Salicylic acid mitigates clopyralid-induced oxidative stress in Camelina sativa by enhancing antioxidant defenses

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Salicylic acid mitigates clopyralid-induced oxidative stress in Camelina sativa by enhancing antioxidant defenses

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ABSTRACT

Salicylic acid (SA) plays a crucial role in mitigating herbicide-induced oxidative stress in crops. This study investigated the potential of exogenous SA (0.5-1.5 mM) in alleviating clopyralid toxicity (0.4-2.4 L ha^{-1}) in Camelina sativa under field conditions. A two-year factorial field experiment examined the effects of foliar-applied SA on plant growth, yield components, oxidative biomarkers, and antioxidant enzyme activities. Clopyralid stress significantly reduced biomass, seed and oil yield, and elevated levels of malondialdehyde (MDA) and hydrogen peroxide (H2O2), indicating oxidative damage. SA treatment improved yield traits, enhanced the activity of key antioxidant enzymes (SOD, CAT, POD, APX), and reduced MDA and H2O2 accumulation. These findings suggest that SA application can effectively mitigate clopyralid-induced oxidative stress and may serve as a practical strategy for enhancing Camelina resilience in herbicide-contaminated environments.

Keywords: 1000-seed weight; antioxidant enzymes; crop yield; malondialdehyde; oil content; proline; soluble protein.

1. Introduction

The increasing global population necessitates higher food production from agricultural land systems. Global food demand is projected to increase by 35% to 56% between 2010 and 2050 (van Dijk et al., 2021). Therefore, agriculture

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plays a pivotal role in addressing sustainability challenges across environmental, economic, and social dimensions. Overall, about 80% of future agricultural production growth in developing nations is expected to arise from intensification practices, including higher yields, multiple cropping, and reduced fallow periods (FAO, 2015).

The agricultural model introduced by the Green Revolution in the 1960s, which relied on a limited range of crops and extensive use of chemical inputs, energy, and capital, is no longer viable for addressing the challenges of the 21st century (Da Silva, 2012). However, conventional agricultural practices would impose significant environmental damage, necessitating a shift toward more sustainable solutions. A new "green revolution" must integrate ecosystem-compatible technologies to enhance crop productivity while reducing reliance on harmful agrochemicals (Da Silva, 2012). Climate change exacerbates these challenges, as rising greenhouse gas emissions contribute to increased surface dryness and more frequent droughts (Dai et al., 2018). In recent decades, drought has emerged as a major constraint on sustainable crop production worldwide, particularly in South Asia (Ali et al., 2017). Given these climatic realities, and considering Iran's location—especially East Azerbaijan Province—within arid and semi-arid regions with limited water resources, the identification and evaluation of drought-resistant crops that can thrive under water-deficient conditions are of paramount importance for ensuring food security.

Presently, Iran imports over 95% of its edible oil requirements (Fallah et al., 2019), highlighting the urgent need to introduce alternative oilseed crops better suited to the country's climatic and agricultural conditions. Oilseeds are nutritionally significant due to their high-energy content, providing more than twice the caloric value of other food sources (Tanveer et al., 2023). The Brassicaceae family is among the most agriculturally and economically valuable plant families due to its diversity and adaptability (Anjum et al., 2012). One particularly promising oilseed from this family is camelina (*Camelina sativa*), an ancient oilseed crop.

Camelina has garnered increased attention in recent years due to its adaptability to diverse environmental conditions, low water and nutrient requirements, high resistance to pests and diseases, unique oil composition, and versatility in food, feed, biofuel, and bioproduct applications (Masella et al., 2014; Iskandarov et al., 2014; Bansal & Durrett, 2016; Berti et al., 2016). As a low-input crop, camelina has been increasingly cultivated on marginal, low-fertility lands (Gehringer et al., 2006; McKenzie et al., 2011; Hergert et al., 2016; Matteo et al., 2020). Studies indicate that camelina can serve as a viable alternative to rapeseed in regions with annual precipitation below 250 mm (George et al., 2018). However, a major challenge in camelina cultivation is weed interference (Berti et al., 2016). The excessive and repetitive use of herbicides in modern agriculture has raised concerns about their ecological impact and phytotoxic effects on crops (Singh et al., 2012). Moreover, herbicide overuse has contributed to environmental pollution and the evolution of herbicide-resistant weed species (kırıcı et al., 2021).

Clopyralid is a widely used selective systemic herbicide belonging to the pyridine family, primarily employed for controlling broadleaf weeds species, particularly those in the Asteraceae, Fabaceae, Solanaceae, Polygonaceae, and Violaceae families (Tu et al., 2001; Hong et al., 2023). However, its application can also cause unintended phytotoxicity to non-target plant species, including crops (Hong et al., 2023). Comparative studies on the phytotoxic effects of four herbicides on six camelina genotypes classified clopyralid among the most detrimental herbicides (Sobiech et al., 2020). Clopyralid treatment has been shown to significantly reduce camelina plant density and seed yield (Serdyuk et al., 2021), as well as impair photosynthetic efficiency, chlorophyll fluorescence, and plant physiological traits (Hong et al., 2023). When applied at concentrations exceeding 1.3 μg L^{-1}, clopyralid inhibited net photosynthesis, reduced chlorophyll content, increased oxidative damage through reactive oxygen species (ROS) accumulation, and disrupted chloroplast and mitochondrial ultrastructure in mesophyll cells of Brassica napus (Hong et al., 2023).

Plant growth regulators play a critical role in modulating plant responses to biotic and abiotic stresses (Chakrabarti & Mukherjee, 2003). Salicylic acid (SA), an endogenous phenolic compound, is a key plant hormone that regulates various physiological processes, including thermogenesis, nutrient uptake, ethylene biosynthesis, protein synthesis, flowering, stomatal function, photosynthesis, germination, respiration, senescence, and enzymatic activity (Hayat et al., 2007; Vicente and Placencia, 2011). SA is integral to plant defense signaling pathways and confers resistance against biotic stresses (Khoshru et al., 2023; Stella de Freitas et al., 2019) as well as abiotic stresses such as drought (Ali et al., 2023; Naderi et al., 2023), salinity (Pirasteh-Anosheh et al., 2021; Korkmaz et al., 2020), heavy metals (Rahmah et al., 2023; Saini et al., 2021), extreme temperatures (Rasheed et al., 2022; Awais Ghani et al., 2020), and herbicide toxicity (Khatooni et al., 2022; Li et al., 2022; Shopova et al., 2021; Deng et al., 2020).

Exogenous SA application enhances stress tolerance in economically significant crops by modulating antioxidant enzyme activity and regulating plant defense responses

(Hayat et al., 2010; Khan et al., 2015; Hasanuzzaman et al., 2017). Notably, SA mitigates oxidative stress induced by herbicide exposure (Radvan, 2012; Singh et al., 2017; Wang et al., 2016; Deng et al., 2020) and prevents the accumulation of pesticides and agrochemicals in plant tissues (Cui et al., 2010; Wang & Zhang, 2017).

Despite extensive research on SA's protective effects against various herbicides, no studies have specifically investigated its role in alleviating clopyralid-induced oxidative stress in camelina. Given the need to introduce camelina as a water-efficient crop in northwest Iran, this study aims to evaluate the protective role of SA against clopyralid-induced oxidative stress by assessing alterations in stress biomarkers and antioxidant enzyme activity.

2. Materials and Methods

2.1. Experimental site and plant material

The field experiment was conducted over two consecutive growing seasons (2020 and 2021) at the Experimental Field of the University of Tabriz, East Azerbaijan, situated at 38°01′15″ N, 46°25′35″ E, at an elevation of 1360 m. The site is characterized by a semi-arid climate with an average annual precipitation of 267 mm and mean temperature of 12.2 °C (Figure 1). Table 1 displays the analysis results of physicochemical properties of soil at experimental site carried out according to standard methods.

2.2. Experimental design and treatments

The study was laid out in a factorial randomized complete block design (RCBD) with three replications. The treatments consisted of:

- **Clopyralid herbicide** at five doses: 0 (control), 0.4, 0.8, 1.6, and 2.4 L ha^{-1}

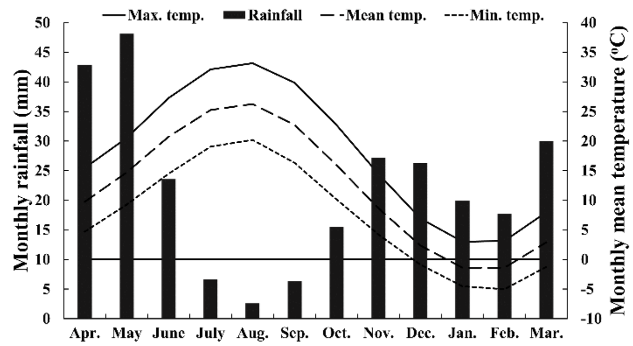

Fig.1 Climatic profile of the experimental site based on 30-year mean temperature and precipitation data

Table 1 Selected physicochemical properties of soil at the experimental site during 2021 and 2022 seasons

Season	Depth	EC	pH	CaCo3	C	N	P	K	Particle size (%)			Texture
	(cm)	(dS m^{-1})	(-)	(%)			(mg kg^{-1})		Sand	Silt	Clay	(-)
2021	0-30	2.71	7.79	7.0	0.74	0.07	15.8	410	64	22	14	Sandy loam
	30-60	2.49	7.96	7.8	0.62	0.06	11.1	400	66	20	14	Sandy loam
2022	0-30	0.26	7.68	5.0	0.61	0.11	4.37	559	26	36	38	Clay loam
	30-60	0.45	7.67	8.4	0.19	0.10	1.5	285	24	30	46	Clay

- **Weeding control**: Hand-weeded (no herbicide)
- **Salicylic acid (SA)** at four concentrations: 0, 0.5, 1.0, and 1.5 mM

A total of 72 experimental plots were established (Table 2).

Table 2 Description of salicylic acid and clopyralid herbicide treatment combinations applied in the field experiment

Weed Control		Salicylic acid (SA) concentrations (mM)			
		0 (S0)	0.5 (S1)	1.0 (S2)	1.5 (S3)
Hand-weeded	W	WS0	WS1	WS2	WS3
Clopyralid concentrations (L ha^{-1})	0 (C0) control	C0S0	C0S1	C0S2	C0S3
	0.4 (C1)	C1S0	C1S1	C1S2	C1S3
	0.8 (C2)	C2S0	C2S1	C2S2	C2S3
	1.6 (C3)	C3S0	C3S1	C3S2	C3S3
	2.4 (C4)	C4S0	C4S1	C4S2	C4S3

Clopyralid was applied at the 4–6 leaf stage (Khatooni et al., 2022) using a backpack sprayer equipped with a boom angle of 80°, delivering a spray volume of 0.56 L min^{-1} at a pressure of 1.5 bar. In the hand-weeding treatment (W), weeds were manually removed and plots were maintained weed-free throughout the growing season. SA was foliar-sprayed three days prior to herbicide application using a backpack sprayer until runoff.

Each plot measured 2 m × 3 m with 20 cm row spacing and 10 cm plant spacing. Camelina was sown in the third decade of April in both years using a hand harrow. The seeds were planted at a depth of 1 cm. Seedlings were thinned at the two-leaf stage to achieve a final plant density of 200 plants m^{-2}. Standard agronomic practices for camelina cultivation were followed throughout the season.

2.3. Physiological and biochemical assessments

To evaluate oxidative stress and biochemical responses, ten leaves per plot were randomly sampled three days after herbicide application and immediately stored at −80°C (Ananieva et al., 2002) for subsequent physiological measurements.

2.3.1. Proline content determination

Proline content was measured following Bates et al. (1973). Approximately 0.5 g of plant tissue was homogenized in 5 mL of 3% aqueous sulfosalicylic acid, followed by heating the supernatant with 2 mL of ninhydrin acid and 2 mL of acetic acid in a water bath at 100°C for 1 hour. The reaction was stopped by cooling in an ice bath, and 4 mL of toluene was added to extract the colored phase. Absorbance was recorded at 520 nm using a spectrophotometer, with proline concentration determined from a standard curve based on fresh weight.

2.3.2. Soluble protein content determination

Camelina leaf tissues were homogenized at 4°C in 1 M Tris-HCl buffer (pH 6.8) containing 5 mM dithiothreitol (DTT), 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 mM NaCl. The homogenate was centrifuged at 13,000 rpm for 30 min at 4°C, and the supernatant was stored at −70°C for protein analysis. Soluble protein content was determined using Bradford's method (Bradford, 1976) with bovine serum albumin (BSA) as a standard. Absorbance was measured at 595 nm.

2.3.3. Hydrogen peroxide (H_2O_2) determination

Leaf tissues (1 g) were homogenized in 3 mL of 50 mM phosphate buffer (pH 6.5) and centrifuged at 6,000 rpm for 25 min. The extract (3 mL) was mixed with 1 mL of 0.1% titanium sulfate in 20% sulfuric acid, then centrifuged at 6,000 rpm for 15 min. The precipitate was dissolved in 5 mL of 2 M sulfuric acid, and absorbance was measured at 410 nm using an extinction coefficient (E = 0.28 μmol^{-1} cm^{-1}).

2.3.4. Lipid peroxidation (malondialdehyde content) determination

Malondialdehyde (MDA) content was measured according to Davey et al. (2005). Leaf tissues (0.5 g) were homogenized in 50 mM phosphate buffer (pH 7.0), and thiobarbituric acid (0.5%) containing trichloroacetic acid (20%) was added. After heating at 95°C for 30 min, the reaction was stopped by cooling on ice. The absorbance of the clear supernatant was measured at 532 and 600 nm, and lipid peroxidation was expressed as nmol g^{-1} fresh weight using an extinction coefficient (E = 155 mM^{-1} cm^{-1}).

2.4. Antioxidant enzyme activity assays

The activity of antioxidant enzymes, including catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and peroxidase (POD), was analyzed. Leaf tissue (0.5 g) was ground using a pre-chilled mortar and pestle on ice and homogenized in 1 mL of extraction buffer. The extraction buffer consisted of 100 mM potassium phosphate buffer (pH 7.0), 1% polyvinylpyrrolidone (PVP), and 0.5% Triton X-100 (Askari & Ehsanzadeh, 2015). The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C, and the resulting supernatant was collected for enzymatic activity assays.

2.4.1. Catalase (CAT)

Catalase (CAT) activity was measured following a modified version of Aebi (1983) by quantifying the decomposition of H_2O_2 using spectrophotometric analysis at 240 nm. The specific activity of catalase was calculated by dividing the volumetric activity by the total protein content in the extract, as determined by the Bradford method (Bradford, 1976).

2.4.2. Ascorbate peroxidase (APX)

Ascorbate peroxidase (APX) activity was assessed according to Nakano and Asada (1981) by measuring the decrease in absorbance at 290 nm. The assay reaction was initiated using 2.95 mL of assay buffer, which contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM H_2O_2, 5 mM ascorbate, and 50 µL of enzyme extract.

2.4.3. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was quantified using the nitro blue tetrazolium (NBT) reduction assay, as described by Giannopolites and Ries (1977). The reaction mixture consisted of 0.3 mL of 13 mM methionine solution, 0.3 mL of 75 µM NBT solution, 0.3 mL of 10 µM EDTA-Na_2 solution, 0.3 mL of 2 µM riboflavin solution, 1.5 mL of 0.05 M phosphate buffer (pH 7.8), 0.25 mL of distilled water, and 0.05 mL of enzyme extract. The samples were exposed to fluorescent light for 20 minutes, after which absorbance was measured at 560 nm.

2.4.4. Peroxidase (POD)

Peroxidase (POD) activity was determined using the guaiacol oxidation method, as described by Hernández et al. (2000). The reaction mixture consisted of 1.0 mL of 0.05 M phosphate buffer (pH 7.0), 0.95 mL of 0.05 M guaiacol, and 1.0 mL of 2% H_2O_2, to which 0.05 mL of enzyme extract was added. The enzymatic reaction was monitored by measuring absorbance at 470 nm using a spectrophotometer.

2.5. Harvesting and yield attributes measurements

Camelina plants were harvested at full maturity (late July) from a 2 × 1.5 m area in each plot. The harvested plants were air-dried for 7 days, and biomass weight was recorded. Plant height, number of pods per plant, and number of seeds per pod were measured on ten randomly selected plants from each plot. Seed yield was recorded, and 1,000-seed weight was determined using an electronic seed counter and a 0.01 g precision balance, with three replications per plot.

2.6. Statistical analysis

Data were subjected to analysis of variance (ANOVA) using MSTAT-C software. Treatment means were compared using the Least Significant Difference (LSD) test at $p < 0.05$. Interactive effects between herbicide and SA treatments were examined using factorial analysis.

3. Results

3.1. Effects of clopyralid and salicylic acid on yield and agronomic traits

The main effects of herbicide stress and salicylic acid on biomass, seed yield, oil yield, plant height, number of pods per plant, and 1000-seed weight were statistically significant ($P < 0.05$) (Table 3). The interaction between herbicide and SA treatments significantly influenced 1000-seed weight ($P < 0.05$) (Fig. 2), but had no significant effects on other yield parameters. Hand weeding significantly increased biomass (10%), seed yield (20%), and oil yield (23%) compared to the untreated control. Clopyralid application at 0.4, 0.8, 1.6, and 2.4 L ha^{-1} reduced biomass by 8%, 10%, 15%, and 11%, respectively, seed yield by 10%, 12%, 18%, and 16%, and oil yield by 6%, 7%, 13%, and 12%, respectively (Table 3). Biomass, seed yield, and oil yield decreased with increasing herbicide concentrations up to 1.6 L ha^{-1}.

Plant height was not significantly affected by clopyralid at any concentration. However, hand weeding significantly increased plant height by 10% compared to the control. Hand weeding also led to a 27% increase in the number of pods per plant, whereas clopyralid at 0.8 and 2.4 L ha^{-1} reduced the number of pods per plant by 22% and 26%, respectively, relative to the control. No significant differences were observed among herbicide treatments in the number of seeds per pod.

Low clopyralid concentrations (0.4 and 0.8 L ha^{-1}) reduced 1000-seed weight compared to the hand weeding treatment but not the control. However, higher clopyralid concentrations (1.6 and 2.4 L ha^{-1}) significantly reduced 1000-seed weight compared to the control.

Table 3 Effects of salicylic acid and clopyralid herbicide concentrations on growth and yield parameters of *Camelina sativa*

Treatment	Biomass (kg ha^{-1})	Seed yield (kg ha^{-1})	Seed oil (%)	Oil yield (kg ha^{-1})	Plant height (cm)	Pod (plant^{-1})	Seed (pod^{-1})	1000 seed weight (g)
W	5931.3 a	1331.2 a	32.6 a	460.0 a	74.6 a	128.7 a	12.7 a	2.03 a
C0	5427.1 b	1106.3 b	31.5 a	372.8 b	68.0 b	101.3 b	11.6 a	1.80 ab
C1	4990.9 c	990.2 c	32.4 a	351.7 bc	70.0 ab	98.6 b	12.9 a	1.64 b
C2	4869.0 c	976.9 cd	33.6 a	346.1 bc	65.6 b	79.0 c	11.5 a	1.68 b
C3	4610.3 d	911.0 e	32.4 a	324.7 c	64.3 b	97.0 b	11.3 a	1.38 c
C4	4842.5 cd	934.0 de	33.9 a	328.1 c	62.5 b	74.5 c	11.2 a	1.31 c
S0	4207.4 d	908.8 c	32.9 a	308.0 c	60.3 c	75.7 c	10.9 c	1.50 b
S1	5142.7 c	1052.3 b	33.9 a	378.2 b	62.9 c	93.9 b	11.2 bc	1.53 b
S2	5438.6 b	1081.2 b	31.5 a	362.8 b	68.6 b	101.5 ab	12.4 ab	1.73 a
S3	5659.1 a	1124.1 a	33.3 a	406.6 a	78.1 a	114.8 a	12.8 a	1.81 a
C.V. (%)	8.1	8.3	12.2	12.3	16.6	34.3	23.8	22.1

Data represent the means within treatments (Clopyralid (n=12) and Salicylic acid (n=18)). Means having different letters are significantly different according to Duncan's tests at $P \leq 0.05$. W (hand weeding), C0 (control, without herbicide use), C1, C2, C3 and C4 (clopyralid application at 0.4, 0.8, 1.6 and 2.4 L ha^{-1}, respectively), S0, S1, S2 and S3 (foliar application of salicylic acid at 0, 0.5, 1 and 1.5 mM respectively 3 days after herbicide application).

Foliar application of SA at 0.5, 1.0, and 1.5 mM significantly increased biomass by 22%, 29%, and 35%, respectively, relative to the control (SA = 0 mM) (Table 3). SA application at 0.5, 1.0, and 1.5 mM increased seed yield by 16%, 19%, and 24%, respectively, compared to the control.

Oil yield increased significantly with SA application, with enhancements of 23%, 18%, and 32% at 0.5, 1.0, and 1.5

mM, respectively, compared to the S0.

All SA concentrations increased plant height compared to the control, except for 0.5 mM SA. The increases in plant height at 1.0 and 1.5 mM SA were 14% and 30%, respectively.

SA also significantly enhanced the number of pods per plant, with 1.5 mM SA resulting in a 52% increase compared to the control. However, the difference between 1.0 mM SA and both 0.5 and 1.5 mM SA was not statistically significant. The number of seeds per pod also increased with rising SA concentrations, with the highest value observed at 1.5 mM SA (17% higher than the control). The highest 1000-seed weight was observed in the hand weeding treatment with 1.0 mM SA, which was 52% higher than the control (without weeding or SA application). Conversely, the lowest 1000-seed weight was recorded under high clopyralid concentration (2.4 L ha^{-1}), both with and without 1.0 mM SA (Fig. 2).

In herbicide-free treatments (hand weeding and control), the highest 1000-seed weight was obtained with 1.0 mM SA. However, in herbicide-treated plots, particularly at 2.4 L ha^{-1}, the highest 1000-seed weight was observed at 1.5 mM SA (Fig. 2).

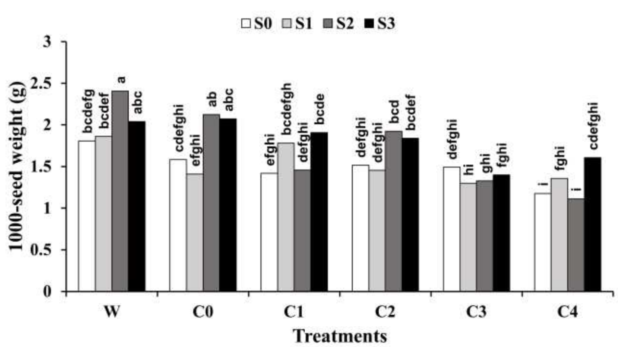

Fig. 2 Effect of foliar-applied salicylic acid on 1000-seed weight of *Camelina sativa* under different clopyralid herbicide concentrations. The results are mean of three replicates (n = 3). Means having different letters are significantly different according to Duncan's test at P ≤ 0.05. W (hand weeding), C0 (control, without herbicide use), C1, C2, C3 and C4 (clopyralid application at 0.4, 0.8, 1.6 and 2.4 L ha^{-1}, respectively), S0, S1, S2 and S3 (foliar application of salicylic acid at 0, 0.5, 1.0 and 1.5 mM respectively 3 days after herbicide application).

3.2. Oxidative stress markers

3.2.1. Leaf proline and protein content

The main and interactive effects of herbicide and SA treatments on leaf proline and soluble protein content were statistically significant (P < 0.05) (Fig. 3). Clopyralid application increased leaf proline content while decreasing soluble protein content, with a greater impact observed at higher concentrations. Compared to the control, proline content increased by 15%, 16%, 31%, and 32% at 0.4, 0.8, 1.6, and 2.4 L ha^{-1}, respectively. Similarly, protein content decreased by 10%, 16%, 17%, and 21%, respectively, at these herbicide concentrations.

SA application significantly increased both proline and protein content, with the effect being concentration-dependent (Fig. 3). Compared to the control (SA = 0 mM), proline content increased by 66%, 131%, and 200% at 0.5, 1.0, and 1.5 mM SA, respectively, while protein content increased by 27%, 34%, and 40%, respectively.

The highest proline content was observed in plants treated with 1.6 and 2.4 L ha^{-1} clopyralid in conjunction with 1.5 mM SA, whereas the lowest content was recorded in control plants that received no SA (Fig. 3). Similarly, the highest protein content was found in plants treated with 1.5 mM SA and no herbicide, while the lowest was observed in plants subjected to high herbicide concentrations without SA application (Fig. 3).

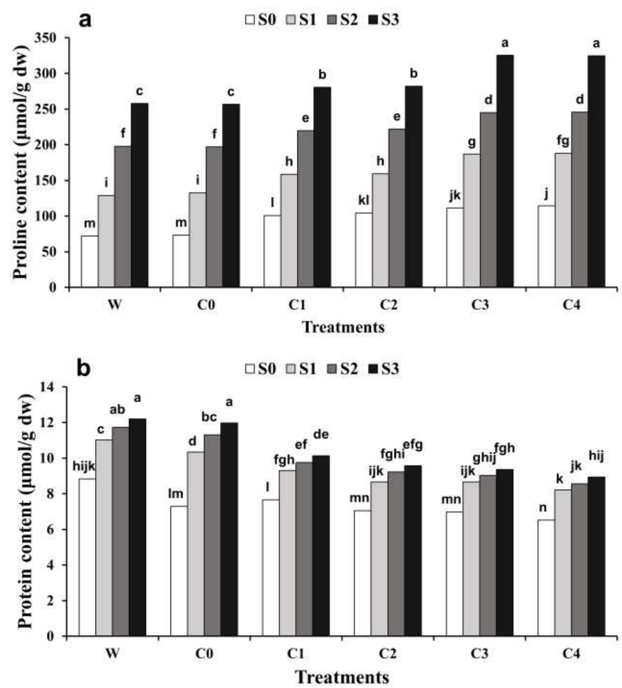

Fig. 3 Effects of salicylic acid application on leaf proline (a) and soluble protein (b) content in *Camelina sativa* under clopyralid herbicide stress. The results are mean of three replicates (n = 3). Means having different letters are significantly different according to Duncan's test at P ≤ 0.05. W (hand weeding), C0 (control, without herbicide use), C1, C2, C3 and C4 (clopyralid application at 0.4, 0.8, 1.6 and 2.4 L ha^{-1}, respectively), S0, S1, S2 and S3 (foliar application of salicylic acid at 0, 0.5, 1 and 1.5 mM respectively 3 days after herbicide application).

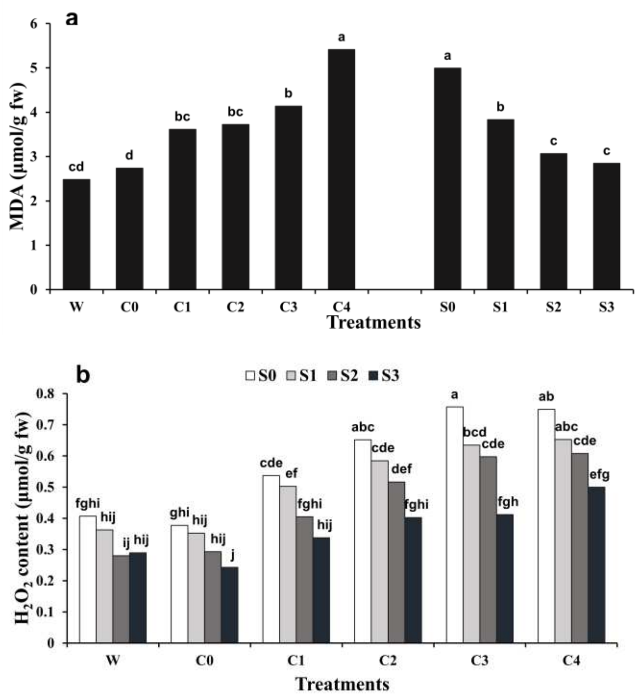

Fig. 4 Effects of clopyralid herbicide and salicylic acid treatments on malondialdehyde (MDA) (a) and hydrogen peroxide (H_2O_2) (b) content in *Camelina sativa* leaves. In case of the MDA, the results are mean of 12 replicates (n=12) for herbicide and 18 replicates (n=18) for salicylic acid and in case of H_2O_2, the results are mean of three replicates (n = 3). Means having different letters are significantly different according to Duncan's test at $P \leq 0.05$. W (hand weeding), C0 (control, without herbicide use), C1, C2, C3 and C4 (clopyralid application at 0.4, 0.8, 1.6 and 2.4 L ha^{-1}, respectively), S0, S1, S2 and S3 (foliar application of salicylic acid at 0, 0.5, 1 and 1.5 mM, respectively 3 days after herbicide application).

3.2.2. Malondialdehyde (MDA) and Hydrogen Peroxide (H_2O_2) Content

The main effects of herbicide and salicylic acid (SA) treatments on malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents were statistically significant at the 5% level, while their interaction was significant only for H_2O_2 ($p < 0.05$). As shown in Figure 4, MDA and H_2O_2 levels in Camelina leaves increased significantly under clopyralid treatment compared to the control. No significant differences were observed between the hand weeding (W) and untreated control (C0) treatments for either parameter. Among the herbicide treatments, the highest MDA content was recorded at the 2.4 L ha^{-1} application rate (Fig. 4 a). Clopyralid applied at 0.4, 0.8, 1.6, and 2.4 L ha^{-1} increased MDA content by 32%, 36%, 51%, and 97%, respectively, and H_2O_2 content by 36%, 64%, 83%, and 91%, compared to the control.

SA application significantly reduced both MDA and H_2O_2 levels relative to the untreated SA control (S0). Specifically, MDA content decreased by 23%, 38%, and 43% at 0.5, 1.0, and 1.5 mM SA, respectively, while H_2O_2 content declined by 11%, 22%, and 37%. The highest H_2O_2 accumulation occurred in the C3S0 and C4S0 treatments (high herbicide doses without SA), whereas the lowest was observed in C0S3 (maximum SA concentration without herbicide) (Fig. 4 b).

3.3. Antioxidant enzyme activities

3.3.1. Catalase

The activity of the catalase (CAT) enzyme was significantly influenced by herbicide and salicylic acid (SA) treatments ($P<0.05$); however, the interaction between these factors did not have a significant effect on enzyme activity. Among the herbicide treatments, the highest CAT activity was observed in treatments C4 (82.0 U.g^{-1}) and C3 (81.0 U.g^{-1}), whereas the lowest activity was recorded in treatments W (75.1 U.g^{-1}) and C0 (74.0 U.g^{-1}). The application of herbicides at concentrations of 0.4, 0.8, 1.6, and 2.4 L ha^{-1} led to an increase in CAT activity by 3.2%, 4.9%, 7.9%, and 9.3%, respectively, compared to the untreated control (Fig. 5 a).

All SA concentrations exhibited significant differences from one another ($P<0.05$), with each concentration belonging to a distinct statistical group (Figure 5 a). Among the SA treatments, the application of 1.5 mM SA (S3) resulted in the highest CAT activity (89.3 U.g^{-1}), whereas the S0 led to the lowest activity (61.1 U.g^{-1}). The application of SA at concentrations of 0.5, 1.0, and 1.5 mM increased CAT activity by 27.2%, 37.5%, and 46.1%, respectively, compared to the control (without SA application).

3.3.2. Ascorbate peroxidase

The activity of ascorbate peroxidase (APX) was significantly influenced by herbicide treatments, salicylic acid (SA) application, and their interaction ($P<0.05$). Among the herbicide treatments, the highest APX activity was observed at herbicide concentrations of 1.6 and 2.4 L ha^{-1}. In contrast, the lowest APX activity was recorded in the hand weeding (W) and untreated control (C0) treatments. Compared to the control, herbicide application at 0.4, 0.8, 1.6, and 2.4 L ha^{-1} increased APX activity by 21%, 28%, 55%, and 60%, respectively.

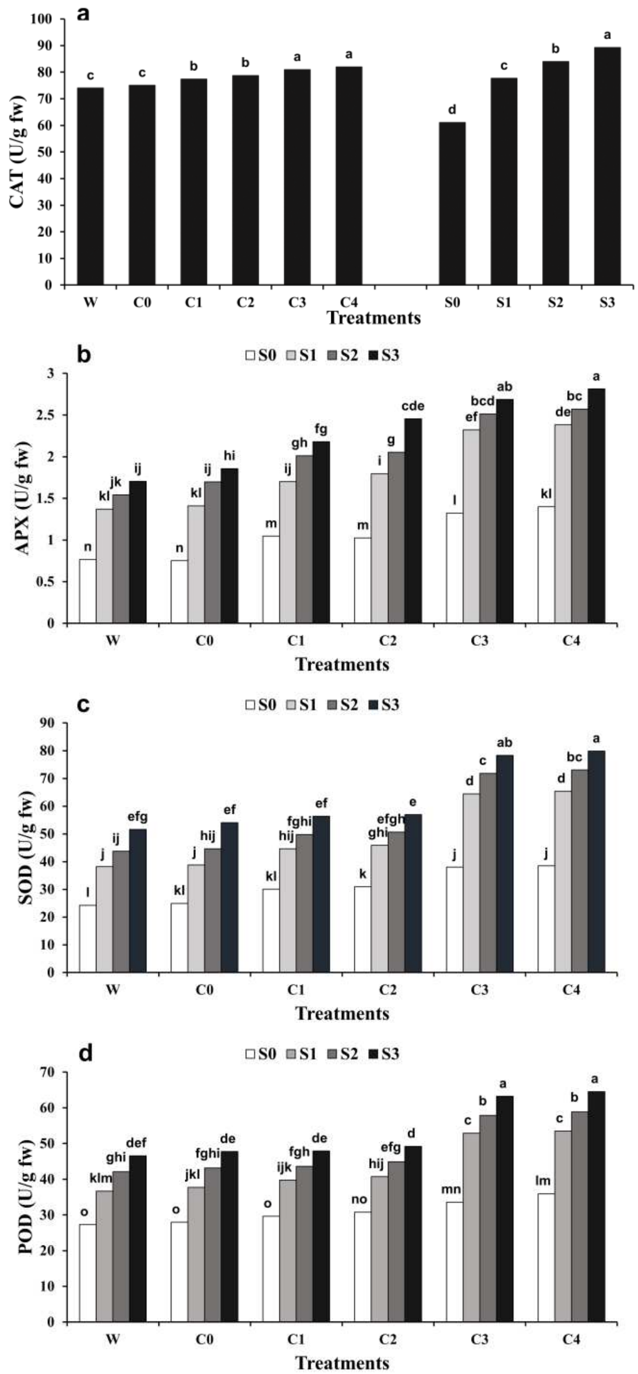

Fig. 5 Effects of clopyralid herbicide and salicylic acid treatments on the activities of antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), and peroxidase (POD) in *Camelina sativa* leaves. In case of the CAT, the results are mean of 12 replicates (n=12) for herbicide and 18 replicates (n=18) for salicylic acid and in cases of APX, SOD and POD, the results are mean of three replicates (n = 3). Means having different letters are significantly different according to Duncan's test at $P \leq 0.05$. W (hand weeding), C0 (control, without herbicide use), C1, C2, C3 and C4 (clopyralid application at 0.4, 0.8, 1.6 and 2.4 L ha^{-1}, respectively), S0, S1, S2 and S3 (foliar application of salicylic acid at 0, 0.5, 1 and 1.5 mM respectively 3 days after herbicide application).

SA treatments also had a significant effect on APX activity, with all treatments differing significantly from one another (P<0.05). The highest APX activity was observed at an SA concentration of 1.5 mM, whereas the lowest activity was recorded in the absence of SA application. Compared to the S0, SA application at 0.5 mM (S1), 1.0 mM (S2), and 1.5 mM (S3) increased APX activity by 74%, 96%, and 117%, respectively. Treatment combinations with higher concentrations of both herbicide and SA exhibited greater APX activity, while those without herbicide application or with low SA concentrations showed lower APX activity. Accordingly, the highest APX activity was recorded in the C4S3 combination, which did not significantly differ from the C3S3. In contrast, the C0S0 and WS0 combinations exhibited the lowest APX activity (Figure 5 b). The increase in APX activity in the C4S3 (highest herbicide and SA concentrations) compared to the C0S0 (no herbicide or SA application) was 274%.

3.3.3. Superoxide dismutase

The activity of the superoxide dismutase (SOD) enzyme was significantly affected by herbicide treatments, salicylic acid (SA) application, and their interaction (P<0.05). Among the herbicide treatments, the highest SOD activity was observed at concentrations of 2.4 L ha^{-1} (C4) and 1.6 L ha^{-1} (C3), while the lowest activity was recorded in the untreated control (C0) and hand weeding (W) treatments. In contrast, herbicide application at concentrations of 0.4, 0.8, 1.6, and 2.4 L ha^{-1} increased SOD activity by 11%, 14%, 55%, and 58%, respectively, compared to the control.

All SA concentrations had a significant effect on SOD activity, with statistically significant differences among them (P<0.01). The highest SOD activity was recorded at an SA concentration of 1.5 mM, while the lowest activity was observed in the absence of SA application. Compared to the control (S0), SA application at concentrations of 0.5 mM, 1.0 mM, and 1.5 mM increased SOD activity by 59%, 79%, and 102%, respectively.

Treatments containing high concentrations of both herbicide and SA, exhibited higher SOD activity, while those without herbicide and SA showed lower SOD activity. Accordingly, the highest SOD activity was observed in the C4S3 and C3S3, whereas the lowest activity was recorded in WS0, C0S0, and C1S0. (Fig. 5 c).

In the absence of herbicide application (control), SA application at concentrations of 0.5, 1.0, and 1.5 mM increased SOD activity by 56%, 79%, and 117%, respectively. Conversely, in the absence of SA, herbicide application at concentrations of 0.4, 0.8, 1.6, and 2.4 L ha^{-1} increased SOD activity by 20%, 24%, 53%, and 54%, respectively. The highest SA concentration combined with the highest herbicide concentration (C4S3) increased SOD activity by 220% compared to the untreated control (C0S0).

3.3.4. Peroxidase

Herbicide treatments, salicylic acid (SA) application, and their interaction had a significant effect on peroxidase (POD) activity (P<0.05). Among the herbicide treatments, clopyralid application at concentrations of 1.6 L ha^{-1} and 2.4 L ha^{-1} resulted in the highest POD activity. Although the hand weeding treatment (W) exhibited lower POD activity, it did not differ significantly from the untreated control (C0) or the 0.4 L ha^{-1} herbicide treatment (C1). The application of clopyralid at concentrations of 0.4 (C1), 0.8 (C2), 1.6 (C3), and 2.4 L ha^{-1} (C4) increased POD activity by 3%, 6%, 33%, and 36%, respectively.

SA application significantly and actively increased POD activity. As a result, each SA treatment formed a distinct statistical group. Compared to the S0, SA application at concentrations of 0.5 (S1), 1.0 (S2), and 1.5 mM (S3) increased POD activity by 41%, 57%, and 72%, respectively.

The mean comparison of the interaction effects demonstrated that SA application enhanced the effect of herbicide treatments on POD activity. Given the additive effects of herbicide and SA, particularly at higher concentrations, it is evident that treatment combinations containing both high herbicide and SA concentrations exhibited the highest POD activity. In contrast, combinations without herbicide or SA application displayed the lowest POD activity. The C4S3 (highest herbicide and SA concentrations) exhibited the highest POD activity, with no statistically significant difference (P<0.05) from the C3S3. Conversely, the WS0 and C0S0 combinations, which did not receive herbicide or SA, exhibited the lowest POD activity, although they were not significantly different from the C1S0 and C2S0 treatments (Fig. 5 d).

In the absence of SA, clopyralid application at concentrations of 0.4 (C1), 0.8 (C2), 1.6 (C3), and 2.4 L ha^{-1} (C4) increased POD activity by 6%, 10%, 20%, and 28%, respectively, compared to the untreated control. Similarly, in the absence of herbicide application (control), SA application at concentrations of 0.5 (S1), 1.0 (S2), and 1.5 mM (S3) increased POD activity by 35%, 54%, and 71%, respectively, compared to S0.

4. Discussion

Clopyralid stress significantly inhibited the growth and productivity of *Camelina sativa*, and triggered oxidative stress. Considering the well-documented role of salicylic acid (SA) in mitigating abiotic stresses, this study investigated the physiological mechanisms underlying SA-mediated modulation of camelina's response to clopyralid toxicity. Oxidative stress is a common physiological disturbance in plants exposed to adverse environmental conditions. It arises primarily from an imbalance between the generation of reactive oxygen species (ROS) and the plant's antioxidant defense systems, ultimately impairing photosynthesis and growth and reducing yield (Ananieva et al., 2002). Multiple studies have reported that herbicides negatively affect plant growth and productivity across species, including camelina (Jha and Stougaard, 2013; Serdyuk et al., 2021; Frizen et al., 2021; Subiech et al., 2020), sugar beet (Li et al., 2022), tomato (Singh et al., 2017), wheat (Shopova et al., 2021), and *Arabidopsis thaliana* (Kim et al., 2003). Our findings confirm that clopyralid significantly reduced plant height, biomass, seed and oil yield, and 1000-seed weight in camelina (Table 3), in agreement with earlier reports (Jha and Stougaard, 2013; Subiech et al., 2020). SA application alleviated the adverse effects of clopyralid, likely by promoting the accumulation of protective molecules that counteract herbicide toxicity and enhance biomass production (Singh et al., 2017). SA is also known to enhance a wide range of metabolic and physiological activities in plants (Askari and Ehsanzadeh, 2015; Maghsoudi et al., 2019). In our study, SA concentrations ranging from 1 to 1.5 mM significantly reduced the detrimental effects of clopyralid on camelina growth parameters. Similar protective roles of SA against herbicide-induced stress have been documented in rice (Deng et al., 2020), tomato (Singh et al., 2017), wheat (Shopova et al., 2021), Arabidopsis (Kim et al., 2003), and sugar beet (Li et al., 2022).

Proline accumulation is a common response to various stressors (Hayat et al., 2012), serving roles in osmotic adjustment, energy storage, and as a carbon and nitrogen source. It also acts as a ROS scavenger, protecting cellular membranes and enzymatic systems (Ruiz et al., 2002; Szabados and Savouré, 2010; Hare et al., 1998). In this study, proline content increased significantly in clopyralid-treated Camelina (Fig. 3 a), corroborating findings that herbicides stimulate proline accumulation (Sergiev et al., 2006). Elevated proline levels under various abiotic stresses—including herbicide exposure (Shopova et al., 2021; Sergiev et al., 2020; Spormann et al., 2019; Kireji et al., 2021; Soarez et al., 2019), drought (Nazar et al., 2011; Tanveer et al., 2023; Ali et al., 2023), and salinity (Yusuf et al., 2008; Kumar et al., 2022; Sunaina and Singh, 2014)—are widely documented. SA treatment further enhanced proline levels under clopyralid stress, especially at 1.5 mM

SA (Fig. 3 a). Similar SA-induced proline increases have been reported under drought and salinity stress (Nazar et al., 2011; Tanveer et al., 2023; Ali et al., 2023; Ahmad et al., 2011). SA can also conjugate with amino acids such as proline and arginine, contributing to enhanced environmental stress tolerance (Baghizadeh and Hajmohammadrezaei, 2011). Additionally, SA promotes the accumulation of osmoprotectants like glycine betaine, soluble sugars, and amines, aiding in osmotic regulation (Khan et al., 2015).

Abiotic stresses such as herbicide exposure impair various metabolic processes during seed germination and development, particularly protein synthesis (Sharp, 1988). Lipid peroxidation damages organelle membranes and disrupts protein functions (Farmer and Mueller, 2013). Plants respond by producing protective compounds, including soluble proteins (Shahzad et al., 2019). In this study, clopyralid significantly reduced soluble protein content in Camelina, whereas SA application mitigated this effect (Fig. 3 b). Similar reductions in protein content were reported under drought (Ahmad et al., 2021a,b; Khalid et al., 2021; Nazim et al., 2023) and salinity stress (Khalid et al., 2021). Herbicides interfere with the shikimate pathway and biosynthesis of aromatic amino acids (phenylalanine, tyrosine, tryptophan), disrupting protein production (Jaworski, 1972). Our findings align with earlier studies in tomato, Arabidopsis, and wheat (Singh et al., 2012; Kim et al., 2003; Cobb and Reade, 2010).

MDA, a byproduct of lipid peroxidation, is a reliable indicator of membrane damage (Belkhadi et al., 2010), and increased electrolyte leakage reflects enhanced membrane permeability. Clopyralid exposure, especially at 2.4 L ha^{-1}, elevated MDA levels significantly (Fig. 4-a), in line with reports linking higher herbicide concentrations to MDA accumulation (Jin et al., 2010; kırıcı et al., 2021). SA application reduced MDA content, highlighting its role in mitigating oxidative damage (Fig. 4-a), consistent with findings in sugar beet (Li et al., 2022), tomato (Singh et al., 2017), chickpea (Singh et al., 2016), and barley (Ananieva et al., 2002).

Increased H_2O_2 accumulation in clopyralid-treated Camelina leaves further indicated oxidative stress (Fig. 4-b), while SA pretreatment reduced H_2O_2 levels below control values. Excess H_2O_2 disrupts photosynthesis and induces oxidative damage (Møller, 2001; Urban et al., 1997). Our findings agree with reports that SA limits H_2O_2 accumulation, enhances antioxidant enzyme activity, and preserves cellular membrane integrity (Popova et al., 2002; Cui et al., 2010). This dual role—detoxification of ROS and restriction of herbicide penetration—underlines SA's protective efficacy (Umezawa et al., 2004).

SA also regulates the activity of key antioxidant enzymes. CAT and APX are central to H_2O_2 detoxification; APX operates effectively at low H_2O_2 concentrations, while CAT acts rapidly at higher levels (Foyer and Noctor, 2009). SOD converts superoxide radicals into H_2O_2, while POD detoxifies H_2O_2 using guaiacol. In this study, clopyralid-induced oxidative stress was marked by elevated MDA and H_2O_2 levels, while SA significantly enhanced the activities of SOD, POD, CAT, and APX (Fig. 5). These findings are consistent with reports that SA strengthens antioxidant defenses in herbicide-stressed plants (Cui et al., 2010; Liang et al., 2012; Hasanuzzaman et al., 2017).

Moreover, SA modulates antioxidant enzyme activity via regulation of their expression and activation status (Apel and Hirt, 2004). Our results also showed that SA increased APX and CAT activities under clopyralid stress, enhancing detoxification of accumulated H_2O_2 (Hong et al., 2023; Monjezi et al., 2015). The rise in APX activity correlated with elevated H_2O_2 and MDA content, decreased photosynthetic pigments (data not shown), and increased proline levels, suggesting a coordinated antioxidant response (Liu et al., 2014; Hasanuzzaman et al., 2021). Overall, the enhanced activity of antioxidant enzymes (SOD, CAT, POD, and APX) and reduced ROS markers (H_2O_2 and MDA) in SA-treated plants confirm SA's role in strengthening the antioxidant defense system under clopyralid-induced stress. These results are consistent with previous findings in various species exposed to herbicide or abiotic stress (Singh et al., 2017; Shopova et al., 2021; Spormann et al., 2019; Kim et al., 2003; Radwan, 2012).

5. Conclusion

In summary, the findings of this study demonstrate that although clopyralid herbicide induced oxidative stress and reduced growth and yield parameters in *Camelina sativa*, exogenous application of salicylic acid (SA) effectively mitigated the herbicide-induced damage by enhancing the plant's antioxidant defense mechanisms. Clopyralid treatments increased the levels of proline, malondialdehyde (MDA), and hydrogen peroxide (H_2O_2), decreased the soluble protein content, and altered the activities of antioxidant enzymes (CAT, APX, SOD, and POD) in a concentration-dependent manner. Moreover, the results suggest that the alleviation of clopyralid's adverse effects is closely related to the concentration of pre-applicated SA. Plants treated with SA exhibited improved tolerance to herbicide stress, characterized by a stronger antioxidant defense system and better growth and yield performance. For recommended doses of clopyralid, 1 mM SA proved sufficient to enhance stress tolerance. However, under higher herbicide concentrations, the application of 1.5 mM

SA may be necessary to bolster the antioxidant defense response.

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