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# Symbiotic performance of *Medicago ciliaris* lines in association with different *Sinorhizobium* strains

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**Abstract**

We analyzed nodulation and nitrogen fixation efficiency for seven lines of *Medicago ciliaris* in association with two references strains of *Sinorhizobium medicae* (M104) and *S. meliloti* (RCR2011). These *M. ciliaris* lines were collected in Northern-East (Enfidha and Soliman) and Western (Rhayet and Mateur) Tunisian areas. The *M. truncatula* reference line Jemalong A17 (JA17) was also included. Plants were harvested after a culture period of 60 days. Two quantitative traits were measured at the harvest including the mean number of nodules per plant and the aerial dry weight (ADW). Analysis of variance showed that nodulation and nitrogen fixation efficiency (NFE) were dependent on the effects of line, strain and their interaction. The highest levels were observed for strain (45.07%) and line (53.24%) effects, respectively, for nodulation and NFE. While studied lines showed generally more number of nodules with RCR2011, they were more efficient with M104 strain. No significant difference in number of nodules was detected between *M. ciliaris* lines and JA17 with M104, whereas RCR2011 was generally most infective with JA17. Overall, JA17 line exhibited the largest NFE with both strains. Furthermore, there was no significant (P>0.05) correlation between nodulation and NFE for *M. ciliaris* line by strain associations.

## 1. Introduction

The genus *Medicago* is one of the most widespread genera of the Fabaceae, including approximately 83 species and 18 infraspecies taxa [1]. Annual *Medicago* species collectively known as "medics" are naturally distributed over a very wide range of environmental conditions in the Mediterranean basin [1, 2, 3]. Nitrogen is a major limiting factor for plant productivity despite the inexhaustible reserve of atmosphere (78% N2) [4]. Biological fixation of molecular nitrogen (N2) from the atmosphere is one of the main sources of nitrogen pool enhancement in agricultural soils [5]. The ability of *Medicago* species to establish a nitrogen-fixing symbiosis with *Sinorhizobium* sp., which can fix N2, makes them excellent candidates for use in sustainable agricultural systems as forage and cover crops [6]. As a result of their nitrogen-fixing capacity, legumes can colonize nitrogen-deficient soils and enhance soil fertility, which makes them optimal candidates for re-vegetation programs. Different *Medicago* species experience variability in nitrogen fixation effectiveness among strains of *S. meliloti* [7]. For instance, the rhizobia of *M. laciniata* and *M. truncatula* do not effectively cross-nodulate even though all these rhizobia have been classified as *S. meliloti* [8, 9]. In Tunisia, the genus *Medicago* comprises an important proportion of the native flora through all bioclimatic stages [10]. The medics can be found in wide-ranging habitats, varying in water availability, temperature and geographical location [3]. Among the *Medicago* annual species, *M. ciliaris* was reported as the most-tolerant species to salt stress [11]. In Tunisia, it grows spontaneously on heavy soils with clay and/or high salinity such as Sebkha edges. Furthermore, *M. ciliaris* has the advantage of belonging to a genus with a model legume, *M. truncatula* [12]. Species closely related to model organisms present the opportunity to efficiently apply molecular and functional tools developed by a larger research community [13]. *M. ciliaris* is an annual, diploid (2n = 16), self-pollinating forage species. In Tunisia, it is a widespread species in northern areas occupying humid, sub-humid and superior and inferior semi-arid bioclimatic stages [14].

It is preferentially nodulated in its natural habitat by the bacteria *S. medicae* [15]. *M. ciliaris* produces significant quantities of biomass directly accessible in pastures. The term "symbiotic efficiency" is used to describe the ability of nodulated plants to fix atmospheric nitrogen [16]. This efficiency of a particular plant line can change depending on the effects of plant genotype, strain genotype and their interaction on the symbiotic relationship [16]. Consequently, it is important to compare the symbiotic relationship between various host plants and strains in order to select the lines showing high symbiotic performances with different strains.

The long term objective of this study is to dissect the genetic basis of symbiotic performance in *M. ciliaris*. The present work aims to (i) analyze the nodulation and symbiotic efficiency of seven lines of *M. ciliaris* in association with two *Sinorhizobium* strains, and (ii) select from studied lines those showing contrasting symbiotic outcomes to be used as parental lines to develop recombinant inbred lines (RILs) segregating populations.

## 2. Materials and Methods

*2.1. Biological material*

Seven lines of *M. ciliaris* sampled from Enfidha (TNC1), Soliman (TNC8), Rhayet (TNC10) and Mateur (TNC11) populations (Fig. 1) were used. Each population consisted of two lines with the exception of Rhayet from which only one line was used. The choice of lines was based on quantitative traits and microsatellite markers to better represent the diversity within a population [3]. The reference line Jemalong A17 (JA17) of *M. truncatula* was also included in this study. Plants were inoculated with two references strains of sinorhizobia: *Sinorhizobium meliloti* (RCR2011) [17] and *S. medicae* (M104) [8].

*2.2. Symbioses growing and harvest*

Seeds were surface sterilized and scarified with concentrated $H_2SO_4$ for 27 min, and rinsed 10 times with sterile-distilled water. Soaked seeds were sown in Petri dishes on water agar (0.9T% m/v) medium before being vernalized at 4°C in the dark for 72 h.

Seedlings were transplanted aseptically in glass tubes (25x200mm) closed with cotton containing 30ml of nutrient medium [18]. The seedlings were inoculated after 48h with single-strain as 300µl of yeast extract mannitol (YEM) broth suspension at early stationary phase ($10^8$-$10^9$ bacteria/ml). Inoculants were prepared by growing the bacterial strains to late exponential phase [Optical Density at

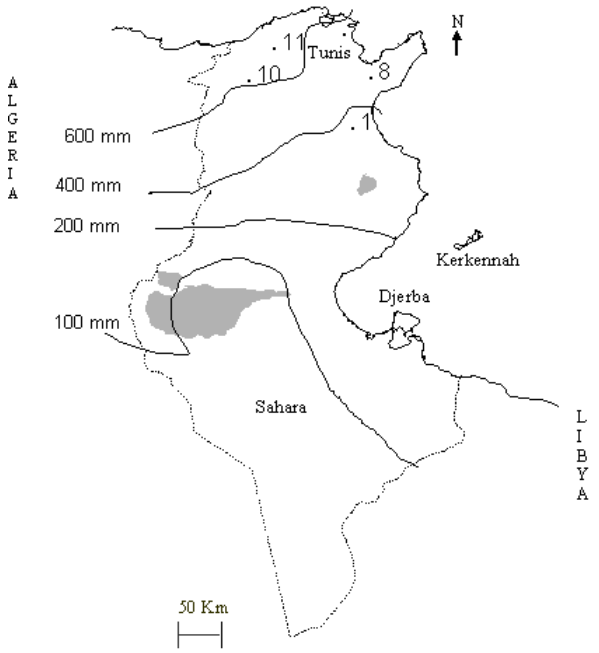


Fig. 1. Map of Tunisia with the location of natural populations of *M. ciliaris* from which studied lines were collected. 1 = Enfidha (latitude (N) = 36°07'; longitude (E) = 10°22'), 8 = Soliman (latitude (N) = 36°41'; longitude (E) = 10°32'), 10 = Rhayet (latitude (N) = 36°39'; longitude (E) = 09°27'), 11 = Mateur (latitude (N) = 37°01'; longitude (E) = 09°40')

620 nm (OD620) = 0.8–0.9] in 100 ml of YEM medium. Uninoculated plants were used as controls. Ten replicates for each line per treatment were used. Plants were cultivated in a growth chamber in controlled conditions: continuous 25°C, 16/8h day/night, and a relative humidity of 100%. Plants were harvested after 60 days to determine the number of nodules per plant and to estimate nitrogen fixation efficiency (NFE) by calculating the ratio of the aerial dry weight of inoculated plants to the aerial dry weight of uninoculated plants. Indeed, the aerial dry weight is positively correlated with the acetylene reduction activity (RAA) [19]. To determine dry weight, plant material was dried in a drying oven at 70°C for 48 hours.

*2.3. Statistical analyses*

Data were subjected to an analysis of variance (ANOVA) using the Statistica software version 5.1 (www.statsoft.com) and the means of measured traits were compared between lines with the Duncan multiple range test. Correlations between nodulation and nitrogen fixation efficiency were estimated for studied *M. ciliaris* lines with M104 and RCR2011 strains by computing the Pearson correlation coefficient (*r*).

## 3. Results and Discussion

Analysis of variance showed that nodulation and nitrogen fixation efficiency (NFE) between studied lines with M104 and RCR2011 strains were dependent on the effects of line, strain and their interaction (Table 1).

Table 1. Proportion (%) and significance levels of the effects of line (L), strain (S) and their interaction (L x S) on the nodulation (Nod) and nitrogen fixation efficiency (NFE) for *M. ciliaris*

| | Nod | | | NFE | | |
|---|---|---|---|---|---|---|
| | df | F | % | df | F | % |
| Line | 7 | 6.29*** | 28.19 | 7 | 71.64*** | 53.24 |
| Strain | 1 | 10.06*** | 45.07 | 1 | 31.09*** | 23.11 |
| L x S | 7 | 5.96*** | 26.73 | 7 | 31.82*** | 23.65 |

The highest levels were observed for strain (45.07%) and line (53.24%) effects, respectively, for nodulation and NFE. Accordingly, numerous studies reported that the variation of these two characters is frequently influenced by both line and strain genotype effects and their interaction [16, 19]. Furthermore, it seems that the importance of the two symbiosis partners' effects on nodulation and NFE is dependent on the characteristics of the experimental design. When several lines were inoculated with only a few strains, generally the strongest effect on symbiotic parameters is attributed to the line factor and vice-versa. Overall, our results suggest that any breeding program aiming the improvement of legume yield productivity through nitrogen symbiotic fixation should take in consideration not only the choice of the best plant genotypes and rhizobia strains, but also their harmonious symbiotic associations.

### *3.1. Nodulation*

Nodulation reflects the infectivity of strains in combination with the studied lines. The number of nodules ranges from 4.89 nodules per plant for TNC11.2 from Mateur to 10.20 for TNC10.11 of Rhayet population inoculated by M104 strain with an average of 7.94 (Fig. 2). Furthermore, it varies between 1.11 nodules per plant for TNC8.5 from Soliman and 14.33 for TNC11.5 from Mateur with a mean of 9.38. Overall, the greatest number of nodules per plant was observed for studied lines with RCR2011 strain, except for Soliman TNC8.5 line.

While no significant difference was detected for

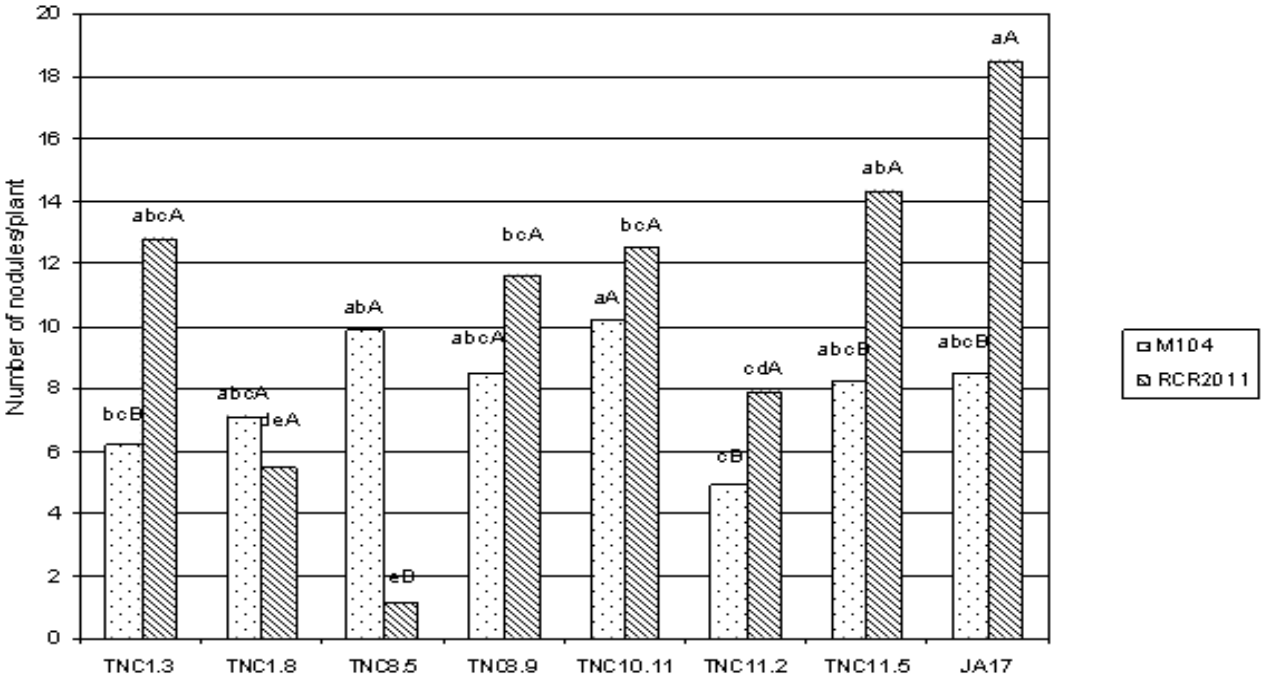


Fig. 2. Mean number of nodules per plant observed for lines of *M. ciliaris* and *M. truncatula* inoculated by M104 and RCR2011 *Sinorhizobium* strains. To compare the nodulation of lines inoculated by only one strain, means followed by the same small letters are not significantly different at *P*=0.05 based on Duncan's multiple-range test. Mean number of nodules formed on each studied line inoculated by the two strains are significantly different (α=0.05) if they are denoted with different capital letters

nodule number between *M. ciliaris* lines and JA17 with M104, higher values were generally observed for JA17 with RCR2011. In contrast to *M. laciniata* species [9], *M. ciliaris* showed a large nodulation spectrum with sinorhizobia strains. Only a few nodules were formed on *M. laciniata*, with the exception of TNL3.6 from Amra, when inoculated by sinorhizobia strains isolated from *M. truncatula*. Moreover, a few nodules were observed on *M. laciniata* lines with the RCR2011 strain. While the lines in this study were generally more infective with RCR2011, they were more efficient with the M104 strain. Similarly, [15] reported that *M. ciliaris* is preferentially nodulated on its original soils by *S. medicae*. Overall, our results suggest that TNC1.3 from Enfidha, TNC10.11 of Rhayet, TNC11.2 of Mateur with M104, and those of TNC11.5 and TNC11.2 from Mateur and TNC8.5 of Soliman with RCR2011 can be used as parental lines to develop RILs populations to analyze the genetic determinism of nodulation in *M. ciliaris*.

*3.2. Nitrogen fixation efficiency*

The term 'symbiotic efficiency' (NFE) is used to describe the ability of nodulated plants to fix nitrogen. NFE observed in this study ranges from 1.06 for TNC10.1 of Rhayet to 2.51 for TNC1.8 from Enfidha with an average of 1.63 for M104 (Fig. 3). It varies between 0.68 for TNC8.9 of Soliman to 1.26 for TNC1.3 from Enfidha with a mean of 1.02 for RCR2011. The greatest NFE was observed for JA17 with both strains. In accordance with the findings of [15], TNC10.11 line exhibited low levels of NFE with both strains. However, while the maximum NFE was only 1.58 for TNC10.11 even inoculated with strains isolated on *M. ciliaris* [15], both studied TNC10.8 and TNC8.5 lines were able to give respectively 2.5 and 2.18 of NFE with M104. This result underscores once again that NFE depends on both plant genotype and *Sinorhizobium* strain. Furthermore, TNC8.9 and TNC11.5 showed NFE less than 1 with RCR2011, indicating a loss of inoculated plant biomass as compared to uninoculated controls. This result has been previously observed for *M. ciliaris* [15] and for *M. truncatula* and *M. laciniata* (Badri *et al.,* unpublished results). This association is not considered a mutualism but as a parasitism [20]. Overall, our results indicate that TNC1.3 and TNC1.8 from Enfidha, TNC8.9 of Soliman and TNC10.11 of Rhayet can be used as parental lines in a future program of crosses to develop RILs segregating populations to analyze the genetic basis of symbiotic performance in *M. ciliaris*.

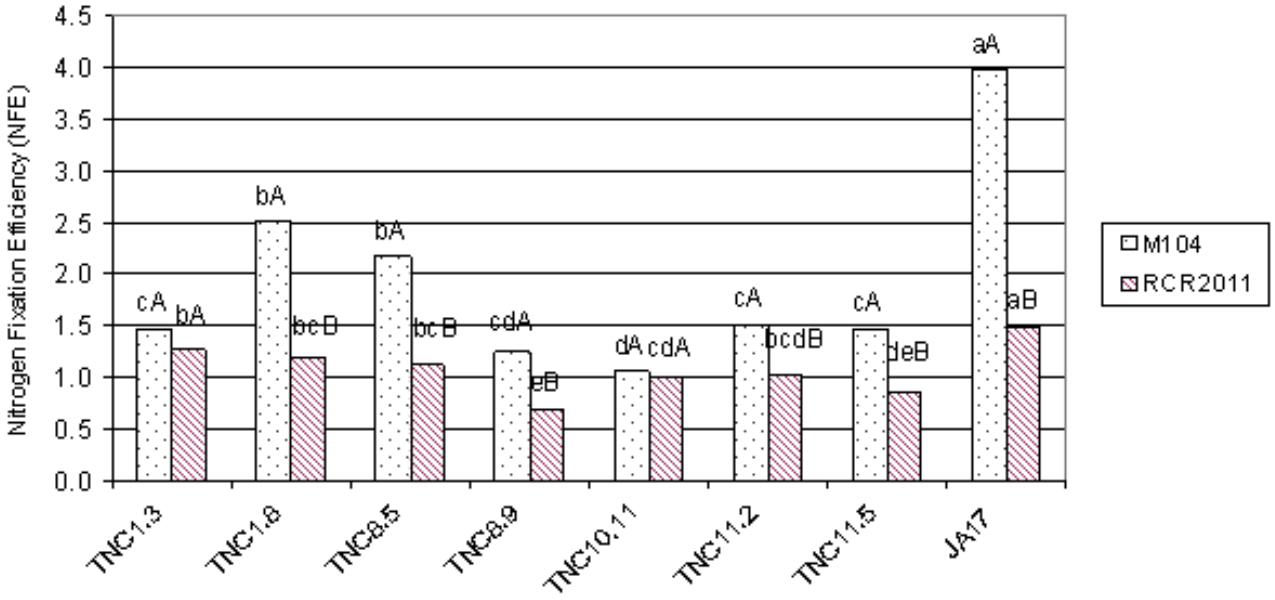


Fig. 3. Nitrogen fixation efficiency (NFE) observed for lines of *M. ciliaris* and *M. truncatula* inoculated by M104 and RCR2011 strains. To compare the NFE of studied lines inoculated by only one strain, means followed by the same small letters are not significantly different at $P$=0.05 based on Duncan's multiple-range test. The NFE values observed for each line inoculated by the two strains are significantly different ($\alpha$=0.05) if they are denoted with different capital letters

*3.3. Correlation between nodulation and nitrogen fixation efficiency*

We found no significant ($P$>0.05) correlation between nodulation and NFE for lines of *M. ciliaris* with both *Sinorhizobium* strains (Table 2).

Table 2. Estimated correlations between nodulation (Nod) and nitrogen fixation efficiency (NFE) for lines of *M. ciliaris* inoculated by M104 and RCR2011 *Sinorhizobium* strains

| | M104 | | RCR2011 | |
|---|---|---|---|---|
| | Nod | NFE | Nod | NFE |
| Nod | 1.00 | -0.029$^{ns}$ | 1.00 | -0.001$^{ns}$ |

This finding has been observed in other studies, no significant association was reported between these two traits for *Pisum arvense* [21], *M. laciniata* and *M. truncatula* [9], and *M. sativa* [16]. One possible explanation of this finding is the high symbiotic specificity of plant-rhizobia genotypes interactions. Indeed, significant variations were detected among rhizobial populations associated with different plant lines of *Vicia faba* [22], *Pisum sativum* [23], and *M. sativa* [16, 24]. On the other hand, large difference was observed between nodulation (Fig. 2) and NFE (Fig. 3) in JA17 line with both strains. While RCR2011 strain induced about twice the nodule number of M104, it had a NFE about one third of the observed for M104, indicating that the latter strain is much more efficient in plant biomass gain per nodule number terms.

Our results suggest that nodulation and NFE in studied lines were essentially dependent, respectively, on strain and line effects but with similar levels for line x strain interaction. While lines were generally more infective with RCR2011, they were more efficient with M104 strain. All lines of *M. ciliaris* in this study can be intercrossed to develop RILs populations as useful backgrounds to analyze the genetic basis of symbiotic performance in *M. ciliaris*.

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