Journal of Agriculture and Crops
ISSN(e): 2412-6381, ISSN(p): 2413-886X
Vol. 9, Issue. 4, pp: 495-502, 2023
URL: https://arpgweb.com/journal/journal/14
DOI: https://doi.org/10.32861/jac.94.495.502

Academic Research Publishing Group

Original Research | Open Access

Citric Acid and Hydro-Priming and Exogenous Application Alleviate Salt-Inhibited Seed Germination and Seedling Growth of Chilli (*Capsicum annuum* L.)

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Article History
Received: 10 July, 2023
Revised: 15 September, 2023
Accepted: 25 September, 2023
Published: 29 September, 2023

Abstract

Salinity is a major barrier for seed germination, plant growth and production. Seed pre-treatment and exogenous chemical usage can effectively confer salt tolerance. We evaluated the potential of citric acid (CA) as priming and exogenous agents to ameliorate salt-inhibited germination and growth of Chilli (*Capsicum annuum* L.) in this study. The seeds were primed with 0.5 mM and 1 mM CA, and soaked in distilled water (hydro-priming) for 30 min. In addition, untreated seeds were used for the control experiment. Finally, primed seeds were subjected to 100 mM NaCl stress. Our results demonstrated that salinity significantly reduced the germination percentage (GP), germination index (GI), germination energy (GE), seed vigor index (SVI), root length (RL), shoot length (SL), shoot–root fresh and dry weight, and plant height and increased mean germination time (MGT). The results also indicated that salinity stress considerably decreased the relative water content (RWC) and photosynthetic pigments such as chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids, and increased relative water loss (RWL). The CA- and hydro-priming improved the GP, GI, GE SL, RL, SVI, and growth of seedlings, and reduced MGT under salt stress. Data also demonstrated that the supplementation of CA enhanced RWC and photosynthetic pigments and lowered RWL in the state of saline condition. When compared to other treatments, pretreatment, and exogenous use of 1 mM CA was determined to be comparatively more effective at imparting the salt tolerance of Chilli.

Keywords: Chilli; Germination; Priming; Photosynthetic pigments; Salinity.

How to Cite: Md. Monir Hossain, Jannatul Ferdous, Kaya Akter. *et.al.* 2023. " Citric Acid and Hydro-Priming and Exogenous Application Alleviate Salt-Inhibited Seed Germination and Seedling Growth of Chilli (Capsicum annuum L.)." *Journal of Agriculture and Crops*, vol. 9, pp. 495-502.

1. Introduction

Among the abiotic stressors, salt is a key limiting factor in growing crops across the world [1-3]. Salinity impacts every area of plant physiology and biochemistry, severely reducing production [4-6], and is a critical environmental influence [7]. Salinity has been estimated to harm 20% of the world's farmed area and half of the world's irrigated areas [6, 8]. During the previous four decades, around 0.551 million acres of new land in Bangladesh have been damaged by varying degrees of salt, resulting in yield loss of various crops [9, 10]

The hot pepper (*Capsicum annuum* L.), also known as the Chilli, is a major vegetable and spice crop in Bangladesh. In 2021-22, Chilli production reached 625 thousand M. tonnes from an area of 243 thousand acres [11]. With a yield threshold of 1.5 dS/m, it has been classified as moderately sensitive to salt [12]. Furthermore, every unit of salt above the threshold reduces Chilli production by up to 14% [13]. Chilli seedlings are more vulnerable to salt than germination, according to study Niu, *et al.* [14]. It has also been stated that under normal and stressful conditions, Chilli seed germination and emergence are delayed and non-uniform [15]. If not effectively controlled, salinity can become a barrier for Chilli production [16]. Recent reports have stated that seeds pre-treatment or plants supplemented with various protective compounds, may have a major role on how plants grow in abiotic stresses [3, 17-19]. Seed priming and exogenous usage of citric acid (CA), alone or in combination with other compounds, can increase salt tolerance and, as a result, crop productivity. Chakrobortty, *et al.* [18] stated that CA priming increased germination percentage and decreased mean germination time of okra seed, as well as increased growth characteristics, and relative water content. It has been also reported that, citrate treatment increased percentage of germination of papaya seeds [20]. Also, CA in addition with ascorbic acid and salicylic acid increased growth and yield under salt stress [19]. Moreover, CA spraying increased photosynthetic pigments and of cotton leaves and ultimately improved the production [21]. In addition, CA supplementation enhanced growth and endogenous CA concentration in the halophytic plant *Leymus chinensis* [22]. In contrast, essential oil constituents of Melissa officinalis improved by CA treatment in the state of saline stress [23]. Again, CA decreased soil salt and increased root yield of sugar beet [24]. In addition, hydro-priming has the potential for seed germination and crop growth enhancement. Damalas, *et al.* [25] reported that hydro-priming enhances the germination and field performance of faba bean. Moreover, it has been reported that hydro priming improved germination indices in wheat [26], and Niger [27]. Recently, Tania, *et al.* [28] reported hydro-primed wheat seeds enhance germination and seedling growth. Though Chilli is moderately sensitive to salt and seedlings are more vulnerable than germination under salt condition, taking into consideration the above statement we conducted this experiment to mitigate salinity effects on Chilli seedlings.

2. Material and Method

2.1. Site of Experiment, Treatments, and Germination Indices Measurement

A Petri dish and pot experiment was carried out at Bangladesh Agricultural Research Institute, Gazipur. A local Chilli (*Capsicum annuum* L.) cultivar Dhani was collected from local market and taken in the experiment. Chilli seeds were primed for 30 minutes in distilled water (DW), 0.5- and 1-mM citric acid (CA). Untreated seeds were used for control. Forty Chilli seeds were pre-treated for every treatment and placed on Petri dishes using tissue papers. Every Petri dish was filled with 8 mL of a 100 mM NaCl solution for the salt treatment, 0.5 mM and 1 mM CA treatments, and a Petri dish with 10 mL of fresh water was used as control for non-saline condition. The treatments were as follows: control (C), 100 mM NaCl (Salt), Hydro-primed+100 mM NaCl (HySalt), 0.5 mM CA+100 mM NaCl (CA0.5Salt), and 1 mM CA+100 mM NaCl (CA1Salt).

Daily records of seed germination were kept. From the initial germination up until the seventh day, the no. of germinated seeds was counted every after 24 hours. Thereafter calculations for GP, MGT, GI, and GE were done. SVI was calculated after measuring RL, and SL at 40 days after sowing. The GP, MGT, GI, GE and SVI were calculated using the corresponding formulae.

Germination percentage (GP) = $\frac{\text{Total no. of germinated seeds}}{\text{Total no. of seeds used}} \times 100$

Mean germination time (MGT) = $\Sigma \ \frac{\text{D}i N}{\text{N}}$

"N" is the seed no. on day D*i* and D*i* is the no. of days calculated from the starting of germination.

Germination index (GI) = $\frac{No.\ of\ germinated\ seeds}{Day\ of\ 1st\ count} + \cdots + \frac{No.\ of\ germinated\ seeds}{Day\ of\ last\ count}$

Germination energy (GE) = $\frac{\text{T}n1}{\text{N}i} \times 100$

Where T*n*1 is the no. of germinated seeds on the 1st day, and N*i* is the total no. of seeds.

Seed vigour index (SVI) = Germination percentage × seedling length (cm)

Where seedling length = shoot length + root length.

2.2. Hydroponic Experiment

A hydroponic system was used to grow uniformly germinated seeds in 2.5-liter pots containing liquid Yoshida's solution under 12-h light/12-h dark, 28^{o}C-day/25^{o}C night, 80% relative humidity conditions. Seedlings of 30 days old were then stressed for 10 days with 100 mM NaCl. Plants were sprayed with 0.5 mM and 1 mM CA over the course of 10 days, starting at the commencement of the stress (double spray each day at 9 am and 7 pm; 3 ml/plant/spray). After 10 days, RL, RFW, SL, SFW, RWC, and RWL were measured. Leaf samples were collected for photosynthetic pigments analysis. After oven drying, RDW, and SDW were recorded.

2.3. Relative Water Content (RWC) and Relative Water Loss (RWL) Determination

Utilizing González and González-Vilar [29] conventional methods, relative water content (RWC) was calculated. After 40 days, leaf samples were taken for the RWC measurement. The leaves were then measured for their fresh weight and submerged in distilled water for 6 hours. The left-over water on leaves was blotted off with a tissue paper, and the turgid weight was promptly taken note of. After oven drying, dry weight was recorded. The milligram (mg) unit was used to measure both weights. The formula below was used to determine the RWC:

$$\text{RWC (\%)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} x100$$

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

$$\text{RWL (\%)} = 1 - \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} x100$$

2.4. Photosynthetic Pigments Estimation

A spectro-photometric technique based on Lichtenthaler [30] method was used to quantify the amounts of photosynthetic pigments. Collected leaves (0.5 g) were put into a small vial with 10 mL of 80% ethanol. In order to extract the pigments, the vials were placed in dark for 10 days. Using a spectrophotometer with wavelengths of 663, 645, and 480 nm (Shimadzu UV-1280, Kyoto, Japan), the amounts of chlorophyll *a*, *b*, and carotenoids were determined.

Total Chlorophyll = Chlorophyll *a* + Chlorophyll *b*

Chlorophyll *a* = (Absorbance at 663 x 0.999 - Absorbance at 645 x 0.0989)

Chlorophyll *b* = {Absorbance at 663 x (-0.328) + Absorbance at 645 x 1.77}

Carotenoids = {(Absorbance at 663 x 0.114 - Absorbance at 645 x 0.638) + Absorbance at 480}

2.5. Statistical Analysis

IBM SPSS Statistics V.25 was used to conduct the statistical analysis. One-way analysis of variance was used to find significant differences, which was followed by Tukey HSD ($p < 0.05$).

3. Results and Discussion

3.1. Hydro and CA Priming Effects on Germination Indices of Chilli Seeds Under Salt Stress

Salinity is a major abiotic component that lowers growth and yield-related features, which lowers agricultural production globally. In contrast, seed priming is an efficient, economical, and biological practice that increases the germination of diverse crops' seeds [20, 31]. Primed seeds exhibit superior germination rates, early and uniform germination, improved growth properties, quicker emergence, and better establishment of crop stand because they are physiologically near to the germination stage [32]. It improves the mean performance of a seed lot by reducing the variation due to staggered emergence within a seed lot. Additionally, this enables the "poor" seeds to overtake the "good" ones during the pre-sprouting germination processes. As a result, direct-seeded crops emerge more quickly, expand quickly, and have a more uniform crop stand. In the end, it leads to the effective establishment of seedlings in an abiotic stress environment by reducing stress during the germination stage [33]. In order to investigate the impacts of hydro and CA seed priming on Chilli germination parameters under salt stress, we evaluated the GP, MGT, GI, GE, and SVI metrics as indicated in Table 1. The study showed that GP considerably decreased with the salt treatment compared to the control condition. Compared to Salt-treated seeds, GP was considerably enhanced by hydro and CA priming in HySalt, CA0.5Salt, and CA1Salt-treated seeds. When it came to MGT, the salt-treated seeds had much greater MGT than the control seeds. In contrast to Salt-treated seeds, MGT declined significantly for HySalt, CA0.5Salt, and CA1Salt. GI and GE were considerably decreased by salt stress compared to the control treatment. Furthermore, GI significantly increased in HySalt, CA0.5Salt, and CA1Salt-treated seeds compared to Salt-treated seeds. Similar to this, GE was greatly improved in HySalt, CA0.5Salt, and CA1Salt-treated seeds as compared to Salt-treated seeds. Contrary to instances of salt stress, hydro and CA greatly increased SVI. The findings demonstrated that HySalt, CA0.5Salt, and CA1Salt-treated seeds had considerably higher SVI as compared to Salt-treated seeds. In the current study, we found that and CA priming greatly reduced MGT, confirming CA's effectiveness in reducing the detrimental effects of salt on germination. The findings of our research are supported by other studies that found that priming seeds with CA significantly improved germination-related parameters in Carica papaya [20] and many field crops [31] and hydro priming improved germination indices in wheat [26], niger [27], and kidney bean [28] under salt stress. However, the seeds primed with CA1 showed more GP, GI, GE, and SVI and lower MGT as compared to hydro-, and CA0.5-primed under saline conditions (Table 1).

Table-1. Effects of hydro-, and CA priming on the germination percentage (GP), mean germination time (MGT), germination index (GI), germination energy (GE), and seed vigour index (SVI) in the state of saline condition. The data are presented as means ±SE, with a sample size n = 3. By using Tukey HSD ($p < 0.05$), different letters between treatments were examined

Treatments	GP (%)	MGT (Days)	GI	GE (%)	SVI
C	68.89 bc	4.37 b	9.19 b	13.33 b	2440.56 b
Salt	28.89 d	5.58 a	2.30 c	1.11 c	651.78 c
HySalt	66.67 c	4.24 bc	8.64 b	12.22 b	2096.00 b
CA0.5Salt	78.89 ab	4.33 bc	10.65 ab	16.67 ab	3272.44 a
CA1Salt	83.33 a	3.95 c	12.57 a	22.22 a	3576.78 a
SE	5.22	0.15	0.95	1.96	278.13

3.2. Exogenous Application Effects on Seedling Growth of Chilli Under Salt Stress

Salinity stress significantly harmed the development characteristics of seedlings [18, 19, 28, 34]. The process of seed priming mitigates the deleterious effects of saline stress on healthy development of seedlings that enhances the addition of Ca^{+2} and K^{+} and decreases Na^{+} and Cl^{-} accumulation in emerging seedlings, causing maximum uptake of water with less osmotic potential [33, 35]. According to reports, under salt stress conditions, exogenous CA administration dramatically improved these parameters [18, 19, 21]. We assessed the RL, RFW, RDW, SL, SFW, SDW, and PH of the Chilli seedlings in order to ascertain the impacts of salt stress and stress-relieving CA activities (Table 2). The findings demonstrated a significant reduction in RL in plants treated with salt. In contrast to Salt-treated plants, RL was greatly improved in HySalt, CA0.5Salt, and CA1Salt-treated plants. When compared to control plants, salt-treated plants had an enormous decrease in RFW. Results also revealed that plants treated with CA0.5Salt, and CA1Salt significantly enhanced RFW in comparison to untreated plants. RDW dramatically decreased under the Salt condition compared to the Control condition, while plants treated with CA0.5Salt and

CA1Salt greatly increased RDW compared to Salt-treated plants. Comparing the salinity stress condition to the control condition, SL was significantly affected negatively. In contrast to Salt-treated plants, SL considerably increased in HySalt, CA0.5Salt, and CA1Salt-treated plants. SFW was considerably increased in CA0.5Salt and CA1Salt-treated plants as compared to Salt-treated plants. Additionally, SDW was greatly raised by CA0.5Salt and CA1Salt-treated plants in comparison to Salt-treated plants, while it was dramatically lowered by Salt treatment in comparison to the control treatment. In terms of plant height, plants treated with HySalt, CA0.5Salt, and CA1Salt had considerably higher PH than plants treated with salt. The findings of our investigation are consistent with those of other studies that showed improved seedling growth and development in maize [19], okra [18], cotton [21], and *Leymus chinensis* [22], when exogenous CA was used. It has been reported that hydro-priming showed increased growth in wheat plants [36]. Additionally, the results showed that CA1 performed better than CA0.5 in terms of seedling development attributes under salt stress condition.

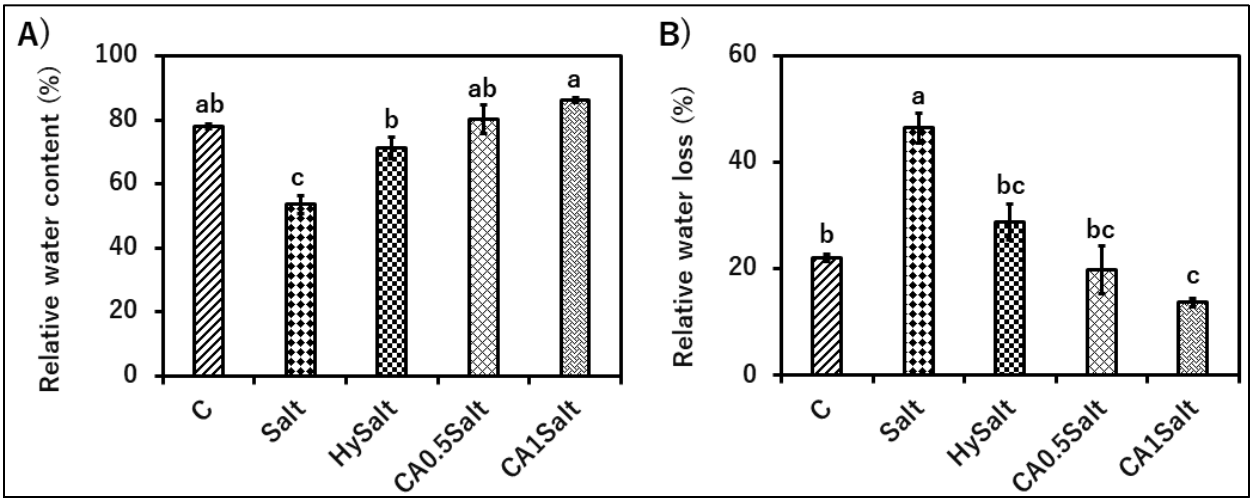
Table-2. Effects of hydro-, and CA priming and supplementation on the root length (RL), root fresh weight (RFW), root dry weight (RDW), shoot length (SL), shoot fresh weight (SFW), shoot dry weight (SDW), and plant height (PH) in the state of saline condition. The data are presented as means ±SE, with a sample size n = 3. By using Tukey HSD ($p < 0.05$), different letters between treatments were examined

Treatments	RL (cm)	RFW (mg)	RDW (mg)	SL (cm)	SFW (mg)	SDW (mg)	PH (cm)
C	13.43 ab	243.33 b	34.33 a	21.97 b	740.00 a	77.67 a	35.40 b
Salt	8.33 c	116.67 c	20.33 c	14.27 d	496.67 b	54.67 b	22.60 c
HySalt	12.93 b	150.00 c	25.67 bc	18.53 c	593.33 b	63.33 b	31.47 b
CA0.5Salt	14.50 ab	293.33 a	30.3 ab	27.03 a	813.33 a	81.00 a	41.53 a
CA1Salt	17.10 a	370.00 a	31.33 ab	25.80 a	840.00 a	86.33 a	42.90 a
SE	0.82	25.26	1.43	1.28	36.51	3.30	2.01

3.3. Exogenous Application Effects on Water Status of Chilli Under Salt Stress

Under salt stress, it's essential to maintain appropriate water content in plants, and RWC is a key sign of how well-hydrated plants are [28, 37, 38]. The results of the current study indicated that salt stress lowered RWC and enhanced RWL (Figure 1), and this was caused by cell wall structural degradation that interfered with adequate water absorption. To check on the water status of the Chilli plants, we measured RWC and RWL. In comparison to the control condition, the results showed that salt stress considerably decreased RWC (Figure 1A). When compared to Salt-treated plants, the addition of CA considerably increased RWC in the CA0.5Salt and CA1Salt-treated plants. The difference in RWC between Salt- and HySalt-treated plants was likewise statistically significant (Figure 1A). In contrast, a sizable rise in RWL was also seen as a result of salt stress (Figure 1B). HySAlt, CA0.5Salt, and CA1Salt-treated plants had considerably reduced RWL than Salt-stressed plants (Figure 1B). These findings showed that CA was more effective at keeping Chilli's water status under salt stress. Similar findings were found in prior research that showed how salt stress raised RWL and decreased RWC in plants [39], and how CA treatment markedly improved RWC and decreased RWL under salinity stress conditions [18, 22]. This finding suggested that CA could be involved in absorbing more water from the soil to change the water content of plant organs [40].

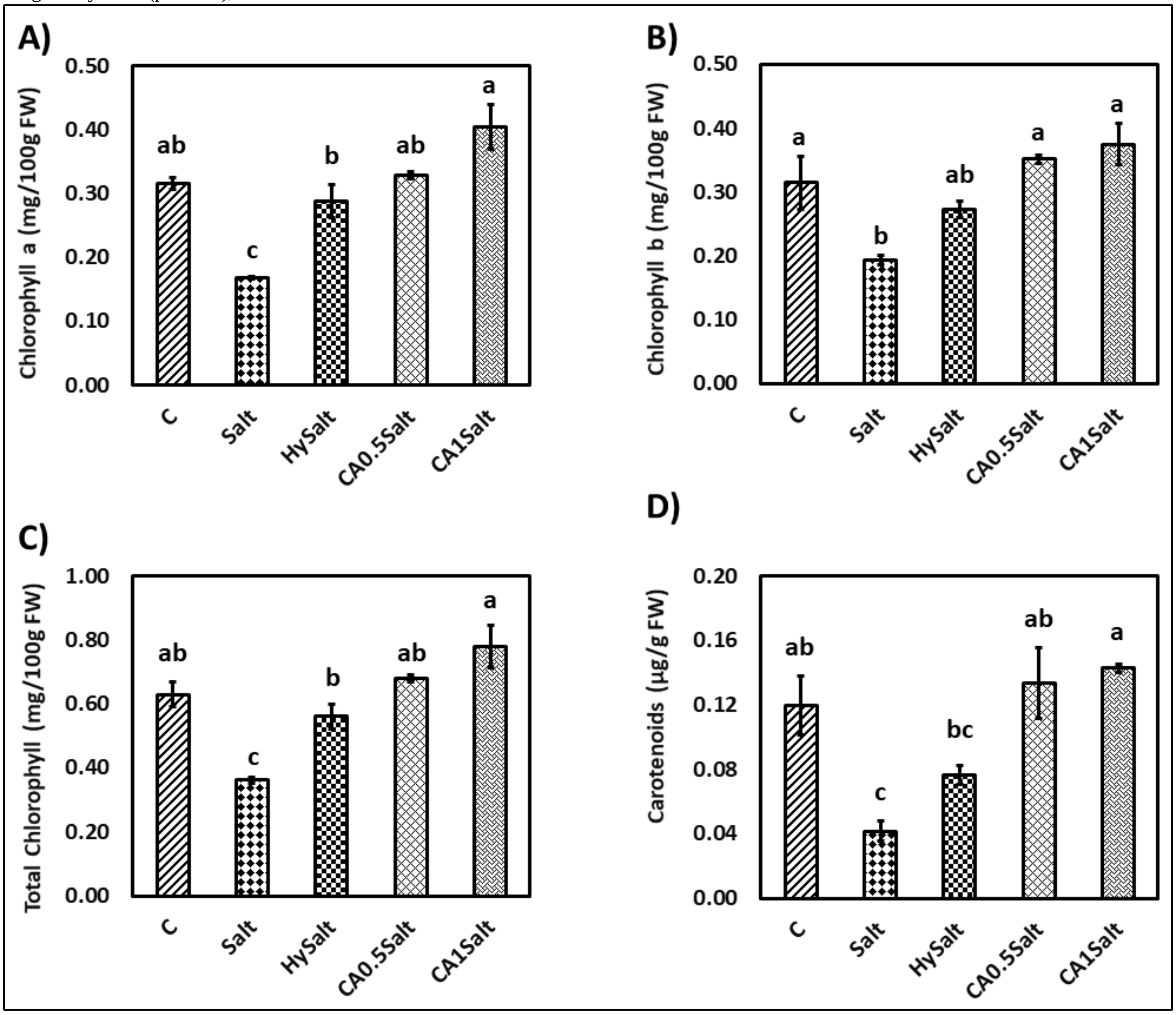
Figure-1. Effects of hydro-, and CA priming and supplementation on A) relative water content; B) related water loss of Chilli seedlings under salt stress. The data are presented as means ±SE, with a sample size n = 3. By using Tukey HSD ($p < 0.05$), different letters between treatments were examined



3.4. Exogenous Application Effects on Photosynthetic Pigments of Chilli Under Salt Stress

The function of chlorophyll is to absorb light for photosynthesis. The main function of chlorophyll a in the electron transport chain is as an electron donor. The function of chlorophyll b is to enable organisms to absorb higher frequency blue light for use in photosynthesis. Plants' photosynthetic pigment levels are considerably decreased due to salt stress [41-43]. The adverse impact of salt stress was observed in the current investigation as a significant variation in chlorophyll pigment levels (Figure 3). Under salt stress, the quantity of total chlorophyll, chlorophyll a, and chlorophyll b in Chilli leaves reduced considerably in comparison to the control (Figure 2A–C). The results revealed that plants treated with HySalt, CA0.5Salt, and CA1Salt had considerably higher levels of total chlorophyll and chlorophyll a than plants treated with salt (Figure 2A–C). Additionally, results demonstrated that CA substantially enhanced chlorophyll b in plants treated with CA0.5Salt and CA1Salt (Figure 2B). The amount of carotenoids in salt-stressed plants was much lower than in control plants. In comparison to salt stress, supplementing with CA0.5 and CA1 showed substantial increasing patterns of carotenoids content (Figure 3D). Our findings support prior reports that showed the foliar application of CA increased photosynthetic pigments in maize [19] and cotton [21]. Previous report also showed that hydro-priming increased photosynthetic pigments in wheat [36]. The data from above also showed that, under saline conditions, CA1 supplementation increased photosynthetic pigments substantially more than CA0.5 supplementations.

Figure-2. Effects of hydro-, and CA priming and supplementation on the photosynthetic pigments- A) Chlorophyll a; B) chlorophyll b; C) total chlorophyll; and D) carotenoids content of Chilli in the state of NaCl stress. The data are presented as means ±SE, with a sample size n = 3. By using Tukey HSD ($p < 0.05$), different letters between treatments were examined



4. Conclusion

After being treated with exogenous bio-active organic acids like citric acid, crops cultivated under a variety of abiotic stress conditions show improved germination and growth. RWC and photosynthetic pigments were both boosted by CA in addition to germination and growth-related metrics. The results of this study lead to the conclusion that, under salt stress, 1 mM CA is superior than 0.5 mM CA in terms of improving germination, seedling characteristics, and photosynthetic pigments. As a result, CA may be used as a seed priming and exogenous agent for Chilli in order to reduce the impacts of salt stress and to encourage early seedling development.

4.1. Data Availability

All the necessary data are included in the manuscript. If additional data are required, the corresponding author can be contacted.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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