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Full Length Article

Potential of *Bipolaris bicolor* in Combination with Ametryn for Inhibition of Glyphosate-Resistant Goosegrass (*Eleusine indica*) Biotypes

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Abstract

Fungal pathogens have been investigated previously for biological control of goosegrass (*Eleusine indica*), but there is little research on the compatibility of herbicides with fungal pathogen *Bipolaris bicolor* for control of glyphosate-resistant *E. indica*. The aims of this study were to 1) examine the *in vitro* compatibility of *Bipolaris bicolor* and three herbicides (ametryn, flumiozaxin and sodium chlorate) and 2) determine the efficacy of a combination of *B. bicolor* and ametryn for inhibiting glyphosate-resistant *E. indica* biotypes in the greenhouse. Mycelial growth inhibition area was measured after *B. bicolor* spores were treated with ametryn, flumiozaxin, or sodium chlorate at one-fifth of their recommended rates. The findings showed that ametryn had the lowest inhibitory effect (10%), while flumioxazin and sodium chlorate had 80 and 30% inhibition, respectively, indicating that ametryn is the most compatible herbicide with *B. bicolor*. *E. indica* seedlings were then subjected to four treatments, including non-treated plants, at the 3 to 4-leaf stage where T1: negative control plant, T2: one-fifteenth of the recommended dose of ametryn, T3: *B. bicolor* spore suspension and T4: ametryn plus *B. bicolor* spore suspension. *B. bicolor* in combination with ametryn was the most effective treatment for *E. indica* inhibition one week after treatment. The treatment reduced seedling height, leaf greenness and shoot dry weight by 50 to 94% and caused plant death seven days after treatment. These findings suggest that *B. bicolor* in combination with ametryn is a viable integrated strategy to control the glyphosate-resistant *E. indica* biotype.

Keyword: Spore; Flumioxazin; Sodim chlorate; Synergistic

Introduction

Eleusine indica L. Gaertn., also known as goosegrass is primarily an agricultural and environmental weed (Randall 2012). It is one of the world's five most problematic weeds, affecting the productivity of 46 different crop species in over 60 countries (Holm *et al.* 1977). Since it has no root at the nodes, *E. indica* can be easily removed by hoeing in the early stages of growth. However, when the *E. indica* matures, a solid root structure forms in the soil, making manual removing difficult. It can withstand a wide range of salt stress, pH and water stress (Ismail *et al.* 2002, 2003; Chauhan and Johnson 2008). Furthermore, after two years, *E. indica* seed buried at a depth of 20 cm still had 79% viability (Chuah *et al.* 2004a).

Glyphosate in combination with fluzifop or sethoxydim (Chuah *et al.* 2004b), ametryn combined with glufosinate (Chuah *et al.* 2008a) and tank mixing of monosodium methanearsonate and diuron (Sim *et al.* 2018) have been effective against *E. indica*. However, due to antagonistic behaviour, a mixture of glyphosate and glufosinate is not recommended for *E. indica* (Chuah *et al.* 2008b). Herbicide resistance in *E. indica* has evolved as a result of the excessive use of the same herbicides. For example, paraquat, glufosinate, fluazifop-butyl and glyphosate resistance has been demonstrated in *E. indica* (Dilipkumar *et al.* 2020). Adopting a diverse integrated weed control strategy that combines chemical, biological, and physical approaches could help to delay the evolution of herbicide resistance (Harker and O'Donovan 2013). Several studies have been done to minimize herbicide use for delaying the evolution of herbicide-resistant *E. indica* (Chuah and Kent 2021a, 2021b). For instance, the emergence and shoot growth of *E. indica* were inhibited by 85 to 100% when treated with oil palm frond (OPF) residues at 3 t/ha, implying the possibility of using OPF residues as organic mulches for the weed management programme (Dilipkumar *et al.* 2015). Further study by

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Chuah *et al.* (2018) revealed that the residue of OPF have the potential to reduce the application rate of herbicide S-metolachlor without compromising on the excellent control obtained in combating the herbicide-resistant biotypes of *E. indica*. However, to date, there is still limited study on a combination of biological and chemcial methods for *E. indica* control.

Determining the impact of herbicides on fungal pathogens is a key factor in developing integrated weed control strategies (Charudattan 2001). When applied sequentially or as tank mixture, herbicides with synergistic potential cannot be harmful to fungal pathogen spores and mycelium (Hoagland 1996). Herbicides have been thoroughly investigated as methods for combining mycoherbicides to improve the efficacy of weed management. Peng and Wolf (2011) documented that herbicide have been shown to disrupt protective mechanisms of weeds, rendering them more vulnerable to mycoherbicide infections.

Bipolaris setariae, *Pyricularia grisea* (Figliola *et al.* 1988), *Phoma herbarum* and *Bipolaris sorokiniana* (Maizatul-Suriza *et al.* 2017) and *Bipolaris bicolor* have all been isolated from diseased *E. indica* (Fakri *et al.* 2019). Pathogenicity tests revealed that *Bipolaris sorokiniana* infected *E. indica* on day five after artificial inoculation and had the highest disease severity as compared to *P. herbarum* and *C. aeria* after 40 days of treatment (Ismail *et al.* 2020), none of these fungal pathogens, however, have been evaluated in conjunction with herbicides to combat *E. indica*. The genus *Bipolaris* consists of a variety of well-known fungal pathogens that are found all over the world. Leaf spots, leaf blight, dense melting, lateral root rots, and other disease symptoms are common in this species, particularly in crop fields for the Poaceae family, which includes rice, corn, wheat, and sorghum (Berbee *et al.* 1999).

For all pathogens, there is no universal chemical synergist (Hoagland 1996). There is also a lack of studies on the interactions of herbicides with *E. indica*-fungal pathogens. Thus, the goals of this study were to 1) assess *in vitro* compatibility between *Bipolaris bicolor* and three selected herbicides and 2) evaluate the *in vivo* efficacy of a combination of *B. bicolor* and a selected herbicide on inhibition of *E. indica* seedling.

Materials and Methods

Isolation of putative *Bipolaris bicolor*

The putative *Bipolaris bicolor* was isolated from diseased *E. indica* plants based on the modified method of Maizatul-Suriza *et al.* (2017). Leaf samples of *E. indica* infected with leaf spot disease were collected 4 meters apart from each other in corn fields at Research Farm, Bukit Kor campus, University of Malaysia Terengganu. The leaf samples were cut into 0.5 cm × 0.5 cm squares using a sterilized scalpel in a designated leaf region (two-thirds of contaminated area, one-thirds of healthy tissue). For 5 min, the samples were surface sterilized in sodium hypochlorite (NaOCl) (10–20% Chlorox). In sterile conditions, the selected leaf samples were surface-dried on sterilized filter paper. The leaf samples were put in a petri dish with potato dextrose agar (PDA) media, sealed with parafilm and incubated at room temperature for 10 days at 27°C. To obtain the pure culture, actively growing mycelium was sub-cultured onto fresh PDA using a sterilized cork borer or scalpel. After 15 days of incubation, the developing mycelium was looped, then placed on a microscope slide and covered with a cover slip to observe the morphology of the spore under 400× magnification.

Identification of putative Bipolaris bicolor

The total genomic DNA was extracted from overnight culture of fungi isolates using DNeasy Plant Mini kit (Qiagen, Hilden, Germany). Fragment of the gene of interest, was amplified using standard PCR protocol and the universal primers with Thermal Cycler machine. Then, the PCR products was analysed by electrophoresis on a 1% agarose gel, stain with SYBR Safe DNA gel stain. The bands were visualized under Life Technologies E-Gel Imager (Thermo Fisher Scientific, Inc., United States). The PCR products were then further analysed by Apical Scientific Sdn. Bhd. Sequence similarity was estimated by searching the homology in the GenBank DNA database and the National Centre for Biotechnology Information (NCBI) using Basic Local Search Tool (BLAST).

Compatibility of *Bipolaris bicolor* and selected herbicides

The sub-cultured fungi were flooded with 0.8% Tween 80 solution and scraped on the 15th day of incubation to carry spore into suspension (Kimaru *et al.* 2018). To extract the spores, the suspension was filtered through a double layer of muslin fabric. A haemocytometer was then used to determine the spore concentration. The spore suspension at 1.7 x 10^8 spores/mL was mixed at one-fifteenth recommended rate with flumioxazin, sodium chlorate, or ametryn. This herbicide rate was selected based on our preliminary testing that revealed the chosen rate could provide partial injury to goosegrass seedlings according to the method of Peng and Byer (2005). The mixture was swabbed onto a PDA plate with a sterilized cotton swab and incubated at 27°C for 3 days to see if mycelial growth was inhibited. Mycelial growth was expressed as percent of inhibition. A 0.8% Tween 80 solution with spore only acted as a negative control, while other treatments included 0.8% Tween 80 solution containing spore mixed with flumioxazin, sodium chlorate, or ametryn. The experiment was set up as a five-replicate complete randomized design.

Inhibition of mycelial growth was estimated as follows:

$$\text{Inhibition area} = \frac{MG - TMG}{MG} \times 100$$

Where,
MG – Area of PDA plate covered by mycelium in control
TMG–Area of PDA plate covered by mycelium in treatment

Post emergence application of ametryn in combination with *Bipolaris bicolor*.

Seeds of glyphosate-resistant biotypes of *E. indica* were provided by Dr. Cha Thye San (Franci *et al.* 2020), scarified by using sandpaper and soaked overnight with 0.2% potassium nitrate. The seeds were planted in trays with commercial soil potting mixture (28 cm × 56 cm). *E. indica* reached the 3 to 4-leaf stage after two weeks. The seedlings were transplanted into a 6 × 9-inch polybag filled with a 3:2:1 mixture of topsoil, cow dung and sand. Using a hand sprayer, *E. indica* seedlings were sprayed with 2 mL of 0.8% Tween 80 solution, *B. bicolor* spore suspension at 1.7 × 10^8 spores/mL, ametryn at one over fifteen recommended rate or a combination of the spore suspension and ametryn. To serve as an adjuvant, 10% edible palm oil was combined with all treatments. The *in vivo* experiment was arranged as complete randomized block design with five replicates. After spraying leaf greenness was measured using SPAD meter while plant height of *E. indica* was accessed daily for one week. The leaf greenness was expressed as a SPAD value. One week after treatments, the shoot dry weight of *E. indica* seedlings was determined. Each plant was carefully removed, washed under running water, and cut to separate the shoot from the root. The above-ground tissues of the plants were then dried for one week at 60°C in a digital oven.

The following formula was used to measure the dry weight reduction of the shoot (Chuah *et al.* 2008a).

$$\text{Shoot dry weight reduction} = 100\text{-}[(\text{treated shoot dry weight}/\text{untreated shoot dry weight}) \times 100)]$$

Colby's approach is a useful method for evaluating the efficacy of pathogen-herbicide combinations (Peng and Byer 2005). The shoot dry weight reduction of *E. indica* seedlings was used to assess the association of the natural mycoherbicide, *B. bicolor* with ametryn. The interaction was considered synergistic when the actual percentage shoot dry weight reduction was at least 5% greater than predicted. The relationship was classified as additive or antagonistic if the change was less than 5% or the actual percentage shoot dry weight reduction was lower than predicted (Grant *et al.* 1990).

The equation used for calculating the expected response was as follows (Colby 1967):

$$E = 100 - [(100 - x) \times (100 - y)/100)]$$

Where,
E = Expected response of shoot dry weight reduction as a percentage of control
x = Shoot dry weight reduction as a percentage of control from ametryn treatment
y = Shoot dry weight reduction as a percentage of control from *B. bicolor* treatment

Mycelia growth inhibition data were analysed using a one-way ANOVA. Tukey's Honestly Significant Difference test was used to compare means at a 5% level of significance. The shoot dry weight reduction data was transformed into an arcsine square root. Data of plant height, leaf greenness, and transformed shoot dry weight reduction data before subjected to a one-way ANOVA, with means compared using Tukey's Honestly Significant Difference test at a significance level of 5%.

Results

Isolation and identification of *Bipolaris bicolor*

Two strains of fungal pathogens (BR and BF) were successfully isolated from *E. indica* and identified as *Bipolaris bicolor*. All samples were blasted from NCBI and showed high similarity ranging from 99.46 to 99.65% from all GenBank descendent throughout world region. *Bipolaris bicolor* strain CPC 28825 (Accession Number: MF490805.1), which was isolated from the Poaceae family in Thailand, had 99.65% similarity to strain BF. Meanwhile, strain BR was found to be 99.65% similar to *B. bicolor* strain CPC 28811 (Accession Number: MF490804.1), which was isolated from *E. indica* in Thailand (Table 1). Thus, the strain BR was chosen to be studied in the following experiments. After 15th days of growth in PDA, the morphology of mature *B. bicolor* spore was observed (Fig. 1). The spores of *B. bicolor* are 1.006 mm long, oblong in shape, and have 2 to 3 septa in the centre at 400× magnification. The spore has septa and is flat, upright, and rarely branched.

Effects of selected herbicides on mycelial growth inhibition of *Bipolaris bicolor*

Fig. 2 presents the inhibitory effects of flumioxazin, sodium chlorate, and ametryn on *B. bicolor* mycelial growth *in vitro*. These herbicides were tested on *B. bicolor* mycelial growth at a concentration of one-fifth of their respective recommended rates. Flumioxazin was found to be the most phytotoxic herbicide to the *B. bicolor* fungal pathogen, followed by sodium chlorate and ametryn. Flumioxazin inhibited mycelial growth by 80%, while ametryn inhibited mycelial growth by 10%. This result suggests that ametryn is the most compatible herbicide with *B. bicolor* for integrated management of *E. indica* using *B. bicolor* as a biocontrol agent.

Efficacy of a combination of *Bipolaris bicolor* and ametryn for inhibition of *E. indica* seedling

Fig. 3 shows the height of *E. indica* seedlings after being treated with ametryn, *B. bicolor* spore suspension, or a

Table 1: Identification of *Bipolaris bicolor* using 16S rDNA sequencing by depositing the sequences into GeneBank in NCBI

Strain	Accession number	ID from NCBI	Source	Origin	Similarity (%)
BF	MF490805.1	*Bipoaris bicolor* strain CPC 28825	Poaceae	Thailand	99.65
	KY047110.1	*Bipolaris bicolor* strain HMC3	*Lolium perenne*	China	99.64
	KY047109.1	*Bipolaris bicolor* strain HMC2	*Lolium perenne*	China	99.64
	KY047105.1	*Bipolaris bicolor* strain HMC1	*Lolium perenne*	China	99.64
	MF490804.1	*Bipolaris bicolor* strain CPC 28811	*Eleusine indica*	Thailand	99.47
BR	MF490804.1	*Bipolaris bicolor* strain CPC 28811	*Eleusine indica*	Thailand	99.65
	MF490805.1	*Bipoaris bicolor* strain CPC 28825	Poaceae	Thailand	99.47
	KY047110.1	*Bipolaris bicolor* strain HMC3	*Lolium perenne*	China	99.46
	KY047109.1	*Bipolaris bicolor* strain HMC2	*Lolium perenne*	China	99.46
	KY047105.1	*Bipolaris bicolor* strain HMC1	*Lolium perenne*	China	99.46

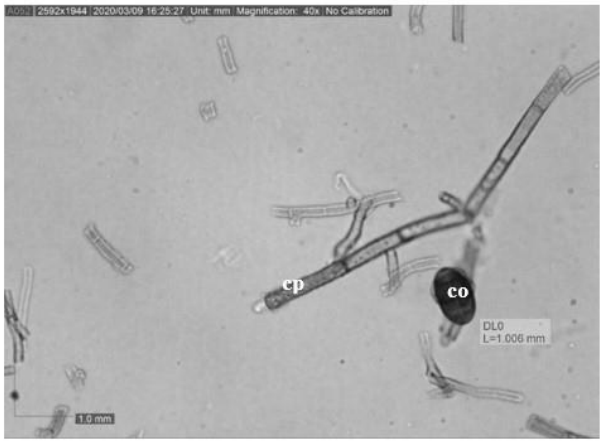

Fig. 1: Conidia (co), Conidiophore (cp) of *Bipolaris bicolor* grown on a PDA plate at 26 ± 2°C

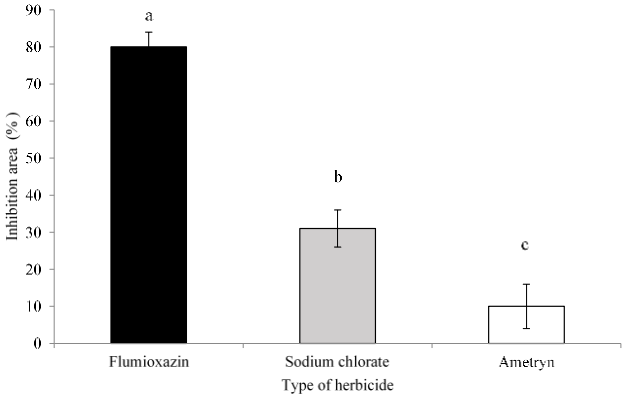

Fig. 2: Inhibitory effects of flumioxazin, sodium chlorate and ametryn on mycelial growth of *Bipolaris bicolor* three days after incubation at 26 ± 2°C. Error bars represent the standard deviation of the mean. Mean followed by similar letters have significant difference after analysed by Tukey test at 5% of significant level

combination of *B. bicolor* spore suspension and ametryn for seven days. Both ametryn and *B. bicolor* alone, as well as a combination of the two, were phytotoxic to *E. indica* seedlings, with plant height reaching a plateau on day 3 after treatment and thereafter, implying that treated *E. indica* seedling's growth was stunted. Non-treated plants, on the

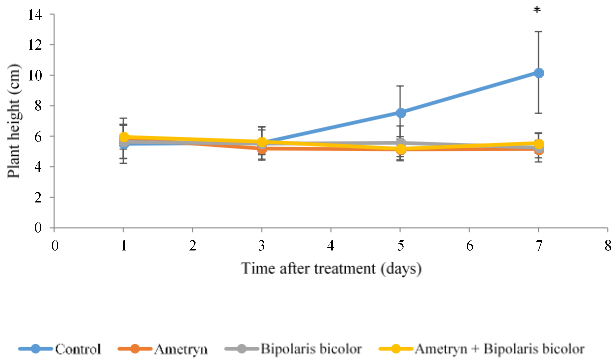

Fig. 3: Changes in *E. indica* seedlings height after subjected to ametryn, spore suspension of *Bipolaris bicolor* and ametryn in combination with spore suspension of *B. bicolor* throughout 7 days of experimental period. Vertical bars represent standard deviation of mean. *Denotes significant difference between treated and non-treated plants within the same day after analysed by Tukey test at 5% of significance level

other hand, grew steadily in height from day 3 to day 7. The non-treated plants reached a maximum height of 10.2 cm, while the plants treated with *B. bicolor*, ametryn, and *B. bicolor* in combination with ametryn reached a maximum height of 5.2, 5.3 and 5.6 cm, respectively, on day 7 after treatment. The Tukey test also showed that on days 5 and 7, after treatment, there was a substantial difference in height between treated and non-treated plants ($P < 0.05$). During the 7th days evaluation period, however, there was no significant difference in height between all treated plants ($P > 0.05$).

Fig. 4 depicts the greenness of *E. indica* leaves as measured by SPAD after treatments with ametryn, *B. bicolor* spore suspension, and ametryn in combination with *B. bicolor* spore suspension over the 7-day duration. Both ametryn and *B. bicolor* alone, as well as a combination of the two, were found to be phytotoxic to *E. indica* seedlings. The amount of chlorophyll present in treated plants decreased on day 3rd and thereafter, with the combined treatment showing a faster decline than the other treatment, indicating that all treatments were able to reduce the green colour of leaf. The SPAD value for non-treated plants, on the other hand, remained constant over the course of the 7th

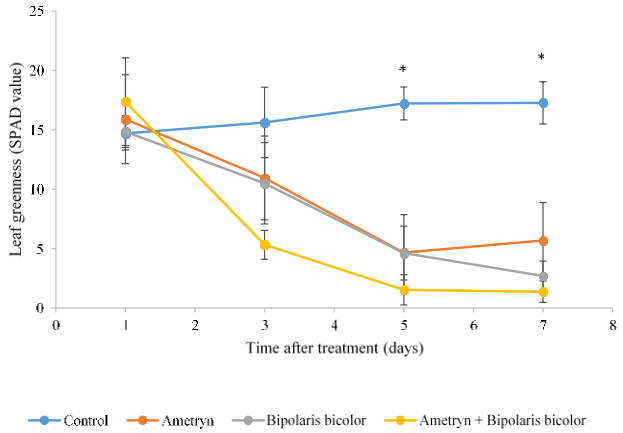

Fig. 4: Changes in *E. indica* SPAD value after exposure to ametryn, *Bipolaris bicolor* spore suspension, and ametryn in combination with *B. bicolor* spore suspension over a 7-day period. The standard deviation of the mean is represented by vertical bars. *Denotes significant difference between treated and non-treated plants within the same day after analysed by Tukey test at 5% of significance level

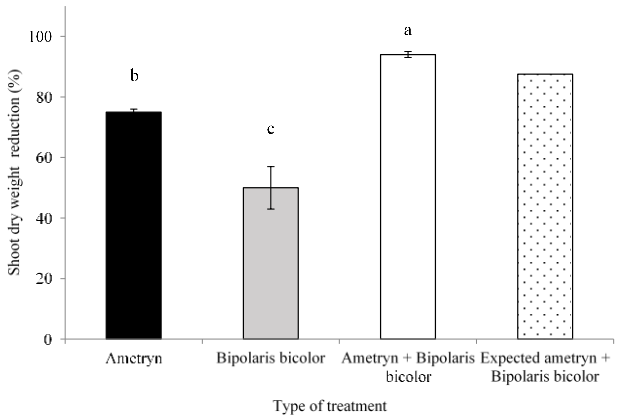

Fig. 5: Shoot dry weight reduction of *E. indica* seedlings at 7 days after subjected to ametryn, spore suspension of *Bipolaris bicolor* and ametryn in combination with spore suspension of *B. bicolor*. Vertical bars represent Standard deviation of mean. *Denotes significant difference between treated and non-treated plants after analysed by Tukey test at 5% of significance level

days assessment period. On days 5 and 7 after treatment, the Tukey test showed a substantial difference ($P < 0.05$) in SPAD value between treated and non-treated plants. However, the SPAD values of all treated plants did not vary significantly ($P > 0.05$) over the course of the 7 days of experiment.

Fig. 5 shows the reduction in shoot dry weight of *E. indica* seedlings subjected to various treatments. At 7 days after treatment, the phytotoxic effects of ametryn alone, *B. bicolor* spore suspension alone, and the combination of *B. bicolor* spore suspension and ametryn varied significantly ($P < 0.05$) in reducing shoot dry weight of *E. indica* seedlings.

Discussion

Bipolaris bicolor has a wide range of distribution including Australia, India. Africa, Brazil, Canada, Cote d'Ivoire, Denmark, New Zealand, Nigeria, Swaziland, Zimbabwe (Farr and Rossman 2013), China (Liang *et al.* 2019) and Thailand (Marin-Felix *et al.* 2017). The fungal pathogen is commonly found in host plants from Poaceae such as *Zea mays*, *Eleusine coracana*, *Pennisetum clandestinum*, *Oryza sativa*, *Panicum maximum*, *Sorghum vulgare*, *Triticum aestivum*, *Urochloa panicoides* and *Zizania aquatica* (Farr and Rossman 2013) and *Eleusine indica* (Marin-Felix *et al.* 2017). The septation characteristics of *B. bicolor* exhibited in this work are consistent with those found by Manamgoda *et al.* (2014) who stated that the fungus produced a slightly cobwebby nearly black growth in pure culture on PDA. However, *B. bicolor* colonisation was fluffy, cottony, whitish dark grey, and undulated in the present study. This fungal pathogen was first reported as causal agent of leaf spot disease on rubber tree (Liang *et al.* 2019).

Fakri *et al.* (2019) reported that diuron is more compatible with 0% inhibition than other herbicides including oxyfluorfen and imazethapyr, which gave 90 and 50% inhibition of mycelial growth, respectively, when tested on *B. bicolor*. Similarly, in comparison to sodium chlorate and flumioxazin, ametryn inhibited mycelial growth of *B. bicolor* the least. Both ametryn and diuron inhibit photosystem II in plants, which is a similar mechanism of action. By contrast, flumioxazin had a strong inhibitory effect on the fungal pathogen. Flumioxazin is a photo-dependent peroxidising herbicide that inhibits protoporphyrinogen oxidase, an essential enzyme in chlorophyll biosynthesis, causing photo-toxic porphyrins to accumulate (Shaner 2014). Porphyrins are a common alternative to traditional antibiotics, and they have already been shown to inhibit bacteria, viruses, fungi, and protozoa *in vitro* (Singh *et al.* 2016).

On day 7th after treatment, the non-treated plant had the highest SPAD value of 17.3, while the plants exposed to *B. bicolor* plus ametryn had the lowest SPAD value of 1.4. The plants treated with *B. bicolor* alone and those treated with ametryn alone had SPAD values of 5.7 and 2.7, respectively. When *E. indica* seedlings were treated with a combination of *B. bicolor* and ametryn on day 7th, the chlorophyll in the leaves rapidly degraded, thereby leading to plant death. Changes in photosynthesis were a major contributor to both impeded light capture and decreased mesophyll carbon dioxide fixation, as shown by the lower SPAD value. A similar symptom has been identified in the infectious phase of *Bipolaris oryzae* in rice, which has harmed the physiology of the leaf, primarily due to cell damage at the membrane level (Dallagnol *et al.* 2011).

The findings of this study on reducing shoot dry weight of *E. indica* seedlings, agree with those of Ismail *et al.* (2020), who found that the fungal pathogen *Bipolaris sorokiniana* inhibited *E. indica* development 40 days after treatment. They also reported that non-target plants were not

affected by *B. sorokiniana*, but the fungal pathogen consistently infected *E. indica*. Meanwhile, according to Rusli *et al.* (2015), *Phoma herbarum* caused 80% of *E. indica* mortality 35 days after treatment.

The combination of ametryn and *B. bicolor* inhibited the most, with 94% inhibition, compared to ametryn alone and *B. bicolor* alone, which inhibited 75 and 50%, respectively, based on shoot dry weight reduction of *E. indica*. For ametryn plus *B. bicolor* treatment, the estimated dry weight reduction value was 88%. This estimated value is 7% lower than the actual value of shoot dry weight reduction, meaning that *B. bicolor* and ametryn work together synergistically. Ametryn is a non-selective, systemic pre-emergence or post-emergence herbicide that inhibits photosystem II at the chloroplast's thylakoid membrane of plants. By binding to D-1 proteins, the herbicide prevents electron transfer and can damage photosynthetic tissues by disrupting the formation of cell membranes and pigments, resulting in nutrient leakage and cellular dysfunction (Reade and Cobb 2002). In other words, ametryn may have damaged *E. indica* seedlings by compromising their defence mechanism, exposing them to infection by the fungal pathogen, *B. bicolor* (Peng and Wolf 2011).

The present findings are consistent with those of Peng and Byer (2005), who used the same multiplicative survival model (Colby 1967) to investigate the synergy of fungal pathogen-herbicide interactions on green foxtail. According to Peng and Byer (2005), propanil and quinclorac interacted synergistically with *Pyricularia setariae*, resulting in a pattern of increased effectiveness and nearly 100% mortality on green foxtail. Peng and Wolf (2011) looked at a variety of herbicides in conjunction with the fungal pathogen *Colletotrichum truncatum* and discovered that herbicides including 2,4-D, MCPA, clopyralid, and metribuzin greatly synergized the fungus on scentless chamomile.

Conclusion

It is clearly indicated that ametryn was the most compatible herbicide because it had the lowest inhibitory effect on *Bipolaris bicolor*. Subsequent greenhouse study revealed that that *B. bicolor* in combination with ametryn acted synergistically and inhibited the glyphosate-resistant biotype of *E. indica* seedlings, with the treated plants showing a dramatic and greater reduction in shoot growth as compared to those provided by single application of ametryn or *B. bicolor*. However, the efficacy of ametryn combined with *B. bicolor* is possibly affected by growth stage of *E. indica* and environmental factors, glasshouse findings cannot be specifically extrapolated to field conditions. Further research is needed to determine whether the combination of ametryn and *B. bicolor* can control mature *E. indica* in the field.

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Author Contributions

ZS, MSAH, and CTS designed the research flow; MAF and NFG performed the research and wrote the manuscript. MAF and CTS edited the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a reasonable request

Ethics Approval

Ethical approval is not applicable in this study.

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