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Glyphosate-Resistant Rigid Ryegrass (*Lolium rigidum*) Populations in the Western Australian Grain Belt

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Glyphosate-resistance evolution in weeds is evident globally, especially in areas where transgenic glyphosate-resistant crops dominate. Resistance to glyphosate is currently known in 16 weed species, including rigid ryegrass in Australia. Following the first report of glyphosate resistance in 1998, there are now 78 documented glyphosate-resistant populations of rigid ryegrass in grain-growing regions of southern Australia. In some regions where glyphosate-resistance evolution has already occurred in rigid ryegrass, transgenic glyphosate-resistant canola was introduced in 2008, further highlighting the need to monitor glyphosate-resistance evolution in weeds. A rigid ryegrass population (WALR70) was collected in 2005 from a crop field in Esperance, Western Australia, after it had survived applications of glyphosate. Dose–response experiments confirmed resistance in the population, with the glyphosate rate resulting in 50% mortality (LD_{50}) for WALR70 being 11 times greater than that for a susceptible biotype. The WALR70 population also had low levels of resistance to some acetyl coenzyme A carboxylase (ACCase)- and acetolactate synthase (ALS)-inhibiting herbicides (diclofop, fluazifop, clodinafop, tralkoxydim, chlorsulfuron, and imazethapyr), but was susceptible to other herbicide modes of action, such as atrazine, trifluralin, and paraquat. Two other rigid ryegrass populations assessed in this study were also confirmed to be resistant to glyphosate. The increasing number of glyphosate-resistant rigid ryegrass populations in Australia is of concern to growers because of the importance of glyphosate in intensive cropping systems and the introduction of glyphosate-resistant canola to this region.
Nomenclature: Glyphosate; rigid ryegrass, *Lolium rigidum* Gaudin.
Key words: Herbicide resistance, glyphosate, annual ryegrass, multiple resistance.

En la maleza, la evolución de la resistencia al glifosato es evidente mundialmente; en especial, en áreas donde dominan los cultivos transgénicos con resistencia al mismo. En Australia, la resistencia al glifosato es actualmente conocida en dieciséis especies de maleza, incluyendo *Lolium* (rigid ryegrass). A partir del primer reporte de resistencia en 1998, en el sureste de Australia existen hasta ahora 78 poblaciones documentadas de *Lolium* resistente. En algunas regiones en donde la evolución de la resistencia al glifosato ha ocurrido en *Lolium rigidum*, en 2008 fue introducida la canola transgénica resistente al glifosato, lo que resalta la necesidad de monitorear en el futuro la evolución de resistencia a esta sustancia en la maleza. Una población de *Lolium rigidum* (WALR70) que sobrevivió aplicaciones de glifosato fue recolectada en 2005 de un campo de cultivo en Esperance, al sureste de Australia. Experimentos de dosis-respuesta confirmaron la resistencia en la muestra, con la dosis de glifosato resultando en un 50% de mortalidad (LD_{50}) para WALR70, lo cual es una tasa 11 veces mayor que lo normal para un biotipo susceptible. La población WALR70 también registró bajos niveles de resistencia a algunos ACCase y herbicidas inhibidores de ALS (diclofop, fluazifop, clodinafop, tralkoxydim, clorsulfuron e imazethapyr) pero fue susceptible a otros herbicidas de diferente acción como atrazina, trifluralin y paraquat. Otras dos poblaciones de *Lolium rigidum* evaluadas en este estudio también confirmaron ser resistentes al glifosato. El creciente número de poblaciones resistentes al glifosato en Australia constituye una gran preocupación para los agricultores, debido a su importancia en la aplicación de sistemas de cultivo intensivos y a la introducción de canola resistente, en mencionada región.

Glyphosate is the world's most important herbicide, used globally to control a broad spectrum of weed species in a wide variety of situations, especially for weed control before crop seeding and in conservation tillage systems (Duke and Powles 2008). The first reports of glyphosate-resistance evolution in weed species occurred in the herbicide-resistance–prone species rigid ryegrass in Australia (Powles et al. 1998; Pratley et al. 1999), 16 yr after the first report of resistance in this species to the ACCase-inhibiting herbicide diclofop-methyl (Heap and Knight 1982). Many thousands of rigid ryegrass populations evolved multiple herbicide resistance across all grain-growing regions of Australia, but resistance to glyphosate remained rare (Broster and Pratley 2006; Llewellyn and Powles 2001; Owen et al. 2007; Powles and Matthews 1992). As rigid ryegrass populations evolved resistance to selective herbicides, there has been increased reliance on glyphosate for rigid ryegrass control and, subsequently, glyphosate-resistant populations have increased in frequency. Most of these have evolved resistance to other herbicides and therefore display multiple resistance to many herbicides, including glyphosate (Neve et al. 2004). Currently there are 78 populations of rigid ryegrass known to be glyphosate resistant (Preston 2008). These populations exist in a variety of situations, including crop fields, horticultural and viticultural sites, and road and fence margins.

The evolution of glyphosate-resistant weeds is becoming a very significant issue in parts of North and South America, where transgenic glyphosate-resistant crops are widely grown and few other herbicides besides glyphosate are used for weed control (Duke and Powles 2008). In these regions, glyphosate-resistant crops are grown, with glyphosate often being the only herbicide used. The increased use of glyphosate has increased the selection pressure for this herbicide, which has resulted in

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the evolution of many weed populations resistant to glyphosate (reviewed by Powles 2008).

Prior to 2008, there have not been any glyphosate-resistant crops in crop-growing regions of southern Australia. Therefore, the use of glyphosate in this region has been restricted to nonselective weed control prior to crop planting, as well as out-of-season weed control. However, glyphosate-resistant canola is now being introduced in cropping regions of southern Australia. In the 14-million-hectare grain belt of Western Australia, glyphosate-resistant canola crops will be present for the first time in 2009. In this cropping region our previous surveys have revealed that the most important weed is rigid ryegrass, and that although the majority of rigid ryegrass populations are glyphosate susceptible (Owen et al. 2007), isolated glyphosate-resistant rigid ryegrass populations do occur (Hashem and Borger 2009; Neve et al. 2004). With the introduction of glyphosate-resistant canola from 2009, it is important to monitor the evolution of glyphosate resistance in weed species in this region. Here, we report three rigid ryegrass populations that have evolved glyphosate resistance prior to the introduction of glyphosate-resistant canola in Western Australia, and demonstrate that these populations also exhibit resistance to several other herbicides (multiple resistance).

Materials and Methods

Plant Material. In May 2005, rigid ryegrass seedlings surviving a glyphosate application of 400 g ae/ha (hereinafter referred to as population WALR70) were collected from a field in Esperance, Western Australia. Another population (WALR80) was collected in 2006, 30 km south of WALR70. This population survived three applications of glyphosate in the field (total 1,600 g/ha) in the year of collection. A third population (WALR90) survived 600 g/ha of glyphosate applied during the summer/autumn period prior to collection. This population was collected in 2003 from a field near Nyabing, Western Australia (230 km NW of Esperance). Populations came from fields that had been continuously cropped over a number of years and from farming systems using no-till practices since the late 1990s. All three populations have a history of glyphosate use with one or two applications of glyphosate per year applied for summer weed control. A further application of glyphosate just prior to seeding is usually followed by pre-emergent and postemergent herbicides. It is common for areas in southern Western Australia to use a number of applications of glyphosate during the summer months, as these areas typically receive extended periods of summer rainfall.

After collection in each year, seedlings were trimmed, planted in plastic pots containing potting mix (50% composted pine bark, 25% peat, and 25% river sand), and placed outdoors at the University of Western Australia during the winter growing season for rigid ryegrass. After 5 d, the seedlings were treated with 1,080 g/ha glyphosate with the use of a custom-built, dual nozzle (TeeJet® XR11001 flat fan) cabinet sprayer delivering herbicide in 110 L/ha water at 200 kPa, at a speed of 3.6 km/h. Plant survival was assessed 21 d after treatment, and surviving plants were grown to maturity and allowed to cross-pollinate in an enclosed area. Mature seeds were collected, and then stored in a glasshouse (average daily temperature 26 C) over summer to ensure dormancy release (Steadman et al. 2003). The seeds from these three populations (WALR70, WALR80, and WALR90) were used in all subsequent experiments. Known glyphosate-susceptible (VLR1) and glyphosate-resistant (NLR70) rigid ryegrass populations (Powles et al. 1998) were used as controls. Population WALR60 was also included in this study. This population came from a vineyard in the Margaret River region of Western Australia. It was originally confirmed to be glyphosate-resistant (Neve, unpublished data); however, its herbicide-resistance profile had not previously been studied.

Whole-Plant Dose Response to Glyphosate. In May 2006, seeds from WALR70 (possible glyphosate resistant), VLR1 (known glyphosate susceptible) and NLR70 (known glyphosate resistant), and WALR60 (known glyphosate resistant) were sown in 180-mm plastic pots containing 20 seeds per pot. The pots were maintained outdoors, watered, and fertilized as required during the winter growing season. At the two- to three-leaf stage, seedlings were sprayed with glyphosate at 0, 135, 270, 540, 1,080, 2,160, 4,320, and 8,640 g/ha, with three replicate pots per treatment. Seedlings were assessed 28 d after treatment and were scored as dead or alive (plants that had strongly tillered were scored as alive). Plant material was harvested and dried for 3 d at 70 C. Total aboveground dry biomass was recorded for each treatment. The experiment was repeated in August 2006.

In May 2008, seeds from the possible-resistant WALR80 and WALR90 populations, and the VLR1 and NLR70 controls were grown in pots and treated with glyphosate at 0, 135, 270, 540, 1,080, 2,160, and 4,320 g/ha with four replicates per treatment. Seedlings were assessed 21 d after spraying and plant material was harvested and dried as previously described. The experiment was repeated in August 2008.

Resistance Profile. To determine whether the glyphosate-resistant populations were resistant to other herbicide modes of action, seedlings were sprayed with label rates of a range of herbicides with known activity on rigid ryegrass (Table 1). All treatments were applied with a full dose of recommended adjuvants. In May 2007, 20 seeds of each population were sown in 180-mm pots containing a standard potting mixture with four replicate pots per herbicide treatment. The known susceptible population VLR1 was included for all herbicides. Plants were grown outdoors during the winter growing season and watered as required. Herbicides were applied at the two- to three-leaf seedling stage, as described previously. Plants were scored as dead or alive, 21 d after herbicide treatment.

For the pre-emergent herbicide trifluralin, 50 seeds of each population were planted at a depth of 0.5 cm in seedling trays (30 by 40 by 10 cm) containing potting mix, lightly covered with soil, watered, and left for 1 d to allow the seed to imbibe water before herbicide treatment. Trifluralin was applied at 960 g/ha. Immediately after treatment, 1 cm of untreated soil was placed on the soil surface to prevent trifluralin volatilization. Seedling emergence was recorded 21 d after treatment.

Data sets from repeated experiments were pooled and analyzed by ANOVA (GenStat[8]) with independent experiments

Table 1. Percentage survival of the herbicide-susceptible VLR1, and the glyphosate-resistant WALR60 and WALR70 ryegrass populations at the recommended field rates of a range of herbicides used for ryegrass control (percentage survival figures are the mean survival ± SE of four replicates, 20 plants/pot). Populations that are significantly different ($P = 0.05$) from the susceptible control are indicated by asterisks. Herbicides applied to ryegrass populations in 2007 are included.

		Rate	VLR1		WALR60		WALR70	
Herbicide class	Herbicide	g/ha	Mean	SE	Mean	SE	Mean	SE
Aryloxyphenoxypropionate	Diclofop[2a]	375	4	4	14	3	33*	9
	Fluazifop[3a]	53	1	2	1	1	23*	8
	Haloxyfop[4a]	52	0	0	0	0	0	0
	Clodinafop[3a]	50	0	0	8	7	43*	7
	Propaquizafop[2a]	45	0	0	0	0	0	0
Cyclohexanedione	Clethodim[5b]	60	0	0	0	0	0	0
	Sethoxydim[2a]	93	0	0	0	0	0	0
	Butroxydim[6b]	45	0	0	0	0	0	0
	Tralkoxydim[6d]	152	0	0	6	4	34*	8
Phenylpyrazoline	Pinoxaden[3c]	30	0	0	0	0	0	0
Sulfonylurea	Chlorsulfuron[1a]	40	13	2	13	6	43*	5
	Sulfometuron[1a]	15	0	0	0	0	3	2
Dinitroaniline	Trifluralin[1d]	960	0	0	0	0	0	0
Imidazolinone	Imazethapyr[1a]	70	23	7	16	10	70*	8
Bipyridyl	Paraquat[3a]	250	0	0	0	0	0	0
Triazine	Atrazine[3b]	750	0	0	23*	3	0	0

[a] Nonionic surfactant (alcohol alkoxylate 1 000 g/L).[6]

[b] Crop-oil surfactant (esterified seed-oil adjuvant).[7]

[c] Crop-oil surfactant (methyl esters of canola fatty acids 440 g/L).[3]

[d] Herbicides did not require addition of adjuvant according to manufacturer's label.

included as a main factor (experiment). When the experiment factor between repeated experiments was not significant, pooled data were used for subsequent nonlinear regression analysis.

Further statistical analysis was carried out with the use of the R programming language (R Development Core Team 2008). The herbicide rate causing 50% mortality (LD_{50}) or growth reduction (GR_{50}) of plants was calculated with the use of a logistic regression model

$$Y = d/1 + \exp[b(\log x - \log e)], \qquad [1]$$

where the parameter d is the upper limit, b is the slope of the curve, and e is the dose producing a response halfway between the upper and lower limit (50% reduction). The R program was used to compare dose–response curves (Knezevic et al. 2007) and LD_{50} values were calculated accordingly. The level of resistance was measured as the R : S (resistant : susceptible) ratio of estimated LD_{50} values. Mortality dose–response graphs are presented with untransformed data. Shoot dry weight was expressed as a percentage of the mean of the nontreated controls. To compare the resistance profile of VLR1, WALR60, and WALR70, data were analyzed by one-factor ANOVA at the 5% level of significance, and differences between pairs of treatment means were assessed by least significant tests.

Results and Discussion

Whole-Plant Dose Response to Glyphosate. As expected, the glyphosate dose–response study established 100% mortality of the known susceptible (VLR1) rigid ryegrass biotype at rates of 580 g/ha or higher (Figure 1). In contrast, putative-resistant populations WALR60 and WALR70 were established to be glyphosate-resistant (Figure 1) and were found to be as resistant as the known glyphosate-resistant (NLR70) rigid ryegrass population (Powles et al. 1998). There was very little mortality of glyphosate-resistant populations at the Australian label rate for glyphosate (490 g/ha), and the LD_{50} value for the resistant populations was established to be 11 times greater than that of the susceptible population (Table 2). The glyphosate rate causing 50% mortality for WALR60 was 3,260 g/ha, and for WALR70 it was 3,280 g/ha. On the basis of the R : S ratio (LD_{50}), these populations are confirmed to be resistant to glyphosate (Table 2).

Populations WALR80 and WALR90 were examined in 2008 (Figure 2) and were similar in survival percentage to the known glyphosate-resistant population WALR50 characterized by Neve et al. (2004). Both these populations had a lower level of resistance than the known-resistant NLR70. The rate causing 50% mortality for WALR80 was 550 g/ha and for WALR90, 290 g/ha. Based on the LD_{50} R : S ratios, these populations are also confirmed to be resistant to glyphosate. WALR80 and WALR90 are two- to threefold more resistant than VLR1 (Table 2). The LD_{50} values for the control populations (VLR1 and NLR70) were lower in 2008 than in 2006. The differences may have resulted from slight variations in environmental conditions (temperature was on average 1 C cooler in 2006 during the same period) experienced at the time the experiments were conducted, which can influence the efficacy of glyphosate (Powles et al 1998).

The growth–response curves showed a marked difference in the populations' response to glyphosate (Figures 3 and 4), with glyphosate dramatically reducing shoot dry mass production in the susceptible biotype (VLR1). The glyphosate rate causing 50% reduction of growth (GR_{50}) of the WALR70 population was 2,150 g/ha, for WALR60 it was 3,800 g/ha, and for the known-resistant NLR70 it was

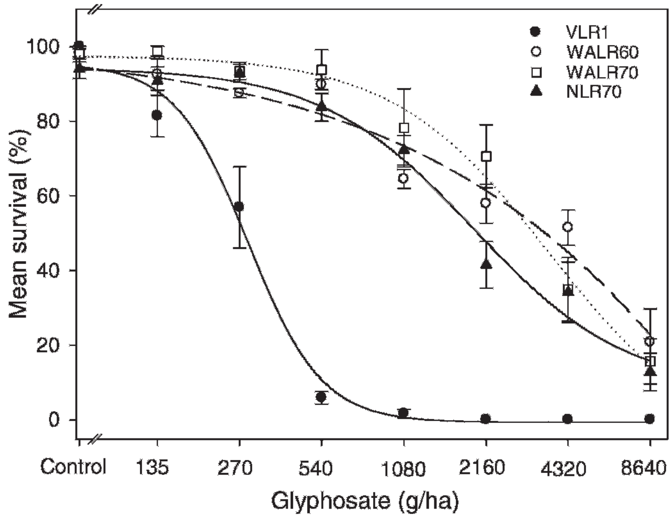

Figure 1. Dose–response curves for survival of a glyphosate-susceptible rigid ryegrass population VLR1 (●), and glyphosate-resistant populations WALR60 (○), WALR70 (□), and NLR70 (▲) treated with a range of glyphosate doses in 2007. Each data point represents mean percentage survival ± SE of three replicate treatments.

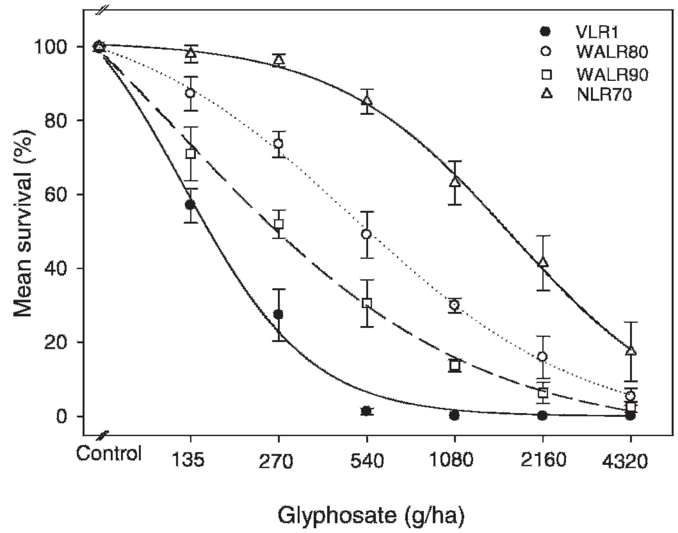

Figure 2. Dose–response curves for survival of a glyphosate-susceptible rigid ryegrass population VLR1 (●) and glyphosate-resistant populations WALR80 (○), WALR90 (□), and NLR70 (△) treated with a range of glyphosate doses in 2008. Each data point represents mean percentage survival ± SE of four replicate treatments.

2,010 g/ha. To reduce growth by 50%, WALR70 and WALR60 required 17 to 30 times more glyphosate than the susceptible biotype (120 g/ha) (Table 2). In contrast, much lower rates of glyphosate (910 and 500 g/ha) are required to reduce the growth of populations WALR80 and WALR90 (Table 2).

The differences between these glyphosate-resistant populations in terms of growth and survival responses with varying levels of glyphosate could be due to the involvement of different resistance mechanism(s) and whether individuals are homozygous or heterozygous for the resistance mechanisms (Michitte et al. 2007; Perez-Jones et al. 2007; Powles and Preston 2006; Wakelin and Preston 2006; Yu et al. 2007a).

WALR70 and WALR60 Herbicide-Resistance Profile. In addition to confirmed resistance to glyphosate, the WALR60 and WALR70 populations displayed multiple resistance to field-applied rates of several ACCase- and ALS-inhibiting herbicides (Table 1) which controlled the herbicide-susceptible population VLR1. The WALR60 and WALR70 populations had slightly different resistance profiles. Population WALR60 displayed relatively low-level resistance to the ACCase-inhibiting herbicides diclofop, clodinafop and tralkoxydim (compared with VLR1), and remained susceptible to the ALS-inhibiting herbicides chlorsulfuron and imazethapyr. Population WALR70 displayed resistance to both ACCase- and ALS-inhibiting herbicides (Table 1). Resistance to these herbicides was not fixed within the populations, and approximately 65% of WALR70 exhibited a susceptible phenotype at the dose applied. In contrast, nearly 90% of WALR60 exhibited a susceptible phenotype to the same herbicides. WALR60 also had plants with resistance to atrazine. These populations have a different resistance profile to the population WALR50 (Neve et al. 2004) which has the ALS Pro-197-Arg mutation (Yu et al. 2008). The resistance profiles for WALR80 and WALR90 were not studied.

Both the WALR60 and WALR70 populations were found to be susceptible to paraquat, trifluralin, sulfometuron, sethoxydim, butroxydim, haloxyfop, and pinoxaden. Research

Table 2. LD_{50} and GR_{50} values and standard errors in parentheses of ryegrass populations VLR1, NLR70, WALR60, WALR70, WALR80, and WALR90 treated with glyphosate. R : S ratios were calculated as a ratio of LD_{50} and GR_{50} values of resistant and susceptible populations.[a]

Biotype	LD_{50} (g/ha)	R/S ratio of LD_{50}	GR_{50} (g/ha)	R/S ratio of GR_{50}
		2006 experiment		
VLR1 (S)	290 (22)	n/a	122 (42)	n/a
NLR70 (R)	2 271 (496)	7.8	2 015 (459)	16.5
WALR60 (R)	3 260 (107)	11.2	3 809 (291)	31.2
WALR70 (R)	3 285 (305)	11.3	2 152 (548)	17.6
		2008 experiment		
VLR1 (S)	159 (10)	n/a	150 (16)	n/a
NLR70 (R)[b]	1 632 (63)	10.3	1 291 (73)	8.6
WALR80 (R)[b]	553 (20)	3.5	916 (310)	6.1
WALR90 (R)[b]	292 (19)	1.8	503 (8)	3.4

[a] Abbreviations: LD_{50}, the dose lethal to 50% of the population; GR_{50}, the dose required to reduce growth by 50%; R, resistant; S, susceptible.

[b] The LD_{50} and GR_{50} ratio is compared to that of VLR1 used as the susceptible control in the 2008 experiments.

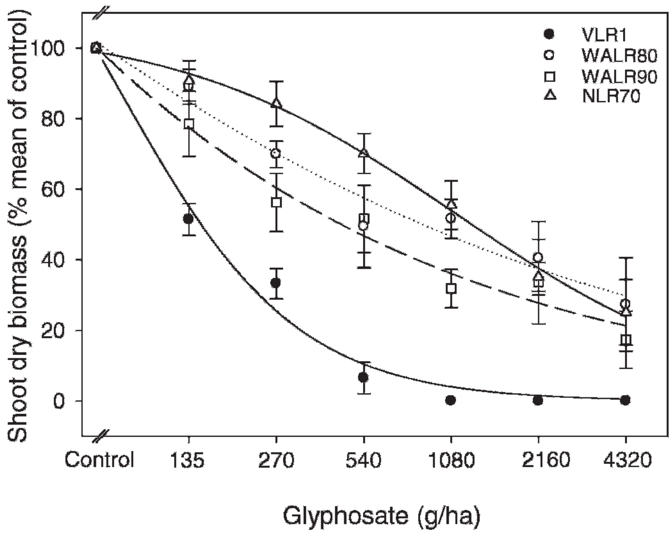

Figure 3. Dose–response curves for aboveground biomass of a glyphosate-susceptible rigid ryegrass population VLR1 (●) and glyphosate-resistant populations WALR80 (○), WALR90 (□), and NLR70 (△) treated with a range of glyphosate doses in 2008. Data points represent mean dry biomass (expressed as percent of control) ± SE of four replicate treatments.

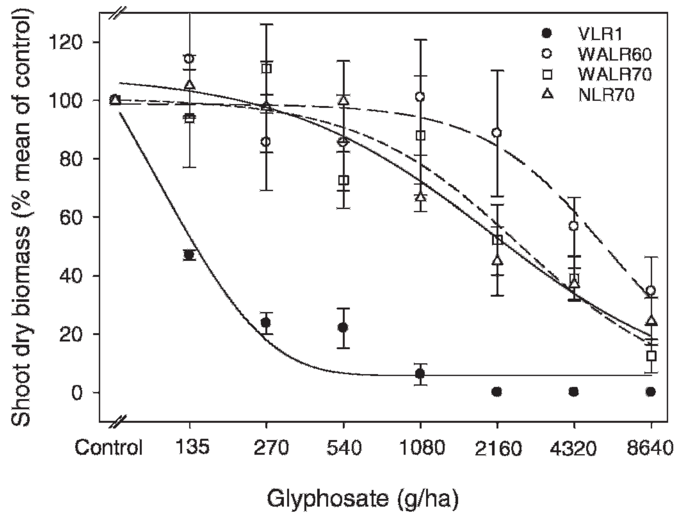

Figure 4. Dose–response curves for aboveground biomass of a susceptible rigid ryegrass population VLR1 (●) and resistant populations WALR60 (○), WALR70 (□), and NLR70 (△) treated with a range of doses of glyphosate in 2007. Data points represent mean dry biomass (expressed as percent of control) ± SE of three replicate treatments.

has revealed that different mutations of the ACCase or ALS gene and/or enhanced rates of herbicide metabolism can endow resistance to ACCase- and ALS-inhibiting herbicides in rigid ryegrass (Yu et al. 2007b, 2008). As these populations survived diclofop and clodinafop (which can be metabolized by plants) and not the other herbicides (e.g., sethoxydim, which cannot be rapidly metabolized), it is postulated that these populations have a metabolism-based resistance mechanism endowing resistance to ACCase-inhibiting herbicides. The mechanisms of multiple resistance in population WALR60 and WALR70 is investigated in another study (Yu et al. 2009)

In conclusion, dose-response studies confirm that an additional three glyphosate-resistant ryegrass populations have evolved in the Western Australia grain belt. Along with glyphosate resistance, these populations exhibit multiple resistance to other herbicide groups. Management options for glyphosate-resistant populations may vary depending on the mechanisms of resistance; therefore, understanding the different response of these populations to glyphosate is imperative. With the introduction of transgenic glyphosate-resistant canola to the southern Australian cropping regions, from 2009 there is likely to be an increase in glyphosate usage and it is important to recognize that alternate strategies should be initiated before resistance becomes prominent. Maintaining diversity in weed management and herbicide usage will be most important to help preserve glyphosate efficacy.

Sources of Materials

[1] Glyphosate, chlorsulfuron, sulfometuron, trifluralin, imazethapyr, Nufarm Australia, Ltd., 4 Laverton Rd., North Victoria, Australia.

[2] Diclofop, sethoxydim, propaquizafop, Bayer CropScience, Pty. Ltd., 391-393 Tooronga Road, East Hawthorn, Victoria, Australia.

[3] Fluazifop, clodinafop, pinoxaden, paraquat, atrazine, nonionic surfactant, Syngenta Crop Protection Pty. Ltd., Level 1, 2-4 Lyonpark Road, Macquarie Park, NSW, Australia.

[4] Haloxyfop, Dow Agro Sciences Australia Ltd., Locked Bag 502, Frenchs Forest, NSW, Australia.

[5] Clethodim, Sumitomo Chemical Australia, 501 Victoria Avenue, Chatswood, NSW, Australia.

[6] Tralkoxydim, butroxydim, nonionic surfactant, Crop Care Australasia Pty. Ltd., Portal North, Unit 15, 16 Metroplex Avenue, Murarrie, QLD, Australia.

[7] Crop-oil concentrate, Victorian Chemicals, 83 Maffra St., Coolaroo, Victoria, Australia.

[8] GenStat version 9.1.0.147, VSN International, Oxford, United Kingdom.

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