Journal of Oil Palm Research Vol. 34 (3) September 2022 p. 465-474
DOI: https://doi.org/10.21894/jopr.2021.0053

EFFECTS OF *Phoma herbarum* AS A BIOLOGICAL CONTROL AGENT OF GLYPHOSATE RESISTANT *Eleusine indica*

RUSLI, M H[1]*; SHARIFFAH MUZAIMAH, S A[1]; MAIZATUL, S M[1] and IDRIS, A S[1]

ABSTRACT

Goosegrass (Eleusine indica) *is one of the weeds that has a problem of herbicide resistance to glyphosate. This study investigated the potential use of* Phoma herbarum *as a biological control agent of glyphosate resistant* E. indica. *Nursery and field experiments showed that the application of* 10^6 *conidial suspension of* P. herbarum *demonstrated biofungicidal activity whereby 91.70% of treated* E. indica *died whilst for field experiment the mortality rate was recorded at 80.00%. The effect of* P. herbarum *was noticeable at 14 days after treatment and continued to increase at 21 days and 28 days after application. This study also investigated the direct effects of a few common herbicides that were used to control* E. indica *in oil palm plantations. The study found that the* P. herbarum *was compatible with herbicide diuron and was able to cause 80.00% mortality to* E. indica *when diuron was applied at full strength. The percentage mortality of* E. indica *increased to 91.67% when half strength diuron was applied. Thus, this study was to report the effectiveness of* P. herbarum *as a potential biological control agent against resistant* E. indica *and compatible with herbicide diuron.*

Keywords: biological control, *Eleusine indica, Phoma herbarum*.

Received: 18 February 2021; **Accepted:** 14 November 2021; **Published online:** 23 December 2021.

INTRODUCTION

Goosegrass (*Eleusine indica*) is a noxious weed in oil palm and fruit trees cultivations in Malaysia (Barnes and Chan, 1990). *Eleusine* is a member of the tribe Eragrosteae, family Poaceae (Bisht and Mukai, 2002). Goosegrass is an all-season prolific grass that is widely distributed in the tropics, particularly in Asia, Africa, South America, and the southern parts of North America (Holm *et al.*, 1977). *Eleusine indica* was reported as one of the five most troublesome weeds in the world and caused problems to 46 different crop species in more than 60 countries (Chuah and Lim, 2015). In Malaysia, *E. indica* was found to be the most occurred grass (82.2%) followed by *Imperata cylindrical* (81.2%), *Ishaemum muticum* (71.8%) and *Pennisetum polystachion* (67.1%) in immature oil palm plantation (Maizatul-Suriza and Idris, 2012).

The only practical way to control *E. indica* is by using herbicide. However, excessive and repeated use of herbicide such as glyphosate to control *E. indica* has caused the weed to become resistant. Glyphosate resistance in *E. indica* has been reported in many countries and could seriously affect future control of this weed if the resistance problem is left unsolved (Chen *et al.*, 2017; Lim and Ngim, 2000; Teng and Teo, 1999; Tran *et al.*, 1999).

Due to the repeated use of herbicides with the same mode of action, populations of *E. indica* have evolved resistance to acetolactate synthase inhibitors (Valverde *et al.*, 2000), acetyl CoA carboxylase inhibitors (Leach *et al.*, 1995), bipyridiliums, glycines (Lim and Ngim, 2000),

[1] Malaysian Palm Oil Board,
6 Persiaran Institusi, Bandar Baru Bangi,
43000 Kajang, Selangor, Malaysia.

* *Corresponding author e-mail: mohd.hefni@mpob.gov.my*

and dinitroaniline herbicides (Mudge *et al*., 1984), all of which are important herbicides for controlling *E. indica* in crops. In Malaysia, the first case of glyphosate-resistant annual grassy weed *E. indica* was reported in an orchard at Teluk Intan, Perak, Malaysia in 1998, where glyphosate failed to give adequate control of goosegrass in a four-year-old orchard (Lim and Ngim, 2000).

Thus, an alternative method such as using a biological control formulation, which is environmental-friendly for controlling weed species and reducing environmental pollution compared to using herbicide formulations that can affect soil microorganisms is sought after (Savita, 2019). Nevertheless, an effective microbial formulation plays a significant role in order to work as an alternative to chemical application (Lacey *et al*., 2001). Biological control of weeds is seen as an economical, effective and environmentally sound method of weed control. Many phytopathogenic fungi such as *Fusarium oxysporum* and *Puccinia komarovii* var. *glanduliferae* have been found to effectively control weeds (Currie *et al*., 2020; Dutta and Ray 2017).

Phoma herbarum Westend is an ubiquitous saprobe and toxigenic pathogen to plants and animals (Hamayun *et al*., 2009). Previous study has reported that three species of *Phoma* have been identified as potential biological control agents to various weeds (Harding and Raizada, 2015). Previous studies showed *P. chenopodia* could control weeds such as *Chenopodium album, Cirsium arvense, Setaria viridis*, and *Mercurialis annua* (Cimmino *et al*., 2013) whilst *P. macrostoma* was reported as biological control agent of many wide host range predominantly dicot plants (Bailey *et al*., 2011). Zhou *et al.* (2004) also isolated a number of *P. macrostoma* strains from necrotic lesions on Canada thistle [*Cirsium arvense* (L.) Scop.] plants collected from fields and roadside ditches.

It was reported that disease symptoms known as photobleaching whereby chlorosis and bleaching on the leaf tissues occurred on broadleaved weed species such as dandelion (*Taraxacum officinale* Weber ex F.H. Wigg.) when applied with mycelial fragments of *P. macrostoma* strain (Bailey *et al*., 2011). Todero *et al.* (2018) found out that culture filtrate of *Phoma* sp. showed significant phytotoxic efficiency against three weeds namely *Bidens pilosa, Amaranthus retroflexus* and *Conyza canadensis* when combined with specific adjuvants. Furthermore, Zhao and Shamoun (2005) reported that *Phoma exigua* can control salal (*Gaultheria shallon*), a perennial evergreen shrub.

Whilst, *P. herbarum* was reported as a biological control agent of dandelion weed *Taraxacum officinale* and *Trianthema portulacastrum* (Ray and Vijayachandran, 2013). *P. herbarum* also was reported to possess strong adaptability to diverse environments, including salty and chilly surroundings (Yang *et al.* 2005), and that it is also a versatile producer of many potent natural products (Cruz *et al*., 2003). To date, there is no report on the utilisation or application of *P. herbarum* as a biological control agent of *E. indica*. The potential of *P. herbarum* for biological control of weeds such as on turfgrass (Hahn *et al*., 2020) and other species of *P. dimorpha* on *Echinochloa* sp., *Amaranthus cruentus, Senna obtusifolia* and *Bidens Pilosa* (Neto *et al*., 2021) have made it as a promising candidate to control *E. indica*, consequently could be a key to solve the herbicide resistant problems in the field. Therefore, this study aimed to investigate *P. herbarum* as a potential biological control agent of resistant *E. indica* and its sequential application with herbicides.

MATERIALS AND METHODS

The resistant goosegrass seeds (Rusli *et al*., 2014) were collected from MPOB nursery in Section 15, Bandar Baru Bangi, Selangor, Malaysia and pre-germinated in trays containing a mixture of 3:2:1 (top soil: peat: sand) with a total weight of 3.0 kg. After two weeks, the one seedling of goosegrass was transferred into each of the terracotta plant pots (20 × 8 inches). The soil medium was made up of sandy loam topsoil in a proportion of 2:1 (top soil: sand). Ten g of rock phosphate was added to the soil medium in each pot to enhance and promote healthy plant growth and approximately 1.5 kg of soil medium was added to the pots, which were placed in the nursery with 50% polyethylene shading net. They were watered from below on alternate days. Other weeds were removed manually when necessary. Every month, approximately 5 g of N.P.K Green 15: 15: 15 (Nifosk Green) was applied to each pot.

Preparation of *P. herbarum* (PH81) Inoculum

Single spore isolates of *P. herbarum* (PH81) isolated from diseased *E. indica* from Malaysian Palm Oil Board (MPOB) nursery in Section 15, Bandar Baru Bangi, Selangor were used in the pathogenicity experiments. Isolates were stored at -80°C in 20% glycerol and were then cultured on potato dextrose agar (PDA) and incubated at 28°C for five days. Three plugs of five-day-old fungus were inoculated onto solid media containing 50 g of semi-fine corns (0.1-0.2 cm), 1% starch, 1% sucrose and 30% water solutions (containing 0.01% Tween 20 and 0.1% glycerol). After 14 days of incubation at room temperature, the *P. herbarum* (PH81) spores were subjected to harvesting through filtration and counted using a haemocytometer. The *P. herbarum* (PH81) spore concentrations were adjusted to 10^6 conidia per mL upon spraying.

Standard Inoculation Procedure for *P. herbarum* (PH81)

Ten mL of conidial suspension was applied to the leaves and around the base of each *E. indica*. The inoculated *E. indica* were watered with sterile distilled water (SDW) for two weeks. Un-inoculated *E. indica* served as controls. *Eleusine indica* seedlings were inoculated at one month of age.

Colonisation of *P. herbarum* (PH81) on *E. indica*

Re-isolation of *P. herbarum* (PH81) from the plant leaves and roots of each inoculated and un-inoculated *E. indica* was attempted. For qualitative re-isolation, 3-cm fragments of plant materials (leaves and roots) were surface sterilised in 2% (v/v) sodium hypochlorite for 10 min (5 min for tissue cores) before rinsing twice in SDW. The materials were then plated onto PDA and incubated for four days at 28°C.

Deoxyribonucleic Acid (DNA) Extraction

Phoma herbarum (PH81) DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) method described by Manicom *et al.* (1987). First, 500 µL of isolate suspension (10^6 spores/mL) and 10-15 glass beads were vortexed in 1.5 mL Eppendorf tubes for 45 s to disrupt the cells. Then, 500 µL of CTAB buffer (2% w/v CTAB, 1 M Tris-hydrochloric acid (Tris-HCl) pH 8, 5 M sodium chloride (NaCl) and 0.5 M EDTA pH 8 was added to the suspension and vortexed again before incubating the samples at 65°C for 40 min. An equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added to extract the DNA. The suspension was vortexed thoroughly to mix the layers and centrifuged at 4000 rpm for 10 min. The aqueous supernatant was transferred to a clean Eppendorf tube, and 500 µL of cold isopropanol was added to precipitate nucleic acids overnight at -20°C. The DNA was pelleted at 13 000 rpm for 30 min, washed with 100 µL of cold 70% ethanol by centrifuging twice and taken up in 100 µL of sterile MilliQ water. DNA purity and concentration were determined using the ND-100 Nanodrop Spectrophotometer (Thermo Scientific) according to the manufacturer's protocol.

Polymerase Chain Reaction (PCR) for Target Sequence Amplification

The PCR was performed with a PTC-100™ (MJ Research Cycling) in reaction volumes of 25 µL. Each reaction consisted of >20 ng genomic DNA (or a 2.5 µL culture suspension), 0.2 µM of each primer, 0.4 mM dNTP mix (10 mM, Promega), 0.5× GoTaq buffer (5× GoTaq buffer, Promega), 2.5 mM $MgCl_2$ (25 mM, Promega), 0.02 u/µL GoTaq® DNA polymerase (5 u/µL, Promega), and sterile Milli-Q water. The PCR cycle conditions were as follows: one cycle at 94°C for 5 min, followed by 40 amplification cycles at 94°C for 0.05 s, 57°C for 0.05 s, and 72°C for 0.05 s. A final extension at 72°C for 2 min was done after 40 cycles followed by cooling at 14°C until recovery of the sample. Amplification products were assessed on a 1% w/v agarose gel stained with ethidium bromide run for 20 min at 80 volts and visualised under ultraviolet (UV) illumination.

Nursery Trial

The nursery trial was carried out in Bandar Baru Bangi, Selangor, Malaysia (2° 57' 56.4120" N and 101° 45' 3.2688" E). The experiment comprised of two treatments (treated and untreated) carried out where each treatment consisted of 20 pots with three replicates using a randomised complete block design (RCBD). Each plant was inoculated with 10 mL of 3×10^6 *P. herbarum* (PH81) conidia/mL. The determinations of weed mortality were carried out at 7, 14, 21 and 28 days post-inoculation, and sample re-isolation was performed at the end of the experiment, after 28 days.

Field Trial

A field trial was conducted in Bandar Baru Bangi, Selangor, Malaysia (2° 57' 56.4120" N and 101° 45' 3.2688" E). This experiment made use of the available *E. indica* to simulate the real weed conditions in the field. It was set up in plots (2 m × 5 m) with approximately 20 *E. indica* aged between 1-2 months old (based on the last cycle of herbicide application) in each plot. Two treatments were replicated in five RCBD plots whereby the first treatment consisted of 200 mL of 10^6 *P. herbarum* (PH81) conidial/mL suspensions sprayed in each plot using a hand-operated sprayer (a knapsack hand pump) with a cone (green) nozzle and the second treatment was a control plot (*E. indica* sprayed with water). The determinations of weed mortality were carried out at 7, 14, 21, 28 and 35 days post-inoculation and *P. herbarum* (PH81) sample re-isolation was performed at the end of the experiment after 35 days.

The Percentage of Weed Mortality in Nursery and Field Trials

The weed mortality percentage was taken at one, two, three and four weeks after treatment (WAT) by counting plants with all tissues completely dead from the point of growing to the surface of soil using a non-destructive sampling method.

Eleusine indica Dry Weight

The growth parameters and dry weight were assessed. The weeds planted in the pots were washed twice with tap water before constant dry weight (g) of the whole plant (roots and aerial parts) were determined, following ≥72 hr at 80°C in a drying oven (Rusli *et al.*, 2014).

Sequential Application of Herbicide and Bioherbicide on Goosegrass Mortality

The experiment was conducted in an open nursery area in Bandar Baru Bangi, Selangor, Malaysia (2° 57' 56.4120" N and 101° 45' 3.2688" E). Five commonly used herbicides to control *E. indica* and a stock solution of each herbicide was made based the product's field recommendation (*Table 1*). Each treatment consisted of 20 pots with three replicates using a RCBD. Each plant was inoculated with 10 mL of 3×10^6 *P. herbarum* (PH81) conidia/mL, 12 hr after application of herbicide at full strength and half strength. The determinations of weed mortality were carried out at 7, 14, 21 and 28 days post-inoculation.

Statistical Analysis

The data collected in the nursery and field studies were subjected to Fisher's exact test. Means were separated using a significance level of $p<0.05$. Comparisons between the treatment means were made using Tukey's HSD test.

RESULTS

Efficacy of *P. herbarum* (PH81) on *E. indica* in the Nursery Trial

Based on the nursery trials, the effects of *P. herbarum* (PH81) application were noticeable after 14 days with 30% mortality and continued to increase significantly with mortality rate at 80% after 21 days post application. It was noticeable that formation of *P. herbarum* (PH81) mycelia on the treated *E. indica* leaves. Overtime, it was observed that *P. herbarum* (PH81) incite photobleaching on the infected leaves and gradually became chlorotic before eventually die (*Figure 1*). The same symptoms was observed when *Conyza canadensis* was treated with mycoherbicides derived from *Phoma* sp. (Todero *et al.*, 2018). At the end of the experiment, 90% of the *E. indica* treated with *P. herbarum* (PH81) was dead 28 days after treatment, whereas no dead signs were observed in the control treatment during the same period (*Table 2*). Twenty fungal isolations from samples that showed black spot discolourations and dried and colonised roots of *E. indica* were carried out at the end of the experiment. The samples were subjected to PCR amplifications using universal primers (White *et al.*, 1990) and were sent out for sequence analysis. Based on the Basic Local Alignment Search Tool (BLAST) sequence analysis, it was found that the isolated fungus was *P. herbarum*. This showed that a stable formulation of *P. herbarum* that can sustain the viability of a potential biocontrol agent has been developed and can be delivered to the target weed.

Field Efficacy Test of *P. herbarum* (PH81) on *E. indica*

Seven days after treatment, no mortality was observed in all treated quadrats. However, the mortality of *E. indica* increased significantly 14 days after treatment to 30% (*Table 3*). The mortality pattern was similar to the previous nursery trial. It was also observed that from day 14 to day 21, a sharp trend of *E. indica* mortality was recorded whereby the percentage of mortality jumped from 30%-70%. The mortality rate of *E. indica* continued until 28 days and stabilised at 80% (*Table 3*). This shows that *E. indica* are susceptible to *P. herbarum* (PH81) infections. The treated *E. indica* showed black spot discolourations and dried in all quadrats. These are typical symptoms of *Phoma* infections which range from necrotic spots, chlorotic halos and wilting (Deb *et al.*, 2020). The study also recorded that 10% of the untreated *E. indica* died due to ageing

TABLE 1. SELECTION OF HERBICIDES WITH DIFFERENT ACTIVE INGREDIENTS AND MODE OF ACTIONS USED IN EXPERIMENTAL STUDIES

Herbicide active ingredient	Mode of action	Product rate per ha (L/ha) Full strength	Product rate per ha (L/ha) Half strength
Glyphosate isopropylammonium (41% w/w)	Systemic	3.0	1.50
Glyphosate monoamonium (52% w/w)	Systemic	5.0	2.50
Diuron (42% w/w)	Systemic	0.9	0.45
Gluphosinate – ammonium (13.5 w/w)	Contact	3.3	1.65
Paraquat dichloride (13% w/w)	Contact	6.0	3.00

TABLE 2. PERCENTAGE OF *Eleusine indica* MORTALITY DUE TO *Phoma herbarum* (PH81) INFECTION AT 7, 14, 21 AND 28 DAYS AFTER TREATMENT IN NURSERY TRIAL

Treatment	% of dead *E. indica*			
	7 days	14 days	21 days	28 days
T1 – *E. indica* treated with *P. herbarum* (PH81) (10^6 conidia/mL)	0a	30a	80a	91.7a
T2 – Untreated *E. indica* (control)	0a	0b	0b	0b

Note: Different letters denote a significance ($p<0.05$) between different treatments on *E. indica* analysed by Fisher's exact test. n - 20; replicate - 3; experimental design - RCBD.

TABLE 3. THE EFFICACY OF *Phoma herbarum* (PH81) ON RESISTANT *Eleusine indica* IN A FIELD TRIAL

Treatment	Mortality of *E. indica* (%)				
	7 days	14 days	21 days	28 days	35 days
T1 – *P. herbarum* (PH81) at 10^6 conidia/mL	0	30a	70a	80a	80a
T2 – Untreated *E. indica* (control)	0	0b	0b	0b	10b

Note: Different letters denote a significance ($p<0.05$) between different treatments on *E. indica* analysed by Fisher's exact test. n - 5; replicate - 3; experimental design - RCBD.

Figure 1. The photobleaching and chlorosis effects of P. herbarum *application at seven days interval in field trial.*

factors. Further investigation found that the weed's root system were still active and regenerated after 42 days post treatments in all quadrat. Figliola *et al.* (1988) also reported similar findings when two leaf-spotting pathogens, *Bipolaris setariae* (Saw.) and *Pyricularia grisea* (Cke.) Sacc., were applied to *E. indica*. In comparison, *E. indica* regeneration was recorded 21 days after treatment when conventional herbicide mixtures were used (Rusli *et al.*, 2014).

This experiment exhibited significantly reduced dry weight compared to the control treatment as shown in *Table 4*. Field efficacy treatments of *P. herbarum* as a potential biological control agent on *E. indica* have been repeated on a number of occasions and giving similar results. The effectiveness of *P. herbarum* treatment indicated the fungus is a potential biological control agent whereby it could suppress the weed growth compared to control.

TABLE 4. THE EFFECT OF *Phoma herbarum* (PH81) TREATMENT ON *Eleusine indica* DRY WEIGHT 35 DAYS AFTER APPLICATIONS

Treatment	Dry weight (g)
T1 – *P. herbarum* (PH81) at 10^6 conidia/mL	115a
T2 – Untreated *E. indica* (control)	250b

Note: Different letters denote a significance ($p<0.05$) between different treatments on *E. indica* analysed by Fisher's exact test. n - 5; replicate - 3; experimental design - RCBD.

Sequential Herbicide Application and Bioherbicide (PH81) on Goosegrass

The bio herbicide fungus, *P. herbarum* (PH81) was tested alone and 12 hr after full strength or half strength selected herbicides application. Based on the results obtained, treatment with *P. herbarum* (PH81) and full strength gluphosinate ammonium (T4) recorded the highest *E. indica* mortality as early as seven days post application (*Table 5*). The weed mortality continued to increase at day 14 (93.33%), day 21 (98.33%) and day 28 (100.00%). Treatment of *P. herbarum* (PH81) with half strength gluphosinate ammonium recorded a lower mortality rate at 43.33% during the whole course of the experiment. The treated *E. indica* was then sampled and fungal re-isolation was carried out in order to confirm the presence of *P. herbarum* (PH81). Nevertheless, no *P. herbarum* can be re-isolated from *E. indica* in T4.

It was also recorded that the *E. indica* mortality in T3 also high with treatment *P. herbarum* (PH81) and full strength diuron shows 71.67% mortality after seven days post application before stagnated at 80.00%. However, treatment *P. herbarum* (PH81) and half strength diuron killed 50.00% of treated *E. indica* and the mortality rate increased to 60.00% (day 14) and 91.67% at day 21 dan 28. This was the highest percentage of mortality recorded compared to other treatments. Based on fungal re-isolation and sequence analysis, the presence of *P. herbarum* (PH81) was identified and confirmed. It was recorded that, weed mortality was significantly improved when *P. herbarum* (PH81) was applied with reduced diuron rate.

For T5, no *P. herbarum* could be re-isolated from treatment with paraquat although 80.00% weed killed was achieved with full strength paraquat application. This could probably due to the herbicide toxicity that inhibited or killed *P. herbarum* (PH81). Nevertheless, *P. herbarum* (PH81) could be isolated from T1 and T2 when the herbicides were applied at half strength though the percentage of mortality were low. T1 recorded weed mortality at 11.67% at full strength glyphosate isopropylammonium in combination with *P. herbarum* (PH81) while half strength application recorded weed mortality at 28.33%. T2 also showed low weed control whereby full strength application of glyphosate monoammonium with *P. herbarum* (PH81) only showed 20.00% mortality whilst 10% weed mortality was recorded with half strength application of the herbicide. Treatment with full strength *P. herbarum* (PH81) (T6) showed similar pattern as observed in previous field trial whereby 28.33% *E. indica* mortality due to PH81 infection was recorded. The highest *E. indica* mortality rate was recorded at 21 days post inoculation at 80.00% and maintained when assessment was carried out at 28 days. Nevertheless, half strength application of PH81 was only able to kill 50.00% of the resistant *E. indica*.

TABLE 5. BIOHERBICIDAL ACTIVITY OF *P. herbarum* (PH81) IN COMBINATION WITH FULL STRENGTH AND HALF STRENGTH OF SELECTED HERBICIDES AGAINST *E. indica*

Treatment	Active ingredient	Percentage of *E. indica* killed (%)							
		7 days		14 days		21 days		28 days	
		FS	HS	FS	HS	FS	HS	FS	HS
T1	*P. herbarum* (PH81) + Glyphosate isopropylammonium	11.67bC	25.0aBC	11.67bCD	25.0aC	11.67bCD	28.33aBC	11.67b	28.33aC
T2	*P. herbarum* (PH81) + Glyphosate monoamonium	13.33aC	10.0aC	20.0aC	10.0bD	20.0aC	10.0bD	20.0b	10.0aD
T3	*P. herbarum* (PH81) + Diuron	71.67aB	50.0bA	80.0aAB	60.0bA	80.0bB	91.67aA	80.0b	91.67aA
T4	*P. herbarum* (PH81) + Gluphosinate–ammonium	91.67aA	43.33bA	93.33aA	43.33bB	98.33aA	43.33bB	100.0a	43.33bBC
T5	*P. herbarum* (PH81) + Paraquat dichloride	80.0aAB	35.0bAB	80.0aAB	35.0bBC	80.0aB	35.0bB	80.0a	35.0bC
T6	*P. herbarum* (PH81) (positive control)	0aD	0aD	28.33aC	0bF	80.0aB	35.0bB	80.0a	50.0bB
T7	Control (water only; negative control)	0aD	0aD	0aE	0aF	0aF	0aE	0a	0aE

Note: *FS - Full strength of herbicide rate application; HS - Half strength of herbicide rate application. Different lowercase letters denote a significance (p<0.05) between different treatments strength of each herbicides (between columns) and between treatment (between rows) on *E. indica* analysed by Fisher's exact test. Different uppercase letters denote a significance (p<0.05) between treatment 1 to treatment 6 by Fisher's exact test. n - 20; replicate - 3; experimental design - RCBD.

DISCUSSION AND CONCLUSION

Chemical control represents an effective way to control weeds for many decades. In Malaysia, the agriculture sectors remains reliant on herbicides despite some efforts to integrate with physical, mechanical and biological methods (Dilipkumar *et al*., 2020). The heavy dependence to herbicides has affected the environment, people, animal and furthermore causing weeds to evolve and become resistant; hence higher use rate is needed for control.

In this study, a stable formulation of *P. herbarum* (PH81) that is able to sustain the viability of potential biocontrol agent and can be delivered to the target weed has been developed for both nursery and field applications. According to the nursery trials, *P. herbarum* (PH81) showed an effective potential as a biological agent to control goosegrass (*E. indica*) with 91.7% efficacy while the mortality rate of *E. indica* recorded in the field trials was 80.00% and recorded the lowest dry weight which showed the effectiveness of the treatment. It is interesting to note that wild *E. indica* around the field trial was reported to be resistant to glyphosate (Rusli *et al*., 2014). This suggested that application of *P. herbarum* (PH81) could be a feasible alternative to control glyphosate resistant *E. indica*.

For both the nursery and field trials, the infection began 14 days after *P. herbarum* application. This result agrees with that of Bailey *et al.* (2011), who also recorded *Phoma* sp. colonisation and mycelial growth on the epidermis of barley roots and root hair seven days and 28 days after treatment, respectively. *Phoma* sp. was recently reported to significantly reduced *Verticillium* wilt of olive disease severity caused by *Verticillium dahlia* 12 weeks post application (Ana López-Moral *et al*., 2021).

Hynes (2018) stated that the ubiquitous genus of *Phoma* had been widely reported as the fungus that was responsible for saprophytic, phytopathogenic and recently bio herbicidal activity, therefore it was not surprising that the *P. herbarum* could also control resistant *E. indica*. This is the first report of *P. herbarum* as a potential biological control agent of resistant *E. indica*. Vikrant *et al.* (2006) identified one particular toxin 3-nitro-1,2-benzenedi-carboxylic acid (3-natrophthalic acid) from *P. herbarum* that can be applied as a phytotoxin against target weed *Parthenium hysterophorus*.

Phoma species have also previously been linked as biological control agents for many invasive weeds such as Canada thistle (Guske *et al*., 2004), and dandelion (Neumann and Boland, 2002). This study recorded that photo-bleaching and chlorosis symptoms appeared 14 days after inoculation. Similarly, Hynes (2018) reported that *Phoma* sp. causes photo-bleaching, significantly reduced the overall weed biomass and weed death whilst Johnston (1981) reported root inhibition by *Phoma* sp. in susceptible plants when broadcast onto the soil as granules.

The delivery of the product formulation of *P. herbarum* through soil application further enhanced its ability to infect the target weed. Pedrasand Yu (2008) stated that *Phoma* affected the target weed by secreting macrocidins that was able to deteriorate the weed's cortex. The application of the fungus at or below soil surface was also found effective and thought to overcome environment detrimental caused by the application of foliar bioherbicides (Boyette *et al*., 1991); the persistent and survival of the biological agent was also found to be increased and therefore provide longer term weed control (Boyette *et al*., 1984). Previous study also reported that soil application of *P. macrostoma's* mycelium was more effective in controlling dandelion compared to using its spores (Bailey *et al*., 2011).

Nevertheless, it is important to note that many of the previous bioherbicides restricted in their use (Jones and Hancock, 1990). Host range studies have shown that the use of *Phoma* sp. as bioherbicide affects several plant species in the Asteraceae, Brassicaceae, and Leguminosae (Bailey *et al*., 2011). Hynes (2018) continued to report that no *Phoma* spp. infection was observed on species from the plant families Poaceae, Pinaceae and Lamiaceae, such as *Agrostis palustris* (bentgrass), *Poa pratensis* (Kentucky bluegrass), *Picea mariana* (black spruce), *Pinus* spp. (pine), *Salvia coccinea* (Crimson sage). Bailey *et al.* (2009) stated that it was vital to demonstrate that the potential bioherbicide does not pose risk to non-target species particularly in the surrounding area of the targeted weeds. It is important to note that *P. herbarum* (PH81) was also inoculated on various crops such as oil palm, maize, chilli and okra and the study (unpublished) found that no infection occurred on these crops that are usually used in oil palm integration.

The incompatibility between mycoherbicide and chemical herbicides is still under study, but herbicides are known to interfere with disease development, either because of a direct toxicity to the pathogen or indirectly by triggering defence responses in the plants (Sanogo *et al*., 2000). The negative effect of gluphosinate ammonium, paraquat dichloride, glyphosate isopropylammonium and glyphosate monoammonium towards *P. herbarum* showed that herbicides can inhibit disease progress because of their direct toxicity to the fungus. Therefore, it is not recommended to use these herbicides in combination with the application of *P. herbarum* since the herbicides will have a tendency to kill the fungus. Moreover, in the field, the fungus exists as a weak, opportunistic, or wound pathogen occurring mostly on woody hosts, especially on members of the Rosaceae (Farr *et al*., 1989). However, the combination of *P. herbarum* with diuron could yield encouraging results for the potential of fungus-

herbicide combinations. Nevertheless, a field trial needs to be conducted to verify this assumption about fungus-herbicide combinations.

Lastly, it was also noted that the application of *P. herbarum* was effective when weeds were at the pre-emergence stages, then it became difficult to control over time as the weeds become persistent and well established. Indeed, one of the most important aspects for successful biocontrol is an even distribution of the bioherbicides. Therefore, the application of bioherbicide at the pre-emergence stages is crucial and essential in order to control this noxious weed.

ACKNOWLEDGEMENT

This research was supported by the Malaysian Palm Oil Board (MPOB). We thank many colleagues at MPOB for providing the materials for this study. This work was conducted at the Malaysian Palm Oil Board headquarters in Bandar Baru Bangi, Selangor, Malaysia.

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