Morphological and Physiological Analysis of Salinity Stress Response of Carob (*Ceratonia siliqua* L.) in Morocco

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**ABSTRACT**

**Background:** Salinization usually plays a primary role in soil degradation, which consequently reduces agricultural productivity. **Method:** In this study, the effects of salinity (0, 40, 80, 120 and 240 mM of NaCl) on growth parameters, chlorophyll, proline content and sugar content of seven populations of carob (*Ceratonia siliqua* L.), eco-geographical origins different from the collection of four areas of collaboration (Fez, Meknes, Marrakech and Khemisset). **Result:** The results show that the influence of salt stress on the growth of the vegetative apparatus is visible for concentrations of 120 and 240 mM. Sodium chloride (NaCl) concentration in the medium leads to a reduction in overhead and underground biomass and slow elongation of stems and roots. The influence of salt stress was confirmed by highly positive correlations after analysis of variance to one way. The study also shows that the mean chlorophyll (a+b) of the 7 populations studied responds variably to the intensity of salt stress. She experienced a decrease in function of stress and it is very significantly reduced by the salinity to the NaCl concentration of 240 mM. The analysis of variance (ANOVA) showed that there was a highly significant difference between the average measured chlorophyll content (a+b) during saline stress for 7 carob studied populations. Proline content is an important physiological parameter for studying the behavior of people towards carob salt stress. **Conclusion:** Species that have shown the most sensitive behavior towards salt on the morpho-physiological react by accumulating proline, by cons, those that have proven tolerant, have a relative stability or low accumulation of their proline content. The same behavior is observed for the leaf level soluble sugar content. The effect of different concentrations of the salt resulted in a highly significant accumulation of the levels of soluble sugars in the leaves of the carob tree populations.

**Key words:** Carob tree, chlorophyll, morphological parameter, physiological parameter, proline, salt stress, soluble sugar

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**INTRODUCTION**

Soil salinization is one of the most severe causes of yield reduction in modern agriculture¹. More than 20% of arable land in arid and semi-arid areas are or will be affected by increased salinization, about 16 million hectare of salinization soils in the Mediterranean, of which 350,000 ha in Morocco². In Morocco, most of the studies in the different irrigation areas showed unsaltable initially became salty soil after irrigation³. Salinization soils extend along the coast in the low coastal plains but also in arid and Saharan and Presaharic⁴. The presence of salt in the soil results in a reduction in the availability of water that the soil is dry and salinization is high. The north eastern region of Morocco is characterized by low annual rainfall as well as variations of very large seasonal and daily temperature⁴.

Moreover, the effects of salinity in addition to the toxic effect due to Na⁺ and Cl⁻ ions are very similar to those of the drought. They result in adaptations of the plant which is seeking to reduce water loss and maintain its vital functions⁴.
One of the possible solutions to address the problems of salinization is the selection of plant material tolerant to salinity, would remain the most effective way for the economic exploitation of land affected by salinity. Particular attention was paid to Carob, dicotyledonous plant grown in semi-arid regions in Morocco. The carob tree (*Ceratonia siliqua* L.) is a sclerophyllous leguminous in evergreen, grown in Mediterranean ecosystems, notably in marginal and calcareous soils. Water-use strategies and nutrients (N, P and K) dynamic are already documented but some ecological traits of this species are not yet known.

Carob appears to grow successfully in saline soils (tolerance to soil salt content of up to 3% NaCl). Correia *et al.* studied several nutrients interactions used to overcome the adverse effect of NaCl but there is no information about mechanism of salt tolerance including impacts on nutrient balance and biomass allocation.

In the recent study, the aim of this investigation was to evaluate the impact of different concentrations of NaCl on ecophysiological response of seven populations of the carob tree (*Ceratonia siliqua* L.).

**MATERIALS AND METHODS**

**Plant material:** The plant material used in this study consists of seven seed accessions species of carob (*Ceratonia siliqua* L.) from the collection of present four domain of collaboration (Fez, Meknes, Marrakech and Khemisset) (Table 1).

**Experimental design:** The germination seeds of these populations were conducted in the month of October 2013. After dipping in sulfuric acid 95% for 30 min, followed by a series of rinse (10, 10 and 15 min), the seeds are germinated in Petri dish containing sterile filter paper moistened with sterile distilled water at ten seeds per dish, incubated in the dark at 30°C for one week of incubation. Pre-germinated seeds were transplanted into plastic bags filled with 1 kg (1/3 peat and 2/3 sand). The plants were placed outdoors from November 2013 until the experiment began in April 2014. The experiment ended July 2014. The site had a typical Mediterranean climate, with hot summers and mild winters. During the experimental period (April-June) the mean air temperature varied between 19°C (minimum) and 37°C (maximum). The experiment consisted of five treatments using: T0: 0 mmol NaCl L\(^{-1}\) (control) and solutions with T1: 40 mmol NaCl L\(^{-1}\), T2: 80 mmol NaCl L\(^{-1}\), T3: 120 mmol NaCl L\(^{-1}\) and T4: 240 mmol NaCl L\(^{-1}\). Each treatment comprised four plants, giving to a total of 140 plants in the experiment. The different concentrations used were chosen with reference to the work of Correia.

**Morphological parameters:** Plants were harvested after two or more weeks and data was collected for stem length, root length, root fresh weight and stem fresh weight. Dry mass of root and stem was determined after drying the plant sample in a fan-forced oven at 75°C at 3 days.

**Physiological parameters**

**Total leaf of chlorophylls:** The determination of chlorophylls was performed using MacKinney method. Reading the optical density is achieved in a spectrophotometer (Biochrom Libra S12) at two wavelengths: 663 and 645 nm, after calibration of the device. Concentrations of Chl (a), Chl (b) and Chl (a+b) are determined from the following formulas (μg/MF).

\[
\text{Chlorophyll a} = \frac{12.7 \times DO(663) - 2.59 \times DO(645) \times V}{1000 \times m} \\
\text{Chlorophyll b} = \frac{22.9 \times DO(645) - 4.68 \times DO(663) \times V}{1000 \times m} \\
\text{Chlorophyll t} = \text{Ch a} + \text{Ch b}
\]

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

**Total leaf soluble sugar:** Soluble sugars determination was performed by Dubois method. The absorbance

| Accession | Sex | Origin | Latitude N | Longitude W | Altitude (m) | Geographic region | Rainfall (mm) |
|-----------|-----|--------|------------|-------------|--------------|------------------|--------------|
| P2        | Female | Fez    | 34°03’00” | 4°58’59”  | 579          | Plateau Saïs     | 600          |
| P3        | Female | Meknes | 33°53’42” | 5°33’17”  | 560          | Plateau Saïs     | 600          |
| P4        | Female | Khemisset | 33°49’0”  | 6°40’0”   | 490          | Plateau Central  | 456          |
| P5        | Female | Marrakech | 31°37’48” | 8°00’00”  | 450          | Haut Atlas       | 500          |
| P6        | Female | Marrakech | 31°37’48” | 8°00’00”  | 450          | Haut Atlas       | 500          |
| P7        | Female | Marrakech | 31°37’48” | 8°00’00”  | 450          | Haut Atlas       | 500          |
is read in a spectrophotometer (Biochrom Libra S12) at a wavelength of 490 nm and according to the calibration curve established before assay:

\[ y = 0.009x - 0.005 \]

The results are expressed as \( \mu g/g \) MF.

**Total leaf proline:** Proline is quantified using the technique of Troll\(^{14}\), simplified and developed by Dreier and Goring\(^{15}\) and amended by Monneveux and Nemmar\(^{16}\). Test tubes containing samples of 100 mg of fresh material and 12 mL of 40% methanol were placed in heated water bath at 85°C for 60 min. To avoid volatilization of alcohol, the tubes were covered with an aluminum foil during 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/sample). The mixture was boiled for 30 min, until the solution turns red. The whole is cooled and 5 mL of toluene were added per sample. Two phases separate after shaking, the upper phase containing proline and a lower phase without proline. After retrieving the upper phase, \( Na_2SO_4 \) was added using a spatula to remove water contained therein.

The optical densities of the samples were determined using a spectrophotometer (Biochrom Libra S12) set at wavelength of 528 nm. The reading is taken according to the calibration curve and values are given in \( \mu g/g \) MF:

\[ Y = 0.156x - 0.001 \]

**Statistical analysis:** The experimental design was Completely Randomized Design (CRD) with three replicates. For all attributes, data was analyzed by three way analysis of variance (ANOVA) using SPSS and the mean difference was compared by the Duncan’s test at 95 or 99% levels of probability. The graphs presented and the average groups of tables were constructed by the software Excelstat 2008-2009.

**RESULTS**

**Morphological parameters:** Salt stress induces cell dysfunction up to senescence of plants. Analyses of the result show that the concentration of \( NaCl \) in the medium influences the growth in length of the aerial part (Stem) and also leads to a reduction in aerial and subterranean biomass. The results illustrated in Fig. 1a-f indicate that the concentration 240 mM of \( NaCl \) induces senescence populations 2, 3, 4, 5 and 6 after 35 days of application of the salt stress, whereas the concentration 120 mM of \( NaCl \) induces senescence populations 2 and 4 after 43 days of application of salt stress. These results are highly significant after taking analysis of variance to a single criterion (Table 2).

Figure 1a-b shows the average values of the height of the stem and root plants stressed by the different treatments. The average length of stems and roots of control plants is higher compared to those stressed. In fact, the length of stems which is 16.47 cm (P1) in the control plants, decreases to 12.05 cm (P5), in those treated with a concentration of 120 mM \( NaCl \) and the length of the roots which is 29.33 cm (P1) in the control, decreased to 22 cm (P5) in treated plants by 120 mM \( NaCl \). The roots of the seven populations studied carob keep more or less stable length despite the intensity of salt stress.

The average values of the fresh biomass of the subterranean part and the aerial part of the plants stressed by the various treatments (Fig. 1c-d), show a lower value of 0.62 g (P3) for fresh biomass individuals who received the highest concentration of salt (120 mM), while the highest value, 2.54 g (P6) to the fresh biomass is given by the control plants. But for the fresh biomass of the subterranean part which is 1.93 g (P1) in the control, decreases to 0.53 g (P7) in plants treated with 240 mM \( NaCl \).

Data analysis of the dry biomass of the aerial part and the subterranean part is shown in Fig. 1e-f, shows that the control plants exhibit the highest values of the dry biomass. For dry biomass of the aerial part which is 1.32 g (P1) in the control, decreases to 0.3 g (P3) in plants treated with 120 mM \( NaCl \) and for the dry biomass subterranean varied between 0.15 g (P7) having received treatment 240 mM \( NaCl \) to 0.42 g of the plants P6 (control).

**Physiological parameters**

**Total leaf of chlorophylls:** The average of the chlorophyll content (a) and (b) and the average total chlorophyll content (a+b) are illustrated in Fig. 2a-c. These contents show that the seven populations respond in a variable manner to the intensity of salt stress. Chlorophyll (a) in all populations, suffered a decrease with increasing \( NaCl \) concentration. The analysis of variance (ANOVA) shows a very highly significant
Table 2: Analysis of variance (ANOVA) at one way

| Parameters                        | Sum of squares | ddl | Mean of squares | F     | Signification |
|----------------------------------|----------------|-----|-----------------|-------|---------------|
| Length of stems                  |                |     |                 |       |               |
| Inter-group                      | 50.377         | 4   | 12.594          | 15.801| 0.000         |
| Intra-group                      | 23.912         | 30  | 0.797           |       |               |
| Total                            | 74.289         | 34  |                 |       |               |
| Length of root                   |                |     |                 |       |               |
| Inter-group                      | 1840.428       | 4   | 460.107         | 6.831 | 0.000         |
| Intra-group                      | 2020.550       | 30  | 67.352          |       |               |
| Total                            | 3860.978       | 34  |                 |       |               |
| Weight fresh aerial part         |                |     |                 |       |               |
| Inter-group                      | 5.113          | 4   | 1.278           | 5.531 | 0.002         |
| Intra-group                      | 6.934          | 30  | 0.231           |       |               |
| Total                            | 12.047         | 34  |                 |       |               |
| Weight fresh roots part          |                |     |                 |       |               |
| Inter-group                      | 0.930          | 4   | 0.233           | 3.893 | 0.012         |
| Intra-group                      | 1.792          | 30  | 0.060           |       |               |
| Total                            | 2.723          | 34  |                 |       |               |
| Weight dry aerial part           |                |     |                 |       |               |
| Inter-group                      | 1.817          | 4   | 0.454           | 4.169 | 0.008         |
| Intra-group                      | 3.269          | 30  | 0.109           |       |               |
| Total                            | 5.087          | 34  |                 |       |               |
| Weight dry roots part            |                |     |                 |       |               |
| Inter-group                      | 0.151          | 4   | 0.038           | 7.943 | 0.000         |
| Intra-group                      | 0.143          | 30  | 0.005           |       |               |
| Total                            | 0.294          | 34  |                 |       |               |
| Chlorophyll a                    |                |     |                 |       |               |
| Inter-group                      | 131.892        | 4   | 32.973          | 9.243 | 0.000         |
| Intra-group                      | 107.016        | 30  | 3.567           |       |               |
| Total                            | 238.908        | 34  |                 |       |               |
| Chlorophyll b                    |                |     |                 |       |               |
| Inter-group                      | 185.591        | 4   | 46.398          | 11.453| 0.000         |
| Intra-group                      | 121.538        | 30  | 4.051           |       |               |
| Total                            | 307.129        | 34  |                 |       |               |
| Chlorophyll t                    |                |     |                 |       |               |
| Inter-group                      | 629.667        | 4   | 157.417         | 11.506| 0.000         |
| Intra-group                      | 410.421        | 30  | 13.681          |       |               |
| Total                            | 1040.089       | 34  |                 |       |               |
| Total leaf proline               |                |     |                 |       |               |
| Inter-group                      | 90.531         | 4   | 22.633          | 3.852 | 0.012         |
| Intra-group                      | 176.246        | 30  | 5.875           |       |               |
| Total                            | 266.776        | 34  |                 |       |               |
| Total leaf sugar                 |                |     |                 |       |               |
| Inter-group                      | 39231.051      | 4   | 9807.763        | 8.479 | 0.000         |
| Intra-group                      | 34702.031      | 30  | 1156.734        |       |               |
| Total                            | 73933.082      | 34  |                 |       |               |

The same towards the stress behavior is observed for chlorophyll b in all seven populations of Carob studied. The analysis of variance (ANOVA) also showed a very highly significant (Table 2) between the averages of the content of chlorophyll b (Chl b) depending on the salt stress. The analysis of variance to one classification criterion of the sum of the average of the content of chlorophyll (a+b) of seven populations the Carob tree shows a highly significant difference (Table 2).

**Total leaf soluble sugar:** The results of the various recorded averages for the sugar levels in the leaves of the populations studied are shown in Fig. 3. They vary in their entirety in accordance with the intensity of the salt stress. The analysis of variance (ANOVA) showed that there was a very highly significant difference between the different measured averages of the content of soluble sugars in the leaves of the carob tree populations in the presence of salt stress (Table 2).

**Total leaf proline:** Metabolic functions of plants, in particular the metabolisms of free amino acids are often
Fig. 1(a-f): Effect of salt stress on the growth of seven population’s carob tree of the Moroccan: (a) Length of the aerial part, (b) Length of subterranean part, (c) Comparison of the fresh weight of the aerial part, (d) Comparison of the fresh weight of the subterranean part, (e) Comparison of the dry weight of the aerial part and (f) Comparison of the dry weight of the subterranean part. The data represent Mean±SE of replicates (n = 3). Values in the same rows carrying different letters are significantly different between treatments and control by Duncan’s multiple range test at p≤0.05.
Fig. 2(a-c): Effect of salt stress on the physiological response of seven populations of carob tree: (a) Chlorophyll a (Chl a), (b) Chlorophyll b (Chl b) and (c) Total chlorophyll (chl (a+b)). The data represent Mean±SE of replicates (n = 3). Values in the same rows carrying different letters are significantly different between treatments and control by Duncan’s multiple range test at p≤0.05.

Disrupted in saline stress conditions. These free amino acid like proline is for many species the main element affected by salinity. Figure 4 shows the changes.

In the proline content of the different populations studied in function of the intensity of the salt stress. Species that have shown the most sensitive to salt on the morpho-physiological reaction by accumulating prolin, by cons those that have proven tolerant, have a relative stability or low accumulation of their proline content. The analysis of variance for single (ANOVA) showed that there was a highly significant (Table 2) difference between the mean of the different measured content of proline in the leaves of seven populations of carob in the presence of stress salt.

DISCUSSION

In this study, several morphological parameters (length of aerial and subterranean part, fresh weight of aerial and subterranean part and the dry weight of the aerial and subterranean part) and physiological (Proline, chlorophyll and soluble sugars) were used to study the behavior of salt stress seven populations of the Carob
Abiotic stress is responsible for a loss of yield estimated at 50% for the more answered cultures. They are, therefore, significant limiting factors. Salt tolerance is usually expressed in terms of growth, performance or survival. It is translated by morphological, physiological, biochemical and molecular changes that affect plant growth and productivity.

The results show that the aerial part is more sensitive to salt stress effect than the root part. The effect on the vegetative growth of the plants tested in is observed especially at concentrations of 120 and 240 mM. Salt stress also leads to a reduction in aerial and subterranean biomass and a slowdown in the elongation of stems and roots. Several studies are consistent with the present results. According to Muhling and Lauchli, increased salinity, leading to the decrease in the yield of the aerial part of maize shoots; however, it has no significant effect on root growth. Similar results obtained by Cicek and Cakirlar, these authors show that the effect of salt stress on two maize cultivars is manifested by a marked decrease of the total fresh and dry weight, stem length and leaf area.

Chlorophyll content was significantly reduced by salinity especially for the harsh treatment (240 mM). These results are in agreement with the observations of Kaya et al. According to Feigin et al. and Grattan and Grieve, NaCl has an antagonistic effect on the absorption of Nitrogen (N), which is an essential component of the structure of the chlorophyll molecule. The results obtained show the depressive effect of salt on the seven populations studied with 240 and 120 mM NaCl concentrations. According to Ashraf and Foolad and Khan et al., the chlorophyll levels experienced a decrease in function of salt stress. In three cultivars of *Lycopersicon esculentum*, average chlorophyll a, b was reduced under the effect of salt stress. Regarding soluble sugars, several studies have focused on the study of their accumulation in several species subjected to stress. Significant and negative correlations were found in saline conditions, between the production of dry biomass aerial and leaf total soluble sugar content of some species such as sunflower, beans and rice. Whereas in other species such as wheat, barley and triticale and cotton and soybean, it is rather the reverse. Alleged varieties more tolerant of these species appear to accumulate higher amounts of soluble sugars.

El Midaoui found in sunflower under the action of the salt stress, the soluble sugar content increases recorded over the control were of the order of 15, 31 and 59% at respective concentrations of 50, 75 and 100 mM NaCl.

These preliminary results must be continued to clarify the coping mechanisms involved in resistance ecotypes of Carob (*Ceratonia siliqua* L.), including the exploration of other morphological and physiological
factors. The generalization of our study in comparative perspective for the study of the effect of salt stress on the dynamic capacity of cellular adaptation would require the study of the ultrastructure of cell membranes and those of chloroplasts (thylakoids). To complement this work, a genetic study to identify the genes responsible for the salinity tolerance was conducted.

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