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Altered Translocation Pattern as Potential Glyphosate Resistance Mechanism in Blackgrass (*Alopecurus myosuroides*) Populations from Lower Saxony

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Abstract: Glyphosate is a broad-spectrum herbicide widely used. After years of extensive usage, many weed species have developed resistance due to both target-site (TSR) and non-target-site resistance mechanisms (NTSRs). *Alopecurus myosuroides* is a competitive weed species. Greenhouse monitoring trials in Germany have revealed reduced glyphosate efficacy against some populations of *Alopecurus myosuroides*. In a foregoing dose–response study, individual plants from four out of six tested populations survived full (1800 g a.i. ha^{-1}) or double (3600 g a.i. ha^{-1}) glyphosate dose rates permitted, suggesting the presence of tolerant biotypes with yet unknown resistance mechanisms. Our aim was to investigate the absorption and translocation patterns of glyphosate in these biotypes. The plants were first treated with ^{14}C-glyphosate, and ^{14}C-glyphosate absorption and translocation were subsequently visualized by phosphorimaging and finally quantified by liquid scintillation counting. The results showed significant differences in the distribution of glyphosate in different plant organs, with significantly more being translocated out of the treated leaf in glyphosate-resistant compared to sensitive (S-) biotypes. The study's findings are partly in contrast to previous studies that have found reduced translocation. Our study demonstrates the complex nature of glyphosate resistance and suggests further experiments to finally elucidate the underlying resistance mechanisms in the biotypes of the *Alopecurus myosuroides* studied.

Keywords: absorption; ^{14}C-labeling; EPSPS; NTSR; phosphorimaging; TSR; weed

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1. Introduction

Glyphosate is a highly effective, non-selective broad-spectrum, systemically active herbicide [1]. It has become the most widely used herbicide worldwide since its introduction into the market in 1974 [1,2]. It is used throughout the world in crop areas for controlling weeds, preparing seed beds, desiccating cover crops, and facilitating harvests [3,4]. Furthermore, glyphosate is used worldwide in tree, nut, and vine cultivation besides various non-crop areas like railway grounds for weed control [1,3,5]. Since the creation and subsequent introduction of glyphosate-resistant crops into agriculture in various countries, glyphosate has been widely used in these regions as a selective in-crop herbicide for weed control [1,3,6,7].

After application and subsequent absorption into plants, glyphosate is translocated to the meristematic tissues via phloem transport [8,9], where it finally passes through the

plastid membrane into the plastids of cells [8]. There, it inhibits the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS). This translocation is essential for the efficacy of the herbicide [8,9], as the highest contents of EPSPS are found in plant meristems [9].

Glyphosate highly effectively inhibits EPSPS in the shikimate pathway. Normally, the EPSPS binds phosphoenolpyruvate (PEP) and combines it with shikimate-3-phosphate (S3P) to form 5-enolpyruvyl-shikimate-3-phosphate (EPSP). Due to the similar structure to PEP, glyphosate binds to the EPSPS and thus blocks the binding site, which is no longer available for PEP. As a result, the biosynthesis of the aromatic amino acids Tyr, Phe, and Trp in the shikimate pathway was inhibited and instead the intermediate shikimate accumulates in the treated plants [1,4,5]. The resulting metabolic disturbances cause first growth inhibition (hours to days after glyphosate application), followed by the yellowing of leaves several days later [10], before leading finally to plant death [5].

Following years of extensive glyphosate usage, the first naturally evolved resistance to glyphosate has been reported in a population of rigid ryegrass (*Lolium rigidum* Gaud.) in Australia [11]. Since then, the number of weed species developing resistance to glyphosate has steadily increased. Today, resistant populations of numerous different weed species according to the criteria defined by the Herbicide Resistance Action Committee (HRAC) [12] were documented [13].

As mechanisms, both target-site resistance (TSR) as well as non-target-site resistance (NTSR) to glyphosate were discovered so far. TSR mechanisms include mutations in the EPSPS gene, leading to amino acid substitutions in the EPSPS. In most of the reported cases linked to this mechanism, proline at position 106 in the EPSPS molecule is substituted by another amino acid (e.g., Ser, Ala, Thr, or Leu), which leads to a structural change in the glyphosate binding site. This decreases the affinity between the EPSPS and glyphosate [5,14,15]. Furthermore, additional mutations together with the Pro-106 mutation [16–19] or alone as a single different mutation (Thr-102-Ser) are known today [20].

Another TSR mechanism is an increased EPSPS gene duplication/amplification. In this case, the resistant plant contains more copies of the EPSPS gene compared with a susceptible plant, leading to an increased synthesis/higher expression of the EPSPS. As a result, higher concentrations of EPSPS are present than the recommended doses of glyphosate [5,14,15,21]. Today, two distinct mechanisms are known, which lead to this type of TSR: I) a tandem duplication mechanism and II) a large extrachromosomal circular DNA (eccDNA) that is tethered to the chromosomes and passed to gametes at meiosis [21].

Known NTSR resistance mechanisms include a lower foliar retention of glyphosate [22] and reduced glyphosate absorption through the leaf surfaces [22–26]. Differences in the leaf cuticle can contribute to the lower retention and absorption of the glyphosate spray solution [27]. Besides the amount or thickness of the cuticular wax mass [28] or their chemical properties [27], presumably, differences in the morphology of the leaf surface itself, such as different numbers of trichomes (hair-like structures with different functions), can also lead to lower absorption [28].

Another NTSR mechanism is the alteration of glyphosate translocation in the plant; most of the absorbed glyphosate is retained in the treated leaves, and consequently, less is translocated to the rest of the plant [29–32]. This mechanism correlates in many cases with the rapid sequestration of glyphosate into the vacuole of plant cells so that less glyphosate is available for translocation. Behind this vacuolar sequestration, an active transport process through the tonoplast is assumed, in which one or more tonoplast transporters—probably ABC-transporters—are involved [8,14,33].

Further NTSR mechanisms discovered to date include the following: (I) enhanced metabolic degradation of glyphosate through an increased synthesis of the enzyme aldo-keto reductase, which degrades glyphosate to aminomethylphosphonic acid and glyoxy-

late [34], (II) rapid necrosis of glyphosate-treated mature leaf tissue, resulting in the reduced translocation of glyphosate to meristems which continue growth [35,36], and (III) extrusion of glyphosate from the cytoplasm into the extracellular space, likely by an up-regulated plasma membrane-localized transporter [37].

In addition, there is evidence of still unknown resistance mechanisms [35,36,38–44]. All glyphosate resistance mechanisms can occur alone or in combination within populations or individuals [14,44].

Glyphosate-resistant weed populations have not been a problem in Germany so far. However, two recent studies from Rhineland-Palatinate/Germany show that (I) a population of the weed species perennial ryegrass [*Lolium perenne* (L.)] can no longer be controlled with the recommended glyphosate field rate in Germany [45] and (II) a possible resistance development to glyphosate occurred in a population of annual fescue [*Vulpia myuros* (L.)], respectively [46,47].

Since 2015, regularly conducted greenhouse monitoring trials on herbicide resistance occurrence and their potential spread in Lower Saxony/Germany revealed a reduced glyphosate efficacy against some populations of Blackgrass [*Alopecurus myosuroides* (Huds.)]. *Alopecurus myosuroides* is probably the most important herbicide-resistant weed in Germany [48] and across Europe [49,50]. It is a competitive weed species that can cause high yield losses if not adequately controlled [49]. Individual plants in recent bioassays occasionally survived the recommended field rate of 1800 g glyphosate ha^{-1} (g a.i. ha^{-1}) or even higher rates. Among survived plants, neither Pro-106 mutations in the EPSPS gene nor EPSPS gene duplication were detected [51]. In various countries, mainly in Europe, to date *Alopecurus myosuroides* evolved resistance to herbicides with six modes of action [13], according to the criteria established by HRAC, among them also against glyphosate with the mode of action of EPSPS inhibition HRAC Group 9.

In 2019, the authors conducted a dose–response study with six *Alopecurus myosuroides* populations from Lower Saxony, for which a reduced glyphosate sensitivity is suspected [52]. No statistically significant reduction in sensitivity in any of the tested populations compared to a sensitive (S) reference population could be detected. However, some individual plants from four out of six tested populations survived the full (1800 g a.i. ha^{-1}) or double (3600 g a.i. ha^{-1}) glyphosate dose rate permitted in Germany without any visible damage and continued to grow vitally. These survivors are assumed as tolerant individuals with an unknown resistance mechanism. The aim of this study was to investigate the absorption and translocation patterns of glyphosate in these selected individuals to confirm or disprove these possible, known NTSR mechanisms. Due to the controversial nature of the topic, an independent public institution was entrusted with the investigation.

These observed survivors are explicitly not plants that grew after glyphosate application—the emergence of additional plants in the pots of the dose–response study was regularly controlled, and newly emerging plants were removed. The survivors examined in the present study must therefore be postulated as tolerant biotypes with a yet unknown resistance mechanism. As these are therefore only (initial) individual plants (further called *individuals*), no plant material was or is currently available for further scientific investigations. However, we believe that the economic relevance and scope of these observations require early publication, as follows:

(I) Should *Alopecurus myosuroides* develop widespread tolerance to this broad-spectrum herbicide (including in Germany), this would have massive economic consequences—as a further challenge among the challenges in agriculture, such as climate change.

(II) The scientific understanding of the mechanism (s) is the first step and thus the basis for all subsequent reactions and decisions—from changing application recommendations to any necessary substitution of the herbicidal active ingredient. In the event of the next

indications of glyphosate resistance development in weed populations, the present study should help to classify this and to mitigate or prevent it through suitable measures in crop production.

For highly sensitive and spatially resolved characterization, radioactive ^{14}C-glyphosate was applied. First, "his translocation" was tracked non-destructively using phosphorimaging, and subsequently quantified by liquid scintillation counting (LSC) after the combustion with an oxidizer of all before separated plant compartments.

2. Materials and Methods

2.1. Plant Material

Plants that survived the dose–response study mentioned above at 1800 (P11, P39, and A1.3) and 3600 g a.i. ha^{-1} (A1.2 and A2.2), as well as plants of a sensitive reference population each without (S-0) and with previous treatment at 225 g a.i. ha^{-1} (S-0125), were vegetatively propagated (cloned) to obtain enough identical plant material for the subsequent studies (Table 1). To differentiate between the variants of S, the population ID was extended by an additional number (e.g., S-0125 for 0.125 × full glyphosate dose rate permitted in Germany).

Table 1. Alopecurus myosuroides plants used for the examination of ^{14}C-glyphosate absorption and translocation with previously survived glyphosate dose and BBCH-stage at the time of propagation.

Plant Clones from Population	Survival at Previous Dose Rate (g a.i. ha^{-1})	Growth Stage [a]	BBCH Stage No.	Replications
S-0	0	Beginning of shooting—1 node stage	30–31	3
S-0125	225	9 or more tillers	29	3
P11	1800	9 or more tillers	29	2
P39	1800	9 or more tillers	29	3
A1.3	1800	7 tillers	27	3
A1.2	3600	9 or more tillers	29	2
A2.2	3600	Beginning of shooting—1 node stage	30–31	3

[a] According to the Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie (BBCH)-scale for weeds [53].

2.2. Propagation and Growing Conditions

First, plastic foil (low density polyethylene) and hereon irrigation fleece were laid out in plant trays. Plastic pots (polypropylene, 9 cm diameter) were filled with a mixture of three parts soil (strong loamy sand, pH 6.8, organic matter 1.9%, sterilized by steaming at 100 °C for 30 min) and one part perlite (pH 7, air pore volume 95%), subsequently placed in the plant trays.

When the selected precursor plants reached the 8 to 9 tillers stage (BBCH 28-29), they were carefully removed from the pots (Jiffypot®, round, 250 mL volume, Jiffy Products International BV, Lindtsedijk 20a, Zwijndrecht, The Netherlands) in which they were previously cultivated and separated from soil by washing their roots under water. The tillers of each plant were then separated with a razor blade. All except two or three leaves on each tiller were cut off. Roots as well as the remaining leaves were cut back, and the separated tillers were planted one per pot in the prepared plastic pots. The plants developed in a greenhouse (mid-April to mid-May). The moisture level was checked daily

and subirrigation was conducted as needed. Twice (21 and 30 days after propagation), a nutrient solution Hakaphos® Red ((8% N, 12% P_2O_5, 24% K_2O, 4% MgO, 0.01% B, 0.02% Cu, 0.075% Fe, 0.05% Mn, 0.001% Mo, 0.15% Zn; 1 g/L), COMPO EXPERT GmbH, Krögerweg 10, Münster, Germany) was added.

2.3. ^{14}C-glyphosate Treatment

As (radiolabeled) treatment solution, ^{14}C-glyphosate ([glycine-2-^{14}C], 99 % radiochemical purity, declared by calibration certificate 1.85 MBq in 500 µL water, American Radiolabeled Chemicals, 101 Arc Drive, Saint Louis, MO 63146, USA), together with 9.375 µL of a non-labeled, commercially available potassium salt formulation of glyphosate (Roundup® PowerFlex, 480 g a.i./l, MONSANTO Europe S.A./N.V, Haven 627 Scheldelaan 460, Antwerp, Belgium) and 300.625 µL distilled water, was prepared to reach the final concentration equivalent of 2898 g a.i. ha^{-1} in 200 L of water, which corresponds to the 1.61 x full glyphosate dose rate permitted in Germany.

Aliquots of this solution, containing 21.7 kBq each, were applied for the treatment of one selected leaf each per plant. Two to three clones each of the surviving plants were used (Table 1). After plants reached their 7 tillers to 1 node stage (BBCH 27-31, Table 1), the first one of the younger, fully expanded leaves of each plant was completely wrapped in aluminum foil. Subsequently, each plant was sprayed with non-radioactive glyphosate at 1800 g a.i. ha^{-1} using a fine sprayer (Hobby 05 Flex, GLORIA Haus- & Gartengeräte GmbH, Därmannsbusch 7, Witten, Germany) in a spiral sprayer movement from the plant center to outside. Thereby, three to four manual spray shots were applied depending on the individual plant size. After this first, cold herbicide treatment, sprayed plants were left for 30 min in a fume hood to dry. Subsequently, aluminum foils were removed. The second application onto the previously wrapped leaves took place with 10 µL of the above blended ^{14}C-glyphosate treatment solution, applied with a Hamilton syringe as 20-30 finest droplets to the adaxial surface. This special, different approach is necessary for reasons of radiation protection (prevention of inhalation during spraying) and for precise dosing of the labeled herbicide. In addition, an even application of the pure chemical on the wax surface of plants is not possible directly, only as a surfactant-containing formylation.

2.4. Harvest and Sample Preparation

Plants were harvested 240 and 264 h after treatment, respectively (HAT, Table 2). First, the treated leaf (TL) of each plant was removed at the ligula. The leaf blade was defined as a leaf, the leaf sheath as belonging to the shoot (pseudostem). The shoots of some grasses, such as *Alopecurus myosuroides*, consist of many nested leaf sheaths. The TL was immersed in a polypropylene tube filled with 10 mL distilled water and alternately 20-25 s vortexed, gently shaken and swung to remove adsorbed, i.e., non-absorbed ^{14}C-glyphosate from the leaf surface. This leaf wash solution was mixed with 10 mL scintillation cocktail (Ultima Gold XR, PerkinElmer Inc., 940 Winter St, Waltham, MA, USA), then measured using a liquid scintillation counter (LSC, Tri-Carb 3110 TR, PerkinElmer Inc., 940 Winter St, Waltham, MA, USA), freshly calibrated daily in the single dpm mode, with background correction by the IPA standard. The TL was then wrapped in a paper towel to dry.

The roots of each plant were carefully washed free of soil using successive water baths, and adherent perlite was mechanically removed by hand. Aliquots of the subsequently filtered root wash solutions were taken, mixed with 10 mL of scintillation cocktail and measured by LSC, too.

Above-ground plant organs were divided from the roots (ROs). The roots were blotted dry and wrapped in paper towels. Similarly, the (complete or subdivided) above-ground

plant organs were evenly spread for later imaging between paper layers. All such prepared plant samples were oven dried at 60 °C for 168 h in a horizontal position.

Table 2. Mean plant weight after oven drying, absorption, translocation (radioactivity outside of TL), and distribution (radioactivity in TL, UL, PS, and RO) of ^{14}C-glyphosate after application on glyphosate-susceptible and -tolerant *Alopecurus myosuroides* biotypes [a, b, c, d].

						^{14}C-glyphosate Distribution							
Plants (Clones) from Individuals	**HAT**	**Biomass Mean Plant Dry Weight**	**^{14}C-glyphosate Absorption**	**^{14}C-glyphosate Translocation**		**TL**		**UL**		**PS**		**RO**	
	h	**mg**	**% Applied**			**% Absorbed**							
sensitive S-0	264	401 ± 112	79 ± 5	17 ± 13	a	83 ± 13	a	3 ± 2	ad	14 ± 11	a	0.8 ± 0.4	a
sensitive S-0125	240	1283 ± 371	81 ± 9	28 ± 22	ab	73 ± 22	ab	5 ± 4	abd	17 ± 14	a	5.6 ± 6.1	ab
tolerant P11	240	699 ± 54	76 ± 1	27 ± 8	ab	73 ± 8	ab	4 ± 4	abd	16 ± 5	a	7.3 ± 8.6	ab
tolerant P39	240	1739 ± 131	80 ± 15	23 ± 7	ab	78 ± 7	ab	3 ± 0	abd	10 ± 7	a	8.7 ± 6.8	ab
tolerant A1.3	264	938 ± 158	77 ± 2	52 ± 32	bcd	48 ± 32	bc	11 ± 6	bcd	16 ± 10	a	26 ± 28	b
tolerant A1.2	264	1075 ± 60	76 ± 10	63 ± 15	cd	37 ± 15	c	18 ± 9	c	37 ± 1	b	8.3 ± 5.3	ab
tolerant A2.2	240	358 ± 40	77 ± 9	61 ± 8	cd	39 ± 8	c	8 ± 4	d	44 ± 9	b	9.2 ± 2.7	ab
p-value			0.9874	0.0418		0.0418		0.0344		0.0085		0.049	

[a] Abbreviations: HAT, hours after treatment; TL, treated leaf; UL, untreated leaves; PS, pseudostem; RO, roots. [b] Similar and different letters indicate no difference and significant difference, respectively, between mean values within each column according to Fisher's LSD tests at 5% level of probability. [c] Mean values ± standard deviation of the mean. [d] Radioactivity from root exudates has not been determined for each individual plant—on average for each plant, 8.5% can be assumed.

2.5. Phosphorimaging

Phosphorimaging was used to obtain an overall picture of the plants and to visualize the ^{14}C-glyphosate distribution in the plant. Our previously optimized, analogous to [54] two-step erasing process was conducted in order to reset the storage capacity and to reach a low blank of the phosphor imaging plates used (DÜRR NDT CR 35, 20 × 40 cm and 35 × 50 cm, DÜRR NDT GmbH & Co. KG, Höpfigheimer Straße 22, Bietigheim-Bissingen, Germany; Fujifilm BAS-MS 2040, 20 × 40 cm, FUJiFILM, Akasaka, Minato City, Tokyo, Japan), as follows: the imaging plates were first erased for 30 min under a high-energy white light (Erasing Unit of a Bio-Imager FUJI BAS 1000, FUJiFILM, Akasaka, Minato City, Tokyo, Japan) and immediately before starting exposure with the Bioimager CR35 Bio (Elysia-Raytest GmbH, Benzstraße 4, Straubenhardt, Germany).

To protect the plates against humidity, dust, and any contaminations, they were wrapped first into very thin cellophane foils. All parts of each plant were placed together onto the so protected imaging plate (s), evenly spread, covered successively by paper, soft foam, and an acrylic glass on the top to optimize the contact between plant parts and the imaging plate. The exposure time (in darkness) was previously optimized to 42 h. After exposure, all coverings including the plants themselves were removed, and the imaging plates were—still in the dark—immediately scanned with the Bioimager CR 35 Bio (before optimized: sensitive mode, 100 µm resolution), subsequently analyzed with the data analysis program Aida Image Analyzer v.5.0 and presented as rainbow color images (using the same optimized gain factor), exported to power point for publication (Elysia-Raytest GmbH, Benzstraße 4, Straubenhardt, Germany). The panel quality is routinely tested using a series of commercially available ^{14}C polymer references (ELYSIA-Raytest, Straubenhardt, Germany).

2.6. Absorption and Translocation

After phosphorimaging, above-ground plant organs were further divided in the untreated leaves (ULs) and the pseudostem region (PS), respectively. All plant parts of each plant were weighed out, divided into subsamples, and combusted in a biological oxidizer

(Hidex Oxidizer 600 OX, Hidex, Lemminkäisenkatu 62, Turku, Finland). Evolved $^{14}CO_2$ was immediately trapped in OxySolve C-400 cocktail (Zinsser Analytics, now Gardner Denver Thomas GmbH, Fürstenfeldbruck, Germany) and subsequently measured by LSC as well. All values were corrected for the daily parallel estimated instrument recovery rate (consistently proven over 95%.).

The proportion of ^{14}C-glyphosate absorbed/translocated was calculated with the following equation:

$$(TL + UL + PS + RO)/[(TL + UL + PS + RO) + \text{leaf wash solution}] \times 100$$

whereby (TL + UL + PS + RO) represents the sum of radioactivity in the plant parts. The ^{14}C-glyphosate in the root wash solutions was determined as several aliquots, and a calculated mean value was used for further calculations.

2.7. Statistical Analysis

Analysis of variance (one-way ANOVA) was conducted using GraphPad Prism 8.4.3 (GraphPad Software, 2365 Northside Dr. Suite 560, San Diego, CA 92108, USA) to test for significant differences between biotypes in ^{14}C-glyphosate absorption, translocation, and recovery. The model assumption of normal data distribution was inspected graphically (QQ-Plot) and with several normality tests (D'Agostino–Pearson omnibus K2 test, Anderson–Darling test, Shapiro–Wilk normality test). The model assumption of homogeneity of variance was inspected graphically (homoscedasticity plot) and with a Brown–Forsythe test. The data for absorption, translocation, and radioactivity levels within the treated leaf, untreated leaves, and the pseudostem were normally distributed ($p > 0.05$).

In contrast, the data for radioactivity levels within the roots and recovery of radioactivity were not normally distributed and therefore transformed (arcsine of the square root) prior to ANOVA to meet model assumptions. Means were separated using Fisher's LSD test at a 5% level of probability ($p = 0.05$).

3. Results

The ^{14}C-recovery in our study was on average 85.8% of applied radioactivity (aR) for all biotypes investigated. The remarkable difference of 14.2% to the originally applied ^{14}C may have two main reasons, as follows: (I) The plants have most likely excreted some of the ^{14}C-glyphosate via their roots. Subsequently, the ^{14}C-glyphosate is (partly) microbially degraded/mineralized—thereby partly incorporated back into microorganisms too—and partly converted to released $^{14}CO_2$ [55–58]; various studies have already shown that glyphosate is used by microorganisms in the soil as a carbon and phosphorus source [55,57,58]. We did not investigate this part further. (II) We filtrated the root washing solutions after harvest for LSC measurements. Therefore, we assume to lose adsorbed ^{14}C-glyphosate on solids, namely on soil, but especially on perlite, well known for its huge surface with numerous pores, which was included initially for ventilation purposes in the experimental conception.

No statistically significant differences in measured ^{14}C-glyphosate absorption patterns were found among the *Alopecurus myosuroides* biotypes tested (Table 2). This is consistent with several studies on other plant species—in many cases, no differences in glyphosate absorption between sensitive and resistant plants have been found [29,59–62].

The recorded images show clear differences in the ^{14}C-glyphosate translocation pattern between the biotypes studied (Figure 1). In all plants of biotypes S-0 (glyphosate-susceptible) and A1.3 (glyphosate-tolerant), the treated leaves (TLs) contain a very high radioactivity distribution over the entire leaf.

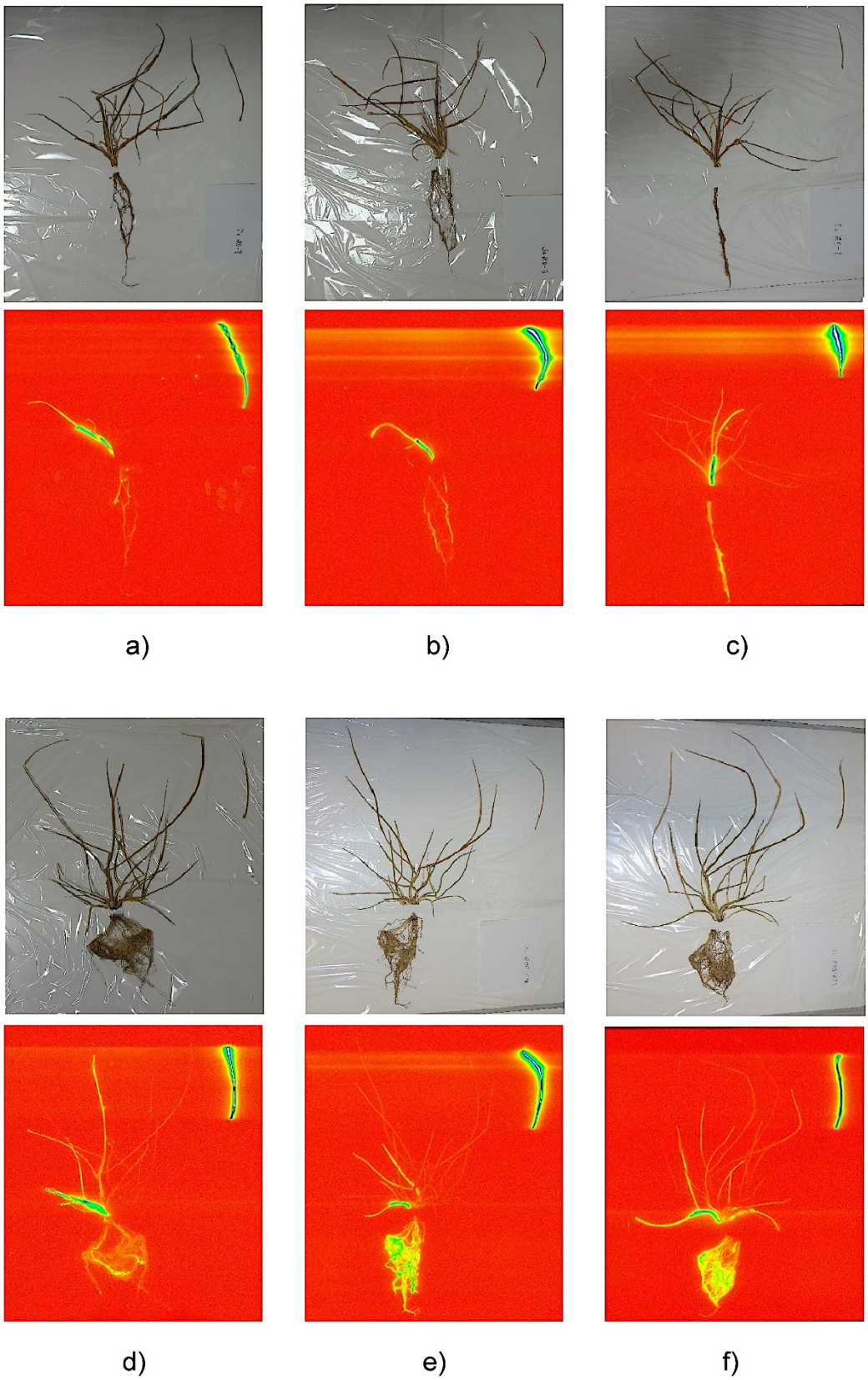

Figure 1. Plants with corresponding phosphor images of glyphosate-susceptible S-0 ((**a**–**c**), 264 h HAT) and -tolerant A1.3 ((**d**–**f**), 264 h HAT) *Alopecurus myosuroides* biotypes, respectively. Image plates are expressed in full size (350 mm × 500 mm); photographs are zoomed for better detail visualization. Example images between S and A1.3 showed large differences in the translocation pattern. Treated leaf is always illustrated in the top right. Red color indicates low activity and yellow, green, blue color indicates raising activities, respectively, white is overexpressed.

This is consistent with the measured high ^{14}C-glyphosate levels in the treated leaves of all biotypes, ranging from 36.8 to 82.9% of aR. S-0 (Figure 1a,b), which shows only a low degree of translocation of the absorbed ^{14}C-glyphosate into the above-ground plant organs. In both plants, only the pseudostem (PS) of the originally treated leaf is powerful visible in imaging. In Figure 1c, the remaining associated leaves are visible on the pseudostem. In Figure 1a,b, only a marginal ^{14}C-glyphosate translocation took place into certain parts of the roots. In contrast, in Figure 1c, an overall higher translocation into all plant organs is visible. In all plants of A1.3 in Figure 1d–f, a much higher ^{14}C-glyphosate translocation took place into almost all above-ground plant parts. Also, the roots are completely visible with varying degrees of radioactivity in all three plants. The differences visible on the exemplary phosphor images (Figure 1) confirm the differences in measured ^{14}C-glyphosate translocation patterns. In some glyphosate-resistant biotypes, a statistically significant difference in the ^{14}C-glyphosate translocation pattern from the treated leaf to the remaining plant parts, compared to the two S-biotypes, was observed (Table 2). In A1.3, A1.2, and A2.2, significantly more ^{14}C-glyphosate was translocated out of the treated leaf with 52.4, 63.2 and 61.0% of aR, respectively, compared to the sensitive biotype S-0 with 17.1% of aR. In A1.2 and A2.2, also significantly more glyphosate was translocated out of the treated leaf compared to the sensitive biotype S-0125 with 27.5% of aR.

Consequently, significant differences also occurred in the distribution of ^{14}C-glyphosate in the individual plant parts. In biotypes A1.3 and A1.2, 10.9% and 17.6% of aR, respectively, of the originally absorbed ^{14}C-glyphosate was translocated into the untreated leaves (ULs), approximately 3–5 times more than in S-0 with almost 2.8% of aR. Analogously, this was also shown in the comparison between A1.2 and S-0125 (4.6% of aR). The translocation of ^{14}C-glyphosate from TL to PS was more than 2 times higher for A1.2 and A2.2 with 37.3 and 43.7% of aR, respectively, compared to S-0 and S-0125 with only 13.5 and 17.3% of aR, respectively. The translocation from TL to RO was by far the highest in A1.3 compared to all other biotypes. In A1.3, with 25.9% of aR, almost 32 times as much was translocated into the RO than in S-0 with only 0.8% of aR. Furthermore, large differences between the S-0 and the other biotypes are also evident—despite the lack of statistical significance. In all biotypes, in contrast to S-0, a 7- to 30-fold higher amount of ^{14}C-glyphosate was transferred into the RO (Table 2).

The results of the comparison of ^{14}C-glyphosate translocation patterns between the *Alopecurus myosuroides* biotypes studied here are partly in contrast to the results of other studies [22,25,29–31,62–64]. In all our glyphosate-resistant biotypes A1.3, A1.2, and A2.2, significantly more ^{14}C-glyphosate was translocated out of the treated leaf into the remaining plant parts than in our two sensitive biotypes S-0 and S-0125. In the mentioned studies, only a reduced translocation of ^{14}C-glyphosate from the treated leaves to the remaining plant parts in resistant plants has always been observed so far. Examples are investigated in, e.g., hairy fleabane [*Conyza bonariensis* (L.) Cronquist] [63], Canadian horseweed [*Conyza canadensis* (L.) Cronquist] [64], Italian ryegrass [*Lolium multiflorum* Lam.] [31], perennial ryegrass [*Lolium perenne* L.] [29], rescuegrass [*Bromus catharticus* Vahl] [22], rigid ryegrass [*Lolium rigidum* Gaudin] [30], and waterhemp [*Amaranthus tuberculatus* (Moq.) Sauer] [25].

4. Discussion

To investigate glyphosate absorption and translocation patterns in survivors of prior glyphosate application, the methods used in our study differ from those used in other studies: the *Alopecurus myosuroides* plants studied were (I) obtained by vegetative propagation to obtain sufficient genetically identical plant material compared to obtaining plants from seeds, and, to show more distinct effects, (II) studied at a higher developmental stage, (III) treated with a higher glyphosate dose than the full recommended or permitted glyphosate

dose, and (IV) studied with a longer time interval between glyphosate application and the determination of ^{14}C-glyphosate absorption and translocation. Therefore, the comparability with other studies is limited.

The plants also differ macroscopically: A1.3 and A1.2 (938 and 1075 mg, respectively) had produced more than twice as much biomass in contrast to S-0 (401 mg) (Table 2). Consequently, A1.2 and A1.3 probably have also more meristematic tissue in, e.g., the roots (Figure 1) than S-0. Glyphosate is predominantly translocated to metabolic sinks, such as meristematic tissues [9,65], after uptake into the plants. The highest levels of EPSPS are found in the meristems [9]. Consequently, the more meristematic tissues there are in a plant, the higher the translocation of glyphosate into these tissues could be. But, in our experiment, A2.2 formed with 358 mg less biomass than S-0, and S-0125 formed with 1283 mg more biomass compared to A1.2 and A2.2. Therefore, the significant differences cannot be explained by the presence of larger amounts of meristematic tissue.

Regarding the level of ^{14}C-glyphosate translocation out of the treated leaf, it is striking that the translocation was higher in all the biotypes examined compared with S-0. The same applies to the ^{14}C-glyphosate translocation from the treated leaf into the roots. Even S-0125, which together with S-0 originates from the same population, showed a higher ^{14}C-glyphosate translocation. However, the differences between S-0125 and S-0 were not statistically significant. S-0 was the only biotype that was not treated with glyphosate before the experiments described in this study were conducted. This suggests that prior glyphosate exposure may have induced epigenetic changes in the biotypes tested. The different translocation patterns of ^{14}C-glyphosate that we observed may be caused by these assumed epigenetic changes. Epigenetic changes may have altered the regulation of certain genes that influence the transport and location of glyphosate in the plant. A contribution of stress-induced and partly also heritable (stress memory) epigenetic changes in the development of herbicide resistance in weeds is assumed. Here, a herbicide could induce a signal (a regulatory cascade) that triggers changes in gene expression and/or the activity of RNA-mediated DNA methylation. This could lead to an altered regulation of genes that ultimately cause herbicide resistance [43]. In winter wheat [*Triticum aestivum* L.], different glyphosate concentrations have led to epigenetic changes through an increase in DNA methylation [66]. In mouse ear cress [*Arabidopsis thaliana* (L.) Heynh.], even different sublethal doses of glyphosate led to epigenetic changes by altering the methylation patterns in this genome [67]. In the study of a glyphosate-resistant and a glyphosate-sensitive *C. canadensis* population, epigenetic changes due to different methylation patterns between the genomes of the two populations were found after glyphosate treatment [68].

An alternative explanation for the higher degree of ^{14}C-glyphosate translocation in the resistant *Alopecurus myosuroides* biotypes could be a targeted vacuolar sequestration of glyphosate after translocation into not with ^{14}C-glyphosate-treated tissues. This would limit the amount of glyphosate that reaches the chloroplasts within the cells and thus the site of EPSPS action in the ^{14}C-glyphosate-untreated tissues. Vacuole sequestration of glyphosate has almost always been observed only in leaves treated with glyphosate [8,14,69]. In *Conyza canadensis*, in addition to vacuole sequestration in glyphosate-treated leaves, vacuole sequestration was also observed in untreated tissues following glyphosate translocation [8,70].

The high accumulation of ^{14}C-glyphosate in the roots observed in A1.3 possibly indicates an increased sequestration of glyphosate into the soil via root exudates, too. Individual root exudations were not investigated in this study. If the increased exudation of glyphosate via the roots plays a role in A1.3 and/or in the other biotypes tested, then ^{14}C-glyphosate absorption via the treated leaf should consequently also have been higher in the biotypes concerned. In this case, the results of the investigation of ^{14}C-glyphosate

absorption would not correspond to reality. It is suspected that such a resistance mechanism against herbicides could be caused by overactivity or genetic overexpression of certain transporters like transporter proteins. This includes transporters that cause increased translocation of herbicides via the phloem into the roots beside transporters localized in the roots, which cause increased exudation of herbicides out of the roots. Such a resistance mechanism against glyphosate has not yet been observed in weeds. However, there are weed species such as leafy spurge [*Euphorbia esula* L.] [71] and *Lolium multiflorum* [72] that are known to secrete herbicide molecules into the rhizosphere after herbicide treatment [41]. There are also several species of weeds such as quinoa [*Chenopodium quinoa* Willd.] [73] and tall windmill grass [*Chloris elata* Desv.] [74] that even secrete glyphosate into the rhizosphere. Two other cases are also known, where the root exudation of herbicides has contributed to herbicide resistance [41]—resistance to imazamox in Mexican fire plant [*Euphorbia heterophylla* L.] [75] and resistance to MCPA (4-chloro-2-ethylphenoxyacetate) in wild radish [*Raphanus raphanistrum* L.] [76].

In addition to the hypothetical resistance mechanisms mentioned so far, further mechanisms could also play a role in the resistant biotypes. These include the (I) metabolic degradation of glyphosate by the increased synthesis of a glyphosate-degrading enzyme such as aldo-keto reductase [34]; (II) extrusion of glyphosate from the cellular cytoplasm into the extracellular space by, e.g., an over-regulated ABC-transporter in, e.g., the plasma membrane [37]; (III) mutations in the EPSPS gene, leading to amino acid substitutions in EPSPS at positions other than position 106 [20]; (IV) increased EPSPS gene amplification (gene duplication) [14,21].

It should be noted that no Pro-106 mutations in the EPSPS gene were found among the surviving plants. A possibly present EPSPS gene amplification in the biotypes was not investigated, as no amplification could be found in the *Alopecurus myosuroides* biotypes in a previous study [51].

The described resistance mechanism of rapid necrosis of glyphosate-treated mature leaf tissue, which results in reduced glyphosate translocation [35,36], can be excluded in the resistant biotypes. The results of our ^{14}C-glyphosate translocation study, combined with the response of the plants in the previous dose–response study [52], are not consistent with the symptoms of such a mechanism.

Suspected resistance mechanisms could result from genetic changes in the resistant *Alopecurus myosuroides* biotypes studied here or, alternatively, epigenetic changes in the genome could be the reason. The observed different ^{14}C-glyphosate translocation patterns may also be an effect of various physiological processes not yet understood.

The *Alopecurus myosuroides* populations examined in the dose–response study, from which the biotypes examined here originated, could not yet be classified as glyphosate-resistant [52]. But the surviving and subsequently tested *Alopecurus myosuroides* biotypes showed resistance to glyphosate. It should be noted that the fulfillment of most of the criteria for herbicide resistance defined by the HRAC [12] has not been investigated for the biotypes tested. These criteria are the following: (I) the fulfillment of the Weed Science Society of America and International Survey of Herbicide-Resistant Weeds definition of resistance; (II) data confirmation of resistance; (III) the resistance must be heritable; (IV) a demonstration of practical field impact [12].

5. Conclusions

Our investigations show the first signs of an incipient development of resistance by finding a few resistant individuals in the tested populations. This assumption is strengthened by the fact that *Alopecurus myosuroides* has already developed resistance to herbicides with six different mechanisms of action (among them also against glyphosate) [13]. Fur-

thermore, a study [77] provided clear evidence that the prerequisites for the formation of glyphosate resistance in different populations of *Alopecurus myosuroides* are evident, and that *Alopecurus myosuroides* populations are undergoing selection for glyphosate resistance in the field. The heritability of reduced sensitivity to glyphosate has been demonstrated. Furthermore, a direct epidemiological link between historical glyphosate selection and current population-level sensitivity has been demonstrated. It has also been shown that current field populations respond to further glyphosate selection.

The results and assumptions presented in this study suggest that further experiments are needed to elucidate the underlying resistance mechanisms in the biotypes of the *Alopecurus myosuroides* populations from Lower Saxony studied.

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