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

2 **metabolism**

3 Roberto Busi[1], Aimone Porri[2], Todd A Gaines[3] and Stephen B Powles[1]

4

5 *[1] Australian Herbicide Resistance Initiative, School of Agriculture and Environment, The University of*

6 *Western Australia, Crawley, WA 6009, Australia*

7 *[2] British American Tobacco, Plant Biotechnolgy Division CB4 0WA, 210 Cambridge Science Park*

8 *Milton Rd, Milton, Cambridge, United Kingdom*

9 *[3] Department of Bioagricultural Sciences and Pest Management, Colorado State University*

10 *Fort Collins, CO 80523*

11

12 **Author for correspondence**: Roberto Busi

13 ph +61 8 6488 1423

14 roberto.busi@uwa.edu.au

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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 [^{14}C]-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.

43 **1. INTRODUCTION**

44 In global agriculture, weed control is mandatory to avoid significant crop losses [1]. In most situations
45 synthetic herbicides allow simple and effective weed control practices. However, the evolution of
46 adaptive traits conferring herbicide resistance in agricultural weeds is hampering the efficiency of
47 herbicidal weed control [2, 3]. Evolved herbicide resistance in weed species can be target-site-based due
48 to gene mutations as single nucleotide polymorphisms (SNPs) that cause amino acid substitutions at a
49 herbicide binding site. Target-site resistance (TSR) is usually single-gene inherited resistance [4-8].
50 Conversely, non-target-site resistance (NTSR) encompasses all mechanisms that minimize herbicide
51 injury by limiting toxic herbicide concentrations reaching herbicide sites of action (i.e., reduced herbicide
52 leaf penetration, impaired herbicide translocation andherbicide metabolism allowing herbicide
53 detoxification and recovery from herbicide damage) [9]. Important among NTSR mechanisms are
54 constitutive enzymatic super families responsible for concerted secondary plant metabolism. Herbicide
55 detoxification can schematically occur in four phases: phase I (oxidation), phase II (conjugation), phase
56 III (transport) and phase IV (further compartmentation) [10-12]. Some of these enzymes can mediate
57 herbicide detoxification via herbicide metabolism and inactivation [e.g., cytochrome P450 mono-
58 oxygenases (P450s), glutathione-S-transferases (GSTs; EC 2.5.1.18) or glucosyltransferases (GTs)]
59 followed by herbicide sequestration (.e.g., ABC transporters) [13-15]. Some herbicides that interact with
60 a complex of primary targets (e.g., chloroacetamides, which inhibit a complex system of elongases
61 responsible for the biosynthesis of very long chain fatty acids, VLCFA) have thus far only selected for
62 NTSR mechanisms in weeds [16].

63 The molecular definition and identification of NTSR mechanisms can often be complex, as the P450 or
64 GST enzyme superfamilies containing a multitude of gene family members often interact within a
65 particular detoxification pathway [11]. P450s can facilitate the oxidation or hydroxylation (phase I) of
66 certain herbicide molecules [17] and be responsible for herbicide metabolism in some crop species (e.g.,

67 maize, rice, wheat) and weeds [12, 18, 19]. Glutathione-S-transferases (GSTs) are phase II enzymes that
68 can allow herbicide metabolism through conjugation with the tripeptide glutathione (γ-
69 glutamylcysteinylglycine, GSH) [20]. GST are abundant in plant cells and widely involved in the
70 recognition and transport of reactive electrophilic compounds of both synthetic and natural origins [21-
71 23]. Specifically GSTs are found in the cytoplasm at high concentrations and catalyze the conjugation of
72 GSH to a variety of endogenous and exogenous toxins. Early studies on GSTs were conducted with crop
73 plants to understand the basis of herbicide selectivity. For example, it was shown that expression levels
74 of detoxifying GSTs in certain crops were much greater than in weeds to explain herbicide selectivity [23,
75 24].
76 *Lolium rigidum* (Gaud.) is a genetically diverse, cross-pollinated globally-distributed weed species that
77 has evolved resistance to many different herbicide modes of action [25]. In Australia the first selective
78 post-emergent herbicide deployed for *L. rigidum* control was the acetyl CoA carboxylase (ACCase)-
79 inhibiting herbicide diclofop-methyl introduced in 1978, followed by the acetolactate synthase (ALS)-
80 inhibiting herbicide chlorsulfuron in 1982. Heap and Knight (1986) reported the first case of cross-
81 resistance to ACCase and ALS herbicides evolved by diclofop-methyl field selection. Currently, ACCase
82 and ALS cross-resistance is widespread throughout the southern Australian cropping system [26, 27],
83 whereas lower levels of resistance have been reported for soil-applied pre-emergence herbicides [28, 29].
84 Thus, in response to widespread ACCase and ALS herbicide resistance, there has been an increase in
85 reliance on pre-emergence soil-applied herbicides such as prosulfocarb, pyroxasulfone, triallate, and
86 trifluralin. No field-evolved pyroxasulfone-resistant *L. rigidum* populations have thus far been identified
87 [30]; however, we experimentally evolved pyroxasulfone resistance in *L. rigidum* by recurrent selection
88 with pyroxasulfone over a few generations [31]. As subsequent studies showed, pyroxasulfone selection
89 can result in co-evolution of cross-resistance to the thiocarbamates prosulfocarb and triallate in two

90 distinct *L. rigidum* populations [28, 32], research was warranted to elucidate the mechanistic basis of
91 pyroxasulfone resistance in *L. rigidum*.
92
93 ## 2. MATERIALS AND METHODS
94 ### 2.1. Plant materials
95 The multiple resistant *L. rigidum* population SLR31 (hereinafter referred to as MR) evolved in the field
96 following extensive herbicide selection. MR plants exhibit multiple herbicide resistance to different
97 modes of action including the ACCase-inhibitor diclofop-methyl, the ALS-inhibitor chlorsulfuron [33],
98 the mitosis inhibitor trifluralin [34], and the VLCFAE inhibitor S-metolachlor [35]. This MR population
99 is susceptible to pyroxasulfone (VLCFAE inhibitor) [36], prosulfocarb (VLCFAE inhibitor), and
100 marginally resistant to triallate [37]. MR individuals were exposed to recurrent selection with below-label,
101 sub-lethal doses of pyroxasulfone and experimentally evolved resistance to pyroxasulfone, prosulfocarb,
102 and triallate [31, 32]. Progeny P6 was obtained by six consecutive cycles of recurrent herbicide selection
103 consisting of pyroxasulfone selection at 60 g ha^{-1} (Progeny one, P1), followed by another pyroxasulfone
104 selection at 120 g ha^{-1} (Progeny two, P2) 120 g ha^{-1} (Progeny three, P3), 240 g ha^{-1} (Progeny four, P4),
105 then further subjected to two consecutive selections at 1000 (Progeny five, P5) and 2000 (Progeny six,
106 P6) g prosulfocarb ha^{-1}. The herbicide susceptible *L. rigidum* population VLR1 was the control in all
107 experiments (hereinafter referred to as 'S').
108
109 ### 2.2. Herbicide assays
110 Viable seeds of *L. rigidum* populations P6, MR, and S were germinated on 0.6% (w/v) solidified agar and
111 planted into 2 L pots containing commercial potting mixture (50% peatmoss, 25% sand, and 25% pine
112 bark) when the primordial root was visibly erupting from the seed coat. Approximately 2 hours after
113 seeding the pots were treated with 0 (untreated), 25, or 100 g pyroxasulfone ha^{-1}. For each herbicide dose

114 there were four replicates with 25 viable germinated seeds treated per replicate. Plant survival was
115 assessed 15 days after treatment (DAT). The experiment was conducted twice.
116
117 **2.3. Metabolism study**
118 ^{14}C-labeled pyroxasulfone ([isoxazoline-3-^{14}C]pyroxasulfone) synthesized by Amersham Biosciences Co.,
119 Ltd. (United Kingdom) with specific radioactivity of 1.7 MBq/mg and > 99% purity was used in this study.
120 Pyroxasulfone (white powder, mp 130.7°C (degrees Celsius), water solubility at 20°C 3.49 mg/L, vp
121 2.4×10^{-6} Pa) and the synthetic compounds, 2-amino-5-[1-(carboxylmethylamino)-3-(5,5-dimethyl-4,5-
122 dihydroisoxazol-3-ylthio)-1-oxopropan-2-ylamino]-5-oxopentanoic acid (M-15), 2-amino-3-(5,5-
123 dimethyl-4,5-dihydroisoxazol-3-ylthio) propanoic acid (M-26) and 3-(5,5-dimethyl-4,5-dihydroisoxazol-
124 3-ylthio)-2-hydroxypropanoic acid (M-29) were used. These compounds were synthesized by KI
125 Chemical Research Institute Co., Ltd. (Japan) and their purities were > 98%. Pyroxasulfone treatments
126 were performed as reported by Tanetani et al. [38]. In brief, 13 *L. rigidum* pyroxasulfone-resistant P6 and
127 -susceptible S plants were grown hydroponically up to the 4-leaf stage in 70 ml distilled water. Liquid
128 fertilizer (70µl) with 10% phosphoric acid, 6% nitrogen and 5% potassium was added to growing solution
129 (HYPONex, HYPONex JAPAN CORP., LTD.). Similarly, 1.3 ppm of [^{14}C]-labelled pyroxasulfone was
130 applied to roots (approximately 3.3 µM). Four individual plants were harvested at three different time
131 intervals corresponding to 1, 2, and 4 days after pyroxasulfone treatment and used for extraction and
132 fractionation. The methodology for extraction and fractionation of pyroxasulfone metabolites following
133 pyroxasulfone treatment of *L. rigidum* plants is described in detail by Tanetani et al [38]. In brief,
134 following pyroxasulfone hydroponic treatment, *L. rigidum* plants were weighed, roots washed with 20 ml
135 of acetonitrile, and plants homogenized. Extraction of pyroxasulfone and its metabolites occurred in 150
136 ml of 25% acetone. The extracts were evaporated in vacuo and dissolved in 10 ml of 50% acetonitrile.
137 The radioactivity of the extracts was measured with a liquid scintillation counter (LSC, TRI-CARB

138 2750TR/LL, PerkinElmer, United States). The radioactivity of the residues of the plants was measured
139 with LSC after combustion by a sample oxidizer. Pyroxasulfone and its metabolites were identified by
140 comparison with standards, using thin layer chromatography (TLC) and LC-MS. For TLC analysis, an
141 aliquot of each extract was applied to silica gel. The plates were first developed with a mixture of ethyl
142 acetate/chloroform/methanol/formic acid (60/60/10/10, v/v/v/v) and then the plate was developed a
143 second time in the perpendicular direction with a mixture of ethyl acetate/methanol/distilled water/formic
144 acid (60/40/20/10, v/v/v/v). The subsequent determination of pyroxasulfone and its metabolites by TLC
145 and LC-MS was performed as reported by Tanetani et al. [38].

146

147 **2.4. Molecular study: q-PCR to quantify gene expression levels**

148 Six pyroxasulfone resistant individuals (survivors to 100 g pyroxasulfone ha^{-1}) from the P6 population
149 were identified and individually collected for total RNA extraction and q-PCR analysis. Similarly, six
150 untreated individual plants (n = 6) from MR and S populations were individually harvested for the same
151 q-PCR study, respectively. Two leaf segments of 2 cm were harvested from each individual 5-tiller plant
152 and placed into a 25 mL tube. The individual plant represented the experimental unit as biological
153 replicate. The experiment was repeated with similar methodologies on a greater number of plants. Fifteen
154 days after pyroxasulfone treatment at 100 g ha^{-1} a total of 50 one-leaf surviving resistant P6 plants were
155 harvested (2-cm plant tissue) and divided (n = 2) for total RNA extraction and subsequent q-PCR
156 experiments. Also, 50 one-leaf S plants emerging after pyroxasulfone treatment at 25 g ha^{-1} were
157 harvested. In addition, 50 untreated MR, P6, and S one-leaf plants, respectively, were harvested for q-
158 PCR analysis. Twenty leaf segments of 1 cm were harvested individually from 25 respective plants and
159 pooled into a 25 mL tube.

160

161 Total RNA was isolated from plant tissues by using RNAeasy extraction kit (Qiagen) and treated with
162 DNA-free DNase (Ambion) to remove residual genomic DNA. One µg of total RNA was used for reverse
163 transcription (Superscript III, Invitrogen) in a 20 µL volume reaction. Quantitative PCR was performed in
164 a 384 well-plate using LightCycler 480 (Roche) and all reactions were conducted in three technical
165 replicates and a negative control containing no template with the same reaction mix. Each reaction of 13
166 µL total volume included 6.5 µL of SyberGreen Master Mix, (SensiFAST), 0.25 µL of 0.5 pmol μL^{-1}
167 primers, 3 µL of cDNA (diluted 1:10) and 3 µL of H_2O. Reaction conditions were 3 min incubation at
168 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
169 to confirm single-product amplification. Threshold-cycles (CTs) were obtained for each reaction using the
170 Second Derivative Maximum method in the LightCycler 480 software (Roche). The mean of CT values
171 for the three technical replicates for each sample was used to calculate the relative expression (RE) of the
172 gene of interest using the following equation:

173 $$RE = 2^{-[CT\ gene\ of\ interest - CT\ control]}.$$

174 The control gene used in this assay was *isocitrate dehydrogenase* as described by Gaines et al. [19].
175 The relative expression of *GST-1* Tau class (contig 4546), *GST-2* Tau class (contig 5390), *GST-3* Phi
176 class (contig 8676), *GST-4* Tau class (contig 13326), *GST-5* Phi class (contig 16302), and *P450-1 CYP72A*
177 (contig 1604), *P450-2 CYP72A* (contig 2218), *P450-3 CYP716A* (contig 6783), *P450-4 CYP89A* (contig
178 6759) and *P450-5 CYP71B* (contig 12788) was quantified using primers described by Gaines et al., 2014
179 [19].
180

181 **2.5. Statistical analysis**

182 For all the *L. rigidum* populations analysed in this study graphical data relative to the resistance phenotype
183 are presented as percent (%) of seed germination and plant survival. Gene expression relative to population
184 S is set as equal to 1. Two main types of analysis were conducted to compare and separate population

185 mean values for survival and gene expression levels. Comparisons among survival rates were assessed
186 by chi-square (χ^2) heterogeneity test performed using the statistical software *R* (version 3.02) with the
187 command *prop.test*. Relative gene expression were subjected to ANOVA and population means (P6 vs.
188 MR vs. S) separated by Tukey's HSD ($\alpha = 0.05$). Graphical data were obtained and plotted with GraphPad
189 Prism (GraphPad Software, Inc. La Jolla, CA 92037 USA).

190

191 **3. RESULTS**

192

193 **3.1 Response to pyroxasulfone treatments of resistant P6, MR and S *L. rigidum* plants prior to**
194 **molecular analysis**

195 When treated at the recommended dose of pyroxasulfone (100 g ha^{-1}) there was 54% survival (plant
196 emergence) of the resistant P6 plants. As expected, for the parental MR and the standard herbicide-
197 susceptible S populations there was only 5% survival (Figure 1). The herbicide assay was repeated with
198 48% plant survival observed in P6 plants treated with 100 g pyroxasulfone ha^{-1} Survival observed in MR
199 plants (45%) treated with a low dose of pyroxasulfone (25 g ha^{-1}) was not different from P6 plants (X-
200 squared = 0.07, P = 0.80). Conversely survival of S plants was significantly lower than P6 and MR,
201 respectively (X-squared = 12.9, P = 0.002) (data not shown). (27% survival, data not shown).

202

203 **3.2 $^{[14]}$C-pyroxasulfone metabolites analysis in pyroxasulfone-resistant P6 *L. rigidum* plants**

204 Following root application of parent [^{14}C]-labelled-pyroxasulfone to *L. rigidum* plants at the 3-leaf stage,
205 the total radioactivity was determined over time. Pyroxasulfone-resistant P6 plants absorbed from 8% (1
206 DAT) up to 25% (4 DAT) of parent pyroxasulfone applied, corresponding to a concentration of $^{[14]}$C
207 radioactivity recovered of 10.71 μg eq./g plant tissue harvested (Table 1).

208 The total radioactivity absorbed (recovered) in resistant P6 plants was approximately two-fold higher than
209 in S plants (Table 1). Equally, the total amounts of the [^{14}C]-labelled metabolites measured at one, two,
210 or four days after treatment (DAT) in the pyroxasulfone-resistant P6 plants were larger than in S plants.
211 In P6 pyroxasulfone-resistant plants the decomposition rate of $^{[14]}$C-pyroxasulfone into metabolites was
212 faster and up to 4-fold greater than in the S plants (Table 2). For example, in P6 pyroxasulfone-resistant
213 plants approximately 88% of the parent $^{[14]}$C-labelled pyroxasulfone was metabolized (1 DAT) versus
214 54% in susceptible plants and similarly the percentage of identified pyroxasulfone metabolites were two-
215 fold greater than in susceptible plants (Table 2). This indicates that in P6 plants the parental [^{14}C]-labelled
216 pyroxasulfone was more rapidly metabolized.
217 In the extracts from resistant P6 and S plants, a total of eight metabolites were evident by TLC analysis
218 (Figure 2). Six of these metabolites (TLC spots), namely pyroxasulfone, Uk-1, Uk-3, cysteine conjugate
219 of isoxazoline ring (M-26), the metabolite in which amino group of M-26 was replaced with hydroxyl
220 group by oxidative deamination (M-29) and glucose conjugate of M-29 (M-29-glc) were the same
221 chemical compounds as those detected in wheat Figure 2, Table 2). Considering the ratio of the
222 radioactivity of each metabolite, M-26, M-29, and M-29-glc were the main metabolites identified in wheat
223 and pyroxasulfone-resistant and -susceptible *L. rigidum,* as previously shown elsewhere [38]. The
224 metabolite M-15 corresponding to the GSH conjugate of isoxazoline ring of parental pyroxasulfone was
225 not detected in this study. However, in plants the GSH conjugate of pesticides is generally catabolized to
226 a cysteine conjugate by liberation of glycine and glutamic acid from the GSH moiety [39]. In our
227 preliminary metabolism study of pyroxasulfone in barnyard millet cultured cells, a large amount of M-26
228 and small amount of M-15 were detected (data not shown) as shown for other herbicides [40], indicating
229 that the pyroxasulfone-GSH conjugate (M-15) is immediately metabolized to the cysteine conjugate (M-
230 26). Out hypothesis is that the M-15 pyroxasulfone GSH conjugate is labile and therefore not detected in
231 the TLC assay. The pyroxasulfone cysteine conjugate M-26 was generated by liberating glutamic acid and

232 glycine from the GSH conjugate of the isoxazoline ring (M-15) and M-26 was then metabolized to M-29
233 by oxidative deamination. Subsequently, M-29-glc was generated by glucose conjugation of M-29. These
234 metabolic processes indicated that the main metabolites (M-26, M-29 and M-29-glc) are assumed to be
235 formed *via* the initial metabolic step of GSH conjugation of the isoxazoline ring of pyroxasulfone. Thus
236 the main route of pyroxasulfone metabolism appears to be the cleavage of methylensulfonyl linkage by
237 GSH conjugation of the isoxazoline ring [38].
238

239 **3.3 Transcript levels of genes encoding herbicide-metabolizing enzyme in resistant P6, MR and**
240 **S *L. rigidum* plants**

241 To assess whether pyroxasulfone resistance is associated with increased transcript levels of herbicide-
242 metabolizing genes, the expression levels of five putative *P450s* and *GSTs* previously identified in
243 resistant *Lolium* populations [19] were determined by quantitative real time PCR. The tested *P450s* and
244 *GSTs* were named from 1 to 5 (see material and methods). In this assay the P6 pyroxasulfone resistant
245 individuals were compared with the untreated susceptible MR individuals and susceptible S individuals.
246 The transcript quantification was performed on six different biological replicates and the statistical
247 significance among the different individuals was assessed using Tukey's HSD and ANOVA tests. The
248 mRNA level of *P450-1* was increased around 6 and 4 times in both pyroxasulfone-resistant P6 individuals
249 and pyroxasulfone-susceptible MR individuals compared with S plants, respectively (Figure 3).There was
250 no difference in *P450-1* expression in resistant P6 compared to MR individuals. The mRNA abundances
251 of *P450-2*, *P450-4* and *P450-5* were not different among resistant P6, MR, and S plants, while the
252 expression of *P450*-3 was 5- and 3-fold reduced in resistant P6 and MR, respectively, compared with S
253 plants ($P < 0.01$) (Figure 3). The transcript levels of *GST-1* were around nine-fold higher in R P6
254 individuals compared to both MR and S plants. Likewise, the mRNA levels of *GST-2* were around 6 and
255 3 times more abundant in R P6 plants compared to MR and S individuals, respectively (Figure 4). The

256 upregulation of these two *GSTs* was consistently found in all tested P6 biological replicates. Tukey's
257 multiple comparisons test of *GST-1* and *GST-2* Tau class expression data showed high statistical
258 significance (P value ≤ 0.01). In contrast, the expression levels of *GST-3,* Phi class *GST-4* Tau class and
259 *GST-5* Phi class were not different among resistant P6, MR, and S individuals (Figure 4). Thus, in the
260 resistant P6 plants the increased transcript levels of *GST-1* and *GST-2* correlates with pyroxasulfone
261 resistance. For further confirmation the expression levels of these two *GTSs* were quantified in resistant
262 P6, MR, and S one-leaf stage plants, 15 days after pyroxasulfone pre-emergence treatment. Resistant P6
263 individuals were treated with 100 g pyroxasulfone ha^{-1} whereas susceptible plants (MR and S) were treated
264 with a sub-lethal 25 g ha^{-1}. The rationale to apply a different pyroxasulfone dose to resistant (100 g ha^{-1})
265 versus susceptible (25 g ha^{-1}) plants was to achieve similar and comparable plant survival in both
266 populations as a uniform plant response from a similar herbicide stress level. In addition, to assess whether
267 the expression of *GST-1* and *GST-2* is constitutively increased in the resistant P6 plants independently of
268 the herbicide treatment, untreated resistant P6, MR and S individuals were also collected. The transcript
269 levels of *GST-1* and *GST-2* in untreated resistant P6 plants were higher (P value ≤ 0.01) than in susceptible
270 plants, with a calculated 7- and 4-fold higher relative gene expression, respectively. Similar results
271 indicating *GST1-1* and *GST-2* over-expression were found in the pyroxasulfone treated plants (Figure 5).

272

273 **4. DISCUSSION**

274

275 This study aimed to understand the biochemical basis of resistance to the herbicide pyroxasulfone in *L.*
276 *rigidum*. Pyroxasulfone has become widely used in major agricultural areas such as Australia, U.S.A.,
277 and Canada [41]. Recent studies have reported experimental selection of pyroxasulfone resistance in *L.*
278 *rigidum* [28, 31] and evolved cross-resistance to pyroxasulfone in *A. fatua* through repeated field exposure
279 to triallate *[42]*. In this study pharmacological (TLC / LC-MS) data support a role for glutathione-S-
280 transferases (GSTs) mediating pyroxasulfone-GSH conjugation. The expression level of two *GST*

281 transcripts was higher in pyroxasulfone resistant plants. Thus, the mechanistic basis for pyroxasulfone
282 resistance in *L. rigidum* is likely to be metabolism-based with resistant *L. rigidum* P6 plants with enhanced
283 capacity to detoxify pyroxasulfone *via* a GSH conjugation pathway. It remains to be determine whether
284 the identified *GST* transcripts are responsible for the GSH conjugation through further experimentation.
285 In pyroxasulfone-resistant P6 plants 88% of the parent $^{[14]}$C-labelled pyroxasulfone can be metabolized
286 into several different metabolites within 24 hours after the herbicide treatment. Similar detoxification rate
287 of pyroxasulfone is reported in pyroxasulfone-resistant wheat plants [38]. TLC and HPLC-MS studies
288 indicate that in pyroxasulfone-resistant *L. rigidum* and wheat plants similar metabolic pathways mediate,
289 *via* GSTs, the GSH conjugation of the isoxazoline ring of $^{[14]}$C-pyroxasulfone leading to formation of
290 three main metabolites explaining safety versus toxicity in crops versus grass weeds *[38].* In one grass
291 species (barnyard millet) a study on pyroxasulfone metabolism in cultured cells indicated that the
292 conjugate pyroxasulfone-GSH is labile as the metabolism of pyroxasulfone resulted in a very small
293 amount of the pyroxasulfone-GSH conjugate (Kumiai Chemical Industry, unpublished data). Tanetani et
294 al [38] reported similarly that the pyroxasulfone-GSH conjugate (metabolite M15) was immediately
295 metabolized to the cysteine conjugate (M-26) after the GSH conjugation reaction [38]. The labile nature
296 of conjugates between the tripeptide glutathione and electrophilic xenobiotic substrates such as herbicides
297 has been extensively reviewed [23]. Activity of GST mediating tolerance to thiocarbamate herbicides was
298 first observed in corn plants pre-treated with the specific herbicide safener dichloroacetamide increasing
299 root GSH content and GST activity [43]. Since then it has become clear that a range of plant GSTs can
300 catalyze conjugation of GSH with certain herbicide classes [44]. GSTs belong to an enzyme superfamily
301 which includes two plant specific classes [Phi and Tau] that can be associated with herbicide resistance
302 in weeds [45]. Thus, the electrophilic nature of some herbicide molecules, often after initial P450-
303 mediated oxidation, sulfoxydation or hydroxylation, can bind to the cysteine residue of GSH as the first
304 step in this detoxification pathway [16, 46]. These chemical reactions involving K_3 herbicides and GSH

305 are similar to the covalent binding of the KCS (3-ketoacyl-CoA synthase) enzymatic complex identified
306 as one of the primary target for these VLCFAE-inhibiting herbicides [47, 48]. Crop selectivity to several
307 different chloroacetamide herbicides is similarly mediated by enhanced GST activity, which is also
308 documented to be the result of a change in *GST* expression [22, 49-51]. Here, a faster rate of pyroxasulfone
309 metabolism in pyroxasulfone-resistant *L. rigidum* and resistant wheat plants suggest similarities in
310 metabolic detoxification of pyroxasulfone in the two species [38, 52].
311 This study provides evidence that a significant increase in constitutive *GST* gene expression is correlated
312 with pyroxasulfone resistance. Both *GST-1* and *GST-2*, Tau class, had significantly higher transcription
313 in pyroxasulfone-resistance individuals (P6) than in pyroxasulfone-susceptible MR or S plants. No
314 additional upregulation of *GST-1* and *GST-2* following pyroxasulfone treatment was observed, indicating
315 that the over-expression of these two GST transcripts was constitutive. As a previous inheritance study
316 shows that pyroxasulfone resistance in *L. rigidum* is conferred by a semi-dominant allele segregating at
317 one major locus [53] the upregulation of two different *GST* genes in a trait inherited as a single semi-
318 dominant allele could be explained if *GST-1* and *GST*-2 are closely linked on the same chromosome,
319 thereby producing an inheritance pattern consistent with a single locus. Another possibility is that
320 expression of the two different genes may be co-regulated by a single transcription factor, which would
321 also produce a single gene inheritance pattern [54]. In Arabidopsis increased tolerance to abiotic stressors
322 has been shown to be regulated by one single transcription factor [55]. In wheat plants *GST* (*TaGSTU4*)
323 over-expression induced by the safener fenchlorazole-ethyl mediates resistance to the ACCase-inhibiting
324 herbicide fenoxaprop-ethyl and the K_3 herbicide dimethenamide [56]. BLAST analysis reveals high
325 similarities between *TaGSTU4* and *GST-1* (contig score 205, E-value 1.6 9 10^{-53}) [19]. Other studies on
326 transcriptome analysis provide additional evidence of *GST* over-expression conferring metabolic
327 herbicide resistance in populations of the grass weed *Lolium* from France [57, 58]. . *GST-1* and *GST-2*
328 (Tau class) have up to 35% identity across 250 bp with three different *GST* Tau class transcriptional

329 markers, and 10% identity with one *GST* Phi class transcriptional marker, suggesting some similarity with
330 the Tau class *GST* markers reported in France. We report an increased expression of *GST-5,* (Phi class)
331 only in three P6 individuals surviving pyroxasulfone which resulted in non-significant over-expression
332 when the results were pooled (Figure 4). *GST-5,* (Phi class) has 94.5% similarity across 145 bp to the
333 *LrGSTF1* homologue of *AmGSTF1* endowing fenoxaprop-ethyl resistance in *A. myosuroides* [59].
334 Taken together, the chromatographic work conducted is highly suggestive of pyroxasulfone metabolism
335 *via* a GSH conjugation pathway with production of distinctive GSH-pyroxasulfone conjugate and
336 subsequent metabolites. In addition the *GST* over-expression found in pyroxasulfone-resistant *L. rigidum*
337 via qPCR is consistent with the hypothesis of enhanced GST activity in resistant *L. rigidum* mediating
338 pyroxasulfone detoxification. Further work remains to resolve the links between patterns of herbicide
339 selection and evolved pyroxasulfone resistance mechanism(s) including gene expression and gene
340 regulation that could drive the evolution of herbicide resistance in grass weeds.
341

342 **Table 1**. Amount of radioactivity detected in resistant (P6) and susceptible (S) *L. rigidum* plants treated with [isoxazoline-^{14}C] pyroxasulfone at
343 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
344 amount of parent [isoxazoline-^{14}C] pyroxasulfone equivalent (µg eq.) or concentration (µg eq./g). Data are partially drawn from [38].

Population	**DAT**	**Plants Harvested**	**Plant fresh mass (g)**	**Total ^{14}C radioactivity (µg eq.)**	**Recovery (%)**	**Total ^{14}C recovered (µg eq.)**	**Concentration ^{14}C recovered (µg eq./g)**
P6	1	13	1.51	1099	8	6.76	4.48
P6	2	13	1.76	1099	12	11.13	6.33
P6	4	13	2.08	1099	25	22.06	10.71
S	1	13	1.82	1099	4	4.1	2.3
S	2	13	1.89	1099	6	5.9	3.1
S	4	13	1.87	1099	10	8.8	4.7

345

346 **Table 2**. Percentages of parent [isoxazoline-^{14}C] pyroxasulfone (%) and its metabolites identified (M-26, M-29 and M-29-glc) and metabolites

347 unknown found in resistant (P6) and susceptible (S) *L. rigidum* plants treated with ^{14}C-pyroxasulfone and harvested 1, 2 and 4 days after

348 treatment (DAT)* Values in parentheses indicate the concentration of radioactivity (µg eq./g).). Data partially re-drawn from [38].

Population	DAT	$^{[14]}$Pyroxasulfone (%) (µg eq./g)	M-26	M-29	M-29-glc	Total identified (%) (µg eq./g)	Metabolites unknown	Grand Total
P6	1	12.2 (0.57)*	21.8	7.8	15.7	45.3 (2.03)	26.5	84.0
P6	2	4.5 (0.28)*	22.5	17.1	11.7	51.3 (3.25)	32.1	87.9
P6	4	4.6 (0.49)*	20.0	12.7	20.0	52.7 (5.65)	28.4	85.7
S	1	46.4 (1.07)	13.7	3.6	4.6	21.9 (0.50)	17.1	85.4
S	2	26.4 (0.82)	18.2	8.2	10.0	36.4 (1.13)	27	89.8
S	4	9.1 (0.43)	24.6	10.0	13.7	48.3 (2.27)	35.8	93.2

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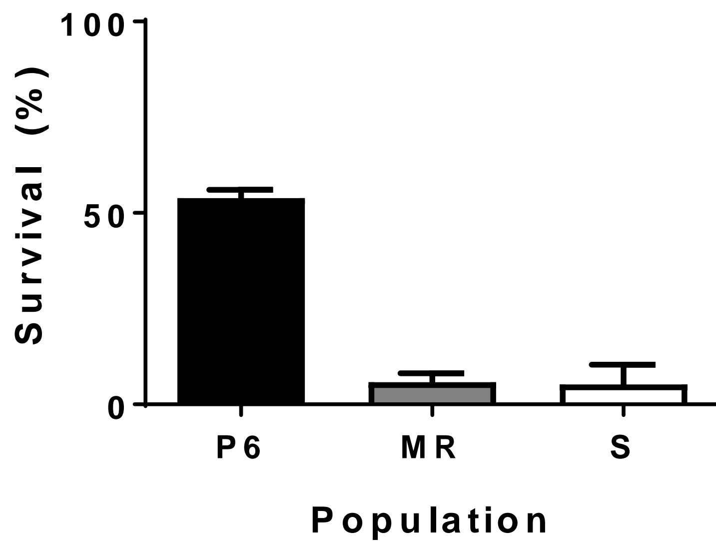


352

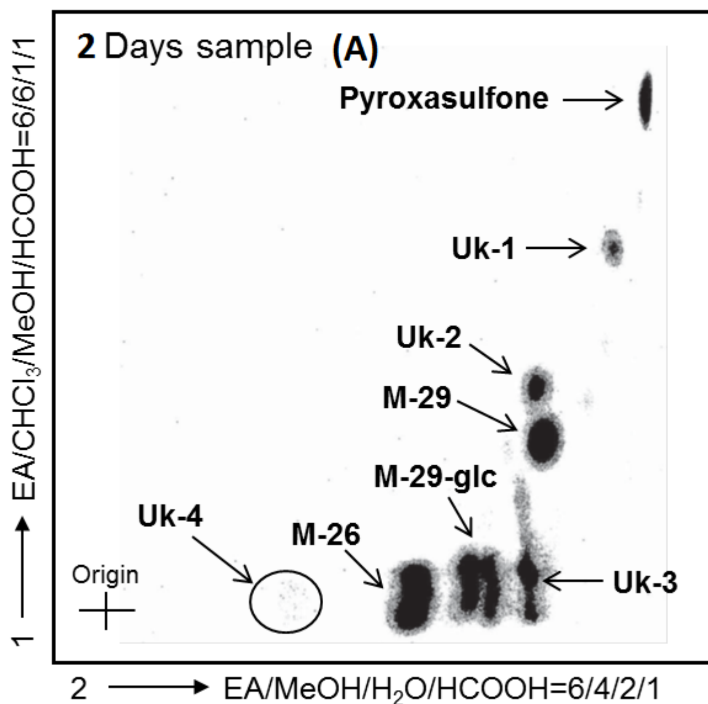
353 **Fig. 1.** Mean plant survival (%) as ratio of actively growing plants versus seeds treated ± standard errors

354 (SE) in pyroxasulfone treated *Lolium rigidum* plants. Survival ± SE (n = 4) assessed as plant emergence

355 in pot cultured plants assessed 60 days after 100 g pyroxasulfone ha^{-1} treatment in pyroxasulfone-

356 resistant progeny P6 (black bar), parental MR (grey bar), herbicide susceptible S population (white bar).

357



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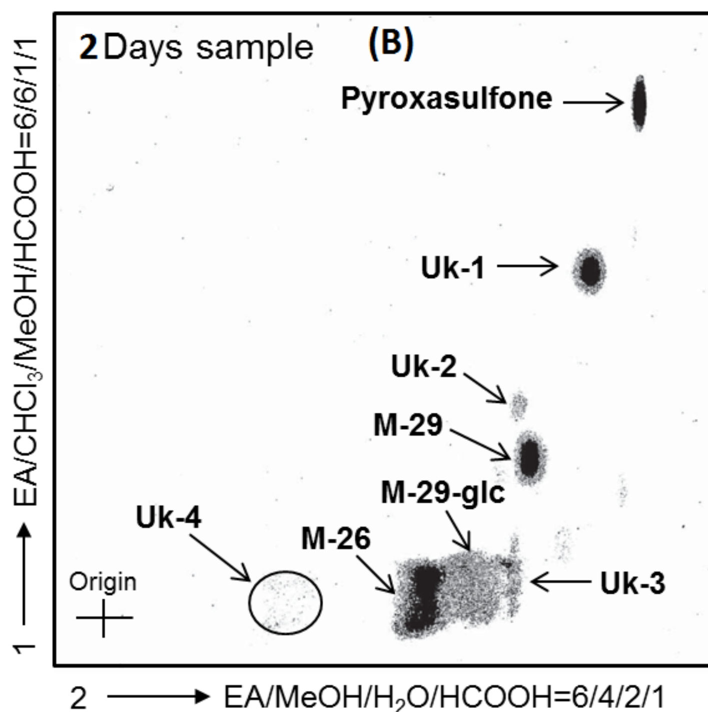
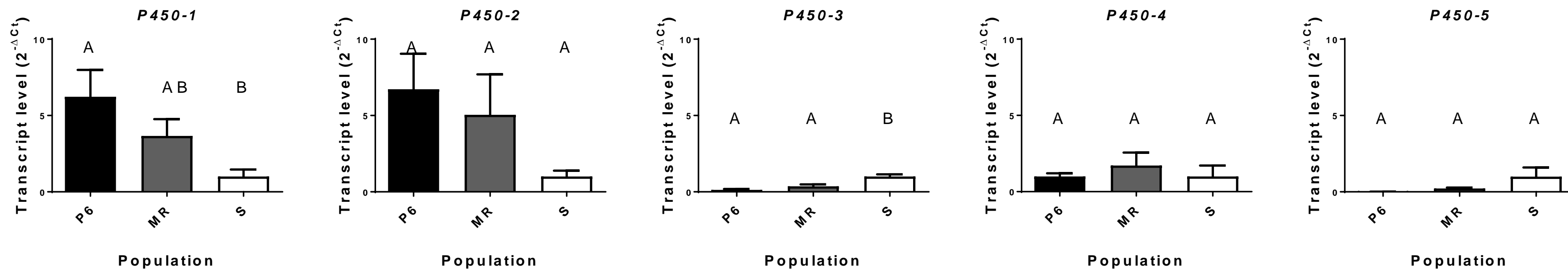


359

360 **Fig. 2**. Two-dimensional TLC of the extract from R biotype of rigid ryegrass after treatment with ^{14}C-
361 pyroxasulfone (4 DAT) in (A) pyroxasulfone-resistant (P6) versus (B) pyroxasulfone-susceptible (S) *L.*
362 *rigidum* plants. Figure (B) re-drawn from [38].

363



364

365 **Fig. 3**. Transcript levels of *P450* genes in *L. rigidum* plants harvested at the 5-tiller stage sixty days after 100 g pyroxasulfone ha^{-1} treatment in

366 pyroxasulfone-resistant progeny P6 (black bars), untreated parental MR population (grey bars), or herbicide untreated susceptible S population

367 (white bars). Transcript levels were assessed by real-time RT-PCR and *Isocitrate dehydrogenase* was used as internal control gene. Transcript

368 abundance (gene expression) was normalized to the level of the S population. Data shown are means of six biological replicates (±standard error)

369 [Different letters indicate significant differences a after ANOVA analysis and *post-hoc* Tukey test P < 0.01].

370

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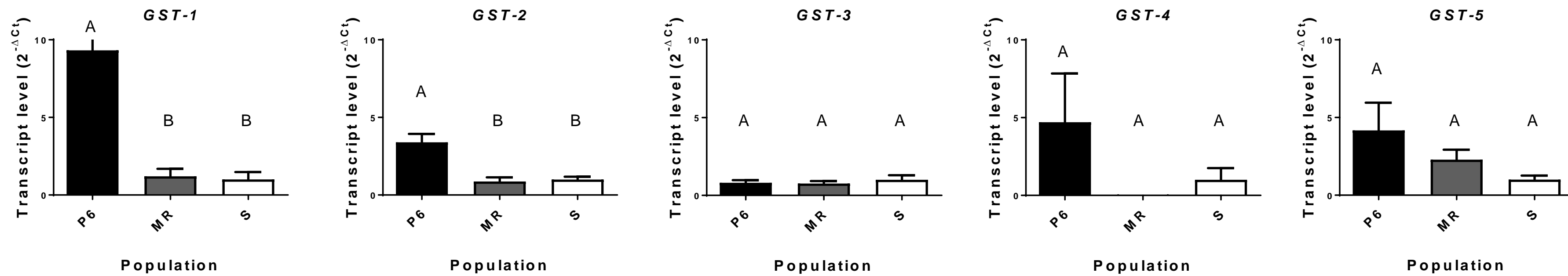

372

373 **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
374 pyroxasulfone-resistant progeny P6 (black bars), untreated parental MR population (grey bars), or herbicide untreated susceptible S population
375 (white bars). Transcript levels were assessed by real-time RT-PCR and *Isocitrate dehydrogenase* was used as internal control gene. Transcript
376 abundance (gene expression) was normalized to the level of the S population. Data shown are means of six biological replicates (±standard error)
377 [Different letters indicate significant differences after ANOVA analysis and *post-hoc* Tukey test $P < 0.01$].

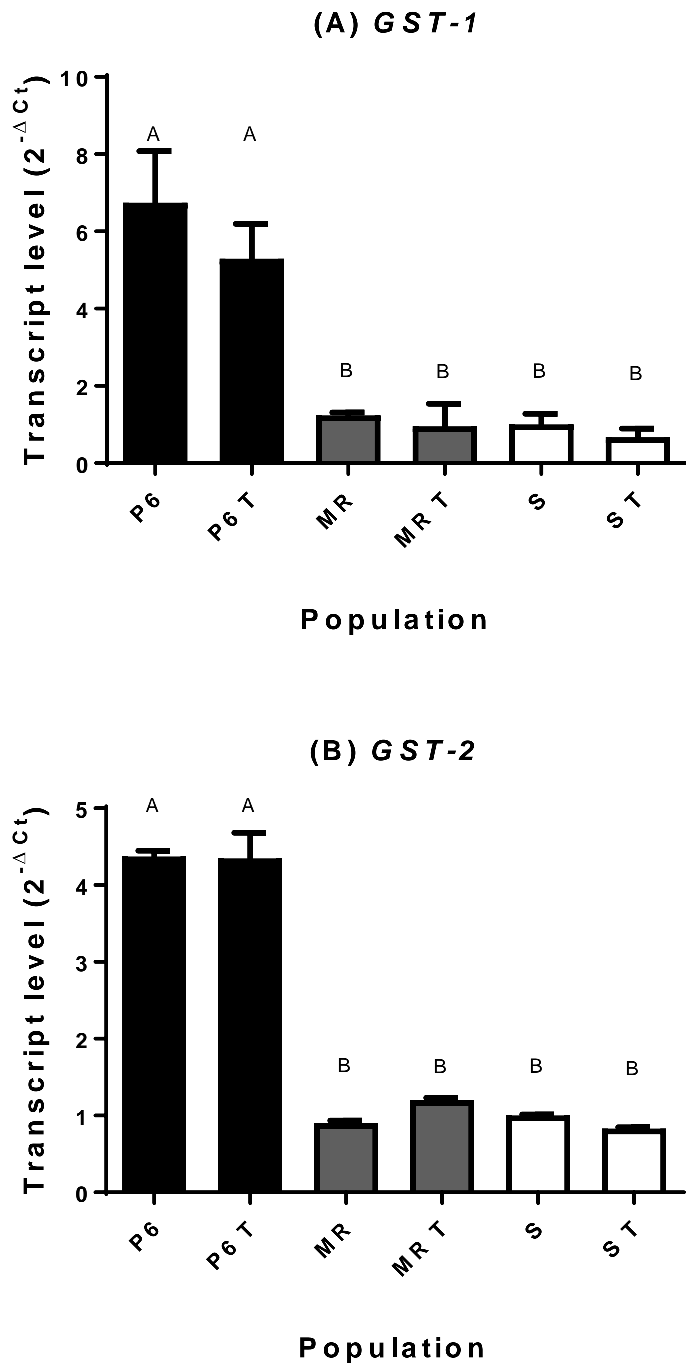

Fig. 5. Transcript levels of *GST-1* (A) and *GST-2* (B) genes in one leaf stage *L. rigidum* plants harvested fifteen days after the application of 100 g pyroxasulfone ha^{-1} treatments (T) versus untreated P6 plants (black bars), treated (25 g pyroxasulfone ha^{-1}) or untreated parental MR individuals (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 $P < 0.01$.

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