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A Mechanistic Action of Salicylic acid on Metabolic Profiling, Antioxidative Potential, and Growth Pattern of *Amaranthus hybridus*

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Authors' contributions

This work was carried out in collaboration among all authors. Author AK conducted the study, analysed the data and wrote the first draft and performed the statistical analysis. Author AS designed the study, revised the manuscript. Author PKY performed literature searches, prepared charts, tables and references. All authors read and approved the final manuscript.

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ABSTRACT

This study has been conducted to investigate the effects of salicylic acid application on the growth and biomass of *Amaranthus hybridus*. The crop was grown in pots during July-August 2023, under natural weather conditions in the botanical garden of Banaras Hindu University, Varanasi. Different doses of SA (0.69 ppm – 13.8 ppm) was sprayed on the shoot of the plants. Compared to the control group, SA application at 0.69 ppm, 1.38 ppm, and 2.76 ppm showed increased growth and yield of the plants. SA applied at 1.38 ppm concentration, particularly, gave maximum increments in the length and biomass of the crop. SA application reduced the formation of oxidative stress markers (H_2O_2, SOR, MDA) by increasing the activities of antioxidants like SOD, POD, APX, total phenol, and proline. Salicylic acid treatment also increased the nutritive value of the crop by

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increasing the level of Ca, Mg, Fe, Zn, and protein, while reduced the levels of oxalic acid. The photosynthetic efficiency of the PSII was strengthened better with 1.38 ppm SA application by increasing chlorophyll content, allocation of absorbed light energy to drive the photochemical process, and quenching of the heat energy. The metabolic profiling via UHPLC-Q-TOF-MS/MS revealed increase in the number and proportion of secondary metabolites belonging to phenolic, alkaloids, terpenoid, flavonoids, betalains, and coumarins group by 240%, 127%, 37%, 182%, 83%, and 90% respectively. Hence, 1.38 ppm of SA has been considered as the suitable dose for spraying on *Amaranthus hybridus* for increased growth and biomass.

Keywords: Phytohormones; vegetable crop; UHPLC; oxidative stress; cellular mechanism; secondary metabolites; salicylic acid; Amaranthus hybridus.

1. INTRODUCTION

Plants are constantly exposed to various environmental stresses. Environmental stress induces crop losses in terms of growth, and yield. The internal defense system of the plant tries to withstand the environmental pressure; however, it can do so up to a certain threshold level of environmental stress. So, the external application of some agrochemicals to support their growth in adverse situations becomes necessary. The application of plant hormones is a simple and straightforward method to boost the defense system in plants. Salicylic acid or ortho-hydroxybenzoic acid is a naturally occurring plant hormone that regulates various growth processes in plants. It is produced in very small quantities in plants. The significance of salicylic acid in plant growth when applied exogenously has been extensively studied and reported to be dependent on the plant species, the state of development, and the concentration level [1].

In recent years, the application of external salicylic acid to the plants has been discovered to operate against different biotic and abiotic stresses [2]. The exogenously applied salicylic acid is reported to have positive outcomes on the growth and yield of crops. The most common route for salicylic acid action is by enhancing the antioxidant activity of the plant. The increased activity of antioxidative enzymes declines the oxidative damage that occurs due to the formation of reactive oxygen species. However, the optimal concentration of salicylic acid needs to be known before applying it to the plant exogenously. Moreover, the optimal level of exogenous salicylic acid varies from plant to plant. Application of higher concentrations of salicylic acid declines the growth and productivity of the plant. Hence, it is necessary to determine an appropriate dose of salicylic acids for essential edible crops.

Amaranthus hybridus is a highly nutritious and medicinal plant. It is one of the cheapest, fast-growing green vegetables in tropical countries. This plant is considered a 'superfood' because of its high nutraceutical value and its ability to grow in hot summer when no other leafy vegetable grows. It is an alternative rich source of proteins, vitamins, carotenoids, and minerals, especially for people in developing countries. Because of its high protein content, it is considered a complete protein supplier when consumed along with other cereals. The presence of betalain improves the antioxidant properties in the plant. Betalain is also reported to have anticancer properties. Many other compounds belonging to the phenolics, flavonoids, terpenoids, and alkaloids groups pose antioxidant and medicinal properties. However, *Amaranthus hybridus* is also known to contain oxalate crystals. Oxalates in food may lead to the development of kidney stones by reducing the absorption of calcium and magnesium in human beings.

Despite its significance in both nutrition and medicine, there remains a scarcity of studies investigating the impacts of salicylic acid application on the photosynthesis, antioxidative activities, and growth of *Amaranthus hybridus*. This study aims to find the optimal concentrations of exogenous salicylic acid by evaluating its effects on growth, photosynthesis, antioxidative activity, and nutritive value of *Amaranthus hybridus*.

2. MATERIALS AND METHODS

2.1 Experimental Design

The experimental site was the Botanical Garden of Department of Botany, Banaras Hindu University, Varanasi, Uttar Pradesh. The experiment was conducted under natural conditions during the month of July-August 2023. The pot experiment was conducted to study the

variations in growth, antioxidant activity, photosynthetic efficiency, nutrient content, secondary metabolites of *Amaranthus hybridus* as influenced with the foliar application of salicylic acid. The seeds were purchased from Indian Institute of Vegetable Research, Varanasi. Salicylic acid, purity 99%, was purchased from Merck, Germany. The experiment involved six different sets of treatment: control (no SA), 0.69 ppm SA, 1.38 SA, 2.76 ppm SA, 6.90 ppm SA, and 13.81 ppm SA. The solution of salicylic acid was prepared in ddH_2O. The dimensions of the pots used were 27.5 cm X 21.5 cm X 12 cm. Each treatment had three replicates. The soil used was sandy-loam in texture. The soil was mixed with farm yard manure (10 T/ha).

The seeds underwent sterilization in sodium hypochlorite (1.0 % v/v) for a duration of two minutes before rinsing with double-distilled water (ddH_2O). Twenty seeds of *Amaranthus hybridus* were sown in each pot. After the growth of the seedlings thinning was done and ten seedlings per pot were grown. At the four-leaf stage the foliar application began and salicylic acid was sprayed three times after every four days in the evenings. Spraying was done on both the sides of the leaves until wet completely. Plant harvesting and analysis was done after forty days of seed germination and studied for different biochemical, physiological, metabolic and growth parameters.

2.2 Estimation of Oxidative Parameters

Oxidative parameters like hydrogen peroxide (H_2O_2), malondialdehyde (MDA) formation, and superoxide radical (SOR) formation were estimated to find the effect of the different treatments on their formation that ultimately leads to oxidative damage in plants. The estimation was done according to the protocols standardized in Yadav et al. [3].

2.3 Estimation of Enzymatic and Non-Enzymatic Antioxidants

The estimation of antioxidative enzymes like superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) were performed according to the standardized protocol described in Yadav et al. [3]. The Ferric Reducing Antioxidant Power (FRAP) assay, as outlined by Vijayalaxmi and Ruckmani [4], was used to determine the overall antioxidant activity. The activity was compared with standard curve prepared by ascorbic acid. The estimation of proline was done according to Bates et al. [5]. The total phenol was estimated according to Bray and Thorpe [6] following the Folin-Ciocalteau method, 1954.

2.4 Oxalic Acid, Minerals, and Protein Determination

The determination of oxalic acid was done by the modified method of Fitriani et al. [7]. Two grams of *Amaranthus* leaves were crushed and heated in 100 mL DDW. After 20 minutes of heating, the extract was filtered. 50 mL of the solution was taken and 1 mL H_2SO4 was added. The solution was titrated with $KMnO_4$.

For the elemental analyses, the leaves were dried in an oven at 70 ºC for 24 hours. The dried leaves were finely crushed and underwent heat digestion in a mixture composed of nitric acid, sulfuric acid, and perchloric acid in a ratio of 5:1:1. The extracted was filtered and diluted with ddH2O. Calcium, magnesium, iron, and zinc levels in the filtrate were estimated through an atomic absorption spectrophotometer.

To estimate the protein content 500 mg leaf was used and the Lowry et al. [8] method was adopted.

2.5 Estimation of Chlorophyll Content and Photosynthetic Metrics

Chlorophyll was measured from the leaf extracts in 80% acetone and contents were calculated following the Arnon's equation [9]. The fluorescence study was performed by PAM-2500 (Heinz Walz GmbH, Germany) to gauge the behavior of the plants photosynthetic system. The plants were dark-adapted for 30 minutes at room temperature. The photosynthetic response to rapid light increase (every 30 s) was recorded to light intensities between 0-2000 µmol photons $m^{-2}s^{-1}$. The data was obtained and processed on PAM Win-3 software. Various parameters including Fv/Fm ratio (maximum quantum yield of photosystem II), Y(II) (effective photochemical quantum yield), ETR (rate of electron transport), Y(NO) (quantum yield of non-regulated energy dissipation), Y(NPQ) (quantum yield of regulated energy dissipation), and NPQ (non-photochemical fluorescence quenching) were derived using the PAM Win-3 software.

2.6 Estimation of Free Salicylic Acid

The free form of salicylic acid was quantified by HPLC following the method of Huang et al. [10].

2.0 g of leaf was crushed in liquid nitrogen and was extracted in methanol by soaking it for overnight. After adding ethyl acetate, it was vortexed and then centrifuged to collect the supernatant. The gathered supernatant underwent purification with primary secondary amine and graphitized carbon black prior to being analyzed via HPLC.

2.7 Estimation of Secondary Metabolites

The control plant was compared for the assessment of variations in the content of secondary metabolites with the salicylic acid-treated plant showing the best response in the parameters assessed so far. For the detection of secondary metabolites, 300 mg fresh leaves were lyophilized to remove the moisture content from the leaves. The dried leaf was ground in methanol and allowed to incubate at room temperature for 72 hours. Following incubation, the mixture underwent centrifugation, and the resulting supernatant was concentrated using a rotary evaporator. A 1 ml aliquot of the resulting concentrated extract was used for the detection of the secondary metabolites by UHPLC system (Dionex ultimate 3000 RS series, Thermo-Fischer Scientific).

The HPLC column used was hypersil Gold™ C18 selectivity boasting a particle size of 1.9 µm, a diameter of 2.1 mm, and a length of 100 mm. All analyses were conducted using Thermo Compound Discoverer 3.3.2.31 in combination with online databases. Prior to analysis, the instrument was equilibrated with water containing 0.1% formic acid (solvent A), and elution was performed using a methanol gradient containing 0.1% formic acid (solvent B). The elution process followed these solvent A and B proportions (v/v): 95:5 for 7 minutes, 70:30 for 15 minutes, 40:60 for 23 minutes, 10:90 for 28 minutes, and finally 95:5 for 30 minutes. A consistent flow rate of 0.3 ml/min was maintained, and the column temperature was kept at 40 ºC throughout the 30-minute run. The eluted metabolites were analyzed using Q-TOF MS in electrospray ionization (ESI) mode, both in positive and negative ion modes. TOF MS data were acquired within the m/z 100–1000 range with a rapid scan time of 0.1 seconds.

2.8 Measurement of Plant Growth

Ten plants were harvested from each treatment for measuring the fresh weight, and length of the plant. To measure the dry weight, the plants were dried in oven at 50 ºC for 72 hours.

2.9 Statistical Analysis

The experimental data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's-b post hoc analysis at $p < 0.05$ using the IBM SPSS software (ver. 21.0). In the figures and tables, the sample variability is given as standard error of the mean value (n=3). The relationship among different attributes has been performed by the Pearson correlation coefficient analysis.

3. RESULTS AND DISCUSSION

3.1 The effect of Salicylic Acid on Oxidative Stress Biomarkers

The exogenous treatment of salicylic acid affects the signaling response in the plants which consequently regulates the production of oxidative biomarkers. In the present study, the H_2O_2 content was lower at lower concentrations of salicylic acid (≤ 2.76 ppm) compared to the control plants. Beyond 2.76 ppm of SA treatment, the level of H_2O_2 increased and reached to the level of the control plant (Fig. 1). The increase in H_2O_2 level at high doses of salicylic acid may be due to the binding of the phytohormone with the catalase enzyme which results in decreased conversion of H_2O_2 to H_2O. The rise in the H_2O_2 level sets the defense system to increase the level of other antioxidative enzymes [11].

The SOR and MDA contents were maximally decreased at 1.38 ppm dose of SA by 17% and 11% respectively compared to control plant (Fig. 1). With subsequent increase in the doses of SA, there was an increase in the values of SOR and MDA. The reduction in the level of MDA, and SOR is because of the improvement in the antioxidant machinery of the plant with the application of salicylic acid at lower doses [12].

3.2 The Effect of Salicylic Acid on Antioxidative Components

The treatment of plants with 1.38 ppm SA rendered maximum increase in FRAP level by 19%, while later concentrations showed insignificant differences in respect to the control (Fig. 2). Further, the activities of enzymatic antioxidants have been estimated (Fig. 3). The SOD activity increased with the application of salicylic acid in a dose-dependent manner. POD

and APX activities were maximally increased up to 6.90 ppm of SA by 75%, and 103% respectively compared to the control. A similar observation has been reported by Moustafa-Farag [13]. The CAT level was found to be lower in plants treated with salicylic acid compared to the control group. The inhibition of catalase activity might be due to the chelation of the iron group present in the enzyme by salicylic acid or due to the donation of electrons from salicylic acid to the enzyme that puts it in an inactive redox state [14].

The activities of non-enzymatic antioxidants were studied by measuring total phenol, and proline content (Fig. 2). It was observed that at lower concentrations of salicylic acid (< 6.90 ppm) the level of total phenol was merely different compared to the control plant. The application of higher concentrations of salicylic acid (6.90 ppm, 13.81 ppm) increased the level of total phenol in the plant. Salicylic acid treatment has been reported to induce the level of phenol. The increase in the phenolic compounds may be due to SA-mediated increase in the activity of PAL (Phenylalanine Ammonia-Lyase), an important enzyme involved in the biosynthesis of phenolic compounds [15]. Phenolic compounds are known to serve antioxidant functions. They counteract oxidative stress by scavenging ROS compounds.

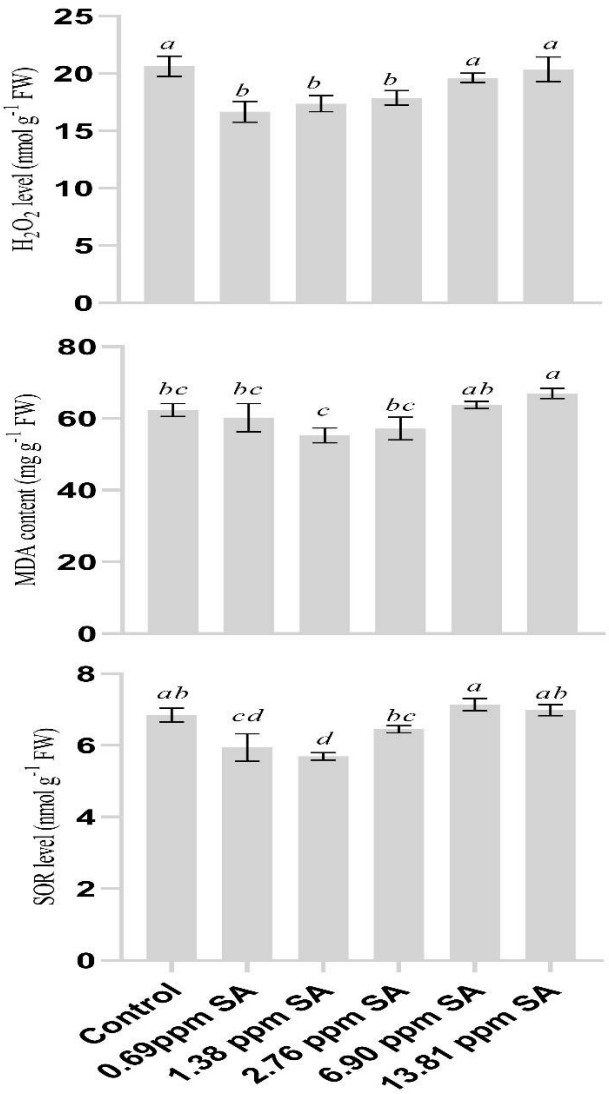

Fig. 1. The level of oxidative stress markers (H_2O_2, MDA, and SOR) under different treatments

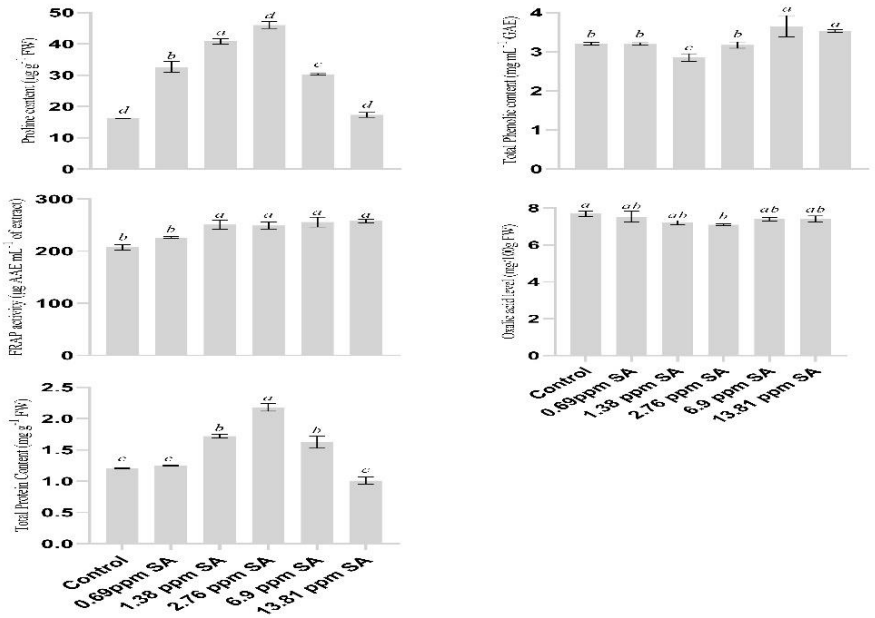

Fig. 2. The level of non-enzymatic antioxidants (proline, phenol), FRAP activity, oxalic acid, and protein content

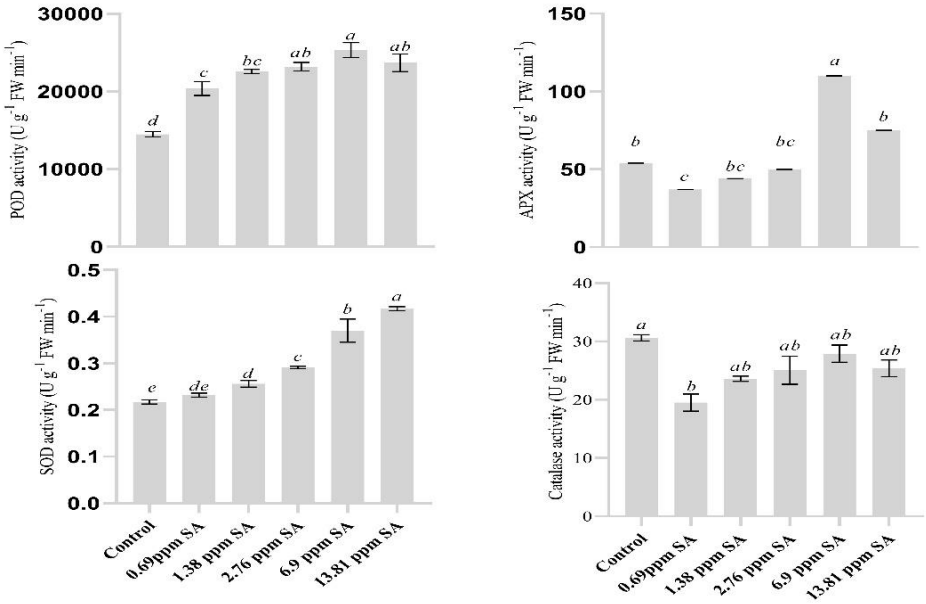

Fig. 3. Enzymatic antioxidant activity (POD, APX, SOD, CAT) under different treatments

The level of proline in the salicylic acid-treated plants was found to be greater up to 2.76 ppm of SA treatment compared to the control plant (Fig. 2). Among the different doses of salicylic acid, the maximum level of proline content was found in plants treated with 1.38 ppm SA (49%) compared to the control plant. The increment in proline content at lower doses may be attributed to the impact of salicylic acid on the proline biosynthetic enzymes including pyrroline-5-carboxylate reductase and γ- glutamyl kinase. Ogunsiji et al. [16] also reported increase in the level of proline in mung bean after the application of salicylic acid. Proline accumulation provides

cellular homeostasis, and acts as a signaling molecule, along with acting as an antioxidant molecule to inhibit lipid peroxidation by scavenging ROS [17].

3.3 The Effect of Salicylic Acid on Chlorophyll Content and Chlorophyll Fluorescence

Among all the plants, the chlorophyll *a*, and *b* content was found to be maximum in plants treated with 1.38 ppm of salicylic acid (Fig. 4). Treatment of plants with 13.81 ppm of salicylic acid resulted significant decline in the chlorophyll contents. The level of carotenoids also declined progressively at increasing concentrations of salicylic acid. Lower concentrations of salicylic acid might be responsible for increasing the biosynthetic enzymes to synthesize more chlorophyll by protecting the chloroplast structure form oxidative damage by increasing the antioxidative enzyme activity as reported by Arruda et al. [18].

Further, chlorophyll fluorescence matrices were estimated to find out the effect of SA application on functioning. In the present study, the Fv/Fm ratio ranged from 0.74-0.81. Control plant showed the minimum value of Fv/Fm (0.74). The supplementation of salicylic acid increased the ratio significantly particularly at 1.38 ppm (0.81). The utilization of salicylic acid enhances the effectiveness of the D1 and D2 proteins, thereby boosting the functionality of PSII, consequently resulting in an elevation of the Fv/Fm ratio in plants [19]. Further, the value of Y(II) increased significantly in plants treated with salicylic acid till 6.90 ppm. The highest dose of SA (13.81 ppm) showed insignificant change in Y(II) value compared to the control plants (0.20). The highest value of Y(II) was recorded in plants treated with 1.38 ppm SA (0.376). The increase in Y(II) may be justified from the increase in the fraction as well as efficiency of the open PSII reaction centres [20] with salicylic acid application. The level of Y(NPQ) showed decrease with the application of salicylic acid till 2.76 ppm as compared to the control plants. Thus, the lower dose of salicylic acid (0.69 -2.76 ppm) may lead to better allocation of absorbed light energy to increase the photosynthetic efficiency over control plants [21]. The Y(NO) did not show any significant difference with salicylic acid supplementation (Table 1).

NPQ which reflects the non-photochemical conversion of light energy into heat showed decline in its value with salicylic acid treatment compared to the control group, suggesting that the exogenous application of salicylic acid strengthened the PSII machinery (Table 1). The value of ETR, the relative electron transport rate, increased with the supplementation of salicylic acid compared to the control plants. However, at higher dose of SA (6.90 ppm, and 13.81 ppm) there was a decrease in the ETR values (Table 1). This suggests that the application of exogenous salicylic acid at lower concentrations (< 2.76 ppm) strengthened the PSII machinery, by quenching the heat energy and facilitating the photochemical process. A similar result has been reported by Moustakas et al. [20] in tomato plants where the exogenous supplementation of salicylic acid has enhanced the photochemistry of PSII.

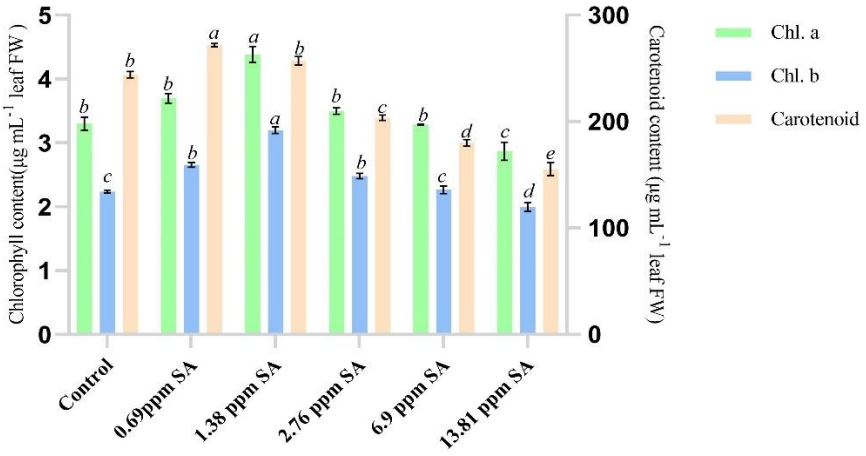

Fig. 4. The level of chlorophyll a, b, and carotenoid

Table 1. Effect of salicylic acid on chlorophyll fluorescence matrix

	Fv/Fm	Y(II)	Y(NPQ)	Y(NO)	NPQ	ETR
C	0.744 *b*	0.208 *d*	0.406 *a*	0.385 *ns*	1.05 *a*	34.75 *c*
0.69 ppm SA	0.774 *b*	0.302 *b*	0.344 *ab*	0.353 *ns*	0.975 *a*	50.47 *b*
1.38 ppm SA	0.812 *a*	0.376 *a*	0.300 *b*	0.323 *ns*	0.928 *b*	62.78 *a*
2.76 ppm SA	0.805 *a*	0.362 *a*	0.302 *b*	0.335 *ns*	0.904 *b*	60.46 *a*
6.90 ppm SA	0.778 *b*	0.243 *c*	0.376 *a*	0.381 *ns*	0.987 *a*	40.58 *c*
13.81 ppm SA	0.764 *b*	0.213 *d*	0.375 *a*	0.411 *ns*	0.914 *b*	35.56 *c*

3.4 The Effect of Salicylic Acid on Oxalic Acid, Nutrient Elements, and Protein Content

It has been reported that oxalic acid in *Amaranthus* is an undesirable trait for human beings as it promotes the formation of kidney stones and also declines the availability of calcium and magnesium in the body [22]. Compared to the control plant, the level of oxalic acid was found to decrease with SA application at all doses. The maximum decline in oxalic content was found in plants treated with 2.76 ppm of SA (Fig. 2). The reduction in the level of oxalic acid may possibly be due to the increased activity of oxalate oxidase due to salicylic acid application which results in breakdown of oxalic acid into CO_2 and H_2O_2, as reported by Walker and Farmiani [23].

$$\begin{matrix} COOH \\ | \\ COOH \end{matrix} + O_2 \xrightarrow{\text{Oxalate oxidase}} 2\,CO_2 + H_2O_2$$

Further, salicylic acid supplementation also promoted the uptake of nutrient elements and increased the protein content in the plant (Fig. 5). The level of two macroelements (calcium and magnesium) and two microelements (iron and zinc) have been estimated in the study. The level of calcium, iron and zinc was found to increase with the application of salicylic acid in a dose-dependent manner over to the control plant. The level of magnesium was found to increase till the application of 2.76 ppm SA, later it gradually declined at other higher concentrations. The increase of calcium content with SA application might be due to the increased activity of the oxalate oxidase that breaks down the oxalate crystals and releases calcium. The increase in nutrient uptake with the supplementation of salicylic acid might be due to increased activity of H^+-ATPase enzymes involved in regulating nutrient uptake. [24].

3.5 Level of free salicylic Acid

The level of free salicylic acid in the plants has been found to be increased after the application of exogenous salicylic acid compared to the control plants. The level of salicylic acid increased in a dose-dependent manner, however, beyond 2.76 ppm there was a marginal increase (Fig. 6). The application of exogenous salicylic acid till 2.76 ppm is able to increase the endogenous level of free salicylic acid beyond which the dose becomes excess for the plant which might have resulted in conversion of salicylic acid into an inactive state by undergoing modifications [25].

3.6 The Response of Salicylic Acid on Growth Attributes

The growth attributes of the plants were measured in terms of total fresh weight, total dry weight, root length, and shoot length (Fig. 7). The shoot and root length, and fresh weight of plants were increased with the application of SA over the control plants (Fig. 8). Over control plants, the total fresh weight was found to be maximum in the plant group treated with 1.38 ppm SA (34.0%), and later on, there were insignificant differences. Similarly, the application of 1.38 ppm SA maximally increased the dry weight of the plant compared to the control group.

All the growth attributes studied in this study have been found to increase with the supplementation of salicylic acid which might be due to the increase in the rate of cell division and expansion. The increase in the content of growth promoting molecules has been found from the analysis of secondary metabolite formation, that is discussed later. A similar response has been observed by Fujikura et al. [26], where low concentrations (< 0.05 mM) of salicylic acid promoted the growth while at higher concentrations (> 0.05 mM) decrease in the growth parameters of the plant was observed. The author explained salicylic acid, depending on

the concentration, to be involved in the activation/deactivation of the expression of the cyclin genes involved in cell cycle. Tan et al. [27] have also documented decreased dephosphorylation of auxin efflux carriers due to salicylic acid binding to the A subunits of protein phosphatase 2A. This interaction ultimately leads to reduced root and shoot length, particularly noticeable with elevated salicylic acid concentrations.

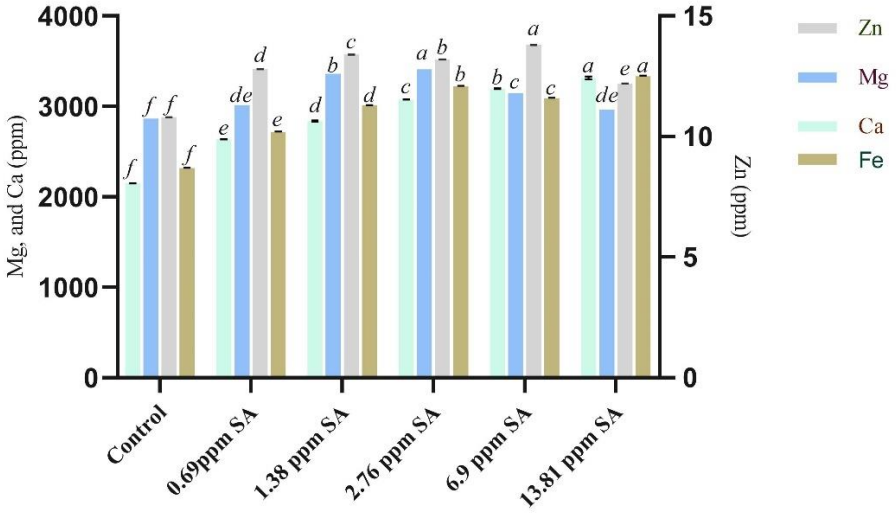

Fig. 5. Level of macroelements (Mg, Ca) and microelements (Fe,Zn)

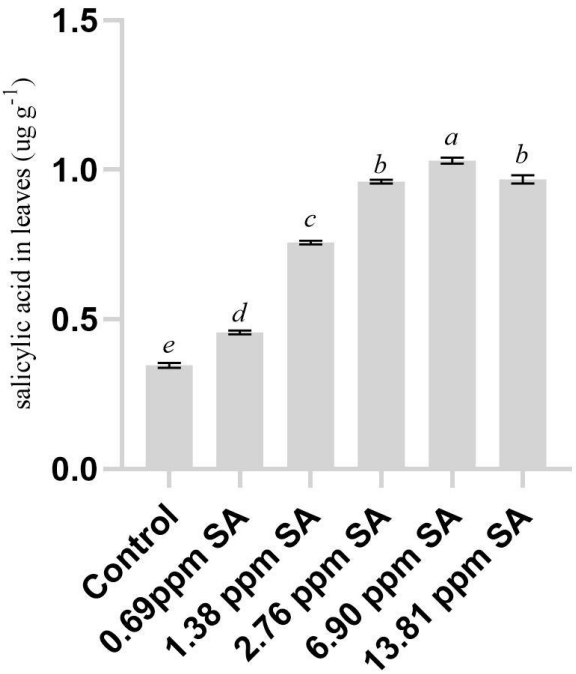

Fig. 6. Endogenous level of the free form of salicylic acid in differently treated plants

Fig. 7. Length of the plant at different treatments

The association between the six different concentrations and 22 different parameters estimated in our study was analyzed through a correlation heat-map matrix (Fig. 9). The application of salicylic acid showed a strong positive correlation with SOD, POD, FRAP, calcium, and zinc ($\geq$ 0.5) while others were moderately influenced. However, magnesium and oxalic acid levels showed a negative correlation with salicylic acid. It showed that with the application of salicylic acid, the quality of *Amaranthus hybridus* increased the antioxidative and nutritive potential. By decreasing the oxalic acid content, it also increases its economic importance.

3.7 The response of Salicylic Acid on Metabolic Profile

The HRAMS study employed UHPLC-Q-TOF-MS/MS for the detection of variation in secondary metabolites between the control and 1.38 ppm SA-treated plants. A total of 94 distinct metabolites were identified in the control plants, while 140 metabolites were identified in the 1.38 ppm SA-treated plants. The cumulative area covered by the identified secondary metabolites was 2.5 times greater in the 1.38 ppm SA-treated plant compared to the control group. The chromatogram of both the treatments is shown in Fig. 10.

The two groups showed variation in the order of the predominant secondary metabolites (Fig. 11). The content of compounds detected in the 1.38 ppm SA-treated plant were ranked as follows: betalains (83.8%)> phenolics (4.2%) > alkaloids (3.9%) > carotenoids (3.5%) > coumarins (1.8%) > terpenoids (1.6%). In contrast, in the control plants, betalains (74%) were followed by carotenoids (10.9%) > alkaloids (4.3%) > phenolics (3.0%) > terpenoids (3.0%)> coumarins (2.4%).

The application of salicylic acid led to a significant increase in the levels of various metabolite groups, including phenols (240.9%), alkaloids (127.9%), indole compounds (164.7%),

terpenoids (37.4%), steroids (341.0%), betalains (182.1%), phenylpropanoid (16.9%), carbohydrate derivatives (101.0%), polyketides (212.2%), flavonoids (83.1%), coumarin (90.2%), fatty acids (60.0%), and oxylipins (29.6%) compared to the control plant (Fig. 12). Phenolic compounds including coumaric acid, phenyl butyric acid, gingerol, cinnamic acid, cinnamaldehyde, eugenol, ferulic acid, guaiacol, hydroquinone, stilbene was identified in the 1.38 ppm SA-treated plant. These phenols are known to serve many purposes in plants including protection from herbivores and pathogens. They also protect the plant cells against UV damage by absorbing the harmful radiation. Many phenolic compounds possess antioxidant properties thereby protecting the cells against oxidative damage from free radicals. The increase in phenolic compounds after treatment with SA is reported in many plant species [28].

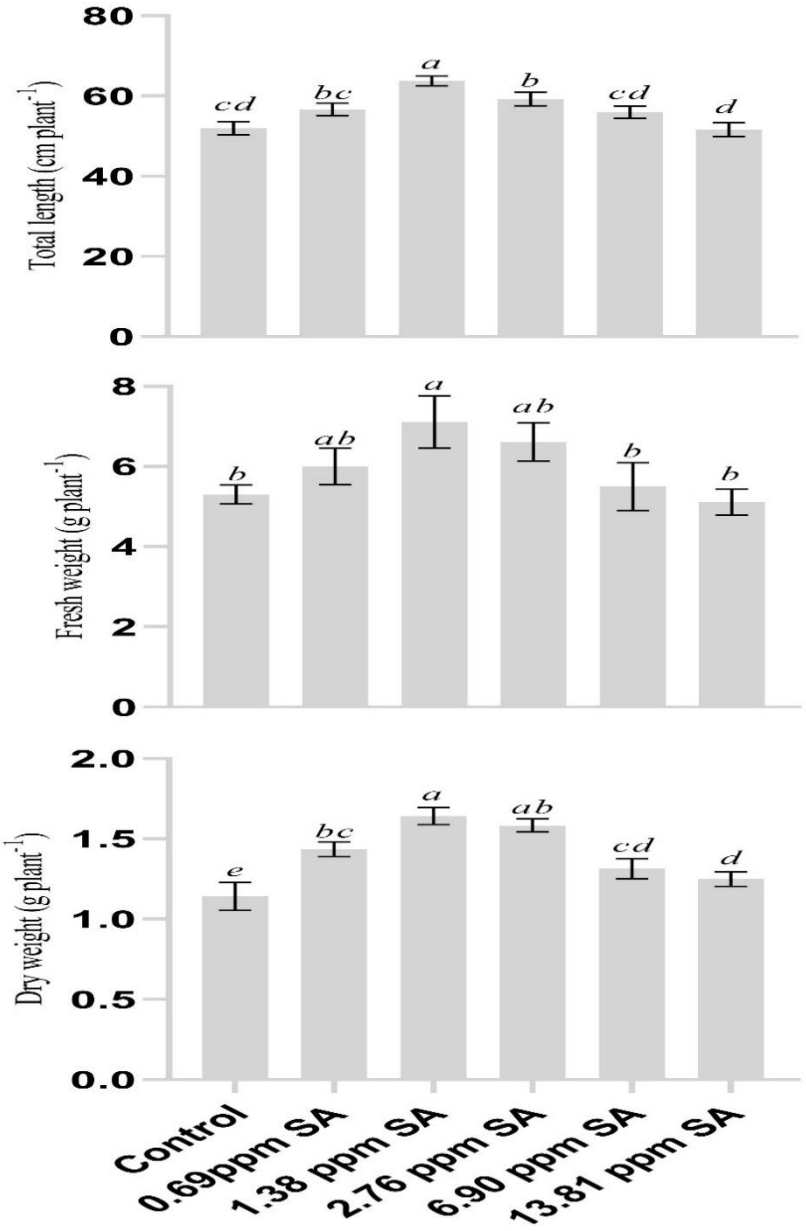

Fig. 8. Growth parameters (total length, fresh weight, and dry weight) of the plant under different treatments

Alkaloids are known to deter herbivores from consuming plant tissues. Eleven different alkaloids were identified in the 1.38 ppm SA-treated plant including trigonelline, harmine, tropinone, tyramine, and harmaline Some of these alkaloids exhibit antimicrobial activity that inhibits the infestation by bacteria, and fungi. The identified alkaloids also influence cell division, elongation, and differentiation. Indole-3-acetic acid, play a crucial role in growth and development. It regulates cell elongation, apical dominance, root formation, leaf expansion, and senescence. The other indole group containing compounds identified in the study like indole-3-acetyl-L-aspartic acid, N-(indole-3-acetyl) leucine, and terpenoids by SA administration has been reported in different plant species [28]. Betaine, belonging to the class betalains, was found to be the most dominant secondary metabolite in both groups. It serves several roles in the plant that improve the overall well-being of the plant. Betaine acts as an osmoprotectant, by maintaining the cell turgor pressure, ion balance, and hydration levels, thereby preventing cell damage. Betaine is also known to enhance photosynthesis and scavenge ROS. It is also involved in the synthesis of methionine from homocysteine. Methionine acts as a precursor for several important proteins and phytochemicals in the plant. Asarone, the only phenylpropanoid detected in the plant, acts as a chemical defense mechanism of the plant against bacterial and fungal infestation. It also contributes to the aroma and flavor of the plant. 7 flavonoids including rutin, tangeritin, kaempferol, quercetin, and trifolin were identified from the 1.38 ppm SA-treated plant. Flavonoids are known for their color-imparting and antioxidative properties. They also absorb harmful5-hydroxyindole-3-acetic acid play a role in the transport and regulation of the activity of indole-3-acetic acid. 35 terpenoids and their derivatives were identified. Terpenoids like perillic acid, pyrethrin I, saikosaponin A, bryonioside C act as chemical defenses against herbivores and pathogens. Other terpenoids like citral, caryophyllene oxide, curcumene, alpha-farnesene, carvone, cymene, turmerone, lupeol, sulcatol bestow the plant with flavor and aroma. The alcoholic terpenoids are involved in the formation of cuticular wax on the leaf surface which helps in reducing water loss and protecting against environmental stress. They also help the plant adapt to adverse conditions by stabilizing cell membranes, scavenging free radicals, and minimizing oxidative damage. The increase in the level of

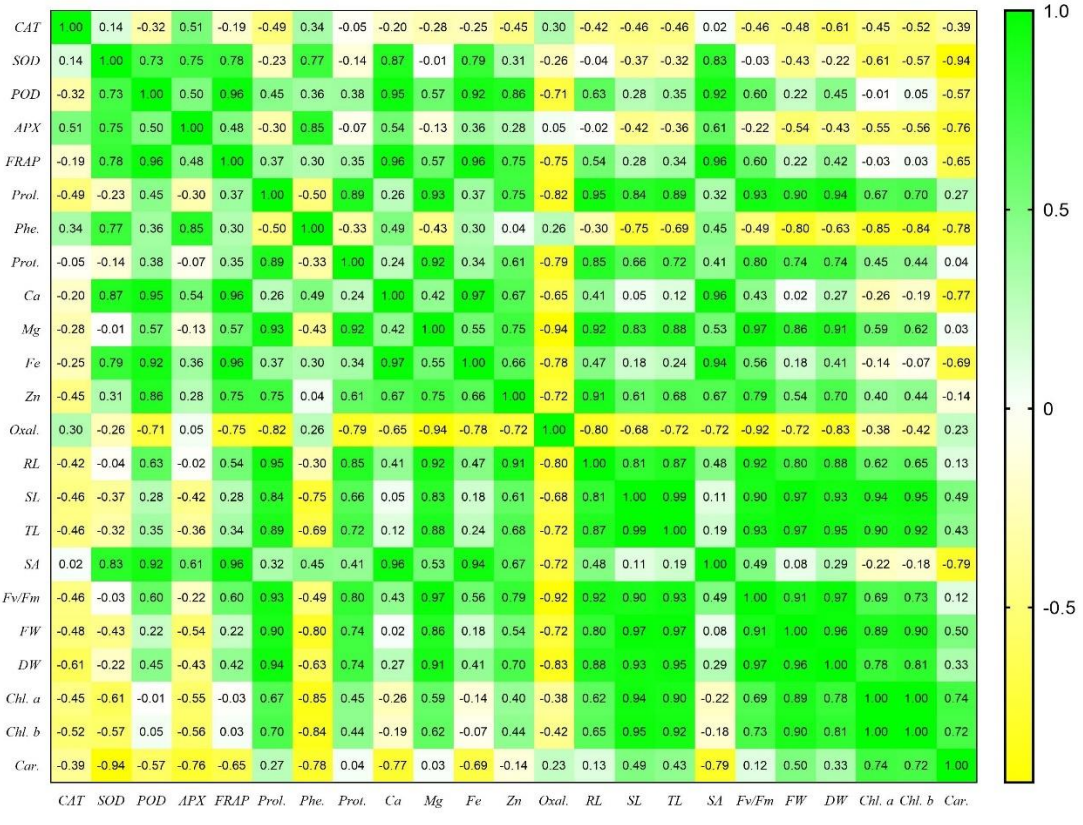

Fig. 9. Heat-map showing the correlation among different parameters

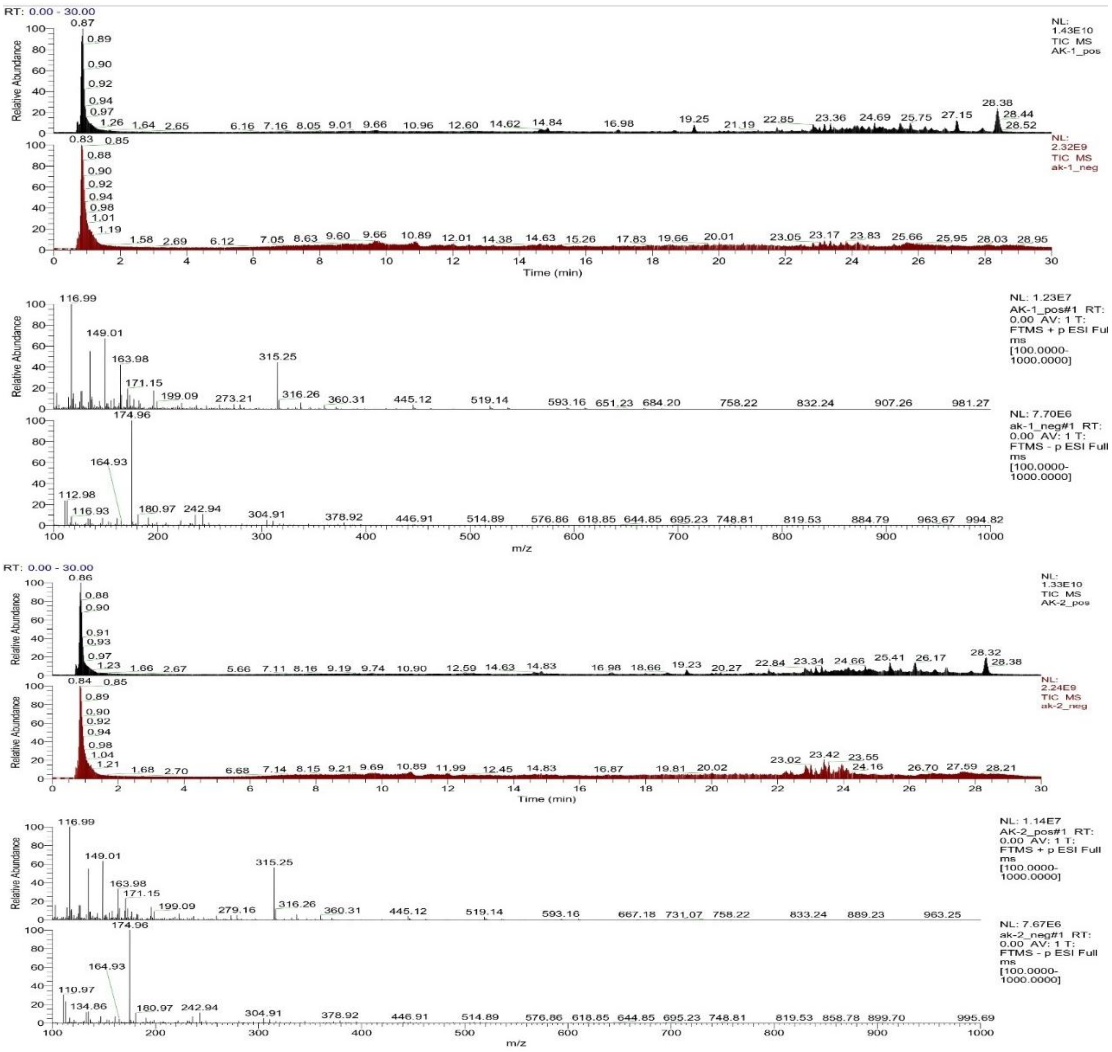

Fig. 10. The chromatogram obtained from UHPLC-HRMS analysis of (a) control treated plant and (b) 1.38 ppm SA treated plant at positive and negative ionization mode

UV radiations to prevent DNA damage and mutations and maintain cellular homeostasis. The increase in the level of antioxidant activity and phenolic and flavonoid compounds has been reported by Ghassemi-Golezani et al. [29]. Coumarins such as 7-hydroxycoumarine, isopimpinellin, scoparone, esculin, and phellopterin also act to deter the herbivores, besides posing antifungal properties. They also play a role in allelopathy. They are also involved in root development, and establishing mycorrhizal associations that improve the nutrient uptake by the roots. Fatty acids are responsible for the formation and integrity of lipid-based structures like cell membranes, cuticles, and storage organelles. Some fatty acids that were identified were eleostearic acid, lignoceric acid, pentadecanoic acid, pinolenic acid. They also act as an energy source in situations of crisis. Oxylipins, derived from the oxidation of fatty acids, serve the role of a signaling molecule. Two oxylipins were detected from the plant extracts: 9S,13R-12-oxophytodienoic acid, and jasmonic acid. 9S,13R-12-oxophytodienoic acid is a precursor in the biosynthesis of jasmonic acid. The conversion of 9S,13R-12-oxophytodienoic acid to jasmonic acid occurs enzymatically when the plant is subjected to stress such as herbivore attack, pathogen infestation, or mechanical damage. Jasmonic acid is categorized as a phytohormone, and it plays a role in the plant's reaction to mechanical damage and injuries. It triggers a cascade of defense responses, including the production of toxic secondary metabolites, proteinase inhibitors, and pathogenesis-related proteins. These responses deter herbivores, inhibit pathogen growth, and

enhance the plant's ability to resist biotic stress. The increase in the different metabolic components by the application of external phytohormones including salicylic acid has also been documented by Lv et al. [30]. The metabolites identified in our study are outlined in Supplementary Table 1.

The metabolic profile thus revealed that the application of salicylic acid strengthens the defense system of the plant by promoting the levels of compounds posing antioxidant activities, reducing the microbe infestation, maintaining the osmolyte homeostasis, and reducing oxidative damage. Salicylic acid also increased the metabolites that strengthened the activity of the root, and promoted growth of the plant. Many of these secondary metabolites also pose medicinal importance and their accumulation increases the merits of the crop.

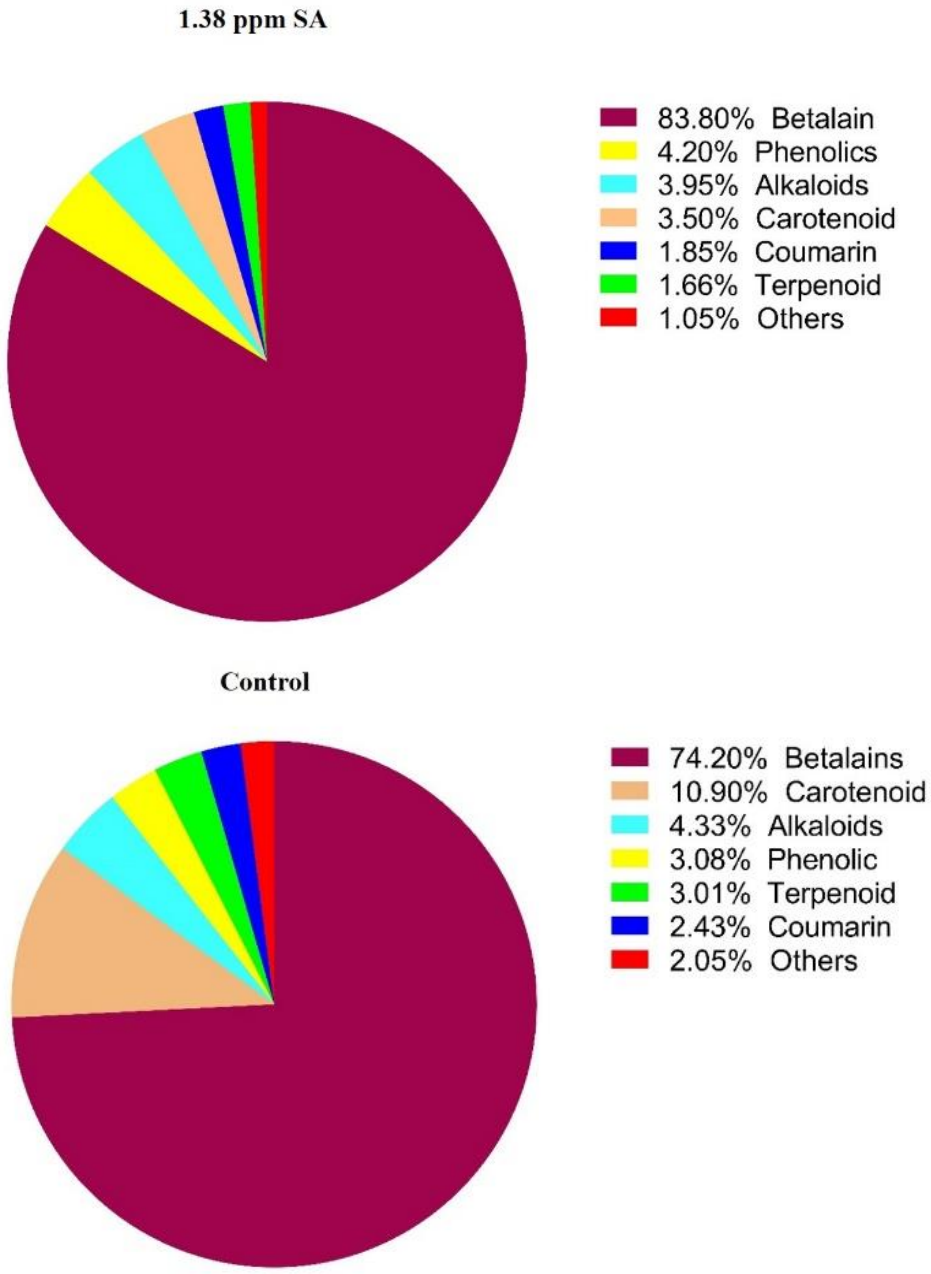

Fig. 11. Proportion of different classes of secondary metabolites in (a) 1.38 ppm SA (b) control plants

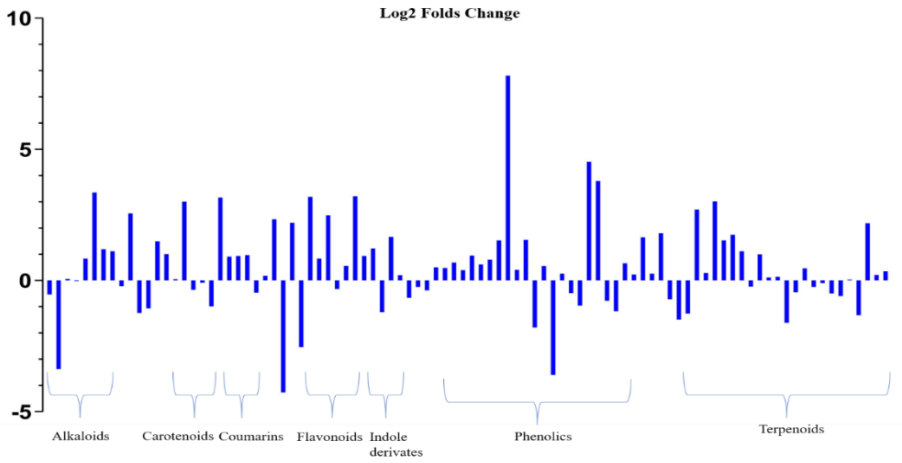

Fig. 12. Log2 folds change of different secondary metabolites of 1.38 ppm SA-treated plant

4. CONCLUSION

By participating in the different signaling responses the salicylic acid could play a promising role in regulating growth and the other functional activities of the plants. In our current study, we have observed that lower doses of SA, up to 2.76 ppm, yield several beneficial effects including an increase in the biomass, maximum photochemical quantum yield, antioxidant properties, protein content, and secondary metabolites production. In comparison to the control plants, the highest increase in fresh biomass was observed at a concentration of 1.38 ppm of SA, with a rise of 34%. Over other laboratory-based studies, the present study has been performed in natural soil conditions, providing pertinent real-world data.

The application of salicylic acid at lower doses demonstrated improvements in the *Amaranthus hybridus* both qualitatively as well as quantitatively. It also revealed the optimal dose and the frequency of application for maximizing the benefits of salicylic acid on plant metabolism. This discovery holds promise for guiding the utilization of salicylic acid in crops encountering challenging circumstances. Nonetheless, further investigation is necessary to ascertain the optimal application techniques and dosages for other economically significant vegetable crops, under various environmental conditions, with or without stress factors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.
Supplementary is available in the following link
https://journalijpss.com/media/galley_proof_2024_IJPSS_115896.pdf

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