South African Journal of Botany 121 (2019) 248–256

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Growth-stage and temperature influence glyphosate resistance in *Conyza bonariensis* (L.) Cronquist

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ARTICLE INFO

Article history:
Received 27 February 2018
Received in revised form 18 July 2018
Accepted 29 October 2018
Available online xxxx

Edited by PN Hills

ABSTRACT

Glyphosate, currently the world's most extensively used herbicide was on the market for more than 20 years since its introduction in 1975 without reported evolution of resistant weed cases. Glyphosate is the only reported herbicide to inhibit 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), and endogenous accumulation of shikimic acid has been used as a biomarker for the herbicide's activity in plants after spraying. Increased levels of shikimic acid indicate sensitivity or injury while a lack or limited accumulation of shikimic acid shows resistance to glyphosate. *Conyza bonariensis* seed germinates in flushes in South Africa and its emergence is staggered throughout the year under conducive temperature conditions. This variability in growth stages in the same field poses a challenge in chemical control. Glyphosate herbicide efficacy is affected by environmental conditions, particularly temperature. A glyphosate resistance screening study was carried out based on reports from farmers that *C. bonariensis* has become increasingly difficult to control especially in conservation tillage systems. This study has confirmed the occurrence of glyphosate-resistant *C. bonariensis* (hairy fleabane) cases in the western and southern Cape regions of South Africa. A glasshouse screening experiment of 24 biotypes indicates resistance levels ranging from 0.6 to 26.9-fold, with GR_{50} values of up to 3908.42 g.ae.ha^{-1} being reported in a population collected from a vineyard in the Piketberg district of the western Cape. The influence of growth stage and temperature on glyphosate resistance in hairy fleabane was evaluated by using a shikimate assay. Results from this study have shown that response of hairy fleabane to glyphosate is influenced by the phenological stage, with sensitivity or injury decreasing with growth stage. The significant statistical tests verified the dependence of the shikimate pathway on temperature, with more shikimic acid accumulating at 15 °C compared to 27 °C across all biotypes, regardless of origin and glyphosate tolerance status. More tolerance to the herbicide was demonstrated under warm temperature conditions. Possible outcomes of applying glyphosate at the bolting stage and under warm temperature conditions are escapes and no control of susceptible and resistant plants, respectively.

1. Introduction

Glyphosate, was commercialized as a broad-spectrum, non-selective, post-emergence herbicide in 1975. It quickly acquired the reputation of being the world's most widely used and fastest growing agrochemical in modern agriculture (Baylis, 2000). Glyphosate, a glycine compound, is a white and odorless crystalline salt. The worldwide importance of glyphosate has been attributed to its rapid environmental degradation and minimal contamination of ground water, low toxicity to mammals, low costs and effective systemic action on most plants (Baylis, 2000). This has resulted in an over-reliance on the herbicide as a sole weed control method, especially in conservation tillage and glyphosate-resistant cropping systems (Nandula, 2010). This situation has given rise to evolution of glyphosate-resistant weeds, with the first case reported in rigid ryegrass (*Lolium rigidum* Gaud.) in Australia (Pratley et al., 1996). More glyphosate resistance cases have since been reported, and according to Heap (2017), 37 weed species have thus far been confirmed to have evolved glyphosate resistance in 29 different countries. In South Africa, *Conyza bonariensis*, *Lolium rigidum* and *Plantago lanceolata* have reportedly evolved resistance to glyphosate (Pieterse, 2010; Heap, 2017).

Glyphosate acts by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Steinrucken and Amrhein, 1980), an enzyme found in the shikimate pathway in plants and microorganisms like fungi and bacteria, but not in animals (Kishore and Shah, 1988; Herrmann, 1995). This pathway is one of the major and most active biosynthetic pathways in higher plants. It is estimated that 20% of all fixed carbon flows through it (Herrmann, 1995; Herrmann and Weaver, 1999; McCue and Conn, 1990). The shikimate pathway is crucial in the manufacture of aromatic amino acids, phenylalanine, tyrosine and tryptophan, which are used as protein building blocks and precursors for

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https://doi.org/10.1016/j.sajb.2018.10.034
0254-6299/

secondary metabolites (Jensen, 1986; Herrmann, 1995; McCue and Conn, 1990). Inhibition of EPSPS by glyphosate is competitive with respect to phosphoenolpyruvate (PEP) binding to EPSPS but uncompetitive with respect to shikimate 3-phosphate (S3P) (Dill, 2005; Boocock and Coggins, 1983; Herrmann and Weaver, 1999). Glyphosate binds to the EPSPS enzyme–S3P complex (Glyphosate: S3P: EPSP synthase enzyme) which forms a dead-end complex that's highly stable with slow reversal rate, which curtails progression of the EPSPS reaction in forming downstream products (Bradshaw et al., 1997; Dill, 2005). This mode of action has been attributed to the unique broad-spectrum control of weeds and overall success of the herbicide, since other enzymes that utilize PEP as a substrate, are not inhibited by glyphosate (Herrmann and Weaver, 1999; Grossbard and Atkinson, 1985). Inhibition of EPSPS by glyphosate results in accumulation of shikimic acid in vacuoles (Holländer-Czytko and Amrhein, 1983; Amrhein et al., 1983). Endogenous accumulation of shikimic acid is used as a biomarker for the herbicide's activity in plants after spraying (Mueller et al., 2003, 2008; Powles and Preston, 2006; Zelaya et al., 2011). Shikimic acid is a good indicator of glyphosate efficacy because it occurs at sufficiently high concentrations for chemical detection and is chemically stable, which allows for ease of sample processing (Mueller et al., 2008). Elevated levels of shikimic acid in treated plant tissues indicates glyphosate injury or sensitivity while a lack or limited accumulation of shikimic acid shows resistance of the plant to the herbicide (Singh and Shaner, 1998).

Conyza bonariensis (L.) Cronquist, a native of South America, belongs to the Asteraceae family (Botha, 2001). In South Africa, hairy fleabane has been described as a weed of cultivated fields, especially in annual crops of conservation tillage and perennial cropping systems such as vineyards and orchards, as well as along roadsides and in disturbed areas (Botha, 2001). The weed is exceptionally prolific, producing more than 100,000 seeds per plant that lack dormancy (Wu and Walker, 2004). Seed germinates in flushes and is staggered throughout the year under conducive environmental conditions. The variability in growth stages in the same field poses a challenge in chemical control (Fig. 1) (Wu et al., 2010). Herbicide efficacy is affected by environmental conditions before, at and after herbicide application (Kudsk and Kristensen, 1992). At constant relative humidity, air temperature may alter permeability of the cutin matrix and rate of spray droplet drying on the plant (Steckel et al., 1997; Grossbard and Atkinson, 1985). Temperature has been reported to have a profound effect on the activity of glyphosate in various weed species; such as *Sorghum halepense* and *Lolium rigidum* (Powles et al., 1998; Vila-Aiub et al., 2013), *Echinochloa colona* Nguyen et al., 2016; Tanpipat et al., 1997), *C. canadensis* (Ge et al., 2011); with better control being reported at low temperature.

Since *Conyza bonariensis* emerges at different times of the year in South Africa, it was important to assess the effect of temperature and growth stage on glyphosate activity at the time of herbicide application. To our knowledge, this is the first study done using a shikimate assay to assess the effect of growth stage and temperature on glyphosate resistance in hairy fleabane from South Africa. Therefore, the objectives of this study were to (1) assess the glyphosate-response of different *C. bonariensis* populations by using a dose–response approach, (2) compare changes in shikimic acid accumulation in glyphosate-susceptible (GS) and glyphosate-resistant (GR) *C. bonariensis* biotypes at two phenological stages; rosette (4–6 leaf stage) and bolting (40–50 leaf stage) phenological stages, and (3) evaluate the role of temperature on shikimic acid accumulation at two temperature regimes; 15 and 27 °C at the rosette stage. The hypothesis that the growth stage and temperature at which glyphosate is applied to *C. bonariensis* has an effect on shikimic accumulation, thus, glyphosate resistance, was tested. From previous evidence, it is expected that shikimic acid will be higher in plants treated at the rosette stage and under cold temperature conditions. If proven, GR *C. bonariensis* biotypes treated at the rosette stage and during cold temperature conditions would be sensitive to glyphosate, resulting in better control.

Fig. 1. Variability in growth stage in *C. bonariensis* in an orchard at Hatfield experimental farm.

2. Materials and methods

2.1. Plant material sources

Seeds of hairy fleabane were collected from 24 different geographical areas in South Africa from February to March, 2013, predominantly from the western and southern Cape provinces. Selection of sampling sites was in part based on field reports of poor weed control with glyphosate, in particular in orchards and vineyards in the Western Cape. Sampling sites were approximately 50 km apart, and included agricultural areas such as vineyards, orchards, wheat fields, alfalfa fields, grass pasture and non-agricultural areas like roadsides (Table 1). At each sampling site, seeds were collected from 30 to 50 mature plants. Seeds were then bulked and put inside labeled brown paper envelopes. Seeds were stored in a dry place at ambient room temperature until use. For each sampling site, the location was recorded using Global Positioning System (GPS) technology.

Table 1
Geographic origin and habitat from which hairy fleabane seeds were collected in western and southern Cape regions of South Africa.

Population code	Location	Latitude (S)	Longitude (E)	Habitat
WP 8	Modder River	29°02.838′	024°40.042′	Alfalfa
WP 10	Orania	29°48.561′	024°24.924′	Orchard: Pecan
WP 12	De Rust I	33°18.482′	022°28.627′	Alfalfa
WP 16	De Rust II	33°29.512′	022°29.346′	Orchard: Olive
WP 19	George I	33°50.955′	022°21.328′	Orchard: Hops
WP 20	George II	33°58.507′	022°24.873′	Pasture
WP 22	Swellendam	34°07.451′	020°21.809′	Wheat
WP 24	Bonnievale	33°56.651′	020°01.179′	Vineyard
WP 25	Montagu	33°47.381′	020°07.111′	Vineyard
WP 26	De Doorns	33°47.381′	020°07.113′	Vineyard
WP 28	Worcester	33°35.713′	019°31.149′	Vineyard
WP 29	Rawsonville	33°35.406′	019°17.543′	Orchard: Peach
WP 31	Wellington	33°35.408′	019°17.543′	Wheat
WP 32	Stellenbosch	33°55.544′	018°47.779′	Vineyard
WP 33	Durbanville	33°47.646′	018°.40.226′	Vineyard
WP 34	Malmesbury I	33°22.235′	018°41.536′	Vineyard
WP 35	Malmesbury II	33°21.636′	018°35.980′	Wheat
WP 36	Riebeeck-Wes	33°20.204′	018°52.666′	Vineyard
WP 37	Porterville	33°08.750′	019°00.341′	Vineyard
WP 38	Piketberg	33°08.752′	019°00.341′	Vineyard
WP 39	Citrusdal	32°36.849′	019°02.006′	Orchard: Citrus
WP 40	Clanwilliam	32°04.146′	019°06.706′	Roadside
WP 41	Darling	32°09.328′	018°37.469′	Wheat
WP 42	Fauresmith	–	–	Pasture

2.2. Glyphosate dose–response

This study was carried out according to Beckie et al. (2000). Seeds were planted on the surface of 12.5 cm diameter plastic pots (Calibre Plastics Ltd.) filled with a potting mix consisting of sand (Silica Quartz (Pty) Ltd.) and coconut coir mixture (Pelemix Ltd.) in the ratio of 6:1 (sand:coir by weight). Previous studies have indicated that hairy fleabane seed emergence is sensitive to soil burial depth and that no emergence occurs at burial depths of more than 2 cm (Wu et al., 2007). The pots were maintained in a glasshouse at the University of Pretoria's Hatfield experimental farm in 2014, with average day temperature of 24.4 °C and photoperiod of 12/12 h under natural sunlight. Plants were watered three times a week with tap water and fertilized with 100 mL Hydroponics fertilizer (NPK 1.6:1:5) (Hygrotech South Africa) in the ratio of 1 g granular fertilizer to 1 L tap water twice per week. Plants were thinned to two plants per pot and at the rosette stage (6–8 cm diameter, with 4–6 leaves), plants were treated with commercial glyphosate formulation (potassium salt), Roundup Turbo® (Monsanto South Africa (Pty) Ltd.), at rates of 0 (untreated control), 225, 450, 900, 1800 and 3600 g.ae.ha^{-1}. These glyphosate application rates correspond to 0, 0.25, 0.5, 1, 2 and 4 times the registered label recommended or normal-use rate of 900 g.ae.ha^{-1}. Glyphosate was diluted in 2% ammonium sulfate (ATP AMSUL-50, Villa Crop Protection) solution prepared with tap water. Plants were sprayed with an Oxford Precision hand-held sprayer that delivered the equivalent of 200 L ha^{-1} at a pressure of 200 kPa using RS-MM 110^{o}/04 nozzles. Spraying was carried out in an enclosed spraying room to prevent contamination of glyphosate to non-target plants. Plants were watered on the evening prior to spraying to avoid having to apply water within 24 h. This time lapse prevented the herbicide being washed off before absorption had taken place. Plants were returned to the glasshouse and watering resumed 24 h after treatment. Seven, 14 and 21 days after treatment (DAT), plants were visually evaluated for herbicide damage. Plants were considered resistant if they survived glyphosate application and produced new growth, and susceptible if they showed severed necrosis, wilting, retarded growth or plant death. Twenty-one days after treatment, all plants were clipped at the soil surface; only green shoot tissue was weighed and fresh mass recorded. The experiment was arranged in a completely randomized design with five replicates.

2.3. Glyphosate resistance map of screened populations

Using results from the screening experiment, a glyphosate resistance weed map was generated using ArcMap 10.3.1 program from ESRI. Information fed into the program included: population, GPS coordinates, habitat and resistance factor. Herbicide resistance across biotypes is compared based on a statistically calculated dose that results in 50% growth reduction (GR_{50}) from regression analyses. The magnitude or level of herbicide resistance is expressed as a ratio of the GR_{50} of resistant to a reference susceptible biotype (R/S). Ideally, responses of multiple susceptible biotypes are compared and an average biotype used as the reference biotype in all R/S calculations (Burgos et al., 2013). A resistance ratio, also called Resistance Index (RI) or Resistance Factor (RF) that is less than 10-fold should be counted as low level or partial herbicide resistance (Heap, 2005). Based on this classification, resistance factors were put into two categories; '1' representing susceptible populations, with resistance factors from 0 to 10 and '2' resistant populations with resistance factors above 10.

2.4. Effect of growth stage on shikimic acid accumulation

One resistant (Swellendam) and one susceptible (George) biotypes were established in the glasshouse at the Hatfield experimental farm. At the 4–6 leaf stage (rosette) and 40–50 leaf stage (bolting), plants were treated with glyphosate at a rate of 900 g.ae.ha^{-1}, as described previously. Aboveground plant tissue was sampled at 0 (before treatment), 2, 4, 6, 8 and 10 days after treatment (DAT) for the experiment at rosette stage and up to the eighth day for the evaluation at bolting stage. One plant was treated as a replicate and three biological replicates were used. Extraction and HPLC analysis of shikimic acid was performed using a modification of the method used by (Singh and Shaner, 1998).

2.4.1. Extraction of shikimic acid

Samples were placed in liquid nitrogen and stored at −80 °C until use. Frozen tissue was finely ground to powder in liquid nitrogen using a mortar and pestle after which 100 mg of tissue was weighed into labeled 1.5 mL Eppendorf microfuge tubes. Shikimic acid extraction was done in 0.25 N Hydrochloric acid (37% Sigma–Aldrich) where 0.9 mL of HCL was added, resulting in approximately 1 mL final volume (assuming the tissue is 100% H_2O). Plant tissue was further ground using a pestle for 5 min, followed by vortexing for 4–5 min. The tubes were placed in a VWR Ultrasonic cleaner (bath) set at 25 °C for 8 min to completely macerate the tissue. The extraction tubes were then spun at 14,000 rpm at 25 °C for 10 min and the supernatant transferred to clean 1.5 mL Eppendorf microfuge tubes. The extract was filtered through a 0.22 μm syringe filter and used directly for analysis by HPLC.

2.4.2. Reversed-phase high-pressure liquid chromatography analysis of shikimic acid

An aliquot of 20 μL of the filtered supernatant was injected into an Agilent Hewlett Packard (Wilmington, DE) 1100 series liquid chromatograph equipped with Chemstation software and a diode array detector using a wavelength of 210 nm. The column used was an Agilent Polaris 5 NH_2 (250 × 4.6 mm, 5 μm particle size) with a flow rate of 1 mL min^{-1}. The isocratic mobile phase consisted of 95% acetonitrile, 1% orthophosphoric acid, and 4% water. The retention time of shikimic acid was approximately 8.3 min with a total run of 20 min. A 5-point calibration curve using pure/analytical shikimic acid (99%, Sigma–Aldrich) standards with known concentrations ranging from 10 to 100 μg.mL^{-1} was used to externally quantify shikimic acid in sample extracts. Shikimic acid in μg.g^{-1} fresh biomass was determined based on the standard curve.

2.5. Effect of temperature on shikimic acid accumulation

2.5.1. Rationale for selection of temperature regimes

Five-year average maximum and minimum temperatures for summer (November–February) and winter (June–August) at Hatfield experimental farm were 28.9/16.1 °C and 21.7/13 °C, respectively. Based on these average temperatures, two temperature regimes were chosen, 27/20 °C (day/night) and 15/10 °C (day/night) for warm and cold temperature regimes, respectively, to mimic the summer and winter average temperatures.

Plants were established as in the previous experiment and at the rosette stage (6–8 cm diameter with 4–6 leaves), plants were transferred to phytotrons set at 27/20 °C (day/night) for the warm temperature regime and 15/10 °C (day/night) for the cold temperature regime. Two biotypes were used: resistant—Swellendam and susceptible—George. Both phytotrons were maintained at 80–90% relative humidity, 13/11 h day length and 500 μmol m^{-2} s^{-1} light intensity. Plants were maintained in the respective phytotrons for one week to allow plants to get acclimatized with the temperatures before being treated with glyphosate herbicide as described in the previous experiment. Aboveground plant tissue was sampled at 0 (before treatment), 2, 4, 6 and 8 days after treatment (DAT) and shikimic acid extraction and evaluation done as described before. One plant was treated as a replicate and three biological replicates were used.

2.6. Statistical analyses

All experiments were arranged in a completely randomized design. Data were subjected to analysis of variance using PROC GLM procedure

in SAS software version 9.3 (SAS 2002–2010, SAS Institute Inc., Cary, NC, USA) and mean comparison done with Tukey's Studentized Range (HSD) test at P = .05 to determine significant differences. A three-parameter non-linear regression model, described by Seefeldt et al. (1995) was used to generate dose–response curves in R version 3.2.3 (R Development Core Team; http://cran.at.r-project.org/) using the 'drc' package (Ritz and Streibig, 2005), using the following equation:

$$Y = d/(1 + \exp.[b(\log x - \log e)]\} \quad (1)$$

where, Y is above-ground fresh-biomass expressed as a percentage of the untreated control, d is the upper limit, e is the herbicide dose required to reduce growth by 50% (GR_{50}), b is the slope of the curve around GR_{50}, and x is the herbicide dose. The relative level of glyphosate herbicide resistance among the populations was determined by calculating the resistance ratio (R/S), which represents the GR_{50} of a population divided by GR_{50} of the average susceptible population.

3. Results

3.1. Glyphosate-resistant hairy fleabane is confirmed in the western and southern Cape regions of South Africa

Significant differences in biomass reduction in response to increasing glyphosate doses were observed. From 5 to 14 days after treatment, phytotoxic effects were visible, with varying degrees of damage on the aboveground tissues. Yellowing of the youngest terminal leaves in sprayed plants was observed in all populations which progressed into chlorosis of the rosettes. In the susceptible populations, necrotic lesions developed on the leaves, followed by wilting particularly in plants that were treated at 900 g.ae.ha^{-1} and above. From 15 to 21 DAT, the susceptible plants had died. In contrast, the resistant plants generated new leaves and the pigmentation changed from pale yellow to green (Fig. 2).

According to Heap (2005), a Resistance Factor (RF) that is less than 10-fold should be counted as low level or partial herbicide resistance. Based on this definition, 37.5% of the biotypes screened showed resistance factors that were greater than 10, with GR_{50} values ranging from 1620.92 to 3908.42 g.ae.ha^{-1} (Table 2). The level of resistance ranged from 0.6-fold for the most susceptible biotype to 26.9-fold for the most resistant biotype.

Hairy fleabane seeds used for screening were collected from diverse cropping and non-cropping areas (Fig. 3). Six out of ten biotypes sampled from vineyards were termed resistant, having resistance factors above 10 (Table 2). The registered normal-use rate in South Africa for control of hairy fleabane is 900 g.ae.ha^{-1}. These biotypes needed approximately 1.8 to 4.3 times the normal-use rate of glyphosate to attain 50% biomass reduction. Biotypes collected from orchards showed varying resistance levels, from 1.0 to 11.5-fold. A biotype from Swellendam collected from a wheat field showed a high resistance level of 20.4-fold with a GR_{50} value of 2958.62 g.ae.ha^{-1}, which required application doses 3.3 times higher than the normal-use rate. A biotype sampled from a roadside in Clanwilliam showed a high resistance level of 13.3-fold and required glyphosate of 1930.40 g.ae.ha^{-1} for 50% biomass reduction. All the biotypes that were collected from pasture and alfalfa fields showed susceptibility to glyphosate, having resistance levels below 1.3 and requiring less than 0.2 times normal-use rate for glyphosate to cause 50% growth reduction (Fig. 3). Significant differences in biomass reduction were also noted in biotypes from the same district. In the two biotypes from Malmesbury, the resistant one from a vineyard had an R/S factor of 18.2 and required 2640.37 g.ae.ha^{-1} glyphosate for 50% biomass reduction, compared to the susceptible biotype from a wheat field whose R/S factor was estimated to be 1.0 and GR_{50} was 144.80 g.ae.ha^{-1}. Similarly, in the two biotypes from De Rust, the resistant one from an olive orchard showed a resistance level of 8.0-fold as opposed to the susceptible biotype from an alfalfa field that indicated low resistance levels of 0.6-fold.

Fig. 2. Hairy fleabane response to glyphosate 21 DAT at 0, 1× and 4× normal-use rate of 900 g.ae.ha^{-1}. WP 22 is the resistant biotype from Swellendam while WP 19 the susceptible biotype from George.

3.2. Hairy fleabane is more sensitive to glyphosate at rosette than bolting stage

A glyphosate dose of 900 g.ae.ha^{-1} caused visible phytotoxic effects, with severe changes in plants sprayed at rosette stage as compared to bolting stage, regardless of biotype. Chlorotic lesions and wilting was

Table 2
Estimates calculated from 3-parameter log-logistic nonlinear regression analyses (Eq. 1) of glyphosate dose response resulting in 50% (GR_{50}) reduction in above ground shoot biomass of hairy fleabane biotypes collected from twenty four locations in western and southern Cape regions of South Africa.

Location	Habitat	GR_{50}[a] (g·ae·ha^{-1})	R/S ratio[b]
Modder River	Alfalfa	85.50 (64.33)	0.6
De Rust 1	Alfalfa	93.29 (65.25)	0.6
George 2	Pasture	97.61 (67.46)	0.7
Malmesbury 2	Wheat	144.80 (58.50)	1.0
George 1	Orchard	145.36 (81.11)	1.0
Darling	Wheat	155.80 (67.78)	1.1
Fauresmith	Pasture	192.65 (41.76)	1.3
Wellington	Wheat	268.04 (65.69)	1.8
Orania	Orchard	344.06 (64.39)	2.4
Durbanville	Vineyard	430.21 (330.87)	3.0
Montagu	Vineyard	998.71 (661.81)	6.9
Riebeeck-Wes	Vineyard	1094.95 (298.73)	7.5
De Rust 2	Orchard	1157.99 (496.54)	8.0
Rawsonville	Orchard	1218.58 (606.34)	8.4
Worcester	Vineyard	1383.78 (392.04)	9.5
Bonnievale	Vineyard	1620.92 (471.62)	11.2
Citrusdal	Orchard	1671.99 (496.51)	11.5
Porterville	Vineyard	1844.16 (592.46)	12.7
Clanwilliam	Roadside	1930.40 (467.50)	13.3
Malmesbury 1	Vineyard	2640.37 (893.06)	18.2
De doorns	Vineyard	2877.40 (513.88)	19.8
Swellendam	Wheat	2958.62 (551.99)	20.4
Stellenbosch	Vineyard	3167.37 (1171.51)	21.8
Piketberg	Vineyard	3908.42 (1458.27)	26.9

Estimates represent mean fresh biomass expressed as a percentage of untreated control ± standard error in g·ae·ha^{-1}.

[a] Abbreviations: GR_{50} – glyphosate dose resulting in 50% reduction in shoot biomass in hairy fleabane 21 days after treatment.

[b] Resistance factor at GR_{50} value of respective hairy fleabane biotype divided by GR_{50} value of average susceptible biotype (George 1).

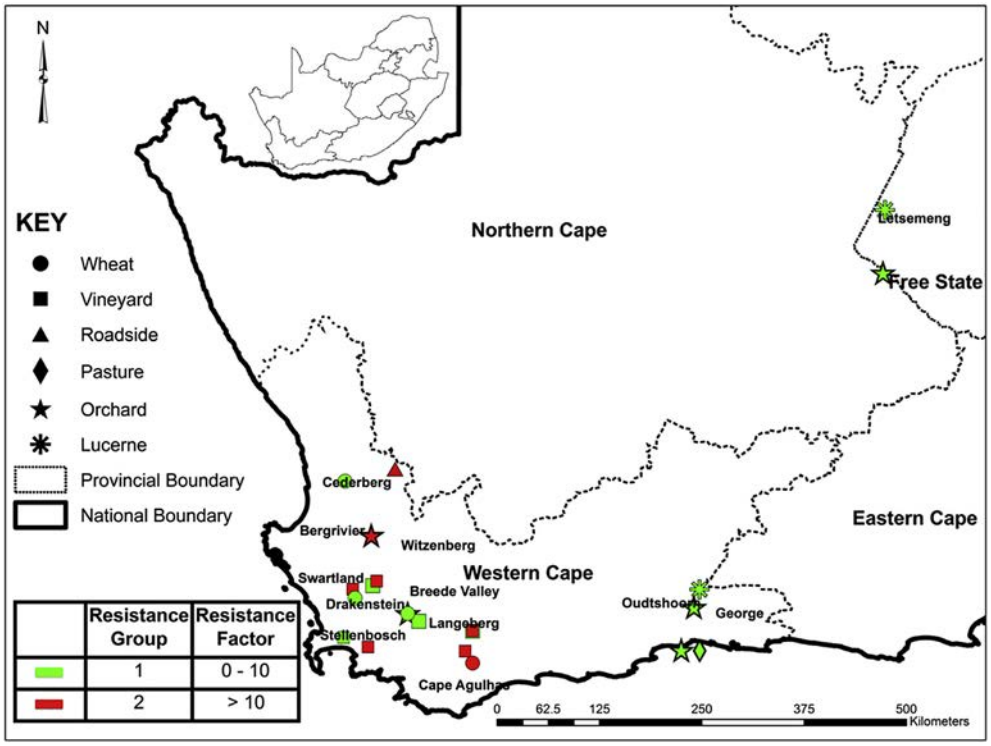

Fig. 3. Map of South Africa showing response of *Conyza bonariensis* to glyphosate herbicide after screening in glasshouse. Symbols indicate cropping system or habitat where seed was collected; colors represent resistance level status or group.

observed in all susceptible biotypes, 5–7 days after treatment, while in resistant biotypes, slight yellowing was replaced by green new growth.

Results from analysis of variance indicated a highly significant interaction effect among biotype, days after treatment (DAT) and growth stage ($P < .0001$). Basal levels of shikimic acid (0 DAT) in all biotypes and both growth stages were below 40 $\mu g.g^{-1}$ above-ground shoot fresh biomass. Both resistant and susceptible biotypes accumulated shikimic acid after treatment at both growth stages. There were no significant differences ($P = .05$) in shikimic acid levels between resistant and susceptible biotypes at 2 and 4 DAT at both growth stages. However, there was a distinct difference in the pattern of shikimic acid levels over time in respective biotypes after 4 DAT (Fig. 4); with a consistent increase in susceptible biotypes and a decrease in resistant biotypes.

In the experiment where plants were sprayed at the rosette stage, the susceptible biotype (George) accumulated shikimic acid of 5872.1 $\mu g.g^{-1}$ fresh biomass, respectively, at 10 DAT (Fig. 4). The resistant biotype (Swellendam) showed a decline in shikimic acid content from 6 to 10 DAT, with the level being 307.5 $\mu g.g^{-1}$ at 10 DAT (Fig. 4).

Shikimic acid levels in plants treated at bolting stage were significantly lower compared to those treated at the rosette stage (Fig. 4). In the susceptible George biotype, shikimic acid level of 2312.1 $\mu g.g^{-1}$ was recorded. On the contrary, in the resistant Swellendam biotype, shikimic acid levels decreased from 4 DAT, and at 8 DAT, the measured amount was 271.2 $\mu g.g^{-1}$ (Fig. 4). A comparison of shikimic acid amounts in respective biotypes at both growth stages revealed higher amounts at rosette as opposed to bolting stages. At 8 DAT, George biotype exhibited a 2-fold increase in shikimic acid at the rosette stage while a 1-fold increase was established in the Swellendam biotype. Although shikimic acid levels were lower at the bolting stage, they were not statistically different in the resistant biotype. Growth stage had an influence on the response of the susceptible biotype to glyphosate. This observation showed a tolerance to glyphosate at the bolting stage in all biotypes but only minimally in the resistant biotype.

3.3. Hairy fleabane is more sensitive to glyphosate under cold than warm temperature conditions

Phytotoxic effects were observed on treated plants with the susceptible biotype exhibiting more sensitivity at 15 °C. A hormetic response was distinct in all biotypes that were kept at 27 °C. While the resistant biotype recovered from the herbicide's lethal effect, the susceptible biotype had severe chlorosis, necrotic lesions, wilting and browning.

There was a significant interaction effect among biotype, days after treatment (DAT) and temperature regime ($P < .0001$). Basal shikimic acid levels were below 11 and 43 $\mu g.g^{-1}$ of above-ground shoot fresh biomass under the warm (27/20 °C) and cold temperature (15/10 °C) regimes, respectively (Fig. 5). Shikimic acid accumulated in all biotypes but the levels were not statistically different at 2 DAT in both temperature regimes ($P = .05$). However, at 4 DAT and onwards, the course was different in respective temperature regimes and biotypes. More shikimic acid was measured under cold compared to warm temperature regimes.

At 15 °C, shikimic acid increased in all biotypes with the susceptible biotype (George) accumulating the highest amount of 5450.3 $\mu g.g^{-1}$, while Swellendam accumulated 2652.5 $\mu g.g^{-1}$ (Fig. 5). On the other hand, at 27 °C, there was a steady increase in shikimic acid in the susceptible George biotype till the last day of sampling while in the resistant Swellendam biotype, a decline was recorded from 4 DAT to 8 DAT (Fig. 5). At 8 DAT, the highest shikimic acid amount of 4950.3 $\mu g.g^{-1}$ was recorded for George biotype, as opposed to 550 $\mu g.g^{-1}$ in Swellendam biotype. The difference in shikimic acid between the two temperature regimes was significantly higher in the resistant biotype than in the susceptible biotype. At 8 DAT, the resistant biotype displayed about five times higher shikimic acid amount under cold compared to warm temperature conditions, which indicated that it was more sensitive to glyphosate under cold temperature conditions. Although the susceptible biotype accumulated more shikimic acid at

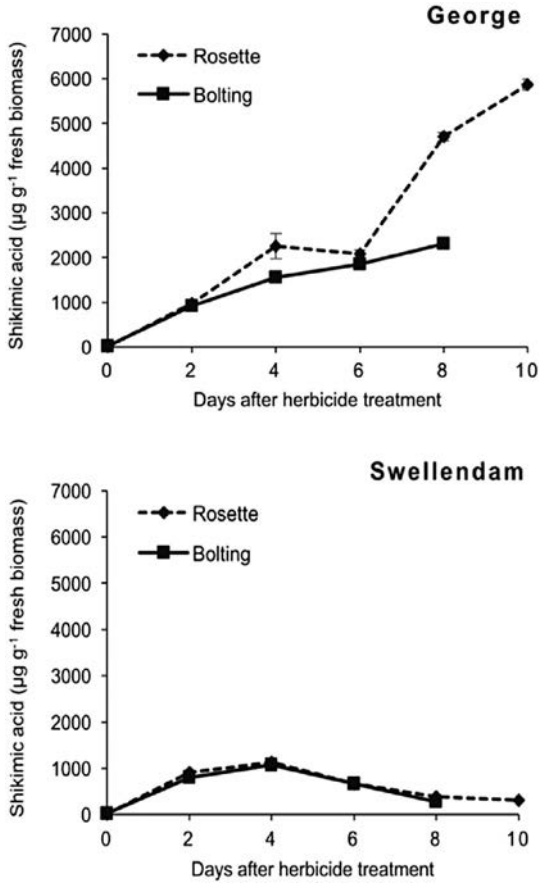

Fig. 4. Time course accumulation of shikimic acid accumulation in above-ground shoot tissue of glyphosate-susceptible and –resistant hairy fleabane (*Conyza bonariensis*) biotypes treated with glyphosate dose of 900 g.ae.ha^{-1} at rosette and bolting growth stages. Vertical bars represent ±standard errors of the mean of three biological replications.

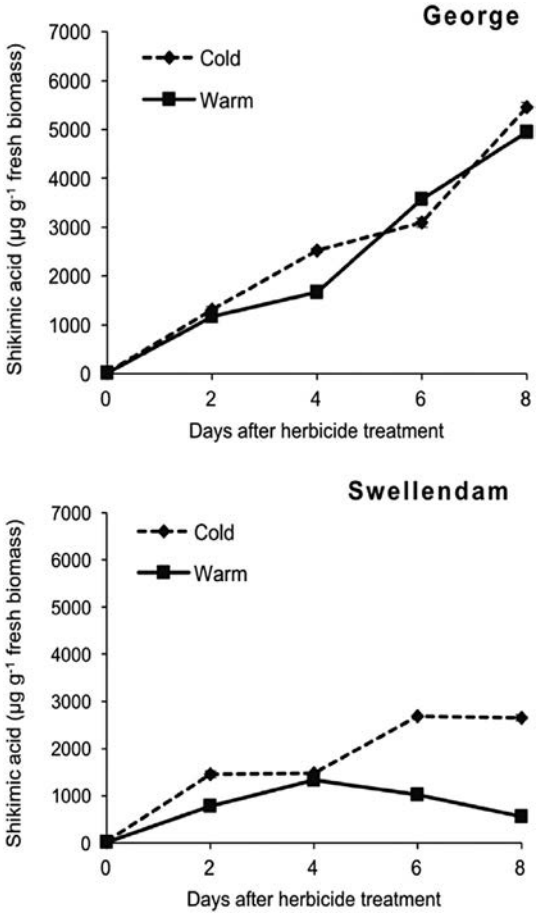

Fig. 5. Time course accumulation of shikimic acid accumulation in above-ground shoot tissue of glyphosate-susceptible and -resistant hairy fleabane (*Conyza bonariensis*) biotypes treated with glyphosate dose of 900 g.ae.ha^{-1} under cold (15 °C/10 °C) and warm (27 °C/20 °C) temperature regimes. Vertical bars represent ±standard errors of the mean of three biological replications.

the cold temperature regime, it was only one times higher as compared to the amount accumulated at the warm temperature regime.

4. Discussion

Phytotoxic effects of the herbicide were detected from 5 to 14 days after treatment with yellowing and chlorosis of the leaves. Although the resistant plants were slightly stunted and displayed minor chlorosis and a few necrotic lesions, they recovered from the herbicide's effects. This observation has previously been reported in glyphosate resistant hairy fleabane from California (Shrestha et al., 2008), horseweed (*Conyza canadensis*) (Dinelli et al., 2006; Mueller et al., 2003) and giant ragweed (*Ambrosia trifida*) (Norsworthy et al., 2010). Glyphosate damages the apical meristems, thus suppressing apical dominance, therefore giving way to development of lateral shoots from axillary buds. Dinelli et al. (2006) proposed that the emission of new growth was a possible mechanism by the resistant biotypes to escape the herbicide's inhibition and lethal effect. This phenomenon could also be a strategy by the resistant biotypes to dilute the herbicide concentration, and increase the number of growth points that the herbicide has to reach in order to cause biomass reduction or plant death.

This study has confirmed glyphosate-resistant hairy fleabane cases in the western and southern Cape regions of South Africa. The level of resistance varied from 0.6 to 26.9-fold compared to the reference susceptible biotype. Moretti et al. (2015) reported glyphosate resistance levels of 5- to 21-fold from field collected *Conyza* spp. Similar resistance levels have been documented in other weed species; Yu et al. (2007) reported a resistance level of 9-fold in resistant biotypes of *Lolium rigidum* from South Africa while Ghanizadeh et al. (2013) reported a 30-fold level of glyphosate resistance in ryegrass (*Lolium perenne*) from New Zealand. Resistance factors of up to 115-fold have been previously documented in horseweed and Palmer amaranth (*Amaranthus palmeri*) growing in glyphosate resistant soybean fields with extensive use of glyphosate (Davis et al., 2008; Norsworthy et al., 2008). Significant differences in biomass reduction were observed in biotypes collected from the same district (Malmesbury and De Rust). These variations in resistance levels in the same district could reflect differences in evolution of glyphosate resistance attributable to the frequency of glyphosate use and tillage systems.

In our study, biotypes from vineyards and orchards were found to be 3 to 26.9 more resistant than the reference susceptible biotype (George 1). Travlos and Chachalis (2010) reported 4- to 7-fold resistance levels in hairy fleabane growing in perennial crops including orchard and vineyards. Similarly, Urbano et al. (2007), established a 7 to 10 resistance level in hairy fleabane collected from olive groves. The cropping systems in related studies are similar to the ones in this study, sharing common features such as adoption of minimum tillage practices, a likely long and repeated history of glyphosate use and lack of crop and herbicide rotation (Dinelli et al., 2006).

Overall, this study has established glyphosate resistance levels of more than 10-fold in 9 out of 24 locations where seed was sampled. Over 50% of the reported resistant cases were from vineyards and orchards; cropping systems characterized by adoption of conservation tillage practices, repeated use of glyphosate and high selection pressures. The ecological adaptability of hairy fleabane to survive in a wide

range of environmental and climatic conditions, production of large quantities of viable wind-dispersed seeds and ability to establish in limited surface moisture, characteristic of conservation tillage systems, poses a challenge in the management of glyphosate-resistant cases. The high magnitudes of resistance levels reported in this study is an indication of presence of a highly effective target-based and/or non-target-site mechanisms of resistance.

Shikimic acid amounts in untreated control plants were below 100 $\mu g.g^{-1}$ fresh biomass. Comparable amounts were reported by Dinelli et al. (2006) in horseweed, and Mueller et al. (2008, 2011) in goosegrasss and nine other weedy species. Shikimic acid accumulated above background levels in all hairy fleabane biotypes tested in both experiments but the levels were not significantly different at 2 and 4 DAT ($P = .05$). This implies that glyphosate was equally loaded in the phloem of both susceptible and resistant biotypes. Similar responses were reported in hairy fleabane biotypes from Spain (Dinelli et al., 2008), horseweed (Mueller et al., 2003; Dinelli et al., 2006; González-Torralva et al., 2012a) and ryegrass (González-Torralva et al., 2012b). In studies conducted by Pline et al. (2002) and Singh and Shaner (1998), no accumulation of shikimic acid was observed in treated tissues of Roundup Ready cotton and soybean, respectively, while shikimic acid rapidly accumulated in glyphosate-sensitive plants. Culpepper et al. (2006), working on Palmer amaranth, did not detect shikimate in leaf tissue of glyphosate-resistant biotypes, irrespective of the glyphosate concentration applied. Although not confirmed, it was suggested that the mechanism of resistance was an altered target site (Culpepper et al., 2006).

Shikimic acid levels differed over time after 4 DAT in respective biotypes. While shikimic acid increased in the susceptible biotype, it declined in the resistant biotype though significantly higher than in untreated controls. This trend was observed at both rosette and bolting growth stages. Similar results at the rosette stage were reported in hairy fleabane (Dinelli et al., 2006), horseweed (Mueller et al., 2003; Feng et al., 2004), goosegrass (Mueller et al., 2011), ryegrass (Michitte et al., 2007) and tall waterhemp (Nandula et al., 2013). At the rosette stage, the highest amount recorded was approximately 5872 $\mu g.g^{-1}$ in George biotype while at the bolting stage, the lowest was in Swellendam biotype, of about 238 $\mu g.g^{-1}$. Comparable amounts were reported in hairy fleabane (Dinelli et al., 2006, 2008), who measured up to 2500 $\mu g.g^{-1}$ in susceptible biotypes and less than 1000 $\mu g.g^{-1}$ in resistant biotypes. Mueller et al. (2003, 2008, 2011), found more than 1000 $\mu g.g^{-1}$ in horseweed, common ragweed and goosegrass, respectively. Differences in the range of shikimic acid documented in previous investigations could be accounted for by differences in quantification methods, plant tissue sampled, species, growth stage and environmental factors. It is theoretically expected that shikimic acid would not accumulate in resistant biotypes or that the levels would be close to those in untreated controls. However, the contrary was observed in the present study, a concern also reported by Mueller et al. (2003), Dinelli et al. (2006) and Huangfu et al. (2007). The fact that significantly higher than background shikimic acid amounts were measured in resistant biotypes indicates that glyphosate reached the target site, EPSPS enzyme. The significantly lower levels of shikimic acid in resistant compared to susceptible biotypes shows that the enzyme is partially inhibited by the herbicide or that the herbicide is not totally excluded from the target site. The decline from 4 DAT to 10 DAT in the resistant biotype could probably be as a result of recovery from herbicidal injury and a possible metabolism of accumulated shikimic acid (Dinelli et al., 2006).

Results from this study have shown that response of hairy fleabane to glyphosate is influenced by the phenological stage at the time of spraying, with sensitivity or injury decreasing with growth stage. Similar observations were reported by Urbano et al. (2007), Kleinman et al. (2015) and Wu et al. (2008), in hairy fleabane, VanGessel et al. (2009), Koger et al. (2004) and Shrestha et al. (2008), in horseweed, González-Torralva et al. (2012b) in ryegrass and Schuster et al. (2007), in common lambsquarters. However, growth stage had a more significant effect on the susceptible, as opposed to the resistant biotype. For example, at the rosette stage, George biotype demonstrated a 2-fold increase in shikimic acid compared to a 1-fold increase in the resistant Swellendam biotype. Additionally, there were no significant differences in shikimic acid levels, thus response to glyphosate, at the bolting stage in the resistant biotype. Walker et al. (2011), Koger et al. (2004) and Pratley et al. (1999) made a similar observation in hairy fleabane, horseweed and ryegrass, respectively. in accordance with Urbano et al. (2007) and Travlos and Chachalis, 2010), the increased tolerance of the biotypes at the bolting stage is due to a lower accumulation of dry matter resulting from a reduction in plant growth. Additionally, since EPSPS enzyme is most active in young growing plant tissues usually in the seedling and rosette stages, plants are more sensitive to glyphosate at these growth stages as compared to later growth stages (bolting to flowering).

The second factor examined in this study was the effect of temperature on shikimic acid levels. The significant statistical tests verified the sensitivity of the shikimate pathway to temperature, with more shikimic acid accumulating at 15 °C than 27 °C across all biotypes, regardless of origin and glyphosate tolerance status. The highest amount of approximately 5450 $\mu g.g^{-1}$ was measured in George biotype under cold conditions, while the lowest amount of about 550 $\mu g.g^{-1}$ was recorded in Swellendam biotype under warm conditions. More tolerance to the herbicide was demonstrated under warm temperature conditions. In a recent study, Nguyen et al. (2016) reported higher shikimic acid accumulation under cool (20 °C) compared to warm (30 °C) temperature conditions in banyard grass. Dose response studies that have confirmed more tolerance to glyphosate under warm temperature regimes include Kleinman et al. (2015), Dennis et al. (2016) and Moretti et al. (2013) in hairy fleabane, Ge et al. (2011) in horseweed, Tanpipat et al. (1997) and Vila-Aiub et al. (2013) in Johnson grass and ryegrass. Since a hormetic response was established particularly under the warm temperature regime, the greater tolerance, as evidenced by lower shikimic acid accumulation, may be attributed to the increased biomass accumulation. Although not tested in our study, Ge et al. (2011) and Vila-Aiub et al. (2013) related the temperature dependence of glyphosate to reduced translocation due to increased vacuolar sequestration under warm temperatures. In their studies, they stated that glyphosate remains in the cytoplasm under cold conditions and is transported to the vacuoles when there is a shift to warm conditions.

5. Conclusion

The presence of resistant biotypes poses a serious problem in annual and perennial cropping systems. It is therefore important that mitigation measures be put in place, and existing ones be improved, to not only contain resistance to glyphosate but also to prevent its evolution in locations where resistance has thus far not been reported. Our studies have shown the relevance of phenological stage and temperature to the efficacy of glyphosate at the time of application in hairy fleabane. The significance of findings from this study are relevant to the South African situation since hairy fleabane is present in farms all year round and the fact that glyphosate is typically applied in fields throughout the planting season in non-GR cropping systems and before planting during summer (October to December) as a burndown. Possible outcomes of applying glyphosate at the bolting stage and under warm temperature conditions are escapes and no control of susceptible and resistant plants, respectively. The temperature-effect results could imply that if all factors are constant, the selection pressure, glyphosate resistance evolution rate and possible mechanism(s) of resistance in the South African hairy fleabane biotypes could become elevated under warm temperature conditions or season. It is pertinent that growth stage and temperature conditions are considered in glyphosate screening tests to avoid incorrect categorization of biotypes, which could ultimately economically have a negative impact on the farmers

dealing with glyphosate resistance challenges. Further studies are underway to elucidate target site mechanism(s) of resistance, by describing the EPSPS enzyme DNA sequence and RNA expression.

Declarations of interest

The project was partially funded by Monsanto South Africa.

Acknowledgements

Technical assistance of Jacques Marneweck, University of Pretoria, is very much appreciated. This work was supported by the Organization for Women in Science for the Developing World (OWSD), the Swedish International Development Cooperation Agency (Sida), National Research Foundation (NRF), South Africa and Monsanto South Africa.

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