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Different Non-Target Site Mechanisms Endow Different Glyphosate Susceptibility in *Avena* Species from Spain

José G. Vázquez-García [1,*], Joel Torra [2,*], Candelario Palma-Bautista [1], Fernando Bastida [3], Ricardo Alcántara-de la Cruz [4], João Portugal [5,6], Jesús V. Jorrin-Novo [1] and Rafael De Prado [1]

[1] Department of Agroforestry, Plant Biochemistry and Molecular Biology, University of Cordoba, 14071 Cordoba, Spain
[2] Department of Forestry and Agricultural Science and Engineering, Agrotecnio-CERCA Center, University of Lleida, 25198 Lleida, Spain
[3] Departmento de Ciencias Agroforestales, Universidad de Huelva, 21007 Huelva, Spain
[4] Centro de Ciências da Natureza, Campus Lagoa do Sino, Universidade Federal de São Carlos, Buri 18290-000, Brazil
[5] Biosciences Department, Polytechnic Institute of Beja, 7800-000 Beja, Portugal
[6] VALORIZA-Research Centre for Endogenous Resource Valorization, Polytechnic Institute of Portalegre, 7300-555 Portalegre, Portugal
* Correspondence: z82vagaj@uco.es (J.G.V.-G.); joel.torra@udl.cat (J.T.); Tel.: +34-957218600 (J.G.V.-G.); +34-973702318 (J.T.)

Abstract: In recent decades, cereal agriculture across main producing areas in Spain has progressively adopted direct sowing, associated with an increased use of herbicides in pre-sowing. Weedy species from genus *Avena* have been observed after the application of glyphosate in wheat. Here, *Avena fatua* (two accessions), *Avena byzantina* and *Avena sterilis* subsp. *sterilis*, one accession each, were taxonomically characterized by a biometric study. Dose-response trials confirmed that one *A. fatua* accession evolved to resistance, because it was four times more resistant (R) than the others, ascribed as susceptible (S). In addition, based on LD_{50}, *A. byzantina* and *A. sterilis* had low susceptibility to glyphosate, with 604 and 458 g ae ha^{-1}. Shikimic acid accumulation was able to discriminate between high susceptible (*A. fatua* (S)), low susceptible (*A. byzantina* and *A. sterilis*), and low resistant (*A. fatua* (R)) populations. On the other hand, the study revealed that *A. fatua* (R), *A. byzantina* and *A. sterilis* had low foliar uptake and decreased movement of glyphosate. In addition, the metabolism study showed less metabolite accumulation in *A. byzantina* and *A. fatua* (S). However, at 96 h after glyphosate application, *A. fatua* (R) and *A. sterilis* were able to convert more than 30% of glyphosate to aminomethylphosphonic acid (AMPA) and sarcosine. Enzyme basal activity and I_{50} values predicted high affinity between the herbicide and the target enzyme for all accessions, thus indicating that a target-site (TS) mechanism is probably not involved in the differences in glyphosate susceptibility. In closing, non-target site (NTS) mechanisms could participate both in *A. fatua* R to glyphosate, and low susceptibility in *A. byzantina* and *A. sterilis* from Spain.

Keywords: EPSPS; weed management strategies; plant evolution; plant resistance; pre-sowing

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1. Introduction

Weeds are an important biological constraint to the production of grain crops [1]. In the Western Mediterranean, *Avena sterilis* L. subsp. *sterilis* and *Avena fatua* L. are among the most important grass weeds, causing revenue losses to grain growers [2]. A recent study reported that 15 to 16 plants/m^2 of *Avena* reduced wheat yield by 50% [3]. In Spain, direct sowing has been commonly used in annual crops for more than 20 years, with a concomitant increased use of herbicides [4]. Compared to traditional intensive tillage, this technique switches mechanical operations for herbicide application, mainly glyphosate, to control early germinating weeds that are already resistant to acetolactate synthase

(ALS) and Acetyl CoA carboxylase (ACCase) herbicides (i.e., *Phalaris* spp. and *Avena* spp.) [5,6].

The controversial glyphosate herbicide interrupt biosynthesis inhibition of three essential amino acids (phenylalanine, tyrosine and tryptophan) by inhibiting the enzyme 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS) [7]. Unlearned lessons by farmers in integrated weed management (IWM) have led to extensive use of glyphosate without rotation of herbicides and/or crops. As a result of this incorrect practice, the selection of tolerant/resistant weeds has been favored [8]. Because of the different agronomic practices implemented in cropping systems, there are two major problems: (a) the composition of the weed flora has progressively changed not only in annual crops managed by direct sowing, but also in Mediterranean perennial crops such as olive groves, fruit orchards and vineyards [9,10]; (b) on the other hand, direct sowing in annual crops could also increase the residuality of herbicides in the soil [11,12].

The term "tolerance" has been defined by the Weed Science Society of America (1998) as the inherent capacity of a species to survive and reproduce after herbicide treatment at field doses. This means that no preselection of herbicide led to low sensibility; therefore, it is naturally an innate tolerant accession. Tolerance to herbicides (in this case to glyphosate) could result from different morphological features or physiological processes, collectively known as non-target site (NTS) mechanisms. These mechanisms participate in reducing or preventing the accumulation of the herbicide in the target meristematic tissues [13]. NTS mechanisms include limited absorption or translocation of the herbicide and/or its sequestration into cellular organelles such as vacuoles. Plant metabolic pathways capable of ending herbicide detoxification also belong to this important group of mechanisms [14,15].

To support the development of IWM strategies in Mediterranean annual crops relying on recurrent herbicide use, the objectives of this study were (a) to determine the level of susceptibility to glyphosate in four accessions of the main weedy *Avena* species sampled in non-cultivated areas and crops of northern Spain and (b) to elucidate the resistance mechanisms involved in these accessions.

2. Materials and Methods

2.1. Plant Collection

Glyphosate susceptibility level was evaluated in three different *Avena* species, including two main weeds of winter annual crops in Mediterranean Spain: two *A. fatua* accessions (one putative resistant (R) and one putative susceptible (S)) and one *Avena sterilis* subsp. *sterilis* population (hereafter *A. sterilis*). In addition, a cultivated *A. byzantina* C. Koch accession, widely used in Spain as livestock feed, was included in the study. In June 2020, seeds of two *A. fatua* and one *A. sterilis* accessions were collected in northeastern Spain. *Avena fatua* (R) was collected under cereal field (41.9102772, 1.005761); *A. fatua* (S) from a field margin (41.864485, 1.007657); and *A. sterilis* was collected around the University of Lleida (non-cultivated habitats). Seeds of all accessions were preserved in a laboratory at 4 °C. Seeds of *A. byzantina* were supplied by the repository of the Department of Agricultural Chemistry, Edaphology and Microbiology of the University of Cordoba.

Seeds of the different accessions were germinated in Petri dishes containing filter paper moistened with distilled water. The Petri dishes were placed in a growth chamber at 28/18 °C (day/night) with a photoperiod of 16 h, 350 μmol s^{-1} of photosynthetic photon flux and 80% relative humidity. After three days, the resulting seedlings were placed in pots (one plant per pot) containing sand/peat in a ratio of 1:1 (*v*/*v*), and the pots were taken into a greenhouse under the same temperature and photoperiod conditions.

2.2. Biometric Study and Taxonomic Identify

Seven to eight plants per population were grown until flowering to confirm species identity. A biometric study was conducted on spikelet and floret diagnostic traits, including lower and upper glume length, number of fertile florets per spikelet, form of disarticulation of the rhachilla at fruit maturity and, for the lemma of the basal floret, length, distribution of hairs, presence of apical awns, and total length and length of the column (basal twisting portion) of the dorsal awn. Traits were measured in one randomly selected spikelet per plant.

2.3. Dose-Response Assays

Glyphosate dose-response assays were conducted to determine the susceptibility level of weedy and cultivated *Avena* species. At third to four leaf stage (BBCH 13–14), the accessions were treated with glyphosate using a laboratory sprayer system (SBS-060 De Vries Manufacturing, Hollandale, MN, USA) equipped with 8002 flat fan nozzles delivering 250 L ha^{-1} at 50 cm height above plants. Glyphosate (Roundup Ultimate® SL, 480 g ae L^{-1} as isopropyl amine salt, Monsanto) doses applied (10 plants per dose) were: 32.25, 62.5, 125, 250, 375, 500, 625, 750, 1000 and 1500 g ae ha^{-1}. Untreated plants were used as control. Plant mortality was recorded after 28 days to determine the effective mean dose suppressing 50% of accession (LD_{50}). In addition, the plants were harvested and subsequently dried (60 °C for two weeks) to determine the effective mean dose reducing growth by 50% (GR_{50}). Data were expressed as percentage in relation to the untreated controls.

2.4. Spray Retention Assays

To determine the amount herbicide retained on leaf surfaces, six plants of each accession of *Avena* were assayed in a completely randomized design. The plants at BBCH 13–14 stage were sprayed with 360 g ae ha^{-1} of glyphosate plus 100 mg L^{-1} Na-fluorescein in the spraying system described in Section 2.3. Subsequently, we followed the methodology described by Yanniccari et al. [16]. Between 1.5 and 2 h after treatment (HAT), *Avena* plants were cut at ground level, and the tissue was submerged in test tubes with 50 mL of 5 mM NaOH for 30 s to remove the spray solution. The washing solution was recovered in glass flasks. Subsequently, plants were placed on labelled filter paper strips, oven dried at 60 °C for 48 h and weighed. Fluorescein absorbance was determined using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) at 490 nm wavelength and an absorbance at 510 nm wavelength. The experiment was repeated, and the results, expressed as μL of sprayed solution retained per g dry weight, were combined for statistical analysis.

2.5. Shikimate Accumulation Assay

For each *Avena* accession, 25 fully extended young leaves (BBCH 13–14) were taken from at least 10 plants and 4-mm diameter discs were cut. Samples of 50 mg leaf discs (pool foliar tissue) were placed in 2-mL tubes containing 1 mL of 10-mM ammonium phosphate monobasic solution (pH adjusted to 4.4 with 0.1 HCl) at different concentrations of glyphosate (0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 mM). The extraction and quantification of shikimic acid was performed according to Vázquez-García et al. [17]. Three samples of each *Avena* accession were assessed per herbicide concentration. The experiment was completely randomized and was repeated. Results were expressed as micrograms of shikimate per mL of HCl solution (μg shikimic acid mL^{-1}) in relation to a calibration curve of shikimic acid.

2.6. Glyphosate Absorption and Mobilization Assays

Ten plants at the BBCH-13,14 stage of each *Avena* accession were used for ^{14}C-glyphosate absorption and mobilization assays, whereas another five plants at the same stage were used for a digital visualization assay. Both experiments were arranged in a completely random design.

A mix (mix A) with labeled glyphosate (^{14}C-glyphosate (glycine-2-^{14}C, 95% radiochemical purity, 273 Mbq mmol^{-1} specific activity, Institute of Isotopes Co., Ltd., Budapest, Hungary) plus a commercial formula (Roundup ultimate 48%, Monsanto, Spain) was prepared with a final specific activity of 100,000 dpm μL^{-1} and 360 g ae ha^{-1}. Thereafter, a drop (1 μL) of mix A was applied on the second leaf of each plant.

A second mix (mix B) of distilled water plus acetone (1:1 *v*/*v*) was prepared to wash the non-absorbed ^{14}C-glyphosate at 48 and 96 h after treatment (HAT), and this procedure was repeated three times per sample (1 mL of mix B per time). The treated leaves of five plants from each population were washed at each evaluation time. The wash solution was recovered in scintillation vials, and 2 mL of scintillation cocktail was added and analyzed by liquid scintillation spectrophotometry (LSS). ^{14}C-glyphosate absorption was expressed as a percentage of recovered radioactivity using the following equation: % of ^{14}C-herbicide absorbed = (^{14}C in combusted tissue/(^{14}C in combusted tissue + ^{14}C in shoot washes)).

Prewashed plants used to determine absorption were removed from the pots, and the root system was washed immediately to remove the substrate. Whole plants were fractioned into treated leaf, shoots and roots, saved in filter paper cones, dried in a stove for two days at 60 °C for 96 h, and thereafter samples were combusted in a sample oxidizer (Packard 307), for 180 s. Evolved $^{14}CO_2$ was trapped and counted in Carbosob/Permafluor E+ (3/7 *v*/*v*) (Packard Instruments Co., Downers Grove, IL, USA.). The radioactivity of plant tissues was quantified by LSS (10 min). Mobilization percentages were calculated according to Vázquez-García et al. [17].

To achieve digital visualization of ^{14}C-glyphosate movement through the plant, *Avena* plants were treated with mix "A" and washed with mix "B", as described above. Whole plants, fixed on filter paper, were oven-dried for four days at 60 °C. Then, the plants were pressed, placed adjacent to phosphor storage film (25 cm × 12.5 cm) and scanned (three hours after) for herbicide movement visualization in a phosphor imager (Cyclone, Perkin-Elmer, Packard Bioscience BV, MA, USA).

2.7. Glyphosate Metabolism

The study of glyphosate metabolism in *Avena* plants comprised three relevant steps: herbicide application, sample harvest and quantification of herbicide and metabolites.

The first step was performed under the same conditions described in the dose-response curves section. A glyphosate dose of 360 g ae ha^{-1} was applied over ten plants of each accession at the BBCH-14 growth stage. Ten additional untreated plants were saved and used as blanks. At 96 HAT, the plants were cut at ground level and washed with distilled water to eliminate herbicide excess from leaf surfaces and substrate residues from roots. Thereafter, the plants were dried with paper towel for three minutes and frozen by liquid nitrogen (N_2). Treated and untreated plant samples were stored in −80 °C until metabolite analysis. Amino methyl phosphonic acid (AMPA), glyoxylate, and sarcosine extraction was performed according to Rojano-Delgado et al. [18]. Calibrations curves were obtained using metabolite standards (Sigma-Aldrich, St. Louis, MI, USA). This study was arranged in a completely randomized design, with ten replications (one replica-one plant) per *Avena* accession. Data were expressed as percentages of the sum of glyphosate plus metabolites recovered.

2.8. Activity of Target Enzyme of Glyphosate

The EPSPS activity was assayed in the four *Avena* accessions. Five grams of young foliar tissue (BBCH-13,14) was collected and immediately macerated in a mortar until obtaining a fine powder. Then, the samples were stored at −40 °C until analyses. The extraction of the target enzyme of the glyphosate, as well as the determination of the total soluble protein (TPS, basal activity without glyphosate) and the EPSPS inhibition rate by adding increased concentrations of glyphosate (0.1, 1, 10, 100, and 1000 μM) were performed following the detailed methodology by Dayan et al. [19]. For each glyphosate concentra-

tion, three technical replicates of each accession were assayed. The experiment was repeated (finally $n = 6$ for statistical analysis), and the results were given as a percentage relative to the control (0 μM glyphosate) of the amount (μmol) of inorganic phosphate (Pi) released per μg of TSP min^{-1} (μmol Pi $μg^{-1}$ TSP min^{-1}).

2.9. Statistical Study

For dose response data, the three-parameter non-linear function, $y = d/\{1 + \exp[b(\log x - \log e)]\}$, was fitted using the "drc" package for the R environment according Ritz et al. [20] to estimate the glyphosate dose necessary to reduce plant growth, lead plant mortality, or inhibit enzyme activity by 50% for each Avena accession (GR_{50}, LD_{50}, and I_{50}, respectively). In the above function, **y** is percentage of growth, survival or enzyme inhibition, parameter b controls the steepness of the curve, d is the upper limit of y, and e is GR_{50}, LD_{50} or I_{50} values. A resistance index (Ri) was calculated for each accession as the quotient of its $GR_{50}/LD_{50}/I_{50}$ value and the corresponding values of the less tolerant/susceptible accession.

For foliar retention, shikimic acid accumulation, herbicide absorption and movement, and metabolism data, normal error distribution and the homogeneity of the variance were graphically verified for each set. Then, ANOVAs were performed to test for significance effects. Post hoc multiple comparisons to separate means were performed using the Tukey's HSD test.

3. Results

3.1. Biometric Study and Taxonomic Identify

Disarticulation of the rhachilla between florets leading to independent dispersal of caryopses separates *A. fatua* from the other two *Avena* species. One-awned lemmas separate *A. fatua* from *Avena barbata* Pott. Ex Link, a species in the flora of the Iberian Peninsula, which also exhibits the above trait. *Avena sterilis* can be easily separated from co-generic species based on its large-sized spikelets and florets, large dorsal awns in lemmas, and rhachillas disarticulating only above the glumes, i.e., with the caryopses produced within a spikelet dispersing as a unit. On the other hand, traits identifying the domesticated species *A. byzantina* include lack of disarticulation of the rhachilla at fruit maturity and dorsal awns in lemmas lacking column, i.e., the basal twisting portion of the awn (Figure 1, Table 1).

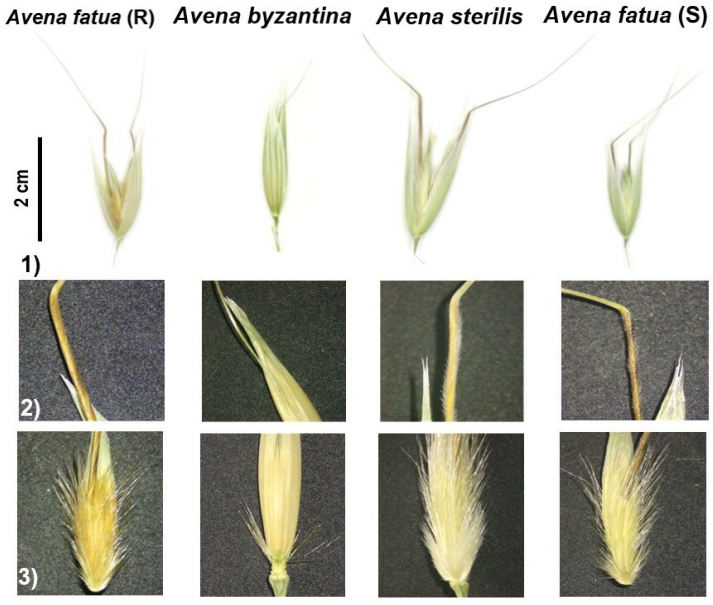

Figure 1. Spikelets and lemmas from flowering plants of the studied *A. fatua* (R), *A. byzantina*, *A. sterilis* and *A. fatua* (S). Rows depict (1) spikelets, (2) basal, twisting portions (columns) of lemma's dorsal awns, and (3) distribution of hairs in the basal half of lemmas of spikelet's lowermost flowers. Note that in *A. byzantine,* the awn lacks a definite column, and hairs are only present at the lemma base.

Table 1. Biometric analysis of diagnostic traits on plants individually grown in pots of the four *Avena* accession studied. Mean values (in cm) and standard deviations are offered for continuous quantitative traits. One spikelet per plant was randomly selected. The number of plants sampled per accession is indicated between parentheses.

	***Avena fatua* (R)** **(*n* = 7)**	***Avena byzantina*** **(*n* = 7)**	***Avena sterilis*** **(*n* = 7)**	***Avena fatua* (S)** **(*n* = 8)**
Spikelet				
Lower glume length	2.5 ± 0.12	2.4 ± 0.08	3.1 ± 0.16	2.5 ± 0.16
Upper glume length	2.6 ± 0.08	2.7 ± 0.13	3.1 ± 0.08	2.6 ± 0.18
Number of fertile florets	2	2	2	2 -3
Disarticulation of the rhachilla at maturity	between florets	no	below the basal floret only	between florets
Lemma of the basal floret				
Length	1.6 ± 0.06	1.6 ± 0.07	2.5 ± 0.11	1.8 ± 0.14
Hairiness	lower half	only at floret callus	lower half	lower half
Apical awns	absent	absent	absent	absent
Dorsal awn, length of the basal twisting portion (column)	1.4 ± 0.12	without a column	2.0 ± 0.14	1.5 ± 0.14
Dorsal awn, total length	3.6 ± 0.33	2.5 ± 0.14	5.7 ± 0.41	3.9 ± 0.20

n = number of individuals.

3.2. Dose-Response Assay

All *Avena* accessions exhibited differential susceptibility to glyphosate (Figure 2). *Avena* fatua labelled as R showed the highest Ri and GR_{50} values (4.2 and 560.5 g ae ha^{-1}), followed by *A. byzantina* (Ri = 3.4, GR_{50} = 467.5 g ae ha^{-1}), and the *A. sterilis* accession (Ri = 2.4, GR_{50} = 288.3 g ae ha^{-1}), which was considered as low susceptible compared to the accession of *A. fatua* labelled as S (117.1 g ae ha^{-1}). Only the *A. fatua* R population had some plants (around 15%) surviving the field dose of glyphosate (1080 g ae ha^{-1}), while all *Avena* accessions were controlled at 1.5x the field dose of glyphosate. Compared to the *A. fatua* (S) accession, *A. fatua* R, *A. byzantina* and *A. sterilis* accessions required a 2–3.5 fold higher dose for achieving 50% mortality (Table 2).

Table 2. Parameters of the log-logistic regression to estimate the mean dose (g ae ha^{-1}) of glyphosate required to reduce the dry weight (GR_{50}) and plant mortality (LD_{50}) by 50% in different *Avena* spp.

Accession	**b**	**d**	**GR_{50}**	**95% CI**		**Ri [a]**
				Lower	**Upper**	
A. fatua (R)	3.1	93.1	560.5	525.5	595.6	4.2
A. byzantina	3.5	93.3	467.5	*439.7*	*495.2*	3.4
A. sterilis	2.5	97.4	314.2	288.3	340.0	2.4
A. fatua (S)	2.9	100.5	130.5	117.1	143.8	-
			LD_{50}			
A. fatua (R)	6.5	97.2	788.8	764.9	812.8	3.5
A. byzantina	5.4	95.7	604.0	582.8	625.3	2.7
A. sterilis	3.9	99.0	458.2	437.5	479.0	2
A. fatua (S)	3.1	98.0	222.1	201.5	242.74	-

[a] Resistance index (GR_{50} *Avena* spp./GR_{50} *A. fatua* (S)).

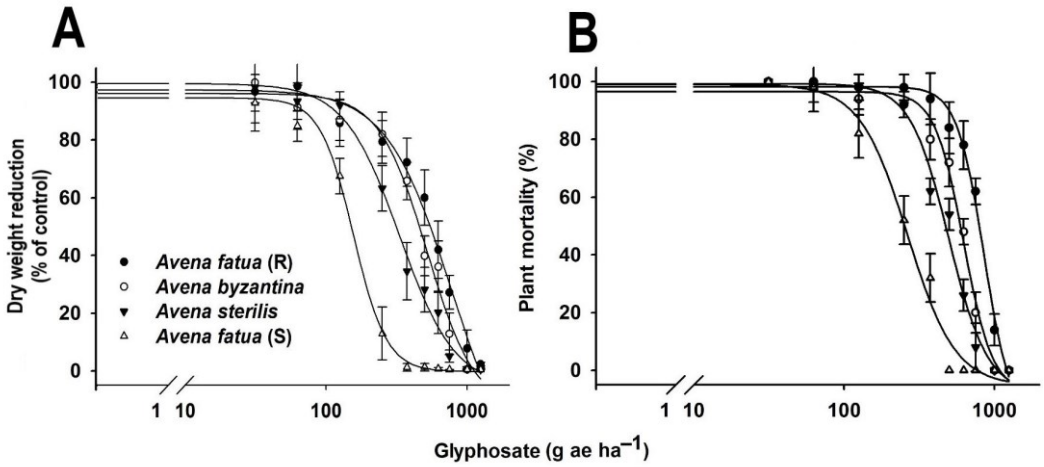

Figure 2. Dose-response curves ((**A**), dry weight reduction; (**B**), plant mortality) for the different *Avena* accessions. Vertical bars represent the standard error of the means (n = 20).

3.3. Herbicide Retention

Foliar retention ranged from 641.5 to 729.8 µL of glyphosate solution per g dry weight among *Avena* accessions. Although the *A. fatua* populations retained ~80–100 µL less than *A. byzantina* and *A. sterilis*, there were no differences in leaf retention (Figure 3).

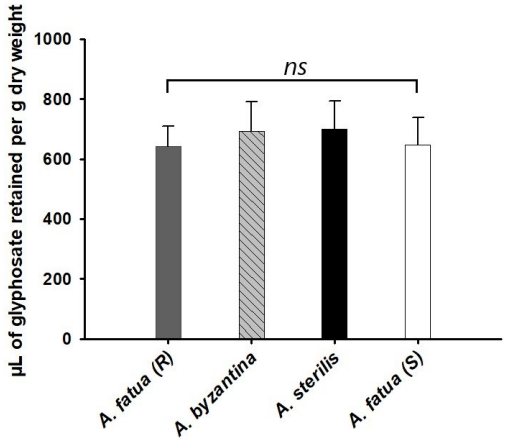

Figure 3. Spray retention of a glyphosate solution on leaves of *Avena* plants. Vertical bars are the standard error of the mean (n = 12); ns = non-significant.

3.4. Shikimic Acid Accumulation

Shikimic acid accumulation increased as glyphosate concentration increased and differed among *Avena* accessions. *Avena fatua* (R) and *A. byzantina* exhibited similarly low levels of shikimate (20–31.5 and 18.5–36.3 µg shikimic acid, respectively). *Avena sterilis* showed intermediate levels (70.8), whereas the *A. fatua* (S) accession accumulated the highest amounts of shikimate (161.4) (Figure 4).

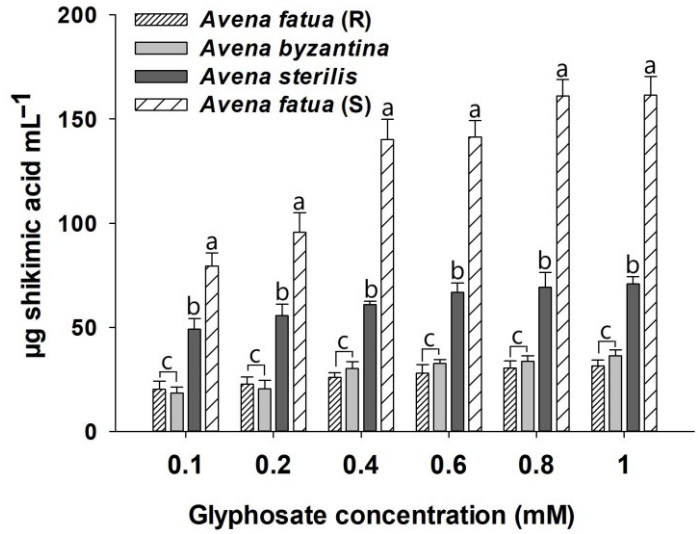

Figure 4. Shikimic acid accumulation in leaves of the four *Avena* accession. Vertical bars are the standard errors of the means (n = 6). Different letters correspond to different groupings according to Tukey's HSD test ($p < 0.05$).

3.5. ^{14}C-Glyphosate Absorption, Mobilization and Visualization

Different patterns of absorption and mobilization of ^{14}C-glyphosate were observed at both evaluation times (48 and 96 HAT) (Figure 5). *Avena fatua* (S) absorbed ~60% of applied ^{14}C-herbicide at 48 HAT, whereas the remaining *Avena* accessions absorbed less than 28%. At 96 HAT, ^{14}C-glyphosate absorption increased between 20 and 30% in all biotypes. In addition, the amount of herbicide moved from the treated leaf to the shoots and roots was higher in *A. fatua* (S) and A. byzantine, while *A. sterilis* and *A. fatua* (R) kept most of ^{14}C-herbicide restricted to the treated leaf, i.e., low amounts of ^{14}C-glyphosate were moved to shoots and roots in these accessions (Figure 5). The qualitative results obtained in the autoradiographs corroborated these patterns of ^{14}C-glyphosate mobilization within *Avena* plants of different accession (Figure 6).

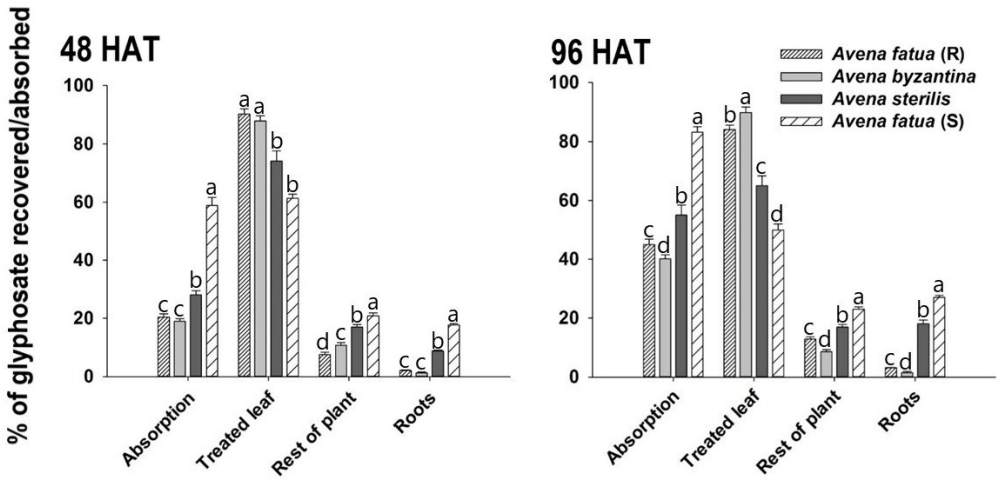

Figure 5. Percentage of glyphosate absorption (%recovered) and movement of glyphosate in plant (% of absorbed) of *Avena* species. Different letters correspond to different groupings according to Tukey´s test (0.05).

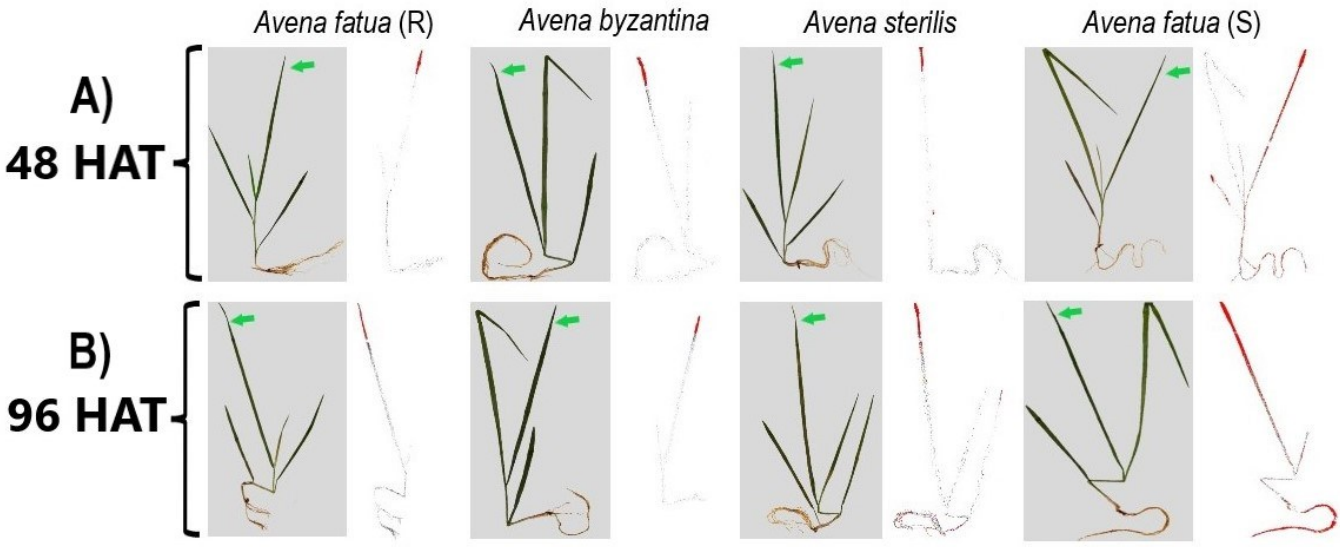

Figure 6. Digital visualization of ^{14}C-glyphosate movement in plants of *Avena* species. (**A**) 48 and (**B**) 96 h after treatment. Green arrow indicates the treated leaf.

3.6. Metabolism Study

Presence of glyphosate metabolites was confirmed in plants of the four accessions. In plants of *A. fatua* (R) and *A. sterilis,* the extent of metabolization of glyphosate to AMPA and sarcosine at 96 HAT was much higher (35–45% of herbicide was metabolized). This resulted in higher percentages (~90%) of unmetabolized glyphosate in *A. fatua* (S) and *A. byzantina* plants (Figure 7).

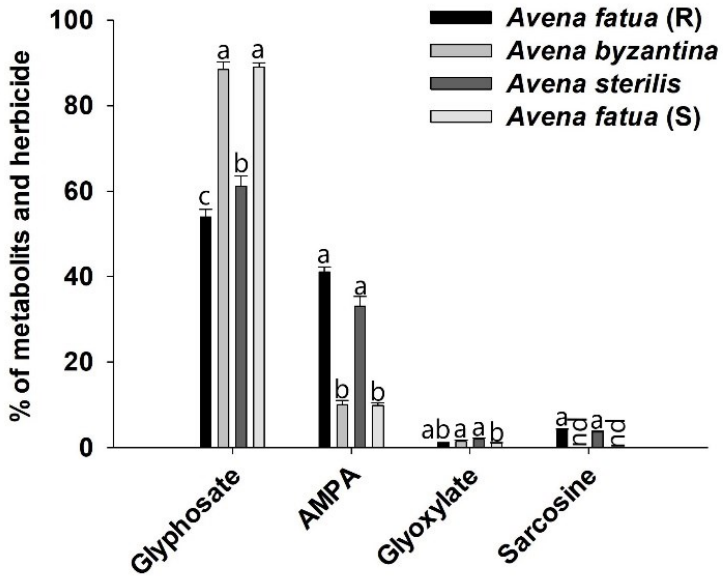

Figure 7. Glyphosate metabolism given as a percentage of total glyphosate and the metabolites in five *Avena* spp. treated with 360 g ae ha^{-1} and harvested 96 HAT. Nd = non detected. Different letters correspond to Tukey's test and mean comparison.

3.7. Activity of Glyphosate Target Enzyme

The basal activity of EPSPS (in the absence of glyphosate) was similar among *Avena* populations/species, ranging from 0.048, for *A. fatua* (S), to 0.055 μmol μg^{-1} protein min^{-1},

for *A. byzantina* (Figure 8A). In addition, the EPSPS enzyme activity was inhibited by glyphosate in a similar proportion among populations (Figure 8B). The I_{50} values ranged from 30.7 to 43.7 μM glyphosate. Although the lowest I_{50} was found for *A. sterilis*, to determine the Ris the I_{50}, *A. fatua* (S) was used; thus the Ris varied from 0.8 to 1.2 (Table 3).

Table 3. Parameter values and resistance index of the log-logistic function used to model enzyme activity (EPSPS) as a function of glyphosate concentration in the four *Avena* accessions.

Accession	b	d	I_{50}	95% CI		Ri [a]
				Lower	Upper	
A. fatua (R)	0.9	96.7	43.7	34.0	53.4	1.19
A. byzantina	0.6	99.9	37.7	26.3	49.0	1.03
A. sterilis	0.8	100.2	30.7	26.8	44.1	0.84
A. fatua (S)	0.8	94.5	36.5	23.7	37.7	-

[a] Resistance index (I_{50} *Avena* spp./I_{50} *A. fatua* (S)).

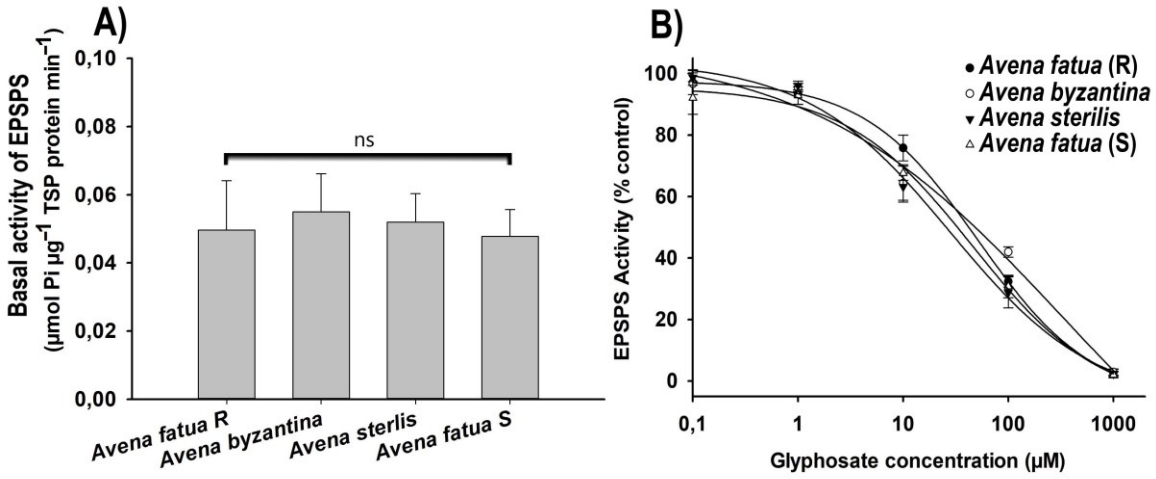

Figure 8. (**A**) Basal activity of 5-enolpyruvilshikimato-3-phosphate synthase (EPSPS) in the absence of herbicide of four *Avena* accessions. (**B**) Glyphosate concentration necessary to inhibit EPSPS activity at 50%. (R) = resistant; (S) = susceptible; ns = non-significant. Bars in plots represent the standard error of the mean (n = 6).

4. Discussion

4.1. Glyphosate-Resistance Confirmation

The four *Avena* accessions characterized in this study showed different levels of susceptibility to glyphosate, as follows (from lowest to highest): *A. fatua* (R) > *A. byzantina* > *A. sterilis* > *A. fatua* (S). The differences between the two *A. fatua* accessions demonstrated an evolution towards a low-level resistance in the R one, while what happened between the three *Avena* species can be explained in terms of inherent differential susceptibility. It is important to consider herbicide tolerance for these cases, because it is necessary to increase the glyphosate doses to kill 50% of the population. On the other hand, it is also true that some studies demonstrate that natural tolerance to glyphosate can evolve into resistance in few years if the required selection pressure is exerted on field populations [21,22]. Differences between other *Avena* spp. accessions were found in Australia; however, resistance mechanisms were not characterized [3]. Furthermore, the levels of resistance to glyphosate in *A. fatua* (R), 789 g ae ha^{-1} and 561 g ae ha^{-1} (for LD_{50} and GR_{50}, respectively), were higher than those reported in Australia (556 g ae ha^{-1} and g ae ha^{-1}), representing a 42% and 60% increase of LD_{50} and GR_{50}, respectively. In addition, the low

glyphosate susceptibility level found in *A. byzantina* and *A. sterilis* suggests that both species have a certain level of natural tolerance to the herbicide, which could evolve into resistance in coming years, as found in previous works [3,8].

EPSPS is the target enzyme of glyphosate that blocks the shikimate pathway [7]; therefore, the presence of glyphosate causes an increase of shikimic acid. The low shikimic acid contents reveal no or low interaction between enzyme/herbicide, i.e., the shikimate pathway is not affected and vice versa. This parameter is used as an early indicator of interaction of glyphosate with its target enzyme EPSPS [23,24]. Here, based on shikimic acid accumulation (at 48 and 96 HAT), we characterized one accession susceptible (*A. fatua* (S)) and one resistant (*A. fatua* (R)), as well as two species with low glyphosate susceptibility (*A. byzantina* and *A. sterilis*). Even if the separation of resistant and susceptible accessions was performed, this result cannot by itself define the mechanisms involved in the response of our *Avena* spp. [25].

4.2. Unraveling Resistance Mechanisms to Glyphosate

For glyphosate to be toxic in plants, it must be present in the cytoplasm and in the growing apical meristems, so mechanisms that reduce the amount of glyphosate to a sublethal level are likely to confer resistance [26]. A lower foliar retention of glyphosate solution is the first event that affects the herbicide effectiveness, because it can reduce the amount that is subsequently absorbed by the plant [27,28]. Here, we provide evidence of no differential foliar retention of glyphosate solution among the *Avena* spp. tested, possibly due to the similarity of the cuticle of the leaves between species/accessions [8,29].

The limited absorption and impaired translocation of herbicide may be involved in different susceptibility of glyphosate in plants [30]. These mechanisms have been identified as responsible for glyphosate resistance in several grass weeds [17,27,31], as well as natural tolerance in different plant species (i.e., Ribeiro et al. [32]). The limited uptake could be due to poor penetration of glyphosate through plant cuticles [29]. In our work, we can separate the tested populations into two groups: the first one corresponds to *A. fatua* (R), *A. byzantina* and *A. sterilis*, and the second group to *A. fatua* (S), which absorbed more glyphosate. The impaired translocation generally prevents the movement of glyphosate within the plant from the treated leaves towards growing apical meristems, as corroborated in digital visualization of plants treated with ^{14}C-glyphosate, showing a small amount of herbicide translocated from 48 to 96 HAT (Figure 6).

An interesting discovery in our work was the ability of *A. fatua* (R) and *A. sterilis* to detoxify glyphosate. Previously, *A. sterilis* from Andalusia was also able to support and metabolize sublethal doses of glyphosate [8]. Our results show that this characteristic is being distributed throughout the Iberian Peninsula in other populations/species of *Avena.* In addition, this is the first work with evidence of enhanced metabolism contributing to glyphosate resistance in *A. fatua*. Different enzyme(s) have been characterized by metabolizing glyphosate: glyphosate oxidoreductase (GOX), aldo-keto reductase (AKR) and Gly oxidase in microbes and plants, both broadleaf and grass weeds [24,26,33–35]. Our results confirm that *A. fatua* (R) and *A. sterilis* can degrade glyphosate into AMPA, glyoxylate and sarcosine; nevertheless, further studies need to be carried out to identify which enzymes could be involved in the detoxification.

Resistance based on target-site alterations resulting in reduced sensitivity to glyphosate could be a point mutation or EPSPS overexpression [36]. We discarded the presence of target-site resistance mechanisms, based on both the basal enzyme activity and the inhibition rate of EPSPS with glyphosate, which were both similar among the four *Avena* spp. accessions. Previous studies have shown that when TSR mechanisms to glyphosate are not present, differences in basal enzyme activity or I_{50} are not expected, both in broadleaf grass weeds [37,38]; however, further studies are necessary to confirm this in our accessions.

5. Conclusions

The results of this work demonstrate different NTS resistance mechanisms governing *Avena* species. First, the low susceptibility in *A. fatua* (R) and *A. sterilis* is due to a detoxification of glyphosate to AMPA, glyoxylate and sarcosine. In addition, reduced uptake and mobilization were confirmed in these two accessions. The different responses between *A. fatua* (S) and (R) accessions confirm the first case of glyphosate resistance evolution of this species occurring in Spain and Europe. Regarding *A. byzantina* accession, this work confirms that the limited uptake and low mobilization endow it low susceptibility to glyphosate. In short, chemical fallow and glyphosate in pre-seeding is an important tool in arable crops for weed control; however, if they are not properly managed, weed accession with high resistance to glyphosate or more modes of action could evolve.

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