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Natural Nodulation of Some Wild Legumes in the South Area of Tunisia

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Abstract: Legumes play a critical role in natural ecosystems, agriculture and agroforestry, where their ability to fix nitrogen in symbiosis makes them excellent colonizers of low-N environments and economic and environmentally friendly crop, pasture and herb species. Few reports exist until now on natural nodulation of legumes. Natural nodulation resource of five pastoral legumes, among which 3 of *Loteae* (*Lotus* sp.), 1 *Psoraleae* (*Psoralea* sp.) and 1 *Trifoleae* (*Ononis* sp.) and prospected from different sites in arid climate of Tunisia, was investigated. Occurency of nodulation and morphology of nodules were observed. Nodulation is reported for the first time on 2 taxas. Three taxas presented globular nodules and two spindle-shaped ones. Nodule shapes are characteristic of legume specie but independent of *Rhizobium* strain. The color of nodules is not related to that of the roots. It depends much more on the soil quality and color and the presence of the leghemoglobin in nodules. The nitrogen fixation intensity seems very significant for the majority of these species. This characteristic enables them to benefit from symbiosis with *rhizobia* to resist to the edaphic aridity of soils.

Key words: Wild legumes, nodulation, nitrogen fixation, *rhizobium*

INTRODUCTION

Nitrogen is the most important element in the nature following carbon, hydrogen and oxygen. Nitrogen status in terrestrial ecosystems markedly affects plant growth and thus vegetation coverage, size, density and phenology (Schlesinger and Lichter, 2001; Shaver and Chapin, 1986) which impacts physical characteristics of land surfaces and thus energy exchanges and water flows. It is well known from theoretical and experimental studies that leaf nitrogen content has a strong influence on photosynthesis (Chen *et al*., 1993; Evans, 1989; Field and Mooney, 1986; Grassi *et al*., 2005; Madsen and Baattrup-Pedersen, 1995; Ripullone *et al*., 2003 and Wright *et al*., 2004) which partially control stomatal conductance. It is because nitrogen is required to replenish leaf photosynthetic enzymes and may be as much as half of the total leaf N content (Warren *et al*., 2003b; Warren and Adams, 2004). Biological N_2 Fixation (BNF) is the major way for N input into desert ecosystems. *Rhizobium*-legume symbioses represent the major mechanism of BNF in arid lands, compared with the N_2-fixing heterotrophs and associative bacteria (Abdel-Ghaffar, 1989; Wullstein, 1989). BNF is an efficient source of fixed N_2, which play an important role in land remediation. Interest in BNF has focused on the symbiotic systems of leguminous plants and rhizobia, because these associations have the greatest quantitative impact on the nitrogen cycle. Deficiency in mineral N often limits plant growth and so symbiotic relationships have evolved between plants and a variety of N_2-fixing organisms (Freiberg *et al*., 1997). The symbiotically fixed N_2 by the association between *Rhizobium* species and the legumes represents a renewable source of N for agriculture. Values estimated for various legume crops and pasture species are often impressive, commonly falling in the range 200-300 kg N ha^{-1} per year (Peoples *et al*., 1995). This underlines the significance of *Rhizobium*-legume symbioses as a major contributor to BNF (Zahran, 1999). In addition to crop legumes, the nodulated wild (herb and tree) legumes have potential for nitrogen fixation, reforestation and to control soil erosion (Ahmad *et al*., 1984). It has been reported that a novel, suitable wild legume-*Rhizobium* associations are useful in providing a vegetational cover in degraded lands (Jha *et al*., 1995). The effectivity of the nitrogen-fixing wild herb legumes and their significance to soil fertility in arid regions, were recently reported (Jha *et al*., 1995 and Zahran, 1998). Nodulation (nodule number, type and structure) and symbiotic nitrogen fixation of five wild herb legumes (*Medicago intertexta*, *Melilotus indicus*, *Trifolium resupinatum*, *Trigonella hamosa* and *Alhagi*

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murarum), collected from cultivated lands of Egypt, has been determined (Zahran, 1998). These wild legumes have shown a great variation in nodulation and nitrogen fixation (acetylene reduction) in respect to site or locality. The wild herb legumes formed effective symbioses with *Rhizobium* strains similar to that established between cultivated legumes and their compatible rhizobia (Zahran, 1998). Since the wild herb legumes are adapted to wild life, they can be cultivated in reclaimed desert soil under arid conditions. The effective rhizobia, isolated from these legumes, have been used for inoculation of another legume crop (Zahran, 1999 and Zahran *et al.*, 1999). Similarly, the spontaneous (wild) forage legumes (*Hedysarum* sp., *Medicago* sp., *Trifolium* sp., *Scorpiurus* sp.), grown in arid regions of North Africa and Mediterranean area (Ouzzane and Abdel-Guerfi, 2000) have shown variation in nodulation (quality, localization and time of appearance) between and within species, symbiotic polymorphism of plant populations and a phenotypic diversity of rhizobia from different origins. Until now, nodulation of wild legumes originating from the mediterranean countries such as Tunisia has been poorly documented and studies have been mainly focused on cultivated species (Nour *et al.*, 1994; Nour *et al.*, 1995; Rome *et al.*, 1996; Squartini *et al.*, 2002). Lucernes (*Medicago* sp.) are of special ecological and agronomic importance and they have a very specific spectrum of LNB, namely *Sinorhizobium* sp. (Jordan, 1984; Eardly *et al.*, 1996; Rome *et al.*, 1996; Villegas, 2002) and *Rhizobium mongolense* (Van Berkum *et al.*, 1998). In the present study, we report on the investigation of wild legumes natural nodulation, some of which are common and play important ecological and pastoral roles, but others are rare and endangered, in the infra-arid zone of South Tunisia, where rainfall does not exceed 180 mm (Jeder *et al.*, 1996).Unlike the rest of the country, this region is characterised by a higher proportion of perennial legume species versus annual ones (Le Floc'h and Grossmann, 1998). The investigated legumes belonged to the *Loteae*, *Psoraleae* and *Trifoleae* tribes. They were frequently nodulated, annual legumes being generally more abundantly nodulated than perennial ones. Nodulation of the species *Lotus pusillus*, *Lotus arabicus* was reported for the first time.

MATERIALS AND METHODS

We prospected in the South region of Tunisia, during the January 2007 period, corresponding to the infra arid climatic zone with rainfall <180 mm (Fig. 1) to identify wild legume plants. We focused on five spontaneous annual

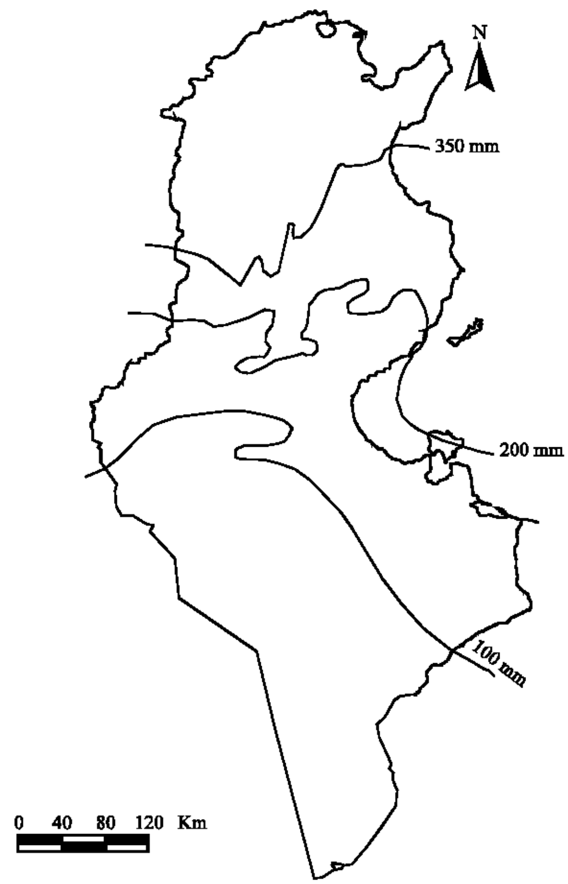

Fig. 1: Bioclimatic map of Tunisia. Rhizobia were all sampled in the infra arid zone (Rainfall between 200 and 100 mm)

Table 1: Tunisian wild legume characteristics

Subfamily Tribe	Genus	Species	Plant charcteristic
Papilionoideae			
Loteae	*Lotus*	*Lotus creticus*	Perennial
		Lotus pusillus	Annual
		Lotus arabicus	Annual
Trifoleae	*Psoralea*	*Psoralea bituminosa*	Perennial
	Ononis	*Ononis natrix* sp. *filifolia*	Perennial

and perennial nodulated legume plant species belonging to the genera *Lotus*, *Psoralea and Ononis* (Table 1). Occurency of nodulation (number of nodules par plant) and morphology of nodules (shape and color) were investigated.

RESULTS

Wild legumes are widely distributed in arid regions of Tunisia and actively contribute to soil fertility in these environments. Five legume species belonging to the

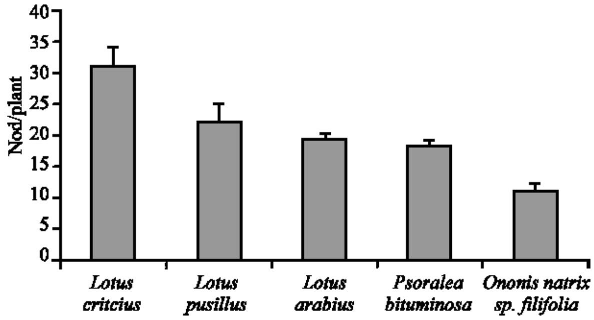

Fig. 2: Number of nodules for each species in natural conditions

Table 2: Morphology and diameter of the specie nodules

Species/nodules	Shape	External colour of nodules	Interior colour of nodules	Diameter
Lotus creticus	Globular	White	Pink-Red	Small/large
Lotus pusillus	Globular	White-brown	Pink-Red	Small
Lotus arabicus	Globular	White-brown	Pink-Red	Large
Psoralea bituminosa	Spindle-shaped	White	Pink-Red	Large
Ononis natrix sp. *filifolia*	Spindle-shaped lobed	White	Pink-Red	Small

genera *Lotus, Psoralea and Ononis* were grown in natural conditions. The objectives were to assess natural nodulation of these legumes and characterize the morphology of their nodules (shape and color). Nodulation of the species *Lotus pusillus, Lotus arabicus* was reported for the first time. Occurency of nodulation of the studied species was given by Fig. 2. Results showed that four species were strongly noduled. Only the species *Ononis natrix* was fairly nodulated. The highest number of nodules (31 nodules) was observed at *Lotus creticus* and the least one (11 nodules) at *Ononis natrix.*

The morphology (shape, colour) and the diameter of nodules were given by Table 2. Results showed that generally, the nodules are rather often rounded white and acquired shapes and sizes characteristic of the host. The size of the nodules varied from the millimetre fraction to the centimetre and can even exceed the diameter of the root of the plant host. The nodules presented also different shapes varying from globular (*Lotus* sp.), spindle-shaped (*Psoralea*) and spindle-shaped lobed (*Ononis*). The pink-red pigmentation in the nodule is related to the presence of a specific chromoprotein: The leghemoglobin. Its presence in the nodule seems to be essential to the nitrogenise activity: Enzymatic complex used by some organisms such as *rhizobia* to fix atmospheric Nitrogen gas (N_2). This pigmentation is consequently the symbol of an intense fixing activity.

Results showed also that certain nodules were attacked either by insects such as the ants or by the predatory microscopic ones, most probably of the protozoa or even of the bacteriophages. The presence of empty nodal structures was sometimes raised at some species. In general the oldest nodules and which reach the stage of dissemination of the plant host make green before their degeneration. It is only after their degeneration that the increase in the nitrogen content of the soil takes place and that the reduced nitrogen is placed at the disposal of the other plants.

DISCUSSION

The nitrogen-fixing leguminous plants are key components of the natural succession in semi-arid Mediterranean ecosystems because these plant species, upon establishing rhizobial and mycorrhizal symbioses, constitute a fundamental source of N input to the ecosystem (Zahran, 2001). Five legume species belonging to the genera *Lotus*, *Psoralea* and *Ononis* were investigated in natural conditions. The objectives were to assess natural nodulation of these legumes and characterize the morphology of their nodules (shape and color). All studied legumes were nodulated, with *Ononis natrix* having the least number of nodules and *Lotus creticus* the highest. This result seems to be in relation to the adaptative aridity character of different species. Indeed, it was showed an absence of fresh nodule in dry period, at arido-passive species (Evenari, 1975). The nitrogen fixation intensity seems very significant for the majority of these species. This characteristic enables them to benefit from symbiosis with *rhizobia* to resist to the edaphic aridity of soils. The nodules are also less frequent at the species which preserve their organs photosynthetic in the bad season (arido-active) (Jeder *et al.*, 1996). Ouzzane and Abdel-Guerfi (2000) mentioned that spontaneous (wild) forage legumes, grown in arid regions of North Africa and Mediterranean area have shown variation in nodulation (quality, localization and time of appearance) between and within species, symbiotic polymorphism of plant populations and a phenotypic diversity of *rhizobia* from different origins. In Egypt, Nodulation (nodule number, type and structure) and symbiotic nitrogen fixation of 5 wild herb legumes (*Medicago* sp., *Melilotus* sp., *Trifolium* sp., *Trigonella* sp. and *Alhagi* sp.) has been determined (Zahran, 1998). These wild legumes have shown a great variation in nodulation and nitrogen fixation (acetylene reduction) in respect to site or locality (Zahran, 1998). The poor nodulation observed at *Ononis natrix* can be expained by the effect of the several environmental conditions. Zahran (1991) showed that legume-*rhizobium* symbioses and nodule formation in legumes are more sensitive to salt or osmotic stress than are the rhizobia. In arid regions, high soil temperature affects both the free-living and symbiotic life of rhizobia (Zahran, 1999). For most rhizobia, the optimum temperature range for growth in culture is 28-31°C and many are unable to grow at 38°C (Graham, 1992). Some strains of the rhizobia surviving under heat stress may loose their infectivity, due to plasmid curing or to alterations in cellular polysaccharides necessary for infection (Zahran *et al*., 1994; Zahran, 1999). High soil temperature (35-40°C) usually result in the formation of ineffective nodules, however, some strains of rhizobia, e.g., *R. leguminosarum* bv. *phaseoli*, were reported to be heat tolerant and to form effective symbioses with their host legumes (Hungria *et al*., 1993; Michiels *et al*., 1994).

Three taxas presented globular nodules and two spindle-shaped ones. Nutman (1956) and Dart (1977) showed that nodule shapes are characteristic of legume specie but independent of *Rhizobium* strain. The colour of nodules varied from white to white-brown. Cloonan (1963) and Dobereiner (1965) mentioned that the colour of nodules is not related to that of the roots. It depends much more on the soil quality and colour and the presence of the leghemoglobin in nodules. The leghemoglobin molecule is a symbiotic product whose vegetal part is synthetized in response to the bacterial infection (Verma and Long, 1983). A physiological relationship has been suggested to exist between nodule leghemoglobin content and nitrogen fixing efficiency and higher nitrogenase activity has been reported in soybean and common bean when leghemoglobin levels were high (Dakora, 1995). On the other hand, the pink-red nodules containing leghemoglobin indicate an active N-fixation so that *Lotus* sp. -*Rhizobia*, *Psoralea* sp. -*Rhizobia* system might prove very valuable for land reclamation, as legumes are known to be good primary successors (Kenny and Cuany 1990; Blundon and Dale, 1990). Viands *et al.* (1979) showed that the absence of leghemoglobin, which results in a white, rather than a pink-red nodule, indicates an inability to support nitrogen fixation.

The efficiency of the assimilation of the fixed nitrogen by the plant could play an important role in plant productivity. Few data exist on the natural efficiency of wild legumes with respect to nitrogen. Values estimated for various legume crops and pasture species are often impressive, commonly falling in the range 200-300 kg N ha^{-1} per year (Peoples *et al*., 1995). Shearer precised that *Prosopis glandulosa* can fix up to 60% of its nitrogen requirements evaluated at 30 kg/ha/an. However, Hogberg and Kvarnstrom (1982) estimated that *Leucaena leucocephala* can fix up to 110 kg/ha/an. *Lotus* sp. and *Psoralea* sp. seems to fix important levels of nitrogen. Their introduction in natural ecosystems provides nitrogen to the system, increasing the quality and quantity of grasses.

Despite the abundance and diversity of *Lotus* sp. legumes in the South area of Tunisia, their ability to form nitrogen-fixing nodules in symbiosis with rhizobia and their response to rhizobial interaction, remain poorly documented. In the first part of this study the occurrence of natural nodulation and the morphology of their nodules were determined. Of the five plant species, *Lotus pusillus* and *Lotus arabicus* were reported to be nodulated at the first time. Genetic study of some legumes reported the role of plant genes in the nodular organogenesis, host specificity and the symbiosis efficacity (Tan *et al*., 1999). Our study must be more deepened to improve the output of nitrogen fixation, especially in the arid areas, where the absence of humus is the principal obstacle for the cultures development. The rhizobia from wild leguminous had been classified into one of the following genera of rhizobia: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Allorhizobium* after extensive analysis of the phenotypic, genotypic and molecular characteristics (Haukka *et al*., 1996; Nick *et al*., 1999; Tan *et al*., 1999; Tighe *et al*., 2000; Wang *et al*., 1999; Zahran, 1997b and Zahran *et al*., 2000). Therefore, these bacteria are very important from both economic and environmental points of view. Our study will shed light on the characterization, the genetic diversity and phylogeny of environmentally important genes, such as rhizobial nod and nif genes, in relation to the mechanisms of co-evolution with their host plants. It will also produce genetic resources that can contribute to developing agriculture based on ecological principles, maintaining biodiversity and, in future, restoring marginal lands affected by drought and salinity.

CONCLUSION

The study of *Rhizobium*-Legume symbiosis raises particular methodological difficulties because the resulting nodules are complex organs resulting from the interaction of two organisms: A higher plant and bacteria. A better comprehension of the role of each partner is a necessary condition for the improvement of the output of this functional unit. Genetic study of some legumes reported the role of plant genes in the nodular organogenesis, host specificity and the symbiosis efficacity. Our study must be more deepened to improve the output of nitrogen fixation, especially in the arid areas, where the absence of humus is the principal obstacle for the cultures development.

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