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Salinity stress induced changes in germination, morpho-physiological, and stomata parameters of black mustard (*Brassica nigra* (L.) Koch)

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Abstract

Background: Soil and irrigation water salinity is a major threat to plant growth. Identifying tolerant species or cultivars is essential for sustainable agriculture.

Questions and Hypotheses: This study investigated the response of black mustard (*Brassica nigra*) to salt stress during germination, emergence, and morphophysiological development. We hypothesized that *B. nigra* tolerates high salinity, with stagespecific responses and physiological traits indicating tolerance.

Studied Species: Black mustard (*Brassica nigra*) was tested under NaCl concentrations of 50, 100, 150, and 200 mM, corresponding to electrical conductivities of 5.5, 10.5, 15.6, and 20.1 dS m^{-1}.

Methods: Experiments were conducted under controlled conditions, assessing germination, emergence, morphological and physiological traits (chlorophyll content and cell membrane stability), and stomatal characteristics (number and area on adaxial and abaxial surfaces).

Results: Salinity reduced shoot and root length and seedling fresh weight, but did not affect germination percentage. In pot experiments, increasing salinity decreased plant height and biomass, while emergence remained unaffected. Conversely, salinity increased dry matter and chlorophyll content. Cell membrane stability declined linearly with salinity. At 100 mM NaCl and above, stomatal number and area decreased on both leaf surfaces. The most pronounced effects on plant height and fresh weight occurred at concentrations exceeding 100 mM NaCl.

Conclusions: Salinity primarily impaired seedling growth rather than germination. Plant height and cell membrane stability are reliable indicators of salinity tolerance in *B. nigra*. Concentrations above 150 mM NaCl represent the critical threshold for germination and early growth.

Keywords: Cell membrane stability, NaCl, plant growth, stomata density.

Resumen

Antecedentes: Una de las mayores amenazas para el crecimiento vegetal es la salinidad del suelo y del agua de riego. Identificar especies o cultivares tolerantes es esencial para una producción agrícola sostenible.

Preguntas e hipótesis: Se evaluó la respuesta de *Brassica nigra* al estrés salino durante la germinación, emergencia y desarrollo morfofisiológico. Se planteó que esta especie tolera altos niveles de sal y responde de forma diferenciada en cada etapa, con ciertos rasgos fisiológicos como indicadores de tolerancia.

Especie estudiada: *Brassica nigra* fue expuesta a concentraciones de NaCl de 50, 100, 150 y 200 mM, con conductividades eléctricas de 5.5, 10.5, 15.6 y 20.1 dS m^{-1}.

Métodos: Bajo condiciones controladas se evaluaron germinación, emergencia, rasgos morfológicos y fisiológicos (contenido de clorofila, estabilidad de membranas celulares), y características estomáticas (número y área en superficies adaxiales y abaxiales).

Resultados: La salinidad redujo longitud de tallo y raíz y peso fresco de plántulas, sin afectar la germinación. En macetas, la salinidad disminuyó altura y biomasa, pero no la emergencia. Aumentó la proporción de materia seca y clorofila. La estabilidad de membranas decreció linealmente con la salinidad. A partir de 100 mM NaCl, el número y área estomática disminuyeron. Los efectos más marcados sobre altura y peso fresco ocurrieron por encima de 100 mM.

Conclusiones: La salinidad afectó más el crecimiento que la germinación. Altura y estabilidad de membranas son buenos predictores de tolerancia. Concentraciones superiores a 150 mM NaCl marcan el umbral crítico para la germinación y crecimiento temprano.

Palabras clave: Estabilidad de la membrana celular, NaCl, crecimiento de las plantas, densidad de los estomas.

Black mustard (*Brassica nigra* (L.) Koch) is a diploid plant that is taxonomically classified within the *Brassicaceae* family. All parts of the plant, including the leaves, stem, buds, inflorescences, and seeds, are consumed (Raza *et al.* 2020). Notably, mustard seeds are one of the important sources of oil, with concentrations ranging from 28 to 34 % (Karydogianni *et al*. 2023). Additionally, the seed meal of mustard seeds is characterized by a substantial protein content after oil extraction in (Negawoldes 2018). The importance of black mustard in agriculture derives from its utilization as a medicinal and home remedy. Its seeds have antibacterial (Obi *et al*. 2009, Markose *et al*. 2016), anti-diabetic (Anand *et al*. 2007), antimicrobial (Tomar & Shrivastava 2014), anti-cancer (Amri 2014), anti-flu (Mazumder *et al*. 2016), anti-spasmodic and aphrodisiac (Rahman *et al*. 2018), appetite-stimulating effects (De Zoysa & Waisundara 2020), and more recently discovered, diuretic properties (Shristy *et al*. 2023). It is also grown for industrial oil production and as a nutritious seed meal (Augustine *et al*. 2014). Currently, black mustard is traditionally grown on a much smaller scale than other amphidiploid species, such as *Brassica napus*, *Brassica juncea*, and *Brassica carinata* (Ashraf & McNeilly 2004).

Salinity excess is caused primarily from soil or irrigation water and is a detrimental abiotic stress that limits plant growth and yield (Roy *et al*. 2014, Leblebici *et al*. 2021, Kulan *et al.* 2021, Ergin *et al.* 2021). Salinity is estimated to affect nearly 10 % of the world's irrigated and rainfed agricultural areas, corresponding to approximately 1.38 billion hectares (FAO 2024). Similar challenges have been reported across many countries, including Türkiye, where salinity affects about 1.52 million hectares of agricultural land (Deliboran & Savran 2015). In salt-affected areas, tolerant plants play a key role in successful crop production (Kaya *et al.* 2019). Species in the *Brassicaceae* family are projected to become more important for future agricultural sustainability due to their ability to adapt to saline and drought conditions (Zhang *et al*. 2022). There are significant interspecific variations within the *Brassica* genus in terms of salt tolerance (Purty *et al*. 2008), yet more experimental work is needed to establish the salt-tolerance of different species and varieties.

Most *Brassica* species are moderately salt-tolerant, but the amphidiploid species (*B. napus*, *B. juncea*, and *B. carinata*) are relatively more tolerant than the diploid species (Kumar *et al.* 2015, Quezada-Martinez *et al.* 2021). The salt tolerance of the amphidiploids suggests that they acquired this trait from the A (*Brassica campestris*) and C (*Brassica oleracea* L.) genomes (Ashraf & McNeilly 2004). He & Cramer (1992) compared the resistance to seawater of *B. napus*, *B. campestris*, *B. nigra*, *B. juncea*, *B. oleracea*, and *B. carinata*, and *B. carinata* was found to be susceptible to salt, while *B. napus* was highly tolerant. Similarly, Kumar (1995) found that *B. napus* was the most salt-tolerant species, while *B. nigra* and *B. campestris* were the most salt-sensitive at the vegetative and adult stages. Öztürk *et al.* (2006) determined that *B. nigra* tolerated the salinity level of 1%, but its tolerance decreased by 32 % at the 1.5 % salinity level.

Tolerance to salinity stress can also change depending on the developmental stages of plants, specifically *Brassica* species. Kaya *et al.* (2005) found significant differences in germination and emergence among *Brassica* species (*B. napus* ssp. *oleifera* L.*, B. campestris* L., and *B. oleracea* L.) under salinity stress, and *B. campestris* was found to be more tolerant to salinity up to 10 dS m^{-1}. Furthermore, Sharma *et al.* (2013) and Singh & Prasad (2025) determined an increased germination-stress index in *B. juncea* under salinity stress, and the lower index values were obtained from the higher salinity levels. Ashraf *et al*. (2001) classified diploid and amphidiploid species of *Brassica* based on salinity tolerance, and they found that amphidiploid species had significantly greater shoot fresh and dry weight, as well as seed yield, compared to the diploid species. In that study, the most salt-tolerant species was *B. napus*, which was followed by *B. carinata* and *B. juncea*, but the salt-sensitive species was the diploid species *B. nigra*, followed by *B. oleracea* and *B. campestris*. However, the response of *Brassica* species to salinity can vary at later plant growth stages. For instance, *B. juncea* exhibits reduced cell–membrane stability (as indicated by increased electrolyte leakage) under saline conditions (100 mM NaCl) (Singh & Prasad 2025). Therefore, there is limited knowledge about the response of *B. nigra* to salinity stress. To utilize increasing saline arable lands and maintain sustainable plant production, it is necessary to identify plant species and varieties that can withstand salinity. The present study specifically focused on examining the salinity tolerance levels of *B. nigra* by considering germination, emergence, and morphophysiological and stomata traits at the flowering stage under artificially salinized conditions.

Materials and methods

A laboratory experiment was conducted in the Seed Science and Technology Laboratory at the Department of Field Crops, Faculty of Agriculture, Eskişehir Osmangazi University, Türkiye, in 2025. Seeds of black mustard (*Brassica nigra* (L.) Koch.) were procured from a vendor of medicinal herbs in Eskişehir and utilized as the material. Salinity levels were prepared at 50, 100, 150, and 200 mM lab-grade NaCl (99 % purity) with electrical conductivities of 5.5, 10.5, 15.6, and 20.1 dS m^{-1}, respectively. Distilled water (0 mM) served as a control.

Germination experiment. Two hundred seeds divided into four replicates (4 × 50) were used for each salinity level. The fifty seeds were counted and inserted into three sheets of sterile filter paper with 21 mL of the respective salt solutions. As soon as the papers were gently rolled, they were put into sealed plastic bags to prevent water loss. These bags were transferred to an incubator at a constant temperature of 25 ± 1 °C in the dark. Seeds with a 2 mm radicle were considered to have met the germination criteria and were counted daily for 7 days. The mean germination time (MGT) was calculated as described by Ellis & Roberts (1981):

$$\mathrm{MGT} = \left(\frac{\sum(Dn)}{\sum n}\right)$$

where n is the number of germinated seeds on day D and D is the number of days from the beginning of the germination test.

Germination index (GI) was also determined using the following formula (Salehzade *et al*. 2009):

$$\mathrm{GI} = \frac{\text{Number of germinated seeds}}{\text{Days of the first count}} + \cdots + \frac{\text{Number of germinated seeds}}{\text{Days of the final count}}$$

Germination Stress Index (GSI) was quantified as a percentage and computed using the following formula provided by Ahmad *et al*. (2009):

$$\mathrm{GSI} = \frac{\left[\left(\mathrm{nd2} \times (1.00)\right) + \left(\mathrm{nd4} \times (0.75)\right) + \left(\mathrm{nd6} \times (0.50)\right) + \left(\mathrm{nd8} \times (0.25)\right)\text{in stressed seeds}\right]}{\left[\left(\mathrm{nd2} \times (1.00)\right) + \left(\mathrm{nd4} \times (0.75)\right) + \left(\mathrm{nd6} \times (0.50)\right) + \left(\mathrm{nd8} \times (0.25)\right)\text{in control seeds}\right]} \times 100$$

where nd2, nd4, nd6, and nd8 = the number of seeds germinated on the 2nd, 4th, 6th, and 8th days, respectively.

Root and shoot length, seedling fresh and dry weights were measured on ten randomly selected seedlings at the end of the experiment. The percent reduction due to salinity stress was determined using the method described by Shirani Rad & Abbasian (2011):

$$\%\ \text{Reduction} = \frac{(\text{Value of control plant} - \text{Value of salt} - \text{stressed plant})}{\text{Value of control plant}} \times 100$$

Pot experiment. The pot experiment was conducted to simulate soil salinity, as the seeds were directly placed into salty growing medium, which were prepared by mixing peat (Potgrond H, EC: 0.40 dS m^{-1}, pH: 5.5-6.5, and organic matter 90 %) with respective amounts of NaCl equal to 50 (2.92 g L^{-1}), 100 (5.84 g L^{-1}), 150 (8.76 g L^{-1}), and 200 (11.68 g L^{-1}) mM by weight. Distilled water (~ 0 dS m^{-1}) was used as a control. After filling the plastic pots (0.5 L volume) with this mixture, 25 seeds were sown at a depth of 1 cm in each pot. The experiment was conducted with four replicates in a growth chamber. The photoperiod was 18 h day and 6 h darkness, and the temperatures were 23 and 18 °C, respectively. The pots were irrigated with distilled water every other day to maintain water content. The amount of water evaporated was applied at each irrigation. Once the seedling emergence, indicated by the appear-

ance of cotyledonary leaves on the surface, was complete after eight days of sowing, one plant was allowed to grow in each pot. After the 30-day growth period, the plants were harvested to measure plant height, plant fresh and dry weights, dry matter, relative chlorophyll content (Chl), relative water content (RWC), and electrolyte leakage (EL).

Leaf relative Chl was measured using a Konica Minolta SPAD-502 meter as a SPAD unit on the third leaf from the top of each plant. Leaf RWC was assessed on fully enlarged leaves of five plants per replicate. The leaf was immediately removed from each plant and weighed to determine its fresh weight (FW). It was then immersed in distilled water in a Falcon tube for 24 h to recover turgidity, and the turgor weight (TW) was measured after gently drying excess water from the leaf surface with paper towels. The dry weight (DW) was weighed after the samples were dried at 70 °C for 48 h. RWC was calculated following the formula (Batool *et al*. 2020):

$$\text{RWC}\,(\%) = \left[\frac{(FW - DW)}{(TW - DW)}\right] \times 100$$

Electrolyte leakage (EL) was determined in the second leaf from the top of the plants, as described by Gulen & Eris (2003). After leaf collection, they were washed with deionized water to remove surface contamination. Six 10 mm diameter discs were excised, weighed immediately, and placed in a glass tube filled with 30 mL of deionized water. After a 24-hour incubation period at 20 °C, the initial electrolyte leakage (ELi) of the solution was measured directly using an EC meter (WTW 3.15i, Germany). The discs were then incubated in a water bath at 90 °C for 60 min. After cooling, the final electrolyte leakage (ELf) was recorded. EL was calculated as .

Cell membrane stability (CMS) was derived from the EL calculation and was determined by using the following equation (Bajji *et al.* 2002):

$$\text{CMS} = \left[\left(1 - \frac{\text{ELi}}{\text{ELf}}\right) \times 100\right]$$

Higher electrolyte leakage indicates lower cell membrane stability.

Determination of moisture content (MC) and salinity of growing medium. After harvest, the weights of the pots were equilibrated with distilled water. The soil pore water electrical conductivity (ECp) and moisture content (MC) of the growing medium were determined using a WET-2 portable moisture meter probe and an HH2 digital reader (Delta-T Devices Ltd., Cambridge, UK).

The third leaf was carefully covered with clear nail polish from the midrib to the leaf margin and then left to dry for 1-2 minutes.

Determination of stomata characteristics. The number, size, and area of the stomata were assessed using the impression technique. The upper side (adaxial) and lower side (abaxial) of the third leaf were carefully coated with transparent nail polish from themidrib to the leaf margin and then allowed to dry for 1-2 min (Grant & Vatnick 2004). The dried varnish was carefully peeled from the lamina. The stomata number (stomatal number per mm² of leaf area) was quantified visually using a light microscope (Zeiss Axiophot microscope, 40 × 10) in conjunction with the image acquisition and digitizing program AxioVision 4.3 software, along with Canon EOS camera eyepieces. Three to five samples from each field were randomly picked from different areas of each sample, and the counting process was repeated four times. The stomata dimensions in the photographs were measured using an ocular micrometer calibrated with an object micrometer (Kaya 2021).

The stomata size was computed by considering the width and length of stomata via the following formula (Kaya & Ergin 2025):

$$\text{Stomata size}\,(\mu\text{m}^2) = \left[\left(\frac{\text{Stomata width}}{2}\right) \times \left(\frac{\text{Stomata length}}{2}\right)\right] \times \pi$$

The stomata area (the area of stomata in 1 mm² of leaf area) was calculated by considering the number and size of stomata via the following formula:

$$\text{Stomata area (mm}^2\text{)} = \text{(Stomata number)} \times \text{(Stomata size)}$$

Statistical analysis. The experiment was planned in a one-way ANOVA completely randomized design with four replications. Data were subjected to variance analysis using the MSTAT-C software (Michigan State University, v 2.10 after checking for homoscedasticity and normality test via Minitab software. Significant differences among the mean values were compared with the LSD test ($P < 0.05$). Correlation analysis was performed in R.

Results

Germination experiment. A germination experiment was conducted to determine how black mustard responds to salinity stress in terms of its germination properties and subsequent seedling growth. The results of the experiment are presented in Table 1. Increasing salinity stress did not affect germination percentage, although mean germination time was prolonged at 150 mM NaCl. The germination index increased at 50 mM NaCl, but it decreased significantly at 150 mM NaCl. The germination stress index increased with higher NaCl levels, reaching the highest level at 200 mM. Root length was severely inhibited by NaCl levels, while shoot length and seedling fresh and dry weights were unaffected up to 100 mM.

Table 1. Effects of salinity stress on germination and seedling characteristics of black mustard

	NaCl							
	Control	**50 mM**	**100 mM**	**150 mM**	**200 mM**	**% Reduction**	***F* value**	***P* value**
GP (%)	97±2.6	97±1.9	95±1.2	97±2.5	96±1.6	1.03	0.56	-
MGT (day)	1.59^{c}±0.07	1.26^{d}±0.10	1.50^{c}±0.08	1.86^{b}±0.12	2.24^{a}*±0.12	-40.8	55.6	0.0000
GI	34.9^{c}±1.3	42.7^{a}±1.4	38.0^{b}±1.7	31.2^{d}±3.0	23.7^{e}±0.7	32.1	65.6	0.0000
GSI	101.9^{c}±2.7	101.8^{c}±1.7	105.8^{b}±1.9	105.6^{bc}±3.1	109.8^{a}±3.1	-7.7	6.75	0.0026
RL (cm)	13.4^{a}±0.13	12.3^{b}±0.05	11.6^{c}±0.09	10.2^{d}±0.05	7.3^{e}±0.07	45.5	3,082	0.0000
SL (cm)	4.54^{b}±0.03	5.02^{a}±0.08	4.99^{b}±0.12	3.71^{c}±0.05	2.54^{d}±0.10	44.1	626	0.0000
SFW (mg plant^{-1})	45.5^{c}±0.8	58.6^{b}±0.9	64.1^{a}±0.9	45.2^{c}±1.0	32.3^{d}±2.5	29.0	320	0.0000
SDW (mg plant^{-1})	2.50^{b}±0.3	2.53^{b}±0.2	2.80^{ab}±0.1	3.03^{a}±0.3	2.73^{ab}±0.2	-9.2	3.65	0.0286

Data represent mean ± standard deviation (SD) of four replicates, *: Means followed by the different letter(s) in each row are significant at $P < 0.05$. GP: Germination percentage, MGT: Mean germination time, GI: Germination index, GSI: Germination stress index, RL: Root length, SL: Shoot length, SFW: Seedling fresh weight, SDW: Seedling dry weight.

Pot experiment. The growth of black mustard seedlings was investigated through a pod experiment conducted in a growth chamber. The phenotypes of black mustard plants are represented in Figure 1, and changes in morphological parameters of black mustard under salinity stress are shown in Table 2. Plant height was severely inhibited by increasing NaCl levels, with a 50.9 % reduction from 31.0 cm to 15.2 cm (Table 2). Similarly, both fresh and dry weights began to decrease at 50 mM NaCl. With each subsequent increase in NaCl concentration, there was a further decline in the fresh and dry weight of the black mustard plant. The fresh and dry weights steadily decreased under NaCl stress, reaching maximum reductions of 36.0 % and 52.1 %, respectively, at 200 mM. The dry matter of black mustard plants was lower in salt-stressed plants compared to the control plants.

Figure 1. Phenotypes of black mustard plants subjected to increasing salinity stress in the pot experiment.

Table 2. Effects of salinity stress on the morphological characteristics of black mustard

NaCl	Plant height (cm)	Fresh weight (g plant^{-1})	Dry weight (mg plant^{-1})	Dry matter (%)
Control	31.0^{a} ± 0.82	4.25^{a} ± 0.03	539^{a} ± 3.46	12.7^{a*} ± 0.08
50 mM	19.0^{b} ± 1.15	3.84^{b} ± 0.01	369^{b} ± 8.43	9.63^{c} ± 0.17
100 mM	19.7^{b} ± 0.96	3.55^{c} ± 0.03	338^{c} ± 5.51	9.55^{c} ± 0.24
150 mM	15.6^{c} ± 0.63	2.96^{d} ± 0.06	300^{d} ± 8.66	10.1^{b} ± 0.42
200 mM	15.2^{c} ± 0.50	2.72^{e} ± 0.06	258^{e} ± 2.87	9.50^{c} ± 0.14
% Reduction	50.9	36.0	52.1	25.1
F value	229	929	1.185	129
P value	0.0000	0.0000	0.0000	0.0000

Data represent mean ± standard deviation (SD) of four replicates, *: Means followed by different letter(s) are significant at $P < 0.05$.

Increased NaCl levels improved the chlorophyll content of black mustard plants, as shown in Table 3. However, it reached its maximum level (54.8 SPAD) at 150 mM NaCl; it declined at 200 mM NaCl. Although NaCl significantly impacted leaf temperature, it was not considered a meaningful indicator of salinity stress.

Salinity stress caused a decrease in the relative water content of black mustard leaves, dropping from 70.0 % at control to 61.7 % at 100 mM NaCl. There was a noticeable decrease in cell membrane stability, which decreased linearly and reached the minimum level (21.7 %) at 200 mM NaCl.

As salt concentrations in the growing medium increased, so did the moisture content and electrical conductivity (Figure 2). Each increase in NaCl levels considerably raised the MC and ECp values.

In black mustard, there were more stomata on the abaxial side of the leaf than on the adaxial side. The number of stomata decreased with salinity levels higher than 50 mM NaCl (Table 4). The lowest number of stomata was found on the adaxial side (174 stomata mm^{-2}) and the abaxial side (276 stomata mm^{-2}) of black mustard plants grown under 150 mM NaCl. The reduction in stomata number was more severe on the abaxial side (12.1 %) than on the adaxial side (19.3 %).

Stomata size was significantly larger on the adaxial side than on the abaxial side, but this variation was insufficient to evaluate salinity tolerance. The stomata area per mm² responded similarly to salinity as the stomata number. On the abaxial side, the stomata area was larger than on the adaxial side. It increased once at 50 mM NaCl and then decreased prominently at 100 mM NaCl and above. Salinity resulted in a 17.5 % reduction in the stomata area on the abaxial side and a 10.9 % reduction on the adaxial side. Images from stomata on the abaxial and adaxial sides of black mustard leaves are displayed in Figure 3.

Table 3. Effects of salinity stress on physiological characteristics of black mustard

NaCl	Chlorophyll content (SPAD)	Leaf temp. (°C)	Relative water content (%)	Cell membrane stability (%)
Control	$48.7^{d} \pm 0.1$	$21.65^{a} \pm 0.1$	$70.0^{a} \pm 0.5$	$75.1^{a*} \pm 0.5$
50 mM	$51.5^{bc} \pm 0.2$	$21.33^{ab} \pm 0.3$	$62.3^{d} \pm 0.9$	$66.6^{b} \pm 1.0$
100 mM	$53.8^{ab} \pm 2.4$	$21.35^{ab} \pm 0.2$	$61.7^{d} \pm 0.9$	$49.9^{c} \pm 1.2$
150 mM	$54.8^{a} \pm 1.4$	$20.83^{c} \pm 0.3$	$63.8^{c} \pm 0.6$	$31.4^{d} \pm 0.7$
200 mM	$50.9^{cd} \pm 2.0$	$21.25^{b} \pm 0.2$	$68.7^{b} \pm 0.5$	$21.7^{e} \pm 0.5$
% Reduction	-4.51	1.84	1.85	71.1
F value	9.87	6.24	121	3.196
P value	0.0004	0.0036	0.0000	0.0000

Data represent mean ± standard deviation (SD) of four replicates, *: Means followed by different letter(s) are significant at $P < 0.05$.

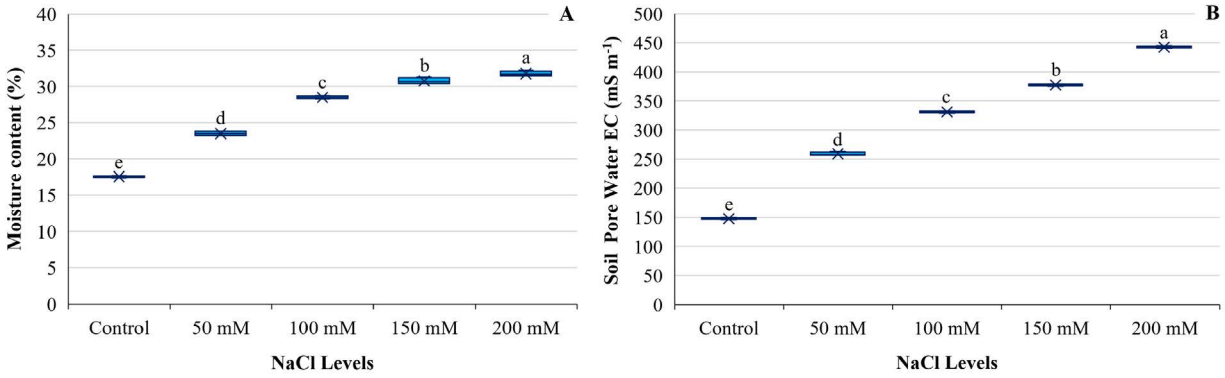

Figure 2. A) Changes in moisture content (MC) and B) soil pore water electrical conductivity (ECp) values of growing medium (peat:salinity mixture) after harvest.

Table 4. Effects of different salinity stresses on the number, size, and area of stomata in black mustard

	Stomata number (number mm^{-2})		Stomata size (µm^2)		Stomata area (mm^2 mm^{-2} leaf area)	
NaCl	**AD**	**AB**	**AD**	**AB**	**AD**	**AB**
Control	$223^{a} \pm 8.5$	$352^{b} \pm 2.5$	$304^{c} \pm 1.3$	$264^{c} \pm 2.1$	$0.678^{a} \pm 0.03$	$0.934^{b*} \pm 0.01$
50 mM	$226^{a} \pm 6.2$	$375^{a} \pm 6.9$	$313^{a} \pm 1.0$	$258^{d} \pm 1.8$	$0.709^{a} \pm 0.02$	$0.969^{a} \pm 0.02$
100 mM	$208^{b} \pm 1.0$	$294^{c} \pm 1.2$	$307^{b} \pm 0.5$	$267^{b} \pm 1.9$	$0.561^{b} \pm 0.15$	$0.787^{c} \pm 0.01$
150 mM	$174^{d} \pm 3.5$	$276^{e} \pm 3.5$	$306^{b} \pm 1.0$	$264^{c} \pm 0.8$	$0.532^{b} \pm 0.01$	$0.784^{d} \pm 0.01$
200 mM	$196^{c} \pm 8.1$	$284^{d} \pm 9.0$	$307^{b} \pm 1.3$	$270^{a} \pm 1.7$	$0.604^{ab} \pm 0.02$	$0.771^{e} \pm 0.03$
% Reduction	12.1	19.3	-0.98	-1.64	10.9	17.5
F value	48.0	269	44.9	29.7	4.90	124
P value	0.0000	0.0000	0.0000	0.0000	0.0100	0.0000

Data represent mean ± standard deviation (SD) of four replicates, *: Means followed by different letter(s) are significant at $P < 0.05$. AD: Adaxial, AB: Abaxial.

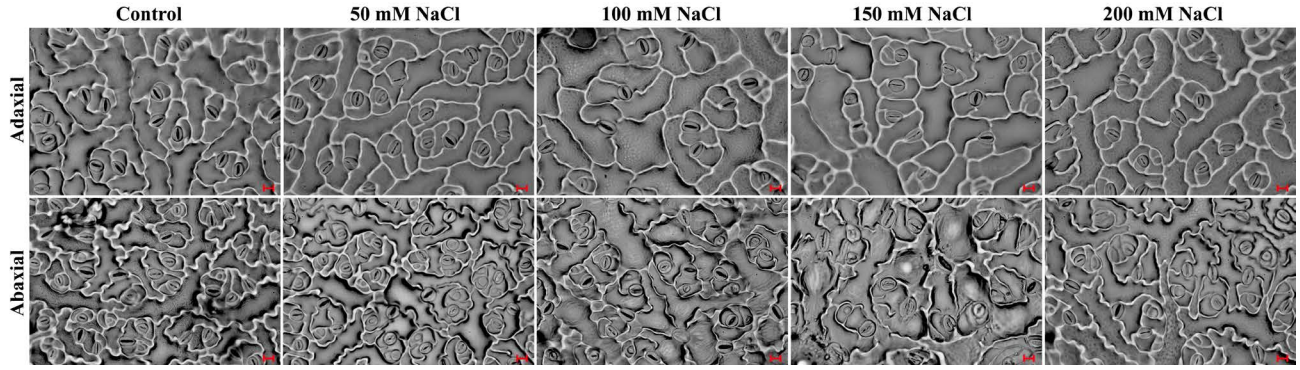

Figure 3. Images of stomata on the adaxial and abaxial sides of black mustard leaves subjected to increasing salinity stress. Scale bar (⊢) is 1 µm.

As shown in Figure 4, the strongest positive correlation was found between the germination index (GI) and shoot length (SL). A significant positive correlation (r = 0.87***) was also observed between GI and SFW. However, the highest significant negative correlation (r = -0.99***) was observed between MGT and GI.

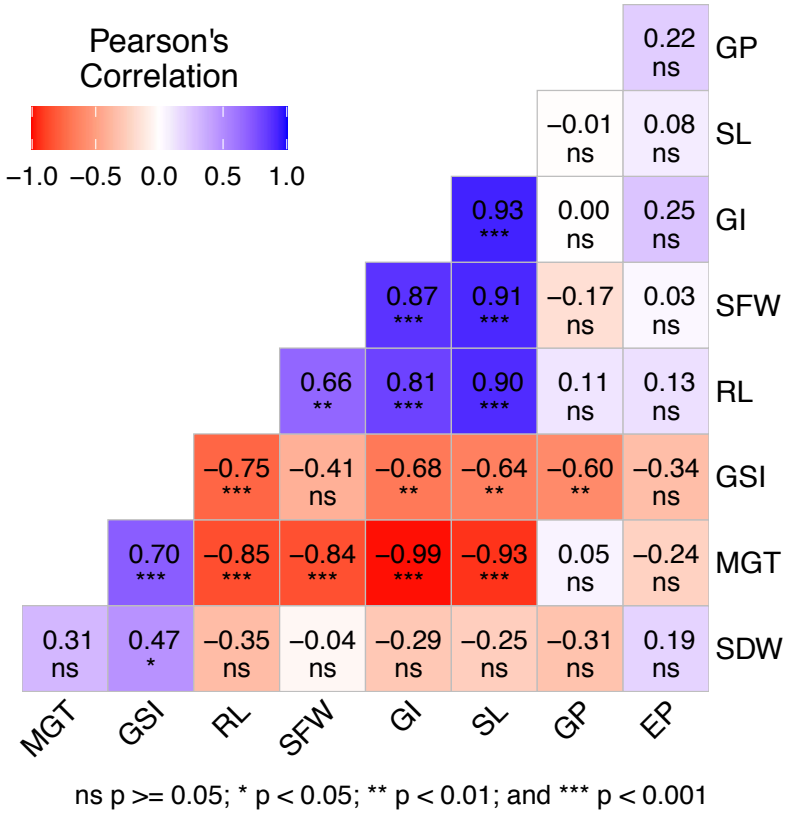

Figure 4. Correlation coefficients (r) between germination characteristics of black mustard seeds under various salinity stresses. GP: Germination percentage, MGT: Mean germination time, GI: Germination index, GSI: Germination stress index, EP: emergence percentage, SL: Shoot length, RL: Root length, SFW: Seedling fresh weight, SDW: Seedling dry weight.

There was a significant negative correlation between soil moisture content and plant height, as well as the fresh and dry weights of black mustard (Figure 5). Additionally, similar correlations were observed between soil electrical conductivity and plant growth parameters. Plant dry and fresh weights were significantly and negatively correlated with ECp and soil moisture content.

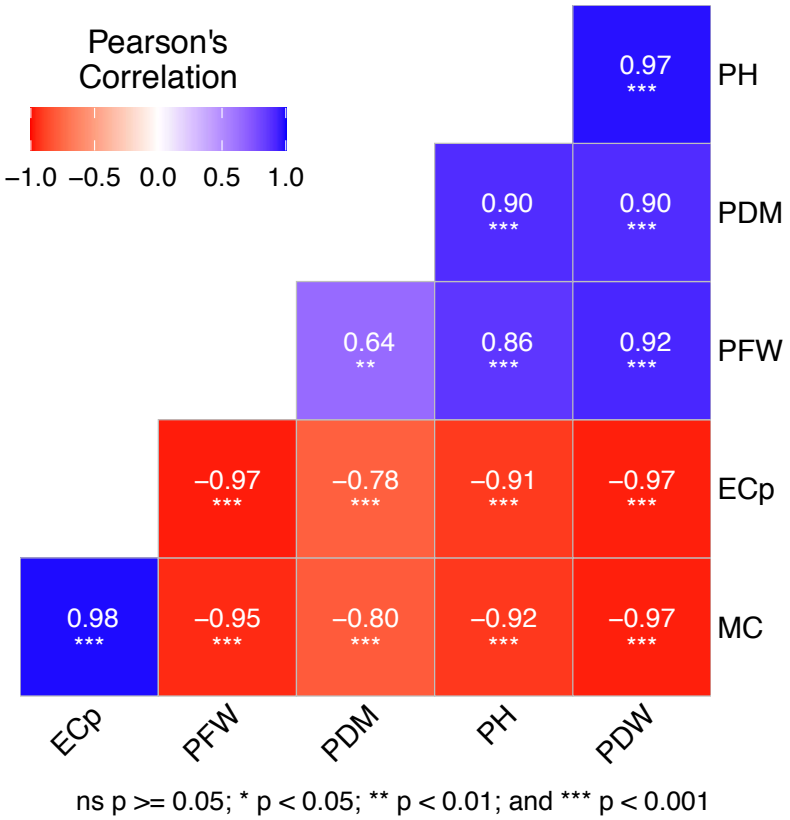

Figure 5. Correlation coefficient (*r*) between plant growth parameters and moisture content (MC) and electrical conductivity (ECp) of the growing medium. PH: Plant height, PDM: Plant dry matter, PFW: Plant fresh weight, PDW: Plant dry weight, ECp: Soil pore water electrical conductivity, MC: Moisture content.

A positive and significant correlation was found between CMS and PFW (r = 0.99***), PDW (r = 0.88***), ABSN (r = 0.88***), and PH (r = 0.82***). Furthermore, a positive and strong correlation (r = 0.83***) was identified between ABSN and PFW to explain the inhibition of black mustard plant growth caused by salinity stress (Figure 6). This correlation suggests that an increase in the number of stomata enhances the fresh weight of black mustard.

Discussion

Germination experiment. The findings indicate that black mustard exhibits partial tolerance to salinity during the germination stage, as germination percentage remained unaffected even at high NaCl concentrations. This may mainly be due to osmotic pressure created by salts in saline water, because germination time was prolonged without changes in germination rate. However, mean germination time increased, and germination index declined at higher salinity, suggesting delayed and less vigorous germination. Similar results were reported by Zia *et al*. (2023), who found a decrease in the root and shoot length of *B. nigra* under salt stress (25-100 mg L^{-1}). The results of a previous study conducted by Santo *et al*. (2017) demonstrated that *Brassica insularis* seeds could germinate at concentrations of up to 200 mM, which supports our findings. Contrarily, Sarker *et al*. (2014) found that when the dose of NaCl increased from 0 to 16 dS m^{-1}, germination and seedling growth in *B. juncea* decreased significantly. The correlation for germination traits revealed strong negative associations between salinity and germination rate, germination index, and seedling vigor, indicating severe inhibition of early development under salt stress. Similar negative correlations were previously reported in *B. napus*, where salinity reduced seedling emergence and vigor indices (Wu *et al.* 2019).

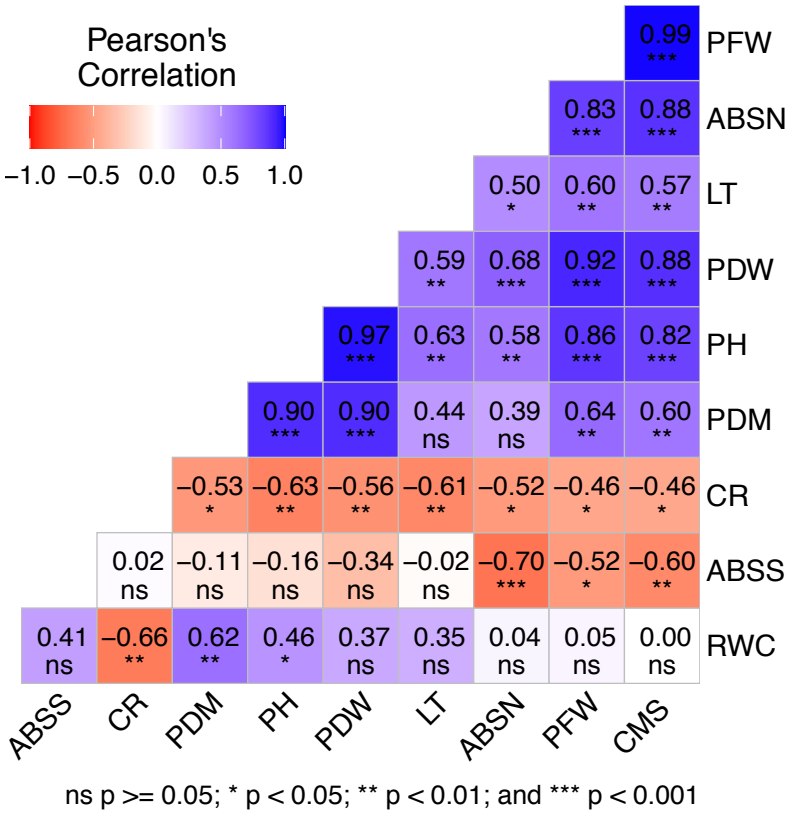

Figure 6. Correlation coefficients (*r*) between morpho-physiological characteristics of black mustard plants grown under various salinity stresses. CR: Chlorophyll rate, LT: Leaf temperature, PH: Plant height, PFW: Plant fresh weight, PDW: Plant dry weight, PDM: Plant dry matter, ABSS: Abaxial stomata size, ABSN: Abaxial stomata number, CMS: Cell membrane stability.

Pot experiment. Reductions in plant height and biomass under salinity were also consistent with previous studies reporting substantial growth inhibition in *B. napus* and *B. campestris* (Uddin *et al*. 2005) and *B. juncea* (Shirazi *et al*. 2011). Genotypic variation in salt sensitivity has been well documented (Hooks *et al*. 2019), suggesting that black mustard may possess intermediate tolerance relative to other *Brassica* species. Hooks *et al*. (2019) also suggested that the different responses of cultivars to salinity stress could be used as a decisive criterion in breeding for salt tolerance.

Chlorophyll content initially increased under moderate salinity before declining at higher levels. This biphasic response may reflect stress-induced pigment accumulation followed by degradation at severe stress levels. In contrast, Hussen *et al*. (2018) reported that increased salt concentrations (from 0 to 100 mM NaCl) led to a reduction in the total chlorophyll content in the leaves of *B. carinata*. This may be due to the methods used for measuring chlorophyll content or the leaves' position on the plant. As is known, the color of the plants darkens when they are exposed to any stress, and then chlorosis begins if the stress continues for a long time. Also, salinity stress first affects the lower leaves. In our study, SPAD readings were performed on the second leaves of the top of the plants.

Salinity also reduced relative water content and cell membrane stability, consistent with osmotic stress leading to impaired water relations and increased electrolyte leakage (Akhtar *et al*. 2017, Singh *et al*. 2019). The linear decline in cell membrane stability highlights its potential as a sensitive selection criterion for salt tolerance in black mustard.

Stomata density and area decreased with salinity, especially on the abaxial surface, in agreement with observations in *B. juncea* (Khan *et al.* 2020, Rasheed *et al.* 2022) and *B. napus* (Hezaveh *et al.* 2019). This reduction likely represents an adaptive mechanism to limit water loss through transpiration under salt stress. The strong positive correlations between stomata traits and biomass production further suggest that stomatal regulation plays a critical role in growth performance under salinity.

Salinity stress adversely affects germination and growth stages, disrupting physiological processes, reducing crop development, and lowering yield. However, salinity tolerance mainly depends on genotypic factors, such as plant species and varieties. In the present study, black mustard was exposed to different levels of salinity stress induced by NaCl during germination, emergence, and further growth stages. The results revealed that germination percentage was unaffected by NaCl; however, the germination index and stress index were negatively impacted, resulting in notable restrictions in root and shoot growth. In the pot experiment, cell membrane stability decreased linearly with increasing salinity. The number and area of stomata also declined due to increased salinity. These changes reduced the growth of black mustard. Black mustard responded to salinity by decreasing stomata number and size, which leads to water loss through transpiration. A NaCl level of 150 mM had a particularly strong inhibitory effect, disrupting the water balance in tissues and increasing electrolyte leakage. Therefore, cell membrane stability could serve as a selection criterion for salt-tolerant black mustard plants, with 150 mM NaCl representing a critical salinity stress threshold.

In conclusion, black mustard (*B. nigra*) can be considered a moderately salt-tolerant plant up to the flowering stage alongside genotypic divergence. It is an important crop for maintaining sustainable production in saline areas. Plant height and cell membrane stability should primarily be evaluated for salinity tolerance. Further studies are needed to determine the seed yield performance and oil quality parameters of *B. nigra* under salinity stress.

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