Arch Biol Sci. 2023;75(4):407-418 https://doi.org/10.2298/ABS230804033I

Seed priming and exogenous application of citric acid enhance seedling growth and photosynthetic pigments and mitigate oxidative damage of soybean (*Glycine max*) under salt stress

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Received: August 4, 2023; **Revised**: September 15, 2023; **Accepted**: September 18, 2023; **Published online**: October 2, 2023

Abstract: Seed priming and citric acid (CA) supplementation on germination and seedling growth of soybeans were investigated. Soybean seeds were primed with distilled water (control), 1 mM CA (CA1), or 2 mM CA (CA2) and then placed for germination in Petri dishes containing distilled water or 150 mM NaCl (SS), alone or in combination with 1 mM or 2 mM CA. Germinated seeds were placed in hydroponic pots using a similar treatment regimen to that specified for the Petri dishes to obtain seedling growth and biochemical parameters. Salt stress significantly lowered germination, growth traits, relative water content (RWC), and photosynthetic pigment. When soybean seeds were primed with CA under salt stress, the germination rate, final germination percentage, seed vigor index, and number of lateral roots significantly increased. Moreover, supplementation of CA significantly increased fresh and dry shoot and root weight, plant height, RWC, and photosynthetic pigments compared to salt-treated plants. The results also displayed that salt stress considerably increased hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents compared to control plants. Spraying of CA1 and CA2 significantly lowered the levels of H_2O_2 and MDA in salt-treated plants. Both hierarchical clustering and PCA revealed that the effects of salt stress and CA on germination, growth characteristics, photosynthetic pigments, H_2O_2, and MDA concentrations strongly interacted with one another. According to the findings, CA could be applied as a seed priming and exogenous agent to help soybeans grow more quickly when exposed to salt stress.

Keywords: citric acid; germination; photosynthetic pigments; seed vigor index; salt stress

INTRODUCTION

One of the main abiotic stressors, soil salinity, affects crop development and yield through morpho-physiological, biochemical, and molecular changes [1-4]. Excessive soil salinity negatively affects crop survival through ionic toxicity, reactive oxygen species (ROS) formation, and water imbalance [5-7]. Numerous investigations reported that salinity stress causes the reduction of different traits in plants, such as stomatal conductance, pigment content, transpiration rate, and photosynthesis rate [8-9]. Moreover, elevated salt in soil contributes to nutritional imbalance, inhibiting plant development and productivity [10-11].

It was found that legumes are more susceptible to salt stress [12]. To ensure sustainable agriculture,

How to cite this article: Imran S, Mahamud A, Chandra Paul N, Chakrobortty J, Sarker P, Paul S, Tahjib-Ul-Arif, Saidur Rhaman M. Seed priming and exogenous application of citric acid enhance seedling growth and photosynthetic pigments and mitigate oxidative damage of soybean (*Glycine max*) under salt stress. Arch Biol Sci. 2023;75(4):407-18.

soybeans (*Glycine max* L.) constitute the most significant source of vegetable protein in both human diet and cattle forage. Soybean, a noteworthy, farmed legume cash crop, is rich in protein, carbs, minerals, and oil [13-14]. In addition to 40-48% protein and 18-22% oil, soybean accounts for 30% of the world's production of edible oils [15]. According to a previous study, soybean growth and output were reduced when soil salinity exceeded 50 mM and could not produce seeds at a saline level of 80 mM [16]. Recent reports have demonstrated that the pretreatment of seeds or plants with various exogenous protective compounds, such as plant hormones, may significantly impact how plants react to specific abiotic stresses [17-22]. Seed priming and exogenous application of citric acid (CA), a low molecular weight organic acid, has been reported to promote plant growth in the presence of abiotic stressors [18,23]. For instance, the germination rate of papaya (*Carica papaya*) increased under salt stress conditions due to the seed pretreatment with citrate [24]. Yadav et al. [25] reported that field crop germination rates increased by up to 50% when CA was applied as a priming agent under saline conditions. In comparison to the control condition, CA considerably lowered the mean germination time of okra seeds and at the same time stimulated germination, the germination index, germination energy, and the seed vigor index [19]. CA foliar application to the leaves of cotton plants (*Gossypium barbadense*) increased salt tolerance by enhancing photosynthetic pigment contents, total soluble sugars, reducing and non-reducing sugars, proline, and total phenolic contents [26]. According to El-Hawary and Nashed [18], the foliar application of CA in conjunction with ascorbic and salicylic acid improved the growth and productivity of maize (*Zea mays*) under saline conditions. It is evident from several studies that CA increased the activity of different antioxidant enzymes, including superoxide dismutase, peroxidase, catalase, glutathione peroxidase, polyphenol oxidase, and ascorbate peroxidase in sugar beet, maize, roselle, cotton, and Chinese ryegrass [18,26-29]. In glycophytic plants and a halophytic plant, *Leymus chinensis*, exogenous CA improved growth features and endogenous CA concentration [27]. In addition, CA with thiamin and ascorbic acid augmented salinity tolerance in *Hibiscus sabdariffa* and *Melissa officinalis* by enhancing the nonenzymatic antioxidants (total phenolics, and proline), and lowering enzymatic antioxidants, including catalase, peroxidase, and phenylalanine ammonia-lyase [29]. In contrast, the essential oil content (monoterpene hydrocarbons and oxygenated sesquiterpenes) of *Melissa officinalis* increased after CA treatment under saline stress [30]. When CA was applied to sugar beet (*Beta vulgaris*), either alone or in combination with banana and tomato peel extracts, the amount of salt in the soil decreased, which improved the production of sugar roots [28].

To our knowledge, there are few studies on how different plant growth regulator supplements affect soybean growth and development when exposed to salinity stress. However, a previous report showed that soybean seed priming with brassinosteroid in combination with nitrogen improved seedling growth, net photosynthetic rate, total chlorophyll, carotenoid content, RWC, secondary metabolites, and antioxidant activities, and mitigated oxidative damage under salt stress [21]. Recently, it was reported that jasmonic acid enhanced seedling growth of soybeans and improved salinity tolerance by promoting the net photosynthetic rate, transpiration rate, stomatal conductance, total chlorophyll contents, relative water RWC, and antioxidant activities [22,31]. Similarly, salt stress was alleviated in soybeans after exogenous application of benzyladenine [32]. It has been reported that melatonin application increased plant growth, biomass accumulation, photosynthesis, mineral uptake, chlorophylls, and carotenoids and improved salinity tolerance in soybean seedlings [33]. Like melatonin, supplementation of salicylic acid improved the salinity tolerance of soybean by improving the net photosynthesis rate and lowering the H_2O_2 and MDA contents [34]. However, there is no previous research on the effects of CA priming or exogenous application on soybean growth and development under salt stress. Thus, the research question of this study is whether seed priming and exogenous supplementation of CA can improve soybean's morphological and physiological traits under salt stress.

MATERIALS AND METHODS

Experimental site and treatment conditions

Petri dish (for germination) and pot (for seedling) experiments were conducted in the laboratory of the Agronomy Department at Khulna Agricultural University, Khulna, Bangladesh. Popular soybean

(*Glycine max* L.) seeds (variety BARI Soybean-6) were obtained from the Bangladesh Agricultural Research Institute, Gazipur, for the experiment. The seeds were treated for 5 min with 1% NaOCl to eliminate microorganisms from the seed surface. Soybean seeds were primed for 60 min in distilled water (W), 1 mM or 2 mM citric acid (CA) (Merck, Mumbai 400079, Maharashtra, India). Thirty soybean seeds were primed for every treatment and placed on Petri dishes 150×25 mm in diameter using three layers of tissue paper. Every Petri dish was filled with 10 mL of a 150 mM NaCl solution for the salt treatment, 1 mM and 2 mM CA treatments; 10 mL of water was used as the control for the non-saline condition. Three Petri dishes were filled with 10 mL of a 150 mM NaCl solution for the salt treatment and combined with 30 seeds primed with water in one Petri dish (SS), 30 seeds primed with 1 mM CA were placed in another Petri dish (CA1+salt-stress or SS), and 30 seeds primed with 2 mM CA were in the third Petri dish (CA2+SS). Three more Petri dishes were filled with 10 mL of water and combined with 30 seeds primed with water per one Petri dish (Control), 30 seeds primed with 1 mM CA were placed in another Petri dish (CA1), and 30 seeds primed with 2 mM CA were placed in a third Petri dish (CA2). The experiment consisted of three replications. CA at 1 mM and 2-mM concentrations were used in the priming and foliar treatments; pre-optimization was based on our preliminary experiment, and several concentrations were used as follows: 0.5, 1, 2, 3, and 5 mM. The pre-experiment showed that soybean seed germination significantly improved at 1 mM and 2 mM CA compared to other CA concentrations (Supplementary Table S1). The 150 mM NaCl stress was chosen based on previous literature [35].

Seed germination parameters measurement

Daily records of seed germination were kept. From the initial germination until the 7th day, the number of germinated seeds was counted every 24 h. Thereafter, calculations for germination rate (GR), final germination percentage (FGP), mean germination time (MGT), germination index (GI), and germination energy (GE) were performed. On the 10th day of germination, the radicle length (RaL), radicle weight (RaW), hypocotyl length (HyL), hypocotyl weight (HyW), and number of lateral roots (NLR) of the germinated seeds were measured from 10 randomly selected seedlings. The seed vigor index (SVI) was calculated after measuring root length (RL) and shoot length (SL) on day 35 after sowing. The GR, FGP, MGT, GI, GE, and SVI were calculated using the corresponding formulae:

$$GR= \frac{GP1}{1} + - - - - - + \frac{GPx}{x}$$

where GP1 is the germination percentage on the 1st day after sowing, and GPx is the germination percentage on the xth day after sowing.

$$FGP= \frac{\text{Total no. of germinated seeds}}{\text{Total no. of seeds used}} \times 100$$

$$MGT=\Sigma \frac{Di n}{n}$$

where "n" is the seed number on day D*i* and D*i* is the number of days from the start of germination.

$$GI= \frac{\text{No. of germinated seeds}}{\text{Day of 1st count}} + \cdots + \frac{\text{No. of germinated seeds}}{\text{Day of last count}}$$

$$GE= \frac{Ti1}{Ni} \times 100$$

where T*i*1 is the number of germinated seeds on the 1st day, and N*i* is the total number of seeds.

$$SVI=FGP \times \text{seedling length (cm)}$$

where seedling length=shoot length+root length.

Plant growth conditions

A hydroponic system was used to grow uniformly germinated seeds in 2.5-liter pots, as described previously [19]. Three pre-germinated healthy seeds were placed in each hydroponic pot labeled as Control, SS, CA1, CA1+SS, CA2, and CA2+SS. The experiment consisted of three replications. Twenty-day-old seedlings were then stressed for 15 days with 150 mM NaCl (for SS, CA1+SS, and CA2+SS pots). Plants were sprayed with 1 mM or 2 mM CA (for CA1, CA1+SS, CA2, and CA2+SS pots) over 15 days, starting at the commencement of the stress (double spray each day at 9 am and 7 pm; 3 mL/plant/spray). After 15 days, RL, RFW, SL, SFW, RWC, and RWL were measured from one seedling from each pot, and leaf samples were collected to analyze photosynthetic pigments, H_2O_2, and MDA contents. Then, the roots and shoots were oven-dried for 72 h at 60°C. After drying, the RDW and SDW were recorded.

RWC and RWL determination

Utilizing conventional methods [36], the RWC was calculated. After 35 days, leaf samples were taken for the RWC measurement. After the measurement of fresh weight (FrW), the leaves were submerged in distilled water for 1 and 2 h. The remaining water on the leaves was blotted off with tissue paper, and the turgid weight (TuW) was noted. The leaves were then oven-dried for 72 h at 60°C. The dry weight (DrW) was then recorded. The gram (g) unit was used to measure both weights. The formula below was used to determine the RWC:

$$RWC\ (\%)=(FrW-DrW)/(TuW-DrW)\times 100$$

Following the RWC calculation, the RWL was determined using the following formula:

$$RWL\ (\%)=1-[(FrW-DrW)/(TuW-DrW)\times 100]$$

Measurement of photosynthetic pigments

A spectrophotometric technique based on the Lichtenthaler [37] method was used to quantify the amounts of photosynthetic pigments. The third leaf of every seedling was collected and kept in an ice box. Collected leaves (0.5 g) were placed in a small vial with 12 mL of 80% ethanol. To extract the pigments, the vials were kept in the dark for 10 days. Using a spectrophotometer (Shimadzu UV-1280, Kyoto, Japan) at wavelengths of 663, 645, and 480 nm, the amounts of chlorophyll *a*, *b*, and carotenoids were determined.

$$\text{Total Chlorophyll}=\text{Chlorophyll } a+\text{Chlorophyll } b$$

$$\text{Chlorophyll } a=(A_{663}\ x\ 0.999-A_{645}\times 0.0989)$$

$$\text{Chlorophyll } b=[A_{663}\ x\ (-0.328)+A_{645}\times 1.77\}$$

$$\text{Carotenoids}=[(A_{663}\times 0.114-A_{645}\times 0.638)+A_{480}]$$

Determination of H_2O_2 and MDA contents

Following the procedures of Zhang and Huang [38] and Alexieva et al. [39], respectively, lipid peroxidation (as MDA) and H_2O_2 were assessed in soybean seedlings for all treatments. The absorbance was measured using a UV-VIS spectrophotometer at 532 nm (T80, PG Instruments, China), and the level of MDA was estimated at an extinction coefficient of 155 $mM^{-1}cm^{-1}$. Absorbance was measured at 390 nm, and the level of H_2O_2 was estimated at the extinction coefficient of 0.28 $\mu M^{-1}cm^{-1}$.

Statistics

IBM® SPSS® Statistics V.25 was used for statistical analysis. Significant differences were determined using one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) ($P<0.05$). The heatmap package was used to construct a heatmap and conduct hierarchical clustering analysis using Euclidean distances in R 4.2.2. Principal component analysis (PCA) was performed using the GGally and factoextra packages.

RESULTS

CA priming augments germination of soybean under salt stress

We assessed the germination rate final germination percentage (FGP), mean germination time (MGT), germination index (GI), germination energy (GE), and seed vigor index (SVI) (Fig. 1) to evaluate the effects of CA seed priming on soybean germination under NaCl stress. The results of this study demonstrated that GR was significantly reduced in SS-treated seeds compared to control seeds. However, CA1- and CA2-primed seeds under SS stress significantly enhanced the GR (Fig. 1A). In the case of FGP, SS significantly lowered the FGP below the control value. Seeds primed with CA had a significantly increased FGP in CA1+SS- (66.66%) and CA2+SS- (82.22%) treated seeds, compared to SS- (31.11%) exposed seeds (Fig. 1B). In the case of the MGT, exposure to SS caused a significantly higher MGT compared to control seeds. However, the MGT was reduced by 28.8% and 35.8%, respectively, in CA1+SS- and CA2+SS-treated seeds when compared to SS-exposed seeds (Fig. 1C). SS significantly decreased the GI and the GE when compared to the control treatment. Moreover, CA1+SS- and CA2+SS-treated seeds showed significant increments in the GI and the GE compared to SS-treated seeds (Fig. 1D, E). Priming with CA boosted the SVI as opposed to SS, which significantly decreased it. Compared to SS-exposed seeds, the SVI was significantly increased by 75.75% and 85.42% in CA1+SS and CA2+SS seeds, respectively (Fig. 1F).

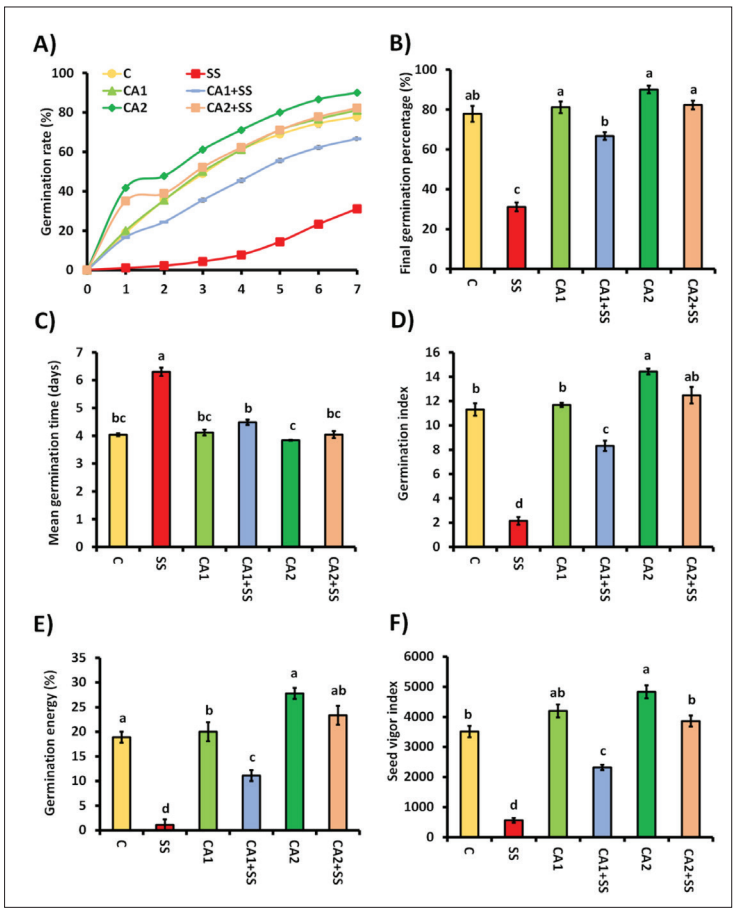

Fig. 1. Effects of citric acid on the germination indices of soybean seeds under saline stress. **A** – Germination rate; **B** – final germination percentage; **C** – mean germination time; **D** – germination index; **E** – germination energy; **F** – seed vigor index. C – control (distilled water), SS – 150 mM NaCl, CA1 – 1mM citric acid, CA1+SS – 1 mM CA+150 mM NaCl, CA2 – 2 mM CA, CA2+SS – 2 mM CA+150 mM NaCl. The data are presented as means of 3 replicates±SE, with a sample size n=30 for each replicate; Tukey's HSD (P<0.05), different letters between treatments.

Table 1. Treatment conditions

Treatments	Denotations
Control	C
150 mM NaCl	SS
1 mM CA	CA1
1 mM CA+150 mM NaCl	CA1+SS
2 mM CA	CA2
2 mM CA+150 mM NaCl	CA2+SS

C – control (distilled water), SS – 150 mM NaCl, CA1 – 1mM citric acid, CA1+SS – 1 mM CA+150 mM NaCl, CA2 – 2 mM CA, CA2+SS – 2 mM CA+150 mM NaCl

In a subsequent study, we measured the effects of CA priming on the radicle length (RaL), radicle weight (RaW), hypocotyl length (HyL), hypocotyl weight (HyW), and the number of lateral roots (NLR) under NaCl stress (Table 2). According to the results of our investigation, salt stress significantly reduced RaL, RaW, HyL, HyW, and NLR. Moreover, CA priming showed a significant increment in RaL, RaW, HyL, HyW, and NLR in CA1+SS- and CA2+SS-treated plants compared to plants exposed to SS. Together, these results indicated that CA2 performed better for germination metrics than CA1 under SS.

CA priming and supplementation accelerates plant growth traits and water status of soybeans under SS

To determine the effects of SS and stress-relieving CA actions, we measured the soybean seedling root length (RL), root fresh weight

Table 2. Effects of citric acid on radicle length, hypocotyl length, radicle weight, hypocotyl weight, and the number of lateral roots in soybean seedlings sown under saline conditions.

Treatments	Radicle length (cm)±SE	Radicle weight (mg)±SE	Hypocotyl length (cm)±SE	Hypocotyl weight (mg)±SE	Number of lateral roots±SE
C	6.97±0.39 bc	90.00±5.77 c	9.27±0.19 b	376.67±8.82 bc	9.33±0.88 b
SS	3.60±0.17 d	33.67±4.17 d	5.13±0.49 c	166.67±14.53 d	3.33±0.33 c
CA1	9.03±0.48 ab	133.33±8.81 ab	9.13±0.38 b	410.00±11.55 ab	11.67±0.88 ab
CA1+SS	6.03±0.46 c	99.67±0.88 c	8.27±0.32 b	316.67±18.56 c	8.67±0.33 b
CA2	9.67±0.68 a	170.00±15.27 a	13.23±0.81 a	483.33±18.56 a	14.00±1.53 a
CA2+SS	8.70±0.55 ab	153.00±3.05 a	10.53±0.75 b	436.67±28.48 ab	12.33±0.88 ab

Seeds were primed in distilled water, 1 mM or 2 mM CA for 60 min and then exposed to 150 mM NaCl alone or in combination with 1 mM or 2 mM CA for 15 days. C – control (distilled water), SS – 150 mM NaCl, CA1 – 1mM citric acid, CA1+SS – 1 mM CA+150 mM NaCl, CA2 – 2 mM CA, CA2+SS – 2 mM CA+150 mM NaCl. The data are presented as means of 3 replicates±SE, with a sample size n=10 for each replicate. Tukey's HSD (P<0.05), different letters between treatments.

Table 3. Effects of citric acid on seedling growth characteristics of soybean during NaCl stress

Treatments	RL (cm)±SE	RFW (mg)±SE	RDW (mg)±SE	SL (cm)±SE	SFW (mg)±SE	SDW (mg)±SE	PH (cm)±SE
C	17.57±0.994 bc	293.33±17.638 bc	31.00±2.082 bc	27.57±0.742 b	766.67±24.037 bc	84.67±5.364 ab	45.13±0.260 c
SS	6.80±0.513 d	99.00±10.536 d	11.67±1.202 d	11.10±1.041 d	410.00±5.774 d	33.67±1.764 c	17.90±1.353 e
CA1	18.90±1.553 abc	376.67±37.565 bc	41.33±3.756 ab	29.40±0.404 ab	710.00±34.641 c	86.67±8.838 ab	51.70±0.814 ab
CA1+SS	13.13±0.433 c	256.67±20.276 c	22.67±2.728 c	21.67±0.745 c	726.67±17.638 c	64.33±5.487 b	34.80±0.351 d
CA2	24.27±1.898 a	550.00±58.595 a	43.33±1.202 a	32.80±1.234 a	896.67±17.638 a	96.67±2.333 a	53.67±1.648 a
CA2+SS	19.60±1.253 ab	413.33±12.019 ab	39.33±1.667 ab	27.33±1.267 b	853.33±37.565 ab	94.00±2.646 a	46.93±1.431 bc

C – control (distilled water), SS – 150 mM NaCl, CA1 – 1mM citric acid, CA1+SS – 1 mM CA+150 mM NaCl, CA2 – 2 mM CA, CA2+SS – 2 mM CA+150 mM NaCl. The data are presented as means of 3 replicates±SE, with a sample size n=3. Tukey's HSD (P<0.05), different letters between treatments. Root length (RL), shoot length (SL), root fresh weight (RFW), root dry weight (DRW), shoot fresh weight (SFW), shoot dry weight (SDW), and plant height (PH).

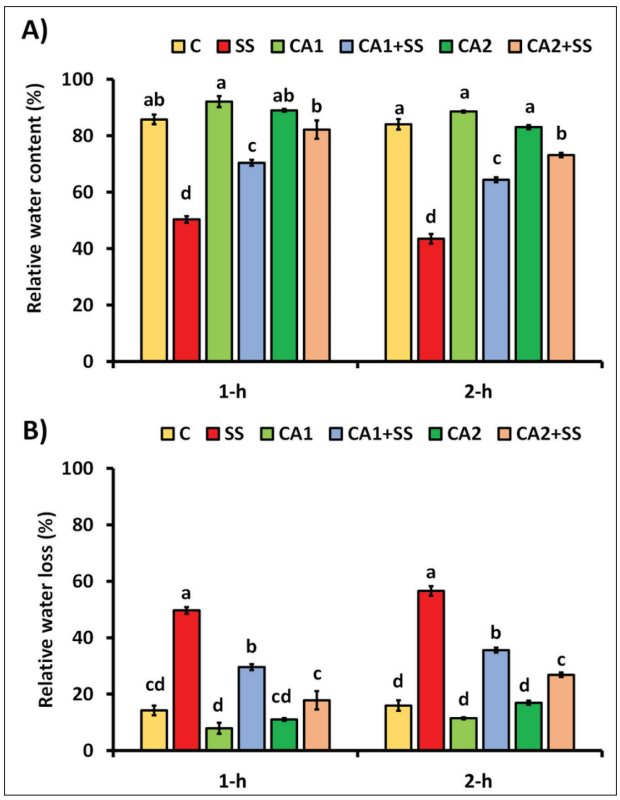

Fig. 2. Effects of citric acid on **A** – the relative water content (1 h and 2 h); **B** – related water loss (1 h and 2 h) of soybean seedlings under salt stress. C – control (distilled water), SS – 150 mM NaCl, CA1 – 1mM citric acid, CA1+SS – 1 mM CA+150 mM NaCl, CA2 – 2 mM CA, CA2+SS – 2 mM CA+150 mM NaCl. The data are presented as means of 3 replicates±SE, sample size n=3; Tukey's HSD (P<0.05), different letters between treatments.

(RFW), root dry weight (RDW), shoot length (SL), shoot fresh weight (SFW), shoot dry weight (SDW), and plant height (PH) (Table 3). The results showed that SS-treated plants significantly decreased RL, RFW, RDW, SL, SFW, SDW, and PH in soybean seedlings compared to the control. However, CA1+SS and CA2+SS plants exhibited significantly enhanced RL, RFW, RDW, SL, SFW, SDW, and PH compared to SS plants. CA2 outperformed CA1 in seedling growth traits under SS.

We measured the relative water content (RWC) and relative water loss (RWL) with or without SS using CA to examine the water status of soybean plants. SS significantly reduced RWC compared to the control after 1 h and 2 h (Fig. 2A). Supplementation with CA significantly improved the RWC after 1 h (by 28.58% and 38.7%) and 2 h (by 32.5% and 40.6%) in CA1+SS and CA2+SS plants, respectively, compared to SS-exposed plants (Fig. 2A). On the other hand, a significant increase in the RWL was also recorded in response to SS at both 1 h and 2 h (Fig. 2B). Compared to SS plants, CA1+SS and CA2+SS plants showed significantly lower RWL both at 1 h (40.4% and 64.1%) and 2 h (37.02% and 52.5%), respectively (Fig. 2B). These results indicated that CA2 maintained a better water status in soybean than CA1 under SS.

CA supplementation increases photosynthetic pigment contents under SS

An enormous fluctuation in chlorophyll pigment content l was noted during SS (Fig. 3). The amount of total chlorophyll in soybean leaves (41.6%), chlorophyll *a* (41%), and chlorophyll *b* (41.3%) decreased significantly under SS compared to the control (Fig. 3A-C). Compared to SS plants, supplementation with CA greatly enhanced the total chlorophyll, chlorophyll a, and chlorophyll b levels in CA1+SS and CA2+SS plants. According to pigment data, SS plants had significantly reduced carotenoids (71.8%) compared to control plants. Supplementation with CA1 or CA2 indicated significant increases in carotenoid levels compared to SS plants (Fig. 3D). The above results also showed that CA2 supplementation increased the photosynthetic pigment contents more than CA1 supplementation under SS.

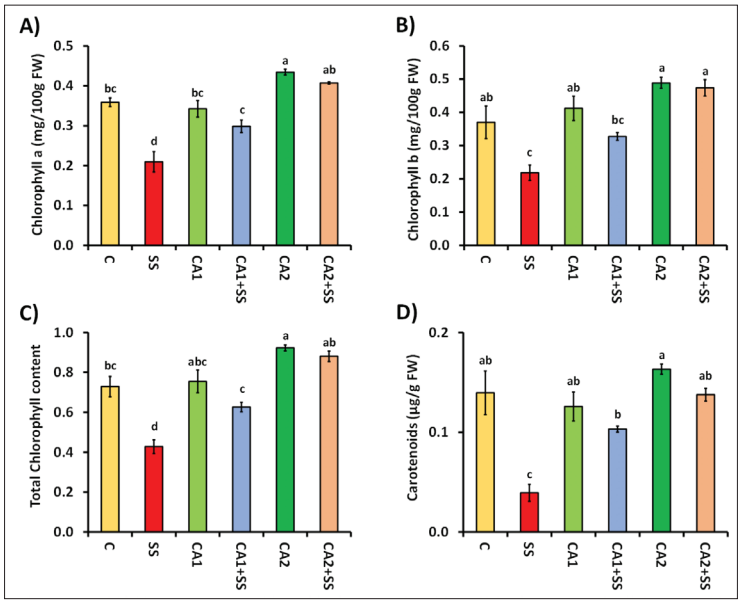

Fig. 3. Effects of citric acid on photosynthetic pigments. Soyabean content during NaCl stress: **A** – chlorophyll *a*; **B** – chlorophyll *b*; **C** – total chlorophyll; **D** – carotenoid. C – control (distilled water), SS – 150 mM NaCl, CA1 – 1mM citric acid, CA1+SS – 1 mM CA+150 mM NaCl, CA2 – 2 mM CA, CA2+SS – 2 mM CA+150 mM NaCl. The data are presented as means of 3 replicates±SE; sample size n=3; Tukey's HSD (P<0.05), different letters between treatments.

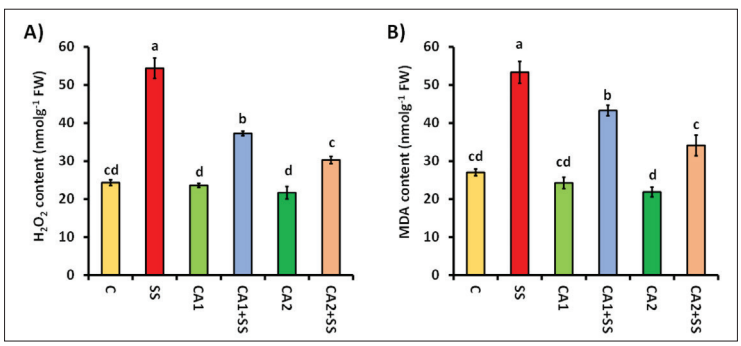

Fig. 4. Effects of citric acid on the **A** – hydrogen peroxide (H_2O_2) and **B** – malondialdehyde (MDA) contents of soybean under NaCl stress. C – control (distilled water), SS – 150 mM NaCl, CA1 – 1mM citric acid, CA1+SS – 1 mM CA+150 mM NaCl, CA2 – 2 mM CA, CA2+SS – 2 mM CA+150 mM NaCl. The data are presented as means of 3 replicates±SE; sample size n=3; Tukey's HSD (P<0.05), different letters between treatments.

Supplementation of CA degrades H_2O_2 and MDA contents under SS

To examine the mitigating effect of CA on salt-induced oxidative damage in soybean leaves, we measured the H_2O_2 and MDA contents (Fig. 4). SS plants exhibited a significantly enhanced production of H_2O_2 and MDA compared to control plants (Fig. 4A, B). In contrast, spraying with CA prevented the onset of oxidative damage in CA1+SS and CA2+SS plants, as shown by the significantly lower levels of H_2O_2 (31.5% and 44.4% for CA1+SS and CA2+SS plants, respectively) compared to SS plants (Fig. 4A). In the case of MDA content, SS plants had a significantly increased MDA content (49.3%) compared to control plants (Fig. 4B). However, spraying CA significantly decreased the MDA content by 18.8% and 36% in CA1+SS and CA2+SS plants, respectively. These results indicated that CA2 performed better in reducing oxidative damage caused by H_2O_2 and MDA than CA1 under SS.

Treatment-variable interaction measurement using hierarchical clustering and PCA

For constructing a heatmap with hierarchical clustering and PCA, the average values of all morphological and biochemical characteristics were used (Fig. 5). Three clusters (I, II, and III) were established in the variable axis using hierarchical clustering (Fig. 5A). The MGT, MDA, H_2O_2, relative water loss after 1 h (RWL1), and after 2 h (RWL2) are presented in Cluster-I. As opposed to C, CA1, CA1+SS, CA2, and CA2+SS plants, the Cluster-I parameter exhibited an upward trend in SS plants. The variable RWC after 1 h (RWC1) and after 2 h (RWC2) were included in Cluster-II. In C, CA1, CA1+SS, CA2, and CA2+SS plants, the Cluster-II parameters showed an ascending trend, but in SS plants, the Cluster-II parameters showed a descending trend. In addition, group III was made up of the variables – total chlorophyll (TChl), chlorophyll b (Chlb), chlorophyll a (Chla), RFW, HyL, SFW, root length (RL), GE, carotenoids (Caro), HyW, GI, SDW, FGP, PH, SVI, SL, the number of laterals roots (Roots), RaW, RDW, and RaL. C, CA1, CA1+SS, CA2, and CA2+SS plants had the highest values of Cluster-III characteristics, followed by plants subjected to SS. Additionally, PCA was also carried out to assess the interactions between the experimental parameters and the treatments (Fig. 5B). According to the positive and negative values of PC1

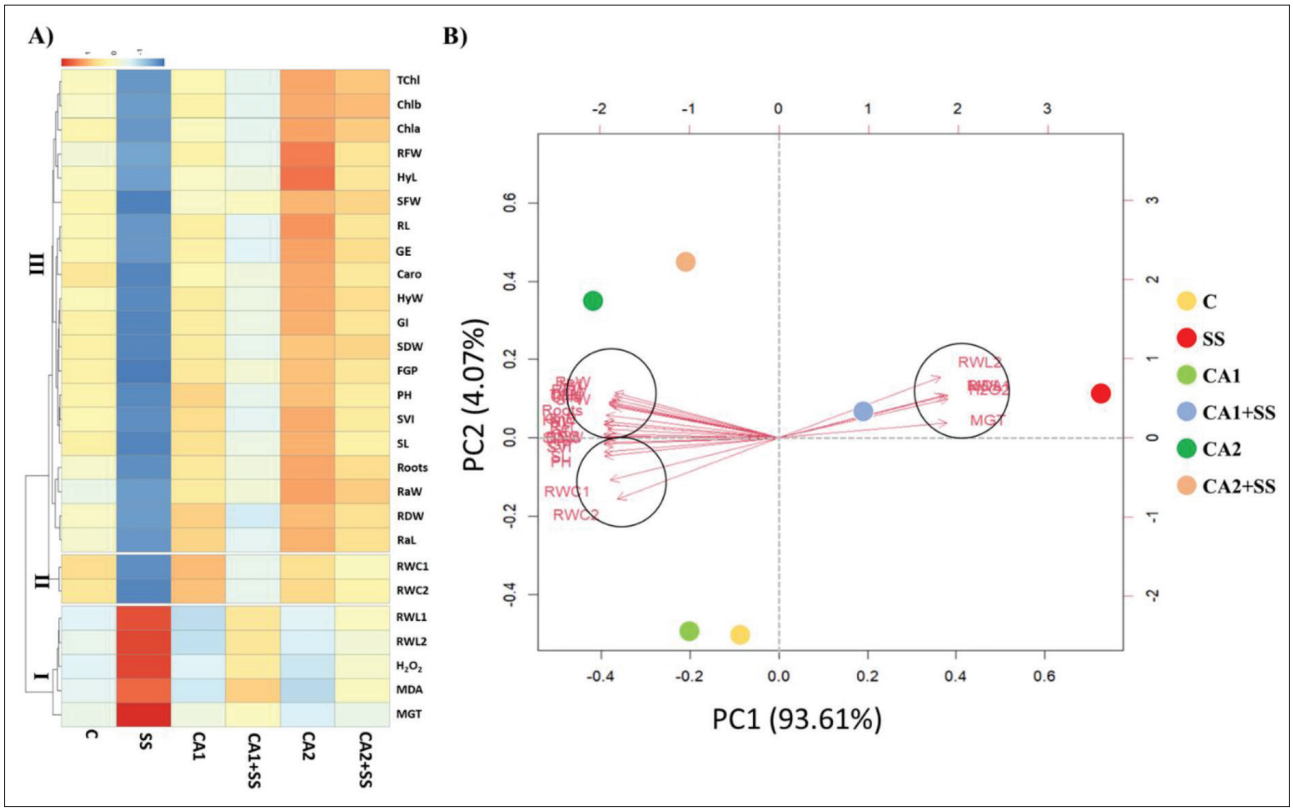

Fig. 5. Principal component analysis (PCA) and a hierarchically clustered heatmap used to visualize the interactions between the treatments and the factors that were studied. The scaled average values of every parameter that was examined for the soybean are shown in **A** – heatmap with a clustering approach. **B** – PCA performed on all data. C – control (distilled water), SS – 150 mM NaCl, CA1 – 1mM citric acid, CA1+SS – 1 mM CA+150 mM NaCl, CA2 – 2 mM CA, CA2+SS – 2 mM CA+150 mM NaCl. The studied parameters were the final germination percentage (FGP), mean germination time (MGT), germination index (GI), germination energy (GE), seed vigor index (SVI), radical length (ReL), radical weight (ReW), hypocotyl length (HyL), hypocotyl weight (HyW), number of lateral roots (Roots), relative water content after 1 h (RWC1), relative water content after 2 h (RWC2), relative water loss after 1 h (RWL1), relative water loss after 2 h (RWL2), root length (RL), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), shoot fresh weight (SFW), shoot dry weight (SDW), plant height (PH), chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll (TChl), carotenoids (Caro), hydrogen peroxide (H_2O_2), malondialdehyde (MDA).

and PC2, PCA scores split the nine treatments: PC1 and PC2 together displayed 97.68% of the data variability across the treatments and all examined soybean components. PC1 demonstrated 93.61% data variability in this case and separated C, CA1, CA2, and CA2+SS from CA1+SS and SS treatments in their positive and negative PC scores, respectively (Fig. 5B). Aside from this, PC2 displayed only 4.07% data variability (Fig. 5B).

DISCUSSION

Salinity is a key abiotic factor that reduces agricultural production globally by decreasing growth and yield-related traits. Protective plant metabolites like CA can be applied exogenously to increase plant tolerance to environmental challenges and maintain food production [23]. It is well-known that exogenous application of CA confers abiotic stressors and stimulates plant growth and development by increasing internal citric acid levels and antioxidant enzyme activities [27]. On the other hand, seed priming is an affordable and promising physiological and biochemical practice that boosts seed germination and improves plant growth and development under salinity stress [24-25,40]. In a recent publication, we stated that the exogenous administration of CA improved seedling growth traits under salinity stress [19]. Similarly, under salt stress conditions, exogenous applications of jasmonic acid, brassinosteroid, benzyladenine,

glutathione, and melatonin increased soybean seedling root and shoot lengths, fresh weight, dry weight, and seedling length [21-22,31-33,41]. The current study was executed to reveal the effect of CA priming and exogenous application of soybean under salt stress. The results of the present study indicated that CA application significantly improved germination and seedling growth parameters, demonstrating its efficacy in decreasing the negative effects of salinity on germination and early seedling growth. The results of our study are consistent with previous reports showing that the priming of seeds with CA significantly increased germination-related traits in *Carica papaya* [24], and different field crops [25] and increased seedling growth in maize [18], okra [19], cotton [26], and *Leymus chinensis* [27]. Additionally, heatmap analysis demonstrated that under salt stress, priming and CA supplementation increased germination and growth characteristics, and PCA demonstrated that germination and seedling traits are strongly associated with priming and exogenous use of CA. Cell growth is the most important process affected by salt stress, which affects plant growth [42], and the application of CA may improve cell growth, which consequently increases plant growth (Table 3) [19,26-27].

Maintaining a sufficient water content in plants under salt stress is crucial for preserving the proper development of plants [43-44], and RWC is an important indicator of water status in plants [45]. It has been reported that salt stress decreased RWC and increased RWL in soybean and *Leymus chinensis* leaves, but CA application significantly increased RWC and decreased RWL under salinity stress [19,27]. According to reports, jasmonic acid and brassinosteroid application significantly improved RWC in soybean seedlings under saline stress [21-22]. It has been reported that decreased RWC and increased RWL may occur due to structural damage to the cell wall, which interferes with water uptake [46]. The presented results also showed that CA application significantly improved RWC and decreased RWL in soybean leaves (Fig. 2). This result indicated that CA may be involved in the uptake of extra water from the soil to alter the water level within plant organs [47].

This experiment also showed that salt stress significantly reduced photosynthetic pigments like chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids. The priming and supplementation of CA1 and CA2 significantly increased these pigments under salt stress. Our results are consistent with previous reports where the foliar application of CA has been demonstrated to increase photosynthetic pigments in maize [18], and cotton [26]. In addition, previous reports showed that the application of brassinosteroid, jasmonic acid, benzyladenine, gibberellic acid, and melatonin also significantly improved photosynthetic pigment contents in soybean under salt stress [21,31-33,48]. Tahjib-Ul-Arif et al. [23] concluded that crops grown in various abiotic stress situations exhibit improved growth and yields after CA treatment by promoting increased chlorophyll content with higher photosynthetic rates. The PCA and heatmap also elucidated their interaction with the treatment agent and stress condition. The outcomes also showed that CA2 outperformed CA1 by increasing the pigments under salt stress.

It is well known that oxidative stress occurs due to the overproduction of ROS under different stresses and increases H_2O_2 and MDA contents [49-50]. The application of CA on salt-stressed soybean seedlings prevented the onset of oxidative damage by lowering the levels of H_2O_2 and MDA compared to plants exposed to salt stress. CA could increase the activities of enzymatic and nonenzymatic antioxidants, helping to reduce the harm caused by stress-induced ROS and MDA and improving the ability of the plants to withstand stress. According to previous reports, the use of jasmonic acid, brassinosteroid, glutathione, melatonin, and salicylic acid under salt stress reduced the initiation of oxidative damage by lowering H_2O_2 and MDA concentrations in soybean, which our findings support [21,33-34,41]. Taken together, the results of this study show that 2 mM CA is superior to 1 mM CA in terms of improving germination, seedling characteristics, and physiological parameters under stress or in the absence of stress. Large-scale experiments should be performed in the field to validate this result.

CONCLUSIONS

Crops grown under numerous abiotic stress conditions exhibit better germination and growth after receiving exogenous bioactive organic acids like CA. The treatment with CA improved germination and growth-related parameters but also decreased H_2O_2 and MDA contents in soybeans exposed to salt stress. According

to the findings of this study, it can be concluded that 2 mM CA is superior to 1 mM CA in terms of increasing germination, seedling traits, and physio-biochemical parameters under salt stress. Therefore, CA can be utilized as a seed priming and exogenous soybean agent to reduce the effects of salinity stress and promote early seedling growth. This experiment is very effective for soybean seedling establishment, even in salinic areas.

Funding: This research was funded by the Khulna Agricultural University Research System (KAURES), Grant: KAU/Reg.Off./Admin.-1/KAURES/74/2020/1646(02).

Author contributions: S. Imran and M. A. Mahamud contributed equally. Conceptualization, S. Imran, M. A. Mahamud and M. S. Rhaman; methodology, S. Imran and M. S. Rhaman; experiment conduct, S. Imran, M. A. Mahamud, N. C. Paul, and J. Chakrobortty; formal analysis, S. Imran; writing-original draft preparation, S. Imran, M. A. Mahamud, N. C. Paul, J. Chakrobortty, P. Sarker and S. Paul; writing-review and editing, S. Imran, M. Tahjib-Ul-Arif and M. S. Rhaman; visualization, S. Imran and M. S. Rhaman. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: Data underlying the reported findings have been provided as a raw dataset and is available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Imran_Dataset.xlsx

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Screening for citric acid (CA) doses

Treatments	Germination percentage (%)±SE
Control	74.44±1.11[b]
0.5 mM CA	75.56±1.11[b]
1 mM CA	85.56±1.11[a]
2 mM CA	91.11±1.11[a]
3 mM CA	75.56±2.22[b]
5 mM CA	65.56±1.11[c]

The data are presented as means±SE, with a sample size n = 30; Tukey's HSD ($P<0.05$), different letters between treatments were examined.