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Natural variation of response to glyphosate in *Sinapis alba* (Brassicaceae): Physiological effects, shikimate accumulation and genetic mechanisms

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Abstract

Glyphosate is the most widely used herbicide worldwide, and glyphosate-resistant weeds have increased globally. Resistance mechanisms, including target-site resistance (TSR) and non-target-site resistance (NTSR), are well documented, but their extent and importance are poorly understood among biotypes. This study examines the natural variation in glyphosate sensitivity and its genetic basis in white mustard (*Sinapis alba* L.) across 192 accessions in Southern Spain. We conducted a multi-population screening to evaluate plant performance, shikimate accumulation, survival and yield under glyphosate exposure and analysed the spatial response pattern. We also assessed the expression of two genes linked to glyphosate resistance, *EPSPS* and *ABCC8*, sequenced *EPSPS* to detect potential mutations and quantified its copy number. Results show glyphosate significantly reduces physiological parameters, with a 90% decrease in photosynthetic rate, a 22% reduction in chlorophyll concentration and a 38% decrease in specific leaf area. However, 32% of accessions survived and produced seeds under glyphosate exposure (1598 g.a.e. ha⁻¹) in the vegetative stage. Survivors accumulated 13% less shikimate, indicating a reduced glyphosate effect on the shikimate pathway. Overexpression of the NTS *ABCC8* gene was the most common mechanism in 39% of non-susceptible accessions. Yet, about 20% of survivors were not linked to *EPSPS* duplication or overexpression of *EPSPS* or *ABCC8*, suggesting other genetic mechanisms may contribute to glyphosate resistance in *S. alba*. Resistant phenotypes were widely dispersed, not confined to specific areas, highlighting the potential role of gene flow in the evolution of herbicide response in *S. alba*. Our study suggests that the plant resistance to glyphosate may arise from multiple and non-exclusive genetic mechanisms.

KEYWORDS

ABCC8, Brassicaceae, *EPSPS*, gene amplification, gene expression, glyphosate, herbicide resistance, white mustard

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1 | INTRODUCTION

Herbicide resistance and weed management are major agricultural concerns, causing significant economic losses and environmental impacts (Pannell et al., 2016). Over the past few decades, there has been a dramatic increase in weed species evolving resistance to a wide range of herbicides, with more than 500 unique cases of herbicide resistance (Heap, 2024). This increasing issue has sparked significant scientific interest in understanding the genetic and molecular mechanisms that lead to plant herbicide resistance, a natural selection-driven evolution determined by population dynamics and environmental pressures. The continuous use of herbicides has accelerated the evolution of herbicide-resistant weeds under this consistent selective pressure, providing evidence of parallel evolution (Bolnick et al., 2018).

Because a population's survival is partially dependent on the sensitivity variation among individuals and the frequency of individuals with similar sensitivity (Leon, 2024), screening for natural variation in response to herbicides between and within populations can help us understand how herbicide resistance develops and persists (Kersten et al., 2023). Traditionally, plant physiology, biochemical and reproduction analyses have been carried out after herbicide exposure. However, there is limited information about how physiological and biochemical effects vary across plant populations. This knowledge is crucial for understanding herbicide resistance's genetic and molecular mechanisms and developing effective management strategies.

Glyphosate (N-phosphonomethyl glycine) is the most extensively used non-selective, broad-spectrum herbicide for weed management (Beckie et al., 2020; Duke & Powles, 2008). Although glyphosate remains highly effective, its widespread use has created intense selection pressure, increasing the number of resistant weeds (Heap, 2024). Glyphosate resistance can occur through target-site resistance (TSR) by modifying the target enzyme EPSPS. Several genetic mechanisms, such as gene amplification and duplication, contribute to increased EPSPS production and are associated with glyphosate resistance (Aulakh et al., 2024; Brunharo et al., 2019; Gaines et al., 2020). Also, missense mutations within the EPSPS gene can lead to resistance to glyphosate in particular species (Sammons & Gaines, 2014; Yu et al., 2015). On the other hand, several non-target-site resistance (NTSR) mechanisms, such as vacuolar sequestration (Ge et al., 2010), reduced absorption (Menendez et al., 2014), or herbicide translocation (Gaines et al., 2019), prevent it from reaching the site of action in quantities that would cause plant death. In particular, an ATP-binding cassette transporter, ABCC8, is associated with glyphosate resistance through the active extrusion of the herbicide into the apoplast (Amrhein & Martinoia, 2021). However, the relative importance of prevalence in each mechanism underlying glyphosate resistance remains unclear.

The Brassicaceae is a large family with approximately 3700 species and includes a diverse group of economically important food and energy crops (Gugel & Falk, 2006). Within the family, glyphosate resistance has been observed in *Brassica rapa* and *Raphanus raphanistrum* (Heap, 2024). Yet, the full extent of this resistance, its molecular and physiological effects and its genetic basis are still poorly known. As part of a research programme to uncover the genetic basis for

glyphosate resistance, we conducted a multi-population screening across a geographical gradient in Southern Spain to examine the natural variation pattern in response to glyphosate exposure in 192 natural accessions of the white mustard, *Sinapis alba* L., an important crop for the agroindustry sector (Ciubota-Rosie et al., 2013) and also a weed that naturally grows in many Mediterranean woody-crops orchards (e.g., olive orchards, vineyards and almond groves). *S. alba* has natural-wild genotypes, cultivars (dedicated to the food industry, e.g., mustard) and naturalised cultivars (Winter, 2011). Therefore, significant genetic variation in herbicide sensitivity is expected according to the likely variation in the regime and recurrence of herbicide application among populations.

In particular, we focus here on the effects of glyphosate application on plant survival, reproduction and essential physiology measurements such as gas exchange, chlorophyll concentration and shikimic acid, a specific metabolic biomarker of glyphosate resistance (Dayan et al., 2015; Shaner et al., 2005). In addition, we investigate the genetic basis of glyphosate resistance variation in *S. alba* by analysing (i) the copy number variation and gene expression of EPSPS, (ii) the gene expression of ABCC8 and (iii) the screening of EPSPS target mutation sites in resistant plants. Specifically, we address the following questions: (1) Does natural variation exist in the physiological, biochemical and genetic response to glyphosate exposure within and between *S. alba* populations? (2) What specific mechanisms does the glyphosate application have on the primary plant physiology, shikimate accumulation and plant fitness? Finally, (3) is the occurrence of glyphosate resistance related to mutations, duplications, or overexpression of the EPSPS gene and/or overexpression of the ABCC8 gene?

2 | MATERIALS AND METHODS

2.1 | Study system

Sinapis alba is an annual plant native to the Mediterranean but currently found worldwide (Winter, 2011). In the Iberian Peninsula, it is present throughout the entire territory, with higher abundance in the south (Jaime et al., 2018). It naturally grows in habitats such as roadsides, waste dumps, fallow lands, crops, quarries, abandoned urban lots and alluvial deposits, preferentially on limestone soils with excess nitrogen (Winter, 2011). It is also found in agricultural areas such as olive groves, grapevines and almond trees, where it is considered a weed. The species has been regularly exposed to herbicides such as oxyfluorfen and glyphosate over the last 60 years, particularly in southern parts of the EU. This is based on observations of the highest concentrations of glyphosate and its primary metabolite, AMPA, in soil, with a maximum concentration of 2 mg kg⁻¹ (Silva et al., 2018).

2.2 | Plant material

Seeds of *S. alba* were collected from 25 different populations across a geographical gradient in Southern Spain (Figure 1 and Table S1).

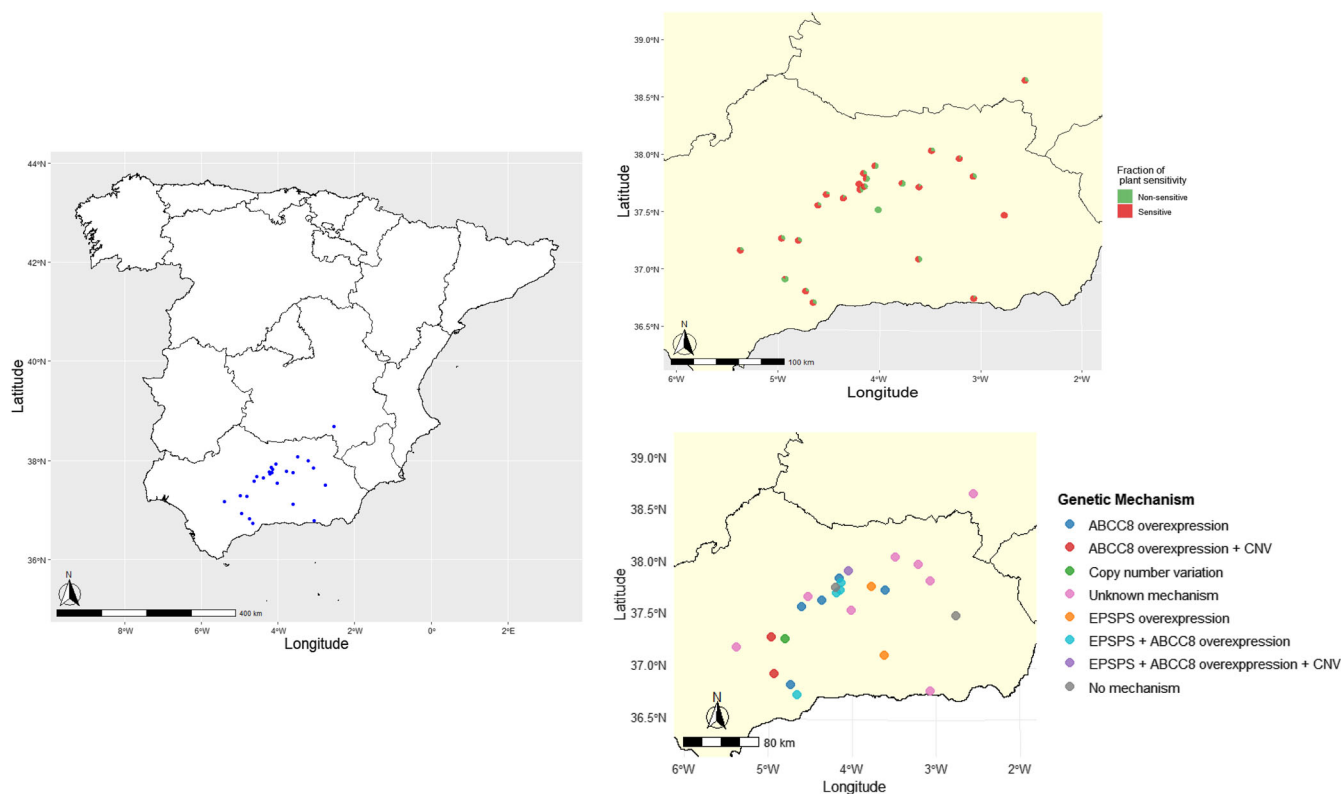


FIGURE 1 Left: Map of the location of the 25 populations of *Sinapis alba* in southern Spain used in this study. Right above: Fraction of glyphosate-sensitive/non-sensitive plants at each population. Right below: Predominant genetic mechanism underlying glyphosate resistance at each population.

Sampling populations were selected based on the nature of their habitat, including olive groves, urban areas, abandoned orchards, forest edges and other croplands (Table S1). The size of the *S. alba* populations varies across the study sites, ranging from a dozen individuals to thousands, typically hundreds (Table S1). We collected from 3 to 10 individual plants (= accessions) at each population, normally averaging between eight and nine (Tables S1 and S2). We spaced the plant collection approximately 10 m apart during sampling each population. This plant species does not exhibit rhizomes or any other form of vegetative growth. We collected seeds (from 5 to 6 siliques per plant) produced from each accession to conduct the experiments. Seeds (half-sibs) were germinated in Petri dishes and planted individually in 1-L pots (a single plant per pot, following a randomized scheme) filled with a mixture of perlite, sand and universal organic substrate (0.5:0.5:1, v/v). A list of accessions used in the experiments is given in Table S2.

2.3 | Experimental design

In 2021, seedlings were grown in an experimental garden at the University of Jaén (Spain) from February to June. After 6 weeks of growth, two groups were established following a split-plot design: plants treated with glyphosate and control plants (Figure S1). In glyphosate treatments, a single foliar application of glyphosate was applied

to the plants during their vegetative growth stage (BBCH 2–3) in early spring to mimic the common agronomic application in the region, such as in permanent olive crops and vineyards. Each treated plant received the recommended dose by the manufacturer for most crops where glyphosate is used in the region (Glifochem®, 360 g a.i. L⁻¹, Spachem), which was applied at 1598 g.a.e. ha⁻¹ (36% w/v) by using a single nozzle manual sprayer (Pressol®) equipped with a flat fan at 150 kPa and the spray rate of 6 L/ha (total application area 45 m²). Herbicide was applied at a 50 cm distance from the plants. No adjuvants were employed. Control plants were not treated during the entire study period and were separated several meters away from treated plants. In both treatments, plants were watered regularly. A total of 192 accessions for each treatment, each with three biological replicates, were used in the experiment, resulting in a total of $N = 1152$ plants assayed.

2.4 | Physiological measurements

We recorded gas exchange measurements in both treatments 1 week after glyphosate application by using a LI6800 portable photosynthesis system (LI-COR Biosciences, Lincoln, NE, USA) for the measurement of CO₂ assimilation rates (A : $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). All plants were measured between 10 AM and 1 PM. Chlorophyll content (μmol of chlorophyll m⁻² of leaf surface area) was also measured on three

leaves from each plant using the MC-100 Chlorophyll Concentration Meter (Apogee Instruments, North Logan, UT, USA), which estimates it indirectly by analysing the ratio of radiation transmittance. Concurrently, in fresh tissue, specific leaf area (SLA), a functional trait indicator of response to environmental changes (Wright et al., 2004), was also measured for three leaves per plant present at glyphosate exposure using a LI-3000C Portable Leaf Area Meter (LI-COR Biosciences, Lincoln, NE, USA).

2.5 | Survival and seed yield

Throughout the experimental period, we recorded the survival of both treated and untreated plants for 30 days. However, some plants (e.g., non-exposed ones) lived longer, completing their life cycle in up to 50 days. Our criterion for determining plant survival was based on the completeness of the entire life cycle; specifically, for this annual plant, dead means the plant dies without producing flowers and siliques, while alive means it completes the cycle and can grow flowers and seeds. We also monitored the production of flowers and fruits. Plants were openly accessible to pollinators (bees, hoverflies, flies, etc.). For plants that completed the entire growth cycle, we harvested all the seed pods (siliques) and counted the total number of seeds per plant.

2.6 | Shikimic measurements

We assessed the impact of glyphosate on leaf shikimate accumulation in the same 192 *S. alba* accessions to confirm glyphosate sensitivity. Each accession was tested with three biological replicates, each with three technical replicates. First, we measured baseline shikimate levels before glyphosate application. Then, on the same plants, glyphosate was applied using the same spraying protocol described earlier, and shikimate accumulation was quantified 48 h post-treatment. Lower shikimate accumulation indicates glyphosate resistance, as glyphosate inhibits *EPSPS* in the shikimate pathway (Dayan et al., 2015). Shikimate analysis followed the protocol described by Shaner et al. (2005) with minor modifications (detailed protocol is given in Supporting Information). Shikimate levels were quantified by fitting the data to a standard curve and subtracting background absorbance. For this batch, we again measured the plant survival of glyphosate-exposed and non-exposed plants for 30 days.

2.7 | Genetic analyses

2.7.1 | DNA-based analyses

A CTAB protocol was used to extract genomic DNA (Doyle & Doyle, 1987) from 192 accessions to quantify the copy number of the *EPSPS* gene and the Sanger sequencing of critical regions. To estimate the copy number in *S. alba*, primers were designed to amplify a

200 bp fragment of the *EPSPS* gene (5'-GCGTGCAGAACCATGTGTT-3', 5'-GCACAATCTCCGACGCTTTC-3') and a 123 bp fragment of the *LFY* gene (5'-TGTAATAGGTGACGAACCAAGT-3', 5'-AGCTTCTTCGCTAGGCAGT-3'), used as a single copy gene. Quantitative Polymerase Chain Reactions reactions were carried out using a mixture containing 5 μ L of SsoFast Evagreen Supermix (Bio-Rad®), 0.25 μ L of each primer (10 μ M) and 2 μ L template DNA (5 ng μ L⁻¹) in a 10 μ L final volume. Amplification was performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad®). It consisted of an initial cycle of denaturation at 98°C for 3 min, 40 cycles of denaturation at 98°C for 15 s, annealing at 56°C for 30 s and extension at 65°C for 5 s. Subsequently, melt curves were generated. PCR products were subjected to electrophoresis on a 2% agarose gel to determine fragment size. We used the comparative 2^{- $\Delta\Delta$ Ct} method (Schmittgen & Livak, 2008) to quantify copy number variations among these 192 accessions. This method measures relative changes in copy number by normalizing Ct values to a reference.

To sequence mutations in the *EPSPS* gene, we designed primers to amplify a 550 bp genomic region containing exons 2 and 3 (5'-TAATTCGTGATGTCATTTTGAGAGG-3', 5'-ACAAGGAATCAAGATAGAAAG-3' and 5'-GGCTGAAGAAAGGTTAACTTTG-3', 5'-TTCATAAAGTAAATGTCAGAGATTG-3'). The selection and design of the critical genomic region were based on sequences previously reported (Nandula et al., 2013), and cDNA sequences from Brassicaceae species, along with the latest available genome (JGI: *S. alba* v3.1) using Primer-BLAST (NCBI). PCR amplification was performed with GoTaq G2 Flexi DNA Polymerase (Promega), and amplicons were Sanger-sequenced and aligned using *Clustal Omega* (EMBL-EBI) and *Geneious Prime* to detect polymorphisms.

2.7.2 | RNA-based analyses

We extracted RNA from the same plants we utilized for shikimate accumulation analyses. RNA was collected both before glyphosate spraying (i.e., constitutive) and 48 h after spraying (i.e., induced). RNA was extracted using the trizol (TRIzol Reagent, Invitrogen) protocol (Chomczynski & Sacchi, 1987). RNA integrity was measured using a Bioanalyzer (2100 Agilent Technologies®). Only RNAs with RIN values ranging between 5.5 and 7 (indicating an RNA integrity moderate to good) were included in the analyses. Thus, we used a subset of 78 accessions for the expression analyses that achieved this minimum quality standard. We measured gene expression levels in 21 accessions for *EPSPS* expression (18 glyphosate-non-sensitive and 3 glyphosate-sensitive) and 64 for *ABCC8* expression (34 glyphosate-non-sensitive and 30 glyphosate-sensitive), respectively (Table S2). Three pairs of primers were designed to quantify the expression of candidate genes. One primer pair was used to quantify *EPSPS* gene expression (5'-GGGGCTTAATGTGGAACGTG-3', 5'-TAAGTGGACGCATGGCTGTT-3'), another pair targeted *ABCC8* gene (5'-TCTCAGGGGAAAGGCATCAGA-3', 5'-AAACCCTGCTGTTGCTAACCTGT-3') and the third pair was designed for the housekeeping gene *ACTIN* (5'-GGAATCGCTGACCGTATGAG-3', 5'-ACCCTCCAATCCAGACACTG-3'), which

served as an internal control (Yang et al., 2014). The RT-PCR mix and program were the same as described above for copy number detection. The default efficiency value used in gene expression calculations is 100%. For each reaction, we run three technical replicates. We used the comparative $2^{-\Delta\Delta C_t}$ to calculate the expression levels of *EPSPS* and *ABCC8* genes. This method measures relative changes in expression by normalizing C_t values to a reference and then comparing the results between treated (induced) and control (constitutive) samples.

2.8 | Statistical analyses

To analyse the variation in the effects of glyphosate exposure on plant physiology among accessions within and between populations, we fitted Generalized Linear Mixed Models (GLMMs) with restricted maximum likelihood estimation using R software (version 4.4.3; R Core Team, 2025). In these analyses, the dependent variables were photosynthesis, chlorophyll concentration, SLA and shikimate accumulation. Glyphosate treatment, with two levels: 'plants exposed to glyphosate' and 'non-exposed plants' (control hereafter), population and their interactions were the main fixed effects factors. We also included the interaction 'accession by treatment' as a random factor in these models to investigate the possibility of accession variation in response to glyphosate exposure. We conducted these analyses using the 'lme4' package (Bates et al., 2015) and the statistical significance of random factors (using likelihood ratio tests, LRT) was evaluated using the 'lmerTest' package (Kuznetsova et al., 2017). Effects of primary factors and contrasts were summarized by obtaining the least-squares means (i.e., the model estimated marginal means) using the 'emmeans' function implemented in the 'emmeans' package (Lenth, 2023). Response variables were log-transformed when necessary to achieve normality and homoscedasticity requirements.

Additional GLMMs were fitted to investigate the effect of glyphosate application on plant survival and seed production. For survival, we considered binomial error and logit link function, where the response variable was 1 for those plants that reached reproductive status and could produce seeds and 0 for those plants that died after glyphosate application or did not produce seeds. Similarly, we evaluated the impact of glyphosate exposure on seed production per plant. In both models, glyphosate treatment, population and their interaction were the main fixed effects factors. We included the interaction 'accession by treatment' as a random factor in these models to investigate the possibility of individual variation in response to glyphosate exposure. In the case of seed production, the variable response was log-transformed and modelled using a log link function.

To analyse whether between-populations spatial structure exists in sensitivity to glyphosate exposure, we first examined the fraction of plants that could produce seeds after glyphosate exposure per population using a chi-square test. In addition, we performed spatial autocorrelation analyses using the 'ape' package (Paradis & Schliep, 2019). In these analyses, we used Moran's I index to elucidate whether spatial correlation underlies the frequency of sensitivity occurrence (the fraction of glyphosate-sensitive plants per population), plant survival,

seed production and shikimate accumulation. In this test, the null hypothesis states that there is no between-population spatial autocorrelation and that sensitivity frequency is distributed randomly. Briefly, this index is interpreted similarly to other correlation tests; if $R = 0$, there is no association between the space and the occurrence of resistance, suggesting a random distribution. Conversely, if $R > 0$, there is a positive association between resistance occurrence across the space, indicating spatial clustering. Lastly, if $R < 0$, there is a negative association between the spatial occurrence of resistance, indicating spatial dispersion.

For exposed plants, we examined the direct and indirect relationship among all physiological measurements (accession mean), shikimate accumulation (Δ : treatment – control), survivorship and fecundity (seed production) by fitting a structural equation modelling (SEM) using the R package 'piecewiseSEM' (Lefcheck, 2016). In this model, we hypothesize that shikimate accumulation influences physiology and this impacts on survival and fecundity (Figure 3).

3 | RESULTS

3.1 | Effects of glyphosate on plant physiology

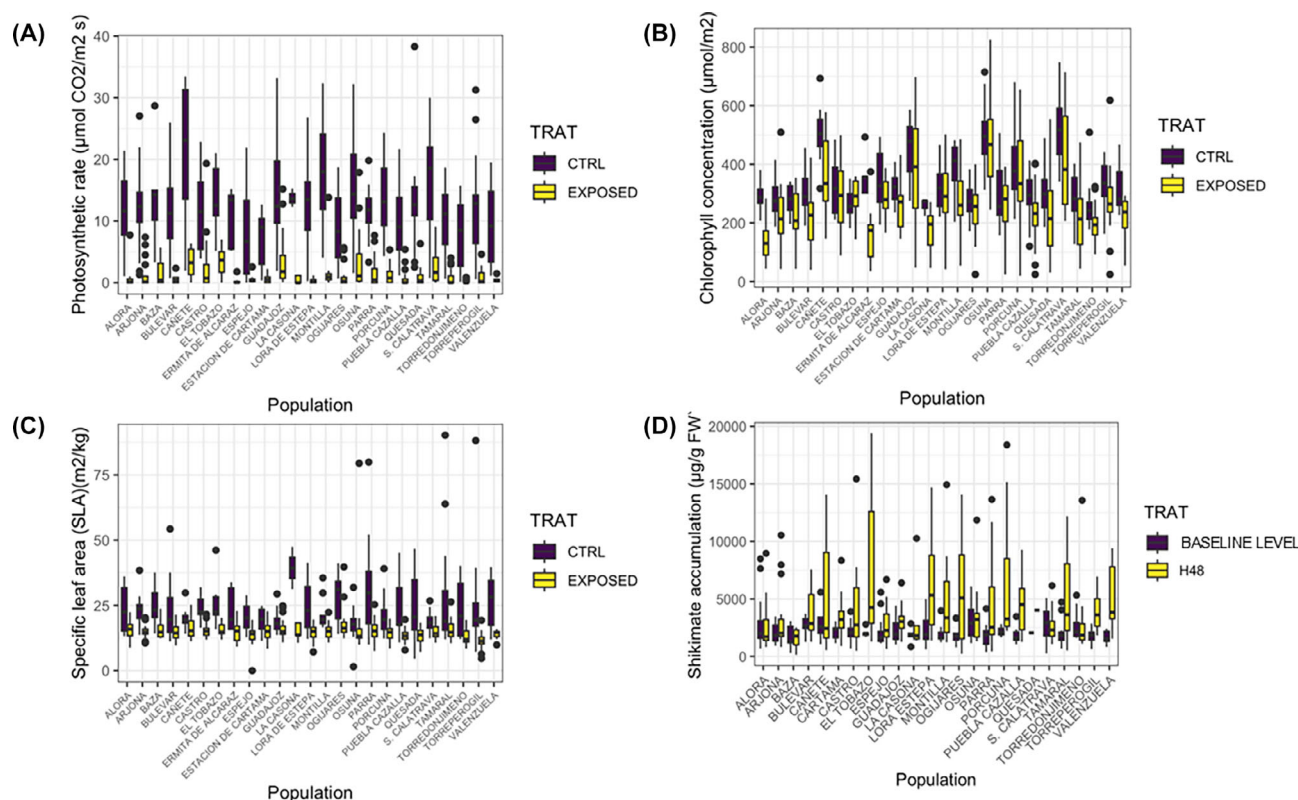
After 7 days, exposure to glyphosate significantly reduced the photosynthetic rate, with an average reduction of about 90% compared to non-exposed plants ($12.37 \pm 0.28 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for non-exposed plants and $1.35 \pm 0.26 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ for exposed plants). This reduction was consistent among populations, but there was individual variation in response to the treatment, as indicated by the significant interaction between accession and treatment (Table 1 and Figure 2A). Accessions such as GUAD_01, CAST_19 and OSUN_03 exhibited relatively high photosynthetic values and maintained high photosynthetic values despite being exposed to glyphosate (Table S3). Similarly, glyphosate exposure significantly impacted chlorophyll concentration, and this was consistent across populations (Table 1 and Figure 2B). On average, chlorophyll concentration was reduced by 21.7% in plants exposed to glyphosate compared to non-exposed plants ($340 \pm 5.69 [\mu\text{mol m}^{-2}]$, $266 \pm 7.31 [\mu\text{mol m}^{-2}]$; model-adjusted means for non-exposed and exposed plants, respectively). Likewise, the significant interaction between accession and treatment indicated inter-accession variation in response to the treatment (see Table 1). Notably, 36 out of 192 accessions (18.75%) exposed to glyphosate showed chlorophyll concentration values higher than the average of non-exposed (Table S4).

Finally, glyphosate exposure also significantly affected SLA. On average, SLA was reduced by 38.43% in plants exposed to glyphosate compared to non-exposed plants ($24.2 \pm 0.64 [\text{m}^2 \text{ kg}^{-1}]$, $14.9 \pm 0.33 [\text{m}^2 \text{ kg}^{-1}]$; model-adjusted means for non-exposed and exposed plants, respectively). However, the significance of the interaction term of the fixed effects (Table 1) suggested that there is variation between populations in the magnitude of such an effect (Figures 2C and S2). For example, accessions exposed to glyphosate from Osuna populations did not reduce SLA compared to non-exposed ones (Figure S2).

TABLE 1 Summary of the Generalized Lineal Mixed Models performed to examine physiological variation across populations between exposed and non-exposed *Sinapis alba* plants to glyphosate.

Factors	Variable								
	Photosynthetic rate			Chlorophyll concentration			SLA		
Fixed effects	df	F	p	df	F	p	df	F	p
Population	24 167	5.9	<0.0001	24 161	11.66	<0.0001	24 146	1.47	0.085
Treatment	1178	1139	<0.0001	1164	82.32	<0.0001	1177	182.72	<0.0001
Population × Treatment	24 162	0.96	0.515	24 150	1.20	0.25	24 162	1.95	0.008
Random effects	df	LRT	p	df	LRT	p	df	LRT	p
Accession × Treatment	2	11.32	0.003	2	27.64	<0.0001	2	60.16	<0.0001

Note: Significant factors ($p < 0.05$) are highlighted in bold type. For each statistical analysis, degrees of freedom (df), the F -distribution (F), the significance level (p) and the likelihood ratio test (LRT) are presented. Significant (at least $p < 0.05$) effects are highlighted in bold.

**FIGURE 2** Box-plot graphs depicting the variation between populations in photosynthetic rates (A), chlorophyll concentration (B), (C) SLA in two treatments (CTRL: non-exposed plants, dark bars vs. EXPOSED: plants subjected to the glyphosate treatment, yellow bars) and (D) box-plot graph depicting the shikimate accumulation per population before and after 48 h of the glyphosate treatment.

Again, the significant interaction between accession and treatment indicated inter-accession variation in SLA in response to the treatment (Table 1).

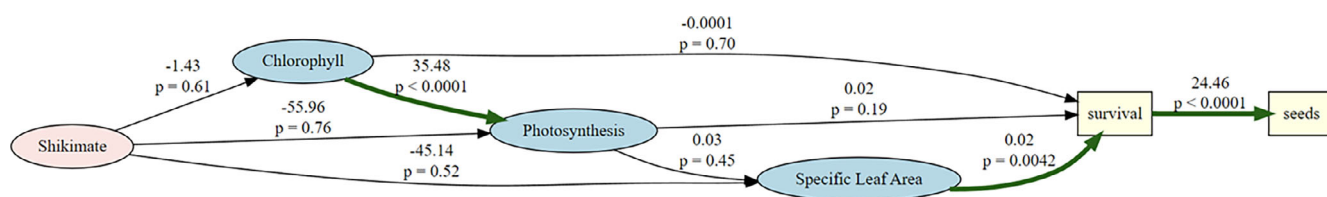
3.2 | Effects of glyphosate on shikimate accumulation

Glyphosate application significantly impacted leaf shikimate accumulation ($F_{1,270} = 48.84$, $p < 0.0001$). The concentration of

shikimate increased by 92% in plants exposed to glyphosate compared to baseline levels from non-exposed plants (4229 ± 264 [$\mu\text{g g}^{-1}$ FW], 2202 ± 205 [$\mu\text{g g}^{-1}$ FW]; model-adjusted means for exposed and non-exposed plants, respectively). Furthermore, shikimate accumulation exhibited significant variation among accessions (LRT = 32.04, $df = 1$, $p < 0.0001$; Figures 2D, S3 and S4). A fraction of glyphosate-treated plants did not increase the shikimate concentration compared to the average of non-exposed plants, among them ALOR_19, BAZA_01, MONT_17 or OSUN_05 (Figure S4). Across accessions, shikimate accumulation was

TABLE 2 Fraction of glyphosate-sensitive/non-sensitive plants per population.

Populations	Fraction of glyphosate non-sensitive plants (%)	Average survival after glyphosate exposure (%)	Number of assayed accessions
El Tobazo	100	33.0 ± 0.17	3
Cañete	83.33	33 ± 0.12	6
Bulevar	62.5	32 ± 0.1	8
Torredonjimeno	60	28 ± 0.12	5
Arjona	55.56	26 ± 0.09	9
Guadajoz	44.44	26 ± 0.08	9
Ogíjares	44.44	23 ± 0.09	9
Quesada	37.5	16 ± 0.08	8
S. Calatrava	37.5	17 ± 0.08	8
Estación de Cártama	33.33	23 ± 0.1	9
Ermita de Alcaraz	33.33	22 ± 0.15	6
Lora de Estepa	33.33	13 ± 0.07	9
Osuna	33.33	16 ± 0.07	9
Porcuna	30	14 ± 0.07	10
Tamaral	27.27	11 ± 0.06	11
Álora	25	12 ± 0.07	8
Montilla	25	17 ± 0.09	8
Espejo	22.22	13 ± 0.09	9
Puebla Cazalla	22.22	9 ± 0.06	9
La Casona	14.28	9 ± 0.07	3
Castro	11.11	4 ± 0.04	9
Torreperogil	11.11	8 ± 0.06	9
Parra	10	7 ± 0.05	10
Baza	0	0	5
Valenzuela	0	0	7

**FIGURE 3** Path analysis diagram that illustrates the effects of shikimate accumulation on physiological traits, and the influences of physiology on plant survival and seed production. This model includes chlorophyll content, photosynthetic rate and specific leaf area as direct survival and seed production predictors. Overall goodness-of-fit of the model: Fisher's C = 11.12, $p = 0.518$, $df = 12$.

negatively associated with 30-day survival ($r = -0.30$, $p = 0.001$, $N = 108$).

3.3 | Resistance occurrence, survival and seed production

After the glyphosate application, 64 out of 196 accessions (32%) completed their life cycle (i.e., they could produce seeds; Table S5). We found that such resistance arose in most populations (Figure 1 and

Table 2), although with variable frequency ($\chi^2 = 37.8$, $df = 24$, $p = 0.036$; Table 2). However, glyphosate exposure strongly reduced plant survival (LRT = 678.03, $df = 1$, $p < 0.0001$). The average plant survival of glyphosate-exposed plants was $17.9\% \pm 1.07$, compared to $86.7\% \pm 1.66$ for control plants. The effect of the treatment on survival was generally consistent across populations (LRT = 36.29, $df = 1$, $p = 0.051$), although some populations showed higher survival after glyphosate exposure than others (Table S5).

The exposure to glyphosate significantly impacted seed production per plant ($F_{1,847} = 1813.1$, $p < 0.0001$). However, the extent of

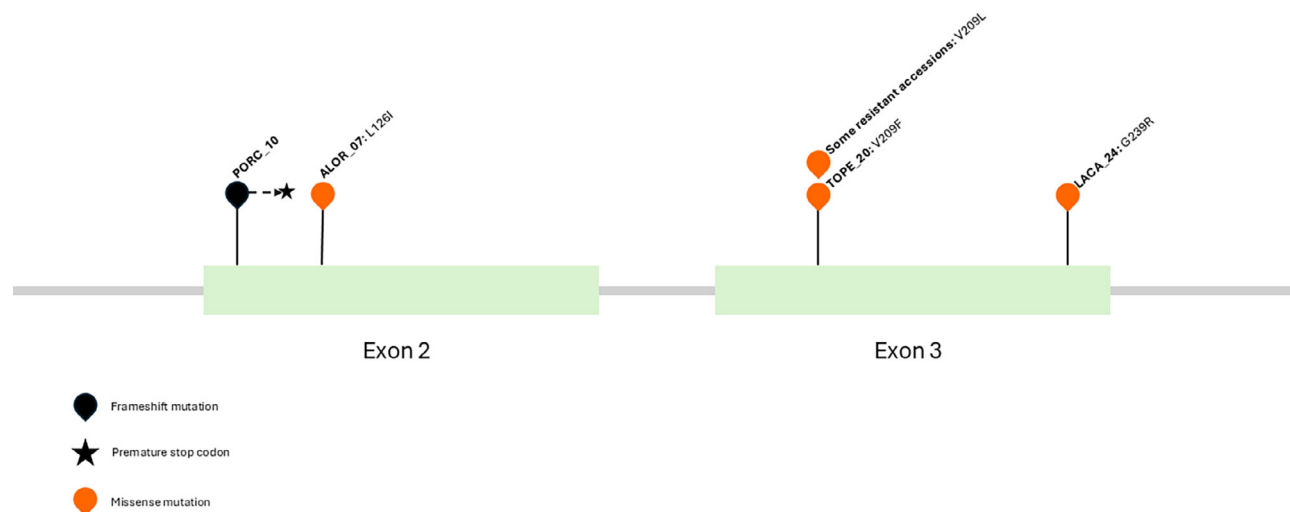


FIGURE 4 Schematic representation of mutations identified in *EPSPS* gene. Green boxes represent exons, with mutations mapped to exons 2 and 3, grey bars represent intronic regions.

this effect varied across different sampled populations ($F_{24,847} = 5.37$, $p < 0.0001$). On average, the seed production of treated plants decreased by 92% to 100% compared to non-exposed plants (Table S6). Significant variation was also detected among accessions (LRT = 4.48, $df = 1$, $p = 0.034$), with some accessions even increasing yield after glyphosate exposure. For example, PORC_15 exhibited a 5.2-fold increase in seed production when exposed to glyphosate compared to its non-exposed siblings. Similarly, TAMA_20 showed a 33% yield increase in exposed plants compared to control plants (Table S7). Variation was significant for the rest of accessions, and the seed production of treated plants decreased by 4.27%–100% compared to non-exposed plants (Table S7).

The spatial distribution of the resistance occurrence frequency, survival and seed production under treatment showed no evidence of spatial autocorrelation (Moran's $I = -0.05$, $p = 0.85$; Moran's $I = -0.043$, $p = 0.97$; Moran's $I = -0.018$, $p = 0.61$, for resistance occurrence, survival and seed production, respectively). Likewise, shikimate accumulation varied randomly across the study populations (Moran's $I = -0.059$, $Z = -0.32$, $p = 0.74$).

3.4 | Relationship between survival, seed production and functional traits under glyphosate exposure

SEM indicated that the only trait directly influencing exposed plants' survival and reproduction is the SLA (Figure 3). Chlorophyll content was positively associated with the photosynthetic rate (Figure 3). Finally, survival was positively linked to higher seed production.

3.5 | *EPSPS* sequencing

We conducted a comparative analysis among 16 sensitive and 62 glyphosate-non-sensitive accessions. We did not find any mutation

at Pro106 (177 in *S. alba*), Gly101 (172) and Thr102 (173) in any accession sequenced. However, in two glyphosate-non-sensitive accessions, PORC_10 and ALOR_07, demonstrated differences at exon 2 (Figure 4). In PORC_10, a thymine insertion at the beginning of the exon introduces a frameshift mutation, which disrupts the downstream reading frame. In ALOR_07, a cytosine-to-adenine substitution results in a missense mutation that changes a leucine to an isoleucine. In exon 3, two missense mutations have been identified. At position 209, the consensus sequence and sensitive accessions encode a valine. However, in the glyphosate-non-sensitive TOPE_20, a mutation changes this codon to encode phenylalanine, while other glyphosate-non-sensitive accessions exhibit a leucine. A second missense mutation occurs in LACA_24 at position 239, where arginine replaces glycine. These sequence variants are detailed in the alignments provided in the Supporting Information (Figure S5).

3.6 | *EPSPS* gene amplification and expression

Across accessions, the copy number variation of the *EPSPS* gene tended to be positively linked to survivorship after glyphosate exposure ($r = 0.25$, $p = 0.053$, $N = 60$). However, only about 10 glyphosate-non-sensitive accessions showed clear copy number values (above 2.5) higher than glyphosate-sensitive plants (Figure 4). Nonetheless, several glyphosate-non-sensitive accessions (e.g., LOES_17, OSUN_05 and OSUN_18) showed elevated copy numbers of the *EPSPS* gene, ranging from 6 to 9 copies, along with elevated survival, suggesting *EPSPS* gene duplication as a resistance mechanism for those lines (Figure 5). In contrast, the rest of the glyphosate-non-sensitive accessions showed lower values, ranging between 1 and 2.5 copies (Figure 5), suggesting that duplication is likely not the primary genetic mechanism of resistance to glyphosate in these accessions. Accessions with higher copy numbers tended to exhibit lower shikimate accumulation, although this relationship was not statistically significant ($r = -0.22$, $p = 0.08$, $N = 60$).

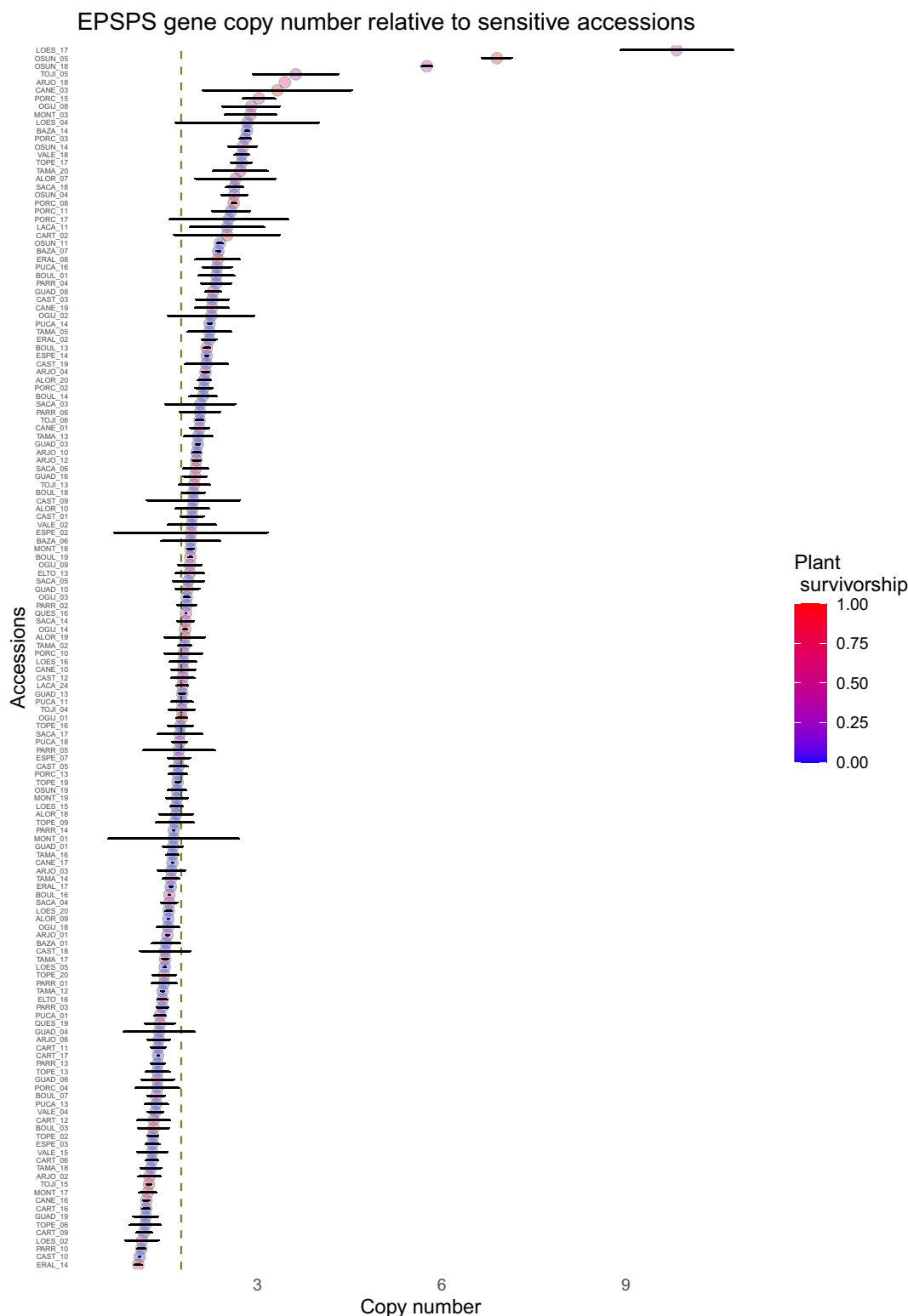


FIGURE 5 Quantification of *EPSPS* gene copy number variation across *Sinapis alba* accessions. Copy number was determined using the $2^{-\Delta\Delta C_t}$ method and normalized with a single-copy gene *LFY*. Each dot represents a single accession and colours indicate plant survivorship proportion after glyphosate exposure, ranging from blue (survival = 0) to red (survival = 1). The green dashed line indicates the mean copy number of sensitive accessions (1.76).

Concerning expression, the relative expression of *EPSPS* varied significantly among accessions (ANOVA: $F_{1,45} = 20.06$, $p < 0.0001$). The expression of *EPSPS* was relatively high or very high for TOJI_13,

OJIG_01, CART_12 and ARJO_12 (Figure 6), suggesting that overexpression of *EPSPS* may underlie glyphosate resistance in these accessions. However, overall, overexpression of *EPSPS* was not related to

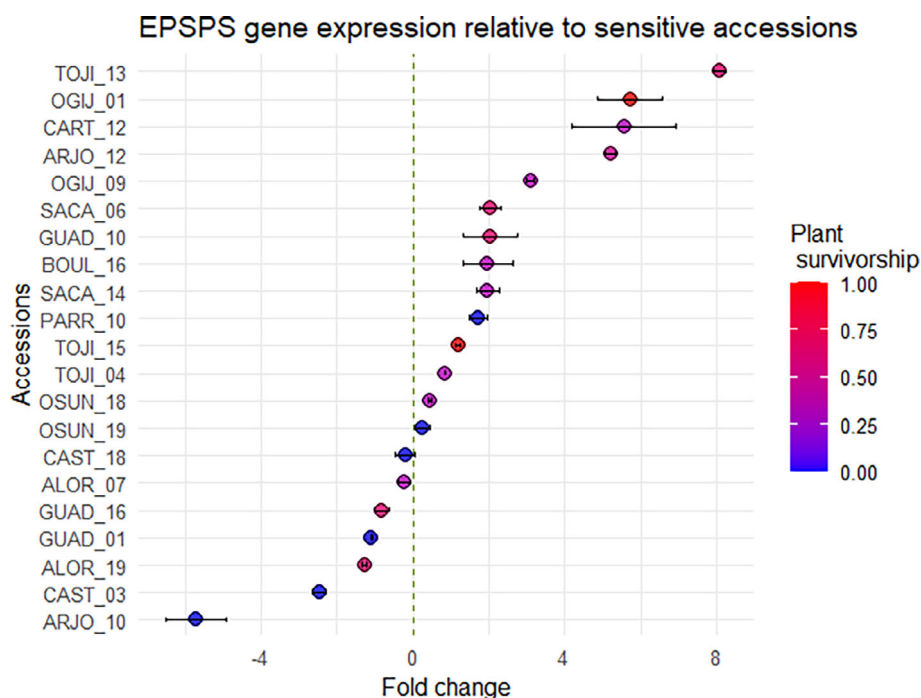


FIGURE 6 EPSPS gene expression levels in *Sinapis alba* accessions. Gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Dots represent the expression levels for each accession and colours indicate plant survivorship proportion, ranging from blue (survival = 0) to red (survival = 1). The green dashed line marks the mean expression in sensitive accessions (0.86).

survival or shikimate accumulation after glyphosate exposure ($r = 0.25$, $p = 0.27$, $N = 21$; $r = -0.11$, $p = 0.61$, $N = 21$, respectively).

3.7 | ABCC8 expression

The relative expression of ABCC8 varied significantly among accessions (ANOVA: $F_{1,63} = 7.89$, $p < 0.0001$). Interestingly, the relative expression of ABCC8 varied among glyphosate-non-sensitive and glyphosate-sensitive accessions (ANOVA: $F_{1,164} = 11.63$, $p < 0.0001$). In glyphosate-non-sensitive plants, the average ABCC8 expression level was 76.7 ± 14.1 , compared to 1.7 ± 16.9 for glyphosate-sensitive accessions (Figure 7). Overall, overexpression of ABCC8 was not related to shikimate accumulation or survival after glyphosate exposure ($r = -0.04$, $p = 0.74$, $N = 62$; $r = -0.013$, $p = 0.92$, $N = 62$, respectively).

4 | DISCUSSION

Glyphosate is a widely used herbicide that effectively controls weeds worldwide. Our results confirm that glyphosate application impacts *S. alba* physiology and metabolism, reducing overall plant performance and survival. However, a notable fraction of assayed accessions (64 accessions from 23 different populations, ca. 32%) could survive, complete the life cycle and reproduce under glyphosate exposure at the manufacturer's recommended doses, which are typically applied in olive crops for the study area, suggesting resistance to glyphosate.

The occurrence of such resistance is not broadly spatially autocorrelated. Our study suggests that resistance to glyphosate likely originated from multiple population sources and spread between populations, supported by the different genetic bases underlying glyphosate resistance in our study populations (Figure 1). In particular, our genetic analyses indicate that ABCC8 expression seems to be a more prevalent mechanism underlying glyphosate resistance in *S. alba*. In contrast, EPSPS amplification and/or expression, or missense mutations in that gene, explain resistance only in some accessions.

4.1 | Glyphosate and physiology

Our study supports that glyphosate exposure of *S. alba* plants at manufacturer-recommended doses decreases, on average, photosynthetic rate by 90%, chlorophyll concentration by 22% and SLA by 38%. This is an expected result given that glyphosate mainly acts at the chloroplast level (Gomes et al., 2016, but see Cedergreen, 2008), where photosynthesis occurs. Furthermore, exposure to glyphosate leads to strong oxidative stress, accumulating reactive oxygen species and a subsequent reduction in physiological parameters (Gomes et al., 2016). Similar results have been described previously (Krenchinski et al., 2017), but this is the first study to evaluate glyphosate effects on the physiology of a Brassicaceae species, an essential economic plant family.

Interestingly, the SEM model indicated that SLA was the only physiological trait influencing plant fitness for plants exposed to glyphosate. Thus, SLA was positively and significantly linked to survival and, subsequently, to seed production (Figure 4). Because SLA is

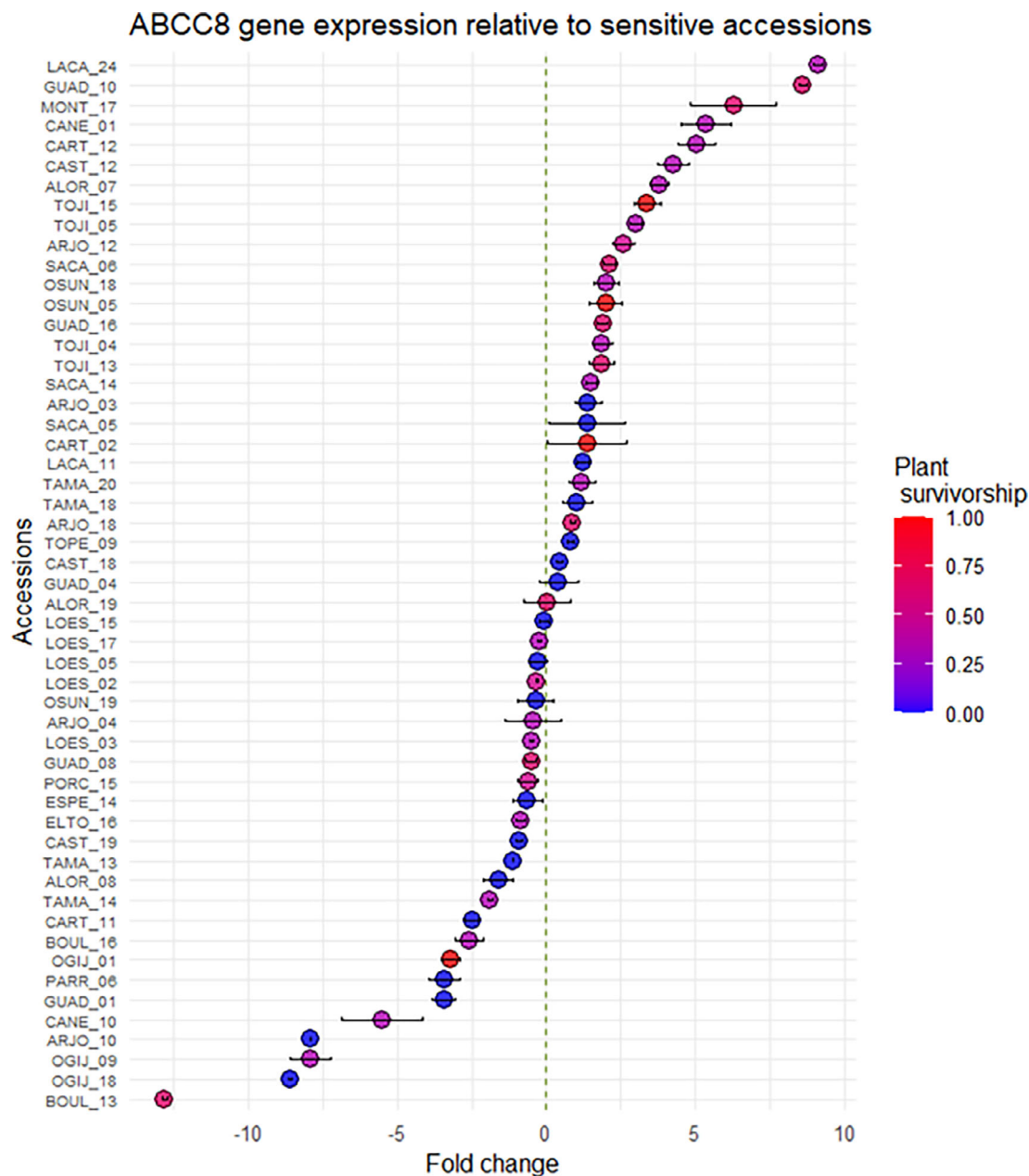


FIGURE 7 ABCC8 gene expression levels in *Sinapis alba* accessions. Expression levels were analysed using the $2^{-\Delta\Delta C_t}$ method. Each dot represents the expression level for a specific accession, with colours indicating the proportion of plant survivorship: blue represents a survival of 0, while red indicates a survival of 1. The green dashed line marks the mean expression level in sensitive accessions, which is 1.06.

typically related to a rapid resource acquisition strategy in annual plants (e.g., fast growth, high photosynthesis and/or short lifespan; Wright et al., 2004), maintaining a higher SLA could reflect a fast-growing strategy to produce seeds to overcome the stressful environment resulting from herbicide exposure.

4.2 | Shikimate

Glyphosate inhibits the shikimate pathway by blocking the synthesis of aromatic amino acids (Franz et al., 1997). Our results confirm that

shikimate is accumulated in glyphosate-sensitive *S. alba* accessions within 48 h of exposure. In contrast, glyphosate-non-sensitive accessions show low or no accumulation within the same period and exposure treatment. These results confirm the feasibility of using this metabolite as a biomarker of glyphosate resistance (Brunharo & Streisfeld, 2022; Singh and Shaner, 1998) in white mustard. They align with previous studies that have reported significant shikimate accumulation after glyphosate application in several plant species (e.g. González-Torralva et al., 2012; Henry et al., 2007; Yu et al., 2009).

As expected, shikimate accumulation was associated with survivorship. Accessions that do not accumulate shikimate have

significantly higher 30-day survival than sensitive ones. This finding aligns with previous studies conducted on the Brassicaceae family that have examined shikimate accumulation and lower survivorship due to toxicity derived from shikimate accumulation and the collapse of essential amino acid synthesis. The Brassicaceae family research has demonstrated that resistant plants of *Brassica napus* do not accumulate shikimate after exposure to varying concentrations of glyphosate (Shaner et al., 2005). This reduced accumulation has resulted in higher survival rates than plants that accumulated shikimate. Similar results were observed with sublethal concentrations in this species (Petersen et al., 2007). *B. juncea* also showed similar outcomes (Huangfu et al., 2007), indicating that shikimate accumulation can be a sensitive glyphosate resistance biomarker.

4.3 | Pattern of resistance

We detected resistance to glyphosate in 23 out of 25 populations examined and in 32% of accessions assayed. The lack of significant spatial autocorrelation (Moran's *I*) suggests that occurrence, survival and seed production in *S. alba* under glyphosate exposure are not clustered across the studied area. Instead, these features appear randomly distributed, suggesting that resistance might have evolved independently in different regions and spread between populations (Figure 1). One potential reason for this pattern could be a high level of gene flow, consistent with the outcrossing mating system described in *S. alba* (Winter, 2011). In fact, insect-mediated pollination has been shown to be effective in this system (Cano et al., 2022). The pollination promotes gene flow, which could homogenise genetic variation and result in the widespread dissemination of resistance alleles, which could explain the absence of spatial autocorrelation. This suggests that natural variation in response to glyphosate exposure is driven more by genetic than geographic factors. As a result, the resistance traits are widely dispersed, not confined to specific areas, underscoring the significant role of gene flow in the spread of herbicide resistance in *S. alba*. Alternatively, we cannot rule out that resistance might also arise from ancestral genetic variation in some populations.

4.4 | Glyphosate resistance and candidate genes

Two main genetic mechanisms, involving *EPSPS* and *ABCC8* genes (Pan et al., 2021), underlie glyphosate resistance. However, the prevalence and coexistence of each mechanism for explaining natural variation are poorly known. Here, we have found that both mechanisms are important for explaining resistance in some populations, although the overexpression of the *ABCC8* gene seems to be more prevalent.

In this study, only three glyphosate-non-sensitive accessions exhibited *EPSPS* gene copy numbers ranging from 6 to 9, indicating *EPSPS* amplification (Figure 5). In contrast, the remaining glyphosate-non-sensitive accessions displayed lower copy numbers, suggesting that gene amplification likely is not the primary genetic mechanism in these accessions. The phenomenon of glyphosate resistance via *EPSPS* gene

duplication was first identified in *Amaranthus palmeri* (Gaines et al., 2009). Since then, numerous resistant genotypes exhibiting *EPSPS* amplification have been reported (Aulakh et al., 2024; Gaines et al., 2010; Salas et al., 2012). In addition, our findings reveal a significant variation in the relative expression of the *EPSPS* among accessions. Four glyphosate-non-sensitive accessions exhibited high levels of *EPSPS* expression without associated gene amplification (Figure 6), suggesting that *EPSPS* overexpression itself can confer glyphosate resistance in some accessions of *S. alba*, indicating an alternative resistance mechanism distinct from gene amplification. The observed overexpression is consistent with previous studies in *A. palmeri* (Gaines et al., 2010; Manicardi et al., 2023), *L. perenne* (Salas et al., 2012) or *Salsola tragus* (Yannicari et al., 2023), which have been shown to overexpress *EPSPS* as a resistance strategy due to duplications. Thus, our study demonstrates that *EPSPS* overexpression can occur independently of gene amplification in *S. alba*, emphasizing the importance of considering multiple resistance mechanisms.

In addition, mutations in the *EPSPS* coding region can also confer glyphosate resistance. We did not observe any canonical *EPSPS* mutation, such as Pro106 substitutions that are widely reported in other species (e.g., Nandula et al., 2013). However, we identified several novel mutations in exons 2 and 3 (Figure 4). These novel mutations include a frameshift mutation, which results in a premature stop codon, a missense mutation in exon 2, and three missense mutations in exon 3. One of these mutations in exon 3 introduces a phenylalanine residue, which contains an aromatic ring. These mutations may be implicated in conformational changes in *EPSPS* that alter its affinity for the herbicide. Future functional genomic studies should reveal the specific mechanisms of action of these novel mutations described here, which are present in 15 out of 62 glyphosate-resistant plants (Figure S5).

On the other hand, our results indicate that the overexpression of the *ABCC8* gene may constitute a primary factor driving resistance to glyphosate in *S. alba* since 25 out of 64 assayed glyphosate-non-sensitive accessions showed elevated expression of this gene under glyphosate exposure. In addition, glyphosate-non-sensitive plants exhibited notably higher average *ABCC8* expression levels than sensitive plants, underscoring the importance of *ABCC8* overexpression in developing resistance (Figure 7). Although *ABCC8* overexpression has been observed in various species such as rice, soybean, and *Echinochloa colona* (Pan et al., 2021), identifying it as a pervasive resistance mechanism in a considerable number of resistant *S. alba* accessions highlights a novel and potentially widespread strategy for glyphosate resistance.

Interestingly, two glyphosate-non-sensitive accessions, ARJO_12 and CART_12, from relatively distant populations, showed elevated expression of both candidate genes, suggesting that these two accessions may have evolved resistance to glyphosate from these independent genetic mechanisms.

5 | CONCLUSIONS

Our research has uncovered the significant physiological effects of glyphosate exposure in *S. alba* for the first time and identified candidate genetic mechanisms associated with resistance. Additionally, we

have verified that low shikimate accumulation is an effective biomarker for resistance to glyphosate exposure. This study reveals that resistance to glyphosate in *S. alba* is a complex trait driven by both TSR and NTSR mechanisms. Among these, overexpression of the *ABCC8* gene has been identified as the major mechanism, occurring in many resistant accessions and contributing significantly to resistance. Additionally, *EPSPS* gene duplication and expression and missense mutation in that gene are significant mechanisms in some accessions. However, 13 out of 64 resistant accessions assayed (approximately 20%) were not associated with *EPSPS* duplication, *EPSPS* coding mutations, *EPSPS* overexpression, or *ABCC8* overexpression, suggesting that other unknown genetic mechanisms are also involved in glyphosate resistance in *S. alba*. Further studies based on genome-wide association should help unravel novel candidate genes that may contribute to a better understanding of the origin of glyphosate resistance in this species. In these studies, maternal effects should be better accounted for, which is a current limitation of our findings. Finally, the absence of spatial autocorrelation in resistance occurrence and fitness-related traits under glyphosate exposure suggests that resistance likely has multiple independent origins and that gene flow has facilitated the dispersion of alleles for resistance across *S. alba* populations.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/wre.70027>.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available in the ZENODO repository <https://doi.org/10.5281/zenodo.15697367>.

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SUPPORTING INFORMATION

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