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ARTICLE

Nontarget Mechanims Involved in Glyphosate Tolerance Found in *Canavalia ensiformis* Plants

Hugo Cruz-Hipolito,*,† Maria D. Osuna,§ Antonio Heredia,# Juan Pedro Ruiz-Santaella,† and Rafael De Prado†

†Department of Agricultural Chemistry and Edaphology, University of Cordoba, Cordoba, Spain, §Department of Hortofruticulture, Research Center "Finca La Orden", Badajoz, Spain, and #Department of Molecular Biology and Biochemistry, University of Malaga, Malaga, Spain

A glyphosate-tolerant population of *Canavalia ensiformis* was collected in a cover crop in citrus orchards in Veracruz (Mexico), where glyphosate had been used for the first time. A susceptible *Amaranthus hybridus* L. population was collected from a nearby field that had never been treated with glyphosate. Dose−response experiments indicated a glyphosate tolerance ratio [ED_{50}-(*C. ensiformis*)/ED_{50} (*A. hybridus*)] of 7.7. The hypothesis of a high level of glyphosate tolerance was provisionally corroborated on the basis of shikimate accumulation in both species. The susceptible population accumulated 6 times more shikimic acid in leaf tissue 96 h after glyphosate application than the tolerant leguminous crop. Two different physiological factors were involved in the glyphosate tolerance of this *C. ensiformis* population, which were confirmed by [^{14}C]glyphosate, being a lack of penetration of glyphosate through the cuticle of the leguminous plants and an impaired herbicide translocation to the roots and the rest of shoots. This paper reports that two different nontarget site-based mechanisms, limited absorption and reduced translocation, contribute to the glyphosate tolerance found in *C. ensiformis*.

KEYWORDS: Glyphosate; tolerance; shikimate; *Canavalia ensiformis*

INTRODUCTION

One of the main factors that decrease crop productivity and increase yield losses is, without any doubt, the presence of weeds. Conventional weed management practices (tillage, crop rotation, herbicides, etc.), among other agricultural activities, have caused water pollution, erosion, and the resulting desertization of extensive agricultural areas (*1*). Although agricultural systems considerably reducing the need to till soils and to massively use fertilizers have been developed, dependence on herbicides for controlling weeds persists, mostly due to the wide genetic diversity conferred on weeds by their strong ability to compete and survive (*2*). One alternative for preventing or reducing intensive herbicide use in agriculture is so-called cover crops, which drastically reduce the germination, emergence, and/or growth of weed populations, thus diminishing water pollution and soil losses from erosion (*3*, *4*). Some studies have indicated that investigating cover crops should be a priority in the new weed management technologies in sustainable agriculture (*1*).

In large citrus orchard areas in the Mexican tropics, leguminous crops have traditionally been used as cover crops in strips 2−3 m wide between each row of trees. One of the varieties most used is *Canavalia ensiformis*, an annual, robust, and drought-resistant plant immune to most pests. It is extensively grown as forage or as an environmentally friendly fertilizer. According to ref (*5*), among the main advantages of using this legume as a cover crop can be highlighted (a) weed control, as this plant's vigorous growth stops the entry of light to the weeds, thus limiting their growth; (b) increase in organic matter levels in the soil; (c) fixation of atmospheric nitrogen as its use contributes up to 231 kg of N ha^{-1}; and (d) erosion control and maintenance of moisture levels in the soil.

During the first two years of establishing these plant coverings, different undesirable weed populations have to be controlled. Among the most injurious and widespread varieties in the Mexican tropics are *Amaranthus hybridus*, *Chenopodium album* L., *Cynodon dactylon* L. Pers., *Cyperus esculentus* L., *Cyperus rotundus* L., *Digitaria* spp., *Rottboellia cochinchinensis* L., *Sorghum halepense* L., and others, all of them completely controlled at early stages after glyphosate application at agricultural doses of 195−400 g of ae ha^{-1} (*2*). Glyphosate has become the leading postemergence, systemic, nonselective, broad-spectrum herbicide for the control of annual and perennial weeds (*6*). Today, it is used as a noncrop, plantation crop (e.g., orchards and vineyards), and spring cover crop herbicide and for selective weed control in transgenic glyphosate-resistant crops (*7*). Inhibition of growth occurs almost immediately, followed by chlorosis at the newest growing points and necrosis throughout the entire plant within 1−2 weeks (*7*, *8*). It is well established that glyphosate exerts its herbicidal effects through inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) (*9*). This inhibition prevents the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan that are required for protein synthesis (*10*). However, a more rapid and dramatic effect

*Corresponding author (e-mail g32crhih@uco.es; telephone +34957218600; fax +34957218600).

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is the increase in shikimic acid, which has been related to a decline in carbon fixation intermediates and a reduction of photosynthesis (*11*).

Glyphosate tolerance/resistance in crops and weeds is due to two main mechanisms defined as nontarget sites as a reduced absorption and/or translocation and target site resistance. Glyphosate resistance due to limited translocation has been described in GR biotypes of horseweed (*12*, *13*), hairy fleabane (*14*), rigid ryegrass (*15*), and Italian ryegrass (*16*, *17*). The pattern of glyphosate movement in these GR biotypes differs from that in GS biotypes; less glyphosate translocated out of the treated leaf in the GR biotypes compared with the GS biotypes (*18*). Different mutations on the EPSP synthase gene have been reported to confer resistance in *Eleusine indica* (Pro106Ser/Thr) (*19*, *20*), *Lolium rigidum* (Pro106Thr/Ala), (*21*, *22*), and *Lolium multiflorum* (Pro106Ser) (*17*). On the contrary, metabolism of glyphosate has not been found to be a mechanism of resistance (*12*, *23*).

The objective of this research was to understand the differential glyphosate sensitivity between *C. ensiformis* and *A. hybridus*. For that, the specific objectives of the present study were to (i) confirm tolerance to glyphosate in *C. ensiformis* cv. and (ii) determine the mechanism(s) of tolerance by evaluating differential shikimic acid accumulation and absorption, translocation, metabolism of [^{14}C]glyphosate in *C. ensiformis* and *A. hybridus* plants.

MATERIALS AND METHODS

Chemicals. The following herbicides and reagents were used in this study: [^{14}C]glyphosate-(phosphonomethyl), specific activity = 52 mC$_i$ mmol^{-1} (ARC, American Radiolabel Chemicals, Inc.). Commercial herbicide formulation (Roundup 360 g/L) was used for dose−response assays, and all other reagents were purchased at analytical grade.

Plant Material and Growing Conditions. A glyphosate-tolerant leguminous cultivar, *C. ensiformis*, and a glyphosate-susceptible *A. hybridus* biotype were used in the experiments described below. Seeds of both species were germinated on moistened filter paper in Petri dishes. Seedlings were planted in pots (three plants per pot) containing peat and sandy loam potting mixture (1:2, v/v) in a growth chamber at 28/18 °C (day/night) in a 16 h photoperiod under 850 μmol m^{-2} s^{-1} photosynthetic photon-flux density and 80% relative humidity.

Dose−Response Assays. Treatments were applied to plants of both species (*A. hybridus*, third pair of opposite true leaves; *C. ensiformis*, third pair of trifolium leaves), using a laboratory track sprayer equipped with a Tee Jet 80.02.E.VS flat-fan nozzle delivering a spray volume of 200 L ha^{-1} at 200 kPa. Glyphosate was applied at rates of 0, 25, 50, 100, 150, and 200 g of ai ha^{-1} for *A. hybridus* and 0, 50, 100, 150, 200, 250, 300, 350, 400, and 500 g of ai ha^{-1} for *C. ensiformis*. Above-ground fresh weight per pot was determined 21 days after spraying, and data were expressed as percentage of the untreated control. Herbicide rates to inhibit plant growth by a 50% decrease in growth with respect to the untreated control (ED_{50}) were determined for each species as described in ref (*24*). The tolerance/susceptibility ratio was computed as ED_{50} (*C. ensiformis*)/ED_{50} (*A. hybridus*). Experimental treatments were replicated four times, and each experiment was conducted three times. Data were pooled and fitted to a nonlinear, log−logistic regression model

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

where Y is the fresh above-ground weight expressed as a percentage of the untreated control, c and d are coefficients corresponding to the lower and upper asymptotes, b is the slope of the line, g is the herbicide rate at the point of inflection halfway between the upper and lower asymptotes, and x (independent variable) is the herbicide dose. Regression analysis was conducted using Sigma Plot 8.0 statistical software (*25*).

Whole-Plant Shikimic Acid Assays. Plants of *C. ensiformis* and *A. hybridus* were sprayed as previously described with commercially formulated glyphosate at 500 g of ai ha^{-1}. Plants of both species (same stage as described before) were harvested for shikimic acid extraction 6, 12, 24, 48, 72, and 96 h after treatment. Leaf tissues were homogenized (0.1 g of fresh weight), and samples were frozen in liquid nitrogen following the protocol of ref (*17*). Shikimic acid accumulation was optically determined using a Beckman DU-640 spectrophotometer. The standard curve was determined using untreated plants and a known concentration of shikimic acid. The experiment was repeated three times with 10 replications per harvest time per species.

Absorption and Translocation Assays. [^{14}C]Glyphosate was mixed with commercially formulated glyphosate to prepare emulsions with a specific activity of 1.85 kBq μL^{-1} (both absorption and translocation studies) and a glyphosate concentration of 3.6 g of ai L^{-1} (corresponding to 720 g of ai ha^{-1} at 200 L ha^{-1}). The labeled herbicide was applied to the adaxial surface of the second leaf of each plant in four 0.5 μL droplets using a microapplicator (Hamilton PB 600 TA, Hamilton Co.). A total of 3.7 kBq was applied on each plant. Plants of both species at the three pairs of true leaves were harvested in batches of three replications of plants at 12, 24, 48, and 96 h after treatment (HAT) and separated into treated leaf, upper leaf, root, and rest of shoot. Unabsorbed [^{14}C]glyphosate was removed from the leaf surface by rinsing the treated area with 3 mL of methanol 80% (v/v). Rinses from each batch were pooled and analyzed by liquid scintillation spectrometry (LSS) (Scintillation Counter, Beckman LS 6500 TA, Beckman Instruments Inc., Fullerton, CA). The plant tissue was dried at 55 °C for 72 h and combusted in a sample oxidizer (Tri Carb model 307, Packard Instrument Co). The $^{14}CO_2$ evolved was trapped and counted in a 10 mL mixture of Carbo-Sorb E and Permafluor E+ (3:7, v/v) (Perkin-Elmer, Packard Bioscience BV, Groningen, The Netherlands). The radioactivity was quantified by LSS, and percent herbicide absorbed was expressed as [kBq in combusted tissue/(kBq in combusted tissue + kBq in leaf washes)] × 100 (*26*). The experiment was repeated three times. In the translocation studies, the treated plants of both species were removed from pots at the same times. Roots were rinsed, and whole plants were oven-dried (50 °C, 4 days), pressed against a 25 cm × 12.5 cm phosphor storage film during 6 h, and scanned (Cyclone, Perkin-Elmer, Packard Bioscience BV) for radiolabel dispersion. Means and standard errors (of the mean) were computed for all parts of plants, and means were tested for group differences and compared using an analysis of variance (ANOVA) and a Tukey HSD posthoc test.

RESULTS

Dose−Response Assays. Fresh shoot biomass in both *C. ensiformis* and *A. hybridus* decreased when the glyphosate rate increased (**Figure 1**). However, there was a different dose−response between the glyphosate-tolerant *C. ensiformis* cv. and the *A. hybridus* susceptible population. At 200 g of ai ha^{-1}, shoot growth was reduced 95% in the case of *A. hybridus*, whereas in the case of *C. ensiformis* it was reduced 30% (**Figure 1**).

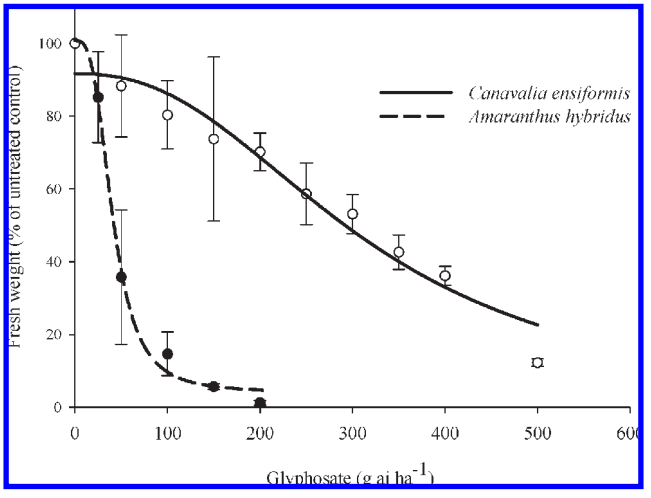

Figure 1. Dose−response assays of *C. ensiformis* (○) and *A. hybridus* (●). The plant fresh weight was determined 21 DAT, and data are expressed as percentage of the untreated control; each point is the mean ± standard errors (SE) of three experiments.

Table 1. Parameters of the Log-Logistic Equation Used To Calculate the Herbicide Dose Required for 50% Reduction of the Fresh Weight (ED_{50}) of *Canavalia ensiformis* and *Amaranthus hybridus*

species	*c*	*d*	*b*	ED_{50} (g ha^{-1})	*P*[b]	RF[c]
A. hybridus	4.09 ± 2.14	100 ± 7.07	3.11 ± 1.12	40.93 ± 6,82	<0.001	
C. ensiformis	1.07 ± 0.17	91.65 ± 6.41	2.40 ± 0.34	315.05 ± 3.25	<0.001	7.69

[a] Equation $Y = c + \{(d - c)/[1 + (x/g)^b]\}$, where *Y* is the percentage of plant injury, *x* (independent variable) is the herbicide rate, *c* and *d* are the lower and upper asymptotes, *b* is the slope of the line, and ED_{50} is the effective dose required for 50% plant injury. Data were pooled and fitted to nonlinear regression model. Data are means of four replicates. [b] Probability level of significance of the nonlinear model [c] RF, resistance factor = ED_{50} of tolerant/ED_{50} of susceptible biotype.

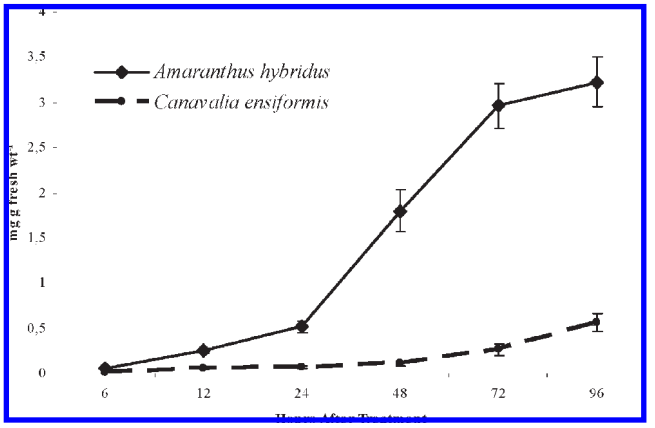

Figure 2. Shikimic acid accumulation in shoots of *A. hybridus* and *C. ensiformis* plants following the application of glyphosate at 500 g ha^{-1}. Vertical bars represent ± standard errors of the mean.

This bioassay concluded that the cultivar (ED_{50} = 315.05 g of ai ha^{-1}) was 7.7 times more tolerant to glyphosate than the susceptible weed (ED_{50} = 40.93 g of ai ha^{-1}) (**Table 1**).

Whole-Plant Shikimic Acid Assays. Shikimic acid concentration increased in both *C. ensiformis* and *A. hybridus* leaf tissues after glyphosate application (**Figure 2**). Similar levels of shikimate were obtained in both species 6 h after treatment (HAT). Nevertheless, it significantly increased at 48, 72, and 96 HAT in *A. hybridus*, accumulating 10, 12, and 6 times more shikinic acid, respectively, than the tolerant *C. ensiformis* cultivar.

Absorption and Translocation Assays. Large differences were found between the glyphosate-susceptible weed and the tolerant cultivar in [^{14}C]glyphosate leaf uptake (**Figure 3**). The pattern of uptake was maximum 48 HAT for *A. hybridus* (93%) population, whereas for the *C. ensiformis* cultivar it was 50% less. After 96 HAT, 63% of the recovered radioactivity had penetrated into the leaf tissue of the *C. ensiformis* with a large increase from 6 (15%) to 48 HAT (52%) and a small increase from 48 to 96 HAT, whereas 93% had penetrated into *A. hybridus* at 96 HAT (**Figure 3**). There were also differences in the percentage of [^{14}C]-glyphosate translocated from the treated leaf to the rest of the plant (**Table 2**). In the case of *A. hybridus*, 24 HAT, 80% of the [^{14}C]glyphosate absorbed remained in the treated leaf, 8 and 12% being translocated into the rest of the plant and roots, respectively (**Table 2**). By contrast, in the case of *C. ensiformis*, 93% of the [^{14}C]-glyphosate absorbed remained in the treated leaf and only 3.3% moved into the rest of plant (**Table 2**). The translocation of [^{14}C]-glyphosate increased with the passing of time, this being greater in the susceptible weed than in the tolerant cultivar. The differences in [^{14}C]glyphosate translocation between *A. hybridus* and *C. ensiformis* were confirmed with phosphorimaging (**Figure 4**).

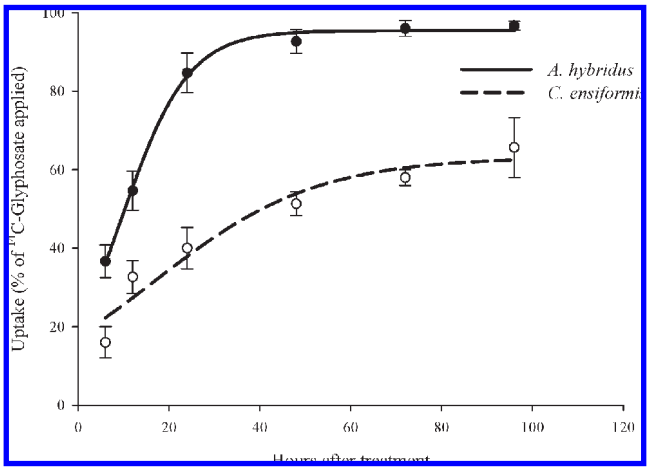

Figure 3. Foliar absorption of [^{14}C]glyphosate in *A. hybridus* and *C. ensiformis* over 96 h. Vertical bars represent ± standard errors of the means.

Table 2. Translocation (Percent of Absorbed Radioactivity) of [^{14}C]Glyphosate in *Amaranthus hybridus* and *Canavalia ensiformis*

h after treatment	species	[^{14}C]glyphosate (% of absorbed)[a]		
		treated leaf	root	rest of plant
24	*A. hybridus*	80.00 ± 2.00 B	8.33 ± 1.53 JK	11.66 ± 2.08 IJ
	C. ensiformis	96.66 ± 3.06 A	0.00 ± 0.00 L	3.33 ± 0.58 KL
48	*A. hybridus*	64.00 ± 2.30 C	16.00 ± 1.40 HI	20.00 ± 1.80 GH
	C. ensiformis	84.00 ± 2.40 B	2.00 ± 0.20 KL	14.00 ± 0.80 HIJ
72	*A. hybridus*	40.00 ± 3.4 D	28.00 ± 4.80 EF	32.00 ± 4.30 E
	C. ensiformis	68.00 ± 2.10 C	8.00 ± 0.80 JK	24.00 ± 2.3 FG

[a] Means within a column followed by the same letter are not significantly different at the 5% level as determined by the Tukey test. Values ± standard error of the mean; 0 = nondetected.

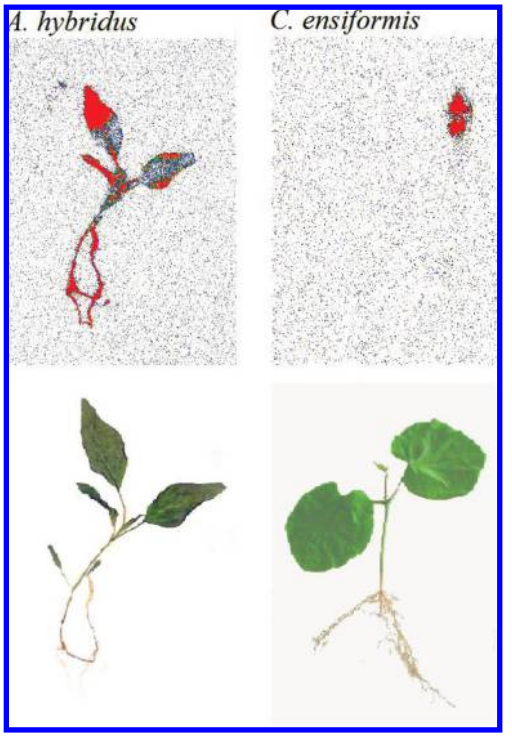

Figure 4. Phosphorimaging visualization of [^{14}C]glyphosate translocation of *A. hybridus* (left) and *C. ensiformis* (right), 24 HAT.

[^{14}C]Glyphosate remained in the treated leaf and moved poorly out of the leaf in the cultivar compared to the weed 24 HAT.

DISCUSSION

Dose−response assays confirmed the tolerance of the *C. ensiformis* to glyphosate. The observed tolerance ratio (ED_{50} *C. ensiformis*/ED_{50} *A. hybridus*) was within the range (2.5−11.8) found for other tolerant and resistant biotypes (*27*−*31*). Some authors demonstrated the efficacy of Petri dish bioassays for glyphosate resistance screening (*32*), but there is some disagreement regarding the resistance ratios obtained. However, the dose−response assays provide a more accurate quantification of glyphosate resistance level as it is reproduces the conditions under which plants developed resistance. Shikimic acid accumulation in leaf tissue 96 h after glyphosate application was 5 times greater in *A. hybridus* than in *C. ensiformis*. The greater accumulation of shikimic acid in the susceptible weed further confirms that the *C. ensiformis* plants are glyphosate-tolerant. However, this result does not define which mechanisms are involved in the tolerance of *C. ensiformis*, as has been demonstrated for other glyphosate-resistant biotypes (*32*).

At least two different physiological factors may be involved in glyphosate tolerance of this *C. ensiformis* population. The first factor is impaired penetration of herbicide across the cuticle of the leguminous plants. After 48 h, 92.6% of the recovered radioactivity had penetrated into the leaf tissue of the *A. hybridus*, whereas < 50% had penetrated into the *C. ensiformis*. These values are in general agreement with the available literature for other specie tolerant to glyphosate (*33*) and some resistant biotypes (*26*). The second feature involved in the tolerance of *C. ensiformis* was the differential translocation as the patterns of [^{14}C]glyphosate translocation were significantly different. These results can be observed in the phosphorimaging visualization of [^{14}C]glyphosate translocation of *A. hybridus* and *C. ensiformis*, when, at 24 HAT, there was no appreciable acropetal and/or basipetal glyphosate translocation in the case of *C. ensiformis*. Altered glyphosate symplast transport has been also observed in several resistant biotypes with decreased translocation to roots, shoot meristematic zones, and untreated young leaves (*12*, *15*, *17*).

In conclusion, we found that glyphosate tolerance in *C. ensiformis* is conferred by two different nontarget site-based mechanisms, limited absorption and reduced translocation. Glyphosate tolerance has been an important topic for many years as some weeds have been described as having inherent tolerance to various herbicides, but it was brought to prominence with the adoption of glyphosate-resistant crops. An early assessment suggesting that resistance to glyphosate would not evolve was wrong and also provided some prediction of tolerant weed species (*35*). The term tolerance is frequently used not only to refer to variations in ability to withstand herbicide application between different species but also when there is variability within a population of the same species (*36*). Tolerance and resistance are also regarded as terminologies denoting differences in the intensity of the same phenomenon. Resistance is considered to be an extreme, but less frequent, case of tolerance (*37*, *38*). Some consider tolerance as being a polygenic mechanism and resistance as monogenic (*39*). A number of mechanisms by which weeds could be tolerant to glyphosate were predicted, and subsequent reports and anecdotal observations validate the early assessment (*40*). Differential absorption of glyphosate, the chemical composition of the epicuticular wax, leaf angle, and other mechanisms can account for tolerance in various weed species.

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