Journal of Environmental Studies [JES]
An International Journal
https://jesj.journals.ekb.eg/
doi: *10.21608/jesj.2025.375403.1126*

Ascorbic acid mitigates oxidative stress caused by fusilade herbicide in wheat plants

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Citation: *Radwan, D.E.M., Salem, J.M., Nasr S.A.(2025). Ascorbic acid mitigates oxidative stress caused by fusilade herbicide in wheat plants. Journal of Environmental Studies, Vol. 38(1): 61-72.*

Article Information
Received 14 April 2025,
Revised 15 May 2025,
Accepted 18 May 2025,
Published online. 1 June 2025

Abstract:

The purpose of this study was to determine the role of ascorbic acid in the mitigation of fusilade herbicide-induced oxidative stress in wheat plants. Various physiological and biochemical parameters were assessed, including contents of pigments, carbohydrates, protein, proline, total free amino acid, antioxidant enzyme activity (peroxidase, superoxide dismutase, ascorbate peroxidase, and catalase), antioxidant metabolites (phenolic, flavonoids, hydrogen peroxide content, and lipid peroxidation levels). fusilade application resulted in chlorosis and yellowing of leaves and reduced pigment, carbohydrate, protein, proline and total free amino acid content. Moreover, fusilade treatment significantly increased lipid peroxidation, by increasing malondialdehyde (43.3%) and hydrogen peroxide (23%) contents over the control. Under fusilade treatment, ascorbate peroxidase and catalase activities decreased, superoxide dismutase and peroxidase activities were increased. Moreover, fusilade treatment had a deleterious impact on flavonoids, phenolics, and overall antioxidant activity. Results showed an enhancement in the content of pigments, carbohydrates, protein, proline, total free amino acid, and the activity of antioxidant enzymes. The combination treatment of fusilade and ascorbic acid decreased lipid peroxidation by 56% compared to that only treated with fusilade. Using ascorbic acid could be a good way to lessen the negative effects of oxidative damage from herbicides, which would increase plant resistance against fusilade stress.

Keywords: herbicides, fusilade, antioxidant, ascorbic acid, oxidative stress, wheat.

1. Introduction

Wheat (*Triticum aestivum* L.*)* is conceder as one of the most significant crops both nationally and globally, particularly as the source of staple foods, and provides approximately 20% of calories and protein in the human diet (El-Megeed *et al*., 2022; Mohammed & Faisal, 2021). With increasing population in the world, the effort of scientists are focusing to increase the productivity and quality of wheat grains, but cultivation of wheat plants faces many problems that affect its productivity, including harmful herbs. So, farmers use different types of herbicides in wheat fields to get rid of the herbs without affecting wheat plants. One of these ways is the use of antioxidant chemicals that are used with herbicides treated plants.

A common selective post-emergence herbicide used to manage annual and perennial grass weeds in a range of broadleaf crops is fusilade, such as wheat, corn, soybeans, sugar beets, vegetables, and fruits *(Fayez et al*., 2014). Fluazifop-P-butyl, the active component of fusilade, is a member of the aryloxyphenoxypropionate (AOPP) class of herbicides which also includes haloxyfop-P-methyl, quizalofop-P-ethyl, and diclofop-methyl (Isah *et al*., 2020). The acetyl-CoA carboxylase (ACCase) enzyme, which is necessary to produce fatty acids in the targeted weed species, is inhibited by these herbicides. Fusilade inhibits the synthesis of essential

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fatty acids, which prevents sensitive grassland weeds from growing and eventually results in their death(Rana & Rana, 2015). Fusilade affects well against a variety of grass weeds, such as foxtail, barnyard grass, brome grass, and ryegrass (Eytcheson & Reynolds, 2019),lawns, golf courses, vegetable gardens, orchards, and other turfgrass regions (Altland *et al*., 2003).. Because fusilade is primarily harms grass species and keeps broadleaf of crop Usually sprayed fusilade into plant foliage, after the appearance of grass weeds (Rokich *et al*., 2009). For the best weed control in wheat, fusilade can be applied alone or mixed with other herbicides (Wrage *et al*., 1989). Fusilade may induce stunted growth, leaf chlorosis (yellowing), necrosis, wilting, or other symptoms of herbicide damage on wheat plants if used at higher doses than recommend one or in an unstable weather conditions, (Keul *et al*., 1990). Several symptoms are frequently seen in the leaves, shoots, and roots such as decreased of photosynthesis rat , nutrient uptake, and water use efficiency due to fusilade application. Fusilade herbicide could cause oxidative stress in plants by decreasing antioxidant defense levels and increasing the production of reactive oxygen species (ROS),. The plant may eventually die because of this oxidative stress, which may harm biological components like cell membranes and chloroplasts (Morderer *et al.*, 2020).

Exogenous application of ascorbic acid (AsA) is consider a one of antioxidant compounds that reducing the detrimental effects of oxidative stress in plants which results from of certain herbicide treatment (Bhardwaj *et al*., 2022; Sacała & Roszak, 2018; Wang *et al*., 2018; Yaman & Nalbantoğlu, 2020; Xu *et al*., 2015; Naz *et al*., 2016). An organic substance that is found naturally, ascorbic acid functions as an antioxidant to assist plants in resisting oxidative stress brought on by harsh environments (Foyer, 2017; Kaviani, 2014). Research has demonstrated that AsA can make a plant more resistant to glyphosate, paraquat, isoproturon, atrazine, and other herbicides (Wang *et al*., 2018; Sacała & Roszak, 2018; DeRidder *et al*., 2002). It could enhance photosynthesis and lessen lipid peroxidation, a prominent sign of oxidative stress. Moreover, AsA could increase the activity of antioxidant enzymes that are essential for detoxifying reactive oxygen species, including peroxidase, catalase, and superoxide dismutase (Madhava Rao & Sresty, 2000; Reichert *et al*., 2023; Latif *et al*., 2016; Mukhtar *et al*., 2016; Naz *et al*., 2016). It could scavenge ROS, reducing their harmful effects (Akram *et al*., 2017). Some studies have shown that pre-treatment with AsA can mitigate the effects of herbicide toxicity in plants by enhancing antioxidant defenses and reducing oxidative damage (Wang *et al*., 2018). Therefore, it may be possible to use AsA as a protective agent against fusilade herbicide toxicity

Applying AsA as a preventive agent against herbicide toxicity has some possible side effects. Among these effects, is the changes in antioxidant activity, where AsA with its antioxidant properties, can help plants cope with various stress conditions (Wang *et al*., 2018). Abdallah *et al*. (2021) reported that AsA may increase or decrease the effectiveness of herbicides in managing grass weeds. Moreover, interaction could depend on multiple factors, such as the concentration of AsA, timing of application, and environmental conditions (Abdallah *et al*., 2021). Depending on specific conditions and timing of application, AsA treatment might alleviate or exacerbate the phytotoxic effects of herbicides on wheat plants (Wang *et al*., 2018; Yaman & Nalbantoğlu, 2020). The interaction between fusilade and AsA in wheat is complex and not well understood. Therefore the purpose of this study was to investigate AsA's protective role against oxidative stress in wheat plants caused by fusilade herbicide applied topically.

2.Materials and Methods

2.1. Experiment design and treatments:

Grains of wheat (*Triticum aestivum* L. cv. Sohag 3; Poaceae) were obtained from the Agriculture Research Centre, Ministry of Agriculture, Giza, Egypt. Wheat grains were surface sterilized and sown in pots containing 2kg of clay/sand (3:1) soil at 18°C ±2°C. Water was used to irrigate these pots according to the soil field capacity twice a week. Four weeks later, identically growing plants were selected, distributed into three sets of pots each, and given the following treatment:

1- Control: Plants sprayed with water.
2- AsA: Plants sprayed (0.01%) ascorbic acid.
3- Low FL: Plants sprayed with a low dose of fusilade (360 g ha^{-1}).
4- Mid FL: Plants sprayed with a mid-dose of fusilade (720 g ha^{-1}).
5- High FL: Plants sprayed with a high dose of fusilade (1440 g ha^{-1}).
6- AsA&Mid FL: Plants sprayed with AsA one day before spraying with a mid-dose of fusilade.

Note: Commertial Fusilade (Syngenta Fusilade max Forte) was used. (12.5% active ingredient) from Syngenta (Egypt) and frequently used for weed control. Fusilade was applied by spraying 100ml/ pot prepared according to the concentrations of low, mid and high doses. Water was sprayed on the controls at the same time as the treatment. Samples were taken for analysis ten days after treatment with the herbicide.

2.2. Biochemical components analyses:

2.2.1. Photosynthetic pigments content analysis:

Regarding the assessment of photosynthetic pigments (carotenoids, chlorophyll a, and chlorophyll b), Lichtenthaler Buschmann (2001) method was used. The leaf sample was extracted at 4 °C in acetone 80% (v/v), and the extract was centrifuged at 15000x*g* for 5 min. The absorption of supernatants was recorded at 663 and 647nm for chlorophyll a and chlorophyll b, respectively, and at 470 nm for carotenoid content. The extract solution concentrations were expressed in milligrams per milliliter using the following formulas

$Chlorophyll\ a = 12.25*A_{663} - 2.79*A_{647}$

$Chlorophyll\ b = 21.50*A_{647} - 5.10*A_{663}$

$Carotenoid = \{1000*A_{470} - (1.82Chl\ a - 95.15lChl\ b)\}/225$

2.2.2. Carbohydrates content:

The anthrone sulfuric acid method developed by Fales (Fales, 1951) was used to extract the soluble and total carbohydrates from the plant tissues; the dried tissue of the shoots was extracted using distilled water for the soluble carbohydrates and HCl for the total carbohydrates. In a test tube, 1 mL of the carbohydrate extract and 9 mL of the anthrone sulphuric acid reagent were combined, and the mixture was heated in waterbath for 7 minutes at 100°C. Using spectrophotometry, the absorbency was measured at 620 nm in comparison to a blank that contained only anthrone reagent and distilled water. The amount of carbohydrates was stated as mg g^{-1} DW.

2.2.3. Protein content:

Using a standard curve made of bovine serum albumin, the Lowry technique (Lowry *et al*., 1951) was used to quantify the total protein content and the soluble portion of the leaf. For total protein, dry weight (0.03 g) leaf sample was extracted for 2 hours at 90 ◦C using 10 mL NaOH (0.1 N) and 10 mL distilled water, respectively. After centrifuging the extracts, the supernatants were measured at 700 nmagainstblank. The amount of protein was stated as mg g^{-1} DW.

2.2.4. Proline content

According to Bates *et al.* (1973), the proline content of leaves was established. Overnight, a known dry weight of leaves (0.03 g DW) was extracted in 10 mL of 3% aqueous sulfosalicylic acid. Proline content was computed as mg g^{-1} DW using a standard curve.

2.2.5. Total free amino acids content

Plant tissues were used to extract total free amino acids, which were then measured using stannous chloride, citrate buffer, and Ninhydrin solution, by Moore and Stein's method (Moore & Stein, 1948). The amounts of free amino acids were determined as mg g^{-1} DW.

2.3. Antioxidant capacity analyses:

2.3.1. Antioxidant enzymes

The activity of peroxidase (POD, EC 1. 11. 1. 7) was determined using Zhang's technique (Zhang, 1992). POD, reaction mixture containing 0.4% H_2O_2, 1% guaiacol, 50 mM phosphate buffer (pH 6.1), and enzyme extract. At 470 nm, an increase in absorbance (E=25.5 mM^{-1} cm^{-1}) was observed as a result of guaiacol oxidation. At a temperature of 25 ± 2 ˚C, the enzyme activity was measured in terms of µmol of guaiacol oxidized min^{-1} g^{-1} fresh weight.

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined by the method of BeauchampFridovich (1971). β-mercaptoethanol of0.05% (w/v), 0.1% (w/v) ascorbate, and 50 mM phosphate buffer at pH 7.8 were used for the extraction of wheat leaves. 50 mM phosphate buffer (pH 7.8), 9.9 mM L-methionine, NBT, 0.025% (w/v), and 0.0044% (w/v) riboflavin make up the SOD assay medium. At 560 nm, the photo-reduction of NBT (purple formazan production) was measured. The extract volume that resulted in a 50% inhibition of NBT photoreduction was considered one unit of SOD activity.

Nakano Asada (1981) method was used to determine ascorbate peroxidase (APX EC 1.11.1.11) activity. Wheat leaves were extracted using phosphate buffer (PH 7.0), centrifuged for 10 minutes at 4°C at 4000 rpm. 0.1 mM EDTA, 0.3 mM ascorbate, 0.06 mM H_2O_2, and 50 mM phosphate buffer (pH 7.0) make up the test media for APX. Following a drop in ascorbate content, absorbance at 290 nm decreased, and activity was determined using the extinction coefficient (E = 2.8 mM^{-1} cm^{-1}).

Measurements of catalase (CAT, EC 1.11.1.6) were determined according to the method of

Chandlee Scandalios (1984). The absorbance at 240 nm (E= 0.036 mM^{-1} cm^{-1}) of a reaction mixture containing 10 mM H_2O_2, 25 mM potassium phosphate buffer (pH 7.0), and enzyme extract was measured to track the elimination of H_2O_2 and determine the CAT activity.

2.3.2. Antioxidant compounds

2.3.2.1. Phenolics content:

The determination of total phenolic contents in leaves involved the use of Folin-Ciocalteau reagent (Singleton & Rossi, 1965). The gallic acid standard solution (2.0 mg/mL) was made by precisely weighing and dissolving 0.01 g in 50 mL of purified water. The solution was then diluted to yield working standard solutions at 1.0, 1.5, 0.2, and 0.1 mg/mL concentrations. 40 microliters of either the gallic acid

standard or the leaf extract (in 80% methanol) were mixed with 1.8 mL of Folin-Ciocalteu reagent, which had previously been diluted ten times with distilled water. After allowing the mixture to stand at room temperature for five minutes, 1.2 mL of sodium bicarbonate (7.5% w/v) was added. Following a 60-minute standing period at room temperature, the absorbance at 765 nm was determined. The outcomes are given in milligrams per gram of gallic acid equivalents.

2.3.2.2. Flavonoids content:

The amount of total flavonoids in various wheat samples was evaluated using the method of Dewanto *et al.* (2002). Wheat dried powder was extracted using methanol, and after that, it was mixed with 75µl of 5% $NaNO_2$ and left for seven minutes. The obtained solution was then mixed with 0.5 mL of NaOH (1 M) and 150µl of $AlCl_3$ (10%). After that, the mixture was completed to 2.5 ml with distilled water At 510 nm, the produced color was measured against a blank. The results were calculated and reported as µg catechin equivalents per gram of dry weight, based on a standard curve created using catechin.

2.3.2.3. MDA contents:

The 2-thiobarbituric acid (TBA) reactive metabolites assay was utilized to quantify the MDA level to measure lipid peroxidation, as Zhang (1992) explains. One gram of fresh leaves, devoid of veins, were ground in 5% trichloroacetic acid (TCA) and centrifuged for ten minutes at 3000 rpm. To enable the creation of the (TBA) 2-MDA adduct, two milliliters of the resultant supernatant were mixed with two milliliters of 0.03 thiobarbituric acid (TBA) and incubated at 94°C for fifteen minutes. After that, the mixture was cooled with tap water, and the absorbance at 532 nm was determined. The extinction coefficient, E = 155 mM cm^{-1}, was used to express the amount of lipid peroxidation as nmol (g FW)$^{-1}$.

2.3.2.4. H_2O_2 content:

Hydrogen peroxide (H_2O_2) level was measured using spectrophotometry according to Jana Choudhuri (1981) method. To extract H_2O_2, 0.5 g of leaf tissue was homogenized in 3 mL of phosphate buffer (pH 6.5, 50 mM). For twenty-five minutes, the homogenate was centrifuged at 6000g. Three milliliters of the extracted solution and one milliliter of 0.1% titanium sulfate in 20% sulfuric acid (H_2SO_4) were combined to measure the H_2O_2 level. Once more, the mixture was centrifuged for 15 minutes at 6000g. A UV-VIS spectrophotometer (Model T80, PG Instruments, United Kingdom) was used to assess the intensity of the yellow color of the supernatant after the pellet had been dissolved in 5 milliliters of 2M H_2SO_4. Utilizing the extinction value of 0.28 µmol^{-1}, the H_2O_2 level was calculated.

2.3.3. Antioxidant activity analysis:

Using 2,2-diphenyl-1-picrylhydrazyl (DPPH) in a free radical-scavenging experiment, the antioxidant activity of leaf extracts was evaluated, by the protocol outlined by Shimada *et al.* (1992). Leaves were extracted using methanol (0.2–10 mg ml^{-1}) and combined with an equivalent volume of recently made methanolic solution that contained 80 parts per million of DPPH radicals. After giving the concoctions a good shake, they were left in the dark for half an hour. The samples' absorbance was then calculated at 517 nm. Using the following formula, the proportion of DPPH scavenging activity was then determined:

DPPH scavenging ability = (1- (Ai-Aj)/Ac) * 100.

Ai is the absorbance of extract + DPPH, Aj is the absorbance of extract + methanol, and Ac is the absorbance of DPPH + methanol. A lower absorbance indicates a higher scavenging effect.

2.4. Statistical analysis

This study employed a completely randomized design (CRD). The ANOVA test was used to determine if the collected data were significant. The least significant differences (LSD) test was used to compare means at P <0.05 and P <0.01 levels. The statistical program SPSS (v. 22.0) was used to perform all tests.

3. Results

3.1. Biochemical components

3.1.1 Pigments content

It was noticed that plants get stunted by the application of fusilade. Leaves showed chlorosis and yellowing (Figure 1). In this study, photosynthetic pigment content (Table 1) in different fragments appeared negatively affected by fusilade herbicide treatments. In detail, Chlorophyll A content was decreased gradually by increasing fusilade concentration. Where decreasing percentages were 4.5%, 30%, 70%, and 15% in Low FL, Mid FL, High FL, and AsA&Mid FL, respectively, compared to control wheat plants. On the other hand, Chlorophyll *A* content in plants sprayed with AsA&Mid FL was increased by 30% compared to unsprayed plants with fusilade (Mid FL). Also, chlorophyll *B* appeared to decrease in its content when wheat plants were sprayed with fusilade. The decreasing percentages were 4.3%, 18.6%, 56.45%, and 15.79% in Low FL, Mid FL, High FL, and AsA&Mid FL, respectively, compared to control wheat plants. It was found that wheat plants sprayed with AsA&Mid FL the chlorophyll *B* content increased by 2.8% compared to unsprayed plants with AsA at the same level of fusilade. Carotenoid content was reduced

by spraying wheat plants with fusilade, especially in Mid FL and High FL concentrations. Where, decreasing percentages were 22.16% and 61.61% in Mid FL and High FL, respectively, compared to control wheat plants. On the other hand, wheat plants sprayed with AsA&Mid FL, carotenoid content significantly increased by 38.4% compared to that only sprayed with fusilade (Mid FL). It was noted that in wheat plants sprayed with AsA, the content of the photosynthetic pigment was increased in all fragments, where increments percentages were 1%, 7%, and 19% in chlorophyll *A*, chlorophyll *B*, and carotenoid respectively, compared to that of the control wheat plants. The chlorophyll *A*/ *B* ratio decreased in Mid FL (2.67) and High FL (2.29) compared to control (3.16) and AsA & Mid FL (4.15) plants. Chlorophyll *A* affected more than Chlorophyll *B* by fusilade doses. The sum of chlorophyll *A*&*B* recorded the highest value in plants that sprayed with AsA (1.33) and the lowest value in High FL treated plants (0.43). The sum of chlorophyll *A*and*B* value of AsA&Mid FL plants (1.12) was higher than the value of Mid FL treated plants (0.94).

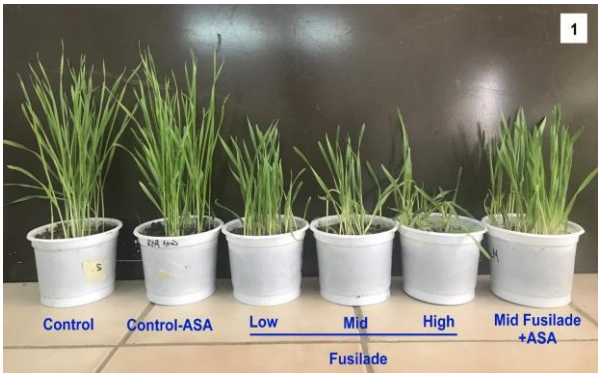

Figure (1): Effect of Fusilade herbicide on growth of wheat (*Triticum aestivum* L.). Stunting of plants, chlorosis and yellowing of leaves due to Fusilade application.

3.2 Biochemical Analysis

3.2.1 Carbohydrates and proteins

Carbohydrate content (Figure 2a) increased in wheat plants sprayed with fusilade, especially in soluble and total carbohydrate content. In detail, there was a significant increase in soluble fragments by 3.4%, 47.4%,78%, and 83% in Low FL, Mid FL, High FL, and AsA&Mid FL, respectively. Moreover, increasing percentages in total carbohydrate content were 19%, 9.4%, 17%, and 55.5% in Low FL, Mid FL, High FL, and AsA&Mid FL, respectively. On the other side, insoluble carbohydrate content was affected in a negative way, where there was a significant decrease in Mid FL and High FL were 20% and 44.5%, respectively. Plants sprayed with AsA&Mid FL significantly increased in all carbohydrate content fragments by 82.8%, 34%, and 55.5% in soluble, insoluble, and total carbohydrate content, respectively. Also, these plants showed an increase in carbohydrate content compared to plants that only sprayed with the same level of fusilade. Increment percentages were, 35.4%, 45.57%, and 46.12% in soluble, insoluble, and total carbohydrate content, respectively.

Protein content (Figure 2b) in sprayed plants with fusalide was affected in different ways, where soluble protein content was significantly increased by 7%, 7.8%, 12.6%, and 37% in Low FL, Mid FL, High FL, and AsA & Mid FL, respectively. However, insoluble and total protein content showed a significant decrease by 43%, 36%, 31%, and 28.6% at Low FL, Mid FL, High FL, and AsA & Mid FL, respectively. Decreasing percentages in total protein of wheat were 22.5%, 12.7%, and 10.9% in response to Low FL, Mid FL, and High FL, respectively, compared to control wheat plants.

In general, plants sprayed with AsA & Mid FL, had protein fragments increase compared to that only sprayed with the same level of fusilade they were 29.3%, 7.5%, and 19% in case of soluble, insoluble, and total protein content, respectively.

Table (1): Effect of fusilade herbicide on pigments content (mg g^{-1} FW) of wheat (*Triticum aestivum* L.cv. Sohag 3) leaves. Values are means (M) of three replicates ± standard deviation (SD).

Treatments	Chlorophyll *A*				Chlorophyll *B*				Carotenoids				*A*/*B*	*A*&*B*	Total
	M	±	SD	%	M	±	SD	%	M	±	SD	%			
Control	0.98 ^{c}	±	0.13	100	0.32 ^{b,c}	±	0.04	100	0.26 b,c	±	0.03	100	3.16	1.30	1.56
AsA	0.99 ^{c}	±	0.01	101.08	0.34 ^{c}	±	0.04	106.69	0.31 b,c	±	0.03	119.17	2.96	1.33	1.64
Low FL	0.94 ^{c}	±	0.02	95.54	0.30 ^{b,c}	±	0.03	95.73	0.26 ^{c}	±	0.03	101.05	3.12	1.24	1.50
Mid FL	0.69 ^{b*}	±	0.04	69.84	0.26 ^{b*}	±	0.00	81.41	0.20 ^{b}	±	0.03	77.84	2.67	0.94	1.15
High FL	0.29	±	0.10	29.48	0.14 a**	±	0.04	43.55	0.10 ^{a*}	±	0.04	38.39	2.29	0.43	0.53
AsA&Mid FL	0.85 ^{b,c}	±	0.02	86.82	0.27 ^{b,c}	±	0.01	84.21	0.30 ^{c}	±	0.05	116.24	4.15	1.12	1.42

Statistical significance of differences compared to control: *, significant *at P<0.05*; **, significant at *P<0.01*. Duncan's composition between variants at α=0.05, the variants with the same letters show the same reaction.

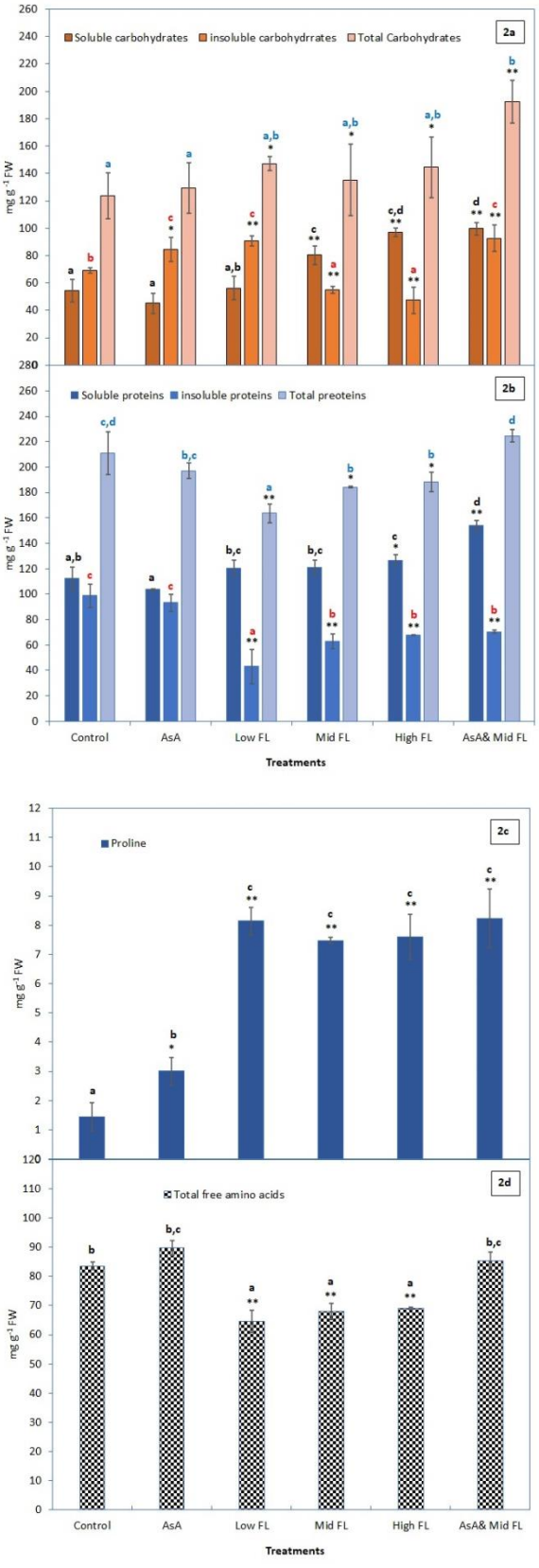

Figure 2 (a, b, c & b): Effect of fusilade herbicide on carbohydrates, proteins, proline and total free amino acids content (mg g^{-1} DW) of Wheat (*Triticum aestivum* L) leaves. Values are means (M) of three replicates ± standard deviation (SD). Statistical significance of differences compared to control: *, significant *at P<0.05*; **, significant at *P<0.01*. Duncan's composition between variants at α=0.05, the variants with the same letters show the same reaction.

Protein content (Figure 2b) in sprayed plants with fusalide was affected in different ways, where soluble protein content was significantly increased by 7%, 7.8%, 12.6%, and 37% in Low FL, Mid FL, High FL, and AsA&Mid FL, respectively. However, insoluble and total protein content showed a significant decrease by 43%, 36%, 31%, and 28.6% at Low FL, Mid FL, High FL, and AsA&Mid FL, respectively. Decreasing percentages in total protein of wheat were 22.5%, 12.7%, and 10.9% in response to Low FL, Mid FL, and High FL, respectively, compared to control wheat plants.

In general, plants sprayed with AsA & Mid FL, had protein fragments increase compared to that only sprayed with the same level of fusilade they were 29.3%, 7.5%, and 19% in case of soluble, insoluble, and total protein content, respectively

3.2.2 Proline and total free amino acids

Proline content (Figure 2 c) was significantly increased in plants sprayed with fusilade, and AsA. Increment percentages were 464.9%, 418.74%, 426.9%, and 470% in Low FL, Mid FL, High FL, and AsA&Mid FL, respectively. In contrast, total free amino acids (Figure 2 d) when plants were treated with fusilade, they expressed significant decrease , by 23%, 19%, and 17.4% in Low FL, Mid FL, and High FL, respectively,. Plants sprayed with AsA&Mid FLshowed an increase in proline and total free amino acid content than plants sprayed with fusilade by 51.4%, and 20.9%, respectively.

3.2. Antioxidant capacity:

3.2.1. Antioxidant enzymes

We examined the activities of peroxidase, superoxide dismutase, ascorbate peroxidase and catalase in treated and untreated wheat plants. Peroxidase and superoxide dismutase activities (Figure 3 a & b) increased significantly with spraying fusilade. In the case of POD, the increase reached 92%, 130.6%, 350.8%, and 217.4% in Low FL, Mid FL, High FL, and AsA&Mid FL, respectively. At the same time, SOD increased by 9.8%, 37.8%, 50%, and 14.5% for the same treatment levels. POD activity in AsA&Mid FL plants increased more than MF plants by approximately 73%. SOD activity in AsA & Mid FL plants is less than MF plants by 83%.

It was noticeable that ascorbate peroxidase and catalase activities (Figure 3 a & b) were decreased significantly by applying fusilade. APX activity decreased gradually by increasing the fusilade dose by 11%, 62%, 72%, and 38% in Low FL, Mid FL, High FL, and AsA & Mid FL, respectively. Also, CAT activity decreased with

sprayed fusilade by 3.5%, 33.7%, 40.9%, and 27.6% in Low FL, Mid FL, High FL, and AsA & Mid FL, respectively,. For plants sprayed with AsA & Mid FL, the enzyme activity was enhanced than those sprayed only with fusilade by 24%, and 6% in APX, and CAT, respectively.

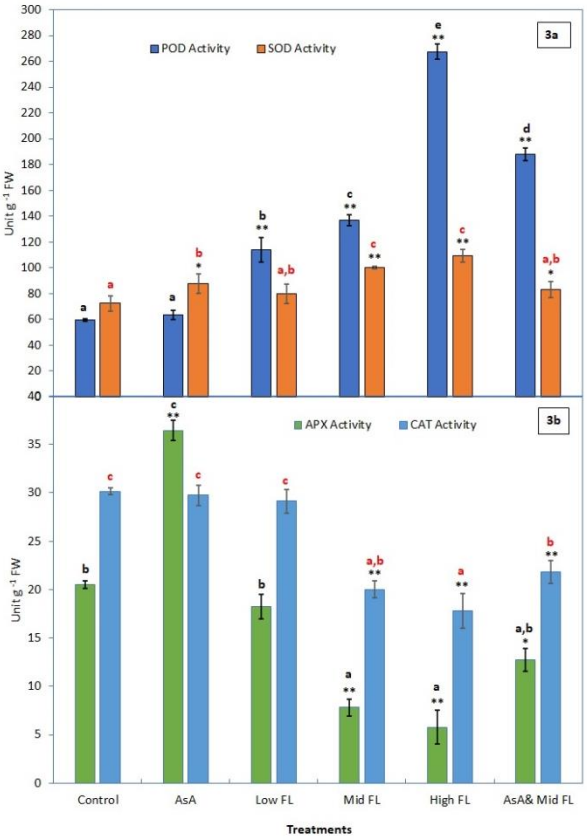

Figure 3 (a & b). Effect of fusilade herbicide on Peroxidase and Superoxide dismutase activities, Ascorbate peroxidase and Catalase (Unit g^{-1} FW) of Wheat (*Triticum aestivum* L) leaves. Values are means (M) of three replicates ± standard deviation (SD). Statistical significance of differences compared to control: *, significant *at P<0.05*; **, significant at *P<0.01*. Duncan's composition between variants at α=0.05, the variants with the same letters show the same reaction.

3.2.2. Antioxidant metabolites

3.2.2.1. Total phenolic compound, flavonoid contents, and Total Antioxidant activity:

The total phenolic compound and flavonoid contents (Figure 4 a) gradually increased by increasing the fusilade dose. Significant increase in phenolic content in Low FL, Mid FL, and High FL, 99.5%, 242.8%, and 338.3%, respectively, compared to control plants. In flavonoid content, significant increases were 59%, 77%, and 154.8%, in Low FL, Mid FL, and High FL, respectively. In contrast, the total antioxidant activity (Figure 4 b) of sprayed plants with fusilade gradually

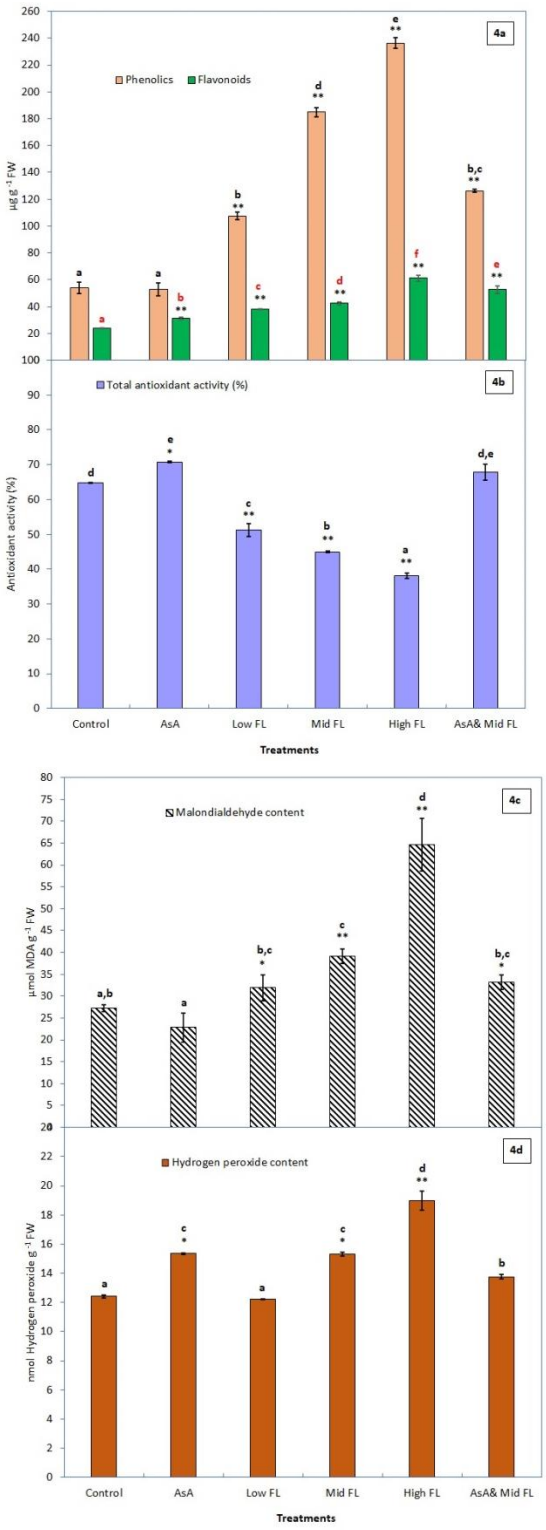

Figure 4 (a, b, c & d): Effect of fusilade herbicide on Total phenolics (µgg^{-1}FW), flavonoids contents (µgg^{-1}FW), total antioxidant activity (%), MDA content (µmol MDA g^{-1}FW) and H_2O_2 content (nmol H_2O_2 g^{-1} FW) of Wheat (*Triticum aestivum* L) leaves. Values are means (M) of three replicates ± standard deviation (SD). Statistical significance of differences compared to control: *, significant *at P<0.05*; **, significant at *P<0.01*. Duncan's composition between variants at α=0.05, the variants with the same letters show the same reaction.

decreased by increasing fusilade concentration. Significant decrease was 21%, 30.6%, and 46.3 in Low FL, Mid FL, and High FL, respectively. Flavonoids content and total antioxidant activity in plants sprayed with AsA & Mid FL appeared to increase in their content and activity related to those unsprayed in the same level of Fusilade by 42%, and 35.24% in Flavonoids content and total antioxidant activity, respectively. However, the total phenolic compound content showed a decrease in its content by 108%.

3.2.3. MDA and H_2O_2 content

MDA and H_2O_2 contents (Figure 4 c & d) increased significantly under the influence of Mid FL and High FL by 43.3% and 136.9%, they were lower in control (23%, and 52.8%, respectively). MDA and H_2O_2 contents were decreased in sprayed plants with AsA and MF less than those treated with the same level of fusilade by 56%, and 12.4%, respectively

4.Discussion

In this work, the toxicity of fusilade herbicide, at certain concentrations, can be mitigated by using (AsA) in wheat plants. Ascorbic acid is known for its antioxidant properties and its ability to mitigate oxidative stress in plants (Jalili *et al*., 2023; Hasanuzzaman *et al*., 2023). Similar symptoms were reported by SalemEl-Sobki (2022), Fayez *et al.* (2014) , and Fayez *et al.* (2013) who reported that fusilade herbicide can cause deformation and leaf chlorosis in peanut plants. Moreover, marjoram plants showed a reduction in leaf number and shoot length when subjected to fusilade herbicide (Hussein *et al*., 2013). These symptoms occurred probably due to oxidative stress by fusilade herbicide application. Numerous studies have demonstrated that fusilade can induce (ROS), which can harm cellular constituents like lipids, proteins, and DNA. (Singh *et al*., 2017; Agostinetto *et al*., 2016). This oxidative stress by fusilade herbicide can cause the degradation of chlorophyll pigment molecules appearance of leaf chlorosis (Carve *et al*., 2018; Leng *et al*., 2023; Parween *et al*., 2016).

Measurement of photosynthetic pigments for plants under stress is important to evaluate the effect of these stresses on plant growth. In this experiment, high fusilade doses caused a 70% reduction of chlorophyll *A* and a 55% reduction in chlorophyll *B*. This reduction in main photosynthetic pigments could reduce production of carbohydrates needed for normal growth. The observed growth reduction of fusilade-treated wheat might be due to reduced photosynthesis and degradation of photosynthetic pigments. Lowered consumption of the stored carbohydrates and reduced energy for growth and other physiological processes. On the other hand, plants sprayed with AsA alone or AsA followed by fusilade showed normal and healthy growth in both treatments. AsA spray strengthens the antioxidant system in untreated plants, resulting in healthier plants, and in treated plants, it can counteract the oxidative stress that is generated.

Oxidative stress increased free radicles that require more antioxidants to capture them before they cause denaturation of cell components. AsA as a known antioxidant can control free radicles and balance the antioxidant status of plants under stress.

In the present work, there is a significant increase in proline content and a clear decrease in the percentage of total free amino acids in plants treated with fusilade at different doses. Higher proline content of fusilade treated plants than that of the control is considered an indicator of plant exposure to stress. Many studies concerning herbicides treated plants exhibited a significantly increase in proline content (Fayez *et al*., 2011; Fayez *et al*., 2014) Proline has been implicated in antioxidant defense mechanisms. It can scavenge ROS, including hydrogen peroxide and superoxide radicals, which are generated during stressful conditions. By reducing oxidative stress, proline helps a protection of macromolecules cellular components (proteins, lipids, and DNA) from damage. In this work, the application of AsA, with or without fusilade, induced more proline that overwhelmed oxidative stress caused by the applied herbicide. Moreover, proline has a role in stabilizing proteins and cellular structures under stress. It can directly interact with proteins, stabilizing their structure and preventing denaturation. Proline has been suggested to function as a signaling molecule in stress responses. It can alter the expression of genes that respond to stress and take part in signal transduction pathways that aid in the adaptation to stress. (Mansour & Salama, 2020; Shafi *et al*., 2019).

Amino acids are the raw material for synthesizing protein and enzymes required for plant. From the present results, it can be deduced that lowered amino acid contents with herbicide application can lead to lowered protein contents and application of AsA played a protective role by modulating these contents to normal levels similar to control. Free amino acids can serve as a reservoir of nitrogen. Protein degradation may occur into amino acids under stress conditions. Some amino acids play a role in antioxidant defense mechanisms. They aid in the production of antioxidants like glutathione, which guards against oxidative damage to cells during times of stress by neutralizing reactive oxygen species (ROS) (Batista-Silva *et al*., 2019).

Activities of antioxidant enzymes were highly affected by fusilade and AsA applications. SOD and POD enzymes were significantly enhanced while APX and CAT were lowered in all treatments. SOD plays a

crucial part in preventing oxidative damage to cells by lowering the concentration of superoxide radicals, which are highly reactive and can initiate chain reactions leading to cellular injury. SOD catalyses the dismutation of superoxide radicals into oxygen and hydrogen peroxide. By scavenging superoxide radicals, SOD helps prevent the formation of more harmful ROS and reduces oxidative stress. POD participates in the scavenging of hydrogen peroxide (H_2O_2), a reactive oxygen species generated during herbicide stress. By catalyzing the reduction of H_2O_2, peroxidases contribute to the elimination of this ROS and prevent its harmful effects on cellular structures. SOD and POD, along with other antioxidant enzymes, contribute to the regulation of cellular redox homeostasis. By maintaining a balanced redox environment, antioxidant enzymes help prevent oxidative damage to biomolecules, such as proteins, lipids, and nucleic acids (Sharma *et al*., 2012).

Besides antioxidant enzymes, the antioxidant metabolites reduce the toxicity symptoms caused by fusilade herbicide. Phenolic and flavonoid compounds, which significantly increase in wheat leaves treated with fusilade or AsA or both, can scavenge ROS and protect cells from oxidative damage. Previously noticed that phenolic and flavonoids were highly accumulated in plants subjected to herbicides (Lydon & Duke, 1989). Phenolic compounds can interact with AsA and other molecules to modulate the antioxidant activity and redox status of plants (Azizi *et al*., 2021).

Fusilade inhibits ACC-ase activity which is involved in lipid biosynthesis. Fusilade causes oxidative stress that increases lipid peroxidation. Malondialdehyde (MDA) is a product of lipid peroxidation and a marker of oxidative damage. Fusilade can increase the MDA content in wheat plants, indicating that it affects their cell membranes and different cellular components. AsA lowered the content of MDA indicating the antioxidant protective action of AsA treatment. AsA can scavenge ROS and reduce MDA accumulation in plant cells. AsA can also transport electrons across the plasma membrane to external electron acceptors such as MDA (Yoshimura & Ishikawa, 2017). Higher production of H_2O_2 indicates oxidative stress in plants under stress. As a defense mechanism against superoxide accumulation, the dismutation of H_2O_2 brings about the accumulation of H_2O_2 which is easy to degrade by many antioxidant enzymes. Moreover, the present results showed that the total antioxidant activity was reduced in plants treated with fusilade and the reduction was gradual from low to high dose. Plants treated by AsA with or without fusilade herbicide showed normal levels of total antioxidant activity indicating that AsA activates defence mechanisms against fusilade toxicity.

5.Conclusion

This study demonstrated that ascorbic acid has potential to alleviate oxidative stress in wheat plants induced by fusilade herbicide in wheat plants. Fusilade application resulted in oxidative damage, as evidenced by chlorosis, increased lipid peroxidation, H_2O_2 content and altered antioxidant enzyme activity as well as carbohydrates, protein, proline and amino acids contents. However, when ascorbic acid was applied alongside fusilade, it effectively improved the activity of antioxidant enzymes and decreased lipid peroxidation and H_2O_2 contents. Furthermore, wheat plants treated with ascorbic acid exhibited improved growth and biochemical parameters compared to those treated with fusilade alone. These results imply that ascorbic acid is an effective shield against oxidative damage caused by herbicides, with the potential to enhance crop productivity and plant health. Further research is needed to explore the underlying mechanisms and optimize the use of ascorbic acid in agricultural practices.

6.References

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