EFFECTS OF *SINORHIZOBIUM MELILOTI* STRAINS (1021 AND NitR) ON NITROGEN ASSIMILATION OF ALFALFA PLANTS UNDER CONDITIONS OF MINERAL ELEMENTS SHORTAGE

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Summary. Two *Sinorhizobium meliloti* strains (1021 and NitR) were used for inoculation of alfalfa plants to study nitrogen assimilation under nutrient deficiency conditions in hydroponics experiments. The wild type *Sinorhizobium meliloti* 1021 was compared with a mutant strain – *S. meliloti NitR*. NitR protein was found to be a regulator of *S. meliloti hmgA* expression under nitrogen deprivation conditions, suggesting the presence of a *ntr*-independent nitrogen deprivation regulatory system. *nitR* insertion mutations were shown not to affect bacterial growth, nodulation of *Medicago sativa* plants, or symbiotic nitrogen fixation under the physiological conditions examined. The relationship between free living and symbiotic bacterial forms was revealed indirectly by the changes of nitrogen fixation and assimilation under conditions of nutrient deficiencies. Before seeds inoculation, bacteria were cultivated in Vincent minimal media with limited nitrogen source. The alfalfa plants were grown on a nutrient solution in the presence or absence of molybdenum. The differences between the two symbiotic systems were established by the variations of nodule formation and enzyme activities participated in nitrogen fixation and

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assimilation (nitrogenase – NG: EC 1.7.99.2; glutamine synthetase – GS: EC 6.3.1.2; glutamate synthase – NADH-GOGAT: EC 1.4.1.14 and nitrate reductase – NR-NADH: EC 1.6.6.1). Negative effects of Mo shortage on the rate of nitrogen fixation and nitrate reduction in both symbiotic systems were found. When plants were inoculated with strain *S. meliloti NitR* and grown under nitrogen limiting conditions, the highest stability of nitrogen fixation and nitrogen reduction was determined in conformity with characterization of this mutant strain.

Key words: alfalfa; strain effect; nitrogen assimilation, mineral elements shortage. VA multifactor analysis.

INTRODUCTION

The role of biological nitrogen fixation is well-known for a long time as a nonpolluting and cost-effective way to improve soil fertility. Rhizobia, such as *S. meliloti*, must be able to persist and compete for scarce nutrients in the bulk soil, compete for colonization of the rhizosphere and plant infection, and adapt their metabolism to the nutritionally more favorable, distinct conditions within the plant cells in the nodule. These three different modes of existence exemplify the need for a high degree of physiological adaptability, specific genetic mechanisms to sense changes in environmental conditions, and the ability to respond rapidly. These characteristics led to a search for *S. meliloti* genes specifically expressed under nutrient limitation conditions (Milcamps et al., 2001). Recently, a gene, named *nitR,* involved in direct or indirect regulation of *hmgA* gene expression in response to various stresses, including starvation, have been identified in *S. meliloti* (Davey and de Bruijn, 2000; Milcamps *et al.*, 2001). *hmgA* gene expression, is involved in the degradation of tyrosine as an alternative nitrogen source (Milcamps, A., and F. de Bruijn. 1999). The mutant strain referred to as NitR is with reduced expression of *hmgA* under nitrogen deprivation conditions.

It is well known that legumes possess a systemic regulatory control able to detect the presence of combined nitrogen in the rhizosphere and

block nodulation in response (Salminen and Streeter, 1990). The results presented by López-Garciá et al., (2001), Dusha et al. (1999), Parniske et al. (1993), indicated that rhizobial N starvation has a positive influence on the symbiosis, through parallel effects on the EPS and CPS structure, *nod* gene induction (Mylona et al., 1995), all of which resulted in increased nodulation efficiency and competitiveness.

Insufficient molybdenum supply leads to significant reduction of nitrogen-fixing activity. The negative effect of Mo exclusion from the nutrient media on activity of the enzymes is related to the primary nitrogen assimilation (NR, GS, GOGAT) and biomass accumulation. It was found that alfalfa is more sensitive to Mo starvation than the pea plants (Hristozkova, 2008). The role of Mo as a plant nutrient is related to its function as a metal component of some enzymes that catalyze nitrogen fixation, nitrate assimilation and reduction (Campbell, 2001).

A significant variation of plant growth, rates of dry matter and N accumulation as well as the total N content of mature plants in response to inoculation with *Rhizobium* strains as a result of differences in N_2 fixation rates was observed by Rodríguez-Navarro et al. (1999) and Neves et al. (1982). Lawn and Bushby (1982) shown that effects of *Rhizobium* strains were associated primarily with specific nodule activity in four Asiatic *Vigna* species. Careful selection of inoculant strain is essential for any legume grown under stress (Graham, 1992).

The present study was designed to investigate the differences between two symbiotic systems (*Medicago sativa/ Sinorhizobium meliloti* 1021 and *Medicago sativa/ Sinorhizobium meliloti* NitR) in response to Mo deficiency (for alfalfa plants) and N limitation (for *S. meliloti*).The differences were established by the variations of nodule formation and enzyme activities participated in nitrogen fixation and assimilation: nitrogenase, glutamine synthetase, glutamate synthase and nitrate reductase.

MATERIAL AND METHODS

Seeds of alfalfa (*Medicago sativa* L. var. Prista 4) were germinated on Fahräeus agar at 25 °C according to Journet et al. (2001). Three days old seedlings were inoculated with a bacterial suspension of *Sinorhizobium*

meliloti strains 1021 TLS and NitR (the strains is provided by prof. F. de Brjiun, LIPM, Toulouse). On the 5th day, seedlings were transferred to 2 L pots (25 plants per pot) containing liquid nutrient solutions of Helriegel and were grown in phytotron chamber at 12 h photoperiod, day/night temperature 25/18 °C and photon flux density of 95 μmol $m^{-1}s^{-1}$ until the 38th day. The solution was aerated continuously and replaced twice a week. Helriegel nutrient solution supplied to plants contained 0,5 mM NO_3^-. Complementation of the solution with micronutrients was done according to Hoagland and Arnon (1950). The following variants were tested: control plants; plants inoculated with *S. meliloti* (1021 and NitR), grown in limited nitrogen source conditions; Mo deficient plants; Mo deficient plants inoculated with *S. meliloti*, grown in limited nitrogen source conditions. In order to prepare crude extracts for determination of nitrate reductase (NR-NADH: EC 1.6.6.2), glutamine synthetase (GS: EC 6.3.1.2) and glutamate synthase (NADH-GOGAT: EC1.4.1.14), the extraction medium containing 50 mM Tris-HCl (pH 8.0), 1 μM Na_2MoO_4, 10 mM $MgSO_4.7H_2O$, 1 mM EDTA, 10 mM L-cysteine, 1 % PVP-40, 1g Dowex (Frechilla, 2002). The extract was filtered through one layer of cheesecloth, centrifuged at 10 000 x *g* for 20 min (4 °C), and the supernatant was used for the following assays: NR activity was measured according to Hageman and Reed (1980), GS activity was determined by a biosynthetic assay based on γ-glutamil hydroxamate synthesis (O'Neal and Joy, 1973), GOGAT activity was assayed according to Chen and Cullimore (1988). Protein content was determined according to Bradford (1976) with BSA as a standard. Nitrogenase activity (NG: EC 1.7.99.2) of root nodules was assayed by the acetylene (C_2H_2) reduction technique immediately after harvest according to Hardy et al. (1973). The acetylene (C_2H_2) reduction assay (ARA) was expressed as μmol C_2H_4 g^{-1} FW nodules h^{-1}. The results are expressed as means ± standard error where n=4 (four replications of analyses run with a single sample, derived from 10 plants). Comparison of means regarding enzyme activity was performed by the Fisher LSD test ($P \leq 0.05$) after multifactor ANOVA analysis.

RESULTS AND DISCUSSION

Our study is based on the special features of stress responses in the

studied *S. meliloti* strains (1021 and NitR) and consequence differences in nodule formation and nitrogen fixation rates. The genotype of the strain had an important effect on the effectiveness of symbiotic system, affecting variables like the number and nitrogenase activity of nodules. The behavior of both N_2-fixing systems under Mo and N deficiency conditions is considered.

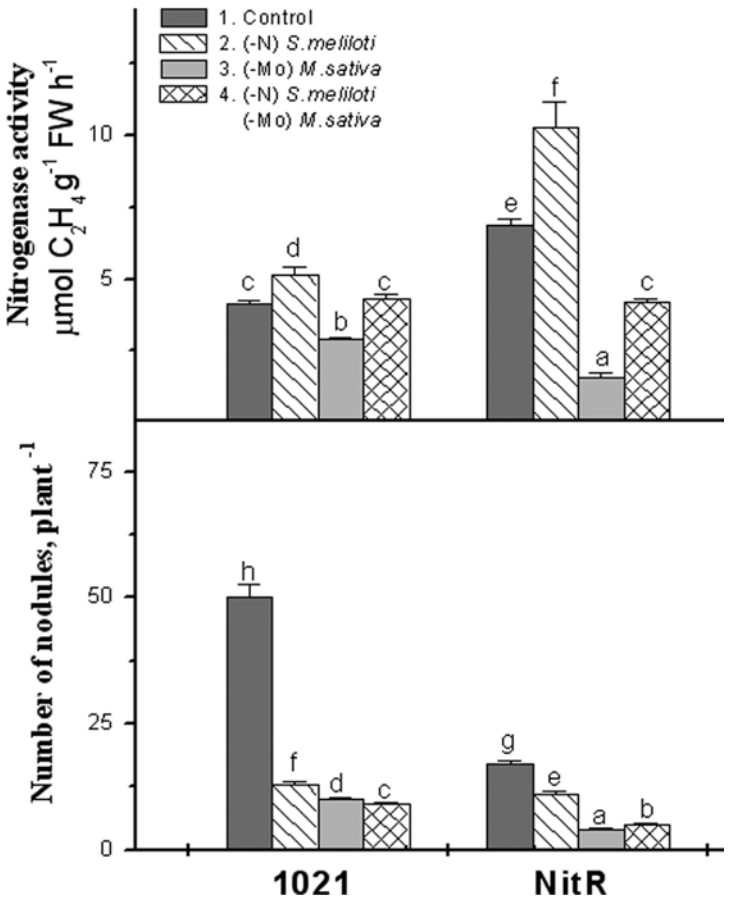

Fig. 1. Nitrogenase activity and nodule number in alfalfa plants inoculated with *S. meliloti* (strains 1021 and NitR) grown under various conditions. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

The nitrogen fixation capacity of the mutant strain NitR is higher than 1021 in normal conditions and insufficient N in the media. In case of Mo deficiency, nitrogenase activity is lower and number of nodules declined in comparison to variants with N limitation and control plants in both symbiotic systems. The symbiotic bacterial enzyme nitrogenase is comprised of two subunits one of which is the MoFe protein directly involved in the reduction of N_2 to NH_3. Supply of molybdenum and Fe to bacteroids is therefore an important process and most likely a key regulatory component in the maintenance of nitrogen fixation in legumes (Kaiser et al., 2005).

The lack of correspondence between the nodule number and nitrogen fixing activity was observed in the symbiotic systems (Fig. 1). According to Skot et al. (1983) and Puppo et al. (2005), different combination between bacteria strains and host plant enable plasticity in nodule formation, for example decreasing of nodule number lead to bigger nodules with higher nitrogen fixation activity.

Lower nitrate reductase activity in the roots of NitR plants compared to 1021 plants, both in conditions of Mo deficiency in plants and lack of N for bacteria could due to the suppression of high GS/GOGAT activity in the roots of these treatments as a consequence of increased NG activity. The products of NO_3^- assimilation including NH_4^+ and amino acids, especially glutamine and glutamate, repressed the transcription of *nia* genes and by this way regulate NR activity in the pathway (Hoff et al., 1994; Dzuibany et al., 1998). The highest activity of NR was found in the shoots of NitR plants in normal conditions (Fig. 2). Pate (1973) pointed out that at low levels of nitrate supplied NR is found to be located in the nodulated roots, but at higher levels of nitrates more nitrate becomes reduced in the shoots. Comparatively higher levels in conditions of Mo deficiency in roots could be explained with lower nitrogen fixation.

GS/GOGAT enzyme activity in plants increased in the progress of nodule development and its ability for atmospheric N_2 fixation, that is, they increased in parallel with the capacity of nodules to convert N_2 to glutamine and asparagines (Oaks, 1994; Parsons and Sunley, 2001). Reduced nitrogen fixing activity under conditions of Mo shortage resulted in decline of root GS/GOGAT enzyme activities in both symbiotic systems. The highest value of shoot GS/GOGAT activity was found under combination of Mo deficiency

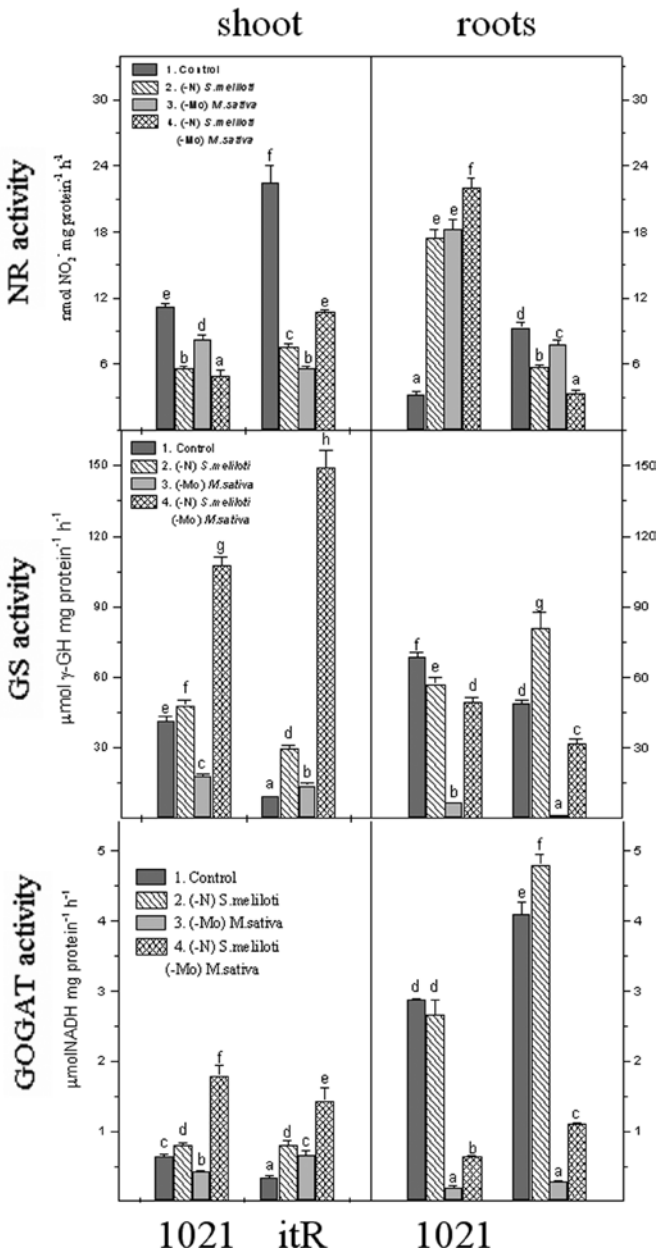

Fig. 2. NR, GS and GOGAT activities in alfalfa plants inoculated with *S. meliloti* (strains 1021 and NitR) grown under various conditions. Different letters indicate significant differences assessed by Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

for plants and lack of N for bacteria both in 1021 and NitR variants (Fig. 2). GS functions as the major assimilatory enzyme for ammonia produced from N fixation, and nitrate or ammonia nutrition. It also reassimilates ammonia released as a result of photorespiration and the breakdown of proteins and nitrogen transport compounds (Miflin and Habash, 2002).

The results connected with nitrogen assimilation in alfalfa showed that the efficiency of nitrogen fixation and assimilation could be improved in normal conditions and lack of N in the growth media by inoculation with the mutant strain NitR. The negative effect of Mo deficiency on the nitrogen fixation and assimilation in alfalfa plants was higher in the symbiotic system alfalfa-*S. meliloti* NitR.

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