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Research Article

Comparative study of the effect of salt stress, *Alternaria alternata* attack or combined stress on the *Cakile maritima* growth and physiological performance

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Abstract

Cakile maritima is a halophytic plant model that is well known by its ability to tolerate high salt concentrations. Salinity was reported to improve the tolerance of halophytes to several abiotic stresses; however, the involvement of salt in the tolerance to biotic stress is still scant. In the present work, the effect of salt on *C. maritima* responses towards the pathogenic *Alternaria alternata* was investigated. For that, *C. maritima* seeds were germinated for four weeks. Plants were then divided into four groups: i) Plants irrigated with salt (200mM NaCl); ii) Plants infested by fungus; iii) Plants irrigated with salt and infested by fungus and finally control plants (0mM NaCl, without inoculation). Our results showed that upon salt stress or fungal attack, plants reduced biomass production, hydration status and photosynthetic performance which were associated with a decrease in the gas exchange and chlorophyll fluorescence parameters, with a more pronounced effect upon fungal attack. However, under combined stress, a significant increase of these parameters was noticed, with a level close to that of control. Concerning nutrient contents, K, Zn, Fe, Cu and Mg decreased in the *C. maritima* leaves exposed to both stresses applied individually. In contrast, all these nutrients were increased in plants grown under combined stress. Taken together, we can conclude that plants

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grown under combined stresses had better growth rate and physiological performance compared to all other treated plants, and that salt may be the key in improving the *C. maritima* ability to tolerate fungal attack.

Keywords: *Alternaria alternata*; *Cakile maritima*; combined stress; fungal attack alleviation; salt stress

Abbreviations: FW: fresh weight; DW: dry weight; A: net CO_2 assimilation rate; gs: stomatal conductance; E: transpiration rate; Ci: internal CO_2 concentration; WUE: Water use efficiency; MDA: malondialdehyde; D: dissipation of excess photon energy in the PSII antenna; NPQ: non-photochemical quenching; Fv/Fm: the maximum quantum efficiency of photosystem II photochemistry; q_P: photochemical quenching

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Introduction

Cakile maritima (family: Brassicaceae) commonly known as sea rocket, is an obligate halophyte frequently found in littoral sandy dunes along the Mediterranean seashore, acting as soil stabilizer (Flowers and Colmer, 2008, 2015). This halophyte is considered as oilseed (40% oil on seed dry weight basis) (Zarrouk *et al.*, 2003) and it has been eaten traditionally as an antiscorbutic, diuretic and purgative and its leaves were used for salads, amongst other culinary uses (Davy *et al.*, 2006).

C. maritima has a small size genome (1C = 719 Mb) and a short life cycle, and recently is considered as model system for halophytes to address salt stress tolerance mechanisms (Arbelet-Bonnin *et al.*, 2019; Debez *et al.*, 2013). Besides being necessary to its growth, salinity (salt stress) was reported to exhibit beneficial effects on their tolerance to abiotic stress (Hamed *et al.*, 2013). In literature, several studies have investigated salt-enhanced tolerance to other abiotic stresses in halophyte plants. For instance, in *Hordeum maritimum*, phosphorus deficiency was alleviated following the application of moderate salinity (Zribi *et al.*, 2012). Similarly, Glenn *et al.* (2012) reported that salinity improves *Atriplex* spp. tolerance to water deficiency (Glenn *et al.*, 2012). Furthermore, beneficial salt effects on *Sesuvium portula castrum* responses to drought stress (Slama *et al.*, 2007) and heavy metals (Ghnaya *et al.*, 2005) were also reported.

The adaptation of halophytes to abiotic stress after an exposition to salt stress could be due to either cross-tolerance, anticipation or stress memory (Hamed *et al.*, 2013). In *C. maritima*, a proposed mechanism was due to the development of a relatively long-term stress memory in plants pre-exposed to salinity which resulted in a lower oxidative stress when subsequently exposed to others abiotic stresses or salt stress (Ellouzi *et al.*, 2013). In addition, the co-existence of salinity with other stresses leads to the amplification of certain physiological and biochemical tolerance traits commonly associated with salinity such as the accumulation of osmolyte, K: Na selectivity, Na^+ exclusion and vacuolar compartmentalization, antioxidant capacity (enzyme and antioxidant molecules) (Gil *et al.*, 2011; Shiri *et al.*, 2015; Hassan *et al.*, 2017). Moreover, the adaptation of halophyte plants involves a variety of protective mechanisms that stabilize photosystems, preserve the photosynthetic apparatus, secure the Calvin cycle enzymes, and control the redox state of many components (Ben Amor *et al.*, 2020; Farhat *et al.*, 2021).

Overall, these investigations indicate that salt has a beneficial effect on halophytes adaptation to abiotic stress. However, in their habitats, halophytes were exposed to multiple stresses among them the biotic factors. Hence, here it is important to know if salt has the same role on the response of halophytes to biotic stress. In fact, in their habitat, *C. maritima* are colonized by several fungal strains of Alternaria (Chalbi *et al.*, 2020), with *Alternaria alternata* being the most pathogenic. Indeed, it affects the adequate development of the crops and also leads to a reduction of post-harvest quality (Akimitsu *et al.*, 2014; Meena and Samal, 2019; Somma *et al.*, 2019). Despite the identification of different pathogenic strains of Alternaria from the different organs (seeds, stem and leaves) of *C. maritima*, it is worth noting that plants spontaneously grown on saline habitats seem to be less affected by Alternaria than those cultivated under laboratory experiments conditions (Chalbi *et al.*, 2020). Since salinity is the most important factor in the halophyte habitat, we may postulate that its presence may confer some degree of tolerance to biotic stress. Thus, the objective of the present work was to study the effect of salt stress or *A. alternata* attack applied separately, or in combination, on the physiological performance of *C. maritima* plants. Thus, the investigation of salt involvement on the mitigation of fungal attack should be the focus of future studies that aim at developing transgenic crops tolerating naturally occurring environmental conditions.

Materials and Methods

Fungal material

Alternaria alternata was isolated from *C. maritima* plants and identified by (Chalbi*et al.*, 2020) as *Alternaria alternata* strain (ITEM17830). Conidial suspensions were prepared from 2-week-old cultures by adding 10 mL of distilled water to each plate and rubbing the surface of each culture with a spreader bar. Conidial suspensions were adjusted to 10^6 conidia mL^{-1} following hemocytometer counts.

Plant culture and treatments

C. maritima seeds were collected from coastal dunes in Djerba, an island in Southeast of Tunisia characterized by temperate winters (10 °C and 17 °C represented the minimal and maximal average temperatures, respectively) and annual rainfall of 100-200 mm (arid region). For salt treatment, we have used 200 mM NaCl as was reported previously by our group to induce salt stress (Farhat *et al.*, 2021; Ben Amor *et al.*, 2020; Megdiche *et al.*, 2007). Seeds were sown in pots of 0.5 L (four seeds per pot) filled with inert sand and daily watered with tap water until germination. The experiment was performed in a growth room under controlled conditions: 16-h light/8-h dark photoperiod; 25±1 °C temperature; 60 ± 2% relative humidity and 440 $\mu molm^{-2} s^{-1}$ photosynthetic active radiations (PAR). Plants were then daily watered with 100 mL of Hewitt nutrient solution (pH 7.3, EC 2.7 mS cm^{-1}) (Hewitt and Eden, 1953) for four weeks. Twenty-eight-day-old plants were partitioned into two lots, which were grown under salt-free conditions and salt conditions (200 mM of NaCl) for two weeks. Then, the different lots of plants previously reported were separated into two groups: the first one was sprayed with 20 mL of H_2O (non-inoculated plants) in leaves; whereas for the second one leaves were sprayed with 20 mL H_2O of a conidial suspension at a concentration of 10^6 CFU mL^{-1} of *A. alternata* until run-off (inoculated plants). Each group was covered with transparent plastic bags to keep an atmosphere saturated with humidity to promote infection, and then they were placed in the dark for 48 hours at 25 °C. The experimental design consisted of two treatments (Figure 1), four blocks, and five plants per block (one plant per pot, supplementary Figure 1).

Plant infection assessment

To assess the inoculation process of *A. alternata* on *C. maritima* leaves, inoculated and non-inoculated leaf samples were twice properly washed with distilled water. The monitoring of the infection process begins from 2 to 96 hours post inoculation (hpi). The Section crosswise was then cut into transparent slices with the help of a sterilized blade or scalpel. Thin cross sections of leaf samples were decolorized with 0.15% (w/v) TCA in ethanol: chloroform (3:1) and then wet mounted with a drop of 0.01% lactophenol cotton blue on a glass slide and covered with a cover slip. Slides were observed under light microscope (Zeiss AX10 Star Plus) at 50-60 Hz and 65 V. More than fifty samples were examined to confirm the results. Conidia numbers were counted by image analysis methods.

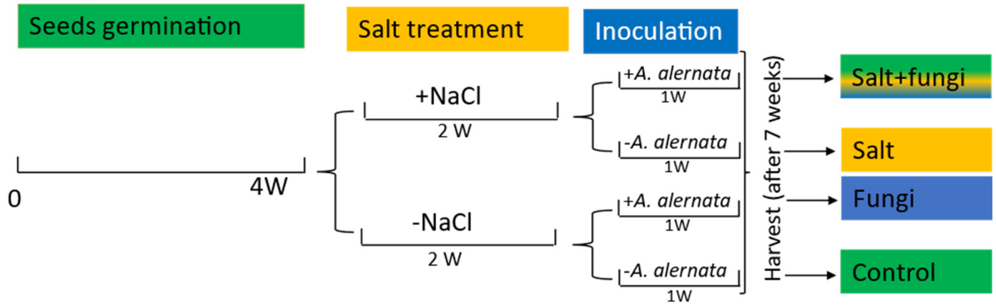

Figure 1. Schematic overview of the experimental design, Salt +fungi; infested plants with *A. alternate* and grown under salt, Salt; plants grown under salt stress, fungi; plants infested by the fungi, Control; plants grown under 0mM NaCl without inoculation, W: week

Biomass parameters and water status

Leaf fresh weight (FW) and dry weight (DW) were determined at the end of the experiment (after 7 weeks). DW was obtained after oven-drying the leaves at 60 °C for 72h. Measurement of the water content (WC) was determined according to the following formulate: [(FW - DW)/DW].

Gas exchange measurements

The determination of leaf gas exchange parameters was conducted using an infrared gas analyzer (LCi portable photosynthesis system, ADC BioScientific Ltd., Hoddesdon, Herts, UK). The following parameters were recorded at saturating light: net CO_2 assimilation rate (A), stomatal conductance (gs), transpiration rate (E) and internal CO_2 concentration (Ci). All the parameters were measured at a photosynthetic available radiation of 800 μmol m^{-2} s^{-1} (saturated light intensity), 65 ± 5% relative humidity, 28 ± 2 °C leaf temperature and 350 μmol mol^{-1} CO_2 concentration. The water use efficiency (WUE) was estimated using the following formula: WUE = A/E(Christophe *et al.*, 2018). All measurements were performed between 09:30 am and 10:30 am in fully expanded leaves (the same leaf stage from the bottom). Values of the above-mentioned parameters were taken after the stabilization of the photosynthetic levels.

Chlorophyll fluorescence determinations

The chlorophyll fluorescence measurements were made using a fluorometer type DUAL-PAM-100 (Walz, Germany) on the upper surface of the intact plants, five replicates were performed, one leaf (from the middle) from each plant. The minimum fluorescence (F_0) was determined one hour after the adaptation of leaves to dark that were then light saturated to determine the maximum fluorescence (F_m) and the maximum quantum efficiency of photosystem II photochemistry (F_v/F_m) = (F_m- F_0)/F_m. Non-photochemical quenching of fluorescence (NPQ) was determined with the equation (F_m – F_m')/F_m'. Photochemical quenching of fluorescence (q_P) was determined using the equation (F_m'–F_s)/(F_m'-F_0'). The determination of the intrinsic efficiency of open photosystem II (Φexc) was calculated as the ratio between F_v'/F_m'. The actual photosystem II efficiency was calculated as F_q'/F_m' = (F_m' – F_s)/F_m'. The efficiency of dissipation of excess photon energy in the photosystem II antenna (D) was determined using the equation 1 -Φexc = 1 - (F_v'/F_m'). All these equations were reported by (Schreiber and Ulrich, 2004).

Leaf macronutrients and micronutrients concentrations

Oven-dried leaves were ground into a fine powder using a mortar. About 0.3 g of dried leaves were digested with 0.5% of nitric acid (HNO_3) at room temperature for four days. Then, the extracts were filtered and used for the determination of nutrient concentration. Chloride was assessed by colorimetry (Büchler), K and Na by flame spectrophotometry (Corning, United Kingdom), and Mg and micronutrients concentration by atomic absorption spectrophotometry (Perkin Elmer 4000).

Lipid membrane peroxidation

Lipid peroxidation was measured to assess the oxidative stress induced membrane damage by determining malondialdehyde (MDA) content, using the thiobarbituric acid method according to (Draper and Hadley, 1990). Fresh leaf samples (the third leaf from the top) were homogenized in 0.1% (w/v) TCA solution and centrifuged at 15,000 *g* for 10 min. An aliquot of the supernatant was added to 0.5% TBA in 20% TCA and the mixture was heated at 95 °C for 30 min in a shaking water bath. After cooling in an ice bath and subsequent centrifugation (10,000*g* for 10 min at 4 °C), the supernatant absorbance at 532nm was read and values corresponding to nonspecific absorption (600 nm) were subtracted. MDA content was calculated according to the molar extinction coefficient of MDA (155 mM^{-1} cm^{-1}).

Statistical analysis

Data were presented as mean ± SEM of five replicates. Statistical analyses were performed using the Statistix 8.0 software. For multiple group comparisons ANOVAs followed by Student'st-test were used. For all analyses $p < 0.05$ was considered significant. For PCA and cluster analysis of all the parameters measured, the web-based software NIA array analysis tool (Sharov*et al.*, 2005) available at *http://lgsun.grc.nia.nih.gov/anova/index.html* was used. This software tool selects statistically valid parameters value based on analysis of variance (ANOVA). The entire data set was analyzed by principal component analysis (PCA) using the following settings: covariance matrix type, four principal components, two-fold change threshold for clusters, and 0.5 correlation threshold for clusters. PCA results were represented as a biplot, with different treatments used in those experimental situations located on the same area of the graph.

Results

Alternaria leaf infection process

Conidia of *A. Alternaria* was found to be small and septate with short beak (Figure 2A). Light micrograph (40X) showed that the fungus *A. alternate* penetrated to its host via conidia (Figure 2B, D) or mycelia (Figure 2C, E) throughout the stomata (Figure 2B, C) or epidermis (Figure 2D, E). *A. alternata* infected *C. maritima* leaf tissue revealed damage to cell and cell wall as compared to healthy tissue (Figure 2F). The damaged portions were found to be discoloured because of interaction with the fungus and it may be due to production of toxins (Figure 2G).

The monitoring of *A. alternata* multiplication on *C. maritima* leaves was performed via light micrograph observation from 2 to 96 hours post inoculation (hpi) (Figure 3A-F). Results showed an increase from 6 to 49 conidia/mm^2 from 2 to 4 hpi. The number of conidia per surface increased nearly by ten-fold, reaching 436 conidia/mm^2 at 18 hpi. The maximum rate of fungus colonization in leaves was from 48 hpi to 72 and 96hpi with values around 5800 conidia/mm^2 (Figure 3G).

Based on these data, we have showed, for the first time, the process of penetration of *A. alternata* to *C. maritima* and further results will focus on its impact on the growth and physiology of *C. maritima* plants either grown under salt or salt-free conditions.

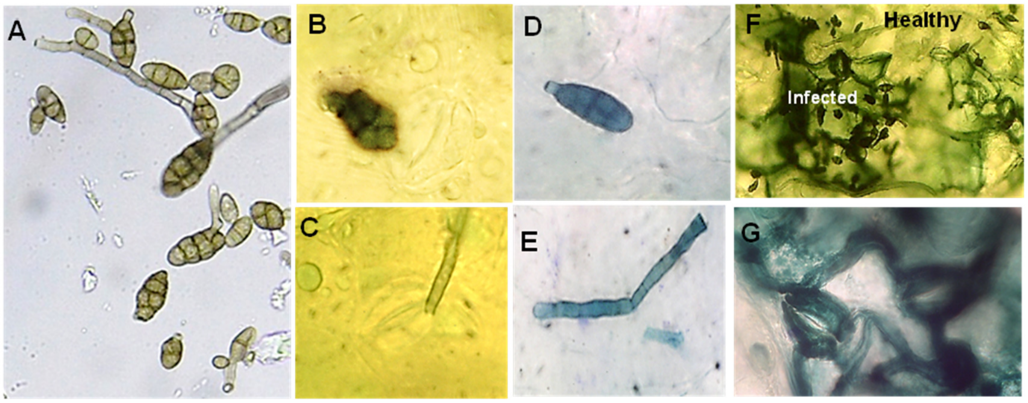

Figure 2. Light micrographs (40X) of conidia and mycelia formed by *Alternaria alternate* fungus (A), penetration of conidia or mycelia on *Cakile maritima* leaf throughout the stomata (B, C) or epidermis (D, E), leaf infected tissue showing difference between healthy and infected portion due to Alternaria toxins (F, G)

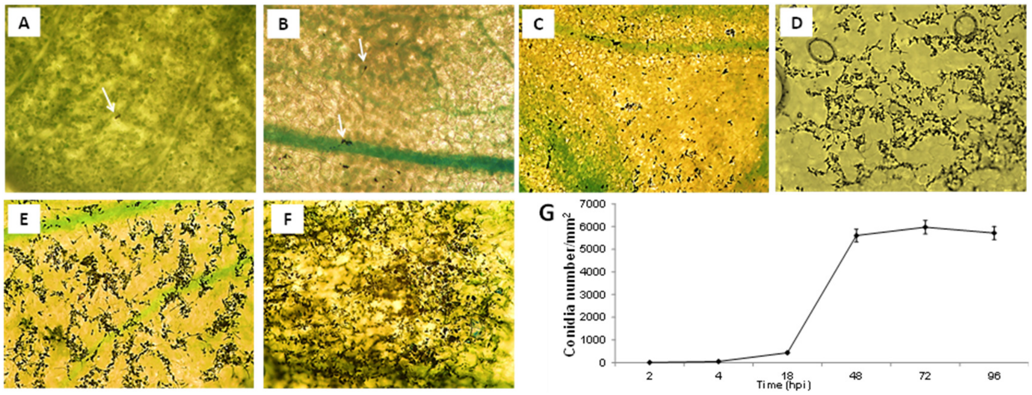

Figure 3. Microscopic observation (40X) of the conidia multiplication at the surface of *C. maritima* leaf upon *A. alternata* inoculation during different hours post inoculation (A;2hpi, B;4hpi, C; 18hpi, D;48hpi, E; 72hpi and F; 96hpi) and the conidia number per mm^2 over time (G), hpi: hours post-inoculation, the row indicates the conidia

Assessment of growth parameters under individual or combined stress

The growth parameters were measured from plants grown either, under salt stress or fungal attack, or under combined stress. Plant biomass was measured as fresh and dry weight (FW, DW). Salt stress or fungal attack significantly impaired growth of *C. maritima* plants compared to controls (0 mM NaCl and without inoculation). In fact, FW, DW and water content (WC) decreased when plants were subjected either to 200 mM NaCl or *A. alternata* (Figure 3). Plants grown under combined stress exhibited similar parameters values to those of plants grown under salt stress and lesser than control plants (Table 1, Figure 4).

Gas exchange determinations and water use efficiency

The application of individual stress significantly reduced the photosynthetic gas exchange parameters. For example, the net CO_2 assimilation rate (A) decreased by 22% under salt stress, compared to control plants; however, upon fungal attack, the (A) decreased by 75%. Plants grown under combined stress exhibited similar level of (A) to that of control plants. Similar trends were observed for the transpiration rate (E), stomatal conductance (gs), and water use efficiency (WUE), where the levels of these parameters decreased upon application of individual stress and reached similar and even higher levels to that of control plants under combined stress (Table 2).

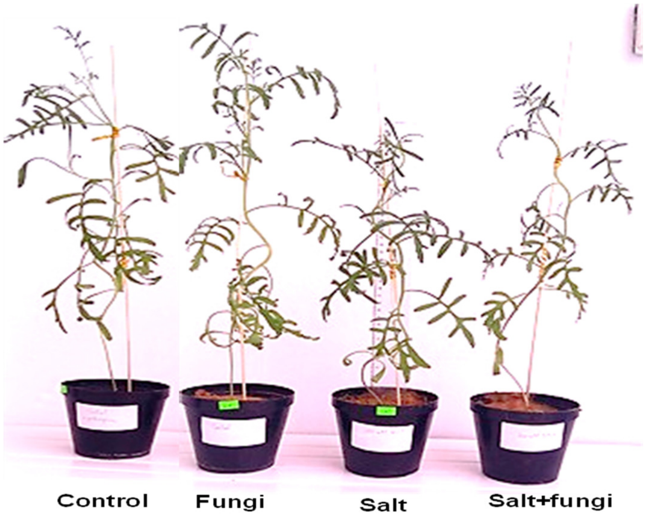

Figure 4. Morphological changes in *C. maritima* plants under different treatments assessed at the end of the experiment: Control; plants grown under 0mM NaCl without inoculation, fungi; plants infested by the fungi, Salt; plants grown under salt stress, Salt +fungi; infested plants with *A. alternate* and grown under salt stress.

Table 1. Effects of different treatments on growth parameters (fresh and dry weight: FW, DW) and water content (WC) of inoculated and non-inoculated *C. maritima* plants with *A. alternata*

Parameters	Treatments	SFC	SC
FW (g)	Non-inoculated	6.41 ± 0.55 aA	2.49 ± 0.30 bA
	Inoculated	5.19 ± 0.58 aB	2.51 ± 0.29 bA
DW (g)	Non-inoculated	0.34 ± 0.03 aA	0.20 ± 0.02 bA
	Inoculated	0.30 ± 0.03 aB	0.19 ± 0.02 bA
WC (g/DW)	Non-inoculated	17.80 ± 0.03 aA	12.47 ±0.63 bA
	Inoculated	16.32 ± 0.03 aB	11.50 ±0.83bA

Values represent means ± standard error of five plants per treatment. Means within a row with the same lowercase letter are not significantly different at $p< 0.05$ (Student's t-test). Means within a column with the same capital letter are not significantly different at $p< 0.05$ (Student's t-test), SFC; salt-free conditions, SC; salt conditions.

Table. 2. Effects of different treatments on gas exchange measurements (net CO_2 assimilation rate (A), transpiration rate (E), internal CO_2 concentration (Ci), stomatal conductance (gs), and water use efficiency (WUE) in non-inoculated and inoculated plants of *C. maritima* with *A. alternata* at the end of the experiment

Parameters	Treatments	SFC	SC
A (µmol CO_2 m^{-2} s^{-1})	Non-inoculated	9.3 ± 0.97 aA	7.3 ± 0.14 bB
	Inoculated	2.46 ± 0.70 bB	8.95 ± 0.68 aA
E (mmol H_2O m^{-2} s^{-1})	Non-inoculated	1.33 ± 0.11 aA	1.05± 0.10 bA
	Inoculated	0.83 ± 0.12 bB	1.28 ± 0.05aA
gs (mmol.$m^{-2}s^{-1}$)	Non-inoculated	0.086 ± 0.01 aA	0.06 ± 0.01 bA
	Inoculated	0.036 ± 0.01 aB	0.05 ± 0.01 aA
Ci (µmol.m^{-2} s^{-1})	Non-inoculated	252.67 ± 21.47 aA	253.67 ± 17.29 aB
	Inoculated	314.81 ± 29.94 bA	361.52 ± 32.98 aA
WUE (µmol CO_2 $mmol^{-1}H_2O$)	Non-inoculated	8.26 ± 0.80 aA	6.43 ± 0.71 bA
	Inoculated	4.40 ± 0.39bB	5.72 ± 0.64 aA

Values represent means ± standard error of five plants per treatment. Means within a row with the same lowercase letter are not significantly different at $p< 0.05$ (Student's t-test). Means within a column with the same capital letter are not significantly different at $p< 0.05$ (Student's t-test), SFC; salt-free conditions, SC; salt conditions.

Chlorophyll fluorescence determinations

Under salt stress, the following fluorescence parameters: the maximal quantum yield of fluorescence (Fv/fm), quantum yield of PSII electron transport (ΦPSII) and intrinsic efficiency of open photosystem II (Φexc) exhibited similar levels compared to control plants. However, the non-photochemical quenching (NPQ), photochemical quenching (q_p) and the efficiency of dissipation of excess photon energy in the PSII antenna (D) decreased compared to control plants. Under *A. alternata* attack, Fv/Fm, NPQ, q_p and D were significantly reduced by 5, 26, 8, and 50%, respectively. However, under combined stress, plants showed an increase in the following parameters: Fv/Fm, NPQ, q_p and D, compared to plants grown under salt conditions (Table 3). Besides, some of the parameters (Fv/Fm, q_p) were close to those of control plants.

Table 3. Effects of different treatments on maximum quantum efficiency of PSII (F_v/F_m), non-photochemical quenching (NPQ), photochemical quenching (q_p), quantum yield of PSII electron transport (ΦPSII), intrinsic efficiency of open photosystem II (Φexc) and efficiency of dissipation of excess photon energy in the PSII antenna (D) in *Cakile maritima* at the end of the experiment

Parameters	Treatments	SFC	SC
Fv/Fm	Non-inoculated	0.75 ± 0.01 aA	0.77 ± 0.06 aA
	Inoculated	0.63 ± 0.04 bB	0.74 ± 0.03 aA
NPQ	Non-inoculated	1.71 ± 0.11 aA	0.14 ± 0.14 bB
	Inoculated	0.45 ± 0.02 bB	0.66 ± 0.04 aA
q_p	Non-inoculated	1.66 ± 0.26 aA	1.28 ± 0.27 bB
	Inoculated	1.38 ± 0.23 bB	1.76 ± 0.29 aA
ΦPSII	Non-inoculated	0.65 ± 0.06 aA	0.71 ± 0.06 aA
	Inoculated	0.73 ± 0.07 aA	0.67 ± 0.06 aA
Φexc	Non-inoculated	0.56 ± 0.03 aA	0.58 ± 0.04 aA
	Inoculated	0.54 ± 0.04 aA	0.53 ± 0.03 aA
D	Non-inoculated	0.97 ± 0.07 aA	0.32 ± 0.05 bB
	Inoculated	0.49 ± 0.01 bB	0.58 ± 0.06 aA

Values represent means ± standard error of five plants per treatment. Means within a row with the same lowercase letter are not significantly different at $p < 0.05$ (Student's t-test). Means within a column with the same capital letter are not significantly different at $p < 0.05$ (Student's t-test), SFC; salt-free conditions, SC; salt conditions.

MDA contents

The MDA content increased significantly by 20% in plants grown under salt stress and by 50% in infested plants, compared to control plants. Under combined conditions, the MDA content was similar to those plants grown under salt stress conditions and lesser than that of infested plants grown under salt free condition but remained higher compared to control plants (Table 4).

Table 4. Membrane lipid peroxidation determination (MDA). Values represent means ± standard error of five plants per treatment

Parameters	Treatments	SFC	SC
MDA (nmol g^{-1} FW)	Non-inoculated	3.74 ± 0.19 bB	5.24 ± 0.66aA
	Inoculated	7.07 ± 0.13 aA	6.0 ± 0.59bA

Values represent means ± standard error of five plants per treatment. Means within a row with the same lowercase letter are not significantly different at $p < 0.05$ (Student's t-test). Means within a column with the same capital letter are not significantly different at $p < 0.05$ (Student's t-test), SFC; salt-free conditions, SC; salt conditions.

Leaf macronutrient concentrations

Under salt stress, an increase in sodium and a decrease in potassium concentrations in *C. maritima* leaves was recorded compared to control plants. Upon inoculation, the K^+ concentration decreased but that of Na^+ remained unchanged compared to control plants. Under combined conditions, Na^+ and K^+ concentrations showed levels that are similar to that of plants grown under salt stress. Concerning leaf Mg concentration, it decreased either under salt stress or upon fungal attack; while, under combined stress, plants showed the highest Mg level compared to all other treatments (Table 5).

Table 5. Effects of different treatments on macronutrients concentrations in *Cakile maritima* at the end of the experiment

Parameters	Treatments	SFC	SC
K^+(mmol g^{-1} DW)	Non-inoculated	61.95 ± 5.83 aA	45.53 ± 4.31 bA
	Inoculated	57.92 ± 1.02 aB	46.68 ± 4.01 bA
Na^+(mmol g^{-1} DW)	Non-inoculated	31.16 ± 3.23 bA	66.65 ± 6.16 aA
	Inoculated	31.85 ± 3.20 bA	69.59 ± 6.15 aA
Mg (mmol g^{-1} DW)	Non-inoculated	1.29 ± 0.03 aA	1.05 ±0.04 bB
	Inoculated	1.08 ± 0.10 bB	1.57 ± 0.15 aA

Values represent means ± standard error of five plants per treatment. Means within a row with the same lowercase letter are not significantly different at $p < 0.05$ (Student's t-test). Means within a column with the same capital letter are not significantly different at $p < 0.05$ (Student's t-test), SFC; salt-free conditions, SC; salt conditions.

Leaf micronutrient concentrations

Leaf Cu, Fe and Zn concentrations were reduced in plants exposed to salt stressor fungal attack, whereas the concentration of Mn increased compared to control plants. The decrease was more pronounced under fungal attack than salt stress. Under combined conditions, all these micronutrient elements concentrations increased compared to control plants (Table 6).

Table 6. Effect of different treatments on micronutrients concentration in *Cakile maritima* at the end of the experiment

Parameters	Treatments	SFC	SC
Cu (µmol g^{-1} DW)	Non-inoculated	0.86 ± 0.08 aA	0.78 ± 0.07 bA
	Inoculated	0.63 ± 0.05 bB	0.93± 0.06 aA
Fe (µmol g^{-1} DW)	Non-inoculated	3.03 ± 0.36 aA	2.81 ± 0.25 aB
	Inoculated	2.35 ± 0.22 bB	3.26 ± 0.23 aA
Zn (µmol g^{-1} DW)	Non-inoculated	1.29 ± 0.12 aA	1.05 ± 0.11bB
	Inoculated	1.01 ± 0.10 bB	1.57 ± 0.12 aA
Mn (µmol g^{-1} DW)	Non-inoculated	2.19 ± 0.19 bB	2.84 ± 0.26 aA
	Inoculated	2.59 ± 0.23 bA	2.93 ± 0.27 aA

Values represent means ± standard error of five plants per treatment. Means within a row with the same lowercase letter are not significantly different at $p < 0.05$ (Student's t-test). Means within a column with the same capital letter are not significantly different at $p < 0.05$ (Student's t-test), SFC; salt-free conditions, SC; salt conditions.

Principal component analysis (PCA) and expression cluster

In order to consolidate our findings, the PCA and cluster analyses were performed by analyzing all the assessed parameters that characterize the response of *C. maritima* to salt stress, fungal attack and combined stress. PC1 and PC2 represented 48.5% and 24% of the biological variability, respectively (supplementary Table 1). Based on the PC1, Control plants were separated from the rest of the treatments; however, they were grouped by PC2 with plants grown under salt stress (Salt) and plants grown under combined stress (Combined), that they share somehow similar common behavior. The separation of inoculated plants from all the rest of treatments, confirm our previous findings that fungal attack was shown to be more deleterious than salt stress (Figure 5a). The cluster analysis showed that the control and inoculated plants were very distant. Nevertheless, control plants and those cultivated under combined stress were very close (Figure 5b). Thus, the results obtained by PCA and cluster analysis validated our hypothesis that salt can improve the plant tolerance against *A. alternata* attack.

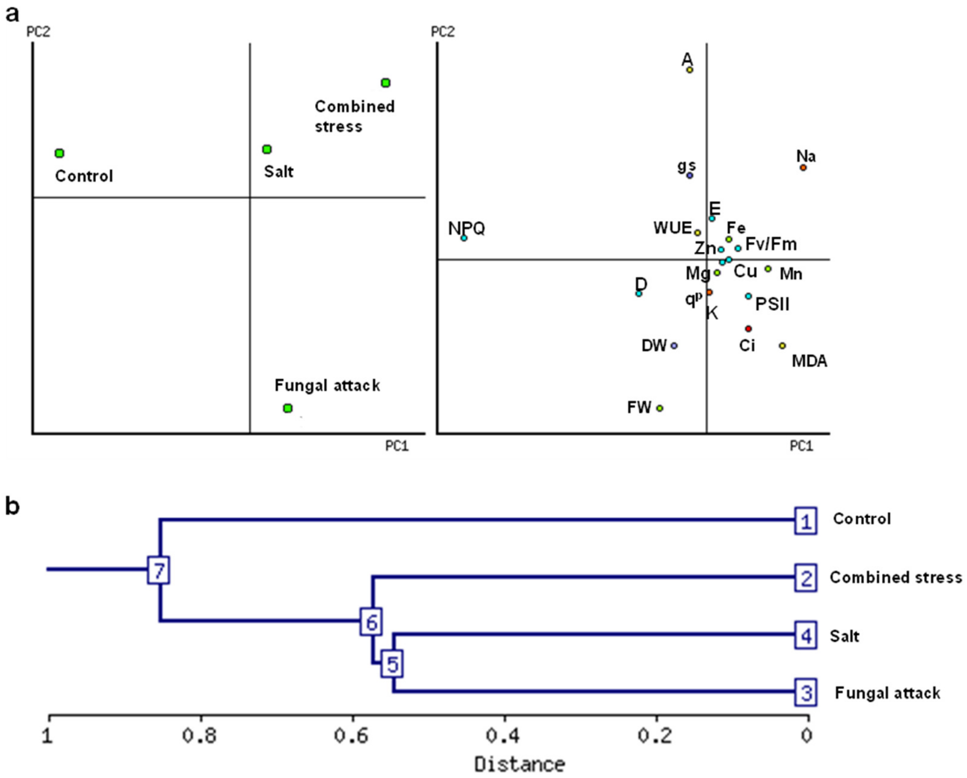

Figure 5. Two-dimensional blots generated by principal component analysis (PCA, a) using all morphophysiological parameters (on the right of the blot) and cluster analysis (b). Control; plants grown on 0mM NaCl without inoculation, Salt; plants grown under salt stress, Fungal attack; plants grown under salt-free conditions and infested by *A. alternata*, and combined stress; plants grown under salt conditions and infested by *A. alternata*

Discussion

Impact of A. alternata attack on C. maritima growth and physiological performances

Recently, we have isolated and identified several strains of *Alternaria* genus from the halophyte *C. maritima* (Chalbi *et al.*, 2020). However, here for the first time the penetration modes, the multiplication rate and the conidia numbers were reported. The microscopic infection process of *A. alternata* on *C. maritima* was similar to that of infection by wild type *A. brassicicola* on cabbage leaf surface (Scott and Lawrence, 2008). *A. brassicicola* on Cauliflower (Sobczak *et al.*, 2020) and *A. brassicae* on mustard (Giri *et al.*, 2013; Mamgain and Biswas, 2020). Light microscopy results showed that the germ tube was found to penetrate the leaf cell through stomata or epidermis. The finding is a common feature of this genus, as reported in *A. porri* (Aveling *et al.*, 1994), *A. linicola* (Vloutoglou *et al.*, 1996), *A. alternata* (Gupta *et al.*, 1998). Our data showed that the cells in the substomatal area were necrotic and the death may be because of diffusible toxins (Sinha *et al.*, 2014; Chalbi *et al.*, 2020). In our present work we found that *A. alternata* conidia number increased rapidly over time and reach their maximum at 48hpi with an increase in necrosis of cells over time. Similarly, the analysis of the impact of *A. brassicicola* on *Brassica oleracea* leaves showed that the number of germinating conidia increased gradually with the post-inoculation time at 20 hpi, indicating fast and massive fungal development on susceptible hosts(Sobczak *et al.*, 2020). The number of conidia of *A. porri* infecting onion was similar to the number of *A. alternata* reported in our work (Mohsin *et al.*, 2016).

At physiological level, *A. alternata* altered the plants biomass and water use efficiency (WUE). In addition, *A. alternata* triggered several negative effects on *C. maritima* plants such as stomatal closure and decrease in the net CO_2 assimilation. Similarly, it has been reported in *Eucalyptus urophylla*, that *Puccinia psidii* infection caused a reduction in stomatal conductance which caused a restriction in photosynthesis process in infected plants by limiting the flow of CO_2 in leaf area (Alves *et al.*, 2011; Mohammadi Alagoz *et al.*, 2016). Riberio *et al.* (2004) showed that in orange seedlings, CO_2 assimilation and stomatal conductance (gs) were higher in healthy plants compared to those infected by *Xylella fastidiosa* (Ribeiro *et al.*, 2004). Polando *et al.* (2014) also pointed out a similar relationship between plants infected with *Colletotrichum lindemuthianum* (Polanco *et al.*, 2014).

Besides to damages occurred on the gas exchange parameters, *A. Alternaria* caused a decrease in the maximum quantum efficiency of PSII (F_v/F_m), indicating that the PSII reaction center was damaged by *A. alternata*, leading to a decline in the overall rate of electron transport (q_p). The decrease in fluorescence parameters may be due to Alternaria mycotoxins secreted in the host (Chalbi *et al.*, 2020) which is considered as an energy-transfer inhibitor (such as the tentoxin) (Arntzen, 1972). Similar to our results, Arntzen (1972) reported that the inoculation with Alternaria species caused an inhibition of photosynthetic electron transport (Arntzen, 1972) and consequently leading to over reduction of the photosystem II (Sobczak *et al.*, 2020). The decrease in photosynthetic efficiency could be a result of the fungus-induced necrotic lesion expansion (Sobczak *et al.*, 2020). The necrotic lesions in leaves induce an increase in the lipid peroxidation (MDA) which is an indicator of membrane integrity damage and is a considerable marker of oxidative stress (Møller and Hansson, 2007). Lipids were shown to be modulated upon inoculation with the tolerant grapevine *Vitis vinifera* cv. Regent with *P. viticola*, particularly at 6 and 12hpi, which decreased when compared to mock-inoculated samples (Cavaco *et al.*, 2021). Altogether, the present findings confirm the damages caused by the *A. alternata* fungus on *C. Maritima* growth and physiology, and the possibility of the involvement of salt in the mitigation of the effect of fungal attack will be discussed in the next section.

Salt involvement in the alleviation of the attack of Altenraria alternata in Cakile maritima

Despite the attack with the pathogen, the growth of *C. maritima* under salt condition led to a positive effect on gas exchange parameters (increase in the net CO_2 assimilation rate, transpiration rate and stomatal conductance) and water use efficiency, compared to those grown under salt-conditions. The water use efficiency was shown to be a good tool for measuring the ability of a plant to adjust its gas exchange parameters under stressful conditions by increasing CO_2 capture and reducing water losses (Gleick *et al.*, 2011). Likewise, the adverse effects of *A. alternata* on the photosynthetic performance of *C. maritima* were reduced when plants were grown under salt stress. In fact, the maximum quantum efficiency of PSII (Fv/Fm) in infested *C. maritima*, grown under salt conditions, remained similar to control plants despite fungal attack. Indeed, it showed normal PSII reaction center functioning which helped in keeping normal levels of PSII photochemical activity and photosynthetic electron transport (Yang *et al.*, 2018). In parallel, an increase in the level of photochemical quenching (q_p), the efficiency of dissipation of excess photon energy in the PSII antenna (D) and the non-photochemical quenching (NPQ), compared to infected plant, were observed. This increase in photosynthetic parameters suggested that the dissipation of excess energy may have played a protective role in the photosynthetic apparatus in plants challenged with stress facing the excess of photo energy (Qiu *et al.*, 2003; Wided *et al.*, 2009).

For non-halophytic plants, the presence of salt and other biotic stresses has effects that are more adverse. For example, in cucumber the synergetic effect of salt and pathogen resulted in a stronger decrease in photosynthetic parameters (Chojak-Koźniewska *et al.*, 2018). Similarly, in *A. thaliana*, the photosynthetic parameters were more adversely affected by the synergetic effect of salt stress and pathogen compared to when both stresses applied separately (Demirbas and Acar, 2017). In our case, the mitigation effect of salt stress on the pathogen attack might be related to the fact that this is a halophyte plant and thus may induce the stress memory phenomena by the enhancement of the abundance of protective proteins involved in photosynthesis activation and protein biosynthesis (Hamed *et al.*, 2013; Shiri *et al.*, 2015). The formation of a stress memory happens when the plant keeps a 'stress imprint' following the exposure to a stress that improves the plant's response to recurrent stress compared to plants without that stress memory (Ellouzi *et al.*, 2013). This suggests that after the exposure to a first stress, plants use a set of defense mechanisms using elements that could persist for several weeks after relief from a first stress. This allows stress pre-exposed plants either to prevent and/or scavenge reactive oxygen species more efficiently than non-pre-exposed plants. Slama *et al.* (2007b) showed that when the halophyte *Sesuvium portula castrum* was exposed to salinity followed by water deficit, photosynthesis machinery and water status were improved (Slama *et al.*, 2007). Similarly, Ellouzi *et al.* (2013) reported the beneficial effect of pre-exposure to high salinity and Cd stress in improving the resistance to high salinity (Ellouzi *et al.*, 2013).

There is a strong relationship between photosynthesis activity and MDA content (Álvarez and Sánchez-Blanco, 2015). In the present work, the level of MDA decreased after dual application of salt-stress and pathogen attack despite the increase following the application of both stresses separately. Similarly, it has been shown previously that the concentrations of MDA reached maximum values at 24 h following salt stress then decreased later on in *C. maritima* when exposed to second abiotic stress (Ellouzi *et al.*, 2013). Our data also showed that there was a close link between the decrease in MDA content and the increase in PSI and PSII function. Similar data was reported by Xu *et al.* (2009, 2011) where it was shown that lipid peroxidation is closely associated with photosynthesis and PSII activities (Xu *et al.*, 2009, 2011). This may suggest that plants were protected from oxidative damage under combined stress that is why they exhibited better functioning.

Concerning nutrient uptakes, the present results showed that salt stress or fungal attack positively impacted the uptake of Mn, but decreased that of K, Fe, Zn, Cu and Mg in *C. maritima* leaves. In contrast, the Mg, Cu, Zn, Mn and Fe concentrations have increased in inoculated seedlings by *A. alternata*, grown under salt stress (combined stress). In fact, Mg as a component of chlorophyll, Fe as a component of ferridoxin and cytochromes; Zn as a component of glutamic, alcohol and lactic dehydrogenase, and carbonic anhydrase; Cu as a component of laccase, cytochrome oxidase, ascorbic acid oxidase, and polyphenol oxidase; and Mn as a component of arginase and phosphotransferase, may be mobilized and utilized by plants and may be influenced by the adverse or favorable environmental growth conditions (Manzoor Alam, 1999; Sogoni *et al.*, 2021). The improvement in the absorption of Mg and Fe can contribute to the maintenance of energy transfer integrity and protein synthesis. In fact, this micronutrient plays an important role in the production of chlorophyll (20 to 25% of the total magnesium of the plant is located in the chloroplasts). Hence, this prevents the ions from interfering with the pathways of growth and metabolism and the reduction of negative impacts of salinity combined to fungal attack (Evelin *et al.*, 2009).

Conclusions

To conclude, in the present work, salt stress negatively affected *C. maritima* growth, water status, mineral nutrition, photosynthesis activity, nutrient uptakes and MDA concentrations. Similar and more pronounced negative effects were obtained when plants were inoculated by *A. alternata*. In the presence of salt conditions, inoculated halophyte plants exhibited better physiological performance than inoculated plants grown under salt-free conditions and even better than those non-inoculated (control). This ability to mitigate fungal attack can be related to the presence of salt in the medium culture of plants, and to the genetic patrimony of halophytes. Molecular investigations are required to unravel this exciting relationship between salt and biotic stress in halophyte which remained until now poorly understood.

Authors' Contributions

Conceptualization: AC and NJ; Data curation: HBJ and IR; Formal analysis: SBMH and HBJ; Funding acquisition: BSH; Methodology: AC and NJ; Supervision: BSH and CA; Validation: BSH and JJN; Writing original draft: BSH and PGC; review and editing: NB, JJN, SBMH, IR, AD. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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