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Biomass production and Symbiotic Nitrogen Fixation in the Legume *Sulla carnosa* in its Natural Biotope (sebkha ElKelbia)

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***Abstract*— *Wild legumes (herbs, shrubs or trees) play a critical role in natural ecosystems, agriculture, and agroforestry, where their ability to fix nitrogen makes them excellent colonizers of low-N environments, and hence an economic and environmentally friendly species. The field natural nodulation of the Tunisian Sulla crnosa, its symbiotic-efficiency and feed production potentiality in its saline biotope (sebkha d'El kelbia) were investigated in this study. A greenhouse experiment was conducted on plants transferred from sebkha with their soil in pots to explore the maximum potentialities of biomass production and nitrogen fixation of this legume when water is not a limiting factor (natural soil salinity was maintained in greenhouse). Obtained field and greenhouse study demonstrated that Sulla carnosa can be a good candidate for saline agriculture regarding its important ability to grow, produce biomass and fix nitrogen under high level of salinity (about 150 mM NaCl). This legume protects its photosynthetic and symbiotic organs against their overload with sodium by an important uptake of potassium and accumulation of Na in the roots. Sulla carnosa can play a goal role in the sustainable development in a region traditionally considered marginal.*
***Keywords*— Feed production, ionic repartition, sebkha, Sulla carnosa, Symbiotic nitrogen fixation.**

I. INTRODUCTION

Salinity leads to several physiological stresses in plants and consequently few plants can tolerate significant salinity levels in their root medium for any length of time. Of all the worlds' species, about 1% are considered halophytes [14], which are defined by Flowers and Colmer [6] as able to complete their lifecycles under saline conditions corresponding to at least 200 mM of NaCl in the root medium.

The negative effects of salt on plant growth are a considerable problem for agriculture and thus the world food production. Currently it is causing problems in many parts of the world (especially in arid and semi arid regions); and it is predicted to get worse under climate change conditions with increasing weather extremes and rising seawater levels [14]. Worldwide, up to 20% of arable land surface is salt affected. Soil salinity relates to the build-up of salts in soil and can be both natural and anthropogenic. A soil is considered saline if the electric conductivity of a saturated paste (equivalent to the available salts in the soil pore water) of that soil is over 4 dS/m (equivalent to ± 40 mM NaCl, which is roughly equivalent to 7% of seawater salinity). Notwithstanding the sensitivity of many plants to salinity, some plants can survive and grow vigorously under saline conditions [15]. The use of such plants would be extremely helpful not only to reclaim salinized areas, but also because it would allow us to use brackish or salt water for irrigation in agriculture. The use of brackish and saline water for irrigation is related to the scarcity of fresh water in the world, especially in areas that receive little annual rainfall. About 1% of the world's water is fresh (>0.05% dissolved salts), about 97% of the water is seawater (<3% of dissolved salts [14]) and the remainder is of intermediate salinity. Humans use large quantities of fresh water (and the demand increases faster than the human population grows) for a variety of activities including industry (20%), domestic use (10%) and, most notably, agriculture which accounts for around 70% of global fresh water consumption. The salinity problems and the scarcity of fresh water point to a solution via the use of salt-tolerant plants in agricultural production. Few crop plants can tolerate even moderate levels of salinity however. Attempts to improve the salinity tolerance of conventional crops have not been successful so far. This is because tolerance to salinity is a complex trait [4], involving multiple genes and having evolved multiple times independently among different lineages [7], leading to different mechanisms of salinity tolerance. A different promising strategy is to focus on the de novo domestication of halophytes. In addition to that, seawater contains many elements required for plant growth. This would reduce the need for fertilization of arable lands under saline irrigation for many nutrients. However, the element plants require in the highest quantities, nitrogen (N), is present in sea water in very small quantities, and would therefore have to be supplemented. In natural ecosystems, the largest input of nitrogen comes from nitrogen fixed by microorganisms, called diazotrophs [18]. These can be either free living or associated to plants from, mainly, the family of the Fabaceae. Nitrogen input from legumes can be a sustainable source of nitrogen in agricultural systems.

In the arid and semi- arid lands of Tunisia, the most limiting factor of livestock and animal production is the availability of feed. The feed crops did not exceed 7% of the ploughable area. For several years we have been working to improve pastures and rangelands of the central and southern regions but it turned out we were on the wrong way. In fact, improving productivity of sown pastures in salinized and drought affected soils is a faulty operation. However, most legumes (of that some spontaneous and halophytic species) are easily able to fix 100 kg N/ha/yr, and this figure fits well with findings in other studies; reported values of 200–300 kg N/ha/yr are no exceptions [13]. About 40–60% of the nitrogen fixed is available for the subsequent crop [16]. Considering a loss of around 50% of N and that 75% of total plant nitrogen comes from atmospheric N2, if we aim for the addition of 100 kg N/ha/yr available for a subsequent crop, the legume used as green manure should fix around 265 kg N/ha/yr. two legumes (*Medicago sativa* and *Vicia faba*) are capable of fixing this amount of nitrogen with the maximum rate of N fixation more than double this requirement. The use of legumes as an alternative to the use of fertilizer, i.e. as green manure, is thus a viable strategy based on these calculations. However, semi arid regions of Tunisia are known by their richness of the natural pastoral flora including some nitrogen fixing legumes. These species could be an alternative to grow food crops and vegetables, regarding the low availability of fresh water and the abundance of saline water and soils. Therefore, in this study we will focus on the importance of using the spontaneous Fabacae, *Sulla carnosa,* in saline agriculture as source of feed and nitrogen. The potentialities of biomass production and nitrogen fixation will be studied in *Sulla carnosa* in its natural biotope (Sebkha d'El Kelbia) and in the Laboratory.

II. MATERIALS AND METHODS

The Sebkha d'El Kelbia is an intermittent lake in Tunisia (Kairouan Governorate) that covers 8000 hectares, in addition to 7000 hectares of surrounding swamps, at 35°50'34" North, 10°16'18" East south of Kondar. it is classified in the lower semi-arid bioclimatic stage.

2.1. Field study: Field study was conducted in October after the beginning of the rainfall season to follow the appearance of new *sulla* shoot after the dry season. Two sites are marked to collect plants and soil samples for analysis. A general description of the landscape and flora was made, the number of *Sulla carnosa* plants and the total number of other fodder and pastoral plants in 1 m^2 were counted in 10 different sites.

2.2. Soil and plant sampling: Sebkha soil was sampled for analysis in the Lab from two different sites. The first one around *sulla* plants (S1) and the second one from the nude area (S2), five samples for each site from the superficial layer (30 Cm deep). In order to express the maximum potentialities of biomass production and nitrogen fixation of *sulla carnosa* and to guarantee nodulated plants, new appeared plantlets are transferred with their soil in 1 Kg plastic pots (1 plant by pot). Precautions are taken to not damage the root system. Twenty pots are then placed in a greenhouse in the Centre of Biotechnology of Borj Cedria and irrigated with salinized tap water. Electric conductivity of the sebkha soil was measured firstly in order to use the same concentration of NaCl for plant irrigation. Each pot is put on a plate to recover the flowing water.

2.3. Analysis: Soil analysis was made in the Laboratory of Soil Sciences in the National Institute of Agronomy (INAT, Tunisia). Samples were dried in the open air for three days, then grind to a fine powder with a mortar (diameter < 2 mm).

pH of the soil solution was measured according to Pauwels *et al.* [12] in a mixture of soil/ water (1/ 2.5). 25 ml of deionized water were added to 10 g of fine soil, shaken for 2 hour, then pH measured with a pH meter Mettler.

Electric conductivity was measured according to Pauwels *et al.* [12] in a soil and deionized water suspension (1/5). 25 ml of deionized water were added to 5 g of fine soil, shaken for 1 hour, then EC measured.

The fodder potentialities of *Sulla carnosa* were estimated by its biomass production after 45 and 90 days of cultivation. Shoot biomass (very appreciated by livestock) was quantified.

90 days old plants were harvested, and separated into leaves, roots and nodules. Nodulated roots were washed thoroughly and successively in 3 baths of ultra-pure water in order to avoid the contamination of roots and nodules with ions and elements from the soil.

For chemical analysis, Samples of fresh material were dried at 70°C for 72 h and ground to fine powder. Nodules were previously numbered at the harvest. After extraction in 0.5% HNO_3, K^+ and Na^+ ions were measured according to Pauwels et al., (1992), using flame emission photometry (Corning, UK). Total nitrogen was determined by the Kjeldahl method.

2.4. Statistical analysis: Analysis of variance (ANOVA), using the AV1W MSUSTAT program with orthogonal contrasts and mean comparison procedures, was performed to detect differences between treatments. Mean separation procedures were carried out using the multiple range tests with Fisher's least significant difference (LSD) (P< 0.05).

III. RESULTS AND DISCUSSION

In its natural biotope (sebkha d'El Kalbia), *sulla carnosa* showed anarchic development. It occupy particularly the sebkha border in which its distribution decreases going deep, sometimes individual and sometimes in association with other halophytes (*Salsola vermculata* and *Atriplex halimus …*) and glycophytes (*Medicago ciliaris, malva sylvestris …*) (picture 1).

Picture 1. *sulla carnosa in association with halophytes and glycophytes in the Sebkha d'El Kelbia*

It is very important to mention the presence of some area where flora disappears and no plant observed. In fact, electric conductivity measured in the sampled soils show two important results. The first one, concerns the soil with important flora including *sulla carnosa* (EC = 13.6 mS/ Cm). It is a clear saline soil regarding its EC exceeding the adopted norms (a soil is considered saline if the electric conductivity of a saturated paste, equivalent to the available salts in the soil pore water, of that soil is over 4 dS/m (equivalent to ± 40 mM NaCl, which is roughly equivalent to 7% of seawater salinity)). This soil is also alkaline regarding its measured pH (7.6). The second result concerns the nude soil (EC = 64.5 mS/ Cm) in which EC is very high explaining the absence of any plant. For this reason EC of the soil of plants transferred in the greenhouse was maintained at 13.5 mS/ Cm.

The study of the frequency of existence of *sulla carnosa* in association with other species of forage and pastoral interest has shown that it represents 28%. The average number of *sulla carnosa* plants observed in this study is about 30 plants / m^2. In fact, Abdelguerfi et *al.* [1] demonstrated that this species appeared on soils with low rate of organic matter, very saline, calcareous and rich in Mg and P. Lachaal et *al*. [10] reported that the biomass production of *Sulla carnosa* decreased by 60% at 300 mM NaCl. Nevertheless, this Legume of Agronomic interest with its fodder quality and ability to improve soil fertility with nitrogen can be used in valorization program of degraded soils particularly in arid and semi arid regions. This Legume offers grazing opportunities during an important period of the year regarding its high capacity of regeneration. Rozema and Schat [15] suggested that these species of halophytes help, not only to exploit saline's zones, but also give opportunities to use sea water in irrigation.

When water is not a limiting factor (plants transferred in greenhouse), *sulla carnosa* can produce 45 g of fresh biomass after 45 days, even maintained on sebkha saline soil (EC = 13.5 mS/ Cm), and 133 g of fresh biomass after 90 days. Taking in account the 30 plants/ m^2 observed in the sebkha, this mean that we can reach a production of 14 tons/ ha of fodder fresh biomass after 45 days in this marginal saline soil when water is available, or in a non saline soil but irrigated with saline water. After 90 days, this production amounts to 40 tons/ ha (fig 1).

Cultivated on its proper saline soil (sebkha), *Sulla carnosa* express high potentialities of nodulation. In fact, fig 2 shows a high number of nodules development on root system reaching 100 nodules per plant after 45 days and exceeding 200 nodules per plant after 90 days of cultivation. We are therefore before a legume species that produce feed biomass and fix nitrogen in a very saline environment (sebkha). The number and the red color of nodules when excised (presence of Leghaemoglobin) reflect an important capacity of symbiotic nitrogen fixation.

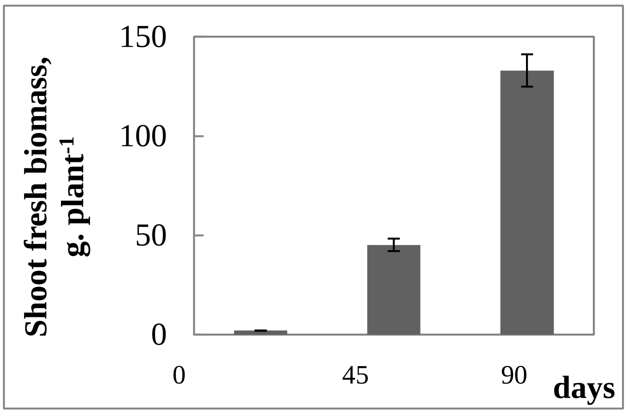

Figure 1. *Fresh biomass production of Sulla carnosa cultivated on sebkha soil (EC = 13.5 mS/ Cm) and irrigated with saline tap water. Data are means of 20 replicates (± standard error; p = 0.05).*

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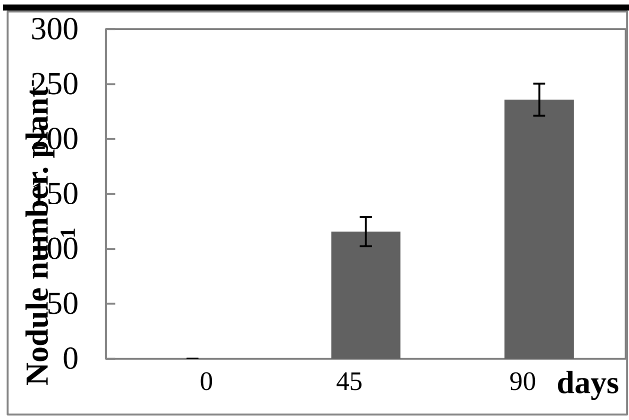

Figure 2. *Nodules number on roots of Sulla carnosa cultivated on sebkha soil (EC = 13.5 mS/ Cm) and irrigated with saline tap water. Data are means of 20 replicates (± standard error; p = 0.05).*

The analysis of nitrogen demonstrates that leaves and nodules accumulates a high levels of this nutrient (4.5 mmol N. g^{-1} DW and 3.3 mmol N. g^{-1} DW, respectively, fig 3). In roots the level of nitrogen is the lowest (1.5 mmol N. g^{-1} DW). If we consider the quantity of nitrogen measured in root and nodule organs (organs remaining in the soil, about 600 mg. $plant^{-1}$) and the number of plants founded in the sebkha (30 plant. m^{-2}), we conclude that this legume can furnish 180 Kg N/ ha within 3 months. It is generally established that the use of legumes as green manure is often equivalent to the application of 30– 80 kg N/ha/yr, with maxima of around 100 kg N/ha/yr [18]. However, for the purpose of comparison we assume 100 kg N/ha/yr as a reasonable approximation of fertilizer needs for most crops. For example, average nitrogen fertilizer use in the United States of America has been a little under 95 kg N/ha/yr (USDA ERS) [3]. Most legumes are easily able to fix 100 kg N/ha/yr, and this figure fits well with findings in other studies; reported values of 200–300 kg N/ha/yr are no exceptions [13].

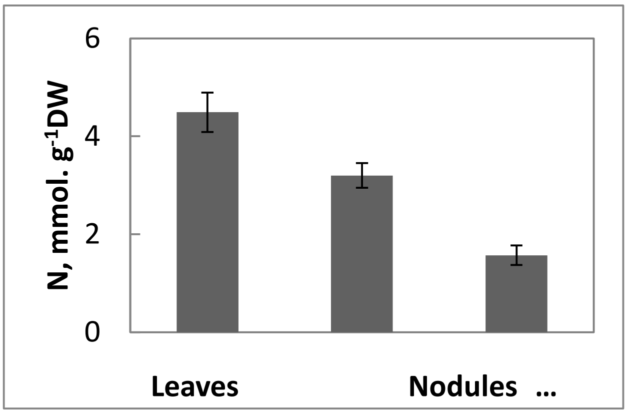

Figure 3. *Nitrogen accumulation in Sulla carnosa plants cultivated on sebkha soil (EC = 13.5 mS/ Cm) for 90 days and irrigated with saline tap water. Data are means of 10 replicates (± standard error; p = 0.05).*

However, about 40–60% of the nitrogen fixed is available for the subsequent crop [16]. This mean that by using *Sulla carnosa* in biosaline agriculture we can produce biomass and improve soil fertility in nitrogen. However, it is well documented that salt stress decreased legume productivity through the inhibition of photosynthesis, nitrogen fixation and C metabolism ([5], [8]). Velagaleti and Marsh [17] demonstrated that the decrease of nodules growth results from the inhibition of carbohydrate allocation to these organs after the reduction of leaves growth and photosynthetic capacity.

By analyzing potassium nutrition we remarks that this nutrient accumulated preferentially in leaves. Concentrations observed in nodules are also important and the roots present the less K concentration (Fig 4a). Sodium analysis repartition show very high level in leaves, then roots and nodules are the less invaded by this element (fig 4b). When calculating the ratio K/ Na (table 1), we observed that values are upper 1 in leaves and nodules and below 1 in roots. This result less think about the strategy adopted by this specie to prevent photosynthetic apparatus (leaves) and symbiotic apparatus (nodules) against the toxicity of sodium.

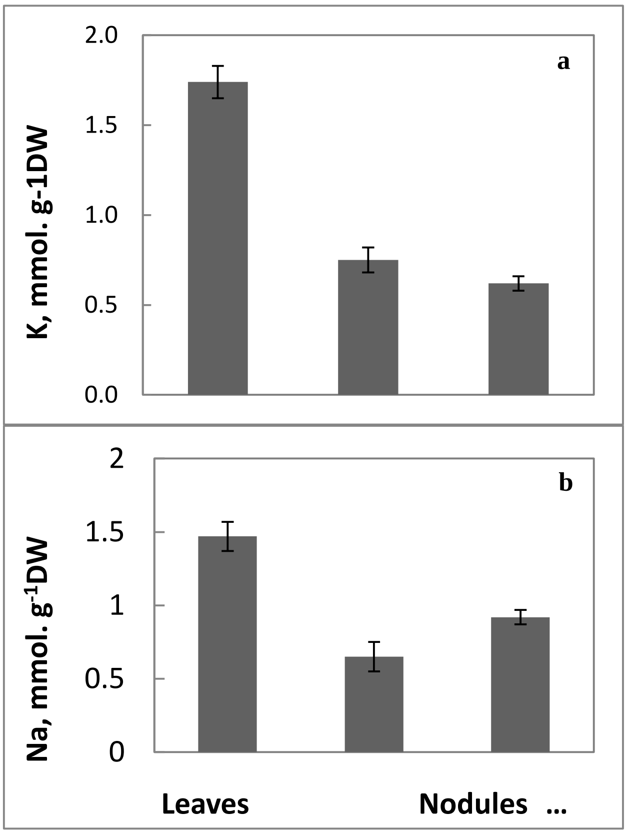

Figure 4. *Potassium (a) and sodium (b) accumulation in Sulla carnosa plants cultivated on sebkha soil (EC = 13.5 mS/ Cm) for 90 days and irrigated with saline tap water. Data are means of 10 replicates (± standard error; p = 0.05).*

Table 1. *Variation of the potassium/sodium ratio in the different organs of Sulla carnosa plants cultivated on sebkha soil (EC = 13.5 mS/ Cm) for 90 days and irrigated with saline tap water. Data are means of 10 replicates (± standard error; p = 0.05).*

Plant organ	Leaves	Roots	Nodules
K /Na	1.2± 0.11	0.7± 0.06	1.1± 0.12

Maintaining plant growth in saline conditions depends in part on the ability to keep cytoplasmic Na^+ levels low to protect the Na^+-sensitive metabolic machinery. Halophytes can utilize at least one of the three mechanisms to prevent Na^+ accumulation in the cytoplasm: reducing Na^+ entry into the cell, active Na^+ efflux from the cell, and active sequestration of Na^+ in the vacuole. In general, the vacuole is the largest sink for toxic Na^+ ions in plant cells. The plants adapt also to the ionic imbalances by osmotic adjustments of the cytoplasm. To avoid Na^+ accumulation in the leaf cytoplasm, there are a number of root-based mechanisms that minimize salt penetration to the foliar tissue ([2], [11]). It seems that our legume species, well adapted to saline environment, is able to protect its photosynthetic and symbiotic apparatus against their overload with toxic ions (Na^+ and Cl^-). In fact, mineral analysis made in this study demonstrated that *Sulla carnosa* concentrate sodium preferentially in roots and potassium preferentially in leaves and nodules. It is clear that the tolerance of *Sulla carnosa* to salinity is linked to its capacity of ions exchange. This means that our legume plant cultivated on saline soil accumulates high levels of potassium in the photosynthetic and symbiotic apparatus protecting them from toxic sodium that is trapped in roots. Bruning and Rozema [3] mention *sulla carnosa* as a xerohalophytic plant that tolerate, with some limits, drought and salinity. Kouas et *al.* [9] demonstrated that this species can tolerate 100 mM NaCl without reduction in plant growth or symbiotic nitrogen fixation. The present study demonstrated that *Sulla carnosa* can grow, produce biomass and fix nitrogen in its natural biotope in the presence of 150 Mm NaCl, but remain a native species not exploited agronomically and its symbiotic mechanism is not elucidated. The use of this Legume in the planning, development and valorization programs of areas considered for a long time marginal, like sebkha, is strategic because its low cost (no fertilizer used, no fresh water, saline abandoned area), environmental aspect (no chemical fertilizer), economic profitability (biomass and nitrogen input) and sustainable (spontaneous plants with high capacity of regeneration). Further studies should focus on the elucidation of the mechanisms of symbiotic nitrogen fixation and isolation of efficient strain of *rhizobia* for lab experiments.

IV. CONCLUSION

Saline agriculture provides a solution for at least two environmental and social problems. It allows us to return to agricultural production areas that have been lost as a consequence of salinization and it can save valuable fresh water by using brackish or salt water to irrigate arable lands. In this study, obtained results demonstrated that *Sulla carnosa* can be a good candidate for this purpose. Legume that grow in sebkha (saline, marginal and abandoned area), produce forage biomass and fix nitrogen. Further studies are in progress aiming to explore the genotypic variability of *Sulla carnosa* response to salinity using other provenance (5 regions of the arid and semi arid area of Tunisia). A special interest is granted to isolation and purification of an efficient strain of *Rhizobia* for hydroponic and La experiments.

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