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Research Article

Nodulation and nitrogen fixation of some wild legumes from differing habitats in Egypt

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ABSTRACT

This study was devoted to exploring the natural nodulation and nitrogen fixation of wild legumes grown in different Egyptian habitats. These habitats are representative to four phytogeographical regions. Sites that inhabited by *Melilotus indicus*, *Medicago polymorpha*, *Trifolium resupinatum*, *Trigonella hamosa* and *Vicia sativa* in each region were selected for study. High nodulation, nitrogen fixation and plant biomass were recorded in plants grown at Nile region and Oases compared with those at Mediterranean region and Sinai. The inhibition in nodulation and potential of nitrogen fixation in legumes at MR and S were attributed to drought and low soil fertility. Differences in species, regions or their interaction have significant effect on nodulation, legheamoglobin, nitroginase activity and biomass of nodules, shoots and roots; the magnitude of effect due to different species was the greatest. Five rhizobial isolates (*Sinorhizobium fredii*, *Rhizobium mesosinicum*, *Rhizobium daejeonense*, *Rhizobium huautlense*, *Rhizobium alamii*) recovered from root nodules of the five species were identified by 16S rRNA gene sequence. The indigenous rhizobia of legumes grown at MR and S expected to be exhibit higher tolerance to the existing harsh environmental conditions. These rhizobia can be used as inoculants for crop legumes under unfavorable environmental conditions of agroecosystems or recently reclaimed desert.

Keywords: Nodulation; Nitrogen fixation; Wild legumes; Legheamoglobin; Rhizobia.

1. INTRODUCTION

The leguminous plants constitute one of the largest families of the flowering plants, consisting of ca. 730 genera and ca. 19,400 species [1]. It is an extremely diverse family with worldwide distribution, encompassing a wide range of life forms, from arctic alpine herbs and temperate or tropical perennial shrubs to annual xerophytes and equatorial giant trees [2].

Legumes play a vital role in agro-ecosystems based on their ability to form a symbiosis with soil rhizobia that fix atmospheric nitrogen [3, 4]. Biologically fixed N_2, either asymbiotic, associative, or symbiotic, is considered a renewable resource that should constitute an integral part of sustainable agro-ecosystems globally [5, 6]. *Rhizobium* spp. are Gram-negative soil bacteria that have a profound scientific and agronomic significance due to their

ability to establish nitrogen-fixing symbiosis with leguminous plants, which is of major importance for the maintenance of soil fertility [7, 8]. Nitrogen fixation by legumes, play an important role in sustaining crop productivity and soil reclamation of the semi-arid areas [9, 10].

It is well documented that wild leguminous plants inhabiting any region show adaptability to the environment and fix the atmospheric nitrogen more efficient than the cultivated legumes in that region [11-13]. Little information has been reported on natural nodulation of wild legumes [14-16]. One fascinating application of rhizobia of wild legumes is using it as inoculums for crop legumes. Many reports proved that some rhizobia of wild legume are more efficient in nitrogen fixation activity with other hosts than their compatible hosts [17, 18]. It has been reported that cross inoculation of crop legumes with rhizobia isolated from wild non-crop legumes enhanced nodulation and nitrogen fixation [19].

Nodule formation and nitrogen fixation of legumes are strongly affected by sub-optimal soil conditions, such as temperature extremes, salt stress, high or low soil pH, low water content, pesticide application and nutrient deficiency [20]. Environmental differences also strongly affect demographic processes like germination and seedling recruitment, which in turn affect genetic differentiation among plant populations [21], often due to random processes such as founder effects or genetic drift [22]. Hence, the hereditary structure of any plant species reflects its interaction with the environment [23]. Genetic diversity is strongly influenced by reproductive mode and mating system [24, 25]. In addition, genetic diversity is assumed to increase with abiotic and biotic heterogeneity and in stressful environments [26, 27]. Thus, ecological components such as temperature and precipitation also affect genetic diversity [28, 29]. The ability of species to respond to changes in the environment will ultimately determine survival in a particular habitat [30, 31].

Egypt extends across large areas of land comprising several geographical regions differ topographically, climatically and environmentally in general. Egypt is the meeting point of floristic elements belonging to at least four different regions: the African Sudano-Zambezian, the Asiatic Irano-Turanian, the Afro-Asiatic Saharo-Arabian, and the Euro-Afro-Asiatic Mediterranean [32]. Egyptian farmers facing a problem in providing crops with required nutrients, especially nitrogen, due to inadequate supply of mineral nitrogen fertilizers or the costs of these fertilizers. However, there is an urgent need to find alternatives on the base that soil fertility strongly depends on metabolic activities of microbes. Efficient symbiotic nitrogen fixation reduces the level of the requirement for external input of mineral nitrogen fertilizers. As nodule activity is known to vary diurnally and seasonally, also nodulation and nitrogen fixation of wild legumes could be varied depending on their habitat. Hence, improving our knowledge around the ecological distribution of wild legumes is a topic of utmost importance to better understand how to preserve it, increase their import and select the most efficient nitrogen-fixing wild legumes. Therefore, the present research aimed to study the biodiversity and biogeography of rhizobia associated with some wild legumes and their ability to fix atmospheric nitrogen.

2. MATERIALS AND METHODS

2. 1. Soil sampling and analysis

Soil samples from each site at four phytogeographical regions were collected for the physical and chemical analyses. Three soil samples were collected from profiles of 0-50 cm depth, pooled together to form a single composite sample, and carried to the laboratory in plastic bags. The samples were then spread over sheets of paper and left to dry in the air. Dried soils were passed through a 2 mm sieve and packed into paper bags for analysis. Soil texture was determined according to Allen et al. [33], organic matter according to Walkley and Black [34], soil sodium and potassium according to Williams and Twine [35], calcium and magnesium according to Johnson and Ulrich [36], chlorides according to Hazen [37], sulphates according to Black et al. [38], phosphates according to Woods and Mellon [39], bicarbonates according to Piper [40], nitrate according to Markus, McKinnon and Buccafuri [41], electric conductivity according to Jackson [42]. The pH value and soil water content also were determined.

2.2. Vegetation sampling and preparation

The current study was carried out along two successive years 2010-2011. The studied stands were chosen at locations inhabited by five wild nitrogen fixing legumes namely: *Melilotus indicus* (L.) All., *Medicago polymorpha* L., *Trifolium resupinatum* L., *Trigonella hamosa* L. and *Vicia sativa* L. One site at each of four Egyptian phytogeographical regions was chosen for this study: Assiut site in NR (27º 08' N, 31º 20' E) Al-Kharga site representing Oases (25º 32' N, 30º 37' E), Burg Al-Arab site in MR (30º 57' N, 29º 37' E) and Saint Katherine site in South Sinai (28º 33' N, 33º 56' E). At each site three individuals (as replicates) from the same population of every plant species were collected, and separated into roots and shoots. The shoots and roots were washed several times with distilled water, blotted gently with filter paper and were quickly weighted for fresh biomass (FW) determination and oven-dried at 70 ^{o}C for 48 hour to determine the dry biomass (DW).

2.3. Assessment of nodulation

Nodulation was assessed by up-rooting the plant, washing away adhering soil particles, and counting the number of nodules present. Nodules fresh and dry biomasses were determined. Some nodules from another individuals were also detached to check for the presence of red pigment (leghaemoglobin).

2.4. Determination of leghaemoglobin in nodule cytosol

One gram of fresh nodules was rinsed thoroughly with distilled water and immediately hand ground in an ice chilled mortar with 5 ml of distilled water. Nodule homogenates were filtered through four layers of cheesecloth and the filtrate was centrifuged at 500 x g for 2 min to remove nodule debris. The resulting supernatant was centrifuged at 12,000 x g for 15 min to sediment the bacteroids. Leghaemoglobin levels in the supernatant, the 'nodule cytosol', were determined colorimetrically as described by LaRue and Child [43], using Unico UV-2100 spectrophotometer. The colorimetric assay was standardized using freshly preapaerd Hemetrol reagent (solution of cyanmethemoglobin titrated exactly according to recommendations of BioMerieux, Marcyletoile, 69260 Carbon nieres les Bains, France)

2.5. Determination of nitrogenase activity

Nitrogenase activity was determined in detached roots, using gas chromatograph as described by Abd-Alla [44], (Thermo Scientific TRACE GC Ultraequipped with FID detector and Capillary column CP-PoraBOND Ufused silica plot 25 m × 0.32 mm, df = 7 m). The excised nodulated roots were placed in 500 ml bottles sealed with a rubber septum. 50 ml of air were taken and the same volume of acetylene gas introduced into the bottle, incubated at 37 $^{\circ}$C then samples from root atmosphere in bottles were with-drawn and injected to the gas chromatograph. Afterwards nodules of each individual root were counted and nodules fresh and dry mass were estimated. A calibration curve was constructed using pure ethylene.

2.6. Determination of proline content

Free proline was determined in fresh tissues according to method of Bates, Waldren and Teare [45], shoots samples (30 mg) were homogenized in 6 ml 3% sulfosalicylic acid, then filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, the resulting mixture was heated for 1 h in water bath at 100 $^{\circ}$C. The reaction was stopped by using ice bath. The mixture was extracted with toluene and mixed vigorously. The chromophore containing toluene was aspired from the aqueous phase and the absorbance measured at 520 nm. Proline concentration was determined using calibration curve.

2.7. Isolation of rhizobial strains

Twenty representative sites inhabeted by the studied species (one site for each species at each of the four phytogeographical regions) were chosen for rhizobial isolation. The strains of rhizobia were isolated from root nodules of *M. indicus, M. polymorpha, T. resupinatum, T. hamosa and V. sativa.* The isolates were grown on yeast extract mannitol

agar (YEMA) medium and incubated at 28 °C [46] on an orbital shaker at 120 rev per min for three days.

2.8. Molecular identification of rhizobia

From bacterial cultures using SDS/CTAB lysis and phenol/chloroform extraction method Ausubel et al. [47], DNA extracted sent to South Korea, Solgent Co., Ltd Bio industry Development for PCR-amplified at using primer pairs 16S (1492R 5' TACGGYTACCTTGTTACGACTT 3' and 27F 5' AGAGTTTGATCMTGGCTCAG 3'). The sequence reads were edited and assembled using BioEdit version 7.0.4 (www.mbio.ncsu.edu/ BioEdit/bioedit.html) and clustal W version 1.83 (http://clustalw.ddbj.nig.ac.jp/top-e.html). BLAST searches were done using the NCBI server at www.ncbi.nlm.nih.gov/blast/Blast.cgi. The rhizobial isolates identified and recoded in GenBank.

2.9. Nucleotide sequence accession numbers

The nucleotide sequences of the rhizobial isolates namely, *Sinorhizobium fredii, Rhizobium huautlense, Rhizobium daejeonense, Rhizobium alamii, Rhizobium mesosinicum* were deposited in the GenBank nucleotide sequence database under accession number [GenBank: KF879914.1, KF87 9916.1, KF879917.1, KF879920.1, KF879921.1, respectively].

2.10. Statistical analysis

Data were subjected to statistical analysis using SPSS package (version 19). One-way ANOVA, followed by Duncan multiple range test were employed and the differences between means deemed to be significant at $p < 0.05$. Correlation analyses were carried between soil variables and some parameters estimated in plants. Factorial ANOVA was carried to achieve the effect of species, regions and their interaction on different parameters estimated in plants and η^2 was calculated as: $\eta^2 = SS_{between} / SS_{total}$.

3. RESULTS

3.1. Soil

The data recorded in Table 1 revealed that there are significant differences between the physical and chemical properties of soils of the four phytogeographical regions. Soil of NR was characterized by the highest values of water content, K^+ and organic matter (OM), while soil of MR was characterized by higher content of HCO_3^- . Compare to other regions, soil of Saint Katherine (S) have high concentrations of Na^+, Mg^{+2} and SO_4^{-2} , and hence the TSS in the soil was high. The soil of the NR and O were rich in NO_3^-, while the soil of the NR and MR were rich in PO_4^{-3}. The pH value of MR and S soil was high as compared with the other regions.

3.2. Nodulation

The present study proved that the nodulation differ significantly among the studied plant species and as affected by the habitats at the four studied phytogeographical regions. Nodules number in all legumes inhabiting NR, O and MR increased significantly compared with those grown at Sinai (Fig. 1). Amongst the studied species, *T. resupinatum* showed the highest nodules number averaging about 223 nodule/individual plant compared with less than 70 in the four other legumes. The highest fresh and dry weight of root nodules was recorded in most species grown at the MR. Across all species, the fresh weight of nodules for plants sampled from MR was more than 1.5-fold of those sampled from other regions. The maximum fresh and dry weight of nodules was recorded in *M. indicus* (non-significant increase) (Fig. 2). Although there was a significant positive correlation between nodules fresh and dry biomass (r-value= 0.891**), both did not depend on the nodules number. Variations between species exerted the greatest magnitude of effect on the number of nodules where $\eta^2 = 0.904$ compared with $\eta^2 = 0.064$ for the effect of regions. Nodules fresh and dry biomass affected by differences between habitats more than their number (Table 3).

3.3. Leghaemoglobin content and nitrogenase activity

Leghaemoglobin content of root nodules and nitrogenase activity (acetylene reduction) differed significantly among the studied plant species as affected by the phytogeographical regions. The significant increases in leghaemoglobin content were recorded in legumes grown at S and NR (Fig. 3). The highest leghaemoglobin content and nitrogen-fixing activity were estimated in *T. resupinatum* (Figs. 3, 4). Despite there are non-significant differences in the content of leghaemoglobin between *T. resupinatum*, *T. hamosa* and *V. sativa*, interestingly the nitrogenase activity in *T. resupinatum* was about 10-fold of the activity in the both other species. Legumes grown at NR were characterized by a significant increase in nitrogenase activity compared with those at other regions. In all studied species, the content of leghaemoglobin was strongly correlated with the concentration of PO_4^{3-} and water content (WC%) of soil, while it negatively correlated with SO_4^{2-}, NO_3^-, TSS, pH, Na^+, Ca^{2+} and Mg^{2+} (Table 2). Nitroginase activity was positively correlated with the concentration of Cl^-, PO_4^{3-} and WC% of soil, while the activity negatively correlated with SO_4^{2-} (weak –ve r-values), NO_3^-, TSS, HCO_3^-, pH, K^+, Ca^{2+} and Mg^{2+}. As shown in Table 3, variation in species have the greatest magnitude of effect on legheamoglobin content ($\eta^2 = 0.878$), while its effect on nitrogenase activity reduced ($\eta^2 = 0.487$) in favor of differences in habitats or regions ($\eta^2 = 0.299$).

Table 1. Some physical and chemical properties of soils at different studied habitats. NR, Nile region (Assiut); O, Al-Kharga Oases; MR, Mediterranean region (Burg Al-Arab); S, South Sinai (Saint Katherine); TSS, Total soluble salts; OM, Organic matter. Values are means± SD, n=5.

Regions					
Parameter		**NR**	**O**	**MR**	**S**
Soil texture		Clay	Clay loam	Loam	Loam
Water content %		30.82±2.29 ^{d}	20.80±0.76 ^{c}	13.73±0.88 ^{b}	8.47±0.70 ^{a}
pH (in 1:5 extract)		7.68±0.13 ^{a}	7.78±0.09 ^{a}	7.98±0.02 ^{b}	8.09±0.02 ^{b}
E.C. (mS/cm)		0.365±0.06 ^{ab}	0.45±0.07 ^{b}	0.33±0.06 ^{a}	0.57±0.09 ^{c}
TSS%		0.117±0.02 ^{ab}	0.144±0.02 ^{b}	0.107±0.02 ^{a}	0.182±0.03 ^{c}
OM %		1.61±0.08 ^{c}	1.23±0.11 ^{bc}	0.82±0.16^{b}	0.47±0.05 ^{a}
Na^+	**mg/g soil**	0.13±0.01 ^{a}	0.15±0.03 ^{a}	0.13±0.01 ^{a}	0.19±0.02 ^{b}
K^+		0.10±0.02 ^{c}	0.01±0.00 ^{a}	0.04±0.00 ^{b}	0.04±0.01 ^{b}
Ca^{+2}		0.34±0.04 ^{a}	0.45±0.03 ^{b}	0.48±0.01 ^{b}	0.48±0.03 ^{b}
Mg^{+2}		0.09±0.04 ^{a}	0.09±0.04 ^{a}	0.12±0.02 ^{a}	0.29±0.04 ^{b}
Cl^-		0.83±0.20 ^{a}	0.89±0.18 ^{a}	1.14±0.18 ^{b}	1.12±0.14 ^{b}
HCO_3^-		2.44±0.30 ^{a}	2.44±0.31 ^{a}	3.25±0.32 ^{c}	2.75±0.26 ^{ab}
NO_3^-		0.37±0.02 ^{c}	0.35±0.06^{c}	0.22±0.02 ^{b}	0.08±0.01^{a}
SO_4^{-2}		0.43±0.05 ^{a}	0.38±0.04 ^{a}	0.34±0.01 ^{a}	0.85±0.04 ^{b}
PO_4^{-3}		0.06±0.001^{b}	0.043±0.001^{a}	0.063±0.004 ^{b}	0.039±0.002 ^{a}

Means with different letters are significantly different according to Duncan comparisons ($P < 0.05$).

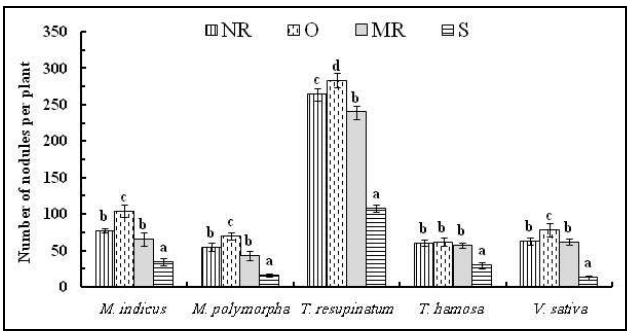

Figure 1. Nodule number (nodule/plant) of the legumes *M. indicus, M. polymorpha, T. resupinatum, T. hamosa* and *V. Sativa* inhabiting different phytogeographical regions of Egypt (NR= Nile region; O = Oases; MR = Mediterranean region; S = Sinai). The values are mean ± SD, n = 3, means of each species with different letters are significantly different at $P< 0.05$.

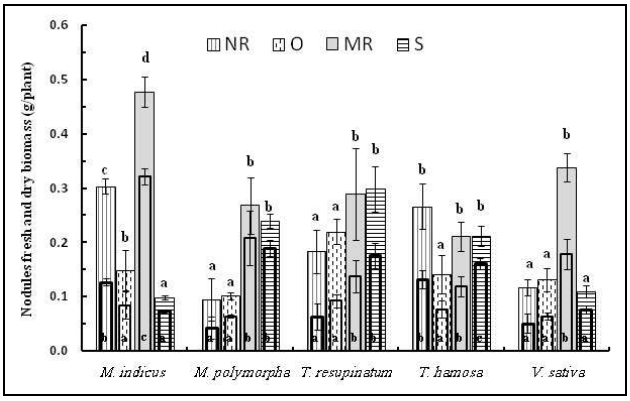

Figure 2. Nodule fresh and dry biomass (g/plant) of five legumes inhabiting different phytogeographical regions of Egypt. Statistics as in Fig. 1.

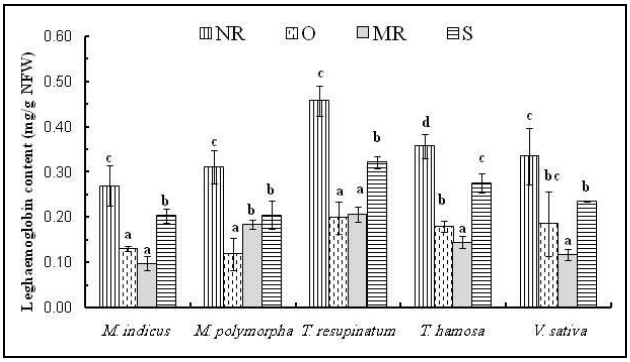

Figure 3. Leghaemoglobin content (mg g^{-1} nodule FW) in five legumes inhabiting different phytogeographical regions of Egypt. Statistics as in Fig. 1.

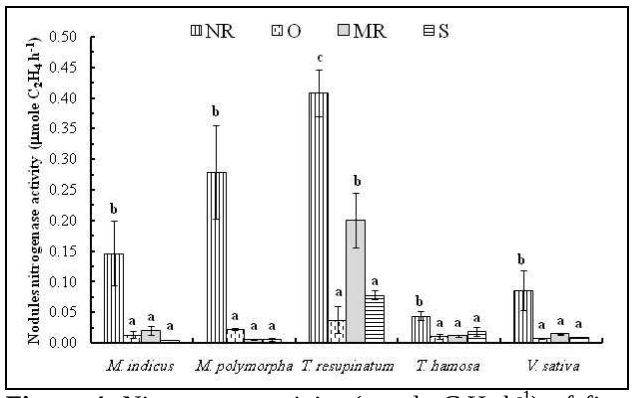

Figure 4. Nitrogenase activity (µmole C_2H_4 h^{-1}) of five legumes inhabiting different phytogeographical regions of Egypt. Statistics as in Fig. 1.

3.4. Proline content

The data recorded in Figure 5 shows that differences in habitat conditions have a significant effect on the proline content in the five wild leguminous plants. High proline content was recorded in plants grown at MR and S compared to those grown at the NR and O. It is clear that *M. indicus* and *T. hamosa* contain high proline content compared with *M. polymorpha*, *T. resupinatum* and *V. sativa*. On the bases of pooled data, proline content in *M. indicus, T. hamosa* and *V. sativa* was significantly higher than that in *M. polymorpha* and *T. resupinatum*. Also, the proline content in plants at MR and S was significantly higher than that in plants at NR and O. Correlation analyses of proline content in shoots of leguminous plants with soil variables showed undefined trend. Proline content negatively correlated with soil K^+ (r-values between -0.796 and -0.870), while it positively and weakly correlated with Na^+. A significant positive correlation resulted between proline content in *M. indica* and concentration of NO_3^- in the soil, but a significant r-value resulted in case of *M. polymorpha*. Against what was expected, a very weak correlation has been found between proline content in all studied plants and TSS in the soil (Table 2). Also, differences between species have the major magnitude of effect on proline content of shoots (η^2 = 0.77) rather than changing habitat conditions (Table 3).

Table 2. r-values of linear correlation analyses between some parameters estimated in different leguminous plants and soil variables (WC = soil water content; TSS = total soluble salts).

Para-meter	Plant species	Soil variables											
		Cl^-	SO_4^{--}	PO_4^{--}	HCO_3^-	NO_3^-	WC	TSS	pH	Na^+	K^+	Ca^{++}	Mg^{++}
Nodule fresh biomass	*M. indicus*	-0.060	-0.511	0.253	0.544	0.313	0.249	-0.310	-0.900*	-0.268	-0.624	-0.166	-0.573
	M. polymorpha	0.984**	-0.596	0.964*	-0.579	-0.999**	0.975*	-0.115	-0.767	-0.430	-0.680	-0.365	-0.255
	T. resupinatum	0.792	-0.289	0.975*	0.155	-0.847	0.720	-0.555	-0.262	-0.047	-0.645	0.160	-0.578
	T. hamosa	0.853	-0.175	0.968*	-0.454	-0.622	0.774	-0.547	-0.148	0.008	-0.588	-0.668	0.005
	V. sativa	0.997**	-0.465	0.993**	0.553	-0.798	0.840	-0.397	-0.690	-0.105	-0.613	-0.252	-0.172
Nodule dry biomass	*M. indicus*	-0.312	-0.303	-0.062	0.677	0.544	-0.056	-0.332	0.670	-0.463	-0.032	0.335	-0.247
	M. polymorpha	-0.896	0.957*	-0.618	0.950*	0.781	-0.915*	-0.847	0.091	-0.358	0.038	0.545	-0.503
	T. resupinatum	-0.374	0.482	-0.598	0.667	0.851	-0.986**	-0.641	0.198	-0.479	-0.109	0.360	-0.368
	T. hamosa	0.187	0.020	0.447	0.643	-0.350	0.108	-0.223	0.956*	-0.624	-0.029	-0.274	-0.003
	V. sativa	-0.347	-0.210	-0.180	0.501	-0.084	-0.266	-0.728	0.292	-0.551	0.016	0.677	-0.588
Leghemoglobin	*M. indicus*	0.964*	0.122	0.808	-0.172	-0.348	0.361	-0.953*	-0.369	-0.937*	0.074	-0.631	-1.000**
	M. polymorpha	0.996**	-0.785	0.862	-0.772	-0.955*	0.999**	-0.178	-0.940*	-0.984**	0.041	-0.588	-0.928*
	T. resupinatum	-0.049	-0.925*	0.644	-0.333	-0.884	0.817	-0.692	-0.769	-0.840	-0.008	-0.357	-0.985**
	T. hamosa	-0.372	-0.982**	0.393	-0.089	-0.869	0.690	-0.998**	0.121	-0.798	0.123	-0.190	-0.699
	V. sativa	0.572	-0.986**	0.660	0.272	-0.954*	0.855	-0.786	-0.861	-0.865	0.118	-0.399	-0.885
Nitroginase activity	*M. indicus*	0.840	-0.495	0.990**	-0.174	-0.495	0.798	-0.099	-0.950*	0.025	-0.174	-0.495	-0.272
	M. polymorpha	0.874	-0.303	0.998**	-0.283	-0.957*	0.851	0.588	-0.594	-0.129	-0.258	-0.721	0.063
	T. resupinatum	0.985**	0.249	0.672	-0.039	-0.525	0.574	0.132	-0.456	0.141	-0.141	-0.327	-0.173
	T. hamosa	0.977*	0.143	0.767	-0.626	-0.267	0.554	-0.282	-0.783	0.287	-0.151	-0.067	-0.183
	V. sativa	0.780	-0.259	0.669	-0.133	-0.468	0.736	0.232	-0.706	0.172	-0.196	-0.736	0.172
Proline	*M. indicus*	-0.193	0.706	-0.409	0.911*	0.995**	-0.863	0.267	-0.849	0.306	-0.825	0.236	-0.025
	M. polymorpha	0.981**	-0.848	0.802	-0.836	-0.917*	0.989**	-0.036	-0.300	0.139	-0.870	-0.036	0.315
	T. resupinatum	0.296	-0.347	0.704	0.811	-0.409	0.045	-0.222	0.210	0.513	-0.796	0.452	-0.043
	T. hamosa	0.600	0.933*	-0.044	0.195	0.612	-0.482	0.007	-0.250	0.548	-0.815	-0.702	0.489
	V. sativa	0.834	0.100	0.771	0.507	-0.332	0.446	0.037	-0.255	0.457	-0.841	-0.025	0.386

**:Correlation is significant at $P< 0.01$.

*:Correlation is significant at $P< 0.05$.

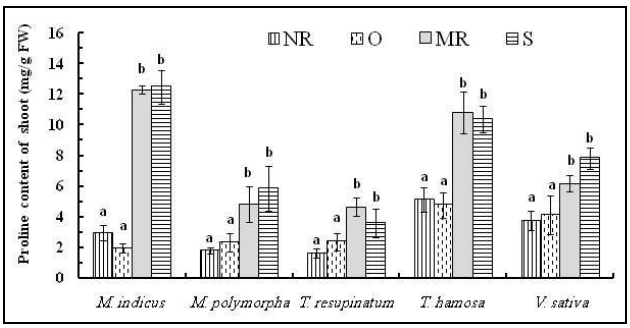

Figure 5. Proline content (mg/g FW) in shoots of the five studied legumes. Statistics as in Fig. 1.

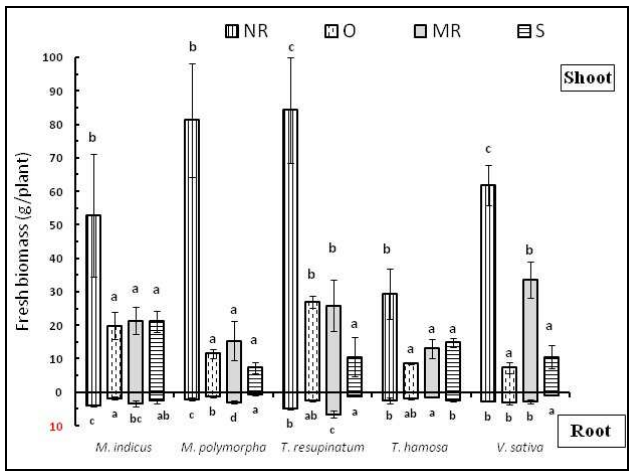

Figure 6. Shoot and root fresh biomass (g/plant) of the five studied legumes. Statistics as in Fig. 1.

Table 3. F-values of factorial ANOVA for the effect of species, regions and their interaction; and eta-square (η^2) calculated for each factor.

	Species		Region		Species*Region	
Parameter	**F-value**	η^2	**F-value**	η^2	**F-value**	η^2
Nodule number/ plant	3848.442	0.904	456.853	0.064	52.825	0.030
Nodule fresh biomass	385.634	0.835	63.956	0.083	12.414	0.065
Nodule dry biomass	421.846	0.776	106.467	0.117	20.856	0.092
Leghemoglobin	700.039	0.878	126.794	0.095	5.310	0.016
Nitroginase activity	178.100	0.487	182.088	0.299	29.166	0.192
Proline	649.648	0.770	218.976	0.156	22.627	0.064
Shoot fresh biomass	212.259	0.634	137.211	0.246	13.410	0.096
Shoot dry biomass	231.130	0.666	135.545	0.235	10.976	0.076
Root fresh biomass	196.687	0.805	28.338	0.070	9.493	0.093
Root dry biomass	136.973	0.770	29.391	0.099	6.414	0.086

All F-values are significant at $p < 0.001$.

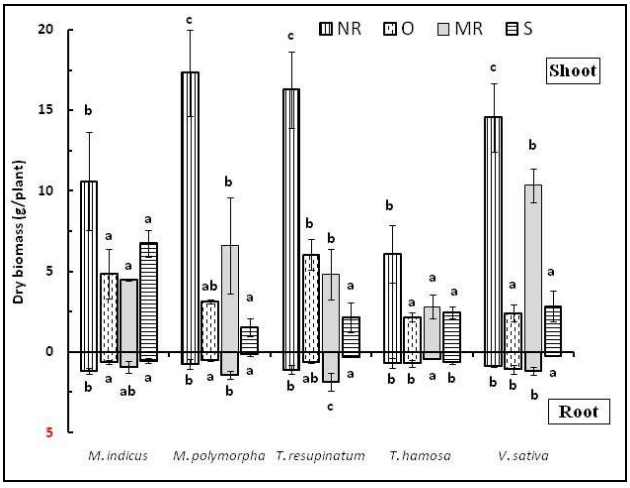

Figure 7. Shoot and root dry biomass (g/plant) of the five studied legumes. Statistics as in Fig. 1.

3.5. Plant biomass

The fresh and dry biomass of collected legumes was affected by phytogeographical regions. The highest fresh and dry biomass of the shoot and root system was recorded in the wild plants collected from NR followed by O, MR and S. Amongst species, *T. resupinatum* grown at NR attained the highest fresh biomass while *M. polymorpha* attained the highest dry biomass (Fig. 6, 7). Calculating the ratio of $root_{dry\ biomass}$: $shoot_{dry\ biomass}$ indicated that *M. indicus* (0.202), *M. polymorpha* (0.214) and *T. resupinatum* (0.384) inhabiting MR and *T. hamosa* (0.323) and *V. sativa* (0.446) inhabiting O have the highest ratios. The lowest root/shoot ratios were recorded for all studied species inhabiting NR (0.042 – 0.113). Averaging across all habitats, *T. hamosa* has the highest ratio (0.211), while *M. indicus* (0.129) and *M. polymorpha* (0.130) have the lowest ratios. As indicated by η^2 in Table 3, the plant, especially root, biomass is greatly related to the variations between species rather than regions or species*regions.

3.6. Rhizobial isolates

The rhizobial isolates recovered from different wild legumes collected from different habitats were identified by 16S rRNA gene sequence. The root-nodule bacteria that was isolated from *M. indicus, M. polymorpha, T. resupinatum, T. hamosa, V. sativa* and had been classified into five species namely: *Sinorhizobium fredii, Rhizobium mesosinicum, Rhizobium daejeonense, Rhizobium huautlense, Rhizobium alamii*, respectively.

4. DISCUSION

The present study clearly indicates that there is a critical role of the habitat conditions on nodulation and nitrogen fixation of wild legumes as indicated by the significant differences in number and biomass of the nodules between individuals of each studied species at different regions. Generally, soils of all habitats at the four phytogeographical regions where the wild legumes studied were not saline and the TSS were less than 0.2% (concentration of Na^+ was less than 0.02%). Soil at Assiut (NR) and for some extent at Kharga (O), with high water content, organic matter, and nutrients such as K^+, Ca^{+2} and PO_4^{-3} represents the best habitat for nodulation, nitrogen fixation and growth of plants. This could be attributes to the clay or clay loamy soil at NR and Oases, respectively. The desert soils of Egypt are inherently low in organic matter due to the arid or hyper-arid climate and historically low vegetation cover, and at all regions the OM were ranging from 0.5-1.6%. The fine textured soils at NR and O were characterized by relatively high level of organic matter, and hence increasing water retention capacity, K^+, Ca^{+2} and Mg^{2+}. Such conditions are essential for survival of rhizobia in the soil and support the process of root hair infection and nodule development. This study supports what have been found by Rao and Venkateswarlu [48] that not the high Indian desert soil temperature but the low organic matter and poor soil moisture were the major factors that reduced the numbers of different micro-organisms.

The poor nodulation and nitrogen fixation of wild legumes grown in sandy soils of MR and S could be attributed to the coarse soil, decreasing content of organic matter and water shortage. However, the nodule fresh biomass positively correlated, while its dry biomass negatively correlated with the soil water content; but both of the fresh and dry biomass negatively correlated with TSS. Another an important factor affecting nitrogen fixation is the temperature. The studied five legumes are annual herbs and their height ranging from 10 to 60 cm [49]; so they complete their life cycle nearly through the winter (the samples collected on April). The average maximum temperature of five months prior to sampling (from December to April) at Assiut, Kharga, Burg Al-Arab and Saint Katherine was 23 ± 2, 23 ± 2, 19 ± 1 and 15 ± 3 °C; while the average minimum temperature was 8 ± 2, 10 ± 2, 10 ± 1 and 4 ± 2 °C, respectively. As Abdel Gadir and Alexander [50] reported in Egyptian sandy soils, the temperature near the soil surface was 59°C when the air temperature was 39°C. However, the soil temperature decreased rapidly with depth, being moderate 35 °C, at 15 cm. Every bacterium has its own optimum conditions, under which it grows at its best. For most rhizobia, the optimum temperature range for growth is 28-31 °C, and many are unable to grow at 37°C [12]. Also, temperature plays an essential role on the exchange of molecular signals between rhizobia and their partners, thus reducing nodulation [51]. However, low temperature may be critical factor reducing nodulation and nitrogen fixation activity in all species at Saint Katherine.

Water, and its availability, is one of the most critical environmental factors that affect the growth and survival of micro-organisms. Drought is one of the most common stresses soil microorganisms have to face. The responses of bacterial cells to drought can be: shrinkage of the bacterial cytoplasm and capsular layers, increase in intracellular salt levels, crowding of macromolecules, damage to external layers (pili, membranes), changes in ribosome structure, and decrease in growth [52]. A shortage of water supply can slow the growth of the nodule and accelerate its senescence. So these results compatible with Ralston and Imsande [53] who reported that nodules in dry soils lose water faster than the vascular system can supply it and hence suffer water stress. Shortage in water supply to the nodules may result in collapse of cells near the surface creating impaired diffusion which reduce the adverse effects of drought. This also reflects why the water content of nodules is too low in plants inhabiting the MR sandy formations and South Sinai. Previous study indicated that harmful effects of water deficit can be alleviated by increasing K^{2+} supplementation [54]. Water stress is quickly reflected as changes in hormonal content [55]. The nodules are an active site of synthesis of auxins and cytokinins. Therefore, it is likely that nodules, besides the supply of organic N, are a source of cytokinins that makes the plant more tolerant to water stress [56]. The results indicated that there is a significant variation in rooting development

between the studied species as reflected on the root: shoot ratios. Plants inhabiting the NR, with the highest soil water content, have the lowest root: shoot ratios and vice versa for those inhabiting Sinai where the soil is relatively dry. However, in dry soil the plants tend to increase the extinction of lateral and sinker roots for mineral uptake and water absorption; and translocation of photosynthats for this vital purpose will affect negatively on that transported to nodules.

Nitrogenase activity is decreased significantly, accompanied by the decrease in respiratory activity of the root nodules [57, 58]. A limitation in metabolic capacity of bacteroids and oxidative damage of cellular components are contributing factors to the inhibition of nitrogenase activity in alfalfa nodules [59]. In addition, the transport of fixed nitrogen out of the nodule is decreased possibly due to an insufficient supply of photosynthates from stems and leaves under stress [60].

Leghaemoglobin content of nodule cytosol was also severely inhibited by drought stress so the leghaemoglobin content in this study was high in NR followed by O and low in MR and S. This decline was attributed to the induction of protease activity [54]. Legumes tend to maintain a level of O_2 within their nodules that can support respiration but is sufficiently low to avoid inactivation of nitroginase [61]. Despite leghaemoglobin act as a buffer for nodule O_2, Denison and Harter [62] adduced that it stores only enough O_2 to support nodule respiration for a few seconds. Gas permeability in nodules decreases under drought or upon exposure to nitrate, and as the permeability decreases may be there is no need to further leghaemoglobin. This may explain why the content of leghaemoglobin increased significantly in nodules of all studied species inhabiting NR, while it negatively correlated (significant –ve r-values in *M. polymorpha* and *V. sativa*) with the concentrations of NO_3^- in the soil.

Proline considered as an indicator for response to environmental stresses and it accumulates in relatively large quantities under stress conditions [63, 64]. Proline content was relatively high in shoots of wild legumes grown at MR and S, and this may be attributed to the low soil water content found at these habitats. Also, this proved that the plants at MR and S, at period of sampling, were more exposed to environmental stresses. The strong –ve correlation between contents of proline in shoots of all studied legumes and K^+ concentrations in the soil, have lead to a suggestion that availability of K^+ may suppress proline synthesis.

Rhizobial isolates recovered from wild legume plants grown in S and MR such as *Medicago polymorpha* (*Rhizobium mesosinicum* ASU8) and *Trigonella hamosa* (*Rhizobium huautlense* ASU3), are of good traits, such as tolerant to high salt, drought and temperature level. Isolation of root-nodules bacteria of wild legumes growing in arid region is very attractive and promising. These indigenous rhizobia are characterized by wide host ranges that offer these legumes ecological benefit. Therefore, successful isolation of rhizobia from such environment will definitely result in obtaining good rhizobia candidates for establishing successful symbioses in extreme environments useful for production of crop legumes. It is well documented that native rhizobia can form nodules with other wild or cultivated crop legumes, and can be utilized for genetic manipulation to improve and perform of symbiotic characters of other root nodule bacteria with crop legumes [19, 65]

5. CONCLUSION

The present investigation revealed that the natural nodulation and nitrogen fixation of wild legumes are drastically affected by habitat. In contrast to Mediterranean region and Sinai, wild legumes inhabiting Nile region and Oases were characterized by high nodulation and nitrogen fixation. *Rhizobium mesosinicum* ASU8 and *Rhizobium huautlense* ASU3 isolated from root nodules of *Medicago polymorpha* and *Trigonella hamosa* grown at Sinai and Mediterranean region are expected to be more tolerant to harsh environmental conditions than rhizobia from cultivated legumes. These rhizobia could be valuable in agricultural practice, specifically in the inoculation of crop legumes grown under unfavorable conditions or in the new reclaimed soil.

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AUTHORS' CONTRIBUTION

MHA-A: Conception, design of the work and Critical revision of the article; AEE: Data collection and revised the manuscript; TRM: Data analysis and interpretation the experimental; ME: Drafting the article; IMN: carried out the practical experiments. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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