Deciphering the broccoli plants response to salinity and boron stress on with a focus on membranes and aquaporins

Micaela Carvajal[1], Juan Nicolas-Espinosa[1], Lucia Yepes[1], Fuensanta Martinez-Bernal[1], and Miriam Fernandez-Pozurama[1]

[1]Centro de Edafologia y Biologia Aplicada del Segura

March 22, 2023

Abstract

Abiotic stresses as salinity, and boron toxicity, and deficiency are commonly found in arid and semi-arid areas were broccoli is abundantly grown. In this work, the physiological response of broccoli leaves (including growth, relative water content, stomatal conductance, and mineral concentration) was studied under salinity and boron stresses (deficiency and toxicity), individually or in combination. Also, the molecular study of PIP aquaporins were studied in relation to their presence in plasma membrane PIP presence and their membrane lipid environment. The results showed that only the combination of salinity and boron deficiency decreased plant biomass, suggesting good adaptation to the other treatments. Changes in stomatal conductance and mineral nutrients suggest that the adaptation was related to water and boron transport through leaves, involving aquaporins since avoidance strategy was observed. Furthermore, changes in aquaporins PIP expression revealed that each individual aquaporin is involved in each treatment, either alone or in combination. However, the discrepancy between the presence of aquaporins in the plasma membrane and microsomal fraction pointed towards the regulation of trafficking and membrane composition (lipids and proteins) should be highly present in this plant under stress.

Introduction

The *Brassicaceae* family includes broccoli (*Brassica oleracea* L. var *italica*), kale, cauliflower, radish, and others, being a widely cultivated crop group. Of all brassicas, broccoli is especially economically significant, with 26,000 kt produced worldwide in 2020 from approximately 1.36 million hectares of harvested land (*http://data.un.org/*). Broccoli is also highly valued for its health benefits, thanks to its rich content of bioactive compounds such as glucosinolates, polyphenols, and vitamin C. Additionally, the byproducts of broccoli cultivation serve as source of different bioactive molecules, such as peptides, for cosmeceutical applications (Picchi et al., 2012; Nicolas-Espinosa et al., 2022).

Abiotic stresses, including drought, waterlogging, salinity, nutrient deficiency, and temperature, pose challenges to broccoli cultivation (Beacham *et al.* , 2017). Salinity stress is one of the major abiotic stress that affects crops globally, particularly in arid and semi-arid regions (Parida and Das, 2005; Yadav S. P. *et al.* , 2019). Approximately 20% of irrigated lands and 6% of the total global land are impacted by salinization, resulting in a reduction of crop yields by a maximum of 70% (El-Badri *et al.* , 2021). Soil salinity results from the accumulation of salts in the soil, which can occur due to a variety of factors, including evaporation, leaching, and irrigation practices. Salinity stress interferes with plant growth and productivity by disrupting water and nutrient uptake, altering osmotic balance, and damaging plant cell tissues (Ma *et al.* , 2020).

In the same way, boron (B) stress, being more relevant B toxicity, is a significant challenge for plant growth and productivity worldwide, especially in region with high soil B content. B is an essential micronutrient for plants, but high levels of B can lead to toxicity symptoms, including stunted growth, reduced crop yields, and even death of the plant (Landi *et al.* , 2019). The toxicity symptoms result from the interference of

excess B with various physiological processes, including cell division and differentiation, protein synthesis, and membrane stability. In this sense, B toxicity can disrupt the transport of water and nutrients within the plant, further exacerbating the negative effects of the stressor (Wimmer and Eichert, 2013).

The simultaneous occurrence of B stress and soil salinity is a common phenomenon in semiarid and arid regions, as high soil salinity concentrations often result in limited leaching and this the accumulation of B in the form of sodium salts. This combination of stress factors is associated with irrigations practices that use water containing high levels of B and salts. The use of desalinated water often contains varying levels of B, leading to increased levels of soil B and further exacerbation of B toxicity in crops (Hilal *et al.* , 2011). Additionally, the use of desalinated water for irrigation could also exacerbate soil salinity, as the desalination process can be limited in its ability to remove all salts, particularly those that are present in high concentrations (Darre and Toor, 2018). This can result in the remaining water still containing high levels of salts. The plasma membrane (PM) of plant cells plays a crucial role in regulation communication between the cell and the environment. As a selective barrier, the PM acts as the main receptor and transducer of external signals, and is critical in maintaining plant homeostasis, providing cellular nutrition, enabling endocytosis, and responding to biotic and abiotic stresses (Gronnier *et al.* , 2018; Morel *et al.* , 2006). The PM is particularly important in responding to salinity stress as it is the first line of defence for the cell. Lipid and transport proteins within the PM play a significant role in regulating membrane permeability and fluidity, triggering responses to salinity (Yepes-Molina *et al.* , 2020). In this scenario, aquaporins (AQPs) have a crucial role in the stress response mechanisms of the plant, acting as key components of the PM and carrying out vital functions within the plant cell. AQPs are integral membrane proteins (MIPs) that are responsible for the regulated transport of water across cell membranes. The AQPs family can be classified into different subfamilies based on their sequence homology and membrane location. Among them, the PIP subfamily is one of the most relevant for maintaining water homeostasis in plants and plays an important role in their ability to cope with environmental stress conditions (Barzana *et al.* , 2021). The PIP subfamily can be further subdivided into two groups, PIP1 and PIP2. This subfamily is primarily located in the PM and acts as water channels, especially the PIP2 group. Additionally, they allow the transport of other neutral molecules such as nitrogenous compounds (e.g., urea and NH_3), boric acid, H_2O_2, and CO_2(Nicolas-Espinosa and Carvajal, 2022). It is well known that AQPs are involved in both stresses, salinity and boron; under saline conditions, the concentration of ions in the soil solution increases which leads to an increase in osmotic potential. This causes a reduction in water uptake by the plant, leading to dehydration and ultimately, plant stress (Martínez-Ballesta *et al.* , 2006). AQPs are involved in mitigating this stress by allowing the plant to control water uptake, reducing the amount of water taken up under saline conditions (Barzana *et al.* , 2021). The presence of a diverse array of plant AQPs suggest that different isoforms may serve distinct functions in various cell types, as their regulation is influenced by specific physiological contexts. Salinity has been shown to alter the expression of AQPs, suggesting that these proteins may play a role in the physiological response that maintains homeostasis under stress. The transcripts levels of PIPshave been observed to decrease under saline stress in Arabidopsis, barley, among others (Boursiac *et al.* , 2005; Horie *et al.* , 2011; Katsuhara *et al.* , 2011). However, in some cases, such as radish seedlings, the mRNA and protein levels of PIPs and TIPs remain unchanged (Suga *et al.* , 2002). Conversely, an increase in PIP certain isoforms expression has been observed under saline stress in Arabidopsis (Jang *et al.* , 2004; Sutka *et al.* , 2011), *Brassica juncea* (Srivastava *et al.* , 2010), and *Brassica rapa*(Kayum *et al.* , 2017). Similarly, many AQPs have been described to be able to transport boric acid, playing a key role in stress conditions, such as AtNIP7;1 and AtNIP5;1 in Arabidopsis (Li*et al.* , 2011), but also AtPIP2;2 and AtPIP2;7 were permeable to boric acid (Groszmann *et al.* , 2023).

To fully comprehend the impact of multiple stress factors on plant growth and development, it is essential to evaluate the interactions between different stressors, including how the plant responds to different stress combinations, and the extent to which each stress factor affects the other. This requires a comprehensive approach that considers the molecular, physiological, and biochemical changes that occur in the plant in response to these stress combinations (Kissoudis*et al.* , 2014).

Consequently, the aim of this study was to assess the physiological (growth, relative water content, stomatal

conductance, and mineral concentration) and molecular (aquaporins) impacts of salinity and boron stresses (deficiency and toxicity) on broccoli leaves. The evaluation has been carried out individually and in combination, in order to identify molecular markers among aquaporins PIP and their membrane lipid environment that could indicate stress resistance coping with water/boron uptake and transport.

Material and methods

Plant growth and treatments application

Brassica oleracea L. var. *italica* cv. Parthenon (broccoli) seeds were germinated and cultivated as reported in Muries et al., (2011) with some modifications. Firstly, a pre-hydration with deionized water was performed for 24 h. Then, seeds were germinated in dark with vermiculite at 28°C for two days and after, the sprouts were transferred to hydroponic conditions in a controlled-environment chamber with a 16-h light and 8-h dark cycle, with air temperatures of 25 and 20 °C, day/night. The relative humidity (RH) was 60–80% and the photosynthetically active radiation (PAR) was 400 µmol m^{-2} s^{-1}, provided by a Pacific LED, WT 470C, LED8OS/840 PSD WB L1600, Philips. After 15 days of growth, treatments were applied consisting in: control conditions (Hoagland, 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 25 µM H_3BO_3, 4 µM $MnSO_4$, 4 µM $ZnSO_4$, 1 µM $CuSO_4$, 0.13 µM $(NH_4)_6Mo_7O_{24}$, and 40 µM $Fe_3{}^+$-EDDHA), salinity (80 mM NaCl), B starvation (no B was added), B excess (250 µM of B), and the combination of both salinity and B starvation and salinity and B excess. Growth medium was renewed after 1 week and plants were harvested 15 days after treatments applications. Finally, when harvested plant growth was measured by determining dry and fresh weight, and leaf area for calculating the leaf per mass area (LMA).

Analysis of Mineral Nutrients

Leaves and roots were collected, dried, and ground into a fine powder dust. Then samples were digested in a microwave oven (CEM Mars Xpress, Stallings, NC, USA), by HNO_3:$HClO_4$ (2:1) digestion. The different elements were detected by a coupled plasma (ICP) analysis (Optima 3000, PerkinElmer, Waltham, MA, USA).

Stomatal conductance

Stomatal conductance was measured every 3-4 days after treatments application. Four leaves were measured in three plants for each treatment. Measures were made with AP4 Delta-T Devices, Cambridge UK.

Microsomal fraction and plasma membrane isolation

A pool of leaves from five different plants of the same treatment was used for four microsomal fraction extractions in order to obtain enough protein and lipid amounts for further analysis. Leaves plasma membranes were purified from microsomal fractions using the two-phase aqueous polymer technique first described by Larsson *et al.* , (1987) and modified by Casado-Vela *et al.* , (2010).

Leaves (50 g) were cut before vacuum infiltrated with 0.5 g of PVP and 160 ml of extraction buffer containing 0.5 M sucrose, 10% glycerol, 20 mM Na_2EDTA, 20 mM EGTA, 50 mM NaF, 5 mM β-glycerophosphate, 1 mM 1,10-phenanthroline, 1 mM Na_3VO_4, 0.6% PVP, 5 mM ascorbic acid, 5 mM DTT, and 0.5 mg/L leupeptin in 50 mM Tris-MES, pH 8.0. After buffer infiltration, tissues were homogenized using a blender and filtered through a nylon mesh (pore diameter of 100 µm). The homogenate was centrifuged at 10,000 *x g* for 40 min at 4°C. The supernatant was collected and centrifuged at 100,000 *x g* for 45 min at 4°C. The pellet was suspended in buffer (FAB) containing 330 mM sucrose, 2 mM DTT, 10 mM NaF, and 5 mM phosphate buffer (pH 7.8). Plasma membranes were purified from microsomal fraction by partitioning in a two phase-system mixture with a final concentration of PEG-3350 (Sigma)/Dextran-T500 (GE Healthcare), 6.0% (w/w), in the presence of 330 mM sucrose, 5 mM KCl, 5 mM potassium phosphate (pH 7.8). The two-phase system was centrifuged for 5 min at 4000 *x g* at 4°C. The upper phase, enriched plasma membranes, was recollected and washed in 300 mM sucrose, 9 mM KCl, 6 mM Na_2EDTA, 6 mM EGTA, 60 mM NaF, and Tris-borate, pH 8.3, and centrifuged at 100,000 *x* g for 45 min, 4°C. The resulting pellet was suspended in resuspension buffer

(FAB). The protein concentration of both, microsomal fraction and plasma membrane-enriched fractions, was determined with Protein assay dye reagent (BioRad), with BSA as standard.

RNA extraction and expression studies, real time qPCR

Primer design

For primer design, general primers for various aquaporin isoforms was followed since the aquaporin family is very extended in broccoli plants, counting with 65 members (Nicolas-Espinosa and Carvajal, 2022), primer design was focused in evaluate the expression of the different subfamilies within PIP1 and PIP2. The primer design was carried out using Geneious prime 2020.1.2 software (*https://www.geneious.com/*), focused in conserved sites of the consensus sequences of PIP1-1, PIP1-2, PIP1-4, PIP1-5, PIP2-1, PIP2-2/3, PIP2-4, PIP2-5, and PIP2-7.

Table 1 . Primer set for aquaporin expression analyses.

Gene name	Forward (3'-5')	Forward (3'-5')	Reverse (3'-5')	Reverse (3
BoiPIP1-1a	BoiPIP1-1a	ACAGCTTCCGGGCTAAGAAC	ACAGCTTCCGGGCTAAGAAC	TTTCGAG
BoiPIP1-2	BoiPIP1-2	GCTCGTGACTCTCATGTTCC	GCTCGTGACTCTCATGTTCC	AGCGTTG
BoiPIP1-4b	BoiPIP1-4b	CCACAACATTCGCTCCTCCA	CCACAACATTCGCTCCTCCA	ATGGGTT
BoiPIP1-5	BoiPIP1-5	TAAGGATCATGCTTGGGGCG	TAAGGATCATGCTTGGGGCG	GGTACAG
BoiPIP2-1	BoiPIP2-1	AAGCTGTTTCCGGAGAAGGG	AAGCTGTTTCCGGAGAAGGG	CAGCTCC
BoiPIP2-2/3	BoiPIP2-2/3	GACGTTCGGCTTGTTCTTGG	GACGTTCGGCTTGTTCTTGG	CCAACTC
BoiPIP2-4	BoiPIP2-4	CAACCAAGAAAAGGCCTGGG	CAACCAAGAAAAGGCCTGGG	CCAGCCG
BoiPIP2-5	BoiPIP2-5	AGCTTGGGACCATCATTGGA	AGCTTGGGACCATCATTGGA	ACCAGCC
BoiPIP2-6	BoiPIP2-6	GCGGTGGATCTTGGTAGTCC	GCGGTGGATCTTGGTAGTCC	CTCAGCT
BoiPIP2-7	BoiPIP2-7	TGGATTCGCTGTGTTCATGG	TGGATTCGCTGTGTTCATGG	ACAGCAG
Boi18-S-RNA *	Boi18-S-RNA *	CCTGCGGCTTAATTTGACTC	CCTGCGGCTTAATTTGACTC	AGACAAA

*(Muries *et al.* , 2011)

Real time qPCR

To assess and compare the expression of aquaporins across various cultivars and treatments, RT-qPCR was conducted using 2 µL of 1:10 diluted cDNA samples in an 8 µL reaction mixture containing 600 nM gene-group primers (**Table 1**) and 5 µL of SYBR Green Master Mix 2X (Applied Biosystems) in an Applied Biosystems 7500 Real-Time PCR system. Amplification was performed in a two-step process, consisting of denaturation at 95°C for 10 min, followed by 40 cycles of 15 s of denaturation at 95°C and 1 min of annealing and extension at 60°C. A dissociation stage was then carried out. Data collection was performed at the end of each cycle's step 2. These conditions were used for both target and reference genes. The reactions were conducted in triplicate for each sample (technical replicates) on a 96-well plate, and four independent samples were tested for each treatment (biological replicates). Transcript levels were calculated using the 2–ΔΔCt method (Livak and Schmittgen, 2001) for both target and reference genes.

Western Blot analyses (Gel electrophoresis and immunoblotting)

Protein (5 µg per lane in plasma membrane fractions and 15 µg for microsomal fractions) was loaded for 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Yepes-Molina*et al.* , 2020). The antibodies used were against *Arabidopsis thaliana* PIP1 group, PIP2 (PIP2-1, PIP2-2, and PIP2-3) and PIP2-7 (Agrisera, Vännäs, Sweden). After checking predicted reactivity against broccoli proteins, the fact that PIP1 antibody had a reactivity against BoiPIP1-1a, BoiPIP1-2a, BoiPIP1-2b, BoiPIP1-2c, BoiPIP1-3a, BoiPIP1-4a, BoiPIP1-4b, and BoiPIP1-5a were determined, whereas PIP2 antibody had a predicted reactivity against (BoiPIP2-1a, BoiPIP2-1b, BoiPIP2-2a, BoiPIP2-3a, BoiPIP2-3b, BoiPIP2-4a, BoiPIP2-4b, BoiPIP2-4c, BoiPIP2-5a, PIP2-5b), while PIP2-7 was specific against BoiPIP2-7 isoforms. Goat anti-

rabbit IgG coupled to horseradish peroxidase was used as the secondary antibody. A chemiluminescent signal was developed using SuperSignal West Pico PLUS substrate (Thermoscientific., Rockford, IL, USA). The intensity of each band was determined by ImageJ software (Schneider *et al.* , 2012).

Lipid analysis

Sterol and fatty acids were determined as described by Chalbi *et al.* , (2015). A mixture of chloroform-methanol (1:2, 0.75 mL) was added in an Eppendorf tube to both, microsomal and plasma membrane fractions (0.5 mL), along with β-cholestanol (20 µL, 0.1 mg mL^{-1}) used as internal standard for sterol analysis. Chloroform ($CHCl_3$; 0.25 mL) was added and the mixture was shaken and centrifuged at 10,000 x g for 6 min. The $CHCl_3$ layer was retained, evaporated to dryness under N_2 and made up to 100 µL with $CHCl_3$. For sterol analysis, 20 µL of the $CHCl_3$ extract was placed in a glass vial (2 mL), evaporated to dryness under $N_2$and acetylated using pyridine (50 µL) and Ac_2O (100 µL). After 2h, the solvents were evaporated under N_2, ethyl acetate was added and the sterols analyzed by GC using an HP5-bonded capillary column (30 m x 0.25 mm x 0.25 µm) coupled to a flame ionization detector (FID), with H_2 as carrier (1mL min^{-1}) and a temperature programmed of 120-260°C at 5°C min^{-1}, then 260-280°C at 2°C min^{-1}, and finally 280-300°C at 6°C min^{-1}. The injector and detector temperatures were 150 and 320°C, respectively. Bound fatty acids were determined by using 20-II portions of the $CHCl_3$ extract; evaporating them to dryness under N_2, transmethylating with sodium methoxide (0.5 N) in methanol (0.5 mL) and heating at 30°C for 7 min. The resultant fatty acids methyl esters were extracted with hexane (1 mL), evaporated under N_2, dissolved in ethyl acetate (20 µL) and analyzed by GC using an HP5-bonded capillary column (30 m x 0.25 mm x 0.25 µm), with FID, He as carrier (1 mL min-1) and a temperature programmed of 150-195°C at 3°C min^{-1}, then 195-220°C at 2°C min^{-1}, and finally 220-300°C at 6°C min^{-1}. The injector and detector temperatures were 280 and 300°C, respectively.

Microscopy, TEM and particle size

Microsomal and plasma membrane enriched fractions from broccoli leaves were pelleted at 100,000 x g. For chemical fixation, pelleted vesicles were sequentially fixed with glutaraldehyde (2.5% in 100 mM phosphate buffer, 2 h at 4°C), osmium tetroxide (1% buffered, 2 h at 4°C), and tannic acid (1% in deonized water, 30 min at 22°C). The pellets were then thoroughly rinsed with water and covered with 2% low melting point agarose, then dehydrated with ethanol and epoxypropane at 22°C and embedded in Epon. Blocks were sectioned on a Leica EM UC6 ultramicrotome, collected on Formvar-coated copper grids and stained with uranyl acetate followed by lead citrate. Sections were examined using a JEOL 1011 transmission electron microscope with digital camera GATAN ORIUS SC200. For each treatment, an average of 5-10 ultrathin sections were examined.

Vesicle size

The average size of the two fractions of vesicles was analyzed using light-scattering technology; through intensity measurements with a Malvern ZetaSizer Nano XL machine (Malvern Instruments Ltd., Orsay, France), as previously described by Barrajón-Catalán *et al.* , (2010). This allowed the measurement of the particles with a size range from 1 nm to 3 µm.

Data analysis

The different statistical analyses were performed using SPSS software (v.26; Chicago, IL). Statistical differences between the experimental groups were determined via *post hoc* analyses, Duncan test was used for parametric data and non-parametric data were analyzed via Kruskal-Wallis analysis of variance test. In all the analyses, $p< 0.05$ was selected to represent statistically significant differences.

Results

Physiological analysis

After measuring the dry weight of the upper part and roots of plants that were treated for 15 days (**Figure 1A**), a decrease in the upper part dry weight (DW) was observed in salinity plus B-deprivation (Comb

(-)) treatment compared to control plants. The salinity (NaCl) and Comb (-) treatments were lower and statistically different upper part values from plants treated with B excess (B (+)), which had the highest upper part total dry mass. Additionally, NaCl and Comb (-) treated plants presented lower root DW than the control plants. The analysis of relative water content showed no differences of any of the treatments with the control plants. However, while B (-) and B (+) showed no significant differences, when they were combined with salinity, Comb (-) were significantly lower than Comb (+). The Leaf per Mass Area (LMA) data (**Figure 1B**) showed that only the salinity leaves were significantly different from the control plants, providing higher values.

We analyzed stomatal conductance (**Figure 2**) every 3-4 days from the beginning to the end of the experiment. The control and B (-) plants showed the highest stomatal conductance that were maintained during all the time of the experiment. Plants treated with B (+) maintained similar values than control until the 3^{rd} day of treatment showing from that day a significant decrease that remain stable from 10^{th} to 13^{th} day of treatment. Salinity (NaCl), Comb (-), and Comb (+) treatments had the lowest stomatal conductance that decreased at first day of treatment, about half that of the control plants, being the lowest those plants treated with NaCl. During the experiment, the stomatal conductance of B (+) treated plants reached similar stomatal conductance to the salinity-related treatments at 10^{th} day of treatment.

The mineral analysis of leaves (**Figure 3A**) showed a clear division between salinity-related treatments and non-salinity treatments along the horizontal axis and between B (-) and B (+) treatments (including Comb (-) and Comb (+) respectively) along the vertical axis, as summarized by a Principal Component Analysis (PCA). The interactions between Na and K, as well as between B and Mn, were negative. However, Cu and Zn demonstrated a positive interaction with NaCl and Comb (-) treatments, but a negative interaction with treatments related to excess B (B (+) and Comb (+)). **Figure 3B-D** illustrates individual plots of B, Na, and K concentrations as mass per DW represented as box plots. The control plants had around 50 mg mg kg^{-1} DW of B, while NaCl, B (-), and Comb (-) treatments had lower concentrations, being Comb (-) leaves the one that presented the lowest concentration of B among all treated plants with around 10 mg kg^{-1} DW. Alternatively, B (+) treated plants had the highest amount of B at 109 mg kg^{-1} DW, doubling the control B concentration, followed by Comb (+) at 87 mg kg^{-1} DW. The three salinity-related treatments (NaCl and the two combinations) had 3.0-3.5 g of Na per 100 g DW, while the rest of the plants had marginal concentrations. In contrast, K levels were lower these salinity treatments, along with B (-), which also had a reduction in K presence compared to control plant leaves, but it was still higher than the salinity treatments.

Aquaporins and lipids molecular analysis

The expression analysis of integral membrane proteins such as AQPs (**Figure 4**) showed that B deficiency had the greatest effect on the expression of these proteins in broccoli leaves. The B (-) treated plants leaf presented generally lower levels of mRNA transcripts compared to control leaves, particularly in PIP2 subfamily isoforms, PIP2-1, PIP2-2/3, and PIP2-7. Similarly, B (+) leaves also resulted in lower expression levels of PIP2-1. Out of the other treatments, only salinity had an impact on the expression of PIP1-2, which showed an almost threefold increase in mRNA transcript levels compared to control.

The vesicles obtained from microsomal fraction and plasma membrane isolation were analyzed to characterize their size and shape (see**Figure 5**), as well as their polydispersity index (PDI). Overall, no differences were observed among the different samples with respect to the treatments applied. However, differences were found between the types of samples. Specifically, the plasma membrane vesicles were more size-stable with a PDI ranging from 0.06 to 0.18 and a mean size around 240-280 nm, while the microsomal fraction vesicles were larger (830 nm) and more polydisperse (PDI of 0.7-0.9).

The immunoblotting analysis (**Figure 6**) displayed the quantification of PIP1, PIP2 subfamilies, and PIP2-7 isoforms in different samples of microsomal fraction and plasma membrane from broccoli leaves of various treatments. In the case of microsomal fractions, changes were only observed in the membrane extractions of the Comb (+) treated plant leaves for PIP1 abundance (**Figure 6A**). However, the levels of PIP1 in plasma membrane increased in the NaCl and B (-) treatments compared to the control, but not in the combination

of these two, Comb (-). There was a general increase in PIP2 subfamily abundance compared to control, but only the Comb (-) treatment had the same levels of PIP2 as the control leaves. The levels of PIP2-7 decreased in the plasma membranes of the two combinations, Comb (-) and Comb (+), while they remained stable in other treatments compared to the control leaves (**Figure 6D-F**).

Finally, during lipids analysis, no changes were observed in fatty acid composition (data not shown), but changes were found in sterol content (**Table 2**). For instance, lower levels of campesterol were observed in all treatments except B (+) when compared to the control in the microsomal fraction samples. Also, in the same fraction, lower levels of stigmasterol and sitosterol were found in salinity and B (-) treated samples. When analyzed the total sterol content of MF, only the combination treatment presented differences, with lower concentration in total sterol content. In plasma membrane sterols, changes were mainly seen in campesterol abundance, with lower concentrations in B (-), Comb (-), and Comb (+) when compared to control leaves. All treatments, except salinity, showed a reduction in sitosterol content, although no changes were observed in stigmasterol. Overall, the total presence of sterols was reduced in all treatment but salinity when compared to control. However, no changes were observed in the stigmasterol/sitosterol ratio in either the plasma membrane or microsomal fraction sample leaves among all treatments and control.

Figure 1 . (**A**) Graphical representation of dry weight (g) root and upper part with the scale on the left and relative water content of the plants with the scale on the right represented with diamonds. Each measure is represented as the mean ± SE (n =6). (**B**) Leaf per mass area (LMA) (g m^{-2}) of treated plants after 15 days of treatment application. Data are represented as box plot. Different letters show statistical differences, the data corresponding both dry weight measures resulted in non-parametric data and *Kluscal wallis post hoc* was selected, on the case of relative water content and LMA statistical differences were evaluated with one-way ANOVA using Duncan test as *post hoc* . Both analyses were conducted with $p < 0.05$.

Figure 2. Stomatal conductance (mmol $m^{-2}s^{-1}$) of leaves from each treatment measured each 3-4 days starting the day of treatment application. Each measure is represented as means ± SE (n = 4). Different letters in lower case represent the statistical differences between treatments each separate day, statistical differences were calculated with one-way ANOVA using as*post hoc* Duncan test when the data were parametric and non-parametric data was evaluated via *Kluscal wallis* test as*post hoc* . Legend of the right represent statistical differences in capital letters of each treatment using repeated measures ANOVA. All analyses were conducted with p <0.05.

Figure 3. Analysis of mineral nutrients of broccoli leaves, (**A**) PCA analysis of micro and macronutrients, (**B**) concentration of B, (**C**) Na, (**D**) and K in in treated plants leaves. Data is represented as boxes (25-75%), error bars represent range within 1.5 quartile, and median line (n = 4). Different letters mean statistical differences of one-way ANOVA with Duncan test as *post hoc* , $p < 0.05$.

Figure 4. Gene expression of different aquaporins in broccoli leaves expressed as fold change (F. C) respect the control of isoforms of PIP1 group (**A-D**) and PIP2 group (**E-I**). Data is represented as means ± SE (n = 4). Different letters show statistical differences one-way ANOVA and Duncan test as *post hoc* . (**E**) PIP2-1 and (**F**) PIP2-2/3 statistical analyses for non-parametric data, Kruskal–Wallis. $p < 0.05$ in all cases.

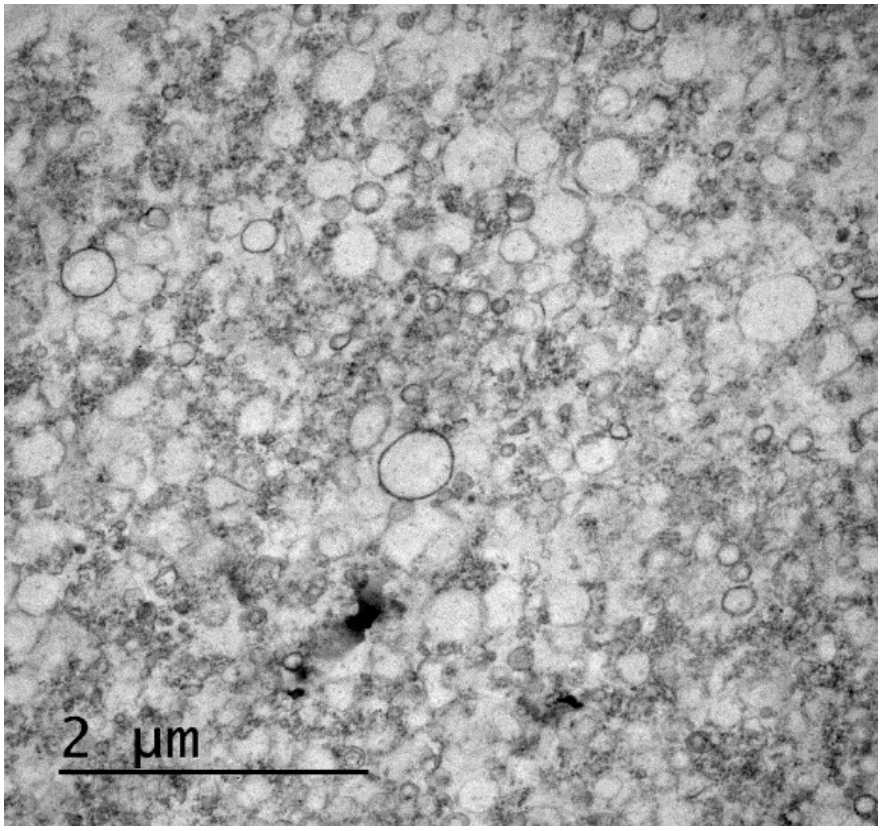

Figure 5. Transmission electronic microscopy images of membrane isolation from broccoli leaves. (**A**) microsomal fraction (MF) with scale bar of 2 µm, (**B**) MF, scale bar of 1 µm, (**C**) plasma membrane (PM) with scale bar of 1 µm, and (**D**) PM, scale bar of 500 nm, isolated from leaves of broccoli plants.

Figure 6 . Broccoli leaves aquaporin protein relative quantification via western-blot analysis of microsomal fraction (MF) (**A-C**), and plasma membrane (PM), **(D-F**). PIP1 (**A** and **D**), PIP2 (**B** and **E**), and PIP2-7 (**C** and **F**) were analyzed in all treated plants representing control as 100%. Data is represented as means ± SE (n = 4). Different letters show statistical differences one-way ANOVA and Duncan test as *post hoc (*$p < 0.05$*)* .

Table 2 . Broccoli leaves sterol content of microsomal fraction (MF) and plasma membrane (PM) (in 125 µg of protein) from broccoli leaves under different stress treatments. Data is represented as means ± SE (n = 4). Different letters show statistical differences $p < 0.05$ with Duncan test as *post hoc* .

		ἀμπεστερολ (µγ)	**Στιγμαστερολ (µγ)**	**Σιτοστερολ (µγ)**	**Ratio Stigma/ Sitosterol**
	C	2.7 ± 0.39 c	2.65 ± 0.37 c	7.93 ± 2.02 c	0.383 ± 0.08 ab
	NaCl	1.78 ± 0.13 ab	1.79 ± 0.28 ab	6.25 ± 0.45 bc	0.285 ± 0.043 a
MF	**B (-)**	1.81 ± 0.17 ab	1.92 ± 0.55 ab	5.85 ± 0.73 abc	0.329 ± 0.083 a
	B (+)	2.41 ± 0.3 bc	2.69 ± 0.48 c	5.38 ± 0.96 abc	0.502 ± 0.008 b
	Comb (-)	1.89 ± 0.22 ab	0.93 ± 0.1 a	3.85 ± 0.41 ab	0.242 ± 0.011 a
	Comb (+)	1.52 ± 0.09 a	0.83 ± 0.06 a	2.82 ± 0.18 a	0.301 ± 0.038 a
	C	7.54 ± 0.35 c	4.87 ± 1.17 a	20.39 ± 2.21 b	0.247 ± 0.076 ab
	NaCl	7.85 ± 0.24 c	4.02 ± 0.84 a	24.04 ± 3.42 b	0.184 ± 0.06 a
PM	**B (-)**	3.78 ± 0.45 a	4.41 ± 1.33 a	9.46 ± 2.28 a	0.437 ± 0.088 b
	B (+)	6.37 ± 1.16 bc	3.57 ± 0.69 a	12.66 ± 0.64 a	0.288 ± 0.061 ab

Comb (-)	5.27 ± 0.44 ab	1.37 ± 0.12 a	12.84 ± 1.05 a	0.111 ± 0.019 a
Comb (+)	4.38 ± 0.16 a	1.23 ± 0.17 a	8.48 ± 0.33 a	0.146 ± 0.022 a

Discussion

Salinity and boron levels have significant impact in crop yield, leading to high economic losses, particularly in semi-arid regions where low quality water is used as the main source of irrigation (Díaz *et al.* , 2011). Our results showed that the only treatment that affected plant (root and shoot) dry weight (DW) (**Figure 1**) was the combination of salinity and boron deficiency (Comb (-)). These results contradicts previous findings that stated that salinity is the stress that produced main impact in plant growth, provoking crop growth inhibition lowering the productivity (Munns and Tester, 2008). In this way, the low impact of the salinity and B stresses, separately, on our broccoli plant growth could be due to the short duration of the experiment (15 days of stress application in 15-day-old plants), since the salt concentration applied (80 mM) has been reported to significantly reduced shoot and root growth (López-Berenguer *et al.* , 2008). The LMA, which represents the relationship between leaf structure and function, is an important factor in determining photosynthetic capacity of the plant and water use efficiency. It is well understood how other stressors such as light and CO_2 impact in LMA, but there is less knowledge about how LMA responds to stresses like salinity (Poorter *et al.* , 2009). Previous studies has shown that plants tend to increase their LMA under salinity conditions, as the increase in LMA was associated with the production of thicker and stronger cell walls that provide structural support and help preserve water (Westoby *et al.* , 2022; Poorter*et al.* , 2009). Our results indicate that only salinity treatment increased LMA (**Figure 1B**), leading to a reduction in water use efficiency by the increased rigidity of the cell walls and elasticity of the plasma membranes.

Stomatal conductance showed reductions with all salinity treatments (NaCl and the two combinations) throughout the experiment duration (**Figure 2**)**.** It is well established that salinity affects water transport and transpiration, and the reduction in stomatal conductance serves as an indicator of stress in plants (Gao *et al.* , 2002). Despite the overall lack of change in plant DW, the reduction in stomatal conductance suggests a decrease in transpiration for all treatments except for B (-). In the case of salinity treatments, both salinity itself and in combination with boron deficiency and excess, decreased the stomatal conductance dramatically from the first day of treatment and remained lower throughout the experiment. The lower stomatal conductance in response to salinity could be the strategy of the broccoli plants to reduce sodium uptake, since its presence generates toxicity (Kronzucker *et al.* , 2013). For B (+), stomatal conductance decreased at the middle of treatment application (after 7 d), reaching the same levels of the salinity-related treatments. This could be attributed to the fact that plants reached toxic levels of boron in their tissues and tried to minimize boron uptake by reducing stomatal conductance. Similar strategies have been reported in *Vitis vinifera* were its stomatal conductance changed after 14 days of NaCl treatments applications (Gunes *et al.* , 2006), in the same way it had been reported that B toxicity reduced stomatal conductance in Arabidopsis plants (Macho-Rivero *et al.* , 2017).

As shown in **Figure 3A** , ion profiles clearly divided the salinity and non-salinity treatments with B (-) and B (+) stresses. High levels of B were found in B (+) and Comb (+) treated plant leaves, while salinity also affected B uptake, lowering levels of B as seen in B (-), with a negative correlation in both combinations. As it can be seen in Comb (+) the plants presented lower presence of B in leaves when compared with B (+), and also, in Comb (-) being the plants with the lowest B concentration. This negative relation between B excess and salinity in B uptake could be attributed to the lower stomatal conductance presented when salinity is applied, trying to reduce the uptake of B and thus increasing its deficiency in Comb (-) plants. The observed decrease in B accumulation in broccoli leaves under salinity stress may be attributed also to lower stomatal conductance and thus transpiration (**Figure 2**), as B is commonly transported through the xylem (Yermiyahu *et al.* , 2008). This hypothesis has been previously tested in other plants, including wheat (Holloway and Alston, 1992), melon (Edelstein *et al.* , 2005), and tomatoes (Ben-Gal and Shani, 2002), where exposure to combined B-salinity resulted in altered B levels in leaves. In this sense, in cases where the concentration of salt is below the tolerance threshold of each crop, the combined stresses of B and salinity

may alleviate B toxicity.

Moreover, only salinity treated plants were found to have high levels of Na when analyzed. As Na^+ movement within the plant is closely related to K^+, the salinity treatment also impacted K^+ levels, reducing them compared to control plants. Interestingly, B deficiency also reduced K^+levels in the leaves of B-deficient plants compared to the control. Although the decrease in K^+ was not as pronounced as with salinity, the reduction was statistically different from the control and B (+). It appears that high presence of B did not affect K^+, but its deficiency somehow lowered K^+ levels. However, it had been shown that B supply does not influence the K^+ content to a major extent, as it is not involved in the K^+ uptake pathway (Wu and Wei-Hua, 2013).

It is well known that Ca^{2+} ion concentrations in plants play a major role in the effect of salinity on B accumulation, as Ca helps maintain the integrity of cell membranes (Cramer, 2006). When analyzing the Ca concentration in the control and the treated plant leaves, a reduction in Ca was observed in the two combined treatments, with the Comb (+) treatment having the lowest Ca presence. On the other hand, salinity treatment by itself did not reduce Ca levels in plant leaves, but B starvation led to an increase of Ca levels compared to control plant leaves (**Figure 3A**). It is reported that salinity stress causes Ca deficiency in plants by disrupting its distribution and reducing its uptake by the plant through disturbance of the K^+/Na^+ balance at membranes (Mohamed*et al.* , 2016), thus inhibiting Ca movement from the root to the xylem and its translocation to upper parts of the plant, like leaves (Läuchli and Grattan, 2007). Although high levels of B are known to positively affect Ca^{2+} transport (Bastías *et al.* , 2010; Läuchli and Grattan, 2007) in this case the opposite occurred, exacerbating the reduction in Ca concentration with B excess and acting as an enhancer of Ca uptake with B starvation. Na^+ competition and high salinity scenarios has been also described to inhibit Mg^{2+} (Syvertsen and Garcia-Sanchez, 2014), but in our case, differences were observed with lower levels in Comb (+), but no differences appeared in the other salinity treatments.

The decrease in stomatal conductance caused by salinity stress and B toxicity reduces the uptake and transport of B in plants, which turn mitigates its potential toxicity. Salinity stress has been reported to affect water relations and reduce transpiration which can limit the transport of B in plants (Yermiyahu *et al.* , 2008). Similarly, boron toxicity can also reduce stomatal conductance and limit the uptake of boron in plants (Barzana *et al.* , 2021). When both stresses are present, their combined effect can further decrease stomatal conductance and water uptake, leading to an even greater reduction in boron uptake and transport. In this scenario, the role of plasma membrane transporters is crucial in determining how plants cope with combined stresses.

The study of aquaporins, which have been shown to transport mainly water but also B, in addition to other neutral solutes, is important in this context of abiotic stresses as salinity (Tyerman *et al.* , 2002). Based on **Figure 4** , it was observed that only B (-) treatment had a significant impact on the PIP2 group, resulting in decreased levels of *PIP2-1* , *PIP2-2/3* , and *PIP2-7* transcripts. B (+) treatment also led to a lower presence of *PIP2-1*transcripts in plant leaves. However, when the presence of aquaporins in plasma membrane were analysed, an increase of expression of PIP2 group was found in both B (-) and B (+) treatments (**Figure 5A and B**). This discrepancy in expression and protein presence could be attributed to the response of plants to B starvation, which modifies AQPs expression to prevent passive transport of B. Thus, the decrease in the expression of several AQPs during B starvation can be interpreted as a strategy to prevent passive transport of B, as the other concentration of B should be lower than the intracellular concentration. Since AQPs act as passive channels, driven by the concentration gradient (Martinez-Ballesta and Carvajal, 2014), this decrease in gene expression helps prevent B leakage through these channels. Furthermore, boric acid tends to easily pass though cellular membranes. In cases of B deficiency, B transporters, formed principally by BOR family, activate to transport boric acid against concentration gradient (Princi *et al.* , 2016), while AQPs inactivate to prevent B leakage though these channels. Additionally, the salinity treatment up-regulated the expression of *PIP1-2* compared to the control after 15 days of the experiment started. Similar results were reported in pomegranate leaves subjected to salinity stress, where *PIP1-4* , *PIP2-3* ,*PIP2-4* , and *PIP2-2* were over-expressed after 3 and 6 days of the treatment application (Kumawat *et al.* , 2021). In contrast to the *Brassica*

rapa PIP genes, which were first up-regulated during salt stress and them down-regulated (Kayum *et al.* , 2017), the lower expression of certain AQPs contrasts with the findings on the plasma membrane presence of these proteins, where a higher signal was observed in the PIP1 group of NaCl and B (-) treated plants. The differences between gene and protein results in broccoli plants under salinity leaded to conclude that mRNA synthesis could be inhibited by the accumulation of the corresponding encoded protein (Muries *et al.* , 2011). However, the regulation at the level of trafficking must be reconsidered and deeply studied.

In addition, the PIP2 group was also found to be present in plasma membrane at higher levels than in the control in almost all treated plants, except for Comb (-). Previous studies have shown that overexpression of PIP AQPs could improve tolerance to salinity in transgenic tobacco (Chen *et al.* , 2022). The increased levels of AQPs may be associated with adaptation to water stress. Studies have also demonstrated that overexpression of PIPs can increase *HKT1*and *SOS1* , transporters that contribute to Na^+efflux and K^+ absorption, respectively, (Horie*et al.* , 2009), to improve tolerance to salt stress and maintain cell ion homeostasis in salinity-stressed transgenic plants (Chen*et al.* , 2022). Overall, overexpression of AQPs has been observed to result in better cell membrane integrity under salt stress. Alternatively, the only treatments that showed a lower presence of AQPs in plasma membrane were the two combinations, specifically in PIP2-7 (**Figure 6F**). Even though, no changes in *PIP2-7*expression were found for the two combinations, this reduction was only observed in the plasma membrane. It has been shown that a cargo receptor, Tryptophan-rich Sensory Protein (TPSO), that is a heme-binding protein induced by abiotic stress (Vanhee *et al.* , 2011), interacts with the intracellular part of PIP2-7, triggering its degradation though the autophagic pathway, downregulating it in the cell, and modulating the osmotic water permeability (Hachez *et al.* , 2014). This recruitment of PIP2-7 into the phagosomes could explain the lower levels of PIP2-7 in the two combined treatments in the plasma membrane while maintaining the protein levels in all membranes together (**Figure 6C**).

The efficiency of B absorption via passive diffusion may depend on the sensitivity of the plant to salt stress, which is influenced by the functionality of AQPs (Bastías *et al.* , 2004). Furthermore, not only AQPs could influence the transport capabilities of the cell, but plants also have the ability to remodel membrane lipids, in addition to protein composition, in plasma membrane to adapt to abiotic stress scenarios (Rawat *et al.* , 2021). In salinity, an increase in sterol content is expected, but in this case, only a reduction in total sterol concentration was observed in microsomal fraction and plasma membrane, particularly under salinity plus B toxicity stress. More changes were observed in the plasma membrane fraction, with a general decrease in sterol content, possibly due to a relocation of sterols to lower compartments of the plant cell. Under saline conditions, the permeability of the plasma membrane has been observed to increase in numerous plant species, such as barley, broccoli, and tomato. This rise in permeability leads to an elevated leakage of electrolytes being a consequence of a reduction in the total lipid content, ultimately resulting in membrane damage (Guo *et al.* , 2019). Salt stress is known to enhance the processes of lipolysis and lipid peroxidation, while also inhibiting lipid biosynthesis pathways, which collectively decrease the overall lipid content in salt-sensitive cultivars. Alternatively, increased in total sterol content induced by NaCl treatment were found in salt-adapted tomato calli (Kerkeb *et al.* , 2001), salt-tolerant wheat (Salama *et al.* , 2007), and the halophyte *Kosteletzkya virginica* (Blits and Gallagher, 1990). In contrast, non-tolerant species/genotypes, such as sensitive wheat cultivar, showed a significant reduction in the amount of sterol lipids reduced (Salama and Mansour, 2015). Based on this evidence, it has been proposed that the ability to increase total sterol content under salt stress may be an important adaptive mechanism in salt-tolerant species/genotypes (Salama and Mansour, 2015). In this way, as no studies have been performed with boron, we could indicate that that maintaining a constant level of sterols in the membrane is essential for plant tolerance (Salama and Mansour, 2015; Guo *et al.* , 2019)**.**

Conclusion

Understanding the plant response to salinity and boron stresses is crucial in developing strategies to overcome these abiotic stresses with resilient cultivars. In our experiments, we observed that the combination of boron deficiency and high salinity has a different impact on plant growth than each stress alone, providing evidence that salinity is not the predominant stress in our conditions. Additionally, the fact that some fewer damaging effects were found under combinations leads us to think that the three stresses applied have strong impact

on broccoli plant physiology, triggering adaptation mechanisms in different directions than individually. In this way, water pass, in relation to aquaporins, could be targeted as an avoidance strategy since mineral nutrients did not appear to change in an avoidance direction. Therefore, the broccoli plant appeared to modulate aquaporins expression towards allowing increase water uptake in case of salinity, for reducing B uptake in case of toxicity and for increasing B uptake in case of deficiency. However, the individual aquaporin involved in each treatment differed, pointing to a difficulty of identifying a marker aquaporin gene since each aquaporin expression changed depending on the individual or combination treatment, demonstrating the enormous complexity of aquaporins response.

As the results can be interpreted as a strategy to prevent the stresses applied, the fact that there are still several gaps in the knowledge constitute a challenge for further investigation. However, shutting down or upregulating the transcription of the gene seems not be enough, since the broccoli leave cells has to deal with trafficking of proteins in route to the plasma membrane and those already active in the plasma membrane. In this sense, the regulation of aquaporins in each membrane fraction need to be addressed, along with the functionality of the aquaporins according to the lipid and other proteins environment.

Data Availability statement

The authors confirm that all data referred to here are available in the body of this article and its supplementary materials.

Acknowledgments

This work was supported by the Spanish Ministry of Science and Innovation with a grant for Juan Nicolas-Espinosa (FPU20/03823) and Lucia Yepes-Molina (FPU17/02261).

Author contributions

Juan Nicolas-Espinosa: conceptualization, investigation, methodology, writing-original draft. Lucia Yepes-Molina: conceptualization, investigation, methodology, writing-original draft. Fuensanta Martinez-Bernal: investigation. Miriam Fernandez-Pozurama: investigation. Micaela Carvajal: conceptualization, funding acquisition, methodology, supervision, validation, writing-original draft.

Conflict of interest

The authors declare that there is no conflict of interest.

References

Barrajón-Catalán, E., Menéndez-Gutiérrez, M.P., Falco, A., Carrato, A., Saceda, M. and Micol, V. (2010) Selective death of human breast cancer cells by lytic immunoliposomes: Correlation with their HER2 expression level. *Cancer Lett.* , **290** , 192–203.

Barzana, G., Rios, J.J., Lopez-Zaplana, A., Nicolas-Espinosa, J., Yepes-Molina, L., Garcia-Ibanez, P. and Carvajal, M. (2021) Interrelations of nutrient and water transporters in plants under abiotic stress. *Physiol. Plant.* , **171** , 595–619.

Bastias, E., Alcaraz-Lopez, C., Bonilla, I., Martinez-Ballesta, M.C., Bolanos, L. and Carvajal, M. (2010) Interactions between salinity and boron toxicity in tomato plants involve apoplastic calcium. *J. Plant Physiol.* , **167** , 54–60.

Bastias, E., Fernandez-Garcia, N. and Carvajal, M. (2004) Aquaporin functionality in roots of Zea mays in relation to the interactive effects of boron and salinity. *Plant Biol.* ,**6** , 415–421.

Beacham, A.M., Hand, P., Pink, D.A.C. and Monaghan, J.M. (2017) Analysis of Brassica oleracea early stage abiotic stress responses reveals tolerance in multiple crop types and for multiple sources of stress. *J. Sci. Food Agric.* , **97** , 5271–5277.

Ben-Gal, A. and Shani, U. (2002) Yield, transpiration and growth of tomatoes under combined excess boron and salinity stress.*Plant Soil* , **247** , 211–221.

Blits, K.C. and Gallagher, J.L. (1990) Effect of NaCl on lipid content of plasma membranes isolated from roots and cell suspension cultures of the dicot halophyte Kosteletzkya virginica (L.) Presl.*Plant Cell Rep.* , **9** , 156–159.

Boursiac, Y., Chen, S., Luu, D.T., Sorieul, M., Dries, N. Van Den and Maurel, C. (2005) Early effects of salinity on water transport in Arabidopsis roots. Molecular and cellular features of aquaporin expression. *Plant Physiol.* , **139** , 790–805.

Casado-Vela, J., Muries, B., Carvajal, M., Iloro, I., Elortza, F. and Martinez-Ballesta, M.C. (2010) Analysis of Root Plasma Membrane Aquaporins from Brassica oleracea : Post-Translational Modifications, de novo Sequencing and Detection of Isoforms by High Resolution Mass Spectrometry. *J. Proteome Res.* , **9** , 3479–3494.

Chalbi, N., Martinez-Ballesta, M.C., Youssef, N. Ben and Carvajal, M. (2015) Intrinsic stability of Brassicaceae plasma membrane in relation to changes in proteins and lipids as a response to salinity.*J. Plant Physiol.* , **175** , 148–156.

Chen, J., Li, J., Huang, Y., Li, Y., Su, C. and Zeng, X. (2022) EuPIP1;2, a Plasma Membrane Aquaporin Gene from Eucommia ulmoides, Enhances Drought and Salt Tolerance in Transgenic Tobacco.*Agronomy* , **12** , 615.

Cramer, G.R. (2006) Sodium-Calcium Interactions Under Salinity Stress. In *Salinity: Environment - Plants - Molecules* . Dordrecht: Kluwer Academic Publishers, pp. 205–227.

Darre, N.C. and Toor, G.S. (2018) Desalination of Water: a Review. *Curr. Pollut. Reports* , **4** , 104–111.

Diaz, M., Bastias, E., Pacheco, P., Tapia, L., Martinez-Ballesta, M.C. and Carvajal, M. (2011) Characterization of the physiological response of the highly-tolerant tomato cv. "poncho negro" to salinity and excess boron. *J. Plant Nutr.* ,**34** , 1254–1267.

Edelstein, M., Ben-Hur, M., Cohen, R., Burger, Y. and Ravina, I. (2005) Boron and salinity effects on grafted and non-grafted melon plants. *Plant Soil* , **269** , 273–284.

El-Badri, A.M., Batool, M., A. A. Mohamed, I., et al. (2021) Antioxidative and Metabolic Contribution to Salinity Stress Responses in Two Rapeseed Cultivars during the Early Seedling Stage.*Antioxidants* , **10** , 1227.

Gao, Q., Zhao, P., Zeng, X., Cai, X. and Shen, W. (2002) A model of stomatal conductance to quantify the relationship between leaf transpiration, microclimate and soil water stress. *Plant. Cell Environ.* , **25** , 1373–1381.

Gronnier, J., Gerbeau-Pissot, P., Germain, V., Mongrand, S. and Simon-Plas, F. (2018) Divide and Rule: Plant Plasma Membrane Organization. *Trends Plant Sci.* , **23** , 899–917.

Groszmann, M., Rosa, A. De, Chen, W., Qiu, J., McGaughey, S.A., Byrt, C.S. and Evans, J.R. (2023) A high-throughput yeast approach to characterize aquaporin permeabilities: Profiling the Arabidopsis PIP aquaporin sub-family. *Front. Plant Sci.* , **14** .

Gunes, A., Soylemezoglu, G., Inal, A., Bagci, E.G., Coban, S. and Sahin, O. (2006) Antioxidant and stomatal responses of grapevine (Vitis vinifera L.) to boron toxicity. *Sci. Hortic. (Amsterdam).* ,**110** , 279–284.

Guo, Q., Liu, L. and Barkla, B.J. (2019) Membrane Lipid Remodeling in Response to Salinity. *Int. J. Mol. Sci.* ,**20** , 4264.

Hachez, C., Veljanovski, V., Reinhardt, H., Guillaumot, D., Vanhee, C., Chaumont, F. and Batoko, H. (2014) The Arabidopsis Abiotic Stress-Induced TSPO-Related Protein Reduces Cell-Surface Expression of the Aquaporin PIP2;7 through Protein-Protein Interactions and Autophagic Degradation. *Plant Cell* , **26** , 4974–4990.

Hilal, N., Kim, G.J. and Somerfield, C. (2011) Boron removal from saline water: A comprehensive review. *Desalination* ,**273** , 23–35.

Holloway, R.E. and Alston, A.M. (1992) The effects of salt and boron on growth of wheat. *Aust. J. Agric. Res.* , **43** , 987–1001.

Horie, T., Hauser, F. and Schroeder, J.I. (2009) HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. *Trends Plant Sci.* , **14** , 660–668.

Horie, T., Kaneko, T., Sugimoto, G., Sasano, S., Panda, S.K., Shibasaka, M. and Katsuhara, M. (2011) Mechanisms of Water Transport Mediated by PIP Aquaporins and Their Regulation Via Phosphorylation Events Under Salinity Stress in Barley Roots. *Plant Cell Physiol.* , **52** , 663–675.

Jang, J.Y., Kim, D.G., Kim, Y.O., Kim, J.S. and Kang, H. (2004) An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in Arabidopsis thaliana.*Plant Mol. Biol.* , **54** , 713–725.

Katsuhara, M., Rhee, J.Y., Sugimoto, G. and Chung, G.C. (2011) Early response in water relations influenced by NaCl reflects tolerance or sensitivity of barley plants to salinity stress via aquaporins.*Soil Sci. Plant Nutr.* , **57** , 50–60.

Kayum, M.A., Park, J.-I., Nath, U.K., Biswas, M.K., Kim, H.-T. and Nou, I.-S. (2017) Genome-wide expression profiling of aquaporin genes confer responses to abiotic and biotic stresses in Brassica rapa.*BMC Plant Biol.* , **17** , 23.

Kerkeb, L., Donaire, J.P., Venema, K. and Rodriguez-Rosales, M.P. (2001) Tolerance to NaCl induces changes in plasma membrane lipid composition, fluidity and H+-ATPase activity of tomato calli.*Physiol. Plant.* , **113** , 217–224.

Kissoudis, C., Wiel, C. van de, Visser, R.G.F. and Linden, G. van der (2014) Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk.*Front. Plant Sci.* , **5** .

Kronzucker, H.J., Coskun, D., Schulze, L.M., Wong, J.R. and Britto, D.T. (2013) Sodium as nutrient and toxicant. *Plant Soil* ,**369** , 1–23.

Kumawat, S., Aggarwal, B., Rana, N., Mandlik, R., Mehra, A., Shivaraj, S.M., Sonah, H. and Deshmukh, R. (2021) Identification of aquaporins and deciphering their role under salinity stress in pomegranate (Punica granatum). *J. Plant Biochem. Biotechnol.* ,**30** , 930–942.

Landi, M., Margaritopoulou, T., Papadakis, I.E. and Araniti, F.(2019) Boron toxicity in higher plants: an update. *Planta* ,**250** , 1011–1032.

Larsson, C., Widell, S. and Kjellbom, P. (1987) Preparation of High-Purity Plasma Membranes. *Methods Enzymol.* , **148** , 558–568.

Lauchli, A. and Grattan, S.R. (2007) Plant growth and development under salinity stress. In *Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops* . Dordrecht: Springer Netherlands, pp. 1–32.

Li, T., Choi, W.G., Wallace, I.S., Baudry, J. and Roberts, D.M.(2011) Arabidopsis thaliana NIP7;1: An anther-specific boric acid transporter of the aquaporin superfamily regulated by an unusual tyrosine in helix 2 of the transport pore. *Biochemistry* ,**50** , 6633–6641.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* , **25** , 402–408.

López-Berenguer, C., Martínez-Ballesta, M.C., García-Viguera, C. and Carvajal, M. (2008) Leaf water balance mediated by aquaporins under salt stress and associated glucosinolate synthesis in broccoli.*Plant Sci.* , **174** , 321–328.

Ma, Y., Dias, M.C. and Freitas, H. (2020) Drought and Salinity Stress Responses and Microbe-Induced Tolerance in Plants. *Front. Plant Sci.* , **11** .

Macho-Rivero, M.Á., Camacho-Cristóbal, J.J., Herrera-Rodríguez, M.B., Müller, M., Munné-Bosch, S. and González-Fontes, A. (2017) Abscisic acid and transpiration rate are involved in the response to boron toxicity in Arabidopsis plants. *Physiol. Plant.* ,**160** , 21–32.

Martinez-Ballesta, M. del C. and Carvajal, M. (2014) New challenges in plant aquaporin biotechnology. *Plant Sci.* ,**217** –**218** , 71–77.

Martínez-Ballesta, M.D.C., Silva, C., López-Berenguer, C., Cabañero, F.J. and Carvajal, M. (2006) Plant aquaporins: New perspectives on water and nutrient uptake in saline environment.*Plant Biol.* , **8** , 535–546.

Mohamed, A.K.S.H., Qayyum, M.F., Shahzad, A.N., Gul, M. and Wakeel, A. (2016) Interactive effect of boron and salinity on growth, physiological and biochemical attributes of wheat (Triticum aestivum).*Int. J. Agric. Biol.* , **18** , 238–244.

Morel, J., Claverol, S., Mongrand, S., Furt, F., Fromentin, J., Bessoule, J.-J., Blein, J.-P. and Simon-Plas, F. (2006) Proteomics of Plant Detergent-resistant Membranes. *Mol. Cell. Proteomics* ,**5** , 1396–1411.

Munns, R. and Tester, M. (2008) Mechanisms of Salinity Tolerance. *Annu. Rev. Plant Biol.* , **59** , 651–681.

Muries, B., Faize, M., Carvajal, M. and Martínez-Ballesta, M.D.C. (2011) Identification and differential induction of the expression of aquaporins by salinity in broccoli plants. *Mol. Biosyst.* , **7** , 1322.

Nicolas-Espinosa, J., Yepes-Molina, L. and Carvajal, M. (2022) Bioactive peptides from broccoli stems strongly enhance regenerative keratinocytes by stimulating controlled proliferation. *Pharm. Biol.* , **60** , 235–246.

Nicolas-Espinosa, J. and Carvajal, M. (2022) Genome-wide identification and biological relevance of broccoli aquaporins.*Plant Genome* , **15** , e20262.

Parida, A.K. and Das, A.B. (2005) Salt tolerance and salinity effects on plants: A review. *Ecotoxicol. Environ. Saf.* ,**60** , 324–349.

Picchi, V., Migliori, C., Scalzo, R. Lo, Campanelli, G., Ferrari, V. and Cesare, L.F. Di (2012) Phytochemical content in organic and conventionally grown Italian cauliflower. *Food Chem.* ,**130** , 501–509.

Poorter, H., Niinemets, U., Poorter, L., Wright, I.J. and Villar, R. (2009) Causes and consequences of variation in leaf mass per area (LMA): A meta-analysis. *New Phytol.* , **182** , 565–588.

Princi, M.P., Lupini, A., Araniti, F., Longo, C., Mauceri, A., Sunseri, F. and Abenavoli, M.R. (2016) Boron Toxicity and Tolerance in Plants. In *Plant Metal Interaction* . Elsevier, pp. 115–147.

Rawat, N., Singla-Pareek, S.L. and Pareek, A. (2021) Membrane dynamics during individual and combined abiotic stresses in plants and tools to study the same. *Physiol. Plant.* , **171** , 653–676.

Salama, K.H.A. and Mansour, M.M.F. (2015) Choline priming-induced plasma membrane lipid alterations contributed to improved wheat salt tolerance. *Acta Physiol. Plant.* , **37** , 170.

Salama, K.H.A., Mansour, M.M.F., Ali, F.Z.M. and Abou-Hadid, A.F. (2007) NaCl-induced changes in plasma membrane lipids and proteins of Zea mays L. cultivars differing in their response to salinity.*Acta Physiol. Plant.* , **29** , 351–359.

Schneider, C.A., Rasband, W.S. and Eliceiri, K.W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* ,**9** , 671–675.

Srivastava, A.K., Suprasanna, P., Srivastava, S. and D'Souza, S.F. (2010) Thiourea mediated regulation in the expression profile of aquaporins and its impact on water homeostasis under salinity stress in Brassica juncea roots. *Plant Sci.* , **178** , 517–522.

Suga, S., Komatsu, S. and Maeshima, M. (2002) Aquaporin Isoforms Responsive to Salt and Water Stresses and Phytohormones in Radish Seedlings. *Plant Cell Physiol.* , **43** , 1229–1237.

Sutka, M., Li, G., Boudet, J., Boursiac, Y., Doumas, P. and Maurel, C. (2011) Natural variation of root hydraulics in Arabidopsis grown in normal and salt-stressed conditions. *Plant Physiol.* ,**155** , 1264–1276.

Syvertsen, J.P. and Garcia-Sanchez, F. (2014) Multiple abiotic stresses occurring with salinity stress in citrus. *Environ. Exp. Bot.* , **103** , 128–137.

Tyerman, S.D., Niemietz, C.M. and Bramley, H. (2002) Plant aquaporins: Multifunctional water and solute channels with expanding roles. *Plant, Cell Environ.* , **25** , 173–194.

Vanhee, C., Zapotoczny, G., Masquelier, D., Ghislain, M. and Batoko, H. (2011) The Arabidopsis multistress regulator TSPO is a heme binding membrane protein and a potential scavenger of porphyrins via an autophagy-dependent degradation mechanism. *Plant Cell* ,**23** , 785–805.

Westoby, M., Schrader, J. and Falster, D. (2022) Trait ecology of startup plants. *New Phytol.* , **235** , 842–847.

Wimmer, M.A. and Eichert, T. (2013) Review: Mechanisms for boron deficiency-mediated changes in plant water relations. *Plant Sci.* , **203** –**204** , 25–32.

Wu, Y.W. and Wei-Hua (2013) Potassium Transport and Signaling in Higher Plants. *Annu. Rev. Plant Biol.* , **64** , 451–476.

Yadav S. P., Bharadwaj R., Nayak H., Mahto R., Singh R. K. and Prasad S. K. (2019) Impact of salt stress on growth, productivity and physicochemical properties of plants: A Review. *Int. J. Chem. Stud.* , **7** , 1793–1798.

Yepes-Molina, L., Carvajal, M. and Martinez-Ballesta, M.C.(2020) Detergent Resistant Membrane Domains in Broccoli Plasma Membrane Associated to the Response to Salinity Stress. *Int. J. Mol. Sci.* ,**21** , 7694.

Yermiyahu, U., Ben-Gal, A., Keren, R. and Reid, R.J. (2008) Combined effect of salinity and excess boron on plant growth and yield.*Plant Soil* , **304** , 73–87.