

Survey of salicylic acid effect on Clopyralid efficiency in control of Field bindweed (*Convolvulus arvensis* L.)

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Abstract

This study investigated the physiological characteristics and fluorescence reflection of Field bindweed after exposure to various doses of Clopyralid alone or in combination with salicylic acid. High doses of Clopyralid increased the concentrations of malondialdehyde and hydrogen peroxide, while decreased the activities of antioxidant enzymes. High concentrations of this herbicide also decreased the amount of protein and photosynthetic pigments but increased the proline content compared to the control treatments. Applying SA three days before Clopyralid spraying intensified oxidative stress and lipid peroxidation due to the disruption of the plant's hormonal balance as a result of the use of high dose of SA. PSII performance index was reduced under Clopyralid + SA. Field bindweed photosynthetic apparatus was further altered after SA application three days before Clopyralid spraying. This study highlighted that, for a better use of Clopyralid, the use of SA could be beneficial, not only for the herbicide efficacy against field bindweed weed, but also for the environment due to lower applied doses of herbicide.

1. Introduction

As a perennial noxious weed with a deep root system (6–9 m long), lateral roots (0.35–1 m long), slender stems (1–3 m long), numerous buds, and rhizomes, field bindweed is difficult to control after establishment (Torrey, 1958; Coombs et al., 2004; Bayat and Zargar, 2020). As a result of competition and interference with harvesting processes, this weed reduces the yield and value of crops (Bayat and Zargar, 2020). Additionally, field bindweed may host pests that cause damage to neighboring crops or serve as an alternative host plant pathogen (Vasilakoglou et al., 2013). Field bindweed poses a severe challenge to winter and summer crops (Hassannejad, 2019). For example, it can reduce winter wheat yields by 33% and summer crop yields by 75% (Ratcliff and Peeper, 1987).

Herbicides are one of the most effective and easy-to-use weed control methods in agriculture (Hassannejad et al., 2023). But chemical control of field bindweed is difficult due to its regenerative ability and extensive root system (Bayat and Zargar, 2020). It is necessary to apply multiple herbicides to control this weed on farms (Grey and Bridges, 2003), for example, picloram, 2,4-D, dicamba (Westra et al., 1992), and imazapyr (Lindenmayer et al., 2013). Clopyralid is a carboxy-methyl derivative that mimics auxin with various modes of action (Cobb and Reade, 2010). Compared to other auxin-mimicking herbicides such as picloram and 2,4-D, this herbicide is more selective (Tu et al., 2001). Clopyralid mimics a plant growth hormone and causes uncontrolled and disordered plant growth, leading to plant death. Similar to other auxin-type herbicides, it has a negligible effect on monocotyledonous plants, some members of the Brassicaceae family, and some other broadleaf plants (Tu et al., 2001). However, Clopyralid controls many broadleaf weeds of the Asteraceae, Fabaceae, Solanaceae, Polygonaceae, and Violaceae families (Tu et al., 2001). It is unclear how Clopyralid affects field bindweed (*Convolvulus arvensis* L.) from the Convolvulaceae family.

Herbicide effects on plants are influenced by several parameters, such as the herbicide's dosage, usage, mode of action, and application timing (Fayez, 2000). Plants can eliminate excessive ROS and keep their cells stable by balancing the activities of enzymatic and non-enzymatic antioxidant systems (Tibor et al., 2003). As a result of herbicide stress, the plant's corrective system for producing and removing reactive oxygen species (ROS) was disrupted. For example, chlorimuron-ethyl changes superoxide dismutase (SOD) and peroxidase (POD) activities as well as malondialdehyde (MDA) levels in wheat (Wang and Zhou, 2006). Numerous investigations have demonstrated that the ROS elimination system is associated with weed tolerance to herbicides. For instance, Zhao et al. (2019) suggested that the free radicals' clearance system of *Alopecurus aequalis* plants was significantly impaired under mesosulfuron treatment in susceptible types compared to resistance types. ROS production is detrimental to polyunsaturated fatty acids, proteins, carbohydrates, nucleic acids, chlorophylls, and carotenoids in plant cells (Philosoph-Hadas et al., 1994). The assessment of chlorophyll *a* fluorescence is a non-destructive, non-invasive, easy, rapid, and highly sensitive method for investigating photosynthetic apparatuses in plants under herbicide stress (Yuan et al., 2013; Porheidar-Ghafarbi et al., 2017; Hassannejad and Porheidar-Ghafarbi, 2018). Herbicide application may alter the photosynthesis apparatus performance in plants by blocking electron transport, decreasing photosynthetically active light, and increasing chlorophyll *a* fluorescence (Hassannejad et al. 2020a, c; Rastogi et al., 2019). According to Feng et al. (2021), acetochlor damages PSII and reduces photosynthesis.

Enhancing herbicide efficacy, particularly at lower dosages, could be considered a strategy for reducing herbicide application rates. By improving the efficiency of herbicides, adjuvants help to optimize their rates and reduce their side effects as well as their costs (Mousavi et al., 2005). Salicylic acid (SA), one of the primary growth regulators in plants, plays a significant role in their systemic responses to environmental stress (Ghassemi-Golezani and Solhi-Khajemarjan, 2021).

This plant hormone is essential for various metabolic processes (Arif et al. 2020) including photosynthesis (Tang et al. 2017), elimination of reactive oxygen species (ROS) (Li et al. 2019), and enhancing the control of the AsA-GSH cycle (Yan et al. 2018). Based on the type of treatment and concentration of SA, its application could affect the growth and performance of the plant (Abdoli and Ghassemi-Golezani, 2021).

Herbicide application promotes physiological changes and stress responses in plants. These alterations are not always accompanied by visible harm. The physiological reactions to many stressors, including herbicides, are comparable. Possible stress-induced markers include changes in pigment content (Yordanov, 1992), proline amount (Reddy and Veerajnegulu, 1991), products of lipid peroxidation (Sergiev et al., 2000), and chlorophyll *a* fluorescence (Hassannejad et al., 2017). However, what physiological-biochemical indices of field bindweed were altered by the herbicide Clopyralid alone and in combination with SA? How did they transform? Solving these issues is crucial for the chemical management of field bindweed, as these weed alterations following herbicide and SA treatment were rarely reported. This study was conducted to determine the effects of Clopyralid herbicide alone and in combination with SA on the early physiological responses of field bindweed following Clopyralid treatment.

2. Materials and methods

2.1. Experimental design

In spring 2021, a two factorial field experiment using a randomized complete block design with three replications was done at the research station of the University of Tabriz. Individual plots (4 m × 3m) infested with field bindweed (*Convolvulus arvensis* L.) were selected to survey physiological and biochemical responses of this weed to SA and Clopyralid. The first factor is the application of salicylic acid (SA) at two levels (0, 1 mM once a day for three days) (Porheidar Ghagarbi et al., 2022), and the second is the spraying of different doses of Clopyralid at five levels (0, 0.4, 0.8, 1.6, and 2.4 L ha⁻¹). Herbicide spraying was done three days after SA application. The control plants (SA=0) were sprayed with distilled water three days before applying herbicides. After the appearance of the 4-5 leaf stage, herbicides were sprayed in the upper portion of the field bindweed seedling leaves using a sprayer equipped with an XR11002-VP TeeJet nozzle with a pressure of 1.5 bars and volume of 200 L ha⁻¹ at a velocity of 4 km h⁻¹ (Hassannejad *et al.*, 2020a).

2.2. Measurement of proline and soluble protein contents

A spectrophotometric measurement of proline content was performed using the ninhydrin method suggested by Bates et al. (1973). In 5 ml of 3% sulfosalicylic acid, fresh leaf samples (500 mg) were homogenized. In a test tube placed in a water bath at 100°C, the mixture was incubated with 2 ml of acid ninhydrin and glacial acetic acid for 1 hour. Then this sample was extracted with toluene, and the absorbance of free proline content (unit mol/g FW) was determined on a BioMate UV-vis analyzer (Model Camspec M330 UV-vis) at 520 nm.

The total soluble protein contents were measured using the Bradford (1976) technique. 1000 mg of fresh leaf samples were homogenized with 4 mL of Na phosphate buffer (pH 7.2), transferred to cold centrifuge tubes (2 mL Eppendorf tubes) and centrifuged at 12,000g for 10 min at 4 °C. The absorbance was measured with a UV-Vis spectrophotometer at 595 nm after supernatants and dye were pipetted into spectrophotometer cuvettes.

2.3. Measurement of antioxidant enzymes

Three days after herbicide treatment, a sample of three field bindweed plants was gathered and stored in an ice-filled container. To eradicate any leftover moisture, the leaves were rinsed with distilled water. Crushing roughly 0.5 grams of fresh leaf pieces from each plot in an ice-cold 0.1 M phosphate buffer (pH 7.5) containing 0.5 mmol L EDTA with a cooled crusher and pestle. The homogenized substance was centrifuged for 15 minutes at 15,000 g and 4 °C (Hettich, MIKRO 200, Germany). The mixture was employed for enzyme testing.

In accordance with Beyer and Fridovich, the superoxide dismutase (SOD) activity was measured (1987). Each unit of the enzyme was determined as the amount of enzyme (unit mg⁻¹ protein) that inhibits 50% of the reduction of nitro blue tetrazolium in light.

The activity of catalase (CAT) was measured by Aebi (1984). Mixing 30 mM H₂O₂ with 50 mM phosphate buffer (pH 7.0) and 0.1 mL enzyme extract produced a 3 mL reaction mixture. The CAT activity (unit mg⁻¹ FW) was assessed by a reduction in absorbance at 240 nm.

Using the Rao et al. (1996) method, peroxidase (POD) activity was determined. The extraction was conducted at 4 °C. One gram of tissue was mixed with 50 mmol/L of potassium phosphate buffer (pH 7.0) in an ice-cold mortar before being centrifuged at 15,000 g for 15 minutes to yield the filtrate for POD detection. At 25 °C, a 50 L solution of enzyme extract was added to a 50 L reaction mixture containing 20 mmol/L guaiacols, 2.8 mL of potassium phosphate buffer (pH 7.0), and 50 L of guaiacol (20 mmol/L). The reaction was beginning by adding 20 L of H₂O₂ with a concentration of 40 mmol/L. Changes in absorbance at 470 nm were detected every 30 seconds. The POD activity was given as 1 mol of tetraguaiacol per minute, whereas the enzyme activity was reported in units per minute and mg of protein.

2.4. Measurement of MDA and H₂O₂

According to Jana and Choudhuri (1982) approach, the concentration of hydrogen peroxide (H₂O₂) was determined colorimetrically. In a 3 mL phosphate buffer (50 mM pH 6.5), homogenize 0.5 g of plant material for 25 minutes at 6000 g. The supernatant was then mixed with 1 milliliter of titanium sulfate dissolved in 20% H₂SO₄ and centrifuged at 6000 g for 15 minutes. A UV-VIS spectrophotometer determined the 410 nm absorbance (T80, PG Instruments, United Kingdom).

Malondialdehyde (MDA), the breakdown result of lipid degradation, is a measure of lipid peroxidation state. Approximately 0.5 g of fresh leaf samples were homogenized in a 20% (w/v) trichloroacetic acid and 0.5% 2-thiobarbituric acid solution to extract thiobarbituric acid-reactive compounds responsible for lipid peroxidation. This mixture was chilled in an ice bath immediately after being heated for 30 minutes at 95 °C to avoid additional reaction. After centrifuging at 5,000 g and 25 °C for 10 minutes, the absorbance of the supernatant was measured at 532 and 600 nm. After removing the undesirable discoloration at 600 nm using a spectrophotometer, the molar extinction coefficient of MDA was determined to be 155 mmol/mol (L cm) (Kosugi and Kikugawa, 1985).

2.5. Measurement of photosynthesis pigment

Utilizing Arnon's (1949) approach, the chlorophyll a, b, and carotenoid concentration was determined. Extracts of fresh leaves (0.2 g) were powdered in 80% acetone (in 10 mL) and centrifuged at 10,000 rpm for five minutes. Chlorophyll a, b, and carotenoids were measured using a spectrophotometer (Model Analytik Jena Spekol 1500 Germany) at 663 nm, 645 nm, and 470 nm, respectively.

2.6. Chlorophyll a fluorescence analysis

Three days following an application of herbicide, the chlorophyll fluorescence (ChlF) of fully grown field bindweed leaves was measured using a Handy PEA fluorimeter (Hansatech Instruments Ltd., England). Before measuring ChlF signals, dark-adapted leaves (30-40 min) were exposed to solid actinic light (8000 mol m⁻² s⁻¹) and fluorescence was measured over a period of 20 s to 2 s. (Abbasvand *et al.*, 2019). Then, Chl fluorescence signals were exported using a PEA plus (1.10) application (Hassannejad *et al.*, 2020b).

2.7. Statistical analysis

Based on the experimental design, SAS 9.1.3 and Microsoft Office Excel 2020 were used to perform an ANOVA analysis on the collected data. To compare the means of each attribute at $p \leq 0.05$, Duncan's multiple range test was utilized.

3. Result

3.1. Antioxidant enzyme activities

Analysis of variance showed that application of Clopyralid, SA, and the interaction of Clopyralid $\times$ SA treatments had a significant effect on proline, soluble protein, antioxidant enzymes activities, malondialdehyde, H_2O_2 and photosynthetic pigments (Table 1).

Comparatively to controls, the activity of the CAT, SOD, and POD increased following an herbicide application in the lowest concentrations and decreased in the highest doses (Fig. 1). Clopyralid in the dose of 0.8 L ha^{-1} increased CAT, SOD, and POD activity to 29.62%, 84.17%, and 57.89% compared to control (Fig. 1). The application of SA three days prior to Clopyralid application influenced the activities of these enzymes. The activity of these enzymes reached the highest value in field bindweed treated with 0.4 L ha^{-1} of Clopyralid three days after SA application (Fig. 1). SA alone was able to induce these enzymes activity at 9.46%, 28.33%, and 35.05%, respectively, compared with control (Fig. 1). SA, with a synergistic effect on herbicide, caused the SOD activity to reach the maximum value of 30.27 U.mg^{-1} protein in the lowest dose (0.4 L ha^{-1}) of Clopyralid treatment. However, with the increase in herbicide dosage, the use of salicylic acid decreased the activity of CAT, SOD, and POD (Fig. 1).

Table 1. Analyses of variance of the data for proline, soluble protein, antioxidant enzymes activities, malondialdehyde, H_2O_2 , and photosynthetic pigments of field bindweed plants in response to different doses of Clopyralid with and without SA application

Mean Squares													
Source of variation	df	Proline	Protein	CAT	SOD	POD	MDA	H_2O_2	Chla	Chlb	Carotenoids	Total Chl	Chla/Ct
Block	2	0.0001 ^{ns}	0.002 ^{ns}	0.0002 ^{ns}	0.024 ^{ns}	0.53 ^{ns}	0.003 ^{ns}	0.0004 ^{ns}	0.001 ^{ns}	0.001 ^{ns}	0.001 ^{ns}	0.001 ^{ns}	0.08 ^{ns}
Salicylic acid (A)	1	0.165 ^{**}	0.36 ^{**}	0.054 ^{**}	132.48 ^{**}	67.48 ^{**}	2.83 ^{**}	0.38 ^{**}	0.43 ^{**}	0.12 ^{**}	0.13 ^{**}	1.84 ^{**}	0.76 ^{**}
Clopyralid (B)	4	0.093 ^{**}	0.44 ^{**}	0.027 ^{**}	33.67 ^{**}	2.01 ^{**}	0.52 ^{**}	0.32 ^{**}	0.07 ^{**}	0.01 ^{**}	0.024 ^{**}	0.27 ^{**}	0.033 ^{**}
A $\times$ B	4	0.002 ^{**}	0.022 ^{**}	0.003 ^{**}	99.83 ^{**}	65.1 ^{**}	0.059 ^{**}	0.01 ^{**}	0.02 ^{**}	0.003 ^{**}	0.01 ^{**}	0.08 ^{**}	0.02 ^{**}
Error	18	0.001	0.001	0.023	1.03	2.44	0.001	0.17	0.001	0.001	0.001	0.001	0.02
CV (%)		0.97	0.99	0.96	0.98	0.92	0.998	0.99	0.996	0.99	0.99	0.99	0.91

ns,*,**: No significant and significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

3.2. Proline and soluble protein contents

In this study, the proline content was significantly different under all treatments (Fig. 2a). The leaf proline content increased in response to Clopyralid spraying alone or three days after SA application compared to the control (Fig. 2a). Initially, proline content increased with increasing Clopyralid dosage from 0.4 to $0/8 \text{ L.h}^{-1}$ in the absence of SA and then declined from 1.6 to 2.4 L ha^{-1} (Fig. 2a). SA application on untreated plants with Clopyralid also increased proline content (Fig. 8a). SA, in combination with Clopyralid, additionally boosted the accumulation of proline. The maximum accumulation of proline (52.34%) was found in plants were treated with 0.4 L ha^{-1} Clopyralid and SA (Fig. 2a). The difference among Clopyralid dosage with or without SA was distinct, except 1.6 and 2.4 L ha^{-1} (Fig. 2a).

The soluble protein significantly decreased with increasing Clopyralid dosage with and without SA application three days before herbicide application (Fig. 2b). The soluble protein content in 0.4 , 0.8 , 1.6 , and 2.4 L ha^{-1} of Clopyralid + SA had declined 7.40%, 15.21%, 22.47%, and 31.92%, respectively, compared to its control (Fig. 2b). The soluble protein content was slightly decreased in untreated plants with SA, so that in 0.4 , 0.8 , 1.6 , and 2.4 L ha^{-1} of Clopyralid without SA had declined 2.13%, 6.93%, 12.67%, and 18.27%, respectively, compared to its control (Fig. 2b).

3.3. MDA content and H_2O_2 concentration

Application of Clopyralid increased H_2O_2 and MAD contents (Fig. 3). The highest amount of MDA was produced in response to the highest concentration of Clopyralid in the presence or absence of SA application (Fig. 3a). High dose (2.4 L ha^{-1}) of Clopyralid caused an increase of 136.84% and 142.08% in the absence or presence of SA (Fig. 3a). Low (0.4 L ha^{-1}) and high doses (2.4 L ha^{-1}) of Clopyralid showed induced H_2O_2 contents by 10.65% and 81.02%, respectively (Fig. 3b). A pronounced increase in H_2O_2 content was observed in field bindweed leaves treated with Clopyralid spraying three after SA application (Fig. 3b). In the presence of SA application, the amount of H_2O_2 in the lowest and the highest doses of Clopyralid was 26.56% and 92.95%, respectively, compared to its control (Fig. 3b).

3.4. Chlorophyll and carotenoids contents

By increasing the concentration of Clopyralid, the chlorophyll *a*, *b*, carotenoid, and total chlorophyll contents of field bindweed leaves were significantly reduced (Table 2). At the highest dose of Clopyralid (2.4 L ha⁻¹), chlorophyll *a*, *b*, carotenoid, and total chlorophyll reduced by 36.4%, 52.38 %, 59.01%, and 44.34%, respectively, as compared to the SA-free control (Table 2). However, the application of SA three days before herbicide spraying significantly reduced this lowering (Table 2). So, compared with SA-applied control, at the highest dose of Clopyralid (2.4 L ha⁻¹), chlorophyll *a*, *b*, carotenoid, and total chlorophyll dropped by 47.49%, 60.20%, 71.67%, and 55.17%, respectively (Table 2). The steep decline in chlorophyll *b* content relative to chlorophyll *a* caused the chlorophyll *a/b* ratio to rise as herbicide dosages increased (Table 2).

Table 2. The photosynthetic pigment of field bindweed plants in response to different doses of Clopyralid with and without SA application

Clopyralid (L ha ⁻¹)	Chlorophyll <i>a</i> (mg g ⁻¹ DW)		Chlorophyll <i>b</i> (mg g ⁻¹ DW)		Carotenoids (mg g ⁻¹ DW)		Total Chlorophyll (mg g ⁻¹ DW)		Chlorophyll <i>a/b</i>	
	SA0	SA1	SA0	SA1	SA0	SA1	SA0	SA1	SA0	SA1
0	1.63 b	1.73 a	0.63 b	0.67 a	0.54 b	0.6 a	2.79 b	3 a	2.58 e	2.58 e
0.4	1.38 c	1.27 d	0.51 c	0.47 d	0.41 c	0.28 e	2.3 c	2.02 e	2.71 de	2.73 de
0.8	1.31 d	1.11 f	0.47 d	0.36 e	0.32 d	0.23 f	2.07 d	1.70 g	2.93 cd	3.06 c
1.6	1.18 e	1.04 g	0.38 e	0.31 f	0.29 e	0.21 f	1.85 f	1.56 h	3.15 bc	3.36 ab
2.4	1.03 g	0.91 h	0.3 f	0.27 g	0.22 f	0.17 g	1.55 h	1.34 i	3.45 a	3.42 a

Each value is the mean of three replicates. Means with the same letter within each trait are not significantly different at $P < 0.05$.

3.5. Chlorophyll *a* fluorescence

Analyses of variance showed significant effects of Clopyralid herbicide alone or after application of SA on chlorophyll *a* fluorescence parameters such as Tfm, Area, F0, Fm, Fv, F0/Fm, Fv/Fm, Fv/F0, and PI (Table 3).

Table 3. Analysis of variance for all chlorophyll *a* fluorescence parameters of field bindweed in response to different doses of Clopyralid with and without SA application

Source of variation	Mean squares									
	df	Tfm	Area	Fo	Fm	Fv	F0/Fm	Fv/Fm	Fv/F0	PI
Block	2	70 ^{ns}	24391 ^{ns}	7.63 ^{ns}	47.63 ^{ns}	47.63 ^{ns}	0.00 ^{ns}	0.00 ^{ns}	0.00 ^{ns}	0.00 ^{ns}
Salicylic acid (A)	1	81120 ^{**}	1132597252 ^{**}	127922.7 ^{**}	125453.33 ^{**}	555424.13 ^{**}	0.054 ^{**}	0.05 ^{**}	20.17 ^{**}	0.02 ^{**}
Clopyralid (B)	4	532.34 ^{**}	1224123998 ^{**}	29779.7 ^{**}	498429.28 ^{**}	565121.8 ^{**}	0.027 ^{**}	0.02 ^{**}	7.79 ^{**}	8.17 ^{**}
A × B	4	7828.33 ^{**}	2682285535 ^{**}	3477.7 ^{**}	89554.58 ^{**}	83562.47 ^{**}	0.001 ^{**}	0.01 ^{**}	0.66 ^{**}	0.88 ^{**}
Error	18	66.30	645641470	10.97	147.74	130.19	0.00	0.00	0.00	0.00
CV (%)		2.34	1.01	0.73	0.63	0.77	0.94	0.36	1.60	1.26

ns,*,**: No significant and significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Tfm and Area

The time required to reach maximum fluorescence (Tfm) (Kalaji *et al.* 2017), which represents the rate of electron transport through the PSII acceptor (Hassannejad *et al.* 2020c), significantly decreased after Clopyralid and SA treatment (Fig. 4a). The Tfm of control plants treated with SA was higher than the other treatments (Fig. 4a). This variable decreased significantly with increasing Clopyralid dose (60 and 41 percent, respectively, with and without SA application at 2.4 L ha⁻¹) (Fig. 1). Reaction centers (RCs) lost energy when the Clopyralid dose increased, particularly after SA application (Fig. 4a). The highest reduction (74.14%) in the PS II pool size or the area parameter occurred at 2.4 L ha⁻¹ of Clopyralid without SA (Fig. 4b). An increase in the Area parameter for herbicide-free control after SA application demonstrates the positive impact of this growth regulator. The application of Clopyralid and SA three days before herbicide spraying significantly reduced the area parameter with increasing herbicide dosage (Fig. 4b). Clopyralid with SA synergistically reduces the plastoquinone pool size in photosystem II (Fig. 4b).

F0 and Fm

A plant's fluorescence level is F0 when all primary quinone acceptors are oxidized and open, but this may change when the plant is stressed (Kumar *et al.*, 2020). Based on the results of this study, the minimum fluorescence (F0) increased with increasing Clopyralid dosage (Fig. 5a), which was described as the switch over from active reaction centers (RC) to a silent form of RC (Hassannejad *et al.*, 2022). By applying SA three days before herbicide application, herbicide efficiency increased significantly. The maximum dosage of Clopyralid (2.4 L ha⁻¹) resulted in a 36.43% increase in the F0 parameter compared to the

control (Fig. 5a). Fluorescence is FM when all primary quinone acceptors (QA) are photo reduced and closed. Fig. 5b shows that this parameter decreased with increasing Clopyralid dosage without SA application. However, this growth regulator changed the maximum fluorescence of field bindweed so that its application increased Fm from 0.4 until 1.6 L ha⁻¹ of Clopyralid and decreased at 2.4 L ha⁻¹ (Fig. 5b).

Fv and F0/Fm

The variable fluorescence intensity Fv represents the amplitude of fluorescence increase from F0 to FM when measured in dark-adapted samples (Kalaji *et al.*, 2017). As Clopyralid doses increased, the variable fluorescence reduced (Fig. 6a). In comparison to the control, the highest dose of Clopyralid (2.4 L ha⁻¹) decreased the Fv parameter by 43.77 percent (Fig. 6a). The synergistic effects of SA and Clopyralid resulted in a reduction of 36.37 percent of this parameter in the minimal herbicide dosage (Fig. 6a). Following the application of SA, no significant differences appeared between the different Clopyralid doses (Fig. 6a). F0/Fm is the proportion of solar energy collected by PSII that cannot be used photochemically and is lost as heat (Hassannejad *et al.*, 2020a). Field bindweed plants treated with Clopyralid demonstrated a significant increase in this parameter as the dosage increased (Fig. 6b). F0/Fm increased significantly by applying SA three days before herbicide (110% increase compared to control) (Fig. 6b).

Fv/Fm, Fv/F0, and PI

Maximum PSII quantum efficiency (Fv/Fm) dropped dramatically in response to increased Clopyralid dose, whereas foliar SA treatment three days prior to herbicide spray altered this parameter slightly (Fig. 7a). Fv/Fm was lowered by 23.75 percent at the maximum dosage of Clopyralid (2.4 L ha⁻¹) compared to the control (Fig. 7a). This study's findings are supported by findings from Force *et al.* (2003). They concluded that the Fv/Fm ratio is the less susceptible than the other fluorescence parameters to stress-induced changes. The water-splitting efficiency on the donor side of the PSII (Fv/F0) ratio decreased by increasing herbicide dosage (Fig. 7b). At the highest dose of Clopyralid with and without SA application, Fv/F0 reduced by 69.99% and 53.25%, respectively (Fig. 7b). Furthermore, this parameter indicates the synergistic effect of SA with Clopyralid in controlling field bindweed. PSII performance index on an absorption basis (PI) increased by 43.9% in control plants treated with SA but untreated with Clopyralid (Fig. 7c). Clopyralid dose-dependently diminished this parameter (92.74%) when sprayed at 2.4 L ha⁻¹ on SA-treated plants (Fig. 7c). This parameter confirms salicylic acid's synergistic effect on Clopyralid (Fig. 7c).

The relationship between dry weight with PSII performance index (PI)

Fig. 8 illustrates the relationship between dry weight (measured at 28 days after herbicide spraying) and the PSII performance index (measured at three days after herbicide spraying) for Clopyralid and Clopyralid + SA, respectively. Clopyralid + SA has a steeper slope than Clopyralid alone. There is a linear relationship between the PSII performance index (PI) and dry weight.

Discussion

Chemical weed control is a crucial aspect of crop management that supports profitable crop production by providing effective weed control. Herbicides frequently interfere with the physiological processes of weeds, resulting in substantial growth reduction or death (Peixoto *et al.*, 2006; Jiang and Yang, 2009). We evaluated the effect of a regularly used herbicide at varying concentrations on the field bindweed weed and the effect of the herbicide on the antioxidant system of exposed plants. We determined that greater herbicide concentrations severely disturbed the physiological processes of plants, and exogenous administration of salicylic acid (SA) amplified these detrimental effects.

Herbicide treatment usually functions as abiotic stress, inducing reactive oxygen species (ROS) production at the cell level (Song *et al.*, 2006; Song *et al.*, 2007). ROS have destructive effects on several physiological processes, resulting in abnormal growth and plant mortality. To prevent the potential damage by ROS, there is a cellular antioxidant system which includes different enzymes and non-enzymatic antioxidant molecules that effectively scavenge ROS to a certain extent (Gill and Tuteja, 2010; Czarnocka and Karpinski, 2018). However, the natural antioxidant system and its regulatory function is often not effective enough to overcome herbicide damage and/or other severe abiotic stresses.

Alterations in antioxidants enzymes have been observed in several crops and weed species in response to herbicide exposure. In the first instance, the plants produce ROS, which cause lipid peroxidation and eventually, membrane damage (Boulahia *et al.*, 2016; Pan *et al.*, 2017). We observed a similar response in the current study where lipid peroxidation increased with the increasing rates of herbicide application. This translated into higher levels of MDA and H₂O₂, especially at higher rates of herbicide (1.6 and 2.4 L ha⁻¹). A similar increase in MDA and H₂O₂ contents was reported in a tomato (*Solanum lycopersicum* L.) plants exposed to the herbicide 2,4-D, which is a synthetic auxin, similar to Clopyralid (used in our study) (Singh and Singh, 2020). Ananieva *et al.* (2002) suggest that herbicide-induced increase in H₂O₂ concentration could be related to enhancing the energy dissipation via photorespiration in the stressed plants. Our results also showed that the use of SA increased the concentration of MDA and H₂O₂ in Clopyralid-treated and non-treated plants (Fig. 3). Generally, low levels of SA (It depends on the plant species, however, < 1 mM SA considered as low concentration.) positively regulate plant development and growth. Nevertheless, the application of high concentrations of SA showed harmful effects on it (Koo *et al.*, 2020). SA's effect as a negative regulator of antioxidant systems is presumably due to an SA-induced oxidative stress. In *Arabidopsis* plants treated with SA (1–5mM), H₂O₂ levels increase up to 3-fold as a result of increased activities of Cu, Zn-superoxide dismutase and inactivation of the H₂O₂-degrading enzymes catalase and ascorbate peroxidase (Rao *et al.*, 1997).

The antioxidant enzymes measured in this study have been strongly associated with stress tolerance. For example, SOD dismutates O₂^{•-} to H₂O₂, avoiding membrane damage. Similarly, certain enzymes, including POD and CAT, play critical roles in catalyzing the reactive H₂O₂ into H₂O or other nontoxic molecules (Zhang and Kirkham, 1994). By converting hydrogen peroxide into oxygen and water, CAT is one of the most important antioxidant enzymes. POD is another indicator of oxidative stress in plants. The SOD, POD, and CAT activity in this study increased up to the herbicide rate of 0.8 L ha⁻¹, but decreased at the higher rates. This was likely because of damage to these enzymes by high H₂O₂ levels or direct damage caused by herbicide residues. However, in plants treated with

SA without herbicide application, the activity of CAT, SOD, and POD increased (Fig. 1). This increment was also observed by the use of SA in plants treated with a dose of 0.4 L ha^{-1} Clopyralid (Fig. 1). The use of SA with a high dose of herbicide further reduced CAT, SOD, and POD activity, although this reduction was not significant in the POD activity (Fig. 1c). This increase in herbicide toxicity following the use of SA can be related to the negative role of SA in inactivating H_2O_2 -degrading enzymes (Rao et al., 1997).

We measured proline content as low molecular weight and highly soluble compound. Proline is involved in cellular water balancing, ROS elimination, membrane permeability preservation, and enzyme function maintenance (Gadallah, 1999). Plants subjected to stress, such as drought, salinity, and extreme temperatures, increased proline accumulation (Ashraf and Foolad, 2007). Application of SA with Clopyralid cause oxidative stress, increasing proline content. Similarly, Nazar et al. (2015) reported that SA enhanced the proline content in mustard plants under stress condition. As a result, proline accumulation in field bindweed can be considered an indicator of Clopyralid + SA-induced stress. Plant soluble protein content is an essential indicator of the degree of injury caused by environmental stress (Liu et al., 2012). All Clopyralid treatments alone or three days after SA application significantly decreased protein content. One possible explanation for decreased protein content under herbicide treatment may be targeting the enzymes synthesizing amino acids and proteins (Singh et al., 2013). The decline of Rubisco activity as a result of use the high dose of SA was attributed to a 50% reduction in protein levels (Pancheva and Popova, 1998).

The present study revealed that treatment of field bindweed plants with Clopyralid herbicide decreased photosynthesis pigment content (Table 2). The photosynthesis pigments degradation is one of the visible symptoms of Clopyralid mode of action in oxidative damage of field bindweed (Table 2). Plants treated with 2.4 L ha^{-1} of Clopyralid + SA showed the minimum values for photosynthesis pigments (Table 2). The decrease in chlorophyll synthesis under stress caused by herbicides is related to the degradation of chlorophyll molecules or inhibition of biosynthesis of the chlorophyll precursor (Radwan et al., 2019). Similarly, Radwan et al. (2019) reported that reduction in photosynthesis results in oxidative stress and increased intracellular ROS production, damaged macromolecules, and lower plant defense levels. In recent years, there has been growing evidence that some herbicides can produce excessive levels of ROS within cells, which can damage them (Wang and Zhou, 2006; Song et al., 2006; Peixoto et al., 2006; Radwan et al., 2019). Again, it has been observed that the effects of exogenous SA on photosynthesis parameters differ depending on the dose and plant species tested. High SA concentrations ($1\text{--}5 \text{ mM}$) cause a reduction in the photosynthetic rate and Rubisco activity in barley plants (Pancheva et al., 1996), and reduced chlorophyll contents in cowpea, wheat, and Arabidopsis (Rao et al., 1997; Chandra and Bhatt, 1998; Moharekar et al., 2003). It was suggested that the harmful effects of this high dose of SA in photosynthesis might be related to the stimulation of auxins oxidation by SA, since decreased auxins levels decreases photosynthetic rate and nitrate reductase activity (Ahmad et al., 2001).

Our results revealed that Clopyralid + SA had a synergistic effect in reducing the PSII electron transport rate (Tfm) (Fig. 4a), the plastoquinone pool size (Area) (Fig. 4b), the variable fluorescence (Fv) (Fig. 6a), the water splitting efficiency (Fv/F_0) (Fig. 7b), and the PSII performance index (PI) (Fig. 7c). There was no difference in Tfm reduction between Clopyralid (0.4 L ha^{-1}) plus SA and Clopyralid (2.4 L ha^{-1}) alone, but Clopyralid (0.8 L ha^{-1}) plus SA was better than Clopyralid (2.4 L ha^{-1}) without SA (Fig. 4a). According to Hassannejad et al. (2020c), application of the Bentazon herbicide decreased the Tfm of common cocklebur (*Xanthium strumarium* L.). Hassannejad et al. (2020b) showed that Basagran, Bromicide, and Gramoxone significantly reduce Tfm in common cocklebur (*Xanthium strumarium* L.). The Area in field bindweed subjected to Clopyralid (0.8 L ha^{-1}) + SA was smaller than Clopyralid (2.4 L ha^{-1}) alone (Fig. 4b). According to the decreasing Area (Fig. 4b) after Clopyralid + SA application, the PQ pool of reduced electron acceptors in PSII and the electron flow at the donor site of PSII was restricted. This result agrees with Hassannejad et al. (2020a), who reported lower values for Area in spiny cockleburs (*Xanthium spinosum* L.) in response to bentazon herbicide.

The number of oxidized Quinone acceptors (F_0) increases significantly when high doses of Clopyralid (2.4 L ha^{-1}) are applied (Fig. 5a). SA enhanced the effectiveness of Clopyralid in silencing reaction centers (RCs) (Fig. 5a). It was shown that Bentazon enhanced F_0 in the spiny cocklebur (*Xanthium spinosum* L.) (Hassannejad et al., 2020a). By applying Lumax (4.5 L ha^{-1}) alone or in combination with castor oil, the F_0 parameter of common cocklebur (*Xanthium strumarium* L.) increased by 35 and 28%, respectively, compared to the control (Hassannejad and Porheidar-Ghafarbi, 2019). Dodder infestation in basil plants at the vegetative stage increased F_0 (Abbasvand et al., 2020). Imazamax and glyphosate boosted the F_0 parameter in sunflower (Balabanova et al., 2016) and soybean (Huang et al., 2012) Clopyralid (2.4 L ha^{-1}), with or without SA, decreased photo-reduced Quinone acceptors (Fm) by 34.39% and 32.69%, respectively (Fig. 5b). According to Hassannejad et al. (2020a) research, the Fm of common cocklebur (*Xanthium strumarium* L.) significantly reduced following Bentazon application. The value of this parameter was reduced in common cockleburs treated with Basagran, Bromicide, Lumax, and Gramoxone (Hassannejad et al., 2020b) and spiny cockleburs treated with Bentazon (Hassannejad et al., 2020a). By converting violaxanthin into zeaxanthin during the xanthophyll cycle and quenching non-photochemically, Fm is reduced (Demmig-Adams et al., 2008).

Fv/Fm is the maximum quantum efficiency of PSII, which is approximately 0.83 under normal conditions (Hassannejad et al., 2020a). Research is frequently conducted using this parameter since it is easy to measure, has a clear physiological meaning, and has well-defined optimum values. In stressed plants, this measure is a good predictor of photoinhibition (Yin et al., 2008). In response to different stress conditions, the Fv/Fm decrease has been often used to identify photoinhibition damage (Brestič et al., 2015), low temperature (Groom and Baker, 1992), excessive light intensities (Živčák et al., 2014), and drought stress (Epron et al., 1992). In this research, Fv/Fm was affected by Clopyralid alone and Clopyralid plus SA (Fig. 7a). According to Hassannejad and Porheidar-Ghafarbi (2018), treating corn cultivars with 75% and 100% recommended dosage of Topik herbicide plus SA significantly reduced Fv/Fm . Clopyralid $\pm$ SA significantly reduced the efficiency of the water-splitting complex on the donor side of PSII (Fv/F_0) (Fig. 7b). A photoinhibition mechanism occurred on the donor side due to the failure of the water-splitting complex (Dayan et al., 2012), which resulted in the oxidation of PSII (Zhang et al., 2016). In a study by Oliveira et al. (2002), they reported that the Fv/F_0 ratio had a similar response pattern to the Fv/Fm ratio but significantly higher values, suggesting better discrimination of minor differences in the PSII quantum yield. In several studies, Fv/Fm has been used to screen for stress (Padhye et al., 2008). However, some authors have suggested that this ratio is not always suitable for stress assessment (Van Heerden et al., 2003; Strauss et al., 2006).

The PSII performance index on an absorption basis (PI) includes the density of reaction centers in the chlorophyll bed, the capture of excitation energy, and the transport of electrons (Strasser et al., 2004). SA had a synergistic effect on field bindweed in response to Clopyralid (Fig. 7c). The decline of PSII activity and the loss of PSI structure and function caused a decrease in PI (Fig. 7c). Clopyralid ± SA reduced PSI ends electron acceptors (Fig. 7c). The values of PI describe the energy flow efficiency of the photosynthetic transport chain beyond PSII (Strasser et al., 2004). According to Force et al. (2003) and our results in this research, PI, which describes the overall vitality of PSII, was the most sensitive indicator for enabled researchers to capture the effect of PAR fluctuations within a short time interval. These results show that Clopyralid, an auxin-type herbicide, interferes with chloroplast electron transport and disrupts the photosynthesis apparatus in field bindweed plants. The steeper slope of the curve between PI and dry weight of field bindweed under Clopyralid plus SA compared to Clopyralid alone showed that SA had a synergistic effect on Clopyralid effectiveness (Fig. 8). According to other researchers, there is a relationship between fluorescence parameters and dry weight for plants treated with Bentazone (Christensen et al., 2003), Metamitron and Terbutylazine (Abbaspoor et al., 2006), Clodinafop (Abbaspoor and Streibig, 2005), Desmedipham and Phenmedipham (Abbaspoor and Streibig, 2007), Sethoxydim ± turnip oils (Hammami et al., 2014), Lumax ± castor oil (Hassannejad and Porheidar-Ghafarbi, 2019), and Lumax ± almond oil (Hassannejad et al., 2022).

Conclusion

Clopyralid triggered oxidative stress in a dosage-dependent manner that was exhibited through elevated lipid peroxidation levels, H₂O₂ production, and soluble protein and proline accumulation. Moreover, this condition's actual mechanism behind ROS formation must be clarified. On the other hand, SA spraying three days before Clopyralid application disturbed the antioxidant system, increased the level of lipid peroxidation and made the plants more sensitive to the adverse effects of Clopyralid. Clopyralid disrupted photosynthetic machinery by interfering with water photolysis, reducing PQ pool size, and slowing electron transport in PSII. SA application three days before Clopyralid spraying enhanced substantial damage to the photosynthetic system. Chlorophyll fluorescence (ChlF) research can replace traditional screening approaches based on current data and experimental results. ChlF methodology is non-destructive, highly sensitive, and quick compared to other detection processes. This study showed that PI is a valuable indicator for assessing herbicide impacts soon after application. Our study represents that classic screening procedures take seven times as long as ChlF approaches. The use of ChlF may be included in future herbicide bioassay investigations. This research proved the best control effect on field bindweed when SA (1 mM) was used in combination with Clopyralid at 75% (1.6 L ha⁻¹) of the recommended dose.

Declarations

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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Figures

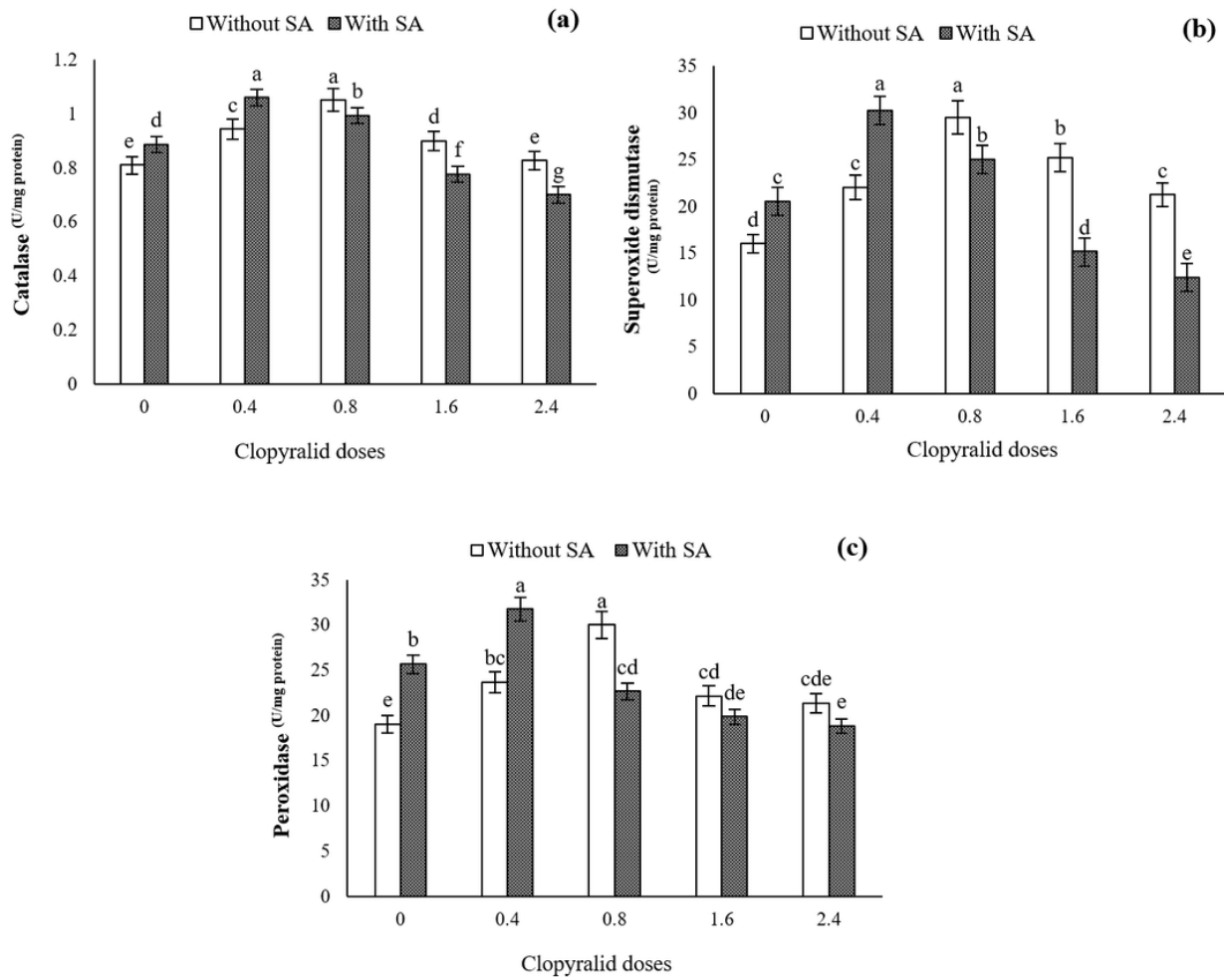


Figure 1

Changes in (a) catalase, (b) superoxide dismutase, and (c) peroxidase activity of field bindweed in response to different doses of Clopyralid with and without SA application. Each value is the mean of three replicates. Different letters represent significant differences ($P < 0.05$).

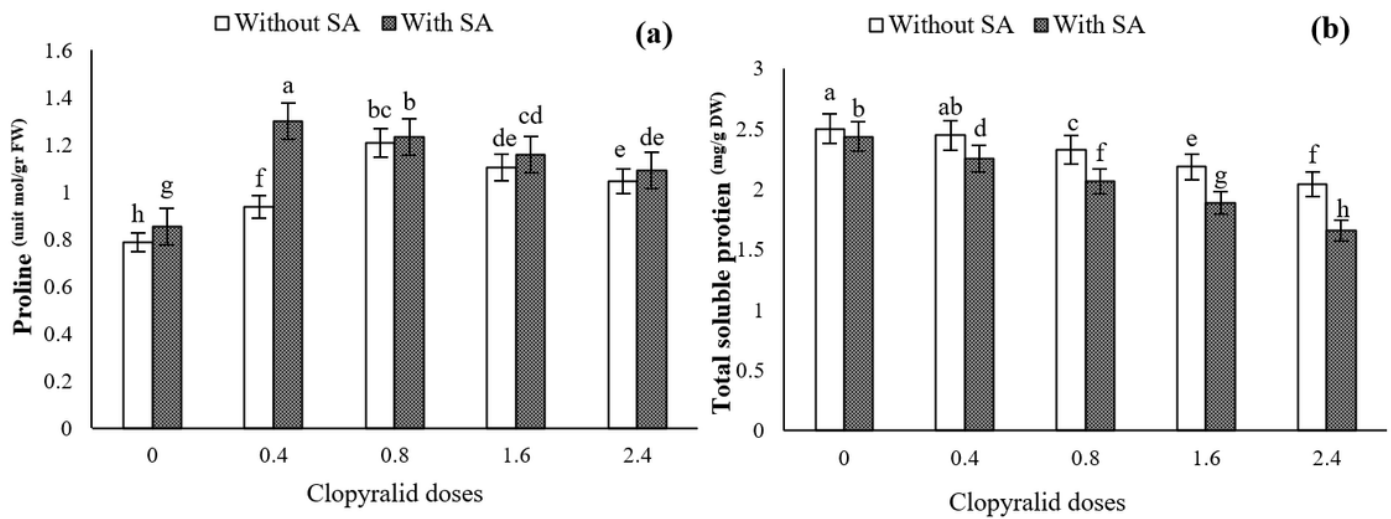


Figure 2

Changes in (a) proline and (b) soluble protein content of field bindweed in response to different doses of Clopyralid with and without SA application. Each value is the mean of three replicates. Different letters represent significant differences ($P < 0.05$).

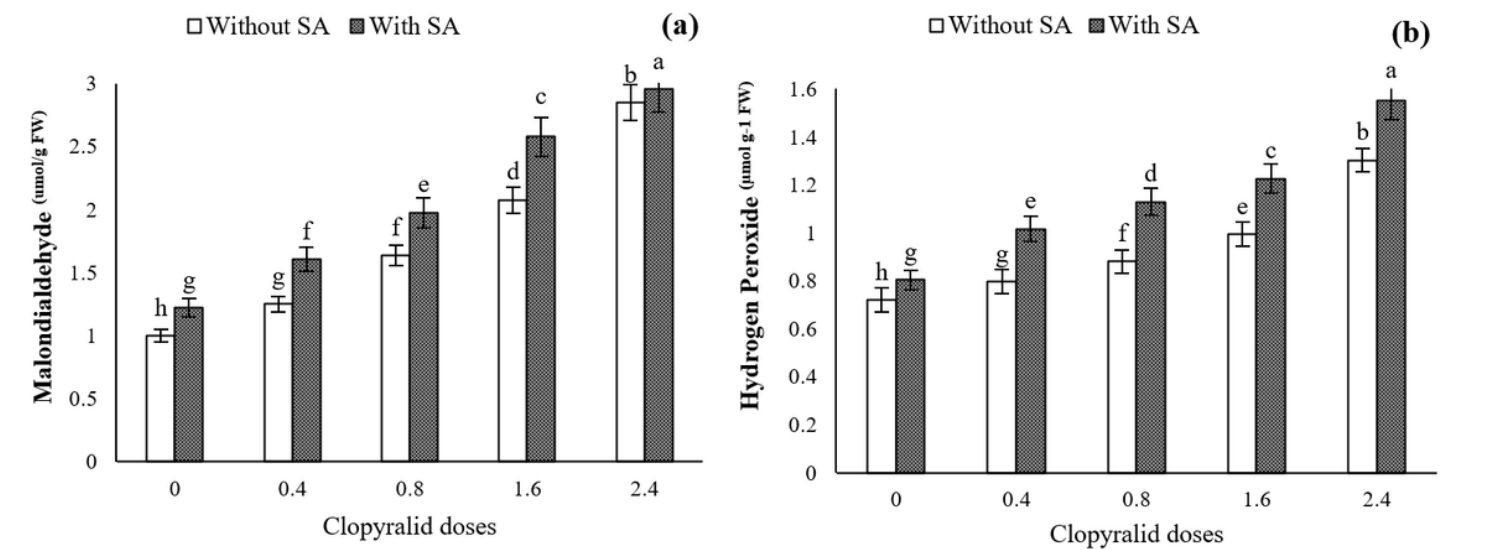


Figure 3
Changes in (a) malondialdehyde and (b) hydrogen peroxide of field bindweed in response to different doses of Clopyralid with and without SA application. Each value is the mean of three replicates. Different letters represent significant differences ($P < 0.05$).

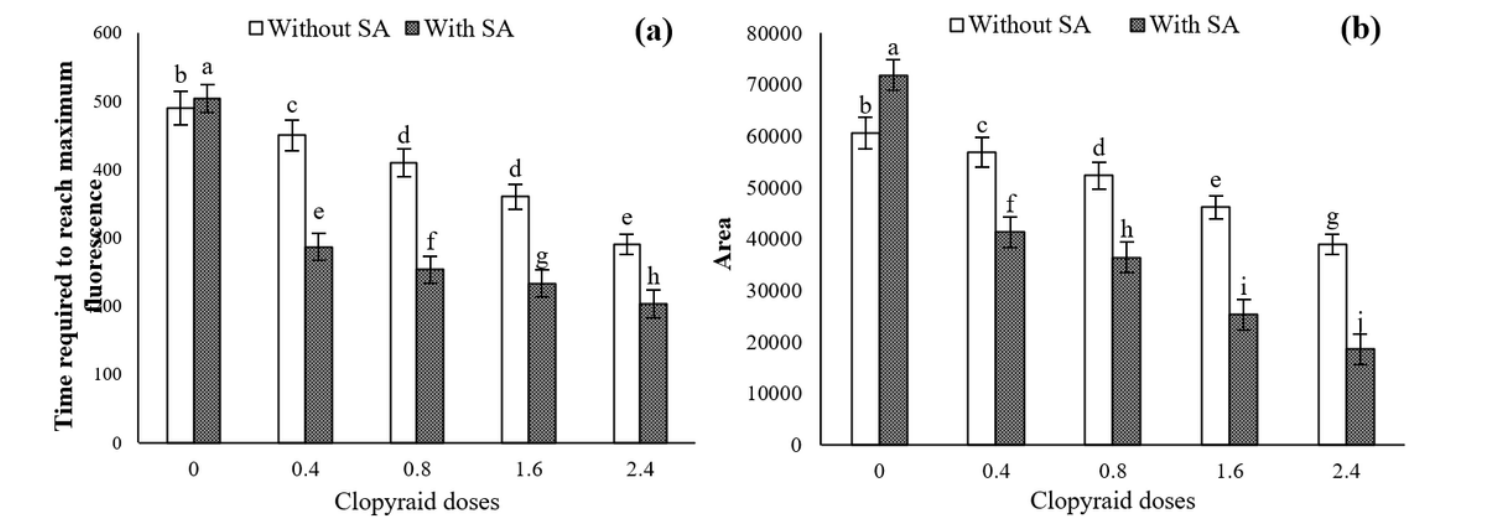


Figure 4
Changes in the time required to reach maximum fluorescence (a) and area (b) of field bindweed 4 days after Clopyralid with and without SA. Each value is the mean of three replicates. Different letters represent significant differences ($P < 0.05$).

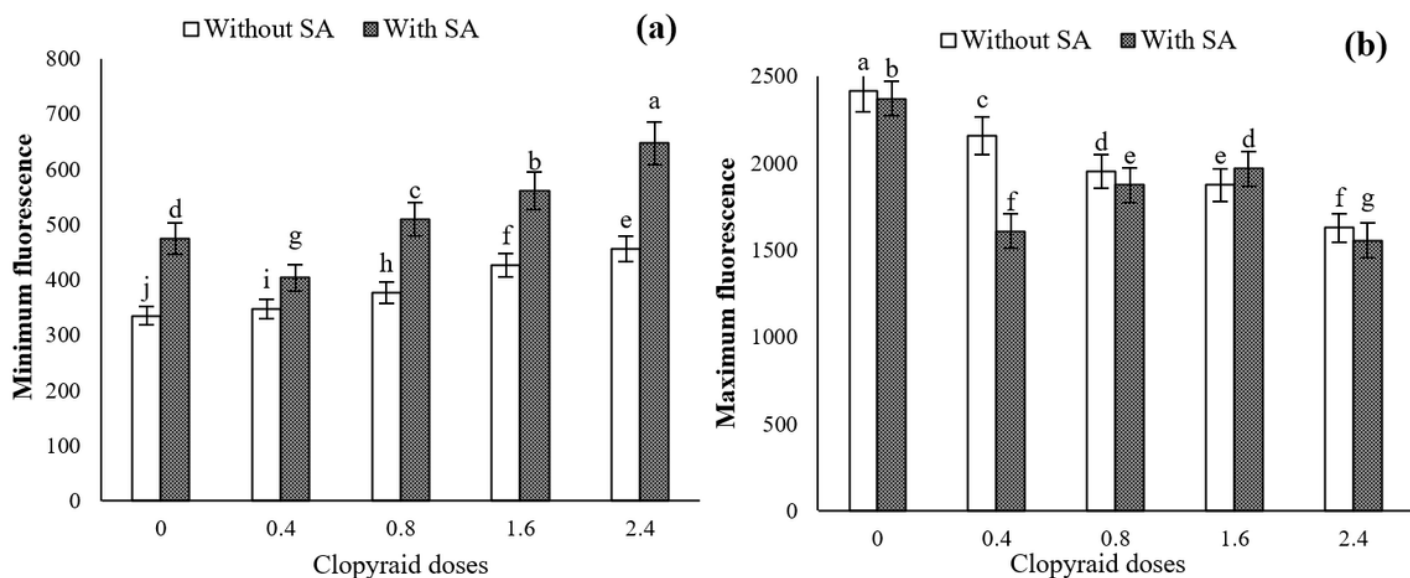


Figure 5
Changes in minimum fluorescence (a) and maximum fluorescence (b) of field bindweed 4 days after Clopyralid with and without SA. Each value is the mean of three replicates. Different letters represent significant differences ($P < 0.05$).

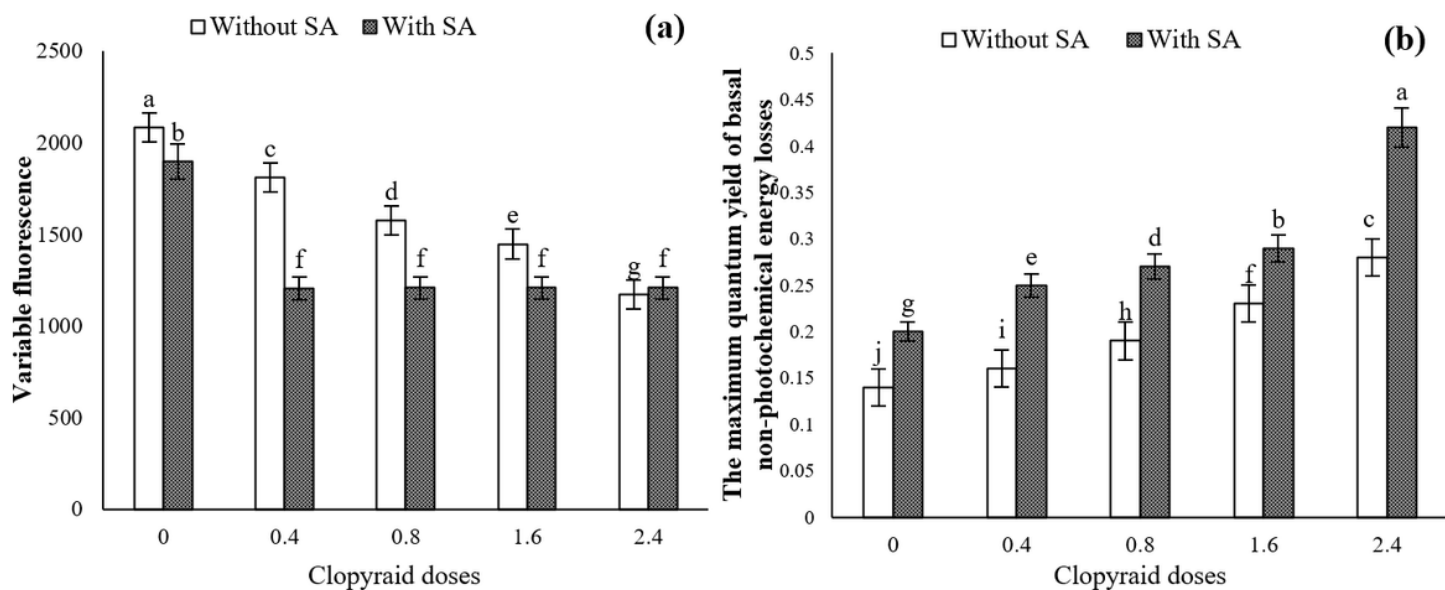


Figure 6
Changes in variable fluorescence (a) and the maximum quantum yield of basal non-photochemical energy losses (b) of field bindweed 4 days after Clopyralid with and without SA. Each value is the mean of three replicates. Different letters represent significant differences ($P < 0.05$).

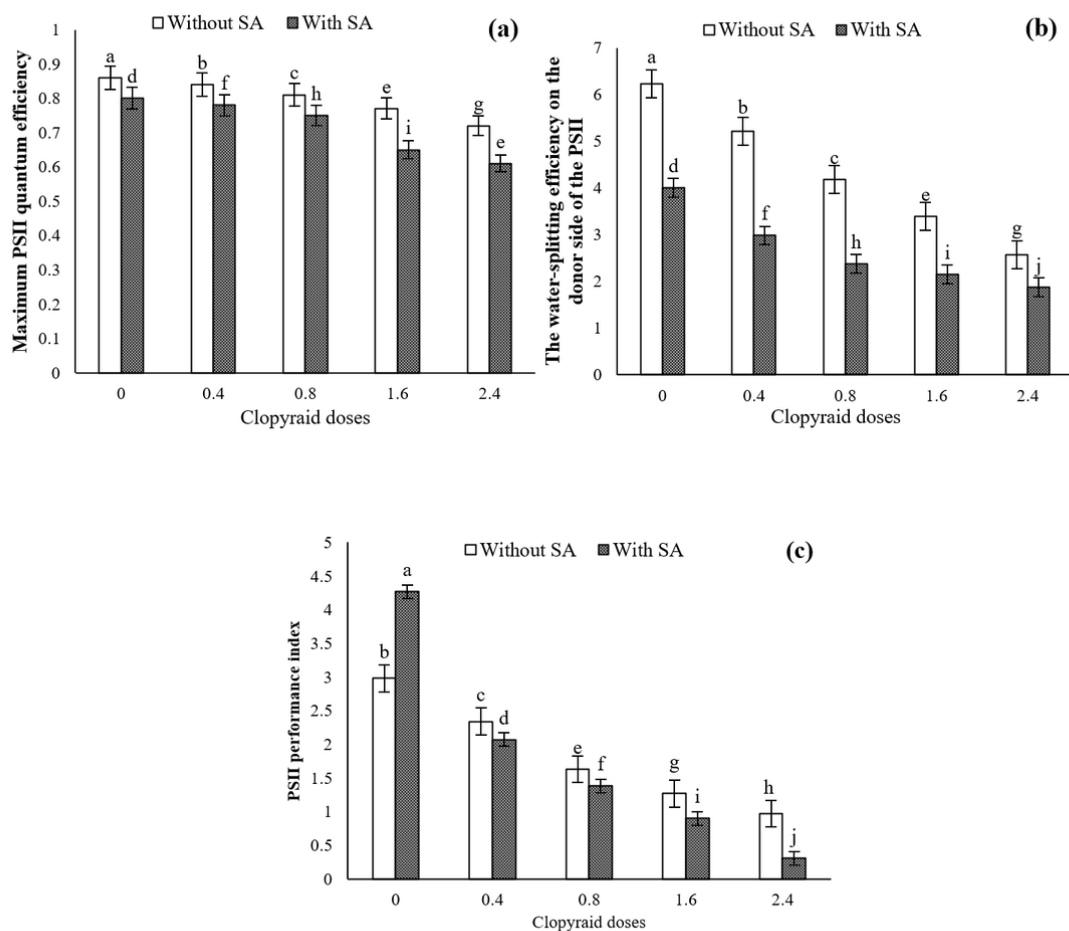


Figure 7
Changes in maximum PSII quantum efficiency (a), the water-splitting efficiency on the donor side of the PSII (b), and PSII performance index (c) of field bindweed 4 days after Clopyralid with and without SA. Each value is the mean of three replicates. Different letters represent significant differences ($P < 0.05$).

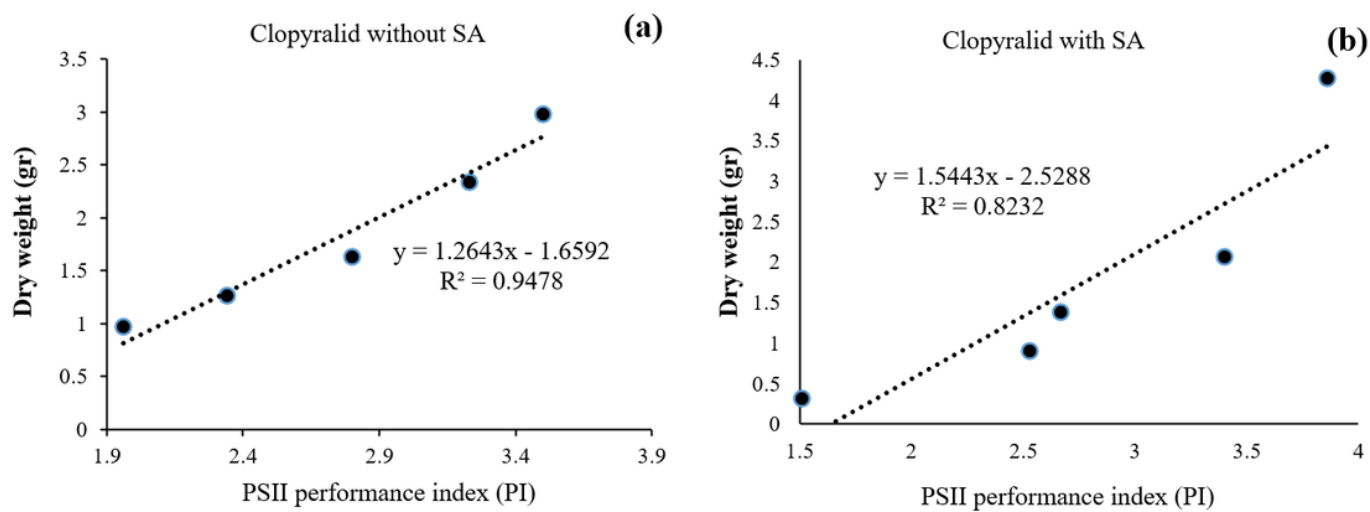


Figure 8

Relationship between PSII performance index (PI) was taken at 3 days after herbicide spraying with dry weight of field bindweed was taken at 28 days after herbicide spraying for Clopyralid (a) and Clopyralid + SA (b).