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Response of *Eleusine indica* and *Paspalum distichum* to glyphosate following repeated use in citrus groves

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ABSTRACT

Eleusine indica L. Gaertn. and *Paspalum distichum* L. are annual and perennial grasses, respectively that are widely distributed in turf and perennial cropping systems throughout Spain. Often, glyphosate is used between rows of perennial crops for control of these grasses, but variable responses have been observed. Sensitivity to glyphosate in each species was examined under greenhouse, laboratory and field conditions. *In vitro* tests on whole plants of both *P. distichum* and *E. indica* revealed no differences in sensitivity to glyphosate for areas with long-term use (treated; T) and no history of use (not treated; NT). The NT population of *P. distichum* (ED_{50} 73.1 g ae ha^{-1}) was 11.6% more sensitive to glyphosate than NT *E. indica* (ED_{50} 81.6 g ae ha^{-1}). No differences between T and NT populations of both species were observed for foliar retention of glyphosate as well as accumulation of shikimate. However, glyphosate retention and shikimate accumulation were up to 64 and 24.4% greater, respectively in *P. distichum* compared to *E. indica*. Within 96 h after treatment (HAT), foliar absorption of ^{14}C-glyphosate was similar among T and NT populations, but 8.8% higher for *P. distichum* compared to *E. indica*. Retention of ^{14}C-glyphosate in treated leaves of *P. distichum* was approximately 55% lower compared to *E. indica*. Translocation from the treated leaf into other shoot tissue (2.8-fold) and roots (8.5-fold) was higher for *P. distichum* versus *E. indica*. This would suggest that differences in *E. indica* versus *P. distichum* response to glyphosate are based upon differential retention in treated leaves and reduced movement out of treated tissue in other shoot and root tissue. In separate field experiments in citrus orchards, glyphosate and other herbicides were applied to assess control of *E. indica* and *P. distichum* over two years. Flazasulfuron and cycloxidim resulted in 90% or greater control of both species by 60 days after treatment (DAT). Only glufosinate, oxyfluorfen, paraquat and iodosulfuron resulted in >85% control of *E. indica*. These corresponding treatments ranged in effectiveness from 73 to 92% on *P. distichum*. Integration of effective herbicides with modes of action different than glyphosate should be used for management of *E. indica* and *P. distichum* and may delay the selection for resistance to glyphosate.

1. Introduction

In the Mediterranean region, no-till practices are adopted commonly to conserve soil resources in perennial cropping systems such as olive (*Olea europaea* L.) groves, *Citrus* spp. orchards and grape (*Vitis vinifera* L.) vineyards (Cerda et al., 2015). In the absence of tillage, living cover crops consisting of barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), and legumes such as vetch (*Vicia* spp.) and lupins (*Lupinus* spp.) are established to deter weed establishment, build soil organic matter, and reduce soil erosion (Gomez et al., 2011; Hartwig and Ammon, 2002). In some cases, grass weeds are allowed to develop in open canopy areas to conserve soil. Growth of cover crops or naturally established weeds is controlled by mowing, non-selective herbicides or animal grazing.

The herbicide glyphosate is frequently applied beneath perennial crops in Spain to manage cover crops or other vegetation (Costa, 1997). Lacking residual activity, glyphosate is non-selective and controls a broad-spectrum of annual and perennial plant

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species (Baylis, 2000). According to Duke and Powles (2008), glyphosate is the most widely sold herbicide in the world.

Glyphosate inhibits the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (EC 2.5.1.19), which catalyzes the conversion of shikimate-3-phosphate and phosphoenolpyruvate to EPSP and inorganic phosphate via the shikimic acid pathway (Geiger and Fuchs, 2002; Reddy et al., 2008). Inhibition of this enzyme prevents biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan as well as other important secondary compounds including auxins and allelochemicals (Harring et al., 1998; Schönbrunn et al., 2001).

Despite the effectiveness of glyphosate, repeated applications within and over numerous years as well as over large areas has resulted in selection of numerous glyphosate-resistant (GR) biotypes (Owen, 2001; Thill and Lemerle, 2001). To date, there are 32 GR biotypes worldwide; five (*Conyza bonariensis* L. Cronq., *Conyza canadensis* L. Cronq., *Conyza sumatrensis* (Retz) E.H. Walker, *Lolium multiflorum* Lam., and *Lolium rigidum* Gaudin) of which are found in Spain (Heap, 2015). In addition, there are other weed species which are difficult to control with glyphosate (Cruz-Hipólito et al., 2009). Two of these species include *Paspalum distichum* L. and *Eleusine indica* L. Gaertn.

P. distichum is a perennial grass introduced from tropical regions of the Americas. *P. distichum* is spread both by seed and rhizomes (Manuel and Mercado, 1977). Aguiar et al. (2005) reported that *P. distichum* exhibits invasive characteristics where water is available such as ditch banks, riparian areas and irrigated crops in the Mediterranean basin. Infestations are commonly reported in perennial crops throughout Spain (Costa, 1997).

Left uncontrolled, established stands of *P. distichum* form monocultures (Guillerm et al., 1990). Mechanical cultivation is effective on seedlings prior to formation of rhizomes, but cultivation spreads perenniating plants by cutting rhizomes into smaller propagules (Huang et al., 1987; Manuel and Mercado, 1977). Moist soil conditions at the time of cultivation renders mechanical control ineffective. Control with glyphosate is challenging; Okuma and Chikura (1985) recommended rates up to 4.9 kg ha^{-1}. Alternative herbicides are necessary to reduce the selection pressure resulting from repeated applications of glyphosate.

E. indica is a summer annual species in the *Poaceae* family. Plants thrive in sub-tropical areas at approximately 50° latitude. Exhibiting a C4 process for photosynthesis, plants are also considered troublesome in temperate areas with hot summers. In climates lacking a killing frost, some *E. indica* plants can survive longer than 1 year. It is an important weed of cultivated crops (*Zea mays* L., upland *Oryza sativa* L., *Saccharum officinarum* L. and many fruit and vegetable orchards), lawns, and golf courses (Holm et al., 1977; Lourens et al., 1989). Eke and Okereke (1990) found 10–16 *E. indica* seedlings competing with a *Z. mays* plant reduced plant biomass approximately 52% compared to *Z. mays* lacking competition.

Once established, goosegrass plants tiller extensively and adapt to frequent mowing. Timely mechanical tillage and herbicide application can be effective for control. However, one consequence in utilizing herbicides is the propensity of some populations to evolve resistance (Vidal et al., 2006; Jalaludin et al., 2010). Recently, glyphosate resistance based upon a Pro-106 point mutation in EPSPS has been identified in a population from Malaysia, with resistant biotypes surviving rates up to 5-fold higher than sensitive populations (Ng et al., 2003; Heap, 2015).

Exclusive, long-term use of glyphosate in irrigated citrus crops for control of *P. distichum* and *E. indica* has led to concerns for selection of resistant biotypes. The specific objectives of this research were: (a) to assess whole plant sensitivity of *E. indica*, and *P. distichum* species to glyphosate based upon comparing T and NT populations; (b) to identify if physical (leaf retention) or physiological (shikimic acid accumulation, ^{14}C-glyphosate absorption and translocation) characteristics may explain differential responses between species; and (c) to determine if herbicide alternatives to glyphosate result in effective control of one or both species.

2. Materials and methods

2.1. Plant production

All seeds from both the treated (T) and non-treated (NT) populations of *E. indica* and *P. distichum* species were collected from mature plants in summer 2009. Plants were considered T if they originated from fields where glyphosate had been applied annually for at least five years. *E. indica* was collected from a field containing citrus crops in the Huelva province (Southern Spain); *P. distichum* was collected from a citrus field located in Castellón province (Eastern Spain). The NT populations of *E. indica* and *P. distichum* were obtained from fields in close proximity to the corresponding T populations, where no documented use of glyphosate was found.

All seeds were germinated in 663 cm^2 trays containing peat saturated at field capacity, then placed in growth chambers. Growing conditions included air temperatures of 28/18 °C (day/night) with a photoperiod of 16 h, 850 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density, and 80% relative humidity. Both T and NT seedlings from each species were transplanted into pots (3 plants per pot) containing a 1:2 (v/v) ratio of sand:peat and placed in a growth chamber under the conditions described above.

2.2. Dose–response assays

Glyphosate applications were made at the BBCH 13–14 stage (3–4 leaves) (Hess et al., 1997). A laboratory spray chamber (DeVries Manufacturing, Hollandale, MN) equipped with TeeJet 8002 flat fan nozzle (Spraying Systems Co., Wheaton, IL) tips delivered 200 L ha^{-1} at 200 kPa at a height of 50 cm. Glyphosate (Roundup Energy SL, Monsanto, Spain) rates included: 0, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, and 600 g ae ha^{-1}. A typical field use rate of glyphosate (1X dose) is 360 g ha^{-1}. Experimental design was completely randomized with four replications of each treatment, where each replicate utilized three plants. Plants were harvested 21 days after treatment (DAT), and immediately weighed to determine fresh weight. Data were expressed as ED_{50} (effective dose to reduce plant fresh weight by 50%) and compared to untreated plants. Assays were conducted twice and results combined.

2.3. Spray retention assays

At the BBCH 13–14 stage, *P. distichum* and *E. indica* were sprayed with 300 g ae ha^{-1} of glyphosate and 100 mg L^{-1} Na-fluorescein using conditions as described above. Once the foliage had dried (20–25 min), shoot tissue was cut at ground level. The tissue was immersed in 50 mL of 5 mM NaOH for 30 s to remove spray solution. Fluorescein absorbance was determined using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) with excitation wavelength of 490 nm and absorbance at 510 nm. Dry biomass of plant tissue was recorded following exposure to 60 °C for 48 h. The experimental design was completely randomized with four replications, where one replicate included three plants of each population and species. Assays were conducted twice and results combined. Spray retention was expressed as mL spraying solution per gram dry matter (González-Torralva et al., 2010).

2.4. Shikimic acid accumulation

Using growth conditions as described above, both *P. distichum* and *E. indica* were grown to the BBCH 13–14 stage, then treated with glyphosate at 300 g ae ha^{-1} using the conditions as described above. At 12, 24, 48, 72 and 96 h after treatment (HAT), leaf tissue (50 mg fresh weight) from treated and non-treated plants was homogenized, placed in separate vials containing 1 mL of 0.25 N HCl, and then immediately frozen in liquid nitrogen. Shikimic acid accumulation was determined according to the method described by Singh and Shaner (1998). Spectral absorbance of the samples was quantified using a spectrophotometer (Beckman DU-640, Fullerton, CA) at 380 nm. Net shikimic acid accumulation was determined as the difference between the treated and non-treated plants for each species. The test was performed in triplicate on five treated and five non-treated plants per population, and the results were expressed as μg shikimic acid per g fresh weight. Assays were conducted twice and results combined.

2.5. Absorption and translocation of ^{14}C-glyphosate

P. distichum and *E. indica* plants from T and NT populations were grown to a BBCH 13–14 stage using conditions described above. A solution of ^{14}C-glyphosate (American Radiolabeled Chemicals, Inc., Saint Louis, MO) was prepared using a commercial formulation to facilitate optimum absorption; specific activity of the solution was 0.834 kBq μL^{-1} and the final glyphosate concentration corresponded to 300 g ae ha^{-1}. A micropipette was used to apply 1 μL of glyphosate solution (0.834 kBq $plant^{-1}$) on the adaxial surface of the second most fully developed leaf. From preliminary studies, maximum glyphosate absorption occurred by 96 HAT (results not shown). At 96 HAT, the treated leaves were excised and carefully washed with 3 mL of water:acetone solution (1:1 v/v) to recover unabsorbed ^{14}C-glyphosate. The rinsate was mixed with 2 mL of scintillation cocktail and analyzed by liquid scintillation spectrometry (LSS) using a scintillation counter (Beckman 6500, Fullerton, CA). The remainder of the plant was removed from the pot and roots washed with distilled water. Plant tissue was sectioned into treated leaf, remaining shoots, and roots. Plant tissues were dried at 60 °C for 96 h and combusted in a biological sample oxidizer (Packard Tri Carb 307, Perkin–Elmer, Waltham, MA). The $^{14}CO_2$ evolved during combustion corresponded to glyphosate and all associated metabolites and was trapped in 18 mL of a mixture (1:1 v/v) of Carbo-Sorb E and Permafluor E+ (Perkin–Elmer). Radioactivity was quantified by LSS. The percentage of absorbed herbicide was expressed as:

$$[\text{kBq in tissue} / (\text{kBq in tissue} + \text{kBq in leaf surface washes})] \times 100 \tag{1}$$

The experiment was designed as completely randomized with three replications, and each replicate was comprised of three plants. Assays were conducted twice and results combined. Recovery of radiolabel applied ranged from 92 to 94.3% for *E. indica* and 90.8–95.1% for *P. distichum*.

2.6. Comparative herbicides for field control

Field experiments with established populations of *P. distichum* were carried out in citrus orchards in the province of Castellón (Eastern Spain), from 2011 to 2012 and again from 2012 to 2013. During this same time period, field experiments with *E. indica* were established in citrus orchards in the province of Huelva (Southern Spain). A total of twelve herbicide treatments (Table 1) and one untreated control were arranged in a randomized complete block design with three replications for each experiment; plot dimensions were 2 by 10 m. Soil composition at the Huelva location was a Vertisol with clay loam texture (38% clay), pH of 7.2 and 1.6% organic matter. At the Castellón location, the soil was also a Vertisol with clay loam texture (41% clay), pH of 7.8 and 2.1% organic matter. At the time of POST herbicide applications, the *P. distichum* and *E. indica* plants were at the BBCH 13–14 stage and total coverage of the soil surface by each respective species was 95%. Flazasulfuron and oxyfluorfen were applied prior to weed emergence. All treatments were applied with a pneumatic backpack sprayer utilizing TeeJet 11002 flat fan nozzle tips and calibrated to deliver 250 L ha^{-1} at 276 kPa. Visual evaluations of treated *P. distichum* and *E. indica* plants were performed at 60 DAT. In previous trials, it was observed that some herbicides induced injury symptoms in as few as 15 DAT. Visual assessments were made at 30 and 45 DAT (data not shown), but were not significantly different than assessments made at 60 DAT. Control ratings were expressed on a 0 (no control) to 100 (plant dead or reduction of cover) scale. *P. distichum* and *E. indica* shoot biomass was harvested at ground level in 0.25 m^2 from each plot at 60 DAT, dried at 50 °C for 5 days and weighed. For comparison, shoot dry weight was converted to a percentage relative to 100% for the untreated control.

2.7. Statistical analyses

Data obtained in the dose response assays were pooled and fitted to a non-linear, log-logistic regression equation:

$$Y = c + \{(d - c)/[1 + (x/g)^b]\} \tag{2}$$

Where Y is expressed as a percentage of the value for untreated plants; c and d are the lower and upper asymptote, respectively; b is the slope of the curve; g denotes ED_{50} (which coincided with the point of inflection halfway between the upper and lower asymptotes); and x is an independent variable representing the herbicide rate. The resistance factor (RF) was computed as:

$$ED_{50\ (T)} / ED_{50\ (NT)} \tag{3}$$

Regression analysis was conducted using SigmaPlot (Version 10.0, Systat Software, San Jose, CA).

Data obtained in spray retention, shikimic acid accumulation, absorption and translocation of ^{14}C-glyphosate, and herbicide field trials were subjected to ANOVA using Statistix (version 9.0; Analytical Software, Tallahassee, FL). A non-parametric Tukey HSD test ($p < 0.05$) was used to separate means.

3. Results and discussion

3.1. Dose–response assays

Within plant species, dose responses are an effective method to elucidate changes in the sensitivity between different populations in response to herbicides (Carvalho et al., 2011). Table 2 shows the parameters from a statistical model used to obtain the ED_{50} (the glyphosate rate needed to reduce plant fresh weight by 50%). The ED_{50} was similar between the T and NT populations of *E. indica* and *P. distichum*. The RF was 1.1 for *E. indica* and 1.0 for *P. distichum* (Table 2). The lack of response differences between T and NT populations of both species suggests no selection of resistant plants was detectable, despite repeated applications of glyphosate over five years. However, among the *E. indica* populations, plants from the T versus NT population required >10% higher glyphosate rates to reach the ED_{50}. Since 2007, *E. indica* biotypes resistant to glyphosate have been detected in eight countries worldwide (Heap,

Table 1
Chemical treatments applied postemergence to populations of *Eleusine indica* L. Gaertn. and *Paspalum distichum* L. under field conditions in citrus orchards in Spain.

Treatment	HRAC (WSSA group)[a]	Timing	Formulated product[b]	Rate (g a.i. ha^{-1})
Control	–	–	–	–
Glyphosate	G (9)	Post	Roundup Energy®	720
Glyphosate	G (9)	Post	Roundup Energy®	1440
Flazasulfuron	B (2)	Pre	Terafit®	50
Oxyfluorfen	E (14)	Pre	GoalSupreme®	500
Clethodim	A (1)	Post	Centurion Plus®	100
Cycloxidim	A (1)	Post	Focus Ultra®	250
Quizalofop-p-ethyl	A (1)	Post	Master D®	125
Fluazifop-p-butyl	A (1)	Post	Fusilade Max®	300
Iodosulfuron	B (2)	Post	Hussar®	10
Paraquat	D (22)	Post	Paratex®	500
Diuron	C_2 (7)	Post	Sumex 80®	250
Glufosinate	H (10)	Post	Finale®	750

[a] Abbreviations: HRAC, Herbicide Resistance Action Committee; WSSA, Weed Science Society of America; A(1), Inhibition of acetyl CoA carboxylase; B (2), Inhibition of acetolactate synthase; C_2, Inhibition of PSII; D (22), Photosystem I-electron diversion; E (14), Inhibition of protoporphyrinogen oxidase; G (9), Inhibition of EPSP synthase; H (10), Inhibition of glutamine synthetase.

[b] Herbicide manufacturers: glyphosate, Monsanto España, Madrid, Spain; flazasulfuron, fluazifop-p-butyl, and paraquat, Syngenta España S.A. Madrid, Spain; oxyfluorfen, quizalofop-p-ethyl, Dow AgroSciences Ibérica S.A., Madrid, Spain; clethodim, diuron, iodosulfuron and glufosinate, Bayer Hispania S.L., Barcelona, Spain; cycloxidim, BASF Agro España, Barcelona, Spain.

Table 2
Estimated parameters to predict the rate of glyphosate necessary to reduce fresh weight of *Eleusine indica* L. Gaertn. and *Paspalum distichum* L. populations by 50% (ED_{50}), 21 days following treatment.[a] T indicates "treated" plants where site had a history of glyphosate usage; NT indicates "non-treated" plants where site did not have a history of glyphosate usage.

Species	Populations	c	d	b	ED_{50} (g ae ha^{-1})	Pseudo r^2[b]	P[c]	R.F.
E. indica	T	1.58	98.19	7.16	90.10 ± 4.25	0.93	<0.0001	1.1
	NT	0.16	99.33	8.01	81.62 ± 7.95	0.96	<0.0001	
P. distichum	T	2.09	98.44	4.69	75.74 ± 3.42	0.94	<0.0001	1
	NT	2.13	98.42	4.64	75.16 ± 2.96	0.99	<0.0001	

c and d are the lower and upper asymptote; b is the slope of the curve.

[a] Data were pooled and fitted to a non-linear, log-logistic regression equation (see Statistical analyses Section).

[b] Approximate coefficient of determination of non-linear models with a defined intercept calculated as pseudo $r^2 = 1 -$ (sums of squares of the regression/corrected total sums of squares).

[c] Probability level of significance of the non-linear model. R.F. (Resistance Factor) = ED_{50} (T)/ED_{50} (NT).

2015). Lee and Ngim (2000) determined an RF of 8–12 to glyphosate for an *E. indica* biotype from Malaysia. This resulted following up to seven applications of glyphosate annually in a fruit orchard for 3 years. Using ED_{50} values for resistant and susceptible biotypes in Tennessee, Mueller et al. (2011) reported an RF of 7.4. Some biotypes of *E. indica* are characterized as GR, but exhibit an RF between 1.3 and 4 (Kaundun et al., 2008; Molin et al., 2013). Although T and NT populations were similar in sensitivity to glyphosate, continued use of glyphosate on T populations of *E. indica* may select for resistant populations. Likewise, the response of T and NT populations of *P. distichum* to glyphosate were essentially identical, indicating susceptibility. To date, no previously published data have characterized the response of *P. distichum* to glyphosate.

3.2. *Spray retention*

On average, *P. distichum* retained an average of 61% more glyphosate than *E. indica* (Table 3). However, no significant difference between T and NT populations was observed within either species. Differences in spray retention between species may partially account for the higher ED_{50} value for *E. indica* compared to *P. distichum* (Table 2). Glyphosate sensitivity between species has previously been attributed to retention on treated tissue, which is influenced by contact angle and leaf coverage; this will ultimately influence the glyphosate uptake and translocation (González-Torralva et al., 2010; Cruz-Hipólito et al., 2009). Carvalho et al. (2011) examined glyphosate retention of a glyphosate-susceptible (GS) and three GR biotypes of *Digitaria insularis* (L.) Mez ex Ekman; spray retention and leaf contact angle were similar and did not contribute to differences in sensitivity. Spray retention is the first in a series of steps that determines efficacy of herbicides: retention; uptake; translocation; and inhibition of the target enzyme (Kirkwood and McKay, 1994).

Table 3
Foliar retention of glyphosate spray by *Eleusine indica* L. Gaertn. and *Paspalum distichum* plants treated at the BBCH 13–14 stage. T indicates "treated" plants where site had a history of glyphosate usage; NT indicates "non-treated" plants where site did not have a history of glyphosate usage.

Populations	mL of sprayed solution retained per g dry weight	
	Species	
	E. indica	*P. distichum*
T	2.42 ± 0.29[a]	3.88 ± 1.02
NT	2.57 ± 0.46	4.15 ± 1.83

[a] Mean values ± standard error of the mean.

3.3. *Accumulation of shikimic acid*

The accumulation of shikimic acid in plant extracts is widely known to reflect specific inhibition of the chloroplast enzyme EPSPS (Bonini et al., 2009; Lydon and Duke, 1988; Singh and Shaner, 1998). As EPSPS is the major enzyme targeted *in vitro* by glyphosate, measurement of shikimic acid levels is a litmus test for glyphosate sensitivity (Tan et al., 2006; Reddy et al., 2008; Amrhein et al., 1980; Lydon and Duke, 1988). At each time point from 12 to 96 HAT, shikimic acid levels between the T and NT populations in both species were similar (Table 4). Also, shikimic acid levels continued to increase from 12 to 96 HAT in both species with final concentrations 6.4- and 7.9-fold higher at 96 versus 12 HAT for *E. indica* and *P. distichum*, respectively. At 96 HAT, *P. distichum* populations on average accumulated 22.9% higher levels of shikimic acid than *E. indica* populations. This may be related to inherent variability in species sensitivity to glyphosate or higher *in vitro* concentrations of glyphosate because of differences in leaf retention.

3.4. Absorption and translocation of ^{14}C-glyphosate

At 96 HAT, no significant differences in foliar absorption of ^{14}C-glyphosate were found between both grass species (Table 5). Within species, T and NT plants accumulated similar levels of glyphosate in both treated leaves and roots. For shoot tissue outside the treated leaf, ^{14}C-glyphosate accumulation was similar in *E. indica*, but 14.6% greater in NT vs T plants of *P. distichum*. Because ED_{50} values in response to glyphosate were similar in NT and T plants of *P. distichum*, translocation differences are not thought to be physiologically important. However, there were marked differences between species regarding ^{14}C-glyphosate translocation. For *E. indica*, 2.2-fold more ^{14}C-glyphosate remained in the treated leaf compared to *P. distichum*. Concomitantly, the percentage of glyphosate translocated (as a percentage of total absorbed) to the rest of the shoots and roots was about 21 and 26% higher for *P. distichum* compared to *E. indica* (Table 5). Differences in retention of ^{14}C-glyphosate in treated leaves and herbicide translocation throughout the remaining shoot tissue can contribute to glyphosate resistance, as shown for *L. multiflorum* by Perez-Jones et al. (2007) as well as *L. rigidum* (Preston and Wakelin, 2008; Fernandez et al., 2015). In this study, greater translocation of ^{14}C-glyphosate for *P. distichum* versus *E. indica* likely underlies whole plant differences in sensitivity.

3.5. Comparative herbicides for field control

Field response of T populations from both *E. indica* and *P. distichum* indicate moderate injury at 60 DAT with glyphosate (Table 6). A rate response to glyphosate was observed for both *P. distichum* and *E. indica*. Mean visual control of *P. distichum* with 1.44 kg ae ha^{-1} was 90 and 83% for the 2011–2012 and 2012–2013 studies, respectively. Control of *E. indica* was somewhat lower, 78 and 75% for the 2011–2012 and 2012–2013 studies, respectively. Farmers would characterize a minimum of 80–85% control necessary for satisfactory weed control. These results contradict claims made by local farmers that *E. indica* and *P. distichum* in respective fields were resistant to glyphosate. Reports of poor control by farmers could be explained by inaccurate application of the herbicide (e.g. inadequate rates, improper growth stage), adverse weather conditions following application, or other factors (extreme water pH, etc.).

Table 5
Absorption and translocation of ^{14}C-glyphosate in *Eleusine indica* L. Gaertn. and *Paspalum distichum* at 96 HAT. T indicates "treated" plants where site had a history of glyphosate usage; NT indicates "non-treated" plants where site did not have a history of glyphosate usage.

Species			Translocation (% of absorbed)[a]		
		% Absorption	Treated leaf	Rest of shoots	Roots
E. indica	T	84.6 ± 3.1	86.3 ± 1.1	10.9 ± 0.9	2.8 ± 1.8
	NT	86.2 ± 7.1	83.4 ± 1.7	12.4 ± 2.2	4.2 ± 0.7
P. distichum	T	92.8 ± 2.9	38.0 ± 2.6	30.2 ± 6.1 b	31.8 ± 2.2
	NT	93.1 ± 4.3	37.8 ± 1.3	34.6 ± 8.4 a	27.6 ± 4.6

[a] Means within a column for each species followed by the same letter were not significantly different at the 0.05 level as determined by the Tukey HSD test. Mean values ± standard error of the mean.

Considering other herbicides utilized, the highest levels of control (90–98%) were achieved with flazasulfuron and cycloxidim for both species at 60 DAT. For *P. distichum*, glufosinate, paraquat and iodosulfuron resulted in 82–92% control, with the remaining herbicides resulting in 52–82% control. *E. indica* was sensitive to oxyfluorfen, iodosulfuron, paraquat, and glufosinate (87% or greater control), but exhibited only 52–83% control with the remaining herbicides. Results with shoot dry weight biomass closely reflected visual control evaluations.

Glyphosate likely exhibited sufficient activity for farmers in the Huelva and Castellón provinces of Spain to make applications twice annually at 720 g ae ha^{-1} on both *P. distichum* and *E. indica*. Glyphosate resistance was not detected for T versus NT populations of each species, but this pattern of herbicide usage strongly correlates with resistance selection for *E. indica* in other countries (Lee and Ngim, 2000). *Leptochloa virgate* (L.) P. Beauv. in Mexico was found resistant to glyphosate in citrus orchards following 3 to 4 applications annually for over 15 years (Perez-Lopez et al., 2014). Similarly, *L. rigidum* in Australian orchards received 2 to 3 applications of glyphosate annually for 15 years before glyphosate resistance was discovered (Powles et al., 1998). It is also concerning that farmers in Spain utilized rates of glyphosate below the optimum rate. In Tennessee, Mueller et al. (2011) reported selection of GR *E. indica* following several years of glyphosate use below labeled dosages.

Under field conditions, four distinct herbicide modes of action other than glyphosate exhibited effective control of *P. distichum* and *E. indica*. Flazasulfuron, cycloxidim, glufosinate, iodosulfuron and paraquat applications are suggested to be used in mixtures or rotation with glyphosate to manage *E. indica* and *P. distichum*, thereby precluding selection of resistant biotypes. The lessons learned in other countries following overuse of glyphosate can be avoided by adoption of prudent management practices.

4. Conclusions

Repeated use of glyphosate to control *P. distichum* and *E. indica* in citrus orchards in Spain did not select for resistance as reported initially by farmers. Estimates of fresh weight reductions, herbicide

Table 4
Shikimic acid accumulation (μg g^{-1} fresh weight) in *Eleusine indica* L. Gaertn. and *Paspalum distichum* plants after glyphosate application. T indicates "treated" plants where site had a history of glyphosate usage; NT indicates "non-treated" plants where site did not have a history of glyphosate usage.

Species		Hours after treatment[a]				
		12	24	48	72	96
E. indica	T	1500 ± 282	3100 ± 410	5600 ± 848	8200 ± 212	9200 ± 158
	NT	1380 ± 70	3250 ± 141	6080 ± 274	8600 ± 424	9250 ± 664
P. distichum	T	1400 ± 311	3800 ± 197	6200 ± 294	9300 ± 551	11200 ± 721
	NT	1480 ± 593	4100 ± 296	6300 ± 127	9250 ± 367	11480 ± 57

[a] Mean values ± standard error of the mean.

Table 6
Mean response of *Paspalum distichum* and *Eleusine indica* L. Gaertn. to herbicides in Huelva and Castellón provinces in Spain, respectively. Separate field trials were conducted in 2011–2012 and 2012–2013. Plant response was measured as visual control (0 = no control; 100 = plant death) and plant biomass (g dry weight) was estimated from a 0.25 m^2 area; evaluations were made 60 days after treatment (DAT).

Treatment	Dose (g a.i. ha^{-1})	*E. indica*				*P. distichum*			
		2011–2012		2012–2013		2011–2012		2012–2013	
		Visual control[a] (%)	Shoot dry weight (g)	Visual control (%)	Shoot dry weight (g)	Visual control (%)	Shoot dry weight (g)	Visual control (%)	Shoot dry weight (g)
Control		–	351.22	–	357.42	–	421.16	–	429.08
Glyphosate	720[b]	73.3 c	91.47 b	68.3 d	100.58 b	78.3 c	87.99 c	78.3 cde	92.37 c
Glyphosate	1440[b]	78.3 bc	57.62 b	75.0 cd	60.40 c	90.0 b	55.37 de	83.3 abcd	55.76 de
Flazasulfuron	50	93.3 a	29.04 d	90.0 a	32.48 d	98.3 a	20.59 f	91.7 a	22.82 f
Oxyfluorfen	500	93.3 a	27.96 d	86.7 ab	29.49 d	83.3 c	88.42 c	73.3 ef	90.67 c
Clethodim	100	83.3 b	55.26 c	80.0 bc	58.43 c	80.0 c	95.97 bc	73.3 ef	99.89 bc
Cycloxidim	250	98.3 a	22.11 d	91.7 a	24.04 d	98.3 a	19.36 f	91.7 a	21.75 f
Quizalofop	125	83.3 b	55.56 c	78.3 c	58.59 c	81.7 c	88.45 c	75.0 def	91.04 c
Fluazifop	300	73.3 c	101.39 ab	68.3 d	99.30 b	78.3 c	109.22 b	68.3 f	112.12 b
Iodosulfuron	10	93.3 a	28.80 d	86.7 ab	29.03 d	90.0 b	58.39 d	81.7 bcde	61.79 d
Paraquat	500	91.7 a	28.55 d	88.3 a	31.27 d	91.7 b	47.60 de	85.0 abc	48.98 de
Diuron	250	63.3 d	114.83 a	51.7 e	115.03 a	58.3 d	191.03 a	51.7 g	193.78 a
Glufosinate	750	96.7 a	18.69 d	93.3 a	20.46 d	91.7 b	41.68 e	88.3 ab	43.92 e

[a] Means within a column for each species followed by the same letter were not significantly different at the 0.05 level as determined by the Tukey HSD test. Mean values ± standard error of the mean.
[b] g ae ha^{-1}.

retention, accumulation of shikimic acid, and absorption and translocation of ^{14}C-glyphosate were similar for populations collected from sites repeatedly treated with glyphosate versus sites with no history of use. The rate of glyphosate necessary to reach the ED_{50} value was 13.8% higher for *E. indica* versus *P. distichum*, which may partially be attributed to greater retention of glyphosate by *P. distichum*. Retention differences also contributed to greater accumulation of shikimic acid in *P. distichum* versus *E. indica*. Absorption of ^{14}C-glyphosate was similar between species, but retention of herbicide in treated leaves of *E. indica* versus *P. distichum* resulted in less translocation to remaining shoot and root tissue. Both *P. distichum* and *E. indica* are sensitive to field applications of flazasulfuron and cycloxidim, suggesting that farmers integrate use of herbicide modes of action to maintain effective levels of plant control.

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