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The biochemical and metabolic profiles of the leaves in *Ziziphus lotus* L. as a potential adaptive criterion to the environmental conditions

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- ✓ soluble proteins

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Abstract

Biomass can be converted to bio-oil, a promising carbon neutral energy source. In this work, bio-oil quality and quantity were optimized by utilizing a fixed-tube reactor's temperature, wood pellet size, and nitrogen flow rate in a central composite experimental design. The highest bio-oil yield (37%±1.3%) and % alkane + aliphatic area from H-NMR (57%±1%) occurred with a temperature of 550°C and low N_2 flow rate. ZrO_2-TiO_2 catalyst, silica catalyst, and ZSM-5 catalyst were tested for upgrading bio-oil. The % alkane + aliphatic area from H-NMR at the same operating conditions were as follows for aqueous-phase bio-oil; uncatalyzed (56%±0.2%), silica bead (56%±1%), ZSM-5 (63%±1%) and ZrO_2-TiO_2 (62%±1%). The major result of this work confirms the different effects between ZSM-5 and ZrO_2-TiO_2 catalyst. ZSM-5 catalyst doubled the amount of aromatics in the organic-phase of the bio-oil from 8% to 17% as measured by GC-MS area while ZrO_2-TiO_2 catalyst increased the cyclopentanones from 3% to 10%. From our analysis, ZSM-5 is the better catalyst for upgrading pyrolysis vapors as it decreases the oxygen containing compounds which is expected to increase the heating value of the bio-oil.

1. Introduction

Ziziphus lotus L. is considered among the medicinal plants widely used in traditional medicine. It presents several interests in nutrition, cosmetic and medicinal fields. Anti-inflammatory, analgesic and antispasmodic activities have been highlighted by previous work [1, 2]. In addition to its pharmacological properties, this plant is also a pastoral and fruit species appreciated by many animals (sheep, camelids and goats) [3]. It is also a shelter for some animals (rodents, insects and reptiles). The fruits of *Ziziphus lotus* L. are well-known by their wealth in alkaloids, flavonoids, sterols, tannins and saponin triterpenoids [4, 5]. These characteristics make of *Ziziphus lotus* L. an universal value colonizing arid and semi-arid ecological surfaces. However, this species is often ignored or even forgotten. Its degradation increased due to the impact of the anthropogenic factors such as overgrazing and grubbing-up by farmers, endangering the survival of the species.

To contribute to a better understanding of physiological behavior of Z. lotus in field conditions and to ensure sustainable production and better utilization of this species threatened, we conducted this study whose investigations were intended to assess the effect of pedoclimatic conditions on physiological and biochemical characters of this species by determining the rate of soluble carbohydrates, soluble proteins, proline and chlorophyll in the leaves. This work was focused on three ecotypes from different bioclimatic zones in Morocco: the regions of Guercif, Fez and Ain Chifa.

2. Material and Methods

2.1. *Plant Material and Study Sites*

The populations of *Ziziphus lotus L*. were collected from three different bioclimatic zones, which are: Ain Chifa (A), Fez (B) and Guercif (C). These regions are characterized by calcimagnesic soils with dominance of the clay fraction.

2.1.1 *Guercif station*

Located in the region of the Oriental to the northeast of the Kingdom, it is characterized by an arid climate with low rainfall not exceeding 185 mm/years, its geographical location is: Latitude: 34 ° 13'32 "North, Longitude: 3 ° 21'12" West, at an altitude of 367 meters (Figure 1). The region of Guercif benefits of fertile soil providing a great agricultural wealth. This region seems to be the ideal environment of proliferation for *Ziziphus lotus*, given that the floundering in areas with rainfall less of 500 mm.

2.1.2 *Ain Chifa station*

It is situated near Imouzzer Kandar. Its geographical location is: Latitude: 33 ° 47'8 North, Longitude: 5 ° 1'45 West, at 1084 meters above sea level (Figure 1). It is characterized by a continental type climate, generally rainy winter, hot and dry summer. The average annual rainfall is about 460 mm, annual mean temperatures of 16º with a maximum of 25 °C and a minimum of 9,5 °C.

2.1.3. Fez-*Saïss station*

Located at the Saïss plateau between the Middle Atlas and the pre-Rif, its geographical location is: latitude: 34 ° 02′13″ North, Longitude: 4 ° 59′59 West to 403 m above sea level (Figure 1). The region of Fez-Saïss enjoys a continental climate, very hot and very dry in summer, cold and damp in winter. The average precipitation is about 375 mm/year, the conditions for that station are favourable to the development of agricultural activities. The temperature ranges between 4°C and 43°C.

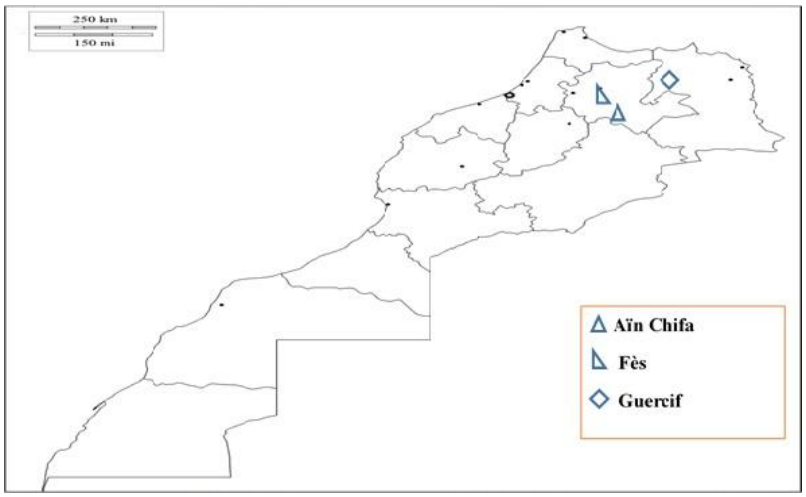

Figure 1: Location of the three study sites

2.2. *Sampling*

Ziziphus lotus L. leaves were randomly collected from thirty trees in each of the regions studied, at the rate of 15 leaves per tree during the month of August 2015. In order to homogenize the choice within a single tree and a representative sampling, several tree dendrometric parameters have been taken into account: height, trunk diameter, middle and the end of the sampled tree. Positioning compared to the four directions (North, South, East, West) as well as their sunshine.

2.3. *Analytical Methods*

The leaf content of different components such as proline, chlorophyll, total soluble proteins, total soluble sugars and free amino acids was determined, taking into account the source of samples. This will help us to highlight the existing relationship between the climatic conditions of the area of harvest, the physiology and biochemistry of the plant.

2.3.1. *Determination of Proline content*

The method used for the determination of proline content in leaves tissues is that of Trolls and Lindsley [6] simplified by Rascio et al [7]. 100 mg of fresh material were placed in a test tube containing 2ml of 80% methanol. The whole mixture was placed at 85 °C in a water bath for one hour. Then, 2ml of the extract, 2ml glacial acetic acid and 2ml of Ninhydrin reagent are mixed and placed in a boiling water bath for 60 min. The ninhydrin reagent was prepared by mixing 60 ml of glacial acetic acid, 30 ml of distilled water and 10 ml of

orthophosphoric acid. After cooling, spectrophotometric measurements (CHROM TECH V1200) were made at 546 nm.

2.3.2. *Determination of chlorophyll pigments content*

The determination of the total chlorophyll content was conducted using fresh leaves material according to Hiscox and Israelstam technique [8]. 4 ml of Dimethylsulfoxide (DMSO) were added to 40 mg of fresh plant material. After incubation in the dark in the oven at a temperature of 65 °C for 15 minutes, the absorbance (A) of the extracts was measured at 663 nm and 645 nm. The concentrations of total chlorophylls are deducted by the formula decriver by Arnon [9]:

Chl a (g/l) = 0, 0127 *A 663 - 0, 00269 *A 645
Chl b (g/l) = 0,0229 * A 645 - 0,00468*A 663
ChlTot (g/l) = 0,0202 *A64 + 0,00802*A 663

With: Chl : Chlorophyll
A : Absorbance

2.3.3. *Determination of the concentration of total soluble proteins*

To determine the concentration of the total soluble proteins, we used the Lowry method [10]. 200 mg of the fresh plant material were ground in 5 ml of buffer sodium phosphate (100 mM, pH 7.5), followed by centrifugation at 4000 rpm at 4°C for 10 min. The supernatant was thus recovered for the dosage. This technique allows us to per-form a particularly sensitive colorimetric determination based on two color reactions:

• Reaction of Biuret, in which Cu2+, in the presence of a base, reacts with the peptide bond giving a deep blue color.

• Chemistry of Folin-ciocalteu reagent, in which a complex mixture of inorganic salts reacts with tyrosine and tryptophan from protein, residues giving a blue-green color. The absorbance was determined by spectrophotometer at 750 nm (CHROM TECH V1200).

Calibration was carried out by known concentrations of BSA (bovine serum albumin).

2.3.4. *Determination of total soluble sugars content in leaves*

For the determination of the content of soluble sugars, 100 mg of fresh material was ground in 4 ml of ethanol 80%. After incubation by stirring for 30 min at 80 °C, the extract was centrifuged at 4500 rpm for 10 minutes. Total soluble sugars were then assayed by using anthrone reagent (anthrone 0,15% (w/v) in sulphuric acid) [11]. The absorbance was determined with the spectrophotometer at 625 nm (CHROM TECH V1200). The calibration range has been prepared by glucose.

2.3.5. *Determination of Total Amino Acids content*

Free amino acids were extracted by grinding of 100 mg of fresh leaves in 2 ml of Sodium-Phosphate buffer (100 mM, pH 7.5). The extract was incubated at 80 °C for 30 min after prior addition of 2 ml of 80% ethanol. 2 ml of 50% ethanol were then added in the tubes and the whole was vortexed.

After centrifugation for 10 min (5000 rpm), the supernatant was recovered and placed in the 80 °C oven. Dry residues with amino acids were solubilized in 500 µl of distilled water. The determination of amino acids was carried out by using the reagent of Ninhydrin [12]. The whole mixture was incubated for 20 minutes in a boiling water bath. After stopping the reaction by cooling in ice, 5ml of ethanol 50% were added and the spectrometric measurements were made at 625 nm. The calibration range was conducted by concentrations of glycine (150,14 mg/L).

2.4 *Statistical Analysis Method*

The data obtained were the subject of a statistical analysis (averaging, analysis of variance ANOVA, SE) to search the variability between different stations. Data were processed using the software "SYSTAT 12". A test of comparison of means was made whenever there was a significant effect of factor studied by ANOVA.

2. Results and discussion

Water stress is the most important of the environmental constraints that may disturb the development of trees and shrubs. These plant species are often developed in marginal soils subjected to long periods of water shortage. This situation tends to worsen because of global warming [13]. The results of our investigations

showed significant changes in the leaves of *Ziziphus lotus* L. in different parameters studied in stations A, B and C. This can be associated with coping mechanisms of the plant to the pedoclimatic constraints imposed in station C compared to stations A and B.

3.1. *Effect on the leaves content of proline in the three populations of Z. Lotus*

The content of proline in leaves from plants originated from the three areas A, B and C were reported in figure 2. For both populations A and B, proline concentrations do not exceed the value of 60,63 mg/g fresh weight (FW). In Stations A and B, the contents of proline in leaves were 58,34 mg/g FW and 60.63 mg/g FW respectively. This difference is not statistically significant, these stations being located in areas characterized by a relatively high rainfall rate. This later is comprised usually between 536 mm/year and 564 mm /year. When the relative rainfall decreases in the case of the region C, relatively significant accumulation of proline was observed (71.48 mg/g FW). A highly significant difference was observed between the station C and the stations A and B (ANOVA: F = 13.627; ddl = 2; P = 0.002). The concentration of proline in the leaves of *Ziziphus lotus* L. from station C was 14,69% higher, in comparison to the station A.

The accumulation of proline in plants has been shown as a mechanism of tolerance to the unfavorable growth conditions [14]. By their performance and physiological adaptation to drought, these biochemical-endemic settings were studied in the three ecotypes of *Z. lotus*. The determination of the leaf proline content reveals that plants confronted with unfavourable rainfall conditions react by increasing its concentration. Thus, to keep turgor potential as high as possible, after the very remarkable fall of moisture due to climatic stress, ecotype C has accumulated some osmotica inside their cells including proline and soluble sugars. Many studies showed that proline and soluble sugars are criteria of adaptation to stress. They allow the plants to withstand the lack of water by a decrease in osmotic potential. This phenomenon is known as the osmotic adjustment [15, 16, 17]. Our results are in agreement with those of Blum [18] having noted that the accumulation of proline has been demonstrated in many species and in different situations of stress (osmotic, water, thermal). In addition, when the level of applied stress increases, the proline concentrations become more marked [19]. This increase in concentration of proline, was very clear in ecotype C compared to the A and B. Proline levels in the cell are determined by the balance between biosynthesis and catabolism. The proline is synthesized from glutamate, which is converted to pyrroline 5-carboxylate (P5C) by the action of the pyrroline-5-carboxylate synthase (P5CS). The intermediate P5C is then reduced to proline by the P5C reductase (PC5R). Under stress conditions, P5CS accumulates in the chloroplasts, leading to enhanced proline biosynthesis [20]. The proline catabolism is enhanced during recovery period from stress. Many functions are attributed to the proline. Beside its role as osmoprotector, proline has a role in the strengthening of the antioxidant system by its ROS scavenging activity and fight against the damage of stress [21, 22, 23, 24]. It would also involve in the regulation of cytoplasmic pH [25], and as a reserve of nitrogen used by the plant after the period of stress [26].

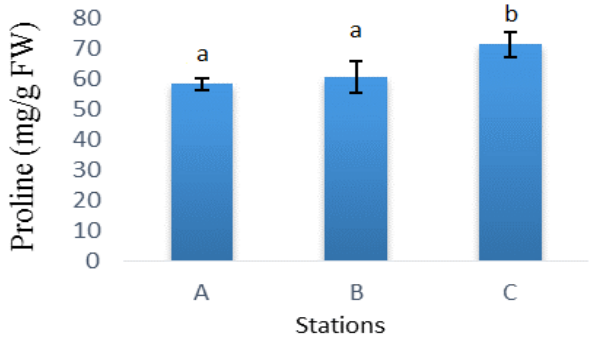

Figure 2: Variation of the proline content between three populations of *Ziziphus lotus L.* leaves: A (Aïn Chifa), B (Fez), C (Guercif). Vertical bars correspond to the SE to n = 4, FW: Fresh Weight

3.2. *Changes of total content of chlorophyll in leaves between the three populations*

The results presented in figure 3 show that in the ecotypes A (Ain chifa) and B (Fez), total chlorophylls levels are more important (2, 2 1 and 2.18 mg/g FW), compared to the ecotype C (1, 5 mg/g FW). The leaves of C population have been developed in an environment that is known by the low rainfall (222 mm/year). The effect of the pedoclimatic factors on the total chlorophyll content is not significant between the populations originated from the zones A (Ain chifa) and B (Sais) (P ≥0, 05). However, statistically significant differences were recorded between ecotype C and the two ecotypes A and B (ANOVA: F = 6, 206; ddl = 2; P <0,05).

The decrease in the rate of chlorophyll pigments is probably due to the decrease in the synthesis of chlorophyll and to the change of the membrane structure of the thyllacoides [27]. The decline of chlorophyll in the population C during the period of drought would also be the consequence of the reduction of the stomata opening as mentioned by Brown and Tanner [28]. This adaptive process would result in a limitation of the water evaporation throughout leaves and unfortunately in a reduction of the influx of atmospheric CO_2 for photosynthesis [29]. The saving of water contributes to the maintenance of a relative turgidity of plant tissues.

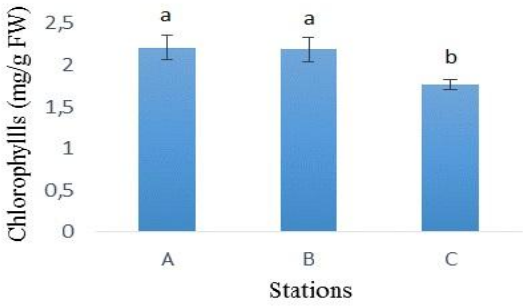

Figure 3: Variation of total chlorophyll of leaves between three populations of *Ziziphus lotus* L: A (Aïn Chifa), B (Fez), C (Guercif). Vertical bars correspond to the SE to n = 4, FW: Fresh Weight

3.3. *Effect of pedoclimatic conditions on the content of total soluble sugars (TSS)*

The results of the changes of the total soluble sugars content in different studied leaves are showed in figure 4. Under the action of the climate constraint, more significant increases in the levels of total soluble sugars were recorded in the C ecotype. The content of TSS in this ecotype was 33.50% and 32.74% higher, when compared to ecotypes A and B, respectively. However, no significant difference was recorded between the ecotypes A and B. These later populations of Z. lotus have shown the lower levels in TSS content. Conversely, highly significant differences between A and C on the one hand, and between B and C on the other hand ($F = 15, 602$; $ddl = 2$; $P < 0.001$) were recorded.

A comprehensive reading of the scientific literature [30, 17, 31] showed that in condition of water deficit, soluble sugars protect membranes against dehydration and participate in large part to the lowering of osmotic potential, such accumulation has been shown by the analyses of the data collected in our study. Indeed, the ecotype C living in stressfull climate (rainfall average: 185 mm/year) Temperature: 18.5 °C; Humidity: 56%; Elevation: 367 m) presents the highest soluble sugars content and statistical analysis will pair with this statement. Soluble sugars have shown to be the indicators of the stress degrees and their accumulation would be a means adopted by plants in order to withstand the stresses [31]. It was also shown that metabolic sugars such as glucose, galactose, sucrose, and fructose allow resistance to various stresses [31]. The stressed plants seem to react by increasing the amount of soluble sugars at the level of their cells [17, 31].

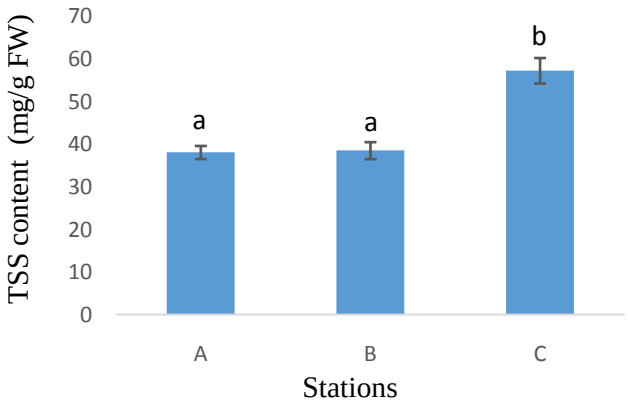

Figure 4 Variation in the leaves content of total soluble sugars between three populations of *Ziziphus lotus* L: A (Aïn Chifa), B (Fez), C (Guercif). Vertical bars correspond to the SE to n = 4, FW: Fresh Weight

3.4. *Effect of pedoclimatic conditions on the total soluble proteins and free amino acids Content*

Changes in the content of total soluble proteins extracted in leaves are presented in figure 5. By its potential for accumulation of proteins, C population exceeds widely both ecotypes A and B by percentages of 22.02% and

14.22%, respectively. The results showed highly significant differences between A and C; on one hand and between B and C on the other hand (ANOVA: F = 34, 087; ddl = 2; P <0.001).

Soluble proteins play a role in the adaptation of the plants and many researchers approach the study of resistance to the stress by the isolation and characterization of these molecules [32]. In response to drought stress, a qualita-tive and quantitative change in the pool of proteins produced by the cells of oat coleoptiles have been reported [33]. These changes in protein synthesis profiles, induced by stress, can be more quantitative than qualitative [34]. Increasing of the soluble proteins content in plant tissues as a response to abiotic stress was described by previous work in soybean, corn and wheat [35, 36]. The negative effect of abiotic stress on the activity of carriers of soil nitrogen and its assimilation by plants has been shown on several occasions [37, 38, 39]. Therefore, the accumula-tion of soluble proteins would be associated with a redistribution of nitrogen molecules in the plant. The remobili-zation of endogenous nitrogen and the modification of the source-sink relationship between the different organs of the plant should be taken into consideration. It would probably result in a modification of the cell proteomic profile. In the ABA deficient mutants of corn, a difference in terms of abundance of 46 protein spots was recorded, when compared to the wild type [40]. The dehydrins buildup has been reported following the application of abscisic acid in sunflower, wheat and poplar. The dehydrins would be the main proteins involved in the protection of the cytoplasmic cellular structures from the effects of dehydration [41; 42].

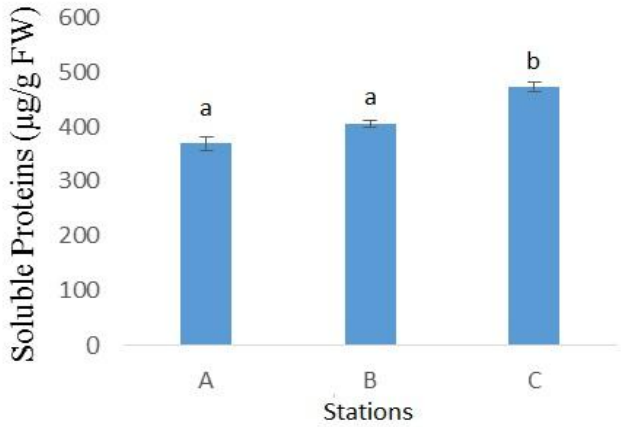

Figure 5: Variation of soluble proteins content in leaves between three populations of *Ziziphus lotus* L.: A (Aïn Chifa), B (Fez), C (Guercif). Vertical bars correspond to the SE to n = 4

The influence of the different pedoclimatic conditions in the three studied areas on the free aminoacids content in leaves is shown in the figure 6. The ecotype A recorded the lower level of free amino acids content (33%) when compared with B and C. Statistical analysis of the results shows of the highly significant differences between the levels of free amino acids of the ecotype A and B on one hand and between B and C on the other hand (ANOVA: F = 33, 682; ddl = 2; P <0.001).

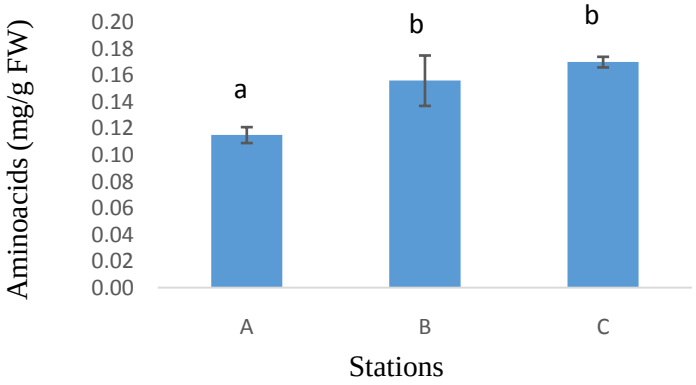

Figure 6: Variation of the content of free amino acids in the leaves between the three populations of Ziziphus lotus: A (Aïn Chifa), B (Fez), C (Guercif). Vertical bars correspond to the SE to n = 4, FW: Fresh Weight

The accumulation of nontoxic molecules of low-molecular weight in the vacuole and cytosol such as aminoacids, water soluble sugars and proline in the tissue of plant is an indicator of the stress occurrence. Biosynthesis and accumulation aminoacids have been reported in many studies on plants exposed to abiotic stress [43, 44]. Branched chain amino acids (valine, leucine and isoleucine) have been shown to accumulate in various abiotic stresses [5]. In our investigations, the accumulation of aminoacids in C population could not be associated to the stress induced protein breakdown. In stressed plant of maize, a consistent rearrangement of the amino acid pool was reported. Compositional changes occurred with a strong decrease in the amount of glutamate concomitant to an increased amount of alanine, valine, serine and aspartate, the glutamate being the amine donor [6]. Authors proposed a function to these accumulated aminoacids as electron donors for the respiratory electron transport chain [47].

Conclusions

Given the wide distribution of this species, *Ziziphus lotus* L. is subject to various climatic conditions. The results obtained allowed us to highlight a significant variation of biochemical parameters. This variation appears to be related to the adaptation of *Ziziphus lotus* L. to its living environment. Thus, in the arid area of Guercif, this species showed important accumulation of different components (soluble proteins, soluble sugars and free amino acids) in the leaves. The arising results from these investigations showed that the climate-induced water stress and the precipitation deficit alter the biochemical composition of the plant organs. To maintain the balance of the osmotic potential in water stress conditions, plants accumulate a number of osmotica such as proline, water soluble carbohydrates and soluble N compounds. As shown by other authors, these changes occur certainly in combination with the reduction in the transpiration rate, the stomatal closure and reduction of the leaf surface to keep turgor and cytosolic volume as high as possible [48; 49]. At the end in this study, we have shown that the three ecotypes used the same strategies to meet the stress but with different degrees in biosynthesis and accumulation of different osmolytes. These criteria may explain the ability of adaptation, development of *Ziziphus lotus* L. and its ability of distribution in various pedoclimatic areas, particularly the arid and marginalized soils.

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