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Herbicide and additive impacts on *Bradyrhizobium japonicum* growth in solution

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Abstract

Plant biostimulants include beneficial fungi and bacteria, and are often applied to foliage to improve crop growth, yield, and/or crop quality. Crop improvements due to biostimulant addition may be modest; therefore, solo applications may not be economical or climate smart. However, biostimulants combined with other postemergence treatments, such as herbicides, may provide an alternative application method, if mixtures do not harm the living organism(s). The growth of *Bradyrhizobium japonicum*, as a biostimulant surrogate, was assessed in solutions of glyphosate [*N*-(phosphonomethyl)glycine] and dicamba (3,6-dichloro-2-methoxybenzoic acid), with and without common spray additives (ammonium sulfate [AMS] and nonionic surfactant) in laboratory studies over 72 h. Solution turbidity, using optical density as a surrogate of bacterial growth, was measured at 600 nm at 24, 48, and 72 h after inoculation, and colony forming units (CFUs) per milliliter were estimated. Growth was not detected in either the glyphosate or AMS solutions, most likely due to the low pH and high electrical conductivity of the solutions, respectively. When herbicides were mixed with a nonionic surfactant, CFUs per milliliter were about 25% greater than the positive control. These data suggest that mixing bacteria with postemergence herbicide + surfactants/additives combinations can hinder or maintain growth when preparing for agrochemical applications. Biostimulant type and the agrichemical combination(s) should be evaluated prior to tank mixing to determine if detrimental interactions occur. After application, an evaluation of the effectiveness of the biostimulant to the crop and efficacy of the agrichemical to the target organism should be conducted.

1 | INTRODUCTION

Climate-smart agriculture objectives are to use "green" technology (reduce natural resource use and environmental degradation, recycle, and have low external inputs), intensify agriculture sustainably, employ cropping systems and crops that are adaptable to climate change, and reduce greenhouse gas emissions (FOA, Climate Smart Agriculture, accessed June 2023). Plant biostimulant applications to crops may fit several of these objectives. Biostimulants are natural or plant-based products that include substances from plant or soil extracts (du Jardin, 2015; Ertani et al., 2014) or microorganisms such as beneficial bacteria (Efthimiadou et al., 2020;

Abbreviations: a.e., acid equivalent; AMS, ammonium sulfate; CFUs, colony forming units; EC, electrical conductivity; OD, optical density.

Saa et al., 2015) and fungi (du Jardin, 2015). Biostimulants stimulate natural growth processes that enhance nutrient uptake, nutrient use efficiency, tolerance to abiotic stress, and climate change (Bhupenchandra et al., 2022), induce defense responses against plant pathogens (Vergnes et al., 2014), or improve crop quality and yield (Brown & Saa, 2015; du Jardin, 2015). In a corn study, the application of a plant extract-based biostimulant during planting reduced grass herbicide injury to corn (Panfili et al., 2019). Biostimulants have been effective when applied in diverse ways, including seed coatings, direct soil application (Ruzzi & Aroca, 2015), and foliar application (Van Oosten et al., 2017). Biostimulants have been reported to increase yields of cereals, legumes, fruits, and vegetables by up to 18% (Jing et al., 2022).

Combining biostimulants as a foliar application with other common crop production management applications would be a climate-smart practice if the correct combinations can be found, as a single application could do "double duty." A few research studies have used preemergent herbicide applications followed by two post-applied biostimulant applications (Ginter et al., 2022; Zarzecka et al., 2022). These authors used several different types of biostimulants and reported that a few of the subsequent biostimulant applications increased potato (*Solanum tuberosum* L.) yield (Zarzecka et al., 2022) and profit (Ginter et al., 2022) over herbicide use alone. The two biostimulants that had the greatest impact on yield and economics differed in composition with one containing living bacteria and actinomycetes, whereas the other was amino acid based with small amounts of macro- and micronutrients.

Since herbicides are applied postemergence in most cropping systems, combining biostimulants with herbicides in a single application may be a common climate-smart practice in the future. Herbicide formulations differ by brand so that while the active ingredient(s) may be the same, each formulated brand may contain a different mix and amount of inert ingredients, such as surfactants, oils, compatibility agents, emulsifiers, and solvents, to enhance the effectiveness of active ingredients. In addition, other chemicals may be added to the tank when preparing for an herbicide application. For example, ammonium sulfate (AMS) is added at 1–2 kg per 100 L of water in glyphosate solutions to overcome hard water impacts on herbicide activity. Other nonionic surfactants may be added to other herbicide mixes to improve droplet spread on the leaf and herbicide sorption in the foliage.

Some biostimulants contain living organisms. The harsh solution environments of the herbicide alone or the mixture with additives, such as surfactants or fertilizers that are used in combination with different herbicides, may reduce the growth potential of living organisms in both the spray tank and on foliage (dos Santos et al., 2005; Jawson et al., 1989), which could result in low or no crop impact. In this study, we assessed the growth of *Bradyrhizobium japonicum* (USDA strain 110), a commonly used beneficial bacteria that enhances nodulation and nitrogen fixation in legumes, as a surrogate compound for a bacterial biostimulant. Solutions containing *B. japonicum* were combined with two postemergent herbicides and surfactants and additives typically suggested for effective application to determine the impact on bacterial growth. The null hypotheses of these trials were that rhizobia growth would not be impacted by herbicides, additives, or the combination of herbicides and additives.

Core Ideas

- Biostimulants may be living organisms applied to improve crop growth, stress tolerance, or quality.
- Biostimulants mixed with herbicides may be climate smart but must not inhibit biostimulant growth potential.
- *Bradyrhizobium*, a microbial biostimulant surrogate, was mixed with herbicides and surfactants.
- Glyphosate and ammonium sulfate halted the growth of *Bradyrhizobium* growth in the solution, whereas dicamba reduced growth by 45%.
- Care must be taken to ensure that biostimulant growth in application solutions is not inhibited.

2 | MATERIALS AND METHODS

The study was performed under aseptic laboratory conditions at 30°C. Treatments were (1) *B. japonicum*, strain USDA 110, in deionized water alone, and mixed with solutions of (2) herbicides used at labeled rates; (3) spray additives; and (4) herbicides mixed with spray additives. Rhizobia growth was monitored every 24 h over a 72-h period (Amajioyi, 2021).

One hundred milliliters of stock solutions for treatments 2, 3, and 4 were prepared using milliQ-water (Millipore Sigma Corp.). The glyphosate stock solution contained 1.4 g acid equivalent (a.e.) of glyphosate using 2.5 mL of Roundup PowerMAX formulation (potassium salt formulation; Bayer AG). The dicamba stock solution contained 0.4 g a.e. dicamba using 1.4 mL of Xtendimax formulation (diglycolamine salt formulation; Bayer AG). Two AMS solutions (21-0-0; Winfield United) were tested at 2 and 20 g AMS per 100 mL, which is in the range of labeled rates used to improve glyphosate activity in spray solutions (Voight, 2017). Duce HSOC (a nonionic surfactant; Helena Agri-Enterprises) stock solutions contained 0.75 (labeled rate) or 7.5 (10× labeled rate) mL per 100 mL. The combination stock solution contained 2.5 mL of glyphosate, 1.4 mL of dicamba, 2.0 g of dry AMS, 7.5 mL of Duce HSOC, and 0.125 mL of Strike Zone LC (labeled rate for herbicide application), a drift reduction and deposition

aid (Helena Agri-Enterprises). Each treatment had three replicates, and the experiment was repeated over time. Positive (water containing *B. japonicum* alone) and negative (sterile water) controls were included with the treatments.

All stock solutions were refrigerated at 4.4°C until needed. The electrical conductivity (EC) and pH of solutions were measured using EC and pH probes (Mettler) at the start of the incubation, and pH was measured after the 72-h incubation.

Bradyrhizobium japonicum was cultured in yeast-extract mannitol broth (Jawson et al., 1989) for 3 days at 30°C and used to inoculate the test solutions when the optical density (OD) reading of the broth was 600 nm (OD_{600}), quantified using a spectrophotometer (Ultrospec 10; Amersham Biosciences Corp.). OD values by treatment were used to estimate the colony forming units (CFUs) per milliliter of solution using a fitted regression line (using regression analysis tool in data analysis of Excel):

$$y = \left(1.18 \times 10^{9}\right) x - 1.2 \times 10^{7} \left(r^{2} = 0.99; F = 0.03\right),$$

where $x = OD_{600}$. The OD of *B. japonicum* was checked after grown in broth solution for 24 h, with CFUs estimated through dilution and counting on agar plates.

The OD_{600} reading of the broth prior to inoculating test solutions was about 0.25 (about 2.7×10^{8} CFUs·mL^{-1}) for herbicides alone and about 0.1 (1.1×10^{8} CFUs·mL^{-1}) for the other solution tests. A 1-mL aliquot of the broth solution was pipetted into a sterile test tube containing 7 mL of milliQ water and 2-mL aliquot of the appropriate test solution. The solutions were incubated at 30°C in a 28-degree orbital shaker (New Brunswick Scientific Excella E24 incubator shaker series; Eppendorf). After 24, 48, and 72 h, a 1-mL aliquot of each solution was pipetted into a cuvette and OD_{600} was quantified as above, using an uninoculated treatment as the blank (Carpenter, 1977; Gonzalez et al., 1996).

Data were analyzed using R-statistical software program (version 4.1.0; R Core Team, 2020) using analysis of variance (ANOVA) for a completely randomized design using the doebioresearch package. The factors were culture media and incubation time. The replicates across runs were combined ($n = 6$) as treatment impacts were similar between the experiment repetitions, and culture media by time interactions were not statistically different. However, starting CFUs differed among the solutions in the positive control treatments for herbicide alone, as compared to the surfactant, AMS, and combination treatment; therefore, these treatments were analyzed separately and compared back to their respective positive control treatment. Least significant difference values at $p = 0.05$ were calculated when the F-values were significant.

3 | RESULTS

3.1 | Impact of herbicides on solution characteristics and *B. japonicum* growth

Solution EC, a measure of solution salinity, and pH can impact the robustness of living organisms. ECs were 5.8 and 2.2 mS·cm^{-1} for glyphosate and dicamba solutions, respectively (Table 1). The pH of the glyphosate solution at 0 and 72 h was 4.27 and 4.0, respectively. The pH of the dicamba solution at 0 and 72 h was 5.65 and 5.16, respectively.

Bradyrhizobium japonicum in glyphosate alone had OD_{600} values near or at 0 h (Table 2) for all three readings, which was below the assay's limit of detection (ASTM International, 1998). This indicated that the starting population from the initial inoculation was killed (Table 2). The *B. japonicum* CFUs per milliliter in the dicamba solution were similar to the initial inoculation CFUs at the 24- and 48-h readings. At 72 h, the CFUs per milliliter in the dicamba solution were about 30% greater than at inoculation, indicating some growth, but about 45% lower than the positive control.

3.2 | Impact of additives on solution characteristics and *B. japonicum* growth

AMS at high (20 g) and low (2 g) concentrations had EC values of 28 and 18 mS·cm^{-1}, respectively (Table 1). The AMS high-concentration solution had pH values of 5.38 at 0 h and 3.13 at 72 h. The AMS low-concentration pH values were 5.12 at 0 h and 3.07 at 72 h. Duce HSOC at both the high and low concentrations had an EC value of 0.55 mS·cm^{-1}. The Duce HSOC at the 7.5-mL treatment had pH values of 7.48 at 0 h and 6.75 at 72 h, and the pH values of the 0.75-mL treatment were 7.48 at 0 h and 5.39 at 72 h.

Low and high AMS concentrations resulted in *B. japonicum* CFUs per milliliter below detection throughout the incubation, indicating that the initial inoculating population was killed (Table 2). At the 24-h reading, Duce HSOC at the 0.75 mL concentration had CFUs per milliliter about 40% lower than the positive control and remained unchanged through 72 h. Duce HSOC at the high concentration, on the other hand, appeared to stimulate *B. japonicum* growth. The CFUs per milliliter were 2.5 to 3× higher at each sampling time than the CFUs per milliliter of the positive controls.

TABLE 1 Herbicide, adjuvant, surfactants, and herbicide mixture concentrations; electrical conductivity (EC) at 0 h; and pH of cultures at 0 and 72 h.

Treatment	Amount of solute added	Electrical conductivity	Solution pH	
			0 h	72 h
	Per 100 mL	mS·cm^{-1}		
Glyphosate (K salt formulation)[a]	1.40 g a.e.	5.80	4.27	4.00
Dicamba (DGA salt formulation)[a]	0.40 g a.e.	2.20	5.65	5.16
Ammonium sulfate[b]	2.0 g	28.00	5.38	3.13
Ammonium sulfate[b]	0.20 g	18.10	5.12	3.07
Duce HSOC[c]	7.50 mL	0.55	7.48	6.75
Duce HSOC[c]	0.75 mL	0.55	7.48	5.39
Glyphosate + dicamba + AMS + Duce + St	1.4 g a.e. + 0.4 g a.e. + 2 g -	32.50	6.98	6.51

[a]Glyphosate and Dicamba—Source: Bayer, AG.
[b]Ammonium sulfate—Source: Winfield United.
[c]Duce HSOC nonionic surfactant—Source: Helena Agri-Enterprises.
[d]Strike Zone drift reduction and deposition aid that contains 95% polyethoxylated hydroxyl aliphatics and carbohydrate polymers was added to the herbicide mixture, but not tested alone—Source: Helena Agri-Sciences.

TABLE 2 Optical density (OD_{600}) values for *Bradyrhizobium japonicum* in water and in treatment solutions that contained herbicide (glyphosate or dicamba), ammonium sulfate, surfactant (Duce HSOC), and a combination treatment solution at 24, 48, and 72 h of incubation.

Treatment	Incubation time					
	Optical density			Colony forming units (CFUs)		
	24 h	48 h	72 h	24 h	48 h	72 h
	OD_{600}			No. CFUs × 10^8 mL^{-1}		
milliQ water + *B. japonicum*	0.40	0.48	0.53	4.1	4.9	5.4
Glyphosate + milliQ water + *B. japonicum*	0.00	0.00	0.01	ND[a]	ND	ND
Dicamba + milliQ water + *B. japonicum*	0.22	0.23	0.30	2.3	2.4	
$LSD_{(0.05)}$		0.02			0.2	
milliQ water + *B. japonicum*	0.17	0.23	0.24	1.8	2.3	2.5
Ammonium sulfate (2) + milliQ water + *B. japonicum*	0.00	0.00	0.00	ND	ND	ND
Ammonium sulfate (20) + milliQ water + *B. japonicum*	0.02	0.00	0.00	ND	ND	ND
Duce HSOC (0.75) + milliQ water + *B. japonicum*	0.10	0.08	0.10	1.1	0.9	1.1
Duce HSOC (7.5) + milliQ water + *B. japonicum*	0.56	0.58	0.63	5.7	5.9	6.4
Herbicides + AMS + Duce HSOC + Strike Zone + milliQ water + *B. japonicum*	0.22	0.27	0.30	2.3	2.8	3.1
$LSD_{(0.05)}$		0.02			0.2	

Note: Due to the difference in the OD of the original broth added to herbicide alone treatments versus the other treatments, the data for the solutions were compared to the appropriate positive control. LSD, least significant difference.
[a]ND indicates that the value was below the assay's limit of detection (LOD < 10 CFUs·mL^{-1} for a 1:10 dilution).

3.3 | Impact of combination treatment on solution characteristics and *B. japonicum* growth

Based on the solution characteristics when the additives were used alone, the low rate of AMS (2 g) and the high rate of Duce (7.5 mL) were used in the herbicide combination. A small amount of Strike Zone (additive for drift control in herbicide mixtures that contain polyethoxylated hydroxyl aliphatics and carbohydrate polymers) was added to the solution. The EC of the combination solution was 32.5 mS·cm^{-1} (Table 1). The pH at 0 and 72 h was 6.98 and 6.51, respectively. The CFU

values for the combination of surfactants and herbicides were about 20% greater at each reading than the positive control.

4 | DISCUSSION

Our data support the previous literature suggesting that rhizobia growth in agitated liquid media can be positively, negatively, or not influenced by herbicide, adjuvant, and surfactant addition (dos Santos et al., 2005; Eberbach & Douglas, 1989; Mallik & Tesfai, 1985; Moorman et al., 1992; Schuls et al., 1985; Singh & Wright, 2002). In our study, the growth of *B. japonicum* was slowed or stopped by both herbicides and AMS over the 72-h incubation period, in comparison to the positive control, as noted by OD values and, by extension, CFU counts. The low solution pH of the glyphosate, dicamba, and AMS solutions may be responsible for the stoppage or decrease in the growth rate of *B. japonicum*, as the optimum pH suitable for the culturing *B. japonicum* has been reported as 6.8 (Maccio et al., 2002; Somasegaran & Hoben, 1994; Vincent, 1970). While we did not examine the effect of solution pH alone on *B. japonicum* growth, Dinkwar et al. (2020) tested 25 *B. japonicum* isolates on media from pH 4 to pH 10. The highest OD for all isolates was observed at pH 7 (OD $\geq$ 0.76), although isolates had some limited growth potential at pH 4 (OD $\leq$ 0.09) and 10 (OD $\leq$ 0.2) (Dinkwar et al., 2020).

The high EC values of the AMS solutions in our study may have resulted in direct toxicity to the microbial population through osmotic stress (Tate III, 2021). Past studies have reported the negative effects of adjuvants and surfactants on the development of soil microorganisms (Berner et al., 1991; Johal & Rahe, 1984; Katan & Eshel, 1973; Sawada et al., 1988). Some adjuvants/surfactants can increase herbicide activity on plants by decreasing or removing the leaf wax layer (Hess, 1985), which increases the amount or speed of foliar herbicide uptake. For microbes, herbicide penetration into the organism may be increased or osmotic balance between the outside and inside of the cell may be disrupted by reducing the surface tension of solutions, resulting in high-doses herbicide that could disrupt growth (Malkomes, 2000) or cause deleterious imbalanced cellular conditions (Tate, 2021).

In our study, Duce HSOC at a higher concentration (7.5 mL) stimulated *B. japonicum* growth over the 72-h period. Duce HSOC is a blend of nonionic surfactant and methylated seed oil, a fatty acid from soybean seed oil esterified with methyl alcohol (Miller & Westra, 1996; Young et al., 2016). Fatty acids and lipid molecules are nutrient sources, forms of carbon and energy storage, and structural components of membranes and hormones. According to Holt et al. (1994), the rhizobia group of bacteria can metabolize glyoxylate, which is a degradation product of fatty acids. The *B. japonicum* strain in our study may have used the surfactant fatty acids as an energy source to sustain metabolic activities throughout the incubation. The surfactant ethylamine used in commercial herbicide formulations (e.g., Roundup Transorb) has been reported to facilitate the growth of beneficial microorganisms including some strains of *Bradyrhizobium* (dos Santos et al., 2005).

In addition to the energy source, Duce alone and in combination with herbicides and surfactants exhibited a more favorable pH (7.4) for growth. The favorable pH combined with an energy source may have been responsible for the higher OD and estimated CFUs per milliliter. Another untested possibility is that Duce surfactant and/or Duce and StrikeZone (which was not tested alone) acted in concert as both osmotic buffers and energy sources in this solution. It should be noted that Duce in the combination solution was at a rate 10 times higher than the labeled rate for herbicide application, and this rate may not be suitable for field application due to unintended consequences of crop injury (Appah et al., 2020).

5 | SUMMARY AND CONCLUSION

The use of biostimulant products containing different combinations of nutrients, plant extracts, and/or living organisms has increased over time. Foliar applications are recommended for some, but single-product applications may not be economical. Combining these with other needed treatments, such as herbicides, can be a climate-smart practice. There are many different formulations of herbicides, and while active ingredients and the amounts are required on labels, inert ingredients are proprietary. Although this study was limited in scope, the results indicate that the herbicide type and the type and amount of surfactants used in the herbicide application mix can negatively impact beneficial bacterial growth in solution. Therefore, specific herbicide mixes that mimic a field application mixture should be tested to avoid adverse impacts on the desired inoculant growth. Alternatively, if biostimulants are mixed with other agrichemicals for application, perhaps choosing biostimulants that do not contain living organisms may be an option, but this has not been tested. In addition, field testing is needed to determine if biostimulants combined with postemergent agrochemicals (e.g., herbicides, fungicides, and insecticides) would enhance crop growth and yield without diminishing efficacy against target pests.

AUTHOR CONTRIBUTIONS

Joy Amajioyi: Data curation; formal analysis; investigation; writing—original draft; writing—review and editing. **Thandiwe Nleya**: Writing—review and editing. **Senthil Subramanian**: Formal analysis; methodology; resources; supervision. **Sharon A. Clay**: Conceptualization;

funding acquisition; investigation; methodology; project administration; resources; supervision; validation; visualization; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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