

# Symbiotic properties and internal antibiotic resistance within new isolates of *Rhizobium leguminosarum* biovar *trifolii*

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**Abstract** - Eight *Rhizobium leguminosarum* bv. *trifolii* strains were isolated from four *Trifolium alexandrinum* L. accessions growing in the same soil of the experimental station of Agricultural High School of Mograne. They were tested for their symbiotic properties and for their internal antibiotics resistance (IAR). The results obtained showed several genetic variability concerning strains resistance to antibiotics. *In vitro* nodulation analysis of the interaction between *Rhizobium*-Berseem showed noticeable variation between strains and efficiency variability of strains of *R. leguminosarum* bv. *trifolii* on the symbiosis yields. Those results showed the differences between the several strains isolated from the same site and same accession of berseem. Aseptic culture of *T. alexandrinum* permitted the selection of two perfuming couples (AK167-Mog5) and (AK101-Mog6).

**Keywords:** *Trifolium alexandrinum* L., Berseem, internal antibiotics resistance (IAR)

## 1. Introduction

*Trifolium alexandrinum* L. (*Fabaceae*) or berseem is one of the most important crops in the Mediterranean agricultural system (Morris and Greene 2001). The geographical area of the berseem is the Mediterranean Basin, the ranging from the Middle East to Iraq and Central Asia (Knight 1985; Chandra and Roy 1995; Sardana and Narwal 2000; Malaviya *et al.* 2005; Badr *et al.* 2008). In Tunisia, berseem covers about 7,000 ha (DGPA, 2014). It is used as autumnal fodder, having high agronomical and economical value in irrigated fields. These several-cut species can be harvested 4 to 8 times per season (September-June) and can reach a yield of 85t/ha and have high nutritional values (20% of proteins and 80% of digestible dry matter) with high palatability (Kaushal *et al.* 2005). It can fix a nitrogen rate of 350 kg.ha<sup>-1</sup>.year<sup>-1</sup> due to the symbiotic bacteria from the genus *Rhizobium leguminosarum* bv. *trifolii* (Williams and Graves 1991). Improving the symbiotic performance can be obtained with the selection of the host plant or the efficient and competitive *Rhizobium* strain (Nutman and Riley 1981; Beck and Duc 1991; Yates *et al.* 2005; Dhane-Fitouri *et al.* 2012).

In this paper, we present the genetic diversity of the indigenous *R. leguminosarum* bv. *trifolii* strains isolated from the same site, and the selection of the combination cultivar-strain with the evaluation of *in vitro* inoculation through the nodulation and efficiency into the four berseem accessions inoculated with *R. trifolii* with several IAR.

## 2. Materials and methods

### 2.1 Strains origin

Eight strains of *Rhizobium leguminosarum* bv. *trifolii* were isolated from root nodules of four berseem accessions (*Trifolium alexandrinum* L.) growing in the experimental station of the Agricultural High School of Mograne (36.428017° N latitude and 10.092097° E longitude, 156m above sea level), according to standard procedures (Mhamdi *et al.* 1999). Strains (Mog1, Mog2), (Mog3, Mog4), (Mog5, Mog6), (Mog7, Mog8) were isolated respectively from accession AK77, AK101, AK142 and AK167 and kept at -80°C in 20% glycerol.

### 2.2 Plants material

Four accessions of *T. alexandrinum* L. belonging to berseem seeds of the Tunisian National Gene Bank (TNGB) have been cultivated during 2010/11. The origin, the lot number, the name of the genotype and the code are summarized in table 1.



Species	Code	Entries	Lot number	Origin
<i>T. alexandrinum</i> L.	AK77	-1645-	83-106-77	Portugal
<i>T. alexandrinum</i> L.	AK101	-1662-	83-106-101	Portugal
<i>T. alexandrinum</i> L.	AK142	-1625-	84-106-142	Turquie
<i>T. alexandrinum</i> L.	AK167	-85-	83-106-167	Palestine

### 2.3 Growth medium and antibiotics

The yeast extract mannitol (YEM) was used as a medium for the growth of *R. leguminosarum* bv. *trifolii* strains. Eight antibiotics were used in ring form (Biomérieux): Amoxicilline (Amx), 10; Chloramphenicol (Cam), 30; Erythromicine (Ery), 15; Gentamicyne (Gen), 10; Kanamycine (Kan), 30; Nalidixique Acid (Nal), 30; Streptomycine (Str), 10; Tetracycline (Tet), 30 µg per ring.

### 2.4 Nodulation and effectiveness tests

Isolates were tested for their ability to induce root nodules on four accessions of *T. alexandrinum*. Plants were grown *in vitro* in test tubes containing The Nicol et Thornton medium (Nicol and Thornton 1941). One presterilized and germinated seed was grown in each tube. Test tubes were covered with cotton to ensure good ventilation. After 10 days, plants were inoculated with 1ml of rhizobial broth culture containing 10<sup>6</sup> cell.ml<sup>-1</sup>. Each strain was tested in three replicates. Plants were kept in a growth chamber with 22 °C and photoperiod of 16 hours. Non-inoculated and nitrogen-fertilized (0.5 mg NH<sub>4</sub>NO<sub>3</sub>.l<sup>-1</sup>) controls were also included. Six weeks after inoculation, plants were harvested and scored for nodulation. Total dry weight and nitrogen were used as indicators of strains effectiveness. Nodulation was evaluated by counting root nodules. Data were analyzed by Plabstat software (Utz 1991), and followed by mean comparison by the LSD test.

## 3. Results and discussion

### 3.1 Internal antibiotic resistance

Six several resistance profiles were found within the eight studied strains (Table 2). All the isolates are sensible to Erythromycine, Kanamycine, Streptomycine and Tetracycline, antibiotic inhibiting the protein synthesis. Mog 7 is sensitive to all the antibiotics. The antibiotic resistance spectrum showed for those strains is similar to results found by Gupta *et al.* (1989). Within the studied strains Mog1 and Mog4 (Nal<sup>r</sup>), Mog3 and Mog6 (Gen<sup>r</sup>, Nal<sup>r</sup>) have the same profile. Despite the fact that the number of the analyzed strains is reduced and coming from the same locality, 50% of those strains showed several IAR profiles. This indicates that studied strains form a heterogeneous group with a reduced parental relationship.

Strains	Antibiotics							
	Amx	Cam	Ery	Gen	Kan	Nal	Str	Tet
Mog1	S	S	S	S	S	R	S	S
Mog2	R	S	S	R	S	S	S	S
Mog3	S	S	S	R	S	R	S	S
Mog4	S	S	S	S	S	R	S	S
Mog5	S	S	S	R	S	S	S	S
Mog6	S	S	S	R	S	R	S	S
Mog7	S	S	S	S	S	S	S	S
Mog8	R	R	S	R	S	R	S	S

R: Resistant; S: Sensitive.

### 3.2 Nodulation

The effect of inoculation on four clover accessions after six week growing *in vitro* is illustrated in Table 3. Statistical analysis of the nodule number per plant shows: *Rhizobium* strains, accessions and interaction accession x strain is high significant at P<0.01. All the strains are Nod<sup>+</sup> within the four accessions. However, the nodules numbers vary from 1 to 9 per plant and are significantly different within the several treatments. Mog2 and Mog5 are the highly infective for the cultivar AK77. A high number of nodules doesn't necessary increase the nitrogen fixation. Mog 4 (1 nodule.plant<sup>-1</sup>) [(8.73 mgN.plant<sup>-1</sup>)] fix three time the nitrogen than the strain Mog 7 associated to AK101 (6 nodules.plant<sup>-1</sup>) [2.75 mg N.plant<sup>-1</sup>].

**Table 3:** Evaluation of the nodulation of *Rhizobium leguminosarum* by *trifolii* strains measured six weeks after plants infection within four accessions of *Trifolium alexandrinum* L.

Strains	Accessions				Mean
	AK77	AK142	AK167	AK101	
Mog1	2	3	1	3	2.25a
Mog2	9	6	1	7	5.75d
Mog3	3	6	1	1	2.75ab
Mog4	3	4	5	1	3.25 b
Mog5	9	1	4	5	4.75 c
Mog6	7	2	2	3	3.5 b
Mog7	7	6	5	6	6.0 d
Mog8	4	5	3	2	3.5 b
Mean	5.5 d	4.13 c	2.75 a	3.5 b	3.97
SEM=0.9375 (P<0.01)					

Means followed by the same letter do not differ statistically at P<0.01

### 3.3 Efficiency of *Rhizobium leguminosarum* by *trifolii* strains

Almost all the strains showed a significant increase (P<0.01) of the dry matter (DM) compared to the control plant not inoculated (Table 4). Mog5 and Mog8 strains gave after the highest mean dry matter weight 40 days after germination for the four berseem accessions with 23 mg.plant<sup>-1</sup> and 21.29 mg.plant<sup>-1</sup> respectively. Mog5 produces twice than Mog1 and the control with nitrogen. Also Mog5 produces twenty times of dry matter than Mog 2 which has little lower dry matter than the control without nitrogen and seems to be more efficient. AK167 and AK101 have the highest dry matter for all the treatments with respectively 28.31 and 22.33 mg DM.plant<sup>-1</sup>.

**Table 4:** Average dry matter yield (mg/plant)

Strains	Accessions				Means
	AK77	AK142	AK167	AK101	
Mog1	21.00	7.00	19.33	20.00	16.83 cd
Mog2	10.00	2.00	18.00	31.00	15.25 bc
Mog3	12.00	7.00	27.00	31.07	19.27 def
Mog4	8.00	8.00	19.00	40.00	18.75 def
Mog5	16.00	9.00	22.00	45.00	23.00 g
Mog6	8.40	9.00	20.00	31.00	17.10 cde
Mog7	24.00	9.00	25.00	21.00	19.75 ef
Mog8	20.00	8.17	36.00	21.00	21.29 fg
Control without nitrogen	8.20	2.00	17.00	21.00	12.05 a
Control with Nitrogen	9.00	2.10	20.00	22.00	13.28 ab
Means	13.66 b	6.33a	22.33 c	28.31 d	17.66
SEM=10.893 (P<0.01)					
Means followed by the same letter do not differ statistically at P<0.01					

### 3.4 Total Nitrogenous Matter

Mog 5 produces the highest quantity of Total Nitrogenous Matter (TNM) for all the population with the value of 5.68 mg significantly higher (P<0.01) than the control with nitrogen (4.62mg) (Table 5). Mog 5 and Mog6 show a TNM significantly higher than the control with nitrogen within the accessions AK101 and AK167. Those entries have the highest TNM compared to all the populations. Into the 32 analyzed combinations, the best ones were AK167 x Mog5 and AK101 x Mog6 which have practically 22 times of nitrogen than the low combination of AK142 x Mog2.

### 3.5 IAR- efficiency

Isolates having the same profiles like Mog1 and Mog4 (Nal<sup>r</sup>), Mog3 and Mog6 (Nal<sup>r</sup>,Gen<sup>r</sup>) don't have the same efficiency with the same cultivars, which confirms the heterogeneity within the *R. leguminosarum* by *trifolii* strains in a small site.

**Table 5:** Nitrogen amount for the 32 combinations cultivars x *Rhizobium leguminosarum* bv *trifolii* strains

Strains	Accessions				Mean
	AK77	AK142	AK167	AK101	
Mog1	3.68	1.53	1.69	3.50	2.60 a
Mog2	2.62	0.44	1.57	6.77	2.85 ab
Mog3	3.15	0.61	5.89	8.15	4.45 d
Mog4	2.45	2.45	6.65	8.73	5.07 e
Mog5	2.80	2.37	9.63	7.91	5.68 f
Mog6	0.73	3.15	5.25	9.49	4.66 d
Mog7	5.24	2.36	4.37	2.75	3.68 cd
Mog8	1.75	0.71	6.30	3.67	3.11 b
Control without Nitrogen	2.51	0.53	4.84	5.51	3.26 bc
Control with Nitrogen	3.15	0.64	7.00	7.70	4.62 d
Mean	2.81 b	1.48 a	5.28 c	6.42 d	4.00

SEM=0.3018 (P<0.01)  
Means followed by the same letter do not differ statistically at P<0.01

#### 4. Conclusion

IAR analysis, nodulation and the efficiency of the symbiotic fixation demonstrate that the eight indigenous strains of *R. leguminosarum* bv *trifolii* are different, even if they are taken from the same accession and the same site. All strains are infective and able for nodulation within the four accessions (Nod<sup>+</sup>) but they don't have the same efficiency. The strain Mog 5 has the highest Dry matter and TNM within the eight studied strains. It forms with the population AK167, an interesting symbiotic couple particularly this population have the higher winter Green and dry matter yield. Selection trials made the retention possible of another couple Mog6-AK101 as efficient as the other one. IAR difference within the studied strains can be used as identification's markers for studying competitive traits within indigenous strains.

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