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Protein identification before and after glyphosate exposure in *Lolium multiflorum* genotypes

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Abstract

BACKGROUND: Weeds reduce crop yields, and among the methods used to control these plants, the use of chemicals is preferred. However, the repeated application of herbicides with the same mechanism of action selects for resistant populations. The aim of this study was to evaluate glyphosate resistance in *Lolium multiflorum* (Lam.) and relate the resistance to protein expression in the absence and presence of the herbicide using a metabolic-proteomic approach.

RESULTS: Glyphosate resistance was confirmed, with a sevenfold difference in resistance between susceptible and resistant genotypes. Among the possible mechanisms affecting resistance, mutations in the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), herbicide differential translocation and overexpression of EPSPS are suggested. Susceptible plants had higher growth than did resistant plants in the absence of the herbicide, in addition to greater expression of protein groups related to photosynthesis and to tolerance to biotic and abiotic stresses. With application of glyphosate, resistant plants maintained their metabolism and began to express EPSPS and other candidate proteins related to herbicide resistance.

CONCLUSIONS: In the absence of glyphosate, the susceptible plants would replace the resistant plants over time, and abiotic or biotic stresses would accelerate this process. Resistance in plants resulted from a combination of target-site and non-target-site resistance mechanisms. We identified several candidate proteins that could be investigated in future studies on glyphosate resistance.
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Keywords: aromatic amino acids; stress tolerance; glyphosate resistance mechanisms; heat shock protein; shikimic acid

1 INTRODUCTION

Weeds are plants that infest agricultural areas and interfere with human activities. To prevent such interference, weeds have been controlled in recent decades almost exclusively by the use of herbicides.[1] However, exclusive use of herbicides, particularly those with the same mechanism of action, selects for resistant populations. One example is the repeated and exclusive application of glyphosate, which has led to selection for increased resistance in several weed species in the last decade.[2,3]

Mechanisms of weed resistance to herbicides can be divided into target-site resistance (TSR) and non-target-site resistance (NTSR). TSR mechanisms occur because of mutations/deletions or overexpression of the target enzyme on which the herbicide acts. NTSR mechanisms involve minimizing and/or preventing the overall effect of the herbicide on the target enzyme of the targeted process in the plant.[2]

Italian ryegrass (*Lolium multiflorum*) is a weed that occurs in several countries and has populations that are resistant to >11 different herbicide mechanisms of action.[3] This ryegrass is a plant from the Mediterranean with a C3 physiology, and in southern Brazil, it primarily infests cereals and perennial crops. When not controlled, *L. multiflorum* produces dense infestations, competes with crops for environmental resources such as water, light and nutrients, and may reduce crop productivity.[4]

Lolium multiflorum plants are resistant to glyphosate because of both TSR and NTSR mechanisms. The TSR mechanism is produced by a mutation in the position of the amino acid Pro-106 of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), in addition to the overexpression of that enzyme.[5,6] The NTSR mechanisms include excluding the herbicide from the site of action as a result of interference with absorption/translocation, storage in the vacuole and detoxification of glyphosate. To date, the metabolism

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of glyphosate to aminomethylphosphonic acid (AMPA) in *L. multiflorum* has not been reported.[7,8]

Although the mechanisms of resistance to glyphosate in *L. multiflorum* are known, the biochemical processes that are involved are not, particularly those related to NTSR, because the genes involved are unknown, and some of them have complex heritability.[9] Glyphosate-resistant *L. multiflorum* plants show apparent damage in development compared with susceptible plants, but the cause of such differences has not been studied.[10] Identification of the proteins expressed in individuals is one approach to measuring such differences and to studying possible processes affected by the resistance of these plants, as the identification of proteins allows different processes that occur in plant cells, tissues and organs to be characterized. [11]

Therefore, the aim of this study was to evaluate possible resistance mechanisms of *L. multiflorum* plants resistant to glyphosate by identifying relevant proteins and relating their functions to plant development in the absence and presence of glyphosate.

2 MATERIALS AND METHODS

2.1 Plant material and growth conditions

Italian ryegrass (*Lolium multiflorum* Lam.) seeds were collected in the state of Rio Grande do Sul (RS), Brazil. Seed samples were collected from at least 40 plants and pooled together at two sites: one without and one with frequent application of the herbicide glyphosate (Ijuí City, 28°23′18.72′ S, 53°55′13.75′ W, and Três de Maio City, 27°47′02.77′ S, 54°14′05.06′ W, respectively). The population collected at the site without glyphosate application was considered the susceptible population. The experiments were then conducted at São Paulo State University in Jaboticabal and Botucatu. The seeds were germinated in polystyrene trays filled with horticultural substrate (Hortimix®; Agristar, Rio de Janeiro, Brazil). After germination, emerged seedlings with two to three fully expanded leaves were transplanted into pots (0.5 L) filled with the same substrate. The trays and pots were maintained under controlled growth conditions at 26/19 °C, with a day/night photoperiod of 12 h, 40% relative humidity and 240 µmol m^{-2} s^{-1} light incidence. The pots were watered daily. A diluted urea solution was applied in water at 5% v/v 14 days after transplanting.

2.2 Dose–response assay

To confirm the resistance and the susceptibility of the genotypes to glyphosate, both populations were treated with herbicide in a dose–response experiment. The experiment was conducted in a completely randomized design (CRD) with four replicates per treatment [except for the commercial dose of the herbicide (720 g a.i. ha^{-1}) with 36 replicates]. Twenty-eight days after transplanting, plants with three to four tillers were treated with glyphosate (Roundup Original®; 360 g L^{-1}; Monsanto, São José dos Campos, Brazil) using a spray bottle pressurized with CO_2 and equipped with four fan jet spray nozzles (8002; Jacto®, São Paulo, Brazil) set to spray 200 L ha^{-1} of spray at a constant pressure of 200 kPa.

Glyphosate was applied at rates of 0, 180, 360, 720, 1,440 and 2,880 g a.i. ha^{-1} in a protected environment with a relative humidity of 84% and temperature of 21.4 °C. After glyphosate application, the plants were returned to the controlled growth conditions. Twenty-one days after treatment (DAT) a visual control assessment of these plants was performed at a scale of 0 to 100%, in which 0% represents no control and 100% represents plant death.[12] Subsequently, the plant shoots were cut at soil level and dried in a forced-air circulation oven (60 °C) for 72 h to obtain dry matter. The percentage mass reduction of treatment plants was calculated in comparison with the mass of control plants. For each population, estimated control values and the percentage mass reduction of dry matter at 21 DAT were subjected to nonlinear log-logistic regression (Eqn 1) when significant differences were detected among treatment means in analysis of variance ($p \leq 0.05$). The program ASSISTAT (http://www.assistat.com) was used to perform statistical analyses, and the program ORIGIN 9 was used to perform regression analyses.[13]

$$y = \frac{a}{1 + e^{-1\left(\frac{x}{xc}\right)}} \quad (1)$$

Here, y represents the visual control or the mass percentage of the reduced dry matter compared with the control; a is the maximum achieved value; k is the curvature of the line; x in the dose, and xc is the herbicide rate used to control 50% of the population.

The herbicide rate that produced 50% control (I_{50}) and a 50% decrease in dry matter (GR_{50}) was determined using Eqn 1. The resistance factor (RF) was calculated using Eqn 2:

$$RF = I_{50R}/I_{50S} \text{or } GR_{50R}/GR_{50S} \quad (2)$$

where R denotes the most resistant population and S the most susceptible population and I50 represents 50% of weed control and GR50, 50% of weed growth reduction. Finally, the percentages of resistant and susceptible plants were calculated for populations using the recommended rate of commercial herbicide in 36 replicates (720 g ha^{-1}).

2.3 Glyphosate resistance mechanisms

In susceptible and resistant biotypes, concentrations of glyphosate, aminomethylphosphonic acid (AMPA), and other important compounds related to the target metabolic pathway of this herbicide were determined. In the treatment with the commercial glyphosate dose of 720 g ha^{-1}, the second fully expanded leaves were collected from each plant before and after the application of herbicide (designated as 0 and 72 h after application, respectively). These leaves were individually identified and stored in a freezer (-30 °C). At 21 DAT, the plants were evaluated for sensitivity to the herbicide. Resistant and susceptible plants were selected within each population for further analysis.

Composite samples of these individuals were used to establish three replicates per population, which were then analyzed for shikimic acid, quinic acid, glyphosate, AMPA, phenylalanine, tyrosine and tryptophan. The samples were macerated with a porcelain pestle in a mortar containing liquid nitrogen. Subsequently, samples were stored in centrifuge tubes, and then subjected to lyophilization in a freeze-drying system (Alpha 2–4 LD Plus; Christ, Osterode am Harz, Germany) at −70 °C (72 h). For each replication, three replicates were used.

The compounds were extracted with the addition of water (10 mL) acidified with acetic acid to pH 3.5, followed by an ultrasound bath at a frequency of 42 kHz for 30 minutes and centrifugation at 4000 ***g*** for 10 min at 20 °C. The supernatant was collected and filtered using a Millex HV filter (0.45 µm) with a Durapore membrane (13 mm) (Merck, Darmstadt, Germany) and stored in an amber bottle for subsequent quantification. The concentration of each compound in the samples was expressed in grams of dry tissue.

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These compounds were quantified using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system with a high-performance liquid chromatograph (HPLC) (Shimadzu Proeminence UFLC; Shimadzu, Kyoto, Japan) equipped with two pumps (LC-20 AD), an autoinjector (SIL-20 AC), a vacuum creator (DGU-20A5), a control system (CBM-20A) and an oven (CTO-20 AC) coupled to a mass spectrometer (3200 Q TRAP; Sciex, Saxonvile, MA, USA) (Applied Biosystems) with a hybrid triple quadrupole.

To identify the compounds and construct calibration curves, analytical standards with purity >99% (Sigma-Aldrich, St. Louis, MO, USA) were used. The optimization of mass spectrometry conditions was performed with direct injections into the equipment (infusions) of 1 mg L^{-1} standard analytical solution of each individual compound. The ionization of the compounds was performed by electrospray ionization (ESI). The voltage calibration conditions and the collision energy were determined individually for the analyses of all compounds.

To perform the analysis, for each compound, the ionization method that generated the greatest signal strength was used. Negative ionization was used to determine the amounts of the compounds glyphosate, AMPA, shikimic acid and quinic acid. For the amino acids phenylalanine, tyrosine and tryptophan, positive ionization was used. The negatively ionized compounds were separated by chromatography using a 5 µm C18 110-Å (150 x 4.6 mm) Gemini column (Gemini C18; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 5 mM ammonium acetate in water (phase A) and 5 mM ammonium acetate in methanol (phase B), with each adjusted to pH 7.0. The gradient used was 0 min, 30% phase B; 2.5 min, 50% phase B; 5 min, 75% phase B; 8.5 min, 90% phase B; and 15 min, 30% phase B with a flow of 0.500 mL min^{-1}. For aromatic amino acids, a 2.5 µm Synergi Fusion RP 100-Å column (Phenomenex, Torrance, CA, USA) and the same mobile phase as described above were used. The gradient used was 0 min, 10% phase B; 2 min, 40% phase B; 4 min, 95% phase B; and 12 min, 10% phase B with a flow of 0.250 mL min^{-1}.[14]

The total run time was 18 min for the negatively ionized compounds and 15 min for the positively ionized amino acids. The retention times for each compound on the chromatographic column were 3.86 min for glyphosate and shikimic acid, 3.69 min for AMPA, 3.79 min for quinic acid, 9.71 min for phenylalanine, 6.84 min for tyrosine and 10.31 min for tryptophan. For each compound, the calibration curve was determined with standard concentrations covering the range of concentrations of that compound found in plant tissues.[14] The experiment was completely randomized with a two by two factorial design for each compound: two leaf collection periods and two glyphosate conditions, absence and presence. The data were submitted to analysis of variance (ANOVA), and the means were compared with Tukey's tests at 5% ($p \leq 0.05$). Statistical analyses were performed using AGROESTAT® software (https://www.agroestat.com.br).

2.4 Proteomic approach

The plants used in the evaluations of the shikimic acid pathway compounds were also used for proteomic analyses. The second leaves of untreated plants of susceptible and resistant populations were collected before and then 72 h after herbicide application. Leaves were immediately placed in liquid nitrogen and were stored at -30 °C until processing.

To extract leaf proteins, plant material was pulverized in liquid nitrogen. Aliquots of 0.5 g were transferred to tubes, and 4 mL of extraction solution was added (6 M urea and 0.1% Dithiothreitol, DTT). The solutions were stirred for 15 min in ice and centrifuged at 3200 ***g*** for 15 min. The supernatant was collected and transferred to a new tube. This process was repeated twice, and then cold acetone at four times the volume of the collected supernatants was added. The precipitation of proteins was conducted 12 h later (after overnight incubation) at -20 °C, with subsequent centrifugation at 3200 ***g***. The pellets obtained were washed twice with 200 µL of cold acetone and dried under laminar flow for 5 min. Proteins were resuspended in 100 µL of sample buffer (6 M urea in 25 mM Ambic). The concentration of the protein extract was determined using known concentrations of bovine serum albumin for the standard curve.[15]

Aliquots of 20 µg for digestion of the protein solution were used for all samples. The digestion was performed for 12 h (overnight) at 37 °C with the addition of trypsin at a 1:50 (enzyme:protein) ratio. Subsequently, the samples were vacuum-dried and desalted using C18 'spin columns' (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations and then resuspended in 0.1% formic acid at the time of the liquid chromatography analyses, with the chromatograph coupled to a mass spectrometer.

Peptides were separated using a C18 column (15 cm; 3 µm; 120 Å) by reverse-phase liquid chromatography with a gradient of 120 min from 5% to 70% of 0.1% formic acid in acetonitrile at a flow of 500 nL min^{-1}. The mass spectrometry analysis was performed using a Q-Exactive (Thermo Fisher Scientific) operated in positive ion mode and data dependent mode (DDA), with cycles consisting of a 'full scan' at 70 000 FWHM (400–2000 m/z), followed by ten cycles of 'DDA scans' at 35 000 FWHM. The peptide fragmentation was obtained by higher energy collisional dissociation (HCD) fragmentation using a collision energy of 27 eV. Ions separately charged were excluded, and peptide ions selected for fragmentation were excluded for 30 s.

Spectral counting was used for the protein relative quantification. Corn (*Zea mays*) protein sequences were obtained from the Phytozome site.[16] The identifications were performed using the integrated PatternLab platform for Proteomics.[17] For the spectral correlation, the COMET tool available on the platform was used.[18] The following modifications were considered during database searches: cysteine carbamidomethylation and methionine oxidation as static and variable modifications, respectively. All spectrum alignments were filtered with SEPRO and adjusted to a false discovery rate (FDR) of 1%.[19] Proteins sharing common peptides were grouped according to the principle of maximum parsimony, and the relative expression of the proteins was evaluated using the normalized spectral abundance factor (NSAF) approach.[20]

Proteins that showed expression differences between the populations that were equal to or greater than twofold ($|\text{fold-change}| \geq 2$) were considered differentially regulated. Additionally, proteins that were identified exclusively in one of the populations were considered differentially regulated.

3 RESULTS

3.1 Resistance level

The tested populations showed differences in susceptibility to glyphosate. The susceptible plants were controlled by 360 g a.i. ha^{-1} of glyphosate (control exceeded 80%). By contrast, the resistant population was not controlled even at 2880 g a.i. ha^{-1} of glyphosate (Figure 1). For determination of the level of resistance in the two populations, it was found that 92% of the plants died and 92% survived in susceptible and resistant populations,

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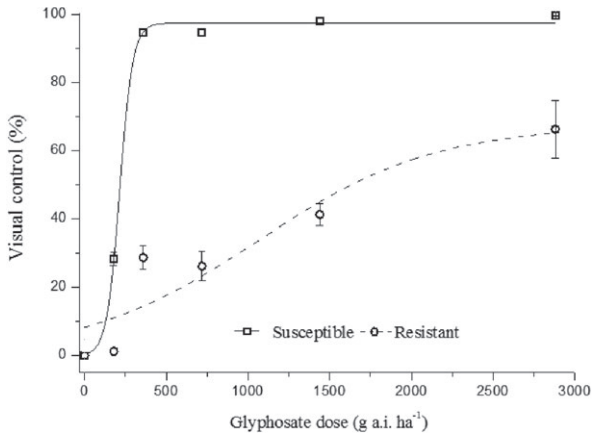

Figure 1. Visual control (%) of resistant (●) and susceptible (■) *Lolium multiflorum* genotypes at 21 days after application of increased doses of glyphosate. Resistant: $y = 67.41/\{1 + \exp[-0.0018(x - 1062.42)]\}$; $R^2 = 0.99$; susceptible: $y = 88.17/\{1 + \exp[-0.021(x - 339.74)]\}$; $R^2 = 0.79$.

respectively, based on the 36 replicates treated with the commercial dose, which showed that resistance was stable and established in the area in which seeds were collected.

According to the I_{50} and GR_{50} values, the degree of resistance to glyphosate in the resistant population was high. The I_{50} values were 218 g a.i. ha^{-1} for the susceptible population and 1635 g a.i. ha^{-1} for the resistant population. The GR_{50} value was 355.3 g a.i. ha^{-1} for the susceptible genotype. However, a GR_{50} value was not obtained for the resistant genotype, because even at the highest dose (2880 g a.i. ha^{-1}), the dry matter reduction did not reach 50%. Thus, the resistant population showed an RF of 7.5 relative to the I_{50} value of the susceptible population and an RF of ≥8.1 for the GR_{50} value.

3.2 Compounds of the shikimic acid pathway

In the analysis of compounds derived from the shikimic acid pathway, the contents of aromatic amino acids were different in the two *L. multiflorum* populations (Table 1). The application of glyphosate changed the metabolism in an idiosyncratic way for each compound. The concentration of phenylalanine was similar between the two genotypes, both before and after herbicide application (0 and 72 h). Tyrosine was detected in high concentrations in the susceptible genotype before herbicide application; however, 72 h after glyphosate application, tyrosine decreased in both genotypes. Tryptophan was also found at high levels in the susceptible genotype before glyphosate application, but herbicide application did not cause a decrease in the concentration of this amino acid.

With respect to compounds derived from the shikimic acid pathway, interaction effects between genotypes and hours after glyphosate application were detected among values for glyphosate, shikimic acid and quinic acid (Table 2). No differences were observed in AMPA values between the genotypes, with only a slight increase in concentration after glyphosate application.

As a product exogenous to plant metabolism, glyphosate was not detected in *L. multiflorum* plants before herbicide application (Table 3). Seventy-two hours after herbicide application, the leaves of resistant plants showed high levels of the herbicide. Before herbicide application, the concentrations of shikimic acid were similar between the genotypes (Table 4), but after glyphosate application, susceptible plants accumulated shikimic acid at levels ninefold higher than those in resistant plants. The behavior of quinic acid was similar to that observed for shikimic acid, which was only accumulated in the susceptible genotype after herbicide application (Table 5). In the comparison of genotypes, quinic acid was accumulated to a lesser degree than shikimic acid.

The analysis of plant biomass without the application of glyphosate (check) reinforced the development advantage of the susceptible genotype compared with that of the resistant genotype. Based on the dose–response curve assay, the dry mass accumulation of susceptible plants was 38% greater than that of resistant plants, indicating that resistant plants incurred a penalty in development for their resistance.

Table 1. Concentrations of essential amino acids (phenylalanine, tyrosine and tryptophan) at 0 and 72 h after the application of 720 g ha^{-1} glyphosate to ryegrass plants

	Amino acid concentration (mg g^{-1})		
	Phenylalanine	Tyrosine	Tryptophan
Genotype			
Resistant	50.06^{a}	21.06^{b}	32.77^{b}
Susceptible	53.86^{a}	33.58^{a}	50.96^{a}
Time			
0 h	53.75^{a}	30.01^{a}	41.63^{a}
72 h	50.18^{a}	24.36^{b}	42.10^{a}
F_g	4.61^{NS}	39.65**	34.01*
F_t	4.07^{NS}	5.48*	0.02^{NS}
$F_{g\times t}$	0.60^{NS}	0.08^{NS}	1.28^{NS}
CV (%)	5.89	14.57	12.90

Means followed by the same letter in columns do not differ by Tukey's test at 5% probability.
F_g, *F*-value for genotype; F_t, *F*-value for time; CV, coefficient of variation.
$^{*}P < 0.05$; $^{**}P < 0.01$. ^{NS}Not significant.

Table 2. Concentrations of shikimic acid pathway compounds (glyphosate, shikimic acid, quinic acid and AMPA) in mg g^{-1} at 0 and 72 h after the application of 720 g ha^{-1} glyphosate to ryegrass plants

	Concentration of compound (mg g^{-1})			
	Glyphosate	Shikimic acid	Quinic acid	AMPA
Genotype				
Resistant	26.15	18.88	47.20	0.08a
Susceptible	21.08	134.25	126.30	0.06a
Time				
0 h	0.00	6.13	33.16	0.00b
72 h	47.23	147.00	140.33	0.15a
F_g	19.05**	36.92**	17.69**	0.50^{NS}
F_t	1655.31**	55.05**	32.48**	40.50**
$F_{g\times t}$	19.05**	40.37**	17.60**	0.50^{NS}
CV (%)	8.51	49.25	37.54	54.43

Means followed by the same letter in columns do not differ by Tukey's test at 5% probability.
F_g, *F*-value for genotype; F_t, *F*-value for time; CV, coefficient of variation.
$^{*}P < 0.05$; $^{**}P < 0.01$. ^{NS}Not significant.

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Table 3. The effects of genotype and time after application on the mean concentration of glyphosate in ryegrass plants at 0 and 72 h after application of glyphosate

	Concentration of glyphosate (g mg^{-1})	
Genotype	0 h	72 h
Resistant	0.00 Ab	52.30 aA
Susceptible	0.00 Ab	42.16 bA

Lowercase letters denote comparisons between genotypes within times and uppercase letters denote comparisons between times within genotypes, with different letters indicating a significant difference between means.

Table 4. The effects of genotype and time after application on the mean concentration of shikimic acid in ryegrass plants at 0 and 72 h after application of glyphosate

	Concentration of shikimic acid (g mg^{-1})	
Genotype	0 h	72 h
Resistant	8.76 Aa	29.00 Ba
Susceptible	3.50 aB	265.00 Aa

Lowercase letters denote comparisons between genotypes within times, and uppercase letters denote comparisons between times within genotypes, with different letters indicating a significant difference between means.

Table 5. The effects of genotype and time after application on the mean concentration of quinic acid in ryegrass plants at 0 and 72 h after application of glyphosate

	Concentration of quinic acid (g mg^{-1})	
Genotype	0 h	72 h
Resistant	33.06 aA	61.33 bA
Susceptible	33.26 aB	219.33 Aa

Lowercase letters denote comparisons between genotypes within times, and uppercase letters denote comparisons between times within genotypes, with different letters indicating a significant difference between means.

3.3 Protein expression

Only one protein group, the 4Fe-4S dicluster domain proteins, was found in both resistant and susceptible plants (Figure 2). Before glyphosate application, these proteins were expressed at levels twice as high in susceptible plants as in resistant plants (Table 6). No protein group was expressed more in resistant plants than in susceptible plants. Other groups were expressed only in plants susceptible to the herbicide, without the application of glyphosate, and most of these were related to plant defenses against biotic and abiotic stresses (Figure 3).

For example, the protein groups PF00012 (heat shock protein 70KDA), PF00168, PF01554 and PF00155 (multidrug resistance), which are found in various organisms to regulate anti-stress processes, were expressed in susceptible individuals. Other groups expressed are related to drug resistance (PF00168, PF01554 and PF00155) and help to ensure the survival of cells under stress.

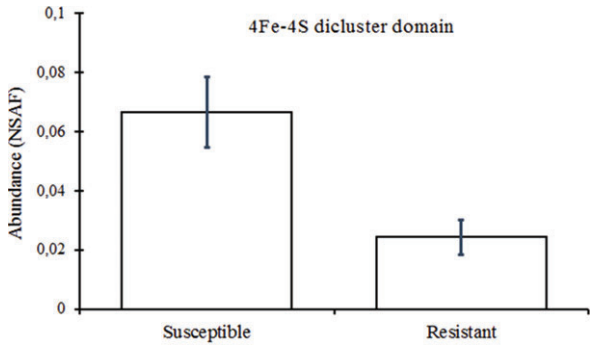

Figure 2. Expression of 4Fe-4F dicluster domain protein group proteins in glyphosate-susceptible and glyphosate-resistant *Lolium multiflorum* plants without glyphosate application. Values are the mean of three replicates ± standard error.

The other expressed proteins were related to protein movement and genetic code editing [PF00076, (splicing factor 3b)], and [5PF039470S, (50S ribosomal protein)] and transfer of amino acids by membranes [PF00155 (Gamma-glutamyltransferase)].

After glyphosate application, the only proteins expressed more in susceptible plants than in resistant plants were phosphoglycerate kinase 1 (PGK1) and photosystem II chlorophyll apoprotein (CP43) (Table 7). Some groups were present in both genotypes and were equally expressed. However, major differences in protein expression were found in resistant plants, with expression of these proteins absent in susceptible plants. The processes observed in resistant plants were related to the metabolism of carbohydrates such as fructose and to carbon sequestration (RuBisCO expression, for example). Resistant plants also expressed several non-active protein groups not found in susceptible plants. The primary processes expressed and identified were the biosynthesis of secondary metabolites and phenylpropanoid, carbon metabolism, biosynthesis of amino acids and carbon fixation (Figure 4).

For example, *L. multiflorum* plants expressed the enzymes glyceraldehyde-3-phosphate dehydrogenase, which is related to plant stress, and transketolase, which is related to photosynthetic activity in plants and relevant to the metabolism of phenylpropanoids.[21] Phenylpropanoid biosynthesis is affected by glyphosate. In resistant plants, compounds were identified that related to this pathway, which did not occur in susceptible plants.

Resistant plants also presented compounds related to carbon metabolism, i.e., proteins related to metabolism and energy generation in plants. Among these proteins, the enzyme malate dehydrogenase was expressed, an enzyme essential for the metabolism of malate. The expression of this enzyme might have occurred in plants as part of normal metabolism or may have been stimulated by the use of glyphosate; the expression of this enzyme was previously shown to be stimulated by herbicides. Another key enzyme expressed in the process was glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in the synthesis of glucose.[22]

In addition to these enzymes, resistant plants showed continued production of the amino acids cysteine, glycine and alanine, whereas these processes ceased in susceptible plants. Expression of the enzyme EPSPS, which is responsible for the synthesis of aromatic amino acids of the shikimic acid pathway (tryptophan, tyrosine and phenylalanine), was also observed, but only in resistant plants. After glyphosate treatment, resistant plants expressed heat shock proteins that were previously expressed only in susceptible

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Table 6. List of differentially expressed proteins in susceptible and resistant plants before glyphosate application

Accession[a]	Protein name	Expression (NSAF)		S:R ratio[b]
		Susceptible	Resistant	
PF14697	4Fe-4S dicluster domain	0.066	0.024	+ 2.75
PF00076	Splicing factor 3b	0.005	0.000	+ 0.005
PF00012	Heat shock protein 70 kDa	0.005	0.000	+ 0.005
PF00168	Multidrug resistance	0.004	0.000	+ 0.004
PF03947	50S ribosomal protein L2	0.003	0.000	+ 0.003
PF00155	Gamma-glutamyltransferase	0.003	0.000	+ 0.003
PF00274	Fructose-biphosphate aldolase	0.035	0.039	- 0.90
PF00162	3-Phosphoglycerate kinase	0.013	0.017	- 0.76
PF01716	Oxygen-evolving enhancer protein	0.067	0.070	- 0.96
PF00004	26S proteasome regulatory complex	0.020	0.031	- 0.65
PF00044	Glyceraldehyde-3-phosphate dehydrogenase	0.024	0.029	- 0.83
PF00016	Ribulose-biphosphate carboxylase	0.135	0.184	- 0.73
PF00542	Ribosomal protein	0.024	0.025	- 0.96

[a] Pfam accession.
[b] Upregulated (+) and downregulated (−) proteins in susceptible plants.

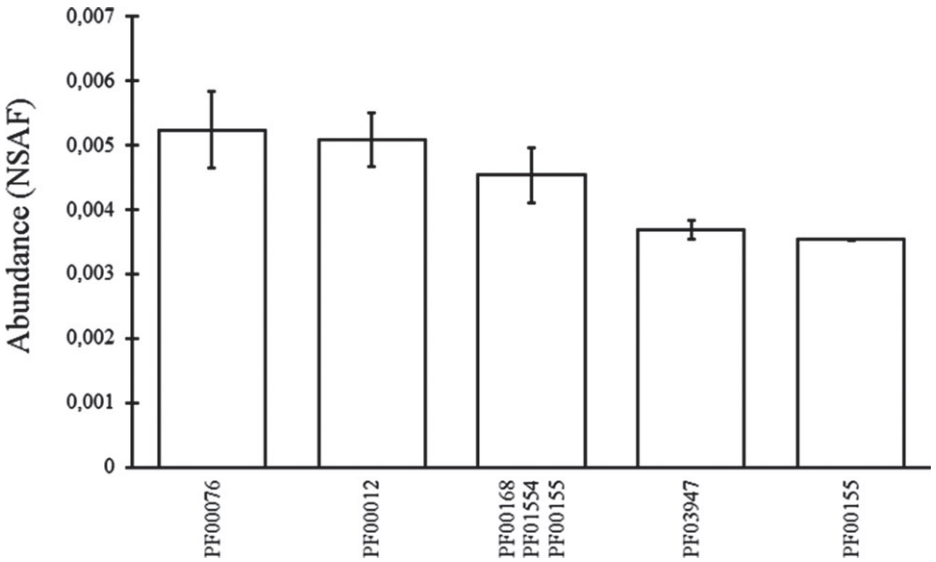

Figure 3. Expression of PF00076 (splicing factor 3b), PF00012 (heat shock protein), PF00168, PF01554, PF00155 (multidrug resistance), PF03947 (50S ribosomal protein) and PF00155 (gamma-glutamyl-transferase) protein groups in glyphosate-susceptible *Lolium multiflorum* plants. Values are the mean of three replicates ± standard error.

plants. EPSPS, which is responsible for the synthesis of EPSP in vegetables, was also expressed.

4 DISCUSSION

The RF obtained in this study is consistent with those reported previously for glyphosate resistance in *L. multiflorum* populations, although it is lower than in some reports, which obtained RF values as much as twentyfold higher. In such cases, resistance mechanisms were detected related to overexpression and mutation of EPSPS. [5,6,8,23]

The application of glyphosate to susceptible plants is known to inhibit the enzyme EPSPS, leading to a blockage of the shikimic acid pathway, which is responsible for the production of the three here evaluated amino acids. Therefore, in the long term, the application of glyphosate should decrease the concentrations of amino acids, as occurred with tyrosine in this study. The responses of the amino acids phenylalanine and tyrosine should be similar when herbicides are applied, because these amino acids have the same precursor in the shikimic acid pathway, i.e., prephenate. However, further assessments must be conducted.[24] The absence of changes in tryptophan levels in this study was probably because that amino acid is less sensitive to the inhibition caused by glyphosate.[25] The aromatic amino acid levels decreased in *Nicotiana plumbaginifolia* 4 days after the application of glyphosate by 59%, 77% and 13% for tyrosine, phenylalanine and tryptophan, respectively, corroborating the results of this study.[26]

The low concentrations of the amino acids tyrosine and tryptophan in resistant plants before the application of the herbicide might be related to the mechanism of target-site resistance to glyphosate. For example, mutations in EPSPS that may alter not only the affinity of the enzyme for the herbicide but also the

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Table 7. List of differentially expressed proteins in susceptible and resistant plants after glyphosate application

Accession[a]	Protein name	Expression (NSAF)		S:R ratio[b]
		Susceptible	Resistant	
PF00162	Phosphoglycerate kinase 1	0.01	0.01	+ 2.53
PF00421	Photosystem II CP43 chlorophyll apoprotein	0.02	0.01	+ 2.19
PF00044	26S proteasome regulatory complex	0.03	0.01	+ 1.92
PF00044	Glyceraldehyde-3-phosphate dehydrogenase	0.02	0.01	+ 1.90
PF00273	Fructose bisphosphate aldolase	0.02	0.01	+ 1.83
PF01716	Oxygen-evolving enhancer	0.05	0.03	+ 1.70
PF14697	4Fe-4S dicluster domain	0.03	0.03	+ 1.03
PF00016	Ribulose-bisphosphate carboxylase	0.02	0.11	+ 0.15
PF00275	Photosystem II 10-kDa phosphoprotein	0.00	0.030	-0.030
PF00504	Chlorophyll A-B binding	0.00	0.024	-0.024
PF02531	Photosystem I reaction center	0.00	0.024	-0.024
PF01789	Oxygen-evolving enhancer	0.00	0.023	-0.023
PF00283	Cytochrome B559 subunit	0.00	0.013	-0.013
PF00542	Ribosomal protein	0.00	0.009	-0.009
PF15511	Histone H4	0.00	0.008	-0.008
PF00033	Plastoquinol-plantocyanin reductase	0.00	0.008	-0.008
PF01333	Apocytochrome F.C-terminal	0.00	0.008	-0.008
PF08534	Peroxiredoxin	0.00	0.007	-0.007
PF00034	Glyceraldehyde-3-phosphate dehydrogenase	0.00	0.007	-0.007
PF00275	EPSPS synthase	0.00	0.007	-0.007
PF01434	Metalloprotease M41	0.00	0.006	-0.006
PF00009	Translation factor	0.00	0.006	-0.006

[a] Pfam accession.
[b] Upregulated (+) and downregulated (–) proteins in susceptible plants (expression higher than 0.005).

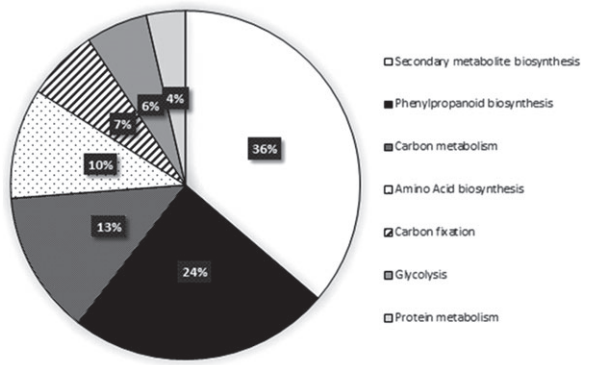

Figure 4. Mean percentages of protein groups separated by metabolic processes expressed in glyphosate-resistant *Lolium multiflorum* plants after herbicide application.

affinity for the substrate, phosphoenolpyruvate (PEP), often cause a change in amino acid metabolism.[27] In general, changes in the amino acid Pro-106 of EPSPS generate low resistance values in affected plants, resulting in RF values between two and three times lower than the levels found in this study. The RF found here suggests a combination of TSR and NTSR mechanisms in this population, as previously reported.[28]

Because the AMPA content did not change, the possibility of metabolism of glyphosate as a resistance mechanism of *L. multiflorum* plants was excluded. AMPA is a by-product of glyphosate degradation in some species as a consequence of the action of the enzyme glyphosate oxidoreductase (GOX), but this mechanism has not been reported for *L. multiflorum*.[29]

The higher concentration of glyphosate in resistant than in susceptible plants confirmed that resistant *L. multiflorum* plants had high retention of the herbicide on leaf surfaces for periods longer than 64 h after application.[30] According to previous authors, the high retention is related to low translocation of the herbicide in the resistant genotype. Therefore, this result supports the theory of multiple resistance mechanisms in *L. multiflorum*, exactly as described for other populations.[5] This species of ryegrass is highly prone to this type of combination of mechanisms because of cross-pollination.[28]

The accumulation of shikimic and quinic acid is a direct result of the inhibition of EPSPS. Accumulation of shikimic acid as a consequence of the application of glyphosate has been reported in individuals sensitive to this herbicide.[31] Other studies report quinic acid accumulation after the application of glyphosate in plants sensitive to glyphosate, because quinic acid is an alternative to the shikimic acid pathway but is less sensitive to glyphosate than is shikimic acid.[24,32]

The higher expression of the 4Fe-4S protein group in susceptible than in resistant plants might be related to many metabolic processes in plants, such as photosynthesis, respiration, nitrogen fixation and DNA repair, which may explain the increased biomass accumulation by these plants. This group of proteins is located at the reaction center of photosystem I and is directly related to the flow of electrons and energy generation. However, reports suggest that this group of proteins is an indicator of oxidative stress.[33]

In this study, *L. multiflorum* showed a strong trade-off between protection from glyphosate and responses to other stresses, given that heat shock and multidrug resistance proteins, for example, were not found in resistant plants. The protein group PF00012

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(heat shock proteins), which functions in protein synthesis, transport and degradation, was observed only in susceptible plants.[34] At least 19 genes are responsible for the expression of that group of proteins in plants, and overexpression is often associated with tolerance to heat, salt and water stresses.[35] Moreover, other proteins found in susceptible plants are responsible for the extrusion of xenobiotics and toxic metabolites from cells. Although an association between resistance to glyphosate and the expression of these proteins in plants has not been confirmed, expression of this group of proteins in bacteria caused resistance to this herbicide,[36] corroborating the presence of these proteins in sensitive plants.

Two hypotheses are proposed to explain these results. First, resistant plants may have lost the ability to express this set of proteins and would therefore be less well adapted to stresses, such as those caused by competition for environmental resources, as demonstrated elsewhere (in fact, after herbicide selection, resistant plants tend to survive in environments free of the abiotic stress provided by crop production). Alternatively, in resistant plants, because the expression of these proteins is not required continuously, those defense mechanisms may be activated only when in competition or exposed to stress conditions. The resistance mechanism, such as enzyme overexpression, is thus adapted for production only with the application of glyphosate and therefore does not compromise the competitiveness of *L. multiflorum*, as previously reported in the literature.[10,37]

With glyphosate application, resistant plants expressed proteins responsible for growth and development, whereas susceptible plants expressed proteins related to responses to herbicide effects. In response to glyphosate, susceptible plants expressed more PGK1 protein (response to oxidative damage) and CP43 protein (related to photoinhibition).[38,39]

In this study, resistant plants expressed glyceraldehyde-3-phosphate dehydrogenase, which is frequently inhibited by stress processes.[22] Additionally, resistant plants expressed the enzyme transketolase,[40] which is responsible for up to 40% of the photosynthetic activity of plants. In susceptible *L. multiflorum* plants, however, these enzymes were not expressed. Furthermore, this protein group is also relevant to phenylpropanoid metabolism.[21]

The biochemical pathway of phenylpropanoid biosynthesis is connected to the production of secondary phenolic and metabolic compounds, including lignin, which are reduced by the application of herbicides in sensitive or inhibited plants, as observed in this study.[41]

Heat shock proteins were expressed in susceptible plants before glyphosate application but then were expressed in resistant plants after herbicide application, which supports the idea that resistant plants may present certain signaling mechanisms for different stresses.

Processes related to the synthesis of secondary metabolites were expected in plants that survived the application of glyphosate, because the shikimic acid pathway is responsible for the synthesis of the amino acids phenylalanine, tyrosine and tryptophan and other compounds involved in carbon and glucose metabolism. These differences can be caused by various geographic adaptations, but in Brazil, this species is located strictly in southern regions. Additionally, because this is a species that reproduces by seed, the benefits from resistant populations, even those not caused by glyphosate resistance as suggested in this study, can clearly enhance the spread of a resistant population over that of a susceptible population, which will also affect the management practices adopted to manage this weed.

Notably, the enzyme EPSPS was upregulated in resistant plants after glyphosate spraying. Therefore, enzyme overexpression is likely to be a glyphosate resistance mechanism in these individuals evidence of such overexpression has been reported for this species in other studies.[6] This is corroborated by the finding that neither of the genotypes expressed this enzyme before the application of glyphosate. Alternatively, related to glyphosate resistance mechanisms previously found in literature, we did not observe different levels of fructose-bisphosphate aldolase or ATPase production, which are found in glyphosate-resistant *Conyza canadensis* and which the authors associated with vacuolar sequestration of the herbicide.[40]

5 CONCLUSIONS

The population of *L. multiflorum* resistant to the herbicide glyphosate was resistant as a consequence of possessing a set of TSR and NTSR mechanisms. Without the application of herbicide, resistant plants had lower growth than susceptible plants and lower expression of proteins related to plant defense against stresses. After glyphosate application, resistant plants expressed proteins related to energy metabolism and synthesis of secondary metabolites, in addition to expressing the enzyme EPSPS, which can also be a resistance mechanism. Several candidate proteins identified in this study could be investigated in the future using the model Arabidopsis to investigate the relationships between these proteins and glyphosate NTSR.

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