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The effect of salt stress on growth, chlorophyll content, lipid peroxidation and antioxidant defence systems in salt-tolerant and salt-sensitive bean genotypes

Khaled TAÏBI ^{*(1,3,4)}, Leila AIT ABDERRAHIM ⁽¹⁾, Fadhila TAÏBI ⁽¹⁾, Amel ENNAJAH ⁽²⁾, Moulay BELKHODJA ⁽³⁾, José Miguel MULET ⁽⁴⁾

¹Faculty of Life and Natural Sciences, Ibn Khaldoun University, Karman Campus, Tiaret, 14000, Algeria.

²Institut National de Recherches en Génie Rural, Eaux et Forêts, Ariana, 2080, Tunisia.

³Laboratory of plant physiology, Faculty of Life and Natural Sciences, Ahmed Benbella University-Oran 1, Oran, 31000, Algeria.

⁴Instituto de Biología Molecular y Celular de Plantas (IBMCP), Universitat Politècnica de València-Consejo Superior de Investigaciones Científicas, Avd. de los Naranjos s/n, Valencia, 46022, Spain.

* Corresponding author: khaledtaibi@hotmail.com

Abstract

Salinity is one of the major abiotic stresses which affect the metabolism of plant cells and reduce plant productivity. The differences in the antioxidant defense systems under salinity among two bean genotypes differing in salt tolerance were investigated. It seems that the difference between the genotypes in response to salinity is more quantitative rather than qualitative since they develop the same strategies with a significant variation in the rate of synthesis and accumulation. For both genotypes, salinity induced a marked reduction in dry matter gain in roots and shoots along with a secondary oxidative stress in plants as indicated by the significant increase in malondialdehyde contents. Besides, all the photosynthetic pigments decreased gradually with the rise of salinity. However, salinity failed to induce appreciable variations in the production of total phenolic compounds in leaves of bean except in the salt-sensitive genotype under high salinity. The salt-tolerant genotype may have a better protection against oxidative damages by increasing, more than the salt-sensitive genotype, the activity of antioxidant enzymes and the amounts of total flavonoids and ascorbic acid under high salinity. These results indicate that bean genotypes respond to salt induced oxidative stress by enzymatic defense systems.

Key words

Phaseolus vulgaris, salinity, lipid peroxidation, antioxidant enzymes, non-enzymatic antioxidants, photosynthetic pigments.

Introduction

Salinity is one of the major factors which affect significantly the metabolism of plant cells, leading to severe damage and a loss of productivity mainly in arid and semi arid regions (Vaidyanathan et al. 2003). Exposure of plants to salinity results in increased generation of reactive oxygen species as by-products, which damage the cellular components (Van Breusegem and Dat 2006). Reactive oxygen species cause chlorophyll degradation and membrane lipid peroxidation, reducing membrane fluidity and selectivity (Verma and Mishra 2005). Chlorophyll loss and lipid peroxidation measured as malondialdehyde content, a product of lipid peroxidation, are considered to be indicators of oxidative damage (Del Rio et al. 2005).

In response to salinity, plants have developed a series of enzymatic (e.g., catalase, glutathione reductase and several peroxidases) and non-enzymatic (ascorbate, carotenoids, flavonoids and other phenolic compounds etc.) detoxification systems to counteract reactive oxygen species, and protect cells from oxidative damage (Sairam and Tyagi 2004). The role of antioxidant enzymes as the main components of the tolerance mechanisms in response to salinity, by the detoxification of superoxide and H_2O_2 , has been already reported (Jaleel et al. 2007; Esfandiari et al. 2007).

Antioxidants such as ascorbate and glutathione are involved in scavenging H_2O_2 in conjunction with monodehydroascorbate reductase and glutathione reductase, which regenerate ascorbate (Horemans et al. 2000). Carotenoids are pigments with several functions in plants, besides their direct role in photosynthesis, including their involvement in the mechanisms of oxidative stress tolerance (Gill and Tuteja 2010). Phenolic compounds also fulfil multiple roles in plants, as structural components of cell walls, participating in the regulation of growth and developmental processes, as well as in the mechanisms of defence against biotic and abiotic stress (Cheynier et al. 2013). Flavonoids represent the main and most complex subgroup of polyphenols with a wide array of biological functions (Di Ferdinando et al. 2012).

Common bean (*Phaseolus vulgaris*) is a major vegetable crop with greater nutritional value, accounts for higher consumption and economic importance all over the world. As the bean is mainly grown in poorly irrigated and partly saline conditions throughout the world, it is exposed to great deal of salt stress. Although much supporting evidences on the role of antioxidants and antioxidant enzymes under salinity are available, there are no specific information pertinent to common bean.

In this study, we hypothesized that an increased antioxidants activities (ascorbate, total carotenoids, phenolics and flavonoids) and antioxidant enzymes (catalase, ascorbate peroxidase, and glutathione reductase) contributes to the protection of bean plants against salt stress. The aim of this work was to evaluate the effects of salt stress on the antioxidant response, lipid membrane

peroxidation, and chlorophyll contents in two bean genotypes; salt-tolerant ‘Tema’ and salt-sensitive ‘Djadida’, in order to better understand their differences in salt stress tolerance.

Material and methods

The experiment was conducted to examine the antioxidant response among the genetic variability of *Phaseolus vulgaris* subjected to salinity and to verify whether differential responses within *Phaseolus* species.

Plant material

Two genotypes, salt-tolerant ‘Tema’ and salt-sensitive ‘Djadida’, commonly cultivated in Algeria, were selected for this experiment on the basis of greenhouse and fields observations that showed differences in growth and yield (Taïbi et al. 2012, 2013a, b).

Plant growth and treatments

Seeds of bean were surface sterilized with 5% (w/v) commercial bleach sodium hypochlorite solution (NaOCl) three times for 30 min with gentle stirring and subsequently washed in deionized water then pre-germinated between wet paper towels at 25°C in the dark for later selection of uniform seedlings.

After 7 days seedlings were individually transplanted in pots containing a mixture of commercial peat and vermiculite (V: V) and were placed in a greenhouse under controlled conditions with light intensity of about 600 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 16h duration, 70% relative humidity and 25/18°C day/night temperature.

Seedlings were irrigated by a Hoagland nutrient solution twice to three times a week. After three weeks of growth, plants of each genotype were arranged into four treatments each one contains ten plants. Salt treatments were set up on three treatments by adding increasing NaCl concentrations of 50, 100 and 200 mM NaCl in the nutritive solution. The control treatment was sustained irrigated by the nutritive solution.

Seven days after salt treatment, five plants of each genotype were harvested and the fresh and dry weights of whole plants were determined. The leaf samples were kept at -80°C for further analyses.

Photosynthetic pigments

Chlorophyll (Chl a and Chl b) and total carotenoids concentrations were determined spectrophotometrically using 100 mg FW of leaf material ground in a pre-chilled mortar in the presence of 8 ml of acetone 80% (v/v). After complete extraction, the mixture was filtered and

the volume adjusted to 10 ml with cold acetone. The absorbance of the extract was read at 663.2, 646.8, and 470 nm and pigment concentrations were calculated according to Lichtenthaler (1987).

Malondialdehyde

Malondialdehyde (MDA), routinely used as an indicator of membrane lipid peroxidation and thus as an excellent marker of oxidative stress damage induced by stress, was determined according to Heath and Packer (1968), using 250 mg of fresh tissue homogenize with 5% (w/v) trichloroacetic acid (TBA). To 1 mL aliquot of supernatant, 4 mL of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid was added. The mixture was heated at 95°C for 30 min, quickly cooled, and centrifuged at 10 000 g for 10 min at 4°C. The supernatant absorbance was read at 532 nm, and the values corresponding to nonspecific absorption (600 nm) were subtracted. Lipid peroxidation products were measured as the content of TBA-reactive substances. The MDA content was calculated according to the molar extinction coefficient of 155/(mM cm). The MDA content was measured as $\mu\text{mol.g}^{-1}$ FW.

Tissue ions concentrations

Dried samples were ground to a fine powder using a mortar and pestle. About 100 mg was ashed at 550°C in a muffle furnace for 5 h and then digested with 2 ml of 20% HCl (6 N) for 5 min at 60°C using a heating block. This hot water extract was cooled and filtered using Whitman n° 42 filter paper and finally diluted to a volume of 50 ml with distilled deionized water. Na⁺ and K⁺ concentrations were determined by using an atomic absorption spectrometer (Perkins Elmer, Norwalk, CT, USA).

Antioxidants enzymes extraction and assay

Enzymes were extracted from 0.5 g of leaf tissue using a mortar and pestle with 5 ml of extraction buffer containing 50 mM K-phosphate buffer, pH 7.6, and 0.1 mM Na₂-EDTA. The homogenate was centrifuged at 15 000 g for 15 min at 4°C, and the supernatant fraction was used to assay various enzymes according to the method of Cakmak and Marschner (1992). Catalase activity (CAT) was assayed in a reaction mixture containing 25 mM phosphate buffer (pH 7.0), 10 mM H₂O₂, and enzyme. The decomposition of H₂O₂ was followed at 240 nm ($E=39.4 \text{ mM cm}^{-1}$). Ascorbate peroxidase activity (APX) was determined by measuring the decrease in the absorbance of the oxidized ascorbate at 290 nm. One unit of APX was defined as the amount of enzyme required to consume 1 μmol ascorbate min^{-1} . Glutathione reductase activity (GR) was determined by measuring the enzyme-dependent oxidation of NADPH by following absorbance

at 340 nm. One unit of GR activity was defined as the amount of enzyme that oxidized 1 μmol NADPH min^{-1} .

Non-enzymatic antioxidant

Total phenolic compounds were quantified by reaction with the Folin-Ciocalteu reagent, according to Blainski et al. (2013). Absorbance was measured at 765 nm and the results expressed in equivalents of gallic acid (mg eq.GA g^{-1} DW), used as standard. Flavonoid contents were determined following the method described by Zhishen et al. (1999); the absorbance was measured at 510 nm, and the amount of flavonoids was expressed in equivalents of catechin (mg eq.C g^{-1} DW), used as standard. Ascorbic acid (ASC) concentration was estimated by using a slightly modified method of Luwe et al. (1993). Plant sample (0.5 g) was ground in liquid nitrogen mortar and then homogenized in ice-cold trichloroacetic acid (TCA, 1 % w/v). The homogenate was then centrifuged at 12 000 g for 20 min at 4 °C and the supernatant (50 μL) mixed with potassium phosphate buffer (0.95 mL, 100 mM, pH 7.0) and ascorbate oxidase (1 μL of 1 Unit μL^{-1}). Oxidation of AsA ($E=14.3 \text{ mM cm}^{-1}$) was then recorded at 265 nm.

Statistic analysis

Prior to the analysis, the Levene test was applied to check the ANOVA requirements. The significance of differences between treatments was assessed using one-way ANOVA, at 95% confidence level. The post-hoc Tukey test was used to estimate homogeneous groups when more than two samples were compared.

Results

Growth

Shoots and roots biomass were adversely affected by salinity in both genotypes ($p\text{-value}<0.01^{**}$). Independently of salinity, salt-tolerant genotype Tema has presented higher shoots biomass weight. Shoots dry weight decreased gradually with the increase of salt concentrations in the growth medium. The decrease is evaluated to be around 30% in salt-tolerant genotype Tema and 27% in salt-sensitive genotype Djadida under 200 mM NaCl. By the same, roots dry weight decreased by 59% in Tema and by 61% in Djadida under high salinity comparing to their respective control (Fig. 1).

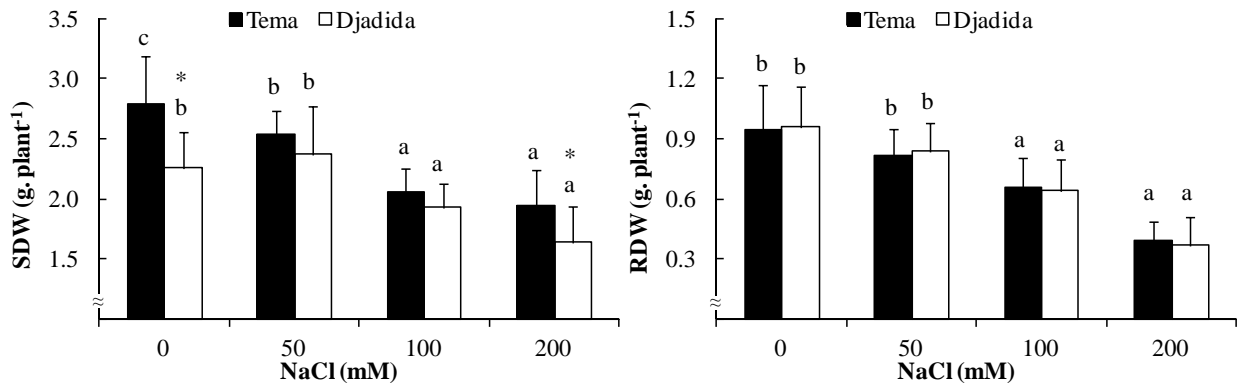


Fig. 1. Shoots (SDW) and roots dry weights (RDW) of two bean genotypes subjected to salinity. Different lower case letters indicate significant differences between treatments, for the same genotype, according to Tukey test ($\alpha=0.05$); asterisks indicate significant differences between the two genotypes for each treatment.

Photosynthetic pigments

Our results show an inverse relationship between salinity and photosynthetic pigments contents. When NaCl concentration increased in the growth medium Chl a contents decreased till 52% in salt-tolerant genotype Tema and 57% in salt-sensitive genotype Djadida under high salinity comparing to control. Besides, Chl b content decrease was evaluated till 33% in salt-tolerant genotype Tema and 43% in salt-sensitive genotype Djadida. By following, total carotenoids contents decreased by 18% in salt-tolerant genotype Tema and by 19% in salt-sensitive genotype Djadida (Fig. 2).

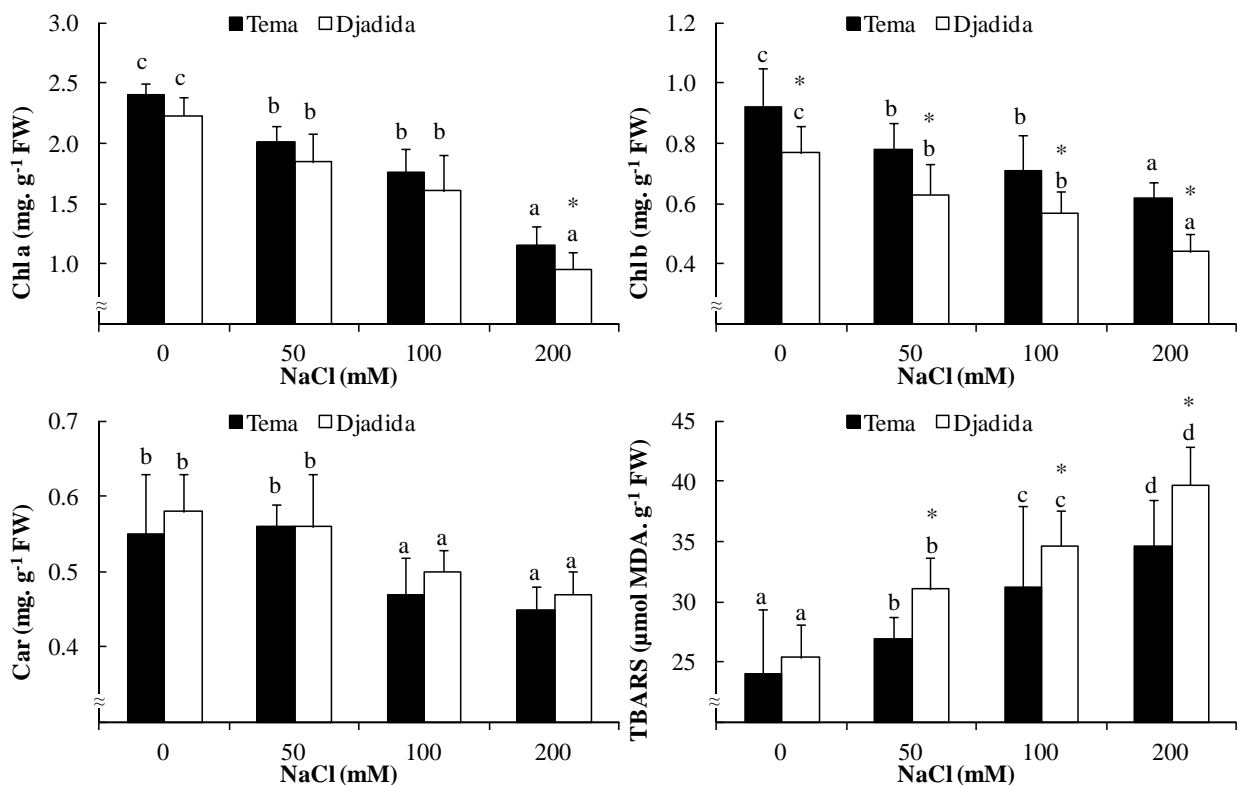


Fig. 2. Chlorophyll a (Chl a), Chlorophyll b (Chl b), Total carotenoids (Car) and Lipid peroxydation (TBARS) of two bean genotypes subjected to salinity. Different lower case letters indicate significant differences between treatments, for the same genotype, according to Tukey test ($\alpha=0.05$); asterisks indicate significant differences between the two genotypes for each treatment.

Lipid peroxydation

The degree of malondialdehyde accumulation was higher in salt-sensitive genotype Djadida in all the treatments indicating higher rates of lipid peroxydation and oxidative damages. NaCl treatments led to a gradual increase in the levels of malondialdehyde in both genotypes. High salinity induced an increase of 44% and 56% of malondialdehyde levels in Tema and Djadida respectively (Fig. 2).

Leaf ionic content

Leaves are the last sink and the most sensitive part of the plant in contact with the atmosphere, and it is here that salt accumulates as a consequence of water evaporating from the plant through stomata. Irrespective of salinity, higher amounts of Na^+ and were measured in the leaves of the salt-tolerant genotype Tema under the treatment control. NaCl treatments significantly affected Na^+ and K^+ contents in both genotypes ($p\text{-value}<0.001^{***}$). High salinity drastically increased leaves Na^+ concentrations to five-fold in salt-tolerant genotype Tema and seven-fold in salt-sensitive genotype Djadida comparing to control plants. However, K^+ concentrations decreased with NaCl treatments by around 32% for both genotypes (Fig. 3).

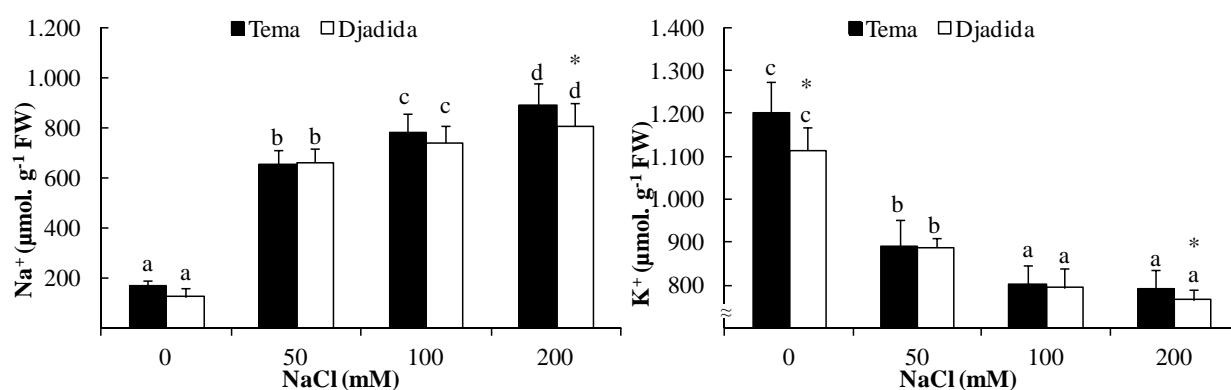


Fig. 3. Sodium (Na^+) and potassium (K^+) contents of two bean genotypes subjected to salinity. Different lower case letters indicate significant differences between treatments, for the same genotype, according to Tukey test ($\alpha=0.05$); asterisks indicate significant differences between the two genotypes for each treatment.

Antioxidant enzymes activities

Both genotypes exhibited a significant increase in catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities with increasing NaCl concentrations in the growth medium (p -value <0.001 ***). There were striking differences in the antioxidant enzymes activities between the two genotypes with increasing NaCl concentrations. The enzymes activities were higher in salt-tolerant genotype Tema under all treatments except for the APX activity which was higher in Djadida genotype under 100 mM NaCl treatment. Both genotypes revealed almost similar antioxidant enzymes activities under the treatment control. However, under high salinity, the rates of increase in GR activity was estimated around 60% in Tema and 20% in Djadida genotypes. By the same, CAT activity increased by four-fold in salt-tolerant genotype Tema and two-fold in salt-sensitive genotype Djadida. In addition, APX activity increased by seven-fold in Tema and six-fold in Djadida under the same conditions (Fig. 4).

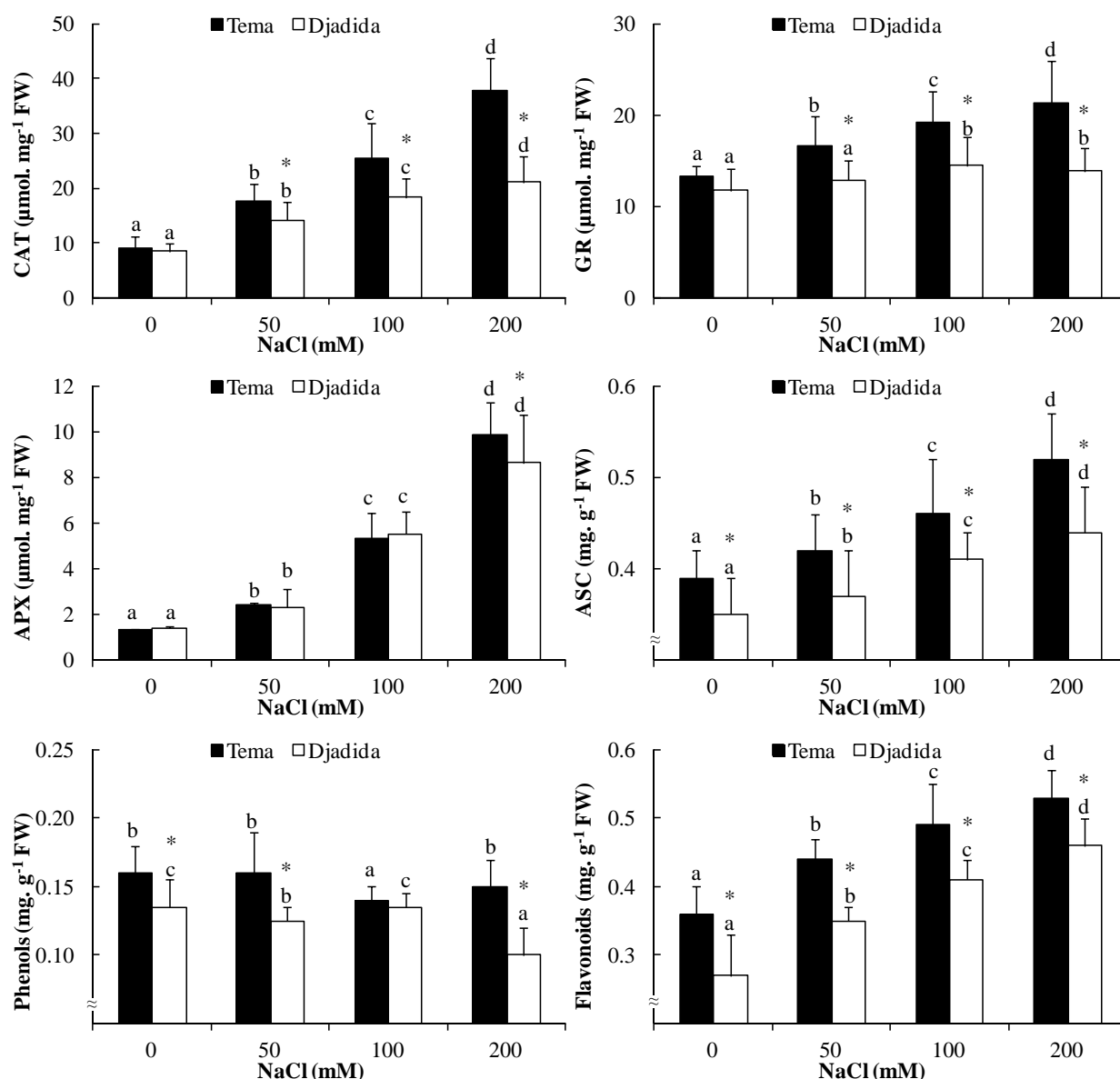


Fig. 4. Enzymatic activities of catalase (CAT), glutathione reductase (GR) and ascorbate peroxidase (APX) and the contents of ascorbate (ASC) and total flavonoids and phenolic

compounds of two bean genotypes subjected to salinity. Different lower case letters indicate significant differences between treatments, for the same genotype, according to Tukey test ($\alpha=0.05$); asterisks indicate significant differences between the two genotypes for each treatment.

Non-enzymatic antioxidants

The salt-tolerant genotype Tema exhibited higher ascorbate and total flavonoids and phenolics contents under all treatments (p-value<0.05*). The contents of ascorbate and total flavonoids increased significantly under salinity for both genotypes (p-value<0.01**) but with different extent. The rates of increase were 33% and 47% in salt-tolerant genotype Tema against 26% and 70% in salt-sensitive genotype Djadida respectively for ascorbate and total flavonoids under high salinity. The total phenolics contents did not change significantly in salt-tolerant genotype Tema under salinity. The significant decrease of total phenolics contents was marked only in Djadida under 200 mM NaCl (p-value<0.05*) (Fig. 4).

Discussion

Salt stress is a major ecological factor which prevents crop plants from realizing their full genetic potential. Reduction of the biomass in beans under saline conditions was indicative of several growth limitations (Gama et al. 2007). The assayed common bean plants are able to grow at salinity levels below 50 mM NaCl but when subjected to 200 mM NaCl both genotypes showed a reduction in plant growth. In term of dry weight, the response of the two genotypes to salinity was found to be different. Salt-tolerant genotype Tema showed better growth than Djadida. Reduction of plant growth and dry-matter accumulation under saline conditions has been reported in several grain legumes, including *Phaseolus vulgaris* (Delgado et al. 1994; Tejera et al. 2006; Taïbi et al. 2012, 2013a, b). Growth retardation could be due to the inhibition of cell elongation (Bandeoglu et al. 2004).

Our results indicated significant decreases in chlorophyll a and b under salinity which agree with previous results of Turan et al. (2007) on *Phaseolus vulgaris* L. and Taffouo et al. (2010) on *Vigna subterranean* L. The decrease in chlorophyll levels in salt-stressed plants has been considered as a typical symptom of oxidative stress (Smirnoff 1996) and was attributed to the inhibition of chlorophyll synthesis, together with the activation of its degradation by the enzyme chlorophyllase (Santos 2004). Reduction of chlorophyll contents, as a result of either slow synthesis or fast breakdown, indicated that there was a photoprotection mechanism through reducing light absorbance by decreasing chlorophyll contents (Elsheery and Cao 2008).

Carotenoids, being antioxidants, have the potential to detoxify the plants from the effects of reactive oxygen species (Verma and Mishra 2005). Salinity induced decline in carotenoid

contents in both genotypes. These results concur with those found by Gadallah (1999) on *Phaseolus vulgaris* L. and Singh et al. (2008) in maize genotypes and wheat genotypes. Carotenoids are known to function as collectors of light energy for photosynthesis and as quenchers of triplet chlorophyll and O₂. Moreover, they dissipate excess energy via the xanthophyll cycle and can act as powerful chloroplast membrane stabilizers that partition between light-harvesting complexes (LHCs) and the lipid phase of thylakoid membranes, reducing membrane fluidity and susceptibility to lipid peroxidation (Demmig-Adams and Adams 2002). The decrease of carotenoid contents indicated that the protection by carotenoid was not one of the most important mechanisms under salt stress.

There is abundant evidence that salinity alters ion transport and contents in plants (Cramer 1997). The increases in Na⁺ in plant leaves of both genotypes under salinity were accompanied by decreases in K⁺. This finding agrees with our previous observations reporting that bean plants exposed to NaCl take up high amounts of Na⁺, whereas the uptake of K⁺ is significantly reduced (Taïbi et al. 2012). Besides, the salt-tolerant genotype Tema accumulated simultaneously higher contents of Na⁺ and K⁺ which agrees with previous finding of Tejera et al. (2006) reporting that variation in ions concentrations has been associated with tolerance of legumes to salt stress.

As lipid peroxidation is the symptom mostly ascribed to oxidative damage, it is often used as an excellent marker of increased oxidative stress under salinity (Hernandez et al. 2000; Khan and Panda 2008). The results reported in this paper showed that NaCl treatments led to a significant increase in the content of MDA in both genotypes. Salt-tolerant genotype Tema showing better growth under salinity had less MDA indicating that salt-tolerance is related to less active lipid peroxidation. The MDA content in *Phaseolus vulgaris* has also been reported to increase under saline conditions (Babu and Devaraj 2008; Kaymakanova and Stoeva 2008).

Plants employ a number of enzymatic and non-enzymatic antioxidants to prevent oxidative damage and keep reactive oxygen species concentrations within a narrow functional range (Ozgun et al. 2013). Antioxidant enzymes such as catalase (CAT) and those of the ascorbate–glutathione cycle, ascorbate peroxidase (APX) and glutathione reductase (GR) are known to substantially reduce the levels of superoxide and hydrogen peroxide in salt-stressed plants (Kaymakanova and Stoeva 2008). Activities of the antioxidant enzymes CAT, APX, and GR were found to be higher in the salt-tolerant genotype Tema than in the salt-sensitive Djadida under NaCl treatments. The elevated antioxidant activity led to the lower lipid peroxidation under salinity as it was reported in salt-tolerant wild tomato *Lycopersicon pennellii* (Shalata and Tal 1998), *Pisum sativum* (Hernandez et al. 2000) and *Beta vulgaris* (Bor et al. 2003). In addition, Scandalios (1993) reported that CAT and APX are the most effective antioxidant enzymes in preventing cell damage. These two enzymes revealed a great importance in regulating H₂O₂ intracellular levels

(Azevedo Neto et al. 2006). Over-expression of the APX gene in plants has been reported to improve protection against oxidative stress (Wang et al. 1999). Our data showed that APX activity was increased by salinity in both genotypes, and the activity of APX was significantly higher in the salt-tolerant genotype Tema under 200 mM NaCl. It is striking that salt-induced APX activation in the salt-tolerant genotype Tema was accompanied by a higher increase in CAT activity. For that reason, it may be supposed that CAT and APX are probably with equal importance in the detoxification of H₂O₂ under high salinity for the salt-tolerant genotype Tema. Our results are in agreement with many reports suggesting that APX activity, coordinated with CAT activity, plays a central protective role under salinity (Hernandez et al. 2000; Demirel and Turkan 2005). The increases in GR activity have been reported also to be involved in salt tolerance in maize (Zacchini et al. 2003) and rice (Maribel and Tobita 2003). Our results suggest that salt-sensitive genotype Djadida exhibited a less active ascorbate-glutathione cycle under high salinity and that the Halliwell-Asada enzymatic pathway may play a key role in salt tolerance in *Phaseolus vulgaris*.

In addition to enzymatic antioxidants, plants possess a variety of non-enzymatic molecules which play a substantial role in counteracting oxidative stress. These non-enzymatic antioxidants include among others ascorbic acid, carotenoids, flavonoids, and phenolic compounds (Schafer et al. 2002). In the present study, salinity led to a significant increase of ascorbate and total flavonoids contents in plants leaves. Flavonoids are frequently induced by abiotic stress and promote roles in plant protection (Grace and Logan 2000). Several flavonoids act as potential inhibitors of the enzyme lipoxygenase, which converts polyunsaturated fatty acids to oxygen-containing derivatives (Nijveldt et al. 2001). These compounds accumulated in plant tissue could help to protect themselves from damaging effects and may help inhibit lipid peroxidation in stressed-plants (Potapovich and Kostyuk 2003). Ascorbic acid has also been shown to play multiple roles in plant growth, such as in cell division, cell wall expansion, and other developmental processes (Pignocchi and Foyer 2003). Panda and Upadhyay (2004) also noticed increase in ascorbate along with increase in the NaCl concentration in *Lemna minor*. Chen et al. (2005) reported an increase in ascorbic acid level with increasing salinity in salt tolerant species, *Spartina alterniflora*.

Antioxidant properties of phenolic compounds arise from their high reactivity as hydrogen or electron donors, from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron, and from their ability to chelate transition metal ions (Huang et al. 2005). When plants were submitted to salinity, variation in antioxidant pools, notably in phenolic compounds, were found (Ksouri et al. 2008). The present data doesn't demonstrate significant variations in total phenolics contents in leaves of salt-tolerant genotype Tema under salinity and

exhibited significant decrease in salt-sensitive genotype Djadida under 200 mM NaCl. Salinity induced disturbances of the metabolic process leading to the increase in phenolic compounds (Ayaz et al. 2000; Keutgen and Pawelzik 2009). Besides, the aptitude to respond to salinity by the synthesis of phenolic compounds has been observed in the tolerant and sensitive strawberry genotypes (Keutgen and Pawelzik 2008). Such a decrease in total phenolic compounds contents under salinity has been reported in bean (Vergeer et al. 1995). In fact, the increase of reactive oxygen species under salinity is usually coupled with changes in net carbon gain which may strongly affect the biosynthesis of carbon-based secondary compounds, particularly leaf phenolic compounds (Radi et al. 2013).

Conclusion

The results of this study showed that there were considerable differences between the salt-tolerant Tema and salt-sensitive Djadida genotypes in their responses to salinity. The differences in response to salinity in bean are closely related to the variation in the extent of the antioxidant response. It seems to be a quantitative more than qualitative difference between these genotypes. NaCl treatments induced secondary oxidative stress in plants of both genotypes as indicated by the significant increase in malondialdehyde contents. Both genotypes develop the same strategies under salinity with a significant difference in the rate of synthesis and accumulation. Our results suggest that salt-tolerant bean genotype Tema may have a better protection against oxidative damages by increasing the activity of antioxidant enzymes along with the total flavonoids and ascorbic acid contents under salinity.

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