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Preliminary characterization of root-nodule bacteria isolated from forage legumes of the genus *Hedysarum* in North of Morocco

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Abstract

Nowadays there is a renewed interest in forage legumes, because of their important role in sustainable feeding systems. Among several species endemic to Mediterranean climate, *Hedysarum* spp., more abundant and diversified, is known to have good forage value and contribute to the enrichment of soil available nitrogen through their root nodules bacteria. In order to assess the diversity among forage species belonging to the genus *Hedysarum*, a prospective study was perform in different regions of Morocco from humid to arid environments, allowed the determination the area of distribution of four *Hedysarum* species: *Hedysarum flexuosum* L., *H. coronarium* L., *H. spinosissimum* L. subsp. *capitatum* and *H. pallidum* Desf. This is the first time a study is made in Morocco to highlight the symbiotic association for the last species. Plants were first evaluated for dry matter and nitrogen content. Then, the diversity of 51 isolates nodulating *Hedysarum* spp. was assessed for their morphological characteristics, and for ability to grow on different culture media. The phenotypic characterization of the root-nodules bacteria associated with those plants revealed a great similarity to strains belonging to *Rhizobium* genera and could be are very good candidates for plant inoculation in a specific region.

Keywords: *Hedysarum* spp., *Rhizobium*, diversity.

Introduction

In Mediterranean pastures, species of the genus *Hedysarum* (*Fabaceae*) represent a valuable agro-economic resource due to their forage quality (Abdelguerfi & Ramdane, 2003; Kadi *et al*., 2011; Errassi *et al*., 2018) and ability to improve soil fertility (Sulas *et al*., 2009; Fitouri *et al*., 2012). The genus (*Hedysarum*) display a high level of species diversity (Duan *et al*., 2015; Liu *et al*., 2017), distinguishable by their biological cycle, morphology, nutritive value, and adapted to large bioclimatic areas from humid to arid conditions (Boussaïd *et al*., 1995; Kadi *et al*., 2011; Errassi *et al*., 2018). Furthermore, those species (*Hedysarum* spp.) were demonstrated to undergone a separate path in terms of interactions with root-nodule Rhizobia (Benhizia *et al*., 2004; Kishinevsky *et al*., 2003). Although only a few of them have been purified and authenticated. In the other hand, the introduction of some species of *Hedysarum* as a forage crop was reported to be hampered by problems of nodulation observed in field trials (Baamrani, 1984; Thami Alami, 1986). The results obtained by Baamrani (1984) and Thami Alami (1986) have shown the need to inoculate Sulla *Hedysarum coronarium* L. in different regions of Morocco where the problem of erosion and feeding is acute. In this framework, the present study was performed to investigate morphological and cultural characteristics as preliminary steps of root-nodule bacteria associated with 4 legumes species of genus *Hedysarum*; *H. flexuosum* L, *H. coronarium* L., *H. spinosissimum* L. subsp. *capitatum* and *H. pallidum* Desf. Good-performing strains will be selected for plant inoculation and prairies restoration.

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Materials and methods

Plants and nodules sampling

The collection of nodulated plants was conducted across northern of Morocco from different climatic and soil regions. Symbiotic concert was estimated at the flowering stage by scoring dry matter weight, nodules abundance and nitrogen content using the Kjeldahl method. For evolution of Rhizobia associated with identified *Hedysarum* species, pink to red nodules were randomly sampled from each field and stored dried in tubes containing Silica gel as described by (Vincent, 1970).

Rhizobia isolation

Rhizobia were isolated from the nodules of *Hedysarum* spp. according to standard procedures as previously described (Vincent, 1970), and incubated in sealed Yeast Extract Mannitol (YEM) agar plates at 28°C.The purity of the isolates was checked by repeated streaking of single colonies on YEM plates. Finally, pure cultures were stored at −20°C in 25% glycerol-YEM broth until analysis.

Rhizobia morphological and cultural characterization

Individual colonies were characterized for morphological characteristics such as, size, color, shape, mucosity, transparency, borders and elevation, by incubation in YEM agar plates at 28°C.Furthermore, isolates were characterized for cellular characteristics using gram staining technique as described by (Somasegaran & Hoben, 1994). Moreover, the isolated Rhizobia evaluated were for some cultural futures to ascertain phenotypic descriptions recommended by (Vincent, 1970; Somasegaran & Hoben, 1994).Thus, all isolates were first tested for their ability to absorb Congo red (CR) dye by incubation in YEM agar enriched with 25 µg.ml-1 of CR, and for their ability to grow on Glucose Peptone Agar (GPA) medium as minimum medium. Moreover, the ability of the rhizobial isolates to produce acid or alkaline were verified in YEM agar plates (pH 6.8) supplemented with (0.025% w/v) bromothymol blue (BTB). Acid producers were classified as fast-growing bacteria, while, alkaline producers were classified as slow-growing bacteria (Jordan, 1984). Finally, the Rhizobia isolates were differentiate for *Rhizobium* and *Agrobacterium* genus using Keto-lactose test according to Bernaerts & Delay (1963). All tests were incubated in dark in 28°C and cheek daily for colony development.

Results and discussion

In order to safeguard and to enhance endemic and indigenous leguminous forage, several surveys were carried out from natural pastures to identify and to collect the species of the genus *Hedysarum*. During these surveys, 4*Hedysarum*species (Figure 1) were located in the North of Morocco. Thus, *Hedysarum flexuosum* L. and *H. coronarium* L. were constantly located in well-drained pastures and alluvial rivers of clay soils near the town of Tangier (Boukhalef) and Ouazan (Khandak Lihoudi) in North-West region of Morocco, when *H. pallidum* Desf and *H.*

Figure 1. *Hedysarum* species identified in the North of Morocco. A: *Hedysarum flexuosum* L.; B: *H. coronarium* L.; C: *H. spinosissimum* L. subsp. *capitatum*; D: *H. pallidum* Desf.

spinosissimum L. subsp. *capitatum* were rarely encountered in the vicinity of Taourirt and Touissit regions, particularly in natural pastures and rocky silty soils of the low mountains in North-East of Morocco. Those results in compliance with observations made by Jahandiez & Maire (1932), Villax (1963) and Abdelguerfi-Berrekia *et al*. (1991), and suggest adaptation to some ecological factors. Moreover, the on-field survey of examined plants showed great efficiency in term dry matter and nitrogen content (Table 1), comparable to those of others species within the genus *Hedysarum* (Fitouri *et al*., 2012) and indicating the presence of highly effective Rhizobial isolates.

Thus, 51 strains from root-nodules of the selected *Hedysarum* species could be isolated and screened for their morphological and cultural characteristics. Morphologically, all strains formed almost white, circular and convex colonies, having 1-5 mm in diameter when grown on YEM plates at 28°C on YEM agar and differed greatly in the amounts of extracellular gum. Furthermore, cellular characteristics show that most of strains are Gram negative bacteria except a few them isolated from *H. coronarium* L. and *H. spinosissimum* were identified as Gram positive (Table 2), suggesting that nodules may colonized internally by endophytic bacteria (non-nodulating). On the other hand, cultural characteristics (Table 3) show that most of rhizobial strains don't absorbed the Congo red dye except those isolated from *Hedysarum coronarium* L. In fact, Rhizobia produce in general white to watery colonies and absorb weakly Congo red dye (Howieson & Dilworth, 2016). Although the later is pH of their medium when incubated in glucose peptone agar (GPA) medium. Although, they are acid producers, turning the medium yellow when incubated in YEM agar medium enriched with bromothymol blue (BTB) as pH indicator. Therefore, theyclassified as fast growers Rhizobium (Jordan, 1984). In addition, the absence of 3-kitolactose, indicate that those isolates belonging to *Rhizobium* genus since colonies were not producing any

Table 1. Infectivityand efficiency of *Hedysarum* spp. evaluated at different collection sites.

Sites	***Hedysarum* spp.**	**Infectivity[1]**	**Total dry matter(%)**	**Nitrogen content (%)**
Boukhalef	*H. flexuosum*	abundant	20.11^{c}±0.85	2.49^{b}±0,04
Kariat Benaouda	*H. coronarium*	extremely abundant	18.22^{d}±0.37	2.12^{c}±0,05
Taourirt	*H. spinosissimum* subsp. *capitatum*		25.28^{b}±0.61	3.64^{a}±0,06
Touissit	*H. pallidum*		41.74^{a}±0.33	2.00^{c}±0,08
S.E.M[2]			3.53	0.201
CV[3]			37.78	25.46
Sig.[4]			***	***

(1)Infectivity of strains was scored using the chart proposed by (Howieson & Dilworth, 2016). (2)Standard error of the means. (3)Coefficient of variation. (4)Sig.: *** Level of significance: $p < 0.05$. Values with different letters in the same column are significantly different ($P<0.05$) according tukey'sHSD test (Spjotvoll and Stoline, 1973).

Table 2. Morphological characteristics of root-nodule isolates from 4 *Hedysarum* species.

Host plant	**Colonial characteristics**						**Cellular characteristics**	
	Number of isolats	**Form**	**Marge**	**Size (mm)**	**Color**	**Mocusity**	**Gram staining**	**Form**
***H. flexuosum* L.**	15	Dome	Entire marging	1-2	White	++	-	Rod
***H. coronarium* L.**	15			3-5		+++	-/+	Rod
H. spinosissimum* subsp. *capitatum	19			1-2		++	-/+	Rod/ Coccus
***H. pallidum* Desf**	16			1-2		+	-	Rod

yellow hallo when incubated in lactose-based medium and flooded with Benedict's reagent (Bernaerts & Delay, 1963)**.**Those phenotypic characteristics developed by our strains, were consistent with the phenotypic appearance previously described by (Kishinevsky *et al*., 2003; Benhizia, 2006; Torche *et al*., 2010) for similar species and exhibiting the basic characteristics of Rhizobia already describe by (Vincent, 1970; Somasegaran & Hoben, 1994;Howieson & Dilworth, 2016).

Conclusion

From an applied perspective, the results of the present survey show the potential use the forage legumes (*Hedysarum* spp.) selected in this study for animal nutrition regarding their nitrogen richness. Their performances could be enhancing by specific inoculation with appropriate microsymbiot. Their performances could be enhancing by specific inoculation with appropriate microsymbiot. The last shows high similarity with *Rhizobium* genera, known for their ability to improve plant biomass and nitrogen production, although, further phenotypic and genotypic characterization should be done to ascertain those isolates to a specific group or genera of Rhizobium bacteria.

Table 3. Cultural characteristics of root-nodule isolates from 4 *Hedysarum* species.

Host plant	YEM-CR[1]	GPA-BCP[2]	YEM-BTB[3]	Keto-lactose
H. flexuosum	-	-	acidic reaction	-
H. coronarium	+++	-		-
H. spinosissimum* subsp. *capitatum	++	-		-
H. pallidum	+++	-		-

[(1)]determined in YEM agar plats supplemented with Congo red (CR). [(2)]Determined in glucose peptone agar (GPA) plats supplemented with bromocresol purple (BCP). [(3)]Determined in YEM agar plats supplemented with bromothymol blue (BTB). (-), no growth; (+), poor growth; (++), moderate growth; (+++), high growth.

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