Pyroxasulfone resistance in Lolium rigidum is conferred by constitutive traits for herbicide metabolism

Roberto Busi¹, Aimone Porri², Todd A Gaines³ and Stephen B Powles¹

¹Australian Herbicide Resistance Initiative, School of Agriculture and Environment, The University of Western Australia, Crawley, WA 6009, Australia
²British American Tobacco, Plant Biotechnology Division CB4 0WA, 210 Cambridge Science Park Milton Rd, Milton, Cambridge, United Kingdom
³Department of Bioagricultural Sciences and Pest Management, Colorado State University Fort Collins, CO 80523

Author for correspondence: Roberto Busi

ph +61 8 6488 1423
roberto.busi@uwa.edu.au

Running title: Pyroxasulfone metabolic resistance

Total word count: 3,934.

Tables: 2

Figures: 5

Keywords: Adaptation, glutathione conjugation, GST, herbicide detoxification, weed resistance.
ABSTRACT

The evolution of resistant weed populations in response to intensive herbicide selection pressure is a global issue. Resistance to post-emergence herbicides is widespread, whereas soil-applied pre-emergence herbicides can often remain effective. For example, in Australia pyroxasulfone is a new pre-emergence soil-applied herbicide which provides control of Lolium rigidum populations resistant to multiple post-emergence herbicide modes of action. A fundamental knowledge of the genetic basis of metabolic resistance in weeds is important for understanding plant evolution pathways under herbicide selection and sustaining long-term weed resistance management.

In this study we define the mechanistic basis of resistance to pyroxasulfone in a L. rigidum population. TLC provides evidence that pyroxasulfone resistance is metabolism-based with approximately 88% of parental [14C]-labelled pyroxasulfone metabolized in resistant plants 24 hrs after the herbicide treatment. HPLC-MS allowed identification of several metabolites of pyroxasulfone formed via a glutathione (GSH) conjugation pathway in pyroxasulfone-resistant L. rigidum plants. However, the initial pyroxasulfone-glutathione conjugate was not found likely due to its labile nature. The observed constitutive over-expression from six to nine-fold of two putative resistance-endowing GST genes was associated with the pyroxasulfone resistance phenotype. The most logical conclusion, based on the data thus far available, is that rapid detoxification of pyroxasulfone mediates pyroxasulfone-resistance in L. rigidum plants. Future research is warranted to confirm the hypothesis advanced by this study of rapid pyroxasulfone metabolism due to GSH conjugation mediated by GST over-expressed in pyroxasulfone resistant plants which similarly leads to the production of distinctive GSH-pyroxasulfone metabolites L. rigidum and wheat plants.
1. INTRODUCTION

In global agriculture, weed control is mandatory to avoid significant crop losses [1]. In most situations synthetic herbicides allow simple and effective weed control practices. However, the evolution of adaptive traits conferring herbicide resistance in agricultural weeds is hampering the efficiency of herbicidal weed control [2, 3]. Evolved herbicide resistance in weed species can be target-site-based due to gene mutations as single nucleotide polymorphisms (SNPs) that cause amino acid substitutions at a herbicide binding site. Target-site resistance (TSR) is usually single-gene inherited resistance [4-8]. Conversely, non-target-site resistance (NTSR) encompasses all mechanisms that minimize herbicide injury by limiting toxic herbicide concentrations reaching herbicide sites of action (i.e., reduced herbicide leaf penetration, impaired herbicide translocation and herbicide metabolism allowing herbicide detoxification and recovery from herbicide damage) [9]. Important among NTSR mechanisms are constitutive enzymatic super families responsible for concerted secondary plant metabolism. Herbicide detoxification can schematically occur in four phases: phase I (oxidation), phase II (conjugation), phase III (transport) and phase IV (further compartmentation) [10-12]. Some of these enzymes can mediate herbicide detoxification via herbicide metabolism and inactivation [e.g., cytochrome P450 mono-oxygenases (P450s), glutathione-S-transferases (GSTs; EC 2.5.1.18) or glucosyltransferases (GTs)] followed by herbicide sequestration (e.g., ABC transporters) [13-15]. Some herbicides that interact with a complex of primary targets (e.g., chloroacetamides, which inhibit a complex system of elongases responsible for the biosynthesis of very long chain fatty acids, VLCFA) have thus far only selected for NTSR mechanisms in weeds [16]. The molecular definition and identification of NTSR mechanisms can often be complex, as the P450 or GST enzyme superfamilies containing a multitude of gene family members often interact within a particular detoxification pathway [11]. P450s can facilitate the oxidation or hydroxylation (phase I) of certain herbicide molecules [17] and be responsible for herbicide metabolism in some crop species (e.g,
maize, rice, wheat) and weeds [12, 18, 19]. Glutathione-S-transferases (GSTs) are phase II enzymes that can allow herbicide metabolism through conjugation with the tripeptide glutathione (γ-glutamylcysteinylglycine, GSH) [20]. GST are abundant in plant cells and widely involved in the recognition and transport of reactive electrophilic compounds of both synthetic and natural origins [21-23]. Specifically GSTs are found in the cytoplasm at high concentrations and catalyze the conjugation of GSH to a variety of endogenous and exogenous toxins. Early studies on GSTs were conducted with crop plants to understand the basis of herbicide selectivity. For example, it was shown that expression levels of detoxifying GSTs in certain crops were much greater than in weeds to explain herbicide selectivity [23, 24].

*Lolium rigidum* (Gaud.) is a genetically diverse, cross-pollinated globally-distributed weed species that has evolved resistance to many different herbicide modes of action [25]. In Australia the first selective post-emergent herbicide deployed for *L. rigidum* control was the acetyl CoA carboxylase (ACCase)-inhibiting herbicide diclofop-methyl introduced in 1978, followed by the acetolactate synthase (ALS)-inhibiting herbicide chlorsulfuron in 1982. Heap and Knight (1986) reported the first case of cross-resistance to ACCase and ALS herbicides evolved by diclofop-methyl field selection. Currently, ACCase and ALS cross-resistance is widespread throughout the southern Australian cropping system [26, 27], whereas lower levels of resistance have been reported for soil-applied pre-emergence herbicides [28, 29]. Thus, in response to widespread ACCase and ALS herbicide resistance, there has been an increase in reliance on pre-emergence soil-applied herbicides such as prosulfocarb, pyroxasulfone, triallate, and trifluralin. No field-evolved pyroxasulfone-resistant *L. rigidum* populations have thus far been identified [30]; however, we experimentally evolved pyroxasulfone resistance in *L. rigidum* by recurrent selection with pyroxasulfone over a few generations [31]. As subsequent studies showed, pyroxasulfone selection can result in co-evolution of cross-resistance to the thiocarbamates prosulfocarb and triallate in two
distinct *L. rigidum* populations [28, 32], research was warranted to elucidate the mechanistic basis of pyroxasulfone resistance in *L. rigidum*.

## 2. MATERIALS AND METHODS

### 2.1. Plant materials

The multiple resistant *L. rigidum* population SLR31 (hereinafter referred to as MR) evolved in the field following extensive herbicide selection. MR plants exhibit multiple herbicide resistance to different modes of action including the ACCase-inhibitor diclofop-methyl, the ALS-inhibitor chlorsulfuron [33], the mitosis inhibitor trifluralin [34], and the VLCFAE inhibitor S-metolachlor [35]. This MR population is susceptible to pyroxasulfone (VLCFAE inhibitor) [36], prosulfocarb (VLCFAE inhibitor), and marginally resistant to triallate [37]. MR individuals were exposed to recurrent selection with below-label, sub-lethal doses of pyroxasulfone and experimentally evolved resistance to pyroxasulfone, prosulfocarb, and triallate [31, 32]. Progeny P6 was obtained by six consecutive cycles of recurrent herbicide selection consisting of pyroxasulfone selection at 60 g ha⁻¹ (Progeny one, P1), followed by another pyroxasulfone selection at 120 g ha⁻¹ (Progeny two, P2) 120 g ha⁻¹ (Progeny three, P3), 240 g ha⁻¹ (Progeny four, P4), then further subjected to two consecutive selections at 1000 (Progeny five, P5) and 2000 (Progeny six, P6) g prosulfocarb ha⁻¹. The herbicide susceptible *L. rigidum* population VLR1 was the control in all experiments (hereinafter referred to as ‘S’).

### 2.2. Herbicide assays

Viable seeds of *L. rigidum* populations P6, MR, and S were germinated on 0.6% (w/v) solidified agar and planted into 2 L pots containing commercial potting mixture (50% peatmoss, 25% sand, and 25% pine bark) when the primordial root was visibly erupting from the seed coat. Approximately 2 hours after seeding the pots were treated with 0 (untreated), 25, or 100 g pyroxasulfone ha⁻¹. For each herbicide dose
there were four replicates with 25 viable germinated seeds treated per replicate. Plant survival was assessed 15 days after treatment (DAT). The experiment was conducted twice.

2.3. Metabolism study

\(^{14}\text{C}\)-labeled pyroxasulfone ([isoxazoline-3-\(^{14}\text{C}\)]pyroxasulfone) synthesized by Amersham Biosciences Co., Ltd. (United Kingdom) with specific radioactivity of 1.7 MBq/mg and > 99% purity was used in this study. Pyroxasulfone (white powder, mp 130.7°C (degrees Celsius), water solubility at 20°C 3.49 mg/L, vp 2.4×10^{-6} Pa) and the synthetic compounds, 2-amino-5-[1-(carboxylmethylamino)-3-(5,5-dimethyl-4,5-dihydroisoxazol-3-ylthio)-1-oxopropan-2-ylamino]-5-oxopentanoic acid (M-15), 2-amino-3-(5,5-dimethyl-4,5-dihydroisoxazol-3-ylthio)-2-hydroxypropanoic acid (M-26) and 3-(5,5-dimethyl-4,5-dihydroisoxazol-3-ylthio)-2-hydroxypropanoic acid (M-29) were used. These compounds were synthesized by KI Chemical Research Institute Co., Ltd. (Japan) and their purities were > 98%. Pyroxasulfone treatments were performed as reported by Tanetani et al. [38]. In brief, 13 \(L.\) rigidum pyroxasulfone-resistant P6 and -susceptible S plants were grown hydroponically up to the 4-leaf stage in 70 ml distilled water. Liquid fertilizer (70μl) with 10% phosphoric acid, 6% nitrogen and 5% potassium was added to growing solution (HYPONex, HYPONex JAPAN CORP., LTD.). Similarly, 1.3 ppm of \(^{14}\text{C}\)-labelled pyroxasulfone was applied to roots (approximately 3.3 μM). Four individual plants were harvested at three different time intervals corresponding to 1, 2, and 4 days after pyroxasulfone treatment and used for extraction and fractionation. The methodology for extraction and fractionation of pyroxasulfone metabolites following pyroxasulfone treatment of \(L.\) rigidum plants is described in detail by Tanetani et al [38]. In brief, following pyroxasulfone hydroponic treatment, \(L.\) rigidum plants were weighed, roots washed with 20 ml of acetonitrile, and plants homogenized. Extraction of pyroxasulfone and its metabolites occurred in 150 ml of 25% acetone. The extracts were evaporated in vacuo and dissolved in 10 ml of 50% acetonitrile. The radioactivity of the extracts was measured with a liquid scintillation counter (LSC, TRI-CARB
The radioactivity of the residues of the plants was measured with LSC after combustion by a sample oxidizer. Pyrazosulfone and its metabolites were identified by comparison with standards, using thin layer chromatography (TLC) and LC-MS. For TLC analysis, an aliquot of each extract was applied to silica gel. The plates were first developed with a mixture of ethyl acetate/chloroform/methanol/formic acid (60/60/10/10, v/v/v/v) and then the plate was developed a second time in the perpendicular direction with a mixture of ethyl acetate/methanol/distilled water/formic acid (60/40/20/10, v/v/v/v). The subsequent determination of pyrazosulfone and its metabolites by TLC and LC-MS was performed as reported by Tanetani et al. [38].

2.4. Molecular study: q-PCR to quantify gene expression levels

Six pyrazosulfone resistant individuals (survivors to 100 g pyrazosulfone ha\(^{-1}\)) from the P6 population were identified and individually collected for total RNA extraction and q-PCR analysis. Similarly, six untreated individual plants (\(n = 6\)) from MR and S populations were individually harvested for the same q-PCR study, respectively. Two leaf segments of 2 cm were harvested from each individual 5-tiller plant and placed into a 25 mL tube. The individual plant represented the experimental unit as biological replicate. The experiment was repeated with similar methodologies on a greater number of plants. Fifteen days after pyrazosulfone treatment at 100 g ha\(^{-1}\) a total of 50 one-leaf surviving resistant P6 plants were harvested (2-cm plant tissue) and divided (\(n = 2\)) for total RNA extraction and subsequent q-PCR experiments. Also, 50 one-leaf S plants emerging after pyrazosulfone treatment at 25 g ha\(^{-1}\) were harvested. In addition, 50 untreated MR, P6, and S one-leaf plants, respectively, were harvested for q-PCR analysis. Twenty leaf segments of 1 cm were harvested individually from 25 respective plants and pooled into a 25 mL tube.
Total RNA was isolated from plant tissues by using RNAeasy extraction kit (Qiagen) and treated with DNA-free DNase (Ambion) to remove residual genomic DNA. One μg of total RNA was used for reverse transcription (Superscript III, Invitrogen) in a 20 μL volume reaction. Quantitative PCR was performed in a 384 well-plate using LightCycler 480 (Roche) and all reactions were conducted in three technical replicates and a negative control containing no template with the same reaction mix. Each reaction of 13 μL total volume included 6.5 μL of SyberGreen Master Mix, (SensiFAST), 0.25 μL of 0.5 pmol μL⁻¹ primers, 3 μL of cDNA (diluted 1:10) and 3 μL of H₂O. Reaction conditions were 3 min incubation at 95°C, 40 cycles of 95°C for 10 sec, 60°C for 20 sec, and 72°C for 10 sec followed by a melt-curve analysis to confirm single-product amplification. Threshold-cycles (CTs) were obtained for each reaction using the Second Derivative Maximum method in the LightCycler 480 software (Roche). The mean of CT values for the three technical replicates for each sample was used to calculate the relative expression (RE) of the gene of interest using the following equation:

\[ \text{RE} = 2^{\frac{\text{CT gene of interest} - \text{CT control}}{}} \]

The control gene used in this assay was isocitrate dehydrogenase as described by Gaines et al. [19].

The relative expression of GST-1 Tau class (contig 4546), GST-2 Tau class (contig 5390), GST-3 Phi class (contig 8676), GST-4 Tau class (contig 13326), GST-5 Phi class (contig 16302), and P450-1 CYP72A (contig 1604), P450-2 CYP72A (contig 2218), P450-3 CYP716A (contig 6783), P450-4 CYP89A (contig 6759) and P450-5 CYP71B (contig 12788) was quantified using primers described by Gaines et al., 2014 [19].

2.5. Statistical analysis

For all the L. rigidum populations analysed in this study graphical data relative to the resistance phenotype are presented as percent (%) of seed germination and plant survival. Gene expression relative to population S is set as equal to 1. Two main types of analysis were conducted to compare and separate population
mean values for survival and gene expression levels. Comparisons among survival rates were assessed by chi-square ($\chi^2$) heterogeneity test performed using the statistical software R (version 3.02) with the command `prop.test`. Relative gene expression were subjected to ANOVA and population means (P6 vs. MR vs. S) separated by Tukey’s HSD ($\alpha = 0.05$). Graphical data were obtained and plotted with GraphPad Prism (GraphPad Software, Inc. La Jolla, CA 92037 USA).

3. RESULTS

3.1 Response to pyroxasulfone treatments of resistant P6, MR and S L. rigidum plants prior to molecular analysis

When treated at the recommended dose of pyroxasulfone (100 g ha$^{-1}$) there was 54% survival (plant emergence) of the resistant P6 plants. As expected, for the parental MR and the standard herbicide-susceptible S populations there was only 5% survival (Figure 1). The herbicide assay was repeated with 48% plant survival observed in P6 plants treated with 100 g pyroxasulfone ha$^{-1}$ Survival observed in MR plants (45%) treated with a low dose of pyroxasulfone (25 g ha$^{-1}$) was not different from P6 plants ($X^2 = 0.07, P = 0.80$). Conversely survival of S plants was significantly lower than P6 and MR, respectively ($X^2 = 12.9, P = 0.002$) (data not shown). (27% survival, data not shown).

3.2 $^{14}$C-pyroxasulfone metabolites analysis in pyroxasulfone-resistant P6 L. rigidum plants

Following root application of parent $^{14}$C-labelled-pyroxasulfone to L. rigidum plants at the 3-leaf stage, the total radioactivity was determined over time. Pyroxasulfone-resistant P6 plants absorbed from 8% (1 DAT) up to 25% (4 DAT) of parent pyroxasulfone applied, corresponding to a concentration of $^{14}$C radioactivity recovered of 10.71 μg eq./g plant tissue harvested (Table 1).
The total radioactivity absorbed (recovered) in resistant P6 plants was approximately two-fold higher than in S plants (Table 1). Equally, the total amounts of the [14C]-labelled metabolites measured at one, two, or four days after treatment (DAT) in the pyroxasulfone-resistant P6 plants were larger than in S plants. In P6 pyrazasulfone-resistant plants the decomposition rate of [14C]-pyroxasulfone into metabolites was faster and up to 4-fold greater than in the S plants (Table 2). For example, in P6 pyrazasulfone-resistant plants approximately 88% of the parent [14C]-labelled pyrazasulfone was metabolized (1 DAT) versus 54% in susceptible plants and similarly the percentage of identified pyrazasulfone metabolites were two-fold greater than in susceptible plants (Table 2). This indicates that in P6 plants the parental [14C]-labelled pyrazasulfone was more rapidly metabolized.

In the extracts from resistant P6 and S plants, a total of eight metabolites were evident by TLC analysis (Figure 2). Six of these metabolites (TLC spots), namely pyrazasulfone, Uk-1, Uk-3, cysteine conjugate of isoxazoline ring (M-26), the metabolite in which amino group of M-26 was replaced with hydroxyl group by oxidative deamination (M-29) and glucose conjugate of M-29 (M-29-glc) were the same chemical compounds as those detected in wheat Figure 2, Table 2). Considering the ratio of the radioactivity of each metabolite, M-26, M-29, and M-29-glc were the main metabolites identified in wheat and pyrazasulfone-resistant and -susceptible L. rigidum, as previously shown elsewhere [38]. The metabolite M-15 corresponding to the GSH conjugate of isoxazoline ring of parental pyrazasulfone was not detected in this study. However, in plants the GSH conjugate of pesticides is generally catabolized to a cysteine conjugate by liberation of glycine and glutamic acid from the GSH moiety [39]. In our preliminary metabolism study of pyrazasulfone in barnyard millet cultured cells, a large amount of M-26 and small amount of M-15 were detected (data not shown) as shown for other herbicides [40], indicating that the pyrazasulfone-GSH conjugate (M-15) is immediately metabolized to the cysteine conjugate (M-26). Out hypothesis is that the M-15 pyrazasulfone GSH conjugate is labile and therefore not detected in the TLC assay. The pyrazasulfone cysteine conjugate M-26 was generated by liberating glutamic acid and
glycine from the GSH conjugate of the isoxazoline ring (M-15) and M-26 was then metabolized to M-29 by oxidative deamination. Subsequently, M-29-glc was generated by glucose conjugation of M-29. These metabolic processes indicated that the main metabolites (M-26, M-29 and M-29-glc) are assumed to be formed via the initial metabolic step of GSH conjugation of the isoxazoline ring of pyroxasulfone. Thus, the main route of pyroxasulfone metabolism appears to be the cleavage of methylenesulfonyl linkage by GSH conjugation of the isoxazoline ring [38].

3.3 Transcript levels of genes encoding herbicide-metabolizing enzyme in resistant P6, MR and S L. rigidum plants

To assess whether pyroxasulfone resistance is associated with increased transcript levels of herbicide-metabolizing genes, the expression levels of five putative P450s and GSTs previously identified in resistant Lolium populations [19] were determined by quantitative real time PCR. The tested P450s and GSTs were named from 1 to 5 (see material and methods). In this assay the P6 pyroxasulfone resistant individuals were compared with the untreated susceptible MR individuals and susceptible S individuals. The transcript quantification was performed on six different biological replicates and the statistical significance among the different individuals was assessed using Tukey's HSD and ANOVA tests. The mRNA level of P450-1 was increased around 6 and 4 times in both pyroxasulfone-resistant P6 individuals and pyroxasulfone-susceptible MR individuals compared with S plants, respectively (Figure 3). There was no difference in P450-1 expression in resistant P6 compared to MR individuals. The mRNA abundances of P450-2, P450-4 and P450-5 were not different among resistant P6, MR, and S plants, while the expression of P450-3 was 5- and 3-fold reduced in resistant P6 and MR, respectively, compared with S plants ($P < 0.01$) (Figure 3). The transcript levels of GST-1 were around nine-fold higher in R P6 individuals compared to both MR and S plants. Likewise, the mRNA levels of GST-2 were around 6 and 3 times more abundant in R P6 plants compared to MR and S individuals, respectively (Figure 4). The
upregulation of these two GSTs was consistently found in all tested P6 biological replicates. Tukey's multiple comparisons test of GST-1 and GST-2 Tau class expression data showed high statistical significance ($P$ value $\leq 0.01$). In contrast, the expression levels of GST-3, Phi class GST-4 Tau class and GST-5 Phi class were not different among resistant P6, MR, and S individuals (Figure 4). Thus, in the resistant P6 plants the increased transcript levels of GST-1 and GST-2 correlates with pyroxasulfone resistance. For further confirmation the expression levels of these two GSTs were quantified in resistant P6, MR, and S one-leaf stage plants, 15 days after pyroxasulfone pre-emergence treatment. Resistant P6 individuals were treated with 100 g pyroxasulfone ha$^{-1}$ whereas susceptible plants (MR and S) were treated with a sub-lethal 25 g ha$^{-1}$. The rationale to apply a different pyroxasulfone dose to resistant (100 g ha$^{-1}$) versus susceptible (25 g ha$^{-1}$) plants was to achieve similar and comparable plant survival in both populations as a uniform plant response from a similar herbicide stress level. In addition, to assess whether the expression of GST-1 and GST-2 is constitutively increased in the resistant P6 plants independently of the herbicide treatment, untreated resistant P6, MR and S individuals were also collected. The transcript levels of GST-1 and GST-2 in untreated resistant P6 plants were higher ($P$ value $\leq 0.01$) than in susceptible plants, with a calculated 7- and 4-fold higher relative gene expression, respectively. Similar results indicating GST1-1 and GST-2 over-expression were found in the pyroxasulfone treated plants (Figure 5).

4. DISCUSSION

This study aimed to understand the biochemical basis of resistance to the herbicide pyroxasulfone in *L. rigidum*. Pyroxasulfone has become widely used in major agricultural areas such as Australia, U.S.A., and Canada [41]. Recent studies have reported experimental selection of pyroxasulfone resistance in *L. rigidum* [28, 31] and evolved cross-resistance to pyroxasulfone in *A. fatua* through repeated field exposure to triallate [42]. In this study pharmacological (TLC / LC-MS) data support a role for glutathione-S-transferases (GSTs) mediating pyroxasulfone-GSH conjugation. The expression level of two GST
transcripts was higher in pyroxasulfone resistant plants. Thus, the mechanistic basis for pyroxasulfone resistance in *L. rigidum* is likely to be metabolism-based with resistant *L. rigidum* P6 plants with enhanced capacity to detoxify pyroxasulfone via a GSH conjugation pathway. It remains to be determine whether the identified GST transcripts are responsible for the GSH conjugation through further experimentation. In pyroxasulfone-resistant P6 plants 88% of the parent [14]C-labelled pyroxasulfone can be metabolized into several different metabolites within 24 hours after the herbicide treatment. Similar detoxification rate of pyroxasulfone is reported in pyroxasulfone-resistant wheat plants [38]. TLC and HPLC-MS studies indicate that in pyroxasulfone-resistant *L. rigidum* and wheat plants similar metabolic pathways mediate, via GSTs, the GSH conjugation of the isoxazoline ring of [14]C-pyroxasulfone leading to formation of three main metabolites explaining safety versus toxicity in crops versus grass weeds [38]. In one grass species (barnyard millet) a study on pyroxasulfone metabolism in cultured cells indicated that the conjugate pyroxasulfone-GSH is labile as the metabolism of pyroxasulfone resulted in a very small amount of the pyroxasulfone-GSH conjugate (Kumiai Chemical Industry, unpublished data). Tanetani et al [38] reported similarly that the pyroxasulfone-GSH conjugate (metabolite M15) was immediately metabolized to the cysteine conjugate (M-26) after the GSH conjugation reaction [38]. The labile nature of conjugates between the tripeptide glutathione and electrophilic xenobiotic substrates such as herbicides has been extensively reviewed [23]. Activity of GST mediating tolerance to thiocarbamate herbicides was first observed in corn plants pre-treated with the specific herbicide safener dichloroacetamide increasing root GSH content and GST activity [43]. Since then it has become clear that a range of plant GSTs can catalyze conjugation of GSH with certain herbicide classes [44]. GSTs belong to an enzyme superfamily which includes two plant specific classes [Phi and Tau] that can be associated with herbicide resistance in weeds [45]. Thus, the electrophilic nature of some herbicide molecules, often after initial P450-mediated oxidation, sulfoxydation or hydroxylation, can bind to the cysteine residue of GSH as the first step in this detoxification pathway [16, 46]. These chemical reactions involving K₃ herbicides and GSH
are similar to the covalent binding of the KCS (3-ketoacyl-CoA synthase) enzymatic complex identified as one of the primary target for these VLCFAE-inhibiting herbicides [47, 48]. Crop selectivity to several different chloroacetamide herbicides is similarly mediated by enhanced GST activity, which is also documented to be the result of a change in GST expression [22, 49-51]. Here, a faster rate of pyroxasulfone metabolism in pyroxasulfone-resistant *L. rigidum* and resistant wheat plants suggest similarities in metabolic detoxification of pyroxasulfone in the two species [38, 52].

This study provides evidence that a significant increase in constitutive GST gene expression is correlated with pyroxasulfone resistance. Both *GST-1* and *GST-2*, Tau class, had significantly higher transcription in pyroxasulfone-resistance individuals (P6) than in pyroxasulfone-susceptible MR or S plants. No additional upregulation of *GST-1* and *GST-2* following pyroxasulfone treatment was observed, indicating that the over-expression of these two GST transcripts was constitutive. As a previous inheritance study shows that pyroxasulfone resistance in *L. rigidum* is conferred by a semi-dominant allele segregating at one major locus [53] the upregulation of two different GST genes in a trait inherited as a single semi-dominant allele could be explained if *GST-1* and *GST-2* are closely linked on the same chromosome, thereby producing an inheritance pattern consistent with a single locus. Another possibility is that expression of the two different genes may be co-regulated by a single transcription factor, which would also produce a single gene inheritance pattern [54]. In Arabidopsis increased tolerance to abiotic stressors has been shown to be regulated by one single transcription factor [55]. In wheat plants *GST (TaGSTU4)* over-expression induced by the safener fenchlorazole-ethyl mediates resistance to the ACCase-inhibiting herbicide fenoxaprop-ethyl and the K₃ herbicide dimethenamid [56]. BLAST analysis reveals high similarities between *TaGSTU4* and *GST-1* (contig score 205, E-value 1.69 x 10⁻⁵³)[19]. Other studies on transcriptome analysis provide additional evidence of GST over-expression conferring metabolic herbicide resistance in populations of the grass weed *Lolium* from France [57, 58]. *GST-1* and *GST-2* (Tau class) have up to 35% identity across 250 bp with three different GST Tau class transcriptional
markers, and 10% identity with one GST-Phi class transcriptional marker, suggesting some similarity with the Tau class GST markers reported in France. We report an increased expression of GST-5, (Phi class) only in three P6 individuals surviving pyroxasulfone which resulted in non-significant over-expression when the results were pooled (Figure 4). GST-5, (Phi class) has 94.5% similarity across 145 bp to the LrGSTF1 homologue of AmGSTF1 endowing fenoxaprop-ethyl resistance in A. myosuroides [59].

Taken together, the chromatographic work conducted is highly suggestive of pyroxasulfone metabolism via a GSH conjugation pathway with production of distinctive GSH-pyroxasulfone conjugate and subsequent metabolites. In addition the GST over-expression found in pyroxasulfone-resistant L. rigidum via qPCR is consistent with the hypothesis of enhanced GST activity in resistant L. rigidum mediating pyroxasulfone detoxification. Further work remains to resolve the links between patterns of herbicide selection and evolved pyroxasulfone resistance mechanism(s) including gene expression and gene regulation that could drive the evolution of herbicide resistance in grass weeds.
Table 1. Amount of radioactivity detected in resistant (P6) and susceptible (S) *L. rigidum* plants treated with [isoxazoline-\(^{14}\text{C}\)] pyroxasulfone at harvest 1, 2 and 4 days after treatment (DAT). Values of plant fresh weight are expressed in grams (g) and radioactivity as equivalent to the amount of parent [isoxazoline-\(^{14}\text{C}\)] pyroxasulfone equivalent (\(\mu\text{g eq.}\)) or concentration (\(\mu\text{g eq./g}\)). Data are partially drawn from [38].

<table>
<thead>
<tr>
<th>Population</th>
<th>DAT</th>
<th>Plants Harvested</th>
<th>Plant fresh mass (g)</th>
<th>([^{14}\text{C}]) radioactivity ((\mu\text{g eq.}))</th>
<th>Recovery (%)</th>
<th>([^{14}\text{C}]) recovered ((\mu\text{g eq.}))</th>
<th>Concentration ([^{14}\text{C}]) recovered ((\mu\text{g eq./g}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6</td>
<td>1</td>
<td>13</td>
<td>1.51</td>
<td>1099</td>
<td>8</td>
<td>6.76</td>
<td>4.48</td>
</tr>
<tr>
<td>P6</td>
<td>2</td>
<td>13</td>
<td>1.76</td>
<td>1099</td>
<td>12</td>
<td>11.13</td>
<td>6.33</td>
</tr>
<tr>
<td>P6</td>
<td>4</td>
<td>13</td>
<td>2.08</td>
<td>1099</td>
<td>25</td>
<td>22.06</td>
<td>10.71</td>
</tr>
<tr>
<td>S</td>
<td>1</td>
<td>13</td>
<td>1.82</td>
<td>1099</td>
<td>4</td>
<td>4.1</td>
<td>2.3</td>
</tr>
<tr>
<td>S</td>
<td>2</td>
<td>13</td>
<td>1.89</td>
<td>1099</td>
<td>6</td>
<td>5.9</td>
<td>3.1</td>
</tr>
<tr>
<td>S</td>
<td>4</td>
<td>13</td>
<td>1.87</td>
<td>1099</td>
<td>10</td>
<td>8.8</td>
<td>4.7</td>
</tr>
</tbody>
</table>
Table 2. Percentages of parent [isoxazoline-14C] pyroxasulfone (%) and its metabolites identified (M-26, M-29 and M-29-glc) and metabolites unknown found in resistant (P6) and susceptible (S) L. rigidum plants treated with 14C-pyroxasulfone and harvested 1, 2 and 4 days after treatment (DAT)*. Values in parentheses indicate the concentration of radioactivity (μg eq./g). Data partially re-drawn from [38].

<table>
<thead>
<tr>
<th>Population</th>
<th>DAT</th>
<th>14C-Pyroxasulfone (%) (μg eq./g)</th>
<th>M-26</th>
<th>M-29</th>
<th>M-29-glc</th>
<th>Total identified (%) (μg eq./g)</th>
<th>Metabolites unknown</th>
<th>Grand Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6</td>
<td>1</td>
<td>12.2 (0.57)*</td>
<td>21.8</td>
<td>7.8</td>
<td>15.7</td>
<td>45.3 (2.03)</td>
<td>26.5</td>
<td>84.0</td>
</tr>
<tr>
<td>P6</td>
<td>2</td>
<td>4.5 (0.28)*</td>
<td>22.5</td>
<td>17.1</td>
<td>11.7</td>
<td>51.3 (3.25)</td>
<td>32.1</td>
<td>87.9</td>
</tr>
<tr>
<td>P6</td>
<td>4</td>
<td>4.6 (0.49)*</td>
<td>20.0</td>
<td>12.7</td>
<td>20.0</td>
<td>52.7 (5.65)</td>
<td>28.4</td>
<td>85.7</td>
</tr>
<tr>
<td>S</td>
<td>1</td>
<td>46.4 (1.07)</td>
<td>13.7</td>
<td>3.6</td>
<td>4.6</td>
<td>21.9 (0.50)</td>
<td>17.1</td>
<td>85.4</td>
</tr>
<tr>
<td>S</td>
<td>2</td>
<td>26.4 (0.82)</td>
<td>18.2</td>
<td>8.2</td>
<td>10.0</td>
<td>36.4 (1.13)</td>
<td>27</td>
<td>89.8</td>
</tr>
<tr>
<td>S</td>
<td>4</td>
<td>9.1 (0.43)</td>
<td>24.6</td>
<td>10.0</td>
<td>13.7</td>
<td>48.3 (2.27)</td>
<td>35.8</td>
<td>93.2</td>
</tr>
</tbody>
</table>
Fig. 1. Mean plant survival (%) as ratio of actively growing plants versus seeds treated ± standard errors (SE) in pyroxasulfone treated *Lolium rigidum* plants. Survival ± SE (n = 4) assessed as plant emergence in pot cultured plants assessed 60 days after 100 g pyroxasulfone ha⁻¹ treatment in pyroxasulfone-resistant progeny P6 (black bar), parental MR (grey bar), herbicide susceptible S population (white bar).
Fig. 2. Two-dimensional TLC of the extract from R biotype of rigid ryegrass after treatment with $^{14}$C-
pyroxasulfone (4 DAT) in (A) pyroxasulfone-resistant (P6) versus (B) pyroxasulfone-susceptible (S) L. 
rigidum plants. Figure (B) re-drawn from [38].
Fig. 3. Transcript levels of \textit{P450} genes in \textit{L. rigidum} plants harvested at the 5-tiller stage sixty days after 100 g pyroxasulfo ne ha$^{-1}$ treatment in pyroxasulfone-resistant progeny P6 (black bars), untreated parental MR population (grey bars), or herbicide untreated susceptible S population (white bars). Transcript levels were assessed by real-time RT-PCR and \textit{Isocitrate dehydrogenase} was used as internal control gene. Transcript abundance (gene expression) was normalized to the level of the S population. Data shown are means of six biological replicates (±standard error) [Different letters indicate significant differences after ANOVA analysis and post-hoc Tukey test $P < 0.01$].
Fig. 4. Transcript levels of GST genes in *L. rigidum* plants harvested at the 5-tiller stage sixty days after 100 g pyroxasulfone ha\(^{-1}\) treatment in pyroxasulfone-resistant progeny P6 (black bars), untreated parental MR population (grey bars), or herbicide untreated susceptible S population (white bars). Transcript levels were assessed by real-time RT-PCR and *Isocitrate dehydrogenase* was used as internal control gene. Transcript abundance (gene expression) was normalized to the level of the S population. Data shown are means of six biological replicates (±standard error) [Different letters indicate significant differences after ANOVA analysis and *post-hoc* Tukey test *P* < 0.01].
Fig. 5. Transcript levels of GST-1 (A) and GST-2 (B) genes in one leaf stage *L. rigidum* plants harvested fifteen days after the application of 100 g pyroxasulfone ha\(^{-1}\) treatments (T) versus untreated P6 plants (black bars), treated (25 g pyroxasulfone ha\(^{-1}\)) or untreated parental MR individuals (grey bars) or treated
(25 g pyroxasulfone ha\(^{-1}\)) or untreated susceptible S plants (white bars). Different letters indicate significant differences after ANOVA analysis and *post-hoc* Tukey test $P < 0.01$. 
Acknowledgements

The AHRI Team is a center of excellence for the study of herbicide resistance funded by the Grains Research Development Corporation. The partial funding from Kumiai Chemical Industry, the Australian Research Council (LP0882758) and Bayer CropScience (Grants4Target #2016-2-22) is acknowledged.
References


