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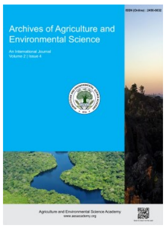

ORIGINAL RESEARCH ARTICLE

Effect of citric acid (CA) priming and exogenous application on germination and early seedling growth of okra (*Abelmoschus esculentus* L.) plants under salinity stress condition

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ABSTRACT

Salinity is a significant barrier to the healthy germination of seeds, the development of seedlings and ultimately the yield of crops. Salinity tolerance can be effectively induced through seed priming and exogenous application of various treatment agents. The vegetable crop okra is a healthy and well-liked one worldwide. Literature shows that salt stress negatively disturbs the growth of okra plants. In the present research, we investigated the effects of citric acid (CA) as priming and exogenous agents to alleviate the salinity-inhibited germination and early growth of okra plants. The seeds were pretreated with CA (1 mM and 2 mM) and soaked in distilled water (control) for 60 min. Germinated seeds were grown in hydroponic solution and subjected to salt stress (50 mM and 100 mM NaCl) with three independent replications and same concentrations of CA (1 mM and 2 mM) were exogenously sprayed. Our results showed that, seed priming with 1 mM CA significantly produced the highest percentage of germination (GP), germination index (GI), germination energy (GE), seed vigor index (SVI), radicle length and weight, hypocotyl length and weight, and number of lateral roots while decreased mean germination time of okra seeds while compared to the control treatment. Additionally, the findings demonstrated that salt stress dramatically reduced root and shoot length, plant height, root and shoot fresh weight and dry weight, and relative water content (RWC). Under salt stress, the addition of 1 mM and 2 mM CA significantly increased the RWC, root and shoot length, root and shoot fresh and dry weight, and plant height. These results provide information that CA priming improves germination parameters and exogenous treatments can improve the salt tolerance, and seedling characteristics of okra. Therefore, our results suggest that 1 mM CA can be utilized as a seed priming and exogenous application agent reducing the impacts of salt stress and promoting early seedling development of okra.

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INTRODUCTION

Okra (*Abelmoschus esculentus* L.) under the family of malvaceae is a widely-grown dicotyledonous plant and popular summer crop throughout the tropical region (Tania *et al*., 2020; Elkhalifa *et al*., 2021). It is commonly known as dheros, lady's fingers, or gumbo in our country. The seed of okra is a source of antioxidants, which is essential to maintain good health. Okra pods are good source of flavonoid antioxidants like beta carotene, xanthine and lutein (Dilruba *et al*., 2009). Also, okra flour has

Table 1. Treatment conditions and their denotation.

Treatment	Denoted
Control	C
1 mM CA	CA1
2 mM CA	CA2
50 mM NaCl	SS1
100 mM NaCl	SS2
1 mM CA + 50 mM NaCl	CA1+SS1
1 mM CA + 100 mM NaCl	CA1+SS2
2 mM CA + 50 mM NaCl	CA2+SS1
2 mM CA + 100 mM NaCl	CA2+SS2

huge potential for use to enrich foods in order to provide adequate nutritional daily needs (Adelakun and Oyelade, 2011). Bangladesh produces about 70242.27 M. tons from 30118.73 acres of land with an average yield of 2.33 M. tons per acre (BBS, 2021). Slow germination and poor seedling emergence might be important reason behind insufficient production. Soil salinity is a major problem in agriculture sector which limits plant growth and causes significant loss of crop productivity worldwide (Allakhverdiev *et al.*, 2000; Munns *et al.*, 2002). Research findings showed that around 20% and 50% of the total cultivated and irrigated land in the world directly affected by salinity respectively (Cheng *et al.*, 2012). However, at present researchers are concentrated on different approaches like seed priming, screening of best varieties, organic fertilizer application, and exogenous applications of different plant growth regulators such as citric acid, auxin, SA, abscisic acid, 5-aminolevulinic acid, and so on to overcome this problem (Rhaman *et al.*, 2016; Yakoubi *et al.*, 2019; Abdel Latef *et al.*, 2021; Rhaman *et al.*, 2021a, b; Tahjib-Ul-Arif *et al.*, 2021). Supplementation of chemicals to plants, either as an exogenous foliar application or seed treatments, may prompt their physiological apparatuses, leading to plant growth enhancement (Vwioko, 2021). For instance, seed priming with plant growth regulators can bring changes in the phenotypes of plants from seed germination to senescence (Rhaman *et al.*, 2021a). Citric acid is an important organic acid for plant growth that has relationship with stress tolerance of salinity (Fougere *et al.*, 1991) and heavy metal (Krotz *et al.*, 1989; Tesfaye *et al.*, 2003; Mailloux *et al.*, 2008; Zeng *et al.*, 2008). The exogenous application of protective plant metabolites like citric acid (CA) have emerged as an effective approach to improve plant resilience to environmental stresses and thus sustain food production (Tahjib-Ul-Arif *et al.*, 2021). Afterward, several studies have debunked and found that the CA not only helps in pH modulation but also in the physiological responses of plants. Such as in cotton application of CA increased TSS, TSP, TPC, FAA and proline, enhanced CAT, POX and SOD activities, and increased salt tolerance (El-Beltagi *et al.*, 2017). Another research on Papaya plant reported that exogenous application on papaya increased germination rate but improved the salinity tolerance (Zanotti *et al.*, 2013). Lopez *et al.* (2000) reported that plants growing in alkaline soils exude CA and malate from their roots which decreasing the pH of the rhizosphere and enables to uptake essential nutrients like iron and phosphorus. Moreover, exogenous application of CA improved physiological parameters in numerous plant species such as Tuberose, Lily flower and Commom bean (Darandeh *et al.*, 2012; El-Tohamy *et al.*, 2013; Eidyan *et al.*, 2014). However, it was found that CA could mitigate salinity, drought, temperature and HM stresses in a variety of plant species (Gebaly *et al.*, 2013; Ehsan *et al.*, 2014; Hu *et al.*, 2016; El-Beltagi *et al.*, 2017). However, it was found that the activities of CAT and APX, which plays a role in scavenging and neutralizing hydrogen peroxide, were stimulated significantly by exogenous application of citric acid and also increased internal citric acid concentrations in stressed plants, which enhanced stress tolerance by improving photosynthesis, relative growth rate and increasing activities of antioxidant enzymes. (Sun *et al.*, 2010). Still, the role of CA in abiotic stress tolerance has not yet been studied exhaustively, additional research is needed but it can be concluded that exogenous CA application by foliar sprays or through rooting medium enhanced growth, photosynthesis and many physio-biochemical parameters that promote crop productivity under abiotic stress conditions and also alleviated the abiotic stress-induced osmotic imbalance by increasing osmoregulators and protecting membranes from damage (Tahjib-Ul-Arif *et al.*, 2021). Therefore, considering the above facts, the present study was therefore undertaken to evaluate the effects of citric acid (CA) as priming and exogenous agents on germination and seedling growth of okra under salt stress.

MATERIALS AND METHODS

Site of experiment, treating conditions, and germination indices measurement

A petri-dish and pot experiment were conducted in the laboratory at the Soil Science department, Khulna Agricultural University, Khulna. The local, high-yielding, popular Okra (*Abelmoschus esculentus* L.) was collected from the local market (Khulna, Bangladesh) and used in the experiment. To prevent the growth of microbial contaminants, present on the seed surface, sodium-hypochlorite (1%) was used for 5 min to sterilize the seeds. The seeds were soaked for 60 min for priming in Distilled water (DW), 1 mM and 2 mM concentrations of CA, each in individual screw-plugged pots for hydro-priming. The concentrations of CA and salinity stress were selected based on previous experiments as well as the literature (Tahjib-Ul-Arif *et al*., 2021). Fifty okra seeds were soaked for each concentration of treatment. The primed seeds were positioned on Petri dishes (150 × 25mm diameter) prepared with 3 layers of tissue papers and kept at room temperature 25±1°C and relative humidity of 96%. The experiment was operated with 3 independent replications. All the chemicals used in this experiment were purchased from Bangladesh. Treatment conditions and their denotation were shown below in Table 1.

Seedling emergence was recorded daily. The number of seeds that germinated was documented at 24 h intervals from the first emergence up to the 3rd day. After that GP, MGT, GI, and GE were calculated. The length and weight of radicle and hypocotyl of germinated seeds were recorded at 5th day of germination. At 21 days after sowing (DAS), SL (cm) and RL (cm) were measured, and SVI was calculated. The respective formulas were used to calculate the GP, MGT, GI, and SVI.

$$\text{Germination percentage (GP)} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds placed in germination}} \times 100$$

$$\text{Mean germination time (MGT)} = \Sigma \frac{\text{Dn}}{\text{n}}$$

“n” is the seed number on day D and D is the number of days calculated from the beginning of germination.

The GI was computed using the following formula:

$$\text{Germination index (GI)} = \frac{Number\ of\ seeds\ germinated}{Day\ of\ 1st\ count} + \cdots + \frac{N\ Number\ of\ seeds\ germinated}{Day\ of\ last\ count}$$

The GE was computed using the following formula:

$$\text{Germination energy} = \frac{\text{T1}}{\text{N}} \times 100$$

Where T1 is the number of seeds germinated on the 1st day, and N is the total number of seeds.

Seed vigor index (SVI) = GP × seedling length (cm)

Where seedling length = shoot length + root length.

Experiment at seedling stage

Uniformly germinated seeds were transferred to 2.5-liter pots and grown hydroponically with the solutions containing 4mM KNO_3, 1 mM $NH_4H_2PO_4.2H_2O$, 1 mM $CaCl_2.2H_2O$, 1 mM $MgSO_4.7H_2O$, and micronutrients (1 ppm Fe, 0,5 ppm B, 0.5 ppm Mn, 0.05 ppm Zn, 0.02 ppm Cu, and 0.01 ppm Mo). After those 12 days old seedlings were subjected to 50 and 100 Mm NaCl stress for 8 days. From the beginning of the stress seedlings were exogenously treated 3 times with 1 mM CA and 2 mM CA in 8 days (Single spray per day at 9am; 5 ml /plant/ spray). After 8 days of CA treatment different morphological data such as root length (RL), root fresh weight (RFW), shoot length (SL) and shoot fresh weight (SFW) were collected from 3 plants of each pot.

Relative water content (RWC) and relative water loss (RWL) measurement

The relative water content (RWC) was finalized using the standard techniques of Mostofa and Fujita (2013). In the case of RWC measurement, leaf samples were collected after 21 days of planting. After that, the fresh weight (FW) of leaves were taken and immersed in distilled water and kept for 2 hrs. Then excess water was removed from the turgid leaves with a tissue paper and turgid weight (TW) was recorded instantly. After taking the fresh weight of root and shoot, those were oven dried at 60 °C for 72 hrs. and dry weight (DW) was recorded. Both weights were recorded in milligram (mg) unit. The RWC was calculated according to the following formula:

RWC (%) = (FW – DW)/ (TW – DW) × 100

After calculating the RWC the RWL was calculated according to the following formula:

RWL (%) = 1- {(FW – DW)/ (TW – DW) × 100}

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics V.25. Significant differences were identified by one-way analysis of variance followed by Tukey HSD ($p < 0.05$).

RESULTS AND DISCUSSION

Seed priming enhances germination parameters of okra

The impacts of CA priming on the germination indices of okra seeds under salt stress are displayed in Figure (1A-1E). The findings show that germination percentages were significantly enhanced by seed priming with CA (Figure 1A). Data revealed that seed priming with 1 mM CA exhibited the highest germination percentage (82%) compared to 2 mM CA (73%), while the lowest germination percentage (68%) was recorded for control. In the case of mean germination time, significant results were found for different priming treatments (Figure 1B). Seed priming with 1 mM CA resulted in the fastest mean germination time (2.3 days) compared to 2 mM CA (2.6 days) and control (2.6 days). Findings from this experiment revealed that the germination index of okra seeds significantly increased in response to CA priming treatments (Figure 1C). The highest germination index (34) was recorded for 1 mM CA compared to 2 mM CA (28) and the lowest germination index (26) was observed for control, respectively. In the case of germination energy, results were found significant for different priming treatments (Figure 1D). Results revealed that seed priming with 1 mM CA exhibited the best performance of germination energy (55 %) compared to 2 mM CA (41 %), while the lowest

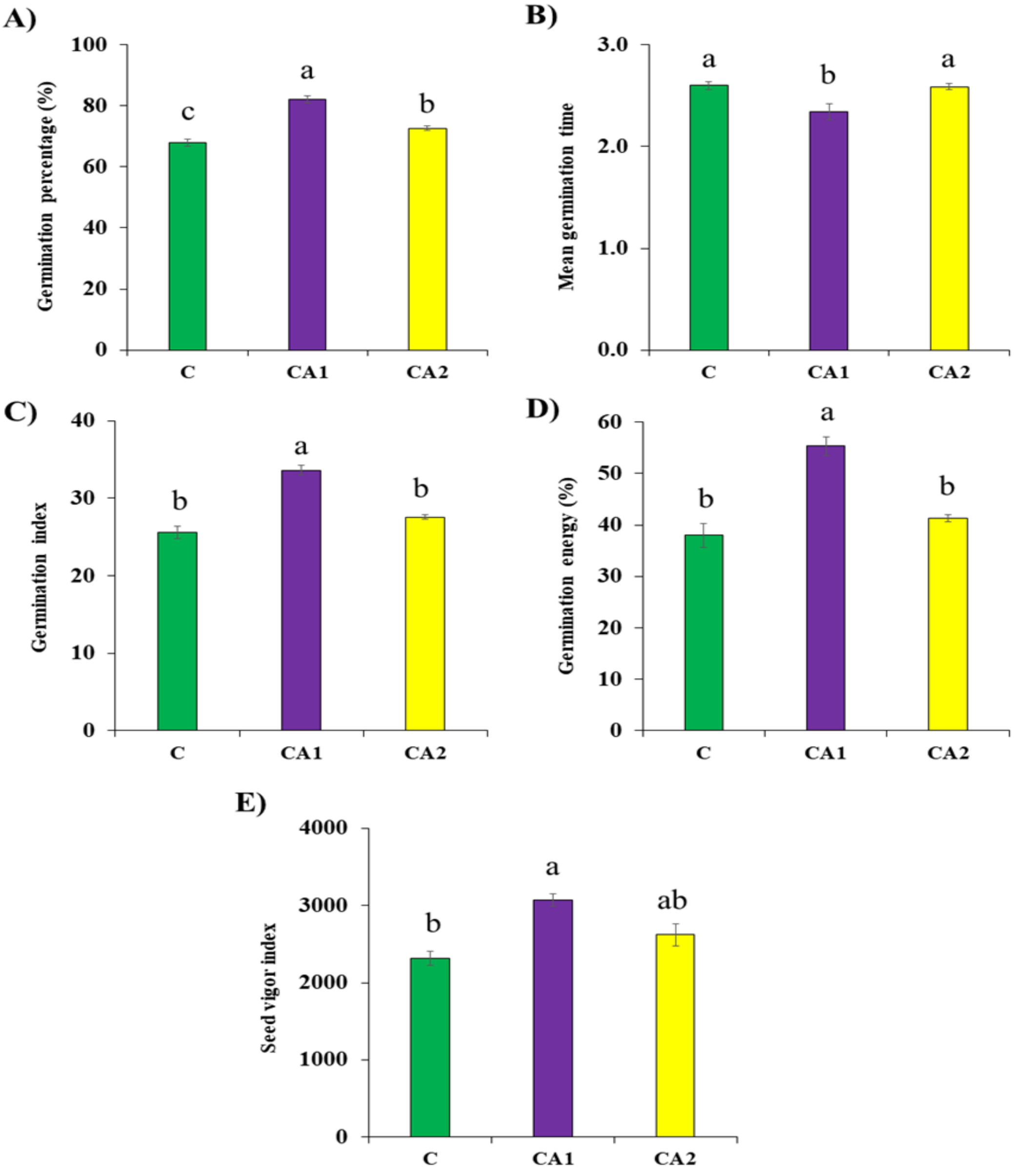

Figure 1. *Effects of CA priming on the germination indices of okra seeds. (a) Germination percentage; (b) mean germination time; (c) germination index; (d) germination energy; (e) seed vigor index. Data are means SE, n = 3. Different letters among treatments were analyzed by Tukey HSD (p < 0.05).*

germination energy (38 %) was recorded for control. Similarly, findings showed that different priming conditions significantly increased seed vigor index (Figure 1E). The results indicated that the seed vigor index increased by 3070 % for 1 mM CA priming compared to 2 mM CA (2622 %), while the lowest seed vigor index (2312 %) was recorded for control.

The process of seed germination is the first and most critical stage in the growth of plants because it helps seedlings adapt to their constantly changing environment and increase their production. Crop production and quality are disturbed by salt stress as it causes a number of physiological imbalances including osmotic stress, nutrient uptake, growth, generation of reactive oxygen species and radicals (Gill and Tuteja, 2010). Salt stress reduces plant growth and development due to its toxic metallic ion activity especially Na$^+$ and Cl$^-$ (Isayenkov, 2012). One of the most popular methods for encouraging seed germination, enhancing morphological characteristics, enhancing plant growth and development under stress is seed priming (Rhaman *et al*., 2020). Seed germination is severely affected by salt stress and seed priming may be a solution in this regard (Afzal *et al*., 2012). In order to increase seed germination, seedling emergence, subsequent plant growth and development, and tolerance to salt stress, seed priming is a shotgun strategy that has been employed successfully (Mansour *et al*., 2019).

Seed priming with CA is very much helpful to increase germination and growth characteristics under different environmental stresses. Xiu *et al*. (2021) found that germination and growth-related traits were improved when tomato seeds were treated with CA. In this experiment, results showed that CA (1mM) significantly improves germination parameters including germination percentage, time required for germination, germination index, germination energy and vigor index under NaCl stress. All the characters are improved due to the seed priming with CA. Ashraf and Foolad (2005) found that CA significantly

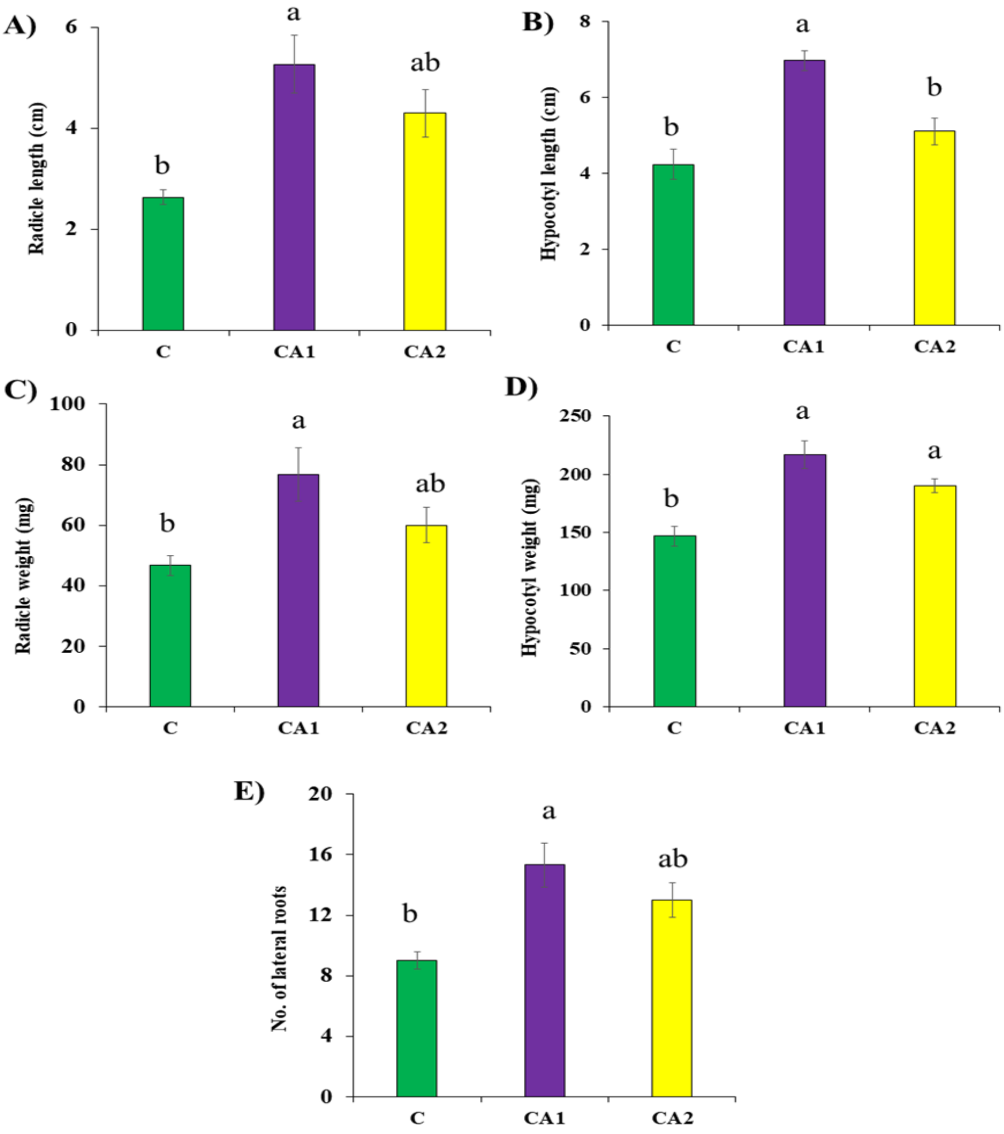

Figure 2. *Effects of CA priming on the (a) radicle length; (b) hypocotyl length; (c) radicle weight; (d) hypocotyl weight; (e) no. of lateral roots. Data are means SE, n = 3. Different letters among treatments were analyzed by Tukey HSD (p < 0.05).*

increases germination and growth parameters under salt stress. Nouri and Haddioui (2021) also found almost similar result in Lepidium sativum under arsenic stress. According to Yadav *et al*. (2018) germination % was increased up to 50% in field crops when CA was used as priming agent under salinity stress. There are many metabolic reactions that occur within seeds and these reactions positively improve germination performance and seedling growth (Sedghi *et al*., 2010).

Seed priming enhances length and weight of radicle and hypocotyl of germinated seeds

The length and weight of radicle and hypocotyl, and the number of lateral roots of the okra greatly influenced the growth of the seedlings. The findings show that radicle length was significantly influenced and increased by seed priming with CA (Figure 2A). Results showed that the maximum length of the radicle (5 cm) was observed because of 1 mM CA priming compared to 2 mM CA (4 cm), while the lowest length of the radicle (3 cm) was recorded for control. Data from this experiment revealed that the hypocotyl length of okra seeds increased due to CA priming treatments (Figure 2B). The maximum hypocotyl length (7 cm) of okra was found for 1 mM CA priming compared to 2 mM CA (5 cm), while the minimum hypocotyl length (4 cm) was observed for control. In the case of radicle weight, priming treatments of 1 mM CA and 2 mM CA produced significantly higher results (Figure 2C). Data revealed that seed priming with 1 mM CA exhibited the highest radicle weight (77 mg) compared to 2 mM CA (60 mg), while the lowest radicle weight (47 mg) of okra was recorded for control. Similarly, the results of the study showed that different priming conditions significantly increased the hypocotyl weight of okra seedlings (Figure 2D). Seed priming with 1 mM CA produced the highest hypocotyl weight (217 mg) compared to 2 mM CA (190 mg), while the lowest hypocotyl weight (147 mg) of okra was observed for control. In the case of the number of lateral roots, results varied significantly and increased for different priming treatments (Figure 2E). The results of the study indicated that the highest number of lateral roots (15) was recorded for 1 mM CA priming which was as good as 2 mM CA (13), while the lowest number of lateral roots (9) was found for control. Yadav

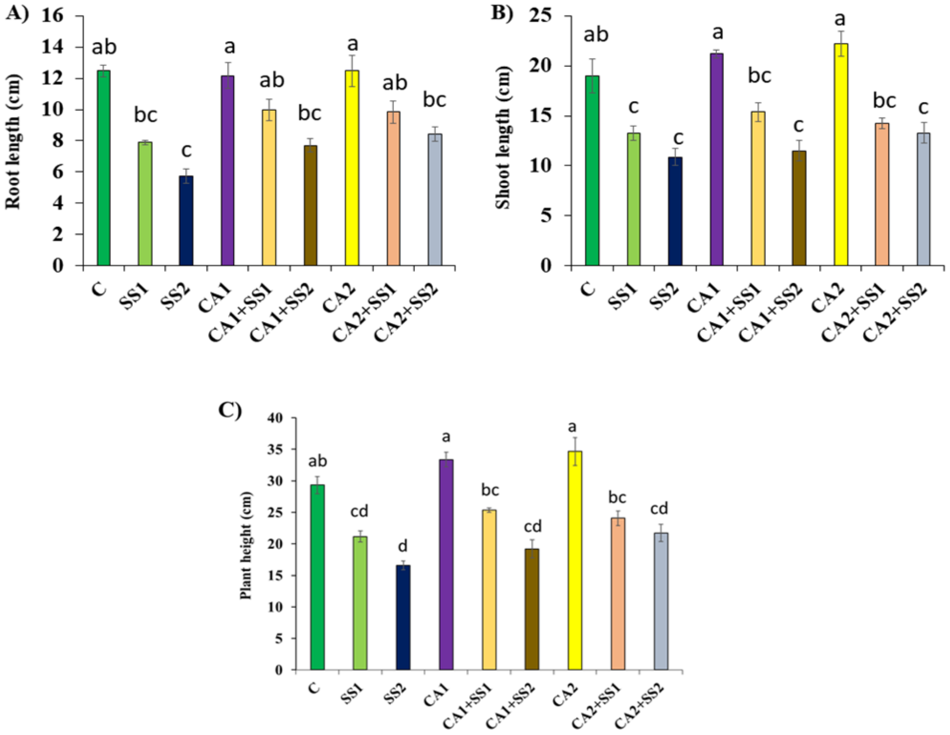

Figure 3. *Effects of CA spray on the (a) root length; (b) shoot length; (c) plant height of okra seedlings under salinity stress conditions. Data are means SE, n = 3. Different letters among treatments were analyzed by Tukey HSD (p < 0.05).*

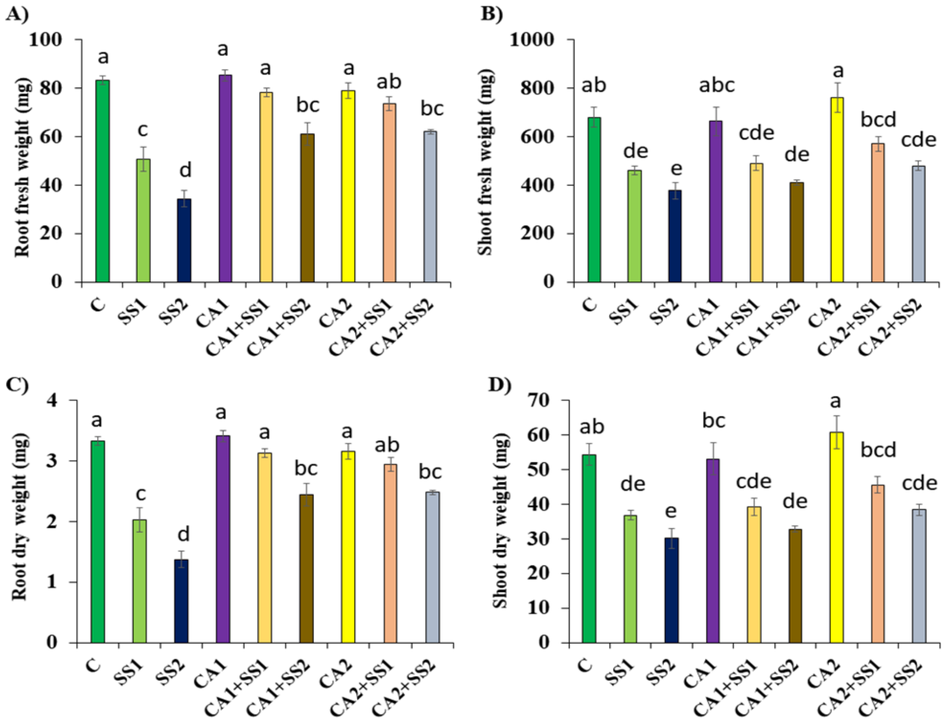

Figure 4. *Effects of CA spray on the (a) root fresh weight; (b) shoot fresh weight; (c) root dry weight; (d) shoot dry weight of okra seedlings under salinity stress conditions. Data are means SE, n = 3. Different letters among treatments were analyzed by Tukey HSD (p < 0.05).*

et al. (2018) found almost same result where radicle length, hypocotyl length and their weights were increased under CA and salt. Again Mer *et al.* (2000) observed that plumule length in wheat, barley, pea and cabbage seeds reduced with increasing salt. But in this study, seed priming with CA (1mM) enhances length and weight of radicle and hypocotyl. This is because CA is a bioactive molecule which mitigates salt stress effects by boosting physiological process (Shaddad, 2010).

Exogenously applied CA enhances seedling traits of okra

Early seedling growth components viz. root length, shoot length, and plant height of wear significantly influenced by the seed priming and exogenous application of various concentrations of CA (Figure 3A-3C). Results of the present study showed that root length, shoot length, and plant height was adversely affected by salt stress. On contrary, the seed priming

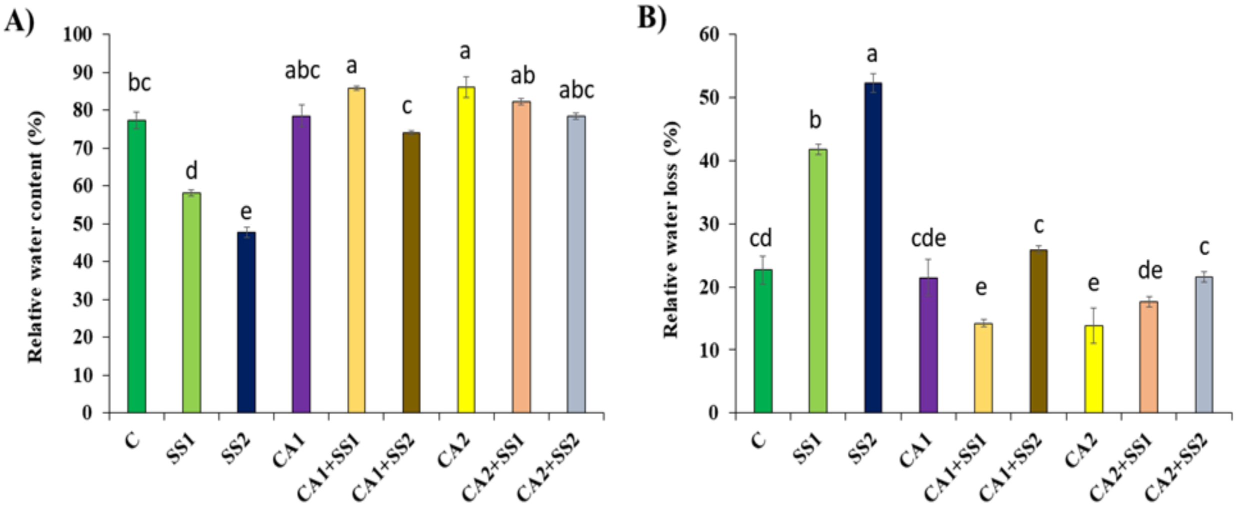

Figure 5. *Effects of CA spray on the (a) relative water content; (b) related water of okra under salinity stress conditions. Data are means SE, n = 3. Different letters among treatments were analyzed by Tukey HSD (p < 0.05).*

and exogenous application of CA evidently increased the growth parameters and decreased the adverse effect of salinity. Considering the root length, the longest root (12.47 cm) was observed from 2 mM CA treatment which was statistically significant with, control; 1 mM CA; 1 mM CA+50 mM NaCl; 2 mM CA+50 mM NaCl, and the shortest root (5.73 cm) was observed from 100 mM NaCl stress. Similarly, the longest shoot (22.20 cm) and longest plant (34.16 cm) were found from 1mM NaCl treatment, which was statistically significant with 1mM CA; and control treatment. On the other hand, the shortest shoot (10.87 am) and shortest plant (100 mM NaCl) were found from 100 mM NaCl.

Results of the current study revealed that root fresh weight, shoot fresh weight, root dry weight, and shoot dry weight of okra seedlings were significantly influenced by seed priming and exogenous application of CA under saline conditions (Figure 4A -4D). Data showed that the application of CA reduced the adverse effect of salt stress. The highest root fresh weight (85.33 mg) was observed from the application of 1 mM CA which was statistically par with control; 2mM CA; 1 mM CA+100 mM NaCl; 2 mM CA+50 mM NaCl and the lowest root fresh weight (34.33 mg) was observed from 100mM NaCl stress. Also, the highest shoot fresh weight (760 mg) was obtained from 2mM CA which was at par with the control and 1mM CA. On contrary, the lowest shoot fresh weight was found from 100 mM NaCl stress. Seed priming and exogenous application with 1 mM CA produced the highest root dry weight (3.41 mg) which was at par with control; 2 mM CA; 1 mM CA+50 mM NaCl and 2 mM CA+50 mM NaCl while the lowest root dry weight (1.37 mg) of okra seedlings was observed for 100 mM NaCl stress. In the case of the shoot dry weight, results varied significantly and the highest number of shoot dry weights (60.80 mg) was recorded for 2 mM CA application which was as good as control, while the lowest shoot dry weight (30.13 mg) was found form 100 mM NaCl stress. CA treatment enhance root and shoot length including lateral root numbers and ultimately plant height. Xiu *et al*. (2021) studied with tomato and found that seed priming with citric acid significantly promote tomato seedling growth, increased biomass accumulation including root and shoot growth. Root and shoot dry weight also increased due to CA seed priming. Cayuela *et al*. (1996) obtained increased root and shoot dry weight in tomato seedlings treated with NaCl under salt stress.

Supplementation of CA regulates the relative water content (RWC) and relative water loss (RWL)

The actual water status of the plant can be determined by the RWC or RWL of the leaves in terms of the physiological effects of cellular water deficit brought on by stress conditions like salinity stress. Results of the current study showed that RWC and RWL of okra seedlings were significantly influenced by the application of CA under different salinity-stressed conditions (Figure 5A-5B). The highest RWC (86.08%) was observed in 2mM CA; which was at par with 1 mM CA+50 mM NaCl; 2 mM CA+50 mM NaCl; 1 mM CA; and 2 mM CA+100 mM NaCl, while the lowest RWC (47.75%) was observed from 100 mM NaCl. On contrary, the highest RWL (52.25%) was observed from 100 mM NaCl and the lowest RWL were found from both 1 mM and 2mM CA.

RWC and RWL are important parameters which are decreased with different environmental stresses (Xie *et al*., 2022). The present study indicates that the RWC is increased and RWL is decreased in seedlings after seed priming with CA under salinity stress. Sheteiwy *et al*. (2021) examined that seed priming and foliar application with jasmonic acid enhance salinity stress tolerance of soybean (*Glycine max* L.) seedlings by increasing RWC and decreasing RWL. Abdelaal *et al*. (2019) studied with sweet pepper and found a considerable increase in RWC when the plants are treated with salicylic acid and proline. Hidayah *et al*. (2022) explained that seed halopriming enhance salt stress tolerance by increasing RWC in IR 64 rice cultivar. Yakoubi *et al*. (2019) studied with the effects of gibberellic and abscisic acids on okra under salt stress and found an increase in RWC. Seed priming with polyethylene glycol increases RWC and decreases RWL in sorghum (*Sorghum bicolor* L.) (Zhang *et al*. 2015).

Conclusion

Our experiment revealed that seed priming and exogenous treatment of CA promote okra seed emergence and early seedling via influencing root and shoot length, fresh weight, dry weight and leaf water contents features. According to the results, it was observed that seed priming with 1 mM CA produced the highest germination percentage (GP), germination index (GI), germination energy (GE), seed vigor index (SVI), radicle length and weight, hypocotyl length and weight, and number of lateral roots. Again, at the same time, it also takes less mean germination time of okra seeds in case of seed priming with 1 mM CA. Therefore, it can be concluded that 1 mM CA can be used as seed priming and exogenous application agent for mitigating salinity stress effects and enhancing early seedling growth of okra. To confirm the findings of the current study, experimentation at the field level is strongly advised.

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