Salinity thresholds and genotypic variability of cabbage (Brassica oleracea L.) grown under saline stress

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ABSTRACT

BACKGROUND: Two botanical varieties of cabbage, namely Savoy (*Brassica oleracea* var. *Sabauda* L.) and White (*Brassica oleracea* var. *Capitata* L.) were used in order to understand the morphological, physiological and biochemical elements of functional salt stress response. Thirteen salt concentrations (range 0 to 300 mM NaCl) were considered in Exp. 1# and out of them three (0, 100 and 200 mM NaCl) were used in Exp. 2#.

RESULTS: Exp. 1# enabled to define two salinity thresholds (100 and 200 mM NaCl), associated with morphological and physiological adaptations. In Exp. 2#, moderate salinity (100 mM NaCl) had lower effects on Savoy than in White cabbage yield (respectively – 16% and -62% from control). Concurrently, 100 mM NaCl resulted in a significant increase of antioxidant enzymes from control conditions, that was greater in Savoy (+289, +423 and +88% respectively) as compared to White (+114, +356 and +28% respectively) cabbage. Ions accumulation resulted to be a key determinant in tissue osmotic adjustment (mainly in Savoy) whereas the contribution of organic osmolites was negligible.

CONCLUSIONS: Higher antioxidative enzymatic activities in Savoy vs White cabbage upon 100 mM NaCl were associated with improved water relations, thus suggesting a possible physiological pathway for alleviating perceived salt stress.

Key words: salt stress, water relations, leaf gas exchange parameters, antioxidative enzymes, plant physiology.

INTRODUCTION

Salinity stress affects crop growth and yield by reduction of osmotic potential, alterations in plant metabolism, inhibition of enzymatic activities, ionic imbalance, disturbances in solute accumulation, specific ion effects or combination of all these factors.[1] Adverse effects on plant growth and development are experienced at physiological and molecular levels.[2,3] Osmotic adjustment helps plant cells to withstand salt stress and water deficit by maintaining a sufficient turgor for growth.[4] It involves the transport, accumulation, and compartmentalization of inorganic ions and organic solutes.[5] Under saline conditions, the osmotic withdrawal of water from growing cells may cause their turgor to drop below yield-stress thresholds. Cells must then develop a sufficiently low osmotic potential to reverse the flow of water, either through the uptake of ions from the medium or by the synthesis and transport of organic compounds; if none of these actions occur, cell expansion will stop.[6] Salt stress reduces gas exchange thereby limiting CO_2 supply to the leaf and causing the over-reduction of the photosynthetic electron transport chain, resulting in production of reactive oxygen species (ROS).[7] ROS are highly unstable compounds that can seriously disrupt normal metabolism through oxidative damage to lipids, proteins and nucleic acids in the absence of any protective mechanism.[8] The generation of ROS, including superoxide radical (O_2•-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), and singlet oxygen ($^1O_2$), is generally enhanced in salt stressed plants.[9] In order to cope with continuous ROS production, plants have a machinery of enzymatic and non-enzymatic antioxidants, which function as an extremely efficient cooperative system. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione reductase (GR), whereas glutathione (GSH), ascorbate (AsA), carotenoids and tocopherols are non-enzymatic antioxidants.[10] SOD is the front-line enzyme in ROS attack since it rapidly scavenges

superoxide, one of the first ROS intermediate to be produced, dismutating it to H_2O_2.[9] However, this reaction only converts one ROS to another, and H_2O_2 also needs to be removed since it promptly attacks thiol proteins and reacts with radicals and transition metals to yield the extremely reactive hydroxyl radical.[11] H_2O_2 is scavenged by peroxidase, especially ascorbate peroxidase (APX) and CAT.[8,12] GR, in the ascorbate/glutathione cycle, has a major role in maintaining the intracellular glutathione pool in the reduced state (GSH).[13] Plants can use the level of steady-state cellular ROS to monitor their intracellular level of stress.[8] However, this steady-state level must be tightly regulated in order to prevent an oxidative burst by over accumulation of ROS, which would ultimately result in extensive cell damage and death.[8] Symptoms of oxidative damage (like lipid peroxidation) have been used to assess the increase in ROS production under abiotic stresses.[8] However, the lack of symptoms is likely to result on the concomitant increase in cellular antioxidant defences. It is generally assumed that salt-sensitive genotypes have low levels of antioxidant enzymes,[14] although these levels are not necessarily an indicator of salinity tolerance.[15] White cabbage (*Brassica oleracea* var. *Capitata* L.) is a relatively salt-tolerant crop,[16] although variability among genotypes has been reported.[17] Consistently, previous reports showed that increased level of salt caused unbalanced nutrient uptake, declined germination, delayed emergence, inhibited seedling growth, root and shoot length, and fresh root and shoot weight in cabbage.[17] The objectives of this study were 1) the identification of salinity tolerance thresholds of a well-known, widely cultivated brassica (White cabbage) and 2) application of these thresholds to define the relative salinity tolerance of a closely related genotype (Savoy cabbage, *Brassica oleracea* var. *Sabauda* L.). This research work addresses the understanding of the adaptive mechanisms responsible for the differential response to salinity, comprising the assessment of the productive performances, the phytochemical, secondary metabolite, and enzymatic antioxidative systems in two botanical varieties of cabbage.

MATERIALS AND METHODS

The present research consisted of two experiments on cabbage. Exp. 1# was conducted in order to identify and confirm salt tolerance thresholds in White cabbage, whereas Exp. 2# used selected salt concentrations for determining differential elements of salt stress enzymatic response in two botanical varieties of cabbage, namely White (same genotype as in Exp. 1#) and Savoy. Both experiments were conducted in environmentally controlled conditions (T° max 23 °C; T° min 13 °C; RH: 60%) in the experimental glasshouse at the University of Bologna, Italy. Seeds were sown in polyethylene trays filled with peat moss and transplanted 20 days after germination onto 1 and 5 liters-pots (Exp. 1# and 2#, respectively) filled with a mixture of perlite and vermiculite (1:1, v:v). Plants were irrigated with a standard nutrient solution[5] having the following composition: NO_3^-: 16.5 mM; NH_4^+: 1 mM; $H_2PO_4^-$: 1.50 mM; SO_4^{2-}: 1.50 mM; K^+: 7.0 mM; Ca^{2+}: 5.0 mM; Mg^{2+}: 1.5 mM; Fe^{2+}: 15 μM; Mn^{2+}: 10 μM; B^+: 25 μM; Zn^+: 5.0 μM; Cu^+: 0.5 μM; Mo^{2+}: 0.5 μM. Salt stress treatments started on seedlings at 20 days after transplanting (DAT), with plants at seedling stage. All morphological measurements were performed at 20 and 50 days after salt (DAS) respectively in Exp. 1# and 2#. Exp. 2# was closed at commercial maturity of the plants. Experimental details are reported below.

Experimental design

In Exp. 1#, thirteen salt concentrations were applied and ranged 0 to 300 mM NaCl, in measure of 0 (2.68 dS m^{-1}), 25 (4.01 dS m^{-1}), 50 (6.33 dS m^{-1}), 75 (7.05 dS m^{-1}), 100 (7.68 dS m^{-1}), 125 (8.04 dS m^{-1}), 150 (8.35 dS m^{-1}), 175 (8.5 dS m^{-1}), 200 (8.72 dS m^{-1}), 225 (8.86 dS m^{-1}), 250 (9.21 dS m^{-1}), 275 (9.28 dS m^{-1}), and 300 (9.33 dS m^{-1}) mM NaCl dissolved in the

nutrient solution, replaced with fresh nutrient solution weekly. The experiment used a completely randomised design, with three replications and three plants per replicate.

In Exp. 2#, three salt concentrations were considered, 0 (2.68 dS m^{-1}), 100 (7.68 dS m^{-1}) and 200 (8.72 dS m^{-1}) mM NaCl dissolved in the nutrient solution that placed in 250 L three separated containers. The experiment used a strip-plot design, with three replications and six plants per replicate.

Plant growth determinations

Morphological determinations included head, root, shoot fresh (FW) and dry (DW) weights (after drying at 60°C until they reached steady weight), and leaf area (LA) on digital images by *Image J* processing software.[4] At harvest, roots and shoots of nine plants per treatment were dried and weighed and the root:shoot calculated. In Exp. 1# measures were limited to shoot and root FW and LA, whereas all morphological determinations were conducted in Exp. 2#.

Plant water relations and leaf gas exchanges

Leaf transpiration (*E,* in *mmol* m^{-2} s^{-1}), stomatal conductance (g_s, in *mmol* m^{-2} s^{-1}) and net photosynthesis (*A,* in μ*mol* m^{-2} s^{-1}) were measured 15 days after salinization on three completely unfolded leaves of nine plants per treatment. Measurements of leaf gas exchange were performed on attached leaf samples using a CIRAS-2 (PPSystem, Hitchin, UK) infrared gas analyser (closed system) with a Parkinson's Automatic Universal Leaf Cuvette (PAR 1000 mmol m^{-2} s^{-1}, 26°C, CO_2 13.63 mmol l^{-1} and 300 cm^3 min^{-1} flow rate) equipped with 18-mm diameter, 2.5-cm^2 area cuvette inserts. Water Use Efficiency (*WUE*) was determined as the ratio between *A* and *E*.

Water (Ψ_w) and osmotic ($\Psi\pi$) potentials were measured on fresh and frozen/thawed leaf samples using a dewpoint potentiometer (WP4, Decagon Devices, Pullman, WA, USA). Osmotic adjustment (OA) was calculated using the equation:

$$OA = \Psi_{\pi 0}\, V_0 - \Psi_\pi\, V \text{ (MPa)},$$

Where $\Psi_{\pi 0}\, V_0$ is the product of (osmotic potential) × (osmotic volume) of unstressed plants and $\Psi_\pi\, V$ is the product of (osmotic potential) × (osmotic volume) of leaves from salinized plants.[5]

In Exp. 1# measures were limited to leaf gas exchanges, whereas all physiological determinations were conducted in Exp. 2#.

Ion accumulation in plant tissue

In Exp. 2# only determination of cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) and anions (Cl^-, NO_3^-, and SO_4^{2-}) was carried out on dry weight basis.[5] Briefly, 500 mg of leaves dry matter were suspended in 50 ml water and homogenized with a stirrer at 150 rpm for 20 minutes. Samples were then filtered using filter paper (589 Schleicher) and then the extracts were further filtered through cellulose acetate syringe filters (0.20 µm). For cations analysis, the filtrated extract was acidified with 65% nitric acid HNO_3 (1:100 ml, v: v) and quantification of cations was performed using Inductive Coupled Plasma Optical Emission Spectrometry (ICP-OES). Anions determination was performed by ion chromatography (IC).

Biochemical determinations

In Exp. 2#, protein[18] and antioxidant enzymes[19] were determined. Extraction was conducted from 10 g of fresh leaves homogenized in 10 ml of 200 mM chilled potassium-phosphate buffer (pH 7.5) containing 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) and 0.1% (v/v) Triton X-100 placed in an ice bath. The homogenate was filtered through a layer of

muslin cloth and centrifuged at 10000 × *g* for 20 minutes at 4 °C. The supernatant was collected and eluted through Sephadex G-25 gel column (NAP-25, Amersham Biosciences, Piscataway, NJ, USA) then re-suspended in 10 mM sodium-potassium phosphate buffer (pH 7.0) and used for the determination of the antioxidant enzymes. All enzymatic activities were assayed spectrophotometrically, the analysis was performed in triplicate and the results were normalized by plant fresh weight.

Soluble proteins concentration of the extract was estimated according to Bradford's method using bovine serum albumin as a standard.[18]

The level of lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid-reactive substance (TBA) method. For MDA extraction, 100 µl aliquot of enzyme extract was mixed with 900 µl thiobarbituric acid solution containing 0.5 % (w/v) 2- thiobarbituric acid and 0.5 M orthophosphoric acid. The mixture was heated in a water bath at 100 °C for 30 minutes then the reaction was quickly stopped by cooling the tubes in an ice water bath. Afterward, the mixture was centrifuged for 1 minute at 13000 × *g* to remove the unspecific turbidity. The absorbance of the supernatant was measured at 532 nm using spectrophotometer Cary-1 (Varian, California, US). Measurements were corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The amount of MDA-TBA complex (red pigment) was calculated from the difference of the two wavelengths based on standard curve of MDA.

Ascorbate peroxidase (APX, EC 1.11.1.11) was determined using an ascorbate reaction solution containing 50 mM sodium-potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid, 0.5 mM hydrogen peroxide and 100 µl enzyme extract in a final assay volume of 1 ml. Ascorbate oxidation was followed at 290 nm. The concentration of oxidized ascorbate was calculated using an extinction coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One unit of APX is defined as the enzyme activity catalysing the oxidation of 1 µmol of ascorbic acid per minute.

Catalase activity (CAT, EC 1.11.1.6) was assayed by measuring the initial rate of disappearance of H_2O_2. Catalase reaction solution contained 50 mM sodium-potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and 20 µl of enzyme extract in a final assay volume of 1 ml. The reaction was initiated by adding the enzyme extract and the decrease in H_2O_2 was measured following the changes in the absorbance of the reaction solution at 240 nm. The concentration of CAT was calculated using an extinction coefficient $\varepsilon = 0.036\ mM^{-1}\ cm^{-1}$. One unit of CAT is defined as the enzymatic activity that catalyses the degradation of 1 µmol of H_2O_2 per minute.

Glutathione reductase (GR, EC 1.6.4.2) was determined using a reaction solution containing 50 mM sodium-phosphate buffer (pH 7.5), 5 mM EDTA, 1mM (NADPH), 1 mM oxidized glutathione (GSSG) and 300 µl enzyme extract in a final assay volume of 1 ml. NADPH oxidation was determined at 340 nm. Activity was calculated using an extinction coefficient $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$ for NADPH. One unit of GR is defined as the enzyme activity that oxidizes 1 µmol of NADPH per min at room temperature.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring its ability to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) to blue formazan by flavins under illumination.[19] Superoxide dismutase reaction solution contained 50 mM sodium-potassium phosphate buffer (pH 8.0), 300 µM methionine, 1.5 mM NBT, 120 µM riboflavin, 100 mM Na_2.EDTA, 300 µM potassium cyanide and 100 µl enzyme extract in a final assay volume of 1 ml. The riboflavin was added last. The reaction was started by illuminating the test tubes under 4 fluorescent lamps for 10 minutes. The absorbance of illuminated solution was measured spectrophotometry at 560 nm. One unit of SOD activity is defined as the amount of enzyme that inhibited 50% of NBT photoreduction versus a blank cell containing no enzymatic extract.

Free proline content was determined using a reaction solution containing 3 mM ninhydrin in 60% (v:v) acetic acid.[20] Samples were heated at 100 °C for 1 h in water bath, then cooled under tap water to stop the reaction and the mixture was extracted with toluene and the absorbance of toluene fraction aspired from liquid phase was read at 520 nm. Proline concentration was determined against a calibration curve and expressed as μmol proline g^{-1} FW.

Photosynthetic pigments chlorophyll *a*, *b*, xanthophylls and carotenes were determined on 0.1 g fresh leaves extracted in 5 ml of chilled 80% (v:v) acetone. The homogenate was centrifuged at 4000 × *g* for 5 min at 4 °C. The absorbance of resulting supernatant was taken at 470, 645, 663 nm. Different pigments were estimated using the following equations:[21]

Chlorophyll a = 12.7 (A_{663}) − 2.69 (A_{645})

Chlorophyll b = 22.9 (A_{645}) − 4.68 (A_{663})

C_{x+c} = =1000 (A_{470}) −1.9×chl *a*−63.14× chl *b* /214 (x= xanthophylls and carotenes)

Statistical analysis

Data were analysed using analysis of variance (ANOVA) by Co-Stat-ANOVA software (CoHort, Monterey, CA, USA). At least three replications per treatment per genotype were used for analysis of all parameters. Treatment means were compared using Student-Newman-Keuls at 5% significance. In Exp. 1#, data were plotted and response functions were identified by datasets with significant linear regression, by limiting the number of data considered when additional data would reduce significance.

RESULTS

Exp. 1#

Plant growth and leaf gas exchanges

In Exp. 1#, upon salinity both plant morphological and physiological performances were decreased (Fig. 1), with exclusion of *WUE*, which increased up to 175 mM NaCl and was decreased beyond. However, in response to the thirteen salinity levels considered, three different linear functions were observed for all studied parameters (Fig. 1). Greatest reducing slopes were observed between 0 and 100 mM NaCl, whereas, above 100 mM NaCl, increasing salinity resulted in lower effects on crop performances. Nevertheless, another threshold could be observed around 200 mM NaCl, above which most of the measured parameters would not be anymore affected.

Exp. 2#

Plant growth and leaf gas exchanges

In Exp. 2#, genotype, salinity and their interaction significantly affected growth parameters (Table 1). Under control conditions, no differences among genotypes were found in terms of head FW (138 g $plant^{-1}$), LA (6892 cm^2 $plant^{-1}$), net photosynthesis (*A*, 17 μmol m^{-2} s^{-1}) and *WUE* (13 μmol CO_2 mmol H_2O) (Tables 1 and 2, Fig. 1). White cabbage presented greater root FW (+44%) as well as leaf transpiration (*E*, +34%) and stomatal conductance (g_s, +150%) as compared to Savoy cabbage (Tables 1 and 2). On the other hand, Savoy cabbage presented greater root/shoot (+17%) as compared to white cabbage.

Yield of both genotypes decreased significantly upon salinity. However, while 100 mM NaCl caused a 62% reduction in head weight in white cabbage, no significant reduction from control was appreciated in the Savoy cabbage genotype. On the other hand, a 70% decline in

head weight was measured in both genotypes when 200 mM NaCl were supplied (Table 1). Root growth was also impaired by 200 mM NaCl (similarly by 83% in the two genotypes). However, under 100 mM NaCl reduction of root FW from control was greater in white (-81%) than in Savoy (-41%) cabbage. Salinity depleted leaf area more dramatically in White cabbage as compared to Savoy cabbage at 100 mMNaCl (-52% and 25%, respectively, from control conditions). However, at 200 mM NaCl no differences among genotypes were appreciated (average reduction 66% from control). Consistently, leaf area was greater (1.6 folds) in Savoy as compared to White cabbage when 100 mM NaCl were supplied (Table 1). Salinity-induced reductions were appreciated in terms of root:shoot ratio in both genotypes (Table 1). Unstressed White cabbage plants exhibited expressively higher values of transpiration rate (E) and stomatal conductance (g_s) as compared to Savoy cabbage plants (1.6 vs 1.2 mmol m^{-2} s^{-1} and 115 vs 46 mmol m^{-2} s^{-1} respectively) (Table 2). However, the transpiration rate of White cabbage plants was more susceptible to 200 mM NaCl as compared to Savoy cabbage (reduction from control conditions in measure of -66% and -49% respectively). Similarly, net photosynthesis (A) was lower in White cabbage plants as compared to Savoy under 100 (-47%) and 200 (-44%) mM NaCl.

Leaf water potential was reduced in both genotypes upon salinization (Fig. 2A). However, such reduction was greater in the Savoy cabbage at both salt levels (-1.40 and -1.67 MPa, at 100 and 200 mM NaCl) as compared to the White one (-0.75 and -1.42 MPa). Similarly, the highest osmotic potential reduction at 100 and 200 mM NaCl was observed in the Savoy cabbage (-1.74 and -2.13 MPa) relatively to the white one (-1.36 and -1.80 MPa) (Fig. 2B). Higher osmotic adjustment values were achieved in Savoy cabbage plants (0.7 and 1.0 MPa) compared to the white one (0.2 and 0.4 MPa) at 100 and 200 mM NaCl (Fig. 2C). Likewise, Savoy cabbage presented higher value of *WUE* under 100 mM NaCl (3-fold higher than White cabbage, 26 versus 9 μmol CO_2 mM^{-1} H_2O) (Fig. 2D).

Mineral solutes accumulation

The concentration of ions varied between the two *Brassica* genotypes and among salinity treatments (Fig. 3). Under control conditions most measured ions (Na^+, K^+, Cl^-, SO_4^{2-} and NO_3^-) were similarly accumulated in both genotypes (Fig. 3). However, Ca^+ and Mg^+ presented greater accumulation in White cabbage leaves (respectively, 3- and 2.7- fold increase, Fig. 3). Imposing salt stress significantly induced an increase in Na^+ concentration in leaves of the two genotypes compared to unstressed plant (Fig. 3A). While a similar Na^+ concentration was found in both genotypes upon 100 mM NaCl, its accumulation was greater in Savoy cabbage (+23-fold as compared to control plants) undergoing 200 mM NaCl, whereas lower increases (+12-fold) were observed in the White cabbage (Fig. 3A). Salinized condition did not affect the Ca^{2+} concentration in Savoy cabbage as shown by the non-significant difference under different salt treatments (Fig. 3B). In contrary, White cabbage plants showed a drastic diminishing in Ca^{2+} ions by -78% and -93% under 100 and 200 mM NaCl in relation to control plants (Fig. 3B). The K^+ concentration of the Savoy cabbage leaves increased by +268% and +116% under 100 and 200 mM NaCl respectively, as compared to control plants, while there was no significant effect of salt treatment in White cabbage leaves (Fig. 3C). Similarly, the Mg^{2+} content in the leaves of Savoy plant increased significantly by +75% and +35% respectively in response to increasing salinity (100 and 200 mM NaCl) (Fig. 3D). Nevertheless, White cabbage plants exhibited a significant reduction in Mg^{2+} content under both salt levels (mean reduction -66% as compared to control conditions). The Na^+/K^+ in Savoy cabbage leaves was not affected by moderate salinity (100 mM NaCl), whereas a dramatic increase was observed at 200 mM NaCl and under both 100 and 200 mM NaCl in White cabbage plants (Fig. 3E). Cl^- concentration was lowest under both 0 and 100 mM NaCl in Savoy cabbage, and under 0 mM NaCl in White cabbage. A

significant increase from control conditions was recorded under 200 mM NaCl in Savoy cabbage and 100 mM NaCl in White cabbage. Finally, greatest Cl^- values (more than 4-fold from control) were observed in White cabbage undergoing 200 mM NaCl (Fig. 3F). SO_4^{2-} concentration was similarly reduced by salinity in both genotypes (Fig. 3G). NO_3^- concentration experienced significant increasing altogether with salinity in both genotypes (Fig. 3H). However, White cabbage leaves accumulated more NO_3^- content by 2.3-fold as compared to Savoy cabbage upon 100 mM NaCl.

Biochemical response to salinity

Under control conditions, APX, CAT and GR enzymatic activities and MDA, proline, protein and photosynthetic pigments were not affected by genotype, whereas SOD was greater in Savoy as compared to White cabbage (1.6 fold increase) (Figs. 4-6).

APX activity in the leaves of Savoy plant was remarkably increased (+123%) as compared to White cabbage plants upon 100 mM NaCl treatment (Fig.4A). However, similar values of APX in the two genotypes were observed under both control and 200 mM NaCl. Similarly, CAT activity was about 2.5-fold higher in Savoy compared to White cabbage leaves at 100 mM NaCl (Fig.4B), whereas no genotypic differences appeared either under control or 200 mM NaCl (Fig.4B). Glutathione reductase (GR) activity showed a rather constant level in White cabbage plants under control and salinized conditions (avg. 69 U g^{-1} FW), while there was registered an increase of GR at 100 mM NaCl (Fig.4C). SOD activity increased similarly with salinity, and no genotypic differences could be observed at the studied salinity levels (Fig.4D). Lipid peroxidation level in Savoy leaves (measured as MDA content) was increased at 100 and 200 mM NaCl (+201 and +94% from control conditions), whereas no changes would be observed in White cabbage plants (Fig.5A). Salinity enhanced leaf proline accumulation similarly under both salt levels (Fig.5B). No significant responses in protein

concentration could be attributed to either genotype or salinity (Fig.5C). Both chlorophyll *a* and *b* were significantly increased by salinity in Savoy plants, whereas no changes were observed in White cabbage plants (Fig. 6A and B). Consistently, xanthophylls and carotenes content decreased significantly in Savoy cabbage (-43%) at 200 mM NaCl as compared to control plant, while no salt-induced changes could be observed in White cabbage (Fig. 6C).

DISCUSSION

Identification of salinity tolerance thresholds in White cabbage

Results from Exp. 1# enabled to identify two salinity response thresholds in White cabbage, respectively at 100 mM NaCl (moderate salinity threshold) and 200 mM NaCl (high salinity threshold). In response to salinity, plants are expected to respond by a linear function where the slope in yield response to EC is associated to species-specific EC threshold.[22] Maggio et al.[23] proposed that the relationship between yield and salinity was represented by a bilinear response function, suggesting the existence of a *second* physiological threshold. Such thresholds may be used to identifying functional shifts between different adaptation mechanisms. A first threshold resulted to be at 100 mM NaCl in White cabbage, where most of the measured parameters showed a change in the decreasing slope when further salt was applied (Fig. 1). Furthermore, in the present study, although yield decline occurred yet at low salinities, another threshold (above which the plant still survives although at basal physiological functions) was identified for cabbage at 200 mM NaCl (Fig. 1). Beyond this concentration, additional salinity would not result in significant decrease in most of the measured parameters. Consistently, in the second experiment 0, 100 and 200 mM NaCl concentrations were used.

Genotypic variability in cabbage physiological and morphological responses to salinity

The greater yield (Table 1) of Savoy *vs* White cabbage plants under moderate salinity (100 mM NaCl) is consistent with the concept of a strict genotype-related response to salinity even within the same family (*Brassicaceae*): even close relatives may show great differences in the capability and means to cope with unfavorable growing conditions.[24] Furthermore, at 100 mM NaCl, not only the cabbage head was preserved (Table 1), but also root biomass and leaf development were scarcely affected in Savoy plants confirming that the stress perceived by these plants was negligible. Recent reports on a wide range of vegetable crops [4,25-28] have pointed out that the capability of the plant to cope with salinity through functional physiological down-regulation may result in preservation of the shoot biomass and, consequently, crop yield. The general reduction of root/shoot upon salinity may be interpreted as a way for restricting the uptake of toxic ions to the shoot while still maintaining plant growth and physiological functions.[26] This may be accomplished by simultaneous reducing root versus shoot development and activating specific metabolic pathways (i.e., osmolyte biosynthesis), both of which occur in saline environments.[15] Based on these considerations, Savoy appeared to be relatively more tolerant than White cabbage at 100 mM NaCl.

Leaf gas exchanges are generally impaired upon salt stress. This reduction is associated with salt damage of the photosynthetic tissue, changes in stomatal features with the consequent restriction of the CO_2 availability for carboxylation or to the acceleration of senescence.[25] The reduced g_s and E observed in Savoy plants, as compared to White cabbage yet under control conditions (Table 2), most likely protected them from tissue dehydration and allowed them to effectivelly adjust to the unfavorable conditions by minimizing transitory cellular turgor loss. [24] The reduction in transpiration with salinity has been associated with reduced g_s and lower stomatal density of leaves developed under saline conditions.[27] Nonetheless, while similar A values were found in the two genotypes grown under control conditions (Table 2), a

greater reduction in photosynthesis was associated to salinity in White cabbage as compared to Savoy. Although salt-induced reductions of *A* are commonly associated to impaired stomatal opening,[4] recent reports suggest that preserved *A* values associated with lower g_s or *E* may be considered as reliable indicators of overall salinity tolerance,[4,26] and this is substantiated by the greater *WUE* (Fig. 2) observed in salt tolerant plants undergoing salinity.[28] The most severe changes in plant water potentials observed in Savoy plants may be the result of the structural-functional changes operated by the plant in order to ensure successful adaptation to salinity.[15] The reduction of the osmotic potential (Fig. 2) would therein be a consequence of the net increase in solute accumulation which occurs through uptake of solute and/or synthesis of organic compounds in a process called osmotic adjustment.[15,29] Osmotic regulation, a phenomena that occurs in both roots and leaves, contributes to maintain water uptake and cell turgor, which are essential to sustain physiological processes such as cell expansion, stomatal opening, photosynthesis.[30] However, a process of osmotic self-adjustment occurs in the plant cells, directed towards the preservation of the water balance by means of accumulation of osmotically active solutes.[15] In the hereby presented experiment, Savoy plants were able to better preserve the turgor and regulate their osmotic adjustment compared to White cabbage (Fig. 2). Thus, also from a physiological perspective, the adaptation of leaf gas exchanges and overall water relations appeared to be more effective in Savoy as compared to White cabbage plants.

Salinity, ion accumulation, and biochemical response in plant tissue: potentials for improved nutritional quality in stressed plants

It is known that deleterious effects of salinity are related to osmotic effects, ion toxicities and ionic imbalance.[15] Under salt stress, plants evolved complex mechanisms allowing for adaptation to osmotic and ionic stress. These mechanisms include osmotic adjustment by

accumulation of compatible solute and lowering the toxic concentration of ions in the cytoplasm by restriction of Na^+ influx or its sequestration into the vacuole and/or its extrusion.[4] In this study, sodium accumulation was enhanced in both *Brassica* genotypes when the plants were exposed to salt and showed similar pattern of Na^+ accumulation (Fig. 3A). However, the vast accumulation of Na^+ in relatively salt tolerant Savoy plant at moderated salt demonstrated that salinity resistance of this species is not linked to their ability to restrict the uptake and/or transport of sodium accumulation into the aerial parts.[27] K^+ has an important role in osmotic adjustment in the guard cell controlling the stomata movement and thus CO_2 assimilation in photosynthesis.[15, 22] Moreover, K^+ is considered to be an effective agent in plant salt tolerance mechanisms through maintenance of Na^+/K^+ homeostasis[31] and osmoregulation.[4] A range of studies indicate that an increase in concentration of K^+ and Ca^{2+} in plants under salt stress could ameliorate the deleterious effects of salinity on growth and yield.[4, 22, 26] In this study, K^+ was the major inorganic ion that accumulated significantly in salt stressed Savoy cabbage while there was no alteration in its concentration in the relatively salt sensitive White cabbage (Fig. 3C). These results suggest that under salt stress, Savoy plant may use K^+ for osmotic adjustment, given that K^+ accumulation plays a key role in salt tolerance mechanism of *Brassica* species by maintaining ion homeostasis.[31] However, under salt stress, plants maintain high concentrations of K^+ in the cytosol by regulating the expression and activity of K^+ and Na^+ transporters and of H^+ pumps that generate the driving force for transport.[32] Ca^{2+} plays an essential role in processes that preserve the structural and functional integrity of plant membranes, stabilize cell wall structures, regulate ion transport and selectivity, and control ion-exchange behavior as well as cell wall enzymatic activities.[32] Salinity dominated by NaCl causes instability of plasma membrane resulting from Ca^{2+} displacement by Na^+,[31] reduces Ca^{2+} availability and mobility to growing regions of the plant, produces extreme ratios of Na^+/Ca^{2+} in the plants which

increase the plants susceptibly to osmotic and specific ion injury as well as to nutritional disorders that result in reduced yield and quality.[26] In the hereby presented experiment, White cabbage exhibited sharp diminishing in Ca^{2+} content associated with increased salt stress (Fig. 3B). The reduction in the concentration of this cation may be related to a lower Ca^{2+} release into the root xylem because of an effect of active loading of these cations into the xylem vessels.[15] However, Ca^{2+} concentration in leaves of Savoy plant was not affected by increasing salt supply, which was consistent with many previous studies pointing out that an increase in Ca^{2+} concentration in plants challenged with salinity stress could ameliorate the inhibitory effects of salinity on growth.[33] In addition, Mg^{2+} was significantly increased in both levels of stressed Savoy cabbage (+55%, as an average), as compared to unstressed plants. Concurrently, the concentration of this ion depressed drastically in White cabbage by 2.6-fold and 3.5-fold respectively under 100 and 200 mM NaCl (Fig. 3D). This result might suggest the presence of membrane selectivity in Savoy cabbage towards ions uptake and accumulation which might be utilized in lowering its osmotic potential as a way to cope with stressed condition. Many studies on halophytes and some tolerant glycophytes showed that a low foliar Na^+/K^+ ratio is a salt tolerance index and a good indicator of salt tolerance.[34] In the present study, leaf Na^+/K^+ was significantly higher in moderately salinized plant of White cabbage by 4-fold as compared to Savoy (Fig. 3E). It might be possible that the internal accumulation of K^+ ions in salt stressed Savoy cabbage reduced the Na^+/K^+ which improved the plant salt tolerance. Besides, there are different factors that affect Na^+/K^+ homeostasis such as different gene resources and over expression of K^+-related genes.[32] Salt tolerance in plants is usually associated with the ability to restrict the uptake and/or transport of saline ions from root to shoot.[5] At moderated salt stress, Cl^- concentration in the leaves of relatively tolerant Savoy plants remained similar to those found under control conditions, while its content increased significantly by 2.4 times in White cabbage as compared to control plants

(Fig. 3F). Therefore, the salinity resistance of Savoy plant could be related to Cl^- exclusion. However, the accumulation of Cl^- in the leaves of both genotypes was considerably enhanced with imposition of 200 mM NaCl to the rooting medium and a considerable difference between cultivars was observed (915 mg kg^{-1} DW in Savoy versus 1445 mg kg^{-1} DW in White cabbage). Excessive accumulation of Cl^- results in ion toxicity and growth inhibition.[15] Accordingly, the higher inhibition of growth parameters that was observed in White cabbage could be related to high concentration of Cl^- (Table 1 and Fig. 3F). There was a considerable reduction in NO_3^- content in White cabbage plant at 200 mM NaCl. This reduction was associated with vast accumulation of Cl^- ions in leaves (Fig 3 F and H). Theoretically, the reduction of NO_3^- uptake might be related to a decrease in the nitrate reductase activity (NAR) as a consequence to the presence of Cl^- salt in the external medium.[35]. According to the hereby presented results, the White cabbage behaved similarly to rocket, a member of *Brassicaeae* family, as a nitrate-accumulation vegetable (up to 4300 mg Kg^{-1} FW).[36] The effects of nitrate and its toxic metabolites on human health have been documented.[36] Therefore, decreasing leaf nitrate concentration is critically important for fresh healthy vegetable production.[36] Under moderated salinity, the turgor was maintained and osmotic adjustment was achieved in the relatively salt tolerant Savoy plant by accumulated higher amounts of Na^+, K^+ and Mg^{2+} values, resulting in lower osmotic potential and higher osmotic adjustment (Fig. 2, 3). Consistently, salt stress tolerance in this species may be associated with ion accumulation.[16]

The alleviation of oxidative damage and increased resistance to salinity and other environmental stresses is often correlated with an efficient antioxidative system.[29] In this study, the activities of the four key antioxidant enzymes (APX, CAT, SOD and GR) appeared to be substantially affected by both salinity and genotypes under assessment (Fig. 4), although each of them showed a specific quantitative and qualitative response. Similarities, as

for other physiological and morphological traits, were generally found among the genotypes either at control conditions or when 200 mM NaCl were supplied. On the other hand, under 100 mM NaCl completely diverse scenarios could be attributed to the two genotypes under study. At this salt concentration, APX, CAT, and GR in Savoy resulted to be about 2.2, 2.5 and 1.3-fold respectively greater than in White cabbage (Fig.4A, B and C). CAT and APX are major enzymes detoxifying hydrogen peroxide.[37] Salinity-induced increase in APX has previously been reported.[37] Low GR values have been associated to stress sensitivity.[37] SOD and CAT activities decreased in roots of a salt-sensitive tomato cultivar while they increased in the roots of a salt-tolerant one under salt stress.[38] Salt tolerant *Brassica juncea* cv. Bio902 had a higher activity of SOD, APX, and CAT and showed higher capacity for the scavenging ROS generated by salt in comparison with cv. Urvashi.[37] Furthermore, it was showed[39] that SOD activity in leaves of cauliflower (*Brassica oleracea* var. *Botrytis* L.) increased first at lower salinity (34, 68 and 102 mM NaCl) and then decreased at higher salinity (136 and 170 mM NaCl), while CAT activities changed reversely with SOD. Other author suggested that increased activities of SOD, APX and GR were responsible of the increased tolerance to salinity in two rice cultivars, while those enzymes declined or were not affected by salinity in salt sensitive rice.[40] The observed increased SOD activity (Fig.4D) in both genotypes might be advocated as a common strategy to scavenge O_2^- and counteract membrane damage. Although antioxidative response is generally associated to greater stress tolerance,[12] higher antioxidant capacity is not necessarily an indicator of the overall plant tolerance. Furthermore, care should be taken when correlating genotypic variability in salt stress response and the relative antioxidant system capability to detoxify ROS.[37] Likewise, salt stress experiments on mutant *Nicotiana tabacum* plants lacking in both APX and CAT showed that plants appeared to be less susceptible to oxidative stress.[41] The observed salt tolerance of Savoy genotype may be partially attributed to greater CAT and APX activity

resulting in improved detoxification of H_2O_2 to H_2O, coordinated by the additional effect provided by the increased GR activity. Consistently, the present study suggests active involvement of at least catalase and peroxidase among the H_2O_2 scavenging enzymes in determining salinity tolerance of cabbage.

Lipid peroxidation (measured as the amount of MDA produced) is the symptom readily ascribed to oxidative damage and is often used as an indicator of oxidative stress which varies in different plant species.[37] Free radicals may induce peroxidation of lipid membrane, which may also reflect stress induced damages at the cellular level.[37] Greater levels of MDA (as observed in Savoy plants under 100 mM NaCl, Fig. 5A) may be associated to the higher osmotic adjustment observed in leaf tissue of the same plants, suggesting that excess salt accumulation triggered the production of ROS which caused the oxidative damage of plasma membrane. Similarly, MDA contents in four cauliflower cultivars were increased gradually with increasing NaCl concentrations.[39]

Proline is generally assumed to serve as a physiologically compatible solute that increases as needed to maintain a favourable osmotic potential between the cell and its surroundings.[24] In response to drought or salinity stress in plants, proline accumulation normally occurs in the cytosol where it contributes substantially to the cytoplasmic osmotic adjustment.[26] In addition to its role as an osmolyte for osmotic adjustment, proline contributes to stabilization of sub-cellular structures (e.g. membranes and proteins), scavenging free radicals, and buffering cellular redox potential under stress conditions. In the present experiment, there was a similar significant increase in proline concentration upon salinization in both genotypes (Fig. 5B). Consistently proline seemed not to play a determinant role as osmoprotectant, confirming that increased proline concentration may not be associated with salinity tolerance in *Brassicaceae*. Although soluble protein content is often claimed to be an important indicator of the plant physiological status,[25] in the hereby presented experiments, the contribution of amino acids

accumulation to osmotic adjustment was not significant (Fig. 5C). Consistently, it may be confirmed that amino acids are not the main organic solutes involved in the osmotic adjustment of cabbage plants. Savoy plants presented greater osmotic adjustment in response to salinity as compared to White cabbage plants, indeed soluble protein and proline concentrations appeared to be not dependent on genotype. These results were consistent with previous authors[18, 26, 32, 42] who mentioned that *Brassica* stress tolerance is associated with ions accumulation only, whereas other report stated about a possible accumulation of organic compounds other than ions towards combined drought and salt stresses.[43]Although higher levels of soluble protein have been reported in salt tolerant cultivars of barley, sunflower, finger millet and rice,[37] it shall be considered that the production of osmolytes is metabolically expensive and limits plant production by consuming significant quantities of carbon.[44] An alternative pathway is provided by the accumulation of a high concentration of ions from the external medium, solution that results in lower energetic cost for the plant, although it may lead to a toxic effect on the normal biochemical activities within the cell.[15] Accordingly, we could correlate the tolerance to salt stress in Savoy plant to its ability to maintain self-osmotic adjustment in terms of inorganic ions substances accumulation through uptake of solute, while the synthesis of organic compound was not achieved as protein and proline accumulated in similar pattern in both the salt-tolerant and -sensitive species.

Savoy plant experienced noteworthy increases in photosynthetic pigments (chlorophyll *a* and *b*), while White cabbage plant showed no variation upon salinization (Fig. 6A and B). Higher chlorophyll content may be associated to greater photosynthetic rates, as well to the functional state of leaf tissues, which depends on the content of photosynthetic pigments, the synthesis of the enzymes taking part in the carbon reduction and the formation of the membrane system of chloroplasts. Chlorophyll *a* content has been related to salt tolerance in *Panicum miliaceum*.[45] Increased total chlorophyll was recorded in *Cucumis sp.,* broad bean

and rice plant undergoing salt stress.[46] On the other hand, many reports associated the salt-induced damages occurring at cell and tissue level to the reduction in photosynthetic pigments, chlorophyll *a* and *b* in different crops such as alfalfa,[47] sunflower,[48] and wheat.[49] In the Savoy genotype, the enzymatic adaptation to salinity may have counteracted the ROS induced damages at cell level, thus resulting in greater photosynthetic pigments in the leaves and overall improved crop response to salinity.

CONCLUSIONS

The response to salinity of two botanical varieties of cabbage, namely Savoy cabbage and White cabbage was hereby addressed. Savoy plants were not only more tolerant to the stress than White cabbage in term of yield, but also operated functional physiological and biochemical adaptation that resulted in improved plant status and increased nutritional value. Higher activities of APX, CAT, and GR were observed in Savoy plants undergoing 100 mM NaCl, resulting in greater detoxification of ROS together with the maintenance of lower water potential and higher osmotic adjustment by accumulation of higher amounts of K^+ and $Mg^{2+}$and lower level of Cl^- and NO_3^-. These combined factors played a functional role in alleviated salt stress in Savoy plant.

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FIGURES LEGEND

Fig. 1. Identification of salinity tolerance threshold in cabbage plants by means of shoot FW (A), root FW (B), Plant LA (C), stomatal conductance (*gs*, D), net photosynthesis (*A,* E), and water use efficiency (*WUE*, F) upon variable salinity (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275 and 300 mM NaCl). Values are the mean ± SE of three replications. Data from Exp. 1#.

Fig. 2. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (White) on Savoy and White cabbage plants at 15 DAS on water potential (Ψw), osmotic potential (Ψπ), osmotic adjustment (OA), and water use efficiency (*WUE*). Values are the mean ± SE of nine independent measures. Data from Exp. 2#.

Fig. 3. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (White) on the accumulation of some cations (Na^+, Ca^{2+}, K^+, Mg^{2+}) and anions (Cl^-,SO_4^{2-}, NO_3^-) and the Na^+/K^+ on Savoy and White cabbage leaves at 50 DAS. Values are the mean ± SE of nine independent measures. Data from Exp. 2#.

Fig.4. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (White) on ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) activities in leaves of Savoy and White cabbage plants. Values are the mean ± SE of three replications. Data from Exp. 2#.

Fig.5. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (White) on malondialdehyde (MDA) and proline level in leaves of Savoy and White cabbage plants. Values are the mean ± SE of three replications. Data from Exp. 2#.

Fig.6. Effect of zero salt (black), 100 mM NaCl (grey), and 200 mM NaCl (White) on chlorophyll *a* and *b* and *x*anthophylls and carotenoid content in leaves of Savoy and White cabbage plants. Values are the mean ± SE of three replications. Data from Exp. 2#.

Table 1. Effect of different salt content (0, 100, and 200 mM NaCl) on some growth parameters of Savoy and White cabbage plants at 50 DAS. Same letters in each column indicate no significant differences among treatments at $P \leq 0.05$ level. Values are the mean ± SE of three replications. Data from Exp. 2#.

Cultivar	NaCl (mM)	Head FW (g plant^{-1})		Root FW (g plant^{-1})		LA (cm^2 plant^{-1})		Root/Shoot	
Savoy	**0**	109±3.51	a	41±4.93	b	7009±239	a	0.7±0.03	a
	100	92±4.36	ab	24±1.00	c	5232±369	b	0.2±0.01	c
	200	33±1.15	d	7±0.58	d	2652±174	cd	0.1±0.01	c
White	**0**	167±7.57	a	59±2.33	a	6775±295	a	0.6±0.07	b
	100	63±1.76	c	11±1.15	d	3227±149	c	0.1±0.01	c
	200	51±3.21	c	10±0.88	d	2082±310	d	0.2±0.03	c
Salt (S)		***		***		***		***	
Var (V)		*		*		**		*	
S × V		***		***		*		ns	

Table 2. Effect of different salt content (0, 100, and 200 mM NaCl) on gas exchange parameters of Savoy and White cabbage plants at 15 DAS. Same letters in each column indicate no significant differences among treatments at $P \leq 0.05$ level. Values are the mean ± SE of eight replications. Data from Exp. 2#.

Cultivar	**NaCl (mM)**	***E*** **(mmol m^{-2} s^{-1})**		***g_s*** **(mmol m^{-2} s^{-1})**		***A*** **(µmol m^{-2} s^{-1})**	
Savoy	**0**	1.2±0.13	b	46±5.7	b	17±0.9	a
	100	0.4±0.04	c	15±1.3	c	9±0.7	b
	200	0.6±0.05	c	17±1.5	c	6±0.2	c
White	**0**	1.6±0.08	a	115±7.4	a	17±1.2	a
	100	0.6±0.08	c	21±3.1	c	5±0.5	cd
	200	0.5+0.03	c	17±1.3	c	3±0.4	d
Salt (S)		***		***		***	
Var (V)		*		***		**	
S × V		*		***		**	

FIGURES

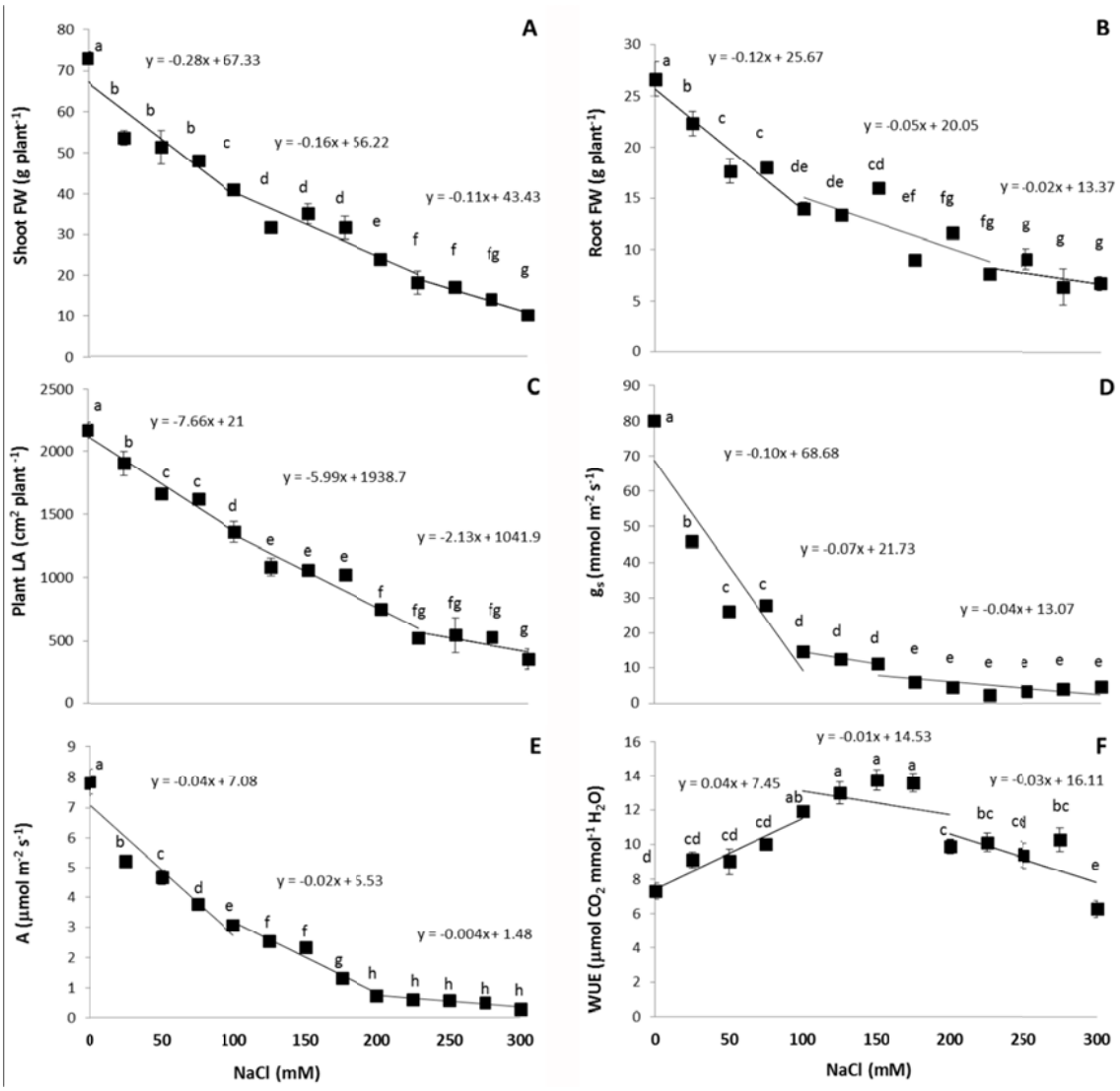

Fig. 1.

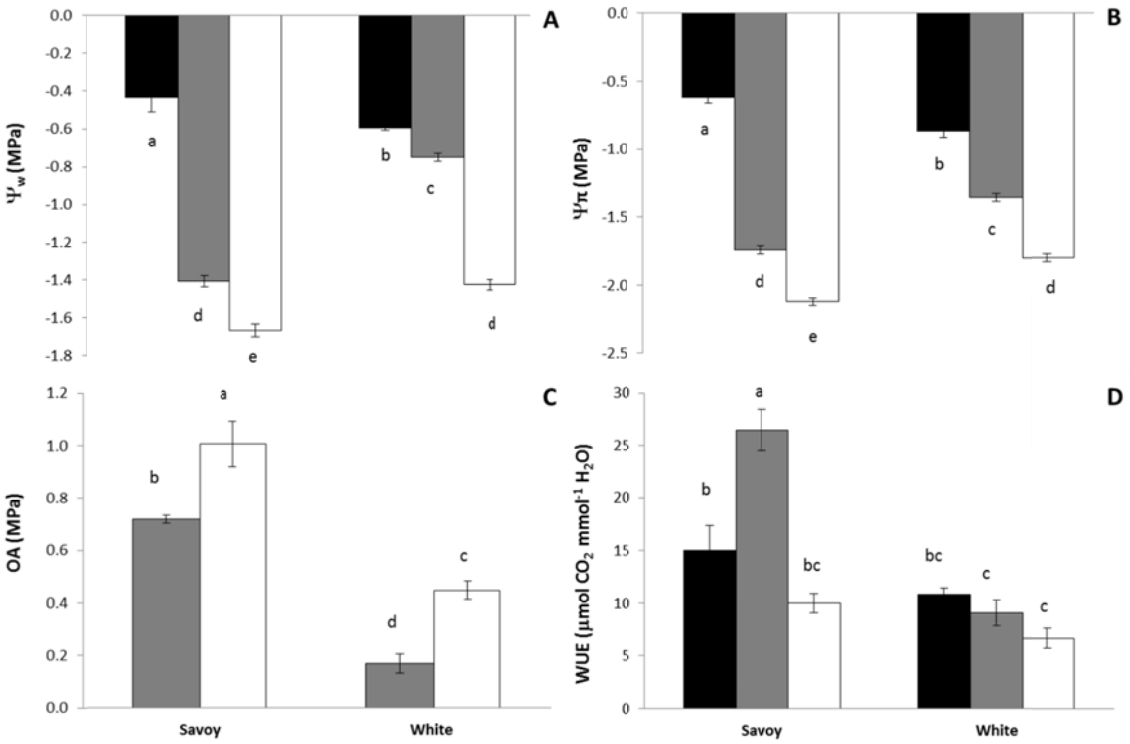

Fig. 2.

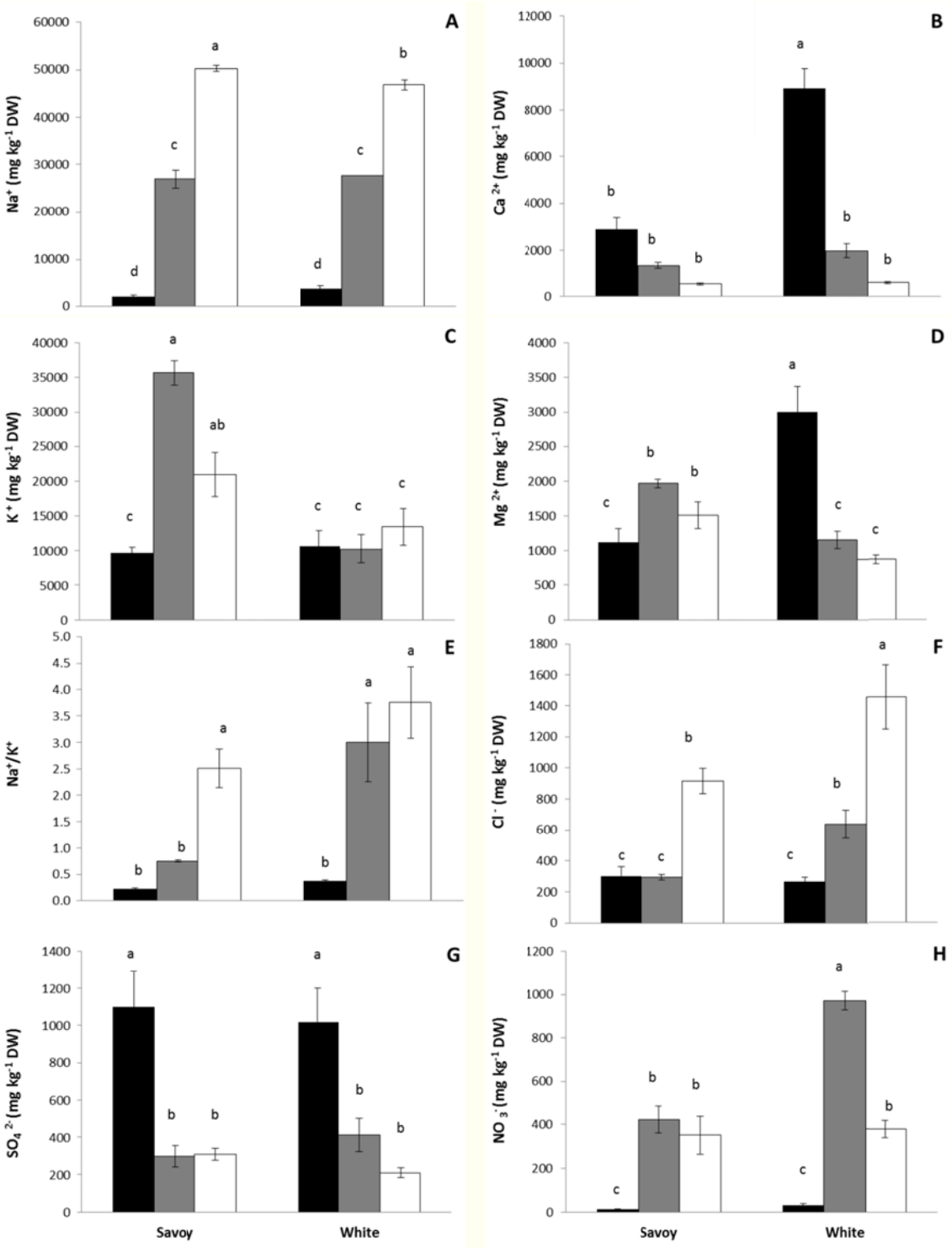

Fig. 3.

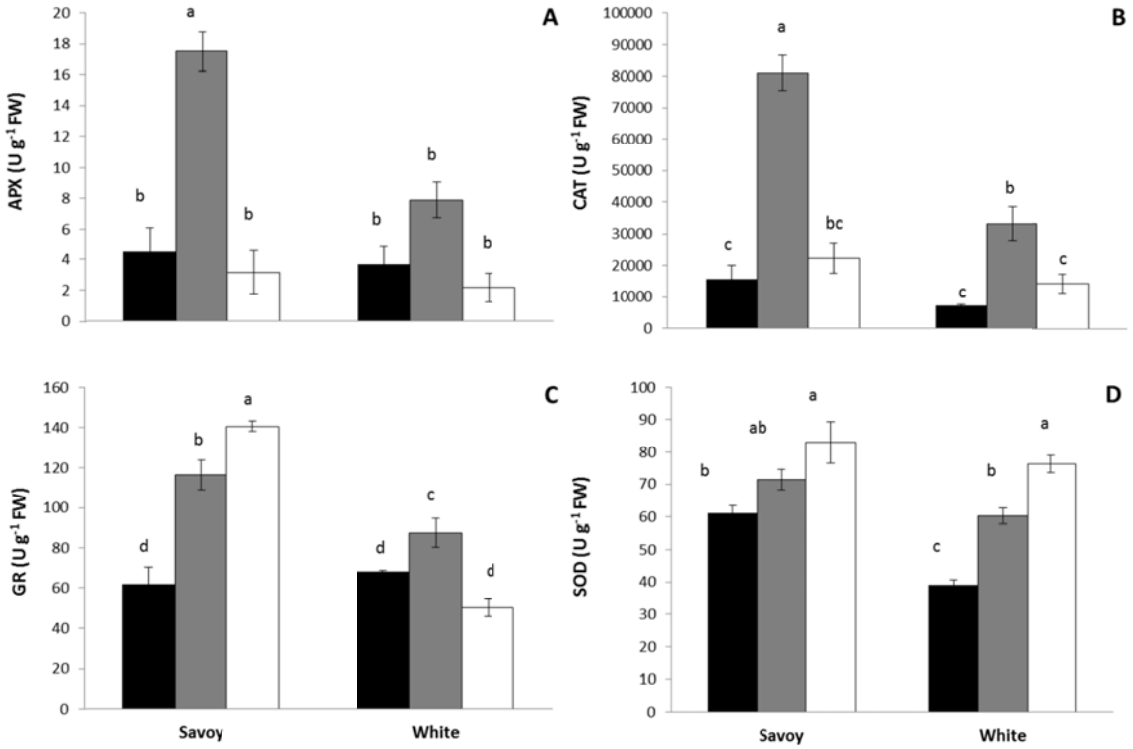

Fig. 4.

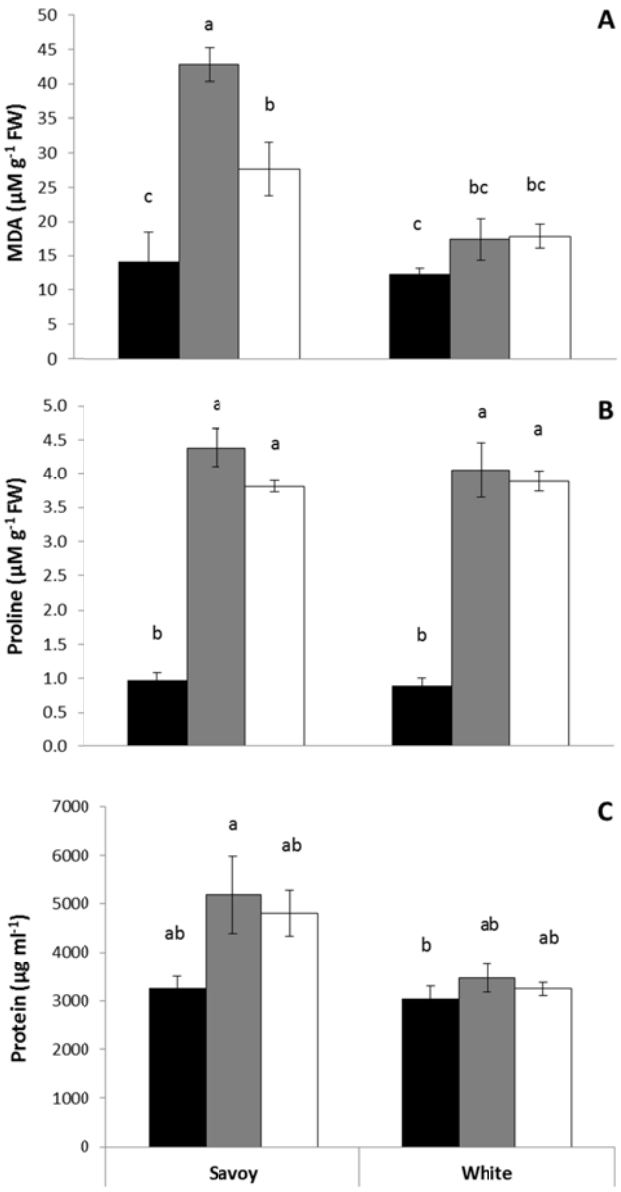

Fig. 5.

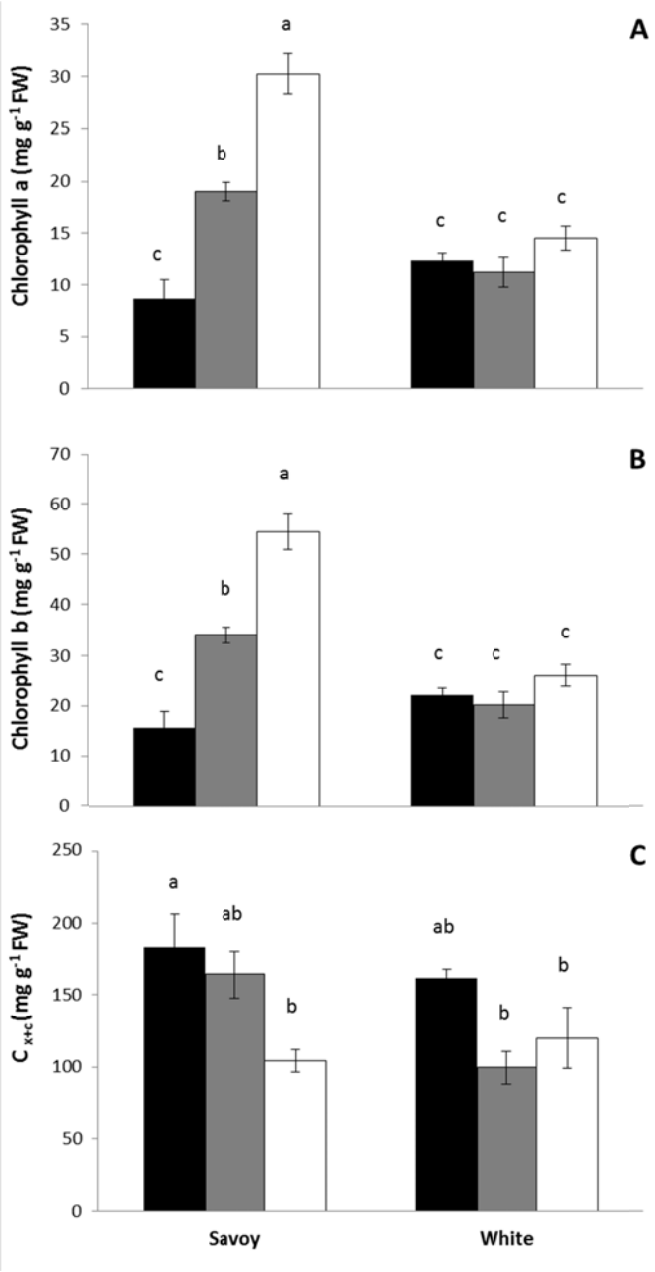

Fig. 6.