Understanding Herbicide Resistance in Grass Weeds Using Metabolic Fingerprinting

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Abstract

Herbicide resistance is a major concern in agriculture. Resistance falls into two categories; target-site resistance (TSR) or non-target-site resistance (NTSR). Enhanced metabolic resistance (EMR) which leads to a decrease in the phytotoxicity of the herbicide is the main type of NTSR in grass weeds. Resistant black-grass (Alopecurus myosuroides), which is a competitive wheat weed widely distributed in the UK and Western Europe, has a negative impact on crop yields causing significant economic losses every year.

This thesis aims to demonstrate that Tandem Liquid Chromatography – Mass Spectrometry (LC-MS) can be used to identify herbicide metabolites and their fragments in complex matrices with the objective to use the metabolic fingerprints to develop a new diagnostic tool for NTSR grass-weeds.

Different MS instruments, low or high resolution, with chromatography or direct injection were tested towards different plant samples treated with herbicides with different chemistries in order to determine optimal LC-MS conditions. Herbicide feeding assays were also carried out over a range of characterised plant systems and were analysed to determine metabolism levels and its correlation with NTSR traits. Treatments of wheat, sensitive and resistant black-grass with individual and mixture doses of herbicides, suggested that there was competition for the detoxification enzymes, especially cytochrome P450s.

In conclusion, this thesis demonstrated that multiple herbicides and their metabolites can be resolved and identified using high resolution LC-MS over a relatively short run time. LC-MS has the power to form the basis of a high throughput analytical technique that can characterise different weed populations based on their metabolism profile and help to further elucidate the favoured routes of primary metabolism in NTSR black-grass.

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Chapter 1. Introduction

1.1 Overview

Alopecurus myosuroides (black-grass) is a wild grass first identified as a problem weed in the 1950s. It is a member of the family Poaceae, which includes more than 10,000 species (Langer and Hill, 1991) including staple cereals such as maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.).

Black-grass is an annual or winter annual grass weed which has adapted to winter cereal production with a seed shedding period between late June and late August and a peak in July (Moss, 1979). It has been demonstrated that black-grass was already present in Neolithic times (4000-2000BC) and was probably native to the Mediterranean area from where it was introduced to Northern Europe (Behrendt and Hauf, 1979). Now, it is a widespread grass weed, present in many wheat producing areas in Europe.

Phenotypically, black-grass is a tall (30-90 cm in height) and tufted or solitary annual grass with purple or greenish leaf sheaths. A single plant can produce up to 150 tillers with between 20-300 heads per plant which can produce more than 1000 seeds per head per m^2 (Chancellor, 1985),(Moss, 1983). The high reproductive capacity makes it a weed that can both propagate rapidly and evolve different types of resistance mechanisms.

Yield losses due to black-grass are more associated to winter-sown cereals, due to germination patterns, the early sowing of winter cereals and by the tendency to keep monoculture crops (Naylor, 1972). Both cereals and black-grass emerge in the autumn and compete for nutrients and light. Despite that, it has been observed that crops and black-grass can have the same growing rates during the winter and early spring, it is in the period between April and June when the development of black-grass accelerates and therefore the competition for nutrients increases, reducing crop head densities and the number of grains per ear (Moss, 1980). It has been shown that around 250 to 500 black-grass plants per m^2 can cause yield losses of 45% but yield losses of up to 66% have been reported in the most severely infested fields. In addition, viable black-grass seeds are left in the soil every year where they will grow and establish in following seasons (Moss, 1979). Once a resistant population is significantly established in the field it can spread to other populations and even to other species if cross-pollination occurs (Rieger *et al.*, 2002; Beckie *et al.*, 2003; Busi *et al.*, 2008). A recent study has determined that the annual lost production in the UK equates to £0.4 billion (2014 prices) with losses of about 0.8 million tonnes of wheat grain

production in the UK. However, globally these numbers reach the £1billion mark and 3.4 million tonnes of wheat loss (Varah *et al.*, 2020).

Presently, an integrated control program that includes chemical control in combination with culturing methods such as crop rotations, use of spring sown crops, reduced herbicide usage, prevention of seed dissemination and establishment of black-grass in the field, is applied to control black-grass

1.2 Herbicides and the Role of Metabolism in Selectivity

Herbicides can be classified as either selective (e.g. pyroxsulam), or non-selective. (e.g. glyphosate) (Mallory-Smith & Retzinger, 2003). Glyphosate is an important non-selective systemic herbicide that inhibits the essential enzyme ,5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS). Plant growth is inhibited soon after its application, accompanied by leaf chlorosis and necrosis, generally four to 10 days after application.

Non-selective herbicides generally kill any green plant tissue following uptake. In contrast, selective herbicides are toxic to some plant species and less toxic or even not toxic at all to others. Pyroxsulam is a post emergence herbicide that inhibits the enzyme acetolactate synthase, that catalyses the first step in branched chain amino acid synthesis. Herbicides such as pyroxsulam do not kill the leaf tissue in the same way as glyphosate. Instead, ALS inhibiting herbicides lead to stunted growth, preventing the weed from competing with the crop for nutrients and light, rather than tissue death as seen with non-selective herbicides. The selectivity of selective herbicides can be enhanced by the use of other compounds, safeners, which cause a protective effect in the crop thus avoiding any herbicide injury in cereal without compromising the effect on the weeds. These compounds were accidentally discovered in 1970s and with their mode of action remain poorly understood. Safeners are usually co-applied with herbicides, modifying the selectivity of these by enhancing the ability of the crop and not of the grass to detoxify the herbicide (Cummins, Bryant and Edwards, 2009; Skipsey *et al.*, 2011; Duhoux *et al.*, 2017). Selectivity is not just ascribed to one route of metabolism being favoured in the crop compared to the weed. Different detoxification pathways can contribute to selectivity. Wheat has been shown to be tolerant to flupyrsulfuron-methyl, an ALS inhibitor. The herbicide is cleaved and undergoes glutathione conjugation rendering it inactive in wheat, while in a herbicide tolerant (*Avena fatua)* populations, the herbicide undergoes glutathione conjugation followed by *O*-demethylation (Koeppe *et al.*, 1997). Whilst herbicide metabolism can be observed and

measured quantitatively it is important to note that the mechanisms by which the herbicide is metabolised contribute to the role of selectivity.

1.2.1 *Herbicide Resistance in Black-grass*

Grass weed control has been dominated since the 1970s by the intensive use of chemicals, driven by the need to increase cereal yields, decrease crop rotations and the adopt monoculture. This herbicide selective pressure has led to the evolution of some plants to develop the ability to survive at the same doses of herbicides that were previously lethal. Consequently, an increase in herbicide resistance in weeds has been shown since 1980, reaching more than 500 unique cases worldwide in 2019 and while now impacting global agriculture. In total, 85 species within the Poaceae have been identified as evolving herbicide resistance, followed by 44 species belonging to the Asteraceae and 22 of the Brassicaceae.

To date there are over 30 resistant weeds species identified in winter wheat globally (Heap, 2014). The weed species of greatest concern in the UK is black-grass due to its large presence across Europe and its reported resistance to multiple herbicides with different modes of action. A survey in 2002 in the UK demonstrated the high frequency (80%) of populations of black-grass resistant to at least one herbicide such as chlorotoluron, which is an inhibitor photosystem II (PSII) (Moss, Perryman and Tatnell, 2007) or ACCase inhibitors such as fenoxaprop-P-ethyl. Grass populations can be divided into different biotypes with respect to herbicide resistance profile. In total thirty-five black-grass resistance biotypes have been identified in 14 countries (UK, Turkey, Switzerland, Sweden, Spain, Poland, Netherlands, Italy, Israel, Germany, France, Denmark, Czech Republic, Belgium), predominantly affecting winter wheat (Heap, 2014).

1.3 Herbicide Resistance Mechanisms

In terms of the resistance mechanisms involved, two dominant types of herbicide resistance in weeds can be identified: target-site resistance (TSR) and multiple herbicide resistance (MHR, also referred to as non-target-site resistance NTSR). NTSR includes more than one mechanism of resistance to different herbicides, that can belong to the same or different chemical classes (Vrbničanin, Pavlović and Božić, 2017). TSR population control can be achieved by alternating the use of herbicides that have different modes of action. NSTR is a more problematic type of resistance, the control of which is a challenge for global agriculture. The two most significant wild grass species with metabolism-based resistance are *Lolium rigidum* (rye-grass) and black-grass. NTSR is distinct from cross

resistance or the ability of an organism to have resistance to more than one herbicide due to multiple TSR evolution. An example of TSR is the case of the amino acid substitution in the ALS enzyme, Asp376Glu, that causes resistance to herbicides belonging to 5 chemical classes (Whaley, Wilson and Westwood, 2007; Beckie and Tardif, 2012; Tétard-Jones *et al.*, 2018).

In the UK some of the best known black-grass biotypes are named after the place of origin, such as Rothamsted (Broadbalk trial site at Rothamsted, UK), Peldon (Essex), Lincoln E1 (Lincolnshire), Oxford (Oxfordshire), Notts (Nottinghamshire), Kent (Kent) and Suffolk (Suffolk). The Rothamsted black-grass population is sensitive to selective herbicides used in wheat as it has never been treated with herbicides (Hall, Moss and Powles, 1997). On the other hand, NTSR populations such as the Peldon population (the site of the first NTSR black-grass population identified in the UK) or the Lincs E1 (highly resistant to fenoxaprop-P-ethyl) have been continuously treated with herbicides for at least 30 years. The Peldon population has been widely used as a model to study NTSR (Tétard-Jones *et al.*, 2018) and is characterised as having NTSR toward different herbicides classes including aryloxyphenoxypropionate (AOPP) herbicides, such as diclofop-methyl, fenoxaprop-P-ethyl and fluazifop-P-butyl, phenylureas such as chlorotoluron, and to ACCase inhibitors such as the cyclohexanedione herbicide tralkoxydim (Hall, Moss and Powles, 1997).

1.3.1 *Target Site Resistance*

Target site resistance accounts for the majority of herbicide resistance cases in weeds (Powles and Yu, 2010). A single nucleotide polymorphism (SNP) is the most common mechanism which results in a change in the enzyme to prevent the herbicide from binding to its target site. Another type of TSR that has been shown in glyphosate resistance is due to overproduction of the target protein (Gaines *et al.*, 2010; Salas *et al.*, 2012).

Herbicides belonging to five chemical classes (imidazolinone, sulfonylaminocarbonyl-triazolinone sulfonylurea, triazolopyrimidine, and pyrimidinyl(thio)benzoate) are used for the inhibition of the ALS enzyme which is necessary for the biosynthesis of branched chain amino acids such as isoleucine, valine or leucine (Tranel & Wright, 2002; Mallory-Smith & Retzinger, 2003). Currently eight amino acid substitutions at different sites have been determined in different weeds: Ala122 (3), Pro197 (13), Ala205 (2), Asp 376(1), Arg377 (1), Trp574 (3), Ser653 (3) and Gly654 (2). Shown in parenthesis are the number of weed species found to have these amino acid substitutions (Yu and Powles, 2014), Pro

197 being the most common mutation followed by Trp 574 and Ser 653 (Tranel & Wright, 2002; Heap, 2020).

TSR is also the most common type of resistance against the herbicides inhibiting acetyl CoA carboxylase (ACCase), necessary in the synthesis of fatty acids to carboxylate acetyl CoA to malonyl CoA such as 'FOPs' (Kaundun, 2014). Black-grass with TSR to microtubule assembly and photosystem II inhibitors have also been identified (Heap, 2020).In terms of TSR, mutations resulting in ACCase and ALS inhibitors mutations have been widely identified, especially in the UK, Germany and France, Belgium, The Netherlands and Turkey (Drobny, Salas and Claude, 2006; Moss, Perryman and Tatnell, 2007).

1.3.2 *Non-target Site Resistance*

Whilst TSR only affects the step in which the herbicide binds to the target site, NTSR mechanisms include changes in the phases of the herbicide action, penetration, translocation and accumulation in the target site. Within this type, enhanced metabolism as well as impaired translocation are the most common mechanisms (Powles and Yu, 2010; Délye, 2013). For example, differences in the cuticle of the resistant plants decrease the penetration level of some ALS-inhibitors (White *et al.*, 2002; Délye, 2013). This type of NTSR has been identified in some *Lolium perenne* populations resistance to glufosinate and glyphosate (Avila-Garcia and Mallory-Smith, 2011). An increase in gibberellin production has been reported in plants resistant to thiocarbamate and pyrazolium herbicides. This change in hormone synthesis causes enhanced shoot growth enough to enable avoiding contact between the herbicide and the site of action (Rashid *et al.*, 1998).

Enhanced metabolism has been identified in many different weed species and is the most studied NTSR mechanism at this time. Multiple correlations between the multiple herbicide resistance mechanisms with the multidrug resistance (MDR) in humans, specifically due to the overexpression of active transporters that pump different compounds out of the cell have been described (Higgins, 2007). Such transport in plants to the vacuole is through an ATP dependant mechanism, usually mediated by ABC transporters, (Cole & Edwards, 2000). These multistep processes that lead to enhanced detoxification and metabolism of herbicides seems to be the most important NTSR mechanisms.

1.3.3 *Detoxification Enzymes and Pathways*

The majority of herbicides applied to plants tend to be chemically modified by plant endogenous enzymes. As each plant species differs in the distribution or activity of the enzymes, the rate of metabolism among species also differs (Iwakami *et al*., 2019). The products of herbicide metabolism are often less phytotoxic to plants and the modifications can allow for further enzymatic modifications to transport the herbicide out of the cell. Four protein superfamilies have been identified as being responsible for herbicide metabolism including cytochrome P450 monooxygenases (CYPs), glutathione S-transferases (GSTs), UDP-dependent glycosyltransferases (UGTs) and ATP-binding cassette (ABC) transporters. These four families are part of a collective known as the xenome (Edwards *et al*., 2005). The plant xenome plays a critical role in the selectivity of the different herbicides.

1.3.4 *CytochromeP450 Mediated Herbicide Metabolism*

CYPs are a superfamily of haem-containing mono-oxygenises involved in the synthesis and metabolism of endogenous and exogenous compounds such as steroids, lipids or xenobiotics (Nebert and Gonzalez, 1987; Mizutani, 2012; Bathe and Tissier, 2019). They use electrons derived from NADH or NADPH, obtained through a protein, cytochrome P450 reductase, to divide the oxygen molecule into single oxygen atoms, one of which is inserted into the substrate with the other reduced to form water (Werck-Reichhart and Feyereisen, 2000; Hamdane, Zhang and Hollenberg, 2008). The reactions catalysed by CYPs are highly diverse, including dealkylation, decarboxylation, dehydration, desaturation, dimerization, C-C cleavage, isomerization, reduction and ring extension (Bak *et al.*, 2011). All CYPs share a common catalytic centre (Werck-Reichhart and Feyereisen, 2000) and have a common three-dimensional fold. Their high abundance and key role in plant primary and secondary metabolism of herbicides (Figure 1.1) make them useful biomarkers to study plant evolution, adaptation and development of new characteristics. In rice CYPs with activity towards the metabolism of herbicides in crops include *Os*CYP81A6, responsible for bentazon and sulfonyl urea herbicide tolerance and *Os*CYP72A31 which confers tolerance to the ALS inhibitor bispyribac sodium (Saika *et al.*, 2014) similarly *Zm*CYP81A9 is responsible of the detoxification of nicosulfuron in maize (Pataky *et al.*, 2008; Liu *et al.*, 2019).

Transcriptome analysis of the Peldon population (Tétard-Jones *et al.*, 2018) has determined the overexpression of CYP clans, including but not limited to, clan 76 (*Am*CYP76), clan 81 (*Am*CYP81-1 and *Am*CYP82-2) and clan 71 (*Am*CYP71-1,

AmCYP71-2 and AmCYP71-3). Several CYPs from those families were reported to be upregulated in resistant weed populations and were shown to have activity against herbicides. Notably, *Lr*CYP81B1 and the *Lr*CYP72A which are up regulated in MHR ryegrass biotypes, share 87% and 90% of identity with *Am*CYP81-1, and *Am*72-1, respectively (Duhoux and Délye, 2013; Gaines *et al.*, 2014). CYP81A12 and CYP81A21 from *Echinocloa phyllopogon* (watergrass) are responsible for cross resistance to ALS and ACCase inhibitors having activities towards bensulfuron-methyl, diclofop-methyl and pinoxaden (Iwakami *et al.*, 2014, 2019). It is important to note that although CYPs with very similar identities (>90%) from different species have been identified as being upregulated and having activity towards specific herbicides, that CYPs can be very species specific with regards to activity towards specific herbicides.

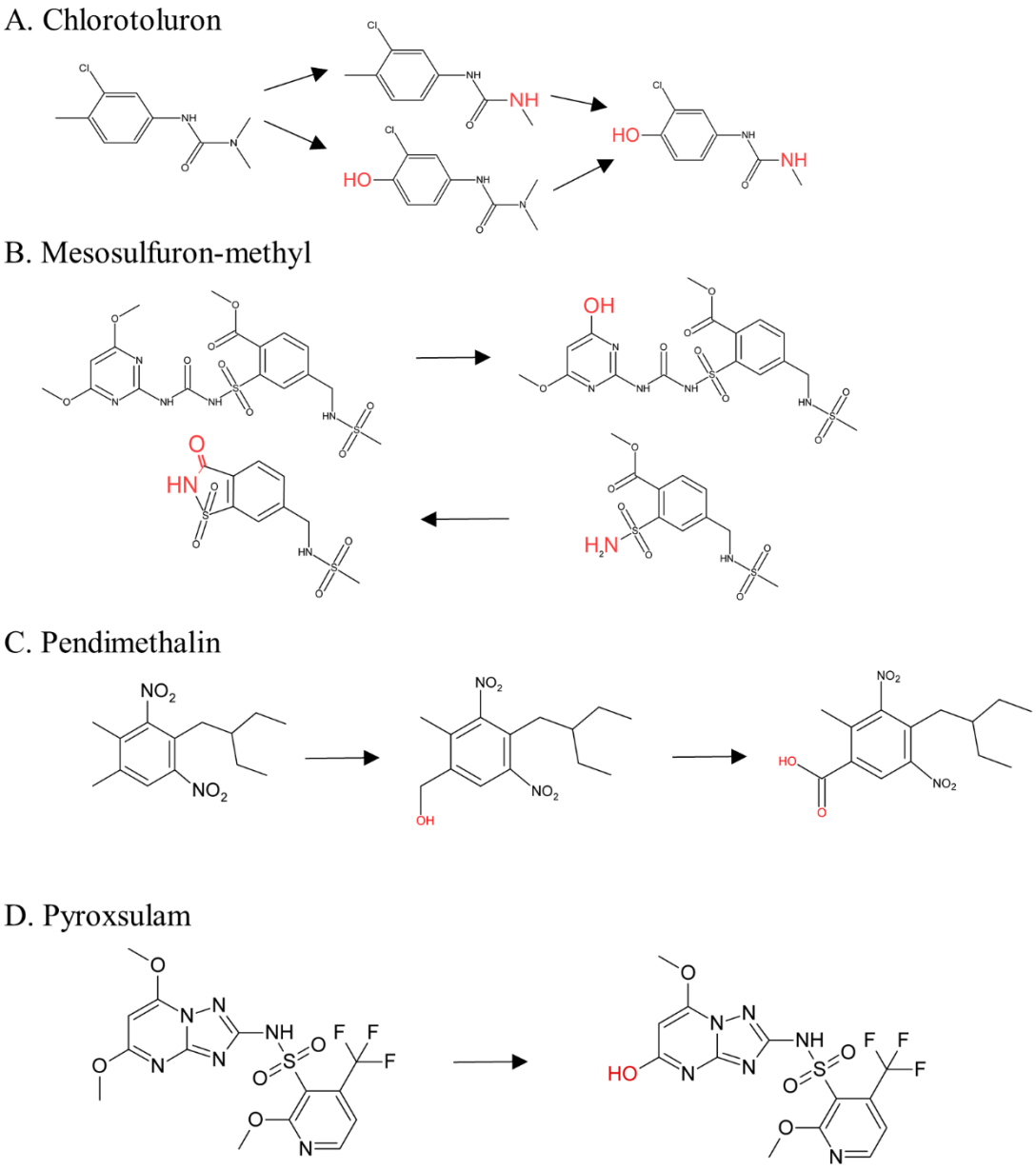


Figure 1. 1 Cytochrome P450 metabolised black-grass herbicides and their primary metabolites. Modifications highlighted in red. A. Chlorotoluron, N-demethylation, ring methyl hydroxylation and N-demethylation plus ring methyl hydroxylation; B. Mesosulfuron-methyl, demethylation, cleavage, cyclisation; C. Pendimethalin, hydroxylation and oxidation; D. Pyroxsulam, demethylation.

1.3.5 *Glutathione S-transferases*

Glutathione S-transferases GSTs, are a superfamily of key enzymes involved in catalysing nucleophilic substitutions and the catalysation of glutathione (g-glutamyl-cysteinyl-glycine) or homoglutathione, to a substrate R-X, forming a product R-SG.

GSTs have been classified using different nomenclatures that consider sequence relatedness, genome organisation, kinetic properties or immunological reactivities. In general, 14 classes of GSTs within kingdoms have been identified including, gamma-subunit classes of the eukaryotic translation elongation factor 1B (EF1B-γ), dehydroascorbate reductase (DHAR), Hemerythrin (H), Lambda (L), Iota, Metaxin, microsomal ProstaGlandin E-Synthase type 2 (mPGES-2), Omega-like or glutathionyl-hydroquinone reductase (GHR), Phi (F), Tau (U), tetrachlorohydroquinone dehalogenase (TCHQD), Theta (T), URE2p and Zeta (Z).

GSTs are homo- or heterodimeric proteins of about 50kDa (Edwards and Dixon, 2005). Each GST subunit possesses two domains with a 5-10 residues linker in between. The N-terminal, formed by β strands and α-helices, is the most conserved domain between families and contains the thioredoxin superfamily fold. The C-terminal, composed of right handed alpha helices, is less conserved and is related to substrate specificity (Frova, 2003; Chronopoulou *et al.*, 2010). In addition, the catalytic subunit includes a conserved glutathione binding site (G-site) in the N-terminal and a substrate binding site (H site) in the C-terminal. Two types of GSTs can be distinguished by the presence of a cysteine or a serine in the G-site, families such as Tau, Phi, Theta and Zeta present a serine, implicated in the formation and stabilisation of the thiolate anion of GSH, while a cysteine is found in the catalytic site in families such as GHR (Sylvestre-Gonon *et al.*, 2019).

GSTZ, GSTU, GSTF, GSTL and DHAR, hemerythrin and iota classes are plant specific GSTs (Liu *et al.*, 2013). GSTU and GSTF are the most abundant and have function related to xenobiotic detoxification (Frova, 2003, 2006). In *Arabidopsis*, these two GST classes have been confirmed to have wide substrate specificity (Dixon *et al.*, 2009) with GSTU/GSTF overexpression related with increased tolerance to abiotic stresses including salt, UV or herbicides, suggesting a protective role for these proteins.

Theta GSTs have a secondary activity, acting as glutathione peroxidases protecting the cells from cytotoxicity caused by hydroperoxides (Basantani and Srivastava, 2007; Chronopoulou *et al.*, 2010). Cummins *et al*., (1999) showed that MHR black-grass has a higher expression of *Am*GST2-2 compared with susceptible populations. This GST was later renamed and classified in the plant-specific family, the phi (F) class, as *Am*GSTF1. This protein has GPOX activity towards hydroperoxidases that were generated after the application of paraquat, chlorotoluron and fluorodifen herbicides. It was then suggested that the enhanced expression of this enzyme can result in resistance to FOPs that also

cause toxicity primary metabolism, that result in hydroperoxide generation (Andrews *et al.*, 2005).

1.3.6 *GSTs in NTSR Black-grass Populations*

GSTs use the products of the phase I herbicide detoxification pathway as substrates (figure 1.2). The first identification of the involvement of this family in herbicide detoxification dates to the 1970s, when the mechanism of detoxification of atrazine was elucidated in *Festucoideae*, *Panicoideae*, and *Eragrostoideae* subfamilies that include species such as *Digitaria*, *Panicum*, *Setaria*, *Sorghum* and *Zea*. Glutathione-atrazine conjugation was discovered in atrazine tolerant species by Jensen *et al.*, (1977). Afterward, glutathione conjugation of atrazine was also observed in velvetleaf atrazine resistant biotypes (Gray, Balke and Stoltenberg, 1996).

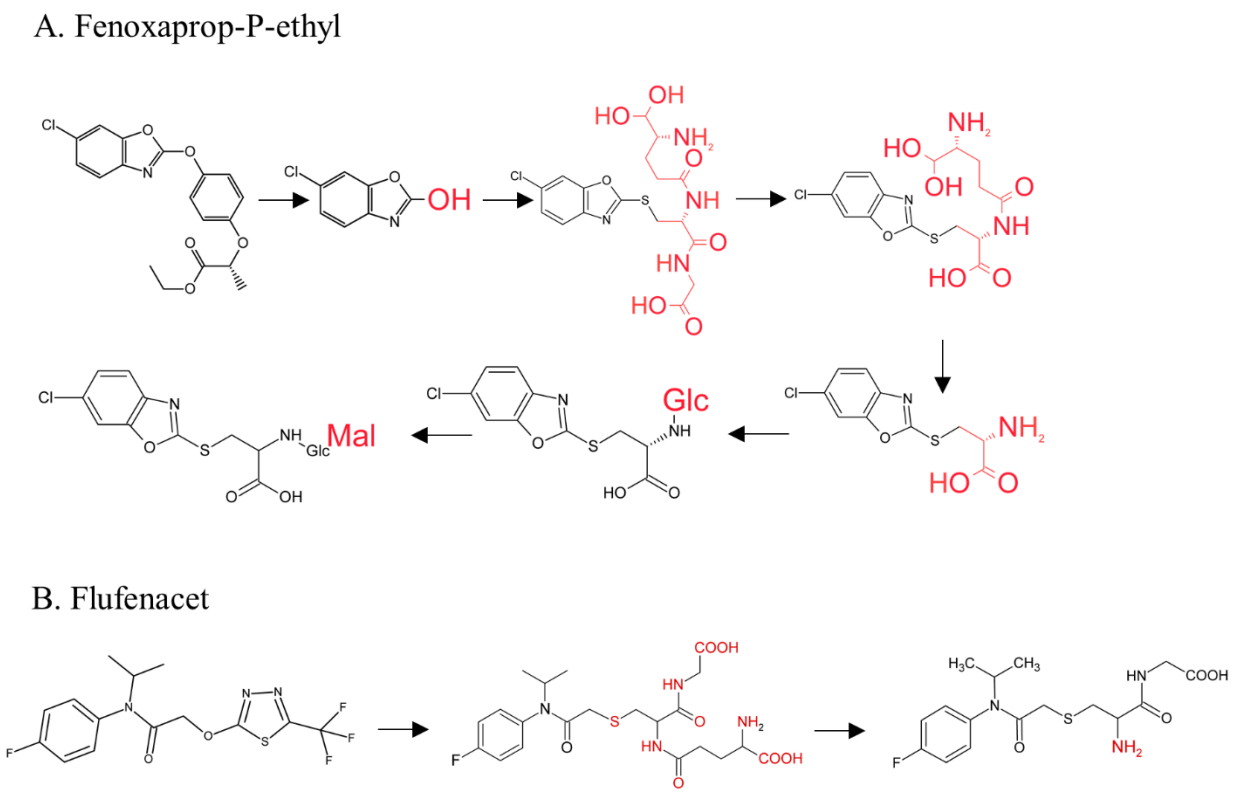


Figure 1. 2. GST metabolised black-grass herbicides and their primary metabolites. Modifications highlighted in red. A. Fenoxaprop-P-ethyl, glutathione conjugation, hydrolysis and further enzymatic activity; B. Flufenacet, glutathione conjugation, hydrolysis and undergoes further enzymatic activity similar to fenoxaprop.

GSTs detoxify major chemical classes of herbicides including triazine, chloroacetanilides, aryloxyphenoxypropionate, sulfonylureas, thiocarbamate and diphenylethers (Cole, 1994).

An overexpression of GSTs has been shown in weed species exhibiting NTSR such as wild oat (*Avena fatua* L.), annual ryegrass (Duhoux *et al.*, 2017), shortawn foxtail (*Alopecurus aequalis* Sobol.), (Zhao *et al.*, 2017) and black-grass (Tétard-Jones *et al.*, 2018).

Cummins *et al*., (1999) concluded that the high concentration of glutathione and an enhanced GST activity in MHR populations, was partially responsible for the resistance to GST detoxified herbicides such as fenoxaprop-P-ethyl. This herbicide is detoxified in crops by its rapid conjugation with GSH as compared with black-grass (Tal *et al.*, 1993; Hall, Moss and Powles, 1997). Whilst GSTF1 is not involved directly in herbicide metabolism Cummins *et al*., (1999, 2009) reported the constitutive expression of the enzyme in all MHR black-grass populations tested. Tétard-Jones *et al*., (2018) shed light on the complexity of the well-characterised NTSR Peldon population through a study that combined transcriptomics and proteomics. Sulphur assimilation, especially GSTs, gluconeogenesis and xenobiotic detoxification genes were enhanced in the NTSR population. Proteomic data also identified two isoforms of *Am*GSTF1 in field NTSR collected populations, as well as in experimentally selected populations resistant to fenoxaprop-P-ethyl; and pendimethalin herbicides. The pendimethalin selected population showed NTSR towards fenoxaprop-P-ethyl, clodinafop-propargyl and cycloxydim but not towards ALS or cell division inhibitors, additionally *Am*GSTF2 and *Am*GSTU2 were also identified through the proteomics in both MHR populations. *Am*GSTU2 showed the highest abundance and this combined to an elevation in the glutathione content has been proposed to give a protective effect from herbicide injury (Cummins *et al.*, 2013).

1.4 Diagnosing Herbicide Resistance

The impact that herbicide resistance has on current food production is only going to expand without intervention by scientists, agronomist and farmers. Weed management is vital to efficient production. Currently the best method of weed control is the use of herbicides alongside integrated weed management practices such as crop rotation and biological controls, to name just two. As new modes of herbicides are not coming onto the market with the regularity they once did in the 1980s, the careful use of the current herbicides is key. Currently predominant methods for the determination of resistance involve glasshouse pot trials, petri dish assays or enzymatic determination with plants propagated from seed, all of which consume considerable time and resources.

1.4.1 *Herbicide Resistance Testing*

Diagnosing herbicide resistance quickly and accurately will be increasingly important. The sooner a resistant population is found and characterised then the sooner alternative chemical controls can be put in place, such as swapping out an ALS inhibitor for an ACCase inhibitor where cross resistance has not been conferred. A common method of diagnosing resistance is a petri based test termed the 'Rothamsted Rapid Resistance Test' for ALS inhibiting herbicides (Hull, R. & Moss, S. R., 2007). This test involves collecting seeds in the field, sorting, cleaning and germinating them on filter paper with KNO_3, with and without herbicide. In this test, sulfometuron and mesosulfuron + iodosulfuron were used to measure ALS resistance. After 2 weeks the shoot length for each germinated seed is measured and the total shoot length calculated for each control and herbicide treated dish for all populations. The percentage reduction in shoot length is then converted to a resistance 'R' rating system. S =susceptible, R?, RR and RRR indicate higher degrees of resistance (Moss *et al.*, 1999). This and other similar methods of testing herbicide resistance are easily accessible and simple to use but take time and they only provide information and whether or not the herbicide has had an effect on the growth of the shoot. Other diagnostic techniques are required to gather more detail about the mechanisms of resistance.

1.4.2 *DNA Amplification*

Polymerase chain reaction (PCR) can be used in the laboratory to amplify potentially mutated gene sequences in TSR black-grass, with the single-nucleotide polymorphisms then identified by sequencing. This process usually take between 2-3 weeks as a commercial service. Loop-mediated isothermal amplification (LAMP) has been developed by as a simple and rapid gene amplification technique (Notomi *et al.*, 2000). This method employs a DNA polymerase and a set of specially designed primers that recognise distinct sequences on the target DNA. Recently, LAMP has been developed for rapid detection of TSR mutations in black-grass (Edwards and Onkokesung, 2020) and other grasses. Using a single-step reaction that utilises four to six probes that bind to DNA regions around the mutation site. Through the quantification of fluorescent products arising from their specific melting temperatures, LAMP generates results within 30–45 minutes. This test is conducted on a portable, battery operated device that can be used in the field. The results from this test can tell whether there is TSR and which mutations are present. This data can then be used to inform the grower about herbicide choices in the future based on previously characterised TSR populations.

1.4.3 *Protein Immunoassay*

A collaborative project between a number of UK universities and research institutes as part of the Black-grass resistance initiative (BGRI) delivered a black-grass resistance diagnostic (BReD) tool that can be used to detect NTSR in the field. Based on lateral flow assay test technology, it reveals if a protein known to be associated with NTSR is present. Such proteins, in this case the '*Am*GSTF1' protein, are called 'biomarkers'.

The test can diagnose the presence of MHR within 10 minutes of taking a black-grass leaf sample. The level of resistance can also be quantified – the stronger the test line on the diagnostic device, the more of the biomarker protein is present. The *Am*GSTF1 protein is now known to play a central role in black-grass NSTR. In fact, it is believed that it is likely to play a similar role in other grasses and further experiments are being carried out to determine if this is the case. The coupling of the LAMP assay with the BReD test kit could allow farmers, researchers and agronomists to enter a field of resistant black-grass and systematically sample the plant tissue and generate a map of TSR and NTSR in a short space of time. This data can then inform the farmer to formulate a herbicide management strategy to efficiently use a range of herbicides based on the effect determined from the current regime.

A limitation of the NTSR test in its current form is that, at this time, it is only able to quantify the presence of one protein associated with NTSR and not any others that may be involved in herbicide detoxification.

Recently, a pilot study was conducted to determine the feasibility of utilising diversity arrays technology (DArT) as a faster and cheaper method for herbicide resistance testing (Preston *et al*., 2014). DArT is a high throughput genotyping technology which utilises microarrays for the discovery of genetic markers. The aim was to identify genetic markers in resistant and susceptible biotypes of annual ryegrass and to assess the ability of DArT to discriminate between herbicide resistant phenotypes in rye-grass. Whilst the study was able to produce a model that could differentiate between sensitive and resistant populations, it was not able to distinguish between TSR and NTSR populations due to the complexities of NTSR.

1.4.4 *Tandem liquid chromatography – Mass Spectrometry*

As discussed, it has been shown that there is a connection between rate of herbicide metabolism and NTSR. In order to explore the measurement of metabolite levels in NTSR weed population, a robust, simple and high-throughput method which is suitable to analyse metabolites from a wide range of herbicides is required. The gold standard way of doing

this would be to use radiolabelled herbicide metabolism assays coupled with high pressure (HPLC) – liquid chromatography (LC) preferably coupled to mass spectrometry (MS). For decades the use of radio labelled herbicides, normally labelled with ^{14}C, allows for complete quantitation of the herbicide, from absorption, translocation, modification and transport(DeBoer *et al.*, 2011), by using a scintillation counter to measure the radioactivity present, in different herbicide solutions, plant tissues and extracts (Roggenbuck *et al.*, 1993). Using HPLC to separate the metabolites of interest and MS to confirm the identity of the metabolite by its accurate mass is a comprehensive technique to detect herbicide detoxification processes (Budde, 2004). Major drawbacks to this technique are that the use of radio labelled chemicals in a laboratory is closely monitored, requiring stringent restrictions on experimental procedures, with separate labs built and dedicated equipment purchased. Radio labelled chemicals are also very expensive and their manufacture and use goes against the principles of green chemistry. In addition, in an agronomy setting being able to demonstrate diagnostics in a field setting such as the LAMP and BReD is key to demonstrating the potential to interested parties.

Using commonly available ^{12}C herbicides, HPLC-MS is routinely used for metabolite identification (Ducker *et al.*, 2019). Ultra-high pressure liquid chromatography (UHPLC) has now been developed, a standard HPLC run time could be from 20 – 60 minutes depending on how many analytes of interest there are to separate. UHPLC uses a solvent gradient as HPLC can be as short as 3 minutes. There are variety of mass spectrometers on the market that are now considered affordable, though with cost there is a correlation with sensitivity, resolution and suitability for the analytes of interest. A single quadropole detector can be purchased in the tens of thousands, where as an orbitrap coupled to a time of flight detector can exceed the seven figure mark. Matching the use of an LC-MS that suits budget, expertise, ability to analyse compounds of interest and developing a robust method is critical as the foundation of exploring a new MS based diagnostic tool.

1.5 Project Aims, Objectives and Hypotheses

As herbicide resistance continues to develop and spread in weed populations worldwide and there is a decreased commercialisation of new mode of action herbicides, weed control using chemicals is becoming progressively more difficult. As such the rapid and accurate diagnosing of resistance is increasingly important. Understanding how non-target site resistance influences enhanced rates of herbicide metabolism and detoxification is key to underpinning the potential of a new mass spectrometry based diagnostic test for NTSR.

The primary focus of this project was to investigate the development of a high throughput LCMS method to separate and identify herbicide metabolites of interest in wheat and black-grass. Then using this method, conduct feeding assays with multiple herbicides to investigate their detoxification rates and routes.

It was hypothesised that as all detoxifying enzymes cannot be up regulated at a single point in time, by treating weeds with multiples herbicide at the same time, dominant detoxification pathways could be identified in NTSR black-grass populations with respect to the herbicides used.

To undertake this investigation *Am*GSTF1 levels were first used as a biomarker of NSTR in black-grass. A suitable high throughput LCMS technique was developed that was capable of detecting metabolites of interest. Using this established method the metabolite levels of multiple herbicides in different plant species were then measured and analysed to determine whether different populations of resistant black-grass have evolved distinct detoxification pathway

Chapter 2. Materials and Methods

2.1 Plant Material

Winter wheat (*Triticum aestivum*, var. Cordiale 2016) was purchased from KWS, UK and used for all wheat experiments. Model herbicide sensitive and resistant (Peldon) black-grass (*Alopecurus myosuroides*) used for the herbicide mixture treatments were purchased from Herbiseed, UK. Herbicide sensitive (Rothamsted 09), resistant (Peldon) and field populations of black-grass (LongC, R30 and Velcourt) used for resistance testing were supplied by Rothamsted Research, Harpenden, UK.

2.2 Cell Cultures

The cell cultures were initiated and subcultured by. Nawaporn Onkokesung, whom I would like to extend my gratitude for doing so *Alopecurus myosuroides* populations used for initiation were , Rothamsted 09 - a herbicide sensitive, pendimethalin-selected population, resistant to 'fops', 'dens', and pendimethalin, a fenoxaprop-selected population, resistant to fenoxaprop-ethyl and Peldon 07- a field derived population resistant to Atlantis (mesosulfuron-methyl and iodosulfuron methyl), 'fops', 'dens', pendimethalin, and chlorotoluron.

A. *myosuroides* seeds were surface sterilised in sterile solution (5% sodium hypochlorite (NaOCl) containing 0.1% tween 20) for 15 minutes with periodic shaking. The solution was discarded, and seeds washed in sterile miliQ water 10 times, before placing on callus-induction medium (CIM) containing Linsmaier and Skoog (LS) basal mix including vitamins (Duchefa, The Netherlands), 3% (w/v) maltose, 7.5 mg L-1 2,4-D and 0.75% (w/v) phytagel (Sigma Aldrich). The medium was adjusted to pH 5.8 with 0.1mM KOH (Hunt et al., 2013). CIM cultures were kept at 4°C in the dark for 2 weeks before moving to a growth cabinet (18°C). After 3-5 weeks, yellowish calli forming around the coleoptile were transferred to the fresh CIM. After 6 weeks, coleoptile calli were divided pieces and 10 small pieces were transferred to 15mL of liquid culture medium containing Linsmaier and Skoog (LS) basal mix including vitamins (Duchefa, The Netherlands), 3% (w/v) maltose, 7.5 mg L-1 2,4-D and 0.75mg L-1 kinetin adjusted to pH 5.8 by 0.1mM KOH (Hunt et al., 2013). Liquid cultures were maintained on an orbital shaker (130rpm), 25°C in the dark. After 2 weeks, 15mL of fresh liquid culture was added. The fresh liquid culture (1:1 ratio) was added every 3 weeks to a final volume of 100mL. At this point, the cell suspension was established and maintained by sub-culture (1:3 ratio) with fresh liquid medium every 10 days. The 10d sub-culture cycles were repeated for 4 cycles before cells

were used for experiments. Cells were used 4 days after subculture (cells entering exponential growth phase) and tested for viability before each experiment by performing 2,3,5-triphenyltetrazolium chloride (TTC) assay. 1% (w/v) TTC (Sigma Aldrich) in 50mM potassium phosphate buffer, pH 7.2 was added to equal volume of suspension culture (1:1 ratio). Cells were kept at room temperature in the dark for 4h without agitation. Metabolically active cells (viable cells) reduce TTC (colourless) to red formazan (1,3,5-triphneylformazan) through the action of dehydrogenases, while inactive cells (non-viable) remain colourless after 4 hours .

2.3 *Wheat and Black-grass Growth Conditions*

Seeds were germinated in 90mm petri dishes on three layers of filter paper with a 90mm glass fibre filter on top, 7mL, 20mM KNO_3, then wrapped in foil and stratified at 4°C for 4 days. After 4 days, the foil was removed, and plates placed into a growth chamber with day cycle 6:00-22:00 (16 hours, 18°C) and night cycle from 22:00-6:00 (8 hours,16°C). During the day cycle, plants were exposed to a light intensity of 125-150 μmol m^2s^{-1} Photosynthetic photon flux density (PPFD), with the night cycle having no light. Germinated seeds of similar size were selected and transferred to pots containing soil from Cockle Park farm, Hebron, Northumberland, 55°13'26.2"N 1°41'09.5"W. Plants were then grown in the growth chambers to the 2-leaf stage of growth and treated after 21 days.

2.4 *Arabidopsis* Transformed with *Am*GSTF1

2.4.1 *Root Culture Growth Conditions*

Arabidopsis seeds, transformed with AmGSTF1 under the control of the CaMV35S promotor (Cummins *et al.,* 2013), were sterilized in 5% bleach, washed five times with sterile water and germinated in half-strength Murashige and Skoog basal salt medium supplemented with 3% sucrose (w/v) and 0.5% Phytagel (w/v), then stratified for 4 days at 4°C. The seeds were then placed in a growth chamber (12hr/12hrs, light/dark, 21/18°C) for 8 days. After 8 days 5 seedlings from each line were placed into 250mL conical flasks containing 100mL full strength Gamborg's B-5 Basal medium with 5% (w/v) sucrose to generate root cultures (Huang and Mā, 1992). The root cultures were then placed in a rotary shaker (dark, 120rpm, 25°C) and treated after 14 days.

2.4.2 *Leaf Tissue Growth Conditions*

Arabidopsis seeds were sterilized in 5% bleach, washed five times with sterile water and planted on half-strength Murashige and Skoog basal salt medium supplemented with 3% sucrose and 0.5% Phytagel (w/v) then stratified for 4 days at 4°C. The seeds were then placed in a growth chamber (12hr/12hrs, light/dark, 21°C) for 8 days. After 8 days the

seeds were transplanted into John Innes No.2 and placed in a growth chamber (12hr/12hrs, light/dark, 21°C) and treated after 21 days.

2.5 Protein Extraction

Root tissue was ground in liquid nitrogen, and the powder placed in 15ml falcon tubes on ice, prior to use. 250mg of ground tissue was transferred to pre-chilled 2mL microfuge tubes and 3x V/W protein extraction buffer (50 mM Tris-HCl, 2 mM EDTA, 1 mM DTT, pH 7.5), together with PVPP (50g/l) added. The sample was vortexed until no clumps were visible and incubated on ice for 10 minutes then centrifuged, 18,000g, 4°C, 15mins. The supernatant was decanted into a clean microfuge tube and stored at -20°C.

2.5.1 *Protein Quantification*

A standard curve of gamma globulin in protein extraction buffer was generated at concentrations 0f 125 to 2000 mg/mL. Twenty µL samples were pipetted into cuvettes and 1mL of Quick Start™ Bradford Reagent added to each cuvette and incubated at room temperature for 5 mins. All samples and standard were analysed in duplicate. The absorbance readings were taken at 595 nm using a Shimadzu UV-1800 spectrophotometer

2.6 *Am*GSTF1 sandwich ELISA

*Am*GSTF1 protein concentration was quantified by Enzyme-Linked Immunosorbent Assay (ELISA) using sheep-antibodies specific to the *A. Myosuroides* GSTF1 protein
A 96 well ELISA plate was coated with 100µl primary antiserum S909-D, (1 ug/mL) in phosphate buffered saline (PBS), and stored at 4°C for 18 hrs. The plate was washed 4 times in a Thermo Scientific™ Wellwash™ Versa Microplate Washer, with 0.1% (v/v) Tween20 in PBS (PBST), blocked with 200ul PBS in 1% bovine serum albumin (BSA) for 1 hr at room temperature. The plate was washed 4 times with PBST. A 1:3 serial dilution of PBST containing 1% BSA was used to prepare recombinant *Am*GSTF1 over a concentration range of 1µg/mL to 1.4 ng/mL. Protein samples were diluted with 100µg/mL in PBST + 1% BSA, then 100µl of *Am*GSTF1 serial dilution and samples added to individual wells. The plate was incubated, 1hr, room temperature, shaking at 150rpm. The plate was washed 4 times with PBST. The secondary antibody conjugated with horseradish peroxidase (S908D-HRP) stock, was diluted 1:50,000 and 100µL added per well. The plate was incubated, 1hr, room temperature, shaking at 150rpm. The plate was washed 4 times with PBST and 100µL 3,3', 5,5; -tetramethylbenzidine (TMB) added to each well then the plate was incubated at room temperature in the dark for 30 min prior to reading on a Bio-Rad iMark™ Microplate Absorbance Reader at 655nm. The reaction was then stopped by the addition of 50µL 1M HCL and the plate reread at 450nm. The

absorbances read at 450nm were used for calculation. The concentration of *Am*GSTF1 protein in samples was calculated from the standard curve, four parametric logistic (4-PL) regression fitting, of recombinant *Am*GSTF1 protein.

2.7 Acetolactate Detection

The ALS activity was determined based on quantifying acetoin formed from acetolactate To extract the acetolactate, 4cm lengths of black-grass leaves were crushed in a 2mL microfuge tube, water added and incubated at 25°C for 45 mins. The sample was centrifuged (5000r.c.f), 5mins, and the supernatant syringe-filtered (0.45µM). The filtrate was placed into a fresh tube and incubated with 10µL 6N H_2SO_4 at 60°C for 30 minutes. A 200µL aliquot of the sample was added to 2mL spectrophotometry reagent (0.1% creatine in water and 1% napthol solution in 2.5M NaOH). After 30 minutes the absorbance of the samples were read at 530nm and the results were quantified using by a standard curve prepared using authentic acetoin (Sigma Aldrich.).

2.8 Genomic DNA Extraction

Black-grass tissue (100mg) was placed in a 2 mL microfuge tube containing 1200 µL 'Edwards' buffer (Edwards, Johnstone and Thompson, 1991), and 5mm stainless steel ball. Samples were homogenised at 30Hz for 2 mins using a Retsch® Mixer Mill MM400, centrifuged, (4 °C, 13500rpm, 5mins), The supernatant (500µL) supernatant transferred to a clean tube with isopropanol (Sigma Aldrich), inverted and stored at 4°C overnight. Samples were then centrifuged again 4°C, 13500rpm, 5mins and the supernatant discarded and the pellet transferred to a DNA separation column (Monarch® gDNA Purification Columns, New England Biolabs) and washed with 70% EtOH. The recovered DNA was dissolved in nuclease free water (Invitrogen™) and was purified (QIAquick PCR Purification Kit) and quantified (NanoDrop™ Lite) as recommended by the respective manufacturers before PCR.

2.8.1 *PCR of gDNA for TSR Mutation Detection*.

Extracted black-grass DNA was diluted to 500ng/20µL and 50µl PCR sample mixture prepared as shown (Table 2.1). The program used for PCR (Eppendorf Mastercycler®) is shown in (Table 2.2). For sequencing (Eurofins Genomics) 100ng of DNA was added to 2.5µL each forward and reverse sequencing primers (Table 5.1). The chromatogram from the sequence sample was then compared to the wild type to identify any mutations using Sequencher v5.4.6.

Table 2. 1. PCR Mixture of gDNA from Black-grass

Mixture	**Volume (µl)**
-RNase free H_2O	31.5
HF Buffer (Phusion, New England Biolabs)	10
dNTPs	1
10mM FWD Primer	2.5
10mM REV Primer	2.5
Phusion® High Fidelity DNA Polymerase	0.5
DNA (50ng)	2

Table 2. 2. PCR Amplification Program

Phase	**Cycle**	**Temperature (°C)**	**Duration**
Initial denaturalisation	1	98	30 sec
Denaturalisation	39	98	10 sec
Annealing		60	20 sec
Extension		72	1 min
Final Extension	1	72	5 min

2.9 Herbicide Metabolism Studies

Analytical standard herbicide stocks (40mM) of flufenacet, chlorotoluron, pendimethalin, mesosulfuron-methyl, fenoxaprop-P-ethyl (Sigma Aldrich) and pyroxsulam (Greyhound Chromatography) were prepared in dimethyl sulphoxide (DMSO). The respective herbicides were then diluted to their final concentrations in the media to be used for the metabolism studies.

2.9.1 *Arabidopsis Root Cultures*

The old media was removed from the roots cultures and fresh media (full strength Gamborg's B-5 Basal medium with 5% (w/v) sucrose), dosed with the corresponding herbicide. After 6 hours the roots were removed from the flasks and washed in LCMS grade acetonitrile, after removing excess herbicide by blotting onto blue roll. The roots were weighed, wrapped in foil, flash frozen in liquid N_2 and stored at -80°C.

2.9.2 *Arabidopsis Leaf Tissue*

Plants grown as described in Section 2.4.2. were removed from the soil with the root intact and weighed. The plants were then placed in 30mL herbicide treatment solution in half strength MS, in a 50mL falcon, for 6 hours. After 6 hours excess herbicide blotted onto blue roll then the foliage wrapped in foil, flash frozen in liquid N_2 and stored at -80°C.

2.9.3 *Herbicide Mixture in Wheat, Sensitive and Resistant Black-grass*

Plant tissue was grown as described in Section 2.3.

2.9.4 *Excised Shoot Assay*

Plants were cut above the soil, weighed and 5mm trimmed from the bottom whilst immersed, and placed into a 7ml amber hydroponic vial contain 5mL of the relevant herbicide/herbicide mixture in half-strength MS . The plants were incubated for the requisite time period. After appropriate time period, plants were removed from the vial, blotted with blue roll, wrapped in foil, flash frozen in liquid N_2 and stored at -80°C.

2.9.5 *Floating Leaf Assay*

Plants were cut above the soil, weighed, 5mm trimmed from the bottom under water, cut in to 3 approximately equal lengths. The tissue was then placed on top of 50mL herbicide mix in half-strength Murashige and Skoog basal salt medium in a 90cm petri dish. The plants were then left under growing conditions for the requisite time period. Plants were removed from the vial, blotted on blue roll wrapped in foil, flash frozen in liquid N_2 and stored at -80°C.

2.9.6 *Herbicide Mixture in Cell Cultures*

Four days after sub-culture, 100mL media was split into three sterile conical 100mL flasks. After sedimenting the cells, the media was removed and 25mL fresh media (LS basal mix including vitamins, 3% (w/v) maltose, 7.5 mg L-1 2,4-D and 0.75mg L-1 kinetin), spiked with herbicide to the final concentration was added to the cells. Cultures were then incubated prior to vacuum filtration through filter paper (47mm, Whatman), then weighed, wrapped in foil, flash frozen in liquid N_2 and stored at -80°C.

2.10 Herbicide and CytochromeP450 Inhibitors in Wheat and Herbicide Sensitive and Resistant Black-grass.

Following the same excised shoot assay procedure, (section 2.9.4), the plants were placed in 5mL 40µM inhibitor, in half-strength Murashige and Skoog basal salt medium, for 1 hour, in a 7ml amber hydroponic vial before transferring to the herbicide mixture in a 7ml amber hydroponic vial for 6 hours. The plants were then weighed, wrapped in foil, flash frozen in liquid N_2 and stored at -80°C.

2.11 Inhibition of CYP81-2 and Effect on Pyroxsulam Metabolism

Recombinant *Ta*CYP81-2 was expressed in yeast by Dr Sara Franco-Ortega and microsome purified by differential centrifugation. The reaction mixture was made up as shown (Table 2.3) with 10µL NADPH. Three replicates were run and a negative control, with 10µL $NaHCO_3$ instead of NADPH. The reaction mixture was incubated for 20mins at 25°C.

Table 2. 3 Microsome Reaction Mixture

Reagent	Stock concentration	Reaction concentration	Volume per reaction
Tris-HCL, pH 7.5	1 mM	50 mM	5 µL
Pyroxsulam	5 mM	200 µM	4 µL
Malathion	5 mM	40 µM	0.8 µL
H_2O	n/a	n/a	63.5 µL
*Ta*CYP81-2	15mg mL^{-1}	2.5 mg mL^{-1}	16.7 µL

The reaction was stopped by adding 100µL acetonitrile:HCl (99:1) and vortexed. The samples were stored at -20°C and thawed before analysis.

2.12 Metabolite Extraction

Cell or plant tissue was ground in liquid nitrogen using a mortar and pestle then placed clean falcon tubes with 3x v/w 80% LCMS MeOH, rotated for 24 hrs in an end over end rotator, centrifuged (5000r.c.f, 5mins,4°C) and the supernatant was stored at -20°C.

2.13 Recovery of Modified Metabolites in Wheat Plants and Black-grass Cell Cultures by Cellulase Assay

Using herbicide metabolites extracts prepared according to 2.12, 500µL was transferred to a 1.5mL tube, x 2, then dried under vacuum. Samples were then resuspended in 0.15M citrate phosphate buffer, pH 5.0, in the presence or absence of 1mg ml^{-1} cellulase (Cellulase from Trichoderma sp., Sigma Aldrich). After incubation at 30°C for 24 hr, samples were partitioned with ethyl acetate, dried down under vacuum and resuspended in 80% LCMS MeOH.

2.14 Herbicide Metabolite Analysis by LCMS

Analysis carried out on the Q-ToF and REIMS was carried out at Newcastle University. Analysis on the IMS-Q-ToF and RADIAN was carried out at Waters, Wilmslow, UK. Samples were prepared for LCMS by centrifugation at 10000xg for 30 seconds and an appropriate amount of supernatant transferred to an amber vial containing a 300µl fixed insert (Chromacol).

2.14.1 *Q-ToF*

For herbicide analysis, liquid chromatography tandem mass spectrometry systems was used, a quadrupole time of flight (Q-ToF) instrument for metabolite identification, During identification, the ultra-performance liquid chromatography (UPLC) instrument used was a Waters Acquity I class flow through needle (FTN) coupled to a Waters Xevo G2-XS quadrupole time of flight (Q-ToF) mass spectrometer (MS). For each sample, an injection volume of 5 µl was taken from the 100 µl sample. Sample separation was performed using an Acquity UPLC Ethylene Bridged Hybrid (BEH) C18 1.7µm reverse phase column, dimensions, 130Å, 1.7 µm, 2.1 mm x 50 mm. Run time 5mins, 0.500mL/min. Solvent A: Waters, B: Acetonitrile. 2.00min, 50.0%A, 50.0%B, 4.50min 5.0% A, 95.0% B, 5.00min 95.0% A, 5.0%B. The solvents used LCMS grade water and acetonitrile (Optima™, Fisher Scientific) each containing 0.1% formic acid (Optima™, Fisher Scientific). When using negative ionisation, a capillary voltage of 2kV was used with 3kV used for positive ionisation together with a source temperature of 120ºC, a desolvation temperature of 600ºC and a gas flow rate of 800L/hr were employed. Full scan, mass range 50 – 1200 amu Identification of mass ions was carried out using MassLynx V4.2.

2.14.2 *IMS Q-ToF*

The same method used in section 2.13.1 was used for the IMS-Q-ToF.

2.14.3 *Metabolomics in* Arabidopsis

The metabolomics study was run on the Acquity I-Class coupled to the Q-ToF with a longer column and run time.

C-18 (100 × 2.1 mm); particle size, 1.7 µm; (Acquity, BEH). Injection volume 5µL. Run time 12 mins. Solvent A: Water, B: Acetonitrile. 12.00min, 0.500mL/min 95.0%A, 5.0%B 0.1% formic acid. ES+, capillary (kV) 0.7, source temp (°C) 120, desolvation temperature (°C) 600, cone gas flow (L/Hr) 50.0, desolvation gas flow (L/Hr) 800.0.

ES-, capillary (kV) 2.0, source temp (°C) 120, desolvation temperature (°C) 600, Cone Gas Flow (L/Hr) 50.0, desolvation gas flow (L/Hr) 800.0.

Full scan, mass range 50 – 1200 amu. Data analysed using Progenesis QI v 2.4.

2.15 Herbicide Analysis by Direct Sampling

2.15.1 *REIMS*

Sampling was performed using a REIMS source on a Xevo G2-XS Q-ToF instrument (Figure 3.12) using a monopolar cutting electrode iknife (Rigano *et al.*, 2019). The diathermy settings were on autocut mode at 20W and the cut time was for 3-5 s. Data was

acquired by Q-ToF MS in sensitivity mode and in negative and positive polarity at a scan rate of 1 scan s-1 over a mass range of 50-1200 m/z. Isopropanol was used as the dopant solvent at a flow rate of 100 µl min-1. Five biological replicates were sampled for each population and three technical replicates (or burns) performed per individual plant. The data was converted for analysis using Progenesis Bridge and analysed using Progenesis QI v 2.4.

2.14.2 *RADIAN*

Samples were introduced into the RADIAN (Figure 3.13) by pipetting 2µL of herbicide extract onto a glass capillary, letting the solvent evaporate for 15-20 seconds and placing the capillary into the source. 10 replicates were sampled to measure reproducibility, three technical replicates were sampled for each extract. The desolvation gas flow temperature and mass range was optimised for each herbicide (Section 3.6.2) Data analysed by MassLynx v4.2.

Chapter 3. Development of Mass Spectrometry in the Detection of Herbicides in Plant Extract

3.1 Introduction

Since the early 1970s, most routine pesticide residue analysis has been conducted by gas chromatography (GC) in combination with electron capture, nitrogen-phosphorous, and/or flame photometric detection (Alder *et al.*, 2006) . In recent years, liquid chromatography (LC) mass spectrometry (MS) has become a popular technique for the analysis of pesticide residues and their metabolites, due to its selectivity, sensitivity and speed of analysis (Tsipi Despina *et al.*, 2015), (Lin, Liu and Liu, 2009). The choice of chromatographic technique to use is based on the analyte. Thanks to the wide range of analysers and operation modes, liquid chromatography coupled with mass spectrometry (LC-MS), or tandem mass spectrometry (LC-MS/MS), enables the analysis of a wide range of compounds (Stachniuk and Fornal, 2016). The main advantages of LC-MS include high sensitivity, the ability to detect analytes of interest at a low concentration, and high selectivity, being able to identify analytes in complex mixtures. Different types of mass analysers and detectors can be combined with an ESI source to facilitate a mass spectrometer to determinate the molecular weights of peptides and proteins and allow the mass spectrometric sequencing of peptides. Since the ionization process takes place at atmospheric pressure with little thermal input, ESI is a soft ionization technique and is therefore especially suitable for biological samples such as proteins, non-covalent interaction complexes, peptides, drugs and DNA fragments (Müller, 2006).

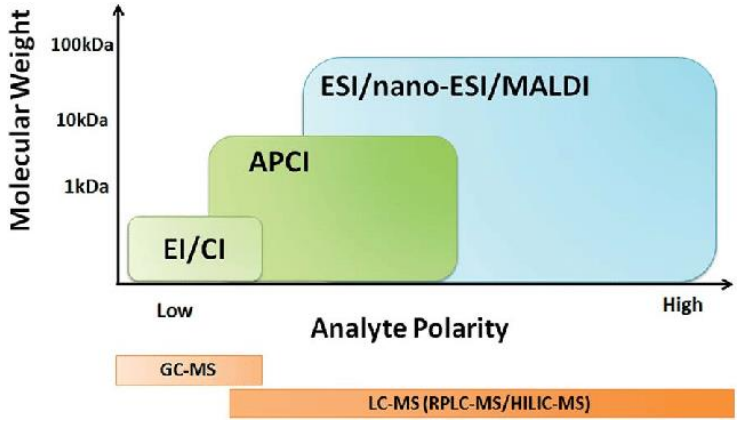


Figure 3. 1. Suitability of different ionisation sources for metabolomic analysis based on metabolite polarity and molecular weight (Wang *et al.*, 2015).

Newcastle University's Mass Spectrometry facility has a variety of GC-MS and LC-MS systems offering both high and low resolution mass detection. As a general statement, high resolution instruments are capable of determining atomic mass units (amu) accurately

up to 4 decimal places, whereas low resolution instruments are capable of accurately determining amu to a single decimal place. High resolution instrumentation allows for the separation of compounds with very similar masses, compared to low resolution . Due to the biological assays planned in this project, it was essential to use an MS system that was sensitive enough to detect low levels of known metabolites from a mixture of herbicides as well as being capable of running hundreds of samples. The LC system chosen to analyse herbicide chemistries that can range from low molecular mass semi-polar compounds to large polar metabolites, whereas GC is only suited to low molecular weight nonpolar molecules (Figure 3.1). The LC-MS used was a Acquity I Class UPLC coupled to a Xevo-G2-XS Quadrupole-Time of Flight (Q-ToF). The Acquity I-class is an ultra-high-pressure LC used to separate analytes of interest. The Q-ToF is designed for the identification and confirmation of the identity of a whole range of compounds in complex samples.

High-definition resolution mass spectrometry (HDRMS) has undergone a new phase of technological evolution. Time of flight instruments with significantly higher resolution and larger dynamic range have become available. Ion mobility spectrometry (IMS), when coupled with mass spectrometry, offers value-added data not possible from mass spectra alone. Separation of isomers, isobars, and conformers; reduction of chemical noise; and measurement of ion size are possible with the addition of ion mobility cells to mass spectrometers. In addition, structurally similar ions and ions of the same charge state can be separated into families of ions using a unique mass-mobility correlation line. Structural isomers of organic compounds such as pesticides can normally only be deduced using carbon-13 nuclear magnetic resonance (C-13 NMR) (Koskela, 2010). A HDRMS was used at Waters, Wilmslow, to investigate the possibility of detecting potential isomers of herbicide metabolites in plant extracts.

Ionisation occurs via electrospray ionisation (ESI), which is suited to ionising compounds spanning a large range of molecular weights and polarities (Siuzdak, 2004). ESI is a ‘soft’ ionisation technique in which little fragmentation is observed, with the parent molecular ion peak being the most abundant. Other ionisation techniques such as electron impact or chemical ionisation led to extensive fragmentation of the molecule. For direct analysis a dilute analyte solution is injected using a mechanical syringe through a capillary at a low flow rate. A very high voltage (2–6 kV) is then applied to the tip of the metal capillary relative to the surrounding source-sampling cone, or heated capillary. This electric field causes the dispersion of the sample solution into an aerosol of highly charged electrospray (ES) droplets (Figure 3.2) (Banerjee and Mazumdar, 2012). A nitrogen flow around the

capillary results in better nebulization. This flow also helps to direct the spray from the capillary tip towards the mass spectrometer. The charged droplets diminish in size by solvent evaporation, assisted by the flow of nitrogen, finally the charged analytes are released from the droplets, some of which pass through a sampling cone into the analyser of the mass spectrometer, which is kept under high vacuum.

One limitation of the LC-MS technique is the sensitivity to the interference by accompanying matrix components. Exogenic and endogenic substances present in the sample can interfere with the analyte in the ion source, resulting in an increased, or reduced, ionization of the analyte. This effect leads to the suppression or, much more rarely, enhancement of the analyte signal. Matrix effects and selectivity issues have long been associated with bioanalytical techniques (Smeraglia, Baldrey and Watson, 2002).However, the high incidence of matrix effects in liquid chromatographic tandem mass spectrometric (LC-MS/MS) methods has led to a greater understanding of the factors which contribute to these effects. A number of approaches have been investigated to improve reproducibility and robustness of LC-MS/MS methods that are subjected to matrix effect (Smeraglia, Baldrey and Watson, 2002).

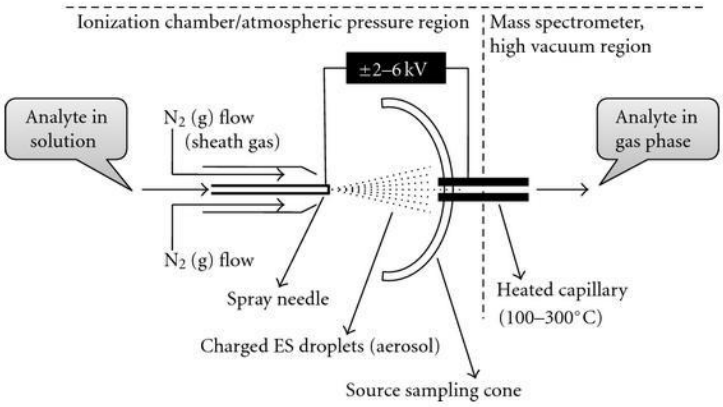


Figure 3. 2. A schematic representation of an ESI source (Banerjee and Mazumdar, 2012).

3.2 Chromatographic Separation

Having chosen to proceed with liquid chromatography to separate the analytes of interest, a reversed-phase chromatography method system was used. In reversed-phase chromatography, alkyl chains are covalently bound to a stationary phase to provide a hydrophobic surface, with the mobile phase being a more polar solvent containing solutes. As the mobile phase passes over the stationary phase, hydrophobic solutes tend to bind to the stationary phase, forming the basis of separation. With UPLC, chromatographic runs capable of separating a mixture of herbicides can be achieved in as short a time as three minutes. An ethylene bridged hybrid (BEH) 50mm column was used with an acetonitrile:

water gradient over five minutes to separate herbicides and their metabolites. The chromatographic settings selected had an isocratic flow of 50:50 acetonitrile: water for 2 minutes, followed by a 2.5 minute linear gradient to from 95:5 to 5:95, then back to 95:5 over 30 seconds, the total run time was 5 minutes, (section 2.13.1). To view the chromatogram, a total ion chromatogram (TIC) was generated. The TIC is a plot of the total ion current vs. retention time obtained from a chromatography experiment using mass detection (Yu *et al.*, 2009). For illustration, the retention times of the herbicides pendimethalin, chlorotoluron, pyroxsulam, mesosulfuron and fenoxaprop are shown (Figure 3.2). They elute at different times depending on their hydrophobic interactions with the stationary phase. The retention times between runs can vary slightly for a variety of reasons such as the condition of the column used, replacement of tubing or pumps and composition of the buffers. The retention times shown here are indicative of the results from the Acquity I-Class at Newcastle University.

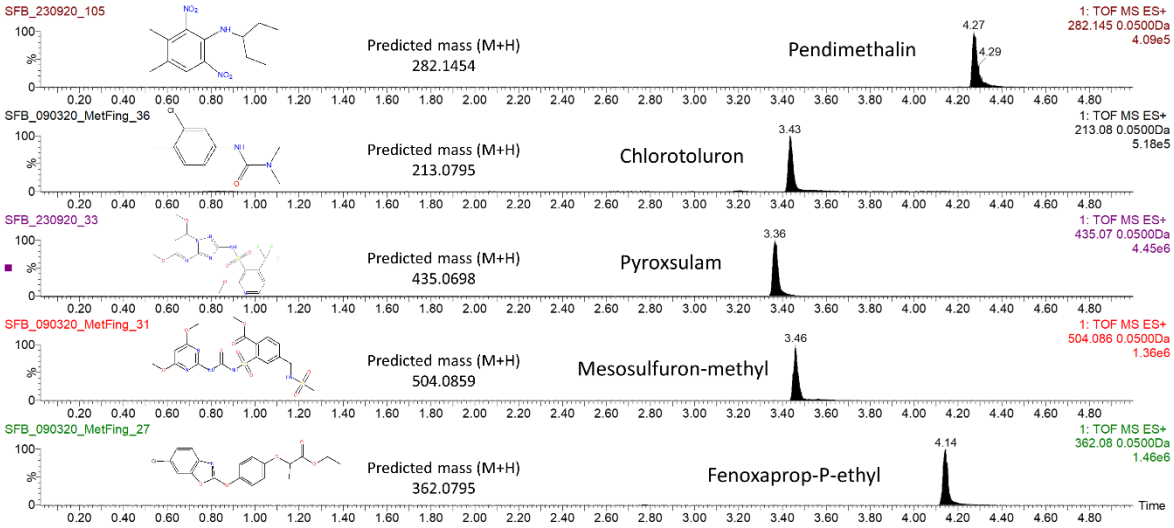


Figure 3. 3. Chromatogram displaying retention times of five herbicide standard; pendimethalin, chlorotoluron, pyroxsulam, mesolfuron-methyl and fenoxaprop-P-ethyl, in minutes. Retention time is shown above the shaded peak. Structures of the relevant compounds are shown on the left along with their predicted mass.

The primary metabolites of P450 detoxified herbicides are often hydroxylated, or demethylated products of the parent compound. These larger and more polar molecules tend to elute earlier than the parent compound. This is illustrated in the chromatograms of pyroxsulam, which elutes at 3.38mins, and its demethylated 5-OH product at 3.14mins, (Figure 3.4).

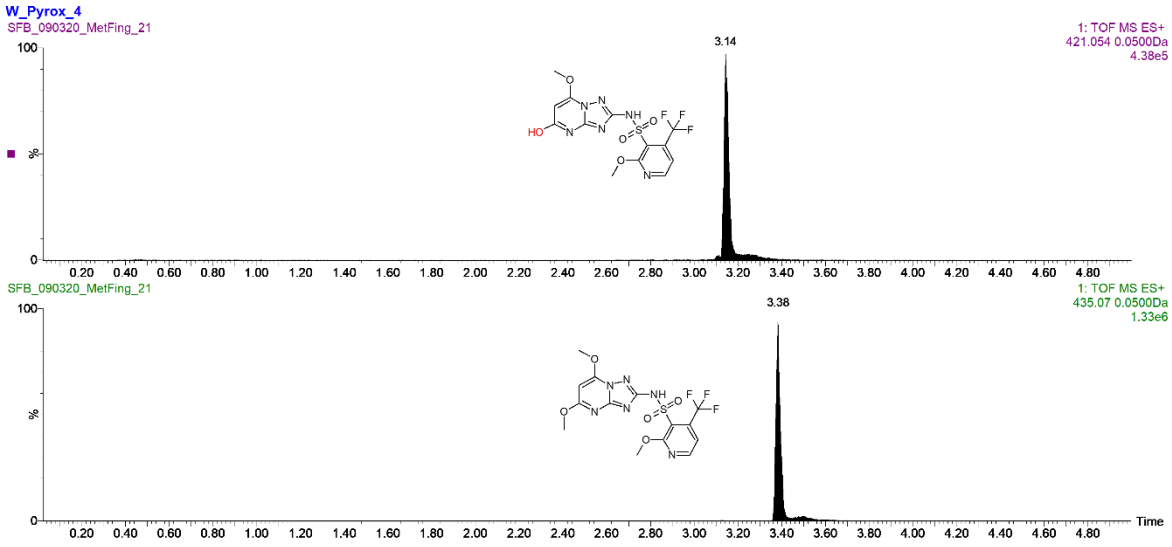


Figure 3. 4. Chromatogram showing peaks of pyroxsulam 5-OH metabolite(top) and pyroxsulam parent (bottom) with accompanying structure on the left of the peak.

3.3 Metabolite Detection

The Q-ToF is a 'hybrid' instrument combining quadrupole with a time-of-flight mass analyser (Figure 3.5) (Allen and McWhinney, 2019) . The first quadrupole (Q1) operates as a mass filter for the selection of specific ions based on their mass-to-charge ratio (m/z). The second quadrupole (Q2) collision cell, where ions are bombarded by neutral gas molecules such as nitrogen or argon, results in fragmentation of the ions by a process known as collision induced dissociation (CID). The resulting ions are pulsed by an electric field and enter a drift space where they separate according to mass, smaller ions having a shorter drift time while larger ions take longer to travel along the drift space. The instrument's reflectron also increases the flight path length which improves mass resolution (Allen and McWhinney, 2019). The mass of the ion can be calculated by rearranging the equation $E = 1/2mv^2$, where E = the energy of the electric field applied, v = the velocity of the ion, measured by the time it takes to travel across the drift field, to find m, which equals the mass of the ion. To establish that the LC-MS was capable of detecting known herbicide metabolites, a range of extracts from plants treated with herbicides was analysed by LC-MS to ensure that they could be detected based on their accurate mass and elemental composition. Wheat plants were treated with six herbicides using an excised shoot assay, as described in section 2.9.4, for 24 hours. In the list of metabolites tested all but one of the metabolites, which resulted from mesosulfuron cleavage (ii) were detected in positive mode (Table 3.1). This meant that all samples had to be run in both positive and negative ionisation mode when mesosulfuron-methyl was used as a treatment.

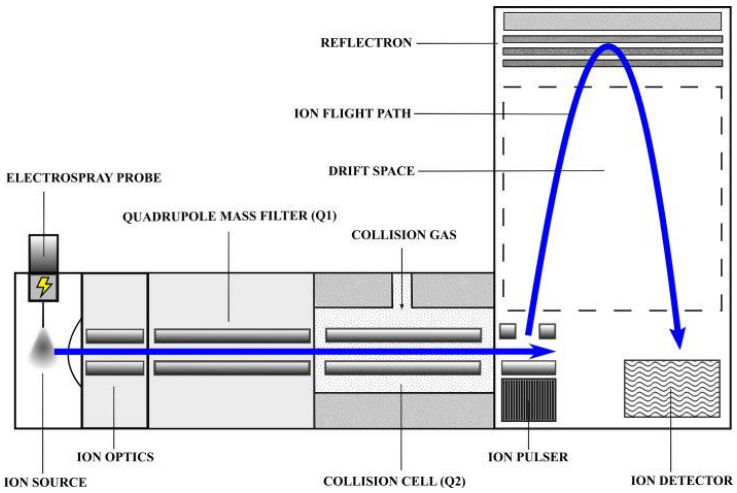


Figure 3. 5. Schematic diagram of a quadrupole time-of-flight mass spectrometer.

Table 3.1. List of herbicide metabolites and corresponding retention time and m/z value.

Herbicide	Metabolite	Retention Time (min)	[M+H] / [M-H]
Chlorotoluron	N-mono-demethylated	3.45	$[199.0638]^+$
	Hydroxylated and demethylated	3.02	$[211.0638]^+$
	Hydroxylated	3.36	$[229.0744]^+$
Pendimethalin	Hydroxylation	3.93	$[298.1403]^+$
	Carboxylic Acid	2.77	$[310.1039]^+$
Pyroxsulam	5-OH	3.16	$[421.0542]^-$
Fenoxaprop-P-ethyl	CBO-SG	3.04	$[459.0741]^+$
	CBO-CE	3.06	$[402.0527]^+$
	CBO-C	2.82	$[273.0101]^+$
Mesosulfuron-methyl	Demethylated	2.94	$[490.0702]^+$
	Cleavage (i)	2.65	$[323.0372]^+$
	Cleavage (ii)	2.88	$[288.9953]^-$
Flufenacet	Cysteine Conjugate	1.02*	$[315.1179]^+$

* Run on a 3 minute gradient compared to 5 for all other runs. Expected peak around 3.02min in a 5 minute run.

The molecular ion peak can vary it terms of its m/z value due to the calibration of the mass spectrometer. For example, 5-OH pyroxsulam, has an experimental m/z of 421.0435 whereas its calculated m/z is 421.0542,(Figure 3.6),. These errors can occur between runs so it was always a sensible approach to routinely check retention times and masses as a metabolite may not be detected, not because it is not present but because the Q-ToF has not been correctly calibrated.

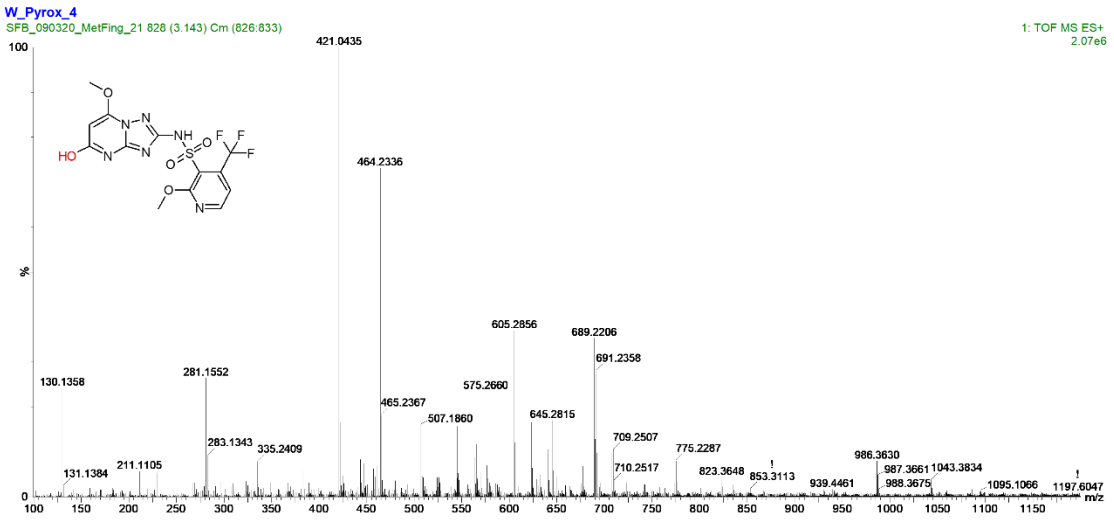


Figure 3. 6. Mass spectrum of pyroxsulam 5-OH, molecular ion peak 421.0435.

3.4. High-Definition High Resolution Mass Spectrometry

In the case of pyroxsulam three possible structural isomers of its 5-OH metabolite have been reported (DeBoer *et al.*, 2011), (Figure 3.7). Here, we wished to see if it was possible to deduce the structural isomers of metabolised pyroxsulam present in plant extracts from pyroxsulam treated wheat using HDRMS.

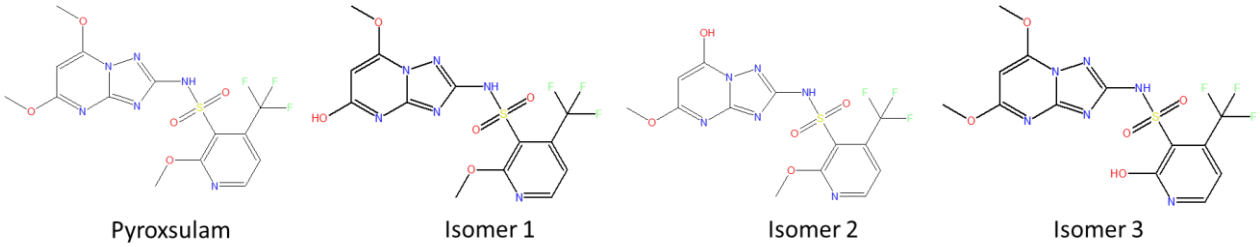


Figure 3. 7. Structures of pyroxsulam and the three potential isomers of pyroxsulam 5-OH. Demethylation (OH) highlighted in red.

Samples of wheat treated with pyroxsulam were analysed by the tandem Acquity I-Class Vion IMS-Q-ToF to observe whether these three isomers could be characterised using ion mobility spectrometry. IMS is used to measure the drift-time of an ion. The drift-time of an ion can be used to calculate the collision cross-section (CCS) in travelling wave ion mobility. Each structural isomer will have a unique CCS score, thus we can detect which isomers are in the plant extract by observing whether or compound has one or more CCS scores. By processing the result through metabolic software (Progenesis QI), it can be seen that the compound with a m/z of 421.0532, the calculated mass of pyroxsulam 5-OH, has a CCS of 184.5, with three possible identifications determined,(Figure 3.6).

Find a compound: Search | Filter compounds... | Filter is active

Compound	Neutral mass	m/z	z	Retention time	CCS	Peak Width	Tag	Accepted ID	Identifications
3.12_421.0532m/z	<unknown>	421.0532	1	3.12	184.50	0.13			3
3.76_334.0474m/z	<unknown>	334.0474	1	3.76	179.75	0.10			1
3.35_434.0617n	434.0617	435.0690	1	3.35	188.84	0.10			1
4.25_282.1442m/z	<unknown>	282.1442	1	4.25	161.08	0.06			1
3.35_435.0681m/z	<unknown>	435.0681	1	3.35	265.58	0.04			1
3.84_297.1321n	297.1321	298.1392	1	3.84	160.55	0.07			1
2.90_490.0693m/z	<unknown>	490.0693	1	2.90	199.50	0.09			1
3.03_402.0515m/z	<unknown>	402.0515	1	3.03	191.89	0.07			1

Figure 3. 8. List of compounds and associated m/z, retention time, CCS score and possible identifications.

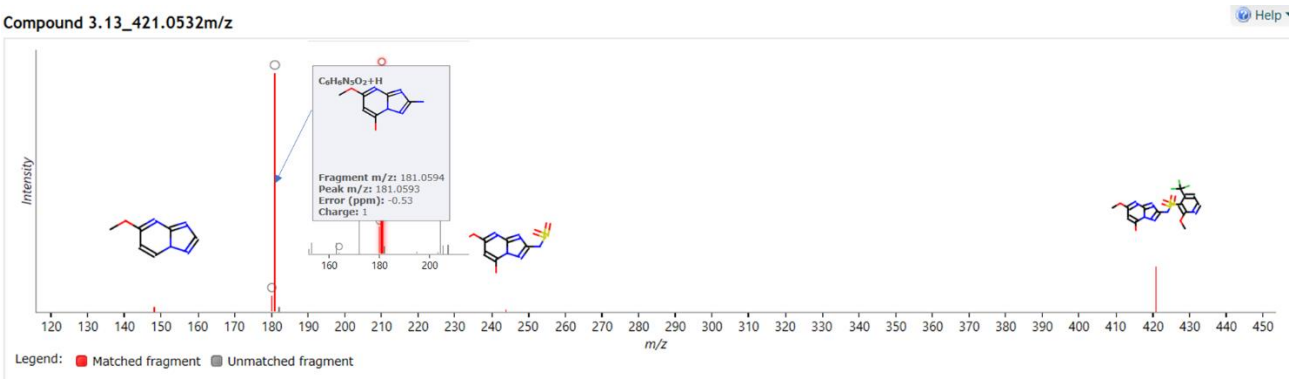


Figure 3. 9. Spectrum of fragments of compound 421.0532 (Pyroxsulam 5-OH).

From this analysis, by looking at the fragmentation pattern, isomer 3 can be ruled out due to the matched fragment at m/z 181.0594, highlighted in blue. This fragment is the result of demethylation at either C5 or C7 of the triazolopyrimidine ring. If the demethylation had occurred at the methylene carbon at C2 of the pyridine ring, a larger fragment, approximately m/z 193, due to the presence of two methoxy groups rather than one, would be observed (DeBoer *et al.*, 2011). The fragmentation pattern however did not discriminate between isomers 1 and 2, as only one CCS score was reported it could be ascribed to either isomer. The fragmentation data provides one very useful piece of information, the dominant fragment ion is a daughter ion, m/z 181.0594, rather than that of the parent, providing another molecular marker of pyroxsulam and its metabolites.

3.5 Matrix Effects of Wheat and Black-grass Extract on Ionisation of Herbicides

As many of the experiments carried will involve extracting herbicide metabolites from plant tissue into methanol, the sample matrix will contains high levels of soluble compunds found in plant tissue. As quantifying the metabolite levels will be based on running serial dilutions of herbicide standards in LC-MS grade methanol it is important to know if the data from metabolites in plant extract has to be corrected due to interference in the detection of metabolites. By comparing the response of herbicide standards in pure

methanol versus plant extract in methanol we can decide whether any data correction is required.

In order to observe any matrix effect in this study, a serial dilution of pyroxsulam was made in 80%MeOH extracts derived from the leaves of wheat, black-grass and methanol with no plant tissue extracted into it. 5µL of each sample was then injected in triplicate and the response measured in peak area units, the measurement of the area under the peak, (PAU). The results show that the ionisation of pyroxsulam in wheat and black-grass extracts is not suppressed when compared to levels seen in 80% MeOH alone, (Figure 3.10). The PAU seen with the 80%MeOH extract were actually lower than in wheat at 10µM and 100nM, this could be down to technical error when pipetting the stock solution. Encouragingly, very little variation was seen between the three biological replicates. This data suggests that the matrix will have a very minimal effect on the ionisation of pyroxsulam in the extract. As no metabolite standards are currently commercially available for the other herbicides used, an assumption was made that the oxidative modifications that form the metabolites will also not have an effect on the ionisation in terms of quantitation.

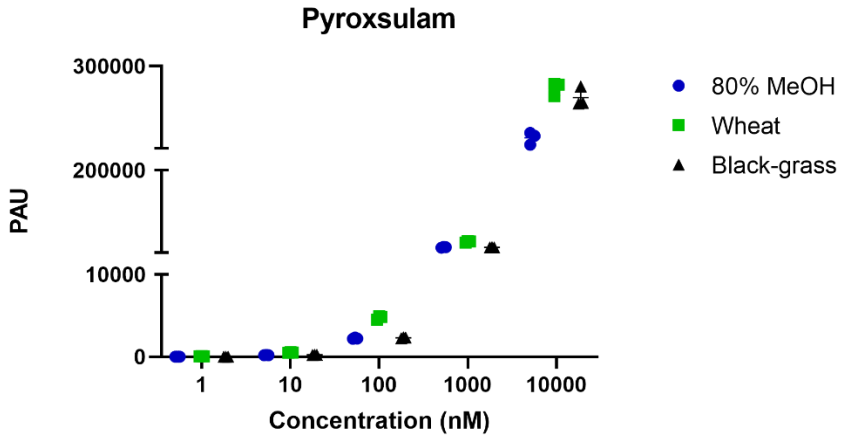


Figure 3. 10 Levels of pyroxsulam (PAU) from standard curves in 80% MeOH, wheat and black-grass extracts.

3.6 Direct Sampling Techniques to Rapidly Detect Herbicide Metabolites – A Future Tool

The current methods used to prepare samples for LC-MS requires time consuming manual preparation and a high expenditure on consumables. Plant samples are ground either manually or homogenised using a mixer mill. The ground tissue then has to be incubated in the extraction solvent of choice to remove as much analyte of interest as possible, before cleaning up the sample in preparation for LC-MS analysis. This can be done by syringe filtering every extract through a fine filter (0.45µM) or as commonly done, when large number of samples are processed, the samples are centrifuged at high speeds, then an

aliquot of the supernatant spun down again to sediment remaining debris before an aliquot is transferred to the sample vial. These lengthy stages of preparation are crucial when using tandem liquid chromatography - mass spectrometry for analysis. Small particles of tissue can block a separation column in the chromatography unit, when operating at high pressure (<16,000 psi) blockages causing expensive and time-consuming damage. Not preparing samples properly can also introduce unknown compounds leading to matrix effects as discussed (section 3.5). Direct mass spectrometry techniques coupled to a variety of instruments are being developed for multiple applications used in clinical medicine, food adulteration and speciation. These new sampling techniques include Direct Analysis in Real Time (DART), RADIAN Atmospheric Solids Analysis Probe (ASAP) and Rapid Evaporative Ionisation Mass Spectrometry (REIMS). These techniques provide an opportunity to explore whether or not they can be developed to replace, or supplement LC-MS analytical methods for herbicide metabolite detection.

3.6.1 *Rapid Evaporative Ionisation Mass Spectrometry*

Rapid Evaporative Ionisation Mass Spectrometry (REIMS) was originally developed by Zoltan Takats as an electrosurgery tool (Figure 3.12) used to differentiate the lipid profile between cancerous and non-cancerous tissue with a real time diagnosis during surgery (Balog *et al.*, 2015). The mass spectral fingerprints are generated by applying a radiofrequency electrical current directly to the sample using a stainless-steel monopolar probe. The resulting vapour, containing gas phase ions of metabolites and structural lipids, is then channelled in to a mass spectrometer (Xevo G2-XS) using the instrument's vacuum system, allowing for mass spectral data to be generated within one second of sample heating (Bolt *et al.*, 2016). The technology has since been acquired by and developed for further applications by Waters Corp. Applications include detecting food fraud, speciation and vegetable quality (Mason *et al.*, 2021). REIMS has been shown to be particularly good at lipid profiling. In the current study it was tested out on wheat treated with herbicides to see whether it could pick up any differences between treated and untreated wheat and identify any small molecules that are indicative of treatment, including the presence of herbicides and their respective metabolites.

Three-week-old wheat plants were cut, weighed, and placed in 40µM herbicide in ½ MS for 24hrs, containing seven herbicides (flufenacet, pendimethalin, pyroxsulam, mesosulfuron-methyl, chlorotoluron, iodosulfuron-methyl and paraquat) using 5 biological replicates and 3 untreated controls.

The output from metabolomics software produced a PCA chart displaying small molecule abundance (Figure 3.11). In this chart the untreated wheat samples (in orange) cluster to the right-hand side of the PCA chart. The wheat samples treated with flufenacet (in blue) clustered to the top and bottom side of the chart. In the case shown, PCA generally is a descriptive chart that can help visualise the statistical difference in the small molecule abundance between flufenacet-treated and untreated wheat. Whilst there is a statistical difference in the small molecule abundance, REIMS was not able to identify any of the herbicides or metabolites. The 162 m/z's, (Appendix A.1) that were statistically abundant (P value <0.05, max fold change >2), were run through the LipidBlast and Chemspider databases. These searches provided no matches, even though it was known that the herbicides and metabolites are present in the plants when analysed by LC-MS using the same ToF detector, (Table 3.1).

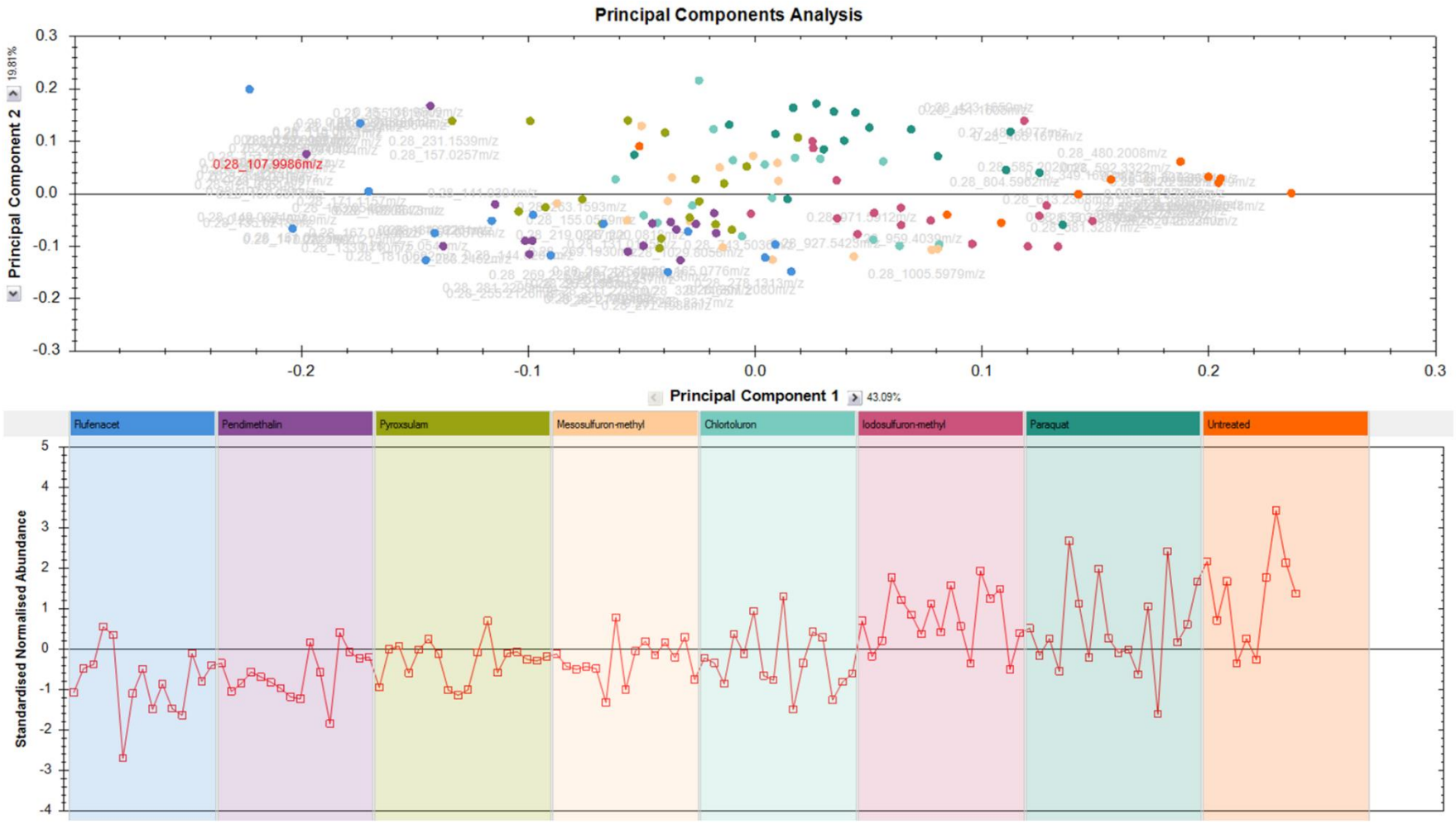


Figure 3. 11 PCA plot (top) and standardised normalised abundance (bottom) of all samples of 15 cuts for each herbicide and untreated samples. Flufenacet, Pendimethalin, Pyroxsulam, Mesosulfuron-methyl, Chlorotoluron, Iodosulfuron-methyl and Paraquat.

One possible explanation is that this experiment was run in negative mode, as this is best for lipid profiling, while the majority of the herbicides and their metabolites used in this experiment ionise primarily in positive mode. Repeating the experiment in positive mode may therefore allow for the detection of the herbicides and metabolites. Another explanation for the lack of herbicide detection could be down to the sampling technique. Burning the plant tissues with the diathermy knife is a destructive technique with a high thermal input that may cause the degradation of herbicides and their metabolites.

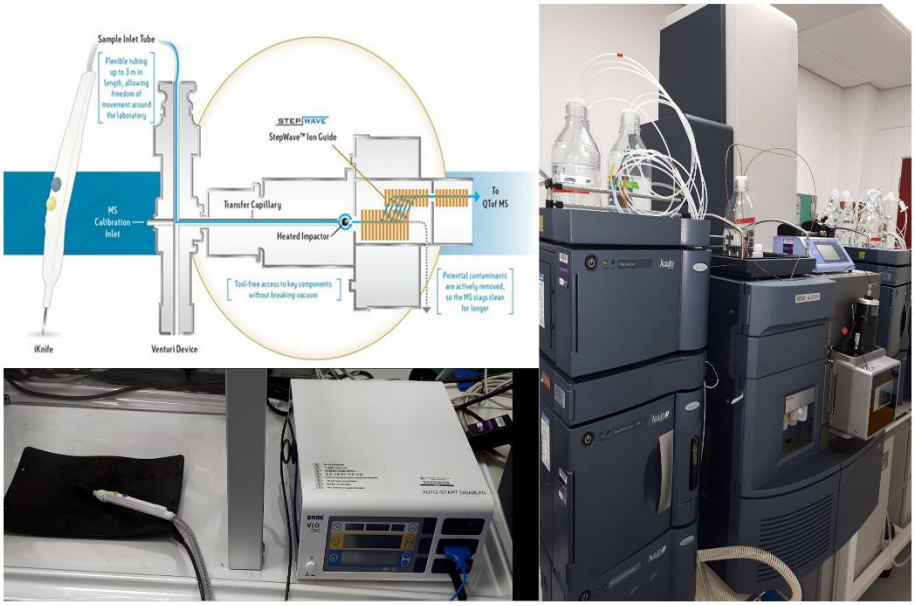


Figure 3. 12. Top left, diagram of REIMS inlet source, Waters. Bottom left, iKnife. Right, Xevo G2-XS Q-ToF, Waters.

3.6.2 *RADIAN ASAP*

The RADIAN ASAP is very recent addition to the market (Figure 3.13) and is based on an established ACQUITY QDa Mass Detector coupled with the Atmospheric Solids Analysis Probe (ASAP) technique for direct sample introduction. The QDa is a single quadrupole mass spectrometer, having a lower resolution and sensitivity compared to the Q-ToF but is smaller, more portable and requires only basic training to be able to operate. The ionisation mechanism in ASAP is Atmospheric Pressure Chemical Ionization (APCI), which is applicable for lower molecular weight, semi-volatile and volatile compounds ranging from the polar to the mid polar.

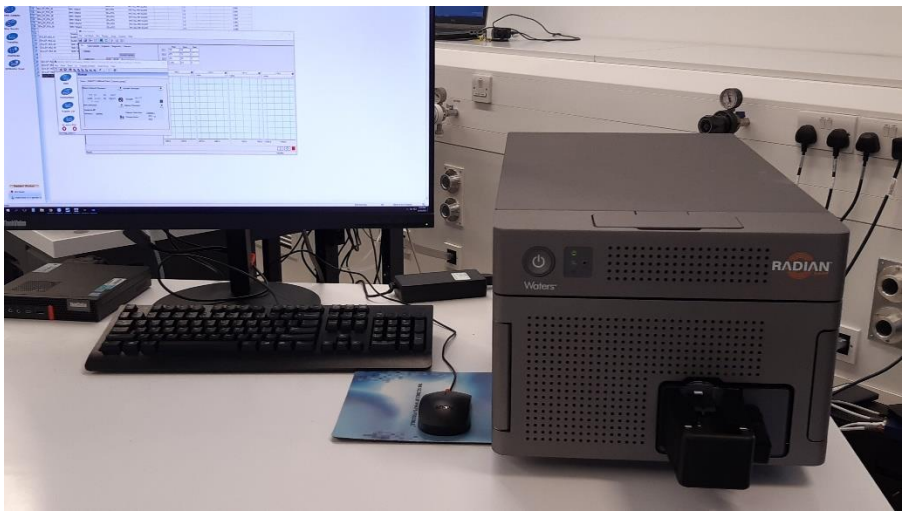


Figure 3. 13. RADIAN ASAP detector, Waters, Wilmslow.

To determine an approximation of the limit of quantitation of a selection of herbicides in the RADIAN, standard curves of pyroxsulam, pendimethalin and chlorotoluron were made in 80%MeOH and black-grass extract and analysed. Sample introduction was performed by pipetting a fixed amount of standard (2µL) on to a glass capillary and letting the solvent evaporate for ~15s. Prior to use, the capillary was loaded into the instrument and cleaned using a 'bakeout' function to remove any contamination on the surface. In order to establish that the sampling method provides a reproducible response, 10 samples of pendimethalin (2.8ug/mL) were analysed and the percentage relative standard deviation calculated with a percentage RSD under 20% considered consistent. The total ion chromatogram (TIC) from sampling 2uL of pendimethalin, after letting the solvent evaporate, provides the response in PAU for each injection (Figure 3.14). The percentage relative standard deviation was 19% so the method was of sufficient quality to repeat for the rest of the samples. To optimise the ionisation of each herbicide, the gas flow temperature and the range of mass detection can be altered based upon its molecular weight (mw) and polarity. Chlorotoluron (mw 212.67) and pendimethalin (mw 281.31) have a significantly lower molecular weight than pyroxsulam, (mw 434.35). Pyroxsulam has more polar substituents than either chlorotoluron or pendimethalin, three methoxy and a sulphonamide group. Based on this chemical information it is predicted that chlorotoluron and pendimethalin are more chemically suited to APCI than pyroxsulam. This prediction was confirmed in the spectra generated by sampling the standards, (Figure 3.15). At 300°C the chlortoluron and pendimethalin standards were detected at levels of 13.3 µg/mL and 2.8µg/mL respectively. Chlorotoluron was detected by its parent ion (m/z 213) and its ^{37}Cl isotope, (m/z 215). Pendimethalin was identified by its parent ion (m/z 282) and its dinitroaniline fragment, m/z 212. Pyroxsulam was only detected down to

107.0µg/mL by identifying its parent ion, m/z 435, (Figure 3.16). Also present in the pyroxsulam samples is a dominant adduct, m/z 449, which is not seen with ESI. At the lower levels of quantitation, chlorotoluron and pendimethalin samples were shown to contain two contaminants, DMSO at m/z 157, while the ion seen at m/z 279, could be a phthalate used in the manufacture of the gaskets, fitted around the seal of the ionisation source.

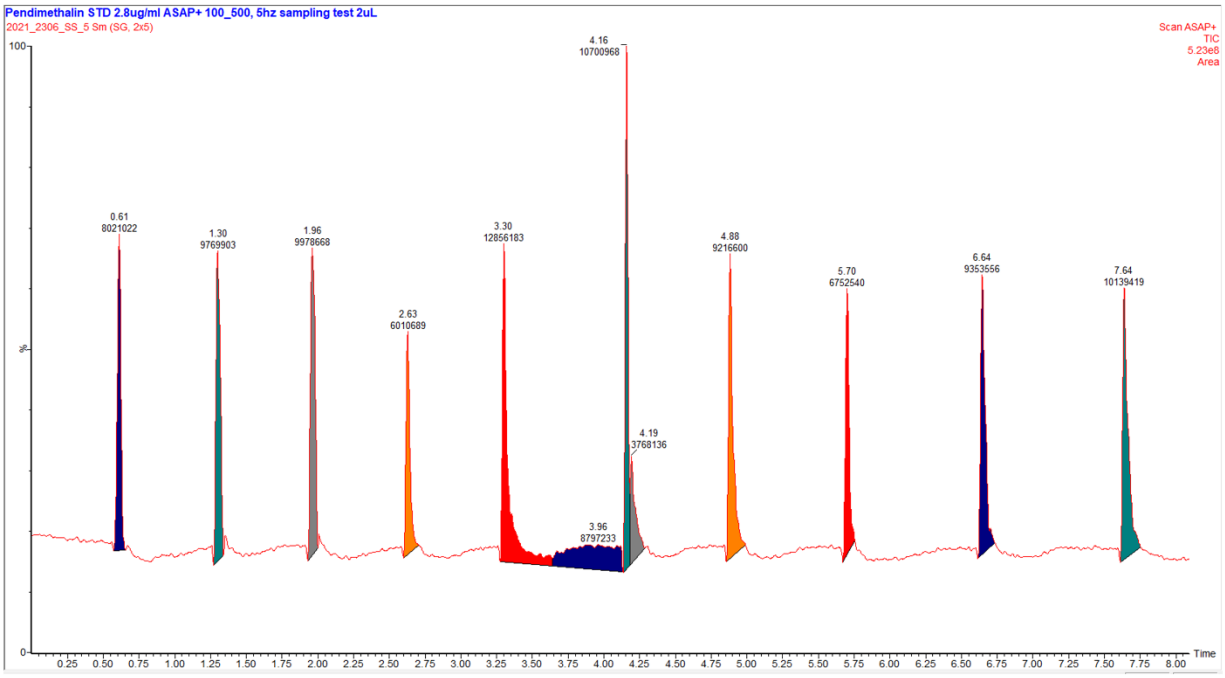


Figure 3. 14 Total ion chromatogram of pendimethalin, (2.8ug/mL) sampled 10 times.

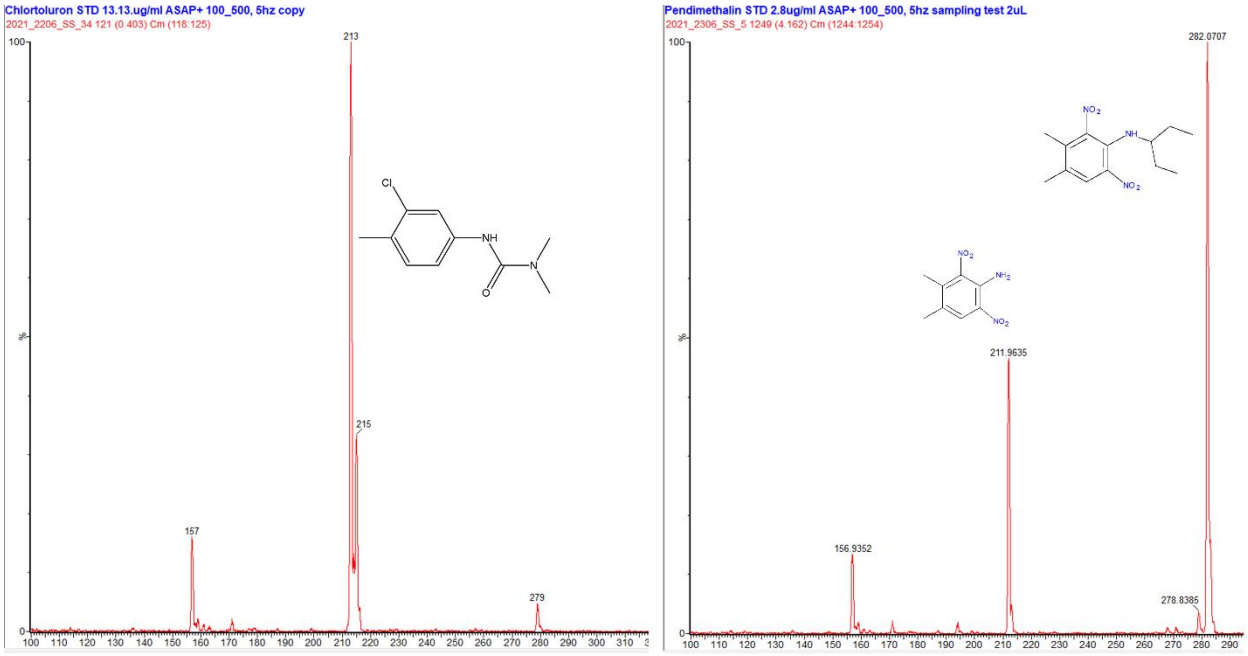


Figure 3. 15. Spectra of (left) chlorotoluron, 13.13µg/mL and (right) pendimethalin 2.8µg/mL, sampled at 300°C.

By comparing the limit of quantitation (LoQ) of chlorotoluron, pendimethalin and pyroxsulam it can be seen that the Q-ToF can detect all three herbicides down to the bottom end of the nanomole scale and that the limits of quantitation for pyroxsulam, chlorotoluron and pendimethalin were 250 µM, 62.5 µM and10µM respectively, (Table 3.2). A range of concentrations can be used when developing herbicide metabolism assays but in this body of work concentrations used range from 8µM to 200µM, which is also typical of other herbicide metabolism assays. This means that although the RADIAN ASAP can detect parent molecules at low levels, down to 10µM in the case of pendimethalin, it is not suitable to then quantify herbicide metabolites which are expected to be in the nanomolar range.

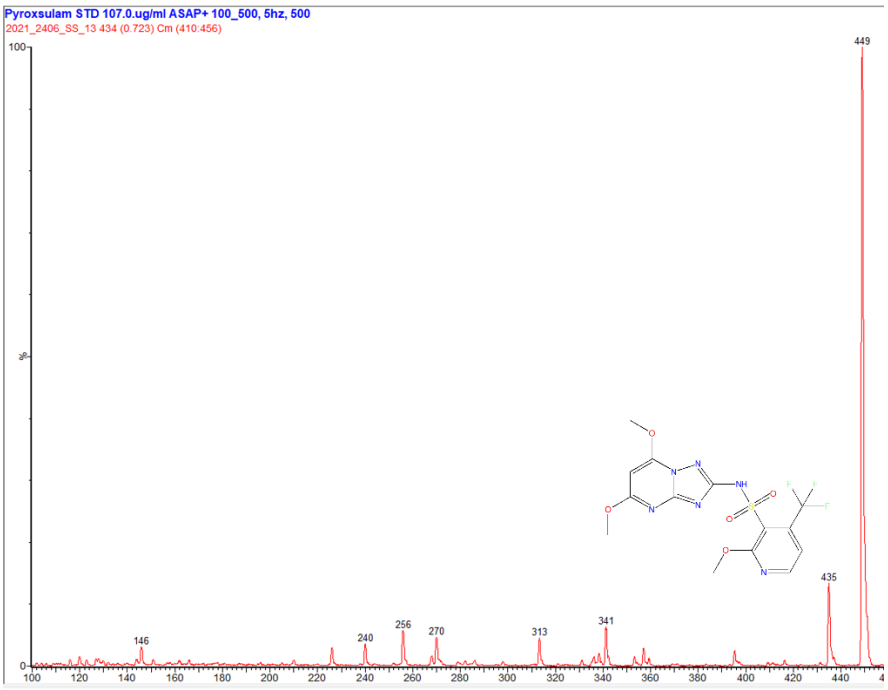


Figure 3. 16 Spectrum of pyroxsulam, 107.0µg/mL, sampled at 500°C.

Table 3.2. Limit of quantitation of pyroxsulam, chlorotoluron, pendimethalin analysed by RADIAN and G-ToF along with respective ionisation temperatures required by the RADIAN..

Herbicide	**LoQ Q-ToF**	**LoQ RADIAN**	**Ionisation Temp (°C) RADIAN**
Pyroxsulam	1nM	107.0µg/ml 250µM	500
Chlorotoluron	1nM	13.3µg/ml 62.5µM	300
Pendimethalin	1nM	2.8µg/ml 10µM	300

3.7 Discussion

No herbicide metabolites were detected when using the REIMS system. The RADIAN ASAP was able to detect herbicide at a level that would be used in herbicide feeding studies, detecting pendimethalin at 10µM, however based on the LoQ of the parent compound RADIAN would not be suitable for metabolite detection in the herbicide assays described here. The use of IMS to explore the detection of structural isomer did provide useful information in that we know the pyroxsulam metabolite was ruled out as being isomer 3. Whilst not necessary for routine analyse it still presents an alternative to NMR. Due to the ESI method coupled with HRMS being able to detect pyroxsulam with no differences in response when using herbicide spiked methanol versus plant extract and was capable of detecting all metabolites with a run time of 5 minutes per sample, this was deemed a suitable method to carry out the analysis.

No herbicides or metabolites were detected using REIMS and that the reasons for this may be due to ionisation mode and analyte destruction, it is clear that the metabolomics data sets generated do contain a large of amount of statistically important mass ion data that warrants further interrogation. In order to validate potential molecular biomarkers two pieces of further study need to come together. The m/z values need to be processed through a greater number of plant metabolite databases to try and identify a match for the parent mass ion or the structure of the compound needs to be deduced by its fragmentation patten. The second piece of work involves setting out the logic as to why one or more identified compounds may be functional biomarkers. In the PCA plot it is clear that wheat treated with flufenacet has a different small molecule profile from untreated but that profile is not down to the presence of herbicides or their metabolites. It is possible that the differences arose from herbicide injury and that these changes in the small molecule profiles was down to some protective but further work analysing the effect of herbicide damage on plants and associate small molecule profiles is required to confirm that possibility. However, for this project further REIMS studies were not pursued in favour of conventional LC-MS analysis of herbicides.

The RADIAN ASAP was very simple to use, maintain and provides chromatograms and spectra within seconds allowing for rapid sampling and analysis. Although there is still a sample preparation stage, the introduction of the extracts into the mass spectrometer is far simpler than for conventional LC. Additional software, such as LiveID, is available that allows the user to create statistical models based on the ratios of abundance of analytes that can inform the user if a certain analyte is present in the sample. A database could be

constructed with a compiled list of herbicides and their m/z's and fragments. This could then be used to determine the presence of unknown herbicides in environmental samples such as water or plant extracts. However as configured RADIAN ASAP is not sensitive enough to detect herbicide metabolites based on the LoQ of the parent compounds. Further sample clean-up such as solid phase micro extraction, with a stationary phase in the coating designed to absorb herbicides based on the chemistry, could be used to concentrate the sample. Having sampled pyroxsulam, chlorotoluron and pendimethalin with ESI and APCI it could be argued that the molecular weight and polarity of pyroxsulam lends itself to ESI based on its LoQ, being in the nanomole range, the same as pendimethalin and chlorotoluron. The higher LoQ of pyroxsulam compared to pendimethalin and chlorotoluron using APCI suggests that it is less susceptible to chemical ionisation than pendimethalin and chlorotoluron. In order to develop high throughput analytical method, based on mass spectrometry to examine herbicides and their metabolites, it was deemed to be of value to stick to one method on a single machine. As the Acquity I-Class coupled to the Q-ToF, which has a ESI source, can detect the chosen herbicides and their corresponding metabolites at the nanomole level, it was the logical choice of instrumentation to use. The RADIAN ASAP was not able to detect herbicides at the low levels required for metabolite analysis. Although an APCI source can be fitted to the Q-ToF and potentially optimised for lower molecular weight herbicides such as chlorotoluron and pendimethalin, the effectiveness of ESI and sensitivity to a range of herbicides make the ESI Q-ToF the instrument of choice for routine analysis of herbicide metabolites. The extra layer of characterisation provided by IMS coupled to the Q-ToF is also useful in elucidating the structures of proposed new metabolites or isomers, in addition to the accurate mass and elemental composition data provided by Q-ToF. There are multiple chemical information databases, such as PubChem and ChemSpider which provide mass spectrometry data about herbicides. A problem with these databases is that they are user generated and herbicide manufacturers are not mandated by government or regulators to provide the data used in mass spec studies to determine the metabolism of these compounds. A consequence of this is the user generated databases come from a wide range of chromatographic and mass spec instrumentation. As such, it can be difficult to find a previously established method suitable for instrumentation at hand. That is why it is important to establish that the planned method for metabolite analysis generates results on the equipment available in the lab, assuming methods can be simply swapped from machine to machine. In conclusion, research publications should provide more and more

mass spectrometry data and researchers encouraged to continue sharing more information about why they have chosen a particular LC-MS method.

Chapter 4. The Effect of *Am*GSTF1 on Herbicide Metabolism in Arabidopsis

4.1 Introduction

Previous studies have shown that high concentrations of glutathione and enhanced GST activity were associated with MHR black-grass populations, and partially contribute to for the resistance to GST detoxified herbicides, such as fenoxaprop-P-ethyl (Cummins, Bryant and Edwards, 2009). Normally fenoxaprop is detoxified in crops by its rapid conjugation with GST as wild grass including black-grass. *Am*GSTF1 is a constituently up-regulated in MHR Black-grass(Cummins, Cole and Edwards, 1999). Though a glutathione transferase, *Am*GSTF1, has little activity in conjugating herbicides but is in fact a glutathione peroxidase (GPOX) and this together with an elevated glutathione content may contribute to protecting MHR black-grass from herbicide injury (Cummins *et al.*, 2013). The discovery of *Am*GSTF1 being present in high levels in MHR black-grass populations as compared to sensitive or TSR plants, lends itself to the protein acting as a biomarker for NTSR (Cummins *et al.*, 2013; Tetard-Jones *et al.*, 2018).

Proteomic data identified *Am*GSTF1 to be elevated in the MHR Peldon population, as well as in selected populations experimentally selected to resistant to the herbicides fenoxaprop-P-ethyl and pendimethalin respectively (Tétard-Jones *et al.*, 2018). The presence of *Am*GSTF1 in field and experimentally derived populations requests the need to measure the *Am*GSTF1 levels in the population of interest. The levels of *Am*GSTF1 are important in identifying NTSR populations as is demonstrating that high levels of this protein do not have an impact on the metabolism of herbicides, both GST and P450 detoxified. The herbicides used in these experiments are chlorotoluron, pendimethalin and flufenacet, whose detoxification pathways have been described earlier (Figures 1,1 and 1,2)

4.2 *Am*GSTF1 Levels in Arabidopsis Root Tissue

*Am*GSTF1 was previously transformed into *Arabidopsis thaliana* using the floral dip method (Bent, 2006). When treated with herbicides, *Am*GSTF1 expressed *Arabido*psis seedlings have been shown to exhibit less damage during growth, when treated with herbicide, compared to an empty vector (EV) control, (figure 4.1).

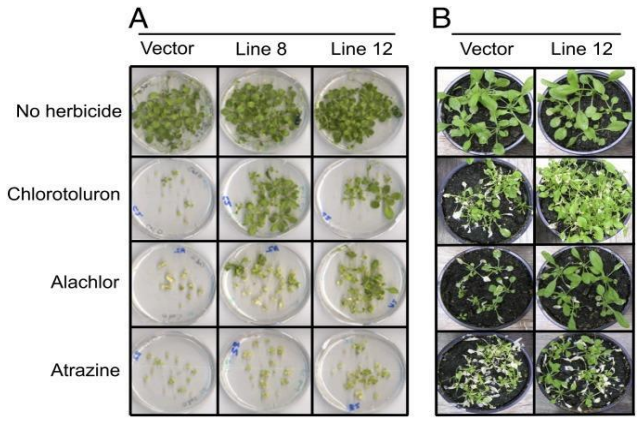


Figure 4. 1 Herbicide resistance of transgenic Arabidopsis expressing Am*GSTF1. (*A*)* Am*GSTF1 expressors and vector-only controls were germinated on agar containing 2 µM chlorotoluron, alachlor, atrazine, or acetone and maintained for 30 d. (*B*)* Am*GSTF1-expressing and vector-only control plants were sprayed with chlorotoluron, alachlor, atrazine, or formulation only at rates of 30 g ai per hectare, 1200 g ai per hectare, and 30 g ai per hectare, respectively, and assessed 9 d after herbicide application*(Cummins *et al.*, 2013).

Arabidopsis was used to express *Am*GSTF1 as its short full life cycle allows for selective breeding and development of homogeneous lines in a matter of months. Homogeneity is big advantage when using *Arabidopsis,* as the variability of many proteins and metabolites in black-grass field populations makes it sometimes hard to statistically validate findings as to their link to MHR as a model plant system of choice, (Meinke *et al.*, 1998).

To categorise *Am*GSTF1 *Arabidopsis* lines as being either high or low expressing, seedlings from three independent *Am*GSTF1 overexpressing lines and two independent EV controls were grown in liquid culture for two weeks and the protein extracted, quantified, diluted and the amount of *Am*GSTF1 quantified by a sandwich ELISA, (Table 4.1). The two over expressing lines F1-1 and F1-2 had an *Am*GSTF1 level more than 100x and 80x higher than the highest 'expressing' EV line, EV-1, respectively. In contrast The F1-3 line has a level akin to both of the empty vector control line. This low level could be ascribed to two factors. The first factor may be due to the transformation efficiency in this independent line or that there was a degradation of protein during the ELISA procedure leading to the lower than expected *Am*GSTF1 level. The cross reaction of *Am*GSTF1 antibodies with the GSTs in *Arabidopsis* might explain the trace of AMGSTF1 detected in two independent EV lines. For the continuation of this study F1-1&2 and EV 1&2 lines were used and classified as high (F1&2) and low (EV1&2) *Am*GSTF1 expressing *Arabidopsis* plants.

*Am*GSTF1 expressed line	*Am*GSTF1 in Arabidopsis roots (mg/g FW)	% RSD
GSTF1-1	6.38	7.7
GSTF1-2	4.05	21.5
GSTF1-3	0.03	14.9
EV 1	0.05	15.4
EV 2	0.01	14.1

Table 4. 1. AmGSTF1 levels measured in three AmGSTF1 expressed arabidopsis root cultures and two empty vector controls. % relative standard deviation, three technical replicates per line.

4.2.1 *Herbicide Metabolism in* Am*GSTF1 Expressed* Arabidopsis *Root Cultures*

*Am*GSTF1 expressed in *Arabidopsis* contributed to resistance to herbicides that have been applied to seeds germinated on agar plates(Cummins *et al.*, 2013). What is less well known is whether the site of herbicide application in *Am*GSTF1 overexpressed in *Arabidopsis* has any bearing on the levels of herbicide metabolites in a specific tissue location. Firstly, it was important to establish that the herbicide can be taken up by the roots and to observe any metabolism that may occur. In the first instance, chlorotoluron, a photosystem II inhibitor, was fed to *Arabidopsis* using a root culture system. Chlorotoluron is metabolised by cytochromeP450s (P450s) in black-grass into three products, a demethylated, hydroxylated and a demethylated and hydroxylated product respectively (Hyde, Hallahan and Bowyer, 1996).

Chlorotoluron was added to make final concentrations of 1.25, 2.5, 5 and 10µM into fresh root culture media into which *Arabidopsis* roots (14 days old) were transferred. These concentrations were used initially in an attempt to span concentrations required to detect metabolism. After 6 hours, the roots were washed in acetonitrile to remove any herbicide that was bound to the outside of the roots. The amount of chlorotoluron in the roots increased in a positive linear pattern, with a doubling in the dose (Figure 4.2). However, no chlorotoluron metabolites were found, nevertheless chlorotoluron uptake was very similar in the *Am*GSTF1 and empty vector root cultures, showing that the presence of *Am*GSTF1 does not have an effect on the uptake of chlorotoluron in root cultures.

This experiment was then re-run with two different herbicides, pendimethalin and flufenacet, a microtubule assembly inhibitor and cell division inhibitor, respectively. Pendimethalin is metabolised by P450s and flufenacet by GSTs, though not by *Am*GSTF1 (Dücker *et al.*, 2019). The primary metabolite of pendimethalin is the 4-hydroxymethyl product, the primary metabolite of flufenacet detected being the glutamyl-cysteine conjugate(Ducker *et al.*, 2019). In each curve dose concentrations were increased to 5, 10,

20 and 40µM. This increase in concentration was to better the ability to observe metabolism without subjecting the plant to toxic levels of herbicide which may cause the plants detoxification system to shut down

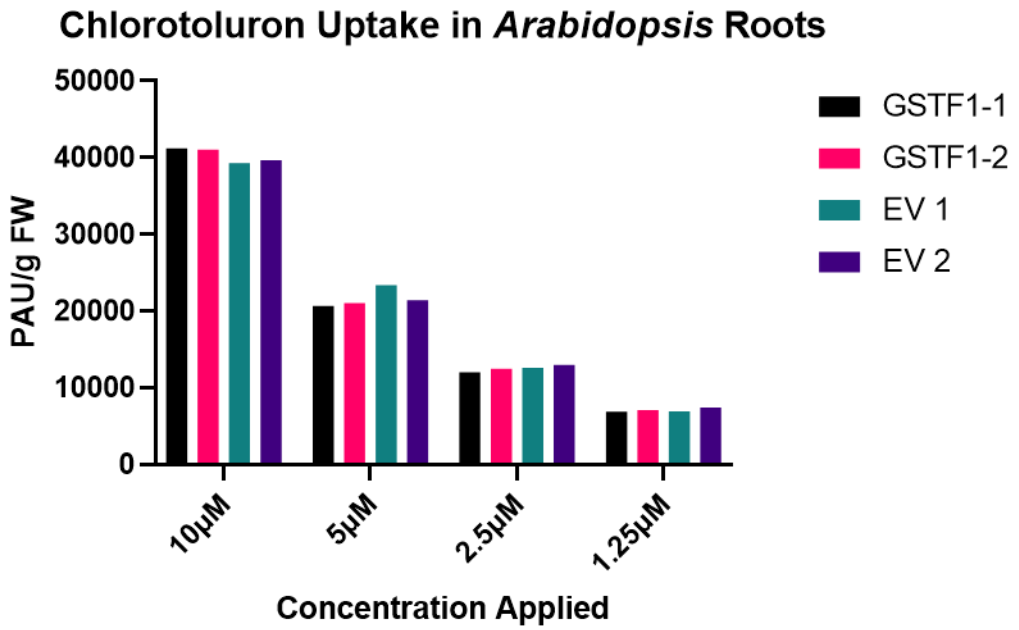


Figure 4. 2. Amount of chlorotoluron, in PAU per gram of fresh weight, extracted from 2 GSTF1 expressing and 2 empty vector Arabidopsis root cultures with each line having 4 root cultures treated with chlorotoluron individually at 1.25, 2.5, 5 and 10µM. 1 replicate per line.

As shown in figure 4.3, we can see that flufenacet was metabolised to the cysteine conjugate at each of the four concentrations tested in both F1-expressing and empty vector control lines. The amount of metabolite recovered increased in a positive near linear fashion with the doubling of flufenacet dose concentration in each of the root culture lines. There is no major increase in the levels of flufenacet conjugate in the F1-expressing lines vs the EV controls. The roots cultures treated with pendimethalin however, suggested a potential increase in the formation of the hydroxylated product in the F1 lines compared to the EV controls, in some of the treatments. Unlike the flufenacet-treated root cultures, there was no positive linear increase in the formation of 4-hydroxymethyl in line with increasing concentration of herbicide parent. In the roots treated at 5µM, the amount of 4-hydroxymethyl-pendimethalin recovered in F1-2 is almost double that was determined in the EV lines. No major differences in metabolite level were observed in the roots treated at 10µM, while at 20µM the amount the amount of 4-hydroxymethyl recovered in F1-2 was almost triple that determined in EV2. No major increases in 4-hydroxymethyl recovery were detected between F1-2 and the EV lines. Interestingly, in the roots treated with 40µM

pendimethalin, both of the F1 lines generated double and triple the amount of 4-hydroxymethyl compared to EV1 and EV2 respectively. The amount of 4-hydroxymethyl metabolite recovered in the EV lines was similar at each of the four concentrations. Moving forward from the initial study treating the root cultures with pendimethalin, a repeat using 3 biological replicates rather than one, was carried out at 40µM. This concentration was used as both F1 expressing lines showed a large increase in the 4-hydroxymethyl metabolites, as opposed to only the F1-2 line in the 20µM treatment.

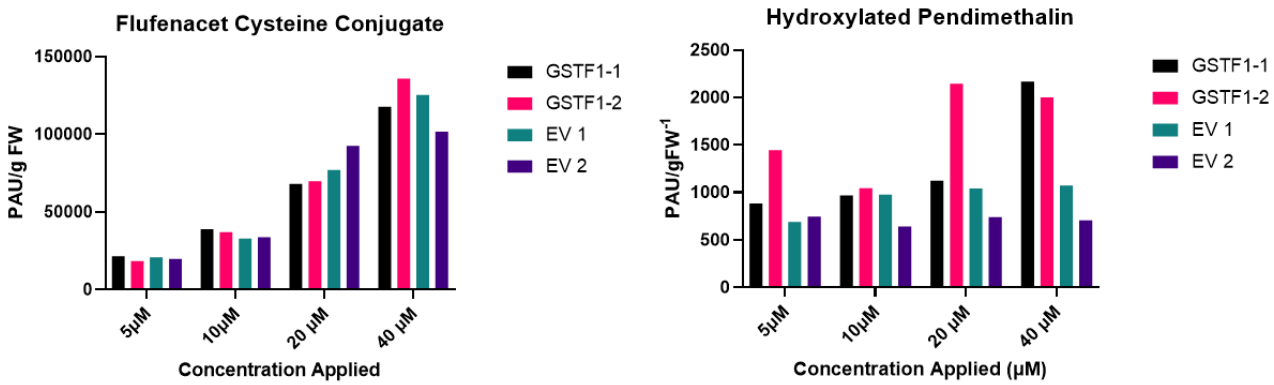


Figure 4. 3. Amount of flufenacet cysteine conjugate (left) and hydroxylated pendimethalin (right) in peak area units per gram of fresh weight, from Arabidopsis root cultures treated with increasing concentrations of flufenacet and pendimethalin respectively. 1 replicate per line.

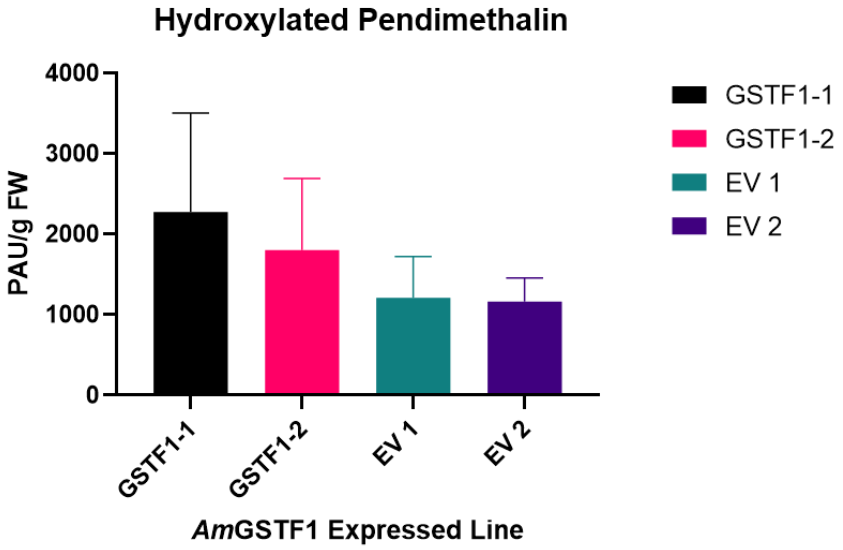


Figure 4. 4 Amount of 4-hydroxymethyl, in peak area units per gram of fresh weight, from Arabidopsis *root cultures treated with 40µM pendimethalin. Data is the mean, error bars are standard deviation, n = 3.*

The results of the replicated study, (Figure 4.4), show that the mean level of the 4-hydroxymethyl metabolite is the same in both empty vector controls and similar to the levels seen in the results from the previous study, (Figure 4.3). There was an increase in

the mean 4-hydroxymethyl levels in both F1 expressed lines, however the large errors bars suggest that high variation leads to the conlusion that the elevated presence of *Am*GSTF1 in *Arabidopsis* roots has no effect on hydroxylation of pendimethalin generated when root cultures are treated with 40µM pendimethalin for six hours.

4.2.2 *Herbicide Metabolism in* Am*GSTF1 Expressed* Arabidopsis *Leaf Tissue*

Whilst enhanced tolerance to the applications of chlorotoluron was observed in *Am*GSTF1 expressing seedlings, these studies focus on observing the growth stages of plants and the measurement of leaf size, root length and dried weight. However, these studies did not investigate the metabolic fate of herbicides *Am*GSTF1 expressed *Arabidopsis* leaf tissue. In the current study three herbicides were used, pendimethalin, chlorotoluron and fenoxaprop-p-ethyl. Fenoxaprop is an acetyl co-enzyme A carboxylase (ACCase) inhibitor, which undergoes ester hydrolysis to form the free acid which then undergoes conjugation to glutathione and then further modification similar to the metabolism of flufenacet (Cummins, Bryant and Edwards, 2009). Each herbicide was fed to *Arabidopsis* leaf tissue derived from the same lines that had been characterised as F1 expressing and EV controls, used in the root culture assay. Due to the variability of metabolite levels seen in the F1 expressed roots, two plants were pooled into one biological replicate, with three biological reps then used per study. The EV samples consisted of one plant per biological replicate, with three biological replicates used. The leaf tissue was treated, on the 21st day after the seedlings were transplanted into soil, by removing the plant with intact roots and then feeding with 40µM herbicide in a 50mL falcon tube for 6 hours.

In figure 4.5, the results of the detected metabolites from the leaf tissue extract are shown. Most noticeable is the lack of fenoxaprop conjugate as no metabolites were found, yet the parent ester and free acid was found in high quantities. With pendimethalin the levels of 4-hydroxymethyl pendimethalin are different when comparing F1 to EV plants depending on which lines were selected for comparison. Comparing the F1-2 line to the EV1 line, it appeared that high level expression of *Am*GSTF1 does have an effect in increasing the levels of 4-hydroxymethyl, yet by comparing F1-2 against EV 2 there is very clearly no difference in 4-hydroxymethyl levels in *Arabidopsis* expressing *Am*GSTF1 leaf tissue versus an empty vector control. The same distinction was made when interpreting the levels of hydroxylated chlorotoluron with regards to F1-2 line as compared with the EV1 line and then subsequently the EV2 line. The levels of metabolites were not significantly increased in plants expressing *Am*GSTF1. By interpreting both assays, root and leaf tissue it can be determined that where metabolism does occur in the corresponding plant tissues,

the presence of *Am*GSTF1 in *Arabidopsis* has no effect on the accumulation of metabolites.

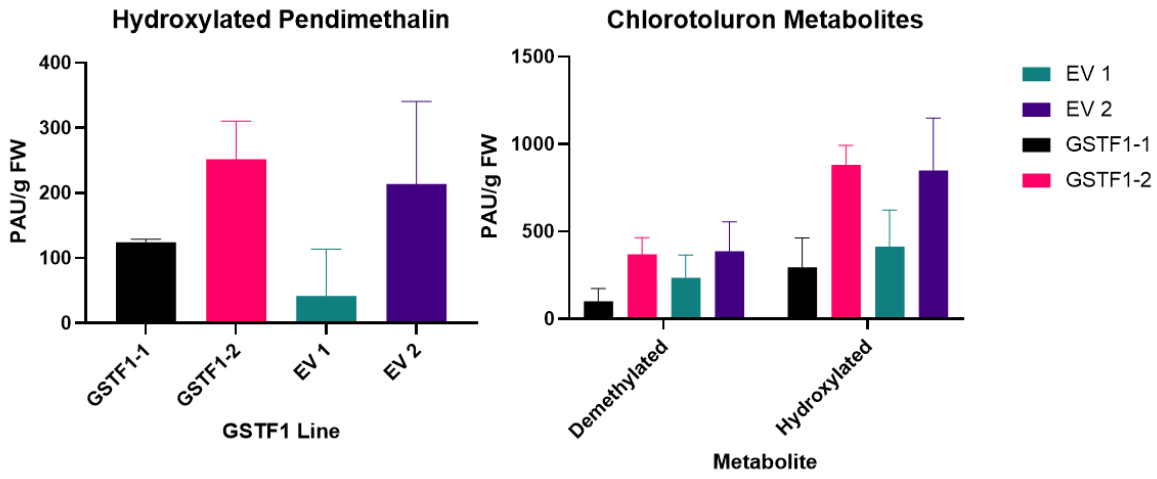


Figure 4. 5 Amount of 4-hydroxymethyl pendimethalin (left), demethylated and hydroxylated chlorotoluron (right), in peak area units per gram of fresh weight, from Arabidopsis *root cultures treated with 40µM pendimethalin and chlorotoluron respectively. Data is the mean, error bars are standard deviation, n = 3*

4.3 Non-targeted metabolomics in *Am*GSTF1-expressing *Arabidopsis* leaf tissue

Having been unable to demonstrate a clear link between *Am*GSTF1 expression and enhanced herbicide metabolism in *Arabidopsis*, the plants were then subjected to non-targeted metabolomics to investigate potential differences in their natural product profile. Targeted metabolomics involve investigating the presence of a chosen set of chemicals in plants such as phytohormone profiling (Floková *et al.*, 2014; Guérard *et al.*, 2017). Non-targeted metabolomics involve the statistical analysis of all detected compounds from plant samples and identifying the chemicals statistically most significant such as the non-targeted metabolomic profiling of wheat under drought stress (Rahman *et al.*, 2017). A non-targeted statistical metabolomics approach was employed to observe the differences in small molecule abundance between *Am*GSTF1 expressing *Arabidopsis* and empty vector controls, with a view to identifying compounds varying in their abundance and exploring their potential as bio-markers. Though the term non-targeted is used, in order to exclude the extraction of long chain fatty acids, proteins and other large molecules 80% MeOH is used as a polar solvent to extract small polar molecules with similar physiochemical properties as herbicides, safeners and synergists etc.

In this experiment 5 biological replicates of previously characterised *Arabidopsis* tissue, namely the two empty vector lines, and two *Am*GSTF1 expressing lines were grown until 21 days old and the leaf tissue ground, extracted into 80% MeOH overnight. The samples were then analysed by LC-MS. In order to assess the quality of the data, a quality control

was made by taking an aliquot of each sample and mixing them to create the QC, five injections of the QC were used as replicates. If the data is to be considered of good quality a pool of all of the samples should cluster together, away from the other samples.

A PCA plot was generated using specialised metabolomics software designed by the LC-MS manufacturer and shows that *Arabidopsis* with higher levels of *Am*GSTF1 has a statistically different small molecule profile from non *Am*GSTF1 expressed *Arabidopsis* (Figure 4.6). The QC group was clustered in the centre of the PCA plots (A) and could be used as measure of quality. As a general trend that the F1 expressing samples, shown in green and peach, clustered above the centre line, with the EV samples, purple and orange, clustering on or below the centre line. This dataset set contained over 12,000 compounds with unique m/zs. The experimental design was set so that all of the EV control samples would be grouped together and compared against all of the *Am*GSTF1 samples grouped together. This grouping shows two very distinct clusters when filters were added to the dataset to include only m/zs that had a max fold change of >2 and a p-value < .05, (Figure 4.5, plot B). The addition of these filters reduced the number of variable m/zs to 129 compounds. The compounds left were run though Lipid Blast, METLIN and ChemSpider databases and from the original 129 mass ions 15 were given tentative ID's. From the 15 compounds two were selected as examples for further analysis as they are the two most significant (lowest p-value) and one has the highest mean in the EV samples and the other has the highest mean in F1 samples, (Figure 4.7).

Table 4. 2. Table showing tentative Id's for the 15 compounds deemed to be significantly different between GSTF1 and empty vector Arabidopsis leaf. Generated by Progenesis Qi.

Compound	Neutral Mass	m/z	z	Retention Time	Peak Width	Accepted ID	Identification ions	Anova (p)	q Value	Max Fold Change	Highest Mean	Lowest Mean	Tags	Isotope Distribution	Maximum Abundance	Minimum CV%
9.50_500.0062m/z	500.0062	1	9.5	2.47			1	2.96E-07	2.95E-04	2.652845	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100 - 20.3	4389.619	25.85
8.19_695.0937m/z	695.0937	1	8.19	0.23			2	7.62E-06	0.002	2.087177	GSTF1	EV	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100	140.557	23.65
1.56_579.0637m/z	579.0637	1	1.56	0.2			2	3.07E-05	0.003	4.305381	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100 - 19.4 - 11.1	397.8338	39.9
6.98_273.1004m/z	273.1004	2	6.98	0.05			2	3.08E-04	0.01	2.338174	GSTF1	EV	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100 - 50.4	416.5512	22.05
6.85_449.2787m/z	449.2787	1	6.85	0.05			1	5.70E-04	0.013	2.339055	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100 - 15.2 - 9.9	161.9446	36.65
1.95_409.2351m/z	409.2351	1	1.95	0.05			1	6.47E-04	0.014	2.226799	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100	127.4403	35.01
7.64_569.1628m/z	569.1628	1	7.64	0.1			2	0.001	0.02	2.146817	GSTF1	EV	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100 - 15.4	330.5281	28.18
5.02_171.0504m/z	171.0504	2	5.02	0.03			1	0.002	0.026	2.465303	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	75.9 - 100	21.61458	25.7
2.10_423.1485m/z	423.1485	1	2.1	0.17			2	0.007	0.043	2.310781	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100 - 17.6	837.1104	13
1.90_393.1228m/z	393.1228	1	1.9	0.07			2	0.013	0.062	2.750953	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100 - 1.14	70.26913	46.96
3.12_215.1442m/z	215.1442	1	3.12	0.14			13	0.019	0.074	3.397385	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100	11.84746	71.21
5.84_605.1862m/z	605.1862	1	5.84	0.03			1	0.035	0.099	2.410003	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100 - 28.9	115.092	55.27
8.73_596.2785m/z	596.2785	1	8.73	0.07			1	0.036	0.1	2.401479	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	100	15.34942	65.08
6.25_167.0604m/z	167.0604	2	6.25	0.05			1	0.044	0.109	2.123842	EV	GSTF1	Anova p-value <= 0.05; Max fold change >= 2; Possible ID's	68.7 - 100	18.25847	69.86

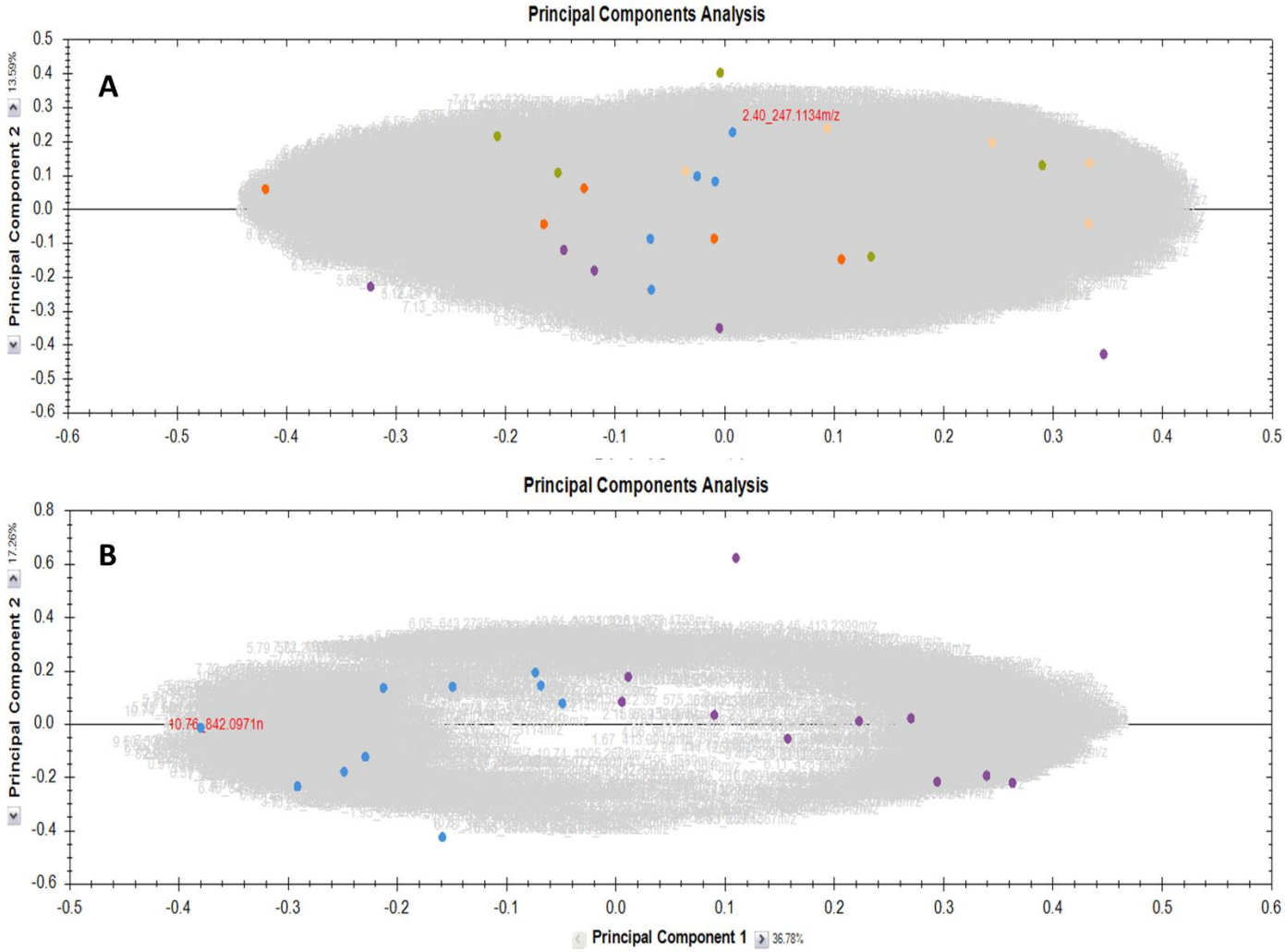
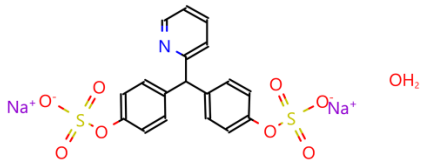
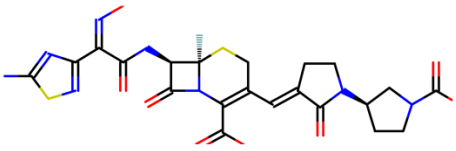


*Figure 4. 6. PCA models constructed in Progenesis QI showing the small molecule distribution in the extract of leaves AmGSTF1; (**A**) EV1 (purple) & EV2 (orange), F1-1 (green), F1-2(peach) and QC (blue); (**B**) EV controls (blue) grouped & GSTF1 expressed (purple) grouped, with ANOVA p value <0.05, generated by Progenesis QI. Values in red are the retention time and mass of the compounds in relation to the sample.*

These database searches can deliver a very small or very large number of matched compunds ID's, however quality checks must be performed on every compound, as the databases are user generated and the data comes from a wide variety of mass spec instrumentats and sources. Having a small number of identified compunds cannot be viewed as a success as they are derived from statistics, which include any errors that may have been generated by the user. Assesing the two compounds selected (Figure 4.7), ceftobirole medocaril is a "novel broad-spectrum cephalosporin with excellent activity against a broad range of pathogens that are important in community-acquired pneumonia." binding to and inactivating penicillin binding proteins (Falcó, Burgos and Almirante, 2018). The othe identified compound was sodium picosulfate, a prodrug metabolised by gut bacteria into an active form of laxative(Krueger *et al.*, 2018). These compounds are commercial pharmaceticals and as such many mass spec based studies will have been carried out in their analysis of these compounds. Clearly general chemical metabolomic databases are biased towards these pharmaceuticals as a consequence of their rigorous MS analysis. All ID's are based on accurate mass, fragmentation patterns, retention times and adducts, properties that differ based on the chromatographic separation, ionisation source, detector and other instrumentation variability. Relying solely on a metabolomics approach to discovering markers of herbicide resistance may not be the most efficient way to undertake the task, however, it can be a useful tool in compound identification if a herbicide metabolite database can be accessed.



Sodium picosulfate
m/z: 500.0062
ANOVA p value: $2.96e^{-7}$
Highest mean : GSTF1



Ceftobiprole medocaril sodium
m/z: 695.0937
ANOVA p value $7.62e^{-6}$
Highest mean: EV

Figure 4. 7. Structures of sodium picosulfate (left) and ceftobiprole medocaril sodium. Compounds IDs matched through the ChemSpider database.

4.4 Discussion

Pendimethalin was P450 metabolised in *Arabidopsis* roots, suggesting its tissue contains a basal level of detoxifying P450 enzyme active towards the herbicide. Statistical analysis demonstrated that *Am*GSTF1 doesn't promote additional P450 activity towards the herbicide. The metabolism levels of chlorotoluron in leaf tissue again suggest that there is a basal level of P450 activity in *Arabidopsis* but that was not enhanced by *Am*GSTF1 expression as there is no major difference when comparing F1-1 to EV 2. What is less clear is the absence of chlorotoluron metabolism in the roots while the herbicide is readily metabolised in leaf tissue. Conversely fenoxaprop was not metabolised in leaf tissue. It can be surmised that, where *Am*GSTF1 has regulatory role in controlling herbicide metabolism in black-grass, this is not the case in transgenic *Arabidopsis.* The differential metabolite levels between the roots and the shoots is interesting as herbicides can be applied to the field before the weeds have emerged such as pendimethalin, which was metabolised in Arabidopsis roots. The ability of *Arabidopsi*s to metabolise the herbicide in the roots may protect the plant from damage during early growth stages yet chlorotoluron is also used as a pre-emergent herbicide yet was not metabolised in the roots. Pendimethalin is a microtubule assembly inhibitor thus may target microtubules in the roots, whereas chlorotoluron is a photosystem II disrupter so may be more effective in the leaves to disrupt photosynthesis. This may provoke *Arabidopsis* to localise its endogenous detoxification enzymes to the site of herbicide. *Arabidopsis* may continue to be the model plant species for many future studies but herbicide metabolism selectivity studies are best undertaken in the crop and weed.

The metabolomics study yielded descriptive charts that help visualise the differences in small molecule abundance between GSTF1 expressed *Arabidopsis* and an empty vector control. Indeed it is quite clear that the metabolic profile of empty vector *Arabidopsi*s leaf tissue differs significantly from GSTF1 expressed *Arabidopsis* by the distinct clustering of the empty vector samples on the left and GSTF1 on the right (Figure 4.6 B). Of the 129 mass ions that were subject to compound searches only 15 came back with tentative ID's. The m/z's of these compounds ranged from 167-695. These are relatively small compounds which is to be expected as electrospray ionisation is suited for ionising lower molecular weight compounds. Of the 15 ID's, 11 had the highest mean in the empty vector *Arabidopsis* samples, this suggests that the difference in the small molecule profiles is due to the absence of these compounds in the GSTF1 expressed plants. Ultimately the 15 mass with tentative ID's were of no significance to this study. These 129 mass ions however

were only matched against the Lipid Blast, METLIN and ChemSpider databases. Other searches can be made against dedicated plant metabolomic databases, such as the Plant Metabolic Network (PMN). Further searching across different databases may potentially yield compound matches of importance. The results of the non-targeted metabolomics study, whilst ultimately of no importance to this study, do however show potential in creating a herbicide metabolism database. The data from the mass spectrometry analysis of herbicide metabolism studies could be collated and a database created containing all of the herbicides and metabolites, identified by their accurate mass, retention time and fragmentation patterns. Obstacles would have to be overcome such as all of the technical details regarding different instrumentation etc and the willingness of institution and industry to share this information freely, yet it seems an achievable goal to create such a database to further mass spectrometry based herbicide resistance research.

Chapter 5. Resistance to Acetolactate Synthase Inhibiting Herbicides

5.1 Introduction

Herbicides are classified by groupings such as by chemical class or by modes of action. Modes of action include, but are not limited to the inhibition of, acetyl CoA carboxylase (ACCase), acetolactate synthase (ALS), microtubule assembly and photosynthesis controlling (photosystems I and II). ALS inhibiting herbicides are currently the most widely used in controlling grass weeds, one reason being the discovery in the 1970s that the ALS-inhibiting, sulfonylureas, were able to stunt the growth of plants when applied at very low concentrations (Whitcomb, 1999). Low application rates, milligrams compared to kilograms, is favourable from a chemical synthesis point of view in that scaling up to commercial production is more economical. Another benefit is that in using much smaller amounts, it is less likely that the product will leach into waterways, which is deemed to be an environmental risk.

ALS catalyses the first step in the biosynthesis of the branched chain amino acids valine, leucine, and isoleucine (Whitcomb, 1999). Two parallel reactions are carried out by acetolactate synthase: synthesis of 2-acetolactate from two molecules of pyruvate and synthesis of 2-aceto-2-hydroxybutyrate from a molecule of pyruvate and one molecule of 2-ketobutyrate, (Figure 5.1).

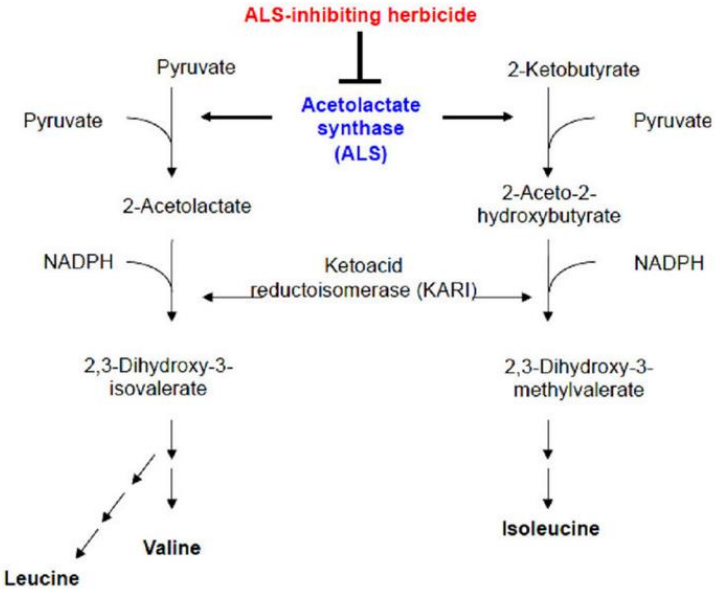


Figure 5. 1 Role of acetolactate synthase in biosynthesis of branched chain amino acids (Whitcomb, 1999).

Grass weed species are now resistant to a greater number of ALS-inhibiting herbicides than to any other herbicide group, this being linked to the widespread use of this class of compound (Tranel and Wright, 2002). To date, resistance to ALS inhibiting herbicides has been reported in 167 weed species (Heap, 2021). ALS inhibitors can be grouped according to their chemical class, namely sulfonylureas, triazolopyrimidines, imidazolinones, sulfonylaminocarbonyl-triazolinone and pyrimidinyl(thio)benzoate. Two commonly used ALS inhibiting active ingredients are pyroxsulam, a triazolopyrimidine co-formulated with florasulam (Broadway Star) which is used to control several grass weed species in North America and mesosulfuron-methyl, a sulfonylurea co-formulated with iodosulfuron-methyl (Atlantis) used in Europe for weed species including black-grass. As described previously, herbicide resistant black-grass were discovered in the UK in the early 1980s at a variety of locations including Faringdon and Peldon in the south of England and since then resistance has spread across England. As mentioned previously, this resistance falls into two categories; target site resistance (TSR) and non-target-site resistance (NTSR) (Powles and Yu 2010). Non-target site resistance to both herbicides have been reported in black-grass populations (Duhoux *et al.*, 2017). Both TSR and NTSR exists within other resistant weed species such as corn poppy (Rey-Caballero *et al.*, 2017). This presents a challenge when trying to diagnose NTSR based on enhanced herbicide metabolism, as NTSR black-grass populations have evolved to upregulate detoxifying enzymes such as *Am*CYP71-1, *Am*CYP71-2 (Tétard-Jones *et al*., 2018). These enzymes and are also present in TSR black-grass, albeit at lower levels. As such, the distinction in the roles of detoxifying enzymes in NTSR resides in their level of expression. Field populations of resistant black-grass also often have large variations in their resistance profile within the population. A random sample of plants germinated from a batch of collected seeds may have 50% TSR mutations. However, repeating the experiment from the same batch may give a mutation rate of 10% or 75%. As weed seeds collected are from the survivors of herbicide spraying, as such the assessment of resistance may not be representative of an entire field.

To help overcome the problem of resistance traits shared due to both TSR and NTSR in field populations of black-grass the series of experiments described in this chapter will use the same genetic material in each experiment. Five black-grass populations were germinated, one sensitive (Roth 09), two NTSR resistant standard populations (Peldon 05 & R30) but with TSR to the ALS inhibitor mesosulfuron and two with previously reported TSR resistance to mesosulfuron, LongC and Velcourt (Marshall *et al.*, 2013).

These resistant populations have been previously known to have SNPs associated with TSR to ALS inhibiting herbicides. To eliminate genetic variation in resistance genes cloned material was used. To this end once the black-grass plants reached the three tiller stage, one tiller was carefully cut from rest of the plant keeping the root intact, the single tiller was placed into fresh soil for two weeks to recover and the original plant returned until a new tiller replaced the taken one. This was repeated for each study.

Black-grass Population	TSR/NTSR	Mesosulfuron Resistance
Roth 09	N/A	No
Peldon 05	NTSR & TSR	Yes
R30	NTSR & TSR	Yes
LongC	TSR	Yes
Velcourt	TSR	Yes

Table 5. 1. List of black-grass populations and their known NTSR/TSR and mesosulfuron resistance status.

5.1.1 *Resistance to Mesosulfuron and Pyroxsulam in Black-grass Populations*

Resistance to mesosulfuron methyl in black-grass is wide spread across the UK (Hull *et al.*, 2014). Mesosulfuron-methyl is detoxified by cytochrome P450 and has a complicated associated metabolic pathway ((EFSA), 2016), (Figure 5.2). The main metabolites of the herbicide found in wheat resulted from demethylation of the parent compound, cleavage of the sulfonylurea bridge to give product (i) which then undergoes cyclisation to form the second cleavage product (ii). Pyroxsulam also undergoes detoxification by cytochrome P450s, resulting in the *O*-demethylation at the 5 position in the triazine ring (Table 3.1) . It has been shown that after 7 days treatment the 5-OH metabolite undergoes glycosylation in wheat (DeBoer *et al.*, 2011). In the current study, however, the focus is on primary metabolism, which occurs in under 24 hours, so the 5-OH product was used as the marker. Current diagnostic tests for TSR and NTSR take between 15-45 minutes so being able to detect a metabolite in the space of hours rather than days makes a potential diagnostic test competitive with existing technologies such as BReD and LAMP assays.

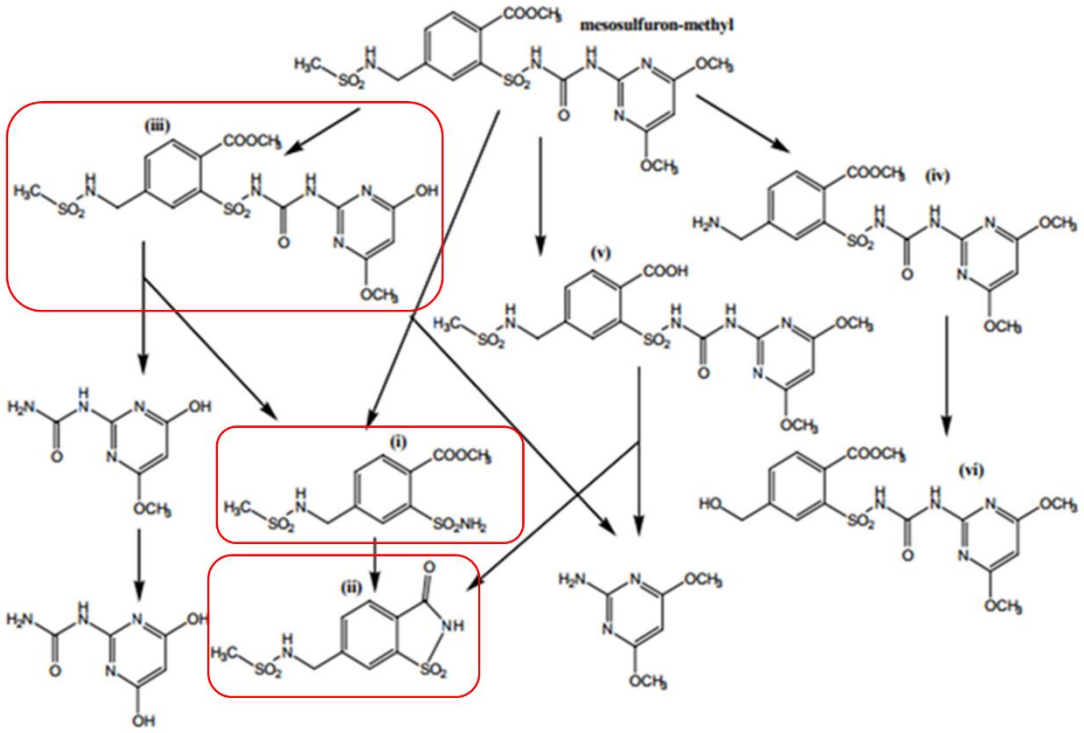


Figure 5. 2. Metabolism pathway of mesosulfuron-methyl, metabolites found in wheat highlighted in red.

Using the cloning procedure, eight tillers from each population, (R30 was not treated with pyroxsulam) were fed with 40µM herbicide, for 24 hours. After 24hr the metabolites were extracted and analysed by LCMS.

Black-grass treated with mesosulfuron generated all four of the suggested metabolites found in wheat however, metabolite (i), whilst identifiable was produced a very low levels and not found in all samples. Therefore the demethylation product and cleavage (ii) were used as primary markers of primary metabolism. The results of metabolism study show the sensitive (Roth 09) population had produced lowest mean amount of the demethylated metabolite (1179 PAU), less than a third compared to the mean of the closest population, R30 (3948 PAU), (Figure 5.3). The mean demethylated metabolite levels were higher in Peldon 05 (6730 PAU), Velcourt (11059 PAU) and LongC (31379 PAU). With the increase in metabolite level there was a corresponding increase in biological variation, due to the heterogeneity of the population. Looking at the secondary metabolite of mesosulfuron, the only population with a greater mean amount of cleavage (ii) than Roth 09 is Peldon 05, (Figure 5.3). Velcourt (2531 PAU) had produced the same amount of metabolite but with a larger variation. LongC and R30 had a mean amount of metabolite less than Roth 09. From this data it can be stated that the resistant black-grass field

populations metabolise mesosulfuron-methyl via demethylation after 24 hours, more actively than then susceptible plants.

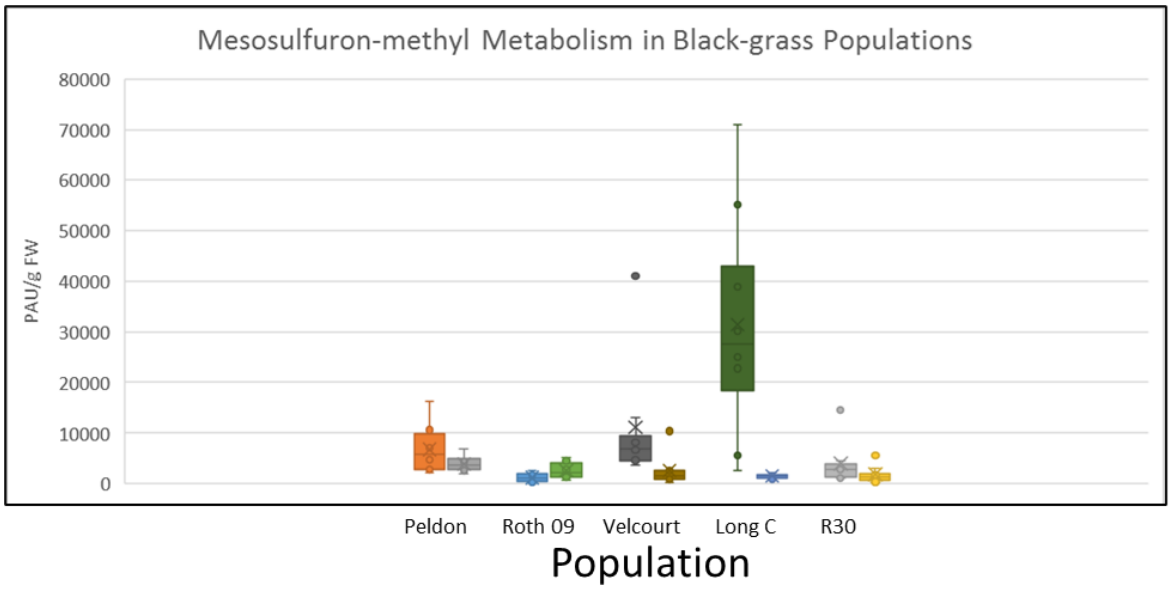


Figure 5. 3.Mesosulfuron metabolism in black-grass. For each population; left bar represents amount of demethylated metabolite and, right bar represents cleavage (ii) metabolites,, PAU/g FW, extracted from 1 sensitive (Roth 09) and 4 resistant black-grass populations treated for 24hrs with 40 µM mesosulfuron-methyl. Error is STD Dev, n = 8

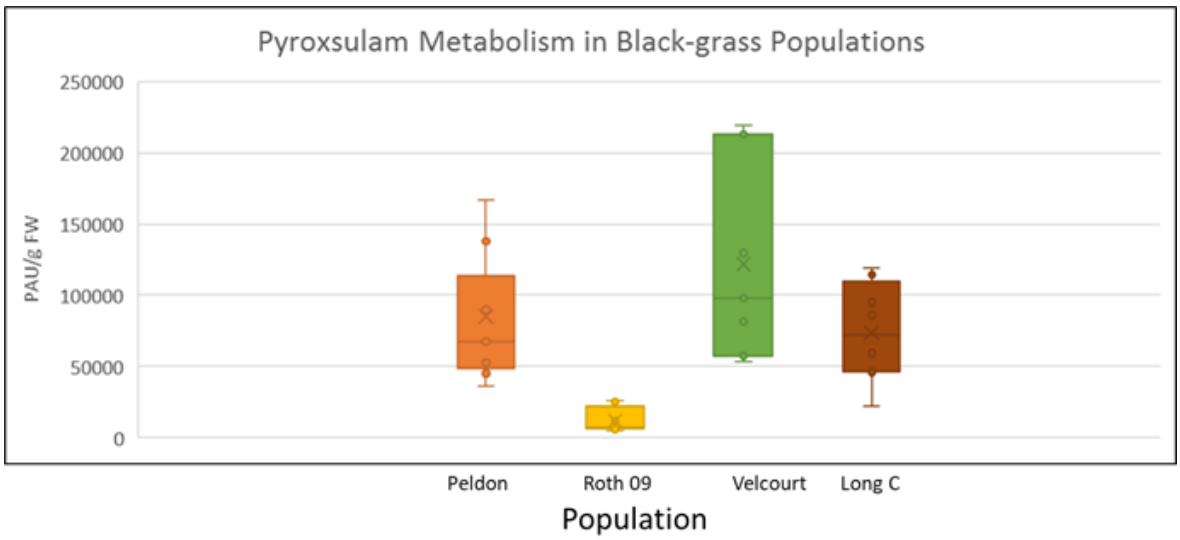


Figure 5. 4. Amount of 5-OH pyroxsulam metabolite, PAU/g FW, extracted from 1 sensitive (Roth 09) and 3 resistant black-grass populations treated for 24hrs with 40 µM pyroxsulam. Error is STD Dev, n = 8

All of the samples from pyroxsulam treated black-grass contained the parent compound and the 5-OH pyroxsulam but in these samples only isomers 1 or 2 were present, (Figure 3.7) .Only All of the resistant field populations had significantly higher levels of the metabolite than the sensitive. The Roth 09 samples have a mean metabolite, less than five times lower than of the TSR population, LongC. The Velcourt samples generated 50% more metabolite than the level determined in Peldon 05. The significantly higher presence of metabolite in the resistant, compared to the sensitive population was associated with

large error bars but the variation seen with pyroxsulam metabolism is less than that seen with mesosulfuron.

5.2 Target Site vs Non-Target Site Resistance

TSR occurs when a mutation in a target protein prevents the herbicide molecule from binding, therefore the herbicide cannot disrupt functional activity and cause damage to the weed. Just one single-nucleotide polymorphism(SNP) can prevent enzyme inhibition. NTSR occurs when other mechanisms are involved, primarily enhanced metabolism, leading to a decrease in bioavailability of the herbicide. When this occurs the amount of herbicide available to access the target site is reduced minimising the toxicity to the plant. An example of a NTSR population in black-grass is the Peldon line which is resistant to herbicides with multiple modes of action including chlorotoluron (Photosystem II inhibitor), diclofop-methyl (acetyl CoA carboxylase inhibitor) and to pendimethalin (microtubule assembly inhibitor) (Moss 1990). This form of resistance is believed to be due to the enhanced metabolism/detoxification of multiple selective herbicides.

5.2.1 Am*GSTF1 in Black-grass Field Populations*

In order to characterise the black-grass populations as being NTSR, the presence of *Am*GSTF1 was quantified by sandwich ELISA, in the same five black-grass plants as used in the herbicide metabolism study. Elevated levels of *Am*GSTF1 in the resistant compared to sensitive populations were used as a biomarker of NTSR. The lowest F1 level (Figure 5.4) was found in the sensitive population, 0.42µg/g. The highest level was determined in Velcourt, 3.65.µg/g followed by R30, LongC and Peldon, 2.96, 1.80, 0.69µg/g respectively. Following the pattern seen in the metabolism data (Figure 5.3), there was a larger variation in *Am*GSTF1 content in the field populations. Although the mean level of *Am*GSTF1 in Peldon is only ~50% higher than in Roth 09 and far lower than the other resistant populations, it is still characterised as demonstrating NTSR in spray trials.

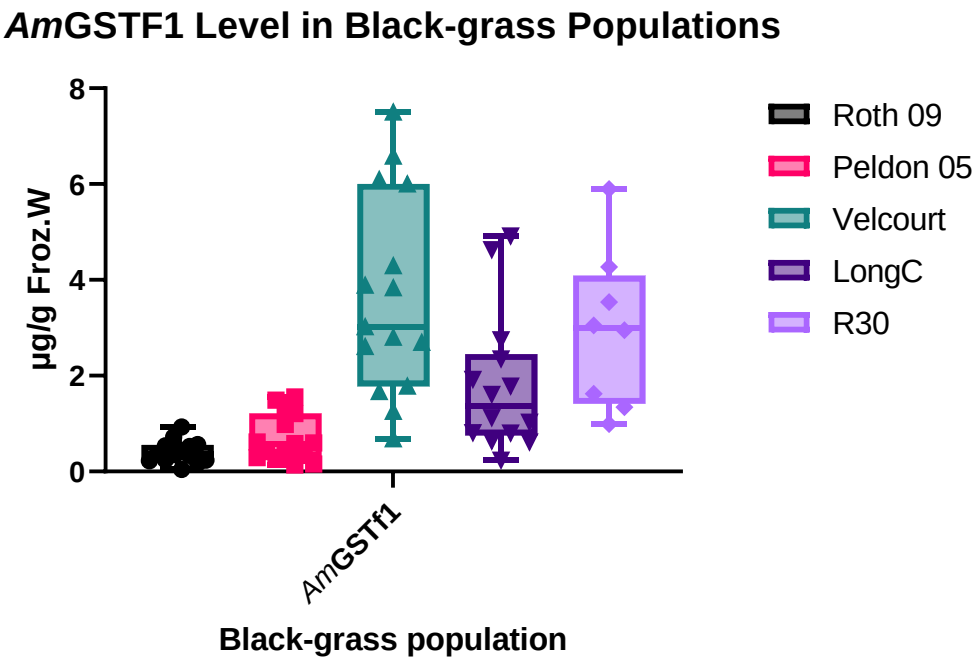


Figure 5. 5 . Am*GSTF1 levels in black-grass populations in µg per gram of frozen weight. Values are mean, error is std. dev. n=16, except R30, n=8.*

5.2.2 *TSR Mutations in Black-grass Field Populations*

Diagnosing TSR in black-grass is well studied and there are many published methods of doing so (Marshall *et al.*, 2013). In this assay one tiller from each of the four resistant black-grass plants was characterised as being TSR or by extracting gDNA, amplifying the ALS gene, (EMBL accession AJ437300), designing amplification and sequencing primers (Table 5.2) then sending the amplified PCR product for sequencing. The P197 and W574 mutations were the only isoforms reported in these black-grass populations that are known to confer with TSR (Table 5.1)(Marshall *et al.*, 2013).

Table 5. 2. List of amplification and sequencing primers.

	Direction	Sequence
Amplification	Forward	CACAGCCACATCCACAGC
	Reverse	GGTGCAATGTGCCTGATCA
Sequencing	Forward	GGCCTTACCCAAACCTACTCT
	Reverse	ATCAGAACTTCGGCAAGAGC

Table 5. 3. P197 and W547 mutations detected in resistant black-grass.

Mesosulfuron	No. of mutations detected		
Population	P197T	W547L	NTSR*
Peldon 05	6	0	2
Velcourt	0	2	6
LongC	6	0	2
R30	0	2	6
Total	12	4	16
Pyroxsulam			
Peldon 05	3	0	5
Velcourt	0	0	8
LongC	6	0	2
Total	9	0	15

*NTSR designation given to all plants with no TSR mutations

The frequency of TSR mutations found in the mesosulfuron treated black-grass was 50%. Noticeably, there was only one SNP mutation found per population. Rarely, two mutations have also been reported (Marshall *et al.*, 2013). The majority of TSR mutations were at P197 as found in the Peldon and LongC populations, six each, whilst the W547 mutation was only found in Velcourt and R30. The mutations found in the black-grass used for pyroxsulam treatment (Table 5.2) were fewer than in the same three resistant populations used for mesosulfuron treatment, with only three P197 SNP mutations determined in Peldon and no W547 in any of the plants tested. Six P197 SNP mutations were found in LongC, mirroring that determined with the mesosulfuron treated plants. The variation in mutations observed are another example of the heterogeneity of field collected samples.

5.3 Use of K**etoacidreductoisomerase Inhibition as an Assay for ALS Activity**

Cyclopropane-1,1-dicarboxylic acid (CPCA) is an inhibitor of ketol acid reducto-isomerase (KARI) the second enzyme involved in the enzymatic pathway that results in the formation of valine, leucine and isoleucine (Figure 5.1). Application of CPCA results in inhibition of KARI, thus KARI catalysis cannot no longer proceed forward resulting in the accumulation of acetolactate, the product of ALS, as the acetolactate cannot be catalysed further. As such the has been used previously to distinguish between sensitive and tolerant weeds (Lovell *et al.*, 1996). Thus, an ALS inhibiting herbicide is applied

along with CPCA in a susceptible plant, acetolactate does not accumulate, as ALS is inhibited by the herbicide. In target-site resistant plants, acetolactate accumulates in the presence of CPCA and the ALS inhibiting herbicide because ALS is no longer inhibited (Gerwick, Mireles and Eilers, 1993). Acetolactate content can be determined by reacting it with H_2SO_4, which produces acetoin which is measurable by spectrophotometry, as acetolactate itself is very unstable and degrades rapidly, (Figure 5.6,. The hypothesis was tested that TSR black-grass treated with CPCA (KARI inhibitor) and an ALS herbicide, that more acetolactate will accumulate than in a NTSR plant treated with CPCA and ALS herbicide. This is because it was predicted that the herbicide will still bind to the ALS enzyme in NTSR black-grass, as is the case in sensitive plants.

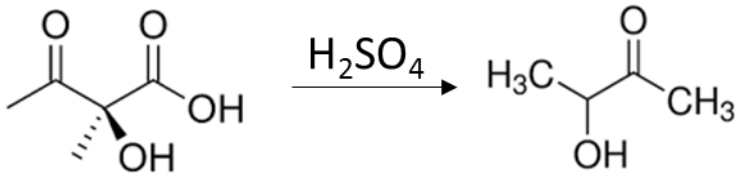


Figure 5. 6. Reacting acetolactate (left) with sulphuric acid produces acetoin (right).

In order to test this hypothesis, first an experiment was run to confirm that black-grass treated with CPCA accumulates more acetolactate than a negative control without CPCA, inferred by the detection of increased levels of acetoin. Levels of acetoin formed following treatment with CPCA were then determined across black-grass populations with different herbicide resistance levels (Figure 5.6).

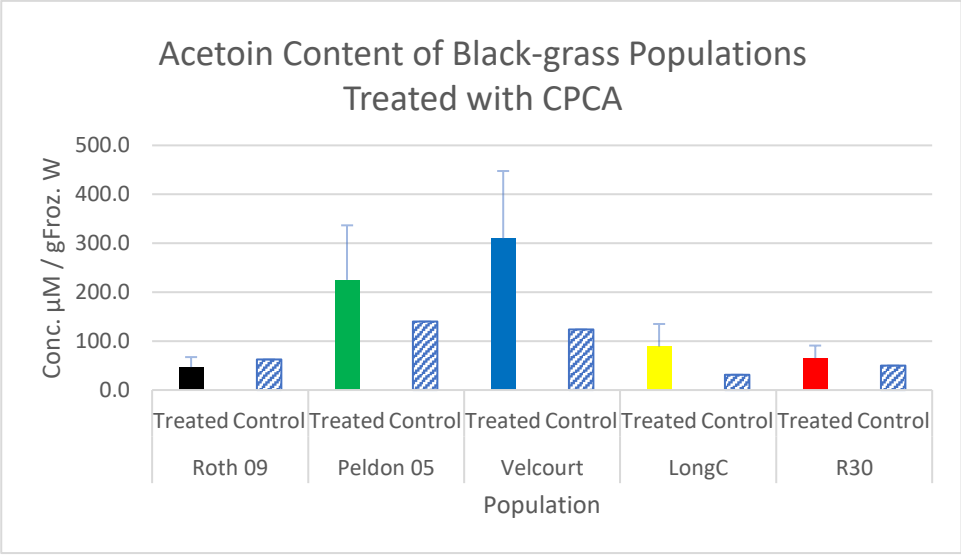


Figure 5. 7 Concentration of acetoin in µM per gram of frozen weight extracted from CPCA treated and untreated (control) black-grass populations, n=8, error bar is std. dev.

The levels of acetoin determined, show that except for Roth 09, the only sensitive population tested, the mean levels of acetoin detected were greater in the CPCA treated than the untreated control (other than R30), (Figure 5.8). Whilst the mean levels were higher in the treated Peldon, Velcourt and LongC populations, following the trends of variation seen in the metabolism assays, in field populations, the variation in acetoin levels was large. As it has already been reported that acetolactate accumulates in TSR weed species when CPCA is applied , the next step was to compare the acetolactate level in TSR vs NTSR black-grass, Eberlein *et al.*, 1997). A tiller from the each of the populations characterised as TSR or NTSR (Table 5.2) was used. Black-grass was treated with CPCA and herbicide (40µM) for 24 hrs, with experiments run side by side, one with pyroxsulam and one with mesosulfuron, with eight biological replicates per treatment. Tillers from the same plant used in the metabolism study were used in the acetolactate study with the corresponding herbicide. The levels of acetoin determined in NTSR black-grass treated with CPCA and pyroxsulam was not significantly different from TSR black-grass that had the same treatment, this was also the case when mesosulfuron was used (Figure.5.8). The results of the experiment show that there were no statistical differences between levels of acetoin measured between TSR and NTSR black-grass following CPCA and mesosulfuron ($p = 0.438$) or pyroxsulam treatment ($p = 0.110$).

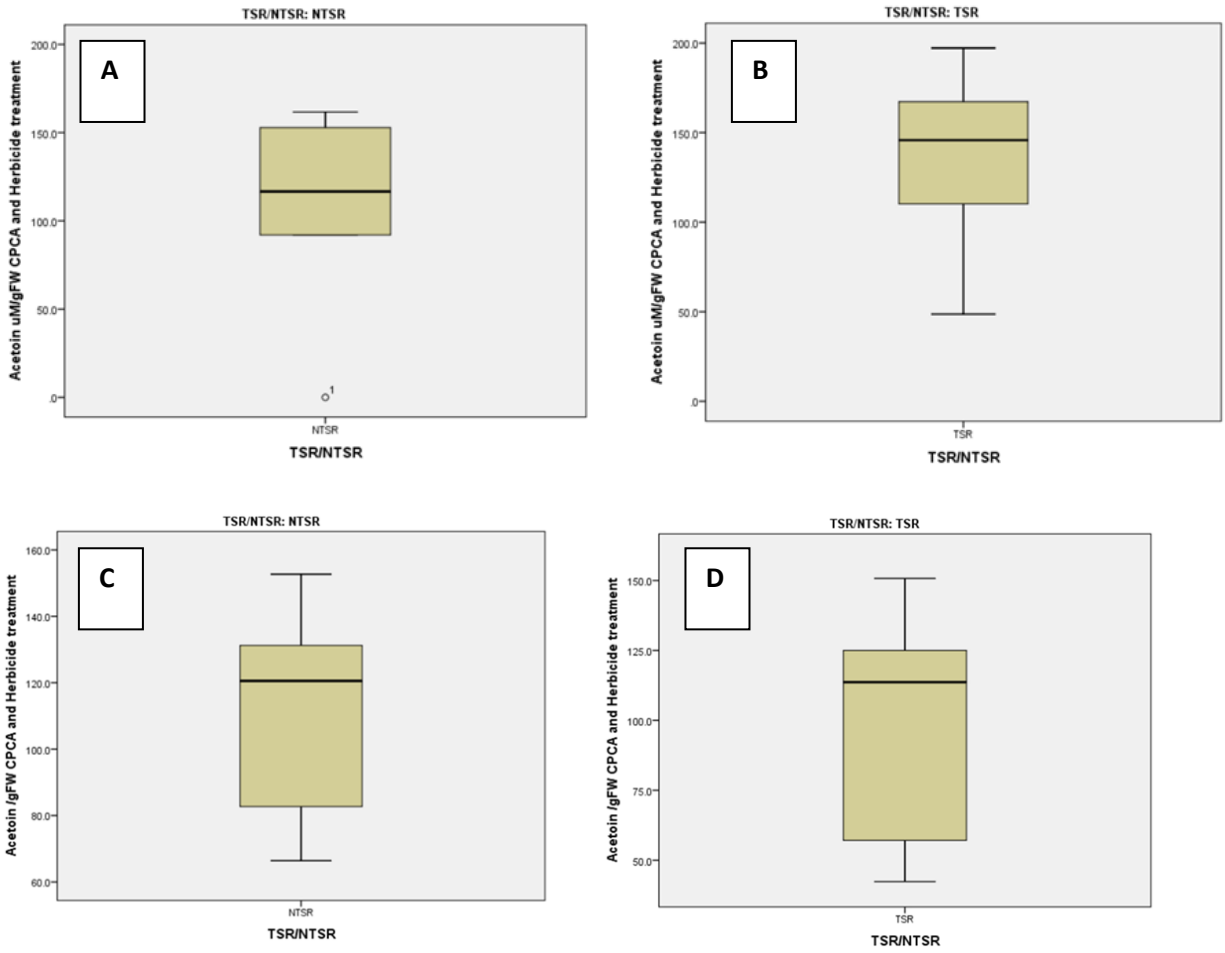


Figure 5. 8. Concentration of acetoin, µM/g fresh weight in ***A*** *NTSR black-grass treated with pyroxsulam and* ***B*** *TSR black-grass treated with pyroxsulam and CPCA.* ***C,*** *NTSR black-grass treated with mesosulfuron and* ***D*** *TSR black-grass treated with mesosulfuron and CPCA.*

5.4 Discussion

A common observation whilst comparing the results from the herbicide metabolism, *Am*GSTF1 level and KARI inhibition studies was that the variation seen in the field populations can statistically render results as being insignificant due to the large error determined. These large differences were often not due to experimental error. The sensitive Roth09 population has never been subject to herbicide treatment, it is homogenous and has long history of its localisation and this produces more consistent results. The resistant plants from the field sites were very heterogenous with populations and individual plants being at different stages in resistance evolution and geographical diversity of parentage. Looking at the SNPs TSR populations were exclusively derived from one mutation in the ALS gene as reported previously (Table 5.3), (Marshall *et al.*, 2013). As such it has evolved other NTSR traits to protect itself from herbicide injury.

Disregarding the deviation in mesosulfuron and pyroxsulam metabolism assays, the Velcourt and LongC populations have a greater level of herbicide resistance based on metabolite levels, than the Peldon 05 NTSR standard. It is probable that these two populations have higher levels of other NTSR traits, based on *Am*GSTF1 level, in addition to TSR. The high levels of *Am*GSTF1 in the R30, however, is anomalous as it is reported to be an ALS TSR standard(Marshall *et al.*, 2013).

The mesosulfuron metabolites that were identified in black-grass are the same ones reported in wheat. This suggests that black-grass metabolises mesosulfuron following the same pathway. Though the first cleavage product (i) was not used as a measure of resistance due it not being detected in all occasions, though it was still present in most samples. Cleavage (i) is then followed by rapid cyclisation to (ii) which may explain why levels of (i) are low (Figure 5.2). The pathway shows two routes to cleavage (i), a breakage of the sulfonyl bridge alone, or demethylation following the breaking of the sulfonyl bridge (Figure 5.2),. It is possible that the P450 that has evolved in selected field populations, Velcourt and LongC, metabolises mesosulfuron primarily by demethylation rather than breaking the sulfonyl bridge. The increase in pyroxsulam metabolism is not as proportionally as great in Velcourt and LongC, as compared to Peldon 05, despite the increase in metabolite levels. As there is only one route of detoxification for pyroxsulam, it could be suggested that the P450 activity responsible is enhanced in the field population.

Although the initial CPCA inhibition test in the absence of herbicide suggests that the ALS enzyme in resistant plants may be more active than in Roth 09 as determined through the increased levels of acetoin, when it came to analysing TSR vs NTSR populations the levels of acetoin were the same. One explanation is that the enhanced detoxification mechanism in NTSR black-grass prevented the herbicide reaching the target site keeping the levels of acetolactate high as seen in a TSR population. Herbicide levels in the acetolactate/acetoin experiments were not analysed by LC-MS due to the amount of sample clean up and dilution that would be required to safely inject. Modifying this assay to include an LC-MS step to look at any metabolite levels would be interesting. Looking at the technical aspects of the assay, the plant is crushed in a tube and incubated in water to release acetolactate, rather than being systematically homogenised. The sample preparation may have to be refined between species due to physiological difference in tissue types to release acetolactate.

From this series of work some conclusions can be drawn and ideas taken forward. The KARI inhibition assay did not provide any information on differences in acetoacetate level between TSR and NTSR populations and this could be down to several factors so is not suitable in assessing TSR. The current methods of using *Am*GSTF1 levels to determine NTSR, whether by ELISA or using the BReD device, are reliable, robust and will continue be used to characterise NTSR in black-grass populations. With regards to identifying TSR, this assay involved extracting the genomic DNA, amplifying the ALS gene, sending to sequence, then interpreting amplification cycles. This was a time consuming method of doing so and the recent development in LAMP technology used to identify TSR mutations in black-grass developed by OptiGene, Horsham, UK, can process up to eight samples in a little as 30 minutes. A combination of rapid TSR and NTSR testing should be encouraged because the sooner a resistance problem is identified, the sooner it can be remedied.

The analysis of the metabolism of mesosulfuron was very encouraging. Though variation in field populations is a challenge, comparing which metabolites are present in black-grass compared to wheat opens a path to examine detoxification pathways and how they differ between crops and weeds and how they give rise to selectivity. It is interesting to speculate that these pathways are better evolving in weeds to mimic crops or are independently evolving totally new mechanisms of resistance.

Chapter 6. Metabolism of Herbicide Mixtures in Wheat, Black-grass and Cell Cultures

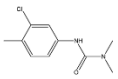
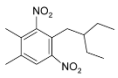
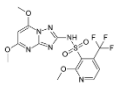
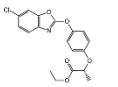
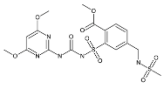
6.1 Introduction

The detoxification of herbicides and other xenobiotics occurs in series of biotransformations involving the introduction, or exposure of reactive chemical groups (phase 1), bioconjugation with GSH or sugars (phase 2) and vacuolar transport (phase 3). Cytochrome P450s, which constitute the largest family of enzymes involved in plant secondary metabolism, are involved in phase 1(Nelson and Werck-Reichhart, 2011). In the case of xenobiotic detoxification the products of CYP-catalysed phase I metabolism are then often acted upon by GSTs. Herbicide metabolites are often less toxic to plants, although some exceptions exist such as is the case with pro-herbicides where the metabolites are more phytotoxic (Jeschke, 2016). As their name suggests cytochrome P450 inhibitors bind to P450 enzymes to prevent them from biotransforming their substrates. There are many compounds known to inhibit cytochrome P450s in plants. Some are called synergists and work by suppressing the resistance mechanisms in weeds and restoring herbicide susceptibility. The applications of these inhibitors has been shown restore sensitivity to herbicides by reversing the growth inhibtion when applied with herbicides that previously stunted plant growth (Yanniccari, Gigón and Larsen, 2020). Some of these compounds have been shown to inhibit the formation of herbicide metabolites, inferring that P450 inhibition is responsible for detoxification. P450s have been the focus of herbicide metabolism research as a result of their ability to endow selectivity in crops and resistance in weeds (Dimaano and Iwakami, 2021). P450s from a single family, such as CYP81A, have been shown to underpin resistance to different herbicides acting on acetyl coenzyme A carboxylase (ACCase) and acetolactate synthase (ALS) (Iwakami *et al.*, 2019). Some P450s from crops and weeds metabolise multiple herbicides with various chemical structures. As such, the rotation of herbicides with different modes of action (Iwakami *et al.*, 2019), the application of herbicide mixtures has been suggested as a technique to combat herbicide resistance (Lagator *et al.*, 2013). Commercial herbicides used in weed control are a typical a mixture of compounds, not just the application of one active ingredient. The active ingredient (a.i.) is the herbicide responsible for phytotoxicity towards the weed. Adjuvants are used to improve various qualities of the a.i. including application, safety and storage (Johnson, Wyse and Lueschen, 1989).

Many herbicide metabolism studies seek to establish if a resistant weed population is evolving enhanced metabolism to a variety of herbicides by treating plants with a single

herbicide and repeating the assay by introducing a new chemistry. Hence, these results do not address the impact of herbicide mixtures on the detoxification of each herbicide in crops or grass weeds. The current studies sought to establish whether or not a resistant weed population can metabolise a mixture of herbicides, also whether the metabolites generated differ between resistant populations. A single P450 capable of metabolising multiple herbicides may have a specificity to a herbicide from a distinct chemical class and this can be observed by measuring the formation of metabolites. In order to assess the above, five herbicides, each associated with resistance in black-grass, from different chemical classes and four different modes of action. Four of the herbicides P450 detoxified and one GST detoxified herbicide (Table 6.1).

Table 6. 1. List of herbicides used in a mixture to treat wheat, black-grass and black-grass cell cultures.

Herbicide	Structure	Mode of Action	Chemical Class	Detoxification Pathway (Figure 1.1/1.2)
Chlorotoluron		Photosystem II Disruptor	Phenylurea	P450
Pendimethalin		Microtubule Assembly inhibitor	Dinitroaniline	P450
Pyroxsulam		Acetolactate Synthase Inhibitor	Triazolopyrimidine	P450
Fenoxaprop-P-ethyl		Acetyl CoA Carboxylase Inhibitor	Aryloxyphenoxy propionate	GST
Mesosulfuron-methyl		Acetolactate Synthase Inhibitor	Sulfonylurea	P450

6.2 *Am*GSTF1 in Wheat and Sensitive and Resistant Black-grass

Wheat has been included in the study as a reference as herbicide metabolism of selective herbicides is faster in the crop than the weed. The sensitive and resistant (Peldon – multiple herbicide resistant) black-grass used in this study were obtained from a commercial company, Herbiseed, UK, rather than from Rothamsted Research. The reason for doing so is that extensively studied and characterised black-grass seeds are in short supply and to overcome variation in populations by increasing replicates a lot of seed would be required in the study. The wheat used was Cordial 2016, at the time a commonly used winter wheat, high yielding bread wheat. It was necessary to determine that the purchased Peldon population had elevated levels of *Am*GSTF1 as compared to the sensitive black-grass, to be confident that it is indeed displaying NTSR. The *Am*GSTF1 levels in wheat were also measured for comparative purposes, even the homologue in the

crop is not known to be associated with metabolic resistance (Figure 6.1). The plants were treated with the five herbicides, individually and as a mixture, alongside a control containing only half strength Murashige and Skoog (MS), a salt media. The purpose of this experiment is to show that none of the herbicides will affect the levels of *Am*GSTF1. The experimental design was set up as follows, following an excised shoot protocol, five plants were pooled as one biological replicate and five biological replicates were treated. The level of GSTF1 was quantified by Sandwich ELISA.

Wheat and herbicide sensitive black-grass contained between 0.1 and 1.2 µg/g *Am*GSTF1, (Figure 6.1). All the mean levels of *Am*GSTF1 in the resistant population (Peldon) were above 1.2µg/g to ~10 µg/g. Although variation is present and mean levels are similar, it is clear that there were outliers, especially seen following treatment with mesosulfuron, or pyroxsulam. However, the control samples treated with salt media alone, the level of *Am*GSTF1 was essentially the same as seen with the herbicide treated samples. The susceptible samples had very low readings that are down to technical error in the technique, except for one sample in the chlorotoluron and mixture treatment. The results in wheat are interesting, no seed misidentification could have taken place due to the physiological difference between wheat and black-grass seed, the variation of GSTF1 seen in some of the samples could be due to cross reactivity of the *Am*GSTF1 antibody with an homologue in wheat.

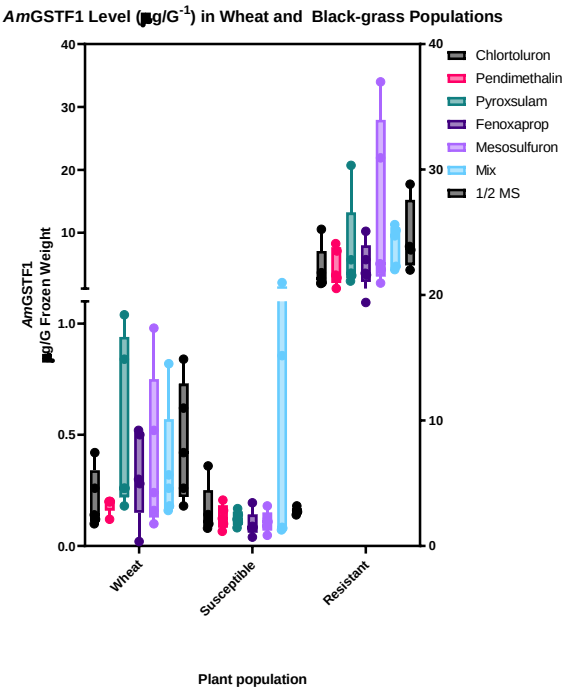


Figure 6. 1. AmGSTF1 levels in µg/g frozen weight, measured in wheat, susceptible and resistant black-grass. N =5, error bars are Std. Dev..

6.3 Metabolism of Herbicide Mixtures in Wheat and Black-grass

ATLANTIS® is an ALS inhibitor containing mesosulfuron-methyl (30g/kg) co-formulated with iodosulfuron methyl (6g/kg), (Bayer, UK). The reasons for doing so are not well reported. Both mesosulfuron and iodosulfuron are ALS inhibitors and it is common advice to rotate the use of ALS inhibiting herbicides where TSR mutations are present (Gerwick, Mireles and Eilers, 1993), so using two different ALS inhibitors in one application may appear counterintuitive. Two reasons for co-formulation can be suggested, firstly that that known TSR mutations in the ALS enzyme towards mesosulfuron may not have an effect on the binding of iodosulfuron so iodosulfuron can target the mutated ALS that mesosulfuron cannot. Secondly, the detoxification enzyme responsible for mesosulfuron detoxification is also responsible for iodosulfuron metabolism and may be more active towards the latter. This could mean that the presence of a second P450 substrate shifts any detoxifying activity away from mesosulfuron allowing the herbicide to inhibit ALS.

6.3.1 *Floating Leaf vs Excised Shoot*

To investigate herbicide metabolism further, we first established a protocol to study herbicide uptake in wheat and black grass tissues. There are many different protocols as to

how best to treat a plant with herbicide. Protocols include but are not limited to, injection, spraying, painting and submersion. When treating black-grass with post-emergence herbicides, it was important that plants were treated in the early growth stages, (1 tiller, 2-3 weeks). The small size of the grass at this stage requires a simple reproducible method that ensures the herbicide can be taken up by the plant, at a rapid rate, in order for metabolism to occur. Spraying, painting or injecting such small plants on a large scale is very time consuming so a 'cut and treat' method was employed here. As individual herbicides move through the plant at different rates (DeBoer *et al.*, 2011), it is important to establish a method that allows efficient uptake and metabolism. Two methods involving placing plants in a herbicide mixture were used containing 8µM each chlorotoluron, pendimethalin, pyroxsulam, fenoxaprop and mesosulfuron .

Firstly, excised shoots were used, whereby a plant was removed from the soil, cut roots placed in a herbicide mixture. The roots are trimmed under water to prevent an air gap forming in the vascular tissue so preventing the herbicide from moving inside the plant. The second method employed excised shoots prepared by cutting the plants into three segments and placing them on a herbicide mixture prepared as for the excised shoot assay. Five plants were pooled in to one biological replicate and five biological replicates were treated each for wheat, sensitive and resistant black-grass. This allows for all the herbicides to move through the plant tissue regardless of their affinity for systemic movement. The results of the comparison of metabolite levels found when using the two methods are shown in Table 6.3. Whilst showing some differences, the results show that the two methods are capable of detecting herbicide metabolism at a relatively short period of time (6 hours) and at a relatively low concentration, (8µM -, 40µM). (Table 6.3).

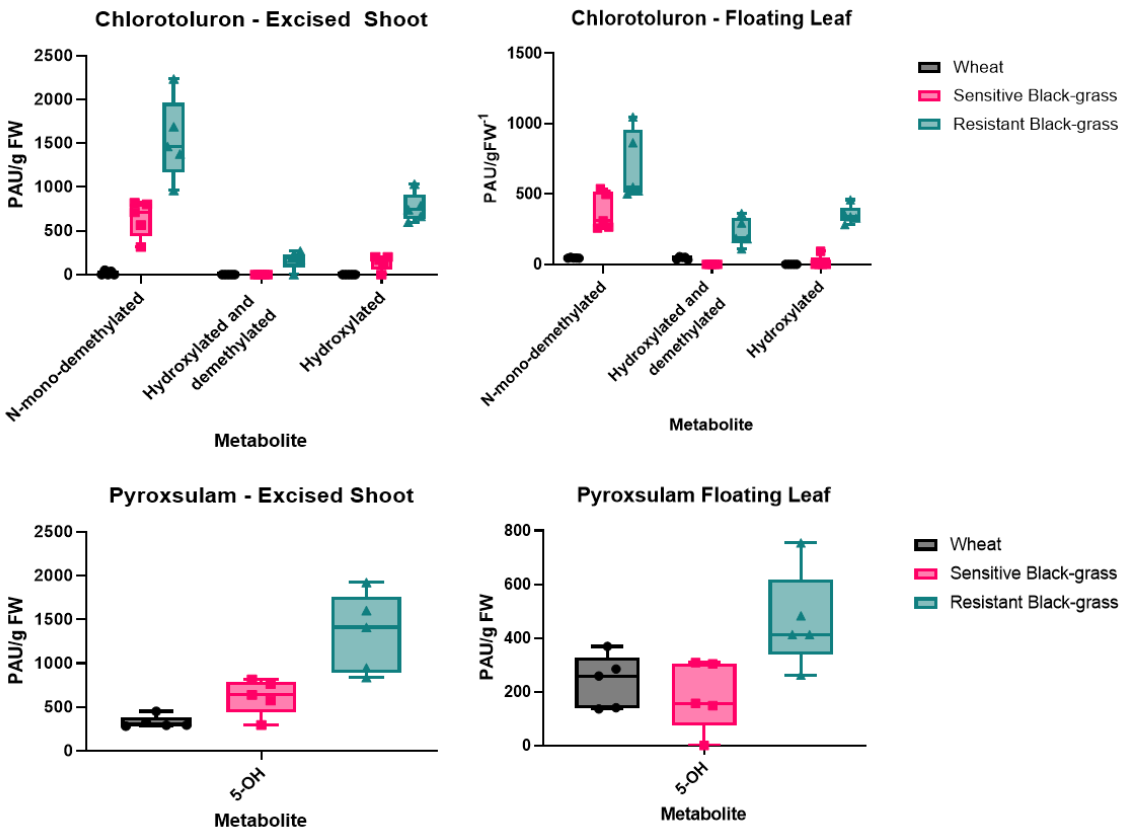


Figure 6. 2. Chlorotoluron (top) and pyroxsulam (bottom) metabolite level from excised shoot (left) and floating leaf (right) herbicide assays. Mean herbicide metabolite levels (PAU per gram of fresh weight) n= 5, ± Std. Dev.

The parent molecule of each herbicide was detected in both the excised shoot and floating leaf assays. The levels of chlorotoluron and pyroxsulam metabolites from the excised shoot assay were higher than in the floating leaf assay, both in wheat and resistant black-grass (Figure 6.2). The levels of pendimethalin were similar in both assays. All three mesosulfuron metabolites were detected in the excised shoot assay in wheat. Only the cleavage (ii) metabolite was found in sensitive black-grass and interestingly no metabolites were detected in the sensitive black-grass population. All of the fenoxaprop metabolites were detected in wheat in both assays, only the CBO-SG and CBO-C metabolites found in resistant black-grass and no fenoxaprop metabolites found in sensitive black-grass (Table 6.3). The levels of detected metabolites were similar across both the floating leaf and excised shoot assay. From these results it was decided to use the excised shoot assay going forward. This decision was based on the higher levels of chlorotoluron and pyroxsulam metabolites generated in the excised shoot assay.

6.3.2 *Individual Herbicide Treatment vs Mixture Treatment*

As the hypothesis of these experiments is that there were will be favoured metabolism routes acting in a mixture of competing herbicides, the effect of treating the plants with a

herbicide mixture as compared to an individual treatment on the metabolism of individual herbicides was determined. Five plants were pooled into one biological replicate and five biological replicates were assayed for wheat, sensitive an resistant black-grass with the five individual herbicides and one mixture of all five herbicides, 90 samples total. Each individual herbicide treatment was at a concentration of 8µM, with the mixture of all representing a total concentration of 40µM. These concentrations were chosen for two main reasons. Firstly, in the previous studies metabolism can be seen at a concentration of 8µM and secondly, whilst greater levels of metabolism can be obtained by increasing the concentration, this could lead to a rapid phytotoxic injury in a way that shuts down any biological detoxification process.

The data resulting from this assay show that all expected metabolites were determined apart from the demethylated and hydroxylated chlorotoluron metabolite and the product of mesosulfuron cleavage (i) (Table 6.2). The pendimethalin carboxylic acid metabolite was only found in the Peldon samples treated individually with pendimethalin.

Most noticeable are the levels of metabolites found in wheat samples. With the exception of demethylated mesosulfuron, they were found to be lower than in either the sensitive or resistant black-grass samples (Figure 6.2). As selectivity is ascribed to the ability of wheat to detoxify herbicides at a much faster rate than in the weed it would be expected to find higher levels of detoxification product in wheat. It may be the case that the metabolites formed in wheat were further modified for further downstream processing.

For each metabolite within a plant population, a paired 2-tailed t-test analysis was run, with a cut off, p-value (<0.05), is to statistically determine if the metabolites with a different level between the individual and mixture treatment. There was no statistical difference in any metabolite level in the sensitive black-grass population, between individual and mixture treatments. There was also no difference observed in the level of the three fenoxaprop metabolites in wheat and resistant black-grass (Table 6.2). The only chlorotoluron metabolite with a difference between the two treatments was the *N*-demethylated product which was seen in increased levels (5668 PAU) in the mixture compared to individual treatment (2495 PAU). This was the only occurrence of a significant increase of a metabolite seen with mixtures as compared to an individual treatment. In both black-grass populations the mean level of *N*-demethylation were lower in the mixture treatment. The second cleavage (ii) product of mesosulfuron metabolism was found in lower levels in the mixture treatment in wheat and resistant black-grass.

None of the pyroxsulam 5-OH metabolite was found at significantly lower levels (p-value <0.05) in the mixture treatment in the three populations. But in wheat and resistant blackgrass the mean levels were noticeably reduced although not significant, (p-value = 0.069 and 0.052) in wheat and resistant black-grass respectively, all five herbicides generated lower metabolite levels in a mixture treatment. Notably only the P450 detoxified herbicides were significantly affected, namely chlorotoluron, pendimethalin, pyroxsulam, and mesosulfuron (Table 6.2) Fenoxaprop, a GST detoxified herbicide, was not significantly affected, suggesting that there may be competition between the P450s responsible for herbicide detoxification.

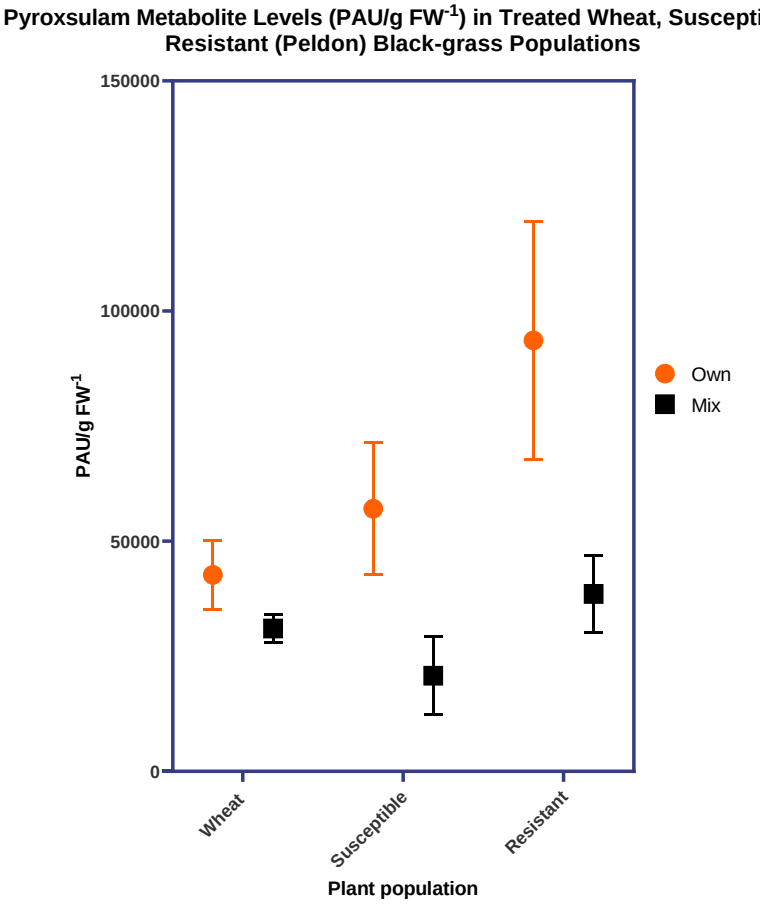


Figure 6. 3. Metabolism of pyroxsulam in wheat, susceptible black-grass and resistant black-grass (Peldon). Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 5, ± = Std. Dev.

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Table 6. 2. Levels of metabolites in wheat, sensitive and Peldon black-grass treated with a mixture of 5 herbicides for 24 hrs. Excised shoot vs floating leaf assay. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 5, ± = Std. Dev. ND - not detected

		Herbicide Metabolites																					
		Chlorotoluron						Pendimethalin				Pyroxsulam		Fenoxaprop-P-ethyl						Mesosulfuron-methyl					
		N-mono-demethylated		Hydroxylated		Demethylated and Hydroxylated		Hydroxylation		Carboxylic Acid		5-OH		CBO-SG		CBO-CE		CBO-C		Demethylation		Cleavage (i)		Cleavage (ii)	
Treatment			±		±		±		±		±		±		±		±		±		±		±		±
Wheat																									
	Individual	3495	154	2312	258	ND	ND	5673	517	ND	ND	42686	7430	174	90	4804	2814	755	25	8588	2250	ND	ND	827	130
	Mixture	5668	617	1938	292	ND	ND	1344	236	ND	ND	31027	3043	270	74	4894	1244	868	89	7850	1621	ND	ND	421	43
Sensitive																									
	Individual	34804	6314	3910	1418	ND	ND	24904	5200	ND	ND	57072	14362	2904	711	3321	948	2891	554	1363	424	ND	ND	2434	845
	Mixture	17672	6624	5193	2126	ND	ND	19743	7679	ND	ND	20789	8441	2871	1097	2362	991	2235	677	1369	235	ND	ND	3137	468
Peldon																									
	Individual	30219	5822	3673	742	ND	ND	21959	2548	284	174	93614	25763	6934	3238	5979	2477	4375	1664	5008	1921	ND	ND	3417	1102
	Mixture	9926	1598	2567	663	ND	ND	10763	3293	ND	ND	38558	8408	4675	751	3580	636	2718	393	1962	532	ND	ND	1538	448

Table 6. 3. Levels of metabolites in wheat, sensitive and Peldon black-grass treated with a mixture of 5 herbicides for 6 hrs. Excised shoot vs floating leaf assay. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 5, ± = Std. Dev. ND - not detected.

		Herbicide Metabolites																					
		Chlorotoluron						Pendimethalin				Pyroxsulam		Fenoxaprop-P-ethyl						Mesosulfuron-methyl					
		N-mono-demethylated		Hydroxylated		Demethylated and Hydroxylated		Hydroxylation		Carboxylic Acid		5-OH		CBO-SG		CBO-CE		CBO-C		Demethylation		Cleavage (i)		Cleavage (ii)	
Assay			±		±		±		±		±		±		±		±		±		±		±		±
Wheat																									
	Floating Leaf	46	3	ND	ND	44	10	440	120	ND	ND	238	100	92	33	203	101	93	37	ND	ND	42	27	17	38
	Excised Shoot	18	25	ND	ND	ND	ND	241	38	ND	ND	329	70	88	13	172	13	82	12	9	19	67	16	24	54
Peldon																									
	Floating Leaf	698	246	352	65	ND	ND	4951	1877	ND	ND	465	181	362	181	ND	ND	29	64	ND	ND	ND	ND	ND	ND
	Excised Shoot	1545	466	766	167	166	100	3112	1461	146	134	1343	453	284	128	ND	ND	78	111	ND	ND	ND	ND	ND	ND
Sensitive																									
	Floating Leaf	374	134	19	42	227	100	1684	552	207	157	184	128	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	42	94
	Excised Shoot	643	209	137	85	ND	ND	684	117	230	117	617	204	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

6.3.3 *Herbicide Mixture Time Course*

The previous herbicide mixture study was conducted over 24 hours, (Section 6.3.2.). Two outputs from the work that were deserving of further study were: firstly, there are lower level of metabolites determined in wheat than in the sensitive or resistant black-grass (Figure 6.3), and secondly, competition appears to exist with regards to the P450 detoxified herbicides. It was conjectured that after 24 hour, wheat is able to modify primary metabolites for further metabolism through conjugation with polar products or through bound residue formation and that may explain why the level of primary metabolites were lower than in black-grass. To investigate that possibility, the same experiment was run with the herbicide mixture over a shorter time course (1, 2, 4 and 6 hours) with wheat, sensitive and resistant black-grass and metabolite levels determined (Table 6.4.).

Continuing the trend seen in the 24 hours treatment, the metabolite levels in wheat were lower than that determined in sensitive black-grass, which were in turn, lower than that of resistant black-grass. Out of the four time points, the 6 hour time point generated most of the metabolites, in the greatest abundance. There is a general, logical, trend that the longer the herbicide treatment, the greater levels of metabolites generated, increasing over 24 hours. All three mesosulfuron metabolites were only detected in wheat after six hours, albeit at very low levels. The three metabolites of fenoxaprop, the only GST detoxified herbicide, were found in wheat after 6 hours, while in sensitive black-grass was seen after only 1 hour. In general, the P450 metabolised products were found in lower levels in the mixture treatment compared to the individual treatments (Table 6.2.) In both wheat and resistant black-grass, were all being observed at either 4 or 6 hours (Table 6.4). These metabolites were either products of demethylation or hydroxylation, two of the most common P450 mediated reactions. Again, following the pattern seen after 24 hours, the pendimethalin carboxylic acid metabolite was only determined in black-grass, not wheat. This may suggest that there is a P450 responsible for pendimethalin oxidation that's exclusive to black-grass. The low levels of mesosulfuron metabolites determined after 6 hours suggest that the optimum time to observe mesosulfuron metabolism in wheat and black-grass was between 6 and 24 hours, while for the other four herbicides, a 6 hour time was sufficient.

Table 6. 4 . Levels of metabolites in wheat, sensitive and Peldon black-grass treated with a mixture of 5 herbicides for 1,2,4 and 6 hrs. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 3, ± = Std. Dev. ND not detected.

		Herbicide Metabolites																					
		Chlorotoluron						Pendimethalin				Pyroxsulam		Fenoxaprop-P-ethyl						Mesosulfuron-methyl					
		N-mono-demethylated		Hydroxylated		Demethylated and Hydroxylated		Hydroxylation		Carboxylic Acid		5-OH		CBO-SG		CBO-CE		CBO-C		Demethylation		Cleavage (i)		Cleavage (ii)	
	Time point (hr)		±		±		±		±		±		±		±		±		±		±		±		±
Wheat																									
	1	ND	ND	ND	ND	118	37	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	108	67	ND	ND
	2	ND	ND	ND	ND	129	28	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	103	50	ND	ND
	4	11	24	40	69	82	61	94	33	ND	ND	294	123	ND	ND	ND	ND	ND	ND	ND	ND	74	36	ND	ND
	6	18	25	ND	ND	ND	ND	241	38	ND	ND	329	70	88	13	172	13	82	12	9	19	67	16	24	54
Sensitive																									
	1	68	97	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	62	85	47	65	194	41	ND	ND	ND	ND	ND	ND
	2	110	73	15	33	ND	ND	14	32	ND	ND	40	55	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	4	493	266	125	157	ND	ND	325	260	ND	ND	3455	223	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	6	643	209	137	85	ND	ND	684	299	230	117	617	204	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Peldon																									
	1	276	81	80	118	ND	ND	ND	ND	ND	ND	83	80	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2	511	198	200	45	ND	ND	501	164	ND	ND	144	152	21	46	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	4	1053	350	478	104	ND	ND	947	374	ND	ND	401	239	135	83	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	6	1545	466	766	167	166	100	3112	1461	146	134	1343	453	284	128	ND	ND	78	11	ND	ND	ND	ND	ND	ND

6.4 Metabolism of Herbicide Mixtures in Cell Cultures

Whole plant feeding studies, are not the only method available to conduct herbicide metabolism studies. A common, yet more technically challenging method of doing so is by treating the respective plant cell cultures with herbicides. Plant cell cultures have been investigated for more than 50 years as a potential source of secondary metabolites to rival extraction processes based on whole plant material (Nuutila and Oksman-Caldentey, 2003). A major advantage of using cell cultures is that there are no limitations with regards to herbicide uptake and availability, as the cells are surrounded by herbicide in media. The black-grass populations used for cell culture initiation were Rothamsted 09 -herbicide sensitive, Pendimethalin – a selected population, resistant to 'fops', 'dens', and pendimethalin, Fenoxaprop - a selected population, resistant to fenoxaprop-P-ethyl without enhanced metabolism and Peldon 07- resistant to Atlantis (mesosulfuron-methyl and iodosulfuron methyl), 'fops', 'dens', pendimethalin, and chlorotoluron (Table 6.6.) The chosen cell cultures were initiated from black-grass, derived from survivors of successive herbicide applications and the respective seeds were then used to establish callus cultures,. For each population four 250mL flasks containing cells were each split in to three 100mL flasks. The cells from each population were treated with the same mixture of herbicides used in the previous whole plant studies, for 1, 2, 4 and 6 hours with 3 biological reps per time point, 48 samples in total. A summary of the data including metabolite levels and standard deviations are shown and results discussed in the following sections (Table 6.7)

Table 6. 6. Cell culture material and anticipated herbicide sensitivity.

Black-grass Population	Cell Culture	Herbicide Sensitivity
Rothamsted 09	Control cell culture (CCC)	Chlorotoluron, pendimethalin, pyroxsulam, fenoxaprop, mesosulfuron
Pendimethalin	Pendimethalin resistant (PCC)	Chlorotoluron, pyroxsulam, mesosulfuron
Fenoxaprop	Fenoxaprop resistant (FCC)	Chlorotoluron, pendimethalin, pyroxsulam, mesosulfuron
Peldon 07	(ACC)	None of the five herbicides used in this study

6.4.1 *Rothamsted 09 (CCC)*

All of the herbicide metabolites were detected at at least one time point except for the mesosulfuron demethylation and cleavage (i) products with, the cleavage (ii) metabolite only detected at low levels (<100PAU). The general trend was for an increase in all herbicide metabolite levels as the time of treatment increased. However, at 6 hours post

treatment there was drastically lower levels of the metabolism of all herbicides compared to 1, 2 and 4 hours. This was seen with all of the herbicides tested. It was concluded that the viability of the cells are reduced after a 6 hr treatment and as such only the earlier time points were used in this study. At all three timepoints (1 – 4hr) the most abundant chlorotoluron metabolite was the demethylated product, followed by the hydroxylated and then the combined hydroxylated and demethylated metabolite. Hydroxylated pendimethalin was present at all time points with the mean level positively increasing with time, 1 hr (1405PAU), 2 hr (2347PAU) and 4 hrs (10698PAU). Hydroxylated pyroxsulam was present at all time points with the mean level positively increasing with time, 1 hr (115PAU), 2 hr (201PAU) and 4 hrs (2624PAU). Formation of fenoxaprop metabolite CBO-SG occurs by 1 hour and increase over time, as does the formation of the CBO-CE but at a much slower rate. The CBO-C metabolite did not appear until 4 hours, this is predictable as the formation of the fenoxaprop metabolites follows the order, CBO-SG > CBO-CE > CBO-C.

6.4.2 *Peldon 07 (ACC)*

All of the metabolites were detected at at least one time point except for the mesosulfuron demethylation and cleavage (i) metabolites. The cleavage (ii) metabolite was only detected in low levels (<110PAU). Mimicking the trend seen in the CCC there was an increase in metabolite level as the time of treatment increases. The exception to this was the 6 hour timepoint. which had drastically lower levels of metabolism compared to 1 ,2 and 4 hours, with the exception of the fenoxaprop metabolites which have similar level at 6 hours and at 1 hour. At 6 hours pyroxsulam also had higher levels of the 5-OH metabolite than after 2 hours (213PAU) but less than determined at 4hours (1513PAU). At all three timepoints the most abundant chlorotoluron metabolite was the demethylated product, followed by the hydroxylated and then the hydroxylated and demethylated metabolite. Hydroxylated pendimethalin was present at all time points with the mean level positively increasing with time, 1 hr (1883PAU), 2 hr (2828PAU) and 4 hrs (6724PAU). Echoing the pattern of fenoxaprop metabolism seen in CCC, production of CBO-SG was determined by 1 hour (3485PAU) and increased over time to 8640PAU after 4 hours. The formation of the CBO-CE occurs by 1 hour (197PAU) and increases over time to 564PAU after 4 hours. CBO-C is formed after 2 hours, this was faster than determined in CCC where it is not seen until 4 hours.

6.4.3 *Pendimethalin-Selected Culture (PCC)*

Unlike the other three populations the only mesosulfuron metabolite detected was derived from cleavage (i) and was seen at all time points. The formation of mesosulfuron metabolites is not consistent across the three resistant cell populations. Unexpectedly, the demethylated metabolite was not detected, which is the product of the first step in mesosulfuron detoxification but only the cleavage (i) product was detected. Even though some levels of metabolite were determined in these experiments, there is not a consistent amount of metabolite to draw any meaningful conclusion, other than the 6 hour time course in cell culture was not the ideal assay with this herbicide. With the other herbicides metabolism levels follow the trend seen in the CCC, there was an increase in metabolite level as the time of treatment increased, except with chlorotoluron and pendimethalin. In both instances the level of metabolites at 6 hours was less than that determined at 4 hours but factors such as cell viability can be ruled out as being the cause the level of fenoxaprop metabolism was at its greatest at 6 hours. Chlorotoluron metabolism peaked after 2 hours, with all three metabolites determined, the overall levels of metabolite, however, are substantially lower than in the other three populations, suggesting that the PCC is more susceptible to chlorotoluron than the other cultures. The levels of fenoxaprop metabolites increased from 1 to 2 hours, then declined after 4 hours, to increase again at 6 hours. The levels of CBO-C at 2 hours suggest that when CBO-C accumulates, the GST reaction slows down until CBO-C is transported away because as the level of CBO-C drops after 6 hours, the levels of CBO-SG increase again. Pyroxsulam 5-OH is seen after 2 hours (141PAU), 4 hours(228PAU) and 6 hours (523PAU). The PCC is the only one in which pyroxsulam metabolism was not seen after 1 hour. The PCC is derived from sensitive black-grass that has been selected to be resistant to pendimethalin so it was not surprising to see the respective hydroxylated metabolite formed in these experiments. What is interesting is that the levels of hydroxylated metabolite were lower seen at 1, 2 and 4 hours in CCC, instead there is a significantly high level of the carboxylic acid metabolite formed. The carboxylic acid metabolite is seen in the CCC, PPC and ACC but the levels seen in the PCC were higher than determined in the other black-grass cultures. The highest level of the carboxylic acid was seen after 2 hours. This suggests that enhanced resistance to pendimethalin is mediated through a second detoxification enzyme responsible for forming the carboxylic acid.

6.4.4 *Fenoxaprop-Selected Culture (FCC)*

Along with the CCC and ACC and populations, low levels of mesosulfuron cleavage (ii) were determined in the fenoxaprop selected culture. There were also very low levels of metabolites found at the 6 hour time point for the other four herbicides so the most meaningful data comes from the 1, 2 and 4 hour timepoints, however, in contrast to the other three populations, the levels of chlorotoluron and pendimethalin metabolites declined over 1 to 4 hours rather than increasing. As the levels of fenoxaprop metabolites do not decline as seen with the chlorotoluron and pendimethalin metabolites it can be assumed that cells are still viable after 4 hours. Though the FCC has known resistance to chlorotoluron and pendimethalin it is interesting that its ability to metabolise chlorotoluron and pendimethalin appears to decrease between 1 and 4 hours. This decrease in detoxification of these two P450 detoxified herbicides may be the result of the plant evolving GST detoxifying enzymes, to further metabolise fenoxaprop, at the expense of the evolution of the P450 detoxifying enzymes. The levels of hydroxylated pendimethalin are far higher than in the PCC, yet the carboxylic acid levels are far lower. Pyroxsulam 5-OH was found at all timepoints and being most abundant at 4 hours. The levels CBO-SG are far higher than found in the other populations. CBO-SG levels were approximately the same at 1, 2 and 4 hours. The CBO-CE levels increased from 1 to 4 hours, with the CBO-C levels staying at roughly the same level. Compared to the PCC population, the levels of CBO-SG and CBO-C were far higher in the FCC, therefore resistance to fenoxaprop in this case was associated with enhanced metabolism of the herbicide.

Table 6. 7. Levels of metabolites in Rothamsted 09, Peldon 07, Pendimethalin and Fenoxaprop cell cultures, treated with a mixture of 5 herbicides for 1,2,4 and 6 hrs. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 3, ± = Std. Dev. ND not detected.

		Herbicide Metabolites																					
		Chlorotoluron						Pendimethalin				Pyroxsulam		Fenoxaprop-P-ethyl						Mesosulfuron-methyl					
		N-mono-demethylated		Hydroxylated		Demethylated and Hydroxylated		Hydroxylation		Carboxylic Acid		5-OH		CBO-SG		CBO-CE		CBO-C		Demethylation		Cleavage (i)		Cleavage (ii)	
	Time point (hr)		±		±		±		±		±		±		±		±		±		±		±		±
Rothamsted 09																									
	1	2575	1490	689	416	274	425	1405	855	ND	ND	115	66	2227	1394	131	94	ND	ND	ND	ND	ND	ND	ND	ND
	2	1602	655	456	110	128	11	2437	225	ND	ND	201	66	3723	841	212	40	ND	ND	ND	ND	ND	ND	ND	ND
	4	5102	2662	1853	1071	334	161	10698	4607	ND	ND	2624	1373	24484	9858	783	323	380	171	ND	ND	ND	ND	ND	ND
	6	21	7	ND	ND	ND	ND	589	361	383	255	7	13	1172	641	838	611	248	189	ND	ND	ND	ND	94	44
Peldon 07																									
	1	3997	1304	1131	455	76	67	1883	291	ND	ND	199	73	3485	1348	197	77	0	0	ND	ND	ND	ND	ND	ND
	2	1963	324	575	154	156	72	2828	1227	ND	ND	213	32	5049	3465	282	173	164	211	ND	ND	ND	ND	ND	ND
	4	2570	2582	1252	597	209	64	6724	3341	ND	ND	1513	584	8640	5861	564	279	272	167	ND	ND	ND	ND	ND	ND
	6	419	212	24	21	259	95	942	524	ND	ND	689	392	3820	3260	189	161	77	65	ND	ND	ND	ND	110	34
Pendimethalin																									
	1	16	28	ND	ND	ND	ND	183	55	966	255	ND	ND	232	49	68	6	99	2	ND	ND	131	28	ND	ND
	2	602	249	327	102	39	68	1469	324	5271	1670	141	135	4184	1832	1650	518	2034	779	ND	ND	378	164	ND	ND
	4	438	184	192	57	ND	ND	2135	701	2988	521	228	117	1343	239	909	75	1379	568	ND	ND	367	63	ND	ND
	6	330	493	25	43	95	165	1376	603	650	215	523	143	6497	2972	1276	628	963	540	ND	ND	47	17	ND	ND
Fenoxaprop																									
	1	3500	2194	850	638	1114	696	13767	10404	630	472	595	513	33991	27952	722	604	6849	5756	ND	ND	ND	ND	ND	ND
	2	2037	332	618	110	603	135	10518	1650	195	41	517	33	34338	7056	913	140	5883	1754	ND	ND	ND	ND	ND	ND
	4	1934	268	311	89	519	89	5565	1636	116	34	835	148	38662	15875	1537	505	5807	2046	ND	ND	ND	ND	ND	ND
	6	250	622	23	25	38	114	1145	852	ND	ND	215	203	1733	1077	176	145	348	371	ND	ND	ND	ND	154	81

6.5 Cellulase Assay; Recovery of Modified Metabolites

As seen in the comparison of the *in-planta* herbicide metabolism assays (Table 6.4) levels of metabolites were lower in wheat than in black-grass. This is in contradiction to the basis of selectivity where higher levels of herbicide metabolites would be expected in wheat. One possible explanation is that wheat has in fact metabolised the herbicide at a faster rate and that hydroxylated metabolites have undergone glycosylation or another conjugation reaction in order to transport the herbicide to the vacuole. In order to determine if this is what was happening in wheat, the plant material treated with herbicides was treated to cellulase digestion then reanalysed by mass spectrometry and the metabolite levels examined. The incubation of the metabolite extract in cellulase will cleave any glucose conjugated to the hydroxylated metabolite. For each sample, where there was enough remaining extract, the extract was split into two and one incubated with cellulase and the other without. An increase in the metabolite level would be expected in the samples incubated with cellulase, if any hydroxylated metabolite underwent glycosylation. The cellulase assay was carried out on the wheat samples from the 6 hour time point. A comparison between wheat and black-grass was not possible due to insufficient amount of the black-grass extract remaining.

6.5.1 *Wheat*

Following cellulase treatment, there was in increase in hydroxylated chlorotoluron, fenoxaprop-cysteine and mesosulfuron cleavage (ii) (Figure 6.4). This suggests that these metabolites have all undergone glycosylation by six hours. The fenoxaprop cysteine metabolite, CBO-C, has previously been shown to undergo glycosylation (Cummins, Bryant and Edwards, 2009). The lack of pyroxsulam and pendimethalin metabolite recovery suggests that they do not undergo glycosylation by six hours.

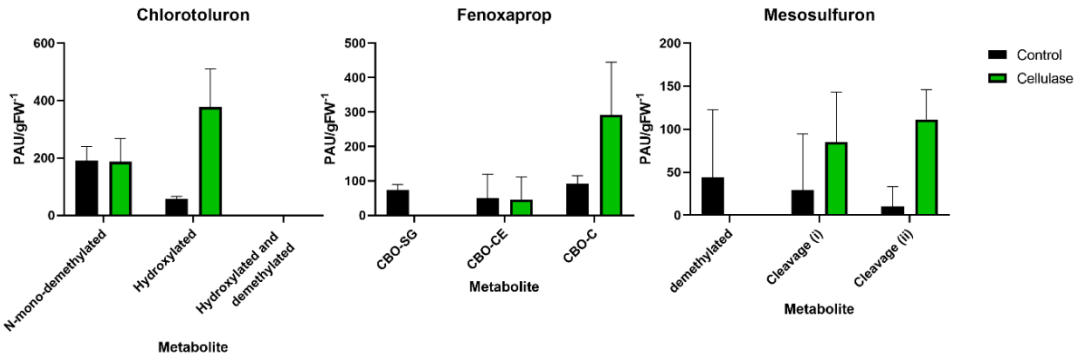

Figure 6. 4. Chlorotoluron, fenoxaprop and mesosulfuron metabolite levels in wheat extract treated with (cellulase) and without cellulase(control). N = 5, ± =Std. Dev.

6.5.2 *Black-grass Cell Cultures*

The cellulase digestion reaction was performed on all of the black-grass cell culture extracts (Section 6.4) and metabolite levels analysed again (Table 6.7.). The only mesosulfuron metabolite recovered was derived from cleavage (ii) and was recovered in the cellulase assay in the extract from the 6 hour timepoint in all four populations. This agrees with what was found in the wheat extract, suggesting that the phase II of mesosulfuron metabolism involves glycosylation of the second cleavage product. In the cell cultures, recovery of the pyroxsulam 5-OH was seen in cellulase treated ACC, PCC and FCC at the 6 hour time point, which was not seen in wheat. Recovery of hydroxylated pendimethalin was seen in all four black-grass populations after 6 hours. A greater level of recovery in the PCC was expected but this is not the case. In the pendimethalin control at six hours a mean level of 1257PAU was determined in the control and 1693PAU following cellulase treatment, an approximate increase in 30%. In CCC there was a mean level of 311PAU in the control and 1013PAU in the cellulase assay an increase of ~200%. The highest levels of hydroxylated pendimethalin were found in the FCC, the only increase in cellulase, is found at the 6 hour timepoint. Demethylated chlorotoluron and hydroxylated chlorotoluron were recovered in the cellulase assay in the three resistant black-grass populations after six hours. The recovery of demethylated chlorotoluron was greatest in the ACC and FCC populations, with the levels increasing around 200% as compared to the control. The increase in the PCC was approximately 40%. The only increase in the combined demethylated and hydroxylated metabolite was found in the cellulase digestion of the Peldon line after one hour but not in all replicates, or in the non-hydrolysed control. Echoing the pattern of metabolite recovery seen in wheat, the amount of CBO-C detected in the cellulase assay is higher than in the non-cellulase treated extract in the CCC, PCC and FCC cultures after 6 hours. Expectedly, the highest level of fenoxaprop metabolites was seen in the FCC.

6.6 Pre-treatment of Wheat and Resistant Black-grass with Cytochrome P450 Inhibitors and the Effect on Herbicide Metabolism

Some P450 inihibitors have been shown to inhibit the formation of herbicide metabolites, inferring that P450 inhibition is responsible for detoxification. To test if herbicide resistance is mediated by P450s, we monitored herbicide metabolite levels upon treatment with P450 inhibitors. The three P450 inhibitors used are aminobenzotriazole (1-ABT), a non-selective mechanism-based inactivator of P450 enzymes, shown to inhibit the metabolism of chlorotoluron in both resistant and susceptible black-grass by reducing the

formation of hydroxylated derivatives and polar conjugates (Menendez and De Prado, 1997). Malathion is a broad-spectrum organophosphorus insecticide used in agricultural, industrial and domestic settings, it has been shown to have synergistic effects in resistant bent-grass (Elmore *et al.*, 2015). Piperonyl butoxide (PBO) is a pesticide synergist that significantly inhibits demethylation and hydroxylation of isoproturon in *Philaris minor* (Singh, Kirkwood and Marshall, 1998). A previous study has shown that the application of malathion can reverse resistance to mesosulfuron-methyl in *A. aequalis* based on the reduction in growth rate (Zhao *et al.*, 2017). The aim of this study was to observe any effects that the P450 inhibitor may have on herbicide metabolism in resistant black-grass, in a herbicide mixture vs a negative control, which is not treated with P450 inhibitor. The study was also carried out in wheat, to observe any differences or similarities in the metabolite profile that may be shared with resistant black-grass.

A treatment with a mixture of all three inhibitors was used because if a single inhibitor caused a reduction in a metabolite level, then it was of interest to determine if further reduction in the metabolite levels within the mixture was observed. If further reduction was seen in the treatment with three inhibitors it could be suggested that the P450 responsible for metabolism is inhibited by multiple synergists, or that there are multiple P450s responsible for detoxification. The pre-treated plant tissue was then placed into a mixture of pyroxsulam, pendimethalin, fenoxaprop, chlorotoluron and mesosulfuron (8µM each, 40µM total), for six hours. No mesosulfuron metabolites were detected in this assay therefor the analysis was based on the detected metabolites of the other four herbicides. The levels of fenoxaprop, pendimethalin, chlorotoluron and pyroxsulam metabolites in the inhibitor treated samples were then compared to the control (Table 6.7).

Table 6. 8 Levels of metabolites in Rothamsted 09, Peldon 07, Pendimethalin and Fenoxaprop cell cultures ± cellulase. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 3, ± = Std. Dev. ND not detected.

			Herbicide Metabolites																							
			Chlorotoluron						Pendimethalin				Pyroxsulam		Fenoxaprop-P-ethyl						Mesosulfuron-methyl					
			N-mono-demethylated		Hydroxylated		Demethylated and Hydroxylated		Hydroxylation		Carboxylic Acid		5-OH		CBO-SG		CBO-CE		CBO-C		Demethylation		Cleavage (i)		Cleavage (ii)	
				±		±		±		±		±		±		±		±		±		±		±		±
Rothamsted09	Time point (hr)																									
		CTRL	ND	ND	ND	ND	ND	ND	48	47	207	52	ND	ND	272	62	39	34	39	34	ND	ND	ND	ND	ND	ND
	1	Cellulase	ND	ND	ND	ND	ND	ND	61	85	301	108	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		CTRL	ND	ND	ND	ND	ND	ND	89	94	369	325	ND	ND	184	253	30	52	30	52	ND	ND	ND	ND	ND	ND
	2	Cellulase	ND	ND	ND	ND	ND	ND	274	89	444	79	ND	ND	346	64	80	11	80	11	ND	ND	ND	ND	ND	ND
		CTRL	ND	ND	ND	ND	ND	ND	516	313	432	117	ND	ND	851	45	192	24	192	24	ND	ND	ND	ND	ND	ND
	4	Cellulase	ND	20	34	ND	ND	ND	82	72	ND	ND	305	62	899	108	191	167	191	167	ND	ND	ND	ND	ND	ND
		CTRL	ND	ND	ND	ND	ND	ND	311	58	ND	ND	ND	ND	345	301	21	36	21	36	ND	ND	ND	ND	ND	ND
	6	Cellulase	70	18	ND	ND	ND	ND	1013	330	ND	ND	ND	ND	815	800	180	180	227	180	ND	ND	ND	ND	405	94
Peldon 07																										
		CTRL	1574	690	881	1057	ND	ND	16	27	113	9	ND	ND	734	915	ND	ND	138	187	ND	ND	ND	ND	ND	ND
	1	Cellulase	678	815	455	546	780	918	521	160	ND	ND	ND	ND	683	951	ND	ND	72	72	ND	ND	ND	ND	ND	ND
		CTRL	811	254	139	42	ND	ND	221	103	346	205	ND	ND	247	237	146	140	48	45	ND	ND	ND	ND	ND	ND
	2	Cellulase	1139	274			335	86	863	208	ND	ND	ND	ND	331	73	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		CTRL	2419	1303	573	254	113	46	516	313	432	117	305	62	851	45	192	65	192	24	ND	ND	ND	ND	ND	ND
	4	Cellulase	2390	573	861	226	135	19	3160	519	ND	ND	305	62	34	59	664	1254	664	1150	ND	ND	ND	ND	ND	ND
		CTRL	585	244	318	66	ND	ND	1197	600	ND	ND	431	134	1751	617	78	26	ND	ND	ND	ND	ND	ND	159	34
	6	Cellulase	1448	224	1191	49	ND	ND	3283	451	ND	ND	1112	289	2075	2427	101	124	29	50	ND	ND	ND	ND	489	118
Pendimethalin																										
		CTRL	ND	ND	ND	ND	ND	ND	606	701	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	1	Cellulase	ND	ND	ND	ND	ND	ND	913	896	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
		CTRL	150	85	17	29	ND	ND	57	7	437	197	ND	ND	450	252	145	60	143	74	ND	ND	ND	ND	ND	ND
	2	Cellulase	181	167	44	76	ND	ND	89	94	369	325	ND	ND	272	190	95	63	165	111	ND	ND	ND	ND	ND	ND
		CTRL	153	100	ND	ND	ND	ND	238	126	344	259	ND	ND	88	30	103	46	140	130	ND	ND	ND	ND	ND	ND
	4	Cellulase	145	161	27	46	ND	ND	266	286	236	246	305	62	85	74	52	47	113	122	ND	ND	ND	ND	ND	ND
		CTRL	1115	356			244	47	1257	567	ND	ND	174	23	763	284	124	35	54	47	ND	ND	ND	ND	293	117
	6	Cellulase	1581	362	709	91	ND	ND	1693	263	ND	ND	429	58	1709	1144	283	191	291	130	ND	ND	ND	ND	454	73
Fenoxaprop																										
		CTRL	243	323	57	99	25	43	16	27	113	9	ND	ND	734	915	ND	ND	138	187	ND	ND	ND	ND	ND	ND
	1	Cellulase	271	291	372	335	ND	ND	61	85	301	108	ND	ND	683	951	ND	ND	72	72	ND	ND	ND	ND	ND	ND
		CTRL	788	94	225	20	124	93	3023	457	ND	ND	ND	ND	3845	557	98	22	606	201	ND	ND	ND	ND	ND	ND
	2	Cellulase	778	78	614	58	153	11	2957	197	ND	ND	ND	ND	4337	886	114	20	698	49	ND	ND	ND	ND	ND	ND
		CTRL	1688	790	449	212	198	93	3773	1613	ND	ND	ND	ND	6587	2390	274	83	1211	288	ND	ND	ND	ND	ND	ND
	4	Cellulase	1640	704	616	291	154	63	2883	1103	ND	ND	305	62	10095	4185	417	151	1755	123	ND	ND	ND	ND	ND	ND
		CTRL	432	245	79	25	ND	ND	1200	761	ND	ND	85	75	853	294	60	54	67	58	ND	ND	ND	ND	330	152
	6	Cellulase	1581	362	709	91	ND	ND	1693	1693	ND	ND	429	58	884	88	68	17	161	38	ND	ND	ND	ND	805	55

Table 6. 9. Metabolism of pyroxsulam in resistant black-grass and wheat, pre-treated with P450 inhibitors. Mean herbicide metabolite levels, n = 5, ± = Std. Dev. ND: not detected.

		Herbicide Metabolites																							
		Chlorotoluron						Pendimethalin				Pyroxsulam		Fenoxaprop-P-ethyl						Mesosulfuron-methyl					
		N-mono-demethylated		Hydroxylated		Demethylated and Hydroxylated		Hydroxylation		Carboxylic Acid		5-OH		CBO-SG		CBO-CE		CBO-C		Demethylation		Cleavage (i)		Cleavage (ii)	
	Inhibitor		±		±		±		±		±		±		±		±		±		±		±		±
Wheat																									
	Malathion	48	107	32	30	23	33	ND	ND	ND	ND	41	47	61	38	122	51	ND	ND	ND	ND	ND	ND	ND	ND
	PBO	ND	ND	7	15	ND	ND	ND	ND	ND	ND	322	116	45	53	115	119	ND	ND	ND	ND	ND	ND	ND	ND
	1-ABT	ND	ND	7	17	13	30	ND	ND	ND	ND	216	40	96	33	35	36	ND	ND	ND	ND	ND	ND	ND	ND
	Mix of 3	ND	ND	17	24	ND	ND	ND	ND	ND	ND	67	85	89	20	101	78	ND	ND	ND	ND	ND	ND	ND	ND
	1/2 MS	25	35	66	45	ND	ND	ND	ND	ND	ND	490	260	87	45	154	123	ND	ND	ND	ND	ND	ND	ND	ND
Peldon																									
	Malathion	657	253	35	78	ND	ND	39	88	629	241	ND	ND	222	55	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	PBO	730	268	407	194	ND	ND	356	151	377	145	398	178	191	55	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	1-ABT	737	92	ND	ND	ND	ND	294	106	344	244	397	179	185	128	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Mix of 3	722	131	394	117	ND	ND	ND	ND	652	207	ND	ND	286	84	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	1/2 MS	898	352	430	163	ND	ND	577	319	430	4053	406	64	127	73	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

6.6.1 *Inhibition of Fenoxaprop-P-ethyl Metabolism*

Fenoxaprop is a GST detoxified herbicide with enhanced metabolism associated with NTSR in herbicide resistant black-grass populations (Keshtkar *et al.*, 2015). The metabolite levels of the herbicide were analysed as a form of control, as P450 inhibition should not supress GST detoxification. Overall the levels of metabolite were low < 300PAU in both Peldon and wheat (Figure 6.5.). The glutathione conjugate was found in Peldon and in wheat along with the glutamyl-cysteine conjugate (CBO-CE). It could be argued that the level of CBO-CE is lower in the 1-ABT pre-treatment but was not replicated in the inhibitor mixture treatment as would be expected, so it can be reasonably stated that the addition of malathion, PBO and 1-ABT had no effect on the detoxification of fenoxaprop.

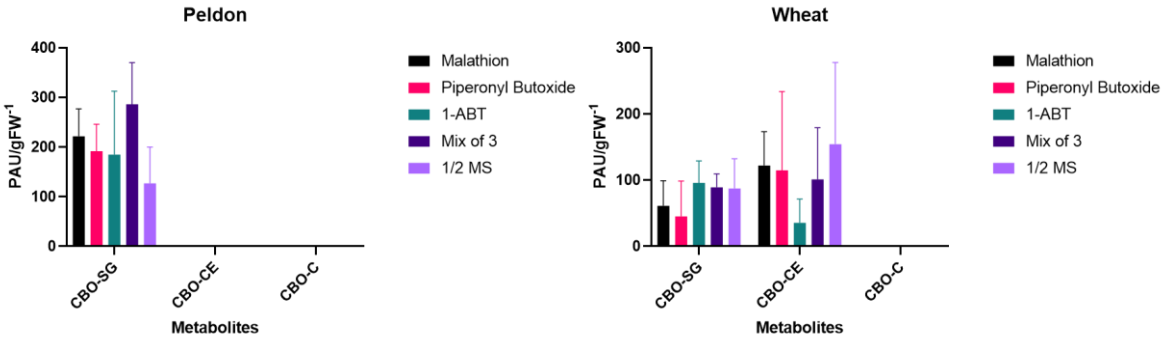

Figure 6. 5. Metabolism of fenoxaprop in resistant black-grass and wheat pre-treated with P450 inhibitors. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 5, ± = Std. Dev.

6.6.2 *Inhibition of Pendimethalin Metabolism*

In the wheat samples no pendimethalin metabolites were detected (data not shown) whereas parent compound was detected in the samples; it is possible that the levels of metabolites present were diluted to below the limit of detection during sample preparation. Metabolites were detected in the resistant Peldon plants (Figure 6.6.). Compared to the control sample, none of the levels of the carboxylic acid metabolite were significantly reduced. The mean levels in the malathion and mixture treatment were ~50% higher than determined in the control further suggesting that the P450s responsible for the oxidation of the hydroxylated metabolites are not inhibited by these three synergists. The hydroxylated pendimethalin metabolite level (~40 PAU) found in the malathion pre-treated sample was significantly lower than that determined compared in the control (~580 PAU), p=0.033. The levels of hydroxylated metabolite were also lower in the PBO and 1-ABT pre-treated

samples. No metabolites were detected when the black-grass was pre-treated with the inhibitor mixture. From this data it can be suggested that malathion inhibits P450s responsible for pendimethalin hydroxylation, with the inhibition of multiple enzymes, suggested by the lack of inhibition of hydroxylated pendimethalin in the PBO, 1-ABT and mixture samples.

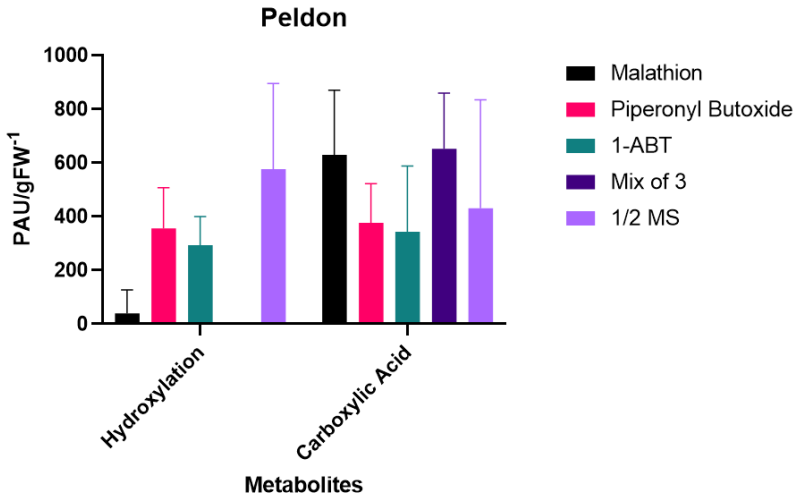

Figure 6. 6. Metabolism of pendimethalin in resistant black-grass pre-treated with P450 inhibitors. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 5, ± = Std. Dev.

6.6.3 *Inhibition of Chlorotoluron Metabolism*

In Peldon the two chlorotoluron metabolites found arise from *N*-mono-demethylation and hydroxylation (Figure 6.7.). The levels of metabolism in the wheat samples were very low in the untreated control group, with the same two metabolites found in Peldon in the controls. As the levels in the ½ MS control are below <100 PAU, the results in wheat are overlooked due to lack of confidence in the detection of metabolites close to the chromatographic baseline. As with the pendimethalin treatment, perhaps the low amount of metabolites present were diluted to below the limit of detection during sample preparation (Figure 6.6.). In the Peldon population, the level of the *N*-mono-demethylation product did not vary significantly across the four inhibitors pre-treatments as compared to the control, therefore the three inhibitors applied did not have an effect on the P450 responsible for *N*-mono-demethylation. The hydroxylated chlorotoluron metabolite level (35 PAU) found in the malathion pre-treated sample was significantly lower compared to the control (430 PAU). In contrast, the levels of hydroxylated metabolite were not lower in the PBO and 1-ABT pre-treated samples compared to the negative control. No hydroxylated metabolites were found when the black-grass was pre-treated with the inhibitor mixture. From this data it can be suggested that malathion selectively inhibits the

P450 that is responsible for chlorotoluron hydroxylation. Previous studies have shown that the application of 1-ABT in plants and cell cultures reduced the formation of the hydroxylated metabolite of chlorotoluron (Menendez and De Prado, 1997).

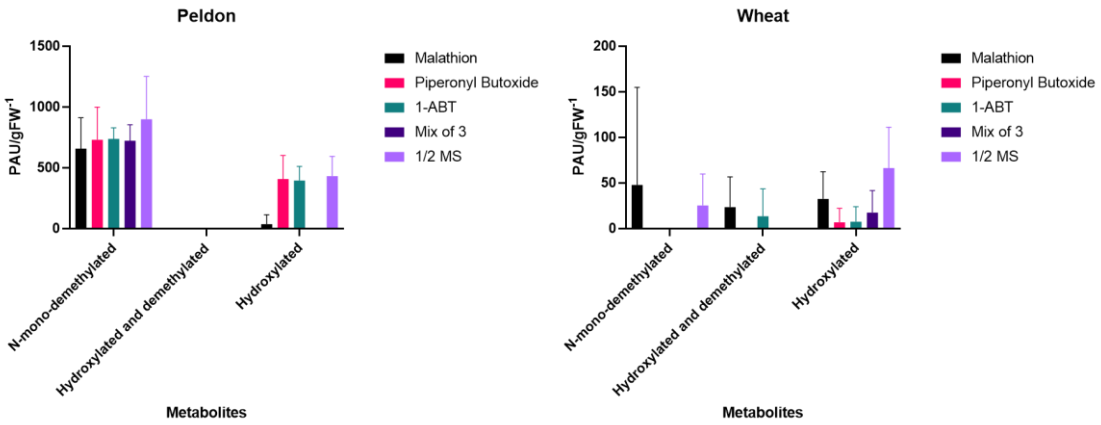

Figure 6. 7. Metabolism levels of chlorotoluron in resistant black-grass and wheat, pre-treated with P450 inhibitors. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 5, ± = Std. Dev

6.6.4 *Inhibition* of *Pyroxsulam Metabolism*

Pyroxsulam has one metabolite, a hydroxylated product (5-OH), that was found in both the wheat and Peldon samples including the controls (~400 PAU). No 5-OH metabolite was found in the Peldon sample that had been pre-treated with malathion, or with the inhibitor mixture (Figure 6.8.). The pre-treatment of malathion completely prevents the metabolism of pyroxsulam in Peldon, suggesting that malathion completely inhibits the detoxifying P450 in Peldon. In the wheat sample there was a 50% reduction in the level of 5-OH formed in the 1-ABT pre-treatment (200 PAU) when compared to the control. There was a greater reduction in the 5-OH level seen in the inhibitor mixture pre-treatment, suggesting another inhibitor was inhibiting the detoxifying P450. Malathion is therefore involved in inhibiting the hydroxylation of chlorotoluron, pendimethalin and pyroxsulam following in the Peldon black-grass population. The level of 5-OH on the malathion pre-treatment in wheat (40 PAU) is lower than determined in the mixture pre-treatment (67 PAU). Interestingly there was still some metabolism of pyroxsulam in wheat following the malathion and mixture pre-treatments, while it was completely suppressed in Peldon.

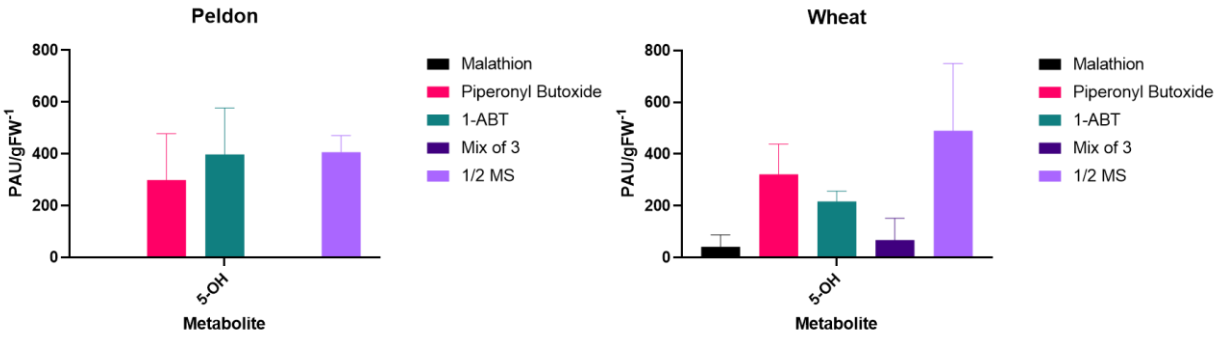

Figure 6. 8. Metabolism of pyroxsulam in resistant black-grass and wheat, pre-treated with P450 inhibitors. Mean herbicide metabolite levels (PAU per gram of fresh weight). n = 5, ± = Std. Dev

6.7. Inhibition of CYP81-2 and Effect on Pyroxsulam Metabolism

Several CYPs have been determined to be overexpressed in resistant weed populations, including *Am*CYP81-2 (Tetard-Jones *et al.*, 2018). In order to determine whether pyroxsulam metabolism was carried out by P450 activity, a P450 inhibition assay was undertaken. An orthologous recombinant CYP450 from wheat (*Ta*CYP81-2) was expressed in yeast and its activity towards pyroxsulam, individually and with malathion, measured. *Ta*CYP81-2 was incubated, for 20mins, pH 6.5, with pyroxsulam(200µM) or pyroxsulam (200µM) and malathion (40µM). Three replicates were run together with a negative control, without NADPH, for both treatments. Activity was measured by quantifying the formation of the 5-OH metabolite.

The mean level of activity of *Ta*CYP81-2 towards pendimethalin was measured as 0.023pkat/mg of recombinant CYP. The activity of *Ta*CYP81-2 towards pendimethalin in the presence of malathion was measured as 0.003pkat/mg of recombinant CYP. This data suggests that malathion inhibits *Ta*CYP81-2 (Figure 6.9.).

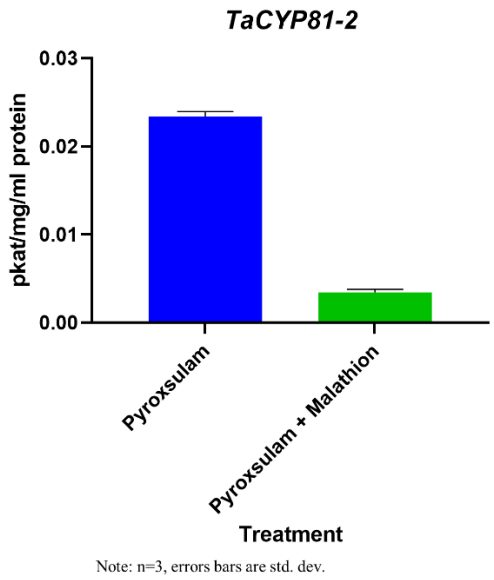

Figure 6. 9 Figure 6.9. Activity (pkat/mg/ml protein) of TaCYP81-2 towards pyroxsulam and mixture of pyroxsulam and malathion n = 3, error bars are Std. Dev

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6.8 Discussion

The optimum time point in which to observe simultaneous multiple herbicide metabolism in whole plants, based on these set of experiments, is between 6 and 24 hours. This is encouraging from a rapid diagnostic point of view. Being able to detect a wide range of metabolites in a relatively short time frame measured in hours opens up the potential for the assay to be expanded to include other herbicides and grass-weeds, assuming similar rates of herbicide metabolism. In the instance of mesosulfuron metabolism, a longer time course was required than for the other four herbicides, in order to determine metabolite formation. The levels of metabolism in resistant black-grass was higher than in sensitive. This agrees with evidence that enhanced metabolism is the main driving force of herbicide resistance in NTSR populations. Counterintuitively, with regards to the basis of selectivity, the levels of metabolites found in wheat were lower than found in resistant black-grass and in several cases as was then determined sensitive black-grass samples. Although the cellulase assay did show that some of the metabolites had undergone glycosylation (hydroxylated chlorotoluron, fenoxaprop-cysteine conjugate and mesosulfuron cleavage (ii) in wheat), the lower levels of metabolites seen, means that suggesting the herbicide detoxification pathways in resistant black-grass have evolved to form the same detoxification pathways in wheat, cannot be proven from these assays. To the contrary, one metabolite is the pendimethalin carboxylic acid metabolite which is only present in black-grass and not seen in wheat (Figure 6.9). These statements suggest that the

differences in herbicide metabolism between wheat and black-grass may be down to the ability of wheat to glycosylate hydroxylated herbicide products.

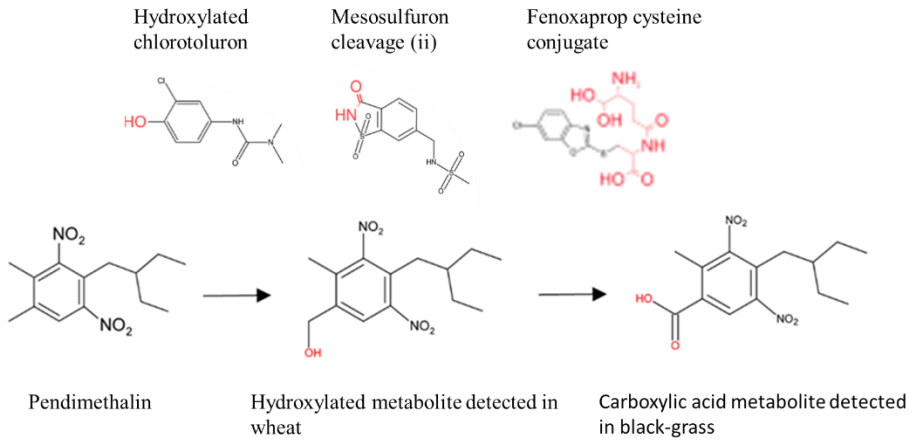

Figure 6. 10. Top; metabolites recovered by cellulase digestion of wheat extract. Bottom; pendimethalin metabolites detected in wheat or black-grass

The cell cultures were a more efficient system in which to view herbicide metabolism than in whole plants. In cell cultures herbicide metabolism was seen as soon as one hour following herbicide application. The optimum time for initial metabolism was between two and six hours. Glycosylation, inferred from the metabolite recovery in the cellulase digestion assay, was seen to occur within one hour after treatment in the resistant black-grass plants but the optimum time to observe this was ix hours in cell cultures. The recovery of glycosylated pyroxsulam 5-OH was only seen in cell cultures and not whole plants. Similar to the whole plant assay, the pendimethalin carboxylic metabolite was seen in all of the cell cultures with the exception of the ACC. The amount generated was greatest in the PCC followed by the FCC. All three resistant cultures have an enhanced metabolic resistance to pendimethalin as seen by the presence of hydroxylated pendimethalin, but only the cultures selected for herbicide resistance formed the carboxylic acid. This could suggest that forced herbicide resistance has caused the expression of an alternate P450, to further detoxify pendimethalin. Further studies across a wider range of grass-weed populations with known resistance to pendimethalin could be carried out to determine whether a high resistance rating correlates with the presence of the ability to from the carboxylic acid. A comprehensive mass balance study in cell cultures, looking at herbicide metabolism, would be an effective way to observe herbicide uptake and modification by measuring the amount of applied herbicide and comparing that to the amount of recovered parent herbicide and metabolites as was undertaken in the study of the herbicide safener, fenclorim (Liu, Brazier-Hicks and Edwards, 2009) . From these

studies, any differences in detoxification pathways could observed . Then, building upon the cell culture data, the assay could be extended to a larger whole plant study to establish whether any of the results can be replicated.

When treating plants with herbicides over a short time periods (1, 2, 4 and 6 hours), lower levels of metabolites are to be expected, this presents a problem when carrying out the cellulase digestion. Due to the procedure of drying the extracts, resuspending them and organic phase extraction, there is a loss of metabolites and the potential for their degradation. Increasing the sample size may reduce the variation caused by these losses by increasing the amount of extract to analyse.

The pre-treatment of whole plants with P450 inhibitors demonstrated, that by treating the wheat and black-grass with the aforementioned inhibitors, the products of three P450 metabolised herbicides can be suppressed. The pre-treatment of Peldon black-grass with 1-ABT and PBO displays some suppression of pendimethalin hydroxylation in the Peldon black-grass population and pyroxsulam hydroxylation in wheat. The pre-treatment of malathion, inhibits hydroxylation in pendimethalin, chlorotoluron and pyroxsulam, in Peldon black-grass, also pyroxsulam hydroxylation in wheat. This suggests that the pre-treatment of resistant black-grass with malathion has a synergistic effect, in that there is suppression of the hydroxylated metabolites detected.

The data from the inhibition of pyroxsulam metabolism (Figure 6.8), could be interpreted that malathion inhibits the same P450 in both wheat and black-grass. However, malathion only completely suppresses the hydroxylation of pyroxsulam in Peldon, in wheat there was still some formation of hydroxylated pyroxsulam. An explanation could be put forward as to explain the results of pyroxsulam hydroxylation. The resistant Peldon black-grass population has evolved a similar P450 detoxification enzyme as found in wheat, as a protective mechanism against herbicide damage, however that P450 in resistant black-grass is more susceptible to the synergistic effect of malathion.

Whilst *Ta*CYP81-2 is inhibited by malathion it still has some, albeit low, activity towards pyroxsulam. In the metabolism study (Figure 6.9.) in wheat there was still some 5-OH formed in wheat. This matches what is seen in the CYP81-2 activity experiment in that the CYP81-2 present in wheat may not be solely responsible for metabolism of pyroxsulam and that there is another P450 that can also metabolise pyroxsulam. In Peldon the metabolism of pyroxsulam was completely inhibited by malathion, suggesting that the

P450 black-grass responsible for pyroxsulam metabolism is a different homologue to that found in wheat, in which pyroxsulam metabolism was still seen, albeit at a reduced level.

In conclusion, it has been demonstrated, that by analysing the products of herbicide primary metabolism there are differences in herbicide metabolism between resistant black-grass and wheat, as shown by the total suppression of pyroxsulam metabolism in black-grass when pre-treated with malathion. Differences are also observed in the metabolism of pendimethalin in resistant black-grass as compared to wheat, in that the pendimethalin carboxylic acid metabolite is only observed in resistant black-grass. Also, the pendimethalin carboxylic acid metabolite is only observed in cell cultures initiated from the sensitive, pendimethalin and fenoxaprop selected black-grass populations, not in the Peldon resistant cell culture, suggesting that forced resistance to herbicides has promoted the evolution of a secondary P450 detoxification enzyme to metabolise pendimethalin. The treatment whole plants of wheat, black-grass and black-grass cell cultures with a mixture of herbicides has highlighted differences in herbicide detoxification pathways.

Chapter 7. General Discussion and Future Work

7.1 Introduction

Since the discovery of herbicides in the 1940's and their initial success in controlling grass-weed populations in crops, the continual research and development of new herbicide chemistries led to new a mode of action herbicide coming to the market approximately every two years, up until the 1980's (Dayan, 2019). This rate of discovery has slowed down dramatically to such an extent that since 1985 only one new mode of action herbicide has come to the market, cinmethyline, a fatty acid thioesterase inhibitor (Campe *et al.*, 2018). This lack of new herbicides has forced growers to rely on the repeated application of existing herbicides. These repeated herbicide treatments has forced the grass-weeds into evolving mechanisms that enable them to develop tolerance to herbicides. The combined problem of herbicide resistance and the lack of the commercialisation of new herbicides has led famers to take an integrated approach to resistance management. One of these approaches is understanding the resistance mechanisms that have developed in the grass-weed species on their farms. There are two distinct types of resistance, target site resistance (TSR) and non-target site resistance (NTSR). NTSR is primarily down to the ability of the weed to detoxify the herbicide preventing it from binding to its target protein. Due to NTSR weeds having the ability to accumulate multiple different mechanisms within a single plant it is difficult to create a detection tool to pinpoint a dominant detoxification pathway (Délye, 2013). Being able to detect and quantify herbicide metabolites gives rise to the opportunity to compare the detoxification pathways between different grass-weed populations with known NTSR and observe any similarities or differences in the metabolites generated. Comparing metabolism levels in black-grass populations to wheat may also reveal different mechanisms that play a role in selectivity, the ability of the crop to metabolise herbicides at a faster rate than weeds.

7.2 Overall Conclusions

The primary focus of this project was to investigate the potential of developing a new tool, based on mass spectrometry, to diagnose NTSR in black-grass and to elucidate the evolution of the herbicide detoxification pathways in black-grass. In order to achieve this the first step was the development of a high throughput LC-MS method capable of separating and identifying the herbicide metabolites of interest, generated in the plant populations used in this study. This was achieved using ultra-high pressure liquid chromatography coupled to a quadrupole time of flight mass spectrometer, that was capable of separating and identifying 6 herbicides and the corresponding 13 metabolites. The metabolite levels measured in the plant

extracts, when found, were all in the nanomolar range when compared to the parent standard. Therefore this creates the requirement for a high-resolution mass spectrometer with high sensitivity in order to be able to detect herbicide metabolites generated in the assays conducted in this study, as it has to be powerful enough to resolve the metabolites from the natural products present in the extracts generated. The presence and quantity of these metabolites differed between wheat, sensitive and resistant black-grass and between black-grass cell cultures with differing resistance profiles.

The main conclusions established from this work were as follows, that both wheat and resistant black-grass are capable of metabolising a mixture of chlorotoluron, pendimethalin, pyroxsulam, fenoxaprop-P-ethyl and mesosulfuron-methyl by 24 hours and that the resistant Peldon black-grass population can metabolise chlorotoluron, pendimethalin, pyroxsulam, fenoxaprop-P-ethyl by 6 hours. The metabolite levels of the cytochrome P450 metabolised herbicides were significantly lower when treated with a mixture of herbicides as compared to individual treatments, suggesting that competition exists between the detoxifying P450 enzymes for the herbicide substrates. The resistant Peldon black-grass, when treated with pendimethalin generates a carboxylic acid metabolite not seen in wheat. The same carboxylic acid is formed in cell cultures initiated from the sensitive, fenoxaprop and pendimethalin selected black-grass populations. This could suggest that black-grass which has been exposed to repeated herbicide application has evolved the expression of an alternate P450, to further detoxify pendimethalin and that the presence of this carboxylic acid metabolite may be a marker of NTSR black-grass with a high resistance rating.

The pre-treatment of wheat and resistant black-grass with malathion before the herbicide mixture treatment, resulted in suppressed levels of pyroxsulam metabolism, completely in wheat but not entirely in black-grass. This could be down to an homologous P450 responsible for pyroxsulam metabolism that may have evolved in resistant black-grass. The microsome inhibition assay using recombinant CYP81-2 from wheat, generated results mirroring the effect of malathion co-application in wheat and black-grass, in that when malathion was co-applied with pyroxsulam, the level of activity of the CYP81-2 towards pyroxsulam dropped drastically in the presence of malathion. The activity is measured by the formation of hydroxylated pyroxsulam. This is the starting point for the development of a mass spectrometry based diagnostic tool to identify NTSR in grass-weeds based on metabolite level.

7.3 General Discussion

7.3.1 *Metabolism of Herbicide Mixtures*

After establishing a method capable of analysing the metabolites of multiple herbicides, wheat, sensitive and resistant black-grass treated with five herbicides were able to metabolise some of the mixture of herbicides by 24 hours. Mesosulfuron-methyl metabolism was noticeably slower than either of the other four herbicides. Its primary metabolites were only detected in significant amounts after 24 hours in whole plants. In cell cultures, where metabolism of the other four herbicides was observed after 1 hour, in the case of the culture initiated from the fenoxaprop selected line, the primary metabolites of mesosulfuron were not detected with any consistency across the four cell cultures. A previous study has shown that the amount of mesosulfuron detected after application to leaf tissue, decreases over 1,3,5 and 7 days in *A. aequalis* (Zhao *et al.*, 2019). That study however did not report the metabolites found, only the disappearance of the parent compound. This study reports the metabolites of mesosulfuron found, something that is lacking in current literature. It is possible that the P450s responsible for enhanced metabolism to mesosulfuron are induced at a later timepoint than 6 hours both in whole plants and cell cultures or that the detoxification enzymes have a higher affinity towards the other four herbicides as substrates in the cultures, thus the decrease in detoxification activity towards mesosulfuron metabolism. In order to characterise mesosulfuron metabolism further, studies using an individual treatment of mesosulfuron in plants and cultures should be conducted and if it is found to undergo primary metabolism at later time point than the other four herbicides used, it should not be used in conjunction with pendimethalin, fenoxaprop, chlorotoluron and pyroxsulam, in which metabolism can be seen in as soon 1 hour in cultures.

The levels of metabolite were higher in the sensitive and resistant black-grass than compared to wheat. The results of the cellulase digestion suggest that in wheat, the metabolites have undergone glycosylation by 6 hours, based on the recovery of hydroxylated chlorotoluron metabolites, which explains why the level of metabolites were lower as only the products of primary metabolism were targeted in the analysis. To explain the incidences of similar metabolite levels in sensitive and resistant black grass it is hypothesised that resistant black-grass follows the same metabolism pathway in wheat, in that the products of primary metabolism are glycosylated at a quicker rate than in sensitive black-grass. The results of the herbicide mixture metabolism studies add to the overall body of herbicide resistance work, in that by being able to demonstrate the ability of resistant black-grass to metabolise five herbicides in a single treatment, it provides a warning for farmers regarding the current state

of herbicide resistance. The rotation of herbicides is now an established practice however the results of this work should be used to push farmers to invest in mapping the resistance types they have on the farm and the herbicides that the resistance traits confer with. There is a very real possibility that some farmers will not be able to rely on commercially available selective herbicides to control resistant grass-weed populations and that they will be forced to let more and more of their arable land lie fallow, depriving them of income and potentially pushing up the prices of cereals for all.

7.3.2 *Mass Spectrometry as a Diagnostic Tool for NTSR*

Mass spectrometry is used for variety of studies in plants, such as target phytohormone profiling, non-targeted metabolomics profiling and herbicide metabolite detection (Floková *et al.*, 2014)(Rahman *et al.*, 2017)(Ducker *et al.*, 2019). Here, a selection of different mass spec analyses were undertaken with regards to identifying potential biomarkers of NTSR. The non-targeted metabolomics study comparing *Am*GSTF1 expressed *Arabidopsis* leaves to empty vector controls, whilst providing a descriptive set of statistical charts demonstrating the difference in the small molecule profile between the two *Arabidopsis* types, did not provide any reliable biomarkers, however, this may have been as result of the approach taken with regard to compound database searching to find any matching identifications. Even if a suitable match could be made for one mass ion, it would still take logic and rationale to interpret why the statically significantly presence of a compound is an explanation to the difference between the two *Arabidopsis* types. The REIMS sampling system is a user friendly mass spectrometry sampling tool owing to the lack of sample preparation that is required. Its rapid sampling combined with instant generation of mass spectrometry data allows for the user to acquire data from hundreds of samples within a few hours. The processing of the data however is akin to the metabolomics approach seen with the non-targeted metabolomics of two types of *Arabidopsis.* It provided a descriptive chart representing the small molecule abundance of wheat treated with different herbicides yet none of the mass ions, deemed to be of statistical significance, were identified as herbicides or their known metabolites. Previous speciation studies have involved lipid identification at the heart of species profiling so it not surprising that employing the same technique here did not yield the detection of small polar molecules as was tested in this wheat study. The chromatographic and mass detection method employed here, UPLC-ESI-Q-ToF was for the most part, successful in identifying all of the known herbicide metabolites in one assay or another. The use of IMS-Q-ToF did add another level of detail to the structure of the hydroxylated pyroxsulam found, in that it agrees with the

current literature that employed the use of NMR elucidate the structure of the metabolite (DeBoer *et al.*, 2011). Using IMS-Q-ToF could be useful in trying to define the structures of previously unreported metabolites however, the extra cost and complexity associated with a high definition high resolution mass spectrometer is not required for the metabolite analysis in the studies discussed here.

7.4 Limitations

Several limitations must be taken into account when interpreting the findings of this study. The method used to observe herbicide primary metabolism was by mass spectrometry detection of known herbicide primary metabolites. In the case of mesosulfuron there is very little published work reporting the primary metabolites. The current studies assessing mesosulfuron resistance use growth inhibition coupled with the disappearance of the parent. More studies into mesosulfuron metabolism need to be carried out in order to expand the current knowledge base. The lower levels of metabolites found in wheat as compared to sensitive and resistant black-grass was suggested not be an effect of the plant matrix often seen with electrospray ionisation but due to the early glycosylation of hydroxylated metabolites. Although cellulase digestion assays were carried out in wheat and black-grass cell cultures, the amount of extract from the black-grass plant assays were not sufficient for cellulase digestion. An increase in plant sample size would provide sufficient extract or ,better still, the number of metabolites anlaysed by LC-MS could be increased to include the conjugated metabolites as the cellulase assay introduces technical error and possible metabolite loss. Additional work on identifying and quantifying all further downstream metabolites would also be advantageous in terms of limiting the amount of radio labelled assays required to quantify the whole metabolic pathway of a herbicide.

The biological variation seen in whole plant metabolism assays presents a challenge when determining herbicide resistance by metabolite levels. Using characterised black-grass plants, when a limited amount of seed is available, in order to observe differences in metabolism levels between black-grass with different resistance profiles is not the most efficient use of the finite material. Initiating cell cultures, whilst a more technically demanding process, allows for the observation of metabolism after short time periods (1 to 4 hours) whilst avoiding potential difficulties with associated with whole plant feeding studies, with a mixture of herbicides, such as uptake and translocation. The preliminary work with regards to identifying a herbicide metabolite as a marker of NTSR is best done in cell cultures from a known NTSR population then extended outwards into whole plants to then assess if the results can be replicated. Only when the results are replicated in cultures and plants should

the assay try to be applied to a different mass spectrometry technique other than the method used for the original metabolite identification.

7.5 Future work

To further explore creating a diagnostic test for NTSR based on metabolite detection, continuing the work based on P450 inhibition could lead to the development of such a test. Having shown that malathion inhibits *Ta*CYP81-2, an orthologue of *Am*CYP81-2 which is overexpressed in NTSR black-grass and this is observed by the reduction in pyroxsulam hydroxylation, the similar suppression of pyroxsulam hydroxylation seen in the black-grass could be used a marker of NTSR. Two pieces of tissue from the same plant, suspected to be NTSR, could be taken and placed into one of two vials. One with pyroxsulam and the other with pyroxsulam and malathion. After a set amount of time, the sample can be homogenised and the levels of hydroxylated pyroxsulam can be determined by LC-MS. If the tissue sample treated without malathion has a significantly increased level of metabolite compared to the sample with malathion then that infers the presence of *Am*CYP81-2 which has been shown to be overexpressed in NTSR black-grass. In order for this to be validated the activities of *Am*CYP81-2 against other P450 detoxified herbicides, to which black-grass displays enhanced metabolic resistance, would have to be measured in order to establish their specificity for the herbicide. The assay could be extended to include other CYP450s, determined by a proteomics approach, to be overexpress in NTSR weed populations such as rye-grass or brome. If this assay was robust then attempts to modify the assay to enable the transfer of the method onto a smaller more portable LC-MS unit, that can be taken in to the field, such as the RADIAN can be made.

Abbreviations

1-ABT	1-Aminobenzotriazole
4-PL	Four parameter logistic
ABC	ATP-binding cassette
ACC	Atlantis cell culture
ACCase	Acetyl-CoA carboxylase
ACN	Acetonitrile
ALS	Acetolactate synthase
amu	atomic mass unit
APCI	Atmospheric pressure chemical ionisation
ASAP	Atmospheric pressure solids analysis probe
BEH	Ethylene bridged hybrid
BSA	Bovine serum albumin
CIM	Callus-induction medium
CCC	Control cell culture
CoA	Coenzyme A
CPCA	1,1-cyclopropanedicarboxylic acid
CYP450	Cytochrome P450
Da	Dalton
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
EPSPS	5-enolpyruvyl shikimate-3-phosphate synthase
FAS	Fatty acid synthase
FCC	Fenoxaprop cell culture
FTN	Flow-through needle
g	gram or relative centrifugal force (context specific)
gDNA	Genomic deoxyribonucleic acid
GCMS	Gas chromatography mass spectroscopy

g / FW	Gram of fresh weight
g / Froz.W	Gram of frozen weight
GPOX	Glutathione peroxidase
GSH	Reduced glutathione
GST	Glutathione-S-transferase
GSTF1	Glutathione-S-transferase, Phi class
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HRMS	High-resolution mass spectrometry
IMS	Ion -mobility spectrometry
KARI	Ketol acid reducto-isomerase
LoQ	Limit of quantitation
LCMS	Tandem liquid chromatography - mass spectrometry
M	Molar
m/z	Mass to charge ratio
min	Minute
mL	Millilitre
mM	Millimolar
MS	Murashige and Skoog or mass spectrometry (context specific)
mw	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
nmol	Nanomole
NTSR	Non-target site resistance
PAU	Peak area units
ppm	Parts per million
PBO	Piperonyl Butoxide
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline ,1% Tween
PCC	Pendimethalin cell culture
PS II	Photosystem II
PVPP	Polyvinylpolypyrrolidone
PCR	Polymerase chain reaction
QDa	Quadrupole dalton

Q-ToF	Quadrupole time of flight
REIMS	Rapid evaporative ionisation mass spectrometry
rpm	Revolutions per minute
SNP	ingle nucleotide polymorphism
TMB	3,3', 5,5;-tetramethylbenzidine
TSR	Target site resistance
VLCFA	Very long chain fatty acid
v/v	Volume to volume
w/v	Weight to volume
°C	Degrees Celsius
µg	Microgram
µg	Microlitre

Chapter 8. References

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Appendix A

Appendix A.1 1. List of 163 tentative compound IDs from REIMS experiment in section 3.6.1.

Compound	Neutral Mass	m/z	z	Retention Time	Peak Width	Accepted ID	Identifications	Anova (p)	q Value	Max Fold Change	Highest Mean	Lowest Mean	Tags	Isotope Distribution
0.28_1113.6013m/z		1113.601	1	0.28	0.03		0	2.13E-10	3.53E-07âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1054.6758m/z		1054.676	1	0.28	0.17		0	3.20E-10	3.53E-07âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_157.0999m/z		157.0999	1	0.28	0.13		0	1.78E-07	1.31E-04âˆž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_227.1799m/z		227.1799	1	0.28	0.17		0	6.34E-07	3.51E-04âˆž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_205.1148m/z		205.1148	1	0.28	0.17		0	3.70E-06	0.002âˆž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_336.2503m/z		336.2503	1	0.28	0.17		0	1.18E-05	0.004	21.93385	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1180.6817m/z		1180.682	1	0.28	0.17		0	3.14E-05	0.01	27.88421	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_219.1323m/z		219.1323	1	0.28	0.17		0	3.57E-05	0.01âˆž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_290.8541m/z		290.8541	1	0.28	0.1		0	4.83E-05	0.011	17.34319	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_698.4148m/z		698.4148	1	0.28	0.17		0	5.11E-05	0.011	5.932463	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_283.2451m/z		283.2451	1	0.28	0.17		0	8.69E-05	0.016	3.319661	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_744.2431m/z		744.2431	1	0.28	0.17		0	1.28E-04	0.016âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_615.0771m/z		615.0771	1	0.28	0.03		0	1.28E-04	0.016âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_771.6747m/z		771.6747	1	0.28	0.17		0	1.28E-04	0.016âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1079.5359m/z		1079.536	1	0.28	0.03		0	1.28E-04	0.016âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1020.0483m/z		1020.048	1	0.28	0.17		0	1.28E-04	0.016âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_332.9909m/z		332.9909	1	0.28	0.03		0	1.28E-04	0.016âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_981.5099m/z		981.5099	1	0.28	0.03		0	1.28E-04	0.016âˆž		Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100

0.28_1105.5525m/z	1105.553	1	0.28	0.03	0	1.45E-04	0.017	950.3365	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_327.2437m/z	327.2437	1	0.28	0.07	0	1.72E-04	0.019	444.8316	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_714.3694m/z	714.3694	1	0.28	0.17	0	1.86E-04	0.02	9.346671	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1005.4814m/z	1005.481	1	0.28	0.17	0	1.97E-04	0.02	7.667875	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_183.1164m/z	183.1164	1	0.28	0.13	0	2.08E-04	0.02	2134.448	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_745.3941m/z	745.3941	1	0.28	0.17	8	2.34E-04	0.021	9.648258	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_702.2087m/z	702.2087	1	0.28	0.17	0	2.35E-04	0.021	789.7996	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_500.1110m/z	500.111	1	0.28	0.17	0	3.43E-04	0.028	257.0928	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_875.4307m/z	875.4307	1	0.28	0.07	0	3.60E-04	0.028	6.10578	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_777.1492m/z	777.1492	1	0.28	0.17	0	3.62E-04	0.028	298.1383	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_985.5487m/z	985.5487	1	0.28	0.03	0	3.65E-04	0.028	6.783543	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1096.6775m/z	1096.678	1	0.28	0.07	0	4.33E-04	0.032	13.29074	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_219.1504m/z	219.1504	1	0.28	0.15	0	4.50E-04	0.032â^ž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_660.2230m/z	660.223	1	0.28	0.17	0	5.50E-04	0.038	15.83854	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_746.6609m/z	746.6609	1	0.28	0.1	0	7.33E-04	0.049	8.178891	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_576.1682m/z	576.1682	1	0.28	0.17	0	8.77E-04	0.057	113.5411	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_134.9524m/z	134.9524	1	0.28	0.13	0	9.63E-04	0.061	7.013208	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_257.2189m/z	257.2189	1	0.28	0.17	0	0.001	0.087â^ž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_823.4433m/z	823.4433	1	0.28	0.07	0	0.002	0.093	3.863362	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_510.1238m/z	510.1238	1	0.28	0.17	0	0.002	0.101	54.34465	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100

0.28_1120.4834m/z	1120.483	1	0.28	0.17	0	0.003	0.137	9.348086	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_714.3314m/z	714.3314	1	0.28	0.17	0	0.003	0.137	6.919194	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_310.2342m/z	310.2342	1	0.28	0.17	0	0.003	0.137	7.314662	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_274.9390m/z	274.939	1	0.28	0.1	0	0.003	0.137	4.988616	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_135.0217m/z	135.0217	1	0.28	0.17	0	0.003	0.137	2.425085	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_241.1961m/z	241.1961	1	0.28	0.17	0	0.003	0.137	16.77894	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_650.3864m/z	650.3864	1	0.28	0.17	0	0.003	0.137	7.262175	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_255.2124m/z	255.2124	1	0.28	0.17	0	0.003	0.154	4.017841	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1167.7777m/z	1167.778	1	0.28	0.07	0	0.003	0.154	5.242388	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_705.2107m/z	705.2107	1	0.28	0.17	0	0.004	0.158	3.331739	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1186.7768m/z	1186.777	1	0.28	0.17	0	0.004	0.158	4.999057	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1023.5438m/z	1023.544	1	0.28	0.1	0	0.004	0.163	8.397151	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_286.9481m/z	286.9481	1	0.28	0.1	0	0.004	0.177	6.146204	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1057.6889m/z	1057.689	1	0.28	0.17	0	0.004	0.177	6.577005	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_152.0498m/z	152.0498	1	0.28	0.17	0	0.004	0.177	8.123206	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_804.3848m/z	804.3848	1	0.28	0.17	0	0.005	0.198	2.96219	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_147.9913m/z	147.9913	1	0.28	0.13	0	0.005	0.207	5.113799	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_232.1576m/z	232.1576	1	0.28	0.17	0	0.006	0.211âˆž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1004.4859m/z	1004.486	1	0.28	0.17	0	0.006	0.211	4.196552	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_281.2288m/z	281.2288	1	0.28	0.17	0	0.006	0.216	2.378665	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100

0.28_724.3342m/z	724.3342	1	0.28	0.13	0	0.006	0.216	5.10063	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_683.4114m/z	683.4114	1	0.28	0.1	0	0.006	0.221	4.098807	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_416.3140m/z	416.314	1	0.28	0.03	0	0.006	0.222	24.4419	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1118.6974m/z	1118.697	1	0.28	0.17	0	0.006	0.225	4.869268	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_146.9480m/z	146.948	1	0.28	0.13	0	0.007	0.23	7.931938	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_709.4173m/z	709.4173	1	0.28	0.17	23	0.007	0.238	3.550709	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_137.0012m/z	137.0012	1	0.28	0.17	0	0.007	0.238	2.395251	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1030.6374m/z	1030.637	1	0.28	0.17	0	0.008	0.263	5.538311	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1138.6285m/z	1138.629	1	0.28	0.17	0	0.009	0.274	10.60408	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_611.2387m/z	611.2387	1	0.28	0.17	0	0.009	0.28	5.635634	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.30_1123.6310m/z	1123.631	1	0.3	0.07	0	0.009	0.28	3.856817	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_394.1342m/z	394.1342	1	0.28	0.17	0	0.01	0.304	3.953928	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_738.6517m/z	738.6517	1	0.28	0.07	0	0.01	0.307	19.0238	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_228.1448m/z	228.1448	1	0.28	0.17	0	0.01	0.309	14.40151	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_184.4819m/z	184.4819	1	0.28	0.17	0	0.011	0.309	5.038121	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_710.3389m/z	710.3389	1	0.28	0.17	0	0.011	0.309	5.37805	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_324.2432m/z	324.2432	1	0.28	0.17	0	0.011	0.319	3.918432	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_565.4463m/z	565.4463	1	0.28	0.17	0	0.012	0.328â^ž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1002.4821m/z	1002.482	1	0.28	0.17	0	0.012	0.334	5.539079	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_597.0664m/z	597.0664	1	0.28	0.03	0	0.013	0.352	16.34949	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100

0.28_1173.5215m/z	1173.522	1	0.28	0.03		0	0.013	0.353	15.29666	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_765.3633m/z	765.3633	1	0.28	0.1		0	0.013	0.353	8.284501	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_269.2288m/z	269.2288	1	0.28	0.17		0	0.014	0.353	12.30182	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1182.6405m/z	1182.641	1	0.28	0.17		0	0.014	0.353	3.862146	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_577.3813m/z	577.3813	1	0.28	0.07		0	0.014	0.353	3.583236	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_134.0003m/z	134.0003	1	0.28	0.17		0	0.014	0.357â^ž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1001.4815m/z	1001.482	1	0.28	0.17		0	0.014	0.357	5.471657	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_335.2477m/z	335.2477	1	0.28	0.17		0	0.014	0.358	2.414633	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_755.2162m/z	755.2162	1	0.28	0.1		0	0.014	0.358	3.82535	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1160.7305m/z	1160.731	1	0.28	0.17		0	0.015	0.364	7.834282	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_726.3287m/z	726.3287	1	0.28	0.17		0	0.015	0.37	6.40732	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_131.9981m/z	131.9981	1	0.28	0.1		0	0.015	0.371â^ž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1059.5942m/z	1059.594	1	0.28	0.03		0	0.016	0.38	3.917796	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1167.6940m/z	1167.694	1	0.28	0.17		0	0.016	0.382	3.582747	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_272.9338m/z	272.9338	1	0.28	0.1		0	0.017	0.384	4.101836	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_858.4353m/z	858.4353	1	0.28	0.17		0	0.017	0.384	12.82289	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_234.1733m/z	234.1733	1	0.28	0.17		0	0.018	0.4	7.716074	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_402.2965m/z	402.2965	1	0.28	0.1		0	0.018	0.4	6.438663	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_185.0553m/z	185.0553	1	0.28	0.13		0	0.018	0.4â^ž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_811.3742m/z	811.3742	1	0.28	0.07		0	0.018	0.4	11.58751	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100

0.28_665.2763m/z	665.2763	1	0.28	0.17	0	0.018	0.4	3.531285	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_314.2248m/z	314.2248	1	0.28	0.17	0	0.019	0.419	6.573275	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_548.4053m/z	548.4053	1	0.28	0.03	0	0.021	0.445	12.89312	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_288.8333m/z	288.8333	1	0.28	0.03	0	0.021	0.446	10.94946	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1120.5831m/z	1120.583	1	0.28	0.17	0	0.021	0.446	7.056536	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_936.9539m/z	936.9539	1	0.28	0.17	0	0.022	0.446	9.989802	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_722.3222m/z	722.3222	1	0.28	0.03	0	0.022	0.446	3.457093	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_684.6226m/z	684.6226	1	0.28	0.17	0	0.022	0.446	2.696046	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_600.1941m/z	600.1941	1	0.28	0.17	0	0.022	0.446	2.949548	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1122.4684m/z	1122.468	1	0.28	0.17	0	0.022	0.446	5.597109	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_395.3766m/z	395.3766	1	0.28	0.17	0	0.022	0.446	2.372126	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_133.0426m/z	133.0426	1	0.28	0.15	0	0.023	0.453âˆž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_228.9668m/z	228.9668	1	0.28	0.07	0	0.023	0.453	9.678025	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_340.0480m/z	340.048	1	0.28	0.17	0	0.025	0.481	4.098211	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_196.1138m/z	196.1138	1	0.28	0.17	0	0.026	0.489	8.035775	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_267.2136m/z	267.2136	1	0.28	0.17	0	0.026	0.489âˆž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1160.6553m/z	1160.655	1	0.28	0.03	0	0.026	0.489	4.281946	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_873.4435m/z	873.4435	1	0.28	0.07	0	0.026	0.496	5.613907	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_652.4903m/z	652.4903	1	0.28	0.17	0	0.027	0.506	3.95059	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_996.5868m/z	996.5868	1	0.28	0.17	0	0.028	0.517	4.608527	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100

0.28_164.0501m/z	164.0501	1	0.28	0.17	0	0.028	0.518	5.188727	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_673.1947m/z	673.1947	1	0.28	0.17	0	0.029	0.521	3.126587	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_288.9467m/z	288.9467	1	0.28	0.1	0	0.03	0.532	7.821857	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_554.3217m/z	554.3217	1	0.28	0.17	0	0.03	0.532	16.64125	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1116.7204m/z	1116.72	1	0.28	0.17	0	0.03	0.533	3.995373	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_192.9689m/z	192.9689	1	0.28	0.1	0	0.031	0.533	9.072533	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_419.3123m/z	419.3123	1	0.28	0.07	0	0.031	0.533	6.754945	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_722.3790m/z	722.379	1	0.28	0.17	0	0.032	0.533	2.3269	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_233.1329m/z	233.1329	1	0.28	0.17	0	0.032	0.533	2.154326	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1060.6828m/z	1060.683	1	0.28	0.17	0	0.032	0.533	6.421922	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_535.3987m/z	535.3987	1	0.28	0.17	0	0.032	0.533	6.049168	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1158.7034m/z	1158.703	1	0.28	0.17	0	0.032	0.533	5.326726	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_854.4420m/z	854.442	1	0.28	0.07	0	0.032	0.536	3.214161	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1184.7886m/z	1184.789	1	0.28	0.17	0	0.033	0.538	3.139256	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_836.4622m/z	836.4622	1	0.28	0.03	0	0.034	0.552	13.64166	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_704.2206m/z	704.2206	1	0.28	0.17	0	0.035	0.554	2.295534	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_992.6273m/z	992.6273	1	0.28	0.17	0	0.035	0.554	4.701018	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_129.0790m/z	129.079	1	0.28	0.13	0	0.035	0.554âˆž		Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_639.3201m/z	639.3201	1	0.28	0.07	0	0.036	0.564	2.565269	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_254.2003m/z	254.2003	1	0.28	0.17	0	0.036	0.564	6.02755	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100

0.28_640.3782m/z	640.3782	1	0.28	0.17	0	0.036	0.564	4.201806	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_294.8440m/z	294.844	1	0.28	0.1	0	0.037	0.566âˆž	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_821.4416m/z	821.4416	1	0.28	0.17	0	0.037	0.567	4.649675	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1159.7122m/z	1159.712	1	0.28	0.17	0	0.038	0.58	3.957078	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1123.4707m/z	1123.471	1	0.28	0.17	0	0.04	0.581	3.519744	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1193.6827m/z	1193.683	1	0.28	0.17	0	0.04	0.581	4.846629	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_590.1765m/z	590.1765	1	0.28	0.17	0	0.04	0.581	3.174999	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_526.1355m/z	526.1355	1	0.28	0.17	0	0.04	0.581	4.675402	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_299.0036m/z	299.0036	1	0.28	0.17	0	0.04	0.581	3.698361	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_701.5195m/z	701.5195	1	0.28	0.17	23	0.041	0.581	4.551045	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1144.7082m/z	1144.708	1	0.28	0.17	0	0.042	0.581	3.167546	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_549.3578m/z	549.3578	1	0.28	0.07	22	0.042	0.581	2.119256	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_571.4106m/z	571.4106	1	0.28	0.17	0	0.043	0.581âˆž	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1141.6356m/z	1141.636	1	0.28	0.17	0	0.043	0.581	3.377081	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_590.1520m/z	590.152	1	0.28	0.17	0	0.043	0.581	2.704795	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_489.3938m/z	489.3938	1	0.28	0.17	0	0.045	0.581âˆž	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_563.2606m/z	563.2606	1	0.28	0.17	0	0.045	0.581	2.480805	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_480.1021m/z	480.1021	1	0.28	0.17	0	0.046	0.581	2.838393	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_239.1816m/z	239.1816	1	0.28	0.13	0	0.046	0.581âˆž	Treated	Untreated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_519.2366m/z	519.2366	1	0.28	0.17	0	0.047	0.581	3.432037	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100

0.28_364.1138m/z	364.1138	1	0.28	0.17	0	0.047	0.581	2.804437	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_1157.7539m/z	1157.754	1	0.28	0.17	0	0.049	0.581	2.510124	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_694.4649m/z	694.4649	1	0.28	0.17	0	0.049	0.581	3.341466	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100
0.28_705.3226m/z	705.3226	1	0.28	0.17	0	0.05	0.581	2.184417	Untreated	Treated	Max fold change >= 2; Anova p-value <= 0.05	100