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Increased Activity of 5-Enolpyruvylshikimate-3-phosphate Synthase (EPSPS) Enzyme Describe the Natural Tolerance of *Vulpia myuros* to Glyphosate in Comparison with *Apera spica-venti*

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Abstract: Rattail fescue (*Vulpia myuros* (L.) C.C. Gmel.) is a self-pollinating winter annual grassy weed of winter annual crops. The problems with *V. myuros* are mostly associated with no-till cropping systems where glyphosate application before sowing or emergence of the crop is the most important control measure. Ineffective *V. myuros* control has been reported following glyphosate applications. Experiments were performed to study the effectiveness of glyphosate on *V. myuros*, and determine the causes of the lower performance of glyphosate on *V. myuros* compared to other grass weeds. Estimated GR_{50} values demonstrated that *V. myuros* was less susceptible to glyphosate than *Apera spica-venti* regardless of the growth stage. Within each species, glyphosate efficacy at different growth stages was closely related to spray retention. However, the low susceptibility to glyphosate in *V. myuros* was not caused by lower retention as previously suggested. A significantly lower shikimic acid accumulation in *V. myuros* compared to *A. spica-venti* was associated with a higher activity of the EPSPS enzyme in *V. myuros*. Nevertheless, the relative responses in EPSPS activity to different glyphosate concentrations were similar in the two grass species, which indicate that EPSPS from *V. myuros* is as susceptible to glyphosate as EPSPS from *A. spica-venti* suggesting no alternation in the binding site of EPSPS. The results from the current study indicate that *V. myuros* is less susceptible to glyphosate compared to *A. spica-venti*, and the low susceptibility of *V. myuros* is caused by an increased EPSPS enzyme activity.

Keywords: chemical control; spray retention; narrow leaves; tolerance

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1. Introduction

Rattail fescue (*Vulpia myuros* (L.) C.C. Gmel.) is considered a problematic weed in Northern European countries [1]. Since first reported in Denmark in 1990, areas infested with *V. myuros* have significantly increased [2]. Initially, *V. myuros* was primarily found in grass seed crops, in particular red fescue; however, with increasing adoption of no-till practices and repeated cropping of winter cereals, *V. myuros* is now considered a common weed in winter cereal crops as well [3,4].

Vulpia myuros is a winter annual grass weed with a life cycle very similar to winter wheat [5]. *Vulpia myuros* is a prolific seed producer and can form a large seed-bank in a single season under poor weed management scenarios [6]. A recent study reported up to 50% winter wheat yield losses at a density of 405 *V. myuros* plants m^{-2} [7]. *V. myuros* has a shallow root system rendering it sensitive to soil disturbance. Furthermore, it has short seed-longevity in soil and persist longer in no-till cropping systems where seeds remain on the soil surface [8]. With the wide adoption of no-till practices, glyphosate has become a widely used herbicide for pre-plant and -emergence weed control [9]. However, *V. myuros*

control with glyphosate is erratic compared to other winter annual grasses [4]. The narrow and linear leaf blades of *V. myuros* constitute a relatively small target area, and that has been suggested as a reason for the poor post-emergence herbicides performance [10,11].

Glyphosate blocks the 5-enolpyruvylshikimate-3 phosphate synthase (EPSPS, EC 2.5.1.19) enzyme. The EPSPS is the sixth enzyme in the shikimic pathway, and essential in the synthesis of three essential aromatic amino acids: phenylalanine, tyrosine, and tryptophan [12]. The inhibition of EPSPS leads to accumulation of shikimic acid, which can be measured to determine glyphosate effectiveness on plants [13]. There are two types of glyphosate resistance reported, target-site and non-target site-based. Target-site resistance is endowed by mutations conferring alterations in the amino acids, which prevents glyphosate from binding the target enzyme [14], or by the amplification of target EPSPS genes [15]. Non–target site resistance is associated with limited uptake or translocation, enhanced glyphosate metabolism or increased vacuolar glyphosate sequestration [16]. There are no reports of resistance in *V. myuros* to glyphosate but tolerance to ACCase and ALS inhibitors is well-known [17]. According to the international weed science society of America [18] herbicide resistance "*is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type*"; and herbicide tolerance is "*the inherent ability of a species to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant*".

It is imperative to understand whether the lower efficacy of glyphosate against *V. myuros* is caused by a low spray deposition that could possibly be overcome by changing application parameters, such as spray volume or inclusion of adjuvants [4,10,19], or whether is it due to an inherent higher tolerance of *V. myuros* compared with other grass weeds. Moreover, previous studies suggested that *V. myuros* can tolerate the recommended rates of glyphosate better than other grass weed species, such as *A. spica-venti*, but there is no information on the level of tolerance in *V. myuros* against glyphosate [4,11,20]. Thus, the objective of the current study was to examine the level and cause(s) of the lower performance of glyphosate on *V. myuros*. *Apera spica-venti*, one of the most problematic grass weeds in winter wheat production systems in Europe [7], was included in the study as a susceptible reference species.

2. Material and Method

2.1. Seed Source

In the summer of 2017, *V. myuros* seeds were collected from non-agricultural areas at Flakkebjerg, Denmark (55.3° N, 11.4° E), where there was no known history of herbicide application. Seeds of susceptible *A. spica-venti* populations originating from six locations across Denmark were mixed to form a meta-population [21].

2.2. Dose-Response and Spray Retention Study

2.2.1. Dose-Response Study

Twenty seeds of *V. myuros* and *A. spica-venti* were planted in 1-L pots, filled with potting mixture containing soil, peat and sand (2:1:1 by weight). Pots were placed in an unheated glasshouse or on outdoor tables depending on the season in which the experiment was conducted. Groups of pots were sown on different dates to ensure that different growth stages could be sprayed simultaneously. After seedling emergence, plant numbers per pot were thinned to a pre-set number (8 plants per pot). Plants were treated with glyphosate (Roundup Bio, 360 g/L, Monsanto Crop Sciences, Hellerup, Denmark) rates ranging from 11.3 to 720 g ha^{-1} using a spray cabinet equipped with a boom and two flat-fan nozzle (HARDI ISO F-110-02). The nozzles were operated at a pressure of three bars and velocity of 5.2 km h^{-1} delivering a spray volume of a 152 L/ha. The dose-response study was repeated three times, plants were sprayed at different plant growth stages in three repeats (Table 1). Experiment 1 was sprayed on 24-01-2019 at growth stages BBCH 11, BBCH 13 and BBCH 21, Experiment 2 was sprayed on 25-04-2019 at BBCH 22, BBCH 23 and BBCH 24, and Experiment 3 on 17-06-2019 at BBCH 22, BBCH 26 and BBCH 29. After spraying, the

pots were placed either in an unheated glasshouse (Experiments 1 and 2) or outdoors (Experiment 3). Each experiment was performed with three replications per treatment plus six untreated controls using a complete randomized design. Dry and fresh foliage weights were recorded 4 to 5 weeks after herbicide treatment.

Table 1. Set-up of the different experiments carried out to examine the level and cause(s) of the lower performance of glyphosate on *V. myuros*.

Study	Experiment	Treatment (Glyphosate Doses; Growth Stage (BBCH))	Environment
Dose-response	Experiment 1	Glyphosate rate ranging from 11.3 to 720 g ha^{-1}; BBCH 11, BBCH 13, BBCH 21	Unheated glasshouse
	Experiment 2	Glyphosate rate ranging from 11.3 to 720 g ha^{-1}; BBCH 22, BBCH 23, BBCH 24	Unheated glasshouse
	Experiment 3	Glyphosate rate ranging from 11.3 to 720 g ha^{-1}; BBCH 22, BBCH 26, BBCH 29	Outdoor under natural conditions
Spray retention	Experiment 1	Glyphosate rate at 90 g ha^{-1} in mixture with fluorescent dye at a concentration of 200 g ha^{-1}; BBCH 11, BBCH 13, BBCH 21	Unheated glasshouse, Laboratory
	Experiment 2	Glyphosate rate at 90 g ha^{-1} in mixture with fluorescent dye at a concentration of 200 g ha^{-1}; BBCH 11, BBCH 13, BBCH 21	Unheated glasshouse, Laboratory
	Experiment 3	Glyphosate rate at 90 g ha^{-1} in mixture with fluorescent dye at a concentration of 200 g ha^{-1}; BBCH 22, BBCH 23, BBCH 24	Outdoor under natural conditions, Laboratory
Whole plant shikimic acid accumulation		Glyphosate rate at 210 g ha^{-1} and 420 g ha^{-1}; BBCH 23	Laboratory
Accumulation of shikimic acid in excised leaves		Glyphosate rate ranging from 0 to 600 µM; BBCH 23 to BBCH 25	Laboratory
EPSPS enzyme sensitivity		Glyphosate rate ranging from 0 to 1000 µM; BBCH 23	Laboratory

2.2.2. Spray Retention Assay

Spray retention was measured at three different growth stages using similar experimental conditions as for the dose-response study. Spray retention study was repeated three times. As plants were sprayed at different plant growth stages and under different environment in three repeats, therefore, to make the interpretations of results straightforward terms Experiments 1–3 will be referred to as three runs of the study (Table 1). Two experiments (Experiments 1 and 2) were conducted on the plants grown in the unheated glasshouse and one (Experiment 3) on outdoor grown plants. Experiment 1 was performed as a part of dose-response Experiment 1, while Experiments 2 and 3 were performed as separate experiments. In Experiments 1 and 2, plants were sprayed at BBCH 11, BBCH 13, BBCH 21. In Experiment 3, plants were sprayed at BBCH 22, BBCH 23, BBCH 24. The spray solution consisted of 90 g ha^{-1} glyphosate in mixture with the fluorescent dye brilliant sulfoflavin at a concentration of 200 g ha^{-1}. Following spray application, five plants of each plant species were cut at soil level and washed in glass bottles containing 50 mL Milli Q water + 0.2% non-ionic surfactant (Contact, AgroDan, Brabrand, Denmark) and shaken well. A representative sample of the solution was taken for analysis. Within 12 h, post treatment the amount of dye in repetitive samples was measured using a luminescence spectrometer (Perkin Elmer model LS50B). The samples were excited at 420 nm and after excitation emission was measured at 518 nm. The actual amount of deposition on the plants was calculated from a standard curve showing the response of concentrations from 3 to 800 µg per liter of the dye. The equation for the standard curve was linear with R^2 of 0.99. Herbicide treated plant samples (sprayed with glyphosate without the fluorescent dye) and untreated plant samples (not sprayed but washed with demineralized water) were also included for comparison. The leaf area of plant samples was measured using a

Licor 3100 area meter and was used for the calculation of per unit tracer deposition. The experiment included 10 replicates per treatment.

2.3. Shikimic Acid Accumulation Assays

2.3.1. Whole Plant Shikimic Acid Accumulation

V. myuros and *A. spica-venti* plants at BBCH 23 were treated with glyphosate at the rate of 210 g ae ha^{-1} and 420 g ae ha^{-1} (Table 1). Application method was the same used in the dose-response experiment. Plant material was harvested 24, 48, 72, and 96 h after treatment, and stored in liquid nitrogen at −20 °C until further analysis. Accumulation of shikimic acid was measured using a method described by Singh and Shaner [22]. A total of 450 mL HCl (0.25 M) was added to 150 mg of plant tissue in a 2 mL Eppendorf tube. The plant material in liquid nitrogen was ground with glass beads using a FastPrep instrument (FastPrep® FP120, Thermo Savant, CA, USA). Samples were then gently vortexed and centrifuged at 10,606× *g* for fifteen minutes at 4 °C. Twenty µL of the supernatant was mixed with 0.5 mL of periodic acid (1%) and incubated at room temperature for 3 h. Then, 0.5 mL of 1 M NaOH and 0.3 mL of 0.1 M glycine were added by pipette to the solution. Spectrophotometric reading of 200 µL samples was performed at 380 nm using a 96 well plate (Epoch, BioTek, Winooski, VT, USA). Six replications were used for each glyphosate concentration and species. Plant morphology for *V. myuros* and *A. spica-venti* differs [5], which could affect spray deposition. Thus, to account for differences in spray deposition on the two species, herbicide spray deposition was determined at the same growth stage that was used to study shikimic acid accumulation, using the method described above. Results were presented as µmol of shikimate per µg glyphosate per g of fresh weight per cm^2.

2.3.2. Accumulation of Shikimic Acid in Excised Leaves

Six leaf segments of 5 cm length were harvested from the youngest fully expanded leaves of *V. myuros* and *A. spica-venti* plants at BBCH 23 to BBCH 25. Fifty Mg of fresh plant material was transferred into a 2 mL Eppendorf tube containing 1 mM NH_4 H_2PO_4 (pH 4.4). Glyphosate was added to the Eppendorf tubes at the range of 0, 0.1, 0.5, 2, 5, 10, 100, 200, 400, 500 and 600 µM (Table 1). Samples were incubated in a growth chamber for 24 h at 24/16 °C day/night with 16 h photoperiod with photosynthetic photon Flux density of 850 µmol m^{-2} s^{-1}. After 24 h of incubation, the Eppendorf tubes were kept at −20 °C until analysis.

Prior to analysis Eppendorf tubes were thawed at 60 °C for 30 min. Then 250 µL of 1.25 N HCl was added, and incubated at 60 °C for fifteen minutes. Thereafter, a 125 µL aliquot from each Eppendorf tube was transferred into a 2 mL Eppendorf tube, and mixed with 500 µL of periodic acid and sodium metaperiodate (0.25% *w*/*v*). Following the incubation at room temperature for 90-min, 500 µL of 0.6 N sodium hydroxide, and 0.22 M sodium sulfite were added to the reaction mixture. The absorbance of samples was measured using a spectrophotometer (Epoch, BioTek, Winooski, VT, USA) at 380 nm. A 96 well plate was used for analysis using 200 µL of each sample. The experiment was performed in three replicates per species and was repeated twice.

2.4. EPSPS Enzyme Sensitivity

V. myuros and *A. spica-venti* plants were established in 1-L pots in an unheated glasshouse. Five g of plant material was collected from the two youngest fully expanded leaves from *V. myuros* and *A. spica-venti* plants at the three-tillering stage (BBCH 23) (Table 1). EPSPS enzyme extraction was conducted using the method described by Sammons and Gaines [14]. Five g of plant material was grounded to a fine powder by pestle and chilled mortar using liquid nitrogen. The powdered plant material was transferred to tubes containing 100 mL cold extraction buffer (100 mM MOPS, 5 mM EDTA, 10% glycerol, 50 mM KCl, and 0.5 mM benzamidine), 70 µL of fresh β-mercaptoethanol, and 1% in polyvinylpolypyrrolidone. Tubes containing samples were continuously stirred and then centrifuged for 40 min (18,000× *g*) at 4 °C. Thereafter, supernatants were decanted into a

beaker through a cheesecloth. Ammonium sulfate ($(NH_4)_2SO_4$) was slowly added to the solution to obtain 45% (w/v) concentration by constant stirring for 30 min and centrifuging at 20,000× g and 4 °C for 30 min. The ammonium sulfate precipitation step was repeated using 80% (w/v) $(NH_4)_2SO_4$. The extract was precipitated with gentle stirring, and the precipitate was then collected with centrifugation (20,000× g, at 4 °C for 30 min). Pellets were dissolved in 3 mL of extraction buffer and dialyzed overnight in 2 L of dialysis buffer by 30 mm, 1000-MWC dialysis tubing at 4 °C on stir plate.

The activity of EPSPS from *V. myuros* and *A. spica-venti* plants was determined using the protocol described by Dayan et al. [13] with on EnzCheckQR phosphate assay Kit (Invitrogen, Carlsbad, CA, USA). The specific activity of EPSPS was determined in the absence and presence of glyphosate. To determine the inhibition of EPSPS activity, the following glyphosate concentrations were used: 0, 0.1, 10, 100, 1000 µM (Table 1). The assay buffer contained 1 mM of $MgCl_2$, 100 mM of MOPS, 10% glycerol, 2 mM sodium molybdate, and 200 mM of NaF. The assay was performed in three replicates for each species, and experiments were repeated twice. The enzyme activity was calculated to measure the phosphate amount (µmol) liberated per µg of total soluble protein (TSP) per minute.

2.5. Statistical Analysis

Glyphosate dose-response, and EPSPS enzyme activity data were analyzed using a log-logistic model [23].

$$Y = \frac{d - c}{1 + \exp[b(\log(t) - log(e))]} \quad (1)$$

where Y is the response that represents the percent fresh biomass of untreated control or EPSPS enzyme inhibition relative to the control treatment; and c and d are regression parameters representing the equation's lower and upper asymptotes, respectively. Parameter e is the glyphosate rate dose providing 50% reduction in fresh biomass (GR_{50}) or inhibition of EPSPS enzyme activity (I_{50}) (midway between the d, and c parameters), b denotes the slope of the curve around parameter e. The model was tested with lack of fit test ($p > 0.05$). If the lower parameter value (c) was equal to zero, the four-parameter equation was reduced to a three-parameter equation:

$$Y = \frac{d}{1 + \exp[b(\log(t) - log(e))]} \quad (2)$$

Non-linear regressions were performed using R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria), with drc package [24]. The species were compared in terms of the parameter e (GR_{50}) using post hoc t-tests. Tolerance index (TI) was calculated as *V. myuros*-to-*A. spica-venti* GR_{50} ratios to compare the responses from a population of *V. myuros* with a meta-population of *A. spica-venti.*

Two-way ANOVA was performed to test differences between *V. myuros* and *A. spica-venti*, and among studied growth stages with respect to spray retention and accumulation of shikimic acid. Means were compared by Tukey HSD test at $p < 0.05$. Assumption of normality and homogeneous variance were visually examined. Data analysis was performed using R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

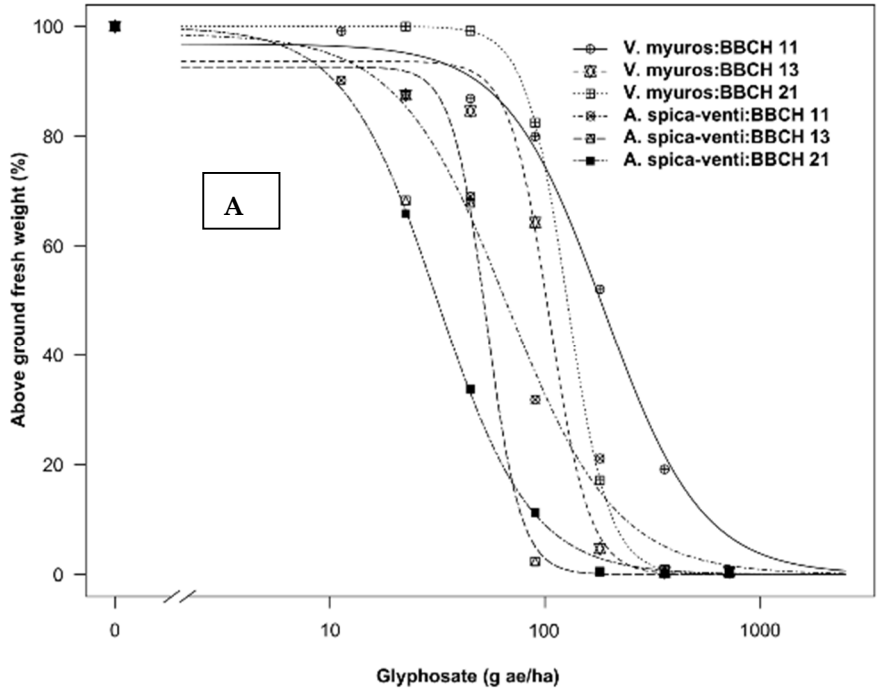
3. Results

3.1. Dose-Response and Spray Retention Study

3.1.1. Dose-Response Assay

The estimated GR_{50} values showed that *V. myuros* was less susceptible to glyphosate than *A. spica-venti* at all growth stages studied (Figure 1; Table 2). The tolerance index (TI), i.e., GR_{50} of *V. myuros* relative to GR_{50} of *A. spica-venti* indicated that *V. myuros* required 2.8, 2.0 and 4.0 times higher doses than *A. spica-venti* for a 50% reduction of biomass at BBCH 11, BBCH 13 and BBCH 21, respectively in Experiment 1. TI's were between 1.3 and 5.4 in Experiments 2 and 3 (Table 2). The TI was not influenced by the plant growth stage studied

but plant growth stage had effect on glyphosate effectiveness. For *V. myuros* the significant effect of plant growth stage on glyphosate activity was noticed only in Experiment 1, where the estimated GR_{50} value was 1.8 and 1.5 fold lower at BBCH 13 and BBCH 21, respectively compared to BBCH 11 (Table 2). Generally, glyphosate activity on *A. spica-venti* was higher at more advanced than at earlier growth stages in all three experiments (BBCH 13 and BBCH 21 than BBCH 11 in Experiment 1; BBCH 23 compared to BBCH 22 in Experiment 2; and BBCH 26 and BBCH 29 compared BBCH 23 in Experiment 3) (Table 2).



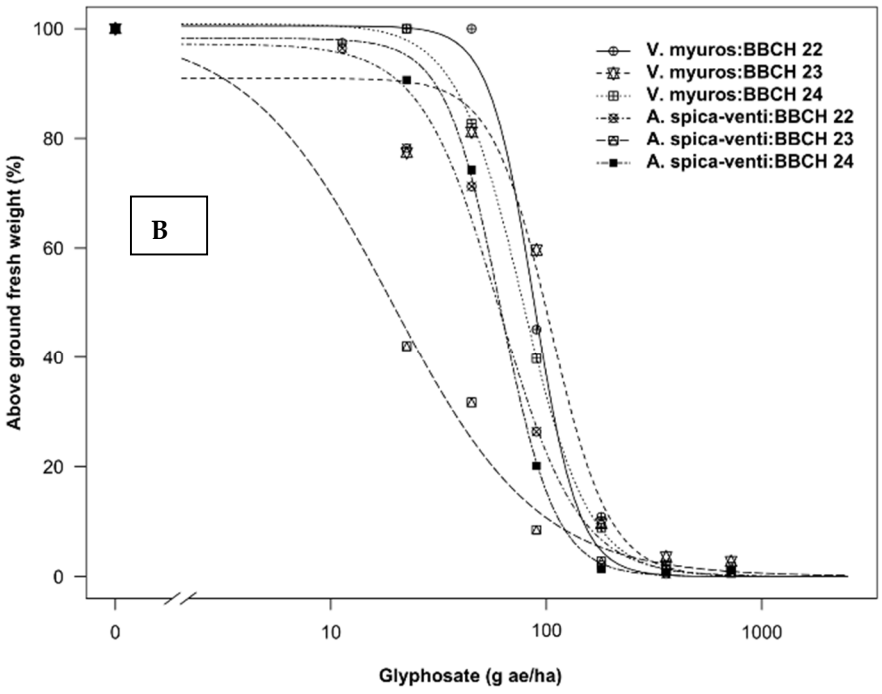


Figure 1. *Cont*.

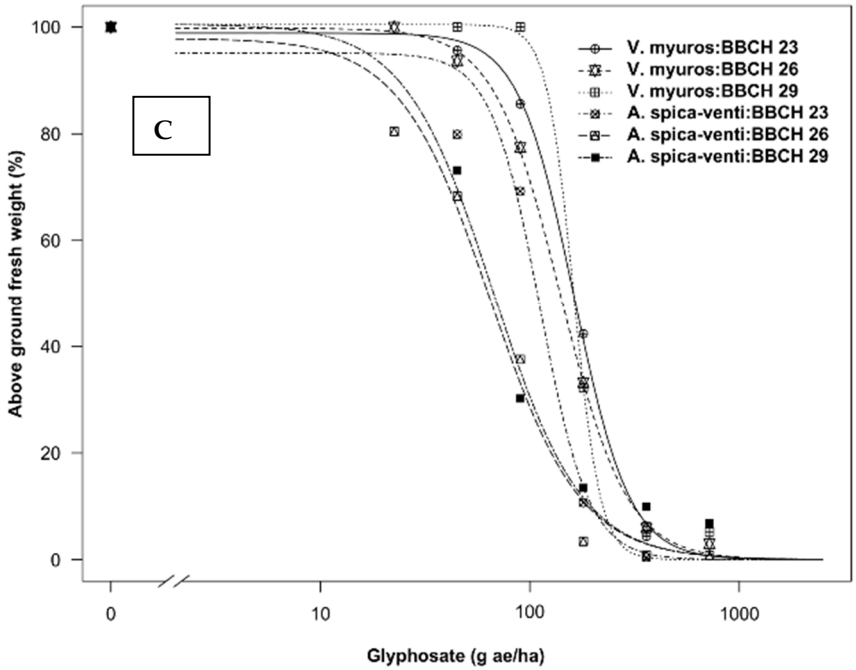


Figure 1. Glyphosate dose-response bio-assay on above-ground fresh weight represented as percentage of untreated control of *Vulpia myuros* and *Apera spica-venti* in Experiment 1 (**A**) Experiment 2 (**B**) and Experiment 3 (**C**).

Table 2. Glyphosate doses (g a.i. ha^{-1}) providing 50% reduction in fresh weight (GR_{50}) of *Vulpia myuros* and *Apera spica-venti* estimated using log-logistic Equations (1) or (2). Standard errors are presented in the parenthesis.

		Growth Stage		
	Species	**BBCH 11**	**BBCH 13**	**BBCH 21**
Experiment 1	*V. myuros*	188 (25.2)	105 (11.5)	127 (10.3)
	A. spica-venti	67 (10.0)	53 (6.2)	32 (3.9)
Tolerance indices	TI (p value)	2.8 (p = 0.002)	2.0 (p = 0.003)	4.0 (p < 0.001)
Growth stages	Species	BBCH 22	BBCH 23	BBCH 24
Experiment 2	*V. myuros*	88 (4.6)	105 (12.8)	78 (4.7)
	A. spica-venti	61 (5.6)	19 (5.8)	61 (3.5)
Tolerance indices	TI (p value)	1.4 (p = 0.006)	5.4 (p = 0.016)	1.3 (p = 0.015)
Growth stages	Species	BBCH 23	BBCH 26	BBCH 29
Experiment 3	*V. myuros*	163 (13.1) 1	140 (10.4) 2	165 (38.6) 3
	A. spica-venti	111 (8.9)	65 (6.5)	61 (2.3)
Tolerance indices [b]	TI (p value)	1.4 (p = 0.0099)	2.16 (p < 0.001)	2.72 (p = 0.011)

Values in the table are GR_{50}, representing the glyphosate required providing 50% reduction in fresh biomass. [b] Tolerance index was compared by t-tests at the 5% level of significance between *Vulpia myuros* and *Apera spica-venti*.

3.1.2. Spray Retention Assay

There was no treatment-by-experiment interaction between the two glasshouse experiments. Therefore, data from the glasshouse studies were pooled and presented together (Figure 2), while the results from the experiment conducted outdoors are presented separately. Spray deposition was significantly higher on *V. myuros* compared to *A. spica-venti* at all growth stages. For instance, in the glasshouse experiment spray deposition was

3.6, 6.4 and 5.5 times higher on *V. myuros* than on *A. spica-venti* at BBCH 11, BBCH 13 and BBCH 21, respectively. Under outdoor conditions, spray retention was 3.6, 2.3 and 2.0 times higher on *V. myuros* compared to *A. spica-venti* at BBCH 22, BBCH 23 and BBCH 24 stage, respectively.

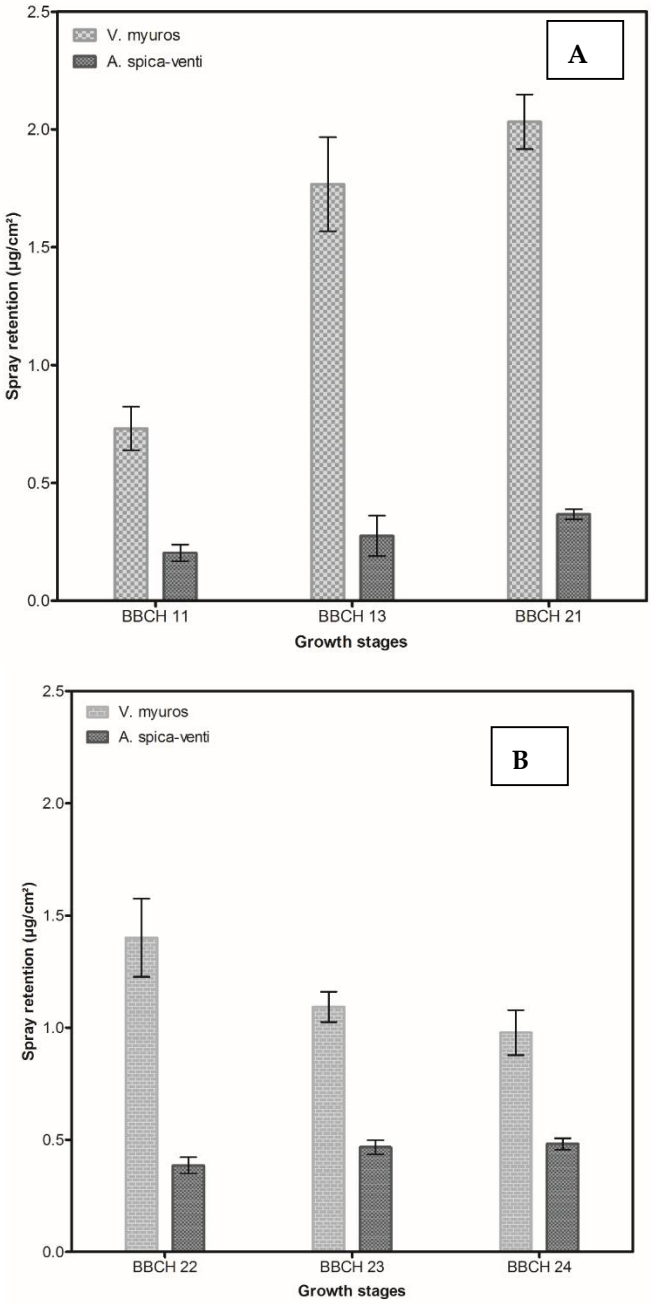


Figure 2. Retention of the glyphosate spray solution on *Vulpia myuros* and *Apera spica-venti* in glasshouse (**A**) and outdoor natural conditions (**B**). Data from two glasshouse experiments are pooled in figure (**A**).

Plant growth stage also had a significant effect on spray deposition. For instance, in the glasshouse study (Experiments 1 and 2) spray retention on *V. myuros* at BBCH 11 stage was significantly lower than at BBCH 13 and BBCH 21, which may explain the lower susceptibility at BBCH 11 compared to the later growth stages (Table 2). A similar trend was observed on *A. spica-venti*; however, significant differences were only detected between BBCH 11 and BBCH 21. In contrast, under outdoor conditions (Experiment 3) spray

retention on *V. myuros* tended to decline with increasing growth stages. For example, the spray retention was significantly higher at BBCH 22 stage than at BBCH 23 and BBCH 24, while no difference was observed between BBCH 23 and 24.

3.2. Shikimic Acid Accumulation Assays

In the whole plant assay, the shikimate concentration in plant tissue of *V. myuros* and *A. spica-venti* increased over time after glyphosate application. The increase in shikimate concentration was significantly higher in *A. spica-venti* than in *V. myuros*. At 96 h after glyphosate application, *A. spica-venti* accumulated approximately 3 and 5 times more shikimic acid than *V. myuros* following treatment with 210 and 420 g ha^{-1} (Figure 3).

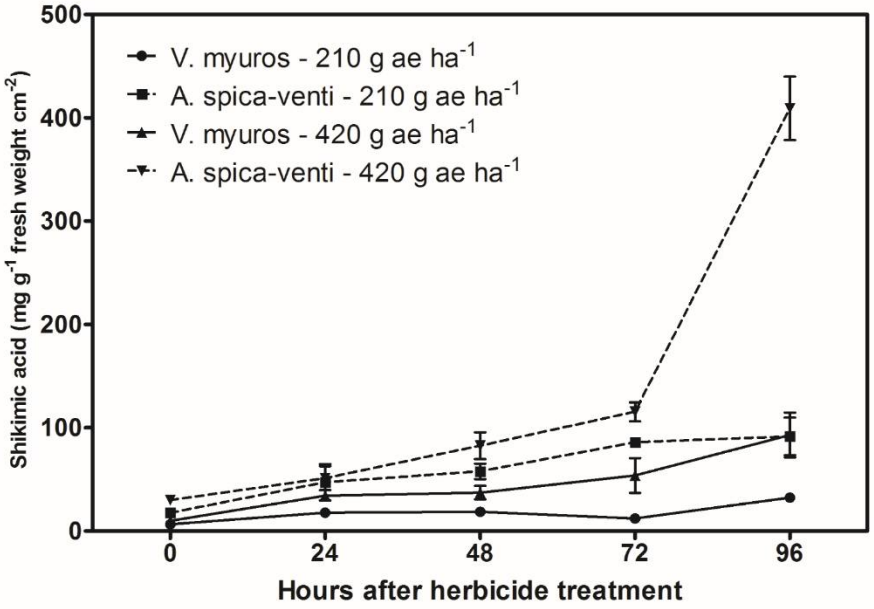


Figure 3. Accumulation of shikimic acid in leaves of *Vulpia myuros* and *Apera spica-venti* following treatment with glyphosate (210 and 410 g ae ha^{-1}). Vertical bars are presenting standard errors of the means.

Similar findings were observed in the assay using leaf segments (Figure 4). At low glyphosate concentrations (0.1 and 0.5 µmol), shikimate accumulation in *V. myuros* and *A. spica-venti* was similar but with increasing glyphosate concentration shikimate accumulation was higher in *A. spica-venti* than in *V. myuros*. Depending on glyphosate concentration, *V. myuros* accumulated 1.2–2.5 fold less shikimate than *A. spica-venti*. Findings from both the whole plant and leaf-segment shikimic acid bioassays were consistent and showed that the susceptible *A. spica-venti* accumulates more shikimic acid than the tolerant *V. myuros* when exposed to glyphosate.

3.3. EPSPS Enzyme Sensitivity

A log-logistic equation was fitted to the EPSPS enzyme activity data for *V. myuros*, and *A. spica-venti*, and regression parameters were estimated (Figure 5; Table 3). The regression parameter values for the slope (*b*), and lower asymptote (*c*) were not significantly different between the two species studied. In contrast, a comparison of the estimates for the upper asymptote (*d*), reflecting EPSPS enzyme activity in the absence of glyphosate (baseline activity) revealed a significant difference between the two species ($p < 0.001$). EPSPS baseline activity for *V. myuros* was 1.3 fold higher than for *A. spica-venti*. The glyphosate rates needed to inhibit EPSPS enzyme activity by 50% (I_{50}) were 264 ± 190 µM and 81 ± 75 µM for *V. myuros* and *A. spica-venti*, respectively ($p = 0.56$) (Table 3).

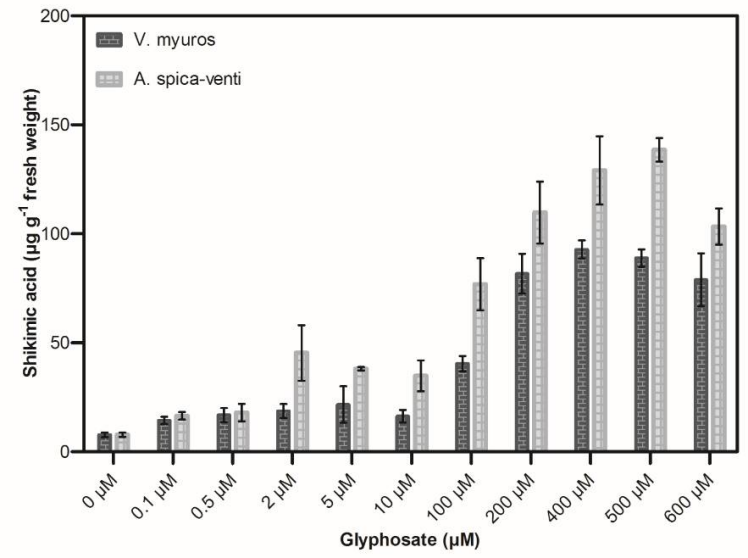


Figure 4. Shikimic acid accumulation in the leaf segment of plants from *Vulpia myuros* and *Apera spica-venti* following incubation with different concentrations of glyphosate. Vertical bars represent standard errors of the means.

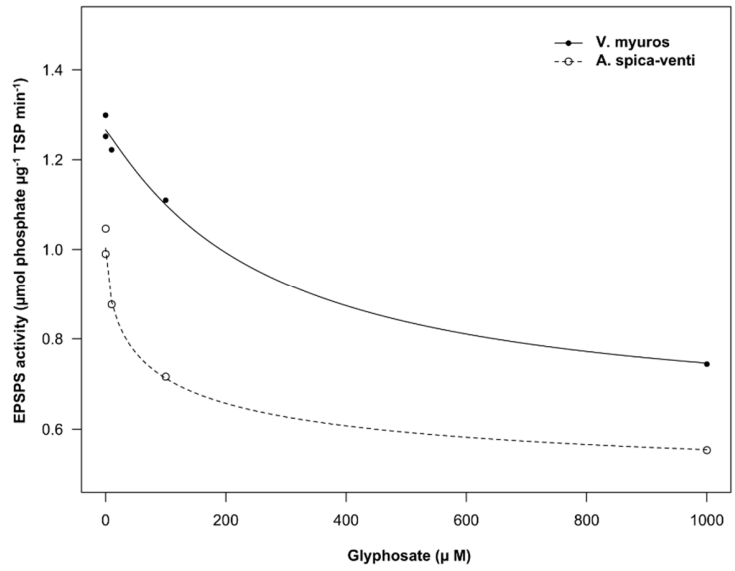


Figure 5. EPSPS activity of *Vulpia myuros* and *Apera spica-venti* exposed to different concentrations of glyphosate.

Table 3. Parameter estimates of log-logistic equations (1 or 2) applied to determine the sensitivity of EPSPS enzyme activity to glyphosate in leaf extracts of *Vulpia myuros* and *Apera spica-venti*. Standard errors are presented in the parenthesis.

	Regression Parameter Estimates [a]			
Species	*b*	*c* (µmol phosphate µg^{-1} TSP min^{-1})	*d* (µmol phosphate µg^{-1} TSP min^{-1})	I_{50} (µM)
Vulpia myuros	1.1 (0.51)	0.6 (0.18)	1.3 (0.01)	264.0 (191)
Apera spica-venti	0.6 (0.21)	0.4 (0.11)	1.0 (0.02)	81.1 (75.8)
Significance level [b]	$p = 0.3957$	$p = 0.444$	$p < 0.001$	$p = 0.3834$

[a] $Y = d - c/(1 + \exp[b(\log(t) - \log(I_{50}))])$. Y represent the EPSPS inhibition relative to the control treatment, *c* and *d* are regression parameters representing the equation's lower and upper asymptotes, respectively. I_{50} is the glyphosate required providing 50% inhibition of EPSPS enzyme. [b] Parameter estimates of non-linear regression (Equations (1) or (2)) were compared by t-tests at the 5% level of significance between *Vulpia myuros* and *Apera spica-venti*.

4. Discussion

V. myuros management strategies in no-till systems primarily relies on pre-sowing glyphosate applications [4]. Farmers have reported that glyphosate is only marginally effective in controlling *V. myuros*, in contrast to other grass weeds, such as *A. spica-venti*. Among scientists and advisors, it is often assumed that ineffective control of *V. myuros* with glyphosate can be attributed to low spray retention due to its narrow and erect leaves [4,10,11]. To elucidate the cause(s) of the higher tolerance of *V. myuros* to glyphosate in comparison to other common winter annual grass weed species, we conducted a series of studies on a *V. myuros* population that has never been exposed to glyphosate and compared the responses to a those of a meta-population of *A. spica-venti*.

4.1. Dose-Response and Spray Retention Study

4.1.1. Dose-Response Study

Results obtained in dose-response studies showed that the GR_{50} values for *V. myuros* were 2 to 3 times higher than for *A. spica-venti*, irrespective of growth stage (Figure S1). However, the actual doses needed to control the two grass weed species were lower than the recommended field doses, which illustrates that pot-grown grass weeds are generally more susceptible than plants in the field. Interestingly, *V. myuros* susceptibility to glyphosate was consistently lower than *A. spica-venti* in three experiments. Field studies have shown that glyphosate is not highly effective in controlling *V. myuros* and that the doses needed for satisfactory control are often higher than the recommended doses [20,25]. Yu et al. [17] studying the closely related species *V. bromoides*, proved its tolerance to ACCase and several ALS inhibitors and concluded that an insensitive ACCase and enhanced metabolism were the likely mechanisms of tolerance to the two modes of action. Hull et al. [3] confirmed that *V. myuros* was tolerant to the two herbicide groups. Here, we report for the first time that *V. myuros* is also tolerant to glyphosate.

4.1.2. Spray Retention Study

Previous studies have shown that variation in plant architecture and leaf characteristics can contribute to differences in herbicide deposition on treated plants [26,27]. In *V. myuros*, the presence of several pubescent veins on the leaf surface and rough leaf margins may result in a higher herbicide retention than on the light and hairless leaves of *A. spica-venti* [28,29]. Differences in the chemical composition and/or structure of the cuticle could also influence spray retention [30]. In contrast to many studies relating the poor performance of glyphosate on *V. myuros* to low spay retention [4,10,11,19], this study showed that reduced spray retention is not the cause of the low performance of glyphosate on *V. myuros*.

In the current study, no correlation was found between spray deposition and herbicide efficacy on the two grass weed species. In contrast, a close relationship was found between glyphosate activity and spray retention comparing growth stages of the same species. For instance, in Experiment 1, *V. myuros* control with glyphosate was low if sprayed at the early plant growth stage (BBCH 11) while control was higher when sprayed at later growth stages (BBCH 13 and BBCH 21), and a similar trend was observed for *A. spica-venti* in three experiments. Overall, our results are in line with those previously reported for *V. myuros*, and other grass weeds in the literature [11,31,32]. In contrast to dicot weeds, grass weeds, due to lower spray retention on the more erect leaves, are generally less susceptible to foliar applied herbicides at very early growth stages than at later growth stages [32]. Ball et al. [11] suggested that the erect leaf orientation of young *V. myuros* seedlings are limiting spray coverage on the leaf surface. The higher retention at later growth stages resulted in a better control than at earlier growth stages in Experiments 1 and 2 (Figure 2; Table 2). Similar to our findings, Koo et al. [33] also found a close relationship between foliar retention of pyribenzoxim and its activity on *Echinochloa crus-galli*.

It can be argued that dose-response and spray retention experiments were carried out at different growth stages and under different growing conditions, but it is important

to emphasize here that the primary aim of these experiments was to study the level and causes(s) of *V. myuros* tolerance to glyphosate. *V. myuros* susceptibility to glyphosate was lower than of *A. spica-venti*, and the reason of low susceptibility to glyphosate in *V. myuros* was not caused by lower retention, as previously suggested. Interestingly, these results were consistent across experiments that were carried out at different growth stages and growing conditions.

4.2. Shikimic Acid Accumulation Assays

Inhibition of the EPSPS enzyme is the mechanism of glyphosate action in plants, which results in the accumulation of shikimic acid in glyphosate sensitive weed plants [15,34]. Shikimate accumulation and EPSPS enzyme activity analysis are considered appropriate parameters to determine tolerance to glyphosate [13]. The findings from the whole plant and leaf-segment shikimic acid bioassays suggest that glyphosate incurs lower inhibition of EPSPS in *V. myuros* than in *A. spica-venti*. The somewhat smaller difference between the two plant species using leaf segments, compared to whole plants, is likely due to the fact that the exposure to glyphosate was the same in contrast to the whole plant assay, where more glyphosate was retained on the *V. myuros* than on the *A. spica-venti* plants. The presence of 200 µM or more of glyphosate did not significantly increase the accumulation of shikimic acid in *V. myuros*. In contrast, in the case of *A. spica-venti*, there was a significant increase in the accumulation of shikimic acid even at concentration of 500 µM. In glyphosate-resistant species, the lower accumulation of shikimic acid can be explained by either a functional feedback control mechanism of the shikimic pathway by the precursor that regulates the 3-deoxy-d-arabino-heptulosonate-7-phosphate (DAHP) synthase activity, and/or a lower interaction with the EPSPS enzyme by glyphosate [35]. The former mechanism prevents further accumulation of shikimic acid and limits the reaction rate early in the pathway, and this scenario can explain the results observed in the leaf-segment shikimic acid bioassays. In vitro methods measuring shikimic acid accumulation have been widely used for detecting glyphosate resistance in plants, where leaf segments are immersed in a glyphosate medium and then incubated [36]. This method has proven to be an effective and quick way to evaluate the differences among glyphosate-resistant and susceptible plants [37]. In our study, the whole plant shikimic acid assay also provided evidence that the interaction of glyphosate with EPSPS was lower in *V. myuros* than in *A. spica-venti.*

4.3. EPSPS Enzyme Sensitivity

Several studies reported high activity of EPSPS as a mechanism of glyphosate resistance [15,34,38]. The larger concentration of EPSPS enzyme in the total protein extracted from sample tissue as a whole/or per unit fresh weight limits glyphosate activity and prevents blocking of the shikimic pathway. Previous studies reported a strong relation between baseline EPSPS enzyme activity and gene copy number [34,39]. Higher baseline EPSPS activity could contribute to *V. myuros* tolerance to glyphosate. Although results from the current study have shown an association between glyphosate tolerance and higher EPSPS enzyme activity in *V. myuros*, it remains to be understood why the higher spray deposition on *V. myuros* did not overcome the effect of the difference in EPSPS activity.

Genome duplication might contribute to gene multiplication, which can lead to the evolution of genes with modified functions [34,40]. The presence of multiple genomes (polyploidy) can provide novel traits to plants [40,41]. For instance, Bunnell et al. [42] has reported that tetraploid individuals of bahiagrass (*Paspalum notatum*) were tolerant to metsulfuron while diploid individuals were susceptible. It is possible that a higher baseline EPSPS activity could be attributed to the higher genome size of the hexaploid *V. myuros* ($2n = 42$) with potentially multiple functional EPSPS alleles compared to the diploid *A. spica-venti* ($2n = 14$). Our study measured the EPSPS enzyme activity at several glyphosate doses in order to determine if a target-site mechanism may have caused the low sensitivity of *V. myuros* [34]. The I_{50} values for *A. spica-venti* were lower but not statistically different from *V. myuros* (Table 3), indicating that EPSPS from *V. myuros* is as susceptible

to glyphosate as EPSPS from *A. spica-venti* suggesting no alternation in the binding site of EPSPS [15]. According to Salas et al. [34] the amino acids present in the catalytic site of EPSPS are very conserved, hence, target-site mutation in plants from natural populations is very rare compared to other herbicide target sites. As there is no evidence of differences in susceptibility within populations of *V. myuros* to glyphosate (Kudsk, pers. comm.), it is very unlikely that any selection for higher EPSPS activity has occurred in natural populations of this species. This assumption is further supported by the fact that *V. myuros* is a new weed species that has been intensively exposed to glyphosate for only a few years. Hence, we suggest that the higher level of EPSPS activity is an intrinsic property of *V. myuros* that might be due to the higher ploidy level, and it is less likely that any resistance mechanisms, such as target-site mutation or reduced absorption, translocation, metabolism and vacuolar sequestration, is the cause of low glyphosate susceptibility in *V. myuros*.

5. Conclusions

The current study confirmed that *V. myuros* is more tolerant than other grass species to glyphosate as it is to other herbicide modes of action and it showed that the tolerance is not attributed to low spray retention as previously anticipated. The EPSPS activity in *V. myuros* was elevated compared to *A. spica-venti* and could explain the lower accumulation of shikimic acid in *V. myuros* and the observed difference in the susceptibility between the two grass weed species. Despite innate tolerance of *V. myuros* to glyphosate, the relative difference in the accumulation of shikimic acid and the estimated GR_{50} values between the two species suggest that *V. myuros* can be controlled by glyphosate but its susceptibility is lower compared to other grass weeds, such as *A. spica-venti*. Because of its tolerance to many selective graminicides, the chemical control of *V. myuros* largely depends on glyphosate. Overuse of glyphosate, due to a lack of other chemical options, may trigger the evolution of resistance to glyphosate in *V. myuros*. To avoid this, the use of glyphosate should be combined with other control strategies, such as a diversified crop rotation and cultivation where possible [42,43].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11080725/s1, Figure S1: Photographic representation of dose-response for the tolerant *Vulpia myuros* and susceptible *Apera spica-venti*.

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