain similar results, by increasing the efficacy of the herbicidal mixtures. This improvement can be achieved by a variety of means, such as increasing the surface area of leaf covered in herbicidal mixture by decreasing the surface tension of the mixture, or by improving the viscosity/adherence of the mixture to the leaf surface (Hazen, 2000; Zhang *et al.*, 2022). Specifically, surfactants aim to reduce the surface tension of the herbicidal mixture so it can cover a larger surface area of the leaf. However, when applied in the field, more than one adjuvant mechanism may be used and affect the biological performance of the herbicide. Both of the adjuvants used in this study were surfactants, and cover a wide range of active ingredients. Tween 20 is more versatile and acts on a large breadth of active ingredient lipophilicities, whilst SLES tends to provide more effective results with active ingredients with medium to low logP's.

3.1.4 Selected species

Plants of *Amaranthus retroflexus*, *Brassica napus*, and *Echinochloa crus-galli* were used for this experiment, due to them common weed species or important crops used in herbicidal screening assays, but they also cover a range of leaf morphologies – broad leaf retentive (*Amaranthus*), broad leaf non-retentive (*Brassica*), and grasses (*Echinochloa*).

Amaranthus retroflexus, more commonly known as red root pigweed, is a crop commonly used in a culinary sense across many parts of the globe, as well as livestock fodder when used in moderation, due to its high nutritional value but also toxicity (Lucian *et al.*, 2018; Weston *et al.*, 2019). Despite its positive uses, *Amaranthus retroflexus* is also a serious weed, affecting the production of over 60 crops, but more seriously having a major effect on the yields of maize, with yield losses can reach almost 90% when *Amaranthus* is present in a great enough density (Brankov *et al.*, 2022; Costea *et al.*, 2004; Dogaru *et al.*, 2012). These decreases in yield can partially be specifically attributed to allelopathy, which is the process of plants releasing harmful chemicals into their environment to inhibit the growth of other plants in the same area (Konstantinović *et al.*, 2014; Rice, 1979).

Brassica napus is a major oilseed crop with it being the second most important source of vegetable oil worldwide, with an estimated 80 million tons being produced in the current harvest season (2022-2023) (Brennan and Bolland, 2007; USDA, 2022). The species initially arose due to the natural hybridisation of *Brassica oleracea* and

Brassica rapa. Aside from its uses as a food crop, *Brassica napus* is also an exceptionally important medicinal plant, prized for its uses in traditional therapies, as well as also being used as a biodiesel and lubricant (Balambica *et al.*, 2022; Friedt *et al.*, 2018; Nath *et al.*, 2016; Soodabeh and Ahmad, 2012).

Echinochloa crus-galli, also known commonly as barnyard grass, is designated as one of the most noxious and serious weeds worldwide, causing substantial yield loss in a number of field crops, including rice and corn, due to it intensely competing for all available Nitrogen in the soil, and removing up to 80% (Bajwa *et al.*, 2015; Chauhan and Johnson, 2011; Holm *et al.*, 1991; Randall, 2012). The effects of *Echninochloa crus-galli* are felt especially so by rice farmers, as at younger stages of its growth the weed looks very similar to the rice crop, and so by the time the farmer can differentiate between them, yield losses may be inevitable. *E. crus-galli* is however sometimes cultivated, due to its uses as cattle-fodder and silage (Heuzé *et al.*, 2020).

3.1.5 Aims

The aim of this study was to assess the efficacies of the six aforementioned herbicides, when used on their own as well as with the addition of the adjuvants SLES or Tween 20, on the terrestrial species of *Amaranthus retroflexus*, *Brassica napus*, and *Echinochloa crus-galli*. The efficacies of the herbicides will be determined using chlorophyll fluorescence imaging, which in turn will be assessed on whether it is a suitable tool for work of this nature. The work within this section is to assess the findings of the initial assay of this project which utilised the aquatic species *Lemna minor*, which determined that CFI was a suitable method for herbicidal MOA screening, whilst building upon the methodologies and adapting them to the different species and task of rating adjuvants.

3.2. Materials and Methods

3.2.1 Plant material and growth conditions

Amaranthus retroflexus (AMARE), *Brassica napus* (BRSNN), and *Echinochloa crus-galli* (ECHCG) were grown in 80 mm pots, in a 50:50 potting mixture of John Innes No 3 compost and coir, with a pH of 6.1. The plants were germinated in the Syngenta growth facilities (glass house, 24^{o}C for AMARE and ECHCG, 20^{o}C for BRSNN, sunlight (~300µmol m^{-2} s^{-1})). When received at the university, the plants were further grown in controlled environments maintained at 23^{o}C and 50% humidity, grown under a light intensity of 300µmol m^{-2} s^{-1}, provided by heliospectra lights with a standard white spectrum, on a 16hr day/8hr night cycle. Plants were kept in well-watered conditions, with their positions under the lights being rotated every day, to ensure an even heterogeneity.

Plants older than 2 weeks since arrival to the university, or dicots past the 4 true leaf stage, were not used as to keep the age of the plants and leaf size somewhat consistent.

3.2.2 Concentrations and application of treatments

In this experiment, three treatments were applied to the plants; 1) the herbicide on its own; 2) the herbicide with the addition of the adjuvant Tween 20; 3) the herbicide along with the adjuvant SLES. In the treatments utilising adjuvants, the herbicide was at the same concentration as the control condition (with no adjuvants). The herbicidal and adjuvant concentrations utilised in this research were both 0.2% by weight, which is equivalent to 400g/ha, for an application rate of 200L/ha. The solutions were made up in 80% by weight Acetonitrile (ACN) in water to provide homogeneous solutions of all the herbicides used, and also to normalise the data for wetting/spreading discrepancies between adjuvants. These concentrations were used as typical of practical application of farmers in the field. The herbicidal mixtures were applied to the leaf surfaces via a micro-pipette, which allowed for droplets of 0.5ul to be applied. Six 0.5ul droplets were applied to the treated leaf. Additionally, the adjuvant concentration was further refined to a point where it was high enough so that differences in treatments could potentially be resolved, however not to too high a concentration to cause any phytotoxic damage itself to the treated leaves. To determine this, precursory tests were performed using adjuvant concentrations of 0.2%, 0.1%, and 0.05% in 80% ACN, and then applied onto the leaf in the same manner that they would be in the experiment. The leaves were then imaged with the same protocol on the CF imager to determine if any damage could be detected. No phytotoxic damage was detected using these concentrations.

Additionally, as the entire leaf of the *Echinochloa* plants could not fit into the imaging area, these were sectioned into a 3-cm section using a marker pen. All treatments would be applied inside this 3-cm section, and the resulting images were edited down as to only include this section of leaf. For *Amaranthus* and *Brassica* plants, the entire leaf was kept, with stems and other peripheral sections of the plant visible being removed.

3.2.3 Chlorophyll fluorescence imaging

Immediately after the treatments had been applied to the plants, the plants were dark adapted for 20 min. To ensure a mostly even dark adaption between samples, the application of treatments to the plants was staggered, by the same length of time that the protocol takes to run, placing plants in and taking out of imager, and saving the file. After the plants had been dark adapted for the suitable amount of time, they were placed inside the CF Imager, and had a piece of non-reflective glass placed over the top, as to ensure that all leaves being imaged were at the same height. The plants then underwent the imaging protocol, which proceeded as follows: 800ms pulse of 6401µmol m^{-2} s^{-1} light to obtain dark adapted values, followed by 3 minutes of exposure to 300µmol m^{-2} s^{-1}actinic light, which was again followed by an 800ms pulse of 6401µmol m^{-2} s^{-1}, this time to obtain the light adapted values. After the protocol had finished, the plant was removed from the imaging system and placed back into the growth facilities, until it was time for the subsequent round of imaging. Samples/plants were imaged at time points of (all time points are given in hours after treatment was applied); 0, 1, 3, 5, and 24.

After the 24-hour imaging protocol had finished, the plants, soil, and pots were disposed of in the appropriate manner, as outlined by the departments risk assessments.

Four replicates were performed of each sample, with there being 54 individual possibilities of combinations, each one a separate sample, imaged across five different time points. This led to the generation of over 1,080 separate chlorophyll fluorescence imager files.

3.2.4 Image editing

All images taken were edited as to only include the leaf which the treatments were applied to, as opposed to the plant stems, soil, or any background reflectance from the imager casing.

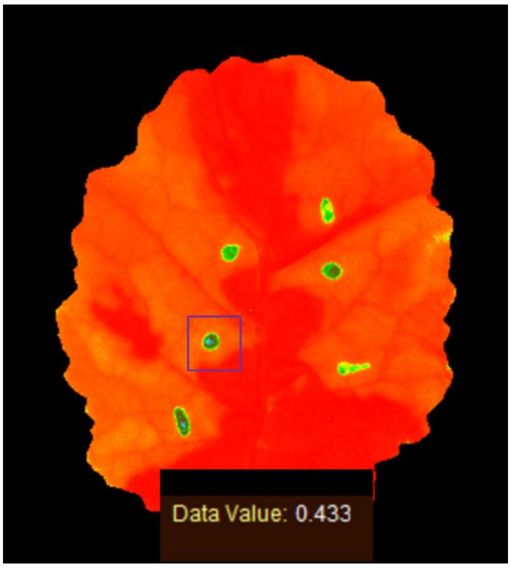


Figure 3.2. *Brassica napus* leaf, 5-hours after the application of a herbicidal mixture containing Glufosinate and Tween 20. Overall leaf F_v/F_m values were equal to ~0.78. When the mouse cursor is placed over the image, the value displayed is the value for that specific pixel. It could be seen that the values inside the blue box above, specifically in the visible 'dot' of affected area, were much lower than the whole leaf value – specifically 0.433.

Further analysis of the collected CF images evidenced that herbicide effects were not always clear – values were much lower in the spots where the treatments were applied compared to the whole leaf. Due to this, for some data, the images were subsequently edited down to a point where only the areas affected by the treatments were included, and treated as a separate data set for comparisons. Section 3.3.4 details this more fully.

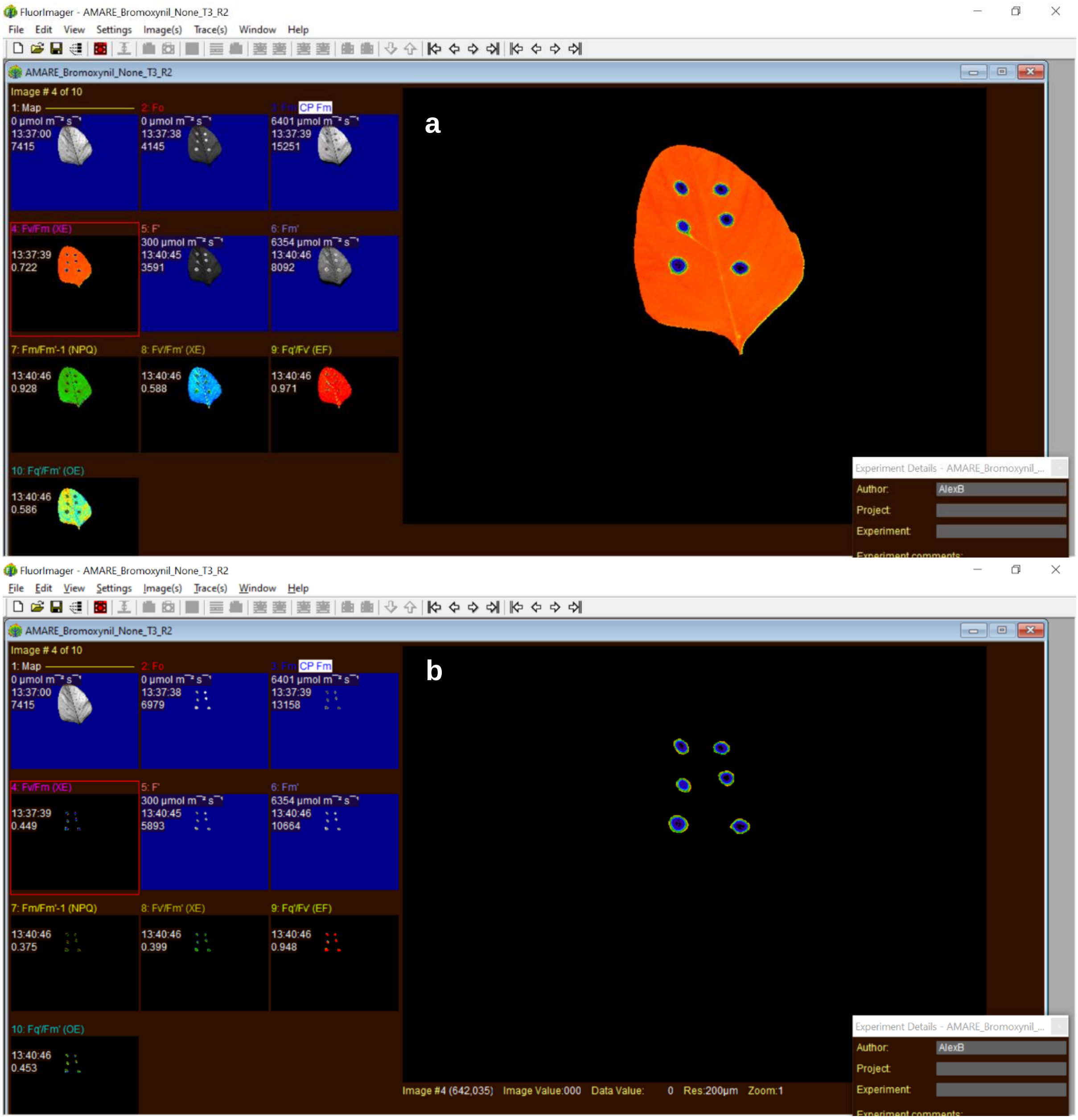


Figure 3.3. Process of deleting leaf data, narrowing to just application site of herbicide, henceforth referred to as 'dot/s'. (**a**) Initial image containing entire leaf, and (**b**) selection of the application 'dot'. The sample included in the images is a leaf of *Amaranthus retroflexus,* 3-hours the application of the herbicide Bromoxynil. Due to this selection process, F_v/F_m values can be seen to decrease from 0.722 for the whole leaf to just 0.449 for the 'dots'. A similar trend is seen for the light adapted data.

3.2.5 Statistical analysis

The Fluorimager programme from Technologica was used to analyse and edit the photos after each image time point. The numerical data was then transferred into

Microsoft Excel and R studio for additional analysis and statistical analyses. Utilizing the R Studio (version 1.1.463) interface for the open-source package R (R Core Team, Vienna Austria 2019, version 3.6.3 for Windows), analyses of variance (ANOVA) and post-hoc Tukey testing were carried out for all statistical studies.

3.3. Results

This study examined 18 treatments, that included different combinations of herbicides and adjuvants. There were 6 herbicides which either had SLES, Tween 20, or no adjuvant added.

3.3.1 Effects of herbicides and adjuvants on the dark-adapted maximum photosynthetic efficiency of photosystem II (F_v/F_m)

Bromoxynil

Little effect of Bromoxynil application was observed on the dark-adapted maximum efficiency of PSII of the AMARE leaves with only a small decrease observed over 24-hours (Fig 3.4a). However, a significant difference was seen between the treatments ($p<0.001$), with those utilising adjuvants being significantly lower than the herbicide on its own (SLES-H $p<0.001$; Tween-H $p<0.05$).

There was no significant difference between the effects of the tested Bromoxynil treatments on the dark adapted maximum photosynthetic capacity of the *Brassica napus* leaves ($p>0.05$) (Fig 3.4g).

A difference in effects of treatments on the *Echinochloa* leaf segments is clearly apparent (Fig3.4m), clearly showing that the treatments utilising the adjuvants SLES and Tween 20 had a much greater effect on the maximum photosynthetic capacity of

PSII than the treatment which solely utilised the herbicide Bromoxynil, and this difference was significant (SLES-H $p<0.001$; Tween-H $p<0.001$). Over the 24 hours, the SLES and Tween 20 treatments decreased F_v/F_m in *Echinochloa* leaves to approximately half of the initial values recorded, whereas the herbicide treatment values stayed relatively consistent over the 24-hours, with only a small decrease of around 0.1.

Diclofop-methyl

The application of Diclofop-methyl, even with the two adjuvants, proved to have little effect on the *Amaranthus* leaves (Fig 3.4b), with F_v/F_m values staying consistently around 0.75 ($p>0.05$).

Diclofop-methyl showed to have little effect on the *Brassica napus* leaves (Fig 3.4h), with no statistically significant differences being observed between the treatments ($p>0.05$).

As with both the *Brassica* and *Amaranthus* leaves, the values recorded for all three of the Diclofop-methyl treatments when applied to ECHCG stayed extraordinarily consistent over the period of the experiment, with little variance being seen between them, and little effect being seen from any of the treatments ($p>0.05$) (Fig 3.4n).

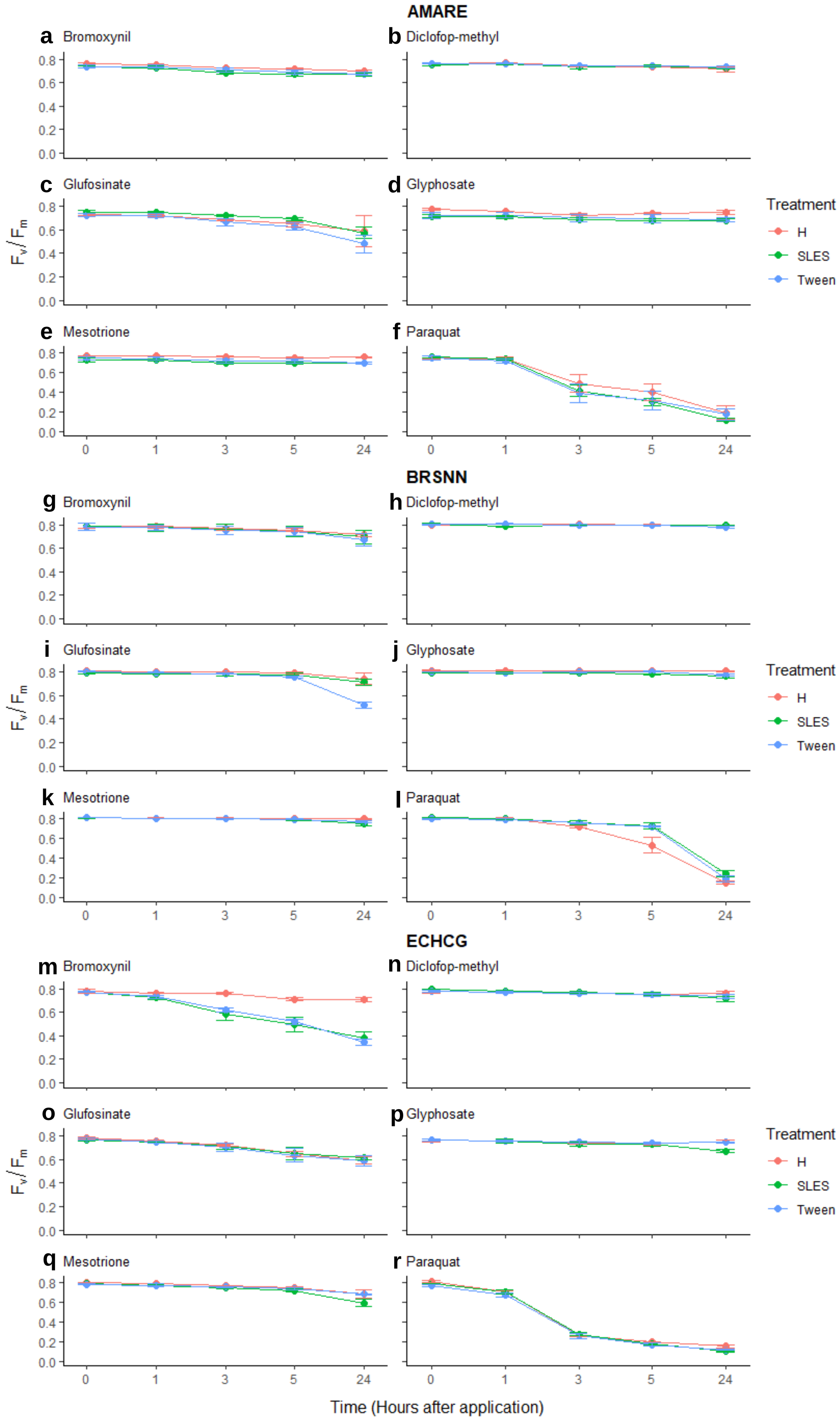

AMARE
a Bromoxynil
b Diclofop-methyl
c Glufosinate
d Glyphosate
e Mesotrione
f Paraquat
BRSNN
g Bromoxynil
h Diclofop-methyl
i Glufosinate
j Glyphosate
k Mesotrione
l Paraquat
ECHCG
m Bromoxynil
n Diclofop-methyl
o Glufosinate
p Glyphosate
q Mesotrione
r Paraquat
F_v/F_m
Treatment
H
SLES
Tween
0 1 3 5 24
0.0 0.2 0.4 0.6 0.8
Time (Hours after application)

Figure 3.4. Mean F_v/F_m of (**a**-**f**) *Amaranthus retroflexus* (AMARE)*,* (**g**-**l**) *Brassica napus* (BRSNN), and (**m**-**r**) *Echinochloa crus-galli* (ECHCG) leaves (or 3-cm leaf segments in the case of ECHCG) after the application of various herbicides and adjuvants, over a time course of 24 hours. The herbicides tested were (**a**,**g**,**m**) Bromoxynil, (**b**,**h**,**n**) Diclofop-methyl, (**c**,**i**,**o**) Glufosinate, (**d**,**j**,**p**) Glyphosate, (**e**,**k**,**q**) Mesotrione, and (**f**,**l**,**r**) Paraquat. Error bars represent ± standard error, n=4-5.

Glufosinate

The three Glufosinate treatments used for this experiment showed similar patterns in effect on the F_v/F_m of the *Amaranthus* leaves, with a subtle decline between 0 and 5 hours after application, and then a larger drop off at the 24-hour mark (Fig 3.4c). However, no significant difference was observed between the treatments ($p>0.05$).

Until the 24-hour mark, little difference is observed between the treatments when using Glufosinate on *Brassica napus* leaves (Fig 3.4i). However, after 24 h following application, the treatment with the adjuvant Tween 20 showed a large decrease ($p<0.01$) in F_v/F_m, which is large enough to cause significance when comparing the treatments SLES-H ($p>0.05$) and Tween-H ($p<0.01$). The differences between the effects of the Tween 20 and SLES treatments were near significance (Tween-SLES $p=0.0659$).

A steady decrease in F_v/F_m over 24 h in the Glufosinate treatments from ~0.8 – 0.6 was observed, however all three treatments showed this trend with similar data values, and hence the results were not significant for ECHCG ($p>0.05$) (Fig 3.4o).

Glyphosate

The treatment of Glyphosate on its own when applied to *Amaranthus retroflexus* leaves (Fig 3.4d) showed significantly higher F_v/F_m values than the treatments making use of the adjuvants (ANOVA p<0.001; Tukey SLES-H p<0.001; Tween-H p<0.01).

When applied to BRSNN leaves (Fig 3.4j), all three treatments showed consistent similar results across 24 hours, however there was a significant difference between them (p<0.001). A difference was found between the adjuvant treatments and the herbicide treatment, however no discernible difference was found between the two adjuvant treatments (SLES-H p<0.001, Tween-H p<0.01, Tween-SLES p>0.05)

Although little effect was seen on the *Echinochloa* leaves due to the application of the Glyphosate treatments (Fig 3.4p), an analysis of variance and subsequent post hoc Tukey tests revealed that a significant difference between them occurred (p<0.05), however only due to the effects of the SLES treatment and not the other adjuvant treatment using Tween 20 (SLES-H p<0.05; Tween-H p>0.05; Tween-SLES p<0.05).

Mesotrione

A significant difference was observed between the effects of the treatments using the plastoquinone synthesis inhibitor Mesotrione on AMARE (ANOVA p<0.001; Tukey SLES-H p<0.001; Tween-H p<0.001) (Fig 3.4e).

After 24 hours of the application on *Brassica napus* leaves, a significant difference was observed over the time series (p<0.01) (Fig 3.4k). The SLES treatment was significantly different from the Tween treatment, but this was not significantly different to the herbicidal treatment alone (SLES-H p<0.01, Tween-H p>0.05, Tween-SLES p>0.05).

A significant difference was observed (p<0.05) between the effects of the various Mesotrione treatments on ECHCG (Fig 3.4q), mainly due to the effects of the

SLES treatment specifically at the 24-hour time point (SLES-H $p<0.05$; Tween-H $p>0.05$; Tween-SLES $p>0.05$).

Paraquat

No significant difference was observed between the various treatments utilising Paraquat ($p>0.05$). With this being said, all three of the treatments caused a very large decrease in the photosynthetic capacity of the *Amaranthus* leaves, decreasing by ~50% from the first data recorded, after just 3 h (Fig 3.4f). Over the entire time series (24 h), F_v/F_m values decreased drastically to values of ~0.1. Due to this no significant difference were observed between the adjuvant treatments and herbicide treatment alone, as without the addition of an adjuvant the herbicidal mixture on its own was shown to have a very potent effect.

No significant difference was observed between the Paraquat treatments on *Brassica* leaves ($p>0.05$) (Fig 3.4l). Although a strong effect was still seen across all treatments after 24-hours, when compared to the other two species being tested (AMARE and ECHCG) in the present research, the effect of the Paraquat took longer to be exhibited in the F_v/F_m of the BRSNN leaves. As mentioned with the ECHCG leaves, the droplets of treatment took up a much smaller area on the BRSNN leaves than the other species, and so the effects of treatments were either less or only apparent after a longer period of time, in order to reach the same effect as seen on the other species.

Similar to the findings in *Amaranthus* leaves, there was a strong effect of Paraquat on the PSII maximum photosynthetic efficiency in the *Echinochloa* leaf sections, with a major decrease in F_v/F_m of ~0.4 being observed between 1 and 3 hours post treatment (Fig 3.4r). However, as before, this effect of Paraquat was observed across all three of the treatments, and therefore no significant difference is observed ($p>0.05$).

3.3.2 Effects of herbicides and adjuvants on the light adapted operating photosynthetic efficiency of photosystem II (F_q'/F_m')

Bromoxynil

There is a clear difference in F_q'/F_m' values of the Bromoxynil treatments on *Amaranthus* leaves ($p<0.001$) (Fig 3.5a), with the data of the herbicide treatment alone being significantly higher than those that included an adjuvant (SLES-H $p<0.001$; Tween-H $p<0.001$). The values across the entire time series for the two adjuvant treatments were similar (Tween-SLES $p>0.05$).

When applied to the leaves of *Brassica napus*, the addition of adjuvants significantly increased the efficacy of the herbicide ($p<0.001$) (Fig 3.5g). The herbicide treatment alone showed a consistently much higher F_q'/F_m' value than both the SLES and Tween 20 treatments, with the latter two showing values of only around ~66% of the herbicide values. There was no statistically significant difference between the two adjuvant treatments (SLES-H $p<0.001$; Tween-H $p<0.001$; Tween-SLES $p>0.05$).

For Bromoxynil, the two adjuvant treatments showed a slight decrease in F_q'/F_m' when applied to the *Echinochloa* leaf segments (Fig 3.5m), and the values recorded were consistently a lot lower (~50%) than the herbicide treatment (SLES-H $p<0.001$; Tween-H $p<0.001$; Tween-SLES $p>0.05$).

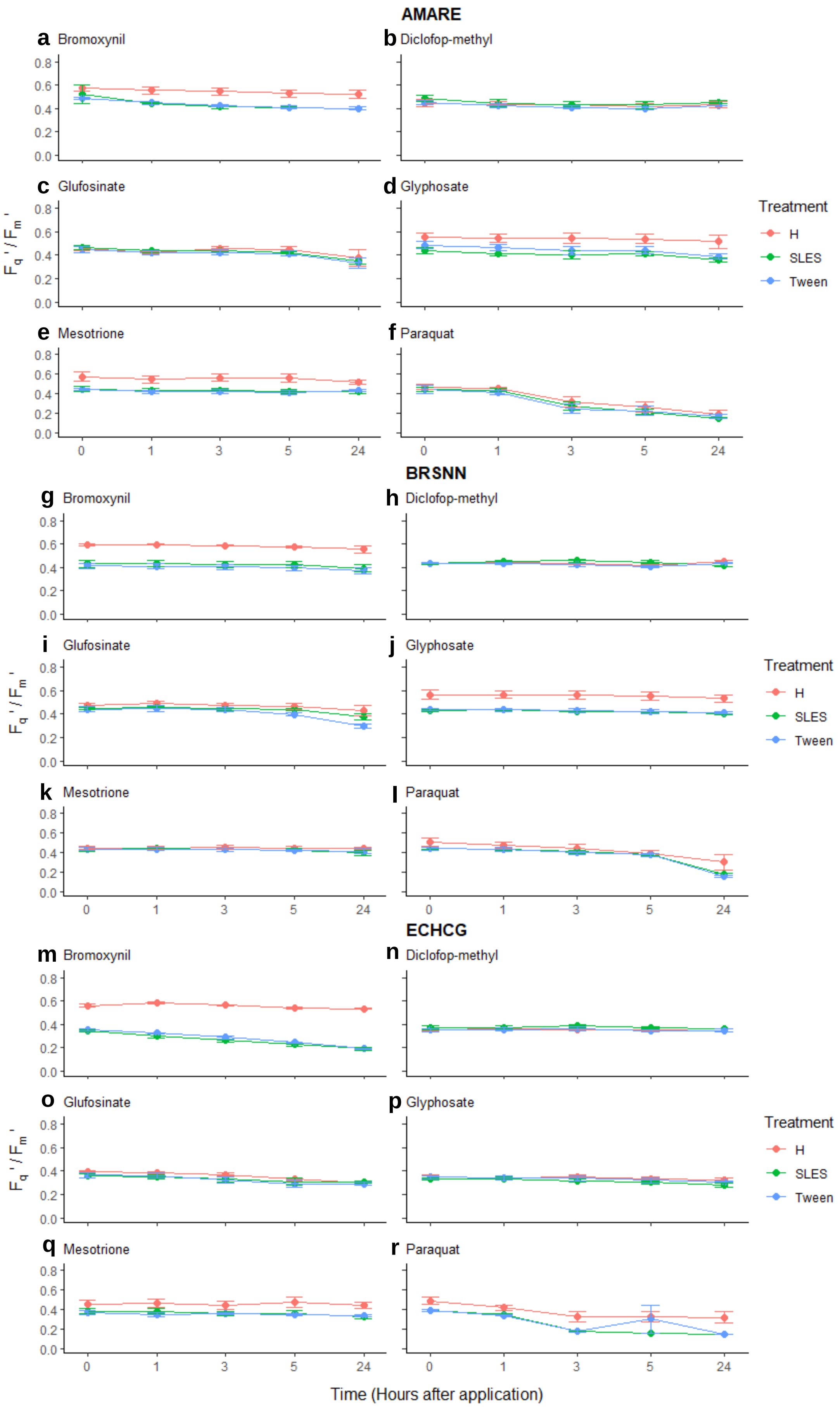

AMARE
a Bromoxynil
b Diclofop-methyl
c Glufosinate
d Glyphosate
e Mesotrione
f Paraquat
BRSNN
g Bromoxynil
h Diclofop-methyl
i Glufosinate
j Glyphosate
k Mesotrione
l Paraquat
ECHCG
m Bromoxynil
n Diclofop-methyl
o Glufosinate
p Glyphosate
q Mesotrione
r Paraquat
F_q' / F_m'
0.0
0.2
0.4
0.6
0.8
0
1
3
5
24
Treatment
H
SLES
Tween
Time (Hours after application)

Figure 3.5. Mean F_q'/F_m' of *Amaranthus retroflexus* (AMARE) (**a**-**f**)*, Brassica napus* (BRSNN) (**g**-**l**)*,* and *Echinochloa crus-galli* (ECHCG) (**m**-**r**) leaves (or 3-cm leaf segments in the case of ECHCG) over 24 hours, having had various herbicide and adjuvant combinations applied. (**a**,**g**,**m**) Bromoxynil, (**b**,**h**,**n**) Diclofop-methyl, (**c**,**i**,**o**) Glufosinate, (**d**,**j**,**p**) Glyphosate, (**e**,**k**,**q**) Mesotrione, and (**f**,**l**,**r**) Paraquat. Error bars represent ± standard error, n=4-5.

Diclofop-methyl

There was no significant difference observed between the treatments of Diclofop-methyl on the F_q'/F_m' of *Amaranthus* leaves (p>0.05) (Fig 3.5b).

After application to the BRSNN leaves, the treatments remain close together in a narrow region of F_q'/F_m' values, and there was no significant differences between treatments (p>0.05) (Fig 3.5h).

The efficacy of Diclofop-methyl was affected by the addition of the adjuvant SLES, with a significant difference between the treatment using that adjuvant, and the other two treatments, on ECHCG (SLES-H p<0.05; Tween-H p>0.05; Tween-SLES p<0.01) (Fig 3.5n). Despite there being a significant difference between the SLES treatment and the other mixtures, contrary to what may be expected, the SLES treatment showed to produce higher F_q'/F_m' values than the others.

Glufosinate

All treatments containing Glufosinate consistently showed lower F_q'/F_m' values when compared to other herbicides – however no significant differences were found between the Glufosinate treatments when applied to AMARE (p>0.05) (Fig 3.5c).

All three of the treatments utilising Glufosinate proved to have an effect on the operating efficiency of the *Brassica napus* leaves (Fig 3.5i), specifically after 24 h, which is the point where most of the differences between the treatments occurs ($p<0.001$). The herbicide treatment alone showed consistently higher F_q'/F_m' values than the two adjuvant treatments, with the Tween treatment exhibiting the greatest effect at the 24-hour mark (i.e. lowest F_q'/F_m' value), resulting in a significant difference between treatments (SLES-H $p=0.054$; Tween-H $p<0.001$; Tween-SLES $p>0.05$).

Without the addition of any adjuvants, and with the addition of SLES to Glufosinate, significantly ($p<0.05$) higher PSII operating efficiency values in ECHCG were observed when compared to the treatment containing Tween 20 (SLES-H $p>0.05$; Tween-H $p<0.05$; Tween-SLES $p>0.05$) (Fig 3.5o).

Glyphosate

When applied to *Amaranthus* leaves, Glyphosate greatly decreased the PSII operating efficiency ($p<0.001$) (Fig 3.5d), with the two adjuvant treatments having routinely lower values recorded than the herbicide treatment alone (SLES-H $p<0.001$; Tween-H $p<0.001$). Although the SLES treatment was also consistently lower than the Tween 20 treatment, this difference was not significant (Tween-SLES $p>0.05$).

The two adjuvant treatments proved to have similar effects on BRSNN (Fig 3.5j), with both having greater effects on F_q'/F_m' than the herbicide treatment alone, recording significantly lower values than the herbicide treatment alone (SLES-H $p<0.001$; Tween-H $p<0.001$; Tween-SLES $p>0.05$).

The values recorded for F_q'/F_m' on the *Echinochloa* leaf sections following Glyphosate treatments remained stable over the 24-hour period (Fig 3.5p). The values for the SLES treatment were consistently and significantly lower than the other two treatments, causing an overall significance between the treatments ($p<0.05$; SLES-H $p<0.05$; Tween-H $p>0.05$; Tween-SLES $p>0.05$).

Mesotrione

The adjuvant treatments resulted in consistently lower values than the herbicide treatment alone on AMARE (SLES-H $p<0.001$; Tween-H $p<0.001$; Tween-SLES $p>0.05$) (Fig 3.5e). For all treatments, the values were consistently around the 0.42 for the adjuvant treatments and 0.6 for the herbicide treatment, without much variance over the entire time course.

There was no significant difference between the treatments across the whole time series when using Mesotrione as the main active ingredient in the herbicidal mixtures applied to BRSNN ($p>0.05$) (Fig 3.5k).

For Mesotrione, the herbicide treatment alone had significantly ($p<0.001$) less of an effect on the operating efficiency of PSII of the *Echinochloa* leaves than the treatments utilising Mesotrione with the addition of adjuvants (Fig 3.5q). The F_q'/F_m' values for the herbicide alone were notably higher across the entire time series (SLES-H $p<0.001$; Tween-SLES $p<0.001$). With both of the adjuvant treatments being consistently lower than the herbicide treatment, there was no significant difference between them (Tween-SLES $p>0.05$).

Paraquat

Most of the herbicides did not show much effect on F_q'/F_m' in *Amaranthus* leaves (Fig 3.5f), however, the PSI inhibitor Paraquat, showed a large effect. Over the 24 hours of the experiment, values were halved, with the greatest decreases observed between 1 and 3-hours. All three treatments showed a similar trend however, and there was no significant differences between them ($p>0.05$).

The separate addition of the two adjuvants SLES and Tween 20 proved to have a significant effect on the efficacy of Paraquat on the *Brassica napus* leaves (SLES-H p<0.05; Tween-H p<0.05) (Fig 3.5l). All three treatments show a steady (albeit only slight) decrease in the F_q'/F_m' of the *Brassica leaves*, however between 5 and 24-hours there was a large decrease in the operating efficiency, with values decreasing from around 0.4 to 0.15.

Across all treatments, Paraquat had the strongest and most rapid effect on F_q'/F_m' in *Echinochloa* leaves, with values being reduced to approximately 0.2 after as little as 3 hours (Fig 3.5r). Despite also having a strong effect, the herbicide treatment alone proved to record higher values than the other two treatments, and significantly so (SLES-H p<0.001; Tween-H p<0.01). There was no significant difference between the two adjuvant treatments (p>0.05).

3.3.3 Effect of herbicides and adjuvants on the quenching parameters F_q'/F_v' and F_v'/F_m'

3.3.3.1 *Amaranthus retroflexus*

Bromoxynil

There was a significant difference in Bromoxynil treatment effects on F_q'/F_v' in *Amaramthus* (p<0.001) with the herbicide treatment alone having greater values across the entire time series than when either of the two adjuvant treatments were added to the treatment (SLES-H p<0.001; Tween-H p<0.001) (Fig 3.6a). There was no significant difference between the two adjuvant treatments (p>0.05).

A significant difference (p<0.001) was found between the treatments, when assessing the F_v'/F_m' of *Amaranthus* leaves after the application of various treatments (Fig 3.6b). The largest differences occurred at time points T0 and T24, with SLES resulting in a F_v'/F_m' lower than the other treatments, and Tween 20 being lower than the herbicide treatment (SLES-H p<0.001; Tween-H p<0.05). Although the two adjuvant treatments showed different responses, the differences were slightly above the 0.05 significance threshold (Tween-SLES $p=0.05432$).

Glyphosate

The results recorded for the treatments containing the herbicide Glyphosate form a bell-shaped response over the 24-h time series, with the highest F_q'/F_v' values recorded after 3 and 5 hours, and the lower values for all treatments either side at 0 and 24 hours after application (Fig 3.6c). The herbicide treatment records consistently higher value than the other two treatments at all time points with the exception of T0, and SLES treatment records consistently lower values than all other treatments (SLES-H $p<0.01$; Tween-H $p<0.01$). However, there was no significant difference between the two adjuvant treatments (SLES-Tween $p>0.05$).

Across the whole time series, the adjuvant treatments with Glyphosate resulted in lower F_v'/F_m' values than the herbicide on its own (SLES-H $p<0.001$; Tween-H $p<0.001$) (Fig 3.6d). Although the SLES treatment values were consistently lower than the Tween 20 treatment, these differences were not significant (Tween-SLES $p>0.05$).

Mesotrione

Similar to the findings for both Bromoxynil and Glyphosate, the Mesotrione treatment alone resulted in higher F_q'/F_v' values than with the addition of the two adjuvants ($p<0.001$) (Fig 3.6e). The two adjuvant treatments had an almost identical effect on the *Amaranthus* leaves (SLES-H $p<0.001$; Tween-H $p<0.001$; Tween-SLES $p>0.05$).

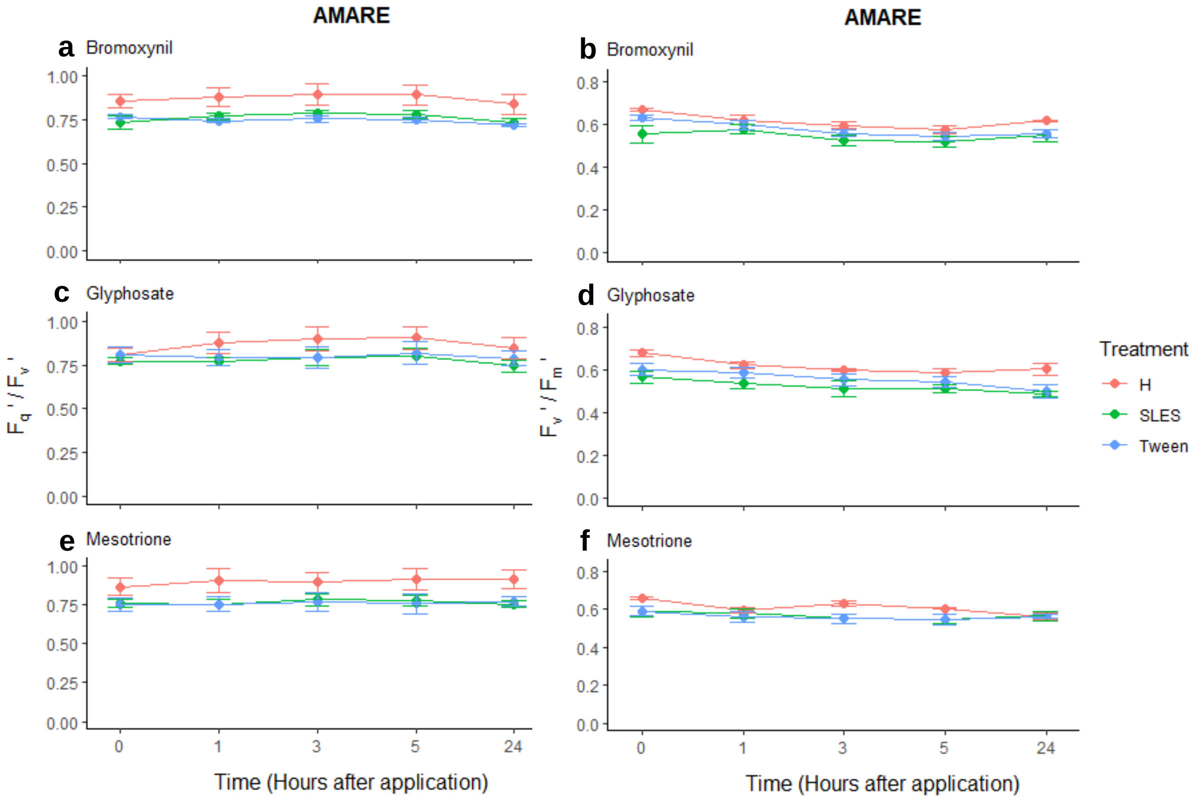


Figure 3.6. Mean F_q'/F_v' (**a**,**c**,**e**) and mean F_v'/F_m' (**b**,**d**,**f**) of *Amaranthus retroflexus* leaves over 24 hours, having had various herbicide and adjuvant combinations applied. The herbicides analysed were (**a**,**b**) Bromoxynil, (**c**,**d**) Glyphosate, and (**e**,**f**) Mesotrione. Error bars represent +- standard error, n=4. Only the treatments which showed significant effects in the corresponding F_q'/F_m' measurements were analysed. Error bars represent ± standard error, n=4-5.

Much like the previous two herbicides mentioned, treatments utilising Mesotrione as an active ingredient showed that those that used an adjuvant in addition to just the herbicide recorded lower F_v'/F_m' values than the treatment using just the herbicide, and to a significant level (SLES-H p<0.01; Tween-H p<0.01) (Fig 3.6f).

3.3.3.2 *Brassica napus*

Interestingly, all four of the herbicides analysed in Fig 3.7 (a-d) resulted in an increase of the F_q'/F_v' in *Brassica* leaves over the 24-hour time series. This effect was often mirrored in the treatments that included either adjuvant, although not always.

Bromoxynil

There was a large difference in F_q'/F_v' between the different treatments, with the herbicide treatment alone having much higher values over the entire time series than when the two adjuvants were added (SLES-H p<0.001; Tween-H p<0.001) (Fig 3.7a). The Tween 20 treatment resulted in lower values than the SLES treatment, however the difference not significant (Tween-SLES p>0.05).

Bromoxynil application on *Brassica napus* leaves was not affected by the addition of an adjuvant, with all three treatments displaying similar results, characterised by a drop in F_v'/F_m' after 24 hours (p>0.05) (Fig 3.7e).

Glufosinate

As no significant difference was found between the SLES treatment and herbicide alone treatment for the results of F_q'/F_m' in *Brassica* leaves, only the results of the treatments 'Herbicide alone' and 'Tween 20' are displayed in Figure 3.7 d&h.

There was no significant difference detected between the effects of the treatments using Glufosinate as a main active ingredient on F_q'/F_v' of *Amaranthus* leaves (p>0.05) (Fig 3.7d).

Both treatments containing Glufosinate have an effect on the maximum efficiency of PSII (F_v'/F_m') in *Brassica* leaves, however the treatment utilising Tween 20 as an adjuvant resulted in lower values after 24-hours with a decrease in values by about 0.2, as opposed to 0.05 observed for the herbicide treatment alone (p<0.001 (Fig 3.7h).

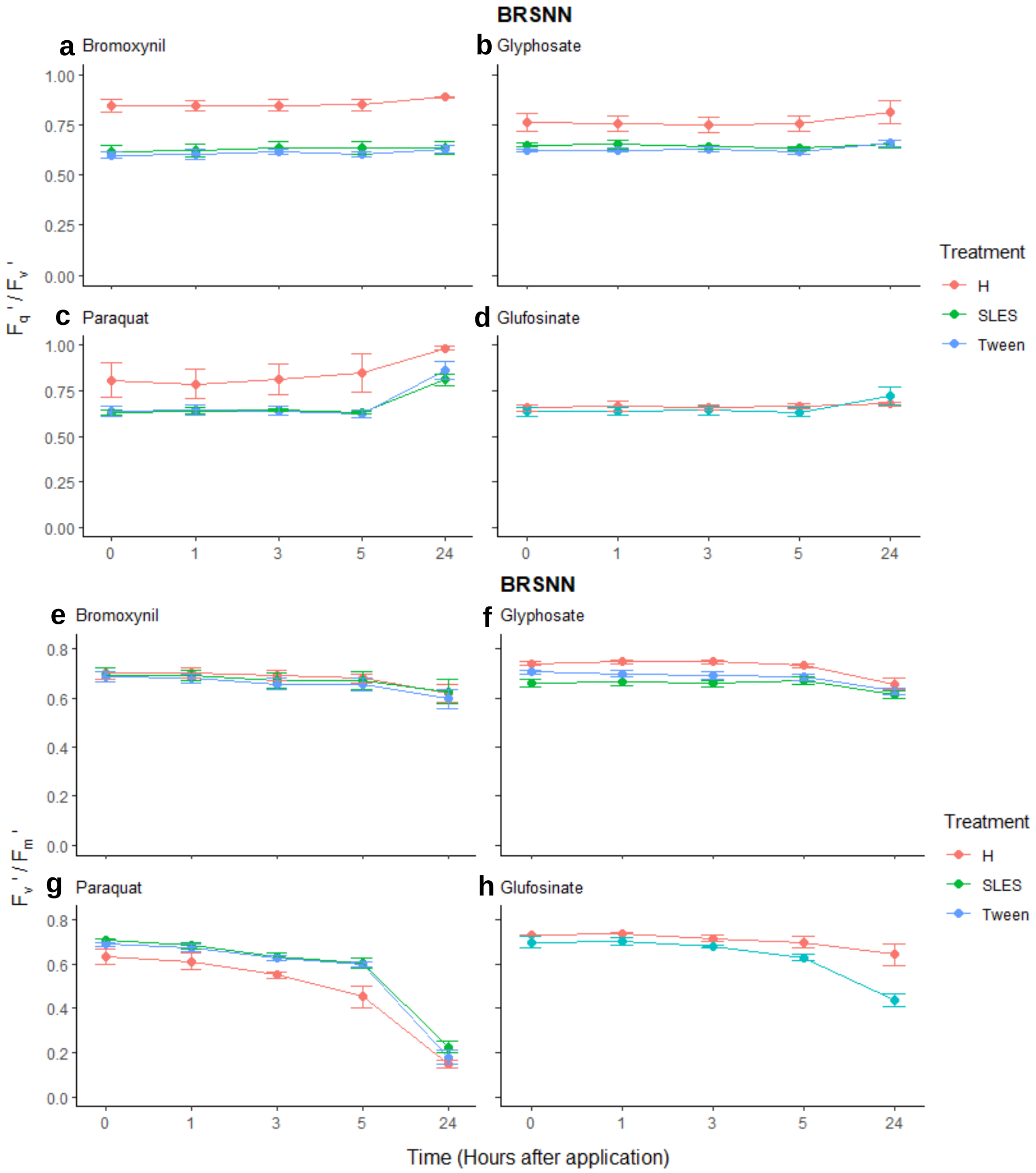


Figure 3.7. Mean F_q'/F_v' (**a-d**) and mean F_v'/F_m' (**e-h**) of *Brassica napus* leaves over 24 hours, having had various herbicide and adjuvant combinations applied. The herbicides analysed were (**a**,**e**) Bromoxynil, (**b**,**f**) Glyphosate, (**c**,**g**) Paraquat, and (**d**,**h**) Glufosinate. Error bars represent +- standard error, n=4. Only the treatments which showed significant effects in the corresponding F_q'/F_m' measurements were analysed. Error bars represent ± standard error, n=4-5.

Glyphosate

A significant difference was observed in F_q'/F_v' ($p<0.001$) between the three Glyphosate treatments (Fig 3.7b). Specifically, there was a clear gap between the herbicide treatment and the adjuvant treatments, with the adjuvant treatments consistently lower than the herbicide on its own, with no difference being detected between the two adjuvant treatments (SLES-H $p<0.001$; Tween-H $p<0.001$; Tween-SLES $p>0.05$).

For the majority of the time series, the treatment without adjuvants maintained higher levels of F_v'/F_m' than the treatments with adjuvants (SLES-H $p<0.001$; Tween-H $p<0.001$) (Fig 3.7f). Additionally, the SLES treatment was significantly lower than the Tween 20 treatment (Tween-SLES $p<0.05$).

Paraquat

After 24 h, F_q'/F_v' increased in all three treatments, with the greatest difference being observed in the two adjuvant treatments, with values increasing by ~40% from approximately 0.6 to almost 0.8 (Fig 3.7c). As this trend was observed with the application of both adjuvants, there was no significant difference observed between the adjuvant treatments, however (as seems to be the case with most of these results), the herbicide treatment alone was consistently greater than when either of the two adjuvants were included (SLES-H $p<0.001$; Tween-H $p<0.001$; Tween-SLES $p>0.05$).

The treatment of solely Paraquat proved to have significantly ($p<0.05$) lower values recorded for F_v'/F_m' than with the addition of any adjuvants (SLES-H $p<0.05$; Tween-H $p>0.05$) (Fig 3.7g). All three treatments had a large effect on F_v'/F_m', with values decreasing to as little as 0.1 after 24 hours. The herbicide treatment alone shows a much more gentle decrease in F_v'/F_m' over the 24 hours, compared with the addition of adjuvants, which lowered F_v'/F_m' between 5 and 24 hours from around 0.6 to 0.1.

3.3.3.3 *Echinochloa crus-galli*

Bromoxynil

The effect of Bromoxynil on the co-efficient of photochemical quenching (F_q'/F_v') of the *Echinochloa* leaf sections is dependent on whether any adjuvants were used in conjunction with the herbicide (Fig 3.8a). When adjuvants were included in the treatments, values were significantly reduced, with F_q'/F_v' values at about half those of the herbicide treatment (p<0.001). Individually, the SLES treatment was significantly different to the herbicide treatment (p<0.001), and so was the Tween 20 treatment (p<0.001), however the two adjuvant treatments were not significantly different from one another (p>0.05).

The treatment mixtures that contained Bromoxynil and either of the two adjuvants showed to steadily decrease the F_v'/F_m' over a 24-hour period, when applied to *Echinochloa* leaf sections (Fig 3.8f). Over this time course, values dropped from ~0.6 straight after the herbicidal mixture was applied, to around 0.3, 24 h after application. The treatment which did not utilise an adjuvant resulted in a decreased light adapted maximum efficiency of PSII over the time period, however this decrease was not large as large as the decrease due to the treatments with adjuvants, and so was significantly different when compared with the other treatments (SLES-H p<0.001; Tween-H p<0.001).

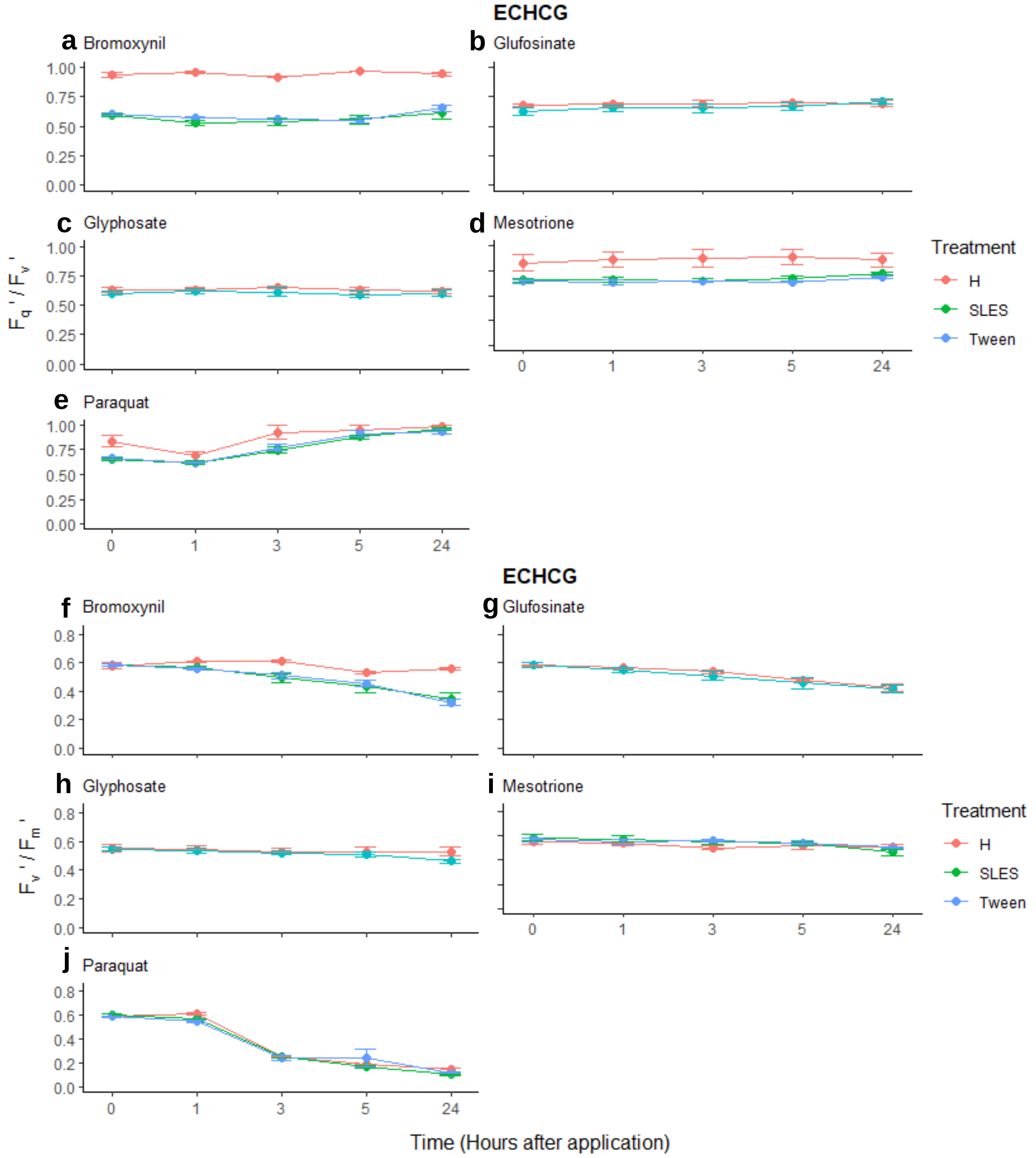


Figure 3.8. Mean *F_q'/F_v'* (**a**-**e**) and mean *F_v'/F_m'* (**f**-**j**) of 3-cm sections of *Echinochloa crus-galli* leaves over 24 hours, having had various herbicide and adjuvant combinations applied. (**a**,**f**) Bromoxynil, (**b**,**g**) Glufosinate, (**c**,**h**) Glyphosate, (**d**,**i**) Mesotrione, and (**e**,**j**) Paraquat. Error bars represent +- standard error, n=4. Only the treatments which showed significant effects in the corresponding *F_q'/F_m'* measurements were analysed. Error bars represent ± standard error, n=4-5.

Glufosinate

As mentioned previously with regards to the Glufosinate applied to BRSNN, only the application of Tween 20 resulted in a significant difference in *F_q'/F_m'* when compared with the herbicide treatment alone, when applied to the *Echinochloa* leaf sections. As such only these results are displayed in Figure 3.8 b&g.

F_q'/F_v' values for all Glufosinate treatments were similar between all treatments, with no statistically significant differences being observed between treatments ($p>0.05$) (Fig 3.8b).

Both of the treatments analysed here, just the herbicide on its own, and with the addition of Tween 20, that included Glufosinate as the main active ingredient of the mixture, showed a steady decrease in F_v'/F_m' with no significant differences between treatments ($p>0.05$) (Fig 3.8g).

Glyphosate

As no significant differences in F_q'/F_m' were found between the Tween 20 treatment and Herbicide alone treatment, only the results for the Herbicide alone and SLES treatments were analysed for the quenching parameters F_q'/F_v' and F_v'/F_m', when applied to ECHCG (Figure 3.8 c&h).

No effect of the Glyphosate treatments on F_q'/F_v' values can clearly be seen, with the values plateauing from T0 until T24, with no significant differences be observable between the treatments ($p>0.05$) (Fig 3.8c).

The F_v'/F_m' values recorded for both the herbicide treatment and SLES treatment remained reasonably similar across the time series, with some differentiation between them starting after 24 hours (Fig 3.8h). However, there was no significant differences between them ($p>0.05$).

Mesotrione

A clear difference was observed in the effects of the various treatments on F_q'/F_v', with the Mesotrione alone treatment staying significantly higher (p<0.001) than the adjuvant treatments for the entire time series (Fig 3.8d) (SLES-H p<0.001; Tween-H p<0.001). There was no difference between the effects of the adjuvant treatments on F_q'/F_v' (Tween-SLES p>0.05).

No statistically significant differences were observed when comparing the effects of the various Mesotrione treatments on the F_v'/F_m' of *Echinochloa* leaf segments (p>0.05) (Fig 3.8i).

Paraquat

A significant difference (p<0.001) in the F_q'/F_v' was observed between the various treatments utilising Paraquat, with the herbicide treatment maintaining higher values than the adjuvant treatments (SLES-H p<0.001; Tween-H p<0.001) (Fig 3.8e). Despite the differences in data, the treatments follow the same pattern, with a dip being observed after 1 h, followed by a steady increase over the rest of the time course, from 3 hours after application until 24 hours after application.

All treatments including Paraquat showed a strong effect on the maximum efficiency of PSII (F_v'/F_m') in the light adapted *Echinochloa* leaf segments, drastically decreasing values from 0.6 at the start of the assay, to a minimal level of around 0.1 after 24 h, for all treatments, with the largest single decrease occurring between 1 and 3-hours (Fig 3.8j). There was no significant difference observed between the treatments (p>0.05).

3.3.4 Whole leaf versus affected area of application data

It can be seen from the results presented above, that the use of a whole leaf collects a large amount of data outside of the application area of the treatments, which can lead to poor judgements on how effective the herbicidal treatments were. The effects of the herbicides and adjuvants get lost and undetected until 24 hours after application, unless the herbicides have an exceptionally strong effect, as seen with the likes of Paraquat (Fig 3.4 f,l,r). This was incidentally partially dealt with when utilising the monocot *Echinochloa crus-galli*, as due to the length of the leaves, the leaves were sectioned down to just a 3-centimetre segment with treatments applied within this reduced leaf area. The following results display this difference in analysis when utilising the whole leaf versus only the affected area of application – dubbed 'dots' henceforth. These 'dots' however were not always visible on the chlorophyll fluorescence images. A smaller subset of the data was chosen, where for the treatments selected, the 'dots' could be seen across all replicates performed of all treatments. This subset only included results for *Amaranthus retroflexus* leaves, 3 hours after the application of the treatments, using only the herbicides Bromoxynil, Glufosinate, Mesotrione, and Paraquat.

3.3.4.1 Maximum efficiency of photosystem II in dark adapted material (F_v/F_m)

Bromoxynil

No significant difference was found between the three treatments when using the 'dot' data ($p>0.05$) (Fig 3.9a). However, a significant difference was found when comparing the results for the 'dot' data to the whole leaf data, when looking at the treatments separately (Herbicide dot vs whole $p<0.001$; SLES dot vs whole $p<0.001$; Tween dot vs whole $p<0.001$).

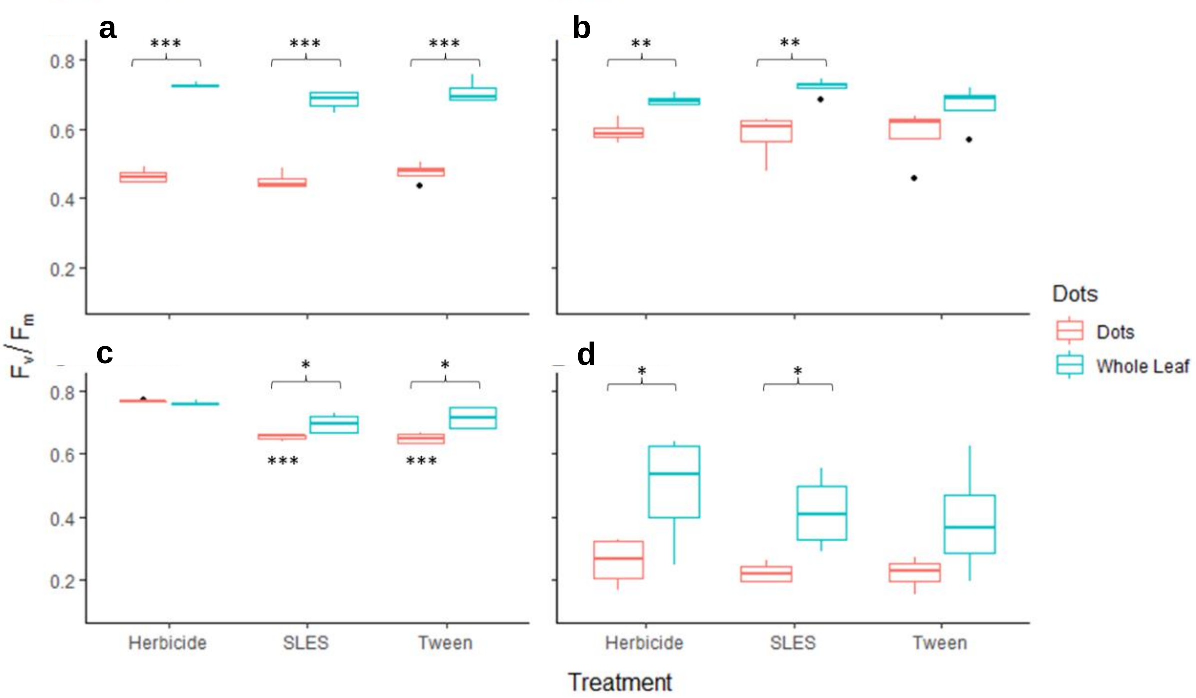


Figure 3.9. Differences between 'dots' and whole leaf dark adapted maximum efficiency of PSII data recorded, for all three treatments, on *Amaranthus retroflexus* plants, 3 hours after application. The results of the following four herbicides were analysed: (**a**) Bromoxynil, (**b**) Glufosinate, (**c**) Mesotrione, and (**d**) Paraquat. Statistical notation above the box plots indicates significance between the 'dot' and whole leaf data; notation below the box plots indicates significance when comparing adjuvant treatments to the herbicide treatment, for 'dot' data.

Glufosinate

No significant difference was found when comparing the adjuvant treatments to the herbicide treatment for this set of 'dot' data ($p>0.05$) (Fig 3.9b). A significant difference was found between the 'dot' and whole leaf data for the herbicide alone and SLES treatments, however not for the Tween 20 treatment (Herbicide dot vs whole $p<0.01$; SLES dot vs whole $p<0.01$; Tween dot vs whole $p>0.05$).

Mesotrione

A significant difference was found between the effects of the three Mesotrione treatments on the F_v/F_m of the *Amaranthus* leaves, three hours after application ($p<0.001$) (Fig 3.9c). Both the SLES and Tween 20 treatments showed significantly lower maximum efficiencies of PSII than the herbicide alone treatment (SLES-H $p<0.001$; Tween-H $p<0.001$). Additionally, both adjuvant treatments showed significant differences between the data recorded over a whole leaf when compared to the data for the 'dots', however this difference was not observed for the herbicide alone treatment (Herbicide dot vs whole $p>0.05$; SLES dot vs whole $p<0.05$; Tween dot vs whole $p<0.05$).

Paraquat

All three treatments utilising Paraquat as an active ingredient, for both the 'dots' and whole leaf data sets, showed substantially decreased F_v/F_m values after just 3 hours, with values as low as 0.2 being recorded (Fig 3.9d). As all three treatments had already had an exceptionally strong response, there are no significant differences between them ($p>0.05$). In contrast, a significant difference was found between the 'dot' and whole leaf data for the SLES treatment, and the difference between the 'dot' and whole leaf data for the herbicide alone treatment was slightly above the significance threshold of 0.05 (Herbicide dot vs whole $p=0.0599$; SLES dot vs whole $p<0.05$; Tween dot vs whole $p>0.05$).

For all four herbicides, the data extracted from the 'dots' exhibited consistently lower F_v/F_m values than the F_v/F_m recorded from the whole leaf data sets – for 66% of the treatments, this difference was significant.

3.3.4.2 Operating efficiency of photosystem II in light adapted material (*F_q'/F_m'*)

Bromoxynil

Significant differences were found between the whole and 'dot' data for all three Bromoxynil treatments (Herbicide dot vs whole p<0.05; SLES dot vs whole p<0.001; Tween dot vs whole p<0.001) (Fig 3.10a). Specifically, a much larger difference was found between the whole leaf and 'dot' data for the two adjuvant treatments, than the herbicide alone treatment. Despite an evidently large difference between the 'dot' F_q'/F_m' values for the three treatments, with the F_q'/F_m' of the *Amaranthus* leaves 3 h after the application of the SLES and Tween 20 treatments being lower than the values recorded for the herbicide alone treatment, no significant difference was found between the treatments (SLES-H p=0.0545; Tween-H p=0.06146). This is probably due to a single value recorded for the herbicide alone treatment that is vastly lower than the rest of the values recorded. This outlier had a value of 0.205 F_q'/F_m', with the rest of the data points having a mean of 0.431. Even when including this outlier, the p-values of the post hoc Tukey tests are close to being significant (SLES-H p=0.0545; Tween-H p=0.06146). The boundary of p=0.05 for significance, whilst widely used, is arbitrary, so these results could prove to be significant when having performed more replicates in the future. Additionally, the F_q'/F_v' results for Bromoxynil (which can be viewed as Fig 6.2 in the appendix) exhibit similar findings, with a clear difference being observed between the three treatments, and a single outlier point for the herbicide alone treatment. However, in this case, a significant difference was found between the treatments (ANOVA p<0.05; Tukey SLES-H p=0.0566; Tween-H p<0.05). Additionally, the results of an analysis between the treatments on F_v'/F_m' in *Amaranthus* leaves (Fig 6.3 in appendix) showed to not being significant (p>0.05). This indicates that if a significant difference were to arise between the Bromoxynil treatment's effects on the F_q'/F_m', then the difference would probably be due to the effect of the treatments on the F_q'/F_v', as opposed to the effects on the maximum efficiency of PSII (F_v'/F_m').

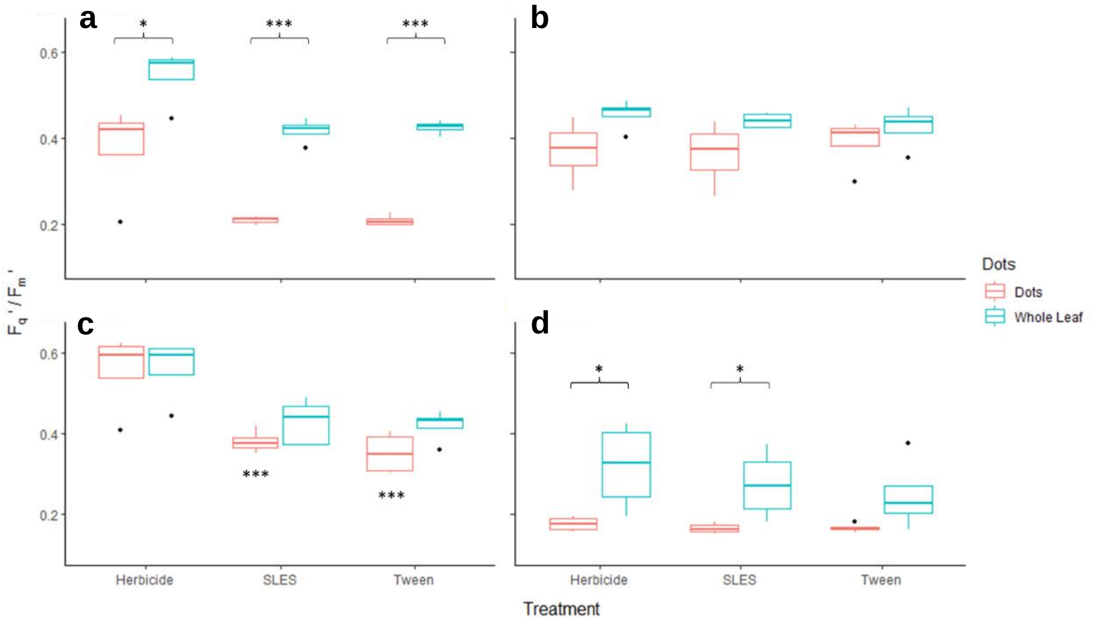


Figure 3.10. Differences between 'dots' and whole leaf light adapted operating efficiency of PSII data recorded, for all three treatments, on *Amaranthus retroflexus* plants, 3 hours after application. The results of the following four herbicides were analysed: (**a**) Bromoxynil, (**b**) Glufosinate, (**c**) Mesotrione, and (**d**) Paraquat. Statistical notation above the box plots indicates significance between the 'dot' and whole leaf data; notation below the box plots indicates significance when comparing adjuvant treatments to the herbicide treatment, for 'dot' data.

Glufosinate

Consistently lower values of F_q'/F_m' were exhibited by the isolated 'dots' of treatment than the whole leaf (Fig 3.10b), however this difference was not large enough to be significant for any of the three Glufosinate treatments (Herbicide dot vs whole $p>0.05$; SLES dot vs whole $p>0.05$; Tween dot vs whole $p>0.5$). Additionally, no significant difference was found between the treatments when comparing data from the ‘dots’ ($p>0.05$).

Mesotrione

Despite visibly lower F_q'/F_m' values being recorded for the adjuvant treatments of the ‘dot’ data (Fig 3.10c), no significant difference was found for any of the treatments when comparing the ‘dot’ and whole leaf data (Herbicide dot vs whole $p>0.05$; SLES dot vs whole $p>0.05$; Tween dot vs whole $p>0.05$). In contrast, a significant difference was found when comparing the treatments ($p<0.001$), with both the SLES and Tween 20 treatments being drastically lower than the herbicide alone treatment, evidenced by values as low as ~55% of the herbicide values (SLES-H $p<0.001$; Tween-H $p<0.001$).

Paraquat

Paraquat evidently has a strong effect on the operating efficiency of PSII, as evidenced by F_q'/F_m' values as low as ~0.25 being recorded for all treatments, irrespective of ‘dots’ or whole leaves (Fig 3.10d). Due to this exceptionally strong effect being seen across all treatments, no significant difference was found between the treatments ($p>0.05$). When comparing the ‘dot’ and whole leaf data, a significant difference was found between the two data sets, for both the herbicide alone and SLES treatments, however not for the Tween 20 treatment (Herbicide dot vs whole $p<0.05$; SLES dot vs whole $p<0.05$; Tween dot vs whole $p>0.05$). Across all three treatments, the utilisation of the ‘dots’ was observed to greatly reduce the error accounted for in the data, with the boxes for the ‘dots’ data being just a fraction of the size of the whole leaf data boxes.

3.3.4.3 Quenching Parameters F_q'/F_v' and F_v'/F_m'

Mesotrione was the only herbicide to display a significance between the treatments when looking at the F_q'/F_m' values of the 'dot' data set (despite some results being near significance e.g. Bromoxynil (Fig 3.10a)). Mesotrione is therefore the only herbicide that will be looked into further with regards to the quenching parameters, F_q'/F_v' and F_v'/F_m'.

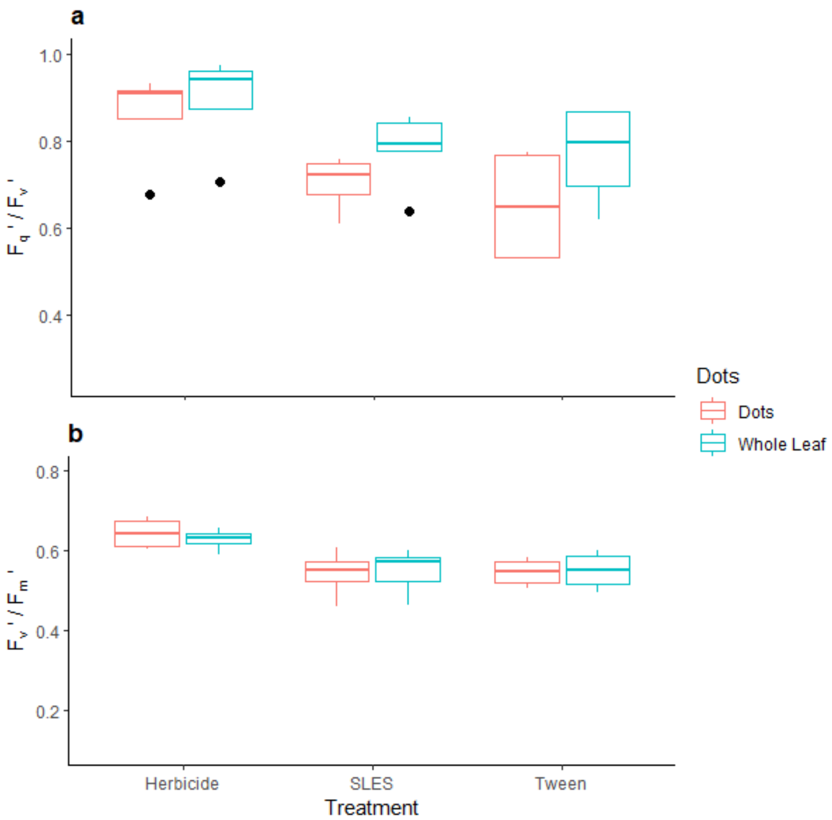


Figure 3.11. Box plot to show the effects of three different treatments, all containing Mesotrione as the main active ingredient, on (**a**) the co-efficient of photochemical quenching (F_q'/F_v') and (**b**) maximum efficiency of PSII (F_v'/F_m') of light adapted *Amaranthus retroflexus* leaves, 3 hours after application, depending on whether a whole leaf was analysed or just the 'dot' where the treatments were applied.

No significant difference was found between the 'dot' and whole leaf data for any of the Mesotrione treatments, with regards to the photochemical quenching coefficient F_q'/F_v' (Herbicide dot vs whole p>0.05; SLES dot vs whole p>0.05; Tween dot vs whole p>0.05) (Fig 3.11a). Additionally, when looking at the 'dot' data set, no significant difference was found between the herbicide alone and SLES treatments, however a significant difference was observed when comparing the herbicide alone and Tween 20 treatments (SLES-H p>0.05; Tween-H p<0.05).

No significant difference was observed between the 'dot' and whole leaf data for any of the treatments (Fig 3.11b), with regards to the maximum efficiency of PSII (F_v'/F_m') in the light adapted *Amaranthus* leaves, 3 h after application (Herbicide dot vs whole p>0.05; SLES dot vs whole p>0.05; Tween dot vs whole p>0.05). A significant difference was found however when comparing the two adjuvant treatments to the herbicide alone treatment (p<0.001), evidenced by both adjuvant treatments exhibiting significantly lower values for F_v'/F_m' than the herbicide alone treatment (SLES-H p<0.01; Tween-H p<0.01).

3.3.5 Principal Components Analysis

A principal components analysis (PCA) was performed on the data for the second study, to identify the most influential variables on the data, with the aim of highlighting the specific essential information, differences, and patterns in the data. For both Figure 3.12 and 3.13, the first principal component (PC1) accounts for approximately 36.5% and 34% of the total variance in the samples, respectively. For both figures, the main attributes of PC1 are the timepoint, the name of the herbicide, and the maximum efficiency of PSII in the light and dark (F_v'/F_m' and F_v/F_m respectively). As some of these variables are non-numeric, they were assigned numerical values for the purpose of the PCA, as seen in Table 3.1. PC2 is mostly a combination of the species and treatment (H, SLES, and Tween) variables, however

PC2 only accounts for ~20% of the variance. The second principal component is primarily in the direction of the variables “Species” and “Treatment”, with smaller shifts towards NPQ, F_q'/F_m' and F_q'/F_v'. PC2 is largely orthogonal to the variables Name, Time, F_v/F_m, and F_v'/F_m'.

Table 3.2. Numerical values assigned to the non-numeric variables of PCA.

Numerical Value	Herbicide	Treatment	Species
1	Bromoxynil	Herbicide	AMARE
2	Diclofop-methyl	SLES	BRSNN
3	Glufosinate	Tween 20	ECHCG
4	Glyphosate		
5	Mesotrione		
6	Paraquat		

The effects of the mode of action of herbicides are not distinctly separated in the Principal Component Analysis (PCA). Paraquat exhibits a significant variance primarily attributed to the amount of time that has passed since its application, demonstrating a notably faster efficacy compared to other herbicides, resulting in a distinct effect observed at T1, while most other herbicides exhibit minimal impact at this stage. The effects of Bromoxynil and Glyphosate can be seen to have been more profoundly influenced by the species to which they are applied, compared to the other tested herbicides, as can be seen in Figure 3.12. The species-specific impact of Bromoxynil surpasses that of other herbicides in terms of magnitude.

Mesotrione, Bromoxynil, and Glufosinate display a greater degree of variation in the parameters F_q'/F_m' and F_q'/F_v' than the other herbicides, with the respective data points being located more towards the lower end of the PCA displayed in Figure 3.12.

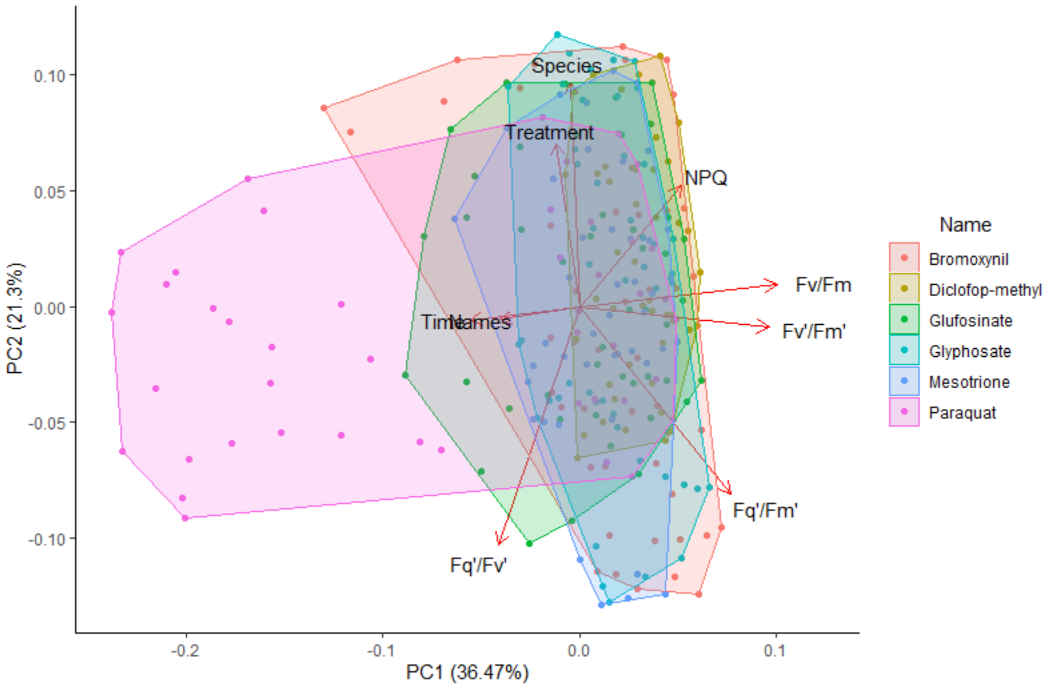


Figure 3.12. Principal components analysis (PCA) of all treatments, species, and parameters utilised in study 2.

Figure 3.13 indicates the variance due to the two largest principal components after having grouped the tested herbicides by similarities in mode of action. Group 1 consists of Paraquat (PSI), Bromoxynil (PSII), and Mesotrione (Plastoquinone), as their MOAs are more directly involved in inhibiting photosynthesis. Group 2 includes Glufosinate (NH_4 assimilation) and Glyphosate (amino acid biosynthesis), as both are linked to the disruption of amino acid biosynthesis. Group 3 consists solely of Diclofop-methyl which has a MOA targeting fatty acid biosynthesis.

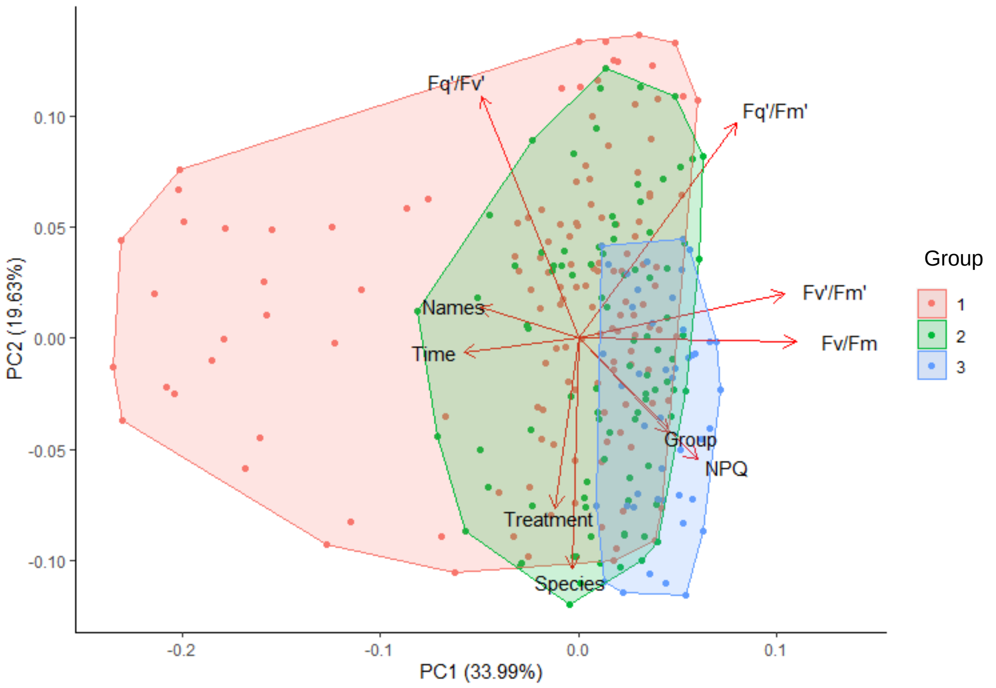


Figure 3.13. Principal components analysis (PCA) of all treatments, species, and parameters utilised in study 2, with herbicides grouped by similar modes of action

Despite this grouping, a large overlap is still seen between them, with Group 1 having the most variance due to the Time point of the data and the Name of the herbicide. This is akin to the indication of Figure 3.12, with these herbicides targeting the process of photosynthesis directly, and therefore having a much more rapid effect than those herbicides with modes of action targeting other metabolic processes, which then feeds back into photosynthesis. The area for Group 2 largely remains central around the origin of the loading arrows, however it does exhibit larger variation due to PC2 than PC1. Group 3 (Diclofop-methyl) has more variance than the other groups in terms of the non-photochemical quenching (NPQ) parameter, which as Diclofop-methyl has a mode of action targeting fatty acid biosynthesis, i.e. not directly affecting photosynthesis, this can be anticipated.

3.4 Discussion

The hypothesis that the efficacies of the 6 tested herbicides will be affected by the addition of one or both of the adjuvants used in the study, and the effects can be detected utilising chlorophyll fluorescence imaging, has been confirmed. Additionally, the results presented here prove that chlorophyll fluorescence imaging is a suitable tool for herbicidal mode of action detection and determination of adjuvant effects. For the dark-adapted results, across all species, four of the six tested herbicides showed improved efficacies when one or both of the adjuvants Tween 20 and SLES was included in the mix. The difference between treatments becomes more evident when examining the light adapted operating efficiency of PSII. In order to determine a mechanistic understanding how these treatments impacted on the operating efficiency of PSII photochemistry, the chlorophyll quenching parameters F_q'/F_v' and F_v'/F_m' (which make up the values for F_q'/F_m') were determined. The results showed that for those herbicides where a significant difference between treatments was observed in the light adapted operating efficiency (F_q'/F_m'), the significance was mirrored in only one of the quenching parameters, F_q'/F_v' or F_v'/F_m', rather than both – most of the time. Crucially, with the utilisation of CFI, these results will help to help determine the MOA of the herbicides, with those that affect F_q'/F_v' more, having more of an effect on processes downstream of the photosystems (CO_2, stomata), and those that have an effect on F_v'/F_m' being related to non-photochemical quenching of energy in the antenna, and therefore affecting protective mechanisms more (Baker *et al.*, 2001; Fryer *et al.*, 2003; Lawson *et al.*, 2002; Murchie and Lawson, 2013). The results for the herbicides and where they target can be seen without the use of the adjuvants, but as well as their effects being analysed separately the effects of the adjuvants aid in detecting herbicidal effects. Both of the adjuvants used in this study were uptake enhancers, and so those herbicides which showed little difference in efficacy in the presence of either SLES or Tween 20, may be more strongly affected by adjuvants with different mechanisms (Arand *et al.*, 2018; Hazen, 2000; Landim *et al.*, 2019; Wang and Liu, 2007).

For most of the herbicides tested in this assay, the lowest values recorded over the 24-hour time series were when applied to *Echinochloa crus-galli* leaves, as opposed to the other two tested species (*Amaranthus retroflexus* and *Brassica napus*). This was due to the natural morphology of the monocot, in that its lengthier leaves would not entirely fit into the imaging area of hardware (unlike the two dicots), and so 3-cm sections were marked onto the *Echinochloa* leaves. All of the herbicide treatments were applied within this 3-cm leaf section, and after imaging, the value for the rest of the leaf was removed. This was done as to have a defined leaf area. Due to this, a comparatively smaller area of leaf material was used for the *Echinochloa* plants than the *Amaranthus* and *Brassica* plants, and hence the ‘dots’ of applied treatment took up a larger proportion of leaf area. This difference in effects due to a difference in leaf area to treatment area ratio is analysed and discussed in further detail below.

The extra analysis focusing on the differences in data when comparing whole leaf values and data taken from just the sites of application (‘dots’) shows that the results acquired and differences in herbicidal effects can be seen more quickly, with greater herbicidal and adjuvant effects being observed after 3 hours in the ‘dots’ data than the whole leaf data. When examining the light adapted results from the ‘dot’ data, the only herbicide which showed significant differences between the effects of the three treatments was Mesotrione. Upon further inspection, a significant difference was found between the treatments for the F_v'/F_m' values, however not for the F_q'/F_v' values.

This suggest that the MOA of Mesotrione targets the protective quenching structures of the plant's chloroplast. This is in line with what is already known about Mesotrione's mode of action, and so provides evidence that this can be a suitable method for detecting an adjuvants effect on the efficacy of a herbicide, as well as determining its mode of action (Mitchell *et al.*, 2001). These results of the quenching parameters F_q'/F_v' and F_v'/F_m' at T3 leads to the logical conclusion that the significant differences found between the F_q'/F_m' values for the three Mesotrione treatments is due to a change in the maximum efficiency of PSII, F_v'/F_m', rather than the operating efficiency of PSII, F_q'/F_v'. Both adjuvant treatments show much more significant results between treatments in F_v'/F_m' than F_q'/F_v'. The operating efficiency (F_q'/F_m') of the *Amaranthus retroflexus leaves* was inhibited by a decrease in the maximum PSII efficiency (F_v'/F_m') when treatments of Mesotrione were applied.

This 'dot' work had a smaller subset of data to work with, and mainly acts as a proof of concept for the rapid data collection with there being a wealth of data possible for obtaining, in comparison to whole leaf data, which is already tremendously faster than traditional whole plant glasshouse tests. Largely, a significant difference was found between the 'dot' and whole leaf data for specific samples. However, when comparing the treatments, those that displayed statistical significance in the whole leaf data, remained statistically significant when looking at the 'dot' data. Likewise, when there was no statistical significance between the treatments. Although no additional significant results were found when comparing the results of the whole leaf and 'dot' data, these results suggest a method that could be utilised in future studies and whilst this method is not without limitations, the 'dots' can give a rapid idea of herbicidal MOA and suggest a likelihood of the MOA targeting photosystems.

A key constraint of the ‘dot’ work is that it focused on one specific time point, T3, and it can be seen from the whole leaf data that more differentiation occurs between treatments after 5 and 24 hours. Additionally, another limitation is that the dots were selected manually – this is not reproducible by various people, as all would have different biases for selection areas, and would take a large amount of time. Work for the future should aim to automate these processes, streamlining the workflow. This would make it compatible for multiple people, multiple imaging systems, and overall make the approaches used in this research a lot more transferable.

Two principal components analyses were performed, with the aim of highlighting the connections between the parameters and variables used in the study, specifically with regard to the groupings of MOAs. Several observations can be made from these PCAs. Firstly, the loadings of variables that are far apart, such as “Time” and “FvFm”, show a negative correlation with each other, meaning that as time progresses, the value of F_v/F_m decreases. Lower values of PSII operating efficiency are observed 24 hours after the herbicide has been applied than after 3 hours, which is expected, as the herbicides have had time to damage the plant. However, in the case of Paraquat, a large amount of variance is exhibited in these aspects compared to the other herbicides, primarily because Paraquat has a more rapid and significant effect on the F_v/F_m values of the leaf, causing it to deviate significantly from the rest of the data points. At T1, Paraquat demonstrates a substantial effect, whereas most other herbicides have shown minimal impact at that point. Paraquat's significant variance can be attributed to its faster action compared to other herbicides.

Additionally, the loadings for F_v/F_m and F_v'/F_m' are very close together in both of the performed PCAs, indicating that the observed variance of the two parameters are positively correlated with one another and closely linked. This finding is logical as these parameters are both analyses of the operating efficiency of PSII, simply with the plant material in different states of illumination. The factors affecting differences in one of these parameters would likely have a similar effect on the other parameter (for example the herbicide Paraquat has a very large effect on both of these parameters due to the nature of its MOA inhibiting PSI).

Additionally, it is observed that a higher treatment and species number (as seen in Table 3.2) is associated with lower F_q'/F_m' and F_q'/F_v' values.This indicates that the combination of, for example, *Echinochloa* plants and SLES would be hypothesised to result in lower F_q'/F_m' and F_q'/F_v' values, as compared to using *Amaranthus* plants and the herbicide on its own. This again suggests that the utilisation of an adjuvant in a herbicidal mixture leads to a greater effect on the photosynthetic processes of a plant than using the herbicide on its own.

Similarly, there are three herbicides which have skews towards the F_q'/F_m' loading in Figure 3.12;Mesotrione, Glyphosate, and Bromoxynil. These three herbicides have largely differing modes of action, with Bromoxynil targeting photosystem II, Mesotrione targeting Plastoquinone biosynthesis, and Glyphosate inhibiting amino acid biosynthesis. The effect of Mesotrione on the quenching parameters of photosynthesis was previously discussed as a result of the ‘dot’ analysis, with the results of this PCA corroborating this finding, indicating that the MOA of Mesotrione has a large effect on the protective quenching structures of chloroplast, which is in line with what is already known about the herbicides MOA.

Despite the differences observed in the PCAs, neither of the largest two principal components account for a great amount of the variance (i.e. >50%). This indicates that the variance between the samples is mainly due to a multitude or combination of the variables, rather than a specific factor (with the exception of the herbicide Name being “Paraquat”, which showed to have the largest amount of variance compared to all other variables). is more different to the rest of the herbicides, than the rest of the herbicides are to anything else).

The causes of the variance seen in the results are elucidated by utilising principal components analysis, and help to tie together and link the effects of variables, which were observed separately earlier in the chapter. Additionally, the similarities of data points, and lack of variance between other variables indicates that whilst significant effects were observed between the herbicides, these differences are the result of a large number of factors in combination, rather than one specific variable.

A limitation of this study, despite the large initial data set and number of samples tested, is that all of the plants were grown at the same consistent temperature and light levels, which does not reflect real-world environmental variation. Previous research has shown that herbicide efficacy decreases under high temperature stress, due to decreased absorption and translocation of the herbicidal active ingredients, as well as low relative humidity’s (Coetzer *et al.*, 2001; Gomes and Juneau, 2017; Varanasi *et al.*, 2016). The research by Coetzer et al 2001 also used the herbicide Glufosinate and the species redroot pigweed (*Amaranthus retroflexus*), both of which were used in the present research. This could be important to take into account for future research, with rising global temperatures, amongst other changing environmental factors due to climate change, and more growth conditions could be tested. An additional inhibiting factor of the present research was that only one plant could be placed inside the imager at a time, due to limited space in imaging area. This meant that slightly different amounts of time passed for each sample post herbicide application – this was combatted by utilising a ‘staggered’ application approach, however this can’t be entirely precise, due to human error as well as other real time issues that may have arisen.

Typically, whole plant tests conducted in glass houses are used to screen for herbicide resistance, however these tests are time and space consuming, as well as being relatively expensive (Kaiser et al., 2013). Whole plant tests relate to the growth of many plants of a species in a specific environment, to be analysed as a whole, as opposed to looking at say specific leaves only. Chlorophyll fluorescence imaging is a suitable method for the detection of modes of action for herbicides, but also could be used to detect herbicidal resistance in common species.

Chlorophyll fluorescence imaging is a suitable method for detecting the efficacies of specific herbicides, as well as determining the effectiveness of adjuvants at improving these herbicides, an important factor when aiming to reduce active ingredient input. This experiment aimed to assess the efficacy of the two adjuvants Tween 20 and SLES at improving the effectiveness of the chosen herbicides, using chlorophyll fluorescence imaging as a detection method. The impact of the herbicides was assessed by utilising both dark and light adapted measurements.

Previous research has been conducted with the aim of determining the effect that adjuvants have on the efficacies of various herbicides (Akhter *et al.*, 2017; Palma-Bautista *et al.*, 2020; Roggenbuck *et al.*, 1990; Travlos *et al.*, 2017). However, the work of Palma-Bautista et al. (2021) used methods other than CFI to determine the effects of the adjuvants on herbicide efficacy (specifically assessing dry weights of treated plants), 21 days after application of the treatments. With the utilisation of CFI, similar results could have been obtained in a much more rapid manner.

This research provides a solid foundation for continuing the research into the viability of using chlorophyll fluorescence imaging as a detection tool for herbicidal modes of action, and adjuvant efficacy, with a large data set with many avenues of potential analysis having been generated. Perhaps more valuably, this research, despite its limitations, has provided an insight into the process required for utilising CFI in this way, as well as transferable approaches and processes that can be used in the future, for other work of a similar nature.

4. Discussion

Chlorophyll fluorescence imaging (CFI) is a technique that provides an assessment of the photosynthetic activity and overall health of a plant, by measuring the photosynthetic efficiency of PSII photochemistry (Baker, 2008; Maxwell and Johnson, 2000; Murchie and Lawson, 2013). Herbicides, through a variety of means, either directly or indirectly, can affect this photosynthetic efficiency, and therefore the effects and efficacy of the herbicide be determined using CFI (Barbagallo *et al.*, 2003).

No new herbicidal modes of action (MOA) had been discovered for approximately 30-40 years, until the introduction of the plant dihydrogen phosphate dehydrogenase inhibitor Tetflupyrolimet in 2019 (Dayan, 2019; Sukhoverkov *et al.*, 2021). This is a major issue, as herbicide resistance is becoming more prevalent, resulting in greater weed numbers, which compete with the same resources as crops, potentially decreasing the yields of the crops (Merfield, 2022; Renton and Chauhan, 2017). Therefore, novel modes of action are needed to combat this problem, as an effort towards increasing crop production to support the predicted changes in global population (Evans, 1999; FAO, 2018; United Nations Department of Economic and Social Affairs, 2022). One major issue with developing these novel herbicidal MOAs is understanding their mechanisms, and having a phenotyping tool with the ability to determine modes of action.

The present research supports the proposal that chlorophyll fluorescence imaging is a suitable tool for research and development of novel herbicidal modes of action, and also provides a method for detecting the efficacy, phytotoxicity, and behaviours of chemical adjuvants. The research here addressed the question of what protocol would be necessary for detecting these various herbicidal modes of action, as well as determining the specificity of the assay. As part of this protocol, the amount of time the proposed method would take was a key factor, which in future work could be compared to more traditional glasshouse methods of herbicidal effect detection (Boutin *et al.*, 2004; Evans, 1999; Kaundun, 2021).

The results presented here support the hypothesis that chlorophyll fluorescence imaging is a useful tool for determining herbicidal mode of action detection. Of the tested herbicides, some greatly affected the photosynthetic efficiency of both the aquatic and terrestrial plants, over the course of 24 h. This effect was noticeable in both the dark adapted and light adapted measurements. The dark adapted results were obtained at a more rapid rate than the light adapted measurements, in as little as 25s, however the light adapted results provided greater information, and gave a better understanding of the mechanistic properties of the various herbicidal MOAs. For all photosynthetic parameters, the greatest decrease in PSII efficiency was caused by herbicides with a PSII inhibiting MOA. However, herbicides with a PSI inhibiting MOA also had a large effect on PSII efficiency, and this can be assumed to be due to the consequential effect that the inhibition of PSI has on PSII. Those herbicides that influenced the light adapted parameter F_q'/F_m' often mainly showed a change in just one of the quenching parameters, F_v'/F_m' or F_q'/F_v'. This information can potentially be used to determine the targeted site and new modes of action of herbicides.

Significant differences between the efficacies of several herbicides were apparent, when used either in conjunction with one of the two adjuvants, or on their own. The first study found that the adjuvant SLES proved to have a greater effect on the herbicidal efficacies than Tween 20, however the second study found that both adjuvants worked well to improve the efficacy – for example as seen in the results for Bromoxynil when applied to *Echinochloa crus-galli* (Fig 3.4m & Fig 3.5m), both adjuvants had a great effect on the efficacy of the herbicide.

The results presented here provide further evidence that chlorophyll fluorescence imaging can be used to detect and determine various herbicidal modes of action, corroborating the work of Barbagallo et al. (2003). Additionally, this technique can be used to assess the efficacies of these herbicides, as well as any additional benefit of using adjuvants, in line with the findings of previous research (Zhang *et al.*, 2022).

Additionally, chlorophyll fluorescence imaging results of those herbicides which do not directly affect the photosystems could still be seen, such as the acetolactate synthase inhibitor Imazapic (Fig 2.6g), and the glutamine synthetase inhibitor Glufosinate (Fig 3.4c,i,o & Fig 3.5c,i,o), decreasing the efficiency of PSII within the 24 h time frame (Loux *et al.*, 2019a; Sebastian *et al.*, 2016). Thus, CFI can be determined to be a useful tool for detecting a wide range of MOAs. This is due to the consequential and indirect effects of the modes of action on the photosystems. However, the indirect effects take a longer period of time to elucidate than the effects of those herbicides which do directly target the photosystems. This aligns with and builds upon the findings of Barbagallo et al. (2003), who reported that whilst using CFI as a screening method does not directly detect any herbicidal effects that have no impact on the photosynthetic efficiency of plants, a surprisingly large amount of herbicides which affect other metabolic processes do consequentially have an effect on photosynthetic metabolism, and therefore can be detected using CFI (Barbagallo *et al.*, 2003). Furthermore, CFI can be used for both higher throughput and lower throughput assays, as evidenced by the two studies described in the present research.

For a large portion of the recorded data, the effects of the herbicides were not evident. Due to the size of the leaves, as well as the comparatively small size of affected plant matter, the effect of the herbicide on the application area was lost due to the affected area being small, relative to the rest of the leaf material. By narrowing down the effect of herbicide treatment application to a small defined area, the effects could be seen more in all CF parameters. This approach will help to narrow down the MOA, as well as giving more rapid results.

The present research did suffer from limitations from a methodological perspective. Study 2 utilised a much smaller set of herbicides than in study 1.

Additionally, the range of species and timepoints were limitations. These were addressed by utilising species with a range of leaf morphologies (broad leaf retentive, broad leaf non-retentive, and grasses), as well as by selecting specific time points to take images at, to gain enough information to develop an understanding of the kinetics of the effects of various herbicide MOAs – 0, 1, 3, 5, and 24 hours after treatment application. Due to these limitations, the specific results for herbicidal MOAs and adjuvants may not be generalisable across all herbicides and adjuvants.

Previous research has shown that differing environmental conditions affect herbicidal uptake, with particular reference to temperature and humidity (Coetzer *et al.*, 2001; Gomes and Juneau, 2017; Varanasi *et al.*, 2016). As the present research only examined one period of growth, this could be considered a limitation.

Future work should look at a broader selection of herbicides, adjuvants, and species as possible, whilst performing more replicates. These would help in improving the robustness of the data recorded and eliminate some of the uncertainties regarding significant differences, specifically in study 2. Additionally, this work should be performed alongside plants maintained in a glasshouse, as whilst photos of leaves were taken at every time point, a direct comparison between CFI and the traditional method of visual assessment in a glasshouse cannot be made. Further studies are necessary to streamline the imaging and data analysis processes, with aims of getting the most out of the collected data. Many of the limitations discussed have simple solutions (e.g. growing plants at different temperatures, performing replicates side by side in a greenhouse and lab) which can be addressed in future studies. Future work could also determine which CF parameters are best utilised for determining specific herbicidal MOAs, as some parameters provide more useful results than others.

Additionally, CFI could be used for further herbicidal research not limited to discovery of novel MOAs – for example, this method could be used to look at herbicidal resistance, or the combinatory effects of herbicides (Sukhoverkov and Mylne, 2021; Zhang *et al.*, 2016). One example of herbicidal synergies is Mesotrione-Atrazine, as described by Sukhoverkov and Mylne (2021), who identified Mesotrione as a standout herbicide for synergistic properties, not just with Atrazine. The present study illustrated that Mesotrione was clearly identifiable utilising CFI. The methods that Sukherkov and Mylne (2021) utilised for detecting these synergies took 14 days – with the use of CFI, this assay time could be dramatically reduced. Third, CFI could be used to monitor the effects of herbicides over time and to track the recovery of the plant after herbicide application. This can provide valuable information about the long-term effects of herbicides on plant health and can help to identify potential issues, such as herbicide resistance, that may arise, whilst giving much more timely results than traditional visual assessments (Menegat and Gerhards, 2014; Wang *et al.*, 2018)**.**

In conclusion, the results of the present research have provided evidence that chlorophyll fluorescence imaging (CFI) is a valuable and suitable tool for studying the effects of herbicides and adjuvants on plants, and can be used in herbicidal discovery and research in several ways. Firstly, by using CFI to measure changes in the amount of chlorophyll fluorescence emitted by the plant in response to different herbicides, it is possible to quickly identify compounds that are effective at controlling the growth of, and killing, invasive plant species. Secondly, CFI can be used to study the mechanisms by which different herbicides act on plants. By comparing the results of CFI measurements with known modes of action (MOA) of different herbicides, it is possible to determine the MOA of an unknown herbicide. This can help to understand the effects of herbicides on plants and predict their effectiveness against specific weed species. Additionally, CFI can provide valuable information about the effects of adjuvants on both herbicidal efficacy and plant health, and can help to optimize the use of adjuvants in herbicide formulations. Despite limitations, CFI remains as a useful and adaptive measure for herbicidal MOA research. Overall, CFI is a valuable tool for herbicide and adjuvant research, providing useful information about the effectiveness and modes of action of different herbicides and adjuvants. Overall, CFI can provide valuable insights into the effects of herbicides on plants, and should be used in conjunction with other techniques, such as traditional greenhouse whole plant assessments, to provide a more complete picture of the herbicidal effects on plants.

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6. Appendix

Table 6.1. Summary of the effects of the 12 herbicides most affected by the addition of an adjuvant of study 1, with “+” indicating a significant difference, and “-“ indicating no significant difference between the adjuvant treatment and herbicide alone treatment.

Name	MOA	SLES	Tween 20	logP
Bromoxynil	PSII	+	+	0.27
Dichlone	Redox mediator	-	-	2.65
Diclofop-methyl	Fatty acid biosynthesis	+	-	0.7
Dinoseb	Uncoupler	+	+	3.69
DMC	Plastoquinone biosynthesis	+	-	4.45
Haloxydine	Homogentisate solanesyltransferase	+	-	2.4
Imazapic	Amino acid biosynthesis	+	-	1.6
Ioxynil	PSII	-	-	2
No name 1	Unknown	+	-	~
No name 2	Cellulose synthesis	+	-	~
PSII standard 1	PSII	+	-	~
PSII standard 2	PSII	+	-	~

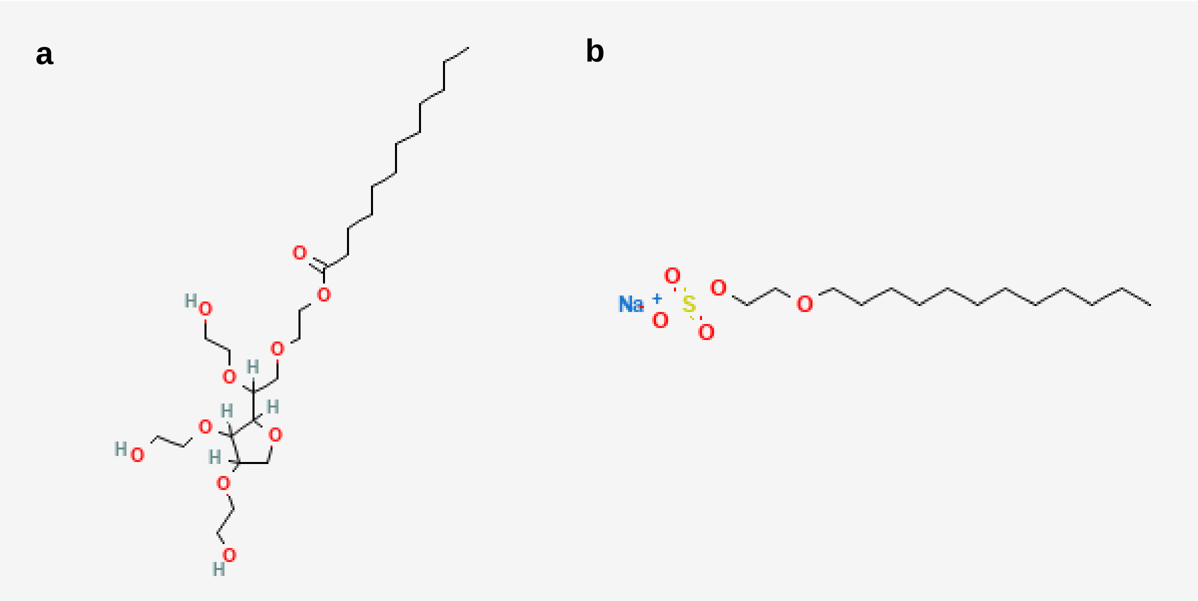

Figure 6.1. 2D chemical structures of the adjuvants utilised in the present research; (**a**) Tween 20, and (**b**) SLES.

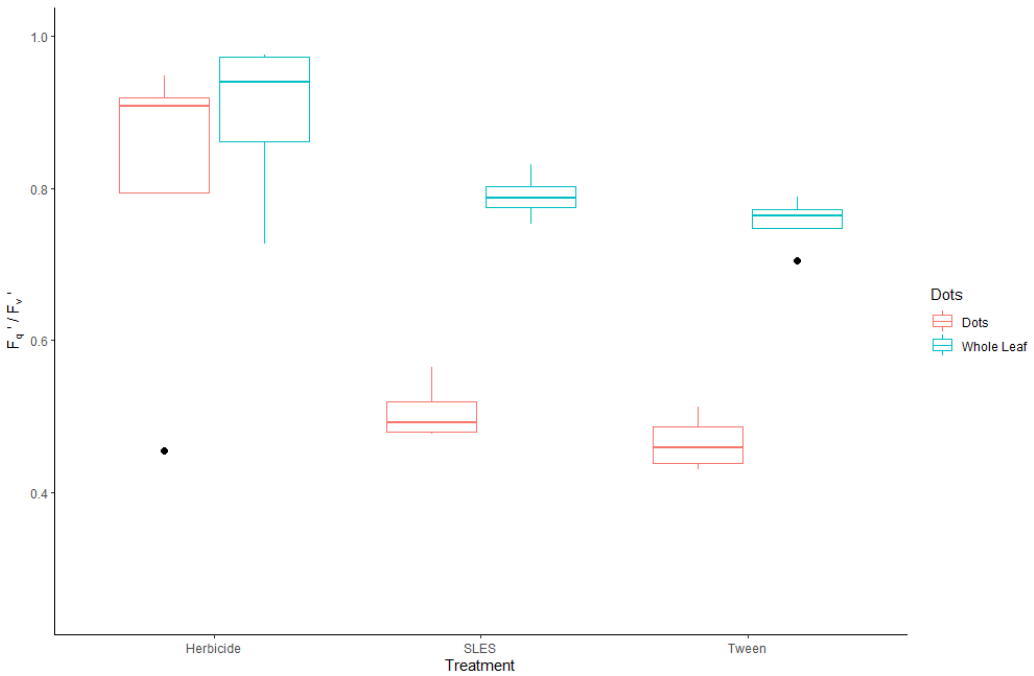

Figure 6.2. A comparison of the effects of the herbicide mixtures containing Bromoxynil on the F_q'/F_V' of *Amaranthus retroflexus* when utilising either whole leaf or 'dot' data. n=4.

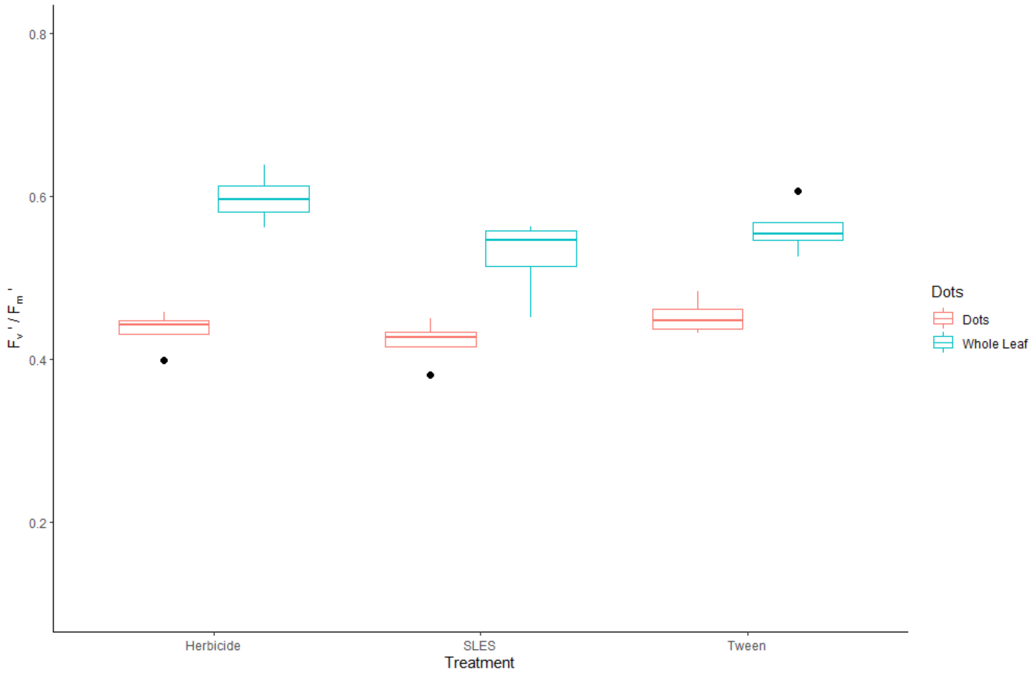

Figure 6.3. A comparison of the effects of the herbicide mixtures containing Bromoxynil on the *F_v'/F_m'* of *Amaranthus retroflexus* when utilising either whole leaf or 'dot' data. n=4.