Glyphosate Driven Selection Strikes Again: Investigating the Mechanism of Resistance in *Echinochloa colona* from California

Rocío Alarcón-Reverte, Alejandro García, Marie Jasieniuk, Thomas Lanini, Bradley D. Hanson, Albert J. Fischer,

University of California, Davis

INTRODUCTION

Glyphosate is a non-selective herbicide that controls a broad spectrum of weeds in a wide variety of situations (Baylis 2000). It has become the most widely used herbicide since its commercial introduction in the early 1970s (Woodburn 2000). The primary mode of glyphosate action is the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Steinrucken and Amrhein 1980), a key enzyme in the shikimate pathway and responsible for the production of the aromatic amino acids phenylalanine, tyrosine and tryptophan, which are required for protein synthesis and are precursors of a wide range of secondary metabolites important for plants' growth (Tzin and Galili 2010). The blockage by glyphosate of the shikimate pathway results in accumulation of high levels of shikimic acid and eventual death of the plant (Amrhein *et al.* 1980).

The first glyphosate-resistant weed, *Lolium rigidum*, appeared in Australia in 1996. Since then, resistance to glyphosate has evolved in a total of 21 weed species and is present in 15 countries around the world (Heap 2011). The mechanisms of resistance thus far elucidated are either target-site mutations in the *EPSPS* gene or non-target site mechanisms such as limited herbicide uptake and translocation, and *EPSPS* gene amplification.

MATERIALS AND METHODS

Plant material

Two suspected glyphosate-resistant *Echinochloa colona* populations, ECHCOL1 and ECHCOL2, were collected in orchard fields of the Northern Sacramento Valley in California and tested, along with the susceptible commercial biotype ECHCOL3 from Herbiseed (Twyford, RG10 0NJ, UK), for resistance to glyphosate using dose-response experiments and shikimic acid accumulation assays.

Dose response to glyphosate

Plants at the six- to seven-leaf or 2-tiller stage were sprayed with glyphosate (Aqua Neat, 0.65 kg ae l^{-1}) at eight different dose rates (ranging from 26.3 to 1,680 g ae ha^{-1}; equivalent to 1/32 to 2 times the field rate). Plants were harvested 21 days after treatment and fresh weight per pot as a percentage of the untreated control determined.

Whole-plant shikimic acid bioassay

Plants were treated at the three- to four-leaf stage with glyphosate (Roundup WeatherMAX, 0.54 kg ae l^{-1}) at 0.42 kg ae ha^{-1}. The youngest fully expanded leaves were harvested 1 hour and 1, 3, 5 and 7 days after treatment. Leaves were chopped and shikimic acid extraction was carried out according to Perez-Jones *et al.* (2007) with some modifications. Absorbance was measured spectrophotometrically at 380nm and the values of absorbance were converted to micrograms of shikimate accumulated per milliliter of solution.

Leaf-segment shikimic acid bioassay

Five millimeter diameter leaf segments were removed from the youngest fully expanded leaf in plants from susceptible and resistant *E. colona* populations and placed in 96-well plates containing different glyphosate concentrations (Roundup WeatherMAX, 0.54 kg ae l^{-1}). Shikimic acid accumulation was then characterized following the methods described by Perez-Jones *et al.* (2005) and Hanson *et al.* (2009) with modifications. Optical density was measured spectrophotometrically at 380nm and the values of optical density were converted to micrograms of shikimate accumulated per milliliter of solution.

EPSP Synthase gene sequencing

Fresh leaf tissue was collected from young leaves of susceptible ECHCOL3 plants and known resistant plants from the populations ECHCOL1 and ECHCOL2. RNA was extracted and reverse transcribed into cDNA, followed by a standard PCR amplification using the primers AW1 and AW2 (Wakelin and Preston 2006). The amplified PCR fragments were directly sequenced.

Statistical analysis

Dose response curves were obtained by a nonlinear regression model calculated with the add-on package drc in the programme R (Ritz and Streibig 2005), using a three parameter log-logistic equation. Shikimate accumulation values were also subjected to nonlinear regression using a three parameter Gompertz equation. The regression parameters were obtained using Sigma Plot®, Version 11.0. ED_{50} and I_{50} values were obtained and level of resistance determined by calculating the resistant : susceptible ratio.

RESULTS

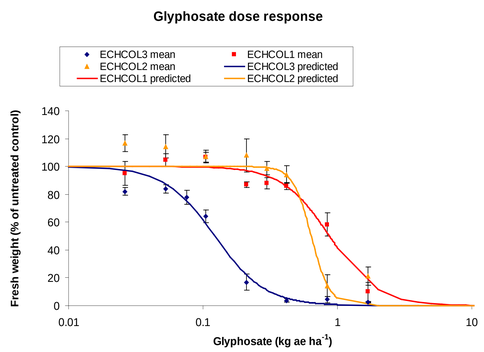

Figure 1. Mean foliage weight as percentage of untreated plants plotted against glyphosate dose for the two glyphosate-resistant populations, ECHCOL1 and ECHCOL2, and the glyphosate-susceptible population ECHCOL3. Symbols and lines represent actual and predicted growth responses, respectively, and vertical bars represent ± standard errors of the mean.

The dose-response experiment revealed that populations responded differently to glyphosate (Figure 1). Populations, ECHCOL1 and ECHCOL2, were 6.6 and 4.4 times, respectively, more resistant to glyphosate than the susceptible standard.

At 0.42 kg ae ha^{-1}, the susceptible standard population, ECHCOL3, accumulated 2.5 times more shikimic than both resistant populations (Figure 2).

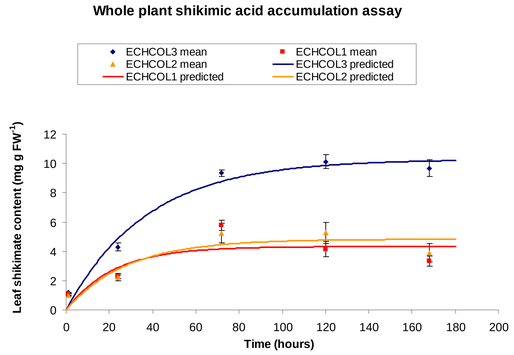

Figure 2. Accumulation of shikimic acid across a timecourse in leaf material from plants sprayed with 0.42 kg ae glyphosate ha^{-1} for the two glyphosate-resistant populations, ECHCOL1 and ECHCOL2, and the glyphosate-susceptible population ECHCOL3. Symbols and lines represent actual and predicted values, respectively, and vertical bars represent ± standard errors of the mean.

The leaf-segment shikimic acid assay was consistent with the dose-response and whole-plant shikimic acid experiments, showing that there is a lower accumulation of shikimic acid in the resistant populations than in the susceptible standard population over a wide range of herbicide concentrations (Figure 3). Results showed that the susceptible standard population ECHCOL3 accumulated 4.6 and 3.2 times more shikimic than ECHCOL1 and ECHCOL2, respectively.

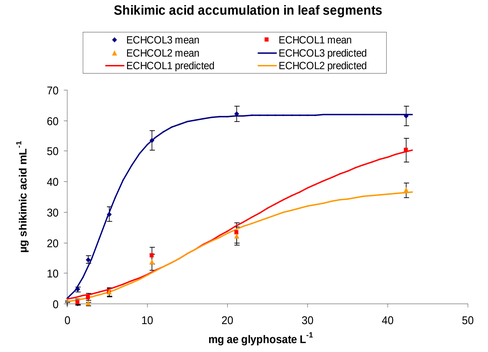

Figure 3. Shikimic acid accumulation in leaf segments of plants from the two glyphosate-resistant populations, ECHCOL1 and ECHCOL2, and the glyphosate-susceptible population ECHCOL3, at different glyphosate concentrations. Symbols and lines represent actual and predicted values, respectively, and vertical bars represent ± standard errors of the mean.

The *EPSPS* gene was amplified and sequenced in all three populations and two different amino acid changes were found in the resistant populations. In the resistant ECHCOL1 population an amino acid change at position 106 from proline to serine (corresponding to a nucleotide change from C to T) was identified, while an amino acid change at the same position from proline to threonine (corresponding to a nucleotide change from C to A) was identified in ECHCOL2. These mutations are potentially responsible for glyphosate resistance in both populations since mutations at this position have been found to confer resistance to glyphosate in other weed species. All the plants sequenced from ECHCOL1 had an amino acid change while not all the resistant plants from ECHCOL2 had a target site mutation.

```
ECHCOL1  GCTGGAACTGCAATGCGGTCATTGACAGCAGCCGTG
           A  G  T  A  M  R  S  L  T  A  A  V
ECHCOL2  GCTGGAACTGCAATGCGGACATTGACAGCAGCCGTG
           A  G  T  A  M  R  T  L  T  A  A  V
ECHCOL3  GCTGGAACTGCAATGCGGCCATTGACAGCAGCCGTG
           A  G  T  A  M  R  P  L  T  A  A  V
```

Figure 4. Amino acid and nucleotide sequences of *EPSPS* cDNA from two glyphosate-resistant populations, ECHCOL1 and ECHCOL2, and the glyphosate-susceptible population ECHCOL3.

CONCLUSIONS

The identification of a target site mutation in plants from both resistant populations might indicate that the mechanism responsible for the resistance to glyphosate in these populations is an altered target site. However, different resistance levels in each population and the presence of resistant plants with no mutation could mean that more than one resistance mechanism is involved. Further experiments are currently being conducted including glyphosate uptake and translocation studies and analysis of *EPSPS* gene expression levels.

BIBLIOGRAPHY

Baylis, A. D. (2000). "Why glyphosate is a global herbicide: strengths, weaknesses and prospects." Pest Management Science 56(4): 299-308.

Hanson, B. D., A. Shrestha and D. L. Shaner (2009). "Distribution of Glyphosate-Resistant Horseweed (Conyza canadensis) and Relationship to Cropping Systems in the Central Valley of California." Weed Science 57(1): 48-53.

Heap, I. (2011). "The International Survey of Herbicide Resistant Weeds." Retrieved Feb 1, 2011, from www.weedscience.com.

Perez-Jones, A., K. W. Park, J. Colquhoun, C. Mallory-Smith and D. Shaner (2005). "Identification of glyphosate-resistant Italian ryegrass (Lolium multiflorum) in Oregon." Weed Science 53(6): 775-779.

Perez-Jones, A., K. W. Park, N. Polge, J. Colquhoun and C. A. Mallory-Smith (2007). "Investigating the mechanisms of glyphosate resistance in Lolium multiflorum." Planta 226(2): 395-404.

Ritz, C. and J. C. Streibig (2005). "Bioassay analysis using R." Journal of Statistical Software 12(5): 1-22.

Steinrucken, H. C. and N. Amrhein (1980). "The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic-acid 3-phosphate synthase." Biochemical and Biophysical Research Communications 94(4): 1207-1212.

Tzin, V. and G. Galili (2010). "New Insights into the Shikimate and Aromatic Amino Acids Biosynthesis Pathways in Plants." Molecular Plant 3(6): 956-972.

Woodburn, A. T. (2000). "Glyphosate: production, pricing and use worldwide." Pest Management Science 56(4): 309-312.