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USE OF PHYSIO-BIOCHEMICAL TRAITS TO EVALUATE THE SALT TOLERANCE OF FIVE *OPUNTIA* SPECIES IN THE ALGERIAN STEPPES

BAHIA LALLOUCHE[1,2*], AMMAR BOUTEKRABT[1], BOUBAKR HADJKOUIDER[2], LEILA RIAHI[3], SALIM LAMINE[4,5] AND NÉJIA ZOGHLAMI[6]

[1]*Department of Agricultural Sciences. Faculty of Nature and Life Sciences. Saad Dahlab University, Blida, Algeria*
[2]*Department of Agricultural Sciences, Faculty of Science, Mohamed Boudiaf University, M'sila, Algeria*
[3]*Laboratory of Biotechnology and Bio-Geo Resources Valorization (LR11ES31), Higher Institute for Biotechnology - University of Manouba, Biotechpole of Sidi Thabet, 2020, Sidi Thabet, Ariana, Tunisia*
[4]*Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Ceredigion SY23 3FG, UK.*
[5] *Laboratory of Management and Valuation of Natural Resources and Quality Assurance, University of Bouira, Bouira 10000, Algeria*
[6]*Laboratory of Plant Molecular Physiology, Biotechnology Centre of Borj-Cédria, Hammam-lif, Tunisia.*
[*]*Corresponding author's email: blallouche@gmail.com; Tel.: +00213 773 563 649; Fax: 00213 35 332 329*

Abstract

In this study, twelve physio-biochemical parameters were estimated to assess the behavior of five *Opuntia* species in the Algerian steppes (*Opuntia ficus indica f. inermis*, *O. amyclea*, *O. streptacantha, O. robusta* and *O. engelmanii*).Herein, the salt stress was induced using three levels of NaCl (200 mM, 400 mM and 600 mM). Based on the analysis of variance (ANOVA), the chlorophyll level for both young cladode and aged cladode was found to be the most discriminant parameter under salt stress concentrations 200 and 400 mM. The species were clustered in three groups with *O. ficus indica f. inermis* and *O. amyclea* being the most tolerant to salinity. For a salt concentration of 600 mM, the ANOVA showed that the chlorophyll content in aged cladode was the most discriminant parameter. The Biplot-based species analysis revealed that *O. engelmanii* was the most salt tolerant species. However, *O. amyclea* and *O. robusta* were found to be the most sensitive. In conclusion, total chlorophyll contents for young cladode and aged cladode, chlorophyll a of aged cladode, and root total soluble sugars can be used as key parameters to identify the salt tolerance for *Opuntia* species.

Key words: Salt tolerance, *Opuntia* species, Physio-biochemical traits, Algerian steppes.

Introduction

Salinity is one of the most important abiotic stresses and constitutes a limiting factor of plant distribution and production (Khan *et al*., 2013). The capacity of the plants to adapt with salinity stress is governed mostly by natural parameters. Thus, understanding the mechanisms underlying salt tolerance is very important (Gilbert *et al*., 1998). Two negative effects can be elicited by salinity stress for plants: osmotic stress and ionic toxicity; Osmotic inhibition is the result of the salt presented in the soil solution which reduces the ability of the plant to take up water, and minerals such as K^+ and Ca^{2+} (Munns *et al*., 2006). Ionic toxicity is caused by an excessive amount of salt entering the transpiration stream which eventually injures cells in the transpiring leaves and may further reduce growth (Munns *et al*., 2006). Reduction in growth and photosynthesis are among the most conspicuous effects of salinity stress. In addition, the primary effect of high salinity in plants is stomatal closure. The latter reduces transpiration and CO_2, and appears to be the main cause of reduced photosynthetic activity (Pelleschi *et al.*, 1997).

Basically, the lack of CO_2 may have a direct effect on the leaf carbohydrate content by generating a translocation pattern (Pelleschi *et al*., 1997). Salt stress causes accumulation of carbohydrates in plant tissues , which contributes in osmotic adjustment (Pattanagul & Thitisaksakul, 2008).

During osmotic adjustment the cells tend to synthesize and accumulate compatible organic solutes in the cytoplasm in order to maintain the osmotic equilibrium (Blum *et al*., 1996; Hazewaga *et al*., 2000). Sugar accumulation in plant tissues and cells due to salinity stress was reported in many studies (Tattini *et al*., 1996).

In many plant species, the absolute osmolyte concentrations maintain osmotic equilibrium (Martínez-Ballesta, 2004). Other advantages of these solutes include buffering the potential of cellular redox and protecting cellular structure under a stress condition. Despite the large number of studies that have been conducted regarding salt tolerance for *Optuntia* species (Murillo-Amador *et al*., 2001; Cony *et al*., 2006; Véliz *et al*., 2007; Ochoa-Alfaro *et al*., 2008; Franco-Salazar & Éliz, 2008; Nieto–Garibay *et al*., 2011, Salas-Muñoz, 2012),the basic mechanisms governing salt tolerance in *Opuntia* are not yet understood and a more advanced work is required in this research area.

The objective of the present work was to evaluate the salt tolerance in five *Opuntia* species and to classify the species as highly salt tolerant (HST), salt tolerant (ST) or moderately salt tolerant (MST). We used three levels of NaCl concentration (200 mM, 400 mM and 600 mM), and the physio-biochemical changes have been estimated using multivariate and cluster analyses.

Materials and Methods

Plant material and salt stress application: The present investigation is based on five *Opuntia* species (*Opuntia ficus indica* Mill*. f. inermis* (O.I)*, Opuntia amyclea* (O.A)*, Opuntia engelmanii* (O.E), *Opuntia robusta* (O.R) and *Opuntia streptacantha* (O.S) growing naturally in the Algerian steppes and belonging to the arid and semi-arid climatic regions (Table 1).

During the random sampling survey, we selected 10 plants from each species and 4 cladodes were sampled from each plant. Thus, a total number of 40 cladodes have been taken from each species. A total of 200 cladodes for the five species were used in this experiment.

The collected cladodes were planted during the period of 2014 using 2L plastic pots, the volume of each pot was filled with sand and placed under natural growing conditions. Experimentation has been achieved in a completely randomized design with ten replicates per species and also per concentration. The pots were watered on a weekly basis by distilled water. Salinity stress was induced to the generated plants after the second year of culture. All the sampleswere exposed to salinity treatments during a period of sixty days (2 months). The treatments included four different NaCl concentration levels (0, 200, 400 and 600 mM), where 0 mM was for the control treatment.

For each species, a total of 40 samples were considered, with 10 samples per concentration were used during the analysis. Variation of tolerance to salt stress within the studied genotypes was evaluated based on different physio-biochemical traits, and after 60 days after of the salt stress treatments. Plant material samples (young cladodes, aged cladodes, and roots) were collected for further analysis (Fig. 1).

Evaluation process: The measure of the levels of chlorophyll a (Chl_a), chlorophyll b (Chl_b) and the total chlorophyll content (TC) for aged and young cladodes was performed using MacKinney method (Mackinney, 1941). Chl_a and Chl_b contents have been measured for each sample, then we obtained the following estimates: $ACChl_a$: aged cladode chlorophyll a, $YCChl_a$: young cladode chlorophyll a, $ACChl_b$: aged cladode chlorophyll b, $YCChl_b$: young cladode chlorophyll b, ACTC: aged cladode total chlorophyll, and YCTC: young cladode total chlorophyll.

The spectrophotometer UV-1800 SHIMADZU was used to measure the Optical Density (OD) at two specific wavelengths 663 nm and 645 nm. Concentrations of Chl_a, Chl_b and TC (μgg^{-1} FW) were estimated separately using the following equations:

$$Chl_a = (12.7\ OD_{663}\text{-}2.59\ OD_{645}) \times V/ (1000 \times m)$$
$$Chl_b = (22.9\ OD_{645}\text{-}4.68\ OD_{663}) \times V/ (1000 \times m)$$
$$TC = Ch_a + Ch_b$$

where, V is the volume of extracted solution, m is the weight of fresh matter and OD is the optical density.

Soluble sugar contents: we adopted the acronyms ACS, YCS and RS respectively for soluble sugars contents of aged cladodes, young cladodes and roots. The measurements were performed following Dubois *et al*. method (Dubois *et al*., 1956). The absorbance was read in a spectrophotometer UV-1800 SHIMADZU at a wavelength of 490 nm and the results were expressed in μgg^{-1} FW.

Proline contents: we adopted the acronyms ACP, YCP and RP respectively for Proline content in aged cladodes, young cladodes and roots. The Monneveux & Nemmar (1986) technique was used for the quantification of Proline. Samples of 100 mg of fresh weight and 2 ml of 40% methanol were placed in test tubes and warm water bath at 85°C for 60 min. In order to avoid the alcohol evaporation, the tubes were covered with an aluminum foil during the heating. After cooling, 1 ml is removed from the extract and added with 1 ml of acetic acid, 80 ml of orthophosphoric acid (H_3PO_4, density 1.7) and ninhydrin (25 mg per sample). The mixture was boiled for 30 min, until the solution turns red. After cooling, 5 ml of toluene were added per sample. Two phases were formed, the upper phase containing proline and the lower phase without proline. After retrieving the upper phase, Na_2SO_4 was added using a spatula to remove water. The optical densities of the samples were determined using a spectrophotometer UV-1800 SHIMADZU adjusted in wavelength 528 nm, and as the previous technique, the results are expressed as μgg^{-1} FW.

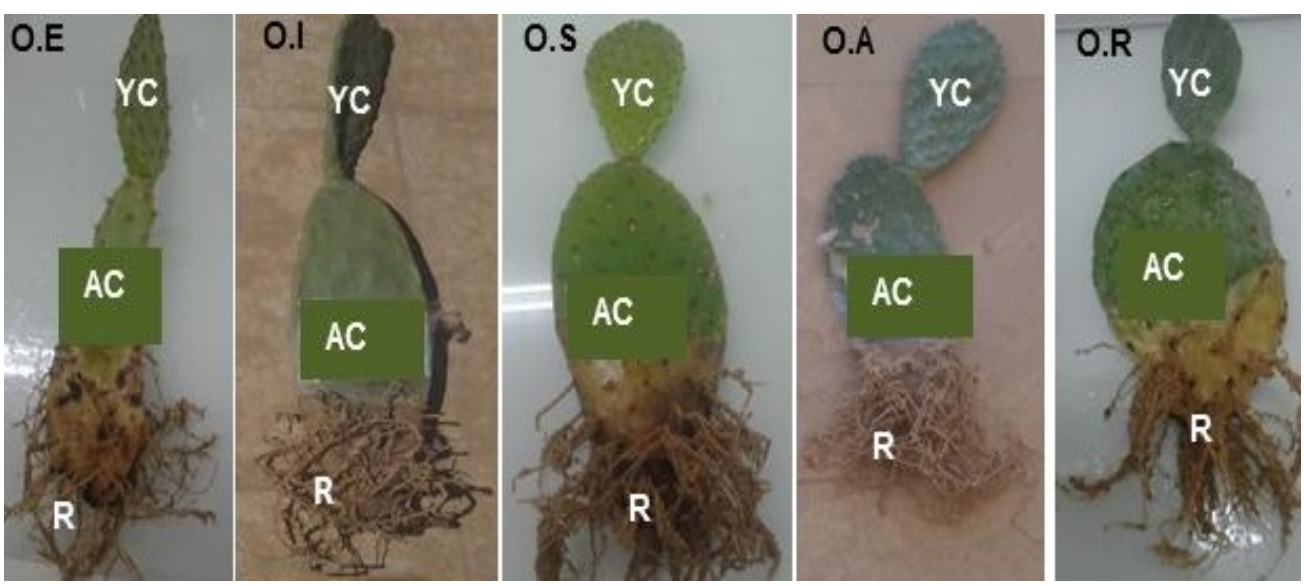

Fig. 1. Illustration of the different *Opuntia* sampling organs used during the experimental analysis (YC for Young cladode samples; AC for aged cladode samples and R for root samples).

Data analysis: The results were analyzed by comparing F ratio values obtained from one-way ANOVA (Fast statistics v 2.0.4). The least amplitude significant differences (PPAS) between the mean at 95% confidence interval, were determined whether there is significant interaction between species and treatments (G x T). Physio-biochemical data were analyzed using multivariate analysis, clustering analysis using XLSTAT software (Addinsoft, www.xlstat.com); principal component analysis (PCA) was performed to identify species groups and to determine the axes and the factors significantly contributing to the variation. In this procedure, the similarity matrix was used to generate eigen values and scores for the species. The first two principal components, which accounted for the highest variation, were then used to plot two dimensional scatter plots. HCA was carried out using Ward's minimum variance method as a clustering algorithm (Williams, 1976) and squared Euclidean distances as a measure of dissimilarity (Ward, 1963).

Results

Four levels of applied stress (0, 200, 400 and 600 mM NaCl concentrations) have been applied in order to test the direct effect of salinity on different physio-biochemical parameters of *Opuntia* species.

Four species (*O. ficus indica* Mill. *f. inermis*, *O. amyclea*, *O. streptacantha* and *O. engelmanii*) were used for the evaluation of salt tolerance (200 and 400 mM). The species *O. robusta* was excluded because it was totally damaged by the first levels of stress (200 and 400 mM) while the three species (*O. ficus indica* Mill. *f. inermis*, *O. streptacantha* and *O. engelmanii*) were used for the evaluation of salt tolerance at 600 mM.

Stress 200 mM NaCl: Statistical analysis revealed significant differences between the species O.I, O.A, .O.S, and O.E 60 days after treatment with 200 mM NaCl (Table 2).One-way ANOVA indicates a significant effect of salinity on the proline contents for root, aged and young cladodes (RP, ACP, YCP), soluble sugar contents for root, aged and young cladodes (RS, ACS, YCS) and chlorophyll a, b and total chlorophyll content for aged and young cladodes ($ACChl_a$, $YCChl_a$, $AGChl_b$, $YCChl_b$, ACTC, YCTC) (Table 2). Young cladode total chlorophyll content (YCTC) and young cladode chlorophyll b content ($YCChl_b$) were found to be the most discriminant parameters, followed by ACTC, $ACChl_b$, YCS, $YCChl_a$, $ACChl_a$, ACS, RS, YCP, ACP and RP (F ratio > F critical). The most discriminant parameter young cladode total chlorophyll content (YCTC) was affected by salinity in all the studied species (Table 2). O.I and O.A exhibited a decrease in young cladode total chlorophyll content of 48.71 and 41.99% respectively. Moreover, O.S exhibited an important decrease in young cladode total chlorophyll content of 62.17%. On the other hand, O.E exhibited an increase of 39.12%. There was a high decrease of more than 57% in the chlorophyll b content of young cladodes in O.I, O.A and O.S, while, , we observed an increase of 23.54% in O.E (Table 2). The matrix of correlations reveals a strong négative correlation of total content chlorophyll in young cladode with total soluble sugar roots (r =- 0.94) and total soluble sugar content of older cladodes (r = -0.968).

PCA revealed that the first and second principal components (PC) accounting for 49.72, and 39.12 % of variation among the traits, respectively . The first PC comprised of RS, ACS, ACTC, YCTC, $YCChl_b$, and $ACChl_b$, while the second PC involved RP, ACP, YCP, $ACChl_a$, and YCS. The PCA plots confirm young cladode chlorophyll total content and young cladode chlorophyll b content as the most discriminant parameters.

This analysis showed that the parameters, soluble sugar contents for root and aged cladodes (RS, ACS) is correlated to the first axis, while the soluble sugar contents for young cladodes (YCS) parameter is correlated to the second axis inversely to what was noted at the control (0 mM) (Figs. 2A and 2B). There were differences in clustering of species in Fig. 2C compared to that observed in Figs. 2A and 2B.

The maximum Euclidean distance of 160.4 was observed between species O.S. and O.E. The lowest Euclidean distance was observed between O.I and O.A with only 36.9. The Euclidean distance between O.I and O.E was moderate with 85.069, due to their genetic similarity. In order to group the species based on 12 physio-biochemical parameters, Cluster Analysis (CA) and Ward method were performed. This analysis grouped the genotypes into three categories: C1 for the category of Highly Salt Tolerant (HST) species, including O.I and O.A. C2 for Moderately Salt Tolerant (MST), including O.S, and C3 Salt Tolerant (ST) for O.E (Fig. 2D). Clusters 1 and 3 had the lowest genetic distance (86.2), while cluster 3 and 2 had the highest genetic distance (160.4). Groups in cluster analysis were similar to the groups of two dimensional plot of PCA. Thus, both analyses validated each other.

Stress 400 mM NaCl: Statistical analysis showed significant differences between the species O.I, O.A, O.S, and O.E sixty days after salt stress treatment at 400 mM NaCl (Table 3).The data of one-way ANOVA based on table 3, indicates a significant effect of salinity on the proline content for root, aged cladodes and young cladode (RP, ACP, YCP), sugar soluble content for root, aged cladode and young cladode (RS, ACS, YCS) and chlorophyll a, b and total content for aged and young cladodes ($ACChl_a$, $YCChl_a$, $AGChl_b$, $YCChl_b$, ACTC, YCTC). Aged cladode total chlorophyll content (ACTC) and young cladode total chlorophyll content (YCTC) were found to be the most discriminant parameters (F ratio > F critical). $ACChl_b$, $YCChl_a$, ACCha, YCS, $YCChl_b$, RS, YCP, ACP, RP and ACS also reflected G×T effects but the F-ratio were relatively low (Table 3). The most discriminant parameters aged cladode, total chlorophyll (ACTC) and young cladode total chlorophyll content (YCTC), were affected by salinity in all species (Table 3). For all species, aged cladode total chlorophyll content and young cladode total chlorophyll content showed significant decrease. This decrease was higher and faster in O.S (from 0.6 ± 1 in control plants to 0.072 ± 1.02 and from 1.15 ± 1 in control plants to 0.083 ± 1 in stressed plants). There was a strong negative correlation (r = -0.971) between aged cladode total chlorophyll content and the total soluble sugar content of older cladodes.

Table 1. Locations and bioclimatic characteristics of the original sampling sites for the five studied *Opuntia* species.

Species	*O. ficus indica* Mill. *f. inermis* (OI)	*O. amyclea* (OA)	*O. streptacantha* (OS)	*O. robusta* (OR) *O. engelmanii* (OE)
Species locality	Belaiba (M'sila)	Doukkara (Tébessa)	Choucha (Laghouat)	Mesrane (Djelfa)
Latitude	35° 36'	35° 58'	34° 8'	34°, 36'
Longitude	05° 17'	8° 14'	3° 01'	3° 03'
Minima (C°)	13,0	10,1	9,36	9,47
Maxima (C°)	24,3	22,3	23,0	23,3
Precipitation (mm)	238,2	406,7	236,4	229
Bioclimatic floor	Semi-arid mild winter	Semi-arid cold winter	Semi-arid cold winter	Semi-arid mild winter

Table 2. Physiological and biochemical parameters for four *Opuntia* species after 60 days of experimental conditions and salt stress (0 and 200 mM of NaCl), Values bearing the same letter in each line are not significantly different at p<0.05.

	One-way ANOVA													
	O. ficus indica Mill. *f. inermis* (O.I)		*O. amyclea* (O.A)		*O. streptacantha* (O.S)		*O. engelmanii* (O.E)		Interaction between all genotypes and treatment (GXT)		O.I under salinity	O.A under salinity	O.S under salinity	O.E under salinity
	Control	200 mM	Control	200 mM	Control	200 mM	Control	200 mM	F-ratio	F-critical	F-ratio > F-critical			
PR (µgg^{-1}FW)	0.32d	0.51a	0.35c	0.5a	0.13f	0.34c	0.23e	0.46b	182.75	4.06	Yes>	Yes>	Yes>	Yes>
ACP (µgg^{-1}FW)	0.86b	0.9a	0.73c	0.88ab	0.3f	0.68d	0.4e	0.75c	190.14	4.06	Yes>	Yes>	Yes>	Yes>
YCP (µgg^{-1} FW)	0.7c	0.89a	0.7c	0.84b	0.3f	0.6d	0.45e	0.84b	510.75	4.06	Yes>	Yes>	Yes>	Yes>
RS (µgg^{-1}FW)	42b	15.8f	16.7f	24.9c	16f	20e	22.5d	50a	706.02	4.06	Yes>	Yes>	Yes>	Yes>
ACS (µg $^{-1}$FW)	44b	17.3e	23.3d	29.2c	53a	28.3c	54a	54a	721.46	4.06	Yes>	Yes>	Yes>	Non<
YCS (µgg^{-1}FW)	42e	44.3d	18.4h	36.1f	23g	64c	89a	73b	875.01	4.06	Yes>	Yes>	Yes>	Yes>
ACTC (µgg^{-1}FW)	0.67e	0.58g	1.42a	0.99d	0.6f	0.50h	1.29b	1.17c	12290	4.06	Yes>	Yes>	Yes>	Yes>
YCTC (µgg^{-1}FW)	0.70e	0.35g	1.23b	0.71e	1.1c	0.43f	1.02d	1.42a	708692	4.06	Yes>	Yes>	Yes>	Yes>
$ACCh_a$ (µgg^{-1}FW)	0.47b	0.42c	0.52a	0.47b	0.4d	0.31e	0.42c	0.32e	723.29	4.06	Yes>	Yes>	Yes>	Yes>
$YCCh_a$ (µgg^{-1}FW)	0.41d	0.23g	0.37e	0.47b	0.8a	0.3f	0.23g	0.45c	790.75	4.06	Yes>	Yes>	Yes>	Yes>
$ACCh_b$ (µgg^{-1}FW)	0.19e	0.15f	0.91a	0.52d	0.2e	0.19e	0.87b	0.85c	12282	4.06	Yes>	Yes>	Non<	Yes>
$YCCh_b$ (µgg^{-1}FW)	0.28f	0.12g	0.85b	0.32e	0.3d	0.13g	0.79c	0.97a	482918	4.06	Yes>	Yes>	Yes>	Yes>

RP = Proline content for root; ACP = proline content for aged cladode proline; YCP = proline content for young cladode proline; RS = soluble sugar content for root; ACS = soluble sugar content for aged cladode; YCS= soluble sugar content for young cladode; ACTC: total chlorophyll content for aged cladode, YCTC: total chlorophyll content for young cladode; $ACChl_a$: chlorophyll a content for aged cladode, $YCChl_a$: chlorophyll a content for young cladode, $ACChl_b$: chlorophyll b content for aged cladode, $YCChl_b$: chlorophyll b content for young cladode

Table 3. Physiological and biochemical parameters for four *Opuntia* species after 60 days of experimental conditions and salt stress (0 and 400 mM of NaCl). Values bearing the same letter in each line are not significantly different at p<0.05.

	One-way ANOVA													
	***O. ficus indica* Mill. *f. inermis* (O.I)**		***O. amyclea* (O.A)**		***O. streptacantha* (O.S)**		***O. engelmanii* (O.E)**		**Interaction between all genotypes and treatment (GXT)**		**O.I under salinity**	**O.A under salinity**	**O.S under salinity**	**O.E under salinity**
	Control	**400 mM**	**Control**	**400 mM**	**Control**	**400 mM**	**Control**	**400 mM**	**F-ratio**	**F-critical**	**F-ratio > F-critical**			
PR (µgg^{-1}FW)	0.32e	0.54b	0.35d	0.54b	0.13g	0.45c	0.23f	0.67a	184.0	4.06	Yes>	Yes>	Yes>	Yes>
ACP (µgg^{-1}FW)	0.86b	0.96a	0.73d	0.95a	0.30g	0.7e	0.4f	0.78c	494.7	4.06	Yes>	Yes>	Yes>	Yes>
YCP (µgg^{-1} FW)	0.7d	0.95a	0.7d	0.91b	0.32g	0.6e	0.45f	0.89c	552.0	4.06	Yes>	Yes>	Yes>	Yes>
RS (µgg^{-1}FW)	42c	18.6f	16.7g	27.4d	16g	50b	22.5e	58a	1031.	4.06	Yes>	Yes>	Yes>	Yes>
ACS (µgg^{-1}FW)	44d	64b	23.3e	54c	53c	71a	54c	70a	182.7	4.06	Yes>	Yes>	Yes>	Yes>
YCS (µgg^{-1}FW)	42d	18.9g	18.4g	27.5e	23f	55c	89a	80b	2311.	4.06	Yes>	Yes>	Yes>	Yes>
ACTC (µgg^{-1}FW)	0.67d	0.50f	1.42a	0.94c	0.6e	0.07h	1.29b	0.33g	402290	4.06	Yes>	Yes>	Yes>	Yes>
YCTC (µgg^{-1}FW)	0.70d	0.33f	1.23a	0.55e	1.16b	0.08h	1.02c	0.23g	116414	4.06	Yes>	Yes>	Yes>	Yes>
ACCh$_a$ (µgg^{-1}FW)	0.47b	0.35f	0.52a	0.45c	0.4e	0.05h	0.42d	0.1g	4455.5	4.06	Yes>	Yes>	Yes>	Yes>
YCCh$_a$ (µgg^{-1}FW)	0.41b	0.20e	0.37c	0.13f	0.8a	0.05g	0.23d	0.03h	18801	4.06	Yes>	Yes>	Yes>	Yes>
ACCh$_b$ (µgg^{-1}FW)	0.19e	0.15f	0.91a	0.48c	0.2e	0.02g	0.87b	0.23d	111798	4.06	Yes>	Yes>	Yes>	Yes>
YCCh$_b$ (µgg^{-1}FW)	0.28e	0.12g	0.85a	0.42c	0.36d	0.02h	0.79b	0.2f	1679.9	4.06	Yes>	Yes>	Yes>	Yes>

RP = Proline content for root; ACP = proline content for aged cladode proline; YCP = proline content for young cladode proline; RS = soluble sugar content for root; ACS = soluble sugar content for aged cladode; YCS= soluble sugar content for young cladode; ACTC: total chlorophyll content for aged cladode, YCTC: total chlorophyll content for young cladode; ACCh$_a$: chlorophyll a content for aged cladode, YCCh$_a$: chlorophyll a content for young cladode, ACCh$_b$: chlorophyll b content for aged cladode, YCCh$_b$: chlorophyll b content for young cladode

Table 4. Physiological and biochemical parameters for three *Opuntia* species after 60 days of experimental conditions and salt stress (0 and 600 mM of NaCl). Values bearing the same letter in each line are not significantly different at p<0.05.

	One-way ANOVA										
	***O. ficus indica* Mill *f. inermis* (O.I)**		***O. streptacantha* (O.S)**		***O. engelmanii* (O.E)**		**Interaction between all genotypes and treatment (GXT)**		**O.I under salinity**	**O.A under salinity**	**O.S under salinity**
	Control	**600 mM**	**Control**	**600 mM**	**Control**	**600 mM**	**F-ratio**	**F-critical**	**F-ratio > F-critical**		
PR (µgg^{-1}FW)	0.32d	0.6b	0.13f	0.44c	0.23e	0.67a	417	5.14	Yes>	Yes>	Yes>
ACP (µgg^{-1}FW)	0.86b	0.985a	0.30f	0.62d	0.4e	0.79c	964.6486	5.14	Yes>	Yes>	Yes>
YCP (µgg^{-1} FW)	0.7c	0.98a	0.32f	0.53d	0.45e	0.93b	1825	5.14	Yes>	Yes>	Yes>
RS (µgg^{-1}FW)	42c	28.7d	16f	69a	22.5e	63b	1418.29	5.14	Yes>	Yes>	Yes>
ACS (µgg^{-1}FW)	44e	87b	53d	98a	54d	63c	961.0	5.14	Yes>	Yes>	Yes>
YCS (µgg^{-1}FW)	42d	28.7e	23f	75c	89b	95a	3469.69	5.14	Yes>	Yes>	Yes>
ACTC (µgg^{-1}FW)	0.67b	0.15f	0.6c	0.47d	1.3a	0.16e	2833.61	5.14	Yes>	Yes>	Yes>
YCTC (µgg^{-1}FW)	0.70c	0.22d	1.16a	0.05f	1.02b	0.15	655.52	5.14	Yes>	Yes>	Yes>
ACCh$_a$ (µgg^{-1}FW)	0.47a	0.04d	0.4b	0.29c	0.42d	0.06b	58273	5.14	Yes>	Yes>	Yes>
YCCh$_a$ (µgg^{-1}FW)	0.41b	0.19d	0.8a	0.03e	0.2c	0.04e	21979	5.14	Yes>	Yes>	Yes>
ACCh$_b$ (µgg^{-1}FW)	0.19b	0.10c	0.2b	0.17b	0.8a	0.1c	4903	5.14	Yes>	Non<	Yes>
YCCh$_b$ (µgg^{-1}FW)	0.28c	0.13d	0.36b	0.01f	0.8a	0.1e	156.00	5.14	Yes>	Yes>	Yes>

RP = Proline content for root; ACP = proline content for aged cladode proline; YCP = proline content for young cladode proline; RS = soluble sugar content for root; ACS = soluble sugar content for aged cladode; YCS= soluble sugar content for young cladode; ACTC: total chlorophyll content for aged cladode, YCTC: total chlorophyll content for young cladode; ACCh$_a$: chlorophyll a content for aged cladode, YCCh$_a$: chlorophyll a content for young cladode, ACCh$_b$: chlorophyll b content for aged cladode, YCCh$_b$: chlorophyll b content for young cladode

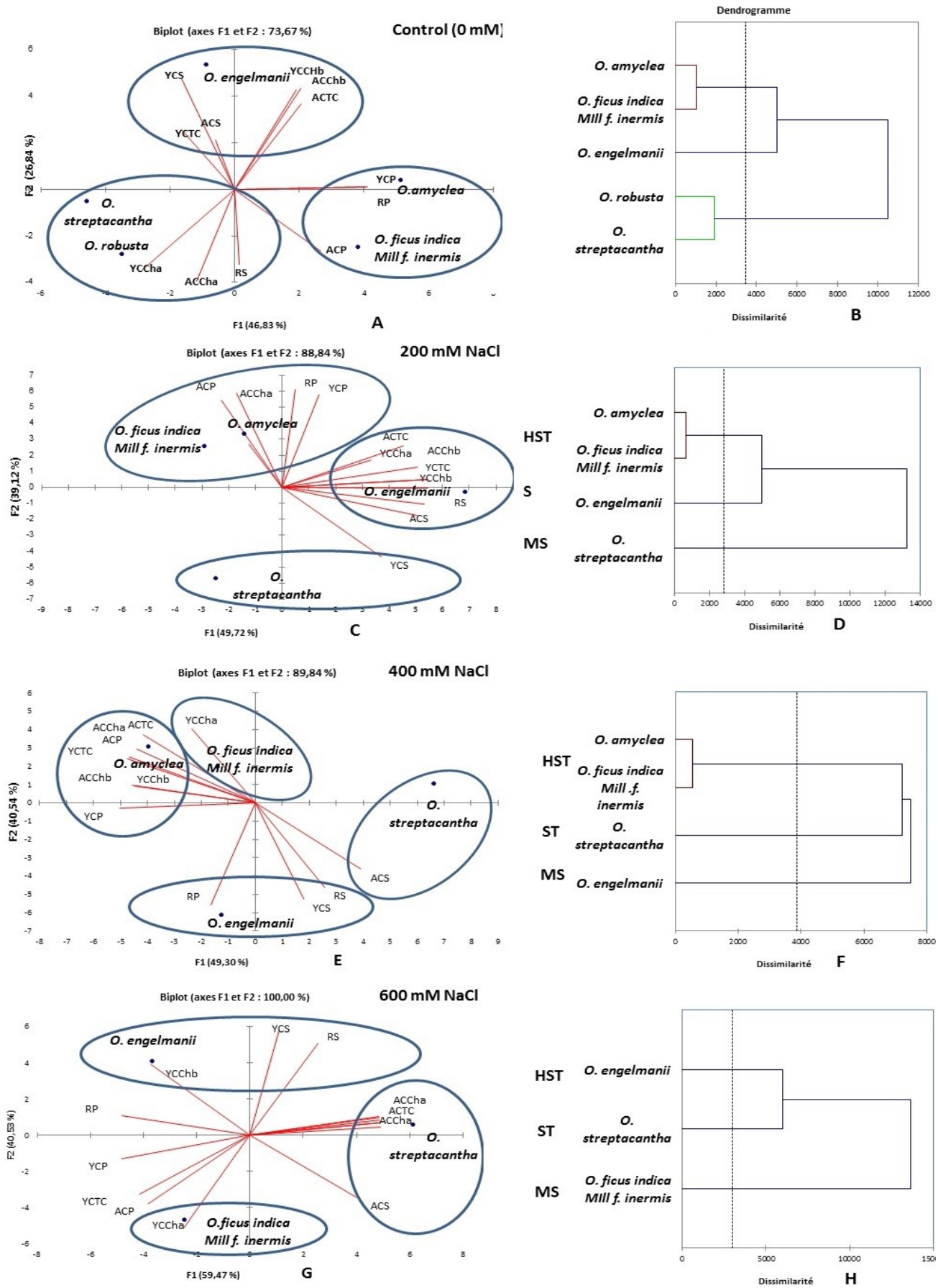

Fig. 2. **Left**: Biplot display of *Opuntia* species according to the first and second PCA components under salt stress conditions (200, 400 and 600 mM of NaCl). **Right**: Cluster analysis using 12 physio-biochemical traits: Highly salt tolerant (HST), salt tolerant (ST) moderately salt tolerant (MST).

PCA revealed that the first, second and third principal components accounting for 49.29, 40.53 and 10.16 % of the observed variation between traits, respectively. The first component comprised of ACP, YCP, ACS, ACTC, YCTC, $ACChl_a$, $ACChl_b$, $YCChl_b$. The second component involved RP and YCS. The third component comprised of $YCChl_a$, and ACL. The PCA plots confirmed that aged cladode total chlorophyll content is the most discriminant parameter.

This analysis showed that the parameters, sugar soluble content for root and aged cladode (RS, ACS) are correlated to the first axis, while the sugar soluble for young cladode (YCS) parameter is correlated to the third axis which was the opposite of what was observed for the control (0 mM) (Figs. 2A and 2B). The distribution of species in the Fig. 2E based on the two axes gave different clustering.

The maximum Euclidean distance was observed between species O.S and O.E (120.4). The lowest Euclidean distance was observed between species O.I and O.A (33.1), which described their genetic similarity. In order to group the species by considering all the attributes, cluster analysis and Ward's method were performed. This analysis generated three clusters: C 1 included HST (O.A, O.I), C2 included ST (O.S) and C3 included MST (O.E) (Fig. 2F). Clusters 2 and 3 had the highest genetic distance (120.4).

Stress 600 mM NaCl: Statistical analysis revealed significant differences between the species O.I, O.A, O.S, and O.E sixty days after treatment with a salt concentration of 600 mM (Table 4). The data of one-way ANOVA (Table 4), showed a significant effect of salinity on the chlorophyll a, b and total content for aged and young cladodes ($ACChl_a$, $YCChl_a$, $AGChl_b$, ACTC, YCTC), proline content for root, aged and young cladode (RP, ACP, YCP), sugar soluble content for root, aged and young cladode (RS, ACS, YCS). Aged cladode chlorophyll a content ($ACChl_a$) was found to be the most discriminant parameter (F ratio > F critical, Table 4). The $YCChl_a$ and RP also reflected G×T effects but the F-ratio was relatively low (Table 4). Aged cladode and chlorophyll a content was affected by salinity in all species (Table 4) O.E, O.S and O.I exhibited a decrease of 26, 84.76, and 89.97 % respectively (Table 4). There was a strong positive correlation (r = 1) between this parameter with aged cladode total chlorophyll content.

In order to study the relationship between all the parameters for two levels of salt stress (control, 600 mM), PCA revealed that the first and second principal components accounted for 59.47 and 40.53% of the observed variation between traits, respectively. The first component comprised of ACS, ACTC, $ACChl_a$ and $ACChl_b$. The second component involved RS, YCS, $YCChl_a$ and $YCChl_b$. The PCA plots confirmed that aged cladode chlorophyll a content was the most discriminant parameter.

In contrast to what was observed at 0 mM of NaCl (control), the parameters, chlorophyll a, b and total content for aged cladode ($ACChl_a$, $ACChl_b$, ACTC), chlorophyll total content for young cladode (YCTC)) (Figs. 2A and 2B). The distribution of species in Fig. 2G based on the two axes has not kept the same grouping.

The maximum Euclidean distance was observed between species O.S and O.I (157.9), the lowest Euclidean distance was observed between species O.S and O.E (109.5), which described their genetic similarity. In order to group species based on all attributes, a cluster analysis and Ward method were performed This analysis clustered the genotypes into three categories such as highly salt tolerance, C1 (O.E), salt tolerant, C2 (O.S) and moderately salt tolerant, C3 (O.I), (Fig. 2H). Clusters 1 and 2 had the lowest genetic distance (109.5). Groups in cluster analysis were similar to the groups of two dimensional PCA plots

Discussion

The ANOVA showed that photosynthetic pigments including total chlorophyll content of young cladode, young cladode's chlorophyll b content, total chlorophyll of aged cladode and chlorophyll a of aged cladode, were the most discriminant parameters. Moreover, the correlation matrix reveals strong négative correlation of total chlorophyll content in young cladode with total soluble sugar roots (r = 0.94) and total soluble sugar content of older cladodes (r = 0.968).

The physiological response, analyzed during the expression of the accumulation of chlorophyll a, chlorophyll b and total chlorophyll content in O.I, O.A, O.E and O.S. for the three NaCl concentrations 200, 400 and 600 mM, showed that these compounds can be accumulated by different plant organs. The level of accumulation varies from one organ to another and also from one species to another, which depends on the applied salt concentration.

In O.E, O.A species, the accumulation of chlorophyll a, chlorophyll b content and total chlorophyll content is the highest for young cladode, aged cladodes, under normal conditions or under salt stress (200, 400 and 600 mM). In the young cladodes, the chlorophyll b content was higher than chlorophyll a content. This result was in agreement with those of Akça&Samsunlu (2012) on walnut genotypes. The total chlorophyll content and chlorophyll b content in the O.E stressed by NaCl (200, 400 mM) increased much more in young cladodes compared to aged cladodes. This result agrees with the study of Silva-Ortega *et al*. (2007) on cactus pear.

In O.I, chlorophyll a, chlorophyll b and total chlorophyll content showed significant reduction in comparison to the control under salt stress both at 200, 400 and 600 mM. However, Chlorophyll b in young cladode was higher compared to chlorophyll a and total chlorophyll in young cladode. In comparison with the control, at 600 mM there was a decrease in chlorophyll a (young cladode), total chlorophyll content (young cladode) and chlorophyll b content (young cladode) with 53.28 %, 67.71 and 88.23 % respectively. In the aged cladodes, the total chlorophyll content was higher than the total chlorophyll in young cladode.

In O.S, there was a significant reduction in chlorophyll a and chlorophyll b of both aged cladode and young cladode, in addition to the total chlorophyll content of aged cladode and young cladode under salt stress at 200, 400 and 600 mM compared to the control. However,

it was observed that this species showed the maximum reduction in chlorophyll content with the increase in salt concentration. In the young cladodes, the total chlorophyll content was higher than that observed in aged cladode.

The results of this study, regarding the decrease in chlorophyll a, b, and total chlorophyll agree with what was obtained by Cha-um *et al*. (2013) who pointed out that the exposure of Cactus (*Echinopsis calochlora*) to zero, 50, 100 and 200 mM of NaCl led to the decrease of chlorophyll a, chlorophyll b and total chlorophyll content. In another study on maize,

Cha-um&Kirdmanee (2009) showed that chlorophyll a, b and total chlorophyll decreased under salt stress. Similar results of decrease in total chlorophyll content when increasing salt concentration were also observed on *Atriplexe halimus* (L.), (Sadder *et al*., 2013), on *Vigna subterranean* (L.) (Taffouo *et al*., 2010), and on beans (*Phaseolus vulgaris* L.) (Stoeva & Kaymakanova, 2008).

Decrease in chlorophyll content with the increase of salt concentrations is a general phenomenon which leads to disordering synthesizing chlorophyll (Parida & Das, 2005). According to Rao & Rao (1981) salinity stress decreases total chlorophyll content by increasing the chlorophylase enzyme activity (Blumenthal-Goldschmidt & Poljakoff-Mayber, 1968). In another study, Ali *et al*. (2004) attributed this reduction in chlorophyll by NaCl to the inhibitory effect of the accumulated ions of various salts on the biosynthesis of the chlorophyll a, b, and total chlorophyll. Salinity affects the forces of bringing the complex pigment protein-liquid into the chloroplast. As the chloroplast stability dependents on the membrane safety (Yeo *et al*., 1990) which under high salinity condition could not remain intact. The reduction in chlorophyll content under salt tolerance is not a function of single organ, but it is the product of all the plant attributes (Ali *et al*., 2004).

The present study showed that the concentration of soluble sugars in the different organs of all *Opuntia* species increased with the increase salt concentration. The accumulation of soluble sugars was higher in cladode than in roots under normal conditions or under salt stress (200, 400 and 600 mM NaCl). Benhassaini *et al*. (2012) reported an increase in the content of total soluble sugars in *Pistacia atlantica* Desf. sub sp. *atlantica* in a salt stress situation. Lallouche *et al*. (2015) observed a higher accumulation of soluble sugars in *O. engelmanii* and *O. streptacantha* at 600 mM NaCl stress. Sugars play an important role in osmotic adjustment, as well as at stabilization of some proteins. The accumulation of sugars seems to induce gelation of the cell contents by saturating the intracellular environment. This phenomenon avoids the crystallization of cell molecules, which limits the damage of cellular structures (Dubos, 2001). Consequently, there is a direct correlation between the amount of soluble sugars and the levels of proline. Therefore, the proline and soluble sugars of the different species correlate reasonably well.

Conclusion

The present study investigated the response of five *Opuntia* species to four sodium chloride concentrations (0, 200, 400, and 600 mM). With regard to physio-biochemical parameters. The tested species in this study, *O. engelmanii* (O.E) and *O. streptacantha* (O.S) were found to be the least affected by salinity stress, followed by *O. ficus indica* Mill. *f. inermis* (O.I). Based on the analysis of the most discriminant parameters, we concluded that *O. engelmanii* (O.E) was highly salt tolerant compared to other *Opuntia* species, while *O. ficus indica* Mill. *f. inermis* (O.I) was moderately salt tolerant. On the other hand, *O. amyclea* (O.A) under salt stress 600 mM and *O. robusta* (O.R) under salt stress 200, 400 and 600 mM were susceptible species. According to our findings, the young cladode, aged cladode total chlorophyll content (YCTC, ACTC), aged cladode chlorophyll a content ($ACChl_a$) and root total soluble sugars can be used effectively as salt tolerance parameters of *Opuntia* species.

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