Seed treatment with chlormequat chloride improves the physiological and biochemical characteristics of *Brassica napus* L. under salt stress

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ABSTRACT

Background: Salinity is a global and increasing problem in agriculture. The response of *Brassica napus* L. (canola or oilseed rape) to salinity and the possible role of chlormequat chloride (CCC) as a seed primer in improving salt tolerance were investigated in a pot experiment. The experiment consisted of four salinities (0, 0.5, 1 and 1.5 g NaCl kg^{-1} soil) and four CCC concentrations (0, 250, 500 and 750 mg L^{-1}) in a complete randomised block design with three replications.
Results: Salt stress decreased relative water content (RWC), stomatal conductance, stomatal density, photosynthetic rate, chlorophyll, potassium and calcium content, K/Na, Ca/Na ratio and seed yield, but increased leaf temperature, intercellular CO_2 concentration, proline, electrolyte leakage, stomatal area and sodium content. Application of CCC, especially 500 and 750 mg L^{-1}, improved all measured traits and Ca and K contents. The increase in K and Ca content and the simultaneous decrease in Na content indicate improved ion selectivity in plants treated with CCC. Stomatal density was reduced by salt stress, while higher concentrations of CCC increased the number of stomata, especially in the abaxial part, thus reducing the adaxial/abaxial stomatal number ratio. Reduced stomatal number due to salt stress increased the resistance of canola to salt stress by preventing transpiration rate and can be considered as a stress avoidance and water saving mechanism in canola. There is no significant difference in all measured traits between non-saline and mild saline stress, but seed yield decreased at this stress level compared to the control condition.
Conclusion: The results suggest that the application of CCC increased the photosynthetic, physiological, and biochemical attributes and seed yield of canola plants. The data show that 500 and 750 mg CCC L^{-1} in saline soils up to 10.5 dS m^{-1} can improve yield and physiological parameters of canola.

Introduction

Climate change and widespread groundwater exploitation are causing soil and water salinisation, especially in arid and semi-arid regions (Shahid et al., 2018; Ma et al., 2020; Khaleghnezhad et al., 2021). Shahid et al. (2018) reported that approximately 10% of all arable land is salinised, and the problem is increasing globally. These conditions are currently causing salt stress and reducing crop growth and production worldwide (Ma et al., 2020; Naheed et al., 2021). Salt stress impairs water uptake and causes ionic toxicity, leading to leaf senescence, reduced pigment synthesis and disrupted photosynthesis (Ansari et al.,

2019b; Jamshidi et al., 2022; Ma et al., 2020). For example, in mustard, salinity negatively affected photosynthesis by reducing leaf area, chlorophyll content, and oxidative damage, and ultimately reduced dry matter production and seed yield (Wani et al., 2019). Salinity also alters nutrient uptake and damages physiological processes (Ansari et al., 2019a; Jamshidi et al., 2022). In one study, salinity stress increased sodium content in canola but decreased potassium uptake (Naheed et al., 2021). Potassium deficiency causes damage to plant cells and photosystem II, reduces the stability of chloroplast membranes and photosynthesis, and ultimately hinders growth rates (Shahid et al., 2020).

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Several measures are used to reduce the deleterious effects of salt stress (Ma et al., 2020). For example, exogenous application of plant growth regulators (PGRs) such as salicylic acid, gibberellins and brassinolide regulate plant adaptation to adverse environmental conditions such as drought and salinity by accumulating osmolytes and improving plant water relations (Gurmani et al., 2011; Wani et al., 2019; Alharby et al., 2021; Biareh et al., 2022).

Chlormequat chloride, also known as chlorcholine chloride or cycocel (CCC), is one of the derivatives of choline, produced by the reaction of trimethylamine and 1- and 2-dichloroethane, in the form of crystals and soluble in water. It belongs to the group of ammonium compounds and is widely used as a plant growth regulator to reduce plant height (Basra, 2000).Chlormequat chloride promotes more vigorous growth, reduces stem length by inhibiting cell elongation and disrupting the biosynthesis of the gibberellin pathway. It also reduces the risk of lodging and can therefore increase crop yield and productivity (Pirasteh-Anosheh et al., 2021). In rice, seed priming with CCC reduced salinity stress damage and it was concluded that CCC treatment can help improve rice plant growth and seed yield under salt stress by reducing plant sodium content and Na/K ratio and increasing potassium and protein content in the plant (Gurmani et al., 2011). It has been reported that foliar application of CCC could increase chlorophyll content, stomatal conductance and photosynthetic rate in wheat grown under salt stress (Barányiová and Klem, 2016). CCC application as a seed treatment also increased proline, total protein, RWC, chlorophyll and antioxidant enzyme activity in mung bean grown under water deficit conditions (Farooq and Bano, 2006). In addition, CCC application increased wheat seed yield, RWC, chlorophyll, potassium content, and plant stress stability (Seyed Sharifi and Khalilzadeh, 2018).

Canola (*Brassica napus* L.) is an important industrial crop grown for oil production and fodder. It has been identified as a salt-tolerant plant, but its yield and productivity are reduced by salinity stress (Bandehagh et al., 2021). Genotypic differences have also been reported in canola cultivars against salt stress (Shekari et al., 2000; Mohamed et al., 2020; Naheed et al., 2021). Considering the detrimental effects of salinity on canola, such as an increase in sodium content and oxidative damage and a decrease in potassium uptake and seed yield (Sabagh et al., 2019; Naheed et al., 2021), it is necessary to investigate the strategies to reduce salt damage on the physiological traits and yield of canola. Although there are several studies on the effects of salt stress on canola, there is no comprehensive information on the effect of CCC application in relation to salt stress. Therefore, this study was conducted to investigate the effect of seed priming with CCC on the stomatal, physiological, and photosynthetic responses and related parameters of canola under salt stress.

Materials and methods

Experimental design, treatments, seed sowing, and greenhouse conditions

A greenhouse experiment based on a randomised complete block design with a factorial arrangement with three replications was conducted at the University of Zanjan, Zanjan, Iran. The treatments consisted of 0, 0.5, 1 and 1.5 g NaCl kg^{-1} soil (control, mild, moderate and severe salt stress) corresponding to electrical conductivity of 3.24, 5.92, 7.4 and 10.2 dS m^{-1}, respectively, and CCC seed priming solutions containing 0, 250, 500 and 750 mg CCC L^{-1}.

Canola seeds (cv. Hayola 401) obtained from Seed and Plant Improvement Institute, Karaj, Iran, were soaked in 100 ml of CCC solutions (Merck, Germany) for 24 h. The seeds were then air dried at room temperature for 48 h to reach their initial weight before priming. The soil used in this study was a mixture of field soil, sand and manure in a ratio of 6:3:1, with a loamy texture, pH 7.4, EC 3.24 dS cm^{-1}, saturation 25, bulk density 1.57 g cm^{-3}, organic matter 2.12%, nitrogen 0.05%, phosphorus 47.8 mg kg^{-1}, potassium 748 mg kg^{-1}, sodium 22.86 mg kg^{-1} and calcium 19.4 mg kg^{-1}. The electrical conductivity of

the soil (3.24 dS m^{-1}) was used as a control. The soil for each pot was mixed separately with the required amount of NaCl and then the pots (35 cm diameter and 50 cm height) were filled with the appropriate soil. Primed canola seeds were disinfected with carboxin tiram and then planted at a depth of 2 cm in each pot with 15 seeds per pot. The pots were placed in a greenhouse with good sunlight and artificial light (10 to 14 Klux) and a 16/8 h photoperiod. The average relative humidity was 60–70% and the maximum and minimum day/night temperatures were 30 and 17 °C, respectively.

The first irrigation was carried out immediately after planting, with subsequent irrigations at intervals of three to four days. To maintain the salt concentration in the pots, the drainage water collected in the saucers was returned to the pots after irrigation. The pots were irrigated with purified tap water (0.36 dS m^{-1}) filtered through a home water filtration system (Rad-Gostar Novin, Aqua Pro_67S1_RO, Iran). Thinning was carried out after emergence and establishment of the plants; only five plants were kept in each pot.

Leaf temperature and relative water content (RWC)

At the pre-flowering stage, leaf temperature was measured randomly in the fourth developed leaf from the upper part of the three plants for each pot using a laser gun thermometer (Minitemp Laser Radiation, China) from 10 to 11 a.m. The fresh, saturated and dry leaf weights were obtained to calculate the leaf RWC using the method of Turner (1981).

RWC = [(Fresh weight - Dry weight) / (Saturation weight - Dry weight)] × 100

Photosynthetic parameters

At the pre-flowering stage, photosynthetic parameters including photosynthetic rate, transpiration rate, stomatal conductance and intercellular CO_2 concentration were measured using an LCi portable infrared gas analyser (ADC -Co. UK) on three plants and the fourth leaf at 25–27 °C and 12 Klux light intensity. Chlorophyll a, b and total chlorophyll contents were measured according to Arnon (1949) using 0.1 g fresh leaf sample and 80% acetone. Light absorbance was read with a spectrophotometer (PerkinElmer, Lambada 25, USA) at wavelengths of 663.2, 646.8 and 470 nm. Chlorophyll content was expressed as mg g-1 fresh weight.

Biochemical parameters

Proline content was measured using a spectrophotometer (PerkinElmer, Lambada 25, USA) on 0.1 g of leaf sample at the pre-flowering stage according to the method of Bates et al. (1973) at 520 nm. Soluble carbohydrates were measured using the Antron reagent method (Irigoyen et al., 1992) on 0.5 g of leaf sample at 625 nm with a spectrophotometer (PerkinElmer, Lambada 25, USA) and soluble carbohydrate content was expressed as mg g-1 fresh weight. To measure electrolyte leakage, leaf samples (0.5 g) were washed with distilled water and incubated in tubes containing 10 ml of distilled water for 24 h at 25 °C. The amount of initial EC was then recorded using an EC meter (CMD 500 WPA conductivity meter, Cambridge, UK). The samples were then placed in a hot water bath at 100 °C for 30 min. The second EC was recorded when the samples reached 25 °C. Electrolyte loss was calculated using the following equation (Lutts et al., 1996).

Electrolyte leakage = (First electrical conductivity / Second electrical conductivity) × 100

Anatomical parameters

At the pre-flowering stage, stomatal area and density were measured using the copying method. The most recently developed leaf was selected from each plant. The adaxial and abaxial sides of the leaf were covered with a thin layer of clear liquid glue. After the liquid glue dried,

slides were made with glass tape. In each sample, the area and density of the stomata were examined in 10 visibilities of a light microscope (Leica Galen III, Germany) equipped with a 40-magnification lens. This lens was equipped with a micrometer to determine the length and width of the stomata. The stomatal area was calculated according to the following formula (Teare et al., 1971):

Stomatal area = Half a large diameter of stomata × Half a small diameter of stomata × 3.14

Potassium, sodium, and calcium estimation

The wet digestion method was used to measure the concentration of potassium, sodium and calcium in the shoot at the pre-flowering stage (Walinga et al., 1995). According to this method, dry samples (0.3 g) were digested with mixed acid (6 g salicylic acid, 100 ml 98% sulphuric acid and 18 ml distilled water). The extract was then used to determine the sodium and potassium content in mg L^{-1} by the flame photometry method using a flame photometer (Jenway, model PFP7/C, UK) and the calcium content in mg L^{-1} using the Varian Spectra AA atomic spectrometer.

Seed yield

At the end of the growing season, the canola plants were harvested at seed maturity and the seeds were separated from the siliques. The weight of all seeds from each pot was then determined using a balance (accuracy 0.001 g) and recorded as seed yield. Seed yield was also reported as g plant^{-1}.

Statistical analysis

The data were analysed using a randomised complete block design in a two-way factorial (4 × 4) experiment with three replications. The general linear model (GLM) procedure was used to analyze variance using SAS statistical software (SAS, Institute Inc. 2009). Where effects were significant ($P \leq 0.05$), differences between means were tested for significance using the Duncan multiple range test ($P \leq 0.05$). Pearson's correlation coefficients were used to calculate the relationships between the characteristics.

Results

The ANOVA results showed that the effects of CCC and salt stress and their interaction were significant ($P \leq 0.01$) on all the traits studied (Table 1). The interaction between CCC and salt stress was not significant for stomatal conductance and adaxial/abaxial stomatal number ratio (Table 1).

RWC was reduced by increasing salt concentration compared to the non-saline treatment. The lowest RWC was recorded at the highest salt concentration in the absence of CCC. There was no significant difference between different levels of CCC under non-stress and mild salt stress conditions. Seed priming with CCC (especially at 500 and 750 mg L^{-1}) significantly improved leaf RWC in canola plants exposed to severe salt stress (Table 2). A positive and significant correlation ($r = 0.73$, $p = 0.01$) was observed between RWC and seed yield.

Leaf temperature increased with increasing salt concentration. The application of CCC at higher concentrations, especially under severe salt stress conditions, caused a significant reduction in leaf temperature (Table 2). The minimum leaf temperature was recorded from non-stressed plants with the application of 250 mg L^{-1} CCC, which was not significantly different from other CCC levels in non-stressed conditions. There was a significant negative correlation ($r = -0.79$, $p = 0.01$) between leaf temperature and RWC. The results showed that the plants with higher RWC had lower leaf temperatures, so that the plants under non-stress conditions had the highest RWC and the lowest leaf temperature (Table 2).

Table 1
Analysis of variance of four levels of salinity and four concentrations of CCC on the studied traits in canola, cv. Hayola 401.

Source of Variation	df	RWC	Leaf temperature	Photosynthetic rate	Transpiration rate	Stomatal conductance	Intercellular CO_2 concentration	Proline content	Content of soluble sugars	Electrolyte leakage	Chlorophyll a	Chlorophyll b
		Mean of squares										
Block	2	2.248**	0.391**	0.192**	0.014**	918.75ns	945.65*	7.966*	0.0001ns	4.245**	0.0001ns	0.0001ns
Salinity (S)	3	70.315**	3.701**	169.373**	0.09ns	2007.63**	44,249.68**	425.114**	0.0001ns	62,123.51**	0.00002**	0.0017**
CCC (C)	3	52.936**	2.424**	21.202**	0.032ns	779.86ns	16,663.78**	11.912**	0.0001ns	11,511.27**	0.0027**	0.00053***
S × C	9	27.38**	0.919**	8.61**	0.0042ns	222.45ns	4877.83**	5.439**	0.0001ns	3686.12**	0.00036**	0.00008**
Error	30	6.223	0.146	1.117	0.053	476.63	220.313	2.51	0.001	171.193	0.00001	0.00003
Coefficient of variation (%)		3.2	2	12.9	24.1	28.6	6.2	14.2	6.7	2	4.4	22.7

S.O.V	df	Total chlorophyll	Adaxial stomatal density	abaxial stomatal density	Adaxial stomatal density / Adaxial stomatal density	Stomatal area	Sodium content	Potassium content	Calcium content	Potassium to sodium ratio	Calcium to sodium ratio	Seed yield
		Mean of squares										
Block	2	0.0001ns	1.68**	122.25ns	0.027**	304.48*	6.969**	105.146ns	9.146**	0.66ns	0.136**	0.266ns
Salinity (S)	3	0.0087**	15.35ns	137.38ns	0.137**	1344.09**	6434.05**	3126.84**	2620.81**	161.192**	115.465**	21.98**
CCC (C)	3	0.0017**	287.79**	2403.77**	0.148**	1373.96**	651.866**	288.299**	346.75**	7.673**	6.081**	8.56**
S × C	9	0.00026**	142.31**	918.79**	0.048ns	724.11**	220.822**	140.65**	107.25**	2.432**	1.264**	1.06**
Error	30	0.00003	33.154	70.028	0.028	77.874	18.014	34.746	10.594	0.429	9.252	0.165
Coefficient of variation (%)		6.5	29	21.5	30	14.1	14.8	7.8	5.1	13	12	13.5

* $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant.

Table 2
Mean comparison of the interaction salinity and seed priming with CCC on the leaf temperature, intercellular CO_2 concentration, relative water content (RWC), chlorophyll a, chlorophyll b, total chlorophyll, adaxial stomatal density, abaxial stomatal density, and stomatal area in canola.

Salinity (g NaCl kg⁻¹ soil)	CCC (mg L⁻¹)	RWC (%)	Leaf temperature (°C)	Intercellular CO_2 concentration (μmole CO_2 mole⁻¹)	Chlorophyll a (mg g⁻¹)	Chlorophyll b (mg g⁻¹)	Total chlorophyll (mg g⁻¹)	Adaxial stomatal density (number mm⁻²)	abaxial stomatal density (number mm⁻²)	Stomatal area (μm²)	Sodium content (mg L⁻¹)	Potassium content (mg L⁻¹)	Calcium content (mg L⁻¹)
0	0	81.4[ab]	18.8[fg]	192.1[e]	1.07[a]	0.69[ab]	1.77[a]	17.7[b-e]	43.7[bc]	52[de]	10.11[de]	85.5[a]	76.2[a]
0	250	84.6[a]	18.35 g	190.4[e]	1.05[a]	0.62[bc]	1.69[ab]	20.3[b-e]	40[bc]	59.9[de]	10.2[de]	90[a]	74.3[ab]
0	500	80.1[ab]	18.85[fg]	188.5[e]	1.05[a]	0.62[bc]	1.69[ab]	18[b-e]	40.3[bc]	60.2[de]	10.02[e]	85.8[a]	75.6[a]
0	750	77.7[abc]	19[g]	185.4[e]	1.07[a]	0.7[ab]	1.7[a]	18.7[b-e]	47.3[bc]	54[de]	9.93[e]	91.6[a]	74.9[a]
0.5	0	79.1[abc]	19.7[cde]	201.2[e]	1.02[a]	0.5[d]	1.53[c]	18.3[b-e]	35[cd]	61[cde]	12.87[de]	91.3[a]	70.1[ab]
0.5	250	78.6[abc]	20[bcd]	194.7[e]	1.05[a]	0.54[cde]	1.61[bc]	20.3[b-e]	36.7[c]	48.6[e]	12.79[de]	87.2[a]	74.4[a]
0.5	500	78.8[abc]	19.5[def]	190[e]	1.05[a]	0.62[bc]	1.67[ab]	17.7[b-e]	35[cd]	48.6[e]	10.08[de]	90.7[a]	75[a]
0.5	750	79[abc]	19.5[def]	188.2[e]	1.03[a]	0.73[a]	1.77[a]	17.7[b-e]	35.3[cd]	47.6[e]	11.73[de]	85.6[a]	71[ab]
1	0	73[d]	20.8[a]	336.7[b]	0.91[b]	0.4[fg]	1.31[de]	10.3[de]	19.3[e]	76.4[bc]	54.41[b]	61.3[b]	52.1[c]
1	250	73.4[d]	20.37[abc]	298[c]	0.88[bc]	0.45[ef]	1.34[d]	12[cde]	21[de]	77.5[b]	45.52[c]	64.2[b]	55[c]
1	500	77[bc]	19.2[ef]	196.1[e]	1.02[a]	0.58[cd]	1.61[bc]	22.3[abc]	53[b]	57[d]	18.21[d]	82.[a]	66.5[b]
1	750	80[ab]	19.17[ef]	191.6[e]	1.06[a]	0.58[cd]	1.64[b]	25[ab]	54.3[b]	66.5[bcd]	17.6[de]	85.6[a]	67.2[b]
1.5	0	70.9[d]	21[a]	375.7[a]	0.6[d]	0.31 g	0.92[f]	8.3[e]	8.7[e]	96[a]	68.23[a]	47.1[c]	30.1[d]
1.5	250	72.2[d]	20.51[ab]	373.3[a]	0.64[d]	0.34 g	0.99[f]	8.7[e]	9.7[e]	107.8[a]	68.06[a]	47.5[c]	33.7[d]
1.5	500	78.3[d]	19.25[ef]	295.7[c]	0.84[c]	0.39[g]	1.24[e]	31.7[a]	70.3[a]	48.2[e]	51.2[bc]	62.2[b]	51.2[c]
1.5	750	79.8[b]	19.21[ef]	227[d]	0.85[bc]	0.48[def]	1.34[d]	32[a]	74.3[a]	44[d]	52.54[de]	62.4[bc]	56.7[c]

For each parameter, data with the same letter are not significantly different (Duncan multiple range test $P \leq 0.05$).

Under unstressed and low salt stress conditions, no significant difference was observed between CCC treatments for electrolyte leakage and proline content (Figs 3a and b). With increasing salt concentration, electrolyte leakage and proline content also increased; however, seed treatment with CCC, especially at concentrations of 500 and 750 mg L⁻¹, reduced electrolyte leakage and increased proline content. The highest electrolyte leakage was associated with the highest salt stress and the treatment without CCC application compared to the control treatment (Fig. 3a). The highest proline content was associated with the 500 and 750 mg L⁻¹ CCC treatments applied under high salt stress. The lowest proline content was also observed in control and mild salt stress plants, with no significant differences between CCC concentrations (Fig. 3b). There was a significant and negative correlation between seed yield and proline content ($r = -0.57$, $p = 0.01$) and seed yield and electrolyte leakage ($r = -0.83$, $p = 0.01$).

Salt stress reduced the rate of photosynthesis (Fig. 1). The lowest value was associated with high salt stress and 0 and 250 mg L⁻¹ CCC treatments. The highest photosynthetic rate was observed in unstressed plants. The photosynthetic rate was reduced by increasing soil salinity, showing a significant reduction at salinities above 0.5 g NaCl kg⁻¹ in the 0 and 250 mg L⁻¹ CCC treatments. The photosynthetic rate was significantly increased by increasing CCC concentration, especially under moderate and severe salt stress (Fig. 1). There was a positive and significant correlation ($r = 0.81$, $p = 0.01$) between photosynthetic rate and seed yield. A similar trend was found for stomatal conductance at control and mild salt stress, but beyond this level and in other salinity treatments, stomatal conductance showed a significant decrease (Fig. 2). Increasing the salt concentration increased the intercellular CO_2 concentration. The highest intercellular CO_2 concentration was associated with high salt stress and 0 or 250 mg L⁻¹ CCC treatments (Table 2). In contrast, the application of CCC, especially at 500 and 750 mg L⁻¹, reduced the intercellular CO_2 concentration (Table 2). Seed yield showed a significant negative correlation ($r = -0.81$, $p = 0.01$) with intercellular CO_2 concentration.

Sodium concentration increased with increasing soil salinity. In contrast, potassium and calcium concentrations in plant tissues decreased (Table 2). The application of CCC increased the status of essential elements in the treated plants. The content of sodium, potassium and calcium in non-stressed plants was not significantly different from the content of these elements in mildly saline stressed plants and the application of CCC did not show any significant change in the concentration of these elements. In contrast, salinity increased sodium and decreased potassium and calcium at higher stress levels. In other words, after mild salinity stress, the concentrations of these elements changed
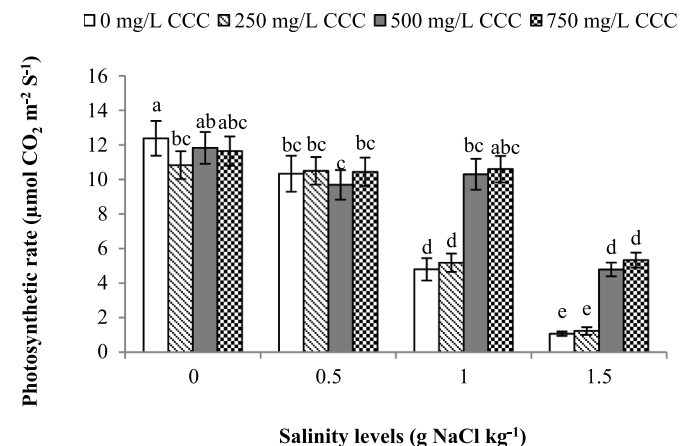
Fig. 1. Mean comparison of the interaction of salinity and seed priming with chlormequat chloride (CCC) on the photosynthetic rate in canola. Vertical T bars indicate standard error. Columns with the same letters do not differ significantly (Duncan multiple range test $P \leq 0.05$).
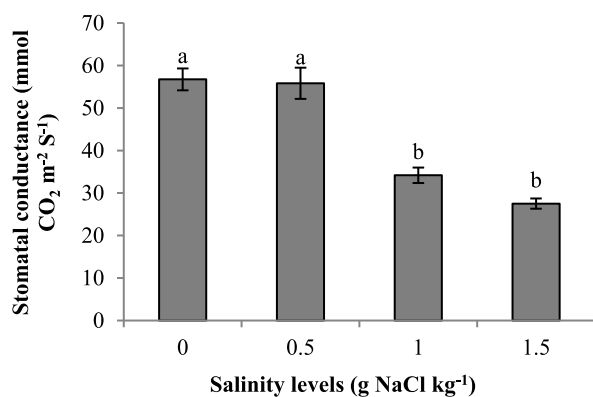
Fig. 2. Mean comparison of the simple effect of salinity on the stomatal conductance in canola plants primed with chlormequat chloride (CCC). Vertical T bars indicate standard error. Columns with the same letters do not differ significantly (Duncan multiple range test $P \leq 0.05$).

dramatically. Seed priming with CCC, especially at 500 and 750 mg L^{-1}, significantly reduced sodium content but increased potassium and calcium content at high salinities (1 and 1.5 g NaCl kg^{-1} soil) (Table 2). The highest sodium content was observed when the 1.5 g NaCl kg^{-1} treatment was applied together with the 0 or 250 mg L^{-1} CCC treatment (Table 2). The Ca/Na (Fig. 5a) and K/Na (Fig. 5b) ratios decreased with increasing salinity. The applying of CCC, especially at 500 and 750 mg L^{-1}, increased the K/Na and Ca/Na ratio at the moderate salinity level. Seed yield was positively and significantly correlated with potassium ($r = 0.8$, $p = 0.01$) and calcium ($r = 0.82$, $p = 0.01$) content, Ca/Na ($r = 0.81$, $p = 0.01$) and K/Na ratio ($r = 0.84$, $p = 0.01$). On the other hand, there was a significant and negative correlation ($r = -0.79$, $p = 0.01$) between seed yield and sodium content. According to the significant relationships in electrolyte leakage and sodium content and the increase in potassium content by application of CCC, seed treatment with CCC increased selectivity in the roots of plants treated with CCC compared to untreated plants. As a result, it can significantly increase salinity tolerance in canola.

Salt stress significantly reduced chlorophyll concentration. The results showed that chlorophyll a is more stable than chlorophyll *b*, because at low salinity the concentration of chlorophyll *b* was reduced compared to the non-stressed condition, but the chlorophyll a content did not differ significantly from the control condition (Table 2). In general, the application of CCC showed no significant effect on chlorophyll a, b and total chlorophyll at no and mild salinity conditions, but with increasing soil salinity an improving effect of CCC on chlorophyll was observed (Table 2). At the higher salinity levels, the application of CCC, especially at 500 and 750 mg L^{-1}, showed positive effects on chlorophyll content.

There was a significant relationship between chlorophyll content, photosynthetic rate, and intercellular CO_2 concentration. Lower photosynthetic rates and higher intercellular CO_2 concentrations were observed in the low chlorophyll treatments. A positive and significant correlation was found between seed yield and photosynthesis and chlorophyll a ($r = 0.76$, $r = 0.91$, $p = 0.01$), chlorophyll b ($r = 0.81$, $r = 0.83$, $p = 0.01$) and total chlorophyll ($r = 0.82$, $r = 0.92$, $p = 0.01$).

Salt stress affected stomatal frequency and size such that stomatal density decreased in the adaxial and abaxial parts of the leaf with increasing salinity. Under non-stressed and mild salinity conditions, CCC application did not significantly affect stomatal density. Surprisingly, application of 500 and 750 mg L^{-1} CCC at higher salinities increased stomatal density on both sides of the leaf (Table 2). Stomatal area increased as stomatal density decreased at higher salinities. In contrast, the application of 500 and 750 mg L^{-1} CCC reduced the stomatal area at the highest salinity level. The highest stomatal area was obtained in non-primed plants and primed plants with 250 mg L^{-1} CCC at the highest

salinity level. The lowest stomatal area was associated with the application of 500 and 750 mg L^{-1} CCC at the highest salinity treatment (Table 2). There was a significant and positive correlation between seed yield and adaxial ($r = 0.35$, $p = 0.05$) and abaxial ($r = 0.49$, $p = 0.01$) stomatal density. In contrast, stomatal area had a significant and negative correlation ($r = -0.65$, $p = 0.01$) with seed yield. The highest ratio of adaxial/abaxial stomatal number was associated with the highest salinity level, with a 54% increase compared to the control (Fig. 4a). The highest ratio of adaxial/abaxial stomatal number also occurred at 0 and 250 mg L^{-1} CCC, and the application of 500 and 750 mg L^{-1} CCC reduced the ratio of adaxial/abaxial stomatal number by 30% compared to no CCC application (Fig. 4b). These results suggest that CCC increased the abaxial rather than the adaxial stomatal number.

Seed yield was influenced by salinity and CCC application. The lowest seed yield was associated with the high salinity treatments with CCC concentrations of 0 and 250 mg L^{-1}. In contrast, in the salinity treatments, the higher CCC concentrations increased canola yield (Fig. 6). In contrast to most traits, which showed no significant difference between unstressed and mild salinity levels, seed yield showed a significant decrease in the mild stress level compared to the unstressed condition. The cumulative effect of CCC at 500 and 750 mg L^{-1} was also found at this level, in addition to higher salinities.

Discussion

Salt stress negatively affects plant growth, biomass production and seed yield (Ansari et al., 2019a, b; Shahid et al., 2020; Jamshidi et al., 2023). RWC is a simple but reliable indicator for assessing environmental stress tolerance (Sarker and Oba, 2020). Salinity stress reduced RWC, especially at higher salinities, but seed priming with CCC declined this negative effect (Table 2). Under salinity stress conditions, water uptake becomes difficult due to decrease osmotic potential in the root zone, resulting in reduced water content in leaf tissues and reduced RWC (Ansari et al., 2019a, b; Ma et al., 2020; Jamshidi et al., 2022;). Higher RWC may be due to an increase in osmolyte content, as an increase in osmolyte content is involved in osmotic regulation, improved water uptake and RWC in stressed plants (Mohamed et al., 2020). In wheat, the application of CCC increased RWC under salinity conditions (Seyed Sharifi and Khalilzadeh, 2018). The improvement in RWC can affect the stomatal aperture and reduce the leaf temperature through transpiration flux.

As shown in Table 2 and Figs. 3a and b, leaf temperature, electrolyte leakage and proline content were lower in none and mildly saline conditions. However, with increasing salinity, these characteristics increased significantly. Seed priming with CCC, especially at 500 and 750 mg L^{-1}, reduced leaf temperature and electrolyte leakage but increased proline content. Leaf temperature is one of the most important parameters in plant physiological and ecological research. It indicates a regulation between plant energy used for transpiration and leaf tissue water content (Ballester et al., 2013). When soil moisture uptake is difficult for plants, some physiological traits such as RWC, stomatal conductance and transpiration rate change in response to the environment (Ru et al., 2020). Chung et al. (2020) found that leaf temperature was much higher in soybean plants treated with NaCl. Salt stress increased leaf temperature with stomatal closure (Ansari et al., 2019b; Chung et al., 2020). Application of CCC to Moldavian balm reduced leaf and canopy temperature under drought stress. This reduction was associated with root growth and development (Jalalvand et al., 2019).

The cell membrane is the first site of damage due to environmental stress. Therefore, electrolyte leakage indicates membrane damage and stress resistance (Ansari et al., 2019b). Studies have shown that cell membrane stability decreases, and electrolyte leakage increases under salinity stress (Ansari et al., 2019b; Sarker and Oba, 2020). It has been reported that salinity negatively affects the membrane integrity and growth of canola. Increasing salinity also increased electrolyte leakage in the cultivars studied compared to the control (Mohamed et al., 2020).
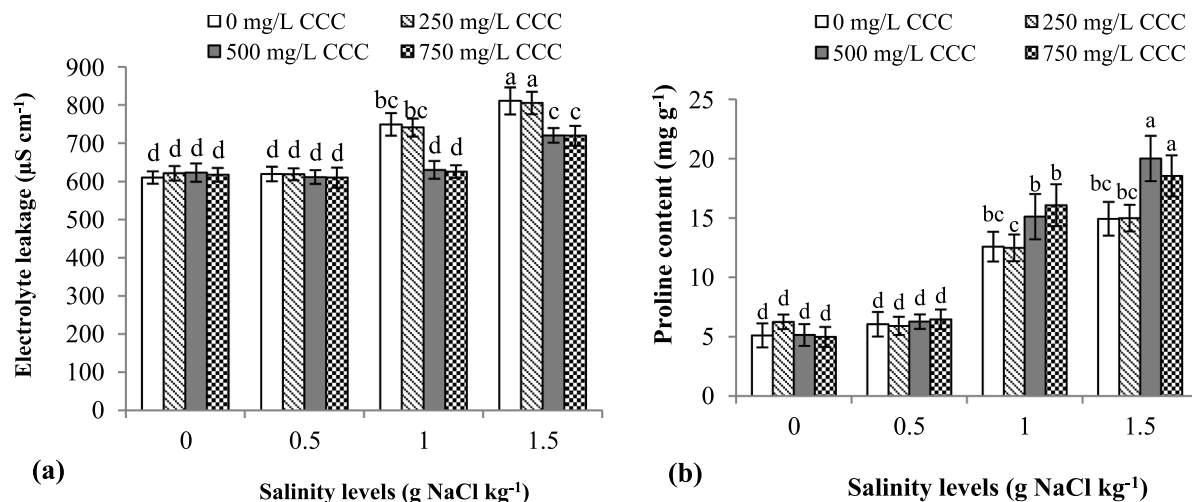
Fig. 3. Mean comparison of the interaction of salinity and seed priming with chlormequat chloride (CCC) on the electrolyte leakage (a) and proline content (b) in canola, cv. Hayola 401. Vertical T bars indicate standard error. Columns with the same letters do not differ significantly (Duncan multiple range test $P \leq 0.05$).
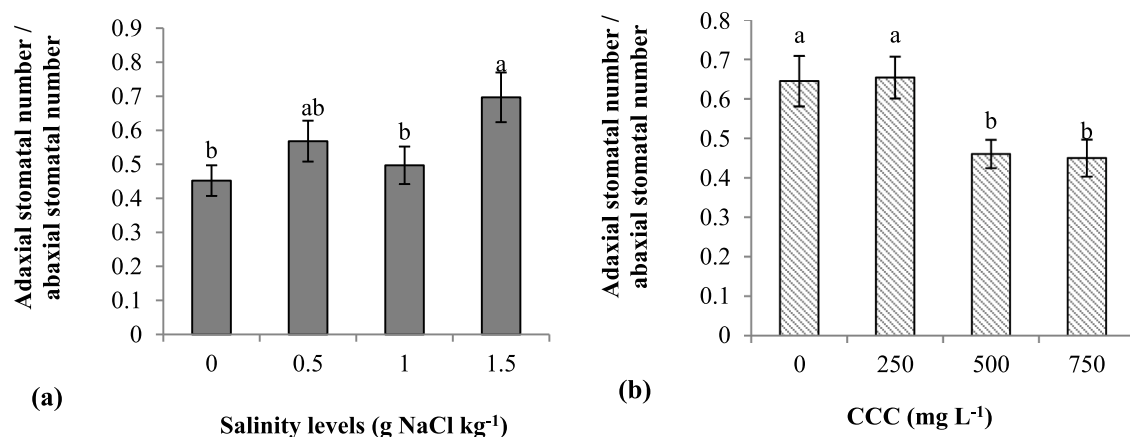
Fig. 4. Mean comparison of the simple effect of salinity (a) and the simple effect of chlormequat chloride (b) on the ratio of the adaxial stomatal number to the abaxial stomatal number in canola, cv. Hayola 401. Vertical T bars indicate standard error. Columns with the same letters do not differ significantly (Duncan multiple range test $P \leq 0.05$).

This increase in electrolyte leakage could be due to increased entry of toxic ions and decreased potassium uptake (Ansari et al., 2019a).

The accumulation of osmolytes, such as proline and sugars, is one of the most important physiological indicators of salt tolerance in plants and is considered an essential mechanism in many plants to overcome salt stress (Ma et al., 2020). The accumulation of these compounds under salt stress protects cells by balancing the osmotic potential of the cytosol with that of the vacuole. Proline is the stabiliser of macromolecular structure and the main source of energy and nitrogen against salinity (Shahid et al., 2020). The increase in proline content of canola under salinity stress compared to non-stressed conditions has been reported in some reports (Mohamed et al., 2020; Naheed et al., 2021). It has been reported that an increase in intracellular proline concentration and plant stress tolerance can be achieved by exogenous application of PGRs such as CCC. Application of CCC increased soluble protein, free proline and antioxidant enzyme activity and improved salt tolerance in wheat (Seyed Sharifi et al., 2016). It was also shown that application of CCC under saline conditions increased proline content and soluble sugars (Gurmani et al., 2011). An increase in soluble sugars and proline increases membrane stability and maintains enzyme activity. These mechanisms help the plant to prevent tissue damage, thus allowing continued growth and development under saline conditions (Mohamed et al., 2020).

As salinity increased, photosynthetic rate and stomatal conductance decreased continuously (Figs. 1 and 2). In contrast, intercellular CO_2 concentration increased with increasing salt stress (Table 2). Seed priming with CCC improved photosynthesis and related parameters. These variations in photosynthetic rate were closely related to the elemental content of the leaf tissue (Fig. 1 and Table 2). Although salinity increased Na content, it reduced K and Ca concentrations in plant tissues (Table 2). Application of CCC reduced Na content and increased Ca and K content. These changes were dramatic at high salinity. The increase in intercellular CO_2 concentration indicates the inefficiency of the photosynthetic system, which reduces the rate of photosynthesis (Shahid et al., 2020). It has been reported that high sodium levels can disrupt intracellular enzymatic processes and photosynthetic electron transfer (Ma et al., 2020). In canola, salinity reduced photosynthesis, transpiration rate and stomatal conductance. Reduced photosynthetic capacity was associated with osmotic stress and relative stomatal closure (Mohamed et al., 2020). Wani et al. (2019) on mustard and Ansari et al. (2019b) on alfalfa also found that salinity stress negatively affected photosynthetic rate by decreasing leaf area, chlorophyll content, and increasing oxidative damage. In mustard, reduced photosynthesis and reduced number of pods, seeds/pod and 100 seed weight led to reduced seed yield (Wani et al., 2019). Salinity also affects the uptake and metabolism of essential nutrients. For example, salinity

affects the uptake of N, P, K, S and Ca in amaranth, leading to changes in photosynthesis and photosynthetic efficiency (Sarker and Oba, 2020).

The uptake and distribution of potassium and sodium ions in tissues are important determinants of salinity tolerance in plants (Sarker and Oba, 2020). High sodium concentrations in the shoot due to salinity can cause metabolic and osmotic problems (Shahid et al., 2020; Jamshidi et al., 2023). Under salinity stress, higher potassium uptake potential increases photosynthetic rate, activates enzymes, reduces ROS and regulates stomatal function, protein synthesis, osmotic regulation and turgor maintenance (Mohamed et al., 2020). Increases in sodium, Na/K, and Na/Ca and decreases in potassium content have been reported in salt-stressed plants such as alfalfa (Ansari et al., 2019a), safflower (Jamshidi et al., 2022), wheat (Seyed Sharifi and Khalilzadeh, 2018), amaranth (Sarker and Oba, 2020), and canola (Naheed et al., 2021). Application of CCC, especially under saline conditions, increased potassium and calcium content in shoot and root and decreased sodium content, Na/K and Na/Ca ratios (Table 2, Figs 5a and b), which is consistent with the findings of Seyed Sharifi and Khalilzadeh (2018). As a secondary messenger, calcium plays a role in signaling stress leading to salinity adaptation (Gurmani et al., 2011). Since one of the main mechanisms of salinity tolerance is to uptake more potassium and calcium against sodium and maintain a high ratio of potassium to sodium (Gurmani et al., 2011; Sarker and Oba, 2020), it seems that seed treatment with CCC can increase photosynthetic rate (Fig. 1), improve growth and increase canola yield (Fig. 6) by inducing salinity tolerance caused by higher potassium and calcium uptake (Table 2) under saline conditions.

Increasing sodium concentration, decreasing potassium and calcium, and decreasing chlorophyll content may be reasons for decreasing photosynthetic rate with increasing salinity (Table 2). The increase in photosynthetic rate and stomatal conductance with CCC application under salinity stress was in agreement with the results of Barányiová and Klem (2016), who found that CCC treatment increased chlorophyll content, stomatal conductance and photosynthetic rate in winter wheat under both normal and stressed conditions. The beneficial effects of seed and plant treatment with PGRs on plant growth and production under stressful conditions may be related to improved nutrient availability and increased plant physiological processes and root development (Biareh et al., 20–22; Barányiová and Klem, 2016).

It was reported that CCC could increase the concentration of chlorophyll and carotenoids, accelerate photophosphorylation and stimulate the rate of photosynthesis in *Solidago canadensis* L. (Osman, 2014). This was consistent with the results of our study on increasing chlorophyll content (Table 2) and photosynthetic rate (Fig. 1), especially under salinity stress. It has been found that increasing the activity of
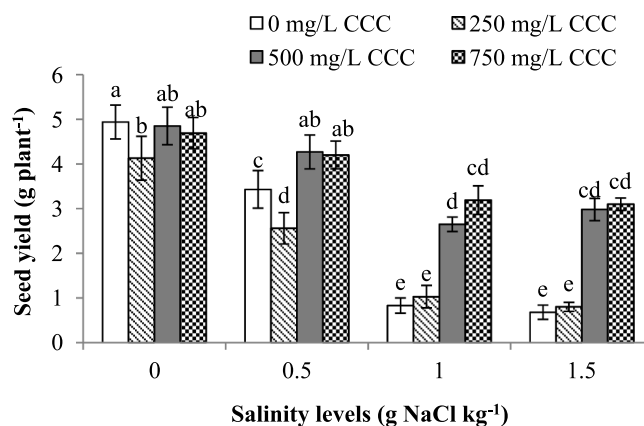

Fig. 6. Mean comparison of the interaction of salinity and seed priming with chlormequat chloride (CCC) on the seed yield of canola, cv. Hayola 401. Vertical T bars indicate standard error. Columns with the same letters do not differ significantly (Duncan multiple range test $P \leq 0.05$).

antioxidant enzymes in response to CCC application can protect photosynthetic systems from damage caused by reactive oxygen species under water deficit conditions (Pakar et al., 2016). Also, the increase in chlorophyll content in wheat plants treated with CCC coincided with an increase in the maximum photochemical efficiency of photosystem II through a higher Fv/Fm ratio (Seyed Sharifi et al., 2016).

Chlorophyll content in plants is one of the most important factors in maintaining photosynthetic potential (Mohamed et al., 2020). Reduced chlorophyll content under stress, such as salinity, is the main factor causing leaf senescence. It has been reported that the decrease in chlorophyll content caused by salinity is due to the production of free oxygen radicals, which cause peroxidation and consequently the degradation of these pigments (Ansari et al., 2019b; Ma et al., 2020; Sarker and Oba, 2020). Salinity leads to the disruption of photosynthesis and the increase in stomatal resistance. This reduction can also be attributed to a decrease in chlorophyll synthesis, increased chlorophyll degradation and activation of chlorophyllase (Shahid et al., 2020). The reduction in seed yield appears to be due to the reduced chlorophyll content and photosynthetic rate. Decreased chlorophyll content, photosynthesis rate and reduced seed yield due to salinity have been reported in mustard (Wani et al., 2019). Increased chlorophyll content has been reported with the application of CCC in wheat plants under both non-stress and stress conditions (Seyed Sharifi and Khalilzadeh, 2018). Increased chlorophyll content in plants treated with CCC may be due to delay in
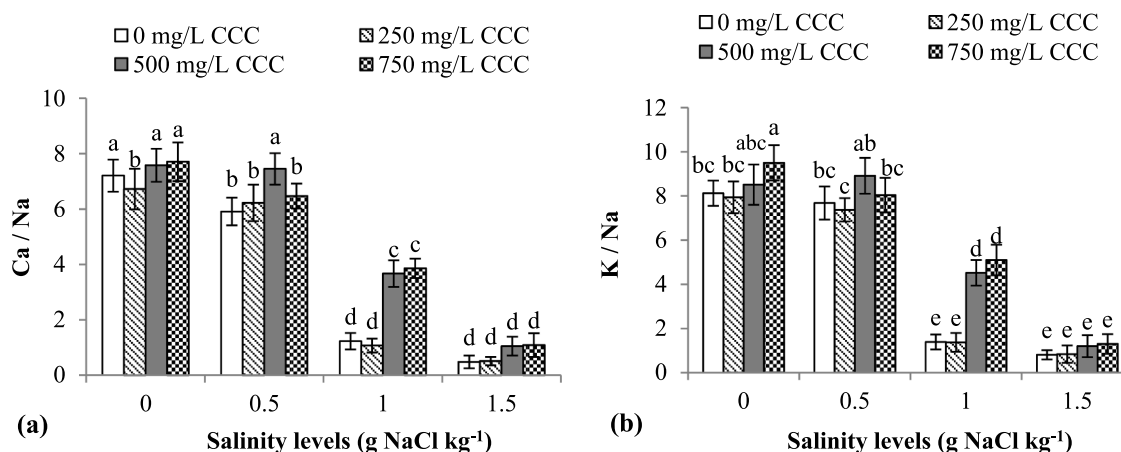

Fig. 5. Mean comparison of the interaction of salinity and seed priming with chlormequat chloride (CCC) on the calcium to sodium ratio (a) and potassium to sodium ratio (b) in canola, cv. Hayola 401. Vertical T bars indicate standard error. Columns with the same letters do not differ significantly (Duncan multiple range test $P \leq 0.05$).

leaf senescence, prevention of chlorophyll degradation, increase in chlorophyll synthesis, and synthesis of soluble proteins and enzymes (Osman, 2014).

Stomata respond to environmental conditions, such as stress, and their number and distribution can change (Mohamed et al., 2020). Salinity altered stomatal size and density in the adaxial and abaxial parts of the leaf (Table 2). As salinity increased, stomatal density decreased but stomatal size increased. In contrast, plants treated with higher CCC concentrations showed increased stomatal frequency and decreased size. Salt-tolerant wheat populations had higher water content and lower stomatal density, and a significant and negative correlation between salinity tolerance and stomatal density was reported (Tao et al., 2021). In barley, salinity tolerance was correlated with reduced stomatal density to retain water (Kiani-Pouya et al., 2020). Tao et al. (2021) reported a negative relationship between stomatal density and plant water content, stating that salt-resistant plants maintain their water status under stress by reducing stomatal density. According to the results of the above studies, reducing stomatal number on both sides of canola leaves under salinity conditions can increase plant resistance to salinity stress by retaining more water and can be considered as a stress avoidance mechanism for canola as water conservation. It has been reported that there is a strong correlation between stomatal area, photosynthesis, transpiration rate and stomatal conductance in canola (Mohamed et al., 2020). In our study, abaxial stomatal area was positively and significantly correlated ($r = 0.34$, $p = 0.01$) with photosynthetic rate. It is reported that there was a significant variation in stomatal area and density of 10 canola cultivars under salt stress. A significant decrease in leaf and stomatal area but an increase in stomatal density were observed in some cultivars under high salt stress (Mohamed et al., 2020). It has been reported that in bean plants under salinity stress, the size of stomata decreased with CCC application, but the number of stomata per unit leaf area increased (Imbamba, 2006). This report was consistent with the results of the present study on the increase in stomatal number with CCC application under high salinity. Improvement in the water status of plants treated with PGRs can be associated with root development, rooting depth, accumulation of osmoregulators and stomatal regulation (Barányiová and Klem, 2016).

CCC application did not significantly affect seed yield under non-stressed conditions. In contrast, higher levels of CCC increased seed yield under all salt treatments compared to no application and 250 mg L^{-1} CCC. These enhancing effects may be due to increased potassium and calcium uptake, improved RWC, chlorophyll concentration, photosynthetic rate and reduced sodium uptake. Reduced grain yield due to salinity has also been reported in mustard (Wani et al., 2019), canola (Naheed et al., 2021), and safflower (Jamshidi et al., 2023). In saffron under non-stress conditions, priming with CCC did not affect the yield of this crop (Heidari et al., 2022). On the other hand, in rice, application of CCC increased panicle dry weight, length and seed yield (Goutam et al., 2018). There are also similar reports on the cumulative effects of CCC on seed yield under salinity stress in wheat (Seyed Sharifi et al., 2016) and *Cyamopsis tetragonoloba* L. (Afria et al., 1999). The results of the above studies were consistent with the results of our study on the positive effect of CCC on seed yield, especially under salinity conditions.

Conclusion

Salinity stress reduced photosynthesis rate and stomatal conductance in *Brassica napus* L. Our data show that salinity reduces RWC and chlorophyll content of stressed plants. These reductions may affect the rate of photosynthesis through stomatal and non-stomatal effects. The decrease in RWC led to a reduction in turgor pressure in leaf tissues and thus to a reduction in stomatal size and its pore dimension. This reduction may affect the CO_2 sink to the mesophyll and act as a limiting factor for photosynthesis. On the other hand, decrease in chlorophyll content and increase in leaf temperature may affect light absorption rate and efficiency of Calvin cycle. In addition, salinity increased electrolyte leakage and sodium content. Disruption of photosynthesis, chlorophyll content, RWC, electrolyte leakage and imbalance in Na/Ca and Na/K ratios may be reasons for seed yield reduction. In contrast, seed priming with CCC mitigated the deleterious effects of salinity stress. It caused a relative improvement in chlorophyll, potassium and calcium content, photosynthesis rate and reduced electrolyte leakage in treated plants. CCC also reduced leaf temperature, which was closely related to RWC values. Seed priming increased proline, potassium and calcium contents, K/Na and Ca/Na ratio. As a result, seed yield of treated plants increased significantly. Stomatal density on both sides of the leaves reduced by in increasing salt levels. This reduction in stomatal density was accompanied by an increase in stomatal size. In contrast, application of CCC increased the number of stomata but reduced their size. This reduction in stomatal frequency under salt stress may be a response of canola plants to the stress by reducing transpiration rate to prevent leaf water content and RWC. Our data show that in canola, water conservation may be an avoidance mechanism to reduce salt stress damage. Under salinity conditions, seed priming with 750 mg L^{-1} CCC was the best treatment in this study to increase photosynthesis rate and yield of canola and to reduce salinity damage.

Author contribution

Maryam Vazayefi: Greenhouse management, plant husbandry, data collecting in greenhouse and laboratory, statistical analyses of original data draft preparation. **Farid Shekari** and **Andrea Mastinu**: Conceptualization, methodology, formal analysis, writing and review of original draft preparation, project administration. **Esmaeil Zangani**: data collecting in the laboratory, formal analysis. **Aria Dolatabadian**: review and editing of the manuscript. **Tibor Janda** and **Andrea Mastinu**: Scientific advisory, review and editing of the manuscript.

All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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