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Growth improvement and pigment composition in cowpea (*Vigna unguiculata* (L.) Walp.) by foliar spray of salicylic acid and ascorbic acid under Nacl stress

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Abstract

Salinity stress is a big hurdle towards growing population as it accounts for a huge loss of crop productivity. So, alternative approaches are to be made to increase crop resistance towards various climatic entities including drought, salt so on. One approach is the exogenous supply of salicylic acid - a potential regulator and ascorbic acid -a strong antioxidant. In this view, a pot culture experiment was stuided to investigate the impact of salicylic acid (SA) and ascorbic acid (AsA) on growth and pigment contents of cowpea at 200mM NaCl stress. Plants were harvested randomly on 30th, 40th and 50th DAS for estimation of growth traits and pigment contents. Salt stress causes a reduction in the growth attributes like root, and shoot length, fresh weight and dry weight, chlorophyll a, b, total chlorophyll and carotenoids. The foliar spray of SA and AsA improved NaCl resistance in cowpea by increasing root length, shoot length, fresh weigh and dry weigh, chlorophyll pigments and carotenoids contents. Overall, SA and AsA foliar application completely counteracted the NaCl stress led deleterious effects at 200mM in cowpea. Thus, it can be concluded that foliar spray of SA and AsA induced tolerance to NaCl stress in cowpea due to enhanced growth and pigment contents.

Keywords: *vigna unguiculata* l., growth regulators, nacl stress, photosynthetic pigments

Introduction

Salinity stress is one of the most devastating abiotic stress factors responsible for huge agricultural loss worldwide and is expected to be drastically enhanced by global climate change (Reddy *et al*., 2017) [52]. The abiotic stresses, i.e., salinity, drought, cold, and heat deleteriously affect the survival, growth dynamics and yield of many essential food crops (El-Ramady *et al*., 2018a) [15]. According to an FAO survey greater than 6% of the world's land has worsen from salinity (FAO and ITPS, 2015) [16]. Therefore, salinity stress has a major effect on crop productivity with adverse effects on seed germination, plant vigor and crop estimation (Munns and Tester, 2008) [44]. The limited fresh water resources and use of brackish water for irrigation of lands has pushed them to salinization and results increase in the demand for food. Thus, to solve the problem of salinity stress, screening of varieties of crops for salt tolerance or to elevate salt tolerance is of great significance (Wu, *et al*., 2015) [62].

The increased concentration of soluble salts results osmotic stress (Tavakkoli *et al*., 2011; Munns *et al*., 2020) [57, 45]. Thus, exposure at high NaCl stress, excess Na^+ ion(s) accumulation in the cytosol or even in the chloroplast may directly target the photosynthetic machinery (Bose *et al*. 2017) [11]. Thus, causes imbalance in the generation and consumption of reducing potentials (NADPH and ATP), which plays active role during electron transport and thus disrupt the carbon reduction cycle (Calvin cycle) (Ogbaga *et al*., 2018) [48]. This oxidative stress (ion toxicity) led to the production of chemical entities Known as reactive oxygen species (ROS) at acceptor end of PSI, thereby causing damage to membrane and other cellular components like DNA, RNA, and proteins (Foyer and Shigeoka 2011; Foyer *et al*., 2017) [20, 21].

Interestingly, plants use inborn strategies to enhance tolerance mechanisms, thereby tackle ROS and maintain an appropriate water status. For instance, stomatal regulation (Tattini and Traversi, 2009) [56]; change in antioxidant defense systems (Gill and Tuteja, 2010; Gautam *et al*., 2017) 23], synthesis of compatible organic solutes like proline, glycine-betaine (GB), sugar alcohols, and alteration in biological membrane structure (Wyn Jones and others 1979; Pirasteh-Anosheh and others, 2016) [63, 51] are common phenomenon that provides adaptability in plants during stress conditions. However, such phenomenon disrupt as the sodium ion(s) concentration goes beyond their threshold level in the cytosol of the cell (Ahmad *et al*., 2019) [2].

Salicylic acid (SA) is a ubiquitous plant phenolic compound that acts as a strong stress ameliorator by playing diverse physio-biochemical and developmental roles in hormonal cross-talks and as an antioxidant. For example, advances germination, improves growth, enhances nutrient uptake, delays senescence, stomatal closure, regulates photosynthesis, maintaining leaf and chloroplast integrity and upsurges enzyme activities during abiotic stress tolerance in plants (Mimouni *et al*., 2016; Agnihotri *et al*., 2018) [41, 1]. It improves salinity tolerance in plants as reported by several authors, for instance, under salinity stress in mung bean, canola plant (Farhangi-Abriz *et al*., 2019; Lotfi *et al*., 2020) [17, 38] and Choysum (Kamran *et al*., 2020) [31].

An ascorbic acid (AsA) a ubiquitous low molecular weight antioxidant possess high water solubility, has the ability to directly or indirectly quenching ROS generated as by-products of photosynthesis. Besides, this acts as a cofactor for enzymes, participates in regulating cell signalling pathways and many other physiological processes such as cell division, cell expansion, primary cell wall growth and in the optimization of photosynthesis (Liso *et al*., 1984; Foyer and Noctor, 2011; Aziz *et al*., 2018) [37, 19, 9]. Nonetheless, externally applied AsA enhances the physiological attributes such as growth, ion transport and photosynthesis rate by increasing the plant's tolerance against abiotic stress (Akram *et al*., 2017) [3]. The exogenous supplied AsA results further enhancement in the internal AsA levels in plant cell systems, and thereby mitigates the stress levels (Billah *et al*., 2017) [10]. The vitamin-C plays a significant role in maintaining photosynthetic machinery in canola and tomato (Bybordi, 2012; Alayafi, 2020) [13, 4] synthesis of phytohormones (Gest *et al*., 2013) [24], ion uptake in okra (Wang *et al*., 2019) [61], tomato by antioxidant system regulation (Siddiqui *et al*., 2019) [55], high yield in millet (Hussein and Alva, 2014) [29], and harvest index in barley (Noreen *et al*., 2021) [47].

The majority of crops especially legumes are highly salt-sensitive, thus reduction in relative growth rate is a common phenomenon, which results in a lower nitrogen demand (Ashraf *et al*., 2018) [9] and reduction of biomass, yield quantity and quality are commonly observed (Zörb *et al*., 2019) [66]. Cowpea (*Vigna unguiculata* L.) Walp. Commonly known as "Lobia" is an annual herbaceous legume belongs to family *fabaceae*. It contains high nutrition value, and essential vitamins and may act as a substitute for soybean-based protein products. This is particularly an important staple crop in arid, and semi-arid regions globally, where other crops don't thrive well. The plant shows moderate resilience to salt stress. However, its yield is greatly affected in saline soils as reported so far (Mini *et al*., 2019) [42]. We hypothesize that foliar supply of SA and AsA can improve growth performance by regulating and enhancing metabolic activities and thereby improves the yield of cowpea under NaCl stress.

Materials and Methods

Plant collection and Chemical reagents

The seeds of Cow pea (*Vigna unguiculata* (L.) Walp) variety vamban-1 (VBN1), were collected from National Pulses Research Centre, Vamban, Tamil Nadu, India. The growth regulators SA, AsA and analytical reagent grade NaCl were purchased from HI-Media Mumbai Ltd.

Experimental design

The pot culture, and physio-biochemical analysis were conducted at botanical garden and experimental part was done in stress physiology lab., Department of botany, Annamalai University. Briefly, Seeds were disinfected with ethanol (70%) (v:v) for 3 minutes, followed by thorough washing with double-distilled water to remove traces of ethanol. Earlier to sowing sterilized seeds were soaked for 6 hours in a glass beaker. Then the soaked dry seeds were transferred to plastic pots (Height = 20 cm and Depth = 20.5cm) filled with 8 kg of homogenous mixture of red soil, sand and farmyard manure in the ratio (1:1:1) for sowing purpose. Subsequently, NaCl treatment 500ml L^{-1} was imposed on 12th days after sowing (DAS) and maintained through soil EC and last up to end of the experiment.

In addition, foliar supply of SA [dissolved in 10ml of warm 95% ethanol for complete dissolution] and AsA solutions were made ready. However, spraying was done twice a week and 80ml $plant^{-1}$ each solution was sprayed uniformly on both the surfaces of leaves, using Tween-20 (0.05%) as a wetting agent. Generally, spraying was done manually using normal spraying bottle and soil surface was covered by a polyethylene sheet during spraying. However, control plants were irrigated with tap water. The pot culture arranged was in a Completely Randomized Block (CRB) manner. Plants were taken into six groups (T0-T5) with three replicates (n=3) each group. The samples were collected for observations on 30th, 40th and 50th days after sowing, respectively. The treatments used in the experiment are as follows (I) Control (0mM Non-Saline) (II) NaCl (200mM) (III) Salicylic acid (0.25mM) (IV) Ascorbic acid (0.5mM) (V) NaCl (200mM) + Salicylic acid (0.25mM) (VI) NaCl (200mM) + Ascorbic acid (0.5mM)

Morphological attributes

Determination of Root length and Shoot length

The cowpea plants were uprooted, washed with tap water and taken to the lab. Immediately to determine the morphological traits. The root length was measuring below the point of root-shoot transition to the farthest point of root tip root. The length between shoot tip and point of the root shoot transition region was taken as shoot length. The values were expressed in cm $plant^{-1}$.

Determination of Fresh weight and Dry weight

The plant roots and shoots were washed with the tap water and blotted gently with tissue paper to remove moisture. The root and shoot fresh weights were taken by using an electronic balance (Model – DS-852J Series) and subsequently, the plants were dried at 70°C in a hot air oven for 48hours. After drying, their weight was measured and the material was kept in same oven until the constant dry weight obtained. the values of both fresh and dry weight were recorded and expressed as gm $plant^{-1}$.

Determination of Chlorophyll and Carotenoid contents

A fresh leaf tissues of 500mg were ground in a pre-chilled mortar by adding 10ml of 80% acetone(v:v). After complete extraction, the mixture was centrifuged at 800 x g for 15 minutes at 4°C. Further, the extraction was repeated again by discarding the supernatant. Finally, the supernatant collected was made up to a final volume of 10ml with 80% acetone. The absorbance was read @ 645, 663, and 480nm using spectrophotometer (Model-118, Systronic India Limited, Gujarat, India, UV–VIS). The chlorophyll and carotenoid contents obtained were calculated followed by coefficients of Arnon (1949) [6], and Kirk and Allen, (1965) [34] and expressed in mg g^{-1} FW.

Statistical Analysis

The data were analyzed statistically using SPSS software (version 22.0) followed by one-way ANOVA. The obtained data represented in bars are mean values of three replicates (n=3), and (±) standard error (SE). The 0.05 % was chosen as significance by Duncan's Multiple Range Test (DMRT).

Result

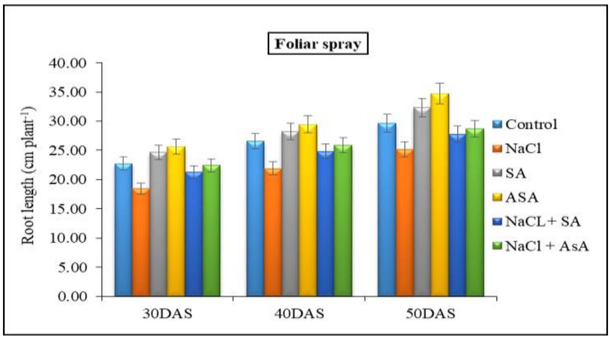

Fig 1: Effect of foliar application of SA and AsA on root length of cowpea (vamban-1 variety) under 200mM NaCl stress. Values represented in Bars are mean of three replicates (n=3) and (±) standard error.

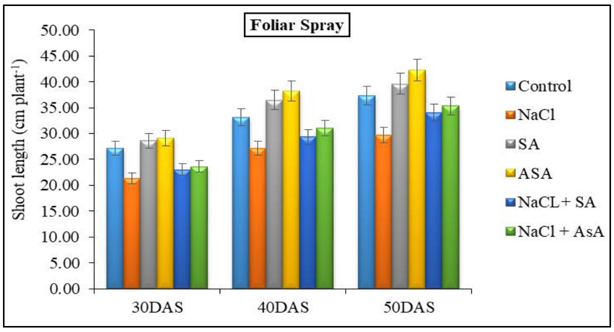

Fig 2: Effect of foliar application of SA and AsA on shoot length of cowpea (vamban-1 variety) under 200mM NaCl stress. Values represented in Bars are mean of three replicates (n=3) and (±) standard error.

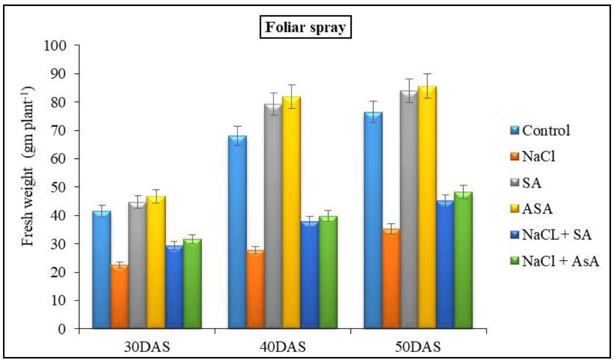

Fig 3: Effect of foliar application of SA and AsA on fresh weight of cowpea (vamban-1 variety) under 200mM NaCl stress. Values represented in Bars are mean of three replicates (n=3) and (±) standard error.

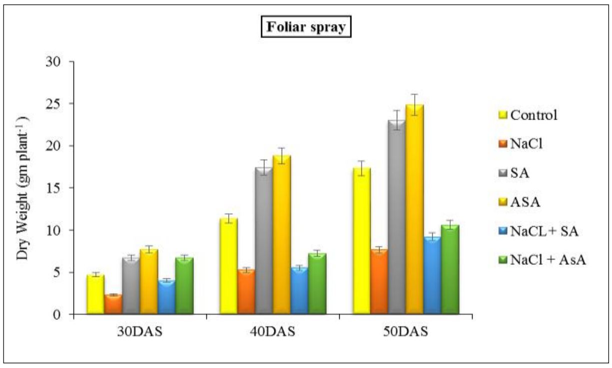

Fig 4: Effect of foliar application of SA and AsA on dry weight of cowpea (vamban-1 variety) under 200mM NaCl stress. Values represented in Bars are mean of three replicates (n=3) and (±) standard error.

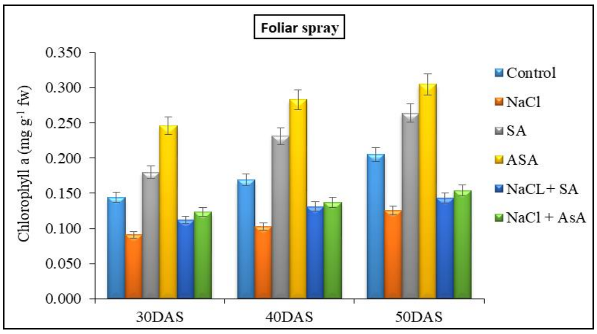

Fig 5: Effect of foliar application of SA and AsA on chl. ‘a’ of cowpea (vamban-1 variety) under 200mM NaCl stress. Values represented in Bars are mean of three replicates (n=3) and (±) standard error.

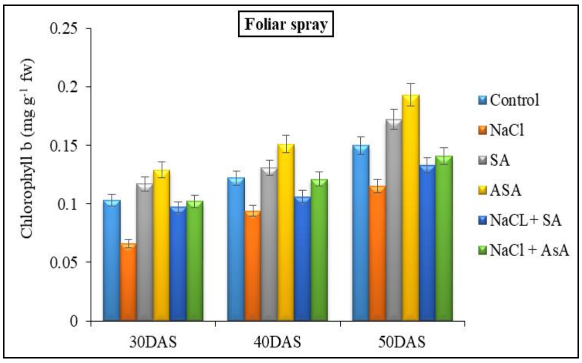

Fig 6: Effect of foliar application of SA and AsA on chl. ‘b’ of cowpea (vamban-1 variety) under 200mM NaCl stress. Values represented in Bars are mean of three replicates (n=3) and (±) standard error.

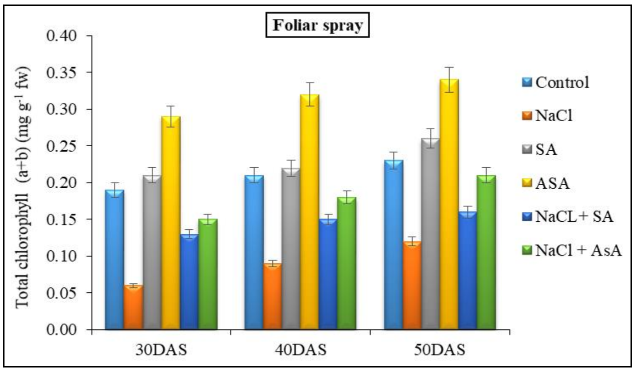

Fig 7: Effect of foliar application of SA and AsA on total chl. ‘a+b’ of cowpea (vamban-1 variety) under 200mM NaCl stress. Values represented in Bars are mean of three replicates (n=3) and (±) standard error.

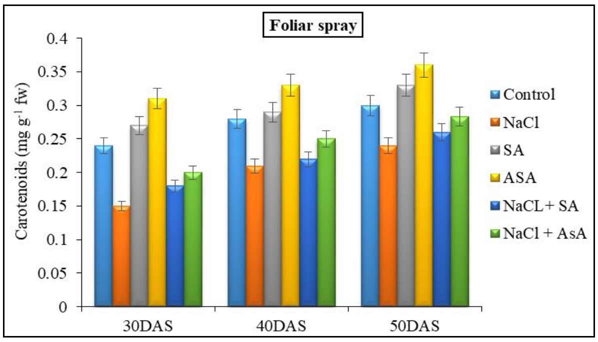

Fig 8: Effect of foliar application of SA and AsA on carotenoids of cowpea (vamban-1 variety) under 200mM NaCl stress. Values represented in Bars are mean of three replicates (n=3) and (±) standard error.

Results

Growth attributes

NaCl stress (200mM) caused a significant reduction in growth related characters like root length and shoot length, fresh weight and dry weight in cowpea plants. The reduction was seen more at 50th DAS NaCl treated plants (200mM) alone compared to control. It was 84.83% and 79.45% in root length (Fig.1) and shoot length (Fig.2) respectively on 50 DAS. But the foliar applications of SA and AsA alone has seen to be enhanced cowpea growth characters maximum in comparison to control one. Moreover, combination of NaCl with SA, and AsA has positive effect on cowpea root length and shoot growth compared to NaCl treated plants alone (Fig. 1- 2). The root length recorded after NaCl + SA treatment was 93.60% and NaCl + AsA 96.76%; while shoot length was NaCl + SA 91.16% and NaCl + AsA 94.61% per cent over control on 50 DAS. Moreover, SA and AsA treated plants also increased root length (108.90% and 117.06%) and shoot length (106.16% and 113.13%) on 50 DAS compared to normal one.

Biomass Reduction

The reduction in fresh and dry weight was 46.02 % and 44.30 % respectively, percent over control in cowpea plant under NaCl stress. (Fig. 3-4). The increase in biomass production recorded after NaCl treated plants combined with SA and AsA was 58.92 % and 63.06% of fresh weight and 53.28% and 61.36% of dry weight, respectively. It represents that foliar sprays of SA and AsA over NaCl stress have made tolerance in cowpea plant against NaCl stress and results maximum improvement of growth in cowpea plant. Moreover, SA and AsA treated plants alone also increased fresh weight (109.73% and 111.84%) and dry weight (133.43% and 143.66%) on 50 DAS compared to control.

Photosynthetic Pigments

Under NaCl (200mM) salt concentration, data on pigment composition-chlorophyll a, b, Total chlorophyll and carotenoids showed maximum reduction in all pigment constituents compared to control on all DAS. The NaCl led reduction in chlorophyll and carotenoids constituents was maximum observed on 50th DAS and was 60.97% (Chl.a), 76.66% (Chl.b), and 52.17% (Chl.t), and 80% (car.) respectively (Fig. 5-8). But the SA and AsA applied foliarly showed enhanced in the pigments, both alone and with salt treated plants. The maximum increase was recorded on 50th DAS and was 69.75% and 75.12% (Chl. a), 88.67% and 94% (Chl. b), and 69.56 % and 91.30% (Chl.t), respectively (Fig. 5-7).

Similar case was observed in carotenoids when cowpea plants were treated with 200mM NaCl stress. However, NaCl salt stress treated cowpea plants showed an increase in carotenoids contents after foliar applied SA and AsA and was 86.67% and 93.32 % (Fig. 8). The aerial spray of SA and AsA alone showed an enhancement in carotenoid contents compare to normal one.

Discussion

Soil salinity has become a major agricultural problem of the modern world (Noreen *et al*., 2021) [47]. Liang *et al*., (2018) reported that if the salinization to agricultural lands continues at the current rate than the yield of important crops may fall around 50%. In the current study sodium chloride stress caused a drastic reduction of plant growth attributes like root length, shoot length, and biomass production. However, this reduction was seen more at 50th DAS compared to 30th DAS and 40th DAS respectively, under 200mM NaCl exposure (Fig. 1-4). Our results agreed with those of Kamran *et al*., (2020) [31], and Noreen *et al*., (2021) [47], Who observed a drastic reduction in plant growth traits and biomass production under exposure to NaCl stress. The reduction in growth of cowpea plant might be due to prolonged effect of severe NaCl stress (200mM) lead drastic damaging effects on root cell activity. Since, roots are first organs to determine growth based on soil environment. Because under salt stress growth reduction is attributed to the toxic ions induced suppression of cell cycle and expansion and finally damage to the root architecture which restricts plants’ efficacy of water uptake and mineral uptake and their subsequent utilization (Kamran *et al*., 2020) [31].

However, applications of SA (0.25mM) by foliar way showed an enhanced growth biomarker in cowpea plant under stressed and non-stressed conditions as well (Fig. 1-2). Our results are in line with the findings of Kamran *et al*., (2020) [31] and Farhangi-Abriz *et al*., (2019) [17]. The authors reported that foliar applied SA can enhanced root growth that in turn promotes growth of the plant. The improved growth of plant after SA treatment could be peculiarity of an enhanced root architecture followed by increased water absorption and nutrient uptake (Kamran *et al*., 2020) [31] and reducing ethylene production in root cells (Khan *et al*., 2014) [32].

Foliar applied ascorbic acid significantly improved root growth and shoot height under non-stressed and salt stressed conditions as well (Fig. 1-2) and thus, enhanced overall growth of plant. Increased root length and shoot length by exogenous applied Vitamin-C (AsA) has found in Okra cultivar (Wang *et al*., 2019) [61], safflower (Farooq *et al*., 2020) [18] and barley (Noreen *et al*., 2021) [47] respectively. Vitamin-C is a strong antioxidant is believed to be beneficial for plants by protecting against stressful conditions. A recent report based on RNA-seq analysis studies of soyabean and other crops showed that down regulation of VTC1 genes among other known regulators participating in the AsA pathways correlated with observed decline in AsA levels (Mellidou, and Kanellis, 2017; Seminario *et al*., 2017) [40, 54]. Thus, foliar application of AsA could enhance root apical meristem activities and ultimately promotes plant growth by regulating root cell-cycle and cell elongation (Akram *et al*., 2017) [3] and thereby declining Na^+ content and increases K^+ content of the tissues. In addition, Ascorbic acid is one of the cheaply available growth promoters that can be taken for farmers to stress tolerance in plants like salt and drought stress (Farooq *et al*., 2020) [18].

Biomass Production

Salinity treatment alone showed decline in the biomass production in cowpea (fig. 3-4). The reduction in biomass of plant due to NaCl stress results by upsurged uptake of Na^+ ions, with concurrent decline in the amount of K^+ uptake by the plant (Noreen *et al*., 2021) [47]. The accumulation of Na^+ ions in the rhizosphere results osmotic stress lead hinder in water and mineral absorption from the soil (Zeeshan *et al*., 2020) [65]. This reduction in water availability of cells causes loss in turgidity that results low performance of physio-biochemical processes. Since, enzymes normally function in their aqueous environment (Vighi *et al*., 2017) [60]. Together the effects of these limitations degrade photosynthetic machinery and ultimately reduced growth and development (Choi *et al*., 2014) [14]. However, biomass of cowpea enhanced upon exogenously applied SA and AsA under control and salt stress conditions as well.

SA-mediated improvement in the biomass and salinity-tolerance was suggested as a result of SA-mediated increase in content of chlorophyll, rate of photosynthetic efficiency, and activity of antioxidant enzymes (Li *et al*., 2014) [35]. The application of SA improved the fresh weight compared to the respective salinity treatment especially with the application of 0.5 mM of SA in *Vicia faba* L (Anaya *et al*., 2018) [5] and in Choysum (Kamran *et al*., 2020) [31].

Vitamin-C is known to benefitted plant growth which may be due to its antioxidant activity that protects plants from abiotic stress lead damages (Beltagi, 2008). Further, Azzedine *et al*., (2011) found that the application of AsA was effective to alleviate salt led negative impact on plant growth. Similarly, Noreen *et al*., (2021) [47] observed foliar applied AsA promotes biomass of cowpea plants under salt stress.

Photosynthetic pigments

Sodium chloride stress has a drastic negative impact on rate of photosynthesis when compared to control. The cowpea plant exposed to NaCl stress (200mM) showed low amounts of green pigments (chlorophyll, a, b, total chl.s) and carotenoids compared to control one. In contrast, foliar applied SA and AsA showed enhanced rate of photosynthetic pigments under non-saline and salt treated plants (Fig. 5-8). The deminish in chlorophyll contents (Chl. a, b, and Chl.t, a + b) might be linked with ROS toxicity lead by excess salt ions accumulated in the cell. This results destruction of chlorophyll molecules, disrupts uptake of essential elements including Mg, K, and thereby upregulating the catabolism of chlorophyll degrading enzyme known as chlorophyllase (Bulgari *et al*., 2019; Sadak *et al*., 2020) [12, 53]. Moreover, carotenoids are known as strong antioxidants and can act as efficient quenchers of free radical caused by ROS. The reason of decrease in carotenoids contents is related to breakdown of β-carotene and its formation to zeaxanthins, which takes part in the protection of pigments against photoinhibition (Sharma and Hall, 1991).

The decline of chlorophyll concentration commonly acts a biomarker of plant stress sensing (Hussain *et al*., 2019) [28]. Our results agreed with the earlier studies that presents decreased chlorophyll contents and carotenoids progressively with increased NaCl concentrations (Manaf, 2016; Wang *et al*., 2019; Frukh *et al*., 2020; Sofy *et al*., 2020) [31, 22].

Probably, the positive effect of SA on pigments could be due to its stimulatory effects on increased K^+ ions and declining toxic Na^+ and Cl^- ions, production of ROS and enhanced Rubisco activity in plants under NaCl stress (Kamran *et al*., 2020) [31]. SA enhanced chlorophyll contents and carotenoids have reported in lemongrass and Choysum plants treated with salt stress conditions (Zaid *et al*., 2019; Kamran *et al*., 2020) [31].

Under limited redox-reaction conditions, ascorbic acid is proposed to act as an e- donor to photosystem-II (PSII) and thereby curbs photo-inactivation (Toth *et al*., 2011; Trubitsin *et al*., 2014) [59]. Thus, prevents chlorophyll damage caused due to oxidative stress. Krupa-Małkiewicz *et al*., (2015) for the tomato varieties and Wang *et al*., (2019) [61] for the Okra found increased chlorophyll contents and carotenoids when treated with AsA under NaCl salt toxicities.

Conclusion

Environmental stresses especially NaCl stress has a negative influence on the productivity of agricultural crops. In our study, NaCl stress showed a negative impact on growth characters, biomass production and pigment composition. However, exogenous applied SA and AsA result in improved growth traits and pigment composition in NaCl stressed plants. Such effects may be due to the preventive role of SA and AsA on cell metabolism by controlling oxidative stress lead by salinity stress in cowpea. Thus, foliar applied SA and AsA can be used to enhance salt tolerance in cowpea results enhanced in the growth and yield.

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