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Original Article

Effects of Salt Stress on Plant Growth, Nutrient Partitioning, Chlorophyll Content, Leaf Relative Water Content, Accumulation of Osmolytes and Antioxidant Compounds in Pepper (*Capsicum annuum* L.) Cultivars

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Abstract

The salinity of soil is among the most important abiotic stresses which limit agricultural productivity worldwide. The effects of salinity on growth, nutrient partitioning, chlorophyll, leaf relative water content, osmolytes accumulation and antioxidant compounds of pepper (*Capsicum annuum* L.) cultivars ('Granada', 'Goliath' and 'Nobili'), widely used in Cameroon, were investigated. Plants were subjected to four levels of NaCl (0, 50, 100 and 200 mM) at early seedling growth stage of plant development. Application of NaCl treatment led to a significant increase in total soluble sugars, proline, soluble proteins, total free amino acids content, peroxydase and superoxide dismutase activity and total phenolic content in salt-tolerant 'Granada' and 'Nobili' compared to salt-sensitive 'Goliath' and untreated plants, on the contrary, decreased in root dry weight, shoot dry weight, number of leaves, shoot length, stem diameter, total leaf area, chlorophyll and leaf relative water content in 'Goliath' at low salinity level. Flavonoid content, K, Ca and Mg concentrations were significantly reduced with increasing salinity in all cultivars. The highest Na concentrations were detected in the leaves while the lowest were recorded in the roots of 'Goliath' at high salinity level. The salt sensitivity of 'Goliath' seems to be increased osmotic adjustment through the strongly accumulation of Na in leaves while the salt tolerance of 'Granada' was related to its induce of antioxidative enzyme system more efficiently, resulting in higher osmolytes accumulation under salinity. 'Granada' was more tolerant and stable in physiological and biochemical traits suggesting that it could be grown in salt-affected soils.

Keywords: antioxidative defense system, compatible solutes, ions distribution, pepper, plant growth, salinity
Abbreviations: Calcium-Ca; chlorophyll-CHL; days after planting-DAP; flavonoids-FLA; magnesium-Mg; number of leaves-NL; peroxydase-POD; phosphorus-P; potassium-K; proline-PRO; relative water content-RWC; root dry weight-RDW; shoot dry weight-SDW; shoot length-SL; soluble proteins-SP; stem diameter-SD; superoxide dismutase-SOD; total free amino acids content-FAA; total leaf area-TLA; total phenolic-TP; total plant dry weight-PDW; total soluble sugars-SS.

Introduction

Salinity is a major environmental factor determining plant productivity in most arid and semi-arid areas of the world. It affects more than 10% of arable land and salinization is rapidly increasing on a global scale, declining average yield for most major crop plants by more than 50% (Bray *et al*., 2000). Salt stress occurs in areas where soils are naturally over salted and precipitation is low and/or where irrigation, hydraulic lifting of salty underground water, or invasion of sea water in coastal areas brings salt to the surface soil that inhabit plants (Neumann, 1995). Globally

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20% of irrigated land and 2.1% of dry land agriculture suffers from the salt problem and NaCl is the predominant salt causing salinization (Munns and Tester, 2008). Salinity adversely affects germination, growth, physiology and productivity by reducing the ability of plants to take up water causing foliage damage and even death of the plants, imbalance in osmotic potential; ionic equilibrium and nutrient uptake (Niu *et al*., 1995). Further, it facilitates severe ion toxicity by depositing high concentration of Na^+ which causes membrane disorganization, inhibition of cell division and expansion. The influence of salinity and mineral nutrient solution, on productivity, photosynthesis and growth has been studies in different plants (Hosseini and Thengane, 2007; Li *et al*., 2008; Taffouo *et al*., 2010). It stated that high levels of Na^+ inhibits K, Ca and Mg in leaves, which results in a K/Na antagonism and net photosynthesis is affected strongly by NaCl conditions, which is related directly to the closure of stomata as to low intercellular CO_2 levels (Al-Karaki, 2000; Turan *et al*., 2007). To develop saline zones and/or the zones having only brackish water resources, it is important to select tolerant varieties. Salinity imposes stress conditions on crop plants and affects growth and chemical contents and has been shown to limit pepper yield (Paridam and Das, 2005). Salt stress severely inhibits plant growth for two reasons: firstly due to an osmotic or water-deficit effect of salinity and secondly due to a salt-specific or ion excess effect of NaCl. Moreover, plants subject to salinity stress conditions produce cytotoxic activated oxygen that can seriously disrupt normal metabolism, through oxidative damage of lipids, proteins, and nucleic acids (Abbaspour, 2012). To defend against such oxidants, plants have evolved specific protective mechanisms, involving antioxidant molecules and enzymes that protect against the potentially-cytotoxic species of activated oxygen.

The responses of plants to high soil salinity and the mechanisms of salt tolerance have been discussed in many works published (Grigore *et al*., 2011; Meguekam *et al*., 2014; Nouck *et al*., 2016). Such salt tolerance mechanisms include ion homeostasis system via salt glands/salt bladders, osmoregulation system such as free proline, glycine betaine, mannitol and soluble sugars, hormonal regulation, and antioxidant machinery (Hasegawa *et al*., 2000; Flowers and Colmer, 2008). Compartmentation of ions in vacuoles and accumulation of compatible solutes in the cytoplasm are commonly proposing mechanisms to salt tolerance of plants (Munns, 2002). In salt tolerant species, the osmotic balance of the cytoplasm is ensured by an active synthesis of the organic and soluble compounds (Grigore *et al*., 2011). In plants, SP are involved in osmotic adjustment. They are stored as nitrogen under salt-stress and re-used when the stress is removed (Singh *et al*., 1987). When different abiotic stresses affect plant functionality, alterations in photosynthesis and carbon partitioning are common features that take place at organ level as well as in whole plant (Gill *et al*., 2003). SS do not only function as metabolic resources and structural constituents of cells, they also act as signals regulating various processes associated with plant growth and development (Jang and Sheen, 1997). Recent studies for increasing tolerance to environmental stresses, through metabolic engineering of compatible solutes, have shown that increases in SS and/or other osmolytes provide optimism to increase plant tolerance to abiotic stresses such as drought, salinity and cold (Cusido *et al*., 1987; Rathinasabapathi, 2000). PRO is probably the most common compatible solutes synthesized by plants as a response to abiotic stress (Ashraf and Foolad, 2007; Meguekam *et al*., 2014). PRO is significantly accumulated under salt stress and performs the positive role in the adaptation of cells to salt and water stress (Kaviani, 2008). Deficit of K induced by salinity increased the levels of FAA, especially of aspartic acid, glutamic acid and PRO (Cusido *et al*., 1987).

The salt-induced disturbance in ionic homeostasis causes a cascade of secondary effects such as oxidative stress due to reactive oxygen species (ROS) production (Ashraf, 2009; Joseph and Jini, 2011). In response to stress, plants activate powerful antioxidant systems, both enzymatic (e.g., superoxide dismutase, catalase, glutathione reductase, peroxidase) and non-enzymatic (vitamins C and E, carotenoids, flavonoids and other phenolic compounds) (Azooz *et al*., 2009). SOD and catalase are known as the most effective enzymes in scavenging of active oxygen species which cause oxidative stress (Karanlik, 2001). However, an active antioxidative defense system comprising enzymatic and non-enzymatic antioxidants reduces the level of oxidative stress in plant cells by scavenging free radicals (Azooz *et al*., 2009; Abogadallah *et al*., 2010). In case of high salinity, oxidative stress occurs due to closure of stomata, interruption of photosynthetic electron transport and disruption of cellular membrane integrity and antioxidative defense systems of plants start work against oxidative damage. SOD is metalloenzyme that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism (Malstrom *et al*., 1975). Numerous studies have reported that salinity treatment increased POD activity in plants (Jebara *et al*., 2005; Mohamed and Aly, 2008; Chookhampaeng, 2011; Sevengor *et al*., 2011). FLA has been recently suggested as playing primary antioxidant functions in the responses of plants to a wide range of abiotic stresses (Brunetti *et al*., 2013). They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants, and for light screening (Pietta, 2000). FLA is formed in plants from the aromatic amino acids phenylalanine and tyrosine, and malonate (Cody *et al*., 1986). However, most interest has been devoted to the antioxidant activity of FLA, which is due to their ability to reduce free radical formation and to scavenge free radicals (Pietta, 2000). FLA in the vacuole of mesophyl cells are in very high concentrations and hence capable of removing H_2O_2 freely diffusing out of the chloroplast under severe excess light stress, when the activity of catalase is strongly depressed (Polle, 2001). Flavonoid glycosides have a much smaller affinity than corresponding aglycones for peroxidases, but their concentrations may allow detoxify H_2O_2 efficiently (Agati *et al*., 2012). The reducing functions of FLA are of primary significance in plants suffering from severe stress conditions (Brunetti *et al*., 2013). FLA compounds are a large group of secondary metabolites, which can play a role in virtually any interaction a plant can have with its environment

(Waterman and Mole, 1994). These compounds have been implicated to stress resistance against biotic and abiotic factors (Bergmann *et al*., 1994). TP compounds are a large group of secondary metabolites, which can play a role in virtually any interaction a plant can have with its environment (Waterman and Mole, 1994). These compounds have been implicated to stress resistance against biotic and abiotic factors (Bergmann *et al*., 1994; Meguekam *et al*., 2014). TP accumulation could be a cellular adaptive mechanism for scavenging oxygen free radicals during stress (Mohamed and Aly, 2008).

Pepper (*Capsicum annuum* L.) is an important agricultural crop, because of its economic importance and the nutritional value of its fruits; it is an excellent source of natural colors, vitamin C and antioxidant compounds worthy for human health (Howard *et al*., 2000). Screening plant species for salinity tolerance or genetic potential to develop tolerance are promising approaches for developing salt tolerant commercial cultivars (Munns and Tester, 2008). The aim of the present work was to study the comparative effects of different concentrations of NaCl on growth, nutrient partitioning, osmolytes accumulation, total chlorophyll, relative water content and antioxidant compounds in pepper cultivars**.** Comparison of these parameters in these pepper cultivars may be helpful to provide additional information on the mechanisms of salt tolerance and develop salt tolerant cultivars for breeding program.

Materials and Methods

Plant materials

Pepper (*Capsicum annuum* L.) is especially productive in warm and dry climates than *C. frutescens* which can tolerate most climates. It also displays a greater resistance to disease and insects, especially to the tobacco mosaic virus. Capsaicinoids chemicals and antioxidants such as carotenoïds provide the distinctive tastes in *C. annuun* variants. The fruit are berries that may be green, yellow or red when ripe. The mature green stage is ideal to acquire maximum pungency due to capsaicinoids, whereas peppers at red ripe stage are best sources of ascorbic acid and dried fruits contain higher levels of total carotenoids (Iqbal *et al*., 2013). Hot peppers are used in medicine as well as food in Africa. Seeds of three pepper cultivars ('Granada', 'Goliath' and 'Nobili'), provided by the breeding program of the Agronomic Institute for Research and Development of Cameroon were used in the study.

Plant growth conditions and salt treatments

The present work was performed in the greenhouse of the Faculty of Science at University of Douala, Cameroon, from October 2013 to May 2014. The seeds were surface sterilized with 3% sodium hypochlorite for 20 min and washed four times with deionized water. One-month-old pepper seedlings were transplanted into 5-L plastic pots filled with 5 kg of sterilized sand. The pots were arranged in a complete randomized design with one plant per pot and four replicates per treatment. All plants were fertilized daily with a modified nutrient solution (in g L^{-1}): 150 g $Ca(NO_3)_2$, 70 g KNO_3, 15 g Fe-EDTA, 0.14 g KH_2PO_4, 1.60 g K_2SO_4, 11 g $MgSO_4$, 2.5 g $CaSO_4$, 1.18 g $MnSO_4$, 0.16 g $ZnSO_4$, 3.10 g H_3BO_4, 0.17 g $CuSO_4$ and 0.08 g MoO_3 (Hoagland and Arnon, 1950). The pH of the nutrient solution was adjusted to 7.0 by adding HNO_3 0.1 mM. For the determination of physiological and biochemical responses of pepper cultivars to salt stress, each cultivar was subjected to 0 (control), 50, 100 and 200 mM NaCl. Plants were watered with deionized water every morning. The daily amounts of water added to the pots were the same for all treatments. Throughout the growth period, average day/night temperatures in the greenhouse were 26 °C/20 °C and the relative air humidity averaged 68.5%.

Plant growth parameters

Plants were harvested 42 DAP. SL, SD, NL and TLA were recorded. Leaves, stems, and roots were separately dried at 70 °C for 72 h, and their dry weights were determined. The RWC ((leaf FW-leaf DW) *100/leaf FW) and TLA (length*width*0.80*total no. of leaves*0.662) were calculated using the methodology described by Kumar *et al*. (2002). Leaf CHL content was determined using Arnon (1949) method. Subsamples (20 mg) of fresh leaves were extracted with 80% alkaline acetone (v/v). The filtrate was analyzed with a spectrophotometer (BECKMAN DU-68, UV/VIS) at 645 and 663 nm wavelengths.

Nutrient contents

For determination of K, Na, Ca, and Mg, subsamples (300 mg) of dried ground leaves (including leaves lost over the growth period) were dry ashed at 550 °C for 4 h and thoroughly mixed with 250 mL of deionized water. The filtrate was analyzed with an atomic absorption spectrophotometer (EPOS 5060, Eppendorf, Hamburg, Germany).

Osmolyte contents

For measurement of SS content, a modified phenol-sulfuric assay was used (Dubois *et al*., 1956). Subsamples (100 mg) of dry leaves were placed in 50 mL centrifuge tubes. 20 mL of extracting solution (glacial acetic acid:methanol:water, 1:4:15 (v/v/v)) was added to the ground tissue and homogenized for 15 sec at 16000 rpm. The homogenate was centrifuged for 10 mn and the supernatant was decanted to a 125 ml Erlenmeyer flask. The residue was resuspended in 20 mL of extracting solution and centrifuged another 5 min. The supernatant was decanted, combined with the original extract, and made up to 100 mL with water. One mL of 5% (v/v) phenol solution and 5 mL of concentrated H_2SO_4 were added to 1 mL aliquots of SS (reconstituted with 1 mL water). The mixture was shaken, cooled to room temperature, and absorbance recorded at 490 nm wavelength with spectrophotometer (Pharmaspec UV-1700 model). The amount of SS present in the extract was calculated using standard curve prepared from graded concentration of glucose.

Pro content was estimated by acid ninhydrin procedure (Bates *et al*., 1973). Subsamples (0.5 g) of fresh leaves were homogenized in 10 mL of 3% (W/v) aqueous sulfosalicylic acid to precipitate protein. The reaction mixture consisted of 1 ml acid ninhydrin and 1 ml of glacial acetic acid, which was boiled at 100 °C for 1 h. After cooling of the tubes in ice, the products were extracted with 2 ml of toluene by vortex mixing and the upper (toluene) phase decanted into a glass basin. The absorbance was read with a spectrophotometer (Pharmaspec UV-1700 model) at 520 nm wavelength.

PR content was evaluated using the Bradford (1976) method. Subsamples (0.1 g) of fresh leaves were homogenized with 4 mL of sodium-phosphate buffer, pH 7.2. The mixture was then centrifuged at 13000 rpm for 4.5 min at 4 °C. One milliliter of the supernatant was poured into a tube containing 5 mL of the Bradford reagent. The mixture was then shaken and incubated in the dark for 15 min. The absorbance of the resulting blue complex was read at 595 nm wavelength with a spectrophotometer (Pharmaspec UV-1700 model). The standard curve was obtained using Bovine Serum Albumin 1 mg mL^{-1}.

FAA content was determined by the ninhydrin method (Yemm *et al*., 1955). Subsamples (1 g) of fresh leaves were ground in 5 mL of ethanol 80%, amino acids were then extracted using reflux technique in boiling ethanol for 30 min. The filtrate was collected and the residue used to repeat the extraction. The two mixed filtrates constituted the raw extract of amino acids that were measured using ninhydrin method. The absorbance of purplish bruise complex was read at 570 nm wavelength. The standard curve was established using 0.1 mg mL^{-1} of glycine.

Antioxidant and non-enzymatic antioxidants

POD activity was determined according to the method described by Jebara *et al*. (2005). The assay mixture of 3 ml contained 1.5 ml of 0.1 M phosphate buffer (pH 7.0), 1 ml freshly prepared 10 mM guaiacol, 0.1 ml enzyme extract and 0.1 ml of 12.3 mM H_2O_2. Initial absorbance was read at 436 nm wavelength and then increase in the absorbance was noted at the interval of 30 s on spectrophotometer (Pharmaspec UV-1700 model). Activity was calculated using the extinction coefficient 26.6 mM^{-1} cm^{-1} for the oxidized tetraguaiacol polymer. Enzyme activity was expressed as μmol guaiacol oxidized min^{-1} g^{-1} protein.

SOD activity was determined according to the method described by Dhindsa *et al*. (1981). Three mL of reaction mixture containing 0.1 mL of 1.5 M Na_2CO_3, 0.2 mL of 200 mM methionine, 0.1 mL of 3 mM EDTA, 0.1 mL of 2.25 mM *p*-nitroblue tetrazolium chloride (NBT), 1.5 mL of 100 mM potassium phosphate buffer (pH 7.5), 1 mL of distilled water and 0.05 mL of enzyme samples. The tube without enzyme was taken as control. Reaction was started by adding 0.1 ml 60 μM riboflavin and placing the tubes below a light source of two 15 W fluorescent lamps for 15 min. The reaction was stopped by switching off the light and covering the tubes with black cloth. Absorbance was recorded at 560 nm wavelength. An illuminated blank without protein gave the maximum reduction of NBT, and therefore, the maximum absorbance at 560 nm wavelength. SOD activity is presented as absorbance of blank minus absorbance of sample, giving the total inhibition, calculated per microgram protein. The activity of SOD was expressed as U mg^{-1} protein.

TP content of the extract was determined by the Folin-Ciocalteu method (Marigo, 1973). Subsamples (1 g) of fresh leaves were ground at 4 °C in 3 mL of 0.1 N HCl. After incubation to 4 °C during 20 min, the homogenate was centrifuged at 6000 g during 40 min. The supernatant was collected, the pellet re-suspended in 3 mL of 0.1 N HCl and centrifuged as previously. The two supernatant are mixed and constitute the crude extract of soluble phenol. The reaction mixture containing 15 μL of extract, 100 μL Folin-Ciocalteu reagents, 0.5 mL of 20% Na_2CO_3 was incubated at 40 °C for 20 min and absorbance read at 720 nm wavelength with a spectrophotometer (Pharmaspec UV-1700 model). A standard curve was established using chlorogenic acid. TP content was expressed as mg g^{-1} fresh weight.

FLA content of crude extract was determined by the aluminium chloride colorimetric method (Chang *et al*., 2002). 50 μL of crude extract (1 mg/mL ethanol) were made up to 1 mL with methanol, mixed with 4 mL of distilled water and then 0.3 mL of 5% $NaNO_2$ solution; 0.3 mL of 10% $AlCl_3$ solution was added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Then, 2 mL of 1 mol/L NaOH solution were added, and the final volume of the mixture was brought to 10 mL with double-distilled water. The mixture was allowed to stand for 15 min, and absorbance was recorded on spectrophotometer (Pharmaspec UV-1700 model) at 510 nm wavelength. FLA content was calculated from a rutin calibration curve, and the result was expressed as g rutin equivalent per g dry weight.

Experimental design and statistical analysis

The experiment was conducted as a factorial completely randomized design with four NaCl treatments and three cultivars in five replications. Data are presented in term of mean (± standard deviation). All data were statistically analysed using Statistica (version 9, Tulsa, OK, USA) and first subjected to analyses of variance (ANOVA). Statistical differences between treatment means were established using the Fisher LSD test at $p < 0.05$.

Results and Discussion

Plant growth

Pepper growth was estimated by measuring RDW, SDW, SL, SD, NL and TLA of three cultivars plants under four NaCl concentrations at vegetative stage (42 DAP). There were statistically significant differences among the cultivars for all salt concentrations and plant growth parameters. Application of NaCl treatment led to a significant decrease in RDW, SDW, SL, SD, NL and NR of 'Goliath' compared to untreated plants (Table 1). The main effect of NaCl on plant growth parameters was that plants of 'Granada' and 'Nobili' under 50 mM NaCl remained almost unaffected for SDW, SL, NL and TLA and presented significantly increased values for RDW and SD as compared to those of untreated plants while the main effect of the cultivar on the majority of growth parameters determined was that 'Goliath' was negatively affected by NaCl treatment and presented significantly lower values compared to 'Granada' whereas 'Nobili' showed intermediate ones. The effect of salt on plant growth inhibition was notably noted at 100 mM NaCl in 'Granada' and 'Nobili' for SDW, SL and TLA but 'Granada' had higher values than 'Nobili' (Table 1). The interaction cultivar x salt treatment was significant for SDW and SL (Table 1). The reduction of growth parameters in salt sensitive 'Goliath' is a consequence of several physiological responses including modification of ion balance, mineral nutrition, stomatal behaviour and photosynthetic efficiency (Hosseini and Thengane, 2007; Li *et al*., 2008; Mudgal *et al*., 2010). This is consistent with the reports that NaCl reduces the ability of the plant to take up water, and this leads to slow growth; then when excessive amounts of salt entering the transpiration stream will eventually injure cells in the transpiring leave and this may further reduce growth (Munns, 2002). In the present study,

the salt inhibition effect in growth parameters studied was significantly noted at 50 mM NaCl in 'Goliath', while 'Granada' and 'Nobili' were significantly ($p < 0.05$) affected at 100 mM NaCl. These results demonstrate that 'Goliath', in common with certain other leguminous plant (e.g. beans), is highly sensitive to salt with severe effects at 50 mM NaCl (Levitt, 1980; Taffouo *et al*., 2009). Under salt stress 'Granada' was observed to have relatively higher tolerance on average of all growth parameters than 'Nobili', intermediate ones. Similar observations for plant growth were reported in 'White seed coat' (Taffouo *et al*., 2010) and 'Fleur 11' (Meguekam *et al*., 2014), described as salt-tolerant cultivars. In the first phase of a biphasic model of growth response to salinity, the vegetative growth is reduced by a decrease in a soil water potential due to water stress effect and may be regulated by inhibitory signals from the roots (Munns, 2002). The fact that salt stress resulted in a considerable decrease of SDW and RDW has been mentioned by other researchers (Nagesh Babu and Devaraj, 2008; Meguekam *et al*., 2014). However, salinity affected root growth less than shoot growth in 'Granada' and 'Nobili' in the present work. These results corroborate the findings of Cordovilla *et al*. (1999) with *Vicia faba* plants but they contradict those of Wignarajah (1992) and Bayuelo-Jiménez *et al*. (2002) with *Phaseolus vulgaris*.

Nutrient uptake

The main effect of NaCl on leaf, stem and root Na concentrations of plants under salt stress showed significant increases as compared to control plants (Table 2). The highest Na concentrations (14.60 g kg^{-1}) were detected in the leaves while the lowest (9.70 g kg^{-1}) were recorded in the roots of 'Goliath' at 200 mM NaCl (Table 2). These results are in accordance with those of Slama (1986) who found that the salt sensitivity of some crops was related to its higher concentration of Na in the leaves and lower in the roots but they contradict those of Taffouo *et al*. (2010) who found that the retention of Na^+ in the roots allows the salt sensitive 'Red Seed Coat' landrace to avoid the invasion of the leaves by toxic elements that are likely to reduce the photosynthetic activity by interfering with the opening of stomata and other metabolic processes (Turan, 2007). Na concentrations in the leaf tissues increased significantly under salt treatment in salt-tolerant 'Granada' and 'Nobili' (Table 2). Na^+/H^+ exchanger (NHXs) in halophytic species has been well established as a major channel to manage the Na influx from the soil solution into root cell and translocate via xylem loading to other organs, leading to increased Na/K ratio (Flowers and Colmer, 2008; Li *et al*., 2008). Similar observations were reported by Taffouo *et al*. (2004) and Theerawitaya *et al*. (2015) with two halophyte legumes (*Phaseolus adenanthus* and *Acacia ampliceps*). In this study, K, Ca and Mg concentrations were significantly reduced with increasing salinity in all cultivars (Table 2). It has been reported that salinity affects plant physiology through changes of water and ionic status in the cells because of ionic imbalance due to excessive accumulation of Na and Cl and reduced uptake of other mineral nutrients, such as K, Ca and Mg (Hasegawa *et al*., 2000). According to Saghir *et al*. (2002), the ionic stress affects plant growth by increasing Na and Cl levels in cells in response to high concentrations of NaCl, and decreased Ca, K, and Mg concentrations. This could be also attributed to the competition of Na with the uptake K, resulting in a K/Na antagonism (Hosseini and Thengane, 2007). The leaf K/Na ratios were found to be significantly highest in 'Granada' and lowest in 'Goliath' (Table 2). The relationship between the degree to which plant tolerate salt stress and their capacity to maintain a high leaf ratio K/Na has been noted by several authors (Al-Karaki, 2000).

Chlorophyll concentrations

In the present study, the depressive effect of salt was less marked on leaf CHL content in 'Granada' compared to 'Nobili', 'Goliath' and untreated plants (Fig. 1). NaCl treatment decreased the leaf CHL content in the salt sensitive 'Goliath' at low concentration (50 mM). This effect of salt was attributed to salt-induced weakening of protein-pigment-lipid complex (Strogonov, 1970) or increased chlorophyllase

Table 1. Effect of salt stress on plant growth in pepper cultivars at the vegetative stage (42 DAP)

Cultivar	Treatment (mM NaCl)	PDW (g $plant^{-1}$)		SD (cm)	SL (cm)	NL	TLA (cm^2 $plant^{-1}$)
		RDW	SDW				
Granada	0	0.17±0.08e	3.33±0.16a	1.85±0.19^e	9.90±0.53d	9.00±0.50a	44.23±2.60a
	50	0.11±0.01f	3.30±0.18a	2.00±0.04c	9.80±0.21d	8.85±0.50a	40.51±1.20a
	100	0.92±0.13a	3.18±0.15b	2.10±0.01b	8.60±0.10f	7.75±0.52d	30.50±2.09c
	200	0.77±0.10c	3.10±0.13c	2.10±0.14b	6.10±0.14j	6.75±0.50f	20.28±1.60d
Goliath	0	0.75±0.02f	3.10±0.13c	2.10±0.11b	11.85±0.11a	8.50±0.40b	28.76±1.42c
	50	0.51±0.07f	2.81±0.20d	1.98±0.09c	7.75±0.81g	7.25±0.51e	23.74±1.40d
	100	0.45±0.11c	1.53±0.09e	1.95±0.10c	7.38±0.50h	6.50±0.57g	22.31±1.38d
	200	0.34±0.03d	0.99±0.03f	2.15±0.18a	6.25±0.63i	6.00±0.50g	21.67±1.10d
Nobili	0	0.16±0.04e	3.20±0.34b	1.85±0.05^e	11.20±0.27b	9.00±0.01a	38.72±2.60ab
	50	0.90±0.15a	3.17±0.21b	1.90±0.08d	10.68±1.10c	9.00±0.01a	36.33±1.60b
	100	0.83±0.25b	3.10±0.18c	1.98±0.09c	9.90±0.90d	8.25±0.01c	35.14±1.01bc
	200	0.50±0.10d	3.08±0.19c	1.95±0.10c	9.00±0.50e	7.25±0.50e	20.55±1.20d
Two way ANOVA results							
Cultivar (C)		*	*	*	*	*	*
Salt treatment (S)		*	**	*	*	*	*
Interaction C X S		NS	*	NS	*	NS	NS

Values shown are means (n=10) ± SD; within columns, means followed by different letter are significantly different ($p < 0.05$).
**, * significant at 1 and 5% probability levels, respectively, NS not significant

Table 2. Effect of salt stress on ions concentrations (g kg^{-1}) in pepper cultivars at vegetative stage (42 DAP)

Cultivar		Treatment (mM NaCl)	Na	K	Ca	Mg	K/Na
Granada	Leaf	0	2.90±0.02g	38.00±0.82a	41.00±0.82a	38.50±0.18a	13.10c
		50	5.60±0.03f	28.60±0.18c	36.00±0.82b	29.00±1.82b	5.10e
		100	8.60±0.07d	25.00±0.82d	28.00±0.82c	21.60±0.18c	2.90f
		200	18.00±0.82a	23.50±0.18d	20.60±0.18d	19.40±0.23cd	1.30f
	Stem	0	2.10±0.08g	34.60±0.18b	29.00±0.82c	20.00±1.82c	16.48b
		50	5.20±0.07f	31.50±0.18bc	18.00±0.58de	15.90±0.52d	6.06e
		100	10.20±0.27cd	27.90±0.50d	9.60±0.18g	10.60±0.18e	2.74f
		200	14.40±0.23b	19.90±0.75de	8.00±0.02g	8.70±0.08e	1.38f
	Root	0	1.80±0.06d	21.80±0.18d	23.90±0.52d	38.90±0.18a	12.11cd
		50	5.40±0.06f	16.90±0.52e	18.00±082de	29.80±0.38b	3.13f
		100	10.60±0.25cd	12.70±0.18f	13.40±0.83e	25.90±0.52c	1.20f
		200	12.80±0.18c	9.50±0.18fg	15.60±0.18e	20.50±0.18c	0.74g
Goliath	Leaf	0	1.60±0.08d	29.90±0.52c	34.00±0.82b	31.50±0.18b	18.70b
		50	4.40±0.08f	18.50±0.18de	29.00±0.82c	27.00±0.58bc	4.20e
		100	7.70±0.03e	14.40±0.18f	26.00±0.82cd	19.50±0.18cd	1.90f
		200	14.60±0.18b	11.50±0.18f	15.60±0.18e	13.50±0.18d	0.80g
	Stem	0	2.20±0.04g	22.00±3.36d	25.30±0.18cd	24.40±0.18bc	10.00d
		50	4.90±0.09f	16.80±0.18e	19.00±0.82de	13.00±0.58d	3.43f
		100	7.60±0.08e	11.30±0.18f	10.90±0.52g	9.20±0.27e	1.49f
		200	12.70±0.18c	9.20±0.27fg	6.40±0.08g	7.00±0.02e	0.72g
	Root	0	1.50±0.01d	16.00±0.82e	18.00±0.82	28.90±0.52b	10.66d
		50	4.80±0.08f	10.60±0.18f	16.09±0.52e	23.00±2.94c	2.21f
		100	8.80±0.08d	8.60±0.08fgh	11.40±0.18g	15.40±0.23d	0.98f
		200	9.70±0.58d	6.20±0.08h	8.00±0.02g	11.00±0.82de	0.64g
Nobili	Leaf	0	1.50±0.08g	34.40±0.18b	43.00±0.82a	35.40±0,23a	22.93a
		50	6.50±0.08ef	25.00±0.18d	34.90±0.52b	29.00±0.82b	3.85f
		100	9.90±0.29d	22.90±0.52d	20.00±0.82d	19.60±0.18c	2.31f
		200	16.40±0.36a	21.00±0.36d	22.40±0.18d	14.60±0.18d	1.28f
	Stem	0	2.20±0.08g	30.90±0.52bc	28.90±0.52c	23.00±0.94c	14.05c
		50	4.40±0.06f	29.00±0.82c	18.40±0.18de	14.50±0.18d	6.59e
		100	9.90±0.19d	22.00±0.36d	12.80±0.18f	11.00±0.82de	2.2f
		200	14.80±0.18b	14.00±0.82f	9.90±0.52g	10.40±0.18e	0.95g
	Root	0	1.70±0.08g	19.00±0.82de	21.00±4.16d	30.70±0.18b	11.18cd
		50	4.70±0.08f	13.90±0.52f	19.40±0.18de	26.60±0.18bc	2.96f
		100	9.40±0.36d	6.60±0.08h	14.00±1.82f	18.00±0.82cd	0.70g
		200	10.40±0.36b	8.00±0.08fgh	10.60±0.18g	12.00±0.02de	0.77g
Two way ANOVA results							
Cultivars (C)			*	*	*	NS	*
Salt treatment (S)			**	*	*	*	*
Interaction C x S			*	*	NS	NS	*

Values shown are means (n=5) ± SD; within columns, means followed by different letter are significantly different ($p < 0.05$).
**, * significant at 1 and 5% probability levels, respectively, NS not significant

enzyme activity (Sivtsev, 1973). In salt tolerant 'Granada' salt affected leaf CHL content at high salt level (200 mM) (Fig. 1.). CHL degrades depending on the degree of salt levels in the soil solution, especially in extreme salt stress (10 dS m^{-1}) (Giri *et al.*, 2003), causing to reduce net photosynthetic rate, especially in extreme salt stress (Takemura *et al.*, 2000). Similar results were reported by Turan *et al.* (2007) with lentil plants.

Leaf relative water content

Leaf RWC of pepper cultivars at different salinity levels is depicted in Fig. 2. There are significant differences between cultivars. The increased RWC values in salt-tolerant cultivars 'Granada' and 'Nobili' under 100 mM NaCl than salt-sensitive 'Goliath' suggest that, accumulation of osmolytes such as SS,

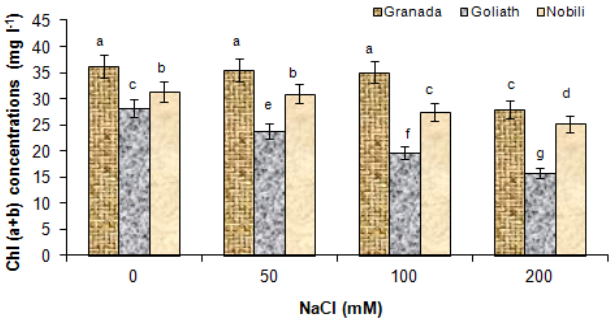

Fig. 1. Effect of salt stress on chlorophyll concentrations in pepper varieties at vegetative stage (42 DAP). Bars are means (n=5) ± SD. Means followed by different letter are significantly different ($p < 0.05$)

PRO, SP, FAA (Fig. 3) makes the surplus of water uptake possible. Similar results were obtained by Salwa *et al.* (2010) with peanut cultivars. On the contrary, a significant decrease in RWC was found at high salinity level (200 mM) in all cultivars. These results may be attributed to the accumulation of toxic ions such as Na^+ and Cl^-, reducing leaf expansion and stomata closure leading to a reduction in intracellular CO_2 partial pressure (Hasegawa *et al.*, 2000). According to Munns (2002) studies, salinity reduces the ability of plants to take up water, and this quickly causes reductions in growth rate, along a suite of metabolic changes identical to those causes by water stress.

Osmolyte contents

The presence of NaCl resulted in a significant increase in SS, SP, PRO and FAA contents in leaves of all cultivars compared to untreated plants, thereby playing a major role as osmotic adjustment (Fig. 3A, B, C and D). The salt tolerant 'Granada' accumulated the highest amount of all osmolytes followed by the moderately tolerant 'Nobili' and the salt-sensitive 'Goliath'. Compartmentation of ions in vacuoles and accumulation of compatible solutes in the cytoplasm are commonly proposing mechanisms to salt tolerance of plants (Munns, 2002). The plants supplied with NaCl showed significantly higher increase and accumulation of SS concentrations in leaves of 'Granada' compared to all other cultivars (Fig. 3A). When abiotic stress affects plant functionality, alterations in photosynthesis and carbon partitioning are common features that take place at organ level as well as in whole plant (Gill *et al.*, 2003). SS do not only function as metabolic resources and structural constituents of cells, they also act as signals regulating various processes associated with plant growth and development (Jang and Sheen, 1997). Recent studies for increasing tolerance to environmental stresses, through metabolic engineering of compatible solutes, have shown that increases in SS and/or other osmolytes provide optimism to increase plant tolerance to abiotic stresses such as drought, salinity and cold (Cusido *et al.*, 1987; Rathinasabapathi, 2000). SP content in plants increased significantly under salt stress in all cultivars compared to untreated plants (Fig. 3B). In salt tolerant species, the osmotic balance of the cytoplasm is ensured by an active synthesis of the organic compounds (Grigore *et al.*, 2011). In plants, SP are involved in osmotic adjustment (Nouck *et al.*, 2016). They are stored as nitrogen under salt-stress and re-used when the stress is removed (Singh *et al.*, 1987). PRO content in plants increased significantly under salt stress in all cultivars compared to untreated plants (Fig. 3C). PRO has been widely considered to be a compatible solute that accumulates in plant in response of wide variety of environmental stresses and confers stress tolerance by contributing to osmotic adjustment, protecting proteins, membranes and quenching reactive oxygen species (Mudgal *et al.*, 2010). Similar findings were reported by Ashraf and Foolad (2007); Grigore *et al.* (2011); Meguekam *et al.* (2014) and Theerawitaya *et al.* (2015). According to Kant *et al.* (2006), halophytes accumulate more PRO than glycophytes, and it has been related to the suppression of PRO catabolism by proline oxidizing enzyme (proline dehydrogenase), and enhanced synthesis of PRO via pyrroline-5-carboxylate synthetase. PRO is significantly accumulated under salt stress and performs the positive role in the adaptation of cells to salt and water stress (Kaviani, 2008). In this study, application of NaCl had a positive effect on leaf FAA content with the highest increase found in 'Granada' and the lowest in 'Goliath'(Fig. 3D). According to Cusido *et al.* (1987) the deficit of K induced by salinity increased the levels of FAA, especially of aspartic acid, glutamic acid and PRO.

Antioxidant compounds

In response to stress, plants activate powerful antioxidant systems, both enzymatic (e.g., SOD, POD, catalase, glutathione reductase) and non-enzymatic (FLA, TP, carotenoids, vitamins C and E) (Ashraf, 2009; Kahrizi *et al.*, 2012). In the present study, NaCl addition led to a significant increase in SOD, POD, TP contents in all cultivars, on the contrary, decreased in FLA content (Fig. 4). Similarly, Azooz *et al.* (2009) showed a positive antioxidant response to salt stress on various crop (eg. *Zea mays* and *Lycopersicum esculentum*, respectively). The inhibition effect of salt stress on FLA content in all cultivars is due to the reduction of functions which are the primary significance in plants suffering from severe stress conditions (Brunetti *et al.*, 2013). According to Agati *et al.* (2012), FLA is located in the nucleus of mesophyll cells, and hence capable of quenching H_2O_2 and H_2O_2-generated hydroxyl radical. In this study, the highest increase in SOD, POD and TP activities was

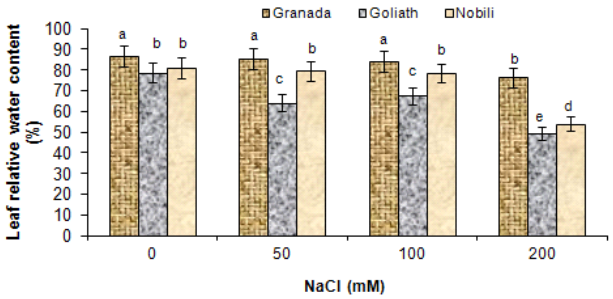

Fig. 2. Effect of salt on leaf relative water content in pepper varieties at vegetative stage (42 DAP). Bars are means (n=5) ± SD. Means followed by different letter are significantly different ($p < 0.05$)

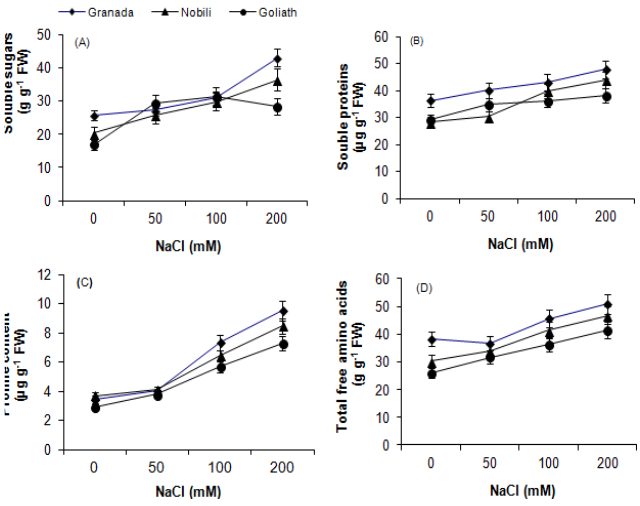

Fig. 3. Effect of salt on accumulation of osmolytes in pepper cultivars (42 DAP). Soluble carbohydrates (A), soluble proteins (B), proline content (C) and total free amino acids (D). Bars are means (n=5) ± SD

found in salt-tolerant 'Granada' and the lowest in salt-sensitive 'Goliath' (Fig. 4B, C and D). Similarly, the level of antioxidant enzymes was higher in salt-tolerant than in salt-sensitive species under various environmental stresses (Demiral and Turkan, 2005). To cope with oxidative damage under extremely adverse conditions like salt stress, plant have developed an antioxidant defense system that includes the antioxidant enzymes SOD, POD and catalase (Karanlik, 2001; Foyer and Noctor, 2005). SOD detoxifies superoxide anion free radicals accompanying the formation of H_2O_2 which is very damaging to the chloroplasts, nucleic acids and proteins (Marschner, 1995). Numerous studies have reported that NaCl treatment increased SOD activity (Ahmad *et al.* 2008; Gama *et al*., 2008; Maia *et al*., 2010; Chookhampaeng, 2011; Kahrizi *et al*., 2012). Accordingly, we also found higher activity of POD in leaves of all the cultivars under salt stress conditions (Fig. 4D). Numerous studies have reported that salinity treatment increased POD activity in plants (Jebara *et al*., 2005; Mohamed and Aly, 2008; Chookhampaeng, 2011; Sevengor *et al*., 2011). An increase in the antioxidative enzymes under salt stress could be indicative of an increased production of ROS and build up of a protective mechanism to reduce oxidative damage triggered by stress in plants. POD in cytosol and chloroplast can perfectly scavenge H_2O_2 (Kahrizi *et al*., 2012).

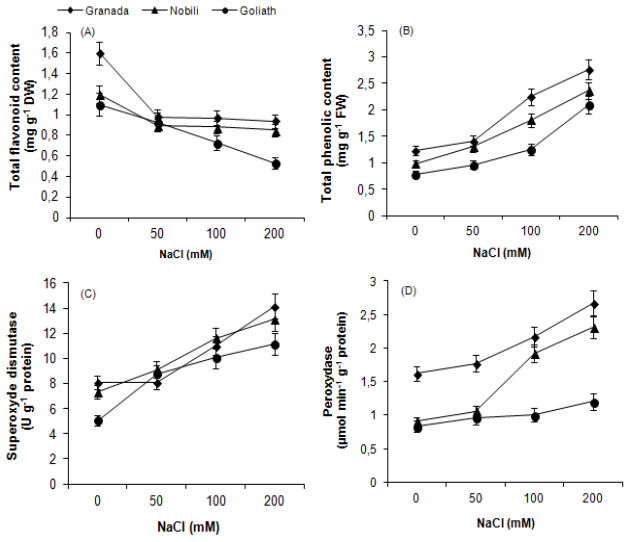

Fig. 4. Effect of salt on antioxidant compounds in pepper cultivars at the vegetative stage (42 DAP). Total flavonoid content (A), total phenolic content (B), superoxide dismutase (C) and peroxidase activities (D). Bars are means (n=5) ± SD

Conclusions

The pepper cultivars in this study showed differential responses for growth, osmolytes accumulation and enzymes activities measured under salt stress. The scavenging system in salt-tolerant cultivar, 'Granada' exhibited higher TP content, POD and SOD activity, than in the salt-sensitive cultivar 'Goliath'. Thus, the salt tolerance of these pepper cultivars seems to be linked to the activities of these antioxidant enzymes. The salt tolerance of pepper cultivars could induce antioxidative enzyme system more efficiently, resulting in higher osmolytes accumulation under salt stress. The conducted study confirmed the genetic variability in salt tolerance among three pepper cultivars which are widely used in Cameroon. 'Granada', followed by 'Nobili', intermediate ones, was found to be the most tolerant cultivar based on the majority of growth parameters, osmolytes accumulation and enzymes activities measured. On the contrary, the most salt sensitive one was 'Goliath', as it presented their greatest salt effect plant growth inhibition at low salinity level (50 mM), low osmolytes accumulation and low enzymes activities assessed. The SS, SP, PRO and FAA were enriched in cellular levels of leaf tissues to play a key role in osmoregulation of salt defense mechanism to protect salt-tolerant 'Granada' from salt-induced toxicity and thus it could be an excellent cultivar to grow in salt-affected soils. FLA content, K, Ca and Mg concentrations were significantly reduced with increasing salinity in all cultivars. The highest Na concentrations under 200 mM NaCl were detected in the roots and the lowest ones in the leaves of the salt-sensitive 'Goliath'.

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