Newcastle University

School of
Natural and Environmental Sciences

The selectivity and metabolism of sulfonamide herbicide safeners in crops

George Giannakopoulos

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“Τῆς παιδείας αἱ μὲν ῥίζαι πικραί, ὁ δὲ καρπὸς γλυκύς”

Αριστοτέλης

Βίοι και γνώμαι των εν φιλοσοφία ευδοκιμησάντων, Βιβλίο Ε, 16-19, Διογένης ο Λαέρτιος

“The roots of education are bitter, but the fruit is sweet”

Aristotle

Lives of the Eminent Philosophers, Book 5, 16-19, Diogenes Laertius

Abstract

Crop protection is increasingly challenged by demanding regulations, limited discovery of new herbicide/pesticide modes of action and increasing pest and weed resistance. Safeners, a diverse group of agrochemicals, have been developed to diversify the application of existing herbicides by selectively enhancing tolerance in large-grained cereal crops. While their exact mode of action remains to be determined, safener treatment results in the induction of xenobiotic detoxifying enzymes and associated transport proteins collectively termed the xenome. Key inducible xenome enzymes include glutathione S-transferases (GSTs) and cytochromes P450s (CYPs).

This study's aim was to investigate the molecular mechanism and selectivity of the recently developed sulfonamide safener cyprosulfamide [N-[4-(cyclopropylcarbamoyl)phenylsulfonyl]-2-methoxybenzamide] in maize (*Zea Mays L.*), wheat (*Triticum Aestivum)* and soybean (*Glycine Max*). The safening activity of cyprosulfamide (CSA) in protecting these crops from herbicide damage was assessed following exposure to herbicides thiencarbazone-methyl (TCM) and tembotrione (TBT) in greenhouse trials, where CSA protected maize, but not wheat and soybean from injury by both herbicides. This correlated with the relative degree of enhanced detoxification observed with herbicide TCM, where CSA enhanced its metabolism specifically in maize. To study the comparative activity with other sulfonamide safeners, the same plants were treated with metcamifen [2-methoxy-N-{[4-(3-methylureido)phenyl]sulfonyl}benzamide]. In contrast to CSA, metcamifen was active in herbicide safening in maize and wheat, but not in soybean when tested in the greenhouse against the same herbicides, which was also associated with enhanced TCM metabolism in both maize and wheat with the safener.

To examine the basis of the differential safening by CSA, its uptake, translocation and metabolism were studied in maize and wheat. The safener displayed increased mobility and translocation in wheat compared to maize but this did not correlate with activity. Metabolism studies showed more rapid metabolism in maize than in wheat, with the presence of a specific metabolite correlating with activity. Primary metabolites of CSA and metcamifen were identified and synthesised for activity testing in the greenhouse and in TCM metabolism studies. With the exception of one metcamifen metabolite, these primary biotransformation products were found to be inactive. Gene expression studies were designed in order to determine if CSA induced xenobiotic detoxifying enzymes prior to its metabolism. *ZmGSTL1*,

a gene widely used as a marker for safening, was induced prior to the appearance of CSA metabolites in maize.

To investigate the roles of CSA metabolism and safening in greater detail, Matrix Assisted Laser Desorption Ionisation (MALDI) Imaging Mass spectrometry was performed in maize, where CSA and TCM were applied on the same and on different leaves. The experiment showed that TCM metabolism was elevated when compounds were applied on the same leaf, indicating that the two compounds should be present in the same tissue for safening to be induced. The characterisation of the relationship between herbicide safeners and plant signalling mechanisms appears to be complex territory, but new insights provided by this study can help lead to the design of improved safeners, which will play an important role in the future of weed control.

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Publications arising from this work

George Giannakopoulos, Jan Dittgen, Wolfgang Schulte, Peter Zoellner, Hendrik Helmke, Andreas Lagojda, Robert Edwards. (2020). Safening activity and metabolism of the safener cyprosulfamide in maize and wheat. *Pest Management Science.*

Michael Kubicki, George Giannakopoulos, Jan Dittgen, Mark Lamshoeft. (2020). Matrix-Assisted Laser Desorption Ionisation (MALDI) Mass Spectrometry Imaging (MSI) as a novel tool for herbicide safener research. *In preparation*

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Chapter 1 – Introduction

1.1 Overview

Ever since the beginning of agriculture around 10000 years ago, crop production has been under threat by pests which mainly include weeds, animal pests and pathogens such as fungi, bacteria and viruses.

Weeds are a major constraint on production, causing higher potential yield losses (34%) globally than animal pests and pathogens in major crops (Oerke, 2005). These losses are minimised (9%) using crop protection, a vital component of food security at a time when agriculture needs to feed a growing population while reducing its environmental impact and preserving scarce natural resources (Oerke, 2005, Godfray et al., 2010, Foley et al., 2011). The use of herbicides, or chemical weed control, is an integral part of crop protection and key in preserving food production through weed management (Yu and Powles, 2014, Norsworthy et al., 2017). This is highlighted by a recent global meta-analysis reporting that yields from conventional agricultural systems that use herbicides are 25% higher than organic ones (Seufert et al., 2012). Chemical weed control is most effectively used when combined with other management methods (cultural, mechanical and biological), in an Integrated Weed Management (IWM) system (Shaner and Beckie, 2014, Norsworthy et al., 2017, HRAC, Beffa et al., 2019). Some of these methods include production of weed-free seeds, appropriate cleaning of farming equipment, crop rotation, maintaining soil fertility, tillage, mowing, stubble burning and introducing insects or pathogens that control weed species (Walker, 1995, HRAC).

The crop protection sector, however, is faced with multiple challenges (Lamberth et al., 2013). Herbicide resistant weeds have become a global problem of increasing severity, with 502 unique cases of resistance to 23 out of 26 known herbicide mode of actions (MoAs), spanning 70 countries now reported (Heap, 2019). New cases of resistance have increased constantly for 35 years (Beffa et al., 2019, Heap, 2019). During the last two decades, the overreliance on simple cropping systems based on Roundup Ready technology has led to approximately 34 glyphosate-resistant weed species globally (Schütte et al., 2017). One very troublesome example of resistance evolution is that of *Lolium rigidum*, often exhibiting cross-resistance to different herbicide chemistries (Yu and Powles, 2014).

Additionally, no new herbicide mode of actions have been introduced for the past 30 years, particularly, as GM-resistance technologies reduced the demand for new compound discovery, coupled with the consolidation of companies carrying out herbicide research (Duke, 2012, Jeschke, 2016a). More than 60% of current commercial herbicide products are based on four modes of action, all of which have reported cases of resistance in various weed species (Kraehmer et al., 2014b). For a new product to be brought to the market, it takes around 286 million USD and 11 years of research and development, to combine product efficacy and a favourable toxicological profile (Kraehmer et al., 2014a, McDougall, 2016). Stricter regulatory requirements (Kraehmer et al., 2014a, Jeschke, 2016a) and high development costs have become a big obstacle to the commercialisation of new herbicides, especially ones without broad activity (Epp et al., 2018). These challenges are even more pronounced in regions like Europe where the use of genetically modified crops is banned in many countries and the use of gene editing technologies such as CRISPR is restricted (EU, 2015, EU, 2018), leaving conventional chemical weed control as the only option for conventional agricultural systems.

1.2 Chemical weed control and selectivity

1.2.1 History of chemical weed control

The use of chemicals for pest management dates back to the 9th century BC, with Homer referring to the use of sulphur for fumigating a chamber in Odyssey (Devendar and Yang, 2017). Crude chemicals like sulfuric acid, copper salts, sodium chlorate, borates and arsenic compounds have been used for total, non-selective removal of plants where they were applied in high doses (Cremlyn, 1991). Most of these herbicides were too toxic to be used without injuring crops. The first important discovery in the field of selective weed control was 2-methyl-4,6-dinitrophenol (DNOC) in 1933, used in cereals (Green et al., 1987). This compound was used extensively during World War II but was very toxic to mammals and humans (Cremlyn, 1991). A lot of other examples of early inorganic herbicides have been reviewed (Timmons, 1970), but were mostly intended for non-selective weed control in areas such as ditchbanks with certain compounds exhibiting some degree of success in selective control in lawns. The herbicidal properties of 2,4-D in the 1940s marked, as described by Timmons (1970), ‘the chemical era of agriculture’.

Targeted herbicide research began in the 1950s, with initial screens consisting of small-scale greenhouse trials aiming at the identification of a satisfactory bioefficacy profile (Kraehmer et al., 2014a). This was further streamlined with the advancements in analytical methods during the 1970s, the sharing of lead compounds between institutes, universities and industry and the introduction of miniaturised high-throughput assays in petri-dishes (Kraehmer et al., 2014a). Finally, advances in organic chemistry and genome biology as well as the introduction of screening robots, enabled industries to screen up to one million compounds a year (Kraehmer, 2012). Despite the advancements in herbicide discovery however, most herbicide mode of actions that are commercial nowadays were discovered between 1971 and 1985, capturing around 80% of the global herbicide market in value (Kraehmer et al., 2014b). Table 1 contains their names, molecular mechanism and market share.

Herbicide groups	Molecular mechanism	Market share
EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) inhibitors	Disruption of the shikimate pathway and aromatic amino acid production	21 %
ALS (acetolactate synthase) inhibitors	Inhibition of branched amino acid biosynthesis	17 %
Inhibitors of VLCFA (very long chain fatty acids	Inhibition of cell division	11 %
PS (photosystem) II inhibitors	Inhibition of CO_2 fixation and production of energy	10 %
ACCase (acetyl-CoA carboxylase) inhibitors	Inhibition of fatty acid biosynthesis	8 %
Auxin herbicides	Overproduction of plant hormones leading to growth inhibition and senescence	8 %
4-HPPD (4-hydroxyphenylpyruvate) inhibitors	Inhibition of carotenoid synthesis and accumulation of singlet oxygen	5 %

Table 1: Most successful commercial herbicides: mode of actions, molecular mechanism and market share. The market share is noted according to Kraehmer et. al. 2014.

1.2.2 Selectivity in weed control

Selectivity is one of the most important characteristics of successful herbicides. When a herbicide is selective in a crop, the crop remains unaffected following treatment, while weeds

are either killed or their growth impaired. Herbicide selectivity results from a complex interaction of different factors involving the plant, the herbicide applied and the environment (Kraehmer et al., 2014a, Blanco et al., 2015). A comprehensive list of important selectivity factors can be found in Table 2.

Factors		**Definition**
Plant	Developmental stage (age)	young/older plants
	Growth rate	fast/slow rate
	Morphology of leaves, meristems and roots	broad/narrow leaves, smooth/rough leaf surface, wax composition, exposed/non-exposed meristem, deeper/more shallow roots
	Physiology	Absorption and movement through xylem and phloem
	Biochemical processes	enzymatic metabolism
	Genetic heritage	genetic characteristics affecting the other intrinsic factors in plants , cultivar
Herbicide	Physicochemical properties	size, shape, lipophilicity/polarity
	Concentration and formulation	high/low concentration, inert mixing ingredients (i.e. wettable powder formulation, spray adjuvants/surfactants)
	Placement and timing of application	pre/post-emergence application, directed application (i.e. between rows)
Environment	Soil properties and environmental conditions	soil type/pH/moisture, temperature, light, radiation

Table 2: Factors influencing herbicide selectivity. According to Blackman (1950), Hatzios and Penner (1982), Kraehmer et al. (2014a), Blanco et al. (2015)

Even though there are a lot of factors that can influence herbicide selectivity (Table 2), its major determinant is a faster rate of metabolism in crops than in target weeds (Carvalho et al., 2009, Rosinger and Schulte, 2019). This has also been described as true selectivity, which refers to a plant's ability to tolerate a herbicide and encompasses other aspects, such as limited uptake and translocation as well as differential metabolic profile (Hatzios and Penner, 1982). Since a herbicide's enhanced metabolic rate is the main component that confers selectivity to that herbicide, the following mainly focuses on this cause of selectivity.

1.3 Herbicide metabolism

1.3.1 Overview of herbicide metabolism in plants

Plants possess a coordinated detoxification system in place to isolate foreign, potentially toxic compounds (xenobiotics), termed the xenome, with its components responsible for the detection, transport and detoxification of herbicides and other xenobiotics (Edwards et al., 2011). When herbicides enter plant cells, they are metabolised by this system into usually more polar metabolites that have reduced, or no phytotoxicity.

The xenome (Figure 1) has two components, a sensing system that regulates metabolism in coordination with transcriptional control and a detoxification system involving all the necessary metabolic enzymes (Edwards et al., 2011). Xenobiotic sensing has been characterised in yeast, vertebrates and invertebrates (Baker, 2005, Dias et al., 2010). Vertebrates for example, possess different receptors according to different xenobiotic responses such as aryl hydrocarbon (AhRs), Toll-like receptors (Ramel et al., 2012) and nuclear receptors (unique in multicellular animals) that bind small lipophilic molecules and regulate various biochemical processes (Baker, 2005). The sensing system in plants has yet to be elucidated, even though there is evidence pointing to its existence since xenobiotics can cause major transcriptional changes (Riechers et al., 2010, Ramel et al., 2012).

The metabolic component of the xenome is organised in four distinct phases (Edwards et al., 2005a, Yuan et al., 2007). Xenobiotics undergo reactions that introduce or reveal functional/reactive groups (phase I), are bioconjugated with sugar, amino acids or peptides (phase II) and actively removed from the cytoplasm to be deposited into the vacuole (phase III). These reactions are very similar to the detoxification reactions in animals, with the only exception being the final fate of the metabolites (Edwards et al., 2011). While these metabolites in animals are typically excreted in urine (Sandermann, 1992), in plants they are further processed in the vacuole and either stored, ultimately mineralised into CO_2 (Ertunc et al., 2004), or incorporated into the cell wall (Phase IV) (Brazier-Hicks et al., 2007).

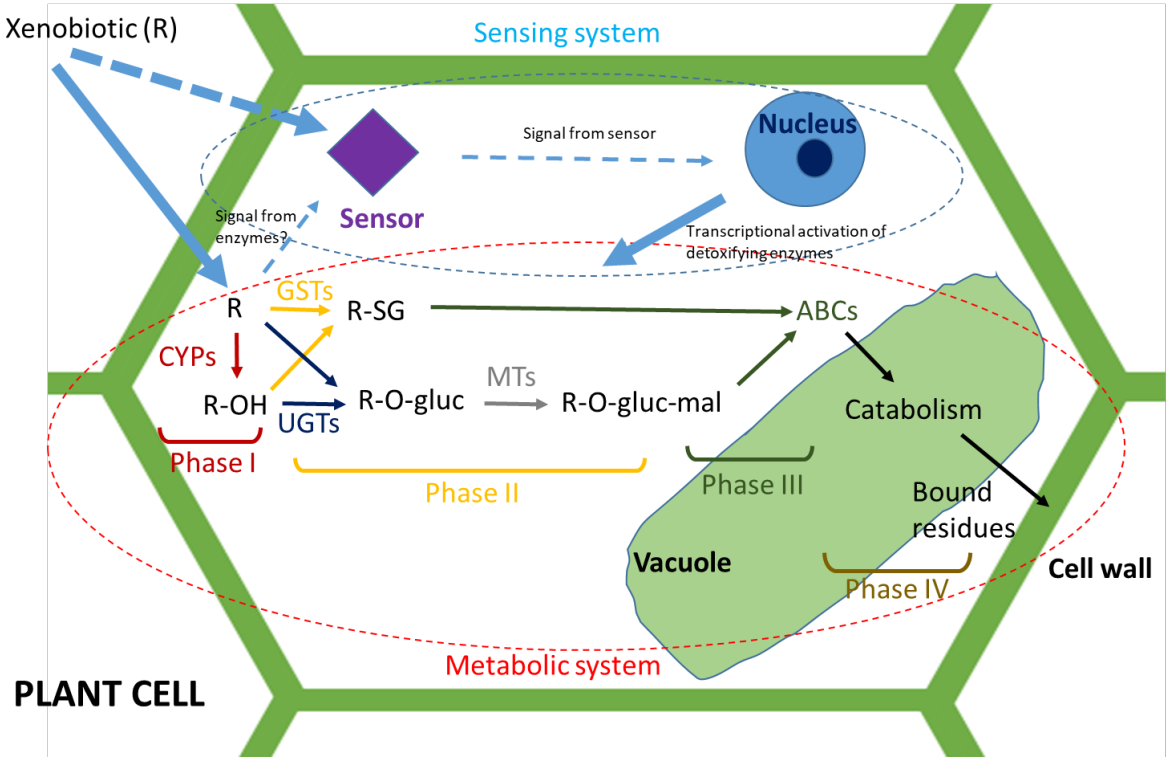


Figure 1: The plant xenome. Visual representation of the sensing system and metabolic systems of the xenome. Bold lines denote characterised events, while dotted lines hypothesised ones. Abbreviations: CYPs: cytochrome P450s, GSTs: glutathione-S-transferases, UGTs: UDP-glycosyltransferases, MTs: malonyltransferases, ABCs: ATP-binding cassete proteins

1.3.2 Phase I – Biotransformation

The first phase of xenobiotic detoxification includes a number of reactions such as hydrolysis mainly catalysed by carboxylesterases (Gershater and Edwards, 2007) and reductions and oxidations, mainly catalysed by cytochrome P450s (Riechers et al., 2010, Edwards et al., 2011). Oxidation reactions are the most common (Davies and Caseley, 1999), with the enzymes associated with these reactions, cytochrome P450s or CYPs, being haem proteins primarily localised on the endoplasmic reticulum. Unlike other detoxification enzymes, they

are able to catalyse a plethora of different reactions such as hydroxylations, demethylations, sulfoxidation, dealkylation and epoxidation, that each reveal, or introduce, reactive or functional groups (Guengerich, 2001, Van Eerd et al., 2003). Their major structural feature is their heme/iron binding site (Phe-X-X-Gly-X-Arg-X-Cys-X-Gly) which together with molecular oxygen and NADPH (Werck-Reichhart and Feyereisen, 2000) is required for their activity. CYPs constitute the largest enzyme family in plant metabolism, with 356 unique CYP genes in rice and 246 in *Arabidopsis thaliana* (Nelson et al., 2004), being associated with the primary metabolism of various herbicide chemistries (Siminszky, 2006). These metabolic reactions are often associated with herbicide selectivity. For instance, CYP-mediated hydroxylation reactions have been demonstrated to confer selectivity in rimsulfuron in maize and diclofop and chlortoluron in wheat (McFadden et al., 1989, Koeppe et al., 2000, Siminszky, 2006). These reactions, however, do not always detoxify herbicides as sometimes the primary metabolic step can yield a more herbicidal product (Casida, 1983, Jeschke, 2016b, Casida, 2017). This has been demonstrated in *Nicotiana benthamiana*, where transformation with CYP105A1 from *Streptomyces griseolus* activated the sulfonylurea pro-herbicide R7402 by *N*-dealkylation (Keefe et al., 1994), a system used as a negative selection marker. Based on the same rationale, CYPs have been primary targets for engineering herbicide tolerance in crops (Werck-Reichhart et al., 2000). Rat CYP1A1 transformation in tobacco plants provided cross-resistance to herbicides with different chemistries and mode of actions, namely chlortoluron, atrazine and pyriminobac-methyl (Ohkawa et al., 1998). Moreover, the overexpression of rice CYP72A31 in rice and *Arabidopsis thaliana* conferred tolerance to ALS-inhibiting herbicides (Saika et al., 2014) and constitutive expression of CYP76B1 from artichoke achieved tolerance to phenylurea herbicides in tobacco and *A. thaliana* (Didierjean et al., 2002).

1.3.3 Phase II – Bioconjugation

Once xenobiotics have been modified with a functional group, they undergo bioconjugation with endogenous biomolecules to form water soluble conjugates, which constitute the second phase of metabolism. Sufficiently reactive xenobiotics do not need to undergo phase I modifications and can directly enter Phase II, or undergo both phases at the same time, like the herbicide atrazine, which can be dealkylated and glutathionylated within single cells (Edwards and Owen, 1986). The most common reactions include conjugation with the tri-

peptide γ-Glu-Cys-Gly, known as glutathione (GSH) (Noctor and Foyer, 1998) as catalysed by glutathione-S-transferases (GSTs) and conjugation with glucose, catalysed by UDP-dependent glycosyltransferases (UGTs). These conjugations result in increased solubility by altering the physicochemical properties of the compounds, rendering them unable to move freely between cellular compartments (Riechers et al., 2010) while facilitating their entry into the third metabolic phase (Bowles et al., 2005, Cummins et al., 2011).

GSTs are one of the most studied plant detoxification enzymes. They are either homo or heterodimer, soluble enzymes located in the cytosol, often associated with membrane bound CYPs (Edwards et al., 2011). Their main function is to catalyse the sulphur attack of GSH to the electrophilic centre of xenobiotics (Armstrong, 1994). There are 54 members in the Arabidopsis family, and around 42 and 25 in maize and soybean respectively (McGonigle et al., 2000, Dixon et al., 2009). GSTs are classified based on their sequence similarity and function, where the main classes involved in xenobiotic detoxification are the plant-specific phi (φ) and tau (τ) classes (Edwards et al., 2000, Dixon et al., 2002b). Members of the lambda (λ) class, which is also specific to terrestrial plants, are strongly induced by xenobiotics and have been suggested to have redox-related roles, without directly detoxifying xenobiotics (Theodoulou et al., 2003, Dixon and Edwards, 2010, Edwards et al., 2011). GSTs are able to detoxify xenobiotics in a selective way which differs between plant species, often determining herbicide selectivity (McGonigle et al., 2000, Edwards et al., 2000). GST-mediated detoxification has been described with multiple herbicide chemistries, including chloracetanilides, sulfonylureas and aryloxyphenoxypropionates (Edwards, 1997, Cummins et al., 2011). Notable examples include atrazine in maize and fenoxaprop-ethyl in wheat where GST-mediated conjugation was shown to be primarily responsible for conferring tolerance (Frear and Swanson, 1970, Tal et al., 1993, Cummins et al., 2009). Due to their well-documented ability to detoxify herbicides, these enzymes have been utilised for engineering enhanced herbicide tolerance in a number of crops. For instance, overexpression of maize GST-27 was demonstrated to increase tolerance against chloracetanilide and thiocarbamate herbicides in wheat (Milligan et al., 2001). Furthermore, rice overexpressing OsGSTL2 were more tolerant against glyphosate and chlorsulfuron (Hu, 2014). A study in tobacco also showed that tolerance against fomesafen required both the transformation of a soybean GST with high activity against the herbicide and an equivalent homoglutathione synthase for the sufficient accumulation of homoglutathione, the major thiol in the crop (Skipsey et al., 2005).

Conjugation reactions with UDP-glucose also belong to the second detoxification phase and are catalysed by UGTs. These can result in *O*-, *S*- and *N*- glucosides either from molecules

that already contain a reactive site (-OH, -SH, -NH_2,, -COOH) or from oxidation products derived from the action of CYPs (Yuan et al., 2007). As CYP-mediated oxidation reactions are very common, the most common of these metabolites are the *O*-glucosides (Van Eerd et al., 2003). UGTs are able to recognise a variety of molecules for glucose conjugation apart from xenobiotics, including phytotoxins, plant secondary metabolites and phytohormones (Bowles et al., 2005). Even though it's the most common metabolic reaction in phase II, the associated UGTs have not been studied as well as CYPs or GSTs. The same goes for malonyltransferases (MTs), another group of phase II detoxification enzymes, that facilitate the conjugation of malonic acid to acceptor 6-*O*-glucosides, anilines and *S*-cysteinated conjugates (Sandermann et al., 1997, Edwards et al., 2011). Glucose conjugation in herbicide metabolism has been previously described for diclofop-methyl, chlorsulfuron, picloram and pyroxasulfone (Shimabukuro et al., 1979, Sweetser et al., 1982, Frear et al., 1989, Tanetani et al., 2013), where in most cases the parent molecule previously underwent either hydrolysis, or oxidation by a CYP. Additionally, malonate conjugation has been reported for metribuzin, picloram and chloracetanilide herbicides (Sandermann et al., 1997). Due to their endocellular homeostatic and detoxification roles, UGTs have been recognised for phytoremediation purposes, with *A. thaliana* UGT72B1 shown to be highly active in conjugating a number of pollutants, including dichloroaniline (DCA) and 2,4,5-trichlorophenol (Loutre et al., 2003).

1.3.4 Phase III – Deposition

During Phase III, the bioconjugated molecules bearing either GSH, glucose or glucose-malonate moieties are recognised and transported into the vacuole by ATP-binding cassete (ABC) transporter proteins, a process facilitated by the use of MgATP and inhibited by Vanadate (Rea, 2007). These ATP-powered primary pump proteins facilitate the transport of a variety of substrates in addition to xenobiotics, including lipids, amino acids, peptides and secondary metabolites (Higgins, 1992, Theodoulou, 2000). They constitute a large family in plants with 120 ABC proteins in Arabidopsis and 121 in rice (Sanchez-Fernandez et al., 2001). ABC transporter subfamily C (ABCC) is primarily responsible for transport of glutathione conjugates into the vacuole, with five ABCC members from *A. thaliana* analysed for potential substrates by heterologous expression in yeast (Klein et al., 2006, Rea, 2007). The transport of glutathione and glutathione conjugates into the vacuole was demonstrated for the first time in barley, with metolachlor and *N*-ethylamaleide glutathione conjugates

(Martinoia et al., 1993). Since then, many studies have shown the involvement of ABC transporters in herbicide metabolism (Rea et al., 1998, Yang et al., 2016, Liu et al., 2018), with a characteristic study demonstrating that overexpression of an ABC protein in *A. thaliana* conferred enhanced tolerance to multiple chemical classes of herbicides (Windsor et al., 2003).

1.3.5 Phase IV – Storage, incorporation or exclusion

The fate of xenobiotic metabolites after vacuolar transport is the least studied phase of xenobiotic detoxification. It was initially thought that further catabolism of glutathione conjugates occurred in the vacuole, where vacuolar peptidases were found to catalyse cleavage of glycine and glutamic acid residues of the glutathione moiety, forming cysteine conjugates in crops (Wolf et al., 1996, Coleman et al., 1997). More recent research however, reported that two distinct catabolism pathways for glutathione conjugates exist in *A. thaliana*, in the cytosol and in the vacuole (Brazier-Hicks et al., 2008). The enzymatic expression data indicated that the vacuolar pathway was predominant in the roots and the cytosolic one in the foliage (Grzam et al., 2007, Blum et al., 2007, Brazier-Hicks et al., 2008). Irrespective of which catabolic pathway is followed, these metabolic products are either stored in the central vacuole or exported into the extracellular space where they are bound to biomolecules such as lignin and cellulose, with some ultimately mineralised to CO_2 (Wolf et al., 1996, Ertunc et al., 2004). A study in barley roots reported that under specific conditions, xenobiotic glutathione conjugates undergo unidirectional long-range transport through the phloem or the xylem, followed by exudation from the root tips (Schroder et al., 2007). Even though these Phase IV reactions are not very well studied, the formation of catabolic products have been found to correlate with enhanced herbicide tolerance, such as with fenoxaprop-ethyl in wheat and blackgrass (Cummins et al., 2009).

1.4 Selective and non-selective herbicides

As described in the previous sections, herbicide selectivity mainly stems from differential metabolism rates between species resulting in some crops being naturally tolerant to specific herbicides. Corn for example, shows extreme tolerance to atrazine, a photosystem II inhibitor,

as it utilises three degradative pathways to detoxify it, *N*-dealkylation, hydrolysis and glutathione conjugation (Shimabukuro, 1967). Plants susceptible to atrazine, like oats, wheat, barley and peas, are devoid of the GST enzyme that catalyses conjugation with gluthathione, which partially explains their susceptibility (Frear and Swanson, 1970, Lamoureux et al., 1972). Other examples include bentazon in rice where tolerance results from hydroxylation and glucose conjugation (Mine et al., 1975, Zhang et al., 2007a), fenoxaprop-ethyl in cereals, which is detoxified through conjugation with glutathione and glucose (Tal et al., 1993, Beffa et al., 2019) and penoxsulam which is *O*-dealkylated in tolerant rice (Johnson et al., 2019).

Some herbicides however have targets whose inhibition is detrimental to most plant species and therefore are classified as non-selective, often used in 'burndown' applications where all plants need to be eliminated (Syngenta, 2019). Such herbicides include diquat, paraquat, glyphosate, glufosinate and indaziflam (Dodge and Harris, 1970, Kraehmer et al., 2014b, Jeschke, 2016a). The use of such herbicides requires engineering of herbicide tolerance traits in crops through genetic modification, breeding or genome editing (Kraehmer et al., 2014b, Green, 2014a, Zhang et al., 2019). Herbicide tolerance has been engineered in crops such as soybean, maize, cotton and wheat, especially towards glyphosate (Shaner and Beckie, 2014, Green, 2014b) with breeding techniques focusing on engineering tolerance to wider chemistries (Kraehmer et al., 2014b).

However, genetic engineering technology is facing big challenges regarding herbicide resistance and biodiversity loss due to glyphosate overreliance (Green, 2014a, Schütte et al., 2017) and is very restricted in Europe due to public opinion regarding GM crops and glyphosate, as well as recent regulations limiting the use of gene editing.

1.5 Herbicide safeners

1.5.1 Definition and history

Apart from genetic engineering, another key way to bestow herbicide selectivity is by the use of herbicide safeners (Kraehmer et al., 2014a), where crop tolerance to certain herbicides can be chemically re-programmed.

Safeners are a diverse group of chemicals used in weed control that enable the use of broad-spectrum herbicides, by selectively protecting crops of interest from herbicide injury, without compromising herbicide activity in the target weeds (Davies and Caseley, 1999). These

compounds are mainly effective in monocotyledonous crops and do not display activity in dicots and weed species (DeRidder et al., 2002, Hatzios and Burgos, 2004, DeRidder and Goldsbrough, 2006, Riechers et al., 2010, Jablonkai, 2013).

Safeners are widely used in weed control, despite the competition with herbicide-tolerant crops. It has been reported in 2011 that approximately 30% of herbicide mixtures in corn and cereals (6% in rice) contained safeners, which amounted to a market of 1.8 billion USD (Rosinger, 2014). From 2006 onwards, most important new selective herbicide products used in cereals and corn contain safeners (Rosinger and Schulte, 2019).

After their serendipitous discovery in 1947 by Otto Hoffman (Hoffmann, 1953) and consequential commercialisation of the first safener compound napthalic anhydride (NA) in 1969, there has been a lot of interest in safener research by universities and industry. As with herbicides, one of the main traits that is required for the development of a new safener is selectivity. This selectivity can be either attributed to selective placement, or due to intrinsic biochemical processes in the crops, as discussed earlier (Table 2). The latter has been termed botanical or true selectivity (Hatzios and Penner, 1982, Hatzios, 1991). The first safeners were selective due to placement, as they were applied by seed treatment to the crops of interest, with NA and oxime ether safeners as prime examples (Hatzios, 1989a, Rosinger and Schulte, 2019). One key disadvantage of this practice was that the use of safener-coated seeds was combined with herbicides from different companies, making 'value capture' for specific products difficult (Kraehmer et al., 2014a). Pre-emergence tank mix safeners were developed afterwards such as dichlormid (Pallos et al., 1972), whose selectivity forced seed treatment safeners such as NA to be withdrawn from the market (Davies and Caseley, 1999, Davies, 2001). With the discovery of post-emergence selective weed control, two significant innovations were introduced into the safener market; (1) the development of post-emergence safener technology and (2) cereal safeners. Up to that point, the monocot crops that could benefit from safeners were maize, sorghum and rice. The first cereal safener was fenclorazole-ethyl, developed for use with fenoxaprop ethyl, which could control weeds without injuring dicot crops but lacked selectivity in barley and wheat. This safener has been replaced by new cereal safeners with stronger safening activity such as cloquintocet-mexyl and mefenpyr diethyl. These have extended the use of herbicides with selectivity limitations in cereals, such as iodosulfuron and mesosulfuron (Hacker et al., 2000). The latest innovations in safener technology are isoxadifen-ethyl, which is active in multiple crops against multiple herbicide chemistries and cyprosulfamide (CSA), a highly selective maize safener which can be used in

both pre- and post-emergence applications (Hacker et al., 2002, Santel, 2012, Kraehmer et al., 2014a, Rosinger and Schulte, 2019).

1.5.2 Applications

Broad spectrum herbicides have selectivity issues (Kraehmer et al., 2014a, Rosinger and Schulte, 2019), so the main use of safeners is to diversify the use of such herbicides. The latest ALS inhibitors depend on safeners for crop selectivity. Two such examples are the broad spectrum herbicides thiencarbazone-methyl (TCM) and pyroxsulam, used for the control of broadleaf weeds and a wide range of grasses (Wells, 2008, Santel, 2012). TCM is combined with either mefenpyr-diethyl for use in cereals, or with cyprosulfamide (CSA) for use in corn, whereas pyroxsulam is used with cloquintocet-mexyl for use in wheat, rye and triticale (Kraehmer et al., 2014b). From the 4-HPPD inhibitors group, isoxaflutole, tembotrione and pyrasulfutole also require safeners to be used effectively. Isoxaflutole controls a broad range of weeds when combined with CSA, as it allows the use of higher application rates, while tembotrione offers control of a broad spectrum of weeds and crop selectivity when combined with isoxadifen-ethyl (Gatzweiler et al., 2012, van Almsick, 2019). Pyrasulfutole controls a specific range of weeds and is normally combined with mefenpyr-diethyl to maximise crop safety for use in cereals (Kraehmer et al., 2014b, van Almsick, 2019). Finally, a notable example from ACCase herbicides combined with safener is pinoxaden, which can be used to control a wide range of annual grasses in cereals when combined with cloquintocet-mexyl (Hofer et al., 2006). These combinations of herbicide and safener pairs can be found in Table 3.

Other advantages of safener applications in weed control is that they provide more chemical weed control options for crops of small market value which are not targeted by herbicide research (Davies and Caseley, 1999, Davies, 2001). Furthermore, they are very useful in cases where crops and weeds are botanically related (i.e. oats and wild oats) or in crop rotation systems where the previous crop constitutes a rotational weed (Davies and Caseley, 1999, Davies, 2001).

The ability of safeners to specifically induce certain detoxifying enzymes has been reported as a useful tool for developing gene expression systems for tissue-specific and developmentally regulated transgene expression (Hershey and Stoner, 1991, De Veylder et al., 1997) and

additional roles have also been proposed for phytoremediation of contaminated soils (Edwards et al., 2011, Taylor et al., 2013). Arguably, however, one of their most valuable application is their use as tools to manipulate herbicide selectivity while identifying the signalling pathways involved (Davies and Caseley, 1999, Jablonkai, 2013).

1.5.3 Commercial safeners

Since the commercialisation of NA, many chemical classes of safeners have been superseded or withdrawn from the market either due to the development of better alternatives (fenchlorazole ethyl vs mefenpyr/isoxadifen) or due to unfavourable market conditions (NA vs dichlormid). Table 3 lists the safeners that are still relevant today, the classes of their herbicide partners, the crop they protect and their application method.

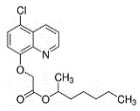
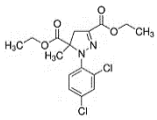
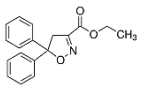
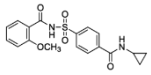
Safener	Structure	Class-chemical	Crop	Herbicides	Application method
Benoxacor		Dichloroacetamide derivative	Maize	Chloracetamides	Pre-emergence spray, Preplant incorporated
Cloquintocet-mexyl		Phenyl pyrazole	Cereals	Clodinafop-propargyl, pyroxsulam, pinoxaden	Post-emergence spray
Mefenpyr-diethyl		Phenyl pyrazole	Cereals	ACCase and sulfonylureas (eg. TCM, pyrasulfutole)	Post-emergence spray
Isoxadifen-ethyl		Isoxazoline	Maize, rice	ACCase, sulfonylureas and 4-HPPD inhibitors (tembotrione)	Post-emergence spray
Cyprosulfamide		Aryl sulfonamide	Maize	ALS and 4-HPPD inhibitors (eg. TCM, isoxaflutole)	Post- and pre-emergence spray

Table 3: The main safener chemistries used in today's market. Adapted from Rosinger (2019). Preplant incorporated application refers to mixing the herbicide in the soil before sowing, pre-emergence refers to application immediately after sowing and post- emergence to after the crops have emerged from the soil.

1.5.4 Mechanisms of safener activity

Successful safeners are characterised by high specificity in both the species protected and in partner herbicide chemistries used (Hatzios, 1991). They need to be applied either before, or at the same time with herbicides for maximum injury alleviation and only protect crops that are already moderately tolerant to the herbicide chemistries applied (Hatzios, 1991).

Safeners protect crops from injury by limiting the action of herbicides, which can happen, in principle, in two mechanisms. Firstly, the safener may antagonistically interact with the herbicide itself, the herbicide's site of action or a receptor able to bind both herbicides and safeners. Secondly, safeners can act as bioregulators, where the safener influences the behaviour of the herbicide in the plant by decreasing the amount of herbicide reaching its target (uptake, translocation and metabolism) (Hatzios, 1991, Davies and Caseley, 1999, Kraehmer et al., 2014a).

1.5.4.1 Antagonism

The interaction of safeners and herbicides at the herbicide site of action derived from the observation that some safeners were very similar in terms of structure and physicochemical properties with their herbicide mixture partners, like safener dichlormid with EPTC and diclofop-methyl with 2,4-D (Stephenson and Chang, 1978, Taylor and Loader, 1984, Kőmíves and Hatzios Kriton, 1991) . Computer aided molecular modelling (CAMM) studies showed that various herbicide-safener combinations share similar degrees of bonding, charge distribution and molecular volume (Yenne and Hatzios, 1990, Bordas et al., 2000). A more recent publication on fenclorim, reported that its ability to rapidly induce detoxifying enzymes was very sensitive to structure variations in both its pyrimidine and phenyl ring, which was indicative of a protein-based recognition system (Skipsey et al., 2011).

In line with these studies, a proposed 'safener receptor' protein (SafBP) was identified in 1998, whose identity was proposed to be an *O*-methyltransferase involved in lignin biosynthesis (Scott-Craig et al., 1998). Competitive binding for this protein was demonstrated between safener R-29148 and herbicides EPTC, metolachlor and alachlor. However, these findings could not be replicated by other research groups and it was been suggested that this

protein binding was not involved in safener-signalling (Edwards et al., 2011). It has also been proposed that safeners could interact with herbicides directly, by forming a complex unable to cause herbicide injury, which is based on the function of adsorbents such as activated carbon and lignin PC 671 (Hoagland, 1989). Such a mechanism has been proposed for the protection of oxime ether safeners against chloracetanilide herbicides (Davies and Caseley, 1999).

The effects of safeners on the ALS enzyme targeted by ALS-inhibitors, have also been the focus of several studies. Safener treatment was able to increase both ALS levels (Milhomme and Bastide, 1990, Milhomme et al., 1991, Davies and Caseley, 1999) and activity (Rubin and Casida, 1985) as well as reduce the enzyme's sensitivity to inhibition (Milhomme et al., 1991). However, these studies are contradicted by a number of following investigations showing that safener treatment either did not affect the levels of the ALS enzyme (Frear et al., 1987, Barrett, 1989, Lamoureux and Rusness, 1992, Burton et al., 1994), or increased its levels but rather decreased its sensitivity (Davies and Caseley, 1999). Several safeners were also unable to reduce herbicide binding at the target site with both ALS and ACCase inhibitors (Hatzios, 1991, Davies and Caseley, 1999).

Due to the conflicting evidence for the antagonism theory, it has been regarded not to be the main mode of action of safeners, even though it could play a minor role, especially for certain herbicide-safener combinations (Hatzios, 1991, Davies and Caseley, 1999, Kraehmer et al., 2014a). The main evidence against this theory is that it fails to explain the lack of safening in dicots, the low safener to herbicide ratios in herbicide products and how some safeners like NA are able to protect against chemically unrelated herbicides (Hatzios, 1991). Some recent reviews on safeners don't acknowledge this theory as a plausible mode of action (Riechers et al., 2010, Jablonkai, 2013, Rosinger and Schulte, 2019). However, an indirect involvement with the target site has been suggested by Davies (1999), where a biochemical stress such as the interactions of safeners with a protein such as SafBP or a herbicide target site (i.e. ALS/ACCase enzymes) could be the start of a signalling cascade able to induce herbicide detoxification enzymes at the transcriptional level. Members of the lambda GST family could potentially have a role in this as they are specifically induced by safeners and herbicides with the latter being hindered in transgenic tobacco transformed with a sulfonylurea resistant form of the ALS enzyme (Hershey and Stoner, 1991, De Veylder et al., 1997).

1.5.4.2 Bioregulation

1.5.4.2.i Effect of safeners on herbicide uptake and translocation

For post-emergence selective weed control, the coleoptile constitutes the main site of action, requiring systemic transport necessary for the applied herbicides to act. Herbicides such as ALS and ACCase inhibitors are very effective when the plants are at a young developmental stage, making protection of the meristems vital for the survival of the plants.

Studies on the effect of safeners on herbicide uptake and translocation have generated conflicting data which is summarised in two very comprehensive reviews, one from Hatzios and one from Davies (Davies and Caseley, 1999, Hatzios, 1991). In Hatzios's review, six safeners either increased or decreased herbicide uptake and translocation, while in Davies's report, 15 safeners produced no conclusive effects, with most of them not affecting uptake and translocation of the applied herbicide. Even in the case of safeners that did affect the uptake of herbicides, it was still doubtful whether that was due to the action of the safener. One such example was the reduction of imazapic uptake after seed treatment of maize seeds with the safener NA. Further investigation reported reduced imazapic uptake even when NA was applied after the herbicide, questioning any correlations of the protective effect with decreased herbicide uptake (Davies et al., 1998).

Further studies on the effects of mefenpyr-diethyl on uptake of sulfonyurea herbicides and the effects of fenchlorazole-ethyl on fenoxaprop-ethyl translocation also reported inconclusive findings (Kocher et al., 1989, Kocher, 2005). In certain cases, reduced uptake and translocation of herbicides is due to corresponding enhancement in their metabolism, as herbicide metabolites tend to be polar and immobile molecules (Davies and Caseley, 1999, Rosinger and Schulte, 2019). It has since been postulated that safener effects on herbicide uptake and translocation are unlikely to account for safener activity (Hatzios, 1991).

Specific safener induced protective effects in meristematic tissues have been reported by several studies. Benoxacor was found to increase metolachlor metabolism mainly in maize coleoptiles by glutathione conjugation (Kreuz et al., 1989), corroborated by similar findings with safener BAS 145138 and herbicide metazachlor (Fuerst et al., 1991). In addition, safeners fluxofenim and cloquintocet-mexyl could induce GST transcript expression, protein levels and activities in the outer cell layers of the coleoptiles in *Triticum tauschii* (Riechers et al., 2003).

1.5.4.2.ii Effect of safeners on herbicide metabolism

Most studies have demonstrated that safeners predominantly act in crops by enhancing the rate of herbicide metabolism (Davies and Caseley, 1999, Hatzios, 1991, Hatzios and Burgos, 2004, Jablonkai, 2013, Riechers et al., 2010, Kraehmer et al., 2014a). This is due to a rapid induction of detoxifying enzymes in the xenome (Davies, 2001, Hatzios and Burgos, 2004, Rosinger and Schulte, 2019). Transcriptome profiling studies in both sorghum and *A.thaliana* report that upon treatment with safeners, genes from multiple phases of the xenome are induced, including CYPs, GSTs, UGTs and ABC transporters (Behringer et al., 2011, Skipsey et al., 2011, Baek et al., 2019). It's worth noting that even though the detoxification machinery is induced in dicot plants, it doesn't result in safener-mediated herbicide tolerance (DeRidder et al., 2002, DeRidder and Goldsbrough, 2006, Behringer et al., 2011, Brazier-Hicks et al., 2018a).

1.5.4.2.iii Effect of safeners on CYPs

There have been a number of studies demonstrating the enhancement of oxidative metabolism of herbicides upon safener treatment (Yun et al., 2001, Davies, 2001, Forthoffer et al., 2001), as confirmed by experiments with CYP inhibitors and microsomal enzyme systems (McFadden et al., 1990, Moreland et al., 1993, Davies and Caseley, 1999). However, reports on transcript expression of specific CYPs upon safener treatment is scarce (Riechers et al., 2010). Several genes encoding CYPs (*CYP71C1*, *CYP71C3*, *CYP72A5* and *CYP92A1*) were induced upon NA treatment in a tissue specific and developmentally regulated manner in corn (Persans et al., 2001) while fenclorim could induce CYPs in both *A. thaliana* and rice cell cultures (Brazier-Hicks et al., 2018a). In addition, CYP81D11 from *Arabidopsis* which is reported to be induced by a range of reactive chemicals (Koster et al., 2012), was also reported to be induced by benoxacor (Baerson et al., 2005). Interestingly, the total CYP content of a plant does not seem to correlate with the rate of oxidative metabolism, and it has been suggested that safeners induce isozymes specific for different herbicide substrates which would explain the lack of CYP transcript expression data in comparison to oxidative metabolism studies (Davies and Caseley, 1999). If this were to be true, the ability of CYPs to metabolise endogenous substrates could be compromised. Accordingly, a study showed that

pretreatments with safener NA enhanced hydroxylation of bentazone in maize microsomes, while it did not affect oxidation of lauric and cinnamic acids (Moreland et al., 1993). The same study reported that this enhancement in hydroxylation was highly specific as it was different between herbicides of the same family, namely nicosulfuron and triasulfuron.

1.5.4.2.iv Effect of safeners on GSTs

The effects of safeners on GSTs are well-established, where safeners are able to induce GST transcript expression and enzyme activity as well as glutathione synthesis (Davies and Caseley, 1999, Hatzios and Burgos, 2004). Initial studies with safeners measured elevated levels of GSH being related to enhanced herbicide tolerance (Breaux et al., 1987). Since then, multiple safeners have been reported to induce different classes of GSTs. For example, chemical and species-specific induction of phi (φ) and tau (τ) GST protein levels was demonstrated with 8 safeners in maize, wheat and *Arabidopsis thaliana* coupled with increased GST activities (Edwards et al., 2005b). GST-27, a maize GST belonging to the theta (θ) class and demonstrated to increase tolerance against chloracetanilide and thiocarbamate herbicides (Milligan et al., 2001), was also highly induced by dichlormid in aerial parts of maize (Jepson et al., 1994). Studies on GSTs in wheat provide further examples, with cloquintocet-mexyl, mefenpyr-diethyl and fenchlorazole-ethyl inducing GSTs from three different classes (phi, tau and lambda) (Cummins et al., 1997, Theodoulou et al., 2003, Taylor et al., 2013). GSTs are also upregulated in dicots despite the lack of safening, and most notably in *A.thaliana*. Benoxacor, fenclorim and fluxofenim increased levels of RNA of phi and tau GSTs, which was corroborated by enhanced GST activity and glutathione content (DeRidder et al., 2002). A number of studies in *A. thaliana* have identified AtGSTU19 as being particularly interesting, as it's highly induced by safeners, highly active towards chloracetanilide herbicides and expressed in an organ-specific manner (DeRidder et al., 2002, Edwards et al., 2005b, DeRidder and Goldsbrough, 2006, Skipsey et al., 2011).

1.5.4.2.v Effect of safeners on UGTs

As glucose conjugation is one of the most common detoxification reactions, (Edwards et al., 2011), safeners have also been reported to induce the action of UGTs. Glucosyl-transferase activity (GT) has been reported with a range of safeners in maize and *A.thaliana* and with cloquintocet-mexyl in wheat (Edwards et al., 2005b), while the transcript expression of four UGTs was also induced by benoxacor and fenclorim treatments (Baerson et al., 2005). Finally, a comparative study between rice and *Arabidopsis* using DNA microarrays found that UGT transcripts are upregulated in both species after fenclorim treatment, identifying the D and L group members as the most safener-inducible UGTs in both species (Brazier-Hicks et al., 2018a).

1.5.4.2.vi Effect of safeners on ABC transporter proteins

The induction of ABC transporter proteins by safeners is commonly reported along with the induction of GSTs. Cloquintocet-mexyl enhanced both GST activity and vacuolar transport of primisulfuron conjugates in barley vacuoles (Gaillard et al., 1994) as well as the protein levels on an MRP protein in wheat along with several GSTs (Theodoulou et al., 2003). Moreover, dichlormid induced a glutathione transferase (ZmGST27), a glutathione transporter (ZmGT1) and a transporter protein (ZmMRP1) in maize (Pang et al., 2012) and safeners benoxacor, fenclorim, and fluxofenim induced the RNA expression of five transporter proteins along with two GSTs in *Arabidopsis* (DeRidder and Goldsbrough, 2006).

Safener effects on Phase IV enzymes are not well established and rarely reported in literature (Riechers et al., 2010, Rosinger and Schulte, 2019).

1.5.4.3 Gene induction and signalling pathway

Despite the fact that safeners have been commercial since 1970 and the evidence of their effects on the transcript expression of xenome enzymes, the biochemical and molecular events

that lead to the induction of these enzymes causing herbicide tolerance are largely unknown (Riechers et al., 2010).

Unlike herbicides, safeners are not known to interact with a specific target site, yet they are recognized by the plant's defense signaling system and effectively up-regulate genes and enzymes involved in detoxification (Zhang et al., 2007b). Since the activation of xenobiotic detoxification enzymes has been linked to multiple stress responses by plants, there has been a lot of theories regarding the mode of action of these compounds, with the main ones summarised by Riechers et al (2010).

A well-established theory is that safeners could tap in the same signalling pathway as oxylipins, oxidised fatty acids and lipids (cyclopentenones, phytoprostanes) created from a-linoleic acid, the first step of jasmonic acid biosynthesis (Schaller and Stintzi, 2009). Gene profiling studies in *A. thaliana* have indicated that oxidative stress can cause accumulation of these oxidised derivatives, which activate plant detoxification enzymes including P450s, GSTs and ABC transporters with specificity, while also exhibiting a protective effect against copper sulfate (Loeffler et al., 2005). In addition, oxylipins are similar to some safeners in terms of structure, which translates into having similar electrophilic centres, lipophilicity and thiol reactivity (Mueller and Berger, 2009).

A study investigating further links between safener and oxylipin signalling showed that safener fenclorim induced a similar subset of genes with phytoprostane PPA1 and allelochemical benzoxazolin-2(3*H*)-one (Skipsey et al., 2011), with the main difference being the kinetics of the upregulation, where safeners induced plant defence and detoxification genes more rapidly. Moreover, certain synthetic compounds inspired by oxylipins were able to induce a rapid transcript regulation of detoxification genes much like safeners in *A. thaliana* but were unable to provide functional safening in rice (Brazier-Hicks et al., 2018b). This highlighted that different responses were dependent on the lipophilicity and electrophilicity of the compounds as well as an inherent species specificity of these chemistries.

Another induction system proposed for safener signalling involves a potential transcriptional repressor system, like the Nrf2-Keap1-ARE complex found in mammals. The Keap1 protein normally destabilises the transcription factor Nrf2, to prevent the activation of the oxidative stress signalling cascade. Upon oxidative stress, however, the Keap1 protein becomes modified and is no longer able to destabilise Nrf2, which then binds to Antioxidant Response Elements (AREs) in the promoters of Phase I and II detoxification genes to activate them

(Nguyen et al., 2009). However, no analogues of the Keap protein or the Nrf2 transcription factors have been identified in plants (Loboda et al., 2016).

Safeners have also been associated with phytohormones. Safener CSA has been shown to work synergistically with abscisic acid (ABA) in rice, protecting against salinity stress and inducing growth such as the formation of new tillers and early flowering (Dashevskaya et al., 2013). A reporter gene analysis in different mutant backgrounds of *A.thaliana*, showed that safener action required salicylic acid (SA) and the action of TGA transcription factors through as-1 regulatory elements through an NPR1 independent pathway (Behringer et al., 2011). Transcriptomic data from the same study revealed that safener inducible genes were mainly TGA/SA responsive, with the rest being possibly regulated by WRKY transcription factors. Since more than 400 genes were found to be induced in *A.thaliana*, it was concluded that the selective activity of safeners in monocots was more likely to be a result of the specific substrates of the induced xenobiotic detoxifying enzymes, rather than the induction of the detoxification machinery itself. Accordingly, herbicide tolerance has been reported to vary between plant species due to the specific substrate of herbicide detoxifying enzymes (Van Eerd et al., 2003, Yuan et al., 2007, Zhang et al., 2007a, Iwakami et al., 2019). This argument is reinforced by the specific induction of detoxifying enzymes in certain organs, tissues and cells, which might also play a part in contributing functional safening (De Veylder et al., 1997, Xu et al., 2002, Riechers et al., 2003, Taylor et al., 2013).

The latest study on safener mode of action was performed in sorghum, where safener action was linked to a single nucleotide polymorphism found in the two safener inducible phi class GST enzymes which was conserved among 761 inbred lines (Baek et al., 2019). The same study, based on transcriptomic data from these lines, showed that a subset of safener inducible genes was also involved in the biosynthesis and metabolism of dhurrin, a cyanogenic glycoside that is an important chemical defence compound in sorghum against herbivores and pathogens (Darbani et al., 2016). This suggested either a common regulator between the safener signalling and the dhurrin biosynthesis pathway or a common detoxification pathway (Baek et al., 2019).

Due to the nature of safener inducible genes, it is hypothesised that safeners utilise multiple signalling pathways in order for functional safening to occur (Riechers et al., 2010, Behringer et al., 2011, Kraehmer et al., 2014a, Rosinger and Schulte, 2019).

1.6 Outline of the project

Safeners are a valuable tool of chemical weed control and even though they have been commercial since 1970, their mode of action remains to be determined. Their specific activity in monocot crops and effects on herbicide metabolism by inducing detoxifying enzymes are well established. There are, however, certain aspects of safeners that remain elusive such as the selectivity among monocots, the link between the induction of detoxifying enzymes and functional safening and safener fate between safened and unsafened crops.

As discussed in section 1.5.2, one of the latest safener innovations is the aromatic sulfonamide safener cyprosulfamide (CSA). CSA is very effective against ALS and 4-HPPD inhibiting herbicide chemistries and can be used in both pre- and post-emergence applications, with its activity being remarkably specific in maize (See section 3.1) (Santel, 2012, Kraehmer et al., 2014a). Following the observation that a closely related structural analogue (Figure 3) exhibited activity in both maize and cereals, the question of what is the selectivity component responsible for CSA's specificity in maize was immediately raised. This provided an ideal model to study the selectivity of safeners in monocots and, based on the above question, this project focused on the characterisation of the early events that follow CSA uptake to eventually lead to its protective effects, including the performance in the greenhouse, uptake, distribution, metabolism and gene inducing capability. This was done by using a combination of phenotypic, biochemical and molecular biology assays.

Overall, this study aims to improve the understanding of safener selectivity between different monocot crop species and identify at which level, between application and safening, it is manifested. Each chapter was designed to answer specific questions, usually following up on findings from the previous chapter.

Chapter 3 investigated the activity profiles of CSA and metcamifen in greenhouse trials, followed by herbicide metabolism studies in order to link the safeners' protective effects in the greenhouse with herbicide detoxification. It was found that CSA's specific activity in maize in the greenhouse translated into safener-mediated enhancement of herbicide metabolism.

Chapter 4 investigated the fate of CSA in maize and wheat, reporting differences in CSA uptake, distribution and metabolism. Uptake and distribution of CSA could not explain its selective activity, but its metabolism could. A CSA metabolite was positively correlated with the specific activity of CSA in maize.

Chapter 5 explored the role of metabolism of CSA in safening by testing the activity of early CSA metabolites as well as the transcript expression of a lambda GST commonly used as a marker for safening. The marker gene was found to be upregulated only in active crop-safener combinations. Additionally, CSA's primary metabolites were found to have no safening activity but new preliminary data on the spatial distribution of CSA metabolism revealed that safener and herbicide co-localisation may be necessary in order for safening to occur.

In Chapter 6, the results of all chapters are discussed in light of new literature in the field and prospective studies are proposed.

Chapter 2 – Materials and Methods

2.1 Plant material

Plants used in this study included maize (*Zea Mays L.*), wheat (*Triticum aestivum*), barley (*Hordeum Vulgare*), rice (*Oryza Sativa*) and soybean (*Glycine Max*) and were provided by Bayer AG (CropScience, Frankfurt, Germany). Seed varieties are listed in Table 4. Maize and wheat calli were initiated from immature embryos of cultivars Z15 and Fielder, respectively.

For studies with maize mutants the commercial sweetcorn variety Merit, containing a 392 bp insertion mutation resulting in the *nsf1* mutation, was used. *Nsf1* is deficient in a gene encoding a cytochrome P450 associated with resistance to various herbicides (Pataky et al. 2008). This variery was more susceptible to herbicides and was used throughout the study as a tool for dissecting the involvement of safeners with cytochrome P450 enzymes.

Apart from the initial spraying trials (See section 2.4 and Chapter 3) which included all the varieties listed in Table 4, the varieties used for maize, wheat and soybean in the rest of the study were Aventura, Triso and Merlin, respectively.

Crops	Varieties			
 Wheat *Triticum Aestivum*	Triso (spring)	Joyau (durum)	Dekan (winter)	
 Maize *Zea Mays L.*	Oldham	Sileno	Aventura	Merit
 Barley *Hordeum Vulgare*	Montoya	Scarlett	Zzoom	
 Soybean *Glycine Max*	Sultana	Merlin		
 Rice *Oryza Sativa*	Ballila (japonica)	IR64 (indica)		

Table 4: Crop varieties used throughout the study.

2.2 Plant cultivation and treatment conditions

2.2.1 Greenhouse Trials

Seeds were planted in 4 cm plastic pots with local sandy loam soil (Riedberg site, Frankfurt, Germany) and grown in the greenhouse facilities of Bayer AG. Maize, rice and soybean plants were grown in a 'warm conditions' greenhouse with a day cycle of 12 hours day at 25°C followed by a night cycle of 12 hours (21°C), with the humidity set at 60% during both cycles. Wheat and barley were grown in a 'cool conditions' greenhouse, with a day cycle of 12 hours at 16°C followed by a 12 hours night cycle at 12°C; the humidity was set at 50%. All plants were watered twice per day and were grown till the second leaf stage (BBCH Scale 11-12).

2.2.2 Metabolism studies

For experiments performed at Bayer AG (sections 3.4, 4.1 to 4.8, 5.3.2) seeds were planted in 7 cm peat pots containing local sandy loam soil (Riedberg site, Frankfurt, Germany) and grown in the greenhouse facilities of Bayer AG (Conditions mentioned in section 2.2.1). All plants were watered twice per day and grown till the second leaf stage (BBCH Scale 11-12). After treatment, plants were transferred to a RuMed, KBWF240 climate cabinet with a light cycle from 06:00 to 19:00 at 25°C and humidity at 60% and a dark cycle from 19:00 to 06:00 at 18°C with humidity set to 80%.

For experiments performed at Newcastle University (sections 4.9, 5.4 to 5.6), seeds were planted in plastic seed trays using John Innes soil (No.2) and were incubated in a climate cabinet (SANYO MLR-351). Maize was grown under a day cycle from 06:00 to 22:00 at 24°C and a night cycle from 22:00 to 06:00 16°C with a light intensity of 5. Wheat was grown in similar conditions apart from the temperature of the day cycle which was 18°C in a climate cabinet of the same model. Both crops were grown till the second leaf stage (BBCH Scale 11-12) and incubation after treatment took place in the same climate cabinet.

2.3 Chemicals

Analytical grade (>95% purity) herbicide and herbicide safeners, along with radiolabelled thiencarbazone-methyl (thiophene-4-^{14}C, specific activity 4130 Bq/µg), radiolabelled cyprosulfamide (sulfonylbenzamide-ring-UL-^{14}C, specific activity 4240 Bq/µg) and safener metabolites synthesised during the course of the study (See Chapter 5) were provided by Bayer AG (CropScience, Frankfurt, Germany). Other chemicals, unless otherwise stated, were also of analytical grade and purchased from Sigma Aldrich.

2.4 Greenhouse trials

2.4.1 Spray application

Herbicide and safener spray applications were carried out with a track sprayer (Höchst AG, Höchst, Germany) equipped with an even flat spray nozzle (TEEJET, TP8003EVS) and calibrated to deliver a spray volume of 800 L/ha at 1.8-2 bar. The compounds used were formulated in wettable powder (WP), or water dispersible granule (WG) premix, as indicated in Table 5 and were dissolved in distilled water containing 0.2% (v/v) liquid Genapol LRO (Clariant) prior to application. Application was performed using a split spraying method at concentrations of active ingredient (a.i) ranging from 2.5 – 2000 g/ha. The trials showing significant safener effects were repeated, as required for trials utilising visual scoring.

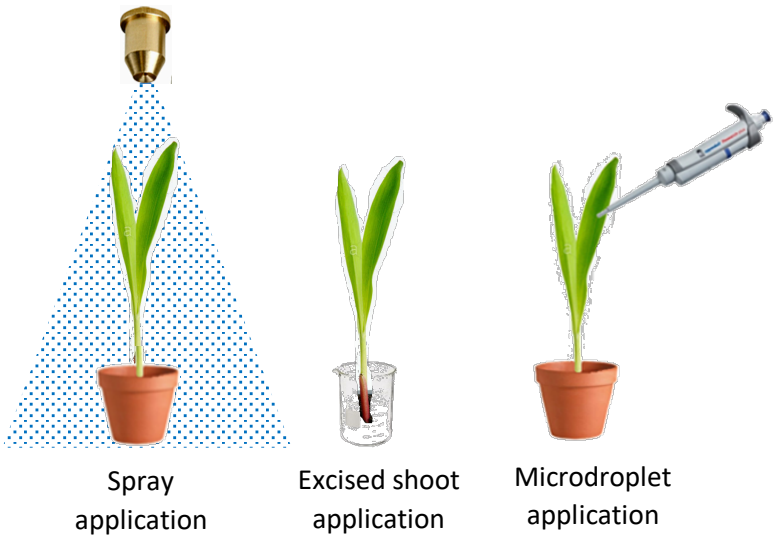


Figure 2: Different application methods used in the greenhouse trials and the metabolism studies.

Compounds	Formulations
Thiencarbazone-methyl	WP10
Tembotrione	WP20
Cyprosulfamide	WP50
Metcamifen	WP50
Flufenacet	WG60
Benoxacor	WP20
Isoxadifen-ethyl	WG50
Dichlormid	WP20
Furilazole	WP20

Table 5: Compounds used in the spraying trials and their formulations. The letters represent the type of formulation used (WP= wettable powder, WG= water dispersible granule) and the number the percentage (%) of active ingredient used in the formulation.

2.4.2 Seed Treatment

Seeds were weighed in sets of 100g and placed in 50 ml falcon tubes, with compounds added at concentrations ranging from 0.25 to 2 g of active ingredient/kg of seeds with the addition of 10 ml distilled water (1 ml for every 10g of seeds). The tubes were put on a roller mixer (Stuart mixer, Speed: 60) until the seed treatment mix was absorbed (average time: 1 hour). Following application, the seeds were planted in 4 cm plastic pots and grown in the greenhouse facilities of Bayer AG till the second leaf stage (BBCH Scale 11-12).

2.4.3 Visual Scoring

Herbicide injury was assessed by visual scoring against untreated control plants, one and two weeks after treatment. Injury scores of every replicate per spraying combination in weeks one and two were averaged to produce the final reported values (Chapter 3). The same regime was followed for all trials. It is important to note that due to the number of the plants in the trial and the complexity injury symptoms, herbicide injury was scored within percentage damage categories (e.g. 50-55% damage) and not as an absolute percentage.

Safener activity was determined by whether or not the compound was able to alleviate herbicide injury, which is referred to throughout this report as a 'safening effect'. In order to account for biological variation, a threshold of more than 10% of herbicide injury alleviation was set in order for the effect to be classified as safening. Based on that, a colour scheme was produced where active safener combination were highlighted in green (Table 6).

Characterisation	Alleviation of herbicide damage	Colour
Severe additional damage	≤ -31%	
Moderate additional damage	-30% ≤ Damage ≤ -21%	
Slight additional damage	-20% ≤ Damage ≤ -11%	
No significant effect	-10% ≤ Damage ≤ +10%	
Slight safening effect	+11% ≤ Damage ≤ +20%	
Moderate safening effect	+21% ≤ Damage ≤ +30%	
Good safening effect	≥ +31%	

Table 6: Colour scheme for the characterisation of the effect of safeners on different crops, the percentages shown refer to absolute changes.

2.5 Metabolism studies

2.5.1 Compound application

Compounds tested for metabolism studies were either applied by incubation via excised shoots, or following application with a pipette on the surface of plant leaves. The shoots of second leaf stage seedlings were excised with a scalpel and placed in vials containing 4 ml of phosphate buffer (1 mM, pH 7), containing compound (5 PPM – 13 µM), unless otherwise stated. At the end of the treatment, the shoots were rinsed in 80% acetonitrile and transferred to fresh water until harvested.

Microdroplet application was performed on the first leaf of second leaf stage seedlings using a pipette with the following formulation: 50µl of compound (2.5 mg/ml, dissolved in 100% acetonitrile), 107 µl wettable powder premix (2.5 mg/ml dissolved in 1 mM phosphate buffer with a pH of 7, obtained from Bayer AG), 40 µl 1% Genapol LRO (Clariant) and 227.3 µl of 1 mM phosphate buffer (pH 7). A volume of 10 µl was applied in 20 droplets on each plant on

a leaf surface area of about 3.3 cm^2, which corresponded to an application rate of 50 g/ha at 300 L/ha.

2.5.2 Radioactivity uptake and distribution

Radioactivity uptake and translocation of ^{14}C-labelled safeners was determined by combusting dried plant parts in a biological oxidizer (OX 500, Zinsser Analytic, Frankfurt, Germany). The plant parts were dried overnight at 60°C in a drying cabinet prior to combustion. The amount of radioactivity present in each part was determined by measuring the ^{14}C carbon dioxide that was trapped in 15 ml of oxysolve C 400 scintillant (Zinsser Analytic, Frankfurt, Germany) following combustion and quantification using a scintillation counter (Perkin Elmer Tri-Carb 2910 TR).

Distribution profiles of radioactivity in plants were obtained by autoradiography; dried plants were placed on a phosphoimager (FUJI Imager X BAS 1000) for 24 hours and relative radioactivity intensities were visually recorded. In each case, the plants were dried between sheets of paper under the pressure of a heavy metal plate prior to phosphoimaging.

2.5.3 Radio-HPLC

2.5.3.1 Extraction

The extraction protocol was optimised for 2 g of plant tissue per sample, which corresponded to 10 wheat, 5 maize and 4 soybean plants per sample. For plant extraction, 20ml of 80% acetonitrile was added to each sample, along with 6 ceramic beads (¼ Ceramic Sphere Cat.No.6540-422,5mm, MP Biomedicals). After securing the lids with parafilm, the samples were disrupted in a high-speed benchtop homogeniser (FastPrep 24, MP Biomedicals, Speed: 6, Time: 2 x 30 seconds, Setting: BG tubes) and put on a roller shaker (Stuart, SRT9D, Speed: 60) for 20 minutes. They were then centrifuged for 20 minutes at 3500 rpm (Heraeus Multifuge 1L-R) and the supernatant collected in separate falcon tubes. The pellet formed after centrifugation was resuspended in 5 ml of 80% acetonitrile and homogenised one more

time (same settings) before a final centrifugation (10 minutes, 3500 rpm). The supernatants were then pooled for further analysis.

2.5.3.2 Concentration of samples

Following extraction, the samples were distributed evenly in two glass test tubes (12.5 ml + 12.5 ml) and put in a nitrogen evaporator (Caliper TurboVap LV) until dried completely (around 2.5 hours). Four glass beads (2mm) along with 1 ml of 50% acetonitrile were added to the dried samples and after being vortexed well they were pooled together again in 2ml eppendorf tubes. These crude extracts were filtered by loading 300 µl of each sample into a filtered microcentrifuge tube and centrifuging for 5 minutes at max speed on a bench-top microcentrifuge (Heraeus Christ Biofuge A). The eluates (~250 µl) were transferred into HPLC vials and 2.5 µl (0.2 % v/v) of formic acid added to each sample for better peak separation, before being loaded on the instrument. Formic acid was added only to the samples containing ^{14}C-TCM.

2.5.3.3 HPLC Protocol

The samples were run on an Agilent 1100 series DAD HPLC instrument coupled with a radio-detector (Ramona Quattro from Raytest) and separated using a Phenomenex column (Specified below) at a temperature of 30°C and a flow rate of 0.5 ml / min. The mobile phases consisted of 0.2 % (v/v) Formic Acid (A) and acetonitrile + 0.2 % (v/v) Formic Acid (B). The eluting conditions for TCM and CSA were as listed below:

<u>TCM metabolites</u>

Column: Phenomenex 250 x 4.6 mm, Luna 3µ C18)

Injection volume: 100 µl

0-10 min	10 % buffer B, isocratic
10-30 min	10 %- 40 % buffer B, gradient

30-35 min	40 %-90 % buffer B, gradient
35-40 min	90 % buffer B, isocratic
40-45 min	90 % -10 % buffer B, gradient
45-50 min	10 % buffer B, isocratic

CSA metabolites

Column: Phenomenex 150 x 3mm, Luna 3µ C18

Injection volume: 50 µl

0- 5 min	20% buffer B, isocratic
5- 40 min	20%-60% buffer B, gradient
40- 45 min	60%-90% buffer B, gradient
45- 50 min	90% buffer B, isocratic
50- 55 min	90%-20% buffer B, gradient
55- 60 min	20% buffer B, isocratic

Non-labelled TCM and demethylated TCM were run along ^{14}C-TCM as reference standards and detected using a built-in diode array detector (UV spectrum between 205 and 270 nm). In each case, peaks were assigned with HPLC software GINA Star (Raytest, Version 5.8). Statistical analysis was carried out by performing multiple t-tests using GraphPad (Version 8).

2.5.4 Radioactivity recovery

Radioactivity measurements of samples, solutions and apparatus were taken at different stages of the experiment to ensure a satisfactory amount of radioactivity was recovered for a reliable HPLC or radioactivity uptake/distribution analysis (indicative measurements included in Appendix). For excised shoot experiments, the incubation solutions were collected in separate falcon tubes and the beakers that originally contained the solutions washed with 80% acetonitrile. In each case, a volume of 100 µl of the beaker wash, the remaining incubation

solution, the water wash after the 3h incubation, the 25ml crude extracts and the 2ml filtered extracts was dissolved in 5ml of LSC universal cocktail (TROTISZINT® ECO PLUS) and measured for trace radioactivity in a scintillation counter (Perkin Elmer Tri-Carb 2910 TR). For most samples, the percentage of radioactivity recovered was over 70%, making the HPLC results an accurate representation of the metabolic profiles of the agrochemicals. In cases where recovery was low, the pellets remaining from the extraction were combusted in a biological oxidizer (OX 500, Zinsser Analytic, Frankfurt, Germany) and the insoluble radioactivity added to the overall recovery.

For microdroplet experiments with ^{14}C-labelled compounds, application leaves were detached from the plants and washed in 15ml falcon tubes containing 80% acetonitrile (each leaf in a separate tube) following another wash in 50 % acetonitrile to remove any residual radioactive compound prior to analysis. The separate tubes were labelled accordingly so radioactivity recovery could be calculated for every single plant.

2.5.5 LC/MS

2.5.5.1 Extraction

For the metcamifen metabolism study (Section 4.7), the extraction protocol used is outlined in section 2.5.3.1. It was followed without the concentration of the samples (section 2.5.3.2).

For the comparison of TCM and CSA metabolism (Section 5.6) and the translocation study of CSA and metcamifen (Section 5.7), plants parts were ground in liquid N_2 and 80% methanol added in a 5:1 ratio (v/w) to plant tissue in 15 ml falcon tubes. Samples were rotated for 20 minutes in a cold room and then centrifuged for 5 minutes at 5000 rcf (Thermo Scientific Heraeus Megafuge 16R). The supernatant was dispensed to Eppendorf tubes and centrifuged for 3 minutes at maximum speed on a bench-top centrifuge (Eppendorf 5415 D) before loading on the LC/MS instrument.

2.5.5.2 LC/MS Run conditions

LC/MS analysis of metcamifen and metabolites (section 4.7) was performed at Bayer AG (Frankfurt), on a quadrupole time of flight mass spectrometer (XEVO TQ-S, Waters) coupled with an Acquity UPLC (I-Class BSM) system with a PDA Detector (UPLC LG 500 nm). Samples were injected at a volume of 0.1 µL and separated using an ACQUITY UPLC® BEH C18 1.7µm column at a column temperature of 40°C and flow rate of 0.4 ml/min. Data was acquired over a mass range of 100-2000 m/z with a scan time of 0.1 sec on positive mode (ES+); the capillary voltage was 3 kV. Cone voltage was set to 33V with 150 L/h of cone gas flow and desolvation gas flow was 1000 L/h with a desolvation temperature of 500 °C. The source temperature was maintained at 120°C and the quadrupole LM resolution was 2.7 while HM was 15. Mobile phase A consisted of water and LC/MS grade acetonitrile, each containing 0.05% formic acid. The gradient was as follows:

Time(min)	Flow Rate	%A	%B	Curve
0.00	0.400	70.0	30.0	0
0.30	0.400	70.0	30.0	6
1.30	0.400	10.0	90.0	6
1.60	0.400	10.0	90.0	6
1.70	0.400	70.0	30.0	6
2.00	0.400	70.0	30.0	6

Quantification of reference compounds was performed by the use of standard curves calculated by 3x serial dilutions prepared from the acetonitrile stock solutions diluted in 80% acetonitrile. Compounds were diluted in plant extracts from untreated plants to correct for any ion-suppression from co-extraction. Identification and quantification of metabolites was performed on Masslynx (v4.1).

LC/MS analysis of the rates of TCM and CSA metabolism (section 5.4) as well as for the translocation study of CSA and metcamifen (section 5.6) was performed at Newcastle University, on a quadrupole time of flight mass spectrometer (XEVO-G2 XS QTOF, Waters) coupled with an Acquity UPLC (I-Class BSM) system with a PDA Detector (UPLC LG 500 nm). Samples were injected at a volume of 0.1 µL and separated using an ACQUITY UPLC® BEH C18 1.7µm column at a column temperature of 40°C and flow rate of 0.4 ml/min. Data

was acquired over a mass range of 50-1200 m/z, with a scan time of 0.1 sec on positive mode (ES+); the capillary voltage was 2 kV. Cone voltage was set to 30V, cone gas flow to 0 and desolvation gas flow was 800 L/h with a desolvation temperature of 600 °C. The source temperature was maintained at 120°C and the quadrupole LM resolution was 4.7 while HM was 15. Mobile phase A consisted of water and LCMS grade acetonitrile, each containing 0.05% formic acid. The gradient used was the same with the Frankfurt protocol. Standard curves were generated after running the five 2x dilutions of the safeners. The concentration range used spanned 500-31.25 nM prepared from 13mM stock solutions diluted in 80% methanol. Identification and quantification of the compounds was performed on Masslynx (v4.1).

2.6 General molecular biology procedures

2.6.1 pH measurements, centrifugations, sterilisations, gel electrophoresis

All solutions and media had their pH determined with a calibrated HANNA edge® instruments as per manufacturer's instructions. Calibrations were performed using buffer solutions from Fischer Scientific (Catalogue #: J/2855/15, J/2825/15, J/2885/15). Centrifugations were performed with an Eppendorf 5415 D bench-top centrifuge for up to 2ml centrifuge tubes and a Thermo Scientific Heraeus Megafuge 16R for qPCR plates and 15 and 50 ml falcon tubes. Tubes, tips, media and solutions were sterilised with a Dixons Vario 1528 bench-top autoclave for 15 minutes at 120°C. Electrophoresis of nucleic acids was performed on 1% agarose gels using a BioRad WIDE MINI-SUB Cell GT gel tank filled with 1xTAE buffer connected to a BioRad Power PAC 300. Agarose was melted in 1xTAE buffer adding GelRed at a final concentration of 0.01% and electrophoretic separation performed at 120V. A 1kb+ DNA ladder was used along with an appropriate volume of 6x Loading dye per sample, both bought from Fischer Scientific.

2.6.2 RNA isolation from plant tissues and calli material

Plants were grown at Newcastle University, as described in section 2.2.2. Plant tissue/calli material was ground in liquid N_2 and 30 mg of tissue used for RNA extraction with the NucleoSpin® RNA 96 RNA kit (Macherey Nagel, April 2014, Rev.05, REF 740709.24), according to the manufacturer's instructions. The samples were processed using the centrifuge protocol. Before proceeding to the binding step, lysates were filtered through a NucleoSpin® RNA Filter Plate for more efficient removal of cell debris.

Quantification and quality control of RNA were performed on an Agilent Tapestation 4200 as per manufacturer's instructions (measurements have been included in the Appendix).

2.6.3 Reverse Transcriptase-mediated cDNA synthesis

cDNA synthesis was performed with the LunaScript™ RT SuperMix Kit as per manufacturer's instructions. For each qPCR experiment, cDNA from a pooled RNA sample was diluted 10 times to a concentration of 5 ng/µl and were subsequently serially diluted 3 more times by a factor of 5 (up to 0.04 ng/µl final concentration), for primer efficiency determination. The rest of the samples taken for qPCR were only diluted ten times.

2.6.4 Primer Sequences used for qPCR

All primer sequences were designed using Primer3 and checked for non-specific binding on PrimerBlast; the primer size, Tm GC% and product size was calculated by the Primer3 algorithm and design settings are provided below:

Primer Size: 18-23 (opt. 20)

Primer Tm: 57-62 °C (opt. 60 °C)

Primer GC%: 45-60 (opt. 50)

Product size Ranges: 80-160

Table of thermodynamics parameters: SantaLucia 1998

Thermodynamic Secondary Structure Alignments and Old Secondary Structure Alignments were set to 0.

Sequences were ordered from Eurofins Genomics and the stocks prepared at a concentration of 0.1 pmol/µl. For qPCR reactions, stocks were diluted 10 times before use.

Gene	Accession number	Additional information	Primer Name	Primer sequence
Zea mays polyubiquitin (MubC5)	NM_001329666.1	Reference	Ubiq3 Forward	TATCTGCGTCGTCTGGTGC
			Ubiq3 Reverse	CGAAGCCACAACCACGATTC
Zea mays actin 2	NM_001154731.2	Reference	Actin1 Forward	CTGAGGTTCTATTCCAGCCATCC
			Actin1 Reverse	CCACCACTGAGGACAACATTACC
Zea mays In2-1	X58573.1	Marker	ZmGSTL1 Forward	GCGGCTCTGGATAAACTCGA
			ZmGSTL1 Reverse	TAACGTACGCTACATCCGCC
Zea mays isoflavone 2'-hydroxylase-like	NM_001319705.1	Marker	NSF1 1 Forward	GGCGACGAGAGCGAAAGTAA
			NSF1 2 Reverse	ATAGGTTCGCGCAAAGAGCA
Triticum aestivum Actin	GQ339780.1	Reference	Actin 2 Forward	TGGACGCACAACAGGTATCG
			Actin 2 Reverse	AGACGAAGGATGGCATGTGG
Triticum aestivum In2-1	Y17386.1	Marker	TaGSTL1 1 Forward	TACTCGCCCTTGATC
			TaGSTL1 1 Reverse	ACGCAATGTCCACCAAGCTA

Table 7: Primer sequences used for qPCR reactions in maize and wheat. The accession numbers were recorded on 07/02/2019.

2.6.5 Quantitative real-Time PCR

Reactions were run on a Roche 96 Lightcycler and were prepared using a Luna™ Universal qPCR Master Mix as per manufacturer's instructions. A volume of 3.5 µl of cDNA was used for the reactions (5 ng/µl concentration) which corresponded to 17 ng of cDNA per reaction. All samples were run in three technical replicates and internal calibrators included for all the primers on every plate. The internal calibrator was pooled from all the samples used in each specific experiment and was always run at the same place on the plate (bottom right).

Cycling conditions were identical for all target sequences and were the following: 95 °C for 90 seconds for the preincubation (1 cycle) followed by a three-step amplification step at 95 °C for 15 seconds, 60 °C for 15 seconds and 72 °C for another 15 seconds (45 cycles) and a final melting curve step at 95°C for 10 seconds and 65 to 95 °C for 1 minute (1 cycle).

In order to express a biomarker's relative change in RNA transcript of a treated sample against an untreated one, the –ΔΔCt method was used (Livak et al. 2001). Since this method assumes uniform primer efficiency for all the primers used, a primer efficiency of 95-105% was required. Three biological replicates were used for each treatment and each of the treated samples was compared individually to all of the control samples; this comparison yielded nine values which were averaged to produce the final expression fold. Standard deviations were calculated in each case. Statistical analysis denoting significance of transcript expression was carried out by performing multiple t-tests using GraphPad (Version 8).

Chapter 3 – Cyprosulfamide's specific activity in maize: Greenhouse trials and herbicide metabolism assays

3.1 Introduction

Sulfonamides are a class of drugs that provide a rich source of bioactive chemistry, with a surprising range of activity. They were first used as antibacterials with the introduction of Prontosil into clinical practice in 1939 (Otten, 1986). They have since been developed into several types of pharmacological agents including ones with antiviral and antitumour activity (Scozzafava et al., 2003). Due to their inhibitory and stimulatory effects observed in bacteria, human phagocytes, yeast and plants (Henry, 1943), these compounds have been developed in fungicides (Zeng et al., 2016), herbicides (Xie et al., 2014) and herbicide safeners. The first sulfonamide safener compounds were patented as early as 1972 (Arneklev and Baker, 1972), shortly after the commercialisation of napthalic anhydride (NA) in 1970. Subsequent patents showed that these compounds had promising protective (Pallos, 1979) or inhibitory effects (Levitt, 1977) in a variety of crops in greenhouse trials, with structural changes dictating the level and range of activity in different crops. In 1991, Hershey and Stoner showed that structural changes in various substituted benzenesulfonamides accounted for differential induction of the genes In2.1 and In2.2 in maize, with safener 2-CBSU able to highly induce both genes, whereas its inactive analog 2-CBSA induced none.

Sulfonamides were not commercialised as safeners until 2008, with the introduction of cyprosulfamide (CSA), an aryl sulfonamide with specific activity in maize against ALS and 4-HPPD inhibiting herbicides when applied pre- and post-emergence(Kraehmer et al., 2014a). CSA was co-developed with the ALS-inhibiting herbicide thiencarbazone-methyl (TCM) to control perennial grasses and problem weeds (Santel, 2012). Due to its remarkable specificity and effectiveness, CSA has been used as a lead compound in studies that have used structure-based bioisosterism design to produce new safeners and fungicides (Zheng et al., 2015, Zhao et al., 2018). Zheng et al. (2015), identified a number of analogs with safening and antifungal activity, with some reported to reduce herbicide phytotoxicity against TCM better than CSA, identifying the cyclopropyl group in its structure as an important pharmacophore for safener activity.

These studies have been a part of a larger attempt to elucidate the structure-activity relationship of herbicide safeners (Pallos et al., 1975, Lay and Casida, 1976, Stephenson and Chang, 1978, Kőmíves and Hatzios Kriton, 1991, Matola and Jablonkai, 2007, Skipsey et al., 2011, Fu et al., 2017, Brazier-Hicks et al., 2018b). To date there is no unifying structural theory for their activity (Jablonkai, 2013). According to the review, motifs important for safening seem to be different according to which class of herbicides they protect against and it has been postulated that for some safeners this might be due to the safener competing with the herbicide's site of action (Yenne and Hatzios, 1990, Bordas et al., 2000). To further elucidate how structural changes bring about differences in activity, future safener studies need to focus on how the structural analogs respond, including what phenotype they cause, what effect they have on the active herbicide, their fate in the plant and how they induce specific enzymes. Safeners exhibit specificity among monocots with some maize safeners not being effective in cereals and vice-versa (Kraehmer et al., 2014a).

Greenhouse trials with metcamifen, a CSA structural analog initially patented by Syngenta (Syngenta Crop Protection AG, development code CGA246783), showed that the compound was active in both cereals and maize (Bayer personal comm.). This was an interesting observation which raised the question of what is the selectivity component responsible for CSA's specificity in maize.

The first step to elucidate this species specificity, was to determine the activity spectrum of the two compounds in a variety of crops and observe how they behave when combined with different herbicides used at various concentrations. This would generate a starting point to study their selectivity and basis of their activity in monocots. After establishing the spectrum of their protective action, the next step would be to identify how the safeners protect the plants. As discussed in section 1.5.4.2, the most widely accepted view is that safeners work by significantly enhancing the metabolism of the applied herbicide in crops, but not in weeds (Hatzios, 1991, Davies and Caseley, 1999, Hatzios and Burgos, 2004, Riechers et al., 2010, Jablonkai, 2013, Kraehmer et al., 2014a). Enhanced metabolism has been demonstrated for seed treatment and pre-emergence safeners (Hatzios, 1988), for fenchlorazole-ethyl, cloquintocet-mexyl, mefenpyr-diethyl, isoxadifen-ethyl and cyprosulfamide (Hacker et al., 2000, Hacker et al., 2002, Kraehmer et al., 2014a, Leonie et al., 2017).

Based on the evidence present in the literature, in the current study it was decided to explore whether the crop-herbicide-safener combinations that showed a protective effect in the greenhouse were associated with enhanced herbicide metabolism through a series of assays. This would connect the safening effect with the enhanced metabolic rate of the herbicide,

setting a solid foundation for elucidating the differential effects produced by two structurally similar safener compounds in monocots. As safeners can depend on unique interactions of specific crop-herbicide-safener combinations (Hatzios, 1988), the main aim was to identify combinations showing consistent safening effects with CSA and metcamifen to draw parallels on how these compounds protect from herbicide injury, while also exploring the activity spectrum of metcamifen and whether CSA's activity could be diversified by altering herbicide concentrations.

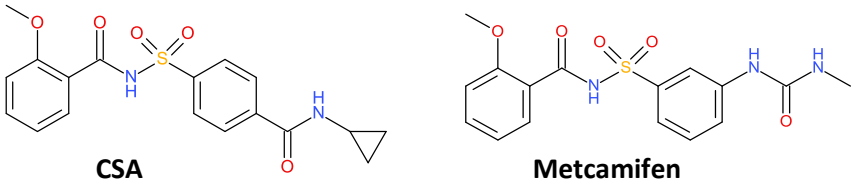


Figure 3: Chemical structures of safeners CSA and metcamifen.

RESULTS

3.2 Characterisation of cyprosulfamide and metcamifen activity in the greenhouse

The trials were designed to highlight the activity spectrum of the safeners in various crops and associated varieties, and identify suitable herbicide and safener concentrations, as well as the ratios of application rate, to accurately compare the specific effect of CSA with the broader effect of metcamifen. The safeners were co-applied with two different herbicide chemistries, each in a separate trial: thiencarbazone-methyl (TCM), an ALS inhibitor, and tembotrione (TBT), a 4-hydroxyhenylpyruvate dioxygenase (HPPD) inhibitor. TCM is a broad-spectrum non-selective herbicide which makes it ideal for the study the effect of safeners and TBT is used in commercial mixtures with TCM to complement its activity. Both ALS and 4-HHPD inhibitors are good representatives of modern agrochemicals (Kraehmer et al., 2014a).

The crop spectrum and herbicide concentrations used in the trials can be found in Table 8. Two concentrations were used per crop (high and low) based on the herbicide field application rates and adjusted accordingly based on crop tolerance or sensitivity to the compounds (personal communication from Bayer informed by previous trials with the chemicals). The majority of the crops used were monocots with the exception of soybean, which was included as a negative control, since safeners are known to be ineffective in dicots (DeRidder et al., 2002, DeRidder and Goldsbrough, 2006). Multiple commercial varieties were used per crop for a more accurate representation of the safeners effect in the field;

Wheat included a spring (Triso), a winter (Dekan) and a durum variety (Joyau) while rice included an indica (IR64) and a japonica variety (Balila).

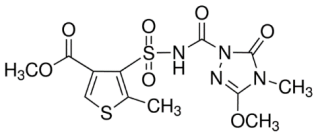
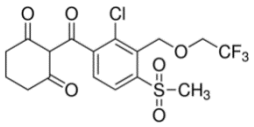
Crop (Varieties)	Herbicide			
	Thiencarbazone-methyl (TCM) Inhibition of acetolactate synthase (ALS)		Tembotrione (TBT) Inhibition of 4-hydroxphenylpyruvate dioxygenase (4-HPPD)	
				
	High dose (g/ha)	Low dose (g/ha)	High dose (g/ha)	Low dose (g/ha)
Maize - *Zea Mays* (Oldham, Sileno, Aventura)	50	5	600	400
Wheat - *Triticum Aestivum* (Triso, Joyau, Dekan)	50	5	400	200
Barley- *Hordeum Vulgare* (Montoya, Scarlett, Zzoom)	50	5	400	200
Soybean- *Glycine max* (Sultana, Merlin)	20	5	20	5
Rice - *Oryza Sativa* (Balilla, IR64)	20	5	400	200

Table 8: Crop species and herbicide concentrations used in initial greenhouse trials. Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). Herbicide injury was assessed by visual scoring, 1 and 2 weeks after treatment, within percentage injury categories (e.g. 50-55 % injury).

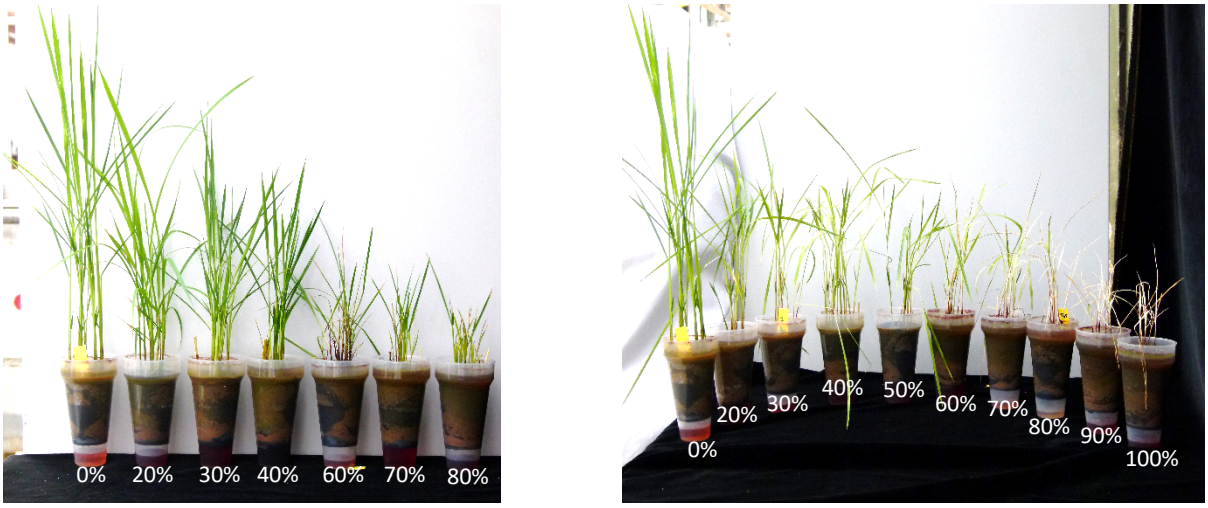


Figure 4: Herbicide injury in rice plants ranging from 0-100%. Injury was induced post-emergence using the ALS inhibitor TCM (left) and the 4-HPPD inhibitor TBT (right).

Herbicides and safeners were both applied post-emergence using a track sprayer with a split application and herbicide injury of treated plants recorded by visually scoring them against untreated control plants one and two weeks after application, as described in section 2.4.3. Figure 4 shows examples of damage scoring with rice plants, where stunting and yellowing of the leaves can be observed on the left and a combination of stunting and bleaching on the right. Every herbicide/safener/crop/variety combination had three pots as replicates, each containing 3-10 individual plants depending on seed size, with large grained cereals containing fewer plants per pot. Herbicide injury values (scored within percentage injury categories – e.g. 50-55% injury) with and without safener treatment are reported in Figure 5, with strong alleviation of injury highlighted in green (colour scheme of herbicide injury in section 2.4.3). Figure 5, 6,***Figure* 7** have also been re-drawn as bar charts and have been included in the Appendix (Figures A4-A6).

Maize displayed the greatest safening effects in the trial with TCM (Figure 5), where both safeners are known to be active. CSA and metcamifen alleviated injury in all maize varieties when TCM was applied at a high dose, but only in one variety at a low dose, with injury being minor at that application rate. Other crops displayed protective effects only with metcamifen, most consistently in wheat (Figure 5). Metcamifen protected all wheat varieties when TCM was applied at a low dose, but not at the high application rate (might have been too damaging for the crop). Effects in other monocot crops were variety dependent, with one variety in barley and one in rice safened at both doses of the herbicide, though the effects at the higher dose were stronger. Soybean on the other hand demonstrated a slight safening

effect with one variety with metcamifen and selected for investigation in further trials. CSA's safening activity was exclusive to maize, with no activity observed in other crops (Figure 5).

		Wheat			Maize			Barley		
Herbicide Dose	**Safener Dose**	*Triso*	*Joyau*	*Dekan*	*Oldham*	*Sileno*	*Aventura*	*Montoya*	*Scarlett*	*Zzoom*
TCM 50 g/ha	-	58 ± 4	60 ± 0	60 ± 0	57 ± 5	43 ± 9	57 ± 5	60 ± 0	72 ± 4	60 ± 10
TCM 50 g/ha	CSA 100 g/ha	70 ± 0	60 ± 0	60 ± 0	18 ± 9	20 ± 14	2 ± 4	60 ± 6	67 ± 11	58 ± 4
TCM 50 g/ha	Metc. 100 g/ha	62 ± 4	60 ± 0	60 ± 0	25 ± 14	10 ± 8	20 ± 6	50 ± 0	47 ± 7	55 ± 5
TCM 5 g/ha	-	48 ± 4	42 ± 4	20 ± 0	32 ± 7	2 ± 4	12 ± 9	53 ± 5	57 ± 11	57 ± 5
TCM 5 g/ha	CSA 100 g/ha	52 ± 4	45 ± 5	20 ± 0	18 ± 7	0 ± 0	3 ± 5	50 ± 0	50 ± 0	53 ± 5
TCM 5 g/ha	Metc. 100 g/ha	20 ± 0	28 ± 4	0 ± 0	3 ± 5	3 ± 5	3 ± 5	43 ± 5	38 ± 9	52 ± 4

		Soybean		Rice	
Herbicide Dose	**Safener Dose**	*Sultana*	*Merlin*	*Balilla*	*IR64*
TCM 20 g/ha	-	77 ± 5	73 ± 9	68 ± 9	65 ± 5
TCM 20 g/ha	CSA 100 g/ha	70 ± 0	77 ± 5	63 ± 7	60 ± 8
TCM 20 g/ha	Metc.100 g/ha	72 ± 4	80 ± 6	62 ± 7	47 ± 7
TCM 5 g/ha	-	58 ± 4	70 ± 0	48 ± 7	47 ± 14
TCM 5 g/ha	CSA 100 g/ha	58 ± 4	63 ± 5	48 ± 3	48 ± 4
TCM 5 g/ha	Metc.100 g/ha	50 ± 8	57 ± 5	45 ± 8	20 ± 8

Characterisation	**Colour**
No effect	
Slight safening effect	
Moderate safening effect	
Good safening effect	
Slight additional injury	

Figure 5: Effects of CSA and metcamifen on TCM injury in various crops. Greenhouse trial with TCM on five crops. Green highlights indicate observed safening effects where intensity is shown according to the colour scheme on the right. The numbers represent herbicide injury in %, scored within percentage injury categories (5-10%), accompanied by the standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). A bar chart version of this table can be found in the Appendix (Figure A4).

The trial with TBT produced less consistent results as most crops displayed were either unaffected by the herbicide at lower doses, or too sensitive at the higher rate, which made visualising any safening effects challenging (Figure 6). Most effects were again observed in maize, where both safeners reduced TBT injury, though the effects were not as strong as in the previous trial and not exhibited by all varieties. Wheat and rice displayed safening effects with metcamifen with one variety and barley showed more consistent safening in two varieties with metcamifen, while only one was marginally protected by CSA. None of the safeners had any effects in soybean.

Out of the two trials, the one with TCM (Figure 5) produced the most informative and consistent results. There was one follow-up trial with TBT (Figure 6) using higher or lower concentrations accordingly (Figure A3-Supplementary) in which safening effects were observed in some of the same combinations as the first TCM trial. Since the TBT trials did not provide extra information and displayed less consistent results compared to the TCM studies, it was decided to use TCM in all the following experiments.

		Wheat			Maize			Barley		
Herbicide Dose	**Safener Dose**	*Triso*	*Joyau*	*Dekan*	*Oldham*	*Sileno*	*Aventura*	*Montoya*	*Scarlett*	*Zzoom*
TBT 600 g/ha	-	-	-	-	33 ± 12	13 ± 12	17 ± 9	-	-	-
TBT 600 g/ha	CSA 100 g/ha	-	-	-	8 ± 9	7 ± 9	5 ± 5	-	-	-
TBT 600 g/ha	Metc. 100 g/ha	-	-	-	10 ± 8	3 ± 5	0 ± 0	-	-	-
TBT 400 g/ha	-	63 ± 5	73 ± 5	50 ± 6	28 ± 15	2 ± 4	15 ± 5	20 ± 10	43 ± 12	47 ± 5
TBT 400 g/ha	CSA 100 g/ha	68 ± 4	67 ± 5	50 ± 6	7 ± 5	0 ± 0	2 ± 4	30 ± 16	48 ± 7	47 ± 9
TBT 400 g/ha	Metc. 100 g/ha	60 ± 8	67 ± 5	53 ± 7	7 ± 5	2 ± 4	3 ± 5	22 ± 13	40 ± 6	**23 ± 5**
TBT 200 g/ha	-	57 ± 5	63 ± 5	40 ± 8	-	-	-	15 ± 16	30 ± 12	35 ± 13
TBT 200 g/ha	CSA 100 g/ha	58 ± 4	65 ± 5	48 ± 7	-	-	-	12 ± 4	18 ± 9	30 ± 10
TBT 200 g/ha	Metc. 100 g/ha	50 ± 6	62 ± 4	**15 ± 5**	-	-	-	10 ± 10	8 ± 7	7 ± 7

		Soybean		Rice	
Herbicide Dose	**Safener Dose**	*Sultana*	*Merlin*	*Balilla*	*IR64*
TBT 20 g/ha	-	62 ± 4	60 ± 6	-	-
TBT 20 g/ha	CSA 100 g/ha	60 ± 6	65 ± 5	-	-
TBT 20 g/ha	Metc. 100 g/ha	62 ± 7	67 ± 5	-	-
TBT 5 g/ha	-	52 ± 4	52 ± 4	-	-
TBT 5 g/ha	CSA 100 g/ha	58 ± 7	60 ± 10	-	-
TBT 5 g/ha	Metc. 100 g/ha	57 ± 5	63 ± 5	-	-
TBT 400 g/ha	-	-	-	65 ± 10	85 ± 9
TBT 400 g/ha	CSA 100 g/ha	-	-	57 ± 9	87 ± 7
TBT 400 g/ha	Metc. 100 g/ha	-	-	47 ± 5	85 ± 10
TBT 200 g/ha	-	-	-	35 ± 10	73 ± 5
TBT 200 g/ha	CSA 100 g/ha	-	-	27 ± 5	77 ± 11
TBT 200 g/ha	Metc. 100 g/ha	-	-	28 ± 11	77 ± 11

Characterisation	Colour
No effect	
Slight safening effect	
Moderate safening effect	
Good safening effect	

Figure 6: Effects of CSA and metcamifen on TBT injury in various crops. Greenhouse trial with TBT on five crops. Green highlights indicate observed safening effects where intensity is shown according to the colour scheme on the right. The numbers represent herbicide injury in %, scored within percentage injury categories (5-10%), accompanied by the standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). A bar chart version of this table can be found in the Appendix (Figure A5).

The combinations that produced a safening effect in maize and wheat were repeated in a separate spraying trial, the results of which are shown in Figure 7. In order to better showcase the safening effect, higher (and lower in wheat) herbicide concentrations were included to ensure satisfactory herbicide injury. The TCM concentrations used for wheat were lower than those used in maize, with the lower rate ranging between 2.5 to 10g/ha and the higher rate being 50g/ha and 100g/ha. Safeners were used at a concentration of 100g/ha in all trials for all combinations, unless stated otherwise.

As in the first TCM trial (Figure 5), all maize varieties were safened by both compounds with all wheat varieties safened by metcamifen (Figure 7). However, effects were mostly prominent at the highest herbicide concentration with both crops. This could potentially be due to this trial taking place during hotter weather conditions, which would have induced plant growth. Varieties Triso (wheat) and Aventura (maize) showed the most consistent safening effects in all the trials so they were selected for subsequent experiments along with Merlin (soybean).

Wheat

Herbicide Dose	Safener Dose	*Triso*	*Joyau*	*Dekan*
TCM 2.5 g/ha	-	47 ± 5	28 ± 7	18 ± 9
TCM 2.5 g/ha	CSA 100 g/ha	38 ± 9	35 ± 8	28 ± 7
TCM 2.5 g/ha	Metc. 100 g/ha	17 ± 5	22 ± 4	13 ± 5
TCM 5 g/ha	-	40 ± 0	38 ± 4	37 ± 5
TCM 5 g/ha	CSA 100 g/ha	33 ± 15	48 ± 4	28 ± 7
TCM 5 g/ha	Metc. 100 g/ha	28 ± 7	33 ± 5	25 ± 5
TCM 10 g/ha	-	58 ± 4	50 ± 0	47 ± 5
TCM 10 g/ha	CSA 100 g/ha	52 ± 4	47 ± 5	57 ± 5
TCM 10 g/ha	Metc. 100 g/ha	42 ± 4	32 ± 4	27 ± 5

Maize

Herbicide Dose	Safener Dose	*Oldham*	*Sileno*	*Aventura*
TCM 50 g/ha	-	20 ± 8	30 ± 8	40 ± 14
TCM 50 g/ha	CSA 100 g/ha	3 ± 5	7 ± 9	0 ± 0
TCM 50 g/ha	Metc. 100 g/ha	10 ± 0	3 ± 5	23 ± 5
TCM 100 g/ha	-	67 ± 5	60 ± 0	62 ± 7
TCM 100 g/ha	CSA 100 g/ha	45 ± 5	40 ± 0	30 ± 10
TCM 100 g/ha	Metc. 100 g/ha	40 ± 0	35 ± 5	48 ± 7

Characterisation	Colour
No effect	
Slight safening effect	
Moderate safening effect	
Good safening effect	

Figure 7: Effects of CSA and metcamifen on TCM injury in maize and wheat-Validation trial. Validation greenhouse trial in maize and wheat with herbicide TCM. Green highlights indicate observed safening effects where intensity is shown according to the colour scheme on the right. The numbers represent herbicide injury in %, scored within percentage injury categories (5-10%), accompanied by the standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). A bar chart version of this table can be found in the Appendix (Figure A6).

The take-home message from the two trials (Figure 5,***Figure* 7**) is summarised in Figure 8. The aim was to establish an activity profile for the two safeners and identify combinations which could help elucidate the difference in their activity. Maize showed a clear safening effect when either safener were applied, whereas with wheat safening was only observed with metcamifen. Safener application in soybean on the other hand did not alleviate herbicide damage with either compound. Having identified crops where both, only one, and no safeners were active, subsequent experiments focused on identifying how the safeners were exerting their phytoprotective effects.

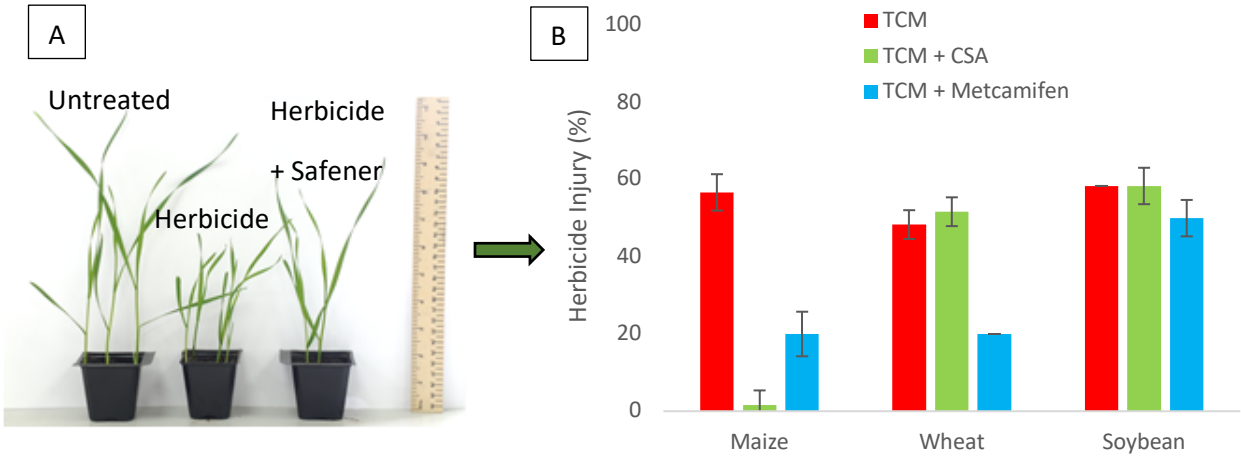


Figure 8: Effect of CSA and metcamifen treatment on TCM injury in maize, wheat and soybean. **(A)** Visual representation of a safening effect in wheat plants. **(B)** Herbicide injury induced by TCM to maize, wheat and soybean after CSA and Metcamifen is applied. The varieties used were Aventura (maize), Triso (wheat) and Merlin (soybean), at a growth stage of BBCH=11-12. Injury values in maize were induced by a concentration of TCM at 50 g/ha and those in wheat and soybean by 5 g/ha. CSA and Metcamifen were applied at 100g/ha for all crops. Error bars represent standard deviations (n=3).

3.3 Herbicide and safener dose responses in wheat and soybean

Soybean generally displayed no effects with safener application in trials with both herbicides (Figure 5,Figure ***6***), apart from a weak effect observed with metcamifen in the trial with TCM (Figure 5). Safeners have been reported to be ineffective in dicots (DeRidder et al., 2002, DeRidder and Goldsbrough, 2006) so it was hypothesised that this effect could be the result of combining a low herbicide application rate with a high safener dose. Following on from that, a trial was designed to investigate whether increasing safener while decreasing herbicide application could induce a safening effect in soybean, which would otherwise not be

observed. Wheat was also included in this trial, to explore any potential effects with increased CSA application or even greater protection using metcamifen. Safeners were applied at concentrations ranging from 100 to 1000 g/ha combined with TCM applied at 5-50g/ha, exploring herbicide:safener ratios of application from 1/2 to 1/200 (Figure 9). The varieties used for wheat were Triso, Joyau, Dekan and for soybean Merlin. Figure 9 has also been re-drawn as a bar charts and has been included in the Appendix (Figure A7).

Wheat, as previously discussed, was safened using several herbicide:safener applications ratios, but there was a lack of consistency with results from previous trials (Figure 5,Figure **7**). Triso, which in previous trials was the most responsive variety, only exhibited weak safening at the highest herbicide and safener concentration (Figure 9A). Joyau on the other hand, exhibited the most consistent safening effects with metcamifen and in some cases with CSA, whereas in previous trials negligible effects were observed. The variety Dekan was safened by metcamifen and by CSA in one combination, but effects were more sporadic than in variety Joyau. Despite these interesting observations as to CSA activity in wheat, there was no clear evidence that the observed safening effects were

A

			Wheat		
Herbicide Dose	**Safener Dose**	**Ratio**	*Triso*	*Joyau*	*Dekan*
TCM 5 g/ha	-	-	20 ± 0	0 ± 0	0 ± 0
TCM 5 g/ha	CSA 100 g/ha	1:20	23 ± 5	23 ± 12	0 ± 0
TCM 5 g/ha	Metcamifen 100 g/ha	1:20	13 ± 5	7 ± 9	0 ± 0
TCM 25 g/ha	-	-	40 ± 0	40 ± 0	37 ± 5
TCM 25 g/ha	CSA 100 g/ha	1:4	40 ± 0	30 ± 0	23 ± 9
TCM 25 g/ha	Metcamifen 100 g/ha	1:4	40 ± 0	33 ± 5	10 ± 8
TCM 50 g/ha	-	-	50 ± 0	50 ± 0	43 ± 5
TCM 50 g/ha	CSA 100 g/ha	1:2	50 ± 0	40 ± 8	60 ± 8
TCM 50 g/ha	Metcamifen 100 g/ha	1:2	43 ± 9	**27 ± 5**	47 ± 5
TCM 25 g/ha	-	-	40 ± 0	40 ± 0	37 ± 5
TCM 25 g/ha	CSA 500 g/ha	1:20	40 ± 0	**23 ± 5**	37 ± 9
TCM 25 g/ha	Metcamifen 500 g/ha	1:20	33 ± 5	23 ± 12	10 ± 14
TCM 50 g/ha	-	-	50 ± 0	50 ± 0	43 ± 5
TCM 50 g/ha	CSA 500 g/ha	1:10	50 ± 0	**37 ± 5**	37 ± 5
TCM 50 g/ha	Metcamifen 500 g/ha	1:10	40 ± 0	**27 ± 5**	37 ± 5
TCM 50 g/ha	-	-	50 ± 0	50 ± 0	43 ± 5
TCM 50 g/ha	CSA 1000 g/ha	1:20	50 ± 0	50 ± 8	47 ± 5
TCM 50 g/ha	Metcamifen 1000 g/ha	1:20	37 ± 5	23 ± 12	**20 ± 0**

B

Herbicide Dose	Safener Dose	Ratio	Soybean *Merlin*
TCM 2.5 g/ha	-	-	0 ± 0
TCM 2.5 g/ha	CSA 100 g/ha	1/40	7 ± 5
TCM 2.5 g/ha	Metcamifen 100 g/ha	1/40	**17 ± 9**
TCM 5 g/ha	-	-	33 ± 5
TCM 5 g/ha	CSA 100 g/ha	1/20	37 ± 5
TCM 5 g/ha	Metcamifen 100 g/ha	1/20	40 ± 8
TCM 10g/ha	-	-	43 ± 5
TCM 10g/ha	CSA 100 g/ha	1/10	40 ± 0
TCM 10g/ha	Metcamifen 100 g/ha	1/10	47 ± 5
TCM 5 g/ha	-	-	33 ± 5
TCM 5 g/ha	CSA 500 g/ha	1/100	37 ± 5
TCM 5 g/ha	Metcamifen 500 g/ha	1/100	30 ± 0
TCM 5 g/ha	-	-	33 ± 5
TCM 5 g/ha	CSA 1000 g/ha	1/200	**57 ± 5**
TCM 5 g/ha	Metcamifen 1000 g/ha	1/200	43 ± 17
TCM 20 g/ha	-	-	57 ± 5
TCM 20 g/ha	CSA 400 g/ha	1/20	63 ± 5
TCM 20 g/ha	Metcamifen 400 g/ha	1/20	65 ± 5
TCM 50 g/ha	-	-	67 ± 5
TCM 50 g/ha	CSA 1000 g/ha	1/20	70 ± 8
TCM 50 g/ha	Metcamifen 1000 g/ha	1/20	67 ± 5

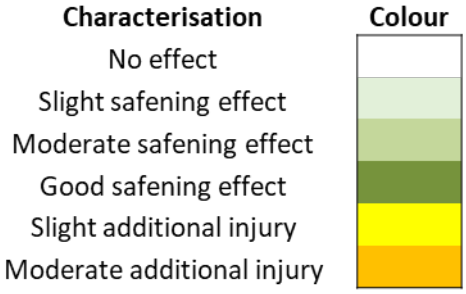


Figure 9: Effect of CSA and metcamifen on TCM injury in wheat (A) and soybean (B) – Dose response and alternating herbicide-safener ratios. Greenhouse trial with various concentrations of TCM and safeners in wheat and soybean. Green highlights indicate observed safening effects where intensity is shown according to the colour scheme on the right, whereas orange and yellow indicate additional herbicide injury upon safener application. The numbers represent herbicide injury in %, scored within percentage injury categories (5-10%), accompanied by the standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). A bar chart version of this table can be found in the Appendix (Figure A7).

stronger when safener application rates were increased (Figure 9A). This was mostly due to the fact that effects were not consistent among varieties. Soybean displayed no safening effects in any of the combinations (Figure 9B), which was consistent with most results from the initial trial (Figure 5), and actually exhibited increased injury at the higher safener concentrations.

The effect by CSA in the wheat variery Joyau was observed at an application rate of 500g/ha, at a TCM application rate of 25 g/ha and 50 g/ha (Figure 9A). This seemed like another case of safening observed through a combination of increased safener, with decreased herbicide application, as in both cases the effect was very mild and in one case the damage induced by

herbicide alone was relatively low (40%). This could potentially mean that CSA was able to induce slight, non-functional safening in that specific wheat variety, which points to the direction of CSA being able to induce the pathway or detoxification machinery involved in safening wheat, just not as strongly or consistently as metcamifen. Another explanation, however, could be that the results observed were affected by hotter weather conditions inducing plant growth, which would explain why responsive varieties in previous trials showed no effects, and vice-versa. In any case, the effect observed with CSA in Joyau was not strong enough and could not be replicated, so it was not followed further.

3.4 Induction of metabolism of [^{14}C]-TCM upon cyprosulfamide or metcamifen application

Having established an activity profile of the safeners in the greenhouse, it was then of interest to identify whether the safeners enhanced the metabolic rate of the applied herbicide which, if confirmed, would link the safening effect to herbicide detoxification. The aim of this experiment was to determine the changes in herbicide metabolism over a time course to identify when detoxification occured and whether it was influenced by safener application.

TCM was selected for this study as it showed the most consistent effects in greenhouse trials. Its rate of detoxification was measured by the quantity of the *N*-demethylated metabolite as the compound loses its herbicidal activity upon *N*-demethylation (Bayer personal comm.).

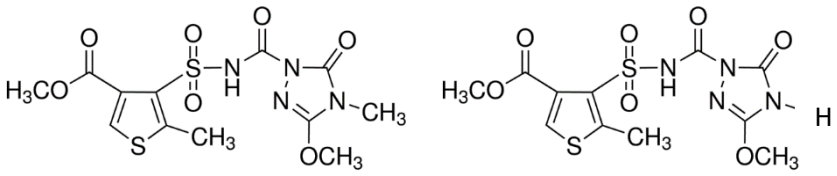


Figure 10: Chemical structures of TCM and *N*-Demethylated TCM. The parent loses its activity upon demethylation.

The metabolism of [^{14}C]-TCM was analysed by radio-HPLC following CSA or metcamifen application at 3, 6, 9 and 24 hours for maize and wheat and at 24 and 48 hours for soybean. Samples consisted of one replicate, each consisting of a number of pooled plants (Section 2.5.3.1). The chemicals were administered through excised shoots (Figure 2) as this ensured

reliable uptake, without needing to formulate compounds to bypass the leaf barrier. The incubation with safener and [^{14}C]-TCM lasted for 3 hours and the plants were transferred to fresh water until harvested.

TCM metabolites were extracted in 80% acetonitrile and run on an HPLC instrument (protocol in Chapter 2) acquiring a chromatogram per timepoint for each crop, in each case the detected radioactivity corresponding to the parent TCM and its metabolites.

The chromatograms showing the metabolism of [^{14}C]-TCM at 24 hours for all the crops under the different treatments were compared in order to check for alterations in the relative metabolic profile of the herbicide (Figure 11). Safener application did not alter the route of metabolism of TCM, producing metabolite peaks of the same identity in all the crops and between all treatments. In maize and wheat, TCM underwent *N*-demethylation , which has been previously reported as the main pathway for TCM metabolism (PMRA, 2010). However, this was not the case in soybean which indicated that the crop could be missing a necessary enzyme, potentially a cytochrome P450, responsible for metabolising the compound.

In order to express the rest of the data in an optimal manner and avoid listing all the

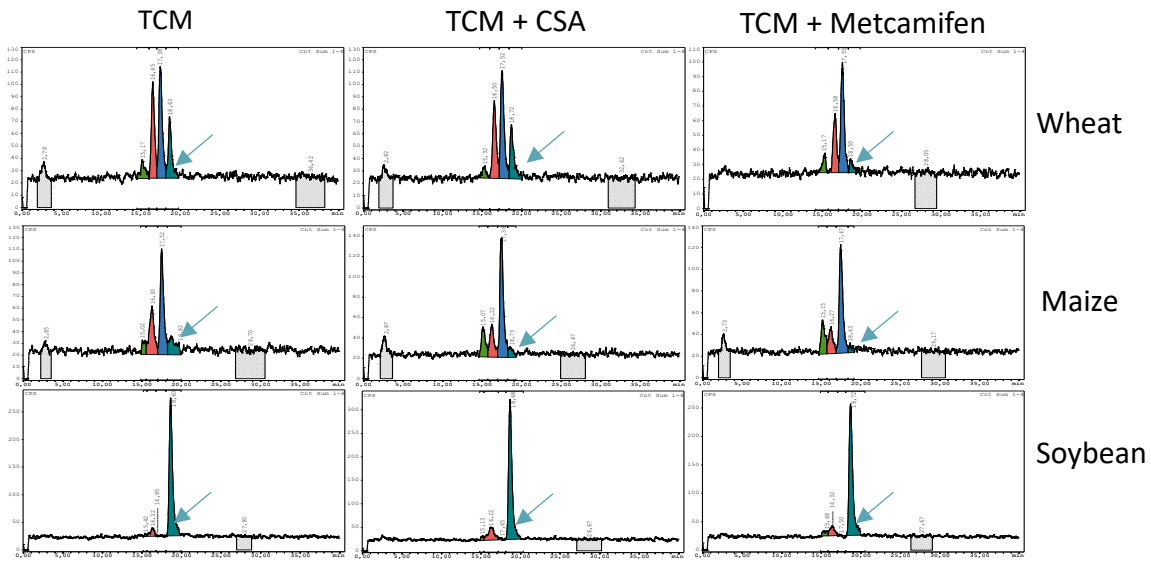


Figure 11: Metabolic profile of [^{14}C]-TCM upon safener application in maize, wheat and soybean shoots - Radio-HPLC. TCM and safeners were administered through excised shoots when plants were 7 days old (BBCH 11-12). The arrow indicates the authentic TCM compound (Ret. Time: 18.7 min, petrol peak) and the peaks on its left represent its metabolites. The blue peak next to TCM (Ret. Time: 17.5 min), represents the *N*-demethylated metabolite which is absent from soybean and the red and light green ones (Ret. Times:16.3 and 15.4 mins, respectively) unidentified TCM metabolites.

chromatograms, two graphs were produced for each crop, one showing the levels of the herbicide parent and the other the levels of metabolites (Figure 12). It is noteworthy that in

maize and wheat, changes in the overall levels of TCM metabolites were principally due to demethylated TCM, as the levels of the other metabolites changed very little over the course of 24 hours.

In wheat, the effects of safener application on TCM metabolism started becoming visible after 9 hours (Figure 12). Metcamifen enhanced TCM metabolism by around 20%, with 40% parent remaining after 9 hours and only 7% by 24 hours, in contrast to 60% and 22% when no safener was applied. TCM metabolites followed a similar pattern, going up to 93% upon metcamifen application and 77% without. CSA application did not affect the metabolism of the herbicide, as parent and metabolite levels did not change as compared to the control (no safener) application.

In line with TCM's selective use in maize (Santel, 2012), it was metabolised more rapidly than in wheat, with 63% parent remaining after three hours without any safener application (Figure 12). Both CSA and metcamifen enhanced TCM metabolism, an effect which was most prominent after 6 hours where parent levels were reduced by 15% and 20% respectively. By 24 hours, they had dropped down to 4% compared to 17% in the control. From a first glance, it seems that herbicide detoxification happened very rapidly even without safener application, questioning the relevance of herbicide detoxification in safener activity. It's worth noting here, that this was an accelerated depiction of herbicide metabolism as compounds were administered by phloem/xylem transport without having to cross the leaf barrier. According to Rosinger (2014), safeners act by keeping herbicidal compounds under a certain threshold to prevent injury to young leaves, as compounds are applied at an early stage of development (BBCH Scale:12). The detoxification graphs pictured here over a few hours (Figure 12), represent what happens over the course of four to seven days in the field, during which protection of young leaves is critical. The results are therefore consistent with the hypothesis that safener application seemed to adequately keep TCM levels under the necessary threshold to prevent herbicide injury.

TCM metabolism in soybean was observed over a longer time period, as the crop had displayed no safening effects in the greenhouse with either safener (Figure 12). After 24 hours the levels of the parent were close to 90%, with or without safener treatment. After 48 hours the levels had dropped by 1% in all treatments and the metabolite levels went up by the same amount.

As discussed above, this was due to the fact that soybean, even though it was able to produce other TCM metabolites at a low quantity, was unable to demethylate the compound which has been reported to be the first step of its detoxification pathway in maize and wheat (PMRA, 2010).

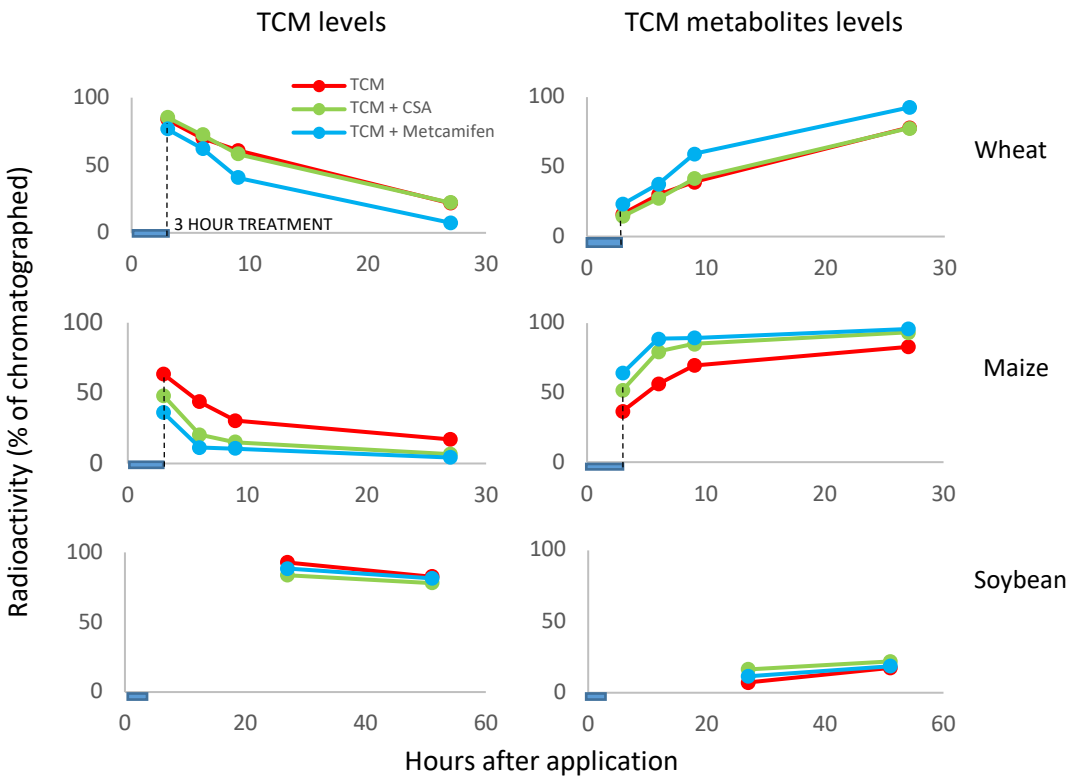


Figure 12: [^{14}C]-TCM metabolism over the course of 24/48 hours upon safener application in maize, wheat and soybean shoots - Radio-HPLC. TCM levels with and without safener application (left). Rate of metabolite appearance with and without safener application (right). TCM and safeners were administered through excised shoots when plants were 7 days old (BBCH 11-12). Chemical treatment lasted for 3 hours before plants were transferred to water before harvest at specified timepoints. The amount of radioactivity detected in the chromatograms of each timepoint was plotted in a line graph for TCM and TCM metabolites.

After determining the detoxification rate of the herbicide in different crops, the study was replicated at one timepoint to strengthen the reliability of the results. The enhancement of TCM metabolism was evident after 9 hours in both maize and wheat so all combinations (crop/treatment) were run in triplicate at that time. The number of pooled plants per sample, type of treatment and extraction protocol were the same as in the previous trial. Soybean was included as a negative control where TCM metabolism was investigated after 48 hours. The metabolite peaks detected on the HPLC chromatograms were visualised in a bar chart with error bars indicating standard deviations among replicates (Figure 13).

The results followed closely what was observed in the previous trial (Figure 12), with metcamifen being the only active safener in wheat in contrast to maize, where both safeners were active (Figure 13). The detoxification rate of TCM was very similar with the one observed in the previous trial (Figure 12) at the equivalent timepoints for all the crops, with soybean displaying no change in TCM metabolism upon application of either safener (Figure 13).

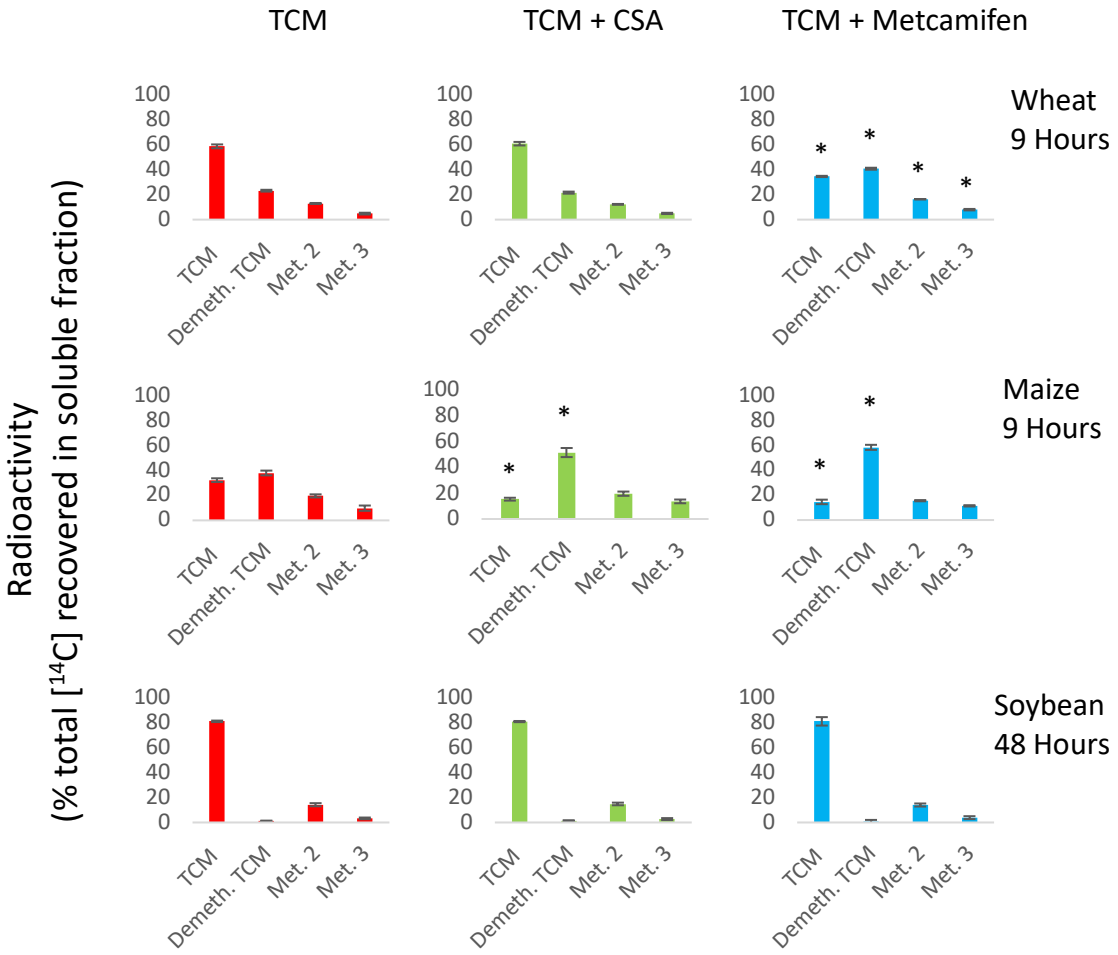


Figure 13: TCM metabolism at one timepoint in maize, wheat and soybean shoots - Replication. Replication of TCM metabolism trial at 6 hours with three replicates per combination. The bars represent the average values of the replicates and error bars show standard deviation. Significant change in metabolite levels upon safener application have been marked with a star (*). TCM and safeners were administered through excised shoots when plants were 7 days old (BBCH 11-12). Chemical treatment lasted for 3 hours before plants were transferred to water before harvest at specified timepoints. The amount of radioactivity detected in the chromatograms of each replicate was plotted in a line graph for TCM and TCM metabolites.

3.5 Discussion

To recap, the aim of this chapter was to establish an activity profile for CSA and metcamifen as well as identify a possible mechanism for how their protective effect was manifested.

The safener activity of CSA was observed only in maize in greenhouse trials, in contrast to metcamifen, which was also active in wheat. Both safeners were inactive in soybean which was consistent with previously reported data on safeners being unable to enhance herbicide tolerance in dicots (DeRidder et al., 2002, DeRidder and Goldsbrough, 2006).

As mentioned in section 3.1, differences in species specificity of the two safeners would be highlighted by crop-herbicide-safener combinations displaying consistent effects with both, one and none of the safeners. Maize, wheat and soybean were very good representative crops of these categories and along with their respective varieties, Aventura, Triso and Merlin, produced the most consistent safening effects in the greenhouse when used with TCM and the safeners. All further experiments focused on those varieties and the herbicide TCM, as they proved to be useful tools in comparing the selectivity of CSA and metcamifen. Another interesting point generated by these trials was the differential safening response between varieties of rice and barley (Balilla-IR64, Scarlett-Zzoom in Figure 5,Figure ***6***) which could be elucidated by genetic approaches, but this was not followed up.

TCM metabolism studies confirmed that the safeners exerted their protective effect by enhancing herbicide metabolism as this was only observed when a protective safening effect was determined in the greenhouse. This was in agreement with the well-established view that safeners mainly act by enhancing herbicide metabolism in monocots (Hatzios, 1991, Davies and Caseley, 1999, Hatzios and Burgos, 2004, Riechers et al., 2010, Jablonkai, 2013, Kraehmer et al., 2014a). The safener-enhancement of TCM metabolism was most marked at early timepoints, which suggested that safening takes place early after application. Subsequent studies focused on events prior to 3 hours after herbicide and safener application in order to identify any species specific patterns in the behaviour of CSA.

Maize and wheat showed some endogenous TCM metabolism which was enhanced by both safeners in maize, but only by metcamifen in wheat. In both species, *N*-demethylation was the primary route of detoxification, as previously reported in toxicological studies (PMRA, 2010). In contrast, soybean was unable to demethylate TCM which could mean that this herbicide is metabolized by a cytochrome P450 (CYP) which is absent in soybean. This could explain

soybean's lack of safening with TCM to an extent, so it was of interest to further explore the role of CYPs in mediating the safener response.

Collectively taken, the results show that CSA's activity was specific to maize, in contrast to metcamifen which was also active in wheat. There was a clear correlation between the safener's action and the enhanced metabolism of the herbicide which confirmed the initial hypothesis that the safener is aiding herbicide detoxification by the crop. This was highlighted by the lack of safener activity and herbicide metabolism in soybean. Since detoxification was observed early, the next step was to study the fate of cyprosulfamide in different crops following uptake, to investigate whether any differences in spatial availability of the compound could account for differential activity.

Chapter 4 – Dissection of cyprosulfamide's specificity: Analysis of cyprosulfamide's uptake, distribution and metabolism

4.1 Introduction

The fate of safeners in crops, has received less attention in the peer-reviewed literature than studies aiming to elucidate their mode of action (Davies and Caseley, 1999, Riechers et al., 2010). While uptake, distribution and metabolism of agrochemicals such as herbicides is usually reported in regulatory dossiers when registering their active ingredients, this is not always the case with safeners as they are classified as "inert" agents. This has resulted in few studies reporting safener fate in plants (Rosinger, 2014, Sivey et al., 2015).

It has been suggested that a combined analysis of transcriptomics, metabolomics and proteomics along with studies of safener fate, can provide important insights into their mode of action (Riechers et al., 2010). Therefore, understanding safener fate is not only valuable from a regulatory perspective, but it can also help predict herbicide - safener interactions when combined with herbicide detoxification studies (Davies and Caseley, 1999, Sivey et al., 2015). There are a number of studies reporting safener action being tissue-specific and developmentally regulated, but without reporting any information on safener distribution and translocation (Kreuz et al., 1989, De Veylder et al., 1997, Riechers et al., 2003, DeRidder and Goldsbrough, 2006). Such studies would give a clearer picture on whether safener bioavailability can affect these observations.

Two extensive studies on the uptake, distribution and metabolism of safeners, focused on compounds oxabetrinil and fluxofenim (CGA133205) in grain sorghum (Yenne et al., 1990) and MG-191 in maize (Jablonkai and Dutka, 1995). Yenne et al. (1990) reported that oxabetrinil and fluxofenim exhibited different uptake rates and minimal redistribution in sorghum. While their metabolism was time dependent, oxabetrinil was metabolised faster than fluxofenim. Although oxabetrinil metabolites were not identified, there was evidence of glutathione conjugation. Jablonkai and Dutka (1995) showed that while a small amount of MG-191 was absorbed by maize, its translocation and metabolism were rapid, with translocation being dose-dependent. To complement these observations, a previous study with MG-191 demonstrated that these low levels of absorption were enough to protect against the herbicides EPTC and acetochlor (Jablonkai, 1991). The author hypothesised that this

phenomenon could be explained by the formation of bioactive molecules during MG-191 metabolism.

It has been proposed that safeners utilise xenobiotic detoxifying enzymes to regulate their own metabolism in addition to the metabolism of other xenobiotics (Breaux et al., 1989, Yenne et al., 1990, Brazier-Hicks et al., 2008). However, evidence on how safener metabolism relates to mode of action remains inconclusive (Riechers et al., 2010). It has also been shown that while some safeners induce GST activity and were metabolised by conjugation to glutathione (Breaux et al., 1989, Brazier-Hicks et al., 2008), others could induce GSTs without being glutathionylated (Roberts, 1998, Riechers et al., 2003). Besides the studies of safener metabolism in crop plants, ecotoxicological studies exploring safener biotransformation on benoxacor and dichlormid, have also been reported (Bart Miaullis et al., 1978, Miller et al., 1996b, Miller et al., 1996a, Sivey et al., 2015, Bolyard et al., 2017).

As shown in the previous chapter, the safening effect of CSA and metcamifen observed in greenhouse trials was linked to safener-enhanced TCM metabolism by both safeners. The next step was to study its fate in maize and wheat to explore whether that could explain CSA's differential activity.

This chapter focused on exploring the fate of CSA in maize and wheat by assessing its uptake, distribution and metabolism, on the basis that one or more of these elements could account for differential activity in these two crop species. An increased uptake rate and mobility could mean that the safener is transported faster to tissues vulnerable to herbicides, such as the coleoptile (Riechers et al., 2010). Furthermore, enhanced metabolism of safeners could mean that metabolic bioactivation was required for safening activity (Rubin and Kirino, 1989). The uptake and disposition of radiolabelled CSA were quantified by an oxidiser analysis in separated plant tissues, distribution was examined in whole plants using autoradiography, and lastly safener metabolism was analysed with radio-HPLC in both separated plant tissues and excised shoots. All these studies were performed using [^{14}C]-CSA (obtained from Bayer AG, Frankfurt), which was labelled on the sulfonylbenzamide ring (Figure 14).

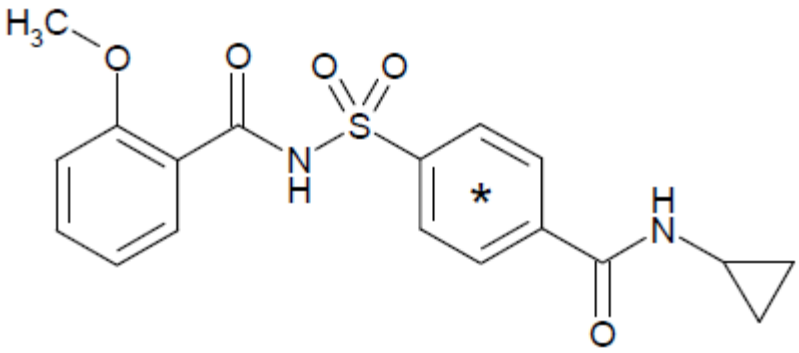


Figure 14: Structure of [^{14}C]-CSA used in uptake/translocation, distribution and metabolism studies. Its specific activity was 4.24 MBq/mg.

RESULTS

4.2 Uptake and translocation of [^{14}C]-CSA

The first experiment aimed to investigate the uptake and translocation of [^{14}C]-CSA in maize and wheat by combusting plant parts in a biological oxidiser after CSA application. The instrument captures the ^{14}C radionuclides in the carbon dioxide generated upon sample combustion, which allows for the quantification of radioactivity by liquid scintillation

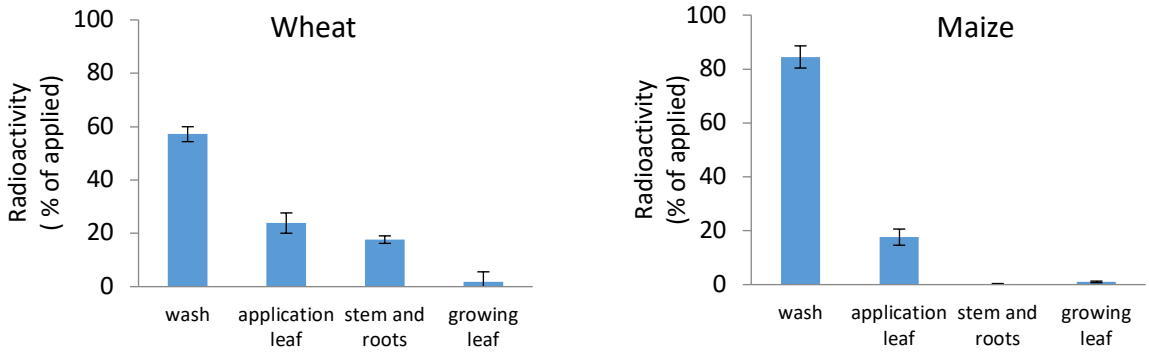


Figure 15: Oxidiser analysis of uptake and translocation of [^{14}C]-CSA in maize and wheat tissues at 24 hours. [^{14}C]-CSA was applied on the first leaf of the plants (BBCH 11-12, n=8 for each crop) by droplet application. The bars represent the average amount of radioactivity contained in each part of the crop relative to the amount applied; the error bars represent standard deviation between replicates. The 'wash' peak represents the unabsorbed amount of radioactivity washed from the surface of the leaf. The amount of absorbed radioactivity is represented by the sum of the rest of the peaks.

counting. Eight plants per crop were grown for 8 days and [^{14}C]-CSA applied on the first leaf in 20 droplets with a pipette, which was equivalent to a spraying application rate of 50 g/ha (See Section 2.4.1). This application aimed to simulate foliar uptake and translocation of CSA after a spray application (Figure 2). This foliar application was used instead of incubation of excised plant shoots, as it accounted for the variability of cuticle permeability, which needs to be taken into consideration when studying uptake and translocation of synthetic compounds in plants. The excised shoot technique is more useful when studying the effect of safeners on the metabolic rate of herbicides, as it allows the uptake of compounds without any barrier (i.e. cuticle). After 24h, each plant was separated into three parts: i) application leaf (local), ii) stem and roots and iii) growing leaf (systemic) and radioactivity measured for each of them as described in Section 2.5.2. The application leaves were washed with acetonitrile prior to measurement, and the leaf wash was also measured to determine the amount of radioactivity washed off (Section 2.5.4). The mean values obtained for each tissue and the leaf wash were determined with the equivalent standard deviations. This analysis showed how uptake and translocation of radioactivity ([^{14}C]-CSA and metabolites) compared in maize and wheat separated tissues (Figure 15). Wheat displayed higher uptake of [^{14}C]-CSA than maize, with 42.8% radioactivity being absorbed by the plants on average, with the rest found in the leaf wash of application leaves (57.2%). The application leaves contained 23.8% of [^{14}C]-CSA and/or its metabolites, while another 17.6% was translocated to the stem and roots of the plants. Growing leaves only contained very small amounts of radioactivity (1.7%).

Maize showed a different uptake and translocation pattern from wheat, with 84.5% of the applied radioactivity found in the leaf wash and the rest (17.6%), detected in the application leaves (Figure 15). The amount of radioactivity translocated to different plant tissues was negligible (~ 2%). Since the measurements were taken after 24 hours, these results suggested that wheat demonstrated better uptake and CSA distribution than maize. This observation did not correlate with CSA's activity, as that was specifically observed in maize. Interestingly, Jablonkai (1991) observed a similar trend with safener MG-191, in which the author reported limited amounts of absorption as enough to induce safening against EPTC and acetochlor in maize.

4.3 Distribution and metabolism of [^{14}C]-CSA in different plant tissues

As the uptake and translocation of CSA in maize and wheat did not correlate with its activity in the respective crop species, the distribution of [^{14}C]-CSA was determined by

autoradiography and complemented by metabolism studies in segregated tissues by radio-HPLC, in order to visualise whether the radioactivity corresponded to [^{14}C]-CSA or its metabolites. For the distribution profile of the safener, [^{14}C]-CSA was applied by microdroplet application as described in the uptake/translocation experiment (Section 4.2), on the first leaves of two maize, and two wheat plants. After 24 hours, the distribution of radioactivity was visually recorded on a phosphoimager (Figure 16), as described in Section 2.5.2.

The metabolism of [^{14}C]-CSA was measured by radio-HPLC at 24 hours in a different set of eight plants. Following microdroplet application of the safener, the application and growing leaves of eight maize and eight wheat plants were cut and pooled as separate samples, to maximize the amount of radiolabel available for chromatographic detection. The application leaves were washed as described in section 2.5.4. to remove residual [^{14}C]-CSA, and the absorbed [^{14}C]-CSA extracted from the pooled plant parts in 80% acetonitrile, as described in section 2.5.3.1-2. The chromatographs obtained were then compared with the autoradiograms (Figure 16).

In wheat, radioactivity translocated from the application leaf to the stem and roots, with only a small amount detected in the growing leaf (Figure 16). These results correlated with the previous oxidizer study (Figure 15). The complementing chromatograms showed that multiple [^{14}C]-CSA metabolites were present in the application leaves, with CSA being the most prominent compound (41.6% detected radioactivity), detected at a retention time of 23 minutes. The growing leaf showed a very small amount of the parent molecule which was also consistent with the previous observations (Figure 15).

Unlike the oxidizer study (Figure 15), radioactivity initially seemed to have translocated to the stem in maize (Figure 16). However, the second autoradiogram showed that [^{14}C]-CSA and its metabolites translocated to the leaf sheath, with little of the radioactivity being detected in other parts of the plant (Figure 16 - inset). The chromatogram of the growing (systemic) leaf contained negligible radioactivity, while the one from the application leaf detected multiple CSA metabolites. The dominant peak (38.5% detected radioactivity) was a CSA metabolite that was more polar than CSA at a retention time of 18.5 minutes. In addition, this metabolite was also detected in wheat but only in a small amount (10.2% detected radioactivity). Interestingly, it seemed that the quantities of the parent and this metabolite were flipped between the crops. While 9% of the parent and 38.5% of the metabolite were detected in maize, 41.6% of the parent and 10.2% of the metabolite were detected in wheat. Downstream metabolism also seemed to be slightly increased in wheat,

which could indicate a slightly different detoxification pathway than the one maize, possibly with increased glutathione or cysteine conjugation (Figure 16).

Even though most of CSA radioactivity was detected in the application leaf, a very small amount of [^{14}C]-CSA was detected in the stems of maize plants (Figure 16). The radioactivity was not detected in the roots according to chromatograms taken from these tissues (data in Appendix). The discrepancy of radioactivity detection between the oxidizer (Figure 15) and this study, could be due to the radio-HPLC approach being more sensitive than quantification by a scintillation counter.

Taken together, these results showed that although [^{14}C]-CSA was taken up faster and was more mobile in wheat than in maize, this phenomenon seemed unlikely to relate to the activity of CSA. On the contrary, the metabolism data showed that CSA was rapidly metabolized in maize while this was not happening in wheat. Enhanced metabolism of CSA which specifically lead to an increased accumulation of a CSA metabolite observed in maize, suggested that the selectivity of CSA might depend on a bioactivation event.

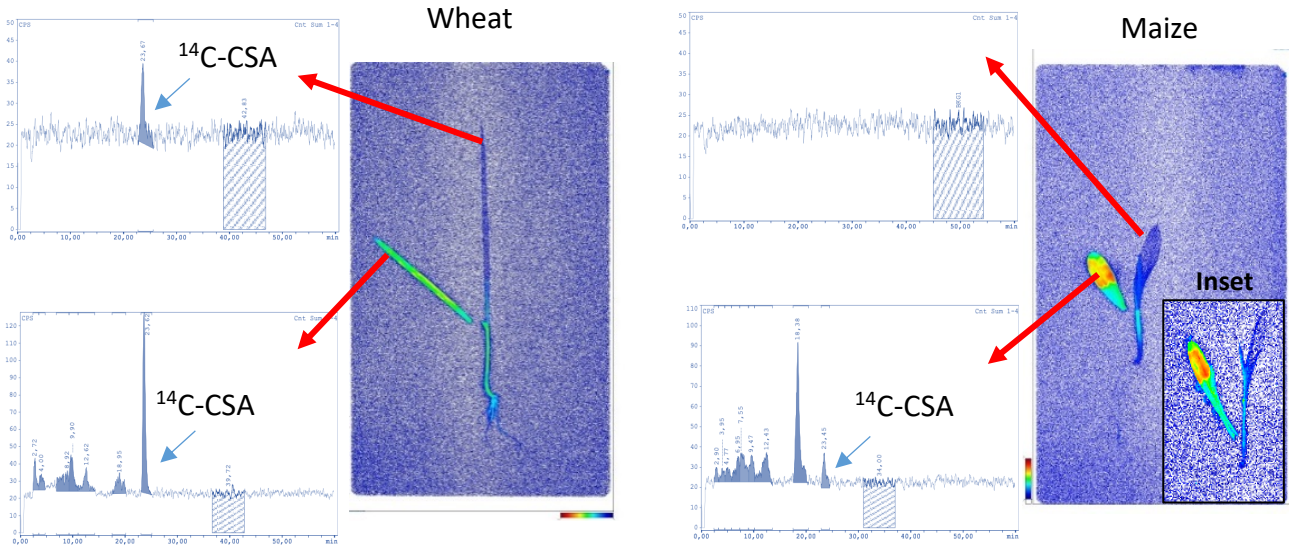


Figure 16: Autoradiograms displaying [^{14}C]-CSA distribution coupled with radio-HPLC chromatograms of [^{14}C]-CSA metabolism in maize and wheat at 24 hours. [^{14}C]-CSA was applied on the first leaf of the plants by droplet application, and the parent compound (CSA) is represented by an arrow in the chromatograms. The leaves were washed prior to imaging/extraction. The rainbow scale denotes the amount of radioactivity contained in the crop, with red being the highest intensity. The inset contains another autoradiogram of a second maize plant, showing that radioactivity translocated across the leaf, rather than to the stem of the plant.

4.4 Metabolism of [^{14}C]-CSA in excised shoots of maize, wheat and soybean

To obtain information on CSA metabolism in excised shoots, a radio-HPLC analysis of [^{14}C]-CSA metabolism was performed in maize, wheat and soybean at 24 hours. Soybean was included in this experiment to explore safener metabolism in a dicot crop that had not displayed any safening effects in the greenhouse or in herbicide metabolism assays. Three biological replicates were analysed for each crop, each consisting a number of pooled plants (Section 2.5.3.1). Since the aim was to study the metabolic rate of [^{14}C]-CSA, this experiment used a similar setup as the TCM metabolism experiments (Section 3.4). [^{14}C]-CSA was applied at a concentration of 5 PPM (13 μM) to excised plant shoots for 3 hours before the shoots were transferred to fresh water. [^{14}C]-CSA metabolites were extracted in 80% acetonitrile and analysed by HPLC (Section 2.5.3.3). A chromatogram per replicate for each crop was acquired to indicate how much of the detected radioactivity corresponded to the parent and metabolites. Since chromatograms among replicates were similar, a representative chromatogram was chosen for each crop to visualise the metabolic profile of [^{14}C]-CSA and standard deviations between replicates for detected compounds are included in a separate graph (Figure 17). The chromatograms of the rest of the replicates have been included in the Appendix.

Similar to the previous experiment, wheat showed little metabolism of CSA, with the dominant peak in the chromatogram being the parent compound (27.1 % of detected radioactivity, Figure 17). The level of parent compound in this experiment was lower than observed when CSA was applied by foliar application (Figure 16), presumably due to its optimized uptake. Furthermore, the dominant peak in maize excised shoots (61.6% of radioactivity detected, Figure 17) was the same metabolite detected in the foliar application experiment (Figure 16), while the parent was significantly lower (14.8 % of radioactivity detected, Figure 17). In addition, the abundance of this CSA metabolite in wheat (13.2 % of radioactivity detected, Figure 17), was significantly lower than in maize. Soybean, on the other hand, did not metabolize CSA, since 100% of the radioactivity detected in the excised shoots corresponded to the parent compound ([^{14}C]-CSA).

Taken together, these results showed that CSA activity positively correlated with enhanced metabolism of CSA and the presence of a specific CSA metabolite, which was most abundant in maize. The ratios between parent compound and the primary abundant metabolite in maize and wheat were similar in two metabolism experiments (Figure 16, Figure ***17***) despite

different application methods. These results indicated that while the route of CSA metabolism was the same in wheat and maize, it was quantitatively different (maize > wheat). The metabolism of CSA could lead to CSA being converted into an active compound, a phenomenon that has been reported before with safeners, which have been referred to as the activation of 'prosafeners' (Rubin and Kirino, 1989). Based on this information, CSA could behave as a pro-drug optimized for uptake, where the formation of an active metabolite was necessary for its phytoprotective action. The lack of safening in wheat and soybean could be caused by the low accumulation of that specific CSA metabolite. This hypothesis was supported by preliminary data (Bayer personal comm.) showing that among a variety of monocots, safening effects caused by CSA correlated with the presence of a specific CSA metabolite.

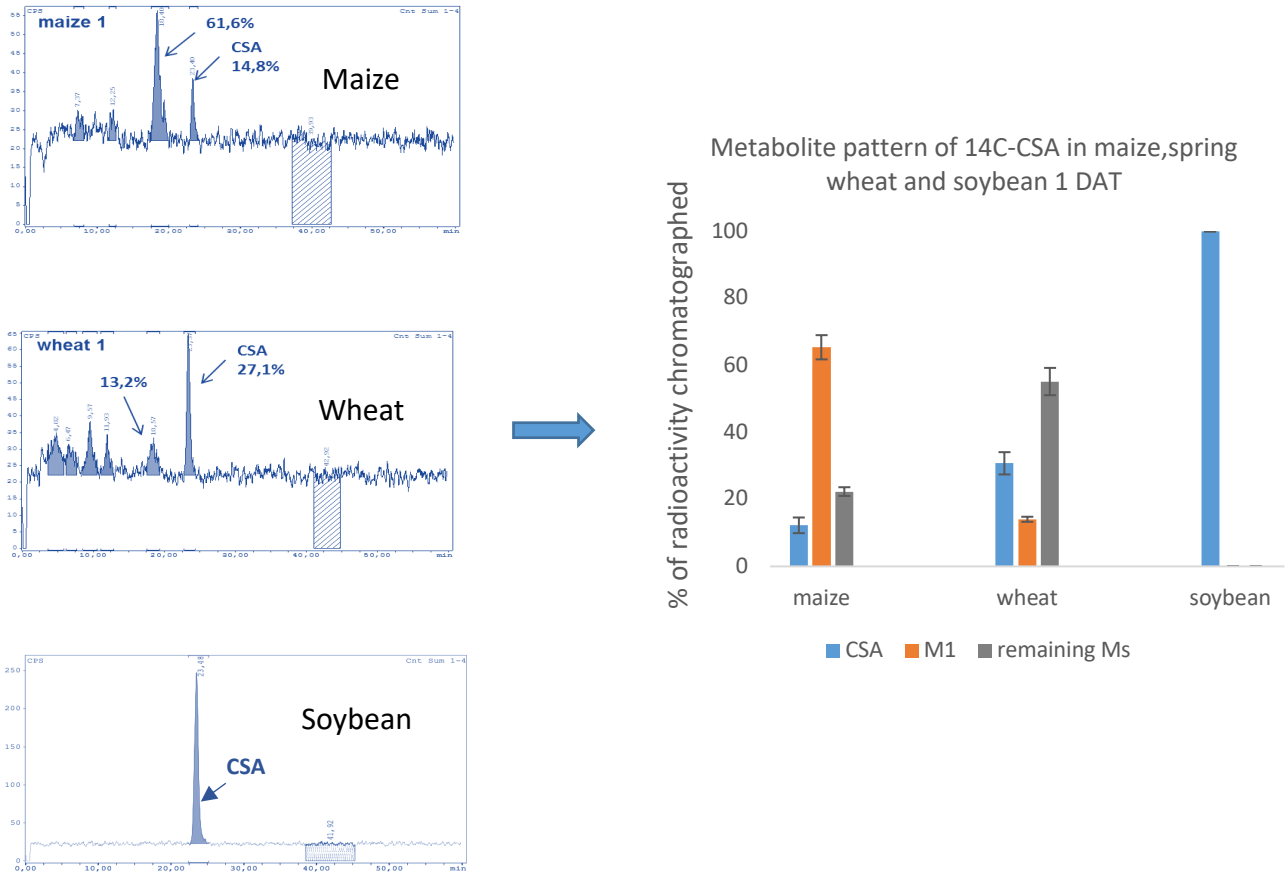


Figure 17: Metabolic profiles of [^{14}C]-CSA in maize, wheat and soybean shoots at 24 hours – Radio HPLC. [^{14}C]-CSA was administered through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted for 3 hours before plants were transferred to water before harvest at 24 hours. The percentage of parent (denoted as CSA by an arrow) and metabolites in the chromatograms (left) are represented in the bar chart on the right. Error bars show standard deviations between replicates (n=3). M1: Metabolite 1 (peak left of the parent)-also denoted by an arrow, remaining Ms: remaining metabolites. The shaded box at the bottom of every chromatogram represents the background of the matrix.

4.5 Identification of CSA metabolites

As a specific CSA metabolite seemed to have a central role in eliciting the safening effect caused by CSA, the identity of CSA metabolites was determined by LC/MS analysis on a triple quad mass spectrometer, via a radioactivity detector and an electrospray interface, done by Dr Peter Zoellner (Analytics group, Bayer AG, Frankfurt, Germany). Excised maize and wheat shoots were incubated with a mix of 10 PPM (13 µM) [^{14}C]-CSA and 40 PPM (52 µM) unlabelled CSA for 3 hours, before transfer to fresh water. Plants were then harvested at 24 and 48 hours after treatment. Plants treated with water were used as negative controls. CSA and its metabolites were extracted in 80% acetonitrile and their atomic composition was

determined by high resolution mass spectrometry (determination of the elemental composition of molecular ions and fragment ions) in the MS and MS/MS mode. For the fragmentation profile, the instrument was tuned according to the parent safener molecule's fragmentation. The metabolic profile of CSA at 24 hours was very similar between maize and wheat but the detected quantities of the identified compounds differed between the species for each metabolite (Figure 18). The CSA metabolites identified in both crops were hydroxylated, demethylated, a hydroxylated and demethylated intermediate, and glycosylated CSA, where hydroxylated CSA was the dominant metabolite in maize.

The elucidation of the position of the –OH group of hydroxylated CSA was performed by Dr Andreas Lagojda (Structure Elucidation group at Bayer AG, Monheim, Germany). After the retention time of the metabolite was confirmed on the radio-HPLC instrument, the metabolite was concentrated and purified. The accurate mass was determined by Electrospray Ionisation (ESI) MS and the position of the –OH group of the isolated metabolite determined by NMR analysis. For NMR measurements, compounds of interest were first subjected to liquid chromatography on an Agilent (Waldbronn, Germany) 1100 Series HPLC equipped with a Macherey-Nagel (Düren, Germany) Nucleodur C18 Gravity column. The flow rate was directed to an Agilent 1260 Fraction Collector (Waldbronn, Germany) and 6 s fractions assessed by LC/HRMS, on an Orbitrap Fusion Tribrid mass spectrometer, (Thermo, Bremen, Germany). The resulting two fractions of interest were dried in a vacuum concentrator, and the sample was dissolved and transferred into the NMR tube. To address the analytical questions, 1H, 1H,1H-COSY-Correlated Spectroscopy, 13C-1H-HSQC-Heteronuclear Single Quantum Correlation, experiments were conducted utilizing a BRUKER AVANCE 600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany).

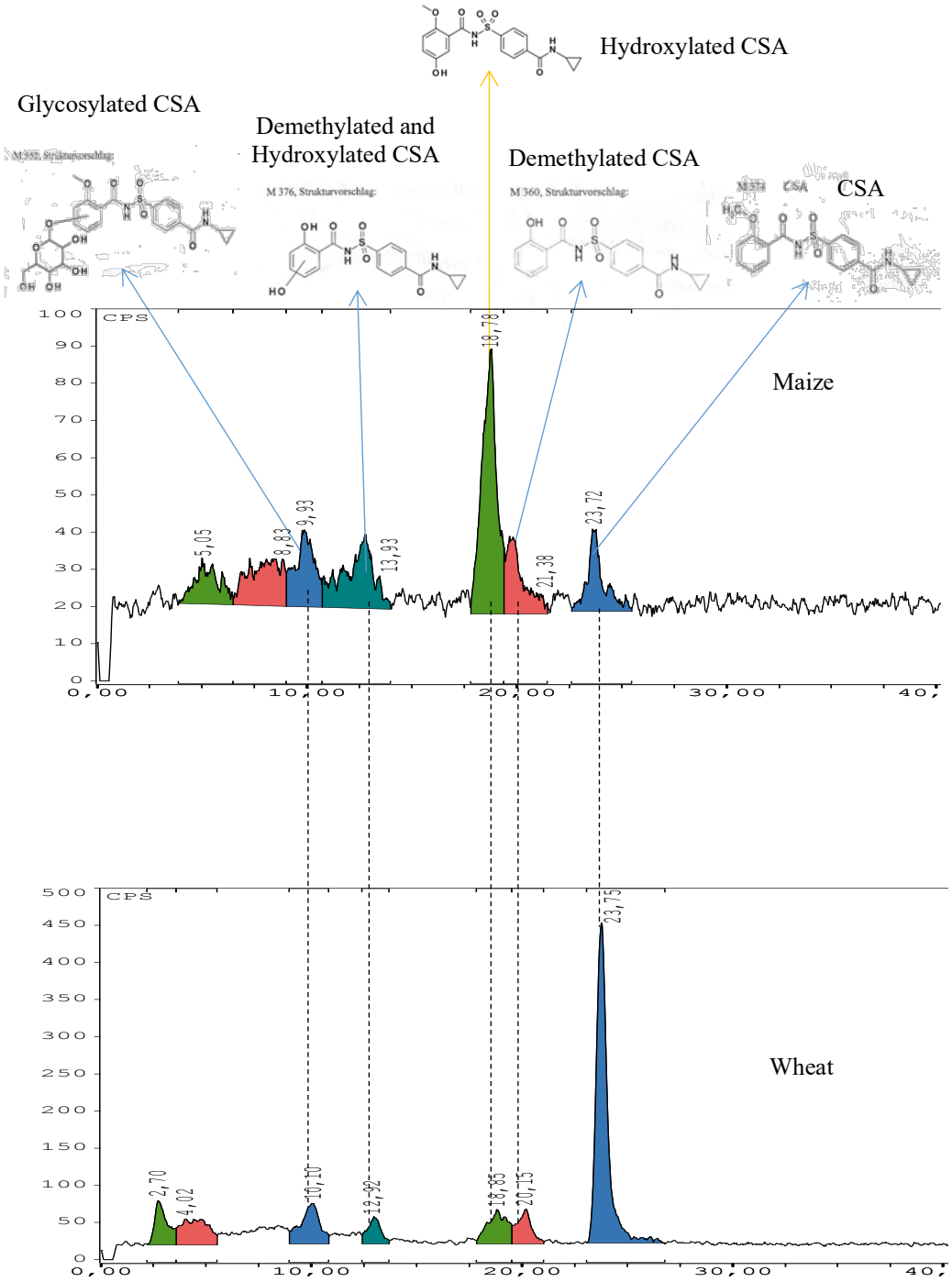


Figure 18: Annotated metabolic profile of [^{14}C]-CSA in maize and wheat shoots at 24 hours – Radio HPLC. [^{14}C]-CSA was administered through excised shoots when plants were 7 days old (BBCH 11-12). Chemical treatment lasted for 3 hours before plants were transferred to water before harvest at 24 hours. Chemical structure of the metabolites was determined by LC/HRMS.

4.6 Rate of [^{14}C]-CSA metabolism in maize

The enhanced metabolism of herbicide TCM by CSA treatment was apparent at 3 hours (Figure 12). To investigate the correlation between the formation of the hydroxylated CSA

metabolite on TCM metabolism, the level of hydroxylated CSA was determined prior to, or at 3h, following treatment. Excised maize shoots were treated with 5 PPM (13 µM) of [^{14}C]-CSA as described in section 4.4, and metabolism monitored by radio-HPLC at 15 minutes, 1, 2, 3, 6, 9 and 24 hours. Samples consisted of one biological replicate from a number of pooled maize plants (Section 2.5.3.1). The data was visualized as described in the first TCM metabolism experiment (Section 3.4); with one scatter graph produced visualizing the disappearance of [^{14}C]-CSA and the appearance of [^{14}C]-CSA metabolites over 24 hours (Figure 19).

Metabolism of [^{14}C]-CSA was observed at 2 hours, with hydroxylated and demethylated [^{14}C]-CSA being the first two metabolites, which increased overtime and reached a plateau at 9 hours. Other metabolites were only detected at 24 hours after treatment, as levels of hydroxylated and demethylated CSA declined. It was hypothesised that CSA was activated upon hydroxylation and was inactivated by glycosylation and stored in the vacuole, which was highly likely since the hydroxylated metabolite positively correlated with safener activity and was detected at early timepoints. Figure 20 shows a proposed metabolic pathway for CSA, based on the data obtained from Figure 18 and Figure 19.

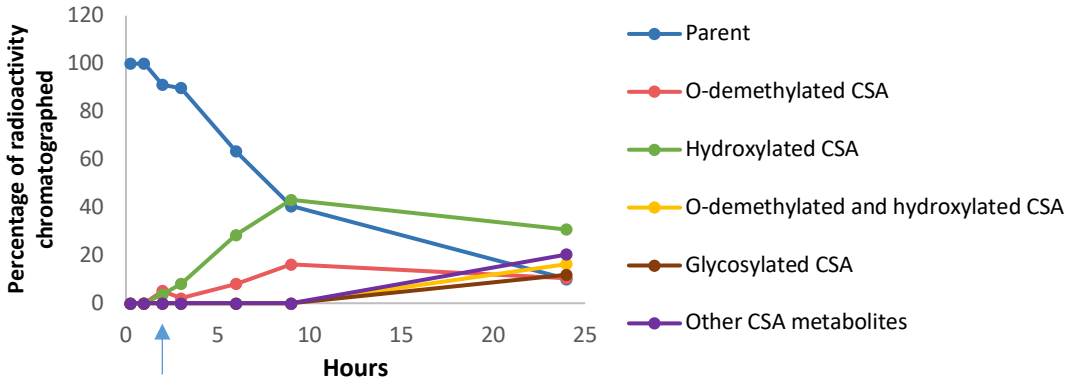


Figure 19: Rate of [^{14}C]-CSA metabolism in maize shoots over the course of 24 hours - Radio-HPLC. [^{14}C]-CSA was administered through excised shoots when plants were 7 days old (BBCH 11-12). Chemical treatment lasted for 3 hours before plants were transferred to water before harvest at specified timepoints. The percentages used for this line graph were derived from the chromatograms of each associated timepoint. The arrow corresponds to the 2 hour timepoint.

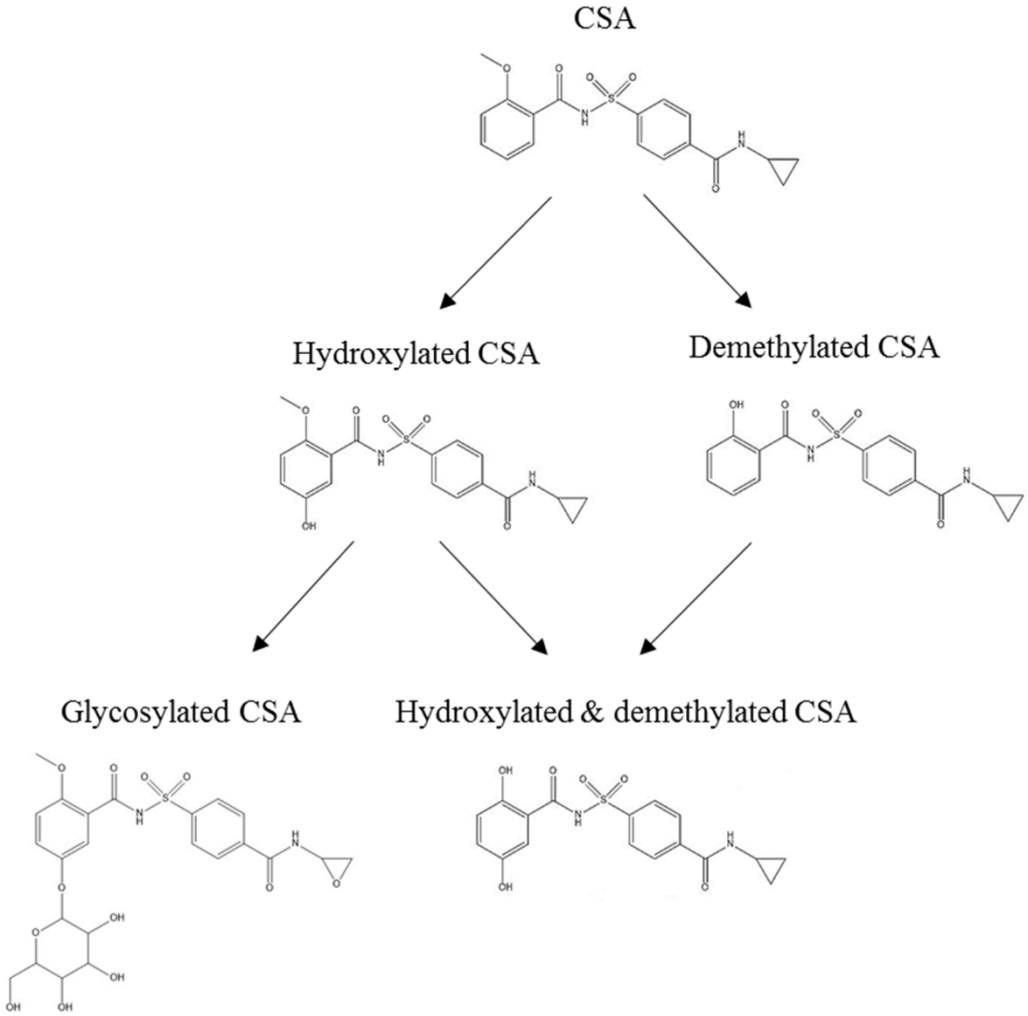


Figure 20: Metabolic pathway of CSA after 24 h. All metabolites were present both in maize and wheat.

4.7 Metcamifen metabolism study

One of the immediate questions arising after elucidating the metabolic profile of CSA was whether metcamifen, which was structurally similar to CSA, could also be undergoing bioactivation as this could explain its activity in both maize and wheat. As radiolabelled metcamifen was not available, this experiment was conducted by LC/MS analysis. As such, metcamifen and its metabolites were quantified in an attempt to make comparisons between crop plants.

Metcamifen metabolism was analysed at 1, 2, 3, 4, 5, 6, 9 and 24 hours following exposure of excised shoots of maize and wheat plants. The experimental setup was as described in the CSA and TCM metabolism experiments (Sections 3.4, 4.4), where excised shoots were incubated in metcamifen solution for 3 hours before being transferred to fresh water until

harvested. One replicate was included per crop, each consisting of a number of pooled maize or wheat plants (Section 2.5.5.1). Metcamifen metabolites were extracted in 80% acetonitrile

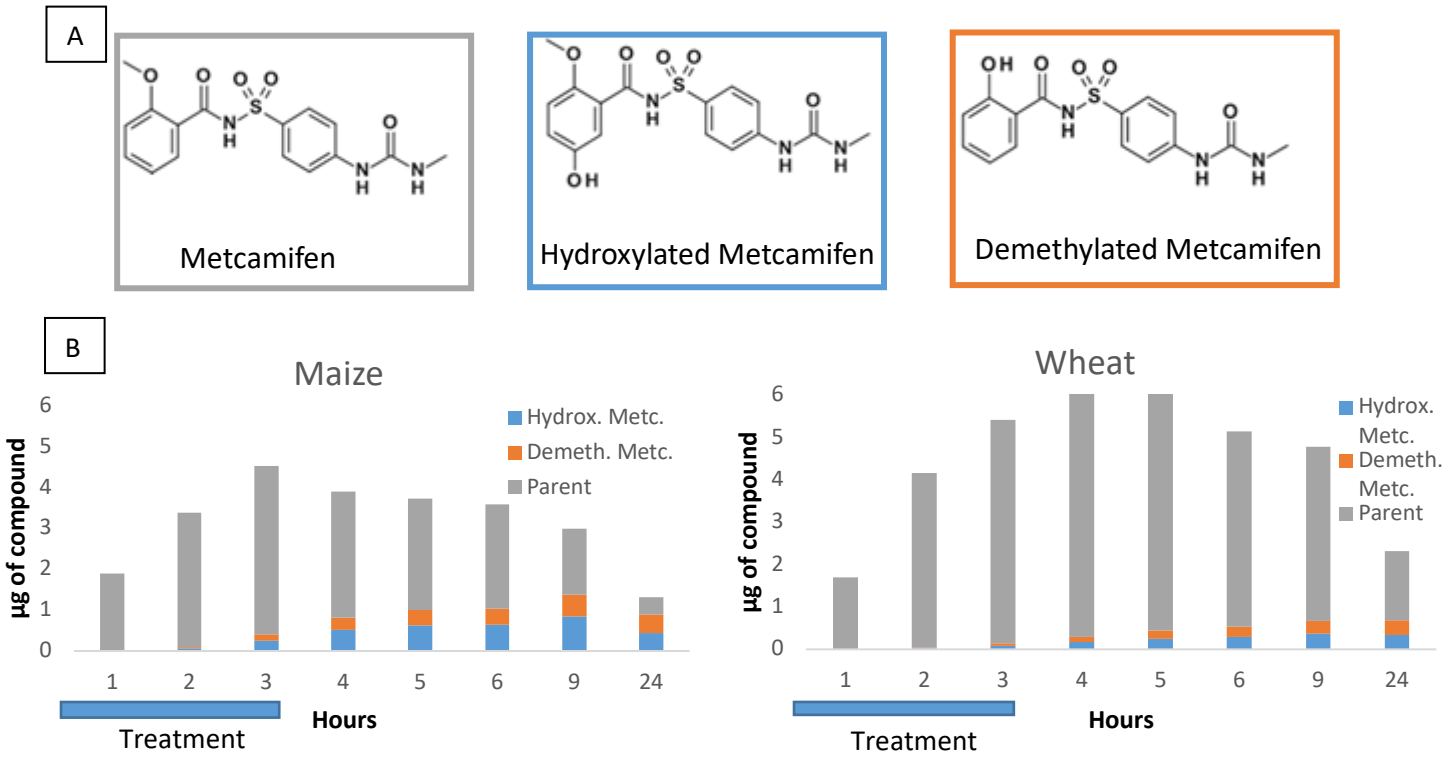


Figure 21: Metcamifen metabolism over the course of 24 hours is maize and wheat shoots – LC/MS. (A). Structures of metcamifen and metabolites**. (B).** Amount (µg) of parent and metabolite compounds over 24 hours. Metcamifen was administered through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted for 3 hours (marked by blue bar) before plants were transferred to water before harvest specified timepoints.

and run on a quadrupole time of flight spectrometer coupled with a UPLC system and a PDA detector for chromatographic separation (Section 2.5.5.2). Figure 21 shows the structures of the compounds analysed and the rate of their appearance over a course of 24 hours in maize and wheat. The structure of the proposed metcamifen metabolites was based on the structures of the corresponding early CSA metabolites (Figure 20).

Metcamifen was detected in higher quantities in wheat than in maize during the 3 hour treatment, reaching its highest levels at 4 - 5 hours (Figure 21). The parent compound levels were expected to decrease after 3 hours due to metabolism, but instead they continued to rise, potentially due to different groups of plants per time point exhibiting various degrees of uptake. Metcamifen metabolism started at 2 hours, with very small amounts (0.02 µg) of both hydroxylated and demethylated metabolites detected (Figure 21). Starting from 6 hours, the reduction in the parent compound did not translate into an equivalent increase of the primary metabolites, a fact that could possibly be attributed to further downstream metabolism (conjugation with either glutathione or cysteine). Metcamifen was reduced to roughly 25% at

24 hours when compared to the amount at the end of the treatment (3 hours). The metabolism of metcamifen at 24 hours was faster compared to CSA's metabolism in wheat (Figure 17).

In maize, metcamifen was detected at its highest level at the end of the treatment (3 hours) with metabolism beginning at 2 hours (Figure 21), which was in line with the [^{14}C]-CSA metabolism experiment (Figure 19). The levels of metcamifen metabolites increased over time, especially hydroxylated metcamifen (Figure 21). After 4 hours, it was likely that downstream metabolism occurred, since the total amount of parent and primary metabolites then decreased over time. The parent compound reached the highest amount directly after treatment (3 hours) and only 10% of that amount remained after 24 hours.

In conclusion, the overall levels of metabolism of metcamifen observed in both crops was low, being more rapid in maize than in wheat as shown at 9 and 24 hours after treatment. As metcamifen was active in both crop species, the results suggested that metcamifen might not need to undergo bioactivation to elicit safening, unlike CSA where activity in maize positively correlated with rapid metabolism. Nevertheless, further experiments on metcamifen metabolism in both crops were required before drawing a final conclusion.

4.8 Merit Trials

Since CSA metabolism was positively correlated with its safening activity, the next question to address was whether safening could be observed if CSA metabolism was blocked. The primary metabolites of CSA were created by hydroxylation and demethylation, processes normally catalysed by cytochrome P450 enzymes (Donaldson and Luster, 1991). Therefore, treating crop plants with cytochrome P450 inhibitors such as tetcyclasis or 1-aminobenzotriazole (ABT) could provide important insights into the role of CYPs in mediating safening activity. However, P450 inhibitors have broad specificity (Gaillardon et al., 1985, Cole and John Owen, 1987, McFadden et al., 1989), which could interfere with the metabolism of TCM as well as that of CSA, since its primary metabolite was demethylated TCM. To overcome this problem, safening was tested in a P450 mutant sweetcorn cultivar, Merit, that contained a mutation in CYP81A9 (Kang, 1993, Bradshaw et al., 1994, Pataky et al., 2008, Pataky et al., 2009, Liu et al., 2015), a gene encoding a cytochrome P450 associated with tolerance against multiple herbicides (Pataky et al., 2008). The mutation has been associated with a 392 bp insertion first occurred in an inbred maize line, that results in a

disruption of the heme/iron ligand site of the encoded enzyme (Fleming, 1988, Kang, 1993, Pataky et al., 2008, Dam et al., 2009). Experiments with safeners had been previously performed on Merit that reported both protective (Darren et al., 2013) and non-protective effects (Burton et al., 1994, Williams and Pataky, 2010), but none of these studies assessed the metabolism of safeners.

To determine whether CYP81A9 was involved in CSA's hydroxylation, CSA metabolism was studied by radio-HPLC in Merit at the same timepoints as described in previous experiments (Section 4.6, Figure 19). Shoots were excised and incubated in [^{14}C]-CSA solution (5 PPM - 13µM) for 3 hours before transfer to fresh water and harvest at the designated timepoints. Samples had one replicate that consisted of pooled maize plants (Section 2.5.3.1). CSA metabolites were extracted in 80% acetonitrile and separated as described in section 2.5.3.3. The chromatograms obtained were compared to the previous chromatograms obtained for CSA metabolism in 'Aventura', the maize cultivar used consistently in the study (Figure 22). Metabolism of CSA started at 3 hours in cultivar Merit and at 2 hours in cultivar Aventura. The level of hydroxylated CSA was compromised and demethylated CSA was almost absent in cultivar Merit (Figure 22). The parent compound was the dominant peak in Merit after 9 and 24 hours, in contrast to Aventura, where hydroxylated CSA was the most abundant at both timepoints. The lack of downstream metabolites in Merit combined with the low

abundance of hydroxylated CSA, suggested CYP81A9 is important in CSA metabolism, where additional CYPs are also involved (Figure 22).

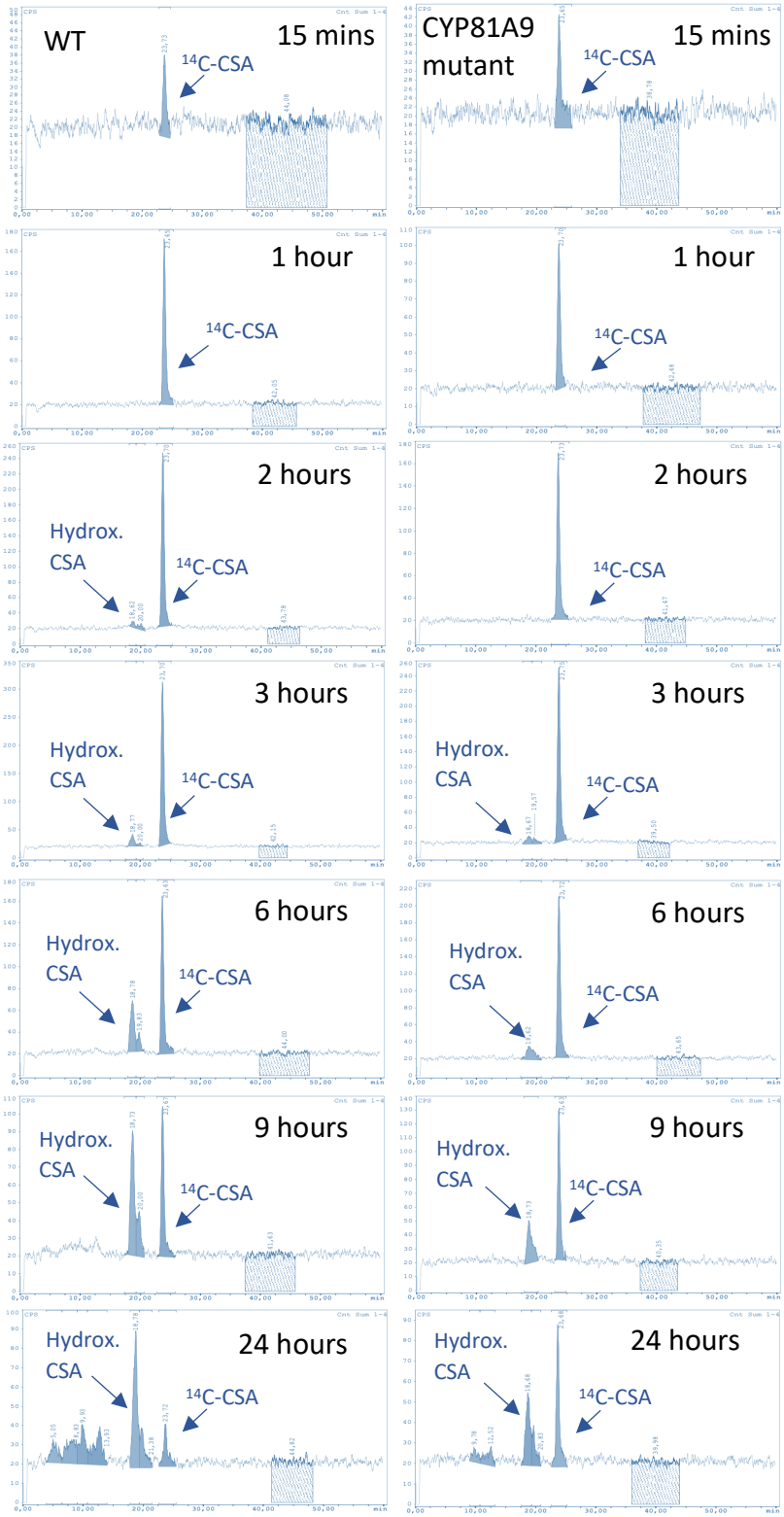


Figure 22: Metabolic profile of [^{14}C]-CSA over 24 hours in WT and CYP81A9 mutant maize shoots - Radio-HPLC. Arrows annotate the parent compound and the primary hydroxylated metabolite in the chromatograms. ^{14}C-CSA was administered through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted for 3 hours before plants were transferred to water before harvest at specified timepoints.

Next, a series of greenhouse trials were designed to explore the effects of compromised CSA metabolism on the safener's protective effects. CSA and metcamifen were tested for safening on Merit and Aventura using the herbicides TCM and TBT, which are both metabolized by P450s (PMRA, 2010, Kupper et al., 2018). The safeners were applied at 100g/ha whereas herbicides were applied at the following concentrations (based on field rate and Bayer personal comm)(Table 9):

Crop (Varieties)	**Herbicide**			
	Thiencarbazone-methyl (TCM) ALS Inhibitor		Tembotrione (TBT) 4-HPPD Inhibitor	
	High dose (g/ha)	Low dose (g/ha)	High dose (g/ha)	Low dose (g/ha)
Maize - *Zea Mays* (Aventura, Merit)	50	5	2000	400

Table 9: High and low concentrations of TCM and TBT used in the trials with Aventura, and Merit. Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). Herbicide injury was assessed by visual scoring, 1 and 2 weeks after treatment. Herbicide injury was assessed by visual scoring, 1 and 2 weeks after treatment, within percentage injury categories (e.g. 50-55 % injury).

All compounds were applied post - emergence with a track sprayer (Höchst AG, Höchst, Germany) as described in section 2.4.1. Every spraying combination included three replicates and herbicide injury was assessed by visual scoring as described in section 2.4.3. Herbicide injury scores for both cultivars were plotted in a bar chart (Figure 23B), accompanied by pictures of the herbicide injury visual assessment (Figure 23A).

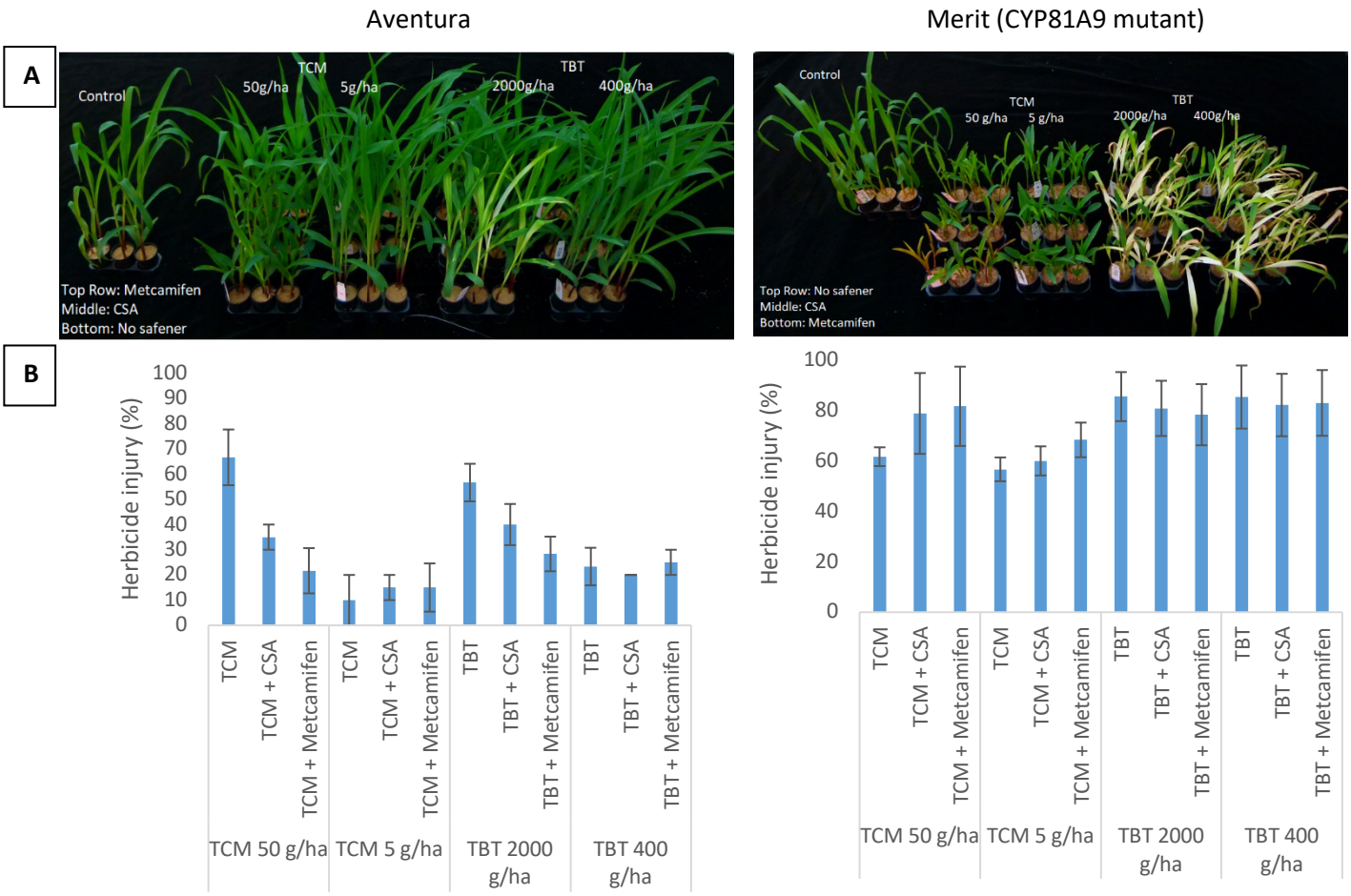


Figure 23: Effects of CSA and metcamifen on TCM and TBT injury in Merit and Aventura. (A): Pictures from greenhouse trial **(B)**: Herbicide injury per herbicide+safener combination was scored within percentage injury categories (5-10%) and error bars represent standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12).

Safening effects were not observed with either CSA or metcamifen in cultivar Merit at any concentration of TCM and TBT used. Furthermore, the cultivar displayed increased herbicide injury (Figure 23), which was in line with its association with herbicide sensitivity in previous studies (Burton et al., 1994, Pataky et al., 2008, Williams and Pataky, 2010). Aventura, on the other hand, showed adequate safening effects with both safeners when high herbicide concentrations were applied, but not at lower ones as they caused inadequate injury. Interestingly, the observed injury in Merit after CSA application (Figure 23) did not correlate with the degree of compromised CSA metabolism (Figure 22). CSA metabolism in Merit was moderate, which according to the hypothesis that CSA metabolism is important for safening, should have resulted in minor safening effects.

To assess whether Merit's sensitivity to TCM and TBT was due to the lack of a functional CYP81A9 enzyme, a second trial was designed with flufenacet, a herbicide metabolised by

GSTs in maize (Bieseler et al., 1997). Since CYPs are not involved in flufenacet's metabolic degradation as it is detoxified by glutathionylation, a lack of safening effects in flufenacet

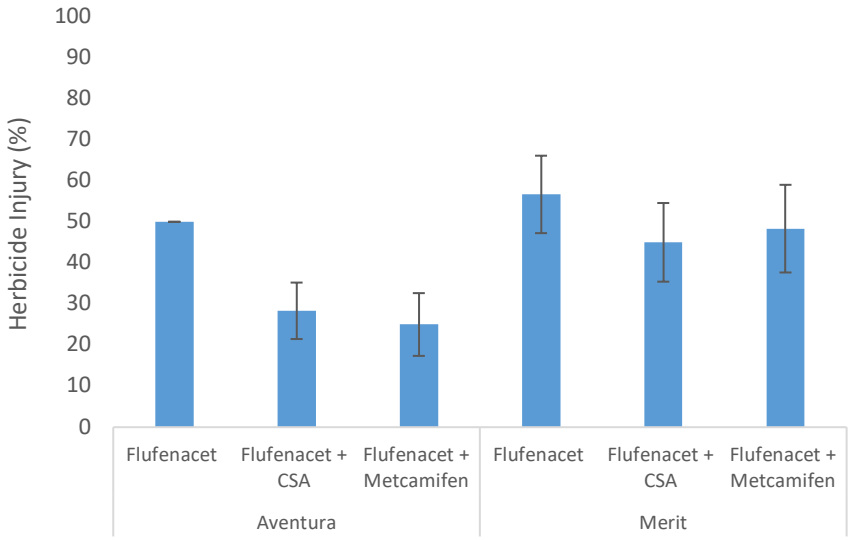


Figure 24: Effects of CSA and metcamifen on flufenacet injury in Merit and Aventura. Greenhouse trial where flufenacet was applied by spray application at 2000g/ha. Herbicide injury was scored within percentage injury categories (5-10%) and error bars represent standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12).

treated plants could provide information on the complex safener signalling mechanism. Flufenacet was applied at 2000 g/ha, with CSA or metcamifen (both applied at 100 g/ha) in the same way as the TCM and TBT trial. Herbicide injury was then determined by visual scoring one week after the spray application (Sections 2.4-2.4.2)(Figure 24).

It was interesting that while cultivar Aventura was safened by both CSA and metcamifen (23% and 25% respectively), no safening effects were observed in cultivar Merit (Figure 24). Flufenacet is metabolized by glutathione S-transferases (GSTs) (Bieseler et al., 1997) which would suggest that CYP81A9 is not involved in controlling safening in maize. However, the absence of safening in Merit indicated that CYP81A9 is controlling, to some extent, protection against flufenacet in Merit.

Multiple safeners were also tested on Merit to check whether herbicide injury could be alleviated by the induction of different sets of detoxification enzymes. TCM was chosen for this experiment, which was applied at 50 g/ha along with benoxacor, isoxadifen, dichlormid and furilazole applied at 100 and 500 g/ha. Untreated controls as well as safener-only applications were also included to ensure no injury was observed due to safener application

(data not shown, no injury at higher rates). Spraying application was performed as described in previous sections (2.4.1, 3.2) and herbicide injury was assessed by visual scoring one week after spraying application (section 2.4.3). Three replicates per sample were included; the trial was not repeated (Figure 25).

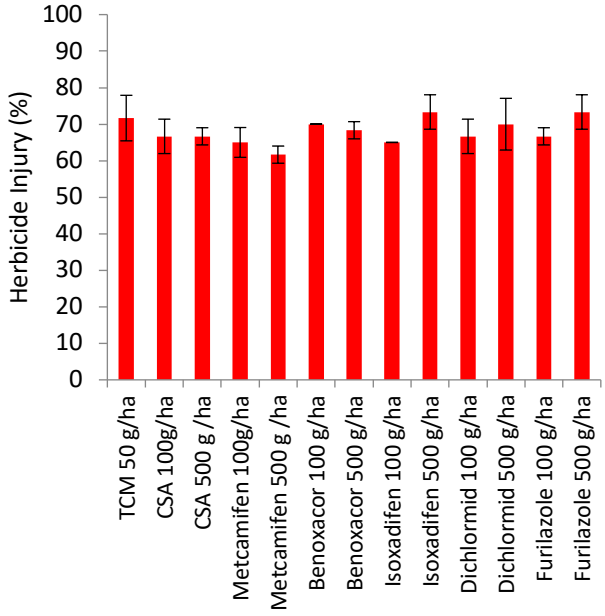


Figure 25: Effects of different safeners on TCM injury in Merit. Greenhouse trial where safeners were applied at the usual rate used throughout the study (100 g/ha) and at 500 g/ha. Herbicide injury was scored within percentage injury categories (5-10%) and error bars represent standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12).

Again, Merit did not show any safening effects with any of the safeners. The injury scores were comparable in all combinations of TCM and safeners (65-73%, Figure 25). A TCM metabolism assay was conducted to explore whether TCM was metabolised endogenously and whether CSA or metcamifen application affected its metabolism. Metabolism was measured at 6 hours in Aventura and Merit by radio-HPLC with the experimental setup as described in Sections 2.5, 3.4 and 4.6. (Figure 26). Radioactivity levels corresponding to TCM and demethylated TCM were plotted with no safener, + CSA and + metcamifen treatments for both cultivars.

The results in cultivar Aventura were very similar to those determined in the previous TCM metabolism experiments (Figure 11,Figure ***12***Figure 13). TCM was endogenously metabolised in maize at 6 hours, primarily by demethylation (30% of detected radioactivity,

Figure 26). Upon CSA and metcamifen application, the levels of demethylated TCM increased to 52% and 57% respectively, followed by an equivalent decrease in the parent compound. In the absence of safener treatment, Merit showed almost no endogenous metabolism of TCM with 80% of the parent compound remaining and only 1% of demethylated TCM detected at 6 hours. TCM metabolism in Merit remained unaffected after CSA or metcamifen application (Figure 26). The levels of downstream TCM metabolites were similar in both cultivars (25% of radioactivity in Aventura and 20% in Merit).

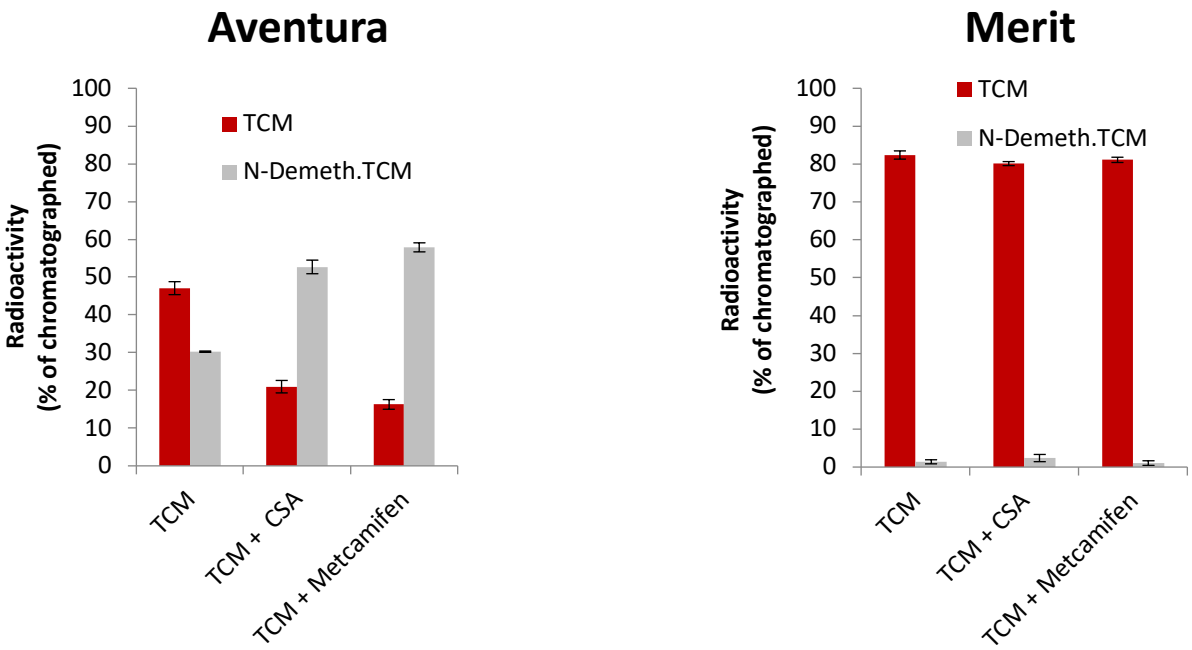


Figure 26: Effects of CSA and metcamifen on [^{14}C]-TCM metabolism study in Merit and Aventura shoots at 6 hours – Radio-HPLC. The levels of the parent (red) are visualised along the levels of the primary inactive *N*-demethylated metabolite (grey). ^{14}C-TCM and safeners were administered through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted for 3 hours before plants were transferred to water before harvest at 6 hours. Error bars represent standard deviation (n=3).

Collectively taken, the experiments with cultivar Merit showed that the lack of safening observed with herbicide TCM was due to the inability of the plants to demethylate the herbicide, probably derived from the lack of a functional CYP81A9 enzyme. This could also explain why no safener effects were observed with the application of different safeners (Figure 25). In addition, these results corroborated previous studies with Merit and sulfonylureas (Green and Ulrich, 1993, Burton et al., 1994). These studies showed that the cultivar was unable to metabolise primisulfuron and nicosulfuron with no enhanced metabolism observed following a treatment with safener BAS 145 138. It has also been reported that a crop needs to exhibit a certain degree of tolerance towards the herbicide for

safening to be observed (Hatzios, 1989b). Based on this information, cultivar Merit is probably not a suitable study model for elucidating safening by CSA, since its main herbicide partners (TCM, TBT) are metabolised by CYPs.

Nevertheless, Merit showed compromised CSA metabolism (Figure 22) which provided evidence for crops utilising the same detoxification system for both herbicides and safeners. According to Davies (1999), such interactions could be used in a predictive framework for determining effective herbicide-safener combinations. However, the lack of safening observed with flufenacet in Merit, could not be explained by the lack of a functional CYP81A9 enzyme. Being a hybrid line that hasn't been well characterised apart from its performance in the field, Merit could possess a more complex genetic background than a simple knockout line (Bayer personal comm.). This could include further disruptions in the xenobiotic detoxification pathway which could either include the GSTs responsible for detoxifying flufenacet or the regulatory elements in their promoters upstream. A more plausible explanation, however, would be that CYP81A9 has additional functions that could indirectly involve the detoxification of flufenacet. CYPs have been demonstrated to have multiple functions, many of them involving plant secondary metabolites (Hamberger and Bak, 2013).

4.9 ZmGSTL1 induction upon CSA treatment in different maize tissues

One important aspect of safener activity is the upregulation of xenobiotic detoxifying enzymes, including GST's (Edwards et al., 2005b). Previous studies in maize have reported tissue-specific induction of GST's after safener treatment (Riechers et al., 2003, Baek et al., 2019). In addition to that, CSA seemed to protect maize plants from flufenacet damage (Figure 24). Therefore, it was interesting to examine whether CSA treatment could induce transcript expression of GSTs in maize in a tissue-specific manner. *ZmGSTL1* is a gene initially identified as being specifically induced by benzenesulfonamide safeners in maize (Hershey and Stoner, 1991) and subsequently reported to not demonstrate GST activity even though it binds glutathione (McGonigle et al., 2000). *ZmGSTL1* belongs to the lambda class of GST's, a class specific to terrestrial plants with a role yet to be determined (Lallement et al., 2014). Enzymes of this class have been demonstrated to possess a cysteine instead of a serine residue like other GST classes (phi, tau), and have been suggested to use glutathione to catalyse redox reactions, including those involved in flavonoid metabolism (Theodoulou et

al., 2003, Dixon and Edwards, 2010). *ZmGSTL1* has been utilised before to study safener signalling (Behringer et al., 2011), so it was selected as a biomarker for the study.

The relative transcript expression of *ZmGSTL1* was quantified at the same timepoints when TCM detoxification had been tested (See section 3.4). CSA was applied by incubation to excised maize shoots for 3 hours before plants were transferred to fresh water. Prior to freezing in liquid N_2, plants were dissected in upper, middle and lower tissues (Figure 27). Every sample consisted of three replicates, each comprising pooled plant tissues from 3 plants, replicating the conditions of the TCM metabolism experiment as closely as possible. After RNA extraction and cDNA synthesis, *ZmGSTL1* induction was determined using the –ΔΔCt method (Section 2.6.2-5). Results are shown in Figure 27.

The induction of *ZmGSTL1* in the upper tissues was observed at 3 hours and the relative expression further increased at a steady rate until it reached 10-fold at 6 hours (Figure 27). In the middle tissues, the transcript induction of *ZmGSTL1* started at 4 hours and increased to 7.8 after 6 hours. The expression of the gene was not increased significantly in the lower tissues after CSA treatment, apart from after 4 hours. Upper tissues displayed the greatest induction

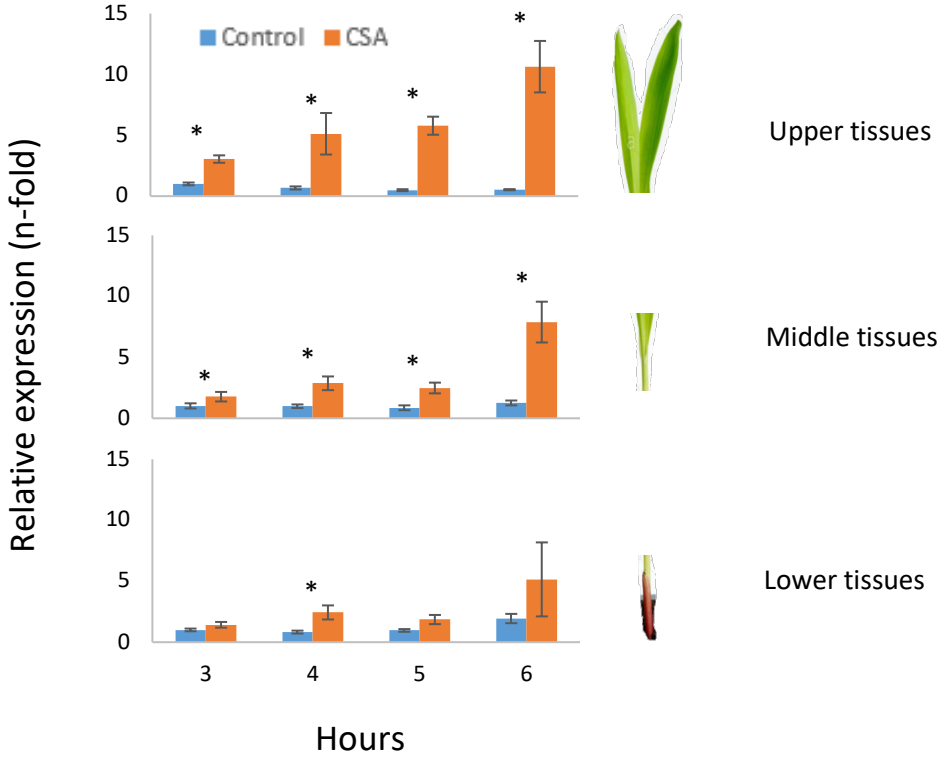


Figure 27: Transcript expression of *ZmGSTL1* over the course of 6 hours after CSA treatment in maize shoots (upper, middle, lower parts) – RT-qPCR. CSA was administered through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted for 3 hours before plants were transferred to water before harvest at specified timepoints. Stars indicate significant differences between control and safener treatments ($p \leq 0.05$) and error bars represent standard deviations (n=3).

of transcript expression of *ZmGSTL1*(Figure 27). This is consistent with GST upregulation in upper tissues being reported as an important factor in protecting developing leaves such as the coleoptile, the predominant site of herbicide action (Riechers et al., 2010). There was also the possibility, however, that the induction of *ZmGSTL1* in the upper tissues might be due to preferential translocation of CSA through acropetal xylem transport (Riederer, 2004).

4.10 Discussion

Following on from the previous chapter where safening was associated with increased TCM metabolism, the fate of CSA was investigated in maize and wheat to explore the effects of safener uptake, translocation and metabolism in protecting crop plants from herbicide injury. CSA displayed higher uptake and mobility in wheat, whereas in maize only a small amount of CSA was taken up and remained localised in the application tissue. While these results were unable to explain the differential activity of the safener, CSA was rapidly metabolised in maize as compared with wheat in the application leaves of the plants. This suggested a CSA metabolite could potentially have an important role in the manifestation of the safening effect in maize. These results were consistent with studies reporting that limited safener uptake is not a compromising factor for safening (Jablonkai, 1991) and safener metabolism can lead to safener activation (Rubin and Kirino, 1989, Taylor et al., 2013, Jeschke, 2016b). As a result of that, subsequent studies explored CSA metabolism in more detail, in an attempt to dissect how it relates to safening by CSA.

The CSA metabolism study was replicated in excised shoots of maize, wheat and soybean where CSA was rapidly metabolised in maize by 24 hours in a more polar metabolite, a similar pattern to that observed when CSA was applied locally on a leaf. Again, wheat displayed minor metabolism of CSA and the major CSA metabolite was present in low quantities. Soybean did not metabolise CSA at all after 24 hours. The correlation of this major metabolite with safener activity led to the identification of CSA metabolites in maize and wheat, with the most prominent one being hydroxylated CSA. Since safening effects appeared to be present in early timepoints in TCM detoxification experiments, hydroxylated CSA had to be present early (before 3 hours) in order to account for safening. A timecourse metabolism assay of CSA over 24 hours revealed that CSA metabolism started at 2 hours, with the appearance of hydroxylated and demethylated CSA. By 9 hours after treatment (where significant TCM detoxification had been observed) only these metabolites could be

detected. These results provided evidence to support the hypothesis that hydroxylated CSA could be responsible for enhancing TCM metabolism and subsequent safening. The latter had been suggested before for safener metabolites such as the respective acids of cloquintocet-mexyl, fenchlorazole-ethyl, mefenpyr-diethyl and isoxadifen-ethyl (Taylor et al., 2013) (Jeschke, 2016b), but no study had proposed that safener metabolism could potentially be the basis for safener selectivity in different species.

LC/MS experiments with metcamifen revealed that metabolism was also starting at 2 hours but was not as fast as determined with CSA. Wheat also metabolised metcamifen more rapidly than CSA, but at 24 hours the most prominent peak was still the parent compound. Considering that metcamifen can protect wheat and maize from herbicide injury, these results did not seem to show any correlation between metcamifen metabolism and safening, which suggested more complex underlying mechanisms of safening by this compound. Nevertheless, metcamifen's enhanced metabolism in wheat compared to CSA could mean that metcamifen caused a stronger induction of wheat's detoxification machinery, which would be consistent with its activity in the crop. Further replication and investigation of metcamifen's downstream metabolism in both crops was required, however, in order to draw that conclusion.

Since hydroxylation is predominantly catalysed by cytochrome P450 enzymes, experiments with a CYP mutant were carried out to examine whether safening could be obstructed by preventing CYP-based metabolism. Compromised CSA metabolism was observed in sweet corn cultivar Merit, which lacked a functional CYP81A9 enzyme. Greenhouse trials with the same cultivar, showed no protective effects with either CSA or metcamifen against P450 and GST metabolised herbicides. However, as compromised CSA metabolism could not account for the increased degree of herbicide injury in the cultivar, a TCM metabolism experiment was carried out to quantify TCM detoxification in Merit. This experiment revealed that Merit was unable to detoxify TCM, which made it challenging to separate the effects of CYP81A9 on safener activity and herbicide detoxification in the cultivar. However, the fact that CYP81A9 was involved in the metabolism of both CSA and TCM highlighted its importance in detoxifying xenobiotics and corroborated the study in soybean which suggested that a CYP was important for mediating TCM metabolism (Figure 11).

Apart from enhancing herbicide metabolism, CSA treatment could also induce transcript expression of *ZmGSTL1*, a safener inducible gene in maize. The transcript expression profile revealed that *ZmGSTL1* was preferentially induced in upper tissues upon CSA treatment, which was consistent with a number of studies reporting tissue specific induction of GSTs with safeners (Xu et al., 2002, Riechers et al., 2003, DeRidder and Goldsbrough, 2006). This

occurred at the same time with CSA-induced TCM detoxification. Future experiments were therefore needed to focus on earlier timepoints to elucidate whether the upregulation of *ZmGSTL1* by CSA happened prior to CSA-enhanced TCM metabolism.

Collectively, these series of experiments uncovered a strong, positive correlation between the safening activity of CSA and the formation of its hydroxylated metabolite in maize. The function of hydroxylated CSA in safener selectivity was further explored in following experiments.

Chapter 5 – Exploring the role of cyprosulfamide's metabolism in safening

5.1 Introduction

The concept of drug or pesticide bioactivation was first observed in the pharmaceutical industry. Prontosil, an antimicrobial sulphonamide, was one of the first compounds that exhibited bioactivation since it needs to be metabolised to sulfanilamide to achieve its therapeutic effect (Tréfouël et al., 1935). While this concept was not well received in the past (Otten, 1986), nowadays, the introduction of biologically degradable groups is commonly used to increase an active ingredient's performance and/or its biological activity. An increased performance of a compound of interest can be achieved by influencing the physicochemical and pharmacokinetic properties of active ingredients to improve systemicity or stabilise reactive/volatile compounds, while increased biological activity refers to improving target selectivity (Jeschke, 2016b). This mainly capitalises on species differences in activation and detoxification reactions, resulting in selective toxicity, an important consideration in environmental safety (Casida, 1983, Rubin and Kirino, 1989, Jeschke, 2016b).

In the agricultural sector, compounds that have undergone such modifications are called propesticides. These are compounds that are biologically inactive as applied and require biochemical (enzymatic), chemical (non-enzymatic) or physical (e.g. photochemical) transformation to generate active derivatives. In the majority of cases, the transformation associated with bioactivation is performed biochemically by hydrolases or cytochrome P450s through reactions involving oxidation or reduction (Casida, 2017). This has been demonstrated in *Nicotiana benthamiana*, where transformation with CYP105A1 from *Streptomyces griseolus* activated the sulfonylurea pro-herbicide R7402 through *N*-dealkylation (Keefe et al., 1994).

Proherbicides are widely used in weed control, comprising 37% of related sales in 2017 (Casida, 2017). The Japanese rice market provides a good example of several proherbicides being used, including pyrazolynate, pyrazoxyfen and benzofenap all of which are activated to provide herbicidal activity over time (van Almsick, 2019). In addition, benzobicyclon is selectively activated in annual grasses, sedge and broadleaf weeds competing with rice (Komatsubara et al., 2009). Similarly the phenoxybutanoic-derivative herbicides 2,4-DB and

MCBP are selectively transformed to their herbicidal phenoxyacetic acids by β-oxidation in susceptible plants (Rubin and Kirino, 1989).

Safeners have been developed according to similar bioactivation principles in order to fully complement their selective use with highly effective herbicides. In fact, some of the latest pro-safener chemistries such as fenchlorazole-ethyl, mefenpyr-diethyl and isoxadifen-ethyl, were developed specifically for increased diffusion permeability (Jeschke, 2016b). These safeners undergo rapid hydrolysis to yield the respective carboxylic acids that are mobile in the phloem, corresponding to the behaviour of their herbicide partners.
Hydrolysis seems to be the main bioactivation step for prosafeners with a few other examples reported in the literature. *N*-phenylmaleimides and *N*-phenylisomaleimides are also hydrolysed rapidly into the *N*-phenylmaleamic acids with safening activity associated with the acids (Rubin et al., 1985, Rubin and Kirino, 1989). The wheat safener cloquintocet mexyl was also rapidly hydrolysed into its respective carboxylic acid, and able to induce GSTs. For that reason, this metabolite was hypothesised to be the active safener (Taylor et al., 2013).

Apart from hydrolysis, cysteine conjugation is another metabolic step demonstrated to have a role in protecting against herbicide injury. L-2-oxo- thiazolidine-4-carboxylic acid (OTC) could alleviate the growth inhibition and GST activity reduction induced by tridiphane by increasing the cellular content of cysteine in sorghum (Hilton and Parthasarathy, 1986). This phenomenon was originally observed with the application of OTC in mice (Williamson et al., 1982).
Furthermore, 4-chloro-6-(methylthio)-phenylpyrimidine (CMTP), a catabolised cysteine conjugate of fenclorim, was shown to have safening activity in growth trials in rice as well as GST-inducing activity in *Arabidopsis thaliana*, even though the previous metabolites had no activity. This phenomenon was a metabolic reactivation of the safener rather than bioactivation, demonstrating the complex catabolic reactions of xenobiotics involved in both detoxification and bioactivation (Brazier-Hicks et al., 2008).
In this thesis, considering that the safening activity of CSA correlated with the formation of hydroxylated CSA in maize while this metabolite was absent in the unresponsive wheat and soybean, it was tempting to speculate that CSA was a prosafener. Moreover, a cytochrome P450 (CYP81A9) was demonstrated to be partly responsible for its hydroxylation (Figure 22), a transformation step common in the bioactivation of other propesticides.

In this chapter, the bioactivation hypothesis for CSA was tested by using the primary metabolites of CSA and metcamifen, which were synthesised and tested for activity in maize by determining their ability to enhance the detoxification of TCM. Metcamifen was included,

as it was structurally similar to CSA, so it was interesting to see whether the same principles of metabolites associated with safening would apply. These trials would provide important insights on whether CSA owed its activity to a bioactivation event.

RESULTS

5.2 Synthesis and lipophilicity of safeners and safener metabolites

Prior to testing their activity, primary CSA and metcamifen metabolites were synthesised by the group of Dr Hendrik Helmke (Chemistry, Bayer AG, Frankfurt, Germany). Their names, structures and logP values, with the latter being an indicator of lipophilicity, can be found in Figure 28. All compounds displayed low logP values which corresponds to their low lipophilicity, a typical characteristic of polar compounds (Bhal, 2011). Both CSA and metcamifen metabolites were more polar than the parent compounds, which suggested that their foliar uptake through the cuticle could be compromised, especially for the hydroxylated derivatives (Bayer personal comm.).

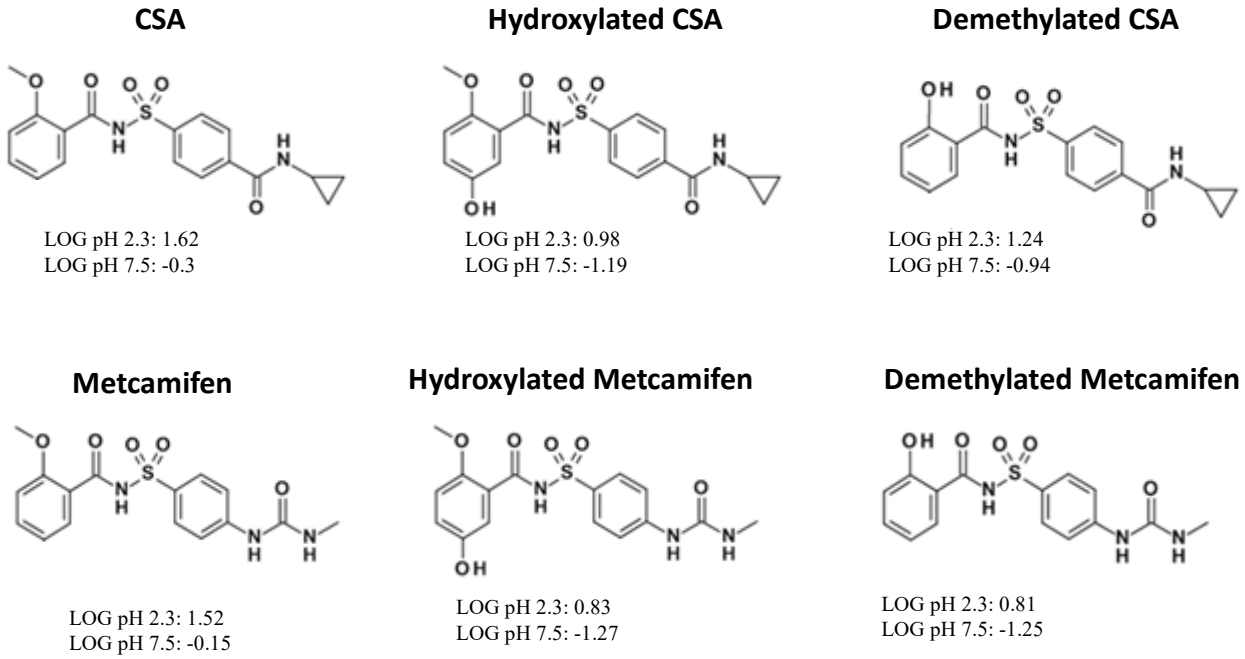


Figure 28: CSA and metcamifen primary metabolites with their respective logP values.

5.3 CSA and metcamifen metabolite trials

To ensure reliable uptake of these polar compounds, a preliminary application method based on the TCM metabolism study (Section 3.4) was tested, where excised shoots were incubated in TCM and safener/safener metabolite solution for 3 hours before transfer to fresh water (section 2.5.1, Figure 2). Injury was assessed a week later by visual scoring (as described in section 2.4.3). TCM is an ALS-inhibitor which induces stunted growth, so injury was assessed based on plant height. As the excised shoots did not grow after excision, the induction of injury was challenging to determine. To overcome this, two other application methods were used. The first application method was through seed treatment with the safeners and respective metabolites prior to sowing, followed by TCM spray application. This application method has been reported previously for testing safener metabolites (Rubin et al., 1985). The second application method was through treatment of excised shoots with safeners/ safener metabolites and [^{14}C]-TCM, where the safening effect was measured based on TCM detoxification.

5.3.1 Seed treatment of safener metabolites

Maize seeds were weighed in batches of 100g. 0.5g of safener or safener metabolite per kg of seed was applied by incubating the seeds in solution containing the compounds (As described in section 2.4.2), prior to sowing. After 8 days, TCM was applied at 50 and 100g/ha with a track sprayer (as described in section 2.4.1) and injury was determined one week after by visual scoring as described in section 2.4.3. Every compound tested consisted of three biological replicates. The spraying formulation of TCM was the same as described in section 2.4.1. Both TCM applications produced similar results, therefore only the results from the 50g/ha application have been reported here (Figure 29).

Treatment with CSA reduced TCM injury whereas no safening was observed with either hydroxylated or demethylated CSA, with injury being 57.5% and 55% respectively compared to the injury observed in the no safener treatment (55% injury, Figure 29). Metcamifen and, interestingly, demethylated metcamifen reduced TCM injury to a similar degree as CSA (by 37% and 35%), but the application with hydroxylated metcamifen had no effect (57.5%

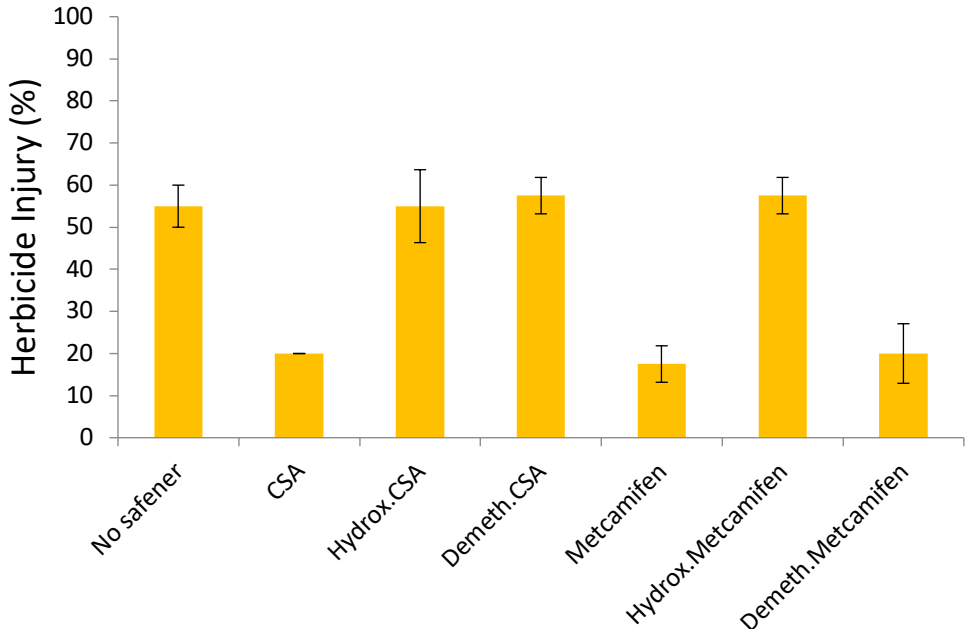


Figure 29: Effects of CSA, metcamifen and their primary metabolites on TCM injury in maize. Greenhouse trial where the safeners/safener metabolites were applied by seed treatment prior to sowing. TCM was applied by post-emergence spray application at 50 g/ha. Herbicide injury was scored within percentage injury categories (5-10%), one week after treatment. Error bars represent standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12).

injury). Even though a strong correlation with the presence of the early CSA metabolites and safening activity was previously observed, CSA metabolites were unable to protect maize from herbicide injury, and this was also true for hydroxylated metcamifen. The role of other metcamifen metabolites in safening was unclear, but demethylated metcamifen seemed to retain safening activity, which was an indication that there were no issues of increased polarity inhibiting activity and the idea of safener metabolites being important for safening was valid. This experiment was not carried out in wheat as only a small amount of the metabolites was synthesised for the trials, which was not sufficient for the quantity required for seed treatment (0.5g/kg of seeds).

These results corroborated trials carried out from the Safener Biology team at Weed Control Biology at Bayer (Frankfurt, Germany) where post-emergence spray applications with CSA metabolites had no effect on TCM-induced injury (Bayer personal comm.).

5.3.2 Induction of radio-TCM metabolism upon safener metabolite application

The safener metabolites were also tested for their ability to enhance metabolism of TCM. Excised shoots of maize and wheat were treated with solutions containing [^{14}C]-TCM +/- safener or safener metabolites, applied at a concentration of 5 PPM (13µM). [^{14}C]-TCM metabolism was determined at 6 hours in both maize and wheat, with three biological replicates per sample. A detailed description of the method can be found in section 2.5. Chromatograms of herbicide metabolism were obtained for each treatment with TCM and demethylated TCM levels were quantified for maize and wheat separately.

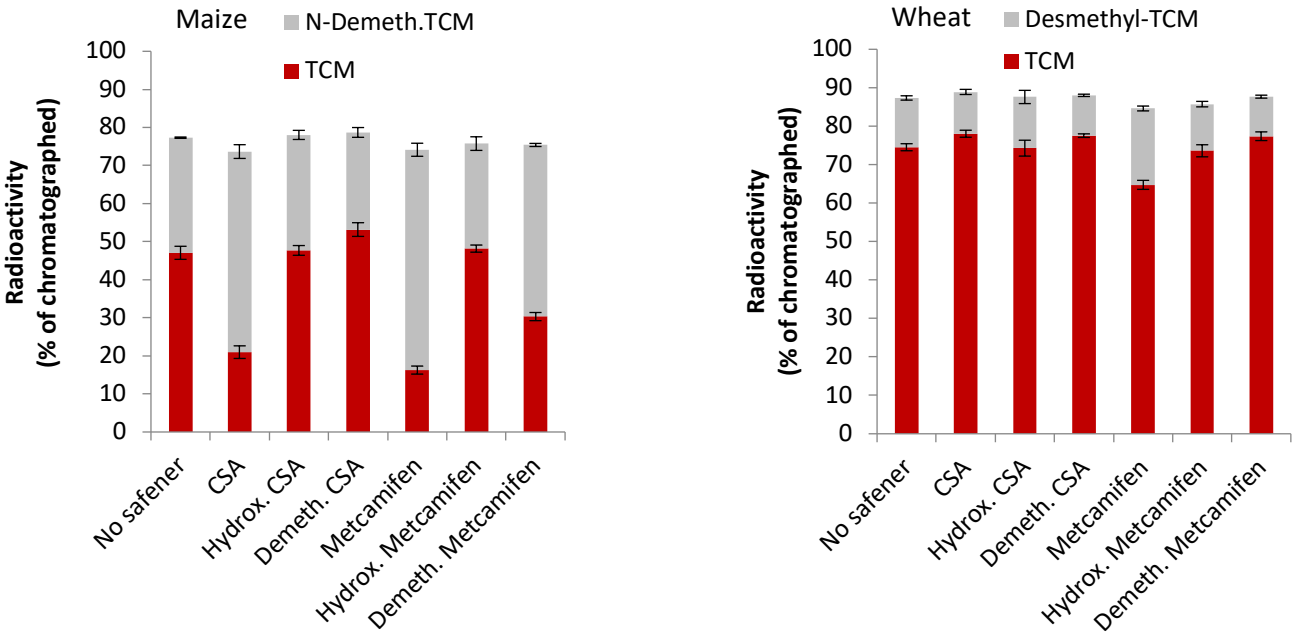


Figure 30: Effects of CSA and metcamifen metabolites on [^{14}C]-TCM metabolism at 6 hours in maize and wheat shoots – Radio-HPLC. The levels of the parent (red) are visualised along the levels of the primary inactive *N*-demethylated metabolite (grey). ^{14}C-TCM, safeners and safener metabolites were administered through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted for 3 hours before plants were transferred to water before harvest at specified timepoints. Error bars represent standard deviations (n=3).

As observed in the seed treatment experiment (Figure 29), only the application of CSA, metcamifen and demethylated metcamifen was able to increase the disappearance of parent TCM in maize (Figure 30). It is noteworthy that demethylated metcamifen was not as effective as the parent compound. As seen from the overall radioactivity levels, the loss of TCM was equivalent to the increase in demethylated TCM (Figure 30). The chromatograms also revealed that CSA and metcamifen had only minor effects on the levels of other detected

metabolites (data in Appendix). CSA metabolites and hydroxylated metcamifen did not enhance the metabolism of TCM in maize.

Wheat underwent very low levels of TCM metabolism by 6 hours, which made the determination of safening effects challenging (Figure 30). Metcamifen, which had been shown to be active in wheat in greenhouse trials and previous [^{14}C]-TCM metabolism assays (Chapter 3), was the only compound that reduced, albeit by a little, the levels of [^{14}C]-TCM at 6 hours. Though it might have been more informative to test the effect of the CSA metabolites at a later timepoint, the trials in maize suggested it would be unlikely to observe any related safening as CSA metabolism is very low in the crop (Figure 18).

Together, these results confirmed the previous results from the seed treatment experiments in maize (Figure 29), where CSA, metcamifen and demethylated metcamifen increased [^{14}C]-TCM metabolism in contrast to CSA metabolites and hydroxylated metcamifen (Figure 30). Along with the observations from the greenhouse trial of the metabolites carried out by the Safener Biology team at Bayer, it appeared that CSA did not retain safening activity upon metabolism. On the contrary, the primary metabolic steps of CSA, hydroxylation and demethylation, seemed to inactivate the safener.

5.3.3 Safener metabolite uptake and translocation

To further confirm that CSA metabolites did not have safening activity, the possibility that CSA metabolites were not translocated to upper tissues of the plant needed to be excluded. Although this was unlikely to occur, since the compounds were administered through excised shoots or seed treatment, the polar nature of the metabolites could limit their translocation. Therefore, the uptake and translocation of CSA and metcamifen metabolites in upper, middle and lower tissues of maize and wheat shoots was assessed. The uptake and translocation of safener metabolites was quantified by LC/MS in maize and wheat at 3 hours (end of the treatment). 10 wheat plants and 5 maize plants were pooled per treatment and incubated in solution containing the safener metabolites (composition described in section 2.5.1) for 3 hours in a climate chamber, as described in section 2.2.2. Following treatment, the plants were dissected into upper, middle and lower tissues (as in section 4.9), and the compounds extracted in 80% acetonitrile (section 2.5.3.1). Replicates were not included in this study. The safener metabolites were dissolved in 80% acetonitrile before being added to the phosphate

buffer solution to a final concentration of 5 PPM (13 µM). Quantification of the compounds was performed by the use of standard curves prepared by 3x serial dilutions from the acetonitrile stock solutions as diluted in 80% acetonitrile. Compounds were also diluted in plant extract from untreated plants for better matrix determination.

As seen in Figure 31, all safener metabolites translocated to all tissues of maize and wheat plants presumably through xylem transport, providing a clear indication that hydroxylated and demethylated CSA as well as hydroxylated metcamifen had no safening activity. The

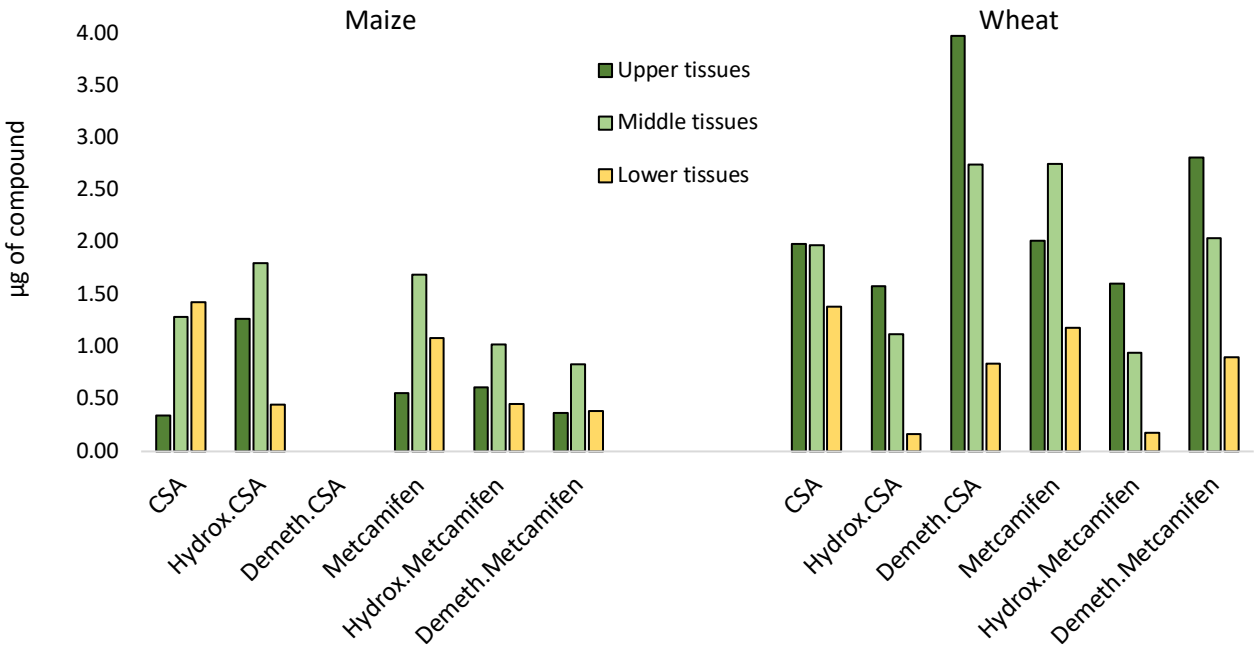


Figure 31: Uptake and translocation of CSA, metcamifen and their primary metabolites at 3 hours in maize and wheat shoots – LC/MS. Safeners and safener metabolites were administered through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted for 3 hours before plants were harvested.

hydroxylated metabolites of both safeners shared a similar translocation profile in the tissues of each crop, which was also true for the demethylated metabolites. Higher overall quantities of all compounds were detected in wheat due to increased uptake compared to maize, which was also observed during data collection for previous excised shoot experiments in the study (section 3.4, 4.5). Demethylated CSA was not detected in maize which was probably due to an experimental error during treatment rather than a result of non-uptake. The same compound displayed the highest translocation in wheat. This experiment was not repeated due to insufficient amounts of safener metabolites.

Despite the inactivity of CSA metabolites, the correlation between CSA metabolism and CSA activity in maize remained. This pointed to the metabolisation of CSA being potentially

important in safener activity, rather than the metabolite itself. Being a complex biochemical process, safening could be dictated by both the timing and the localisation of molecular events leading to it. Another possibility could be that enhanced CSA metabolism in maize was a consequence of its detoxification being induced by CSA, resulting in its metabolism. This has been demonstrated before with fenclorim, which was able to upregulate the detoxification enzymes that consequentially metabolised it (Brazier-Hicks et al., 2008). CSA's metabolism therefore needed to be explored further, temporally and spatially, in order to identify its role in safening.

5.4 TCM metabolism compared to CSA metabolism

Since CSA metabolism had been associated with TCM detoxification, it was interesting to investigate whether CSA metabolism preceded TCM metabolism. This could provide supporting evidence for the existence of a signalling event kickstarting TCM detoxification. In order to correlate these results with the previous TCM and CSA metabolism studies, the compounds were administered through excised shoots.

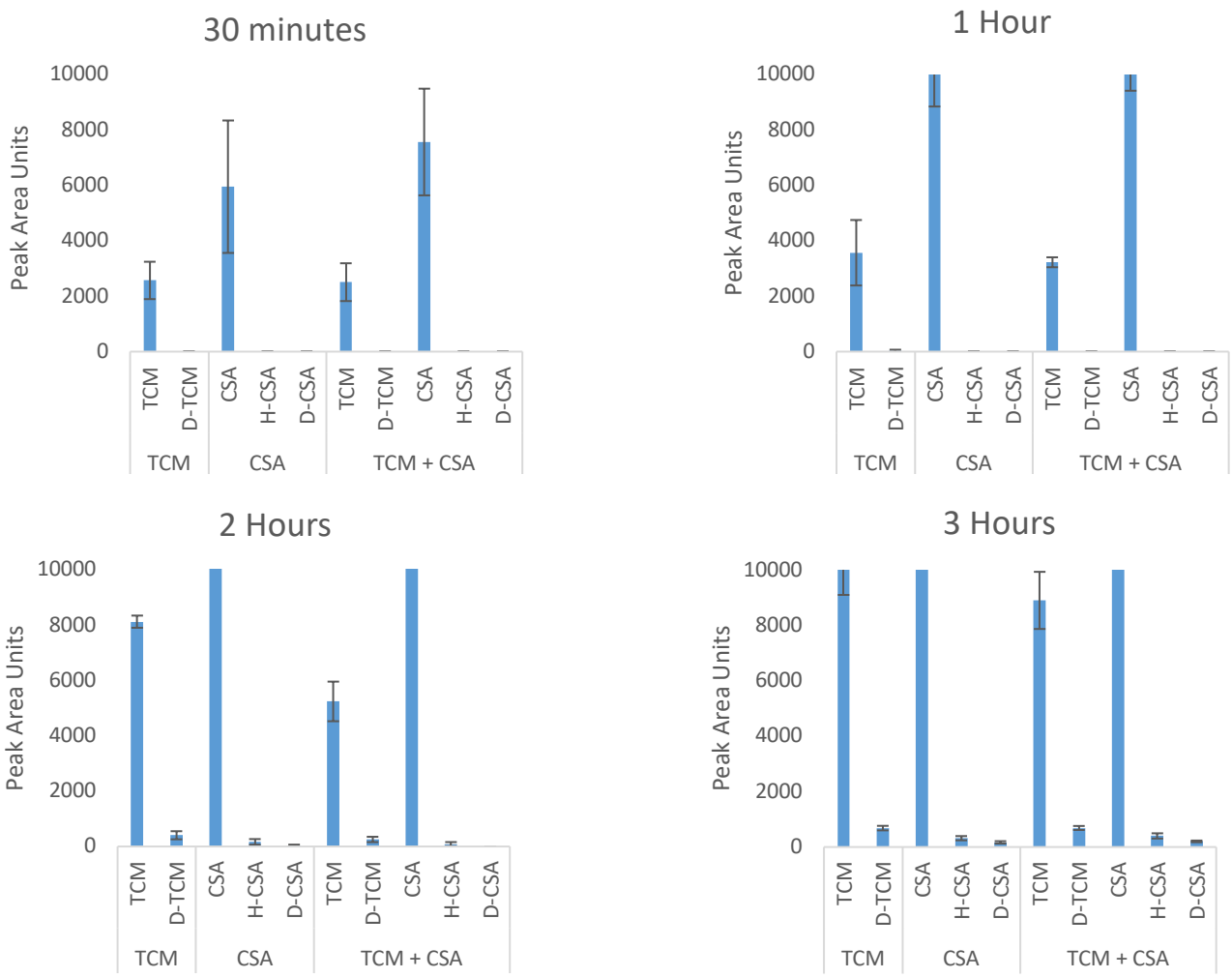


Figure 32: Rates of TCM and CSA metabolism over the course of 3 hours in maize shoots – LC/MS. The compounds were applied either on their own or in co-application. through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted until plants were harvested. Error bars represent standard deviations (n=3).

The metabolism of CSA and TCM was examined in maize shoots using LC/MS analysis at 30 minutes, 1, 2 and 3 hours. The excised shoots were treated with CSA, TCM and CSA + TCM by incubation and the primary metabolites quantified, including demethylated TCM, hydroxylated CSA and demethylated CSA. Every treatment had 3 replicates with 3 maize plants pooled per biological replicate (section 2.5.1). TCM and CSA were dissolved in 80% acetonitrile before being added to the phosphate buffer solution to a final concentration of 5 PPM (13 µM). The compounds were extracted in 80% acetonitrile, as described in section 2.5.5.1. LC/MS analysis was performed as described in section 2.5.5.2. To ensure that the compounds and their metabolites were all ionised adequately, four different analytical protocols using different ionisation modes were tested, with the selected method being the most representative of the results obtained from radiolabelled studies (Appendix). The conditions of the selected analytical method were described in section 2.5.5.2.

As expected, TCM and CSA were detected in higher quantities at later timepoints (2 and 3 hours), indicating higher uptake over time (Figure 32). At 30 minutes and 1 hour, none of the primary metabolites were detected, in neither single treatments, nor through co-application. After 2 hours, all primary metabolites could be detected, which positively correlated with the time-course experiment of CSA metabolism, where related metabolites first appeared after 2 hours (Figure 19). At 3 hours, the quantity of all metabolites increased, being similar in all treatments. These results suggested that TCM and CSA were metabolised at the same time, without mutually antagonistic or synergistic effects on the metabolism of each compound at the same timepoints. Based on these results, the question about the involvement of CSA metabolism in safening could not be resolved.

5.5 GSTL1 induction upon CSA and metcamifen treatment in maize and wheat

Linked to their ability to enhance herbicide metabolism, safeners are able to induce the transcript expression of detoxification genes (Davies and Caseley, 1999, Riechers et al., 2010, Kraehmer et al., 2014a). Since TCM and CSA's metabolism started at the same time, the effect of CSA on relative transcript expression of a safener inducible biomarker was quantified, in an attempt to see if it preceded the appearance of CSA metabolites. Transcript expression was quantified in both maize and wheat shoots. In addition, metcamifen treatment was also included in order to test whether transcript induction of the biomarkers correlated with the safener activity of the compound. The biomarker for maize was *ZmGSTL1* since the induction of this gene upon CSA treatment was confirmed in Chapter 4. Furthermore, *ZmGSTL1* was reported by other studies as a safener inducible biomarker (Hershey and Stoner, 1991). In wheat, the expression of *TaGSTL1*, an orthologue of *ZmGSTL1* was analysed. *TaGSL1* was also reported by literature to be safener inducible and involved in flavonoid metabolism (Theodoulou et al., 2003, Dixon and Edwards, 2010). The induction of the two genes was tested at early timepoints, as described in the CSA metabolism study (See section 4.6). CSA and metcamifen were applied by incubating excised shoots for 3 hours before transferring plants to fresh water. Every sample consisted of three biological replicates, each comprising 3 pooled crop plants.

RNA extraction, cDNA synthesis, qPCR and determination of transcipt induction (–ΔΔCt method) were performed as described in sections 2.6.2-5. Compounds were dissolved in DMSO before application (stocks of 13mM) and applied at a final concentration of 5 PPM

(13 µM) through excised shoots of crop plants (Section 2.5.1). The relative transcript expression of *ZmGSTL1* and *TaGSTL1* is presented in Figure 33.

In maize, the induction of *ZmGSTL*1 expression with both CSA and metcamifen started at 1 hour post exposure. CSA treatment significantly increased *ZmGSTL1* expression in almost all timepoints after 1 hour. The increase ranged from 2.3 to 3.5 times compared to the expression at time 0. The expression of *ZmGSTL1* in the control treatment remained unchanged at all timepoints. Metcamifen induced a stronger expression of *ZmGSTL1* than CSA, starting with an 8.6-fold increase at 1 hour and reaching 48-fold at 6 hours. The control was also upregulated at that time, 4 times more compared to the beginning of the experiment (0 minutes), being stable at previous timepoints.

After CSA treatment in wheat, the transcript expression of *TaGSTL1* was transiently increased, starting at 15 minutes and decreasing after 2 hours. It was interesting that the

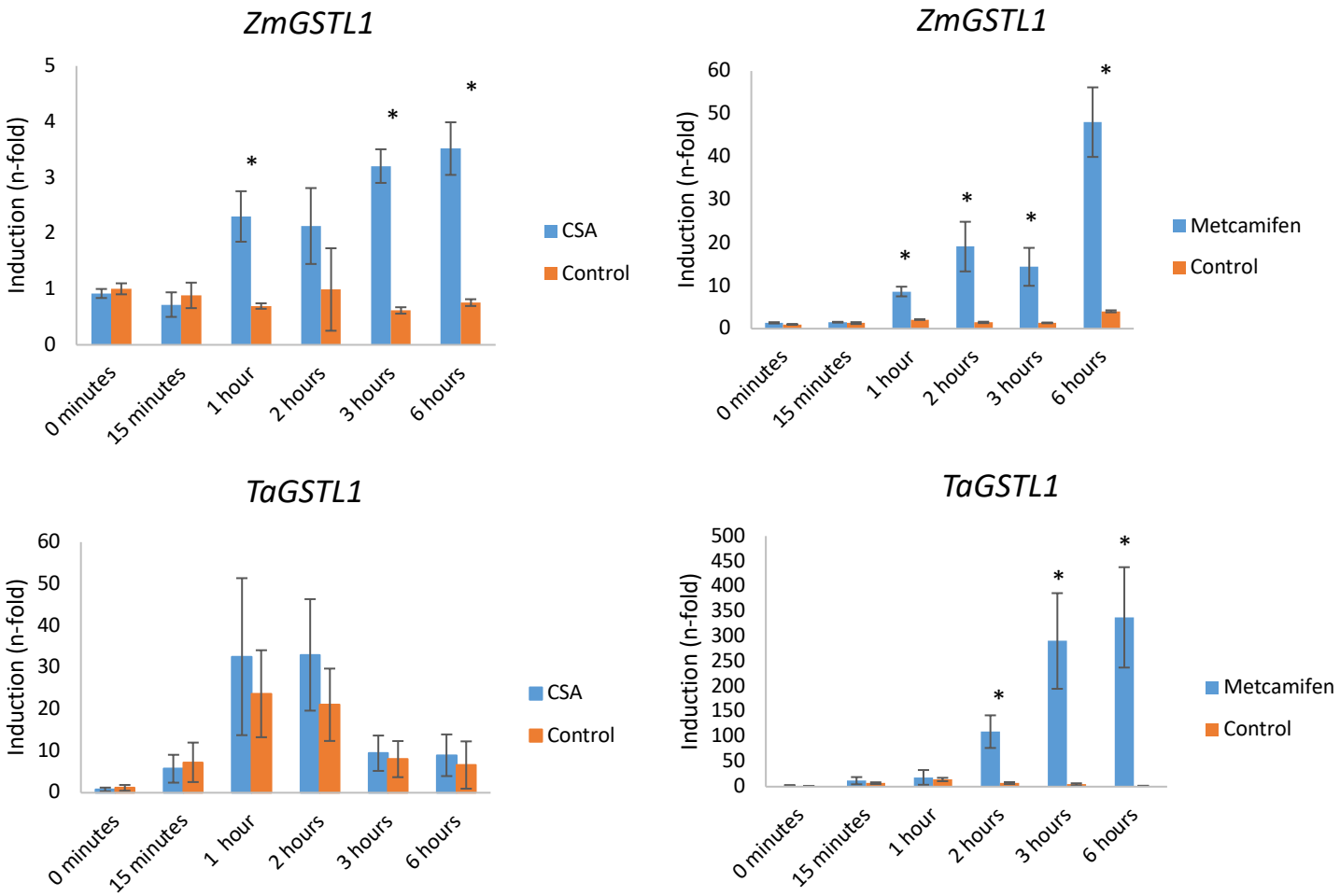


Figure 33: **Transcript expression of *ZmGSTL1* and *TaGSTL1* over the course of 6 hours after CSA and metcamifen treatment in maize shoots and wheat shoots – RT-qPCR**. Stars indicate significant differences between control and safener treatments ($p \leq 0.05$). Control = treatment with phosphate buffer (1 mM, pH=7). through excised shoots when plants were 7 days old (BBCH 11-12). Treatment lasted for 3 hours before plants were harvested. Error bars represent standard deviations (n=3).

transcript expression of *TaGSTL1* was not significantly different between CSA and control treatments. These results suggested that *TaGSTL1* could be a wounding-inducible gene which would explain the upregulation in the control treatment (excised shoots application). A similar trend was observed after metcamifen application in wheat, as well. The transcript expression started to increase after 15 minutes in both control and metcamifen treatments. The relative expression of *TaGSTL1* was increased 109-fold after 2 hours and 337-fold after 6 hours, compared to the expression levels at the start of the treatment (0 hours). The expression of *TaGSTL1* was transiently induced in the control treatment as previously observed. These results suggest that *TaGSTL1* could be induced by both wounding and safener treatments. On the contrary, *ZmGSTL1* seemed to be specifically induced by safener treatment, as reported by Hershey and Stoner (1991).

There are two important messages from the graphs in Figure 33: *ZmGSTL1*'s expression in maize was induced by both safeners starting at 1 hour, while *TaGSTL1*'s was only differentially induced by metcamifen starting at 2 hours. Transcript induction of both biomarkers was only observed in safener-crop combinations where the safener had displayed activity (both safeners in maize and only metcamifen in wheat).

When the transcript expression pattern of *ZmGSTL1* was compared to the CSA metabolism profile in maize (Figure 19), the expression of *ZmGSTL1* started to increase at 1 hour, preceding the appearance of both primary metabolites of CSA (Figure 34). Although the [^{14}C]-CSA metabolism study presented in Figure 19 was not replicated, the appearance of the metabolites at 2 hours had also been observed in a replicated LC/MS study (Figure 32). These results suggested that CSA metabolism was not temporally involved in safening as CSA was able to induce a safener biomarker prior to its metabolism. Even though the involvement of CSA metabolism in safening seemed unlikely, to provide unequivocal proof of its non-involvement would require a link between the expression and the enzymatic function of GSTL1 over time. This could be challenging as GSTL1 in corn and soybean has been demonstrated to have no GST activity even though it binds glutathione (McGonigle et al., 2000) which has also been observed for recombinant TaGSTL1 (the wheat ortholog) (Theodoulou et al., 2003). As discussed in Chapter 4, the function of lambda class GSTs has yet to be fully determined (Lallement et al., 2014).

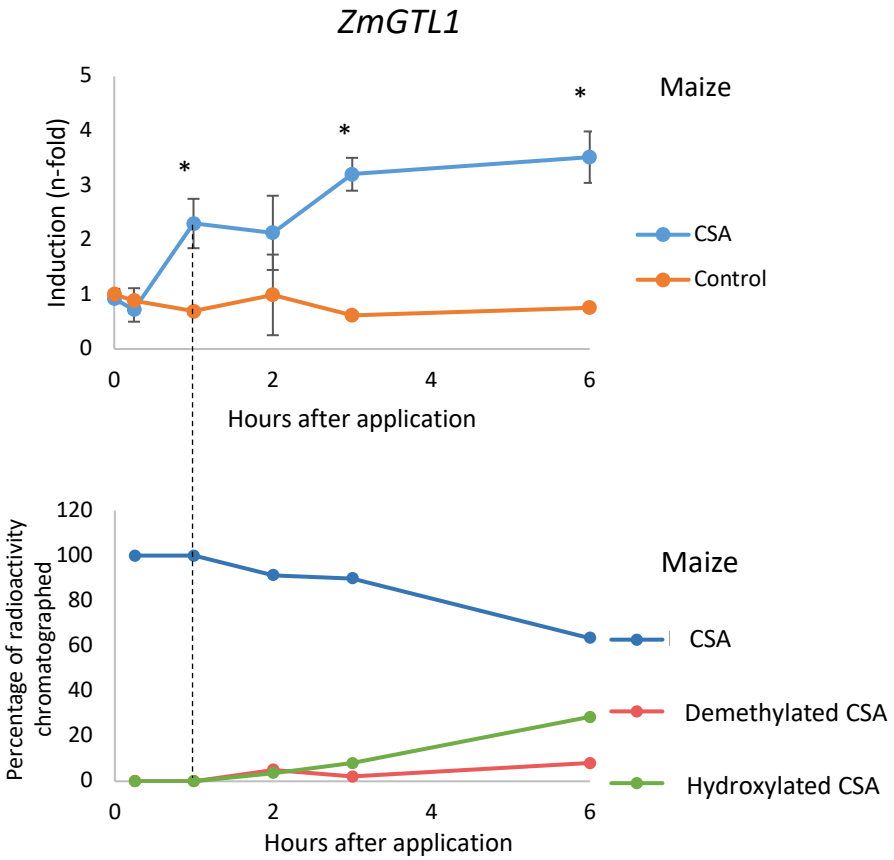


Figure 34: Transcript expression of *ZmGSTL1* after CSA treatment (Top: RT-qPCR) and rate of [^{14}C]-CSA metabolism (Bottom: Radio-HPLC) in maize shoots over 6 hours. Stars indicate significant differences between control and safener treatments ($p \leq 0.05$), with error bars representing standard deviation (n=3). This is a combination of parts from figures 19 and 33.

5.6 Translocation study of CSA/Metcamifen in maize over 72 Hours

The spatial involvement of CSA metabolism in safening was investigated in maize, where the greatest metabolism of this safener was observed. The timing between the metabolism of CSA and TCM had been established in section 5.4 (Figure 32), but this did not provide any information regarding the localisation of their metabolites, partly because compound application was done by incubation of excised shoots.

CSA and metcamifen translocation was studied by LC/MS in maize at 24, 48 and 72 hours in order to establish the timing of metabolite appearance as well as their translocation. This study explored later timepoints as the compounds were applied by microdroplet application,

where associated uptake is not very rapid. This method of application, as explained in section 4.2, was selected to simulate foliar uptake and translocation of CSA after a spray application, accounting for the variability of cuticle permeability. Metcamifen was included in this study to observe how it compared with CSA in terms of systemicity. The safeners were applied on the first leaf of maize plants (as described in section 2.5.1). Maize seedlings were harvested

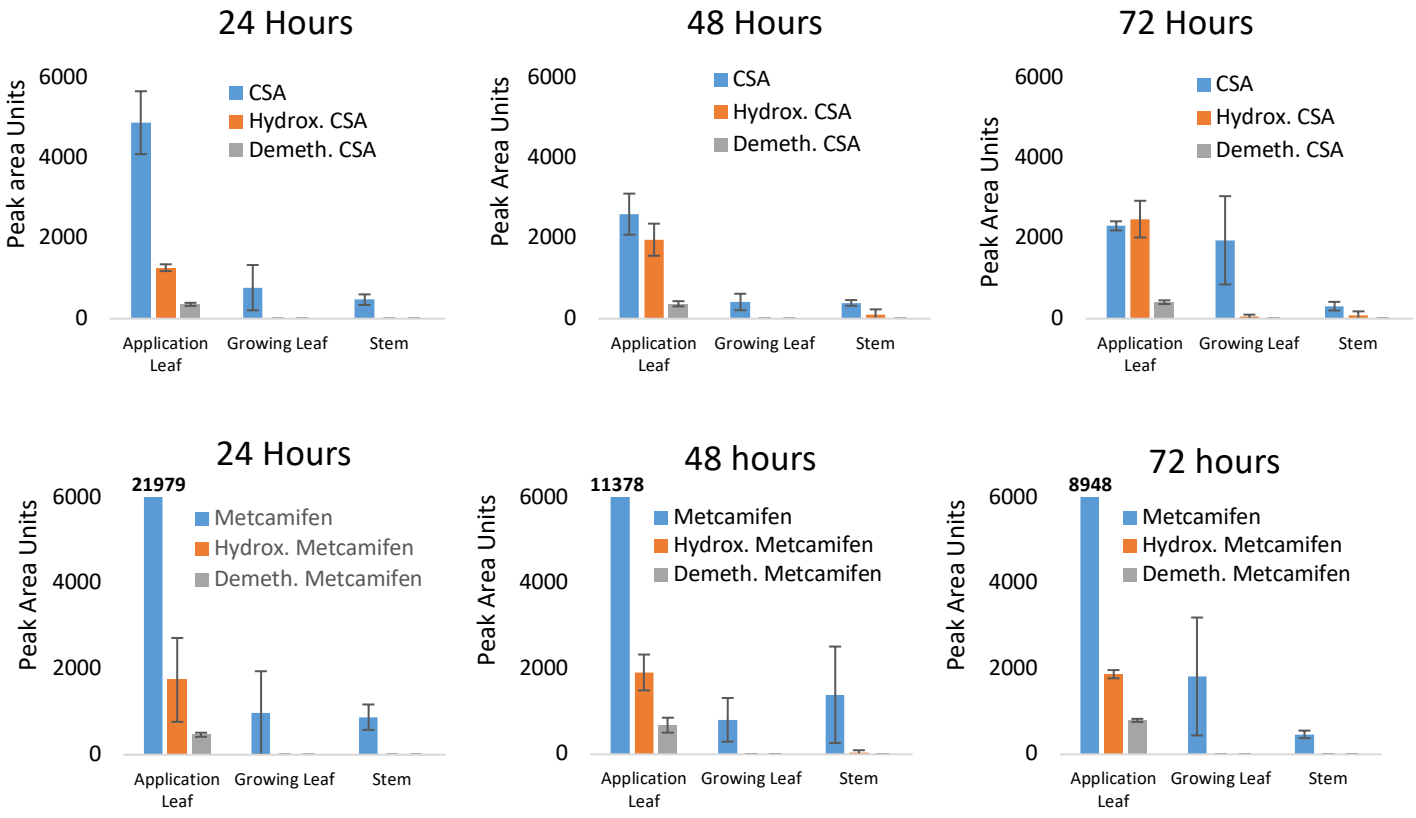


Figure 35: Translocation of CSA, metcamifen and their associated primary metabolites over the course of 72 hours in maize shoots – LC/MS. Safeners were applied on the first leaf of the plants by droplet application. In the graphs with metcamifen, the levels of the parent have been noted in bold above the peaks. Error bars represent standard deviation (n=3).

and segregated into three tissues: i) application leaves (local), ii) growing leaves (systemic) and iii) stem. Three biological replicates were included per sample, each one comprising three individual maize plants. Extraction and LC/MS analysis were performed as described in section 2.5.5. The detected quantities of safeners and their primary metabolites are presented in Figure 35.

CSA was detected in all types of tissues from maize plants at 24 hours whereas its primary metabolites, hydroxylated and demethylated CSA, were only detected in the application (local) leaf. At 48 hours, CSA levels decreased in the application leaf, while its content in other tissues remained stable. Again, the primary CSA metabolites were detected only in the

application leaf. At 72 hours, CSA levels decreased further in the application leaf and increased in the growing (systemic) leaf, with the primary metabolites mainly detected in the application leaf. Hydroxylated CSA levels increased steadily over 72 hours, whereas the levels of demethylated CSA remained unchanged after 24 hours. These results demonstrate that the reduction of CSA did not translate into an equivalent increase of primary metabolites. It is noteworthy that since this study was performed over three days, it is possible that part of the parent (CSA) compound had been metabolised to more polar secondary metabolites, such as conjugated derivatives.

Metcamifen displayed a similar translocation pattern as observed with CSA treatment (Figure 35). At 24 hours, the parent compound could be detected in all tissues, with primary metabolites detected only in the application (local) leaf. This trend was extended to later timepoints (48 and 72 hours). While metcamifen levels decreased overtime, the levels of hydroxylated metcamifen remained unchanged and the levels of demethylated metcamifen were slightly increased. The behaviour of metcamifen metabolites was different from the CSA metabolites, which suggested that different rates of safener metabolism are potentially due to the two safeners upregulating different detoxification enzymes. As with CSA, the decrease of the parent compound levels could not be attributed to conversion into primary metabolites.

Taken together, these results indicate that both safeners were mobile, while their metabolites remained localised in the local leaf over 72 hours. To further obtain information on the high spatial resolution metabolic profile of TCM and CSA in *planta*, a Matrix-Assisted Laser Desorption Ionisation (MALDI) Mass Spectrometry Imaging (MALDI Imaging) study was conducted in collaboration with Michael Kubicki (Technische Universität, Dortmund, Germany).

5.7 Matrix-Assisted Laser Desorption Ionisation (MALDI) Mass Spectrometry Imaging (MSI)

The translocation profiles of CSA and its metabolites were established using traditional analytical approaches (autoradiography, HPLC, LC/MS) in the previous experiments. Although these approaches provide important information, they cannot assess the spatial distribution of target compounds. In recent years, mass spectrometry imaging has been used to study the spatial distribution of natural compounds in plants, as a non-invasive and semi-

quantitative method to visualise spatial profiles of the target molecules inside the plant tissues, with high spatial (< 10 μm) and mass resolution (Kaspar et al., 2011, Boughton et al., 2016). In order to employ mass spectrometry imaging as an approach to study the mechanism of CSA safening, the following work was conducted in collaboration with Michael Kubicki (Technische Universität, Dortmund, Germany) who performed sample preparation and imaging with MALDI technology. The information for experimental design including time-course, compound concentration, and application method were derived from this thesis.

Having demonstrated the inactivity of CSA metabolites, the main question was whether the site of CSA metabolism correlated with enhanced TCM metabolism in maize. To answer this, TCM were applied by foliar microdroplet application on the same and on different leaves of second leaf stage maize seedlings (BBCH Scale: 12) and MALDI MSI was used to obtain spatial distribution images of TCM and CSA metabolism overlaid on leaf morphology images at different times (3 hours to 7 days) after application. These combined images pinpointed CSA's site of metabolism, detailed kinetics and possible interactions with the herbicide TCM and its metabolism. It was of particular interest to investigate whether safening, the enhancement of TCM metabolism in this case, could be induced by CSA systemically from a distant tissue. This would support a model where CSA safening is induced by a mobile signal that doesn't involve herbicide and safener co-localisation, utilising signalling cascades such as waves of calcium or ROS signalling, which has been reported as able to induce xenobiotic detoxification genes in plant responses to abiotic stress (Zhu, 2016).

Each treatment (same leaf or separate leaves) included one biological replicate per timepoint. The samples were scanned once and pixels of every scan were mapped to ion masses in order to create a pixelated distribution of the target compound, then overlaying the scanned area of the leaf. The target compounds in the scans were detected with different adduct ions, which gave an indication of the tissue of detection (H^+: leaf surface, K^+, Na^+: inside of the leaf). This distinction was devised experimentally with a number of different compounds prior to this study (Michael Kubicki personal comm.) and has also been demonstrated by other MSI studies, where it was suggested that the adducts' locations are indicative of their respective roles (e.g. K^+ 's role in metabolism)(LEIGH and WYN JONES, 1984, Mullen et al., 2005, Gerbig et al., 2015).

The target compounds of interest were:

- Cyprosulfamide (CSA): $C_{18}H_{18}N_2O_5S$, Mol. Weight: 374.41
- Hydroxylated Cyprosulfamide: $C_{18}H_{18}N_2O_6S$
- Demethylated Cyprosulfamide: $C_{17}H_{16}N_2O_5S$
- Thiencarbazone-methyl (TCM): $C_{12}H_{14}N_4O_7S_2$
- *N*-Demethylated Thiencarbazone-methyl: $C_{11}H_{12}N_4O_7S_2$

The time course experiment revealed that differences between treatments in terms of TCM metabolism were present after 4 days, so only these scans have been included in the figures (Figure 36, Figure 37); the rest have been included in the Appendix.

Based on the preliminary data, two observations were of particular interest. The signal intensity of demethylated TCM in the same leaf application (Figure 36) seemed to be higher than the different leaf application. Considering that parent compound TCM was adequately absorbed in both treatments and was detected along with its primary metabolite in the tissue, this suggested a positive correlation between co-application of TCM and CSA and increased TCM metabolism.

The second interesting observation was given by the distribution profile of CSA metabolism in different treatments (Figure 37). In the different leaf applications, CSA did not translocate to the TCM application leaf, which positively correlated with the absence of enhanced TCM metabolism. Demethylated CSA was the only compound able to translocate from the CSA application leaf to the leaf where TCM was applied, though previous experiments had shown it was not able to elicit safening (Figure 29, Figure 30).

As mentioned previously in this section, more controls and replicates will be needed to draw conclusions from this experiment. The method needs to be optimised further in order to resolve discrepancies with previous observations from HPLC and LC/MS studies, regarding the quantity of hydroxylated CSA as well as the mobility of CSA. Nevertheless, these studies can give a new dimension in the interactions of TCM and CSA where they can shed light into how these compounds affect each other's metabolism. The data so far indicate that demethylated metabolites are more mobile in maize plants and co-application of TCM and CSA enhances TCM metabolism. Metabolites also tend to be localised in different patterns than parent compounds, but replication is needed before any further conclusions can be

drawn. These results provide a new lead in safener metabolism studies and point towards a model where TCM metabolism is enhanced locally by CSA, indicating that there is no systemic signalling involved in CSA safening.

Future work will require more replicates and control treatments. Data reported here constitute a preliminary analysis and any conclusions derived are suggestions based on what has been observed so far. It is also worth noting that MALDI MSI is a semi-quantitative method and absolute pixel-to-pixel quantification is challenging. The same applies to the penetration depth after laser irradiation, as it is challenging to determine the exact penetration depth of the leaves. However, multiple scans of the same leaf area have given an approximate depth profile for single measurements, which was found to be very similar among different samples and measurements (personal communication with Michael Kubicki).

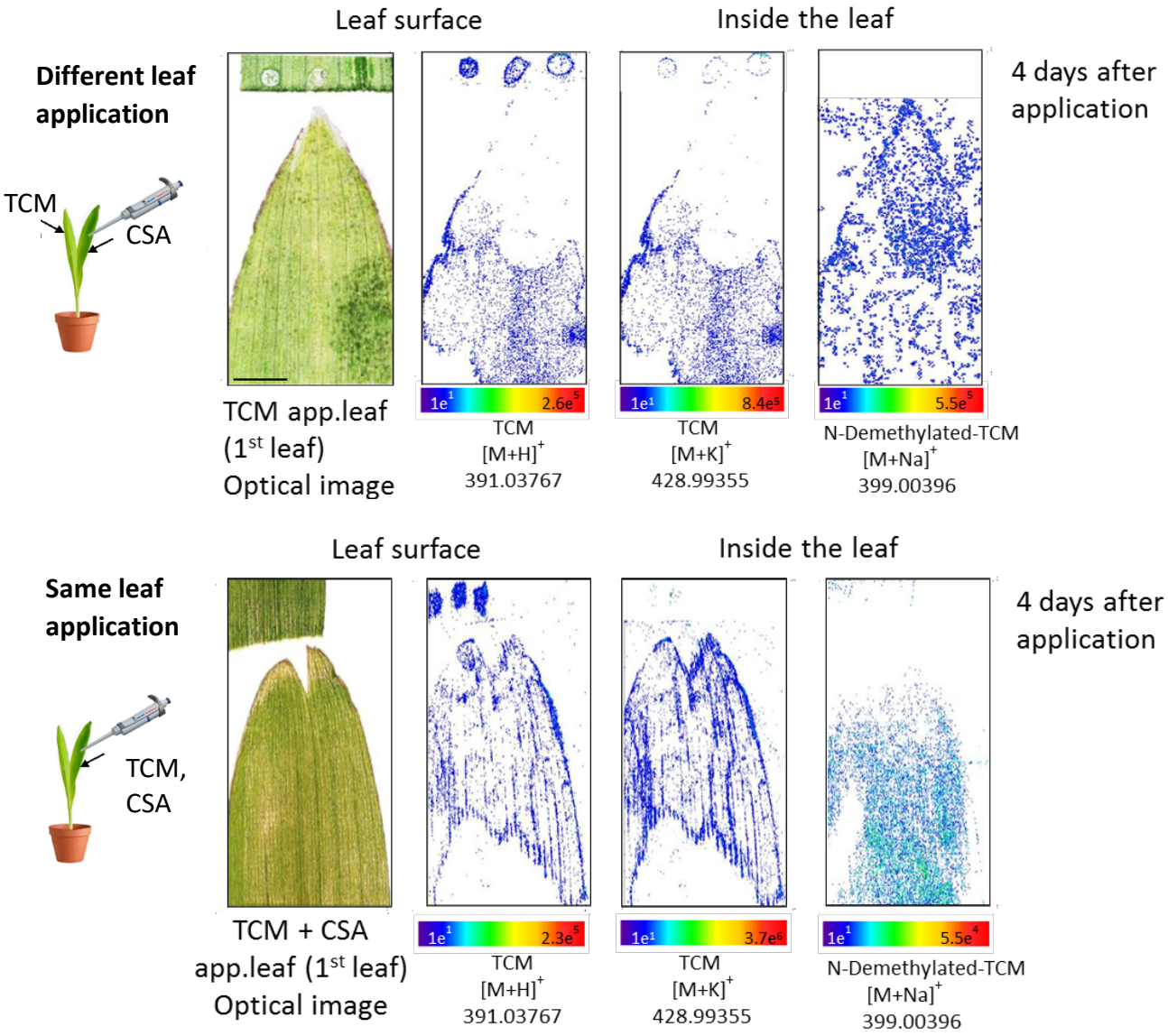


Figure 36: MALDI Imaging of the TCM treated leaf (different leaf application) and TCM + CSA treated leaf (same leaf application) after 4 days visualising TCM metabolites. Scans showing the optical image of the leaf as well as distribution profiles of target compounds (TCM + metabolite) on the surface (non-absorbed) and inside the leaf (absorbed). The non-absorbed and absorbed compounds are differentiated by their adducts, where $+H^+$ is an adduct representative of the surface of the leaf whereas $+Na^+$ and $+K^+$ are representatives of the tissues below the leaf surface. The numbers on the rainbow scales indicate the intensity of the least and most intense pixel in that scan. Below the target compound's name its adduct and molecular mass can be found. 'On tissue' calibration spots on an untreated part of a control plant (as seen on the second scan from the top) give an idea on pixel intensity: 200 PPM (left), 500 PPM (middle) and 1000 PPM (right) acetone/water (1:1, v/v).

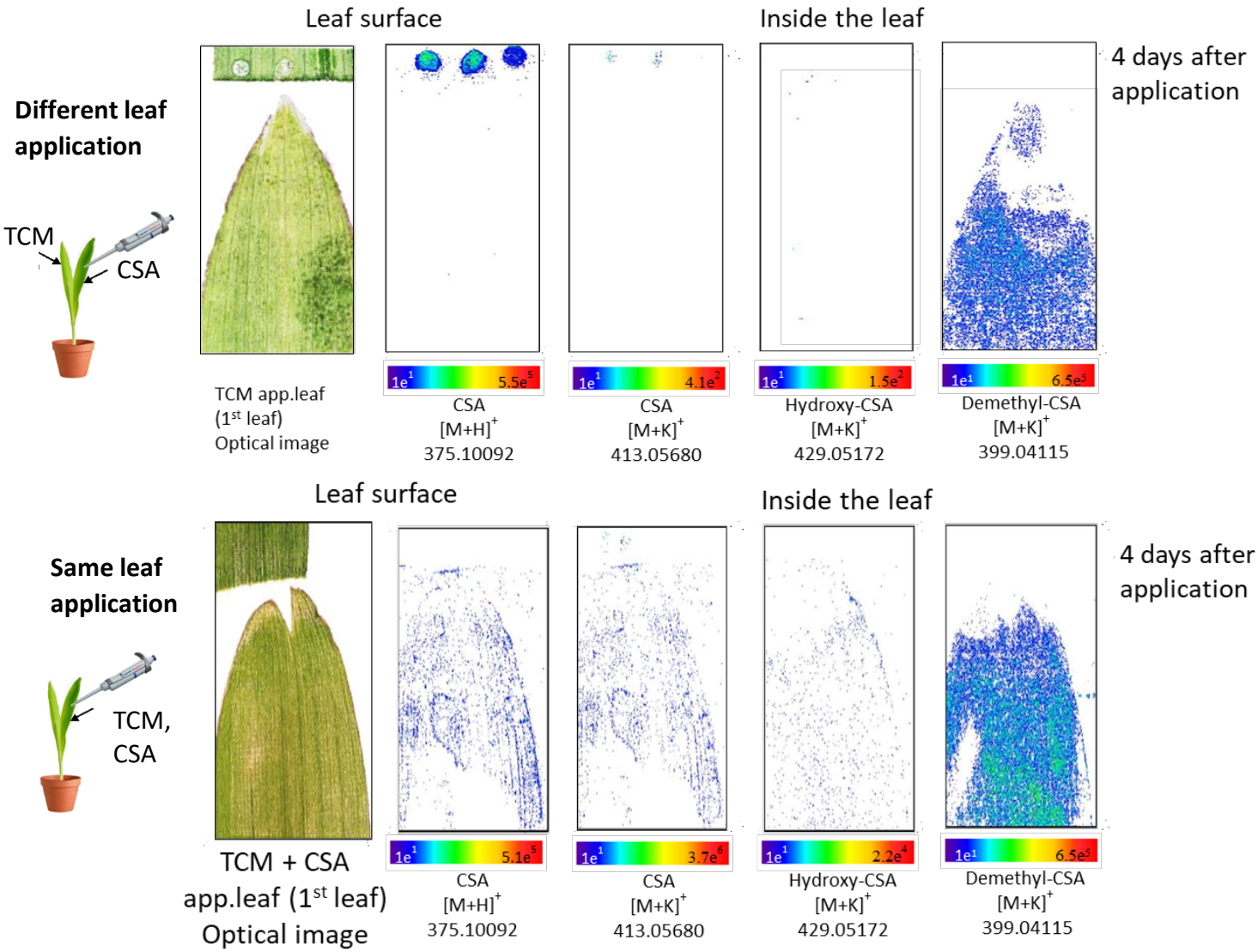


Figure 37: MALDI Imaging of the TCM treated leaf (different leaf application) and TCM + CSA treated leaf (same leaf application) after 4 days visualising CSA metabolites. Scans showing the optical image of the leaf as well as distribution profiles of target compounds (CSA + metabolites) on the surface (non-absorbed) and inside the leaf (absorbed). The non-absorbed and absorbed compounds are differentiated by their adducts, where $+H^+$ is an adduct representative of the surface of the leaf whereas $+Na^+$ and $+K^+$ are representatives of the tissues below the leaf surface. The numbers on the rainbow scales indicate the intensity of the least and most intense pixel in that scan. Below the target compound's name its adduct and molecular mass can be found. 'On tissue' calibration spots on an untreated part of a control plant (as seen on the second scan from the top) give an idea on pixel intensity: 200 PPM (left), 500 PPM (middle) and 1000 PPM (right) acetone/water (1:1, v/v).

5.8 Discussion

This chapter focused on dissecting the positive correlation between CSA activity and metabolism. Primary metabolites of CSA and metcamifen were tested for safening activity in

a herbicide greenhouse trial and in a TCM metabolism study. Both CSA metabolites and hydroxylated metcamifen provided no safening effect in either experiments. In contrast, the parent compounds and demethylated metcamifen provided safening in both experiments. Even though the basis of the pro-drug hypothesis seemed to be valid since demethylated metcamifen could elicit safening, it could not explain the selectivity of CSA in maize. This was supported by a safener uptake LC/MS study, showing that all metabolites could translocate to all tissues of maize and wheat shoots. These experiments clearly demonstrated that CSA, unlike other modern safeners such as cloquintocet-mexyl, mefenpyr-diethyl and isoxadifen-ethyl (Taylor et al., 2013, Jeschke, 2016b), was inactivated upon its metabolism since none of its primary metabolites retained safening activity. This process was partly mediated by CYP81A9 (Figure 22). Despite the fact that CSA metabolites were inactive, CSA's metabolism still correlated positively with its activity, suggesting that either CSA was inducing its own metabolism like the safener fenclorim (Brazier-Hicks et al., 2008), or the process of its metabolism was in fact responsible for triggering the safener response. To elucidate the latter, the relationship between CSA's metabolism and safening had to be characterised both temporally and spatially.

The temporal involvement of CSA metabolism in safening was further explored by assessing the timing of TCM metabolism. An LC/MS study with excised maize shoots, showed that both TCM and CSA were metabolised at the same time which suggested that CSA metabolites might not be required to trigger TCM metabolism. Combined with the fact that CYP81A9 partly mediated both TCM and CSA's metabolism (Figure 22, Figure 26), it was likely that both compounds are treated as xenobiotics and are detoxified through the same mechanisms. This hypothesis has been proposed before but has not been unequivocally proven (Riechers et al., 2010). Finally, the transcript expression profile of a detoxification biomarker, *ZmGSTL1*, showed that the expression of *ZmGSTL1* was elevated prior to the appearance of CSA metabolites. Taken together, these results indicate that safening effects are likely to be mediated by CSA rather than its metabolites.

To further elucidate the role of CSA metabolism in safening, the uptake and translocation of CSA and its metabolites through different parts of the shoot was analysed. The parent compound (CSA) was more mobile than its metabolites since it could be detected in other tissues apart from the treated leaf, over the course of 72 hours. These results further highlight an important function of CSA in mediating a safening effect in maize.

The major drawback of conventional analytical approaches is that they cannot provide information on spatial distribution of target compounds in situ. To overcome this challenge,

mass spectrometry imaging (MSI) with MALDI ionisation technology was applied, through a collaboration with Michael Kubicki, so the spatial distribution of TCM, CSA and their metabolites could be studied. The preliminary results from MSI showed that the CSA was capable of enhancing TCM metabolism when the compounds were applied on the same tissue, while CSA metabolites seemed to not have any role in TCM metabolism. This suggested that an MSI approach has potential in terms of studying the underlying mechanisms of safener selectivity in a non-invasive way. MALDI MSI is an upcoming technique in plant science able to combine both protein and metabolite profiling (Kaspar et al., 2011) and has been employed previously for the imaging of pesticides (Mullen et al., 2005, Anderson et al., 2010). The combined analysis of data generated by traditional analytical methods and by MSI can provide novel insights in the understanding of safening mechanisms in different crop species.

Chapter 6 – Final discussion

6.1 Overview

Chemical weed control is an essential component of crop protection, currently responsible for protecting approximately 1/4 of the global crop production (Oerke, 2005). However, challenges such as herbicide resistant weeds, limited herbicide discovery and strict registration regulations for new chemicals put pressure on the sector (Lamberth et al., 2013), especially in regions such as Europe where the use of GM crops and gene editing technology such as CRISPR is restricted.

Herbicide safeners are a group of diverse agrochemicals used to address selectivity issues of broad-spectrum herbicides by selectively protecting the crops of interest without compromising weed control. Crop safety is mainly ensured by the enhancement of herbicide metabolism through the upregulation of detoxifying xenobiotic enzymes such as CYPs and GSTs, but the mechanism of induction is yet to be determined. Safeners are highly selective, being effective only in monocot crops without having significant activity in dicots and weeds. This selectivity is often present among monocot crops as well, with different safener chemistries commercialised between maize, rice and cereals (Rosinger and Schulte, 2019). This is an important aspect of safeners that is not often investigated, with only limited information contained in patents of safener chemistries (Pallos, 1979, Howe and Lee, 1980).

To improve the understanding of safener selectivity in monocots, this thesis utilised cyprosulfamide (CSA), a commercial aryl sulfonamide safener with specific activity in maize, and metcamifen, a close structural analogue which has activity in both maize and wheat. Since a small structural change could elicit a broader activity profile, the studies presented in this thesis aimed at answering the following question: ‘What constitutes CSA’s selective action?’

The activity of CSA was tested in maize, wheat and soybean in greenhouse trials, where it selectively protected maize against herbicides TCM and TBT. This correlated with CSA enhancing TCM detoxification specifically in maize. In contrast to CSA, metcamifen protected both maize and wheat from the same herbicides, which also correlated with

enhanced TCM metabolism in both crops. Soybean displayed no safening effects with either safener in the greenhouse, which was also associated with no enhanced TCM metabolism.

The basis of CSA's differential activity was further investigated in maize and wheat by assessing its uptake, distribution and metabolism. Uptake and distribution of CSA were limited in maize compared to wheat, which could not account for its protective activity. Its metabolism, however, was more rapid in maize than in wheat, with hydroxylated CSA correlating very closely with activity. Early CSA and metcamifen metabolites were synthesised and tested for activity in greenhouse trials and TCM metabolism assays, but were all inactive with the exception of demethylated metcamifen.

Gene expression studies with CSA showed that the safener inducible biomarker *ZmGSTL1* was upregulated before CSA metabolism in maize, indicating that CSA metabolism was unlikely to be important in mediating the safening response. Subsequent gene expression studies of *ZmGSTL1* and its wheat ortholog *TaGSTL1* showed that the genes were significantly induced with both CSA and metcamifen in maize and only with metcamifen in wheat.

To explore the role of CSA metabolism in safening in more detail, Matrix Assisted Laser Desorption Ionisation (MALDI) Imaging Mass spectrometry was performed in maize, where CSA and TCM were applied on the same and on different leaves. Preliminary results suggested that co-localisation of CSA and TCM was important for the enhancement of TCM metabolism, indicating that the safening response is mediated in a non-systemic way. The MALDI MSI study was carried out in collaboration with Michael Kubicki (TU Dortmund), where he carried out sample preparation and imaging.

6.2 Conclusions and future studies

The results presented above suggested that CSA's selective action in maize depended on two components: (1) tolerance against the applied herbicide due to enhanced metabolism and (2) detection and induction of the appropriate detoxification enzymes in a non-systemic way.

Evidence for the first point can be provided by the studies in soybean and Merit, the CYP81A9 mutant line. As discussed in Chapter 3, TCM was metabolised by *N*-demethylation, a reaction usually mediated by CYPs. Maize and wheat showed endogenous

N-demethylation of TCM, which was enhanced by the addition of safeners (Figure 13). Safening was absent in both soybean and Merit, as both were unable to produce *N*-demethylated TCM (Figure 11, Figure 26). The lack of an appropriate CYP (CYP81A9 in the case of Merit) is highly likely to account for the lack of *N*-demethylation and possibly even safening, in both soybean and Merit. Apart from the fact that the lack of safening in dicots has been attributed to the lack the appropriate enzymes such as CYPs (Behringer et al., 2011), the CYP81A subfamily seems to be unique in monocots (Nelson et al., 2004) and has been associated with herbicide tolerance in maize, rice and *Echinocloa phyllopogon* (Zhang et al., 2007a, Iwakami et al., 2014, Liu et al., 2015). The study in *E. phyllopogon* in particular, showed that CYP81A12 and CYP81A21 were associated with resistance against ALS-inhibiting herbicides, could mediate O-demethylation in both herbicide resistant *E. phyllopogon* plants and yeast microsomes, and their expression could provide herbicide tolerance in transgenic *A.thaliana* and be manipulated by a single unidentified trans-acting element (Iwakami et al., 2014). It is therefore very tempting to speculate that a safener-responsive dicot crop could be engineered by the transgenic expression of a CYP from the 81A subfamily under the control of a safener-responsive promoter such as that of ZmGSTL1. It is also possible that the function of this kind of CYP could also extend further than just the ability to metabolise herbicides, possibly involving the activation/inactivation of plant secondary metabolites. This was highlighted by the lack of safening in Merit against flufenacet (Figure 24), a herbicide detoxified by GST conjugation (Bieseler et al., 1997). Accordingly, CYPs have been reported to have many functions involving oxygenation reactions activating plant secondary metabolites such as terpenoids and cyanogenic glucosides (Hamberger and Bak, 2013), with the latter being recently associated with the safener signalling pathway (Baek et al., 2019). It would also be interesting to test the use on selective herbicide chemistries in soybean such as pyroxasulfone (Kraehmer et al., 2014b). Since in this case soybean would have intrinsic tolerance to the herbicide, this could directly indicate whether the safener signalling is functional in the crop. Even though safeners for dicot crops have not been found despite many efforts by industries (Kraehmer et al., 2014b), it has recently been shown that the use of insecticides like thiamethoxam and phorate could alleviate pyroxasulfone induced injury in soybean and clomazone injury in cotton respectively, providing promising leads for weed control in those crops (Ferhatoglu et al., 2005, Steppig et al., 2018).

The second point mainly relates to the action of CSA in wheat. CSA did not protect wheat from herbicide injury at normal application rates and did not enhance TCM metabolism. In

contrast to soybean, however, wheat had a level of intrinsic tolerance to TCM, being able to produce the *N*-demethylated metabolite with the addition of a safener, a process which was enhanced by metcamifen (Figure 13). It was also shown that CSA in maize and metcamifen in both maize and wheat mainly enhanced the rate of *N*-demethylation for TCM, with no major effects on other metabolites (Figure 13). Taken together, these results suggested that CSA in wheat is either not detected or it fails to sufficiently induce the enzyme(s) responsible for *N*-demethylating TCM, or a combination of both. Interestingly, the safener dose response studies in wheat and soybean suggest a lack of enzyme inducibility rather than a complete lack of CSA detection (Section 3.3). While alterations in TCM and CSA application rates and ratios did not produce any safening effects in soybean, the wheat variety Joyau displayed some slight safening effects with CSA when it was applied at a high rate combined with a low rate of TCM. These safening effects were not consistent, however, as they were not observed in other wheat varieties (Figure 9). Similar slight safening effects have also been reported in monocot weeds with a range of safeners (Hulesch and Dutka, 1993, Jablonkai and Hulesch, 1996, Cummins et al., 2009) and with CSA (Leonie et al., 2017, Rosinger and Schulte, 2019), with no practical effect in weed control. A greenhouse trial in maize, wheat and various monocot weeds testing the effect of CSA against various herbicide chemistries could potentially give an indication on whether CSA's protective effects in wheat and monocot weeds depend on the dose of its application. The sporadic and variety dependent CSA effects in wheat (Figure 9) also raised the question whether the ability of CSA safening could be selected through breeding. An interesting target for testing the safening effects of CSA would be wheat cultivar SR3, that has an elevated ROS content and is tolerant to salinity stress due to the epigenetically enhanced expression of TaCYP81D5 which accelerates ROS scavenging (Wang et al., 2019). Apart from the importance of CYPs in CSA's selective action (discussed above), parallels have been drawn between oxidative and safener signalling, as well as between ABA and CSA signalling (Behringer et al., 2011, Dashevskaya et al., 2013).

Studies in CSA metabolism in maize, wheat and soybean (Figure 17) could also support the above conclusions about CSA's selective action in maize. Wheat was able to produce the same CSA metabolites as maize, but not in the same quantity. Soybean on the other hand, did not metabolise CSA at all. According to the metabolic profiles of CSA in maize and cultivar Merit (Figure 22), CSA was rapidly hydroxylated in maize and CYP81A9 was partly responsible for this hydroxylation. The different metabolic profiles of CSA in maize, wheat and soybean suggest that the substrates of xenobiotic detoxifying enzymes differ between the crops, with maize and wheat being more similar than soybean. Since CSA in maize was

shown to induce detoxifying enzymes prior to being metabolised (Figure 34), its rapid metabolism could suggest that CSA is able to induce this process in maize but not in wheat. Safeners flurazole and fenclorim have also been suggested to be able to induce their own metabolism, as they could induce GST activity and were conjugated with glutathione in maize and *A.thaliana* respectively (Breaux et al., 1989, Brazier-Hicks et al., 2008). The LC/MS study on metcamifen metabolism (Figure 21) also supported a link between metcamifen's metabolism and activity, since wheat was able to metabolise it more rapidly than CSA, albeit not as rapidly as maize. These results further highlighted the importance of sufficient induction of enzymes with appropriate substrates in the selective action of CSA. Future studies should focus on characterising xenome detoxifying enzymes by identifying their herbicide substrates and establishing their induction after safener application, which would provide links between enzyme induction and functional safening of various safener chemistries.

Another interesting observation from the studies in Chapter 5, was that even though CSA was inactivated rapidly (Figure 22), it could lead to rather long-lasting effects as it protected from TCM injury in the greenhouse (Figure 29). While it is possible that this effect was manifested because of the different application methods used (incubation of excised shoots vs foliar spraying application), it could also suggest that a transient activation of the detoxification machinery is sufficient from maintaining herbicide concentrations below the damaging concentration threshold. This is indirectly suggested by early publications on safeners (Kőmíves and Hatzios Kriton, 1991, Hatzios, 1991) that report that effective safeners are applied either prior or together with their herbicide partners. Even though this needs to be further investigated, safeners could act as transient primers of herbicide metabolism, which could be a form of plant defence priming. Priming has been suggested to involve a combination of appropriate defence genes, plant secondary metabolites and epigenetic modifications (Ramírez-Carrasco et al., 2017), so it is tempting to link it to another complex biochemical process such as safening. Establishing a link would require to identify TCM's damaging concentration threshold and monitor TCM and CSA metabolism over time, which would reveal whether the safening effect of CSA is maintained beyond its complete detoxification.

Some insights in the events upstream of herbicide and safener metabolism can be provided by the gene expression studies on *ZmGSTL1* and *TaGSTL1*. While metcamifen induced both genes, CSA selectively induced *ZmGSTL1* in maize, highlighting that CSA's selective action was also present at the transcriptional level. The selective induction of lambda GSTs by

specific safeners has been reported before in maize, wheat and *A.thaliana* (Hershey and Stoner, 1991, Dixon et al., 2002a, Theodoulou et al., 2003, Skipsey et al., 2011). As discussed in Chapter 5, *TaGSTL1* was also transiently induced by wounding in the control treatment due to the application method (incubation of excised shoots), while this was not the case with *ZmGSTL1* (Figure 33), which has been demonstrated induced by benzenesulfonamides but not wounding (Hershey and Stoner, 1991). The subtly different mechanisms of induction of *ZmGSTL1* and *TaGSTL1* could indicate different regulatory motifs in their genes' promoters such as as-1 elements. Interestingly, a study using the promoter of *ZmGSTL1* to study the safening signalling cascade in *A.thaliana* by reporter gene analysis, identified through serial deletions two as-1 elements both contributing to the expression of the reporter gene (Behringer et al., 2011). These elements can be activated by a variety of chemicals and signalling molecules (Xiang et al., 1996) and their introduction to promoter sequences, disruption or cytosine methylation can completely alter responsiveness and regulation of associated plant defence genes (Kanazawa et al., 2007, Pape et al., 2010, Koster et al., 2012). Another study has demonstrated that the promoter of *CYP81D11*, a safener inducible CYP from *A.thaliana* also containing as-1 elements, is activated by both the xenobiotic and the jasmonic-acid signalling pathways in an interdependent manner (Koster et al., 2012). It is therefore plausible that regulatory elements play an important role in the induction of *ZmGSTL1*, *TaGSTL1* and other xenobiotic detoxification genes by CSA and metcamifen, possibly leading to functional safening. While ZmGSTL1 and TaGSTL1 have not been linked to functional safening, their well-documented induction by safeners makes them good models for studying the signalling networks involved in their activation, which could potentially help in the identification of an upstream regulator for safener signalling. With the rapid advancements in crop genome sequencing, it would be of interest to perform whole transcriptome sequencing in maize and wheat after CSA treatment or in wheat after CSA/metcamifen treatment to identify more targets potentially important for safening. After characterising these genes, more insights could be gained by performing deletion series experiments on their promoter sequence to gain further understanding on their mechanism of induction.

6.3 Model for the action of CSA

A potential model for the action of CSA (and safeners in general) based on the results presented above and the current literature, can be summarised in Figure 38. CSA is able to selectively induce xenobiotic detoxifying enzymes, which are able to detoxify both CSA and TCM, leading to TCM tolerance. CYP81A9 partly mediates both CSA and TCM detoxification, but it's not known whether it's induced by CSA. Even though it was attempted to quantify its transcript expression after CSA and metcamifen application in maize, its low abundance made it challenging (data not shown). There is also the possibility that CYP81A9 may interact with a potential receptor or interfere in a different way with the safening response induced by CSA. This was highlighted by the fact that CSA application couldn't alleviate flufenacet injury in Merit (CYP81A9 mutant), which is normally detoxified by GSTs. CSA's selective activity is both dependent on enzymes with the necessary substrates to detoxify TCM and the ability to induce those enzymes following CSA perception by the crop. The preliminary data from MALDI MSI (Figure 36, Figure 37) suggest that the events leading to CSA enhancing TCM metabolism occur in a non-systemic way.

The studies with CSA in wheat demonstrated that there is some degree of selectivity regarding the upstream events of the safening response, but the mechanism leading to the induction of the relevant genes is largely unknown. Since a lot of safener inducible genes have detoxification/general stress response functions and many of them have been associated with as-1 elements (Behringer et al., 2011, Skipsey et al., 2011, Brazier-Hicks et al., 2018a, Baek et al., 2019), one plausible induction mechanism could be through the involvement of SCARECROW-like 14 (SCL14) and class II TGA factors. SCL14 is a GRAS regulatory protein identified in *A.thaliana* that's able to bind class II TGA factors at as-1 regulatory elements, and is associated with SA and xenobiotic signalling in an NPR1 independent manner (Fode et al., 2008, Gatz, 2013). Likewise, class II TGA transcription factors are also commonly associated with the induction of oxidative stress/detoxification genes (Gatz, 2013) and have been reported to be involved in the upstream events of safener signalling in *A.thaliana* (Behringer et al., 2011). While safeners have not been associated with SCL14 directly, the expression of *A.thaliana* safener inducible genes *CYP81D11* and *GSTU7* has been described to be SCL14-dependent, since the SCL14/TGA complex is bound to as-1 elements on their promoters in an uninduced state (Fode et al., 2008). This complex has been reported only in *A.thaliana* so far, but as-1 elements, TGA factors and GRAS proteins have

been identified in various crops (Fitzgerald et al., 2005, Behringer et al., 2011, Guo et al., 2017, Li et al., 2019). CSA selectivity could potentially directly involve this complex or the just the upstream events, but this remains to be determined. Experiments with SCL14 mutants could shed light as to whether the transcript expression of SCL14 associated genes by safeners depends on SCL14, as has been shown for TGA factor and SA mutants which exhibited reduced safener inducibility of the safener-responsive promoter of *ZmGSTL1* (Behringer et al., 2011).

The first signalling event following safener application is arguably the most elusive part of the pathway. It has been suggested that the safener signalling pathway, independent or not, has probably evolved as a result of allelopathy between neighbouring plants of different species or between plants and pathogens, aiming at the protection from a variety of harmful chemicals

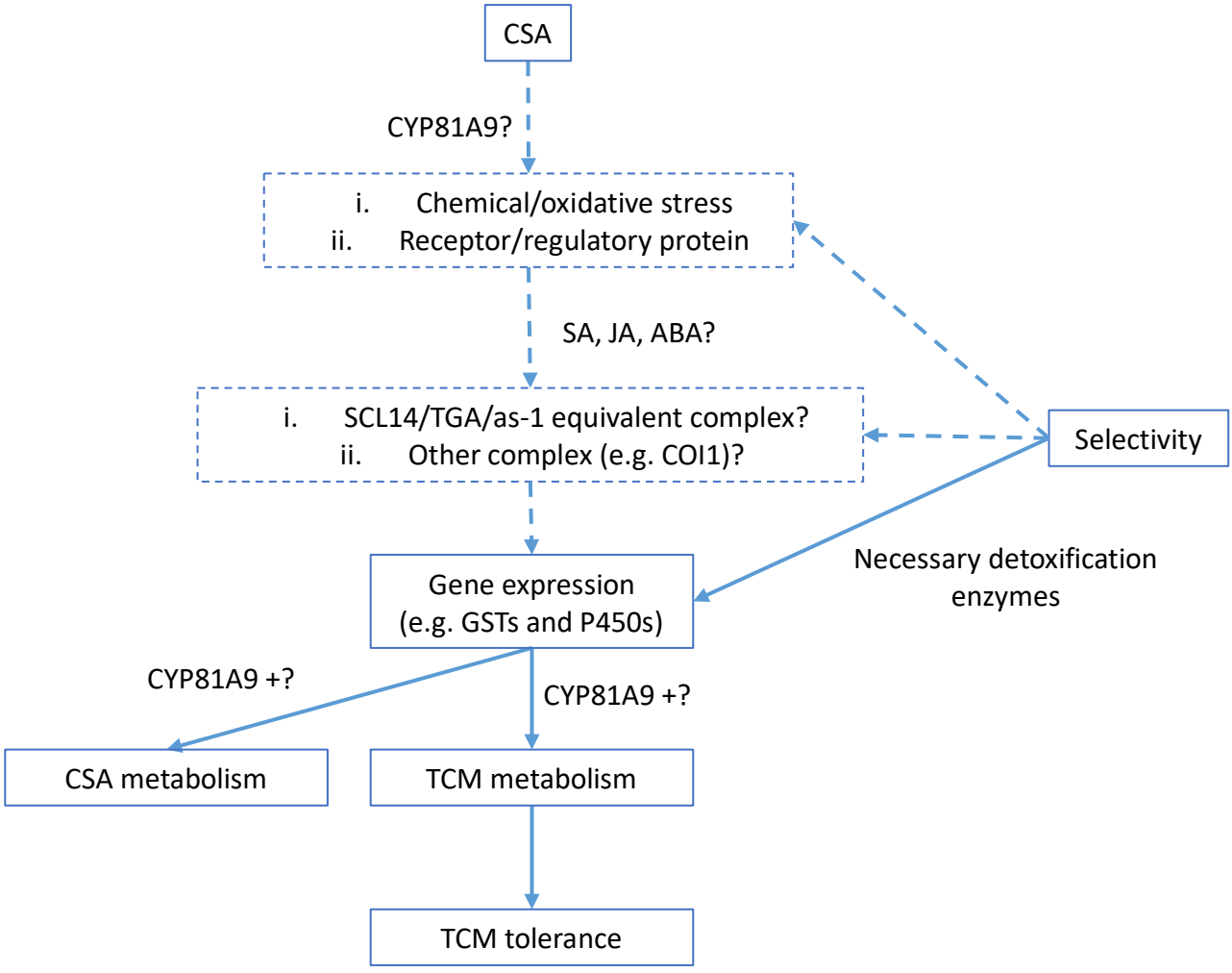


Figure 38: **Model for CSA mode of action against herbicide TCM.** Adapted from Riechers (2010). Bold lines denote characterised events, while dotted lines hypothesised ones. Abbreviations: CSA: cyprosulfamide, TCM: thiencarbazone-methyl, SA: salicylic acid, JA: jasmonic acid, ABA: abscisic acid, SCL14: SCARECROW-like 14, TGA: TGA transcription factors, as-1: activation sequence-1 regulatory elements, COI1: CORONATIVE INSENSITIVE1, CYPs: cytochrome P450s, GSTs: glutathione-S-transferases

(Fode et al., 2008). Accordingly, the genes associated with the SCL14/TGA complex are induced by a variety of chemicals such as SA, 2,4-D, benoxacor, fenclorim, benzooxazolinone, phenobarbital and 2,4,6-trinitrotoluene (TNT) (Ekman et al., 2003, Baerson et al., 2005, Fode et al., 2008, Brazier-Hicks et al., 2018a).

Oxidative stress has been suggested as a more general inducer of the SCL14/TGA complex (Fode et al., 2008, Gatz, 2013). As discussed in section 1.5.4.3, oxidative stress can cause the accumulation of oxylipins, whose associated signalling has been linked to safeners (Riechers et al., 2010, Brazier-Hicks et al., 2018b). Interestingly, transcriptome studies in *A.thaliana* have shown that while oxylipins, benzooxazolinone and fenclorim induced a similar set of genes, safeners mefenpyr-diethyl and isoxadifen-ethyl induced a fundamentally different one (Baerson et al., 2005, Behringer et al., 2011, Brazier-Hicks et al., 2018b). These studies not only suggested that safener responses encompass multiple signalling pathways (Behringer et al., 2011), but also that chemical structure strongly affects gene inducibility (Baerson et al., 2005, Brazier-Hicks et al., 2018b).

A protein recognition system has been suggested based on the effects of safener structure on the kinetics and magnitude of associated gene expression (Skipsey et al., 2011). This could also be supported by the expression patterns of *ZmGSTL1* by CSA and metcamifen as well as *TaGSTL1* by metcamifen (Figure 33), demonstrating both chemical and species specificity. It is challenging to speculate which type of specificity accounts the most for differences between the activity of the two safeners. However, it could be speculated that the urea moiety of metcamifen is necessary for its broader activity, potentially because it is not as reactive as CSA's cyclopropyl group, and could be better at forming interactions with polar aminoacids of a receptor protein (Clow, 1937, DePuy, 1973). While no safener receptor has been identified yet, most studies speculate that it would be part of a chemical sensing mechanism of broad specificity conserved between different plant species, most likely closely related to the Keap1 protein found in mammals (Baerson et al., 2005, Nguyen et al., 2009, Brazier-Hicks et al., 2018b). The observations that CSA's action is both local and rapid combined with the inactivity of its primary metabolites, suggest that mutant screens have a high chance of identifying a direct target of CSA.

6.4 Limitations and closing remarks

Some experiments in this thesis had limitations such as the lack of replication either due to lack of time, resources or to reduce the use of radioactive material. These include the studies of [^{14}C]-TCM, [^{14}C]-CSA (in maize and Merit) and metcamifen metabolism over a time course, the translocation of CSA, metcamifen and their metabolites in different tissues and the MALDI MSI studies. Most of these studies were designed to give an indication of when interesting/important effects were present, which when identified were replicated accordingly (TCM metabolism at 9 hours, CSA metabolism at 24 hours – HPLC, CSA metabolism up to 3 hours – LC/MS). Another limitation was that the qPCR studies were performed with one reference gene in maize and wheat, potentially creating some bias towards the induction profiles of the investigated genes. Finally, the MALDI study indicated that demethylated CSA was the most abundant metabolite, which was not in agreement with the previous metabolism studies with [^{14}C]-CSA. As discussed in Chapter 5, this study needs to be appropriately optimised and replicated before any firm conclusions can be drawn from it.

Despite its limitations, however, this thesis provides valuable insights in the selectivity of safeners among monocots by comparing the activity of two structurally similar safeners while focusing on multiple aspects of the safener response such as the associated phenotype, the effects on herbicide metabolism, safener fate and gene induction capability. It demonstrated that herbicides and safeners are metabolised by similar detoxification system involving the same enzymes (CYP81A9) and reported for the first time, to the author's knowledge, on the inactivation of a safener through xenobiotic detoxification (Figure 29, Figure 30).

Since the safener response appears to be manifested on many levels, it is becoming increasingly clear that the elucidation of the safener mode of action will require the use of integrated transcriptomic, metabolomic and proteomic studies as well as assessment of the phenotype in the greenhouse, safener fate studies and enzymatic assays. The characterisation of the relationship between herbicide safeners and plant signalling mechanisms appears to complex territory as demonstrated by this and other published studies (Behringer et al., 2011, Brazier-Hicks et al., 2018b, Baek et al., 2019). It can, however, lead to the design of improved safeners, which will play an important role in the future of weed control.

Appendix

TCM WHEAT, 5ppm, 0hr						
	incubation medium before incubation	uptake after incubation	vessel 80% ACN-wash	water for rinsing	25 ml crude extra	recovery %
Volume (ml)	4	0.98	5	40	25	
Bq (total)	80395.2	19696.8	446.00	594	16640.25	**92.97**
Measuring volume (µl)	50µl	50µl	100 µl	100 µl	100 µl	
Bq	1004.94	1004.94	8.92	1.49	66.561	

TCM WHEAT, 5ppm, 3hr						
	incubation medium before incubation	uptake after incubation	vessel 80% ACN-wash	water for rinsing	25 ml crude extra	recovery %
Volume (ml)	4	0.85	5	40	25	
Bq (total)	83306	17702.5	487.35	1056.8	13091	**85.84**
Measuring volume (µl)	50µl	50µl	100 µl	100 µl	100 µl	
Bq	1041.325	1041.325	9.75	2.64	52.364	

TCM WHEAT, 5ppm, 6hr						
	incubation medium before incubation	uptake after incubation	vessel 80% ACN-wash	water for rinsing	25 ml crude extra	recovery %
Volume (ml)	4	0.81	5	40	25	
Bq (total)	74073.6	14999.9	502.30	1125.4	14428.75	**111.10**
Measuring volume (µl)	50µl	50µl	100 µl	100 µl	100 µl	
Bq	925.92	925.92	10.05	2.81	57.715	

TCM WHEAT, 5ppm, 24hr						
	incubation medium before incubation	uptake after incubation	vessel 80% ACN-wash	water for rinsing	25 ml crude extra	recovery %
Volume (ml)	4	0.76	5	40	25	
Bq (total)	90583.6	17210.9	345.30	589.6	15131.75	**96.67**
Measuring volume (µl)	50µl	50µl	100 µl	100 µl	100 µl	
Bq	1132.295	1132.295	6.91	1.47	60.527	

CSA WHEAT, 5ppm, 0hr						
	incubation medium before incubation	uptake after incubation	vessel 80% ACN-wash	water for rinsing	25 ml crude extra	recovery %
Volume (ml)	4	0.87	5	40	25	
Bq (total)	84274.8	18329.8	484.00	513.2	14600	**87.50**
Measuring volume (µl)	50µl	50µl	100 µl	100 µl	100 µl	
Bq	1053.435	1053.435	9.68	1.28	58.4	

CSA WHEAT, 5ppm, 3hr						
	incubation medium before incubation	uptake after incubation	vessel 80% ACN-wash	water for rinsing	25 ml crude extra	recovery %
Volume (ml)	4	0.62	5	40	25	
Bq (total)	80171.6	12426.6	330.95	540.4	10098.5	**90.79**
Measuring volume (µl)	50µl	50µl	100 µl	100 µl	100 µl	
Bq	1002.145	1002.145	6.62	1.35	40.394	

CSA WHEAT, 5ppm, 6hr						
	incubation medium before incubation	uptake after incubation	vessel 80% ACN-wash	water for rinsing	25 ml crude extra	recovery %
Volume (ml)	4	0.67	5	40	25	
Bq (total)	88144.8	14764.3	492.45	688.4	11437.75	**88.29**
Measuring volume (µl)	50µl	50µl	100 µl	100 µl	100 µl	
Bq	1101.81	1101.81	9.85	1.72	45.751	

CSA WHEAT, 5ppm, 24hr						
	incubation medium before incubation	uptake after incubation	vessel 80% ACN-wash	water for rinsing	25 ml crude extra	
Volume (ml)	4	0.86	5	40	25	
Bq (total)	82516	17740.9	401.90	964.4	15055.5	**95.93**
Measuring volume (µl)	50µl	50µl	100 µl	100 µl	100 µl	
Bq	1031.45	1031.45	8.04	2.41	60.222	

Figure A1: Radioactivity recovery values for some of the wheat samples used in the TCM metabolism time course study (Figure 10). Radioactivity measurements of samples, solutions and apparatus were taken using a liquid scintillation counter as described in Section 2.5.4. The radioactivity recovery values have been calculated based on the ratio of the radioactivity taken up by the plants over the actual recovery value.

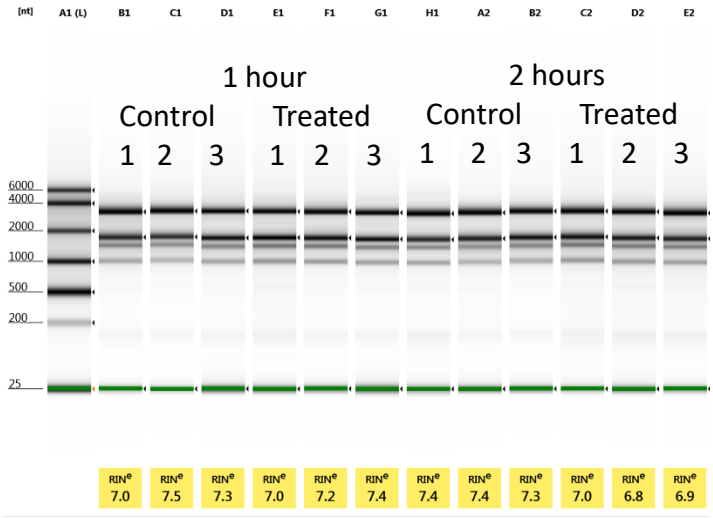


Figure A2: Indicative quality of wheat RNA samples (control and treated) represented by a gel image (top) and RIN^e values between 1-10 (bottom). The gel image is picturing 28S rRNA (~4000 nt) and 18S rRNA (~2000nt). The highest quality RNA samples are represented by value 10. The visualised samples were used in Section 5.5. The treatment was with metcamifen.

Maize

Herbicide Dose	Safener Dose	*Oldham*	*Sileno*	*Aventura*
TBT 2000 g/ha	-	43	7	40
TBT 2000 g/ha	CSA 100 g/ha	0	0	0
TBT 2000 g/ha	Metc. 100 g/ha	7	0	0
TBT 1000 g/ha	-	20	7	23
TBT 1000 g/ha	CSA 100 g/ha	0	0	3
TBT 1000 g/ha	Metc. 100 g/ha	3	7	13

Characterisation	Colour
No effect	
Slight safening effect	
Moderate safening effect	
Good safening effect	
Slight additional injury	
Moderate additional injury	

Wheat **Barley**

Herbicide Dose	Safener Dose	*Dekan*	*Montoya*	*Zzoom*
TBT 1000 g/ha	-	-	33	-
TBT 1000 g/ha	CSA 100 g/ha	-	10	-
TBT 1000 g/ha	Metc. 100 g/ha	-	13	-
TBT 500 g/ha	-	-	10	-
TBT 500 g/ha	CSA 100 g/ha	-	33	-
TBT 500 g/ha	Metc. 100 g/ha	-	23	-
TBT 200 g/ha	-	10	-	43
TBT 200 g/ha	CSA 100 g/ha	23	-	23
TBT 200 g/ha	Metc. 100 g/ha	10	-	17

Rice

Herbicide Dose	Safener Dose	*IR64*
TBT 100 g/ha	-	70
TBT 100 g/ha	CSA 100 g/ha	70
TBT 100 g/ha	Metc. 100 g/ha	63
TBT 50 g/ha	-	63
TBT 50 g/ha	CSA 100 g/ha	67
TBT 50 g/ha	Metc. 100 g/ha	50

Figure A3: Effects of CSA and metcamifen on TBT injury in various crops-Validation trial. Greenhouse trial with TBT on five crops. The numbers represent herbicide injury in %. Green highlights indicate observed safening effects, where significant ones are in bold ($p<0.05$).

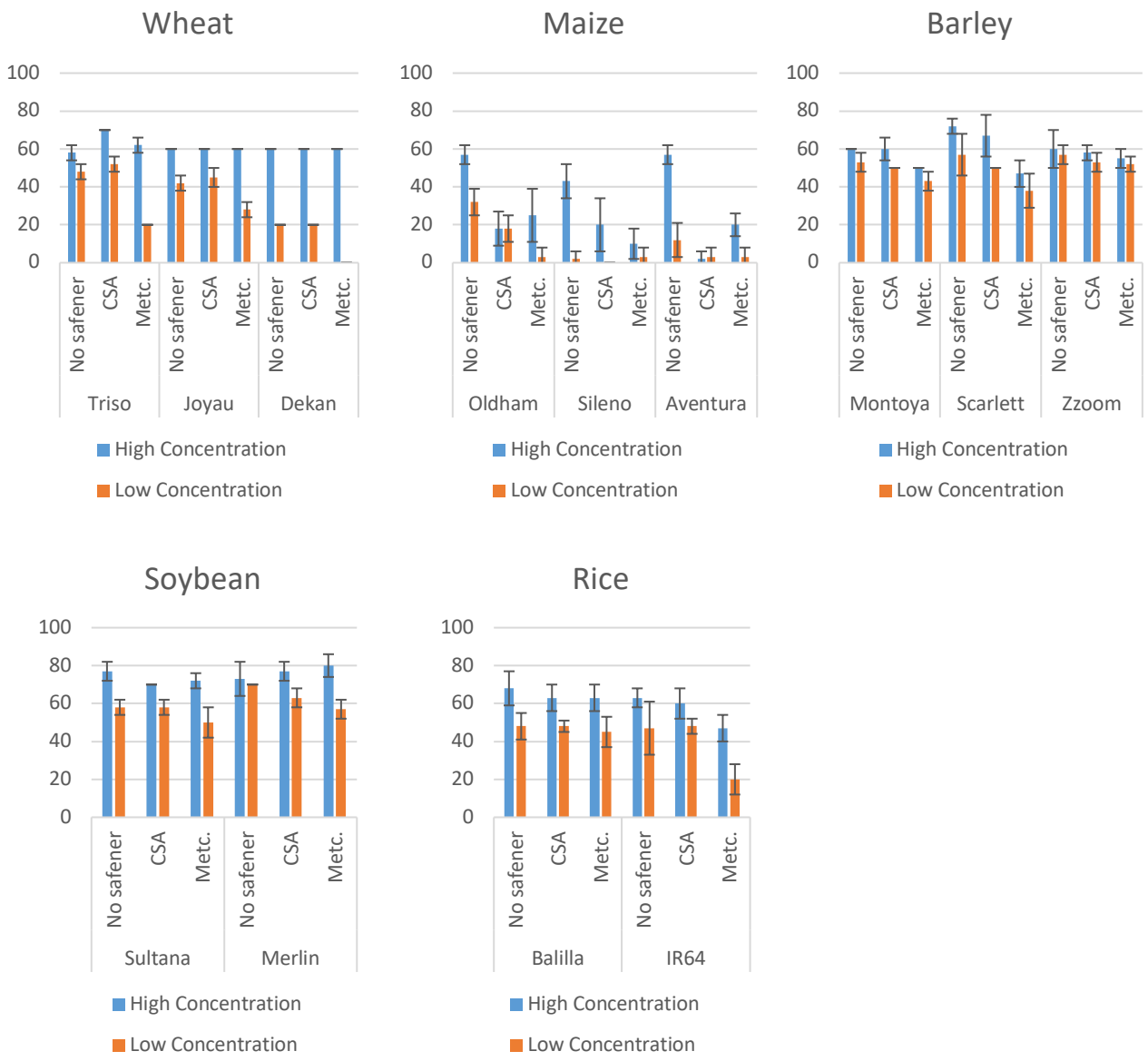


Figure A4: Effects of CSA and metcamifen on TCM injury in various crops – Bar chart of Figure 5. Greenhouse trial with TCM on five crops. Green highlights indicate observed safening effects where intensity is shown according to the colour scheme on the right. The numbers represent herbicide injury in %, scored within percentage injury categories (5-10%), accompanied by the standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). TCM concentrations used – Wheat, maize, barley: 50 g/ha (high), 5 g/ha (low). Soybean, rice: 20 g/ha (high), 5 g/ha (low).

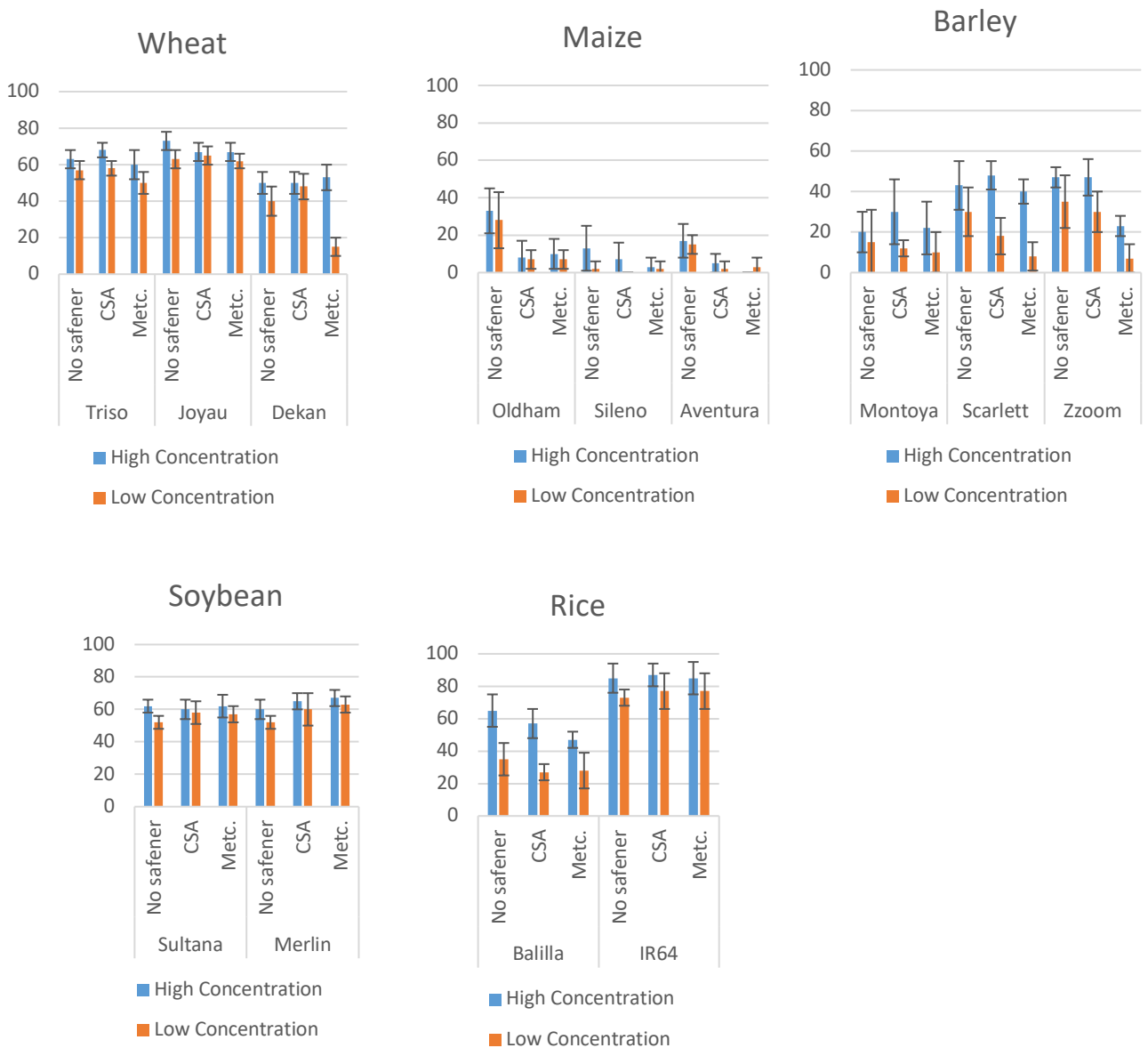


Figure A5: Effects of CSA and metcamifen on TBT injury in various crops. Greenhouse trial with TBT on five crops. Green highlights indicate observed safening effects where intensity is shown according to the colour scheme on the right. The numbers represent herbicide injury in %, scored within percentage injury categories (5-10%), accompanied by the standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). TBT concentrations used - Maize, 600 g/ha (high),400 g/ha (low). Wheat, barley, rice: 400 g/ha (high), 200 g/ha (low). Soybean: 20 g/ha (high), 5 g/ha (low).

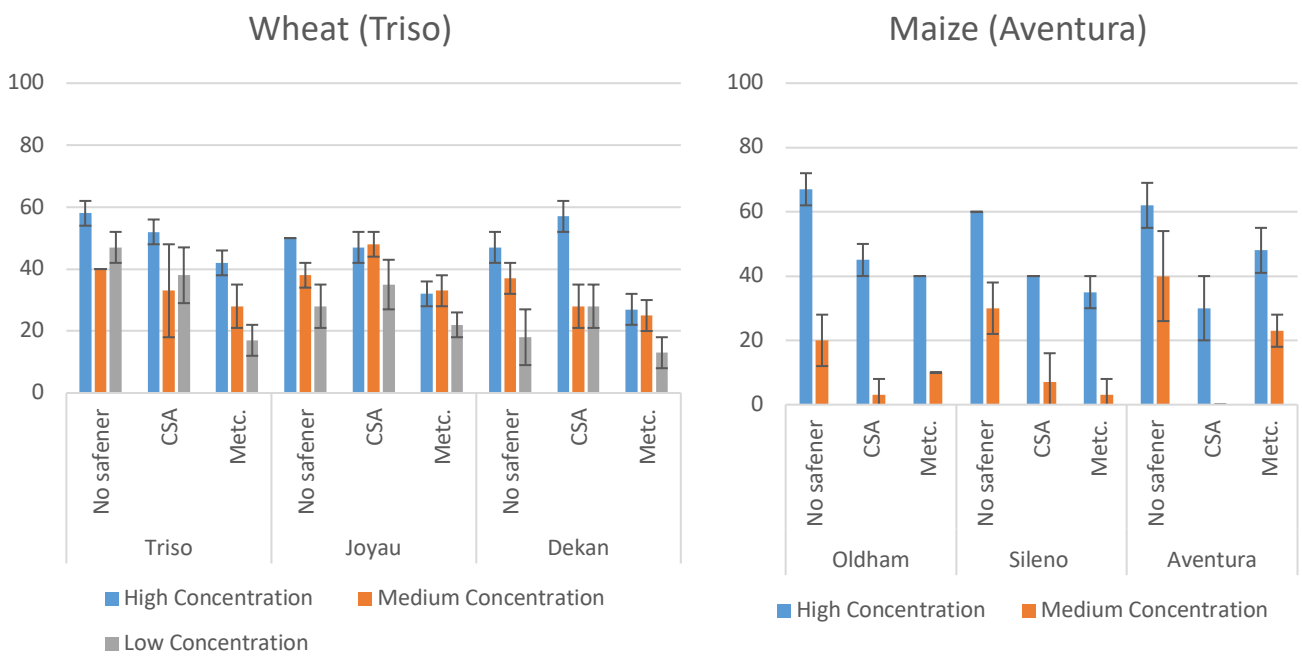


Figure A6: Effects of CSA and metcamifen on TCM injury in maize and wheat-Validation trial. Validation greenhouse trial in maize and wheat with herbicide TCM. Green highlights indicate observed safening effects where intensity is shown according to the colour scheme on the right. The numbers represent herbicide injury in %, scored within percentage injury categories (5-10%), accompanied by the standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). TCM concentrations used – Wheat: 10 g/ha (high), 5 g/ha (medium), 2.5 g/ha (low). Maize: 100 g/ha (high), 50 g/ha (medium).

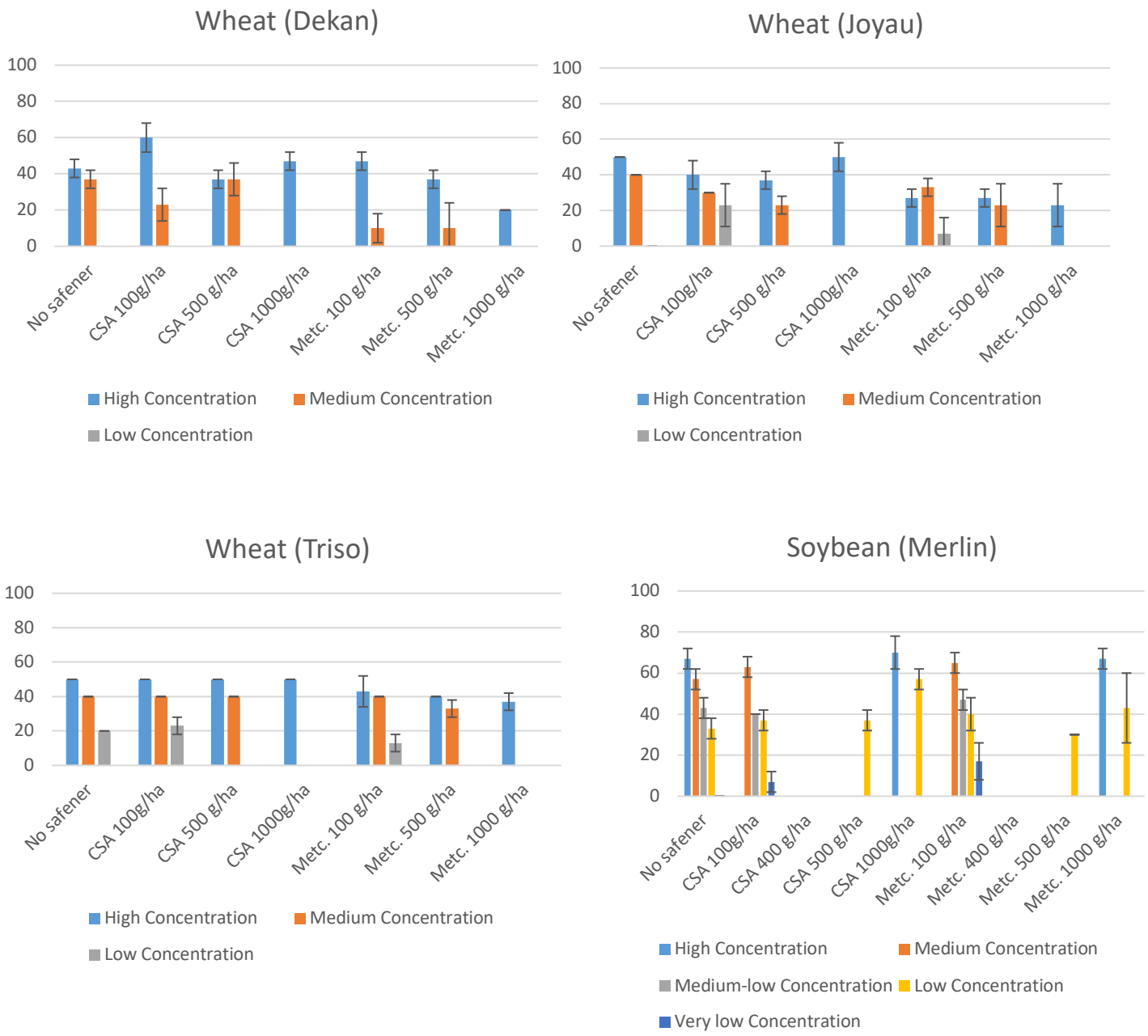


Figure A7: TBT Trial. Effect of CSA and metcamifen on TCM injury in wheat (A) and soybean (B) – Dose response and alternating herbicide-safener ratios. Greenhouse trial with various concentrations of TCM and safeners in wheat and soybean. Green highlights indicate observed safening effects where intensity is shown according to the colour scheme on the right, whereas orange and yellow indicate additional herbicide injury upon safener application. The numbers represent herbicide injury in %, scored within percentage injury categories (5-10%), accompanied by the standard deviations (n=3). Every combination (herbicide/safener/crop/variety) had 3 replicates (3 pots, each containing 3-10 plants) and chemical treatment was applied by spraying application, 7 days after sowing (BBCH=11-12). TCM concentrations used – Wheat: 50 g/ha (high), 25 g/ha (medium), 5 g/ha (low). Soybean: 50 g/ha (high), 25 g/ha (medium), 10 g/ha (medium-low), 5 g/ha (low), 2.5 g/ha (very low).

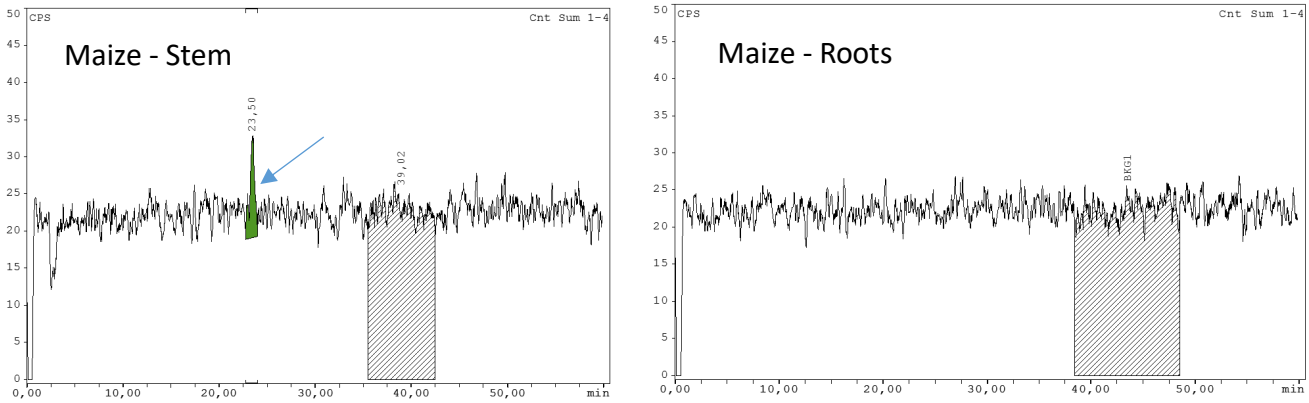


Figure A8: Radio-HPLC chromatograms of [^{14}C]-CSA metabolism in maize parts at 24 hours. CSA was applied on the first leaf of the plants, and the parent compound is represented by an arrow in the chromatograms.

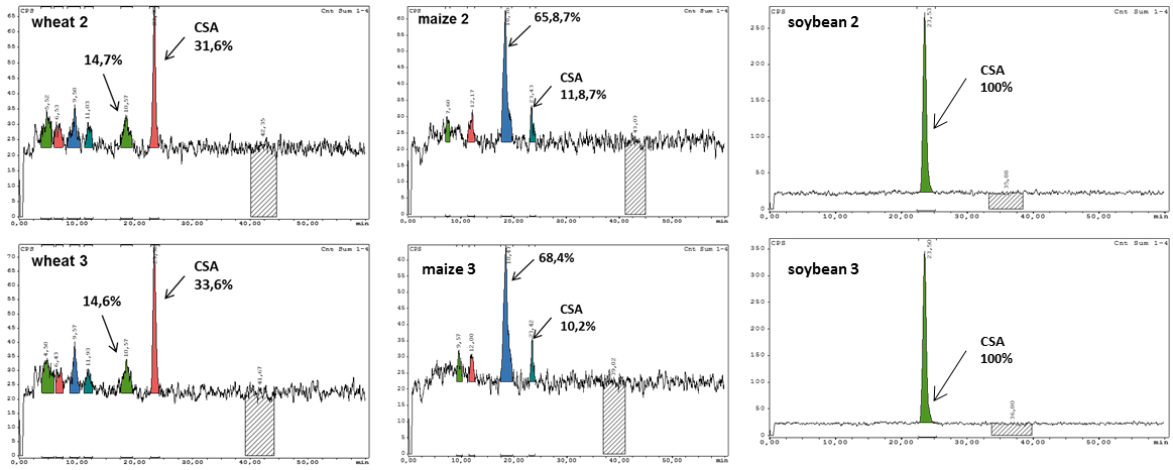


Figure A9: Radio-HPLC chromatograms of [^{14}C]-CSA metabolism in maize parts at 24 hours. CSA was applied by incubation of excised shoots.

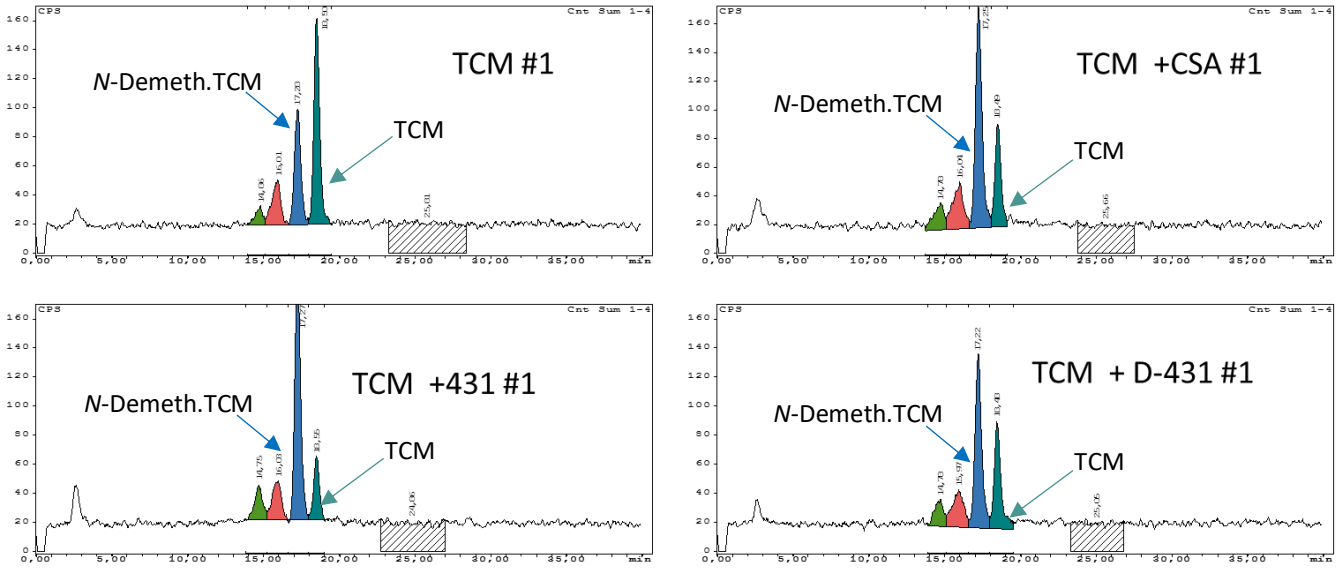


Figure A10: Radio-HPLC chromatograms of ^{14}C-TCM metabolism in maize upon safener/safener metabolite treatment. The chromatograms visualise the metabolic profile of TCM at 6 hours in maize with different treatments (upper right of each chromatogram). The green arrow (right) indicates the authentic TCM compound and the blue arrow (left) the *N*-demethylated metabolite. The red and light green peaks are unidentified TCM metabolites.

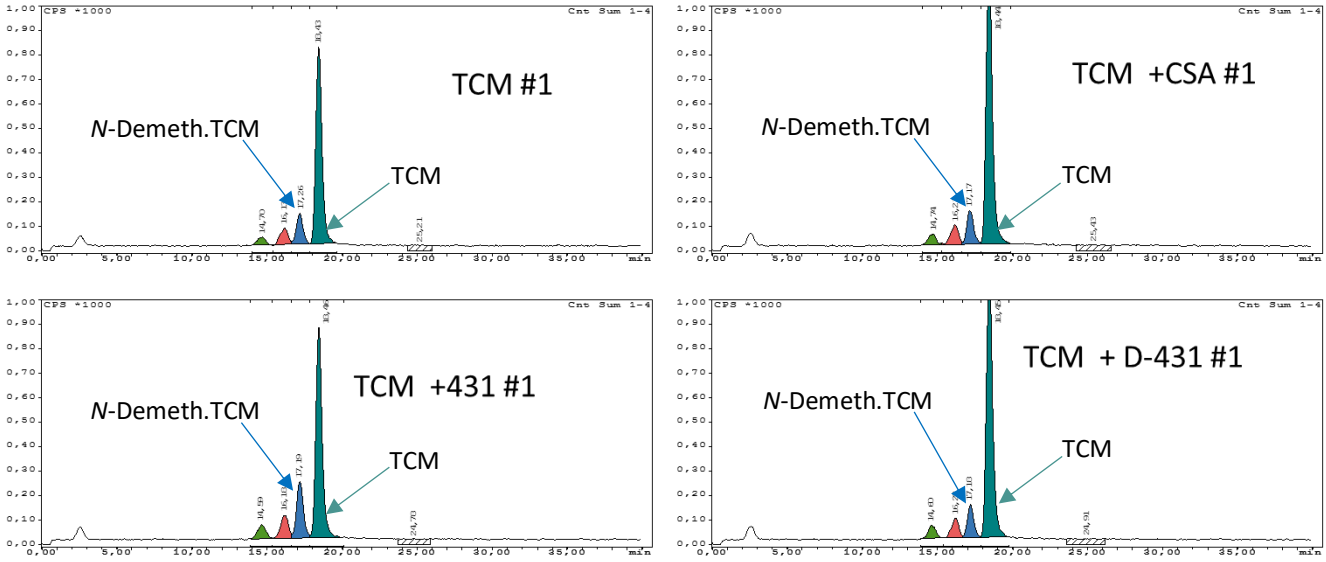


Figure A11: Radio-HPLC chromatograms of [^{14}C]-TCM metabolism in wheat upon safener/safener metabolite treatment. The chromatograms visualise the metabolic profile of TCM at 6 hours in maize with different treatments (upper right of each chromatogram). The green arrow (right) indicates the authentic TCM compound and the blue arrow (left) the *N*-demethylated metabolite. The red and light green peaks are unidentified TCM metabolites.

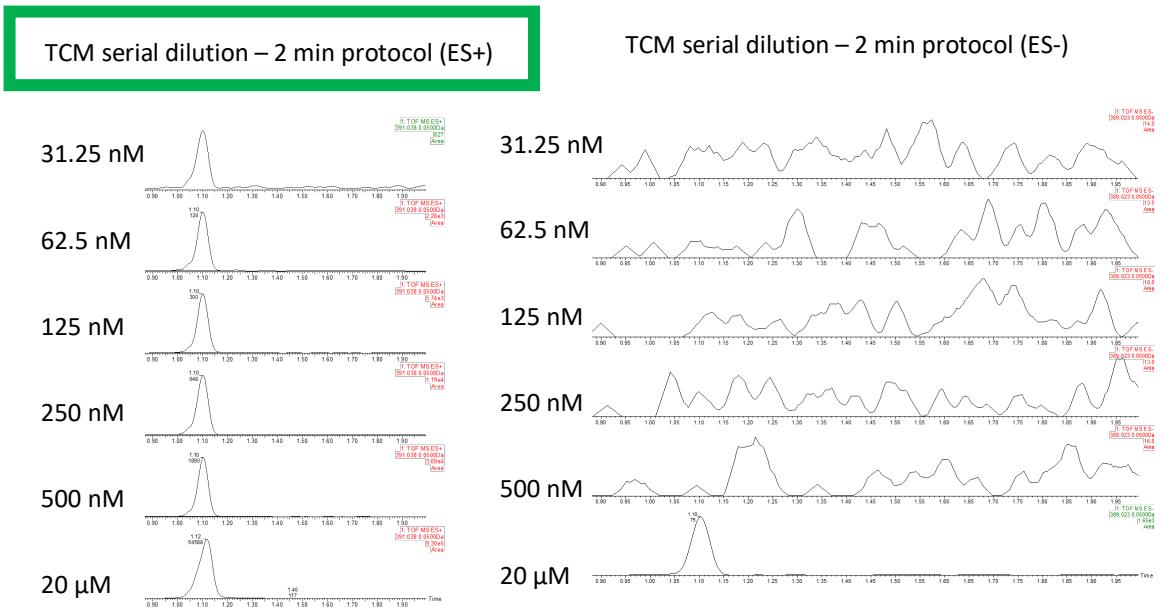


Figure A12: LC/MS chromatograms of TCM serial dilutions run with the same protocol in ESI- and ES+. Apart from the dilution with the highest concentration (20µM), TCM was not flying in ES- (negative mode). The protocol in ESI+ (positive mode) was selected (framed in green). A 3min protocol was tested as well in both modes, but it produced the same results as the 2 min one.

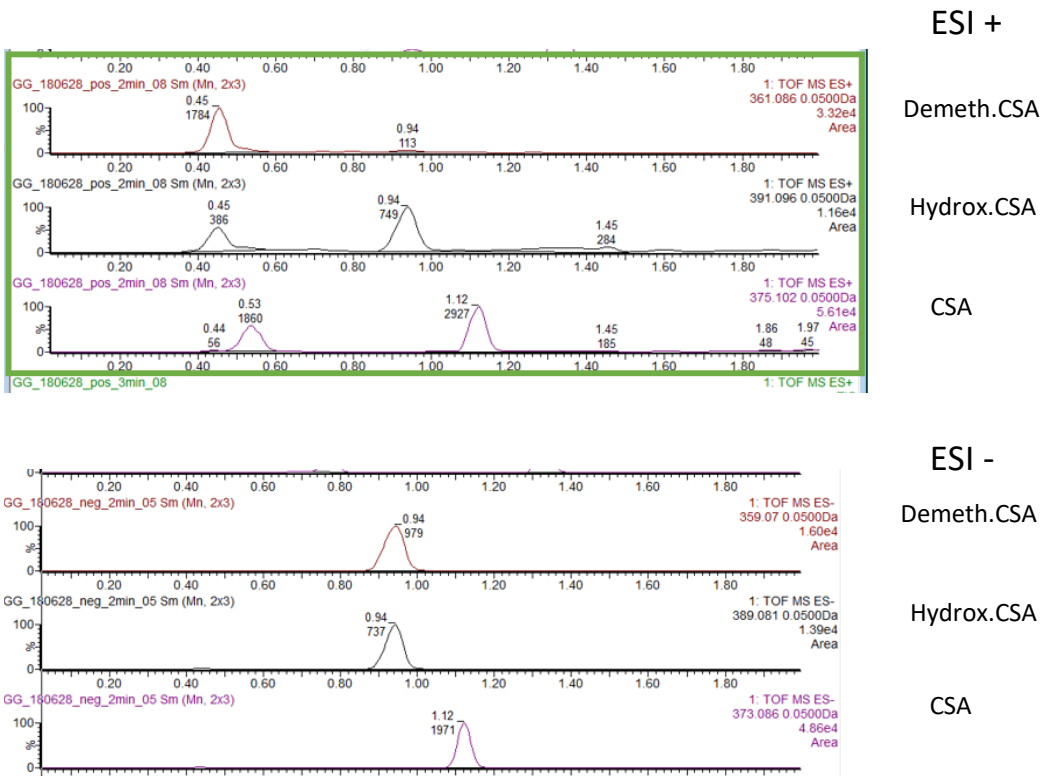


Figure A13: LC/MS chromatogram visualising the peaks of CSA and its early metabolites in ESI- and ES+. When the ESI- protocol was run, the quantity of the CSA metabolites was not in agreement with the CSA metabolism radiolabelled studies (Section 4.5), showing that the demethylated CSA was more abundant instead. The protocol in ESI+ (positive mode) was selected (framed in green).

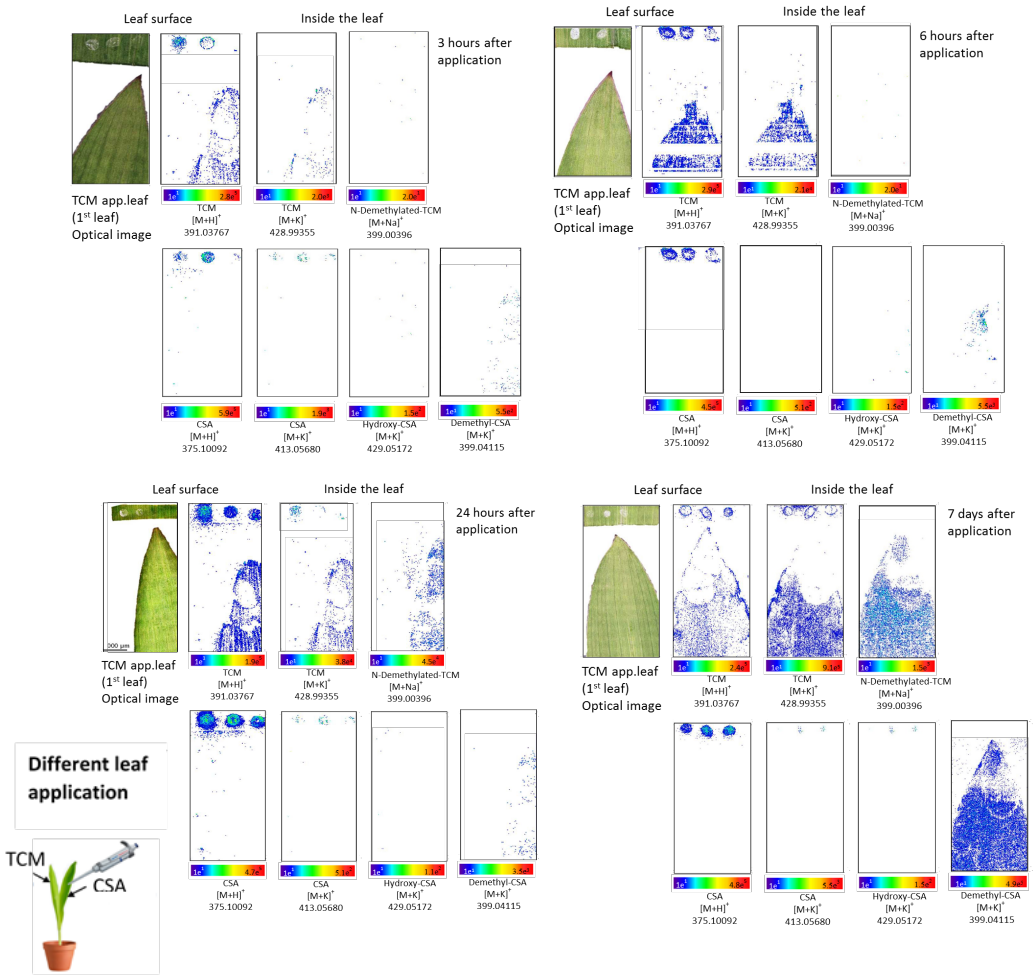


Figure A14: MALDI Imaging of the TCM treated leaf (different leaf application) after 3 hours to 7 days, visualising TCM and CSA metabolites. Scans showing the optical image of the leaf as well as distribution profiles of target compounds (TCM/CSA + metabolites) on the surface (non-absorbed) and inside the leaf (absorbed). The non-absorbed and absorbed compounds are differentiated by their adducts, where $+H^+$ is an adduct representative of the surface of the leaf whereas $+Na^+$ and $+K^+$ are representatives of the tissues below the leaf surface. The numbers on the rainbow scales indicate the intensity of the least and most intense pixel in that scan. Below the target compound's name its adduct and molecular mass can be found. 'On tissue' calibration spots on an untreated part of a control plant (as seen on the second scan from the top) give an idea on pixel intensity: 200 PPM (left), 500 PPM (middle) and 1000 PPM (right) acetone/water (1:1, v/v).

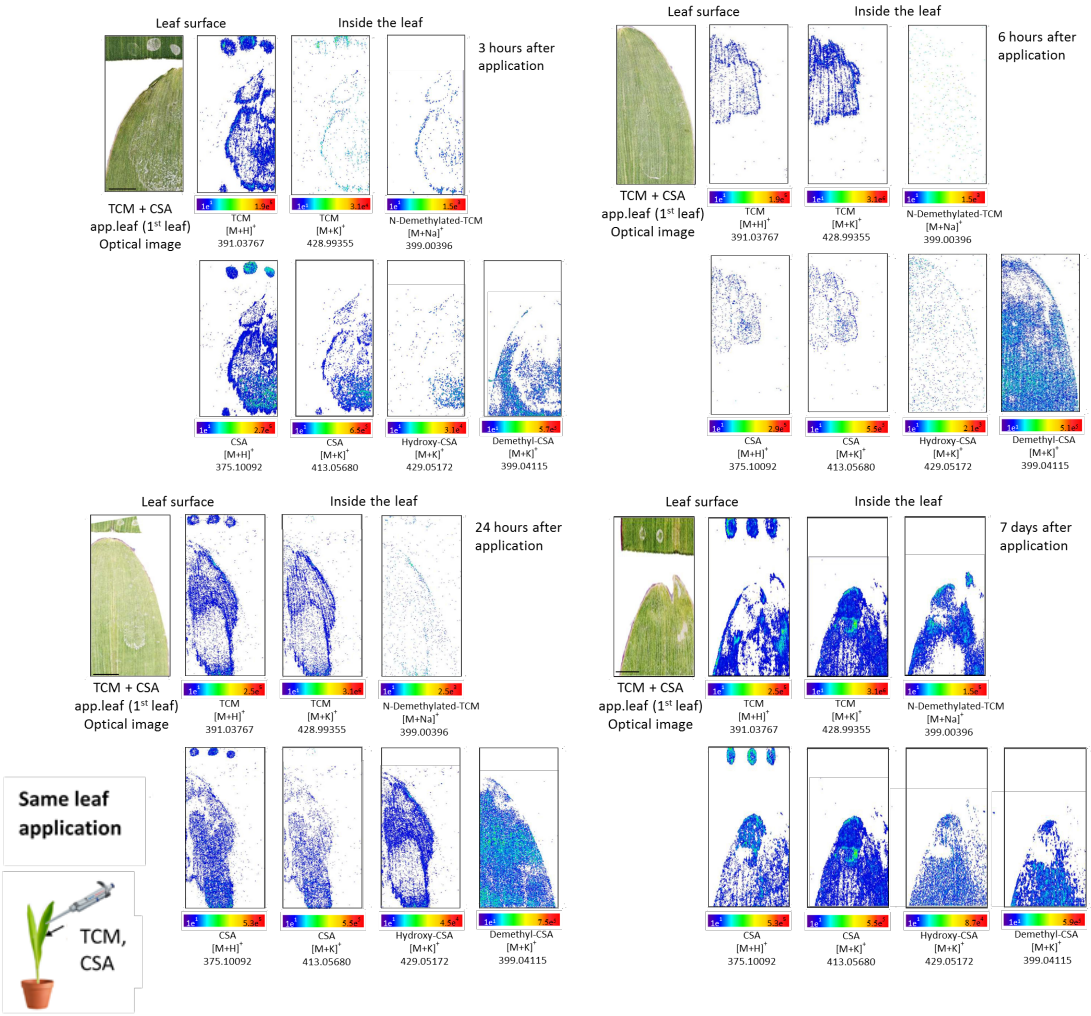
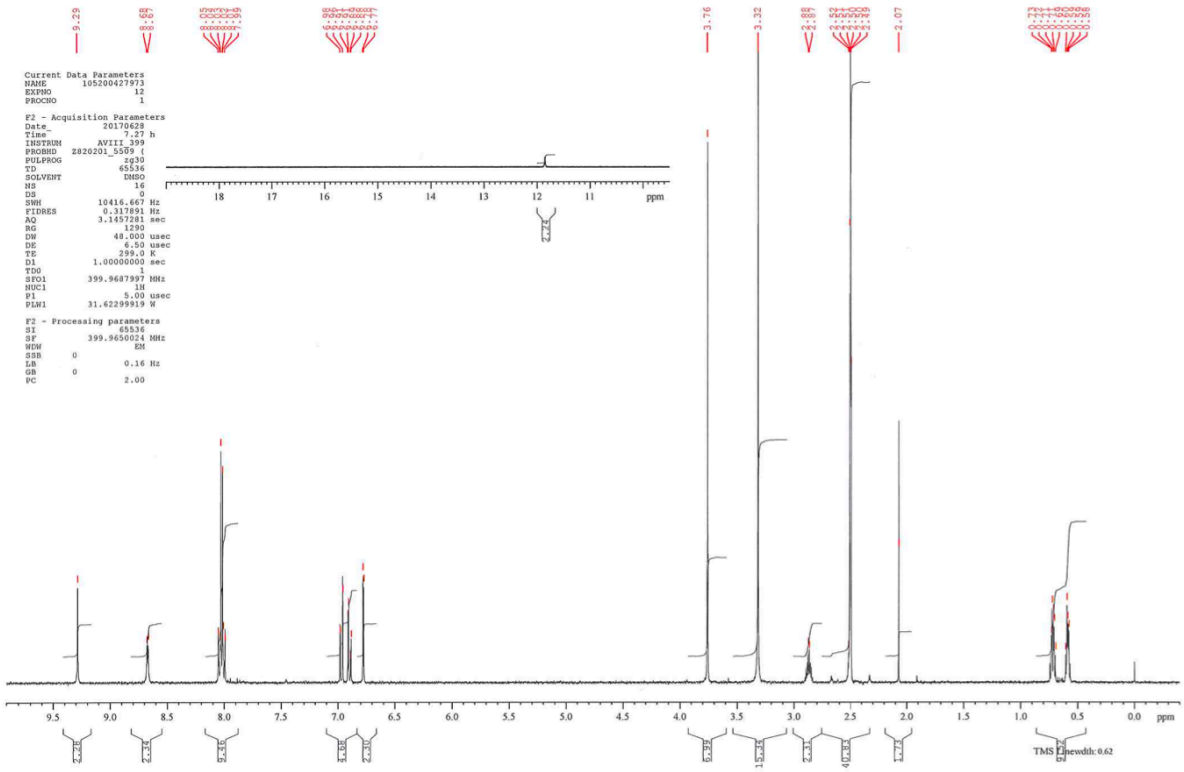


Figure A15: MALDI Imaging of the TCM + CSA treated leaf (same leaf application) after 3 hours to 7 days visualising TCM and CSA metabolites. Scans showing the optical image of the leaf as well as distribution profiles of target compounds (TCM/CSA + metabolites) on the surface (non-absorbed) and inside the leaf (absorbed). The non-absorbed and absorbed compounds are differentiated by their adducts, where $+H^+$ is an adduct representative of the surface of the leaf whereas $+Na^+$ and $+K^+$ are representatives of the tissues below the leaf surface. The numbers on the rainbow scales indicate the intensity of the least and most intense pixel in that scan. Below the target compound's name its adduct and molecular mass can be found. 'On tissue' calibration spots on an untreated part of a control plant (as seen on the second scan from the top) give an idea on pixel intensity: 200 PPM (left), 500 PPM (middle) and 1000 PPM (right) acetone/water (1:1, v/v).



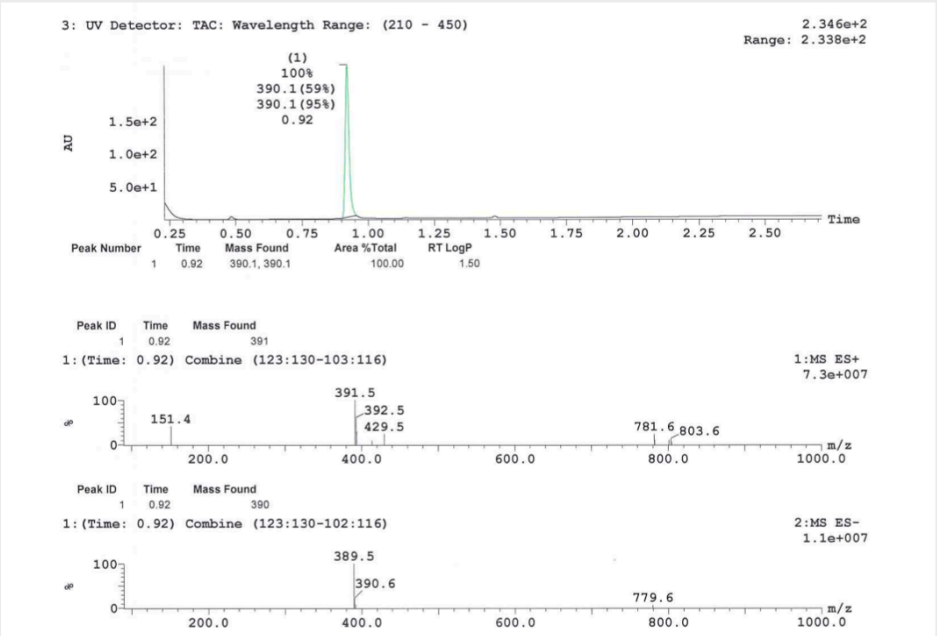


Figure A16: Original data spectra (NMR) of N-{[4-(cyclopropylcarbamoyl)phenyl]sulfonyl}-5-hydroxy-2-methoxybenzamide (hydroxylated CSA).

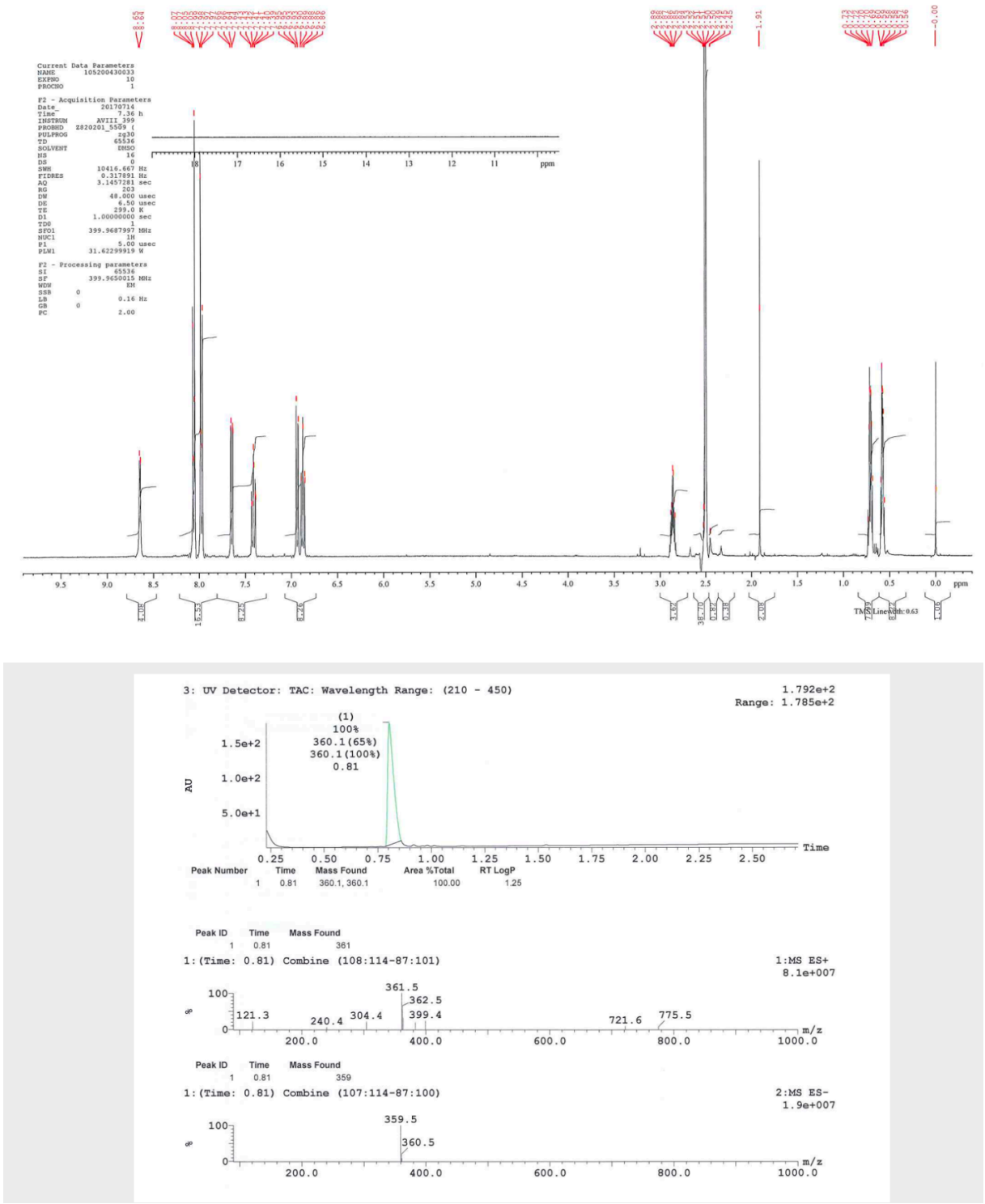


Figure A17: Original data spectra (NMR) of N-{[4-(cyclopropylcarbamoyl)phenyl]sulfonyl}-2-hydroxybenzamide (demethylated CSA).

Abbreviations

©	Copyright
°C	Degrees Celcius
µl	microliters
µM	micromolar
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4-DB	4-(2,4-dichlorophenoxy)butyric acid
4-HPPD	4-hydroxyphenylpyruvate
ABA	Abscisic acid
ABCs	ATP-Binding Cassete transporter proteins
ACCase	Acetyl-CoA carboxylase
ALS	Acetolactate synthase
ARE	Antioxidant Response Elements
As-1	Activation sequence 1
BBCH	Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie
Bq	Becquerel
CAMM	Computer aided molecular modelling
CBSA	chlorobenzenesulfonamide
CBSU	*N*-(aminocarbonyl)-chlorobenzenesulfonamide
CMTP	4-chloro-6-(methylthio)-phenylpyrimidine
COI1	CORONATIVE INSENSITIVE1
COSY	Correlated Spectroscopy
CPS	Counts per second
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSA	Cyprosulfamide

CYPs	Cytochrome P450s
DAD	Diode Array detector
DCA	Dichloroaniline
DNA	Deoxyribonucleic acid
DNOC	2-methyl-4,6-dinitrophenol
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase) inhibitors
EPTC	S-Ethyl-N,N-dipropylthiocarbamate
ES	Electrospray ionization
g/ha	grams per hectare
GC%	Percentage of guanine-cytosine
GM	Genetically-modified
GRAS	GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GA1 (RGA), and SCARECROW (SCR)
GSH	Glutathione
GSTs	Glutathione-S-tranferases
HPLC	High-performance liquid chromatography
HRAC	Herbicide Resistance Action Committee
HSQC	Heteronuclear Single Quantum Correlation
IWM	Integrated weed management
JA	Jasmonic acid
LC/MS	Liquid chromatography-mass spectrometry
LOG	logarithm
LSC	Liquid scintillation counter
MALDI	Matrix Assisted Laser Desorption Ionisation
MCBP	3,3-Di(9H-carbazol-9-yl)biphenyl
min	minutes

ml	millilitres
mm	millimetres
mM	millimolar
MRP	Multidrug resistance-associated protein
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSI	Mass Spectrometry Imaging
MTs	Malonyltransferases
NA	Naphthalic anhydride
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
Nsf1	Nicosulfuron 1
OsGSTL2	Oryza sativa Glutathione-S-transferase lambda 2
OTC	L-2-oxo- thiazolidine-4-carboxylic acid
PDA	Photodiode
PMRA	Pest Management Regulatory Agency
PPM	Parts per million
PSII	Photosystem II
rcf	relative centrifugal force
RNA	Ribonucleic acid
ROS	reactive oxygen species
RT	Retention time
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SA	Salicylic acid
SCL14	SCARECROW-like 14

TaGSTL1	Triticum aestivum Glutathione-S-Transferase lambda 1
TBT	Tembotrione
TCM	Thiecarbazone-methyl
TOF	Time of flight
UDP	Uridine diphosphate
UGTs	UDP-glycosyltransferases
UPLC	Ultra performance liquid chromatography
USD	US Dollars
UV	Ultraviolet
v/v	volume per volume
v/w	volume per weight
VLFCFA	Very long chain fatty acids
WG	Wettable granules
WP	Wettable powder
WT	Wild-type
ZmGSTL1	Zea mays Glutathione-S-Transferase lambda 1
ΔΔCt	delta-delta Ct

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