ASSESSMENT OF OXIDATIVE STRESS AND PROLINE METABOLISM GENES EXPRESSION OF COWPEA PLANTS (*Vigna unguiculata* L.) UNDER SALINE CONDITIONS

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Abstract. This work was aimed to study the effects of salt stress induced by increasing concentrations of sodium chloride (NaCl): 50, 100 and 150 mM on cowpea plants (*Vigna unguiculata* L.). The results showed that higher levels (100 and 150 mM) of NaCl significantly affected the growth by reducing shoot height, leaf area and shoot dry weight of plants.

At the cellular level, the salt induced a decline in relative water content correlated to an accumulation of proline in leaves. This accumulation was shown to be related to an over expression of P5CS gene (Δ^1-pyrroline 5 carboxylate synthase) and to ProDH (proline dehydrogenase) gene expression which remained at a low level for all salt treatments. Increasing salinity had also decreased photosynthetic pigments; both total chlorophyll and carotenoids contents declined by 40 % under 150 mM NaCl.

The effect of oxidative stress induced by salt stress on cowpea plants was determined by evaluating the membrane integrity of cells; the results revealed that salinity increased up to 50% the malondialdehyde content in leaves as well as the electrolytes leakage in plants grown on 150 mM NaCl compared to controls. However, the oxidative damages seemed to be counterpoised by increasing the catalase and ascorbate peroxidase activities in stressed plants. Besides, at transcriptional level, no variation in mitochondrial (AOX2b) and plastidial (PTOX) alternative oxidases genes expression was recorded.

Keywords: *Vigna unguiculata*; salt stress; proline; ProDH; P5CS; antioxidant enzymes.

INTRODUCTION

Cowpea (*Vigna unguiculata* L.) is a warm-season, annual, herbaceous legume grown throughout the African continent, Latin America and parts of Southeast Asia. It is a valuable source of proteins, minerals and vitamins for human diet and it provides fodder for livestock [52]. In addition to its nutritional qualities, cowpea is less demanding in fertilizer and tolerant to drought [10]. It improves soil fertility by its ability to fix atmospheric nitrogen [36]. However, in many parts of the world, crop yields, particularly cowpea, are increasingly difficult to obtain due to constraints related to environmental conditions. Among these, salinity is considered to be one of the most severe environmental factors that limit growth and agricultural productivity [27]. The deleterious effects of salinity on plant growth can be initially associated with the osmotic stress component caused by decreases in soil water potential and consequently, restriction of water uptake by roots. Plant growth is also affected by toxic damages as function of nutritional disequilibrium and high salt levels uptake [12]. Salinity may also generate secondary oxidative stress due to the accumulation of reactive oxygen species like singlet oxygen ($^1O_2$), superoxide anion ($O\cdot^{-2}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), etc. The formation of these molecules is closely related to the perturbation of the photosynthetic and respiratory processes [59]. The ability of cells to manage the consequences of oxidative stress depends on their ability to mobilize their antioxidant defense systems. In case these systems are exceeded, the reactive oxygen species attack various cellular components: lipids, proteins or DNA, causing irreversible cell damages [65].

The intensity of adverse and detrimental effects of salinity stress depends upon the nature of plant species, severity and duration of salt stress, plant developmental stage, and cropping techniques [50]. Thus, under salt stress conditions, all plants do not react in the same way. Some are not able to withstand the presence of salt; others develop multiple biochemical, physiological and molecular mechanisms to protect cell functions, in particular by regulating proper water relations and maintaining ion homeostasis [29]. To balance the water potential, many plants species accumulate high level of osmotically active solutes like proline. Under osmotic stress proline biosynthesis occurs mainly from glutamate pathway [22]. Proline is produced in the cytosol or chloroplasts from glutamate by the rate limiting enzyme Δ^1-pyrroline-5-carboxylate synthase (P5CS). It is degraded in mitochondria by proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) to glutamate [63].

Redox homeostasis is also fundamental for normal cell survival. Its regulation includes control of reactive oxygen species (ROS) generation [29]. Excess ROS amounts are particularly scavenged by antioxidant metabolites such as ascorbate, glutathione, tocopherols, carotenoids and by ROS detoxifying enzymes as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT). SOD converts superoxide to H_2O_2, which is detoxified to water and oxygen by the catalase and/or ascorbate-glutathione cycle. Peroxidase (APX) uses ascorbate as the electron donor for the reduction of H_2O_2 and is important in H_2O_2 detoxification along with catalase [2]. Besides, many studies revealed that during environmental stresses mitochondrial alternative oxidase (AOX) and its analogue in chloroplast PTOX (plastoquinol terminal oxidase) could reduce the ROS generation by maintaining the relative redox balances of the electron

transport chains and then lower the possibility for oxidative damage [41]

Possible approaches to improve crop productivity under adverse environmental conditions require a better understanding on how plants respond to salinity stress at different levels [21]. Combining molecular tools with physiological and biochemical techniques at the cell or whole plant level can therefore be used not only to understand the behavior of plants against stress but also for the development of salt-tolerant plants in salt-affected areas [27].

To contribute to our understanding of the mechanisms underlying salinity stress responses, the present report was aimed to study the impact of salt stress on growth, physiological, biochemical and molecular attributes of drought tolerant plant of cowpea. The problematic was that drought tolerant plant of cowpea could display tolerance to different salinity levels, since drought and salt stress share several common and prompt responses in plants.

MATERIAL AND METHODS

Plant material and experimental design

Seeds of cowpea were collected from palm grove in Timimoun, an arid zone in southwestern Algeria. Seeds are surface-sterilized in diluted sodium hypochlorite (NaOCl) solution (1%, v/v) for 5 min and then rinsed several times with distilled water. Seeds were soaked on filter paper saturated with distilled water, and incubated at 25 °C in the dark. Germinated seeds were transferred to pots containing a mixture of cultivated soil (clay 43.94 %; silt 13.64 %; sand 42.42 %, pH 7.5) and peat mixture (3:1, v/v). The peat (reference V1 mix, Jiffy Products International BV, The Netherlands) is a mix of white and black peat with added fertilizer NFU42001 giving NPK content of 17, 10 and 4 kg/m3 respectively. Then, the pots were dispatched into 4 lots and watered with different concentrations: 0 mM (control irrigated with distilled water), 50, 100 and 150 mM of NaCl. The pots were kept under controlled growth conditions (in long-day conditions 16 h light, at temperature 25 °C) and watered twice a week. Plants were grown until the second trifoliate leaf was fully emerged (plants were 30 days old).

Morphological parameters

Plants were harvested after four weeks of treatment and data was collected for shoot height and shoot dry weight. The latter was determined after drying the plant samples in oven at 65 °C for 3 days. The leaf area (cm^2) was calculated from digitized images taken with a standard scanner, using Mesurim software.

Measurement of relative water content

Relative water content of leaves was estimated according to the method described by Smart and Bingham [53]. Leaf discs were punched from control and each treated plant and the fresh weight **(FW)** was determined. The same leaf discs were kept on water for 24 h and turgid weight **(TW)** was recorded. For dry weight **(DW)** the leaf sample was dried in oven at 80 ºC for 48 h. Calculation of relative water content was done by the following formula:

$$RWC\ (\%) = [(FW\text{-}DW)/ (TW\text{-}DW)]\times 100$$

Determination of photosynthetic pigments

Total chlorophyll and carotenoids contents were determined by the method of Lichtenthaler [37]. Leaf tissue (10 mg) was homogenized in 2 mL acetone (80 %). The homogenate was centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was recorded at 663 and 647 for chl a, chl b and 470 nm for carotenoids. The values are given in $mg.g^{-1}$ dry weight.

Determination of malondialdehyde

Oxidative damage to leaf lipids, resulting from salt stress, was estimated by the content of malondialdehyde (MDA) content. MDA content was measured by the method of Popham and Novacky [47]. Fresh leaf samples (0.1 g) were ground in 300 µL of buffer [Na_2HPO_4-NaH_2PO_4 at 0.2 M and pH 6.5 + 1 % triton x-100 + 0.001 % BHT]. The homogenate was centrifuged at 10000 rpm for 20 min at 4 ºC. An aliquot of 120 µL from the supernatant was added to 450 µL of 0.67 % (w/v) thiobarbituric acid (TBA) in 10 % (w/v) TCA. Samples were heated at 90 ºC for 15 min. Thereafter, the reaction was stopped in ice bath. Centrifugation was performed at 10000 rpm for 10 min, and absorbance of the supernatant was read at 532 nm on a spectrophotometer and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The malondialdehyde content was calculated using its absorption coefficient (ε) and expressed as nmol malondialdehyde g^{-1} dry weight following the formula:

$$MDA\ (nmol.g^{-1}\ dw) = (A532\ nm - A600\ nm) \times V \times 1000/ \varepsilon \times W$$

where ε is the specific extinction coefficient (=155 $mM^{-1}\ cm^{-1}$), V is the volume of crushing medium and W is the dry weight of leaf.

Estimation of membrane integrity by electrolytes leakage measurement

Electrolytes leakage (EL) was determined according to the method of Hubac et al. [31]. Leaf samples were washed with distilled water to remove surface adhered electrolytes and cut into discs of uniform size. Leaf discs were put in closed test tubes containing 20 mL of distilled water and incubated at 25 ºC for 1 h and subsequently electrical conductivity of the solution (C1) was recorded using a conductivity meter (HANNA HI 9932).

Samples were then kept in water bath at boiling temperature (100 ºC) for 1 h and the final electrical conductivity (C2) was obtained after equilibrium at 25 ºC.

The electrolytes leakage was defined as below:

$$EL\ (\%) = (C1 / C2) \times 100$$

Antioxidant enzymes activities measurements

Fresh leaves (0.1g) were homogenized in 1 mL of 50 mM Tris-HCl buffer (pH 8.1). The homogenate was centrifuged at 3000 rpm for 10 min at 4 ºC and the resulting supernatant was used for enzyme assays. The extraction was carried out at 4 ºC. Proteins were

quantified according to Bradford [14] using albumin bovine serum as a standard.

Catalase (CAT, EC 1.11.1.6) activity was assayed by the method described by Luck [38]. The activity of CAT was determined by monitoring the disappearance of H_2O_2 at 240 nm (extinction coefficient of 36 M^{-1} cm^{-1}). One unit of activity was defined as the amount of enzyme required to decompose 1 µmol H_2O_2 per min at 25 °C. The activity was expressed as U mg^{-1} protein.

Method of Nakano and Asada [44] was followed for the determination of ascorbate peroxidase activity (APX, EC 1.11.1.11). Assay mixture contained 0.1 mL of enzyme extract, 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2 and 1 mL of potassium phosphate buffer (pH 7.0). The decrease in absorbance of ascorbate was read at 290 nm, (extinction coefficient 2.8 mM^{-1} cm^{-1}). One unit of enzyme was defined as the amount necessary to decompose 1 µmol of ascorbate per min at 25 °C. The activity was expressed as U mg^{-1} proteins.

Determination of proline content

Proline content was measured according to the method of Troll and Lindsley [60] modified by Magné and Larher [39]. Dry weight (50 mg) was extracted by homogenization in 3 % (w/v) aqueous sulfosalicylic acid. After 20 min of centrifugation at 13000 rpm supernatant collected was mixed with acetic acid and ninhydrin and then was incubated at 90 ºC for 1 h. The reaction was cooled in an iced bath. The chromophore was extracted using 3 mL of toluene and its absorbance was determined by spectrophotometer at 520 nm. The reading is taken according to the calibration curve and values are given in mg.g-1 dry weight.

RT-PCR genes expression

The genes expression of Δ^1-pyrroline-5-carboxylate synthase (P5CS), proline dehydrogenase (ProDH), mitochondrial (AOX2b) and plastidial (PTOX) alternative oxidases were determined by RT-PCR. Total RNA was extracted by method of Salzman [49]. 200 mg of fresh leaf was grounded in liquid nitrogen. The powder was suspended in 0.75 mL extraction buffer (0.2 M Tris-HCl pH 7.5, 0.5 % (v/v) SDS, 0.25 M NaCl, 25 mM EDTA pH 9) and mixed with 0.75 mL of phenol/chloroform (1:1, v/v). The aqueous phase was extracted twice with phenol/chloroform. Total RNA was then precipitated overnight on ice with 2 M LiCl. After centrifugation (10 min, 10000 rpm at 4 ºC), the pellet was resuspended in 2 M LiCl and left to precipitate for 6 h on ice. Then a second centrifugation (10 min, 10000 rpm at 4 ºC) was performed, the pellet was resuspended in ethanol 70 % (v/v) and left to dry for 10 min. Pellet was than resuspended in 50 µL RNase-free water. Traces of DNA were removed by DNase treatment. RNA was quantified by measuring the absorbance at 260 nm and 280 nm using a Nanovue® spectrophotometer. RNA integrity was checked by electrophoresis on a 1 % agarose gel. First-strand cDNA was obtained from 1.5 µg of total RNA using Revert Aid Reverse Transcriptase and oligo(dT) as primer according to the manufacturer's instructions (Thermo Scientific). For RT-PCR, cDNAs were amplified using Dream Taq Green DNA polymerase (Thermo Scientific) and gene-specific primers. For each PCR reaction, 2 µL of cDNA was used as a template, 0.8 µM of forward and reverse primers, 0.2 mM dNTP, 1 unit of DreamTaq in 1× GreenTaq Buffer. PCR conditions were the following: 5 min at 94 °C, followed by a cycle of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C repeated 28−32 times according to each gene expression followed by 10 min at 72 °C. Primers characteristics are described in **Table 1**. EF1α (Elongation Factor 1α) gene transcripts which expression is constant were amplified as a control gene. Amplified PCR fragments were visualized using ethidium bromide stained 2 % (w/v) agarose gels. The results are analyzed by agarose gel electrophoresis.

Statistical analysis

All results were analyzed by one-way ANOVA and significant differences between the means of parameters were determined by using the Student test. The means of different treatments were compared with the control. The data are the mean ± SD. Results were also expressed as percentage of variation: **PV= 100×(S-C)/C.** Where C and S represent the treatments without and with salinity respectively. **Levels of significance: n.s.**: non-significant at $p < 0.05$, **significant** at $p < 0.05$ (*), **very significant** at $p < 0.01$ (**), and **highly significant** at $p < 0.001$ (***).

RESULTS

Effects of different concentrations of NaCl on cowpea growth parameters

The lowest salt concentration (50 mM) did not significantly alter cowpea plants shoot height (SH) and shoot dry weight (DW) (Table 2). However, in the

Table 1. Primers sequences

Gene	Primers 5'-3'	Sequence name, Genebank and publication
EF1α	Primer F: GTAACAAGATGGATGCCACC Primer R: CCACTTTCTTCAAATACGAGGAG	[40]
P5CS	Primer F: TAACTCACCTCAACTCGCTC Primer R: AACAGCACCTGAAGTTACCA	VuP5CS (Genbank AB056452.2)
ProDH	Primer F: ATGCTCGAGAAGATTGCTGA Primer R: ACAGGTCCAAATGGCATGTA	VuProDH (Genbank AB779665)
AOX2b	Primer F: GGATGTCCACTCTTCCAGAC Primer R: GCTCAATGGTAACCAATAGG	(Genbank AJ421015) [20]
PTOX	Primer F: TCCAAATCATCTCCACTTCAC Primer R: CTTTATCACAGAATCCGTGAG	[40]

Table 2. Effects of different concentrations of NaCl on shoot height (cm), shoot dry weight (g) and leaf area (cm^2) of *Vigna unguiculata* L. plants.

Parameters / Concentrations	Shoot height (cm)	Shoot dry weight (g)	Leaf area (cm^2)
NaCl 0 mM	16.01±0.69	0.069±0.010	17.70±1.62
NaCl 50 mM	14.69±0.55 **n.s.**	0.060±0.005 **n.s.**	15.56±1.38 **n.s.**
NaCl 100 mM	12.19±0.36 ***	0.052±0.003 **	10.40±2.21 ***
NaCl 150 mM	9.72±0.59 ***	0.039±0.005 ***	7.50±0.94 ***

Plants were cultured in soils and submitted to 0, 50, 100 and 150 mM NaCl for 30 days. Values are the means ± SD (n=10). The results were statistically analyzed by ANOVA followed by Student test. Levels of significance: n.s.: non-significant at p <0.05,** very significant at p < 0.01,*** highly significant at p < 0.001.

presence of the higher concentrations, the salt effect became conspicuous. At 100 and 150 mM NaCl, the SH was declined by 24 % and 39 % respectively, while 25 % and 42 % of decrease was unregistered for DW. The leaf area was also reduced in plants growing on 100 and 150 mM NaCl to less than 45 % and 60 % of control plants leaf area (Table 2).

Effects of different concentrations of NaCl on relative leaf water content

Relative leaf water content (RWC) is considered as a reliable criterion to measure water status in plant tissues. As compared to the control, the plants subjected to 50 mM NaCl kept substantially the same RWC (Figure 1). However, at 100 mM NaCl the effect of the salt was marked by a highly significant reduction in RWC. In plants treated with the highest concentration (150 mM NaCl), the effect of the salt become even more pronounced, causing a lowering of RWC to 83 % compared to 91 % in control plants (Figure 1).

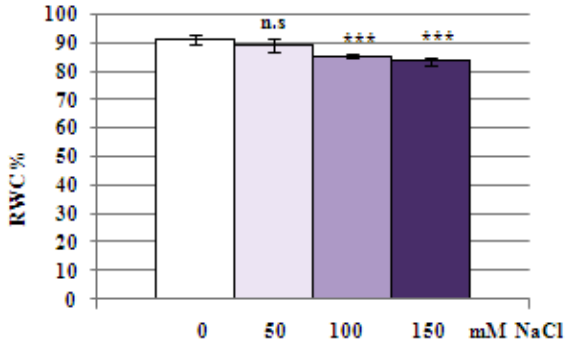

Figure1. Effects of different concentrations of NaCl on relative water content (RWC). Plants were cultured in soils and submitted to 0, 50, 100 and 150 mM NaCl for 30 days. Values are the means ± SD (n=5). The results were statistically analyzed by ANOVA followed by Student test. Levels of significance: n.s.: non-significant at p <0.05,*** highly significant at p < 0.001

Effects of different concentrations of NaCl on malondialdehyde, electrolytes leakage and pigment content

Malondialdehyde (MDA) is the terminal product of peroxidation of polyunsaturated fatty acids, it is widely used as a marker of the damages caused by ROS to cell membranes. Compared to control, plants grown on the lowest 50 mM NaCl did not show an increase of MDA. Under 100 mM NaCl, MDA marked an increment hardly exceeding 30 %. At the highest concentration 150 mM NaCl, plants displayed up to 58 % more MDA compared to untreated plants (Figure 2a).

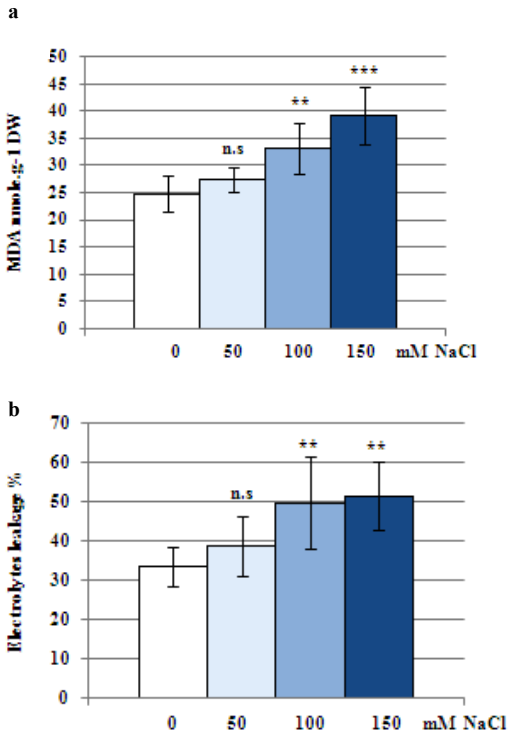

Figure 2. Effects of different concentrations of NaCl on malondialdehyde content (a) and electrolytes leakage (b). Plants were cultured in soils and submitted to 0, 50, 100 and 150 mM NaCl for 30 days. Values are the means ± SD (n=5). The results were statistically analyzed by ANOVA followed by Student test. Levels of significance: n.s.: non-significant at p <0.05,** very significant at p < 0.01,*** highly significant at p < 0.001

The effect of the oxidative stress produced by the excess salt on cell membranes was also evaluated by measuring the electrolytes leakage (Figure 2b). Results showed that electrolytes leakage was higher when salt stress is above 50 mM. Up to 50 % more leakage than in the control was measured for plants grown on 150 mM NaCl (Figure 2b).

Total pigments (chlorophyll a and b, carotenoids) showed the highest value in controls (33.15 $mg.g^{-1}$DW and 6.67 $mg.g^{-1}$DW respectively). Whereas, under salt stress chlorophyll (Figure 3a) and carotenoids (Figure 3b) contents were significantly lower. As compared to

control, total chlorophyll content declined by 28 % and 41 % respectively under 100 mM and 150 mM NaCl (Figure 3a). Besides, carotenoids content decreased drastically with the concentration of salt, with 45.5 % of reduction under 150 mM NaCl (Figure 3b).

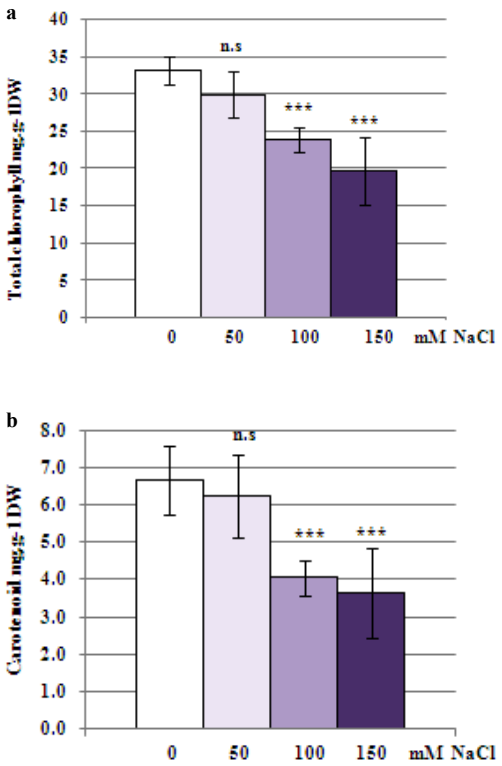

Figure 3. Effects of different concentrations of NaCl on photosynthetic pigments: Total chlorophyll (a), Carotenoids (b). Plants were cultured in soils and submitted to 0, 50, 100 and 150 mM NaCl for 30 days. Values are the means ± SD (n=5). The results were statistically analyzed by ANOVA followed by Student test. Levels of significance: n.s.: non-significant at $p < 0.05$, *** highly significant at $p < 0.001$

Stress responses

Antioxidant enzymes activities

As a consequence of ROS production, salt stress induced antioxidant enzymes activities. In order to evaluate the antioxidant response of cowpea plants to salt stress, we measured catalase and ascorbate peroxidase activities involved in hydrogen peroxide removal from mitochondria and chloroplast. Both catalase (Figure 4a) and ascorbate peroxidase (Figure 4b) activities were enhanced with increasing salinity levels above 50 mM. When plants were exposed to 100 mM and 150 mM NaCl, catalase increased by 32 % and 42 % respectively. Besides, compared to control, the variation was much higher in ascorbate peroxidase activity which increased up to 253 % and 402 % respectively.

Mitochondria and chloroplast ROS production is often associated with an up-regulation of electron transfer chain alternative oxidase such as AOX in the mitochondria and PTOX in the chloroplast. The effects of salt stress on AOX and PTOX, enzymes which could mitigate the ROS generation were evaluated at the transcriptional level. The results (Figure 5) showed that AOX2b and PTOX genes seemed to not be modulated by salt stress, both genes were similarly expressed whatever the NaCl concentration.

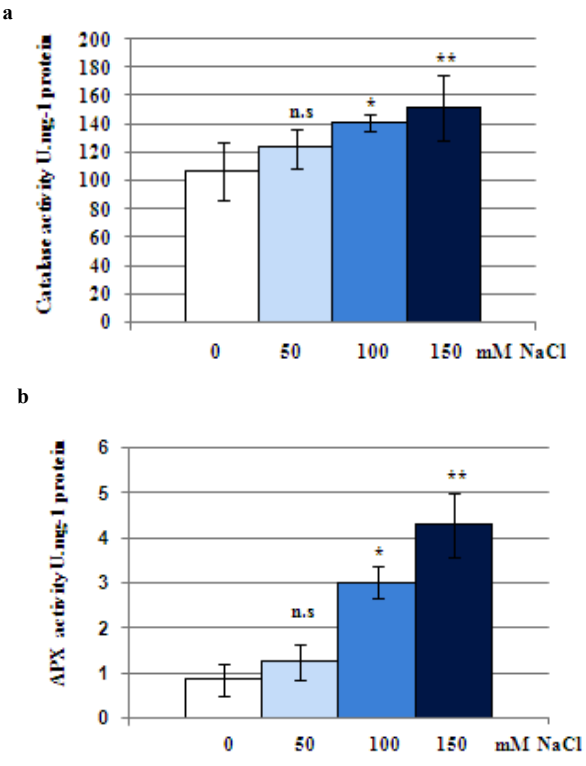

Figure 4. Effects of different concentrations of NaCl on catalase (a) and ascorbate peroxidase (b). Plants were cultured in soils and submitted to 0, 50, 100 and 150 mM NaCl for 30 days. Values are the means ± SD (n=3). The results were statistically analyzed by ANOVA followed by Student test. Levels of significance: n.s.: non-significant at $p < 0.05$,* significant at $p < 0.05$, ** very significant at $p < 0.01$.

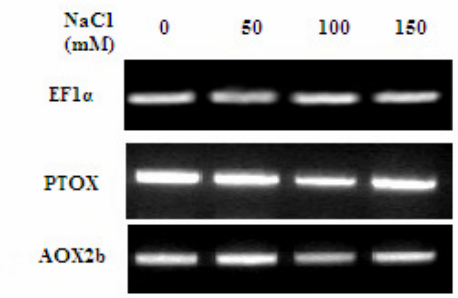

Figure 5. Effects of different concentrations of NaCl on genes expression. Plants were cultured in soils and submitted to 0, 50, 100 and 150 mM NaCl for 30 days. The gene expression levels were tested using RT-PCR analysis. **EF1α** elongation factor, gene transcripts which expression is constant, **PTOX** plastid alternative oxidase, **AOX** alternative oxidase.

Proline content and proline metabolism genes expression

Proline is accumulated in cowpea leaves in response to salt stress. The result showed that salinity

had a marked effect on leaf proline content at NaCl concentration above 50 mM (Figure 6a). As compared to control plants, proline content was enhanced by up 57 % and 106 % respectively under 100 mM and 150 mM NaCl (Figure 6a).

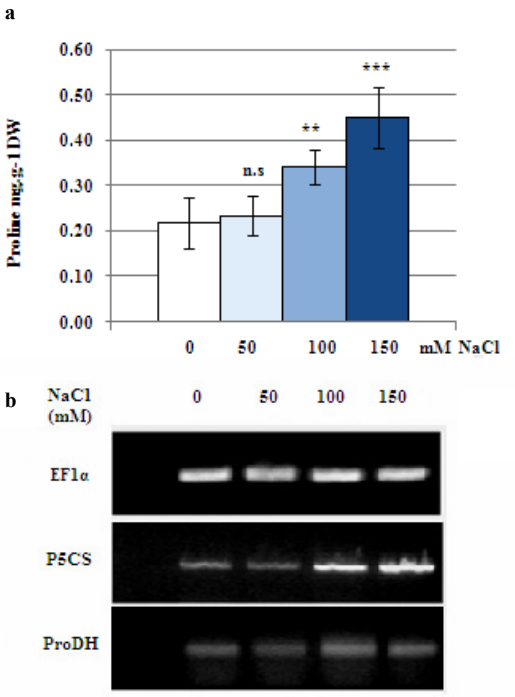

Figure 6. Effects of different concentrations of NaCl on proline content (a) and proline metabolism genes expression (b). Plants were cultured in soils and submitted to 0, 50, 100 and 150 mM NaCl for 30 days. Values are the means ± SD (n=5). The results were statistically analyzed by ANOVA followed by Student test. Levels of significance: n.s.: non-significant at $p < 0.05$, ** very significant at $p < 0.01$, *** highly significant at $p < 0.001$. **EF1α** elongation factor: gene transcripts which expression is constant, **P5CS** Δ^1-pyrroline-5-carboxylate synthetase, **ProDH** proline dehydrogenase

The expression profile of Δ^1-pyrroline-5-carboxylate synthase (P5CS) which is a rate-limiting enzyme involved in the biosynthesis of proline in higher plants, showed that the accumulation of proline was concomitant with an over-expression of P5CS gene (Figure 6b). Whereas, the expression of proline dehydrogenase (ProDH) which is in contrary, involved in the catabolism of this amino-acid remained very low for all treatments and seemed to not be modulated by the salt stress (Figure 6b).

DISCUSSION

The effect of salinity (NaCl) on cowpea growth was detrimental at concentration above 50 mM NaCl. Dry weight, shoot height and leaf area were reduced after four weeks of growth on saline substrate. Our results are in agreement with previous studies reporting growth reduction in salt stressed legume plants as well as cowpea [9, 16]. Salinity decreases plant growth by reducing the ability of plants to take up water and by affecting the availability, transport, and partitioning of nutrients. It may cause nutrient deficiencies or imbalances, due to the competition of Na^+ and Cl^- with nutrients such as K^+, Ca^{2+} and NO_3^- [26]. Belmecheri-Cherifi et al. [11] showed that growth reduction of fenugreek plants under salinity conditions was related to a decrease in stomatal conductance, nutrient deficiency, excessive uptake of Na and, importantly, hormonal imbalance.

The estimation of the water status of the plants by measuring the relative water content of the leaves revealed a close relationship between the presence of high concentrations of salt in the culture medium and the appearance of a physiological water stress marked by water deficit in leaves. These results suggested that the growth reduction in salt stressed cowpea plants could be at least partially a consequence of an inefficient water supply. Earlier studies have shown that salt stress affected water relations in cowpea by decreasing RWC [28].

Salt stress above 50 mM NaCl resulted in an important decrease in photosynthetic pigments. Comparable results have also been found in cowpea [9, 28] and other species such as soybean [24], wheat [4], barley [3], common bean [48] and tomato [8]. The reduction in chlorophyll content could result from the inhibition of certain enzymes involved in their biosynthesis such as protochlorophyllide oxidoreductase [61] and/or acceleration of their degradation by stimulation of chlorophyllase activity [23]. Competitive biosynthesis processes between chlorophyll and proline for their common precursor, glutamate, could likely lead to the decrease in chlorophyll content [57]. Besides, the decrease in carotenoids content under salt stress might be a consequence of the down-regulation of carotenoid biosynthetic genes such as phytoene desaturase [7]. The amount of photosynthetic pigments can be a limiting factor in photosynthesis [8]; its reduction can then affect the synthesis of organic substances necessary for plant growth.

Perturbations of photosynthetic and respiratory processes lead inevitably to the formation of reactive oxygen species [5]. In the absence of an effective defense system, the accumulation of these molecules is proved to be extremely dangerous for the various cellular components, in particular the lipids. In this regard, we assessed the degree of damage caused to the membranes by the determination of malondialdehyde (MDA) content and by measuring the electrolytes leakage. Our results revealed that salt stress had a marked effect on malondialdehyde content and electrolytes leakage which increased concomitantly at medium and high salinity levels. These results would reflect a perturbation of the stability of cell membranes resulting likely from lipid peroxidation. Similar finding was reported by various studies conducted on cowpea [18, 25, 28] and other species such as cotton, sorghum [25], corn [14] and bean [58]. Moreover, it has been demonstrated in some studies conducted on cowpea

plants grown under saline conditions that high malondialdehyde content is often accompanied by injuries in membrane integrity and a deficient defense system against reactive oxygen species [17, 25]. Our study suggests that concentrations above 50 mM NaCl are detrimental to cowpea possibly caused by an excess of ROS.

Antioxidant enzymes play an important role in scavenging ROS induced by salt stress [55, 27]. H_2O_2 is a ROS that is suggested to play a signaling role and a regulator of gene expression [32]. However, at high levels, H_2O_2 becomes deleterious and in subsequent reactions must be mitigated by conversion to H_2O by catalase (CAT) and ascorbate peroxidase (APX) enzymes [1]. CAT and APX activities enhanced markedly at 100 mM and 150 mM NaCl, but were comparable to control at 50 mM. APX was found to increase more than CAT. Furthermore, CAT is known to have lower affinity to H_2O_2 than APX (mM and µM range respectively). Thus, both enzymes are modulated by salt stress and would be involved in ROS scavenging [43, 2]. These results are in agreement with those of Chen et al. [19] and Hashem et al. [28] who showed that increasing salinity enhanced the activity of APX and CAT in cowpea cultivars. Besides, many studies provided evidence that increased tolerance of plants to salt stress is due to higher endogenous activities of CAT and APX, some examples are: green bean [65], maize [6], and corn [14].

Alternative oxidases are a group of enzymes that have the potential to divert electrons from electron transport chains in the mitochondria (AOX) and chloroplasts (PTOX) and use them to reduce O_2 into water [14, 51]. Thus, it has been suggested that AOX and PTOX could act as a safety valve preventing oxidative damage under adverse environmental conditions [45, 35]. Our results showed that PTOX and AOX2b were constitutively expressed in control plants and remained unchanged under different salt treatments. PTOX is shown to be over-expressed in salt stress condition [54, 30], possible regulation of PTOX activity at post-transcriptional level is than suggested. Various studies indicated that AOX genes are expressed either constitutively or are highly induced under stress [46, 54]. Costa et al. [20] have shown that AOX2b was expressed in both cowpea cultivars tested under non stress conditions. Futhermore, in their work on two cowpea landraces submitted to drought stress, Zegaoui et al. [67] reported that AOX2b did not vary after days of progressive dehydration. These authors supposed that AOX could be activated at protein level.

Proline is an amino acid whose presence is often associated with stressful conditions. Its accumulation has been shown in many glycophytes grown in the presence of salt [56]. Proline has many roles; it is able to protect cells from damage by acting as an osmotic compound, a cell protectant and a radical scavenger [34]. The accumulation of proline could result from a stimulation of its biosynthesis and / or inhibition of its oxidation. Our results showed that proline content was enhanced in leaves when plants were exposed to high salinity levels. The accumulation of proline was tightly related to an over-expression of P5CS and a very low expression of ProDH gene. These results are in agreement with many studies which had shown that in salt stress conditions, the accumulation of proline is

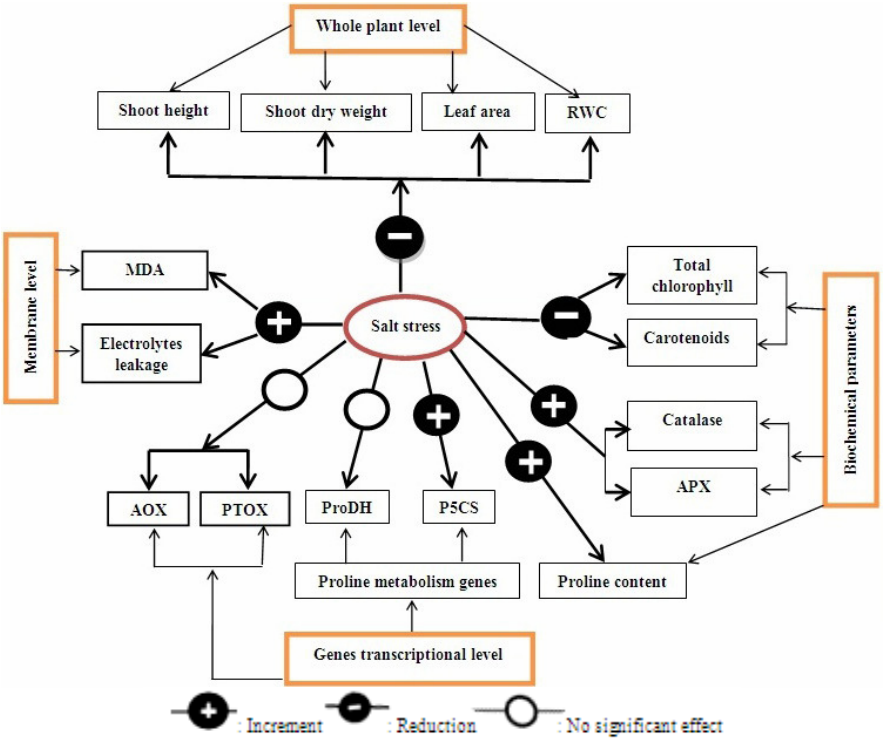

Figure 7. Effects of 100 mM and 150 mM of NaCl on cowpea plants. RWC: Relative leaf water content, MDA: Malondialdehyde, APX: ascorbate peroxidase, ProDH: proline dehydrogenase, AOX: Mitochondrial alternative oxidase, PTOX: plastid alternative oxidase, P5CS: pyrroline-5-carboxylate synthase.

correlated with activation of the enzymes involved in its synthesis (pyrroline-5-carboxylate reductase, Δ^1-pyrroline-5-carboxylate synthase and γ glutamyl kinase). Conversely, the accumulation of proline is often related to the inhibition of proline dehydrogenase (proline oxidase) which is involved in the catabolism of this amino acid [42, 62, 64]. Moreover, the reduction of the use of proline in protein synthesis and / or the degradation of proteins rich in proline or its precursors (glutamate, ornithine) could also result in the accumulation of this amino acid [33].

In present study, we found that low (50 mM) salt stress did not affect plant growth and did not trigger antioxidative enzymes. At 100 mM and above salt stress (Figure 7) caused a significant decrease in growth, relative water content and photosynthetic pigments. However, it increased MDA content along with the electrolytes leakage. Cowpea responded to the adverse effect of salt stress by over-expressing P5CS gene and by increasing the amount of proline and antioxidant enzymes activities (CAT and APX).

In conclusion, despite all the hallmarks of stress tolerance (high antioxidant enzymes activities and high proline content), the drought tolerant plants of cowpea displayed symptoms of sensitivity (growth reduction and damages) to salt stress. Thus, to withstand the detrimental effects of salinity, cowpea plants had to deal not only with the lower water availability but also with the specific ions toxicity generated by the high levels of salt.

Abbreviations:

ROS reactive oxygen species
H_2O_2 hydrogen peroxide
$O^{\cdot -2}$ superoxide anion
$^1O_2$ singlet oxygen
$^{\cdot}OH$ hydroxyl radical
AOX alternative oxidase
APX ascorbate peroxidase
CAT catalase
DW dry weight
EF1α elongation factor
FW fresh weight
SOD superoxide dismutase
MDA malondialdehyde
ProDH proline dehydrogenase
PTOX plastid alternative oxidase
P5CS Δ^1-pyrroline-5-carboxylate synthase

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