

Joint action of malathion with herbicides against *Alhagi pseudalhagi* (Bieb.) Desv.

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Abstract: **Background:** In recent years, the use of simultaneous pesticides in tank mixtures has greatly increased due to cost reduction, improved efficacy and minimizing environmental impact. Additionally, it has been shown that the detoxification of chemicals in plants can be enhanced by the inhibitory capability of some organophosphorus insecticides. **Objective:** This study aims to assess the joint action of binary mixtures of an insecticide and common herbicides using the additive dose model (ADM) as a reference. **Methods:** Four separate greenhouse experiments were conducted on *Alhagi pseudalhagi* (Bieb.) Desv. using seven doses of each of the following herbicides: malathion, 2,4-D, glyphosate, glufosinate-ammonium, and paraquat. Dose-response curves were analyzed with a three-parameter log-logistic model for pure and mixed ratios (100:0, 80:20, 60:40, 50:50, 40:60, 20:80, 0:100) to obtain

ED₅₀ values in R software, and ED₈₀ and ED₉₀ values in Excel®. **Results:** A potent synergism was found for mixtures of malathion with paraquat and/or glufosinate-ammonium on *A. pseudalhagi*, with sums of toxic units for the 50:50% effect mixture ($\Sigma TU_{50:50}$) as low as 0.32 and 0.37, respectively. The mixture of malathion with glyphosate showed moderate synergism with $\Sigma TU_{50:50}$ of 0.75. The binary mixture of malathion with 2,4-D followed ADM (additive effects), though it showed a slight synergism (λ -value > 1). **Conclusion:** This study highlights the importance of understanding synergistic pesticide interactions for more efficient weed management. By optimizing pesticide combinations, we can minimize resistance, reduce environmental impact, and achieve better control. Further research is needed to address limitations of the analysis tools and explore synergistic interactions further.

Keywords: Additive Dose Model (ADM); Binary Mixture; Dose-Response Curves; Malathion; Synergism

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1. Introduction

Alhagi pseudalhagi (Bieb.) Desv., commonly known as camel thorn, is a perennial plant that poses significant challenges in agricultural and rangeland ecosystems due to its invasive nature. This species competes vigorously with native flora, leading to reduced biodiversity and impaired land productivity. Traditional methods of controlling *A. pseudalhagi* include mechanical removal, cultural, biological and the application of herbicides. However, the effectiveness of these herbicides can be limited by the plant's resilience and adaptability (Rashed Mohassel et al., 2002). Among these, employing systemic herbicides during fallow seasons is often considered one of the most cost-effective and efficient approaches due to the risk of its spread during plowing operations (Chitband et al., 2021). While herbicides can be effective in controlling *A. pseudalhagi*, repeated use of the same or similar herbicides can lead to the development of resistance in more species, requiring alternative control methods (Inui, Ohkawa, 2005; Oliveira et al., 2018; Chtourou et al., 2024).

Simultaneous use of pesticides, specifically binary mixtures of herbicides and insecticides, is a common strategy in pest management. This approach is often used in integrated pest management (IPM) programs to minimize pesticide resistance, reduce the amount of pesticides needed, fuel use, labor costs, equipment exhaustion and improve overall pest control (Streibig et al., 1998; Barbieri et al., 2022). The activity of these chemicals can lead to the enhancement or reduction of the effects of one or more compounds due to changes in their physical and chemical properties. Although research on chemical combinations in agriculture began in the early 20th century, the method of chemical mixtures was first used by Loewe et al. (1926) in pharmacology (Streibig, Jensen, 2000).

An isobole (derived from "iso," meaning equal, and "bole," meaning effect) is a statistical analysis method that represents a specified constant level of effect in the system under study. An isobologram, the graphical representation of isobole curves, illustrates the transverse cutting of dose-response curves in mixtures of different ratios (Sørensen et al., 2010). The joint action forms of chemical mixtures are classified by isoboles as synergistic, antagonistic, or additive. Synergism is defined as the combined effect of two or more chemicals is greater than their individual effects, while antagonism refers to the combined effect of two or more chemicals is less than

their individual effects (One chemical reduces or cancels out the effect of the other). Additivity occurs when the combined effect of two or more chemicals is equal to their individual effects (The chemicals act independently, without influencing each other's actions) (Sørensen et al., 2010).

Assessing the joint action of chemical combinations is complex and requires a reference model to determine synergistic, antagonistic, or additive effects. There are three scientific concepts to evaluate the combined effect of chemicals, each summarized as a model. In the Additive Effect Model (AEM), it is assumed that two chemicals do not interact, and their effects are simply additive (Streibig, Jensen, 2000). When an interaction exists between two chemicals, and there is a significant probability in their analysis of variance, the reference model can be grouped as Additive Dose Models (ADM) or Multiplicative Survival Models (MSM) (Streibig, Jensen, 2000). The ADM assumes that the herbicides can replace each other wholly or partly at equivalent biological dose rates, maintaining their relative potency at any response level. Conversely, MSM assumes that the two herbicides act independently of each other (entirely different or dissimilar actions), and their responses are estimated as percentages or proportions of some hypothetical maximum response. Therefore, a fundamental difference between the two models is that ADM considers dose rates, while MSM considers the effects resulting from these dose rates. The criteria for choosing the reference model are based on the existence of interaction at the same site of action. The ADM was used as a reference model to find the departure of mixture ratios from the ADM isoboles (Streibig, Jensen, 2000). Consequently, the relative potency of chemicals, i.e., the sum of their single doses at response levels ED_{50} , ED_{80} , and ED_{90} was created for specific mixture effects.

Cytochrome P450 monooxygenases (CYPs) are a superfamily of enzymes that play a crucial role in the metabolism of xenobiotics, including pesticides such as malathion and herbicides. CYPs are responsible for the oxidation of various substrates, including malathion and herbicides, leading to their detoxification or activation. The interaction between malathion and herbicides may affect the activity of CYPs, leading to altered metabolism of the pesticides. For example, the presence of herbicides may inhibit or induce the activity of CYPs, which can affect the metabolism of malathion and its efficacy as an insecticide (Casida, 2017; Chtourou et al., 2024). Various publications have reported that the activity of CYPs in plants is inhibited when organophosphorus insecticides are applied (Biediger et al., 1992; Kapusta, Krausz, 1992; Baerg et al., 1996; Munkegaard et al., 2008). CYPs are well-known enzymes involved in the degradation and detoxification of pesticides and xenobiotics in higher plants. These enzymes can reduce toxicity before it reaches the site of herbicide action by adding an oxygen atom to the herbicide molecule (Carvalho et al., 2021). Varsano et al. (1992) found that maize plants' susceptibility to triazine herbicides increased

when the activity of P450 monooxygenase was inhibited. Furthermore, Biediger et al. (1992) and Kapusta and Krausz (1992) investigated that other OP insecticides such as malathion, chlorpyrifos, disulfoton, and isozophos also have the potential to cause nicosulfuron or primisulfuron injury to maize when mixed with foliar-applied herbicides. Hence, literature indicates that P450 monooxygenases play a fundamental role in the degradation of pesticides in terrestrial plants.

Numerous studies have reported that interference with P450 activity by organophosphorus insecticides such as malathion may enhance the effectiveness of herbicides for weed control. The use of CytP450 enzyme inhibitors, such as organophosphate insecticides, with malathion being the most commonly used, forms the basis of herbicide metabolism studies. The oxygenation of malathion by the CytP450 enzyme involves releasing a sulfur atom, attaching it to the enzyme complex, and altering its molecular conformation. This process inhibits CytP450 enzyme activity, thereby increasing the sensitivity of weeds to herbicides (Carvalho et al., 2021).

Since 2,4-D, glyphosate, glufosinate-ammonium, and paraquat are widely used for controlling perennial weeds such as *A. pseudalhagi* (Bieb.) Desv. due to their extensive spread in Iran and the potential for resistance development, understanding the joint action of malathion with various herbicides could provide a more effective strategy for managing *A. pseudalhagi* infestations. Hence, the aims of this study were to: i) evaluate the potential increase in sensitivity to organophosphorus insecticides resulting from herbicide exposure, which could inhibit P450 enzymes in weeds and other higher plants, using ADM as the reference model; and ii) examine and compare binary mixtures of these herbicides using two different software approaches to test deviations from the ADM model. The pesticides malathion, 2,4-D, glyphosate, glufosinate-ammonium, and paraquat were selected because they are affected by P450 monooxygenases (Ohkawa et al., 1999; Inui, Ohkawa, 2005; Siminszky, 2006).

2. Materials and methods

2.1 Plant material

Seeds of *Alhagi pseudalhagi* (Bieb.) Desv. were collected from the Astan-e-Ghods fields in Khorasan Razavi, Iran, and sown at a depth of 0.5 cm in 2 L plastic pots, with fifteen seeds per pot. All pots were filled with a mixture of sand, clay loam soil, and peat in equal proportions (1:1:1 v/v) and provided with all necessary nutrients. Prior to sowing, seed dormancy was broken by placing the seeds in boiling water for 3 minutes, followed by washing (Andersen, 1968). Four experiments were conducted in the greenhouse of the Faculty of Agriculture at Lorestan University, Iran (Lat 32° 3' N, Long 48° 21' E; 1117 m altitude), from June to September 2021. The greenhouse was equipped with an intelligent meteorological system to regulate temperature,

relative humidity, carbon dioxide, light, and irrigation, supplementing ambient conditions. This system maintained a photoperiod of approximately 16:8 h (light:dark), with relative humidity levels of 45% during the day and 65% at night, and temperatures ranging from 22–25 °C during the day and 16–18 °C at night. After seedling emergence, the number of seedlings was reduced to four per pot at the cotyledon stage. Each pot received 30 mL of a water-soluble N-P-K (20:20:20) fertilizer solution, prepared by dissolving 3 g of fertilizer per liter of tap water.

2.2 Chemicals and treatments application

Dose–response experiments were conducted using five doses of the commercial formulation of the following pesticides: malathion (Malatox, 570 g a.i. L⁻¹, Mahan, Iran, dose range 53.44–855 g a.i. ha⁻¹), glyphosate (Roundup®, 410 g a.i. L⁻¹, Agrolife, India, dose range 128.13–2,050 g a.i. ha⁻¹), 2,4-D (U46, 720 g a.i. L⁻¹, Atoll, India, dose range 56.25–900 g a.i. ha⁻¹), paraquat (Gramaxon, 200 g a.i. L⁻¹, Partonar, Iran, dose range 18.75–300 g a.i. ha⁻¹), and glufosinate-ammonium (Basta, 200 g a.i. L⁻¹, Ariashimi, Iran, dose range 62.5–1,000 g a.i. ha⁻¹). Each herbicidal treatment was applied either alone or in binary mixtures with fixed ratios. The ratios of malathion to herbicides in the binary mixtures were 100:0, 80:20, 60:40, 50:50, 40:60, 20:80, and 0:100. The initial assumption in the binary mixtures was to obtain a distribution of the overall percentage effect of the insecticide-herbicides in the ratios. Chemical treatments of binary mixtures were sprayed at the four-to-six-leaf stage using an overhead trolley sprayer equipped with a flat-fan nozzle (8002 Tee-jet; Spraying Systems Company, Wheaton, IL, USA), delivering 200 L ha⁻¹ at 300 kPa. Five doses for each insecticide and/or herbicide in mixture ratios with four replications for each dose, plus the ten untreated controls, were chosen for four experiments in a completely randomized design. All aboveground plant parts (both control and treated plants) were approximately cut three weeks after spraying and oven-dried at 75 °C for 48 hours before being weighed.

2.3 Dose–response models

The description of dose-response curves of the alone or in binary fixed-ratio mixture was done with a three-parameter log-logistic model (Streibig, Kudsk, 1993):

$$U = \frac{d}{1 + \exp[b(\log(z) - \log(ED_{50}))]} \quad (1)$$

where U donates the biomass at dose z , d donates the upper limit of the biomass at zero dose, b is proportional to the slope around ED_{50} , z is the herbicide dose and ED_{50} donates the dose reducing biomass by half. Other levels of ED such as ED_{80} and ED_{90} values can be achieved at any point of the fitted dose-response. A Box-Cox transform-both-side approach was performed to estimate the homogeneity of

variance wherever it was needed. Also, graphical analysis of residuals and F-test for lack of fit were applied for the goodness of fit (Ritz, Streibig 2005; Rudemo et al., 1989; Streibig, Kudsk 1993). Data were analysed using R statistical free-software (R Core Team 2021) and the add-on package drc (Ritz, Streibig 2005).

2.4 Joint action model

ADM was used as a reference model of joint action. The following equation can be explained the isobole of the additive, synergy and antagonism under ADM conditions at any response level (Streibig et al., 1998):

$$z_a/Z_a + z_b/Z_b \begin{cases} = 1 & \text{additive} \\ < 1 & \text{synergism} \\ > 1 & \text{antagonism} \end{cases} \quad (2)$$

where z_a and z_b denote the doses of insecticide A and herbicide B in a mixture producing the same biological response as the dose of Z_a and Z_b signifying the ED_x of the insecticide A and herbicide B applied singly. The principle of “biological” exchange rate is the basis for the relative potency, i.e. the insecticide and herbicide effects do not change in the plant when they are applied in mixture. The relative potency (RP), i.e., the horizontal displacement between the insecticide and herbicide Z_a and Z_b was calculated as follows:

$$RP = Z_a/Z_b \quad (3)$$

According to ADM in any ratio for insecticide and herbicide mixture in any pre-defined response level (i.e., ED_{50} , ED_{80} and ED_{90}) given by (Streibig et al., 1998):

$$z_a + rz_b = Z_a = rZ_b \quad (4)$$

$z_a + rz_b$ is the mixture giving ED_x , expressed in the units of Z_a . Eq. (4) describes the relationship between insecticide A and herbicide B in any mixture as a straight line. This line is called an isobole which equals the ADM.

According to ADM, the ED_{50} , ED_{80} and ED_{90} values of the compounds applied singly and the ratio of the compounds in the mixtures can be applied for estimating the ED_{50} dose of the mixtures (ED_{50mix}) or other ED_x (i.e., ED_{80mix} and ED_{90mix}):

$$ED_{50,80 \text{ or } 90mix} = ED_{50,80 \text{ or } 90A} / (\alpha + (1 - \alpha) RP) \quad (5)$$

where $ED_{50,80 \text{ or } 90A}$ are the ED_{50} , ED_{80} and ED_{90} doses of the insecticide/herbicide A, α is the ratio of insecticide/herbicide A in the mixture and RP is the relative potency similar to Eq. (3). Hewlett model (1969) was used as a non-linear model on basis of the curvature of the isobole i.e., the λ value, for evaluation of joint action in R programme:

$$(z_a/Z_a)^{1/\lambda} + (z_b/Z_b)^{1/\lambda} = 1 \quad (6)$$

The interaction parameter is called the λ value. If λ value equals one, the combination effect follows ADM. The combination effects are defined as synergism, if λ value is larger than one, or antagonism, if λ value is smaller than one.

As mentioned earlier, the ADM isobole represents a straight line known as an isobologram. A curved isobole, which best fits the data, was compared to the ADM isobole to assess whether deviation from the reference model significantly improved the data fit (F test, $P > 0.05$) at a 5% significance level. The sum of toxic units (ΣTU) was computed to determine the relative amount of chemical in a mixture. ΣTU values for the 50:50% effect ratio ($\Sigma TU_{50:50}$) can gauge the extent of synergism or antagonism within the ADM model, as this ratio reflects the largest deviation from the ADM for symmetric isoboles. The $\Sigma TU_{50:50}$ for the Hewlett isobole model is estimated as $2^{1-\lambda}$ (Cedergreen et al., 2007; Sorensen et al., 2010).

In Excel, ADM isobolograms were generated by plotting the ED_{50} , ED_{80} , and ED_{90} values of each mixture ratio from single applications on the x and y axes and connecting these values with a straight line. Predicted response ADM isoboles were also represented as straight lines (assuming additivity of doses). The ED_{50} , ED_{80} and ED_{90} values of herbicides used individually were standardized to 1 unit on the x- and y-axes, allowing comparison with the presented doses of treatments on the graph relative to the ADM isoboles. Significant deviations from the straight line isobole (ADM) were classified as synergism if lower and antagonism if higher than the corresponding calculated ED_{50} , ED_{80} and ED_{90} values.

3. Results

3.1 Dose-response assays

A summary of the log logistic slope parameter, upper limit, ED_{50} , lambda value, and the sum of toxic units (ΣTU) of the 50:50% effect ratio for biomass from the four experiments, along with the test for lack of fit, are provided in Table 1. All experiments data (Expt I to Expt IV) were evaluated for their fit to the data at various ratios by the three-parameter and four-parameter logistic logarithmic models. The F-test was used to compare these two models, and it was found that the simpler three-parameter model was a better fit as the test result was not significant at the 5% level. Furthermore, the tests for lack of fit were not significant in all experiments ($P > 0.05$), as indicated in Table 1. This suggests that the logistic model accurately described the data, and it performed as well as an ordinary ANOVA. Therefore, nonlinear regression analysis of data is a more suitable approach than their variance analysis, given the preference for the simpler three-parameter logistic model and the good fit of the logistic model to the data. The lowest relative slopes were

observed in the glufosinate-ammonium and malathion mixture (Expt III), indicating that the ED_{50} and their associated standard deviations were higher than in the other mixtures. Similarly, the lower relative slope values presented in mixture 2,4-D and paraquat combined with malathion (Expt I & IV) suggested that the ED_{50} and their associated standard deviations were higher than in the other mixture (Expt II). Large variations in relative slope values were not observed in the other experiments (Table 1). The substantial variation in isobologram slopes, which describe the relative slope of the response curves, was affected by the response level. Differences in light intensity between spring and summer seasons may have contributed to variations in response levels, particularly in mean biomass, especially in the controls. Consequently, the efficacy of the herbicides could be influenced by variations in plant growth conditions, as illustrated by the RP of the herbicides (Table 1).

3.2 Joint action assays

The parameters calculated from the isobole analyses, such as the ED_{50} , λ -estimates, and the sum of toxic units (ΣTU) of the 50:50% effect ratio, along with related statistics, are listed in Table 1. Isobolograms of the four combinations (Experiments) are illustrated in Figures 1 and 2.

3.3 2,4-D:Malathion

The λ values obtained by R software indicated a more-than-additive effect with a low degree ($\lambda = 1.13$) for the 2,4-D:malathion mixture (Exp I), while based on the shape of the isobole, none of these five mixture ratios, except one, were significantly different from ADM ($P > 0.05$). Hence, it can be inferred that this isobole did not show synergistic interaction when using ADM as a reference model. The calculated isobole closely resembles the ADM isobole due to the small λ estimates in this isobole (Table 1; Figure 1). The degree of deviation from ADM, measured as the sum of toxic units ($\Sigma TU_{50:50}$), equaled 91%, meaning 91% of the chemicals needed to reduce the response to 50% compared to that expected from ADM (Table 1). In Excel, five different mixture ratios were examined as binary mixtures of the ED_{50} , ED_{80} , and ED_{90} values. Turning to the isobolograms, the ED_{50} , ED_{80} and ED_{90} values of the mixture ratios were well distributed along the ADM isobole. For the 2,4-D:malathion mixture (Exp I), all five mixture ratios of ED_{50} were located below the isobole, but only one showed a significant deviation from the assumption of additivity of doses, indicating a synergistic effect. Out of the ten mixture ratios, two produced ED_{80} and ED_{90} doses significantly lower than predicted according to ADM, indicating synergism. The remaining mixture ratios followed ADM.

3.4 Glyphosate:Malathion

The higher λ value presented in R software for the glyphosate:malathion mixture (Expt II) signifies a synergistic action with a higher degree ($\lambda = 1.42$). Turning to the shape of the isobole, only one of the five mixture ratios (20:80) did not differ significantly from the ADM, while the other four mixture ratios were lower than the isobole ADM. Therefore, it can be deduced that this isobole was synergistic. However, synergism was not as strong and

obvious for mixture ratios with higher values of glyphosate. The $\Sigma TU_{50:50}$ value for the glyphosate:malathion mixture was 0.75, describing a reduction of the response to 50% compared with that expected from ADM, equaling 75% (Table 1). In Excel, a smooth distribution along the isobole was observed for the mixture ratios of glyphosate:malathion (Figure 1). With the exception of one observation, all other four mixture ratios were located along the lower part of the isobole at ED_{50} doses. In other words, the ED_{50} response level of this mixture was less than expected according to ADM,

Table 1 - Summary estimates of statistical parameters for experiment (the four binary herbicide combinations tested; malathion with 2,4-D, glyphosate, glufosinate-ammonium and paraquat) on *Alhagi pseudalhagi*. Hewlett isobole model for the λ -values is given with standard errors three weeks after treatment. The sum of toxic units (ΣTU) of the 50:50 effect ratio is given under ADM model as a predefined response of the mixtures.

Experiment Mixtures (Expt I)	Ratios	Slope (b)	Upper limit (d) ^a	ED_{50}	λ	$\Sigma TU_{50:50}$	Lack of fit test (5%)	Relative potency (RP) ^d
2,4-D:Malathion	0:100	1.57 (0.30)**	2.83 (0.61)**	360.26 (48.99)*	1.13 (0.19)	0.91	0.12(NS) ^c	0.62
	80:20	1.38 (0.35)**	2.86 (0.97)**	360.57 (57.71)**				
	60:40	3.00 (0.81)*	2.43 (0.48)**	399.74 (48.71)*				
	50:50	2.26 (0.51)**	2.38 (0.94)*	410.71 (59.64)**				
	40:60	1.52 (0.48)**	2.54 (0.93)**	409.46 (78.61)*				
	20:80	1.24 (0.37)**	2.49 (0.45)**	332.50 (58.37)**				
	0:100	0.91 (0.42)*	2.75 (0.91)*	545.01 (106.39)**				
(Expt II) Glyphosate:Malathion	0:100	1.03 (0.12)**	2.66 (0.05)**	506.14 (78.13)**	1.42 (0.19)	0.75	0.74(NS)	0.38
	80:20	1.78 (0.40)**	2.36 (0.23)*	420.09 (49.89)*				
	60:40	1.76 (0.33)**	2.70 (0.21)**	595.70 (61.69)**				
	50:50	2.28 (0.43)*	2.42 (0.13)*	646.51 (64.01)*				
	40:60	1.26 (0.32)**	2.55 (0.41)**	444.99 (61.70)**				
	20:80	2.03 (0.49)*	2.04 (0.11)**	874.33 (145.01)**				
	0:100	2.08 (0.34)**	2.61 (0.38)*	1325.85 (242.81)*				
(Expt III) Glufosinate-ammonium:Malathion	0:100	1.20 (0.19)**	2.77 (0.07)**	1143.34 (132.07)*	2.43 (0.26)	0.37	0.15(NS)	0.84
	80:20	0.65 (0.17)*	2.89 (0.44)**	526.71 (68.01)**				
	60:40	2.10 (0.51)**	2.43 (0.16)*	455.80 (43.79)**				
	50:50	0.83 (0.21)**	2.85 (0.23)**	485.89 (46.62)**				
	40:60	0.58 (0.14)*	2.78 (0.45)*	571.42 (69.87)**				
	20:80	1.03 (0.52)**	2.79 (0.31)**	1047.79 (147.87)*				
	0:100	0.91 (0.26)**	2.74 (0.49)*	1363.78 (270.55)**				
(Expt IV) Paraquat:Malathion	0:100	3.99 (0.77)*	2.69 (0.05)**	138.15 (0.74)**	2.66 (0.31)	0.32	0.69(NS)	0.10
	80:20	2.10 (0.47)**	2.40 (0.09)*	92.01 (12.57)**				
	60:40	2.54 (0.59)**	2.78 (0.03)**	122.68 (22.99)**				
	50:50	2.03 (0.45)**	2.45 (0.03)*	114.93 (19.70)*				
	40:60	3.46 (0.69)*	2.38 (0.02)**	125.97 (25.27)**				
	20:80	1.54 (0.29)**	2.29 (0.04)*	153.24 (32.45)**				
	0:100	3.08 (0.64)*	2.63 (0.05)**	1334.31 (162.42)*				

In experiment, the test for lack of fit were not significant, indicating that the logistic model was able to describe the data better than an ordinary ANOVA; ^a The upper limit when the dose is zero. ^b Standard errors are in parentheses. ^c NS, * and **: Non Significant at the 5% level, Significant at 5% and 1% levels of probability, respectively. ^d The relative potency is for the two herbicides applied alone.

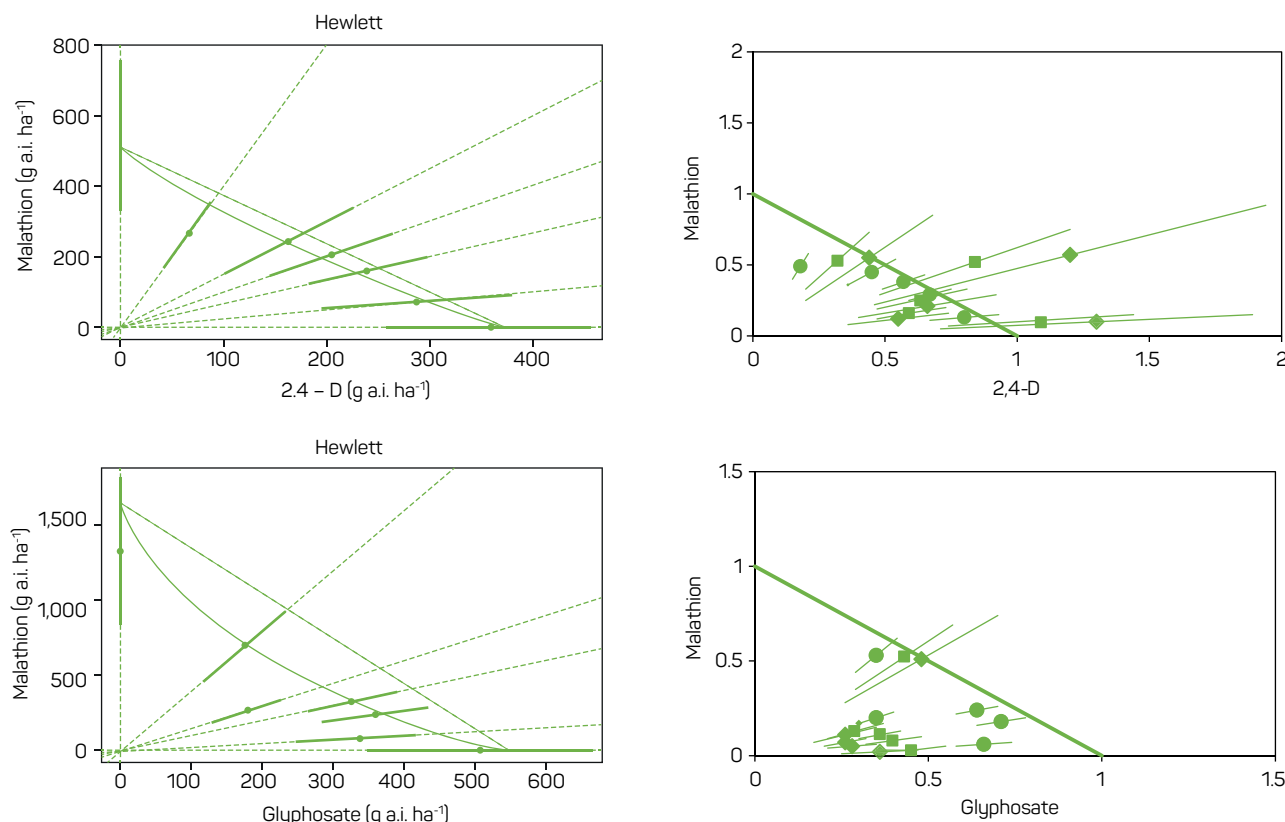


Figure 1 - Isobolograms of mixtures of malathion as an insecticide with two herbicides such as 2,4-D and glyphosate by two approaches for experiment; by R software (Left). The points represent the ED₅₀ and their corresponding standard error at 95% confidence intervals. The straight dot-dashed line is the ADM isobole; the curved solid line (when displayed) is the best fitting isobole model and the estimated ADM line based on all mixtures when there is a significant interaction. By Excel (Right); at the ED₅₀ (●), ED₈₀ (■) and ED₉₀ (◆) response levels. Bars indicate 95% confidence intervals for the estimated ED₅₀, ED₈₀ and ED₉₀ doses. The doses have been scaled so that the doses of the herbicides applied separately are 1.0.

indicating a synergistic response. At the 80% and 90% response levels, only two of eight mixture ratios followed ADM, while the others exhibited synergistic behavior.

3.5 Glufosinate-ammonium:Malathion

The λ -estimate in this isobole was significantly higher than in the previous two isoboles, averaging 2.43 ± 0.26 for the third experiment (Expt III; glufosinate-ammonium:malathion). At their ED₅₀ doses, all mixture ratios showed significantly lower values than the ADM isobole, with synergism more pronounced at higher glufosinate-ammonium concentrations. The $\Sigma TU_{50:50}$ was the lowest for this mixture (0.37), indicating that only 37% of the chemicals are needed for a 50% effect compared to the ADM situation (Table 1). In Excel, unlike the previous two experiments and based on the illustrated results of the isobologram, observations at the 50%, 80%, and 90% response levels were all located inside the isobole, suggesting completely synergistic interactions (Figure 1).

3.6 Paraquat:Malathion

The highest λ value obtained in R software averaged 2.66 ± 0.31 for the paraquat:malathion mixture (Expt IV). All ED₅₀ doses at five mixture ratios were significantly lower than the ADM isobole. Unlike the previous isobole (glufosinate-ammonium:malathion), an asymmetrical isobole based on the Hewlett model was achieved in this experiment due to the accumulation of all mixture ratios on one side of the isobole (paraquat part). The lowest $\Sigma TU_{50:50}$ value was 0.32 for the paraquat: malathion mixture (Expt IV), implying that 32% of the chemicals are needed for a 50% effect compared to that expected from ADM (Table 1). In Excel, the paraquat:malathion mixture at ED₅₀ doses was clearly less active than predicted by ADM, indicating strong synergism between the herbicide and insecticide. Results differed at the ED₈₀ and ED₉₀ response levels. At the 80% effect level, one observation followed ADM, and the other four observations were significantly lower than predicted by ADM, indicating synergism. In contrast, at the 90% response

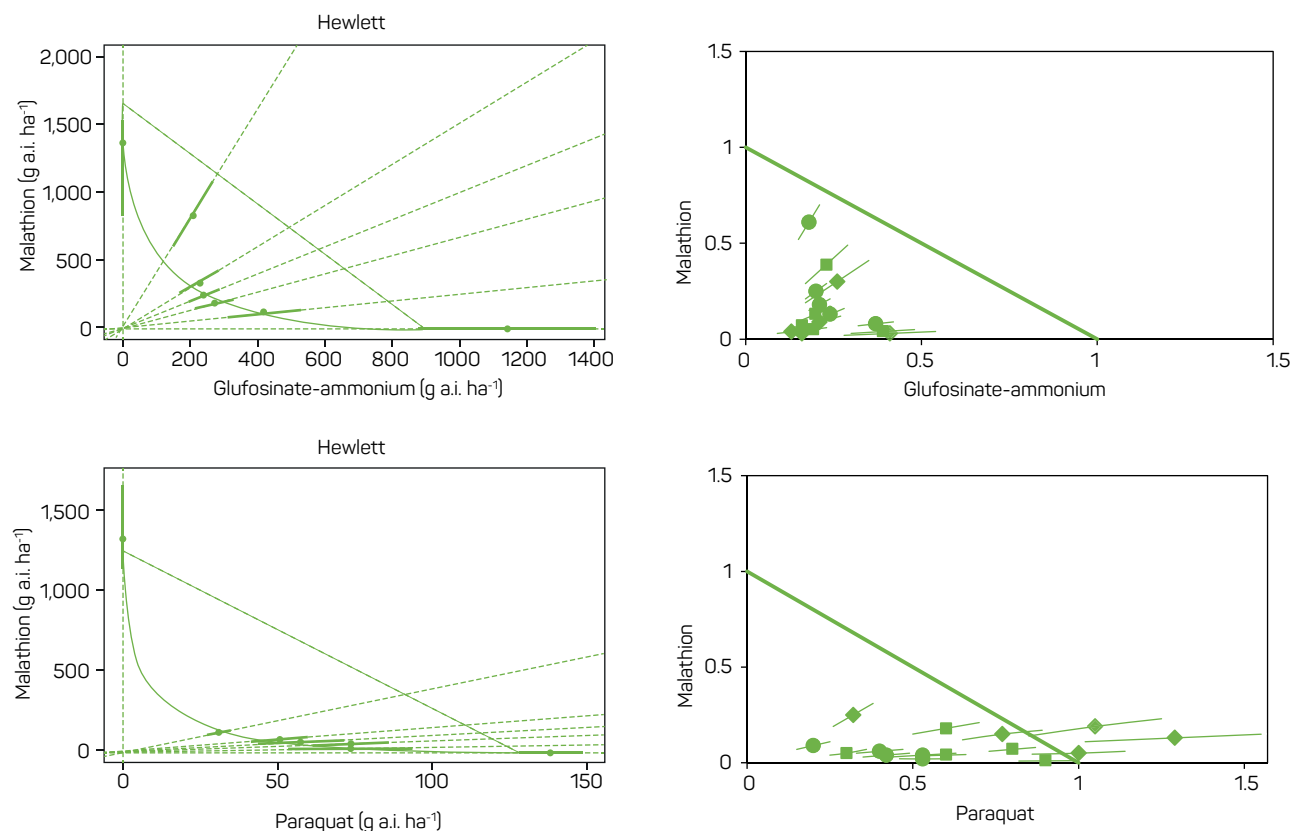


Figure 2 - Isobolograms of mixtures of malathion as an insecticide with two herbicides such as glufosinate-ammonium and paraquat by two approaches for experiment; by R software (Left). The points represent the ED₅₀ and their corresponding standard error at 95% confidence intervals. The straight dot-dashed line is the ADM isobole; the curved solid line (when displayed) is the best fitting isobole model and the estimated ADM line based on all mixtures when there is a significant interaction. By Excel (Right); at the ED₅₀ (●), ED₈₀ (■) and ED₉₀ (◆) response levels. Bars indicate 95% confidence intervals for the estimated ED₅₀, ED₈₀ and ED₉₀ doses. The doses have been scaled so that the doses of the herbicides applied separately are 1.0

level, one was antagonistic, one performed synergistically, and three remaining mixture ratios followed ADM.

4. Discussion

The results of Experiment I, involving the mixture of 2,4-D and malathion, indicated synergism with a low degree of effect on the λ values. However, there were no significant differences observed compared to the ADM isobole at ED₅₀, ED₈₀, and ED₉₀ response levels (see Figure 1). Cedergreen and Streibig (2005), as well as Cedergreen et al. (2007), reported additive effects when plant growth regulators were combined with various herbicide groups. Chitband et al. (2019) demonstrated an additive model effect when clopyralid (an auxin group herbicide) was mixed with desmedipham+phenmedipham+ethofumesate on *Portulaca oleracea* L. and *Chenopodium album* L. In a similar study, an additive effect was observed on *Acroptilon repens* (L.) DC. control when clopyralid was combined with glyphosate (Abbaspoor et al., 2013). Wehtje and Walker (1997) noted a range of responses from additive to synergistic when glyphosate was mixed with 2,4-DB on pitted, palmleaf, and ivyleaf morning glory. Additionally, Sarabi et al.

(2018) found additive effects for redroot pigweed and common lambsquarters when 2,4-D+MCPA was mixed with nicosulfuron and foramsulfuron, respectively.

The higher λ values in Experiments II and III (glyphosate and glufosinate-ammonium with malathion) demonstrate a greater and complete degree of synergistic effects. A possible explanation for these interactions could be directly linked to the enhanced efficiency of amino acid herbicide inhibitors by suppressing cytochrome P450 monooxygenase enzymes, particularly for malathion. Teymourinia et al. (2023) observed the opposite effect when imazethapyr was combined with glyphosate as a binary mixture. They suggested that this reduction in efficacy could be attributed to increased expression of P450 enzymes, thus enhancing the metabolism of EPSPS inhibitor herbicides. Several studies have indicated that organophosphorous insecticides such as malathion contribute to detoxifying xenobiotics and degrading pesticides in higher plants by inhibiting plant CYPs. Hatzios and Penner (1985) noted positive effects of organophosphorus and organochlorine insecticides when mixed with herbicides. Khodayari et al. (1986) studied the synergistic effect of propanil herbicide in rice with various methyl carbamate or organophosphate insecticides.

Numerous publications have demonstrated synergistic effects of mixing organophosphorus insecticides like endosulfan and chlorpyrifos with triazine family herbicides on various algae and invertebrates. Likely mechanisms for the synergistic toxicity between atrazine and organophosphorus insecticides include the formation of more toxic metabolites through increased uptake and biotransformation by cytochrome P450-dependent monooxygenases. This results in the replacement of sulfur with oxygen in the chemical structure of the OP (desulfoxidation), producing acetylcholinesterase as a much stronger inhibitor metabolite. Therefore, there is evidence suggesting that P450 monooxygenases play a crucial role in the biotransformation and inhibition of herbicide metabolism in higher plants. This finding aligns with Carvalho et al.'s (2021) report that organophosphorous insecticides such as malathion synergistically enhance the effectiveness of certain herbicides by inhibiting the metabolization process, enabling herbicides to reach the enzymatic binding site.

Another potential mechanism for interactions in Experiments II and III may be attributed to the presence of an adjuvant in the active ingredients of the herbicide formulation, significantly enhancing the uptake and translocation of the herbicide in the combination. A comparison of the combined responses of commercial glyphosate or glufosinate-ammonium formulations suggests that the binary mixture of these two herbicides with malathion was influenced by their formulation constituents. This finding aligns with the higher λ values obtained in Experiments II and III, indicating a greater degree of synergistic effects. Pankey et al. (2004) demonstrated that the observed synergistic response in the glyphosate and dicotophos mixture could be attributed to the surfactants contained within the glyphosate formulation. Pline et al. (1999) showed that the absorption of ^{14}C -glufosinate with 5% (w/v) ammonium sulfate (AMS) significantly increased the control of *Setaria viridis* (L.) Beauv. and *Senna obtusifolia* (L.) compared to glufosinate alone. Similar results were reported by Maschhoff et al. (2000), where the application of glufosinate with 20 g L⁻¹ ammonium sulfate (AMS) significantly increased the control of *Echinochloa crus-galli*, *Setaria faberi*, and *Abutilon theophrasti*. The authors attributed this increase in efficacy to improved herbicide movement through the leaf cuticle due to the ammonium ion present in AMS, leading to increased foliar absorption and subsequent translocation of glufosinate (Wanamarta et al., 1989; Penner 2000). On the other hand, the addition of an adjuvant in the binary mixture formulation leads to a reduction in the surface tension of the herbicide spray solution, thereby not only increasing the contact area and spread of the droplets on the *A. pseudalhagi* leaf surface but also preventing the spray droplets from bouncing off after impact, increasing the chance for a spray droplet to adhere to the plant surface and consequently enhancing the wetting of the leaf surface cuticle. This process may include greater penetration of the active ingredient, increased herbicide absorption and translocation, and ultimately,

enhanced herbicide efficacy in mixtures. Pratt et al. (2003) found that both glufosinate and glyphosate absorption by velvetleaf (*Abutilon theophrasti* Medic.) was enhanced by the addition of 2% w/v AMS. Moreover, rapid plant death occurs when the overproduction of reactive oxygen species (ROS) happens due to the stress generated under glufosinate application and ammonia accumulation, resulting in chlorosis and necrosis of leaves, suppression of growth, cell lipid peroxidation (CLPO), and membrane damage (Caverzan et al., 2019; Takano, Dayan 2020). Hence, it appears that the performance of glufosinate and glyphosate can be improved either by inhibiting P450 monooxygenases with malathion due to similarities in their molecular structures or by the presence of an adjuvant in their formulation.

Significant synergism was observed when malathion was combined with paraquat (Experiment IV), as evidenced by the highest and lowest λ and $\Sigma\text{TU}_{50:50}$ values, respectively, in Table 1. Previous studies have reported synergistic effects of photosynthesis inhibitors with other mode of action groups against both grass and broadleaf species. For example, Campbell and Penner (1982) mentioned that the mixture of bentazone with organophosphate insecticides such as malathion, parathion, and diazinon enhanced crop injury in soybean (*Glycine max* Merr.) and navy bean (*Phaseolus vulgaris* L.) by inhibiting the hydroxylation of bentazone. Baerg et al. (1996) found that foliar applications of propanil and monuron with OP insecticides resulted in reduced metabolism of propanil and monuron, leading to increased injury in rice and cotton, respectively. Pankey et al. (2004) observed increased injury in cotton seedlings with pre-emergence application of monuron or diuron after a soil application of disulfoton. Similarly, Ma et al. (2013) and Oliveira et al. (2018) documented a clear synergistic response between mesotrione and tembotrione with malathion in waterhemp (*Amaranthus tuberculatus*) seedlings through the inhibition of cytochrome P450 activity.

A probable mechanism underlying this synergism could be the inhibition of detoxification of the herbicide or phytotoxic compounds generated by herbicide usage, such as ROS. Paraquat is known as a fast-acting herbicide due to the accumulation of ROS in cell membranes (Rogachev et al., 1998). Additionally, glufosinate is identified as a fast-acting herbicide, and its contact activity and rapid phytotoxicity are often attributed to disrupting both photorespiration and the light reactions of photosynthesis, leading to photoreduction of molecular oxygen, the accumulation of ROS, and subsequent lipid peroxidation and membrane damage (Takano, Dayan, 2020). Conversely, glyphosate does not directly affect PSII but eventually results in a lack of ROS quencher (Søbye et al., 2011). Therefore, the activity of these herbicides, when combined with malathion, is enhanced due to their protective role in quenching ROS.

Factors other than the sites of action, such as formulation and physico-chemical properties, can lead to mixtures following ADM, rather than indicating likely synergism. Formulated herbicides contain the active ingredient mixed

with the main components of the formulation, which enhances adhesion to sprayed drops on plant leaves, improves spray solution characteristics, enhances retention, modifies spray deposits, or improves penetration and translocation. Therefore, it appears that both the active ingredient and the type of chemical formulation can significantly affect the absorption of other formulation active ingredients in insecticide/herbicide mixtures and increase the activity of the mixture. For instance, Streibig et al. (1998) found that the combination of mecoprop-P potassium salt with MCPA dimethylamine salt followed ADM, whereas synergistic effects occurred when the same herbicide formulation as esters was used. In other words, the type of formulation of these herbicides determines their interaction effects. Thus, the reduction in *A. pseudalhagi* Fisch. phytotoxicity under mixtures of 2,4-D+malathion (compared to the other three mixtures) may result from physical or chemical incompatibility between this mixture in the spray tank.

On the other hand, it is widely acknowledged that solvent-based herbicide formulations activate more effectively than particulate formulations, especially with foliar application herbicides (Green et al., 1995). Paraquat, glufosinate-ammonium, and glyphosate are categorized as hydrophilic herbicides due to their low Logarithm K Oil-Water partition coefficients ($\log(K_{ow})$), with values of -4.50, -4.01, and -3.40, respectively. These herbicides exhibit slow movement and absorption into cuticular waxes because of the lipophilic (fat-loving) properties of the cuticle. This hydrophilic herbicide issue can be addressed by adding surfactants to the tank spray or using herbicides formulated with adjuvants that dissolve surface waxes and prolong the drying time of spray droplets. For instance, Tan and Crabtree (1992) reported a 2.5-fold improvement in 2,4-D absorption by removing epicuticular wax from apple leaves.

Similar to the result in the current study, previous studies demonstrated that the use of some surfactants enhanced the solubility of the herbicide in the wax and induced direct stomatal penetration of the spray solution (Buick et al., 1993; Schreiber, 1995). Hence, the presence of an adjuvant with glufosinate and glyphosate is able to allow more intimate contact between herbicide and plant surface by reducing the surface tension of spray solution. Besides, the absorption and infiltration of the cationic herbicides such as paraquat and glyphosate can be increased because of the ion to ion interaction with the negatively charged cuticle, while 2,4-D absorption and penetration did not show such interaction on cuticle. Cedergreen et al. (2007) found that compounds such as 2,4-D with a low K_{ow} and negatively charged ions (anionic form) at environmental pHs enter plants slowly and therefore have a lower uptake through the lipophilic cell membrane.

Isobolograms were used with both R and Excel softwares to predict the effects of binary combinations. The Isobole model, which can be implemented using the 'drc' package in R, is a great choice if you're comfortable with R and need visual representations of interactions, specifically

isobolograms. However, it requires a specific data format and currently only supports CompuSyn's (CA or ADM) analysis. On the other hand, Excel offers a more user-friendly interface and is more flexible with input data. It supports both CompuSyn's (CA or ADM) and Chou's (IA or MSM) analysis. While it doesn't provide fitted dose-response parameters or a visually appealing presentation of data, it's a solid option if you prefer working in Excel. Mathematically, the two approaches describe deviations from reference models differently, but in practice, the results in terms of the type and degree of deviation are similar, as demonstrated in Cedergreen et al. (2007). Ultimately, the choice between the two models depends on your experimental design and the goals of your study. If you're more comfortable with R and need visualizations, the Isobole model might be the way to go. If you prefer Excel and need more flexibility with data input, it could be a better fit.

5. Conclusion

Two different approaches were employed to predict the joint action of insecticide with certain herbicides (R *versus* Excel). The 'drc' package in R and Excel software offer open-source solutions, ease of use, and greater flexibility in terms of input data. However, selecting between these analyses depends significantly on the experimental design and research objectives. The ADM model served as a reference model for its ease of use and implementation, or for providing more conservative predictions across both software platforms. Furthermore, the results of total binary mixtures from both methods were largely similar. The results from this research suggest that organophosphorus insecticides, such as malathion, exhibited varying degrees of synergistic effects on PSI and amino acid biosynthesis-inhibiting herbicides through the induction of cytochrome P450 monooxygenase inhibition activity. Therefore, the combination of other organophosphorus insecticides with other herbicides from different groups is suggested to be evaluated for the potential of synergistic interactions. Overall, including more information about the role of CYPs, can help to improve the understanding of the efficacy of binary mixtures of these pesticides and their potential for controlling invasive plant species such as *A. pseudalhagi*. Further research is needed in order to identify the compatibility of insecticides with herbicides and to ensure safety and efficacy for weed management practices, as some combinations might not be safe or could lead to unexpected outcomes. Additionally, the presence of an adjuvant or an increase in the rate of one component appeared necessary due to observed additive interactions in the malathion mixture with 2,4-D, facilitating absorption and translocation for effective *A. pseudalhagi* control.

6. Author's contributions

All authors have read and agreed to the published version of the manuscript. AAC, and MR: conceptualization. AAC, MR,

and GAA: investigation. AAC, and MR: collection and analysis of the data. AAC, MR, and GAA: validation. AAC, MR, and GAA: writing– original draft. AAC: writing–review and editing. AAC, MR, and GAA: resources, supervision and funding acquisition.

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