Scientific Research Publishing

American Journal of Plant Sciences, 2018, 9, 2306-2319
http://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Interaction of the Bioherbicide *Myrothecium verrucaria* with Technical-Grade Glyphosate on Glyphosate-Susceptible and -Resistant Palmer Amaranth

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How to cite this paper: Hoagland, R.E., Boyette, C.D., Jordan, R.H. and Stetina, K.C. (2018) Interaction of the Bioherbicide *Myrothecium verrucaria* with Technical-Grade Glyphosate on Glyphosate-Susceptible and -Resistant Palmer Amaranth. *American Journal of Plant Sciences*, **9**, 2306-2319.
https://doi.org/10.4236/ajps.2018.911167

Received: September 26, 2018
Accepted: October 26, 2018
Published: October 29, 2018

Abstract

Previously we found that a strain of *Myrothecium verrucaria* (MV) exhibited bioherbicidal activity against several important weeds, and that some commercial formulations of glyphosate applied with MV resulted in synergistic interactions that improved weed control efficacy. We also found that MV had bioherbicidal activity against glyphosate-resistant Palmer amaranth. We have also reported that some commercial formulations are inhibitory to MV. Our objectives were to test the effect of unformulated glyphosate (high purity, technical-grade glyphosate) alone and in combination with MV for bioherbicidal activity on glyphosate-susceptible and -resistant Palmer amaranth biotypes under greenhouse conditions and to examine technical-grade glyphosate on the growth of this bioherbicide. High purity glyphosate (without adjuvants/surfactants) was not toxic to MV growth and sporulation at concentrations up to 2.0 mM when grown on agar supplemented with the herbicide. Both biotypes were injured by MV and MV plus glyphosate treatments as early as 19 h after application (3 h after a dew period of 16 h). These injury effects increased and were more evident through the 6-day time course, when after 120 h the MV plus glyphosate treatment had killed all glyphosate-susceptible and -resistant plants. The interaction of glyphosate plus MV was synergistic toward the control of Palmer amaranth. Data strongly suggest that the active ingredient is responsible for the synergy previously found when this bioherbicide was combined with some commercial formulations of glyphosate. Results demonstrated that MV can control both glyphosate-resistant and -susceptible Palmer amaranth seedlings and act synergistically with high-purity glyphosate to provide improved weed control.

Keywords

Amaranthus palmeri, Biocontrol Agent, Bioherbicide, Biological Weed Control, Glyphosate-Resistance, *Myrothecium verrucaria*, Palmer Amaranth, Pigweed

1. Introduction

Palmer amaranth (*Amaranthus palmeri* S. Wats.) is an invasive weed that has rapidly spread from its origin (North American southwest), to eastern North America and to Europe, Asia and Australia [1] [2]. In the southeastern U.S. it is a major weed [3], with evolved resistance to several herbicide groups including triazines, acetolactate-synthase inhibitors, dinitroaniline, PPO (protoporphyrinogen oxidase) inhibitors and glyphosate herbicides [4]-[10]. Although originally controlled with the herbicide glyphosate, Palmer amaranth has become resistant to glyphosate, and resistant biotypes are widely distributed [3]. This weed is an abundant seed producer, for example up to 400,000 per plant [11] and herbicide resistance traits can be transferred when Palmer amaranth cross-breeds with the related weed, water hemp (*Amaranthus rudis*) [12].

The molecular site of action of glyphosate is inhibition of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in the shikimate pathway [13], which is responsible for the production of aromatic amino acids and phenolic compounds, some of which are related to plant defense [14]. The extensive use of glyphosate in non-cropping areas and in transgenic crops resistant to glyphosate has resulted in the evolution of many glyphosate-resistant weed biotypes [15]. To date, over 40 weed species are reported to be resistant to glyphosate [10]. Glyphosate resistance in Palmer amaranth plants is due to high copy numbers of the EPSPS gene, relative to that in glyphosate-susceptible plants [16]. This high EPSPS copy number enables the plant to produce adequate EPSPS to support required aromatic amino acid production even when high levels of glyphosate are present in plant tissues. This increased EPSPS gene copy number is a heritable trait when plants are cross-bred [16]. The transfer of resistance through cross-breeding, its aggressive nature and the prolific seed producing capacity of this weed [12] also exacerbate its spread.

Biological control initiatives such as the use of plant pathogens as bioherbicides for weed control have been studied since the early 1970s, as outlined in review chapters and books [17]-[26]. The fungus *Myrothecium verrucaria* (Alb. and Schwein.) Ditmar:Fr. (strain IMI368023) (MV) has been shown to have bioherbicidal activity on several weeds [27] [28] [29]. Other studies in our laboratory demonstrated that MV had bioherbicidal activity against economically important weeds such as: kudzu (*Pueraria lobata* var. montana) [30], purslanes (*Portulaca* spp.) and spurges (*Euphorbia* spp.) [31], morninglory spp. (*Ipomoea* spp.) [32], hemp sesbania [33], and Palmer amaranth (*Amaranthus palmeri*) [34]. Furthermore, synergistic interactions of some commercial formulations of

the herbicide glyphosate and MV for control of certain weeds were discovered [33] [35] [36] [37]. Other bioherbicidal plant pathogens also exhibit synergistic interactions with glyphosate [38] [39].

Commerical glyphosate is available as several different formulated products. Although some of these products have been shown to have positive interactions (synergism) with some bioherbicides, certain glyphosate formulations are toxic to bioherbicides and/or cause antagonistic effects when applied with bioherbicides. For example, Touchdown® ± and Round Up HiTech® formulations were found compatible for tank mixing with MV spores, but Accord XRT II® and Round Up Weather MAX® rapidly killed spores after mixing with low concentrations of these products [40]. Another fungal bioherbicide, *Microsphaeropsis amaranthi,* was incompatible with some commercial glyphosate products [41]. We have previously reported similar incompatibility of some glyphosate products on MV [35].

Adjuvants and surfactants are major components of commercial formulations of herbicides. These inert ingredients can also aid in the absorption and uptake of herbicides into target plants. Various adjuvants including surfactants have also been used to improve the efficacy of many bioherbicides. Many reports in the literature demonstrate that certain adjuvants, invert emulsions and surfactants can improve the efficacy of bioherbicides (see [40] for a brief summation of selected citations). MV also requires a surfactant (Silwet L-77) to increase its infectivity and bioherbicidal effects on weeds [27] [28] [40].

Host range studies of MV spores showed phytotoxic activity on *Amaranthus retroflexus* [27] [28], and on *A. hybridus* and *A. tubercalatus* [28]. More recently MV was found to exhibit bioherbicidal effects on Palmer amaranth in greenhouse and laboratory studies [34]. Due to the severity of Palmer amaranth as a very serious weed problem, and in order to help clarify the role of the formulation ingredients in Touchdown herbicide related to its synergistic action found with MV [37], our objectives were to: ascertain if high purity, technical grade glyphosate (without commercial adjuvants) exhibits a synergistic interaction with MV on glyphosate-resistant and -susceptible Palmer amaranth populations. To evaluate the effects of MV, technical grade glyphosate and the combination of this bioherbicide and herbicide in glyphosate-resistant and -susceptible Palmer amaranth we used sub-lethal technical grade, high purity glyphosate and sub-lethal MV concentrations in order to avoid rapid and severe plant injury that would mask any possible synergistic interactions of these weed control agents. Because MV requires a surfactant such as Silwet L-77 to increase its infectivity and bioherbicidal effects on weeds [27] [28], this surfactant was used in all treatments, including control. We also used Palmer amaranth plant populations that had been characterized for susceptibility or resistance to glyphosate [42] [43].

2. Materials and Methods

2.1. MV Source and Production

MV spores [*M. verrucaria* (IMI 361690)], originally isolated from sicklepod

(*Senna obtusifolia* L.), were grown and maintained in petri dishes on potato dextrose agar (PDA) (Difco Laboratories, Inc., Detroit, MI, USA) at 25˚C. Mycelial cultures of MV used in these experiments were prepared as described previously [37]. Briefly, a fermenter (Model MF-214, New Brunswick Corp., Edison, NJ, USA) charged with liquid media (soy flour-corn meal) was inoculated under sterile conditions with starter inoculum (mycelial preparation grown in shake-flasks). The shake-flask medium (soy flour-corn meal) was inoculated with a 10 mm agar plug (~10^6 spores) from a petri dish of MV spores. The flask was incubated on a rotary shaker (185 - 200 rpm, 28˚C, 7 days) and mycelial fungal growth proceeded without spore production. The MV mycelial product produced via fermentation for 48 - 72 h was harvested and stored at 4˚C until use. Concentrations of the mycelial formulations used in these tests were based on percent (v/v basis) of the fermentation batch as described elsewhere [37]. That procedure consisted of determining the viable propagule density (colony forming units; cfu) of the MV mycelial fermentation product in diluted samples (1.0 ml product: 1.0 L sterile H_2O) after thoroughly mixing under sterile conditions, by plating of aliquots of the mixture onto PDA in petri dishes, incubation of plates (28˚C for 48 h), and then counting colonies. Appropriate dilutions were made to obtain a concentration of 1.0×10^7 cfu mL^{-1}.

2.2. Plant Propagation

Palmer amaranth plants used in these experiments were grown from seeds previously characterized as glyphosate-susceptible or -resistant [42] [43]. Seeds were planted in potting soil, allowed to germinate and grow to about 50 - 60 mm tall and then uniform plants were transplanted into pots (9 × 7 cm) containing a potting soil mixture 70:20 mixture of 1:1 commercial potting mix:soil. Plants were grown in an environmental chamber (20˚C - 24˚C, with a 16 h photoperiod supplied with fluorescent and incandescent bulbs) for 5 - 6 additional days before treatments were applied. Plants were watered with de-ionized water and dilute fertilizer [N:P:K (13:13:13)] was provided.

2.3. Application of *Myrothecium verrucaria* Mycelial Formulation and Glyphosate to Plants

Seedlings from each biotype (4-week-old) were sprayed using hand-held compressed air spray canisters (Crown Spra-Tool, North American Professional Products, Woodstock, IL, USA) to run-off (ca. 300 $L{\cdot}ha^{-1}$), with each treatment [Silwetat 0.20%, v/v (control)], MV at 70% mycelia product (sublethal concentration) plus Silwet (0.20%, technical grade glyphosate (1.0 mM) plus Silwet, or the combination of MV and glyphosate. Treatments were: 1) control, water: Silwet, 2) MV mycelium: Silwet, 3) technical-grade glyphosate: Silwet, and 4) MV: glyphosate: Silwet. All treatments contained 0.20% (v/v) Silwet L-77 surfactant. Seedlings (10-day-old) hemp sesbania (*Sesbania exaltata*) were also sprayed with the MV: Silwet treatment so that we could measure the virulence of the MV my-

celial fermentation batch used in these tests. Hemp sesbania is highly sensitive to MV [27]. After spray treatment, the seedlings were placed in a dew chamber (Percival Scientific, Model No. 1-35 DL, Boone, IA, USA) at 25˚C for 15 h in darkness and then transferred to a greenhouse conditions for further growth, evaluation, and measurements. Greenhouse temperatures ranged from 28˚C to 32˚C, 40% - 60% RH, and a photoperiod of ~14 h, at 1600 - 1800 $\mu E \cdot m^{-2} \cdot s^{-1}$ (photosynthetically active radiation) PAR measured at midday.

2.4. Determination of MV Effects on Plant Growth

After MV application, the plants were visually examined for injury symptoms at various intervals after treatment over a 6-day time course. Plant shoot fresh and dry weights were determined 6 days after treatment on plant shoots excised at the soil level. The excised shoot material was weighed (fresh weight) and then placed in paper bags, labeled, and oven-dried (90˚C to 98˚C for 48 h) prior to weighing for dry weight determinations.

2.5. Disease Progression Tests

Disease progression or injury severity on plants of these Palmer amaranth biotypes (4-week-old) after treatments were applied as a spray [70% MV mycelial fermentation product] prepared in 0.20% Silwet was monitored at several intervals over a 6-day period. A modified visual disease severity rating scale [44], was used and defined as: 0 = no infection, and 1.0, 2.0, 3.0 and 4.0 = 20, 40, 60, and 80% leaf and stem lesion coverage/injury, respectively, and 5.0 = plant mortality. Data were analyzed using standard mean errors and best-fit regression analysis. Disease ratings ≤ 2.0 were considered "slight", 2.1 - 3.9 were considered "moderate", and ≥4.0 were considered severe. Surviving plants were excised at the soil line, their heights and fresh weights measured, followed by oven-drying for 48 h at 85˚C in order to determine dry weights. In all experiments, treatments were replicated three times. The experiments were repeated over time, and data were averaged following Bartlett's test for homogeneity of variance [45]. A randomized complete block experimental design was utilized.

2.6. Toxicity Tests of Technical Glyphosate on *M. verrucaria* Growth

To examine possible toxic effects of high purity glyphosate on MV *in vitro,* the herbicide was incorporated into PDA to achieve agar plates containing various concentrations (0 to 2.0 mM). Aliquots (3 µl) of MV conidia (5×10^4 conidiaml^{-1}) were pipetted onto the center agar surface of each concentration. These tasks were performed under sterile conditions in a bio-safety cabinet (NuAire, Model No. NU-425-400, Plymouth, MN, USA). Inoculated plates were placed in an incubator (Precision Scientific Inc.)at 28˚C under a 12-h alternating light-dark cycle. The test was set up in triplicate and radial growth (colony diameter) of each colony was measured at 24 h intervals over a 7-day growth period.

2.7. Experimental Design and Statistical Treatments

A randomized complete block experimental design was used with each treatment consisting of 2 to 4 plants and all treatments were triplicated and the experiments were repeated. Analysis of variance (ANOVA) at the 5% probability level was used to statistically compare the data. Data values presented are means of replicated experiments. When significant differences were detected by the F-test, means were separated with Fisher's protected LSD test at the 0.05 level of probability. Error bars are ±1 SEM (standard error of the mean). For the disease/injury progression tests, the data were subjected to regression analysis.

3. Results and Discussion

3.1. Effects of MV and Technical Glyphosate on Glyphosate-Resistant and -Susceptible Palmer Amaranth

Both the glyphosate-susceptible and -resistant Palmer amaranth plants showed some injury effects caused by MV and MV plus glyphosate treatments as early as 19 h after application (4 h after dew period of 16 h) (**Figure 1**). These effects on injury increased and were more evident at 43 h (**Figure 1**). Some injury was observed on the indicator plant, hemp sesbania at these early 14 time points (data not shown). Generally, the injury symptoms progressed, and at 6 days after treatment the MV plus glyphosate treatment had killed both glyphosate-susceptible and -resistant plants (**Figure 2**). MV alone caused some necrosis to some leaves and meristem tissue in both biotypes, in addition to retarding growth (height and fresh weight accumulation) (**Figure 2**). Hemp sesbania seedlings were dead at 6 days after MV treatment and exhibited essentially no growth after the fungus was applied, indicating that this fermentation batch of MV was virulent (**Figure 3**).

Detailed analysis of plant height and fresh weight reduction of Palmer amaranth plants 6 days after treatment indicated that MV had an equal effect on seedlings of both biotypes (**Figure 4(a)** and **Figure 4(b)**).

In both biotypes, MV caused a 25% reduction of height and about a 50% reduction of fresh weight accumulation compared to control plants. As expected, the technical glyphosate treatment caused significant damage on the susceptible plants, but no necrotic lesions or chlorosis in the resistant plants. In the susceptible biotype, glyphosate caused a 25% reduction of plant height and an 80% reduction of fresh weight accumulation. The combination of MV and glyphosate caused a synergistic interaction on both parameters in both biotypes, *i.e.*, reducing plant height and fresh weight reduction by ~50% and 90%, respectively. Dry weight reduction caused by these treatments followed a similar trend to that of the fresh weight data (data not shown).

3.2. Disease and Injury Progression

In these test plants, disease progression with MV treatment was very similar in susceptible and resistant Palmer amaranth plants. Disease caused by MV on

both biotypes was observed (1.6 and 1.7 rating) 24 h after treatment (Figure 5(a) and Figure 5(b)). This effect increased slowly over the 6-day (144 h) time course with a final rating of 2.3 and 2.4 for susceptible and resistant plants, respectively. In the susceptible biotype, glyphosate also showed injury after 24 h (2.0 rating) and this effect progressed to a rating of 2.9 at 144 h (Figure 5(a)). In the resistant biotype there were no injury symptoms with glyphosate treatment (Figure 5(b)). In both biotypes, MV plus glyphosate caused moderate disease/injury (2.7 and 3.1 rating in susceptible and resistant, respectively) 24 h after treatment. This effect was more severe than that caused by either MV or glyphosate alone. Disease/injury progressed from 48 to 120 h with 100% mortality (disease rating = 5) of both biotypes after 120 h (Figure 5(a) and Figure 5(b)). Disease development in these plants treated with sub-lethal MV doses was slower than typically found with MV alone at full-strength [34] which corroborates our previous findings.

3.3. Toxicity Tests of Technical-Grade Glyphosate on *M. verrucaria* Radial Growth on PDA

The toxicity of various concentrations of technical grade glyphosate (0 to 2 mM), incorporated into PDA was examined on MV over a 7-day time-course. After inoculation on PDA dishes after 5 days, radial growth of MV colonies was found to be unaffected by the herbicide at any concentration tested; the pooled mean value for colony growth diameters at 0 and 2.0 mM was 27.1 ± 0.51 mm (Figure 6). Similarly, after 7 days radial growth at these two concentrations were 35.1 ± 0.47 mm. Furthermore, there was no effect of glyphosate at any concentration on sporulation or spore production of MV. It is noteworthy that a sector (spontaneous spore mutation) occurred on one MV colony (data not shown). Although we have not pursued testing of this sector, we have previously characterized some

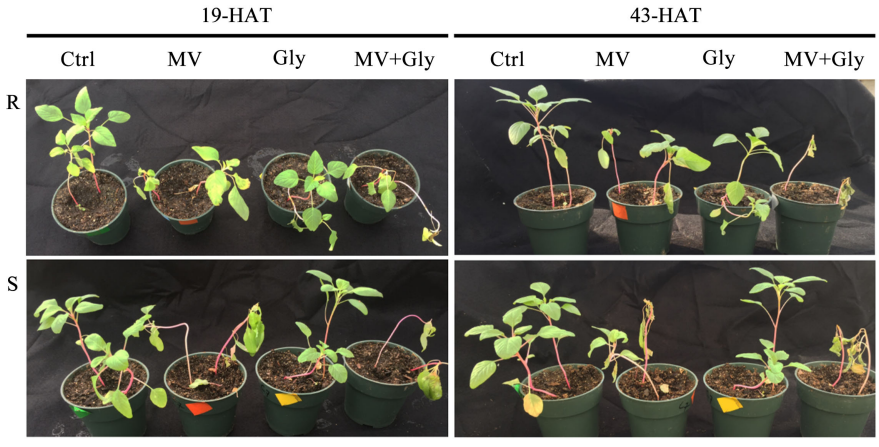


Figure 1. Photographs depicting effects of spray applications of MV, glyphosate and the combination of MV and glyphosate on glyphosate-susceptible and -resistant Palmer amaranth seedlings, 20 and 44 h after treatment. Top photos for both time periods = glyphosate-resistant Palmer amaranth plants; bottom photos = glyphosate-susceptible Palmer amaranth plants.

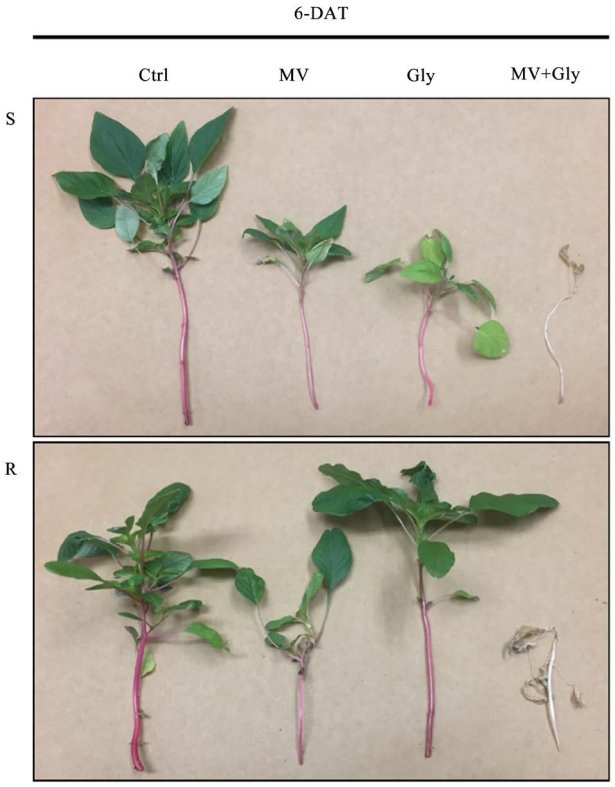


Figure 2. Photographs depicting effects of spray applications of MV, glyphosate and the combination of MV and glyphosate on glyphosate-susceptible (top photo) and -resistant Palmer amaranth seedlings (bottom photo), 6 days after treatment.

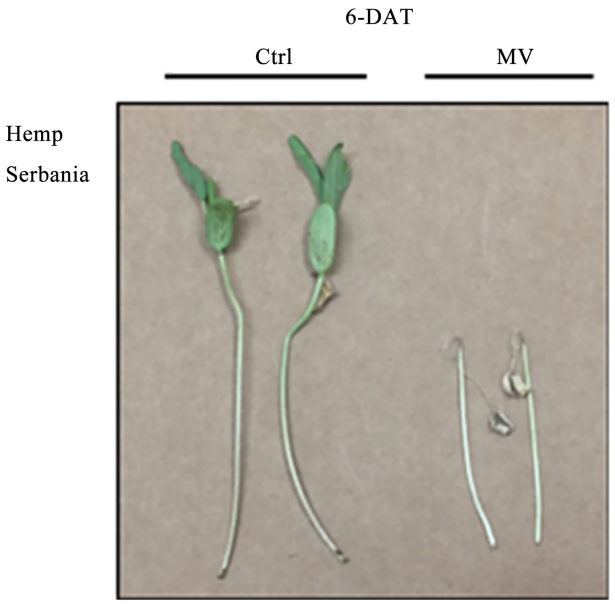


Figure 3. Effects of spray applications of MV on hemp sesbania seedlings, 6 days after treatment. Control plants (two excised plants, left side) were treated with water; Silwet (0.20%, v/v) and MV (right side) was applied in 0.20% Silwet surfactant. Plants were handled in the same manner as the plants described in Materials and Methods, and this species was included as a test plant to verify virulence of the MV mycelial fermentation batch.

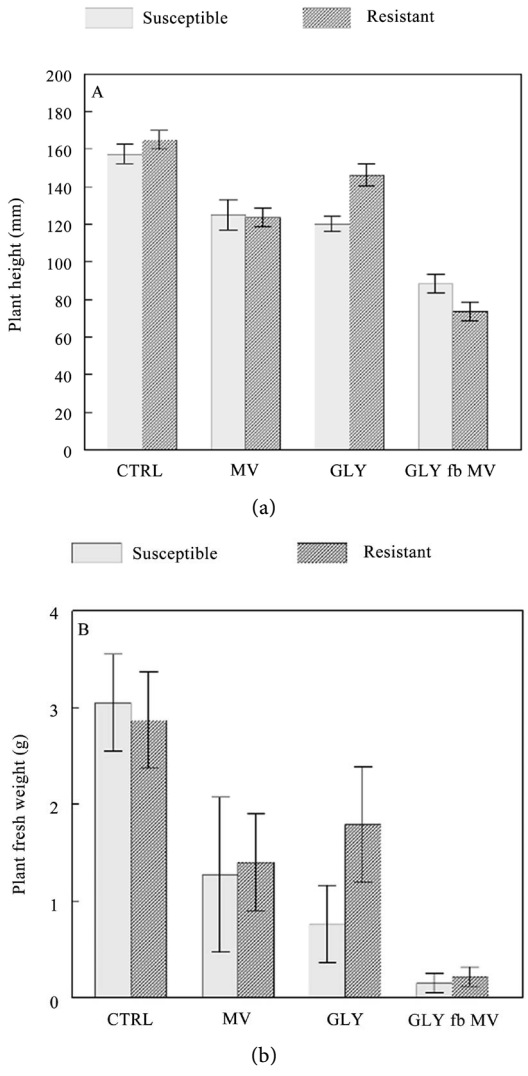
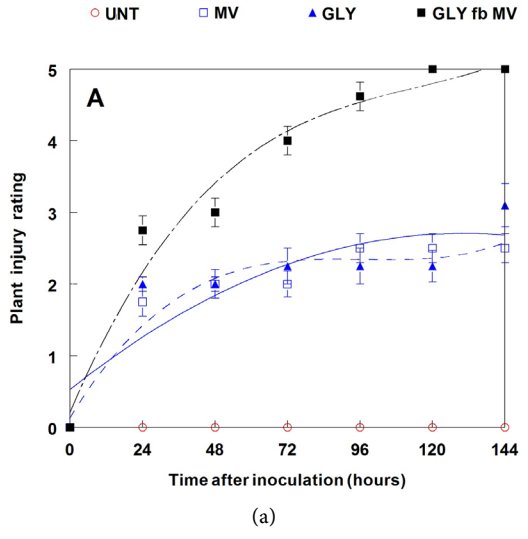
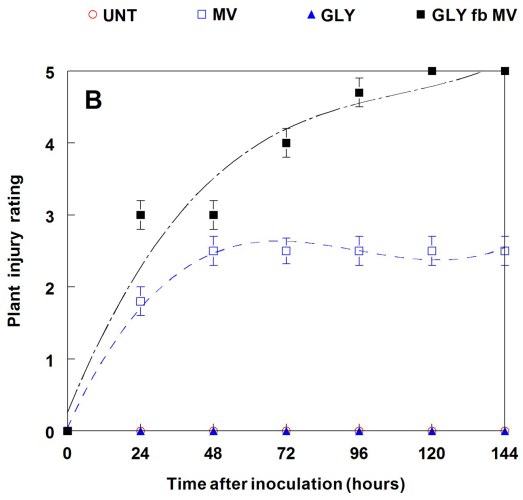


Figure 4. Effects of spray applications of MV, glyphosate and the combination of MV and glyphosate on glyphosate-susceptible and -resistant Palmer amaranth seedlings, on (a) plant height, and (b) plant fresh weight accumulation, 6 days after treatment.





(b)

Figure 5. Disease and injury progression effects on glyphosate-susceptible and -resistant Palmer amaranth treated with spray applications of MV, glyphosate and the combination of MV and glyphosate seedlings, over a 6-day time course under greenhouse conditions. (a) = glyphosate-susceptible plants; (b) = glyphosate-resistant plants. Regression equations relative to the data are as follows: Susceptible: Control -- $Y = 0$, $R^2 = 1.0$; MV -- $Y = 0.13 + 0.07X - 0.01X^2$, $R^2 = 0.97$; glyphosate -- $Y = 0.53 + 0.03X - 0.01X^2$, $R^2 = 0.94$; MV plus glyphosate -- $Y = 0.21 + 0.01X - 0.01X^2$; $R^2 = 0.96$. Resistant: Control -- $Y = 0$, $R^2 = 1.0$; MV -- $Y = 0.05 + 0.09X - 0.01X^2$, $R^2 = 0.98$; glyphosate -- $Y = 1.0$; MV plus glyphosate -- $Y = 0.25 + 0.10X - 0.01X^2$, $R^2 = 0.97$.

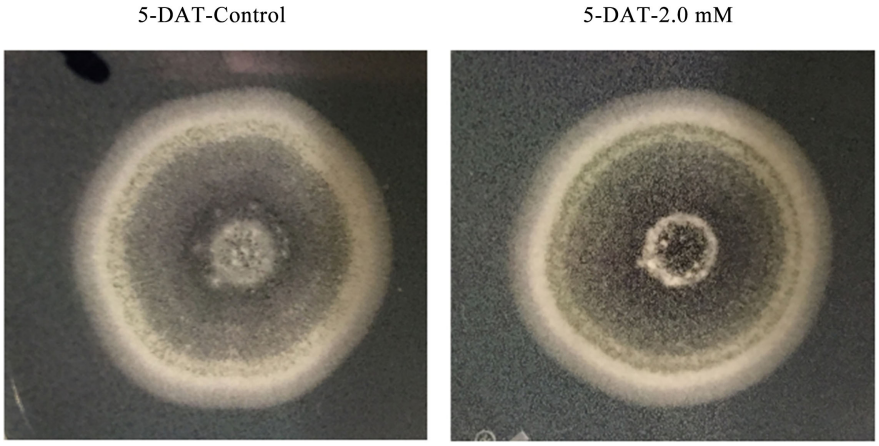


Figure 6. Photographs depicting the radial growth and sporulation of MV on PDA supplemented with technical grade glyphosate at 0 and 2.0 mM, after 5 days incubation.

MV sectors with regard to growth rate, sporulation and virulence on several weeds [46] [47]. Comparative studies were conducted on a whitish sector, isolated and grown in pure culture on PDA and found to be a stable, non-spore producing mutant with phytotoxicity to several weeds (including weeds tolerant or resistant to glyphosate) [47].

Another bioherbicidal fungus, *Microsphaeropsis amaranthi* has been shown to have activity against several weeds in the Amaranthaceae family [41]. Commercial glyphosate products also had inhibitory effects on conidial germination

of this bioherbicide, but inhibition was found to be caused by formulation adjuvants, not the active ingredient. This corroborates our present findings demonstrating that glyphosate can act as a synergist with some bioherbicidal pathogens, and that in cases where commercial products are inhibitory to MV, formulation or herbicidally inert ingredients are most likely the inhibitory components. Since most adjuvants in the commercial formulations of this herbicide are proprietary, we were unable to perform tests directly on those ingredients.

Unlike glyphosate, the mode of action of MV is unknown. Furthermore, the mechanism(s) of the synergistic interaction of MV and glyphosate is also unknown. When considering such interactions, it is important to acknowledge that the site of action or mode of action of a given herbicide may only be remotely related to its ability to interact positively with a bioherbicide. This is especially true if the herbicide and bioherbicide are applied to a weed that is resistant to the particular herbicide. However, as stated above, only rarely is the major site of action known for bioherbicides. Herbicides may affect many secondary and tertiary pathways and/or enzymes in the plant that may be closely related to infectivity and bioherbicidal activity. Herbicides may also directly influence the biochemistry of the bioherbicidal pathogen. More in-depth research on the biochemistry and molecular biology associated with a given bioherbicide, herbicide and weed will be necessary to solve the complexity of such interactions. Future research will address some of these issues, as well as examine the potential of synergistic interactions of MV and technical glyphosate under field conditions.

Conflicts of Interest

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

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