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Identification of Glyphosate Resistance in *Lolium rigidum* Gaudin

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SUMMARY

Glyphosate resistance was found in *Lolium rigidum* Gaudin (Rigid ryegrass, LOLRI) in South Africa. Suspected glyphosate-resistant *L. rigidum* populations were collected and grown under greenhouse conditions. The plants were sprayed with a range of doses of glyphosate 35 days after planting and shoot dry biomass was determined 17 days after herbicide treatment. Based on the dose-response experiment conducted in the greenhouse, one population of *L. rigidum* suspected to be resistant to glyphosate was approximately 5.3 fold more resistant than susceptible population. The other population was 2.8 fold more resistant than susceptible population. Difference between the two suspected resistant populations was 1.9 fold. All plants were treated with glyphosate (1000 g a.i. ha^{-1}) and shikimic acid was extracted 2, 4 and 6 days after treatment. The plants of susceptible populations accumulated more shikimic acid than other two populations.

Keywords: *Lolium rigidum*; Glyphosate; Resistance; Shikimic acid

INTRODUCTION

Glyphosate is the only herbicide reported to inhibit enzyme 5-enolpyruvylshikimate-3-phosphate syntetase (EPSPS) (Steinrucken and Amrheim, 1980). Glyphosate is a broad-spectrum, nonselective herbicide that has been widely used for vegetation control in plantation crops and nonagricultural land since 1970s (Baylis, 2000). After glyphosate use some species of weeds resistant to glyphosate have been reported in several countries (Heap and LeBaron, 2001). Metabolism of glyphosate in higher plants is very limited and not well understood (Rubin et al., 2004). Resistance to glyphosate has been reported in *Lolium rigidum* in Australia (Pratley et al., 1996, 1999; Powles et al., 1997, 1998; Lorraine-Colwill et al., 1999) and California (Simarmata et al., 2003). Additional populations of glyphosate–resistant genera *Lolium* were also reported (Perez and Kogan, 2003). The basis of glyphosate resistance in *L. rigidum* from Australia is not clearly understood. Shikimic acid accumulated in leaf tissue of susceptible population (S) after glyphosate application, but no differences in uptake, translocation, metabolism and EPSPS activity were found between the populations

(Lorraine-Colwill et al., 1999; Perez et al., 2004). Recently Lorraine-Colwill et al. (2003) suggesting that difference in the herbicide translocation and distribution between resistant (R) and S populations might be involved in the resistance.

L. rigidum Gaudin (Rigid ryegrass, LOLRI) is an annual weed up to 0.9 m tall, with acute or short-awned lemmas like perennial ryegrass and blades rolled in bud like Italian ryegrass (Figure 1). *L. rigidum* grows in the same types of habitats as perennial and Italian ryegrass and can hybridize with them. Rigid ryegrass is sometimes cultivated for livestock forage and has occasionally been implicated with the same toxicity problems as perennial and Italian ryegrass (DiTomaso and Healy, 2007). That is diploid out-crossers that seem to cluster into a single variable complex. It is a grass native to the Mediterranean (Powles et al., 1998), present in some cereal crops, orchards, alfalfa and non-arable lands. *L. rigidum* developed resistance to numerous classes of herbicides (Hall et al., 1994; Preston et al., 1996) and ability to accumulate resistance has been attributed to its widespread distribution, prolific seed set, genetic variability and phenotypic plasticity (Powles and Matthews, 1992). Glyphosate inhibits biosynthesis of the aromatic amino acids tryptophan, tyrosine and phenylalanine (Gressel, 2002). In the shikimate pathway, glyphosate competes with substrate phosphoenolpyruvate (PEP) for the binding site of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP, E.C. 2.5.1.19). Singh and Shaner (1998) stated that shikimic acid accumulation may be used as a method to determine whether a plant species is resistant to glyphosate. Harring et al. (1998) stated that this assay was also useful in evaluating efficacy of different glyphosate formulations. Accumulation of shikimic acid is caused only by glyphosate inhibition of EPSPS (Lydon and Duke, 1988). Shikimic acid accumulation can be measured using high-perfomance liquid chromatography (HPLC) (Lydon and Duke, 1988).

The objective of this research was to determine the resistance in populations of *L. rigidum* collected in South Africa and to characterize the level of resistance to glyphosate on a whole plant and shikimic acid accumulation basis.

MATERIAL AND METHODS

Resistant seed of *L. rigidum* (LR pop.) was provided by Institute for Plant Production, Department of Agriculture, Western Cape, South Africa. Susceptible (LS pop.) and presumably resistant (LPR pop.) seed was collected in cereals near the Western Cape. Experiments were conducted at the University of Pretoria, Pretoria, South Africa in 2007. After emergence in Petry plates in phytotrone (7 days on filter paper with 5 ml water in the dark at 5°C, after that at 20°C under day/night regime (12/12 h) until appearance of the seedlings 2-3 cm in length) the seedlings of *L. rigidum* were planted in 1l pots. Pots were filled with soil from the field (Hutton form, Sandy Clay Loam with 23% clay, 0.6 Carbon, and pH of 6.5) typical for the South African region. Plants were placed in the greenhouse at an average temperature of 22.8/10.5°C (day/night), and 54.6% RH, under 12:12 h light:dark period. The plants were watered every other day with tap water and nutrient solution mix (calcium nitrate: CaO-19.5% and NO_3-15.5%; potassium sulfate: K_2O – 42% and S – 18.5%) every 15 days (200 ml per pot). Nutrient solutions were prepared by dissolving 7.5 g calcium nitrate and 32 g potassium sulfate in 50 l of water. In our experiments we applied glyphosate trimesium sulphosate as compound Touch down (500 g a.i. l^{-1}, SC, Syngenta) with Oxford laboratory hand type sprayer equipped with RS-MM 110°/04 nozzles and at 300 l water per ha at 276 kPa. In dose response test herbicide was applied 35 days after planting at following doses: 125, 250, 500, 1000, 2000, 4000, 8000 and 12000 ml ha^{-1} plus untreated control. The experimental design was a fully randomized, with two replications (2 pots with 8 plants per pot per dose). The pots were harvested 17 days after application (DAA). The plants were then oven-dried (75°C) for 48 h to determine dry weight. Three-week old plants were used for measuring shikimic acid accumulation. Plants were sprayed with recommended dose of herbicide of 2000 ml ha^{-1} (1000 g a.i. ha^{-2}). Samples were then collected (whole plants without the roots) from sprayed and unsprayed plants of both varieties 2, 4 and 6 DAA.

Extraction of Shikimate

Plant material was ground in liquid nitrogen by mortar and pestle. About 1.5 g of grounded material was mixed with 10 ml 1M HCl and shaken for 24 h. pH was adjusted with 1M NaOH and 0.1M NaOH to pH = 3.0-3.5. After that, the filtration was performed and the supernatant was kept in a refrigerator (at 4°C) until analysis.

High Performance Liquid Chromatography (HPLC) Analysis of Shikimate

HPLC analysis of shikimate was performed using a method by Mueller et al. (2003). Material extracted as described above was centrifuged at 15000 g for 5 min to

remove any particulate matter. An aliquot (20 µl) of the supernatant was injected into Water HPLC (Hewlett Packard Agilent 1100 series, DAD (Diode Array Detector), Lune-NH2, column of 5 µl diameter, flow 1 ml min^{-1}.

Statistical analysis

Statistical analysis was performed using Sigma Plot 4.0 software (1997). The experimental results of microscopy studies were examined visually. Lethal doses curves were fitted according to the following nonlinear regression model by Streibig et al. (1993) and Seefeldt et al. (1994):

$$Y = c + \{(d - c) / [1 + (x / g)^b]\} \quad (1)$$

Where: *Y*- % growth, *c*-average plant response to high herbicide application rate, *d*-average plant response to application rates close to zero, *b*-slope of the best fitted line, *g*-herbicide dose causing the effect between *c* and *d*, *x*-herbicide application rate.

Index of resistance (IR) was calculated as LD_{50} resistant population / LD_{50} of susceptible populations.

RESULTS

A differential response to glyphosate between three populations of *L. rigidum* was observed (Figure 2). At 2000 ml ha^{-1} in susceptible plants (LS), the growth was reduced up to 90% compared to the untreated control, whereas in resistant (LR) and presumably resistant plants (LPR) it was reduced 10-20%. Index of resistance showed clear differences between tested populations. LR population was 5.3 fold and LPR population 2.8 fold more tolerant to glyphosate vs LS population. LPR population showed only 1.9 fold more susceptibility to glyphosate vs LR. Letal dose (LD_{50}) for LS population was 0.101 kg a.i. ha^{-1}, for LPR population 0.278 kg a.i. ha^{-1} and for LR population 0.538 kg a.i. ha^{-1} (Table 1, Figure 2).

Similar levels of differences in the amount of shikimik acid were observed. Every tested population contained higher amounts of shikimik acid compared to untreated control 2 DAA. At the following evaluation dates (4-6 DAA) the differences in the amount of shikimic acid between the populations were recorded. In LS and LPR populations there was a tendency of increase in the amount of shikimic acid, while in LR population after an increase in shikimic acid, 2, 4 and 6 DAA a lower amount of shikimic acid was recorded. The plants

Figure 1. *Lolim rigidum*

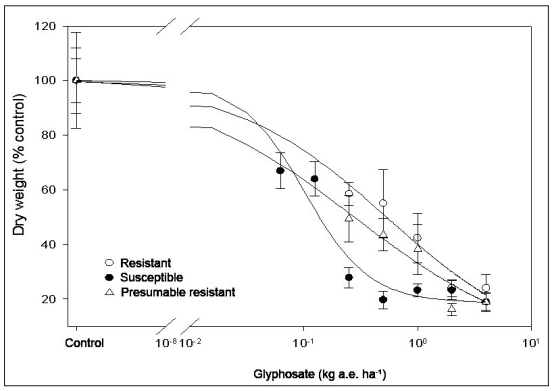

Figure 2. Susceptibility of *L. rigidum* (pop. LS, LPR, LR) vs different doses of glyphosate trimesium sulphosate based on dry mass

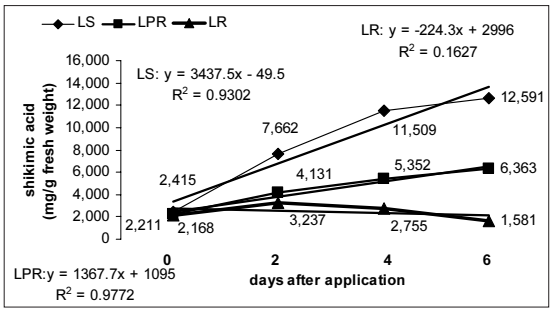

Figure 3. Shikimic acid accumulation in leaves and stem of glyphosate LS, LPR and LR *L. rigidum* following the application of glyphoste trimesium sulphosate (2000 ml ha^{-1})

in untreated control also contained lower levels of shikimic acid. The LS population 2 DAA contained 3.2 fold, 4 DAA 4.8 fold and 6 DAA 5.2 fold more shikimic acid compared to untreated control. Similar levels of differences were observed in plants of LPR population (1.9 fold, 2.4 fold and 2.9 fold more than in untreated

plants 2, 4 and 6 DAA) contrary to differences measured in plants of LR population. Statistical analysis showed clear differences between treated and untreated plants, except in LR population (Table 2). In LR population 6 DAA we recorded decrease in shikimic acid amount (1.4 fold) compared to values measured prior to herbicide application (untreated control). 2 and 4 DAA an increase in shikimic acid in treated plants (1.5 and 1.3 fold) was not observed (Figure 3, Table 2). This confirmed the resistance of resistant (LR) *L. rigidum* to 2 000 ml ha^{-1} of glyphosate trimezium sulphosate, while LS and LPR populations showed starting levels of resistance. Earlier conducted preliminary tests with higher glyphosate dose (20 l ha^{-1}) showed similar results and confirmed the method applicability. The results showed rapid increase in shikimic acid amount in all tested populations compared to amounts measured before herbicide application (data not shown).

Table 1. LD_{50} and IR based on dry matter

Populations	LD_{50}	CI	IR
	kg a.i. ha^{-1}		
LR	0,538	0,480	**LR / LS = 5.3**
LPR	0,278	0,260	**LPR / LS = 2.8**
LS	0,101	0,038	-
	-	-	**LR /LPR = 1.9**

CI - confidence interval from 95% for LD_{50}, IR-index of resistance, LR -resistant pop., LPR - presumable resistant pop., LS - susceptible pop.

DISCUSSION

Visual evaluation of plants treated with 2000 ml ha^{-1} of glyphosate, 17 DAA showed differences between treated and untreated plants of *L. rigidum*. We confirmed the susceptibility of LS population (> 90% of plants showed the damage symptoms), while plants in LPR and LR populations showed 10-20% damage. Based on calculated index of resistance (IR) between LS and LR populations, plants in LR population were 5.3 fold more tolerant to the herbicide, and plants in LPR population 2.8 fold more tolerant. The differences between LR and LPR populations were at IR=1.9.

Measurement of the shikimic acid accumulation is a quick method to identify glyphosate resistance in plants (Sing and Shaner, 1998). Before glyphosate application amount of shikimic acid was similar in all tested plants in three tested populations (LPR 2.207 mg g^{-1} fresh matter, LS population 2.414 mg g^{-1} fresh matter and LR population 2.168 mg g^{-1} fresh matter). Statistical analysis showed that after glyphosate application clear differences between treated and untreated plants were observed, except in LR population (Table 2). At rate of 2 000 ml ha^{-1} the susceptible plants accumulated approximately 3.2 fold (2 DAA), 4.8 fold (4 DAA) and 5.2 fold (6 DAA) more shikimic acid than the LR and LPR populations (Figure 3). In all tested populations the content of shikimic acid increased 2 DAA, and continued to rise over time in LS and LPR populations (Figure 3) contrary to LR population after glyphosate application. In LR population 4 and 6 DAA decreasing amount of shikimic acid accumulation was recorded (Figure 3).

Table 2. Statistical analysis of differences in shikimic acid amounts (LSD test)

parameters	Populations	Control: 2 DAA	Control: 4 DAA	Control: 6 DAA
Sikimic acid	**LR**	NS	NS	NS
	LPR	NS	*	*
	LS	**	**	**

NS-nonsignificant differences, p<0,05*; p<0,01**; LS-susceptible pop., LPR-presumable resistnt pop., LR-resistant pop., DAA days after application

Perez-Jones et al. (2007) obtained similar results while testing resistance in *Lolium multiflorum* to glyphosate. They confirmed 2-3 fold higher shikimic acid levels in resistant populations compared to susceptible ones. Michitte et al. (2007) recorded similar trends as seen in our research: increase in the amount of shikimic acid in S populations of *L. multiflorum* with time, and more or less unchanged levels of shikimic acid in resistant populations (R) after glyphosate application. 6 DAA in S population *L. rigidum* amount of shikimic acid was 8 fold higher than in R populations. Some variations in levels of shikimic acid in R populations can be explained by stress effect after herbicide applications, but the plants recovered quickly. As a result plants sometimes react with increased levels of EPSPS or increase its activity to overcome stress effects caused by glyphosate (Feng et al., 1999; Baerson et al., 2002). This temporary weakness of plants LR population can be explained with the fact that although resistant form of EPSPS enzyme is present in plants, there is also a certain amount of susceptible form of EPSPS enzyme present (Bourque et al., 2002). Another explanation could be that "partial

shot-off of glyphosate" from plastids happened, which results in lower glyphosate effect on EPSPS (Feng et al., 2004). Baerson et al. (2002) explained differences between S and R populations of *L. rigidum* by increased activity of EPSPS enzyme by 2-3 fold in treated vs untreated plants.

Initially high level of shikimic acid in plants of R population (10 fold more than in control plants, Figure 3) was explained by increased plant activity to overcome stress state caused by glyphosate applications.

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Testiranje rezistentnosti *Lolium rigidum* Gaudin na glifosat

REZIME

Rezistentnost na glifosat je potvrđena kod *Lolium rigidum* u Južnoj Africi. Semena rezistentne, očekivano glifosat-rezistentne i osetljive populacije *L. rigidum* su sakupljena u usevu pšenice sa različitih lokaliteta na području Južnoafričke Republike. Testiranje rezistentnosti na glifosat je izvedena u kontrolisanim uslovima staklare Univerziteta u Pretoriji (Južnoafrička Republika). Tridesetpet dana nakon setve (u plastičnim sudovima) biljke su tretirane suspenzijom od 125, 250, 500, 1000, 2000, 4000, 8000 i 12000 ml ha^{-1} glifosata, a suva masa je izmerena 17 dana nakon primene herbicida. Za fitovanje krive i utvrđivanje efektivne doze (dose-response-test) korišćena je jednačina ne-linearne regresione analize (R softver, *drc* paket). Na osnovu dobijenih rezultata utvrđena je 5,3 puta veća otpornost na glifosat kod rezistentne populacije *L. rigidum* (RLR) u poređenju sa osetljivom populacijom (OLR). Kod pretpostavljeno rezistentne populacije (PRP) konstatovana je 2,8 puta veća otpornost u odnosu na osetljivu populaciju. Razlika između pretpostavljeno rezistentne i rezistentne populacije je bila 1,9 puta. Za utvđivanje efekta glifosata na sadržaj šikiminske kiseline biljke su tretirane suspenzijom od 1000 g a.m. ha^{-1}, a sadržaj šikiminske kiseline (HPLC metodom) je meren 2, 4 i 6 dana posle primene herbicida. Sadržaj šikiminske kiseline je bio veći kod osetljive populacije u poređenju sa druge dve testirane populacije *L. rigidum*. Dakle, potvrđena je rezistentnost RLR populacije, odnosno osetljivost OLR populacije, što

obavezuje farmere da sprovode elemente antirezistentne strategije da bi usporili i sprečili ekspanziju rezistentnih populacija *L. rigidum* na području Južnoafričke Republike. Takođe, ta iskustva treba preneti i na druga područja u svetu da bi se sprečilo/usporilo širenje rezistentnosti korova na glifosat.

Ključne reči: *Lolium rigidum;* šikiminska kiselina; rezistentnost