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Comparison of WeatherMax® and Engame™ Formulations of Glyphosate on Cotyledon Surface Structure, Chlorophyll A Fluorescence and Shikimate Levels in Isogenic Cotton Cultivars Differing in Roundup Resistance

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Abstract

The effects of Roundup WeatherMax® and Engame™ formulations of glyphosate were investigated on the cotyledons of glyphosate resistant (GR) and glyphosate sensitive (GS) isogenic cotton cultivars. Engame™ is a mixture of glyphosate and 1-aminomethanamide dihydrogen tetraoxosulfate (AMADS). Fully expanded cotton cotyledons treated with Engame™ or AMADS developed surface lesions within 2 hours after treatment whereas surfactant-treated control or WeatherMax®-treated tissues did not develop lesions. The Engame™ and AMADS damage appeared as depressions which were confirmed by scanning electron microscopy. Light micrographs of cross sections through the depressions revealed collapsed and compressed epidermal and mesophyll cells with congealed cytoplasmic contents in the palisade and spongy mesophyll cells. Changes to photosynthetic electron transport were evident at 4 hours after treatment (HAT) in all treatments as revealed by chlorophyll A fluorescence. In GR cotton, the fluorescence perturbations decreased with time such that at 72 HAT Engame™-treated cotyledons could not be distinguished from the surfactant- or Weathermax®-treated plants. The GS cotton continued to show progressive decreases in the fluorescence parameters Fv/Fm and performance index (PI) to 72 HAT. Shikimate levels increased following glyphosate treatment in glyphosate sensitive cotton and Engame™ caused a two- to three-fold greater increase in shikimate compared to WeatherMax®. These results indicate that the Engame™-based glyphosate formulation involved structural tissue damage which likely increased glyphosate uptake and subsequently increased inhibition of photosynthesis and the shiki-

mate pathway.

Keywords

Glyphosate, Engame™, Weathermax®, Cuticle Damage, Shikimate, Uptake

1. Introduction

Glyphosate-based herbicides are broad spectrum, nonselective, post-emergent products having high unit activity on a wide variety of annual and perennial weeds [1]. Globally, glyphosate-based herbicides are very successful with approximately 6.5×10^5 tons, valued at $6.5 billion (US dollars), used in 2011 [2] [3]. Glyphosate-based herbicides are generally formulated as a mixture of a soluble salt and proprietary surfactants and adjuvants that increase dispersion and retention on the leaf surface, and penetration through the hydrophobic, transport limiting cuticle [4] [5]. Although many glyphosate products are formulated as isopropylamine salts, dimethylamine, potassium, diammonium and trimethylsulfomium salts are also used in some commercial glyphosate products [4] [5]. To achieve maximum retention on the leaf surface, a concentration of approximately 1% of a 15 - 20 EO tallow amine was identified as the benchmark adjuvant for glyphosate [6] [7].

Despite identification of efficient adjuvants, it was evident from uptake and translocation studies that more than 50% of the applied glyphosate remained on the leaf surface within the first 72 HAT indicating there may be room for improvements in formulation [8]-[21]. For example, uptake and translocation of glyphosate on velvetleaf were compared among three commercial formulations (Roundup, Roundup Ultra and Touchdown) [20]. Glyphosate in the Roundup formulation showed immediate uptake and translocation followed by tissue necrosis, appearing as epithelial collapse and congealed cytoplasmic contents, which was visible in cross sections of leaves within 24 HAT [19]. However, at 72 HAT leaf washes contained approximately 45% of the applied glyphosate in the Roundup compared to 70% of the glyphosate in Touchdown [19]. Thus, efforts to identify new glyphosate formulations and additives to maximize glyphosate uptake and translocation, and hence to maximize its benefits were warranted. From 1995 to 1998, there were 32 patents and 76 research papers on the activity of glyphosate formulations, mixtures and formulation effects [22]. Active research into glyphosate formulations may have occurred in anticipation of Monsanto patents on glyphosate expiring outside the USA in 1991 and in the US in 2000. Additives, such as ammonium sulfate, were already known to increase the phytotoxicity of many water-soluble post-emergence herbicides in glyphosate [2] [11] [18]. For example, ammonium sulfate reduced the concentration of glyphosate isopropylamine salt to cause a 50% inhibition of velvetleaf dry weight accumulation by two- to five-fold [10].

Herbicidal compositions outside of the more conventional formulations, such as glyphosate/sulfuric acid mixtures, were claimed to produce more rapid, more thorough, broader spectrum vegetation control, and were more stable chemically and less toxic than isopropylamine formulations of glyphosate herbicides although these were not commercialized [23]. Although the effects of these glyphosate/sulfuric acid formulations on leaf surfaces were not presented, the effects on leaves may be like simulated acid rain treatments applied as dilute sulfuric and nitric acid solutions [24]. These treatments caused cuticular erosion, displacement of leaf surface waxes and reductions in cuticle thickness [24]. Another new formulation of glyphosate diverging from the alkaline counter ion and surfactant-based approach, was Engame™ herbicide [25]. Engame™ was a proprietary mixture of glyphosate and 1-aminomethanamide dihydrogen tetraoxosulfate (AMADS) [25]. AMADS was the reaction product formed upon heating a proprietary combination of sulfuric acid and urea and had a pH of 2. The Engame™ formulation formed necrotic lesions on the plant surfaces like that from acid rain [26]. When applied to weeds, Engame™ improved glyphosate performance and rainfastness which was thought to be the result of faster uptake and translocation [26] [27]. AMADS increased the efficacy of three glyphosate formulations on corn by three to fourfold and was more effective than ammonium sulfate in overcoming the antagonism of hard water on the efficacy of glyphosate in the potassium salt form [28]. The improved performance observed with Engame™, expressed as increased tissue damage, uptake and translocation, may also increase lethality on glyphosate resistant crops as a result of its acidic nature.

The objectives of this research were four-fold: 1) to compare the Roundup Weathermax® and Engame™ formulations of glyphosate on isogenic cotton cultivars differing in resistance to glyphosate (Delta and Pine Land cultivar 491 glyphosate-sensitive (GS) and 494 glyphosate-resistant (GR), 2) to document changes in leaf surface anatomy caused by Engame™, 3) to determine changes in response time in shikimate accumulation between these formulations as an indicator of glyphosate action, and 4) to determine and compare the chlorophyll A fluorescence (Chl A) parameters, the ratio of variable fluorescence to maximum fluorescence (Fv/Fm), and the performance index (PI), as means to confirm physiological injury. These results may better explain formulation limitations of current glyphosate products.

2. Materials and Methods

2.1. Plant Material and Herbicide Application

Isogenic lines of cotton (*Gossypium hirsutum* L.), upland cotton cultivar Deltapine 491 (DP491, glyphosate sensitive (GS)) and Deltapine 494 RR (DP494 glyphosate resistant (GR)) were a gift from Delta and Pine Land Inc., Scott, MS. The pedigree of DP494 was a recurrent parent selection from DP491 [29]. Cotton seeds were sown in a 4:1 (w:w) mixture of soil (Dundee silty clay loam,

fine-silty, mixed thermic Aeric Ochraqualf) and commercial potting mix (Redi-earth Plug and Seedling mix, SUN GRO Horticulture Distribution Inc., Bellevue, WA, USA) in 10 cm diameter pots. Pots were placed in the greenhouse set at 30˚C ± 2˚C and with a photoperiod of 14 h under natural sunlight conditions supplemented with high-pressure sodium lights providing 400 μmol·m^{-2}·s^{-1}. Pots were sub-irrigated as needed.

When cotton cotyledons had become fully expanded, herbicides were applied using a pneumatic track sprayer delivering 187 L/ha water at 179 kPa. All treatments were formulated to contain 0.25% Induce (Helena Chemical) adjuvant. Roundup WeatherMax® and Engame™ were applied to deliver 0.8 kg·ai/ha of glyphosate. AMADS was applied at the same rate as found in Engame™.

An RCB design with 4 replications was used and the experiment was repeated. Data were not significantly different between experiments and were combined.

2.2. Fluorescence

Fluorescence induction curves (Kautsky curves) were recorded from cotyledons with a HandyPEA fluorimeter (Hansatech Instruments, Norfolk, England), for 2 seconds. Cotyledons had been dark adapted for 30 minutes using dark adaption clips supplied with the instrument prior to measurement. Measurements were collected at 4, 24 and 72 HAT. Fluorescence measurements were taken from one half of the cotyledon, and afterward, disks (4 mm diameter) were cut from treated and control cotyledons for shikimate analyses. Fluorescence was measured on 4 plants per treatment and the experiment was repeated. Fluorescence parameters were collected from the software provided by Hansatech (PEA Plus Version 1.00, Copyright © 2007). The parameters measured were the yield of fluorescence in the absence of actinic light (F_o), the maximum fluorescence in the absence of any photochemical quenching (F_m), the difference between F_m and F_o(F_v), the maximum quantum yield of photosystem II (PSII) (F_v/F_m), and performance index (PI).

2.3. Shikimate Leaf Disk Assay

The shikimate accumulation microtiter plate assay [30] was used for shikimate determination with modifications in plate size and disk number to improve performance. Plants were grown as described above in 15 cm^2 pots until the fourth leaf in the whorl was 20 cm in length. Only leaves that were uniformly green and free of chlorotic or necrotic leaf tips were used. From each cotyledon, 4 disks, 4 mm in diameter, were cut with a cork hole borer, and placed in 100 μL of incubation buffer in a 48-well microtiter plate.

2.4. Microscopy

Samples for light microscopy were fixed at 2 HAT in a 6% (v/v) glutaraldehyde solution in 0.05 M PIPES buffer (pH 7.4) for 2 h at room temperature. After two 15 min washes in PIPES buffer, the samples were dehydrated in an ethanol series

at 4 ˚C, with the 75% step held overnight. The next day samples were transferred to 100% ethanol at 4 ˚C and then transferred to −20 ˚C for 24 h. Embedding was carried out by increasing the amount of LR White (Electron Microscopy Sciences, Hatfield, PA) plastic at 25% increment each day until 100% plastic was reached. After 24 h in 100% plastic, the samples were brought to room temperature and agitated on a rocking platform for 24 h. Tissue pieces were placed in flat-bottomed BEEM capsules (BEEM® Cryo Capsule Holders, EMS, Hatfield, PA) and polymerization took place at 50 ˚C for 2 h in a vacuum oven. Samples were flat embedded, and the cotyledon pieces were cut from the blocks and mounted on acrylic stubs so that cross sections were obtained. Sections (0.35 μm) were obtained with a Reichert Ultracut ultramicrotome using a Delaware Diamond Histo-Knife (DDK) and were mounted on chrome-alum subbed microscope slides. Sections were stained with 1% toluidine blue in 1% sodium borate and examined with a Zeiss photomicroscope. Digital images were collected.

2.5. Scanning Electron Microscopy

Cotyledon samples were placed sprayed-side-up on weigh papers in desiccators over calcium chloride and allowed to air-dry. The samples were prepared in this manner to avoid removal of surface waxes that might be lost during normal dehydration and critical point drying procedures. When the samples were dry, they were mounted on aluminum stubs with adhesive tabs and coated with 15 nm gold-palladium with a Hummer sputter coater. Specimens were observed with a JEOL 840 scanning electron microscope operating at 15 kV. Images were collected digitally using a Kevex digital acquisition program.

3. Results

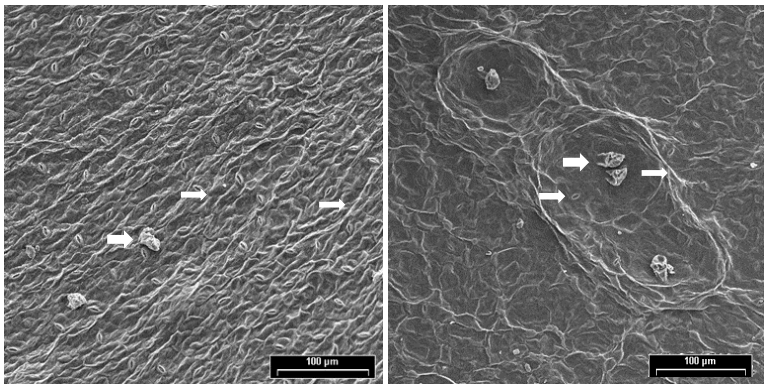
Visually, the cotton cotyledon surface was glossy and smooth indicating a relatively uniform waxy cuticle (**Figure 1(A)**). Treatment of the cotyledons with Engame™ or AMADS (not shown) disrupted the cuticle forming pits on the cotyledon surface on both GR and GS cotton. These pits were visible without a microscope at 24 HAT and resulted in a speckled appearance of the cotyledon surface (**Figure 1(B)**). At higher magnification, the surface had a textured appearance with numerous stomata and irregular structures which were likely trichomes (**Figure 2(A)**). Low magnification microscopy of the pits revealed a ridge of cuticular material along its outer edge which may have been dislodged cuticular material from the center of the pits (**Figure 2(B)**). A secondary structure was found within the pit area which appeared to be disorganized cuticle and/or remnants of trichomes (**Figure 2(B)**).

Cross sections through the pits were prepared at 2 HAT to capture the early effects of Engame™ treatment. The injury caused by the Engame™ formulation of glyphosate or AMADS by itself caused severe cellular disruption which was observed as collapsed palisade and mesophyll cells with congealed, cytoplasmic contents consisting of chloroplasts and other organelles (**Figure 3(A)**). The

formulations infiltrated two or three cell layers into the spongy mesophyll well below the leaf surface. The collapse of the palisade cells was most evident due to their columnar structure in healthy tissues. The contraction of the surface forming a pit or lesion was likely due to membrane disruption and desiccation of the palisade and mesophyll cells caused by the low pH of the AMADS in Engame™. Cells outside the pit area were turgid with chloroplasts appressed to the cell wall and appeared relatively normal (**Figures 3(A)-(D)**). The pits ranged in diameter from 75 μm (**Figure 3(A)**) to 10 μm (**Figure 3(D)**). No pits, wax disturbance or aggregates were noted in control cotyledons or WeatherMax® treated cotyledons prepared in an identical manner (data not shown).

(A) (B)

Figure 1. The effect of surfactant (A) and AMADS (B) on GS cotton cotyledons at 24 h after treatment. The response of cotton was identical (Data not shown). Note the formation of white lesions in B.



(A) (B)

Figure 2. Scanning electron micrographs of control (A) and Engame™-treated (B) cotton cotyledons that have been air-dried and then coated with gold-palladium. (A) Surface of the control cotyledon reveals no pits, the only structure being the abundant stomata (middle size arrow) and a few trichomes (large arrow). (B) Low magnification micrograph revealing numerous craters or pits created by the Engame™ treatment (B). Cuticular material appears to be removed from the center of the crater and deposited towards the edges, leaving a lip around the crater (thin arrow). The crater has accumulated cuticular or trichome material in the center, gathered into an irregular aggregation (large arrow).

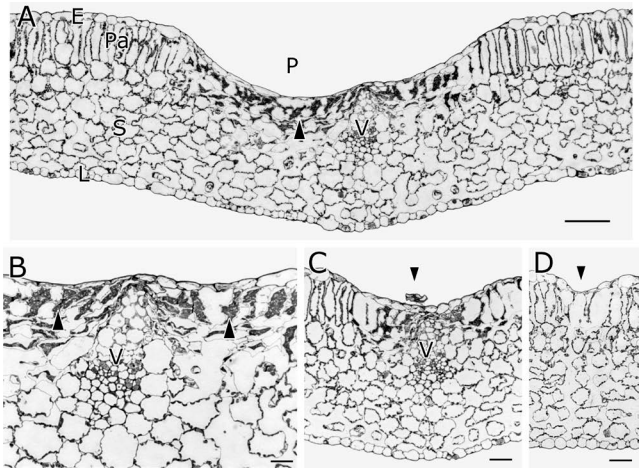


Figure 3. Light micrographs of GS cotton cotyledons treated with the Engame™ formulation of glyphosate. GR cotyledons responded identically (data not shown). A. A low magnification cross-section of an area through a large pits (P) created by spraying with Engame™. Both the epidermal (E) cells and top layer palisade (Pa) cells are affected in the pit area but not elsewhere in the cotyledon. S = spongy mesophyll, V = vascular tissue. B. A higher magnification area in the pit tissue, showing the abnormal congealing of cytoplasmic contents and of chloroplasts (arrowheads). C. Accumulation of cuticular material in a pit (arrowhead). D. A very small pit (arrowhead). Bars = 10 μm.

Engame™ at an equivalent glyphosate concentration as that in Weathermax® resulted in an increase in shikimate levels to nearly twice that of the Weathermax® formulation at 4 and 24 HAT and nearly three-fold the level by 72 HAT in GS cotton (Table 1). Engame™, Weathermax® and AMADS alone did not increase shikimate in GR cotton nor did AMADS increase shikimate in GS cotton (Table 1).

Chlorophyll A fluorescence (ChlA) transients for untreated GS and GR, shown in Figure 4, were identical. At 4 HAT, following Roundup WeatherMax® and Engame™ treatments, the maximum fluorescence, F_m, in both GS and GR cultivars decreased beginning at inflection point D and the initial fluorescence, F_o, increased (Figure 4, Figure 5(A) and Figure 5(B)). The decrease in F_m was slightly greater following treatment with Engame™ in GR (5B). At 24 HAT in GS, F_m continued to decrease and F_o and F_j increased following Engame™ treatment whereas transients indicated a measure of recovery for both herbicides in GR (Figure 5(C) and Figure 5(D)). At 72 HAT, there was nearly complete photoinhibition of PSII with Engame™ in GS and the decrease in F_m following Weathermax® treatment remained unchanged from that at 4 HAT (Figure 5(E)). At 72 HAT, injury in GR was not detectable for either herbicide formulation (Figure 5(F)).

Chl A parameters confirmed the observations highlighted by fluorescence transients (Table 2). In GS, Engame™ increased F_o, and decreased F_m, and F_vF_m at 24 and 72 HAT. Engame™, and to a lesser extent Weathermax®, also caused an increase in F_o relative to the control but the effect from Weathermax® was transient and it decreased to control levels at 72 HAT. Another stress indicator, the

performance index (PI), decreased rapidly in GS cotton in response to Engame™ but the response to Weathermax® indicated recovery at 72 HAT. PI was a more sensitive indicator of herbicide injury than F_v/F_m and may provide a clearer indication of the decline in plant health following glyphosate treatment.

Table 1. Effect of Engame™ and Weathermax® on shikimate accumulation in cotton cotyledon disks from cotton cultivars DP 491 (GS) and DP 494 (GR).

	Control		AMADS		Engame		Weathermax	
Hours after treatment	491	494	491	494	491	494	491	494
	Shikimate (μg/ml)							
4	4.1ab[1]	2.4ab	6.3b	0.6a	38.5d	3.4ab	22.1c	2.2ab
24	2.7a	1.0a	4.8a	0.3a	140.1c	2.7a	69.3b	1.0a
72	3.3a	2.4a	1.7a	2.4a	156.2c	4.4a	55.9b	2.1a

[1]Means within a time followed by the letter were not significantly different according to Tukey's HSD test at $P > 0.05$.

Table 2. Effect of AMADS, Engame™ and Roundup WeatherMax® on fluorescence parameters collected from cotton cultivars DP 491 (GS) and DP 494 (GR). Data were pooled over experiments.

		Control		AMADS		Engame		Weathermax	
	HAT	491	494	491	494	491	494	491	494
Fo	4	689abc[1]	646bc	608c	663bc	740ab	780a	773ab	738ab
	24	653e	679de	775cd	716cde	1024a	852b	818cb	778bcd
	72	677b	620b	618b	682b	1218a	647b	611b	619b
Fm	4	2841ab	2970ab	3152a	2776ab	2788ab	2884ab	2614b	2843ab
	24	3333ab	3613a	3139bc	3025bcd	2331e	2895cd	2679de	2937bcd
	72	2924b	3312ab	3146ab	3050ab	2241c	3258ab	3147ab	3390a
Fv	4	2149abc	2321ab	2541a	2110abc	2046bc	2063bc	1838c	2102abc
	24	2680ab	2934a	2364bc	2309bc	1307e	2042cd	1860d	2159cd
	72	2247ab	2691a	2527ab	2367ab	1057c	2611ab	2535ab	2770a
Fv/Fm	4	0.76abc	0.78ab	0.81a	0.76abc	0.72bc	0.69c	0.70c	0.74abc
	24	0.80a	0.81a	0.75abc	0.76ab	0.55d	0.70c	0.69c	0.73bc
	72	0.75bc	0.81ab	0.80ab	0.78ab	0.44c	0.80ab	0.80ab	0.82a
PI	4	1.43ab	1.70ab	2.21a	1.16b	0.86b	0.86b	0.81b	1.28ab
	24	2.34ab	2.62a	1.23cd	0.92cde	0.09e	0.86cde	0.68de	1.59bc
	72	1.68bc	2.32ab	2.30abc	1.44c	0.04d	2.11abc	2.77a	2.54ab

[1]Means within a time followed by the letter were not significantly different according to Tukey's HSD test at $P > 0.05$.

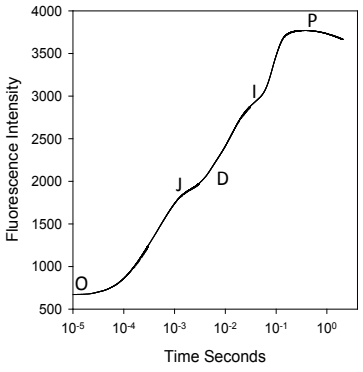


Figure 4. OJIP curve from control GS and GR cotyledons.

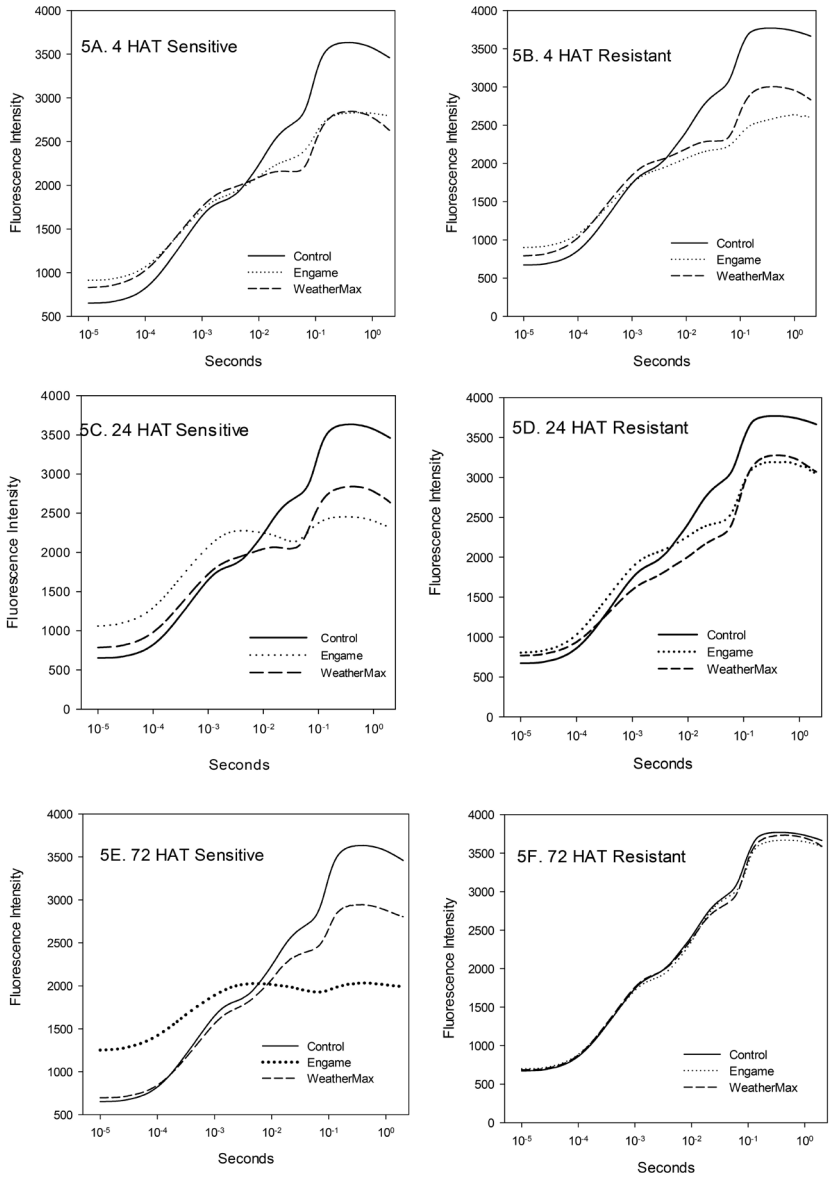


Figure 5. Effect of Engame™, and Roundup WeatherMax® on chl A fluorescence curves collected from cotton cultivars DP 491 (GS) and DP 494 (GR). AMADS treatments were not different than control treatments (data not shown).

4. Discussion

The results presented herein indicated that GS cotton was more sensitive to Engame™ than Weathermax® when treated with equivalent rates of glyphosate indicating a higher unit activity of the Engame™ formulation. Previously, Engame™ was shown to be 2 to 3 times more active than Roundup Ultra, Touchdown or Ultramax formulations of glyphosate in growth inhibition [26] [27]. In addition, absorption of ^{14}C-glyphosate was three to sixfold greater with glyphosate supplemented with AMADS compared to the glyphosate-isopropylamine formulation [10] and approximately three to sixfold more glyphosate was translocated out of the treated leaf with the AMADS formulation [10]. Similarly, the IC_{50} of the glyphosate-AMADS formulation was 3 to 4 times lower than a glyphosate-isopropylamine formulation [10]. The increased activity on GS cotton was likely the result of greater ultrastructural damage thereby allowing deeper penetration into the cotyledon tissue. The greater inhibition of the shikimate pathway, and more complete disruption of photosynthetic electron transport were also consistent with these results. AMADS by itself caused surface damage but did not cause measurable physiological stress as assessed by the methods used here. Similar surface disruptions were observed with simulated acid rain [31] and plant desiccation with sulfuric acid [32]. Tissue damage as epithelial and mesophyll necrosis was observed from Roundup and Roundup Ultra but without the catastrophic cell collapse. The surfactants used in these products caused similar injury without the addition of glyphosate [19]. GR cotton did not accumulate shikimate in response to either herbicide formulation indicating that the resistance trait afforded ample protection from glyphosate.

Utilizing PI values to assess the injury from Engame™ provided a more in-depth assessment of the damage caused by raising the level of glyphosate in GS cotton. The greater accumulation of shikimate indicated that flow through the shikimate pathway was not saturated by the glyphosate in the Weathermax® formulation. However, the dose supplied by Weathermax® was sufficient to kill GS cotton.

The activity demonstrated by Engame™ indicated that the standard salts and surfactants used to achieve high unit activity in Weathermax® might be improved to capture additional benefit from glyphosate. AMADS also increased the rainfastness of glyphosate on corn and several weeds [10] [27] [28]. Furthermore, achieving a more complete inhibition of PSII as demonstrated by ChlA transients and PI values, and greater inhibition of the shikimate pathway, and improved rainfastness may be desired benefits worth pursuing. Despite the potential benefit of achieving higher unit activity with acidic formulations, the acidic nature may not be acceptable for general use due to application incompatibility and safety to handlers. Glyphosate-resistant weeds having the proline to serine substitution in EPSPS may be more susceptible to formulations delivering a higher dose of glyphosate.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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