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$^{-1}$/C$_0$) was 11.6% more sensitive to glyphosate than NT *E. indica* (ED$_{50}$ 81.6 g ae ha$^{-1}$/C$_0$). 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.

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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
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). Aguici et al. (2005) reported that P. distichum exhibits invasive characteristics where water is available such as ditches 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. Experiencing 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).

Exclusively, 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, 14C-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 cm² 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 μmol 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 a.e. 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 ED₅₀ (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 spectrophotometer (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 \( \mu g \) 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 mL 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:

\[
\text{absorption} = \frac{[\text{kBq in tissue} / (\text{kBq in tissue + kBq in leaf surface washes})] \times 100}{100}
\] (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:

\[
Y = c + \left\{ \frac{(d-c)}{[1 + (x/g)^b]} \right\} \quad \text{(2)}
\]

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:

\[
\text{ED}_{50} (T)/\text{ED}_{50} (NT) \quad \text{(3)}
\]

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, 2011).
(2011) examined glyphosate retention of a glyphosate-susceptible
for 3 years. Using ED50 values for resistant and susceptible biotypes
up to seven applications of glyphosate annually in a fruit orchard
srate for an
sums of squares).

in Tennessee, Mueller et al. (2011) reported an RF of 7.4. Some
were characterized as GR, but exhibit an RF be-
intercept calculated as pseudo $r^2 = 1 – (\text{sums of squares of the regression/corrected total}
\text{sums of squares})$.

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% (ED50), 21
days following treatment. 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Populations</th>
<th>c</th>
<th>d</th>
<th>b</th>
<th>ED50 (g ae ha$^{-1}$)</th>
<th>Pseudo $r^2$</th>
<th>$P^a$</th>
<th>R.F.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. indica</td>
<td>T</td>
<td>1.58</td>
<td>98.19</td>
<td>7.16</td>
<td>90.10 ± 4.25</td>
<td>0.93</td>
<td>&lt;0.0001</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>NT</td>
<td>0.16</td>
<td>99.33</td>
<td>8.01</td>
<td>81.62 ± 7.95</td>
<td>0.96</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>P. distichum</td>
<td>T</td>
<td>2.09</td>
<td>98.44</td>
<td>4.69</td>
<td>75.74 ± 3.42</td>
<td>0.94</td>
<td>&lt;0.0001</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NT</td>
<td>2.13</td>
<td>98.42</td>
<td>4.64</td>
<td>75.16 ± 2.96</td>
<td>0.99</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

$c$ 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 $r^2 = 1 – (\text{sums of squares of the regression/corrected total}
\text{sums of squares})$.

$^c$ Probability level of significance of the non-linear model. R.F. (Resistance Factor) = ED50 (T)/ED50 (NT).

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 ED50 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>mL of sprayed solution retained per g dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. indica</td>
<td>P. distichum</td>
</tr>
<tr>
<td>T</td>
<td>2.42 ± 0.29$^a$</td>
</tr>
<tr>
<td>NT</td>
<td>2.57 ± 0.46</td>
</tr>
</tbody>
</table>

$^a$ 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 14C-glyphosate

At 96 HAT, no significant differences in foliar absorption of 14C-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, 14C-glyphosate accumulation was similar in E. indica, but 14.6% greater in NT vs T plants of P. distichum. Because ED50 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 14C-glyphosate translocation. For E. indica, 2.2-fold more 14C-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 14C-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 14C-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 (e.g., inadequate rates, improper growth stage), adverse weather conditions following application, or other factors (extreme water pH, etc.).

Table 5

<table>
<thead>
<tr>
<th>Species</th>
<th>% Absorption</th>
<th>Translocation (% of absorbed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>NT</td>
</tr>
<tr>
<td>E. indica</td>
<td>84.6 ± 3.1</td>
<td>86.3 ± 1.1</td>
</tr>
<tr>
<td>P. distichum</td>
<td>92.8 ± 2.9</td>
<td>38.0 ± 2.6</td>
</tr>
</tbody>
</table>

* 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.

Table 4

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

* Mean values ± standard error of the mean.

Considering other herbicides utilized, the highest levels of control (90–98%) were achieved with flazasulfuron and cycloxydiam 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 Castellon 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 virgata (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 Tennesse, 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, cycloxydiam, 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
retention, accumulation of shikimic acid, and absorption and translocation of 14C-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 ED50 value was 13.8% higher for E. indica versus P. distichum, which may 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 14C-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 cycloxydim, suggesting that farmers integrate use of herbicide modes of action to maintain effective levels of plant control.

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