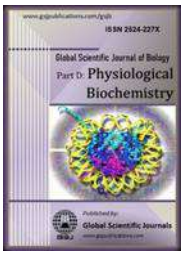
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Some Enzymatic and Non-Enzymatic Antioxidants in Cowpea Vigna Unguiculata L. under the Influence of Foliar Spraying with Vitamin C, Salicylic Acid, and their Interaction

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ABSTRACT

The current study is conducted to find out the effect of leaf spray of vitamin C and salicylic acid along with their combination on various enzymes and non-enzymatic antioxidant contents of the cowpea plant (Vigna unguiculata L.). In this connection, three levels of salicylic acid (0, 40, and 80 mg·L^{-1}) and four levels of vitamin C (0, 50, 100, and 150 mg·L^{-1}) were taken. Various parameters such as peroxidase and catalase enzyme activity and concentration of salicylic acid, glutathione, total ascorbate, and proline were recorded. RCBD technique with three replicates and 36 experimental units was adopted in this study. The results revealed that: 1.Both salicylic acid and vitamin C had a positive effect on the studied traits. 2.Higher concentrations of both substances contributed to increased values for all the studied traits. 3. The interaction of Salicylic acid and Vitamin C levels especially the high levels of both affected the enzymatic and non-enzymatic antioxidant activities of the cowpea plants studied positively.

1. Introduction

Cowpea (Vigna unguiculata L.) is a crop belonging to the highly important Fabaceae (legume) family. It is cultivated for its fresh pods and dry seeds, which are valuable sources of food and animal feed. With a cultivated area of approximately 12.5 million hectares—concentrated largely in Central and West Africa, a region accounting for about 64% of global production (El-Shaieny et al., 2015)—the crop is distinguished by the high mineral, protein, and vitamin content of its seeds and pods. Another way in which it plays an important role in sustaining agriculture is in its symbiotic association with the *Rhizobium* bacteria in terms of their roots; the bacteria form root nodules that enable the conversion of nitrogen to a usable form by the plant. (Kim et al., 2025).

Reasons for growing cowpea in Iraq are mainly economical, nutritional, and environmental

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reasons; given ideal growth conditions such as proper variety selection, quality fertilizer application, and proper irrigation, cowpea production may be up to 5 tons/ha.

The vitamin C is a product of glucuronic acid metabolism and can be found in both free and bound states; the latter is predominant and localized in the mitochondria, taking part in the metabolism of alpha-ketoglutaric acid (Hacisevki, 2009). Vitamin C catalyzes and assists biological processes and enzyme activities in the plants, such as the Krebs cycle enzymes dependent on the alpha-ketoglutaric acid; the vitamin controls cell division, growth, and flowering (Gallie, 2013). Vitamin C is an antioxidant, reducing agent either free or in bound state; it is able to reduce oxidized glutathione to the native state due to the ability of enediol group.

Moreover, it acts as a cofactor for several metabolic enzymes, mainly those involved in the metabolism of nucleic acids and gibberellins. In its free state, it can diffuse throughout the plant tissues, which is made possible by the influx of sodium, and has the capability to block free-radical chain reactions using reactive oxygen species, especially the hydroxyl radical, turning them into monodehydroascorbate (Zhang, 2013). In its free state, it diffuses throughout the plant tissues, which is achieved due to influx of sodium, and blocks free-radical chain reactions using reactive oxygen species, especially the hydroxyl radical, turning them into monodehydroascorbate (Zhang, 2013).

Ascorbic acid is viewed as one of the most vital non-enzymic antioxidants in plant cells; high concentrations improve the efficiency of antioxidant enzymes and make plants more resistant to various environmental stresses (Hassan et al., 2019). Moreover, the spraying of ascorbic acid on turmeric plants has proved beneficial in enhancing the biochemical features of the plants (Ali, 2025). It is a key hormone that boosts the immune system of the body, being very efficient in defending the cell as well as acquiring immunity (Peng et al., 2021).

Treatment with salicylic acid and its derivatives such as acetylsalicylic acid significantly enhances plant tolerance to both biotic and abiotic stresses. This occurs by boosting resistance via the SAR mechanism often through the induction of changes in internal leaf anatomy and by triggering internal signaling pathways that confer tolerance to various stressors, including heat stress; the latter can otherwise lead to increased cell membrane fluidity and plant death resulting from reduced water content and lethal temperatures (Kolupaev et al., 2011). Treatment with salicylic acid and its derivatives serves several important physiological and chemical functions in plant growth and development (Raskin, 1992). These include inducing flowering, facilitating ion and nutrient uptake, regulating stomatal movement and photosynthesis, and inhibiting ethylene biosynthesis. Additionally, it counteracts the effects of abscisic acid, prevents the oxidation of endogenous gibberellins, auxins, and cytokinins, and increases levels of nucleic acids and amino acids (Abdi, 2010). One of the most significant effects of salicylic acid is its stimulation of antioxidant production to counteract Reactive Oxygen Species (ROS) during heat and drought stress; it also facilitates energy provision for the plant via alternative pathways, accompanied by changes in nucleic acid and amino acid levels (Baghizadeh and Hajmohommadrezaei, 2011).

Salicylic acid enhances plant immunity by stimulating peroxidase enzyme activity (Ibrahim and Hammad, 2020). Salicylic acid has the ability to bind with amino acids; this interaction facilitates a mechanism for storing and slowly releasing the acid, thereby sustaining systemic acquired resistance. Furthermore, the association of salicylic acid with specific amino acids—such as proline and arginine—enhances the plant's ability to withstand environmental stresses (Hayat and Ali, 2010). Spraying with salicylic acid resulted in a significant increase in the leaf nutrient content (Al-Azzawi & Abu Al-Mikh, 2026). The study was conducted to determine the effect of treatments with Vitamin C and salicylic acid and their interactions on certain enzymatic and non-enzymatic antioxidants in cowpea plants.

2. Materials and Methods

A field experiment was conducted during the 2018–2019 summer growing season in the Al-Latifiyah area (Al-Mahmoudiyah District, Baghdad Governorate) to investigate the effects of Vitamin

C, salicylic acid, and their interactions on certain enzymatic and non-enzymatic antioxidants in cowpea plants.

The field site was prepared by plowing the soil twice in perpendicular directions using a mechanical plow; the soil was then pulverized and fertilized with Diammonium Phosphate (DAP) across all experimental units.

The experiment involved two factors:

1.Salicylic acid concentrations (40 mg L^{-1}, 80 mg L^{-1}, plus a control treatment).

2.Vitamin C concentrations (50 mg L^{-1}, 100 mg L^{-1}, 150 mg L^{-1}, plus a control treatment).

The Randomized Complete Block Design (RCBD) was selected for the experiment. The experiment involved two factors and three replicates (3 × 4 × 3), resulting in a total of 36 experimental units; each unit measured 3 meters, with a 50 cm spacing between adjacent units.

Determination of Peroxidase (POD) activity (absorbance units·mL^{-1}):

Determined according to the method of Müftügil (1985).

Solutions used:

A-Stock solution: Guaiacol solution, prepared by mixing 1.36 mL in 250 mL of distilled water.

B-Hydrogen peroxide (H_2O_2) solution (0.1% concentration): Prepared by taking 0.4 mL of H_2O_2 and making up the volume to 120 mL with distilled water.

Methodology:

1 mL of guaiacol was combined with 1 mL of H_2O_2. Absorbance was measured at a wavelength of 420 nm using a spectrophotometer. To determine enzyme activity, 0.1 mL of the sample extract was added to 2 mL of the reaction mixture. Changes in absorbance were recorded every 30 seconds for a duration of 3 minutes at the same wavelength. POD

Enzyme activity was then determined as follows:

$$\text{Peroxidase enzyme activity}(\boldsymbol{Absorbance\ unit.\ ml-1}) = \frac{\text{Change in the device reading}}{\frac{\text{Change in time}}{0.1*0.01}}$$

Where:

0.1 = Sample volume.

0.01 = Amount of enzyme causing an increase in light absorbance of 0.01 per minute at the same wavelength.

Determination of catalase (CAT) activity (absorbance units·mL^{-1}):

It was determined according to the method described by Aebi (1974).

Solutions used:

A-Phosphate buffer solution: Prepare a 50 mM phosphate buffer solution at pH 7.

B-30 mM hydrogen peroxide solution: Prepare by taking 0.34 mL of hydrogen peroxide and making up the volume to 100 mL with phosphate buffer.

Procedure: 0.1 mL of the sample filtrate—prepared according to the SOD enzyme preparation method—was mixed with 1.9 mL of phosphate buffer solution. Then, 1 mL of hydrogen peroxide solution was added, and the mixture was shaken. The sample's absorbance was subsequently measured using a UV spectrophotometer at a wavelength of 240 nm, with changes in absorbance monitored over a 3-minute period at 30-second intervals. A blank sample was prepared using the same procedure but omitting the filtrate, substituting it with 0.1 mL of distilled water.

$$\text{Catalase enzyme activity}(\boldsymbol{Absorbance\ unit.\ ml-1}) = \frac{\text{Change in the device reading}}{\frac{\text{Change in time}}{0.1*0.01}}$$

Where:

0.1 = Sample volume.

0.01 = Amount of enzyme causing an increase in light absorbance of 0.01 per minute at the same wavelength.

Determination of salicylic acid concentration (µg·mL^{-1})

Determined according to the method of Warrier et al. (2013).

fresh tissue samples (85 days old) were placed directly into a freezer and frozen at -5°C for 24 hours; subsequently, they were crushed and allowed to thaw. A 100 mg portion of the tissue was weighed and placed in tubes, then centrifuged at 10,000 rpm for 10 minutes. An immediate draw of 100 µL of the supernatant was done, and its volume made up to 3 mL by the addition of ferric chloride solution (that had been prepared during the experiment). The appearance of violet color was noted, and the samples were analyzed in the spectrophotometer with a wavelength of 540 nm. The standards were prepared using salicylic acid dissolved in distilled water at 100 µg/mL concentration.

Determination of glutathione concentration (µmol·g^{-1}):

Determined according to the method of Moron et al. (1979).

Solutions used:

A-Trichloroacetic acid (TCA) solution: Prepared by weighing 10 g of the acid in 200 mL of distilled water.

B-Phosphate buffer solution: 0.2 M.

C-5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) solution: Prepared by dissolving 10.07 mg of DTNB in a portion of phosphate buffer, then bringing the volume up to 300 mL.

Procedure: 0.5 g of fresh plant tissue (78 days old) was ground in a porcelain mortar after adding 2.5 mL of TCA solution. The mixture was centrifuged at 1000 rpm for 10 minutes. Subsequently, 0.1 mL of the supernatant was taken, and 1 mL of phosphate buffer was added, followed by 3 mL of TCA solution and 2 mL of DTNB solution. After adding the DTNB solution, the mixture was left to stand for 15 minutes as the color gradually turned yellow. Absorbance was then measured at a wavelength of 412 nm using a spectrophotometer, and the concentration was calculated using a standard curve.

Determination of total ascorbate concentration (mg·100g^{-1})

Determined according to the method of Hussain et al. (2010)

Solutions used:

1-Ammonium molybdate solution

2-Sulfuric acid (H_2SO_4) solution (5%)

3-Oxalic acid solution (0.05 M): Prepared by dissolving 30 g of the acid and making up the volume to 100 mL with distilled water.

4-Acetic and phosphoric acid solution: Prepared by dissolving 70 mL of acetic acid and 30 mL of phosphoric acid, then making up the volume to 500 mL with distilled water.

Procedure: Soft plant tissue (86 days old) was crushed, and 10 ml of oxalic acid was added. The samples were then stored in the dark at 4°C for 12 hours. Following filtration, 0.5 ml of an acetic+phosphoric acid solution and 2 ml of ammonium molybdate were added to the filtrate, and the volume was made up to 25 ml with distilled water. The samples were analyzed using a spectrophotometer at a wavelength of 760 nm, and the concentration was determined using a standard curve.

Determination of proline concentration (µg·g^{-1}).

Proline concentration was determined according to the method of Bates et al. (1973).

Solutions used:

A- 3% Sulfosalicylic acid.

B- Ninhydrin reagent: Prepared by mixing 1.25 g of ninhydrin with 30 mL of glacial acetic acid and

20 mL of 6M phosphoric acid; the mixture was then stored in the dark at 4°C for 24 hours.

C- Toluene.

Procedure: Weigh 0.5 g of fresh plant tissue (78 days old) and place it in a porcelain mortar. Add 10 mL of 3% sulfosalicylic acid and grind the tissue. Centrifuge the mixture at 2000 rpm for 10 minutes, then filter it. Pipette 2 mL of the filtrate into a test tube and add 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent. Place the tubes in a water bath at 95°C, then transfer them immediately to an ice bath to cool. Add 4 mL of toluene and mix well before allowing the reaction mixture to stand for 30 minutes during which time a red color will be developed. The top red layer of the solution is then removed carefully by using a Pasteur pipette to collect 3 mL of the solution and read the color intensity at 520 nm.

3. Results and Discussion

Peroxidase Enzyme Activity

The results presented in Table (1) demonstrated a significant effect of Vitamin C concentration on peroxidase enzyme activity. An increase in the mean enzyme activity was observed; the highest mean activity was 9.18 absorbance units·mL^{-1} at a Vitamin C concentration of 150 mg·L^{-1}, while the lowest mean activity was 8.61 absorbance units·mL^{-1} at a concentration of 0 mg·L^{-1}. The same table demonstrated a significant effect of salicylic acid on increasing peroxidase enzyme activity; as the acid concentration was raised from 0 to 80 mg L^{-1}, the mean activity increased from 8.71 to 9.24 absorbance units mL^{-1}.

Table (1) Peroxidase (POD) Enzyme in Cowpea (Vigna unguiculata L.) Under the Influence of Foliar Spraying with Vitamin C, Salicylic Acid, and Their Interaction.

Concentrations of Salicylic acid mg.L^{-1}	**Concentrations of Ascorbic acid mg.L^{-1}**				**Average**
	(0)	(50)	(100)	(150)	
0	**8.11**	**8.69**	**8.94**	**9.10**	**8.71**
40	**8.73**	**8.81**	**9.12**	**9.17**	**8.96**
80	**8.98**	**9.27**	**9.41**	**9.28**	**9.24**
(L.S.D : 0.05)	**Salicylic acid × Ascorbic acid =0.064**				**Salicylic acid=0.032**
Average	**8.61**	**8.92**	**9.16**	**9.18**	
(L.S.D : 0.05)	**Ascorbic acid =0.037**				

Catalase Enzyme Activity

The results in Table (2) demonstrated a significant effect of increasing Vitamin C concentration on enhancing catalase enzyme activity. The highest mean enzyme activity was 21.18 absorbance units·mL^{-1} at a Vitamin C concentration of 150 mg·L^{-1}, while the lowest mean was 16.80 absorbance units·mL^{-1} at a concentration of 0 mg·L^{-1}.

The same table also indicated a significant effect of salicylic acid on increasing catalase enzyme activity; raising the acid concentration from 0 to 80 mg·L^{-1} resulted in an increase in mean activity from 18.09 to 20.08 absorbance units·mL^{-1}. Results regarding the dual interaction between Vitamin C and salicylic acid concentrations revealed significant differences in increased enzyme activity; the highest value—21.89 absorbance units·mL^{-1}—was observed at concentrations of 100 mg·L^{-1} for Vitamin C and 80

mg·L^{-1} for salicylic acid, while the lowest value—14.76 absorbance units·mL^{-1}was recorded at zero concentrations for both substances.

Table (2) Catalase (**CAT**) Enzyme in Cowpea (Vigna unguiculata L.) Under the Influence of Foliar Spraying with Vitamin C, Salicylic Acid, and Their Interaction.

Concentrations of Salicylic acid mg.L^{-1}	**Concentrations of Ascorbic acid mg.L^{-1}**				**Average**
	(0)	(50)	(100)	(150)	
0	**14.76**	**18.09**	**19.09**	**20.41**	**18.09**
40	**17.54**	**18.74**	**21.00**	**21.23**	**19.63**
80	**18.10**	**19.22**	**21.11**	**21.89**	**20.08**
(L.S.D : 0.05)	**Salicylic acid × Ascorbic acid = 0.026**				**Salicylic acid= 0.013**
Average	**16.80**	**18.68**	**20.40**	**21.18**	
(L.S.D : 0.05)	**Ascorbic acid = 0.017**				

Salicylic Acid Concentration

The results in Table (3) demonstrated a significant effect of increasing Vitamin C concentration on the level of salicylic acid. The highest mean salicylic acid concentration was 1.41 µg·mL^{-1} at a Vitamin C concentration of 150 mg·L^{-1}, while the lowest mean was 1.26 µg·mL^{-1} at a Vitamin C concentration of 0 mg·L^{-1}.

The same table also indicated a significant effect of salicylic acid concentration itself; increasing the acid concentration from 0 to 80 mg·L^{-1} resulted in an increase in the mean value from 1.12 to 1.51 µg·mL^{-1}. The results of the two-way interaction between Vitamin C and salicylic acid concentrations revealed significant differences associated with increasing salicylic acid levels. The highest value—1.57 µg/mL—was recorded at concentrations of 100 mg/L for Vitamin C and 80 mg/L for salicylic acid, whereas the lowest value1.01 µg/mL was observed at zero concentrations for both substances.

Table (3) Salicylic Acid Concentration in Cowpea (Vigna unguiculata L.) Under the Influence of Foliar Spraying with Vitamin C, Salicylic Acid, and Their Interaction.

Concentrations of Salicylic acid mg.L^{-1}	Concentrations of Ascorbic acid mg.L^{-1}				Average
	(0)	(50)	(100)	(150)	
0	1.01	1.09	1.15	1.22	1.12
40	1.35	1.41	1.40	1.47	1.41
80	1.42	1.51	1.57	1.54	1.51
(L.S.D : 0.05)	Salicylic acid × Ascorbic acid =0.020				Salicylic acid=0.010
Average	1.26	1.34	1.37	1.41	
(L.S.D : 0.05)	Ascorbic acid = 0.014				

Glutathione Concentration

The results in Table (4) demonstrated a significant effect of increasing Vitamin C concentration on the increase in glutathione concentration. The highest mean glutathione concentration was 114.84 μmol·g^{-1} at a Vitamin C concentration of 100 mg·L^{-1}, while the lowest mean was 111.30 μmol·g^{-1} at a Vitamin C concentration of 0 mg·L^{-1}.

The same table indicated a significant effect of salicylic acid on increasing glutathione concentration; as the acid concentration was raised from 0 to 80 mg L^{-1}, the mean value increased from 111.88 to 114.50 μmol g^{-1}. Results regarding the interaction between Vitamin C and salicylic acid concentrations showed no significant differences in the increase of glutathione concentration.

Table (4) Glutathione Concentration

in Cowpea (Vigna unguiculata L.) Under the Influence of Foliar Spraying with Vitamin C, Salicylic Acid, and Their Interaction.

Concentrations of Salicylic acid mg.L^{-1}	Concentrations of Ascorbic acid (mg.L^{-1})				Average
	(0)	(50)	(100)	(150)	
0	108.00	111.00	114.65	113.87	111.88
40	112.41	113.24	114.90	115.00	113.89
80	113.51	114.21	114.98	115.31	114.50
(L.S.D : 0.05)	Salicylic acid × Ascorbic acid = N.S.				Salicylic acid= 1.87
Average	111.30	112.81	114.84	114.73	
(L.S.D : 0.05)	Ascorbic acid = 2.01				

Total ascorbate Concentration

The results (5) highlighted the significant effect of Vitamin C concentration on increasing total ascorbate concentration; the mean value reached 5.01 mg·100g^{-1} at a Vitamin C concentration of 150 mg·L^{-1}, while the lowest mean—3.41 mg·100g^{-1}—was observed at a concentration of zero.

The results also show the significant impact of salicylic acid on the increase of total ascorbate concentration; the more the acid concentration was increased from zero to 80 mg·L^{-1}, the higher the mean value, which ranged from 3.98 to 4.79 mg·100g^{-1}.

Regarding results concerning the joint action of Vitamin C and salicylic acid concentrations, there were significant differences in the increase of total ascorbate concentration. The highest value – 5.22 mg·100g^{-1} – was noted at 150 mg·L^{-1} of Vitamin C concentration and 80 mg·L^{-1} of salicylic acid concentration, while the lowest value – 2.56 mg·100g^{-1} – was at zero concentrations for both components. Table (5): Total ascorbate concentration

in Cowpea (Vigna unguiculata L.) Under the Influence of Foliar Spraying with Vitamin C, Salicylic Acid, and Their Interaction.

Concentrations of Salicylic acid mg.L^{-1}	**Concentrations of Ascorbic acid (mg.L^{-1})**				**Average**
	(0)	(50)	(100)	(150)	
0	**2.56**	**4.00**	**4.45**	**4.91**	**3.98**
40	**3.64**	**4.28**	**4.71**	**5.04**	**4.42**
80	**4.04**	**4.81**	**5.09**	**5.22**	**4.79**
(L.S.D : 0.05)	**Salicylic acid × Ascorbic acid =0.095**				**Salicylic acid =0.047**
Average	**3.41**	**4.36**	**4.75**	**5.06**	
(L.S.D : 0.05)	**Ascorbic acid =0.059**				

Proline Concentration

The findings from Table (6) showed that there is a significant influence of the increase of Vitamin C concentration on the proline concentration. The highest mean value was 26.29 µg·g^{-1} at a Vitamin C concentration of 150 mg·L^{-1}, whereas the lowest mean value was 21.95 µg·g^{-1} at a concentration of 0 mg·L^{-1}.

Table (6) also revealed that there is a significant influence of salicylic acid on increasing the proline concentration; an increase in the concentration of acid from 0 to 80 mg·L^{-1} caused an increase in the mean concentration from 23.06 to 25.50 µg·g^{-1}.

It was found that there were some differences in the increase in the proline concentration due to the relationship between the vitamin C and salicylic acid concentrations. The highest value, which was 27.47 µg·g^{-1}, was obtained from a vitamin C concentration of 150 mg·L^{-1} and salicylic acid concentration of 80 mg·L^{-1}, while the lowest value, which was 19.69 µg·g^{-1}, was obtained from zero concentrations of both materials.

Table (6) Proline Concentration

in Cowpea (Vigna unguiculata L.) as a Result of Foliar Spraying with Vitamin C, Salicy.

Concentrations of Salicylic acid mg.L^{-1}	Concentrations of Ascorbic acid (mg.L^{-1})				Average
	(0)	(50)	(100)	(150)	
0	19.69	23.15	24.12	25.31	23.06
40	22.68	24.00	24.89	26.08	24.41
80	23.49	25.11	25.91	27.47	25.50
(L.S.D : 0.05)	Salicylic acid × Ascorbic acid = 2.44				Salicylic acid = 1.22
Average	21.95	24.09	24.97	26.29	
(L.S.D : 0.05)	Ascorbic acid = 1.35				

Increased Vitamin C concentrations led to enhanced activity of both enzymatic and non-enzymatic antioxidants. Increased enzyme action is because of Vitamin C ability to counteract the negative effects of hydrogen peroxide (H2O2) and free radicals generated from the photosynthetic process involving oxygen reaction in Photosystem I located in chloroplast by controlling the metabolism of antioxidants in growing plants (Noctor et al., 2014). Furthermore, the presence of Vitamin C boosts the metabolic rate, thereby increasing the levels of soluble carbohydrates produced through metabolism; the accumulation of these carbohydrates within the plant is essential for Vitamin C synthesis (Lisko et al., 2014). Increasing the foliar application concentrations of salicylic acid led to enhanced enzyme activity. This increase is attributed to the acid's role in boosting soluble carbohydrate content and nucleic acid (RNA and DNA) levels, inhibiting reactive oxygen species (ROS)—such as hydrogen peroxide (H2O2) and superoxide—and stimulating the production of antioxidants like peroxidase, catalase, and superoxide dismutase when the plant is exposed to environmental stress (Korkmaz et al., 2007). The acid is believed to play a role in suppressing ROS concentrations generated by drought stress—specifically hydrogen peroxide (H2O2) and superoxide (O2•−)—by increasing the levels of H2O2-scavenging enzymes (peroxidase and catalase), thereby neutralizing the toxic, protein-oxidizing effects of H2O2 and converting it into water (Gupta, 2011).

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