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Role of glutathione S-transferases in the mode of action of herbicides that inhibit amino acid synthesis in *Amaranthus palmeri*

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ABSTRACT

Acetolactate synthase inhibitors (ALS inhibitors) and glyphosate are two classes of herbicides that act by inhibiting an enzyme in the biosynthetic pathway of branched-chain or aromatic amino acids, respectively. Besides amino acid synthesis inhibition, both herbicides trigger similar physiological effects in plants. The main aim of this study was to evaluate the role of glutathione metabolism, with special emphasis on glutathione S-transferases (GSTs), in the mode of action of glyphosate and ALS inhibitors in *Amaranthus palmeri*. For that purpose, plants belonging to a glyphosate-sensitive (GLS) and a glyphosate-resistant (GLR) population were treated with different doses of glyphosate, and plants belonging to an ALS-inhibitor sensitive (AIS) and an ALS-inhibitor resistant (AIR) population were treated with different doses of the ALS inhibitor nicosulfuron. Glutathione-related contents, GST activity, and related gene expressions (glutamate-cysteine ligase, glutathione reductase, Phi GST and Tau GST) were analysed in leaves. According to the results of the analytical determinations, there were virtually no basal differences between GLS and GLR plants or between AIS and AIR plants. Glutathione synthesis and turnover did not follow a clear pattern in response to herbicides, but GST activity and gene expression (especially Phi GSTs) increased with both herbicides in treated sensitive plants, possibly related to the rocketing H_2O_2 accumulation. As GSTs offered the clearest results, these were further investigated with a multiple resistant (MR) population, compressing target-site resistance to both glyphosate and the ALS inhibitor pyrithiobac. As in single-resistant plants, measured parameters in the MR population were unaffected by herbicides, meaning that the increase in GST activity and expression occurs due to herbicide interactions with the target enzymes.

1. Introduction

Weeds pose severe problems in croplands due to the competition with crops and pest hosting. Between the many developed methods for weed control, herbicides stand as quick, effective and time-saving tools (Imran and Al-Tawaha, 2021). Herbicides inhibit specific molecular target sites within plant biological pathways. The target of a herbicide can be defined as the biomolecule or process that gets specifically inhibited by herbicidal action, and efficient target-site delivery is a fundamental factor in herbicide activity. Two enzymes involved in amino acid synthesis must be highlighted as important herbicide targets: 5-enolpyruvylshikimate-3-phosphate-synthase (EPSPS) in the aromatic amino acid (AAA) biosynthetic pathway, and acetolactate synthase (ALS) in the branched-chain amino acid (BCAA) biosynthetic pathway. First, EPSPS is the target of glyphosate (Herbicide Resistance Action Committee or HRAC group 9), the most used herbicide in the world. Glyphosate is widely used in agricultural and non-agricultural practice (gardening, for example), especially since the spread of genetically modified glyphosate-resistant crops (Székács and Darvas, 2011; Vereecken, 2005). For its part, ALS-inhibiting herbicides (HRAC group 2) conform a large herbicide group, englobing various chemical families, such as the sulfonylurea herbicides.

The downside of over-usage of herbicides results in some non-desirable economic and environmental consequences (Farhangi-Abriz,

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2016). One of these consequences is the development of herbicide-resistant weed populations. The resistance of weed biotypes to herbicides occurs naturally, and long-term repeated application of the same herbicide or herbicides with the same target accelerates the selection of resistant weeds to that type of herbicide (Neve et al., 2014). Multiple resistant weed biotypes have also arisen, which are simultaneously resistant to two or more different mechanisms of action, and are even more difficult to manage. Recently, weed biotypes resistant to up to nine different mechanisms of action have been found (Heap, 2023).

Glyphosate and ALS inhibitors are two of the herbicide groups for which more resistant weeds have been detected (Heap, 2023). ALS inhibitors conform the herbicide group for which the highest number of weed species of which resistant populations has been detected, and glyphosate-resistant weed populations have also proliferated during recent years (Heap, 2023). Some weed species are especially propense to develop resistance to herbicides, like *Amaranthus palmeri* S. Watson. It is one of the most problematic weeds worldwide, widespread across much of the American continent and also present in Europe (Manicardi et al., 2023; Torra et al., 2020).

In *A. palmeri*, the most usual resistance mechanism to glyphosate and ALS inhibitors is based on target modifications of the enzymatic target-site (TS), this is, herbicide resistance is based on target modifications. In the case of glyphosate, the most common resistance mechanism is *EPSPS* gene amplification, which gives place to an extremely high number of copies of the EPSPS enzyme. In the case of ALS inhibitors, the most common resistance mechanism consists of point mutations in the *ALS* gene that prevent the herbicides from binding to the target but maintain the enzyme's functionality (Heap, 2023; Yu et al., 2010; Zhao et al., 2020). Both EPSPS and ALS activities are well-established target sites, and are well studied and characterised. Nevertheless, how plants die after enzyme inhibition and the sequence of events from herbicide application to plant death (i.e. the herbicide's mode of action) are not fully understood in all the cases. Based on previous studies, physiological effects in response to both types of herbicides, glyphosate and ALS inhibitors, seem really similar. This suggests that, although they target different enzymes of different pathways, both types of herbicides kill the plants by similar mechanisms (Zulet et al., 2013, 2015). These common physiological effects include carbohydrate accumulation due to impairment of carbon metabolism (Orcaray et al., 2012; Zabalza et al., 2004), decrease in soluble protein in favour of free amino acids (Zulet et al., 2013), and also a mild oxidative stress (Eceiza et al., 2022, 2023), all of which are related to the interaction of the herbicides with the respective target enzymes.

Other plant responses to herbicide treatment are less known, like the responses related to glutathione. Furthermore, the possible physiological implications of resistance mechanisms on glutathione metabolism are understudied. Glutathione (γ-glutamylcysteinylglycine) is an intracellular thiol molecule present in almost all living cells in its reduced (GSH) and oxidised (disulphide dimer, GSSG) forms. Glutathione biosynthesis involves two steps: formation of γ-glutamylcysteine (GGC) catalysed by glutamate-cysteine ligase (GCL) (Anderson and Meister, 1983; White et al., 2003), and addition of glycine (Gly) to GGC by glutathione synthetase (Hoefgen and Hesse, 2007).

Glutathione is vital as an antioxidant molecule by protecting cells from oxidative damage by reducing reactive oxygen species (ROS) and other oxidant molecules. In turn, GSH is oxidised to GSSG, which can be reduced back to GSH in a reaction catalysed by glutathione reductase (GR). Other antioxidant functions of GSH include its use as a substrate for glutathione peroxidase (GPX), for maintaining reduced α-tocopherol and zeaxanthin, and for preventing of oxidative denaturation of proteins (Hasanuzzaman et al., 2017). Apart from its antioxidant function, glutathione is the precursor of phytochelatins and is involved in sulphate assimilation, flower development, and signalling responses (Ha et al., 1999; Noctor et al., 2012; Rouhier et al., 2008). Another important function of glutathione is acting as a substrate for several enzymes, such as glutathione S-transferases (GST).

GSTs consist of a diverse enzyme family with several functions, but detoxification of xenobiotics by nucleophilic attack is considered the most important one (Noctor et al., 2012). Among all GSTs, Phi and Tau GST classes are the most abundant GSTs in plants and are plant-specific. GSTs are involved in the detoxification of a wide variety of xenobiotics, and particularly Phi and Tau GSTs are involved in the detoxification of herbicides, can act as antioxidants, and are inducible following exposure of the plant abiotic or biotic stress (Evans et al., 2017; Lee et al., 2014; Munyampundu et al., 2016). They are induced, for instance, by infections or by treatments invoking plant defence reactions, by extreme temperatures, by osmotic stress, and by xenobiotics like herbicides (Basantani et al., 2011; Dixon et al., 2002; Gullner et al., 2018; Marrs, 1996).

In *Amaranthus* species, GSTs have been identified as enzymes that detoxify various herbicides such as fomesafem (Varanasi et al., 2018), atrazine (Ma et al., 2013; Nakka et al., 2017) and s-metolachlor (Brabham et al., 2019; Hwang et al., 2023; Rangani et al., 2021). ALS inhibitors can be detoxified by GST activity in *Echinochloa crus-galli* (Fang et al., 2019) and the bacteria *Klebsiella jilinsis* (Zhang et al., 2020), while glyphosate detoxification by GST has not been demonstrated. Although herbicide metabolization by GST has been widely described, few *GST* genes have been identified to be involved in this process. Different recombinant Phi and Tau GSTs have been described to metabolise flufenacet in *Alopecurus myosuroides* (Parcharidou et al., 2023) and a phi-class GST (*AtuGSTF2*) has been identified to confer resistance to atrazine (PSII inhibitor) in *Amaranthus tuberculatus* (Evans et al., 2017). Phi-class GSTs are also known to confer herbicide selectivity and natural tolerance in maize due to the rapid metabolization of herbicides (Fuerst et al., 1993; Holt et al., 1995; Irzyk and Fuerst, 1993; Jepson et al., 1994).

The availability of *A. palmeri* populations resistant to glyphosate or ALS inhibitors due to modified TS in their addressed enzymes and their respective sensitive populations gives the opportunity to carry out a comparative study on the metabolism of GSH in plants sensitive and resistant to these herbicides. Therefore, the main aim of this study was to evaluate the role of GSH metabolism, with special emphasis on GSTs, in the mode of action of glyphosate and ALS inhibitors. For this purpose, the basal pattern, and the response of five *A. palmeri* populations to herbicide treatment were evaluated: glyphosate-sensitive, glyphosate-resistant, ALS-inhibitor sensitive, ALS-inhibitor resistant, and multiple resistant (to glyphosate and ALS inhibitors).

2. Materials and methods

2.1. Plant material and growth

Amaranthus palmeri plants belonged to five separate populations with known and previously characterised sensitivity/resistance profile: glyphosate-sensitive (GLS, which was also sensitive to ALS inhibitors), glyphosate-resistant (GLR), ALS inhibitor-sensitive (AIS), ALS inhibitor-resistant (AIR), and multiple resistant (MR). The seeds of GLS and GLR biotypes were originally collected from North Carolina (USA) and kindly provided by Dr. Gaines (Colorado State University, Fort Collins, CO, USA). The TS-resistance (TSR) mechanism of the GLR population was *EPSPS* gene amplification, previously characterised (Fernández-Escalada et al., 2016). Apart from glyphosate resistance conferred by *EPSPS* gene amplification, no phenotypic effects have been detected between both populations (Fernández-Escalada et al., 2017, 2019). The seeds of AIS and AIR biotypes were originally collected from Lleida (Spain) and kindly provided by Dr. Torra (University of Lleida, Catalonia, Spain). In AIR plants, resistance is conferred by a point mutation in the Trp-574 position of the *ALS* gene, which makes the ALS enzyme non-sensitive to nicosulfuron and other ALS inhibitors conserving its functionality (Eceiza et al., 2023; Torra et al., 2020). Finally, MR plants were originally collected from Arizona (USA) and kindly provided by Dr. Gaines. The plants belonging to this population contained both the *EPSPS*

amplification (58-fold increase) and the *ALS* mutation at 5 positions conferring resistance to ALS inhibitors (Barco-Antoñanzas et al., 2022).

Plants belonging to all the populations were cultivated and grown under the same growth conditions according to Fernández-Escalada et al. (2016). Seeds were surface sterilised prior to germination with NaClO and HCl (Labhilili et al., 1995). For germination, seeds were incubated for 4 days at 4 °C in darkness. Then, they were maintained for 60 h in a light/darkness cycle of 16 h/8 h at a temperature of 30 °C/18 °C. After germination, plants were transferred to aerated 2.7 L hydroponic tanks in a phytotron (TARRE, Noain, NA, Spain) (day/night, 16 h/8 h; light intensity, 0.5 mmol s^{-1} m^{-2} PAR; temperature, 22/18 °C; relative humidity, 60/70 %). A full-strength modified Hoagland nutrient solution (Hoagland and Arnon, 1950) was provided each 9 days, which contained 15 mM KNO_3. All treatments were applied to three-week-old plants after selecting individuals of similar size and vigour and all experiments were made on plants in the vegetative phenological stage.

2.2. Treatment application

Amaranthus palmeri plants were treated with herbicides depending on the population they belonged to. Consistent with previous studies, GLS and GLR plants were treated with glyphosate as described in Eceiza et al. (2022) at field rate (FR) and 3 times the FR (3FR). Glyphosate is recommended at 0.84 kg ha^{-1} (Culpepper et al., 2006) and Fortin Green® (Key, Tárrega, CAT, Spain) was used as the commercial herbicide. AIS and AIR plants were treated with nicosulfuron as described in Eceiza et al. (2023) at field rate (FR) and 3 times the FR (3FR). Nicosulfuron is recommended at 0.06 kg ha^{-1} (Huang et al., 2019) and Samson® (Key, Tárrega, CAT, Spain) was used as the commercial herbicide. In the four populations, some plants remained untreated ('control' plants).

Apart from that, GLS, GLR, and MR plants were treated with glyphosate, the ALS inhibitor pyrithiobac, and the combination of both herbicides at FR, as described in (Barco-Antoñanzas et al., 2022). Analytical grade pyrithiobac (Dr. Ehrenstorfer LGC, Augsburg, Germany) was applied to leaves containing 5.4% sodium lauryl sulphate adjuvant (commercial formula Biopower 27.65%) (Bayer Crop Science, Madrid, Spain), and is recommended at 0.09 kg ha^{-1}. Plants of the combined treatment were sprayed first with glyphosate and then with pyrithiobac. For comparison with sensitive counterparts, some GLS plants also received these treatments. Control plants were only sprayed with adjuvant.

For herbicide spraying, the required substance was sprayed diluted in 2.5 mL distilled water, using an aerograph (Junior Start model, Definik, Sagola, Spain) coupled to a compressor (Werter One, Beverrato). This applied for all the treatments.

2.3. Analytical determinations

All leaves of GLS, GLR, and MR plants were sampled three days after herbicide treatment, while leaves of AIS and AIR plants were sampled one week after herbicide treatment. Sampled leaves of each plant were collected in 6 mL vials, frozen in liquid N_2, and stored at −80 °C. Leaf samples were powdered with a Retsch mixer mill (MM200, Retsch, Haan, Germany) and the amount of tissue needed for each analytical determination was separated (aliquoted).

2.3.1. H_2O_2 detection

H_2O_2 concentration was determined according to Deckers et al. (2020) using the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) in a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). Results for each herbicide treatment were expressed in % of the respective untreated plants of the same population to remove daily variability.

2.3.2. Antioxidant capacity, glutathione and related compounds

Antioxidant capacity was measured in untreated GLS, GLR, and MR plants, using two different assays: 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), in the same way as in previous studies (Eceiza et al., 2022).

Glutathione was extracted from leaf samples (0.1 g) in 1 mL 1 M HCl, derivatised with 5-iodoacetamide fluorescein, and reduced with tributylphosphine. Glutathione content was measured by capillary electrophoresis (CE) equipped with a laser-induced fluorescence detector (Eceiza et al., 2022, 2023; Zinellu et al., 2005; Zulet et al., 2015). Reduced (GSH) and oxidised (GSSG) glutathione were determined as described before (Eceiza et al., 2022).

2.3.3. Enzymatic activities

For peroxidase and GR activities, proteins were extracted from leaf samples (0.05 g) as described in Eceiza et al. (2022). Peroxidase activity was measured with guaiacol as the substrate by measuring the increase in the absorbance (470 nm) due to oxidation of guaiacol. GR activity was measured using NADPH as a substrate. The reaction mixture consisted of 48 mM HEPES buffer (pH 7.8), 1.4 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid, 0.3 mM GSSG, 1.4 mM NADPH, and sample extract. The decrease in absorbance due to NADPH oxidation was measured at 340 nm.

For GST activity, proteins were extracted from leaf samples (0.05 g), homogenised in a 0.15 mL extraction buffer comprising 100 mM Tris-HCl (pH 6.5), 2 mM ethylenediaminetetraacetic acid, and containing freshly added 2 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride (PMSF), and a minimum of 7.5 mg mL^{-1} polyvinylpolypyrrolidone (PVPP). Extracts for GST activity also contained 14 mM mercaptoethanol. GST activity was measured as described by Miyata et al. (2004), with a reaction buffer comprising 100 mM phosphate buffer (pH 6.5), 0.36 mg mL^{-1} GSH, 28.6 mM 1-chloro-2,4-dinitro-benzene (CDNB), and a sample extract. Decrease in absorbance due to CDNB conjugation (25 °C) was measured at 340 nm.

Enzymatic activities were related to total soluble protein, measured in the same extracts (Bradford, 1976). A Synergy™ HT Multi-Detection Microplate Reader (Agilent Technologies Inc., Santa Clara, CA, USA) was used for absorbance measurements.

2.3.4. Gene expressions and sequencing

Relative transcript level was calculated for genes encoding enzymes related to glutathione synthesis (Glutamate cysteine ligase, *GCL*), the reduction of GSSG (*GR*), and its conjugation (*GST*). RNA extraction and the subsequent cDNA synthesis were performed according to Fernández-Escalada et al. (2017). The cDNA was diluted tenfold and stored at −20 °C until analysis as described before (Hendrix et al., 2020).

Primers (Suppl. Table 1) for genes encoding *GCL* and *GR* were designed using the genomic sequence of *A. palmeri* and crossed with *Amaranthus hypochondriacus*, as in Zulet-González et al. (2020), using Primer3 4.10 software (GitHub Inc., San Francisco, CA, USA). For primers codifying GST, first *GSTF2* (which codifies Phi class GST in *Amaranthus tuberculatus*) and *GSTU1* and *GSTU2* primers (which codify Tau class GST in *Amaranthus tuberculatus*) from the study conducted by Evans et al. (2017) were tested, but only *GSTU2* worked for *A. palmeri*. According to the same study, Phi 2 GST plays an important role in metabolising herbicides like atrazine (Evans et al., 2017), so primers for two pairs of genes of *A. hypochondriacus* that resemble Phi 2 of *A. thaliana* were designed: *GSTF2.1* and *GSTF2.4*, plus one additional primer, *GSTF2.5*, which is supposed to amplify both pairs.

The specificity of all genes was verified in silico using NCBI BLAST (Basic Local Alignment Search Tool). Quantitative real-time PCR (qPCR) was performed using the Quantinova™ SYBR® Green PCR Kit (Qiagen, Hilden, Germany) according to Hendrix et al. (2020). The amplification conditions were set (2 min at 95 °C, 60 cycles of 5 s at 95 °C, 25 s at the annealing temperature) and the product specificity was confirmed as described in Eceiza et al. (2023). Relative gene expression levels were

calculated using the $2^{-\Delta Cq}$ method, and data were normalised against the expression of a set of stable reference genes selected via the GrayNorm algorithm (Remans et al., 2014) listed in Suppl. Table 1; and whose primers were designed using the sequence of *A. palmeri* and crossed with *A. hypochondriacus*, excepting carbamoyl-phosphate synthetase, extracted from Ma et al. (2013). Primer efficiencies were obtained using a standard curve of a two-fold dilution series generated from a pooled sample Eceiza et al. (2023). The optimal annealing temperature (55–62 °C) for each primer was determined by gradient PCR (Suppl. Table 1). Only primers with efficiency between 80 and 120% were used for qPCR analysis.

PCR products of GST-codifying genes were sequenced by the Sanger sequencing method by StabVida company (Salas, Caparica, Portugal).

2.3.5. GST photoaffinity labelling

Photoaffinity labelling was used to detect GSTs in the GLS, GLR and MR populations. The photoaffinity probe used, DB478, contains glutathione, a benzophene photoreactive group, and an alkine minitag that can be labelled with a fluorophore of biotin via click chemistry. Determination of protein concentration and extraction for GST marking were performed as described before (Barco-Antoñanzas et al., 2023). Protein concentration was adjusted to 0.25 mg mL^{-1}. Samples of 0.05 mL were incubated with 0.005 mL DB478 (Font Farre et al., 2023), 4 mM PBS (pH 7.5), 3 mM β-mercaptoetanol and 1 % PVPP (w/v) for 10 min and then exposed to UV light for 45 min on ice. For click chemistry, samples were resuspended in 4 mM PBS (pH 7.4), 1% (v/v) sodium dodecyl sulphate (SDS), 2.5 μM FAM-picolyl-azide (Cy5 fluorophore), 0.1 mM tris(benzyltriazolylmethyl) amine, 2 mM tris(2-carboxyethyl) phosphine, and 1 mM $CuSO_4$. Samples were shaken for 10 min and boiled at 90 °C for 1 min. Samples were incubated for 60 min with shaking, at room temperature, and in darkness. Click was stopped by the adding 0.2 mL acetone and samples were incubated at 4 °C for 30 min and dried at room temperature for 15 min.

Proteins were separated on 12% SDS polyacrylamide gels as described before (Barco-Antoñanzas et al., 2023). The intensity of the detected bands was quantified using an Amersham™ Typhoon™ Biomolecular Imager scanner (GE Healthcare, Munich, Germany).

2.3.6. Chemical composition

Powdered and weighted leaf samples were placed in a −20 °C freezer for 1 h and immediately afterwards vacuum dried in a lyophiliser. The samples were weighed (dry weight), placed in glass heat-resistant tubes (Schott Duran®), and 1 ml of 69% HNO_3 (ARISTAR®) was added. Next, samples were heated at 110 °C until full digestion. This step was repeated three times. The last digestion was performed using 1 ml of 37% HCl (ARISTAR®) (Huybrechts et al., 2020). Finally, the samples were dissolved in a 2% HCl aqueous solution. The concentration of different elements was quantified using inductively coupled plasma optical emission spectrometry (ICP-OES; Agilent Technologies 700 Series, Santa Clara, CA, USA). The analysed elements were Na, K, Ca, S, Zn, Cu, Mn, Fe, Mg, P, and Cd.

2.4. Statistical analysis

Separately maintained individual plants were considered as biological replicates. Plants were separated for statistical analyses by populations: GLS, GLR, AIS, AIR, and MR. Each mean value was calculated using samples from individual plants that belonged to the same population and received the same herbicide treatment. GLS and GLR plants were not compared with AIS and AIR plants in any case: experiments with glyphosate and nicosulfuron were two separate parallel experiments.

For all measured parameters, first possible differences between untreated GLS and GLR plants and between untreated AIS and AIR plants were evaluated using Student's t-test and considered significant when p-value $\leq$0.05. Significant differences between untreated plants to each herbicide group are marked in the figures by asterisks. Student's t-test (p-value $\leq$0.05) was also used for evaluating possible significant differences between plants treated with each dose and the untreated plants of the same population in results related to elemental analysis. One factor ANOVA analyses with the same p-value threshold were used for evaluating possible significant differences between herbicide treatments in plants of the same population. Significant differences between treatments are highlighted in the figures with different letters.

For the experiments involving the MR population, possible differences between untreated GLS and MR plants were evaluated using Student's t-test and considered significant when p-value $\leq$0.05. When comparing untreated GLS, GLR, and MR populations, one factor ANOVA analyses with the same p-value threshold were used for evaluating possible significant differences between untreated plants. One factor ANOVA analyses with the same p-value threshold (0.05) were used for evaluating possible significant differences between herbicide treatments in plants of the same population. Significant differences between treatments are highlighted in the figures with different letters.

Statistical analyses were performed and figures were built using R 4.3.1 software (R Foundation for Statistical Computing, Vienna, Austria).

3. Results and discussion

Previous studies characterised the resistance of GLR population to glyphosate (Fernández-Escalada et al., 2016) and AIR population to nicosulfuron (Eceiza et al., 2023). GLR individuals had a mean 47.5-fold increase in the number of copies of the EPSPS gene, which led to a 25-fold increase in the level of this protein and a 26-fold increase in EPSPS activity, compared to GLS individuals, and all AIR plants contained the Trp-574 mutation. Both populations were resistant only due to TSR mechanisms. Plants belonging to the GLR population had *EPSPS* gene amplification, while plants belonging to AIR population had a point mutation in the Trp-574 position of the *ALS* gene. GLS and AIS plants suffered growth arrest and died a few days after their respective herbicide treatments, while GLR and AIR plants survived all the glyphosate and nicosulfuron doses, respectively.

3.1. Alterations of glutathione synthesis and turnover in response to glyphosate or nicosulfuron

Glutathione is a crucial molecule in response to abiotic and biotic plant stress and, specifically, to oxidative stress, due to its antioxidant properties. Therefore, glyphosate and ALS inhibitors can potentially alter glutathione synthesis and its turnover, which was evaluated in present study. Glyphosate clearly induced glutathione biosynthesis in sensitive plants (Suppl. Fig. 2). Not only did glutathione content increase, but the main precursors Cys and γ-glutamyl-cysteine increased as well (Suppl. Fig. 2). Glyphosate-induced accumulation or enhanced synthesis of GSH has been reported in *A. palmeri* (Eceiza et al., 2022) and other plant species such as *Pisum sativum* (Zulet et al., 2015) and *Arabidopsis thaliana* (de Freitas-Silva et al., 2017). Contents of metabolites related to glutathione were unaffected by glyphosate treatment in resistant plants, as reported before (Suppl. Fig. 2).

On the other hand, glutathione synthesis did not vary significantly in response to nicosulfuron in the AIS and AIR populations, only a slight increasing tendency was seen in AIS plants treated with 3FR of nicosulfuron (Suppl. Fig. 2). This is an already very high dose, so with these results, nicosulfuron cannot be considered to induce glutathione synthesis. The content of cysteine (Cys) slightly increased in AIS while γ-glutamyl-cysteine was not affected by herbicide treatment (Suppl. Fig. 2). These results are consistent with previous research (Eceiza et al., 2023).

An increase of glutathione content in glyphosate cannot be related with an increase of S uptake or translocation to leaves. Indeed, total S concentration in leaves tended to decrease proportionally to glyphosate

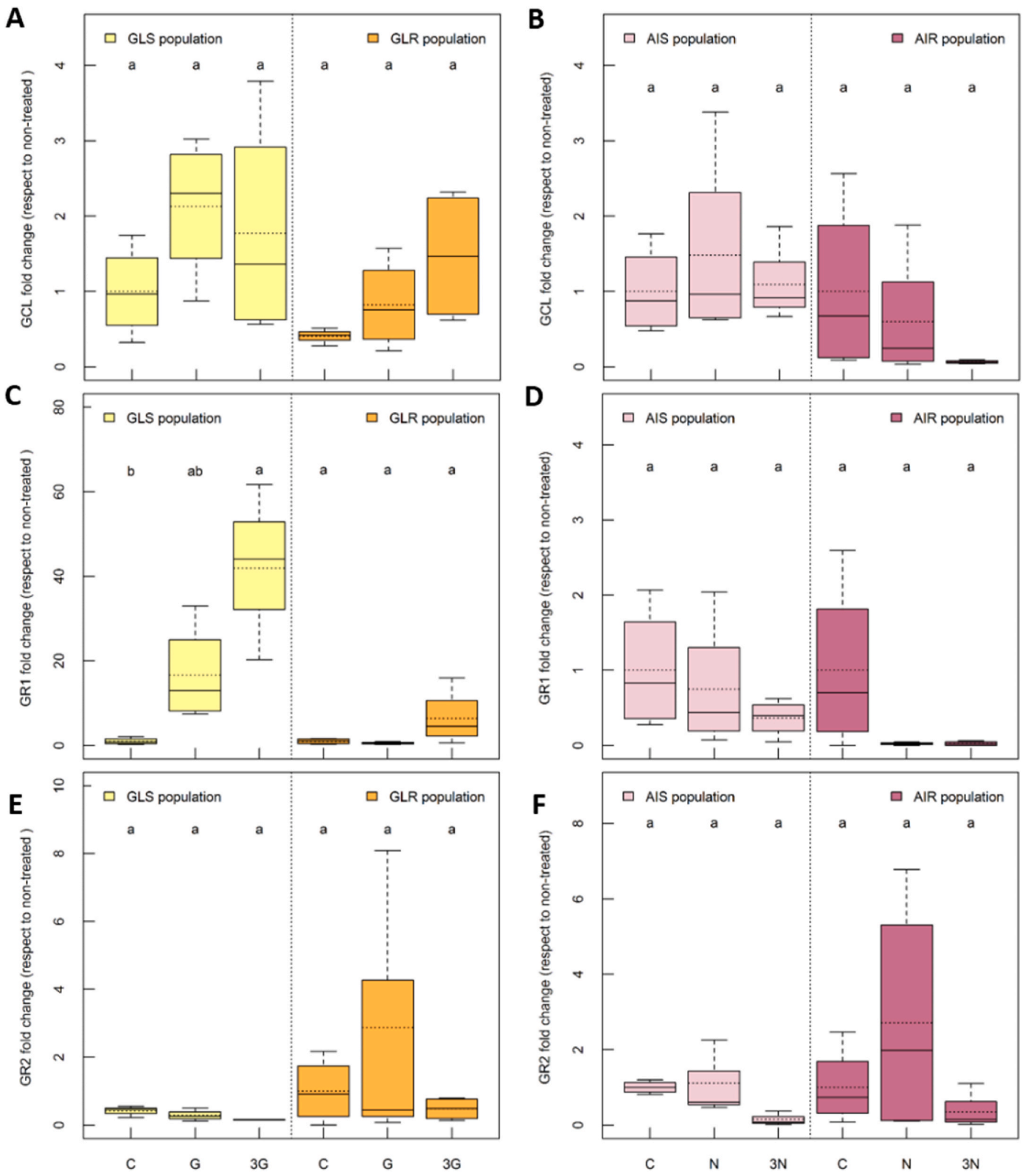

Fig. 1. Glutamate-cysteine ligase (*GCL*) gene expression (A, B) and glutathione reductase (*GR1* and *GR2*) gene expressions (C, D, E, F) in *Amaranthus palmeri*. Glyphosate-sensitive (GLS) and glyphosate-resistant (GLR) populations (A, C, E) were treated with different glyphosate rates (X axis, C is for 'control' or non-treated, G is for glyphosate at the recommended field rate of 0.84 kg ha^{-1}, and 3G is for glyphosate at three times the recommended field rate). ALS-inhibitor sensitive (AIS) and ALS-inhibitor resistant (AIR) populations (B, D, F) were treated with different nicosulfuron rates (X axis, C is for 'control' or non-treated, N is for nicosulfuron at the recommended field rate of 0.06 kg ha^{-1}, and 3N is for nicosulfuron at three times the recommended field rate). Data are 1st, 2nd and 3rd quartiles, the minimum and maximum, and mean (dotted) (n = 4). Gene expressions are expressed in relative fold change with respect to untreated plants of each population. For each population, significant differences between treatments are highlighted with different letters (ANOVA, Tukey HSD test/T3 Dunnet (p value $\leq$ 0.05)).

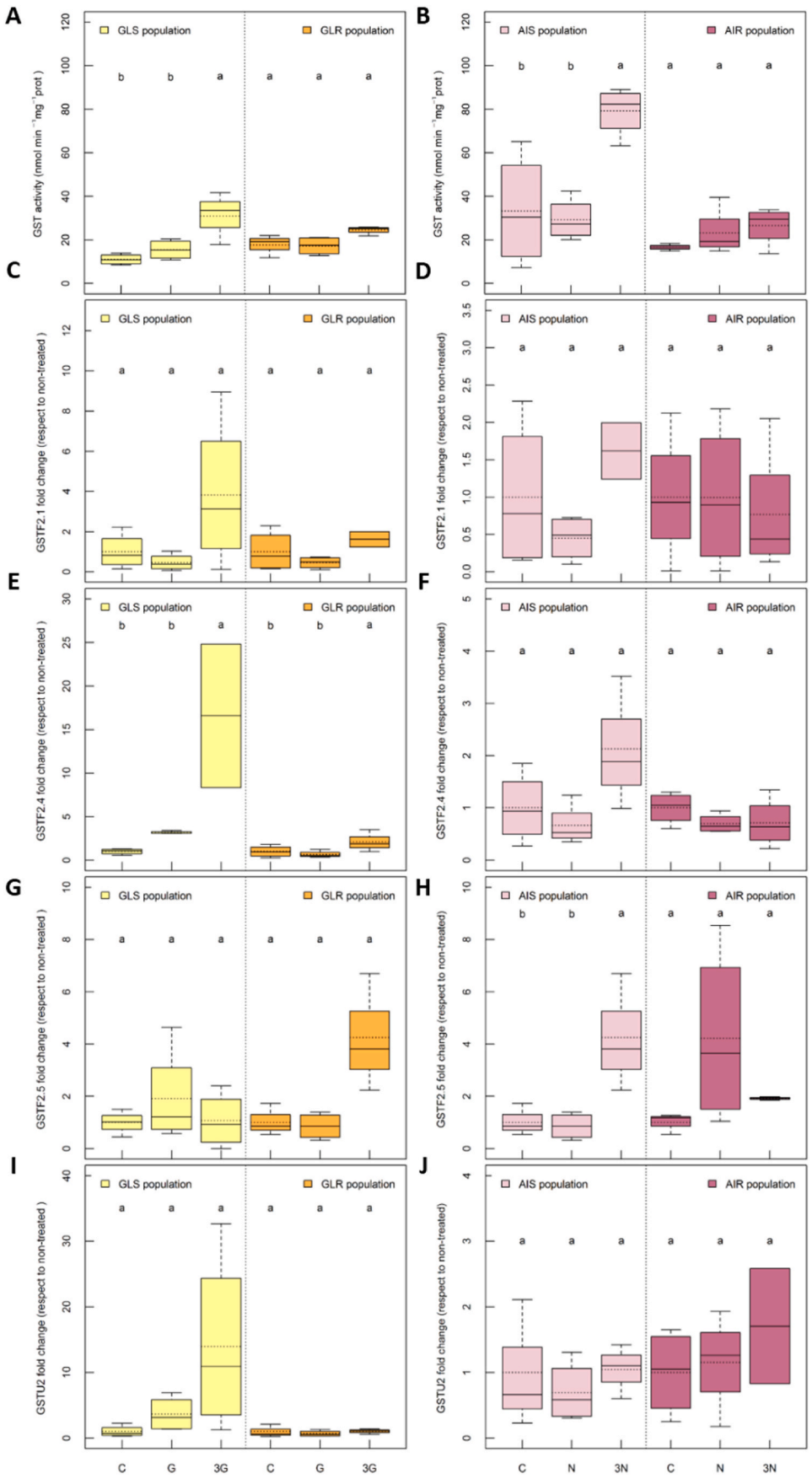

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Fig. 2. Glutathione S-transferase (GST) activity (A, B), GST Phi 2 (*GSTF2.1*, *GSTF2.4*, and *GSTF2.5*) gene expressions (C, D, E, F, G, H), and Tau 2 (*GSTU2*) gene expression (I, J) in *Amaranthus palmeri*. Glyphosate-sensitive (GLS) and glyphosate-resistant (GLR) populations (A, C, E, G, I) were treated with different glyphosate rates (X axis, C is for 'control' or non-treated, G is for glyphosate at the recommended field rate of 0.84 kg ha^{-1}, and 3G is for glyphosate at three times the recommended field rate). ALS-inhibitor sensitive (AIS) and ALS-inhibitor resistant (AIR) populations (B, D, F, H, J) were treated with different nicosulfuron rates (X axis, C is for 'control' or non-treated, N is for nicosulfuron at the recommended field rate of 0.06 kg ha^{-1}, and 3N is for nicosulfuron at three times the recommended field rate). Data are 1st, 2nd and 3rd quartiles, the minimum and maximum, and mean (dotted) (n = 4). Gene expressions are expressed in relative fold change with respect to untreated plants of each population. For each population, significant differences between treatments are highlighted with different letters (ANOVA, Tukey HSD test/T3 Dunnet (p value $\leq$ 0.05)).

dose (Suppl. Table 2). In general terms, elemental composition was barely affected by both herbicides, with some exceptions, such as the Ca decrease in response to both herbicides (Suppl. Table 3). This result was observed before in response to glyphosate due to reduced Ca uptake and translocation after herbicide treatment in *Glycine max* (Cakmak et al., 2009).

Relative transcripts of a gene encoding GCL (*GCL*) were measured as glutathione biosynthesis-related genes (Fig. 1A and B). There were no significant differences among untreated plants of GLS and GLR populations nor AIS and AIR populations (Suppl. Table 4). Gene expression of *GCL* did not follow a clear tendency in response to glyphosate nor nicosulfuron, although in the case of glyphosate it slightly tended to increase with the dose in both populations (Fig. 1A and B).

Results of glutathione content do not entirely match with present results of glutathione biosynthesis-related gene expression. Neither glutathione content nor *GCL* gene expression were affected by nicosulfuron treatment. However, *GCL* does not significantly change with glyphosate in the GLS population, only a slight increasing tendency was observed (Fig. 1A). Meanwhile, these plants displayed a significantly increased glutathione content. GCL activity and Cys availability are the most important two factors regulating glutathione biosynthesis. Accordingly, constitutive increases of glutathione content can be produced either by overexpression of *GCL* or of enzymes involved in Cys synthesis (Creissen et al., 1999; Harms et al., 2000; Noctor et al., 1996, 1998; Noji and Saito, 2002; Strohm et al., 1995; Wirtz and Hell, 2007). In this case, it seems that glutathione biosynthesis correlates more with Cys availability than with *GCL* gene expression: *GCL* gene expression does not change significantly in any population in response to herbicide treatments, while Cys content increases in response to glyphosate, as does glutathione content. The increase of Cys content in response to glyphosate treatments could not be a protective response, but may owe to the fact that free amino pool is increased after EPSPS inhibition, due to the induction of proteolytic pathways and the increased protein turnover (Zulet et al., 2013). Besides that, the disparity between *GCL* gene expression and the actual glutathione synthesis suggests the major importance of posttranscriptional regulation of glutathione synthesis.

For its part, GR is an important antioxidant enzyme in plants, as it indirectly leads to ROS scavenging by converting GSSG to GSH (Elavarthi and Martin, 2010). The expression pattern of two genes encoding this enzyme (*GR1* and *GR2*) was evaluated (Fig. 1C, D, E, F). Both isoforms are cytosolic and, although there are no data for *A. palmeri*, in *A. thaliana* 65% of the total GR activity is attributed to GR1 (Marty et al., 2009). There were no significant differences among untreated plants of GLS and GLR populations or AIS and AIR populations (Suppl. Table 4). *GR1* gene expression increased in GLS plants proportionally to the applied glyphosate dose. No other significant changes regarding GR-related gene expression were found.

With GPX, GR maintains a balanced state of GSH/GSSG by reducing GSSG back to GSH (Hasanuzzaman et al., 2017). Due to its absolute specificity towards GSSG (Mapson and Isherwood, 1963), it is closely linked to GSH oxidation, this is, GR activity increases when GSH acts as an antioxidant. Indeed, as an indicator of the oxidative status, H_2O_2 content was measured (Suppl. Fig. 1). H_2O_2 is one of the most important ROS as it is the most stable, one of the most abundant; and because its overproduction leads to the production of other ROS like hydroxyl radical (Demidchik, 2015; Lenzen et al., 2022). Significant H_2O_2 accumulation is a clear sign of oxidative stress (Demidchik, 2015). In response to both glyphosate and nicosulfuron, H_2O_2 accumulation was induced in sensitive populations, although the increase was only significant in response to 3 FR of both herbicides (Suppl. Fig. 1). Previous studies (Eceiza et al., 2022, 2023) concluded that GLS and AIS plants suffer moderate oxidative stress after glyphosate and nicosulfuron treatment, respectively, when treated with high doses with nicosulfuron inducing a higher H_2O_2 accumulation than glyphosate. On the contrary, resistant populations did not suffer oxidative stress in response to herbicide treatments (Suppl. Fig. 1), as reported before (Eceiza et al., 2022, 2023) and confirming that the induced oxidative stress was provoked undoubtedly by target inhibition (EPSPS or ALS, respectively).

Garnik et al. (2016) reported that expression of genes encoding GR was also induced by ROS in *A. thaliana*. According to the previous studies, GR activity did not vary in function of glyphosate treatment (Eceiza et al., 2022), but increased significantly in AIS plants treated with nicosulfuron (Eceiza et al., 2023). In view of present results, however, gene expression does not behave like the activity in sensitive plants. *GR1* gene expression in GLS increases proportionally to the glyphosate dose, but does not change with nicosulfuron. Resistant plants remain unaffected by herbicide treatments (Fig. 1).

Due to the close linkage between glutathione biosynthesis and GR to oxidative stress, the previous studies attributed the induction of glutathione biosynthesis in response to glyphosate (Eceiza et al., 2022) and the increase of GR activity in response to nicosulfuron (Eceiza et al., 2023) to the oxidative stress generated by EPSPS and ALS inhibition in GLS and AIS plants, respectively. Similarly to glutathione biosynthesis, the disparity between GR activity (presented in the previous studies) and the related gene expressions (presented here) evidences that glutathione turnover is mainly regulated posttranscriptionally.

3.2. GST activity, sequencing and gene expressions in response to glyphosate or nicosulfuron

GST activity and all GST-related measured gene expressions of untreated GLR and AIR plants were similar to their respective sensitive populations (Suppl. Table 4). The enzymatic activity increased proportionally to the glyphosate dose in the GLS population, and to the nicosulfuron dose in the AIS population. Both increases were significant in plants treated with 3FR of the used herbicide. In none of the resistant populations GST activity changed significantly with herbicide treatments (Fig. 2A and B), which means that these changes in GST metabolism are due to the enzymatic target inhibition. Sequencing showed that *GSTF2.1*, *GSTF2.4*, and *GSTU2* amplified what was being sought, while *GSTF2.5* appeared to amplify a similar sequence to *GSTF2.1* (Suppl. Table 5). Generally, the levels of expression reflected a slight increase in response to glyphosate or nicosulfuron rates, but the only significant differences with glyphosate were seen with *GSTF2.4* in both sensitive and resistant plants (Fig. 2E) and the only significant difference with nicosulfuron was seen with *GSTF2.5* in sensitive plants treated with 3FR (Fig. 2H).

On the other hand, GST protein content could not be measured in *A. palmeri*. To have an approach of this parameter, it was measured in sensitive *A. thaliana* treated with glyphosate and the ALS inhibitor imazamox, an imidazolinone, by immunochemical detection of GST proteins (Corpas et al., 1998; Fernández-Escalada et al., 2017), detailed in Suppl. Materials and methods. A slight increase of the protein content was observed with glyphosate in *A. thaliana* plants (Suppl. Fig. 3). While

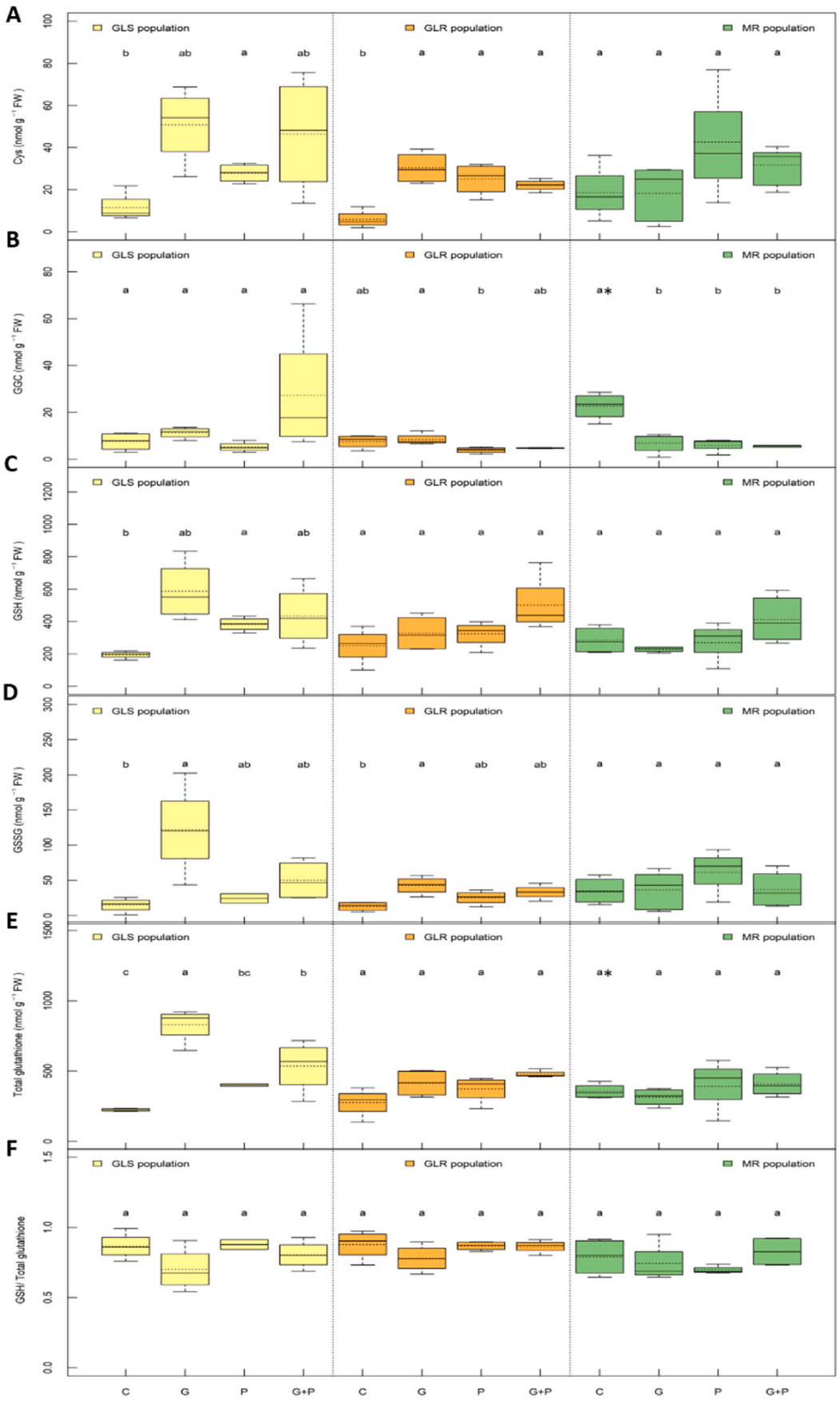

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Fig. 3. Cys content (A), Y-glutamyl-cysteine (GGC) content (B), reduced glutathione (GSH) content (C), oxidised glutathione (GSSG) content (D), sum of GSH and two times GSSG (total glutathione) contents (E), and GSH to total glutathione ratio (F) in *Amaranthus palmeri*. Glyphosate-sensitive (GLS), glyphosate-resistant population (GLR) and multiple resistant (MR) populations received different treatments (X axis): glyphosate (G) (recommended field rate or FR, FR = 0.84 kg ha^{-1}), pyrithiobac (P) (recommended field rate or FR, FR = 89 g ha^{-1}), or the combination of both herbicides (G + P); C is for 'control' or non-treated. Data are 1st, 2nd and 3rd quartiles, the minimum and maximum, and mean (dotted) (n = 4). Significant differences between untreated (C) populations are highlighted with asterisk (ANOVA; Tukey HSD test (p value ≤ 0.05)). Different lowercase letters indicate significant differences between treatments (ANOVA; Tukey HSD test/ Dunnett's test (p value ≤ 0.05)).

it is true that each plant species may behave differently, and that *A. palmeri* and *A. thaliana* are not particularly close phylogenetically, it is highly likely that GST protein content also increases in *A. palmeri*, consistent with enzymatic activity and gene expression.

Due to the characteristics of plant GSTs explained above, it can be assumed that the huge increase observed in GST activity is mainly due to Phi and/or Tau GSTs. The results of gene expressions reveal an increasing tendency of Phi *GST*s in response to both herbicides, and no clear tendency of Tau *GST*s, so most likely, increase of GST activity corresponds mainly to Phi GSTs.

Increase of GST activity in response to glyphosate has been observed before in different species (Basantani et al., 2011; Cataneo et al., 2003; Jain and Bhalla-Sarin, 2001; Miteva et al., 2010; Peragón and Amores-Escobar, 2018; Uotila et al., 1995). A higher GST activity has been positively correlated with glyphosate tolerance, but may not be involved in glyphosate metabolism, as currently there is no evidence for glyphosate degradation by GST. Miteva et al. (2010) suggested that GSTs participate in the detoxification of H_2O_2 and lipid peroxides. More specifically, they correlated the increase of GST activity in response to glyphosate with the oxidative stress caused by EPSPS inhibition. Indeed, some GSTs are induced by H_2O_2 signalling, and several stress-inducible GSTs protect plants from oxidative damage by functioning as antioxidants, more exactly as GPXs (Dixon et al., 2002). Glyphosate is known to induce H_2O_2 formation and oxidative stress via EPSPS inhibition (Ahsan et al., 2008; Eceiza et al., 2022; Gomes et al., 2017; Gomes and Juneau, 2016; Meloni and Bolzón, 2021; Ostera et al., 2016), and the present results for H_2O_2 support this (Suppl, Fig. 1).

GST activity is also clearly induced by nicosulfuron treatment in AIS plants, a result that comes up with studies performed in maize (Liu et al., 2019; Lv et al., 2019). Gene expression of Phi GST also tends to increase. The response of AIS plants to nicosulfuron is similar to the response of GLS plants to glyphosate, and nicosulfuron and other ALS inhibitors also induce H_2O_2 accumulation and oxidative stress by ALS inhibition (Eceiza et al., 2023; Liu et al., 2019; Wang et al., 2018b; Yang et al., 2021). H_2O_2 accumulation with nicosulfuron is actually higher than with glyphosate (Supp. Fig. 1).

The increase of GST activity and the level of Phi GST gene expression in response to glyphosate and nicosulfuron is associated to H_2O_2 signalling and related to the oxidative stress induced by both herbicides in sensitive plants, while in both resistant populations with insensitive herbicide targets no changes in GSTs were detected.

3.3. Glutathione metabolism in the MR population

Before analysing glutathione-related compounds and GSTs in the MR population, some indicators of the oxidative status were measured in untreated plants of this population and compared to the GLS and GLR populations, to discard basal particularities of the MR population like a possibly higher basal antioxidant capacity. For that purpose, the antioxidant capacity as well as enzymatic activities of GR and peroxidases were measured. As can be seen in Suppl. Table 6, the MR population had a similar basal oxidative status as the GLS population. This similar oxidative basal status was also observed in the GLR and AIR populations in respect to their sensitive populations (Eceiza et al., 2022, 2023). Thus, the *EPSPS* amplification and the *ALS* point mutation are not accompanied by an enhanced antioxidant system. GSH-related compounds were also statistically similar in the untreated GLS, GLR, and MR plants (Fig. 3). Also, the MR population presented a similar basal GST activity to the GLS population (Fig. 4A).

In response to herbicide treatments, glutathione metabolism was more altered with glyphosate than with the ALS inhibitor pyrithiobac, coherently with the previously observations (Suppl. Figs. 2 and 3). Glyphosate treatment provoked in the GLS population an increase of both glutathione forms, GSH and GSSG, giving as a result an increase in total glutathione and an unaltered GSH to GSSG ratio (Fig. 3). This tendency was not observed in the GLR or the MR population. On the other hand, pyrithiobac provoked a slight increase of glutathione-related compounds in ALS-inhibitor sensitive populations (GLS and GLR). The combination of glyphosate and pyrithiobac did not provoke a significantly higher increase in glutathione-related compounds comparing with glyphosate applied alone (Fig. 3).

With regard to GSTs, GST activity tended to increase with herbicide treatments in the GLS population, especially with pyrithiobac treatment (Fig. 4A). The similarity with the results of nicosulfuron in the AIS population shown in Fig. 2B, as well as the results of studies using other ALS inhibitors (Balabanova et al., 2018; Liu et al., 2015), corroborates that the increase of GST activity is a response to ALS inhibition. In view of these results and the results presented in Fig. 2, it seems that the combination of glyphosate and an ALS inhibitor at normal rates provokes less activation of GSTs than an increased rate of one single herbicide, discarding a synergetic effect of both herbicides. Again, the tendency to increase GST activity was not accompanied by a clear increase in level of *GST* gene expressions (Fig. 4). These results confirm that this GST class is more related to the effect elicited by glyphosate and ALS inhibitors.

In the MR population GST activity did not significantly increase with glyphosate or the combination, and indeed decreased with pyrithiobac treatment (Fig. 4A). Although a marginal expression of *GSTF2.4* and *GSTU.5* was detected in the MR population after pyrithiobac treatment, GST expression was not affected by the herbicides in general. The behaviour of the MR population in response to glyphosate and/or pyrithiobac was not clear, thus, similar to the behaviour of GLR population in response to glyphosate, and AIR population in response to nicosulfuron (Fig. 2).

To get a deeper approach of the response of GSTs, GSTs were labelled with photoaffinity probe DB478, which targets the glutathione binding site in GSTs (Font Farre et al., 2023). Two bands between 15 and 25 kDa were detected (Fig. 5A). Labeling leaf extracts of other plant species resulted in signals in the 25 kDa region, although signals varied in intensity and MW (Font Farre et al., 2023).

The 25 kDa band was more intense than the 15 kDa band and was detected in all the samples, allowing us to compare between the treatments. Band intensity was generally higher in herbicide-treated samples, especially when the plant was sensitive to the herbicide (GLS to both herbicides, and GLR to pyrithiobac), although there were not any statistical differences (Fig. 5B). The 15 kDa band, when detectable, presented a low intensity that did not vary significantly between treatments. Wheat seedlings treated with the herbicide fenoxaprop-P-ethyl had elevated GST labelling (Font Farre et al., 2023). Due to the observed tendency and the intensity of the 25 kDa band, Phi and Tau GST activities are thought to be mainly represented in this band. Indeed, enrichment and mass spectrometry analysis of labelled proteins from *A. thaliana* with DB478 identified GSTs from the Phi- and Tau-classes (Font Farre et al., 2023).

Seemingly, the MR population behaves similarly to a single resistant population in response to one single herbicide (glyphosate or

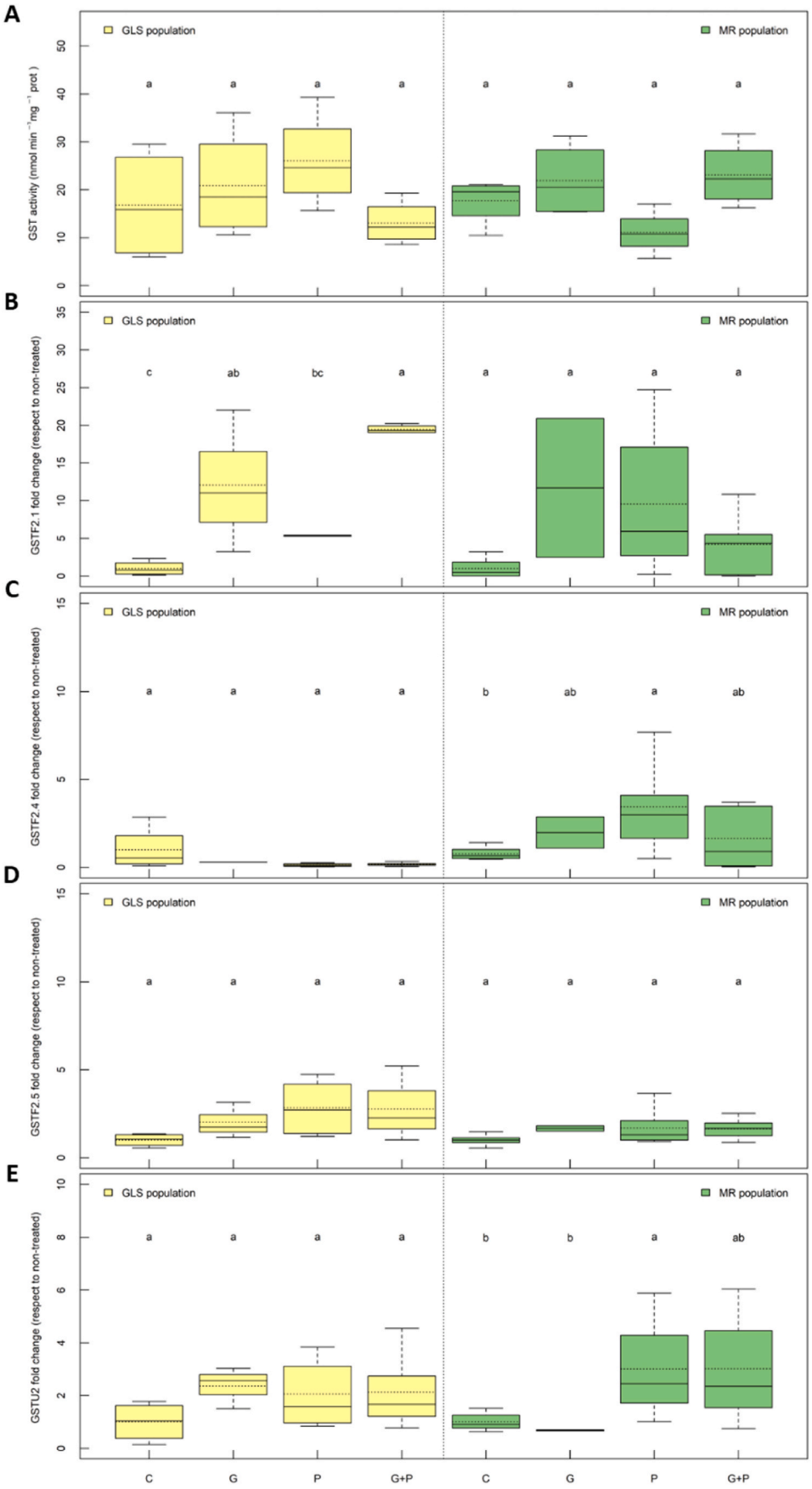

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Fig. 4. Glutathione S-transferase (GST) activity (A), GST Phi 2 (*GSTF2.1*, *GSTF2.4*, and *GSTF2.5*) gene expressions (B, C, D), and Tau 2 (*GSTU2*) gene expression (E) in *Amaranthus palmeri*. Glyphosate-sensitive (GLS, yellow) and multiple resistant (MR, green) populations received different treatments (X axis, C is for 'control' or non-treated, X is for glyphosate at the recommended field rate of 0.84 kg ha^{-1}, P is for pyrithiobac at the recommended field rate of 0.089 kg ha^{-1}, and G + P us for the combination of both glyphosate and pyrithiobac at their recommended field rates). Data are 1st, 2nd and 3rd quartiles, the minimum and maximum, and mean (dotted) (n = 4). For each population, significant differences between treatments are highlighted with different letters (ANOVA, Tukey HSD test (p value ≤ 0.05)).

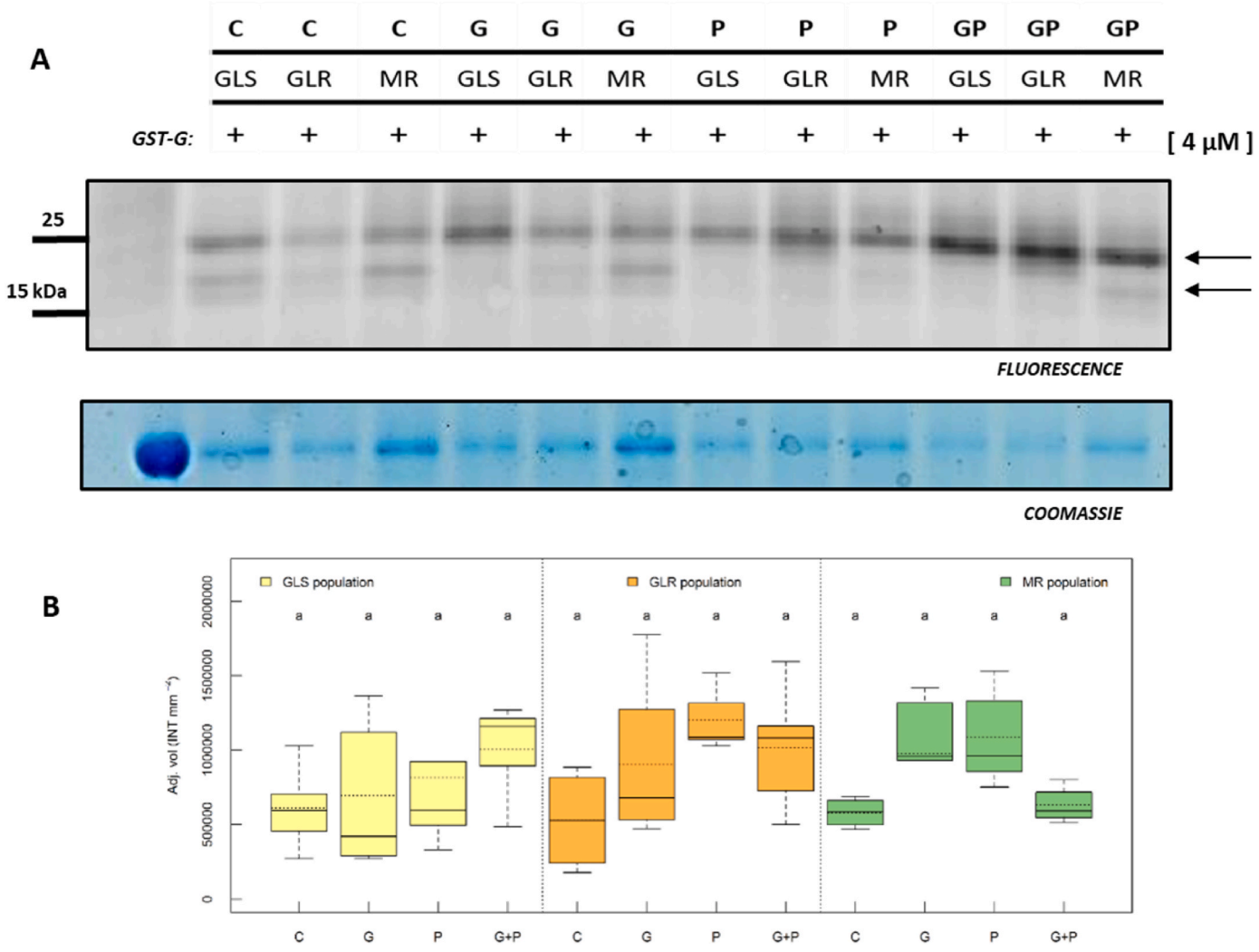

Fig. 5. Labelling of the GST active sites. The measure was determined in leaves of three populations of *Amaranthus palmeri*: Glyphosate-sensitive (GLS), glyphosate-resistant population (GLR) and multiple resistant (MR) populations received different treatments (X axis, C is for 'control' or non-treated, X is for glyphosate at the recommended field rate of 0.84 kg ha^{-1}, P is for pyrithiobac at the recommended field rate of 0.089 kg ha^{-1}, and G + P us for the combination of both glyphosate and pyrithiobac at their recommended field rates). A model representative gel is shown (A), relative intensity (B). Data are 1st, 2nd and 3rd quartiles, the minimum and maximum, and mean (dotted) (n = 4). For each population, significant differences between treatments are highlighted with different letters (ANOVA, Tukey HSD test/T3 Dunnet (p value ≤ 0.05)).

pyrithiobac). This conclusion is in agreement with previous studies suggesting unclear association between physiology of multiple resistant weeds and their resistance. According to Papapanagiotou et al. (2023), if there were variations in fitness or physiology of multiple resistant biotypes, they would not probably be linked with the resistance alleles, but with other attributes: their evolutionary origin, the polymorphisms at non-resistance alleles, the inbreeding depression, the non-random mating, the linkage disequilibrium processes or the genotype by environment interaction.

Two key aspects can be extracted from present study. The first one is related to the response of sensitive populations (GLS, AIS) to the herbicides. Glyphosate, nicosulfuron and pyrithiobac induced an increase of GST activity and gene expression in these populations (Figs. 2 and 4), indicating that GSTs take part in the physiological response to glyphosate and ALS inhibitors. Induction of GSTs, then, may be considered a physiological effect of glyphosate and ALS inhibitors, herbicides that, as explained before, have several physiological effects in common. This response is probably related to H_2O_2 accumulation (Suppl. Fig. 1) and the subsequent oxidative stress, also detected in response to both glyphosate (Ahsan et al., 2008; de Freitas-Silva et al., 2017; Eceiza et al., 2022; Gomes and Juneau, 2016; Ostera et al., 2016) and ALS inhibitors (Eceiza et al., 2023; Lv et al., 2019; Wang et al., 2018a, 2018b; Yang et al., 2021; Zabalza et al., 2007). The second conclusion is that these herbicides barely altered glutathione metabolism or GSTs in TSR populations, even to high herbicide doses. This was observed regardless of the type (single or multiple) of herbicide resistance. Due to the fact that the resistance is target-site specific, it can be said that the alterations in GSTs observed in response to glyphosate

and the ALS inhibitors are undoubtedly due to EPSPS and ALS inhibition, respectively.

4. Conclusion

Glyphosate and ALS inhibitors target different enzymes (EPSPS and ALS, respectively), but there are several physiological effects triggered by both herbicides in a similar way. Present results of glutathione synthesis, its turnover and GSTs thicken this list of physiological effects. Similarity between untreated sensitive and resistant plants in almost all the parameters confirm the nature of the resistance mechanisms of GLR and AIR populations, being solely TS. This was confirmed with a multiple-resistant population endowing the mechanisms of both single-resistant populations, that behaved similarly to them. Resistant populations suffer less stress and do not have to deal with the herbicide-induced oxidative stress. The observed increasing tendencies in GST activity and gene expression only occur in the sensitive plants, meaning that, in the end, they are a secondary effect of EPSPS or ALS inhibition, in line with other common physiological effects shared by both herbicide groups.

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CRediT authorship contribution statement

Mikel V. Eceiza: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Clara Jimenez-Martinez:** Methodology, Investigation, Formal analysis. **Miriam Gil-Monreal:** Writing – review & editing, Conceptualization. **María Barco-Antoñanzas:** Methodology, Investigation. **Maria Font-Farre:** Methodology. **Michiel Huybrechts:** Writing – review & editing, Methodology, Investigation, Formal analysis. **RenierA.L. van der Hoorn:** Writing – review & editing. **Ann Cuypers:** Writing – review & editing, Conceptualization. **Mercedes Royuela:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Ana Zabalza:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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