

Effect of New Auxin Herbicide Formulations on Control of Herbicide Resistant Weeds and on Microbial Activities in the Rhizosphere

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Abstract

Widespread distribution of glyphosate-resistant weeds in soybean-growing areas across Mississippi has economically affected soybean planting and follow-up crop management operations. New multiple herbicide-resistant crop (including soybean) technologies with associated formulations will soon be commercialized. The objectives of this research were to determine the efficacy of new 2,4-D + glyphosate and dicamba formulations on herbicide resistant weeds, and to determine the impact of the new 2,4-D + glyphosate formulation on microbial communities in the soybean rhizosphere involved in nutrient cycling. New 2,4-D + glyphosate and dicamba formulations registered for use on 2,4-D and dicamba-resistant soybean, respectively, adequately controlled glyphosate resistant and susceptible pigweeds (*Palmer amaranth* and tall waterhemp) and common ragweed. The 2,4-D + glyphosate formulation did not significantly impact soil microbial activities linked to nutrient cycling in the soybean rhizosphere. These results indicate these new 2,4-D + glyphosate and dicamba formulations can be effective in controlling glyphosate resistant and other herbicide resistant weeds while not having adverse effects on the activities of beneficial soil microorganisms.

Keywords

2,4-D, Dicamba, Glyphosate, Resistance, Soil Microbiology, Soybean

1. Introduction

Initial efforts in the development of herbicide resistant crops resulted in the release of bromoxynil-resistant cotton in 1995 and canola in 2000, however, these were discontinued because bromoxynil is not a broad-spectrum herbicide [1]. The real turning

point occurred in 1996-1997 with the commercial release of glyphosate-resistant (GR) canola (*Brassica napus* L.), soybean (*Glycine max* L. Merr.), and cotton (*Gossypium hirsutum* L.). Additional GR crops, alfalfa (*Medicago sativa* L.), corn (*Zea mays* L.), and sugarbeet (*Beta vulgaris* L.), were released to growers between 1998 and 2007. Glufosinate-resistant canola, released as early as in 1995, was not as widely adopted as GR canola. Glufosinate-resistant cotton was commercially made available in 2004, but was inferior in yield as compared to GR cotton. A new generation of GR cotton was developed in 2006 that had enhanced tolerance to glyphosate while at the same time allowed in-season glyphosate applications during the reproductive phase of the crop. New GR and glufosinate-resistant soybean germplasm was released in 2009. Glufosinate-resistant corn was commercialized for the first time in 1997 and was combined with GR corn varieties as a “double stacked trait” in the mid-2000s. This allowed control of a broader spectrum of weeds with the two unique modes of herbicide action [1].

An unfortunate consequence of commercialization of GR crops was the evolution of resistance to glyphosate in weed populations. The agrochemical industry, seed companies, and related entities have invested substantial resources in development of the next generation of herbicide resistant crops that have stacked multiple herbicide resistance traits. The aim of these efforts has been to diversify the growers' crop portfolio, as well as combat weed resistance by providing cropping technologies that allow application of more than one mode of action herbicides. These new technologies include the following: cotton (glyphosate + glufosinate + dicamba; glyphosate + glufosinate + 2,4-D), corn (glyphosate + glufosinate + 2,4-D; glyphosate + dicamba; glyphosate + acetolactate synthase inhibitors (ALS) inhibitors), and soybean (glyphosate + glufosinate + dicamba; glyphosate + glufosinate + 2,4-D; glyphosate + 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors; glyphosate + glufosinate + HPPD inhibitors) [1]. Due to stringent regulatory/environmental monitoring and approval requirements, some of these technologies may never be commercialized. For example, a new mechanism of resistance to glyphosate was developed [2] and stacked with a high resistance trait from a different mechanism of action of herbicides, but was withdrawn a year or two from commercialization. Some of the technologies involving dicamba and 2,4-D resistance also have associated formulations specifically developed for application with these new stacked technologies [1]. Such formulations include 2,4-D choline formulation with glyphosate (Enlist Duo®) from Dow AgroSciences and dicamba formulation (Engenia®) from BASF.

Several resistant weed populations exist in Mississippi and neighboring states, some of which have multiple resistances (to more than one herbicide mode of action), cross resistance (to herbicides from different chemical families but similar modes of action), multiple mechanisms of resistance, or metabolism based resistance (endowing ability to detoxify more than one herbicide mode of action) [3]. Information on the efficacy of the above herbicide formulations on glyphosate resistant and other herbicide resistant weed populations infesting soybean growing areas of Mississippi is lacking.

Herbicides, such as glyphosate, applied to plants can be released into soil through

root exudates [4] [5], or via direct contact, and after cultivation and rain events. These herbicides can have an impact on soil microorganisms that play an important role in plant nutrient availability by facilitating nutrient cycling and serving as the source of nodule forming rhizobia that provide fixed nitrogen to soybeans. Glyphosate application can alter microbial community composition [6] [7], and from a nutrient cycling perspective, it can have an inhibitory effect on phosphatase activity in soils [8] [9] and the activity of nitrogenase in some bacteria [10]. While the effects of glyphosate on soil microbial communities appear to be only minor and transient [6] [7] [11] [12], the combined application of multiple herbicides may have a greater impact.

Very little is known about how other herbicides influence soil microorganisms under field conditions. Dicamba and 2,4-D can be used as carbon sources by certain bacteria [13] [14], but these herbicides can have toxic and inhibitory effects on other bacteria [15] [16] [17] [18] [19]. There are also indications that these herbicides could have a direct impact on microbially mediated nutrient cycling. For example, dicamba has been shown to have an inhibitory effect on nitrogenase in the lab [16] and 2,4-D application can inhibit the activities of microbially secreted exoenzymes in soils linked to P and C cycling, including phosphatase [20] [21] and beta-glucosidase [20]. As such, application of these herbicides have the potential to cause shifts in the community composition and activity of bacteria in soil. However, many of these studies were carried out under laboratory conditions, or using toxic levels of herbicides above those used in the field. With the upcoming introduction of crops stacked with tolerances to both glyphosate and dicamba or 2,4-D, it is important to investigate the cumulative effect of these herbicides on the activity of soil microbial communities under agriculturally relevant conditions, since herbicides applied in combination might have a greater impact on soil microorganisms than when applied alone.

Therefore, the objectives of this research were to determine efficacy of new 2,4-D + glyphosate and dicamba formulations on selected herbicide resistant weeds, and to determine the impact of 2,4-D choline formulation on soybean rhizosphere microbial activities involved in nutrient cycling in two soil textures.

2. Materials and Methods

2.1. Seed Sources, Germination, Planting, Growth, and Herbicide Treatment Conditions

GR (biotypes GR1, GR2, GR3) and glyphosate-susceptible (GS) Palmer amaranth (*Amaranthus palmeri* S. Wats.), GR and GS tall waterhemp (*Amaranthus tuberculatus* Moq. Sauer), GR and GS common ragweed (*Ambrosia artemisiifolia* L.) seed was obtained from the USDA-ARS weed seed repository in Stoneville, MS. In the summer-fall of 2015, seeds of Palmer amaranth and tall waterhemp were planted at 1 - 1.5 cm depth in 10-cm by 10-cm by 10-cm plastic pots containing a commercial potting mix (Metro-Mix 360, Sun Gro Horticulture, Bellevue, WA, USA), watered, and placed in a greenhouse (25°C day and 20°C night, 14-h photoperiod under natural sunlight conditions supplemented with high pressure sodium lights providing 400 mmol/m²/s light inten-

sity). Common ragweed seeds were planted and watered as above and incubated for a week at 2°C - 8°C and then kept in the greenhouse as before. Two weeks after emergence, weed seedlings were transplanted into 6-cm by 6-cm by 6-cm pots containing the potting mix. Plants were fertilized once with a nutrient solution (Miracle-Gro, The Scotts Company LLC, Marysville, OH, USA) containing 200 mg/L each of N, P₂O₅, and K₂O one week after transplanting and sub-irrigated as needed, thereafter. All herbicide treatments were applied with a backpack sprayer equipped with 8002E nozzles (Spraying Systems Co., Wheaton, IL, USA) delivering 140 L/ha at 280 kPa to weed and crop (soybean, growing procedure described below) plants. At the time of herbicide treatment with 2,4-D + glyphosate, Palmer amaranth and tall waterhemp plants were ≤12.5 cm and ≥25 cm in height; with dicamba, Palmer amaranth and tall waterhemp plants were ≥25 cm in height. Common ragweed plants were 10-15 cm in height with both 2,4-D + glyphosate and dicamba. Soybean plants were at the two-three trifoliate stage when treated with 2,4-D + glyphosate or glyphosate alone. 2,4-D + glyphosate (choline + dimethylammonium salt, Enlist Duo, Dow AgroSciences, Indianapolis, IN, USA) was applied at a rate of 1.06 kg ae/ha + 1.13 kg ae/ha to both weed and soybean plants. In addition, a separate group of soybean plants were treated with glyphosate (potassium salt, Roundup WeatherMAX, Monsanto Co., St. Louis, MO, USA) alone at 1.13 kg/ha. Dicamba (N,N-Bis (3-aminopropyl) methylamine (BAPMA) salt, Engenia, BASF, RTP, NC) was applied to weeds at 0.7 kg ae/ha. Herbicide efficacy treatments are summarized in **Table 1**. Percent control [visible estimate of injury on a scale of 0 (no injury) to 100 (plant death)] and mortality were recorded for weeds 3 weeks after treatment (WAT).

2,4-D + glyphosate resistant soybean plants (variety GC16146889, provided by Dow AgroSciences), were grown in sandy loam and silty clay loam soils. Both soil types were collected from sites in Stoneville, MS, ground to disrupt clumps, and passed through a 1 cm sieve to remove debris. Pots (15.2 cm diameter and depth) were set up containing either sandy loam (45 pots) or clay loam (45 pots) soil. Soybean seeds were soaked in water at room temperature overnight before planting to stimulate imbibition and germination. Soybeans were planted at a rate of 10 seeds per pot, and covered with a 1:1 sand:soil mixture to a depth of 2.5 cm. Pots were placed in a greenhouse, watered every two to three days as needed, and maintained under 28°C day, 22°C night conditions

Table 1. Summary of treatments used in the herbicide efficacy study.

Weed species	Population	Height (cm)	Herbicide	Herbicide rate (kg ae/ha)
Palmer amaranth	GR1, GR2, GR3, GS	≤12.5, ≥25	2,4-D + glyphosate	1.06 + 1.13
	GR1, GR2, GR3, GS	≥25	Dicamba	0.7
Tall waterhemp	GR, GS	≤12.5, ≥25	2,4-D + glyphosate	1.06 + 1.13
	GR, GS	≥25	Dicamba	0.7
Common ragweed	GR, GS	10-15	2,4-D + glyphosate	1.06 + 1.13
	GR, GS	10-15	Dicamba	0.7

with a 13-hr photoperiod of natural light supplemented with artificial lighting as described above. Nineteen days after planting, seedlings were thinned down to four per pot, selecting to keep plants that were of similar size and developmental stage (early second trifoliolate stage). Soybeans were sprayed 27 days after planting as described above. Plant treatments included no herbicide application, glyphosate alone, and 2,4-D + glyphosate.

2.2. Soybean Rhizosphere Analysis

Rhizosphere soil was collected from plants at 1, 3, 7, 14, and 30 days post application (DPA) of herbicide treatment. Rhizosphere analysis treatments are summarized in **Table 2**. At each time point, the soil-root mass from three replicate pots for each soil type and treatment was removed, gently broken up to dislodge bulk soil, and shaken to remove all but the soil tightly adhered to roots (rhizosphere soil). Approximately 5 g of root + rhizosphere was transferred to a 50 mL centrifuge tube and kept on ice until all pots were sampled. 50 mM acetate buffer (pH 5) was added to each tube until the root sample was submerged (approximately 20 - 30 mL). Each tube was vortexed for 1 min and then placed in a sonicating water bath for 1 min in order to dislodge rhizosphere soil from the roots. Roots were removed from the tubes using sterile forceps. The resulting rhizosphere soil slurries were assayed for the activities of phosphatase, β -glucosidase, and N-acetylglucosaminidase (NAGase) in 96-well plate format using p-nitrophenol (pNP)-linked assays as described previously [22], with some modifications. Briefly, for each enzyme assayed, 150 μ L of rhizosphere slurry was pipetted into six wells in a deep well plate, vortexing vigorously between wells. Next, 150 μ L of pNP-linked substrate solution was added to four replicate samples wells for the enzyme reactions. The remaining two sample wells on each block served as sample blanks and contained only 150 μ L rhizosphere slurry and 150 μ L 50 mM acetate buffer. Substrate solutions were prepared as previously described [22]. Assays were incubated at 25°C with shaking (150 RPM). Incubation times varied by enzyme: 30 min for phosphatase, 1.5 hr for β -glucosidase, 2.5 hr for NAGase, and 16 hr for cellobiohydrolase. Following incubation, deep well plates were centrifuged at 3220 \times g for 5 min. 150 μ L of supernatant was withdrawn from each well and transferred to a 96-well plate containing 150 μ L of 0.067 M NaOH stop reagent. The absorbance of each plate was read at 410 nm. Ab

Table 2. Summary of treatments used in the soybean rhizosphere study.

Soil type	Rhizosphere sampling time (days post treatment)	Herbicide	Herbicide rate (kg ae/ha)
Sandy loam	1, 3, 7, 14, 30	None	0
	1, 3, 7, 14, 30	2,4-D + glyphosate	1.06 + 1.13
	1, 3, 7, 14, 30	Glyphosate	1.13
Silty clay loam	1, 3, 7, 14, 30	None	0
	1, 3, 7, 14, 30	2,4-D + glyphosate	1.06 + 1.13
	1, 3, 7, 14, 30	Glyphosate	1.13

sorbance of soil slurry blank wells was subtracted from reaction wells and compared to a standard curve of known pNP concentrations to determine the amount of substrate consumed. Dry weight determinations for the amount rhizosphere soil per reaction were performed in triplicate, pipetting 150 μ L of slurry from each sample into pre-weighed aluminum tins, incubating overnight at 105°C, and weighing the next morning. Weight of the pan was subtracted from the dry weight to get the weight per reaction.

Enzyme activities were calculated as the nmole of substrate consumed per g dry weight per hour. Analysis of Variance (ANOVA) was performed in JMP version 11.2.0 (SAS Institute Inc., Carey, NC) to determine if there were any significant differences between herbicide treatments in the two soil textures, with an α of 0.05.

3. Results and Discussion

3.1. Herbicide Efficacy

All pigweed plants (GR and GS Palmer amaranth and tall waterhemp) were completely controlled when treated with 2,4-D + glyphosate at ≤ 12.5 cm in height (**Table 3**). When pigweed plants were treated with 2,4-D + glyphosate at a height of ≥ 25 cm two GR Palmer amaranth populations, GR2 and GR3, were completely controlled, but 13% of plants from a third GR Palmer amaranth population, GR1, survived, though severely injured. The GR waterhemp population was also severely injured, but 7% of treated

Table 3. Response of glyphosate-resistant (GR) weed species to 2,4-D + glyphosate and dicamba. Survival rating is based on presence of an actively growing apical or lateral meristem. Percent control rating is based on visible injury to plants^a.

Weed species	Population	Height (cm)	Herbicide	#Plants treated	#Plants survived	Survival %	% Control
Palmer amaranth	GR1	≤ 12.5	2,4-D + glyphosate	15	0	0	100
Palmer amaranth	GR2	≤ 12.5	2,4-D + glyphosate	15	0	0	100
Palmer amaranth	GR3	≤ 12.5	2,4-D + glyphosate	15	0	0	100
Tall waterhemp	GR	≤ 12.5	2,4-D + glyphosate	15	0	0	100
Palmer amaranth	GR1	≥ 25	2,4-D + glyphosate	15	2	13	90
Palmer amaranth	GR2	≥ 25	2,4-D + glyphosate	15	0	0	100
Palmer amaranth	GR3	≥ 25	2,4-D + glyphosate	15	0	0	100
Tall waterhemp	GR	≥ 25	2,4-D + glyphosate	15	1	7	95
Common ragweed	GR	10-15	2,4-D + glyphosate	15	0	0	100
Palmer amaranth	GR1	≥ 25	Dicamba	15	0	0	100
Palmer amaranth	GR2	≥ 25	Dicamba	15	0	0	100
Palmer amaranth	GR3	250	Dicamba	15	15	100	80
Tall waterhemp	GR	≥ 25	Dicamba	15	12	80	40
Common ragweed	GR	10-15	Dicamba	15	0	0	98

^aGlyphosate-susceptible plants of all weeds examined were completely controlled at all heights and with all herbicides.

plants survived (**Table 3**).

Dicamba was applied only to ≥ 25 -cm pigweeds. As with 2,4-D + glyphosate, two GR Palmer amaranth populations, GR1 and GR2, were completely controlled, but a third GR population, GR3, had 100% survival (**Table 3**). Similarly, a GR waterhemp population had 80% survival 3 WAT. GR pigweed populations, Palmer amaranth and waterhemp, which survived dicamba at 25-cm height at the time of treatment, did not put out new healthy growth even by 8 WAT. Plants remained injured or began to senesce without any evidence of new growth (data not shown). GR and GS common ragweed, 4- to 6-inches in height at the time of treatment, were completely controlled by both 2,4-D + glyphosate and dicamba.

3.2. Soybean Rhizosphere Analysis

Due to lack of availability of dicamba resistant soybean, only the new 2,4-D + glyphosate formulation was assessed for its potential impact on the soybean rhizosphere. 2,4-D + glyphosate did not significantly alter the activities of NAGase or cellobiohydrolase in the rhizosphere of soybean plants grown in either sandy loam (**Figure 1(c)** and **Figure 1(d)**) or clay loam (**Figure 2(c)** and **Figure 2(d)**) soils at any of the time points measured. Phosphatase activities also did not differ significantly between treatments in either soil texture for most of the experiment (**Figure 1(a)** and **Figure 2(a)**). One exception was observed 14 days after herbicide application in sandy loam soils, when phosphatase activity in the rhizosphere of 2,4-D + glyphosate treated plants was significantly lower than untreated plants ($p = 0.022$). However, this effect was transient and phosphatase activity had recovered to non-treated levels by the end of the experiment.

β -glucosidase activity in the rhizosphere was largely unaffected by herbicide application in either soil texture (**Figure 1(b)** and **Figure 2(b)**). The only statistically significant difference occurred 7 days after application in clay loam soils, when β -glucosidase activity was 17% higher in 2,4-D + glyphosate than in untreated plants ($p = 0.042$). However, this difference was small compared to the variability between rhizosphere samples at other time points and not likely to be biologically relevant.

Overall, microbial activities varied greatly among samples. However, there was no consistent impact of herbicide application on microbial activities between sandy loam and clay loam soil textures. Most differences in rhizosphere activity observed between herbicide treatments were transient in nature and not statistically significant, an observation that is consistent with previous studies looking at the effects of glyphosate [23] and 2,4-D [20] on glucosidase and phosphatase activities in soil. Previous studies reporting greater impacts on microbial activities used substantially higher dosages than the field rates used in the current study [9] [21]. Given the current results, it appears that the combined application of both glyphosate and 2,4-D do not significantly influence soil activities in the soybean rhizosphere.

In summary, with no new herbicide modes of action on the horizon, multiple herbicide resistant crops with resistance to 2,4-D or dicamba and one or more other herbi-

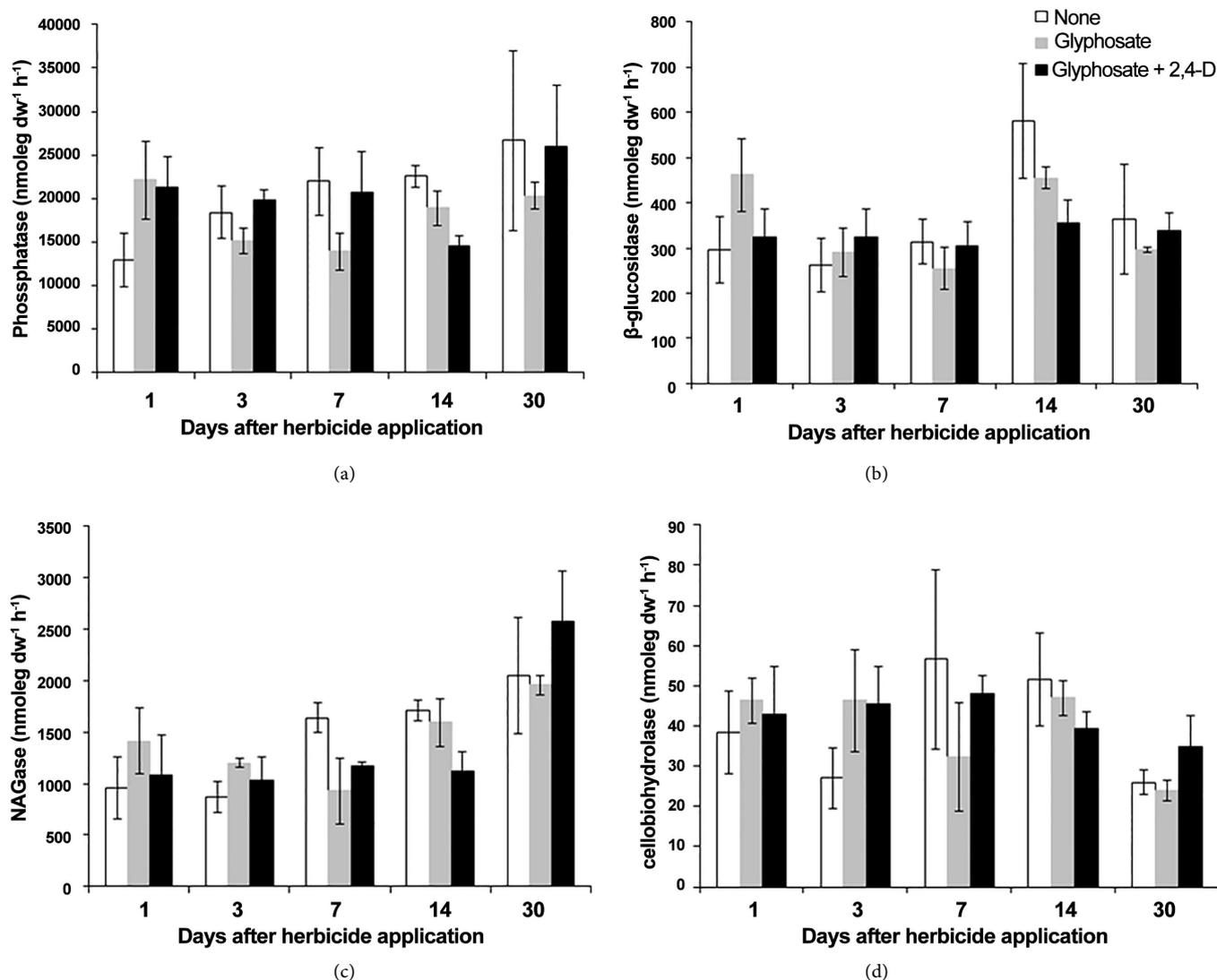


Figure 1. Mean \pm standard error of phosphatase (a), β -glucosidase (b), N-acetylglucosaminase (NAGase) (c), and cellobiohydrolase (d) activities in the rhizosphere of untreated, glyphosate treated, or glyphosate + 2,4-D treated soybean plants grown in a sandy loam soil. The data from each treatment represents three replicates, with each replicate being composed of the pooled rhizosphere soil from four plants, for a total of 12 soybean rhizospheres collected per treatment and time point.

cides can be effective tools in combating herbicide resistant and non herbicide resistant weed populations. Although, several troublesome weed species such as Palmer amaranth and tall water hemp are resistant to glyphosate, the new formulations of 2,4-D and dicamba labeled for multiple herbicide resistant crops tested in this research demonstrated their effectiveness in controlling large sized weed plants. This tool could be effectively used for post harvest weed control. An additional benefit of these formulations is the lack of adverse effects on soil microbial flora associated with crops such as soybean. Soil microbial flora plays a tremendously important role in nitrogen fixation and nutrient cycling essential for adequate crop growth and development.

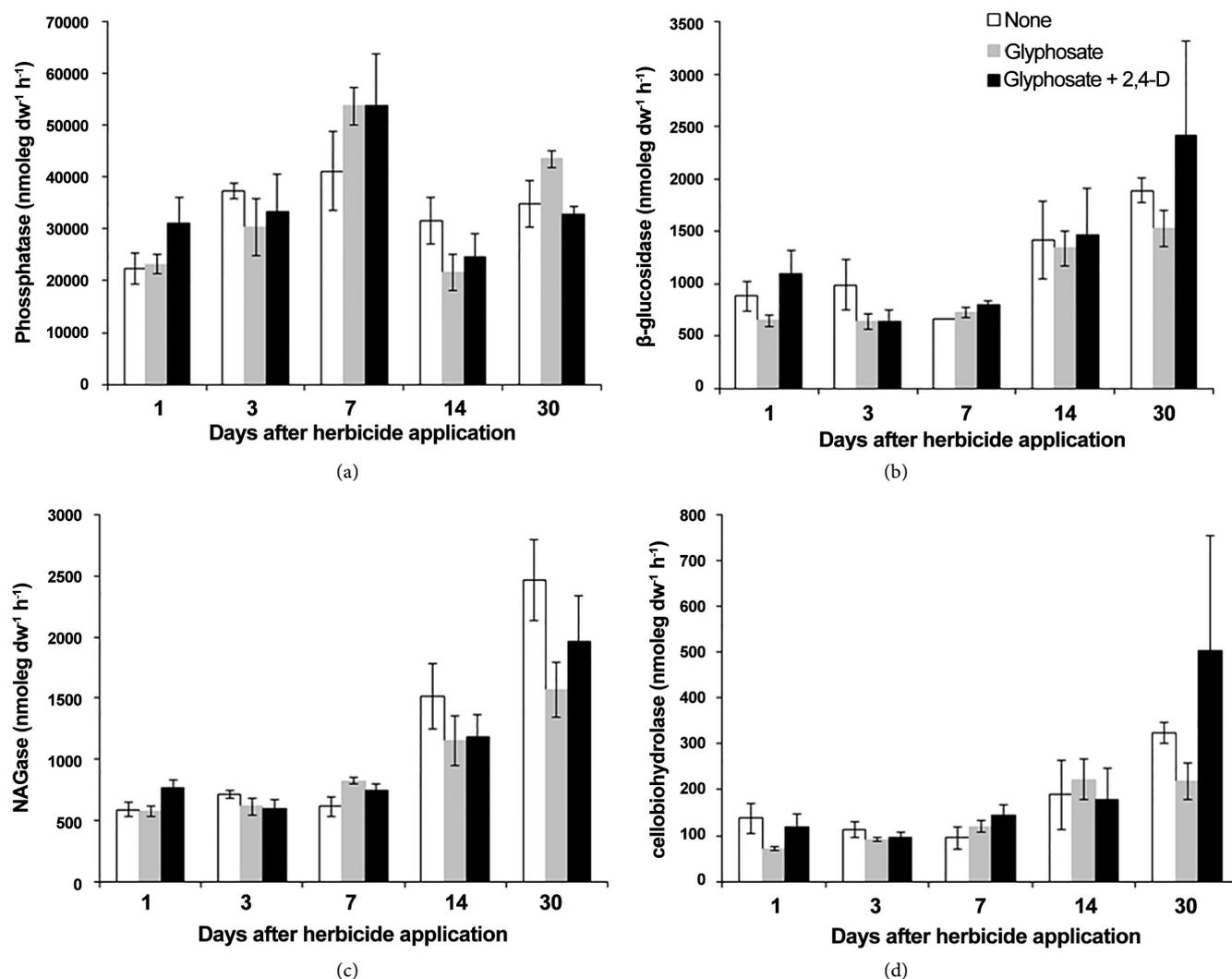


Figure 2. Mean \pm standard error of phosphatase (a), β -glucosidase (b), N-acetylglucosaminase (NAGase) (c), and cellobiohydrolase (d) activities in the rhizosphere of untreated, glyphosate treated, or glyphosate + 2,4-D treated soybean plants grown in a silty clay loam soil. The data from each treatment represents three replicates, with each replicate being composed of the pooled rhizosphere soil from four plants, for a total of 12 soybean rhizospheres collected per treatment and time point.

4. Conclusion

The above results clearly indicate that the new 2,4-D + glyphosate and dicamba formulations registered for use on 2,4-D and dicamba-resistant soybean, respectively, provide a very viable alternative for controlling GR as well as wild types of troublesome pigweeds such as Palmer amaranth and tall waterhemp, and other broadleaf weeds such as common ragweed. Furthermore, the new 2,4-D + glyphosate formulation does not significantly impact the activities of soil microorganisms linked to nutrient cycling in the soybean rhizosphere.

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