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BETTERMENT OF BIOLOGICAL NITROGEN FIXATION IN SNAP BEAN UNDER MEDITERRANEAN SEMI-ARID CONDITIONS

HAYET BELTAYEF[1,2*]; MONGI MELKI[2]; WAFA SAIDI[2]; SARRA SAMAALI[3]; ADELE MUSCOLO[4]; CRISTINA CRUZ[5]; TAWFIK GAROUI[2]
[1]*University of Carthage, Faculty of Sciences of Bizerte, Box 77 - 1054 Amilcar, Tunisia*
[2]*University of Jendouba, Higher School of Agriculture of Kef, 7119 Le Kef, Tunisia*
[3]*University of Sousse, High Agronomic Institute of Chott Mariem, B.P. 47 - 4042 Chott Meriem - Sousse, Tunisia*
[4]*Mediterranea University of Reggio Calabria, Salita Melissari, 89124 Reggio Calabria, CF 80006510806, Italy*
[5]*Universidade Lisboa, Centre for Ecology, Evolution and Environmental Changes, Faculdade de Ciências Campo Grande 1749-016 Lisboa, Portugal*

Abstract

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A greenhouse experiment was implemented to evaluate the influence of *Rhizobia* strain inoculation, on growth, nitrogen purport, nodulation and enzymatic activities of common bean (*Phaseolus vulgaris* L.). The factors studied are two varieties of beans and three strains of *Rhizobium*. The results indicate that the two indigenous strains are low effective than the international strain (*tropici* CIAT 899), which present the best results for all parameters analyzed whatever the variety. A significant interaction was detected between varieties and rhizobia for the number and dry weight nodule ($p \leq 0.05$). In fact, Plants inoculated with *R. tropici* CIAT 899 showed the highest total biomass by fixing the most atmospheric nitrogen.

The enzyme test linked to the nitrogen assimilation process was realized in leaves of bean. It was revealed that the activities of GOGAT and GS were higher than GDH and MDH activities. *R*. Ciat 899 inoculation showed the up mostest values in all enzymatic activity compared to the control and native Rhizobium. However, our study pointed out the high affinity between *Rhizobium tropici* and the Italian variety "Garrafel Enana".

Key words: greenhouse; semi-arid; nitrogen, fixation; common bean

Introduction

A widespread family, Legumes are highly recommended in the culture system not only because of their ability to increase organic matter in the soil, however, as notably for their ability to fix dinitrogen that is transformed to ammonia within a proceeding renowned as biological nitrogen fixing (BNF) through nitrogenase (Postgate, 1998; Bhatia et al., 2001). BNF is a symbiotic affiliation linked specific bacteria nitrogen-fixing (rhizobia) and legumin plants (Bano et al., 2016).

Umpteen symbiotic N-fixing vegetables are of agronomical matter; furthermore by participating in sustainability environmental (Lindström et al., 2010).

Among vegetables, beans are generally renowned for a weak biological N fixing efficiency in comparison to other grain legumes because of limitations by some biotic and abiotic factors (Anteneh,2016).Beans fixes N unreliably, and N fertilization is habitually approved feeble symbiotic performance of field grown beans is generally attributed to lower N-fixation feature of new cultivars, and the inability to establish efficient nodules in the habitual agricultural soil

**Corresponding author:* beltayefhayet@gmail.com

environment (Zephania et al., 2014). Well known, that N fixing legume understates the utilization of fertilizers which are not only expensive but also much damaging to the ecology. From this point of view, the docket of *Rhizobia* has been recognized in agronomic sustainability and the conservation of ecology (Richardson et al., 1988).

Rhizobium tropici strain CIAT 899 establishes effective symbioses with bean that has been shown to tolerate several abiotic stresses, including high temperature, low pH, or salinity (Riccillo et al., 2000). It has been, furthermore discovered the resilience to various pesticides, fungicides and antibiotics (Bernal et al., 2004). These things have guided to CIAT 899's merchant take on to inoculate *Phaseolus vulgaris* in Southern America and Africa too. (Ormeno-Orrillo et al., 2012).

In recent years, a crescent number of investigation have demonstrated that bean crop can avail from BNF, provided that the management of the agent related to seeds inoculation are done (Ferreira et al., 2009).

In Tunisia, although the bean occupies a limited area, it consumes large quantities of ammonium nitrate to cover its nitrogen needs but the yield stay low around 14.1 q/h in 2015 (Tunisian minister of agriculture). Only in soil usually cultivated with bean, was found nitrogen fixing nodulation. Although nodulation was absent in the other soils, this is the resulted of non-specific strains (Mhamdi et al., 1999).

It is widely known that N assimilation is one basic requirement for vegetable growth, the nitrogen fount accessible to plants being ammonium and nitrate ions. Ammonium ion assimilation forms a central metabolic road in many organisms.

GS incorporates this ammonia in the chloroplasts as the amide cluster of glutamine bestows glutamate like a substrate. (Melo-Oliveira et al., 1996)

NADH-GOGAT switch the amide cluster over an 2- oxoglutarate molecule making two glutamates. At the same time with GS, these 2 enzymes mold the major path of ammonia assimilation in plants. The cycle of GOGAT is thus essential not only for elementary nitrogen assimilation but also to steady the broad nitrogen economy of the vegetable.

While the enzyme GDH does not blow a direct part in the assimilation. It safeguards the mitochondrial functions over periods of nitrogen metabolism and participle in nitrogen remobilization.

Within a few terms NADH GDH, it has been proposed that it is an enzyme catalyzes the deamination, by reversible way of glutamate. It can participate uniformly in the assimilation of ammonium (Ferraro et al., 2015).

During this research, a testing was realized to learn inoculation impact on bean crop utilizing autochthon strains comparing to international *R*.CIAT 899; and the performance of the cultivated variety in Tunisia compared to Italian variety under controlled conditions, as well as possible nodulation under semi-arid climate. Moreover, the inoculation effect on enzyme activity attached to the assimilation of ammonium.

Material and Methods

The vegetable material used was two varieties of bean;

1) Contender

2) GarrafalEnana

To sprout, seeds were put in the jars containing sand. They have been inoculated with Rhizobia strains or not for other treatments (see at the bottom).

Biological material

Surface sterilization of seeds had by sodium hypochlorite for 10 min, and then washed by 6 washings with sterile purified water. The inoculation occurred at the time of 2-leaf stage; using three *Rhizobia* strains about ten exhibiting nine for each plant as following:

*Two native strains R1 and R2.

* R.Ciat899

The inoculum was withheld from bacteria suspension which cultured on agar yeast extract mannitol medium well-preserved at 4°C and uphold for one day at 28 °C according to Vincent (1970).

Bacterial strains

● *R. tropici* used in this study included type strain CIAT 899 from CECT (collection Espanola de cultivostipo) which has the designation CECT4654).

● Indigenous strain were isolated from nodules of bean; they were recovered from Tunisian soil in Kef region in the north of Tunisia.

Growth conditions

Seedlings inoculated with *R. tropici* CIAT899 were grown in sterile sand. Jars were watered with sterile distilled water daily until harvest, and received once a week the nutrient solution according to Vadez et al. (1996)

Harvest and data analysis

For greenhouse testing, for each treatment four plants were gathered. The samples were segregated into shoots and roots. Then nodules were extirpated from the roots.

To dry samples they were put in a drying oven at 70°C for 72 h.

For each sample stale weight, shoots, roots and pods were measured individually and the nitrogen term (% N/Pl) was deliberated using the process of Kjeldahl.

Extraction and assay conditions of enzyme

According to the ratio 1:3 w/v (plant material / mixture solution); the solubilization of enzymes from leaves had done by the manual ground of leaves in a pestle mortar. According to Silvia (2015) we added then, the buffer Hepes-NaOH solution (100 mM) at pH 7.5, magnesium chloride solution (5 mM), and dithiothreitol (1 mM); the resulting filtrate had been clarified by centrifugation for 15 min at 20 000 g. Then the supernatant had been kept and had been used in the enzymatic essays. All proceeding was neatly done at 4°C. An aliquot of the extract had been used to define its protein content through Bradford method (1992) involving BSA as standard.

Glutamine synthetase (GS EC 6.3.1.2)

To evaluate the glutamine synthetase activity, the mixture for the assay contained 90 mM imidazole-HCl (pH 7.0), 60 mM hydroxylamine (neutralized), 20 mM Na_2KAsO_4, 3 mM $MnCl_2$, 0.4 mM ADP, 120 mM glutamine and the appropriate amount of enzyme extract. The assay was performed in a final volume of 750 µL. The enzymatic reaction was developed for 15 min at 37°C.

Glutamyl-gamma- hydroxamate was made by colorimetric method. This was done from merely adding of 250 µL of an intermixture (1:1:1) of:

- 10% (*w*/*v*) Iron trichloride.$6H_2O$ in 0.2 M hydrochloric acid,
- 24% (*w*/*v*) trichlorotrichloroethanoic acid,
- 50% (*w*/*v*) Hydrochloric acid.

The optic density was determined at A 540 [51].

NADH-GOGAT EC 1.4.1.14

The essay was made in 1.1 mL final volume and was assayed spectrophotometrically at A 340. It contained the following chemical products:

Chemical products	
Hepes-NaOH pH 7.5	25 mM
glutamine	2 mM
α-ketoglutaric acid	1 mM
NADH	0.1 mM
Na_2 EDTA	1 mM
Enzyme extract	100 µL

Malate Dehydrogenase (MDH)

This essay was made by preparation of phosphate buffer pH 6.7 (94.6 mM), beta-NADH (0.2 mM), oxalacetic acid (0.5 mM) and Magnesium chloride (1.67 mM) in aoverall intermixture 3.17 mL. The MDH activity was deliberated spectrophotometrically at A340 grant to Bergmeyer (1986) by overseeing NADH oxidation.

Phosphoenolpyruvate carboxylase: (PEPC, EC 4.1.1.31)

The activity of PEPC was deliberated by checking the oxidation of NADH at 340 nm at 30°C for 5 min. We put for 1ml assay medium; Tris–HCl pH 8.0 (100 mM), $MgCl_2$ (10 mM), $NaHCO_3$ (10 mM), NADH (0.2 mM) and MDH (1.5 IU) which added by enzyme extract (100 µl).

Ammonium Quantification

In the fresh weight of leaves, NH_4^+ was measured spectrophotometrically at A 630 according to Krom (1980). The findings were uttered as mol g −1 FW.

Statistical analysis

The trial model was a split plot in completely randomized design (CRD). Statistical result was realized by the SAS software (1997). The data were construed involving ANOVA and subsequent comparison of means was made by utilizing the Duncan test at $p \leq 0.05$.

Results

Nodule

The findings on nodule tenure confirmed the choosing of compatible strains depends on growth performance. Utmost nodule taking was perceived with R. CIAT 899, followed by indigenous Rhizobium: native R1 and native R2. The interaction among two factors (rhizobium and varieties) was significant by examining statistical data ($p < 0.01$).The variety "Garrafel Enana" inoculated with the R.CIAT 899 donated preeminent numbers of nodules than the variety Contender (Table 1); The varieties had various total nodule mass: Contender had less and larger nodules than GarrafalEnana . However, there is no proof to propose that many small nodules could be low efficient at nitrogen fixation than the same mass of larger nodules.

We note that nodules with indigenous strains were colorless; while those which were inoculated with *Rhizobium* Ciat 899 had pink color, which suggested that an active nitrogen fixation had been settled between the nodule bacteria and crop bean (Figure 1).

Biomass production

Common bean plants inoculated with R.CIAT 899 have increased significiantly aerial dry biomass by comparing to the control (non-inoculated) regardless varieties. However, inoculation with native Rhizobium 1and native Rhizobium 2was improved aerial dry biomass compared to the control. (Figure 2).

Table 1
Inoculation impact on indigenous rhizobium and R.Ciat 899 on nodulation of bean varieties (35 days after inoculation)

	Nodulation	
	Number (no/pl)[n]	Dry Biomass (mg/pl)
Contender		
-R	–	–
R.CIAT899	64±3.80	0.055±0.009
native R 1	1±0	0.00007±0
native R 2	14±2.8	0.0115±0.001
Garrafal Enana		
-R	–	–
CIAT899	274±11	0.16±0.01
native R 1	10±1.4	0.0063±0.0001
native R 2	55±9.3	0.07±0.002
ANOVA (F-Statistic)		
Main effects		
Var	3.68ns	98.49***
R	11.53***	133.72***
Interactions		
Var*R	10.38**	34.79***

npl in an plant; R: Rhizobium; -R: control plant; Var: for each variety the Values done are means ± SE, n = 4. *; **; *** = significant at P ≤ 0.05, P ≤ 0.01, P ≤ 0.001 respectively, ns = not signficant, SE = standard error. average calculated according to Duncan significance at P = 0.05

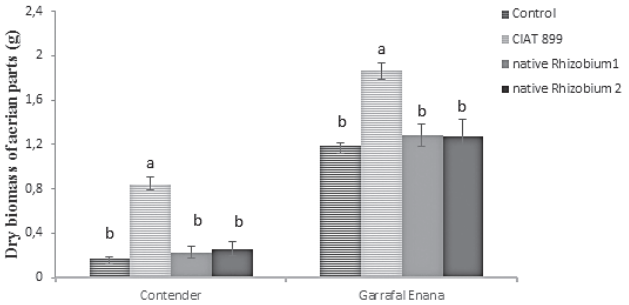

Fig 2. Inoculation effect of rhizobia strains on dry biomass of the air part of bean varieties
Datum are means ± SD of four repetition, plants harvested at 50 days after seedling; various letters point out significant otherness between processing means according to Duncan's test ($p \leq 0.05$)

Similarly, *R*.CIAT 899 induced to a significantly enhanced root dry matter compared with non-inoculated (< 0.001) (Figure 3).

An analogous pattern was recorded in pods dry biomass with native rhizobium showing a similar result to non-inoculated plants (Figure 4).

Nitrogen tenor

The symbiotic plants rely entirely on biological nitrogen fixation to gratify their nitrogen need. In order to rate the amount of N fixed, the nitrogen content was determined in

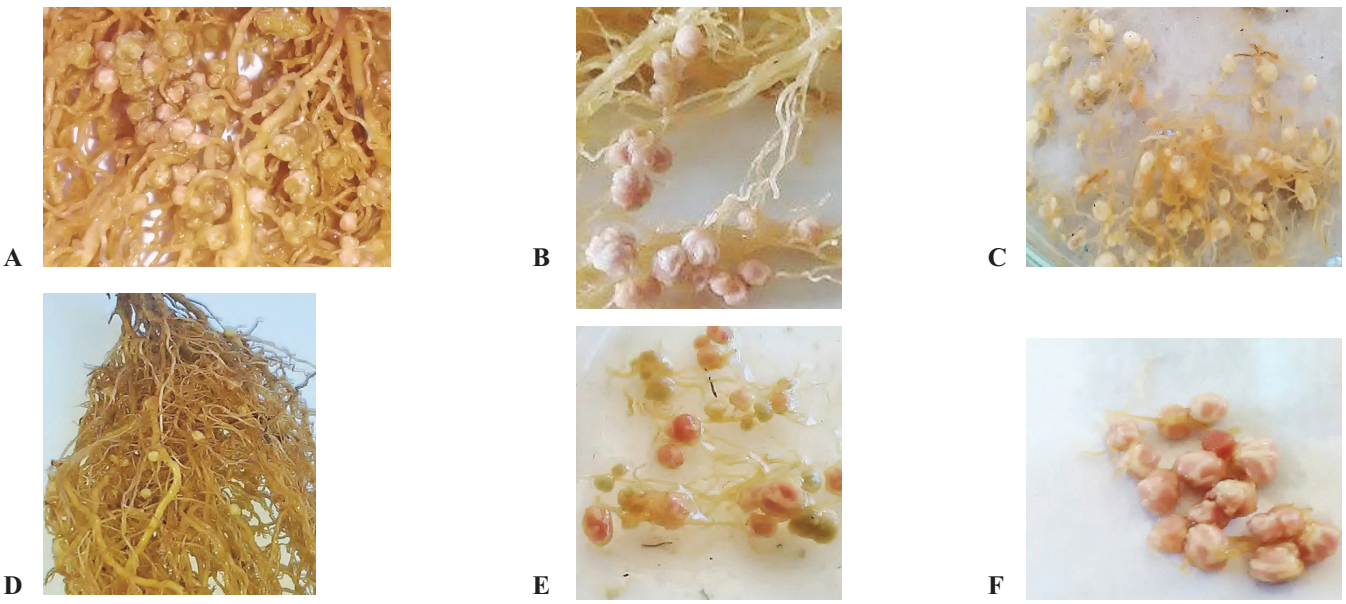

Fig. 1. Feature of nitrogen fixing nodules by different rhizobium strains: R.Ciat 899 root nodules on Garrafal Enana (a) and on Contender (b); root nodules of native R2 (c) and R1 (d); (e) Contender nodules with R.Ciat 899; (f) Garrafal Enana nodules with R. Ciat 899

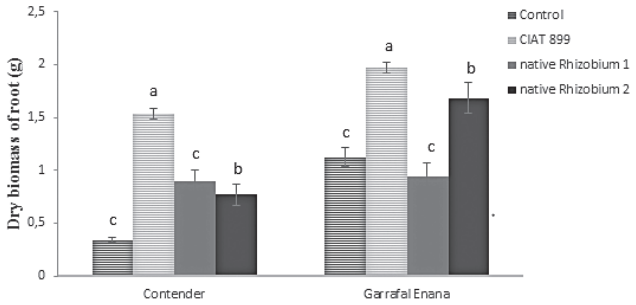

Fig. 3. Inoculation effect of rhizobia strains on dry biomass of root bean varieties bean

Datum are means ± SD of four repetition, plants harvested at 50 days after seedling; various letters point out significant othernes between processing means according to Duncan's test ($p \leq 0.05$)

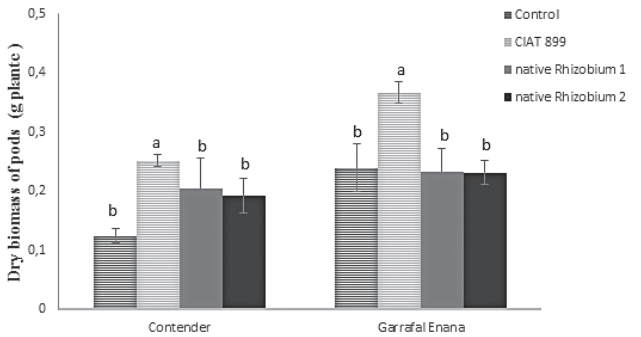

Fig. 4. Inoculation action of rhizobia strains on dry biomass of root of bean varieties

Datum are means ± SD of four repetition, plants harvested at 50 days after seedling; various letters point out significant otherness between processing means according to Duncan's test ($p \leq 0.05$)

shoots, root and pods of Contender and Garrafal Enana after inoculation with either *R. tropici* CIAT899 or indigenous rhizobia strain.

The amount of nitrogen in shoot and root depended on rhizobium ($p < 0.001$) and variety ($p < 0.001$) while the interaction between the two factors (rhizobium and varieties) was statistically significant ($p < 0.001$) in shoot and ($P \leq 0.01$) in root (Table 2).

The nitrogen content in pods was strained by rhizobium ($p < 0.001$) and by variety ($p = 0.37$), although the interaction between the two factors (rhizobium and varieties) was statistically significant ($p \leq 0.001$).

Among the common beans, the large seeded Garrafal Enana accrued significantly more nitrogen than the Contender did.

Activities of ammonium assimilation

The enzyme activities of ammonium assimilation by leaves are showed in Figure 5.

Table 2

N tenor in aerian parts, root and pods of bean varieties at harvesting (50 days after seedling).

`	Nitrogen content (%)		
	Shoot	Root	Pods
Contender			
Control	0.20±0.012	0.12±0.012	0.48±0.025
R.Ciat 899	0.46±0.031	0.48±0.017	0.94±0.037
Native R 1	0.32±0.018	0.17±0.014	0.57±0.030
Native R 2	0.30±0.017	0.16±0.014	0.54±0.042
Garrafal Enana			
Control	0.36±0.026	0.29±0.040	0.48±0.012
R.Ciat 899	0.78±0.029	0.52±0.038	0.87±0.034
Native R 1	0.48±0.017	0.35±0.025	0.52±0.012
Native R 2	0.39±0.012	0.24±0.012	0.51±0.029
ANOVA (F-Statistic)			
Main effects			
Var	542.2***	154.96***	1.16ns
R	340.01***	240.84***	221.63***
Var*R	38.37***	13.71 **	0.40**

R: Rhizobium; R: control plant; Var: for each variety the Values done are means ± SE, n = 4 *; **; *** = significant at $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ respectively, ns = not significant, SE = standard error. average calculated according to Duncan significance at P = 0.05

The GS activity and NADH-GOGAT activity was thereabouts 2 fold higher in plant inoculated with *R.*Ciat 899 than control, and both enzyme activities increased softly with indigenous Rhizobium.

The assimilation of ammonium establishes pivotal metabolic paths in many organisms, and glutamate synthase, in accordance with glutamine synthetase, performs the primary docket in the incorporation of ammonium ion into glutamine and glutamate. Ammonium concentration rises when GOGAT and GS activity stepped up.

Inoculation with Rhizobium don't show a significant increase in glutamate deshydrogenase and malate deshydrogenase activieties, but only a slight one.

The average GDH activity was 0.086, 0.087 µmol min-1 mg-1 protein in leaves of Contender and Garrafal Enana inoculated with *R.*Ciat 899 respectively, but reached about 0.24, 0.17 µmol min-1 mg-1 protein in leaves of Contender and GE inoculated with native Rhizobium respectively; which are comparative to control (Figure 6).

Discussion

This study reveals that *R.*CIAT 899 significantly increased morphologic growth of seedlings of two bean va-

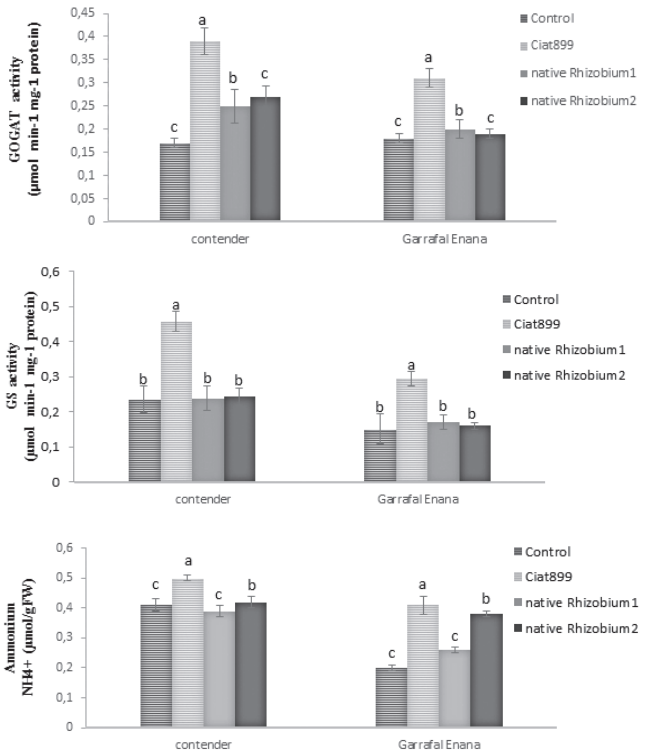

Fig 5. Enzyme activities glutamate synthase (GOGAT, (imol min^{-1} mg^{-1} protein); glutamine synthetase (GS, |imol min^{-1} mg^{-1} protein); in leaves of *P. vulgaris* non inoculated (control), inoculated with the *R. tropici* CIAT 899 strain and indigenous rhizobium (native Rhizobiuml and native Rhizobium 2)

Data are means ± SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range test ($p \leq 0$ 0.05)

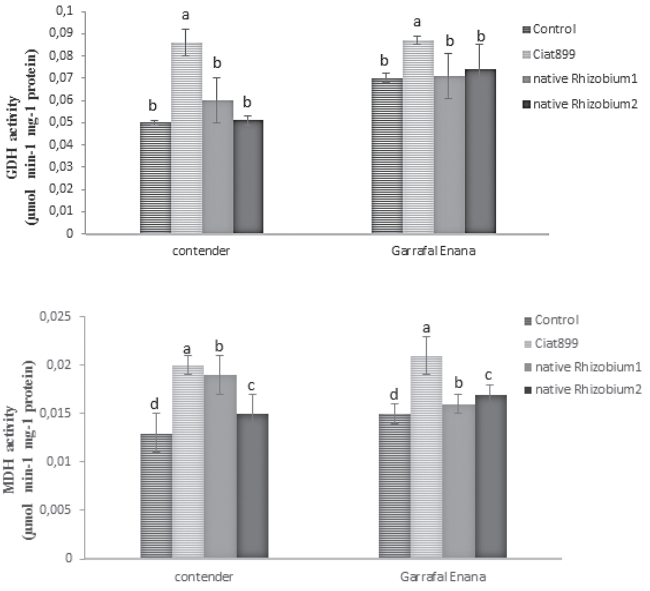

Fig. 6. Enzyme activities Glutamate deshydrogenase (µmol min^{-1} mg^{-1} protein) and Malate deshydrogenase (µmol min^{-1} mg^{-1} protein) in leaves of *P. vulgaris* non inoculated (control); inoculated with the *R. tropici* CIAT 899 strain and indigenous rhizobium (native Rhizobium1 and native Rhizobium 2)

Data are means ± SD of 3 replicates, plants harvested at 50 days after sowing; different letters indicate significant differences between treatment means according to Duncan's multiple range tests ($p \leq 0.05$)

rieties. Concerning enhancement root, shoot and pods dry biomass, beforehand, studies avowed *R*.CIAT 899 induced improvement in many plant species that could be owing to ameliorate photosynthetic activities, leaf turgor and other allied physio-metabolical processes. It has been broadly used in inoculation investigations (Hardarson et al., 1993; Graham and Vance, 2000). In the tropical acidic grounds of Southern America, this strain is an efficient microsymbiont of common. The strain proprieties are marked on wide tolerance of ecological stresses and its wide legume ranging-host (Martinez et al., 1991; Hungria et al., 2000).

Under controlled conditions, the interaction Rhizobium-genotype had a crucial effect on the symbiotic efficiency of various strains. A notable property over this study is the elevated affinity between R.CIAT 899 and the Italian variety „Garrafel Enana". *R*. CIAT899 was greater competitiveness than indigenous strains which have shown in northern Tunisia by Tajini et al. (2008). This point, doesn't sustain the view of Thies et al. (1992) that the native rhizobia are usually high competitive than the introduced ones. So this upholds that strain competitiveness still the key to success the inoculation under field conditions (Boonkerd et al., 1978; Singleton and Tavares 1986).

Looking at the result of plant development, number nodule and mass nodule (Table 1) it might suggest that the efficiency of rhizobia given by umpteen agents from strains such as the interaction between rhizobia – cultivar which perform the crucial mechanism of nitrogen-fixing which could have the capacity to improve nitrogen input in semi-arid ecosystem studied. It's also showed by Hungriaand Neves (1987) which point out the crucial in view of both symbiotic partners while seeking to enhance N2 fixation in crop bean.

The perceivable of inter strain rivalry may serve out to drift effective *Rhizobium* strains which are distinguished by

their capacity of which are also capable of contest with indigenous strains for nodule tenure.

The glutamine synthetase and NADH glutamate synthase perform to internalize symbiotically fixed N (Lea and Forde, 1994).

During testing, the inoculation with *R*.Ciat 899 evinced the highest values in growth parameters and the enzymatic activities were increased more than 25% in leaves compared to control and inherent Rhizobium.

Concerning NADH-GOGAT and GS activities were roughly 3 fold raised than GDH and MDH activities. An intercourse similarly this is proposed by Berberich (1972) showing that physiological processes might involve in regulation levels of these enzymes in an inversed way. Within the limitation proviso of NH_3, the reverse position is true, which would increase GOGAT activity and would reduce of GDH. So NH_3 would be produced increasingly in root nodules by N2-fixing.

In our experiment, a notable increase in GDH activity was observed in parallel with a raise concentration of ammonium. These findings propose that the ammonia ensuing from the reduction of nitrate in the leaves was assimilated into glutamine.

Conclusion

Enhancement agronomical management is fairly necessary to upgrade bean behavor, and preservation agriculture afford matter opportunities for growing production of common beans. This pursuit revealed that R.CIAT 899 could ameliorate *Phaseolus vulgaris* plant growth; increasing enzymatic activity; it may be of wide interest to farmers growing bean.

Further prospective studies are besides re required in field to look up thought into *Rhizobium* symbiosis for getting better seedling growth, nitrogen metabolism linked to leading performance of raise plants and seed goodness.

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