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BRIEF COMMUNICATION

Response of *Zea mays* to the inoculation with *Azospirillum* on nitrogen metabolism under greenhouse conditions

C.M. RIBAUDO, D.P. RONDANINI, J.A. CURÁ* and A.A. FRASCHINA

Cátedra de Bioquímica, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín, 4453-1417, Buenos Aires, Argentina

Abstract

The maize (*Zea mays* L.) plants inoculated by N_2-fixing bacterium *Azospirillum* showed increased activity of glutamate dehydrogenase (GDH) and glutamine synthetase (GS) in root cells free extracts over uninoculated control plants. Maximum differences in NADH-GDH activity were observed during the second and third weeks after sowing. The specific activity of GS showed a greater increase at the end of the assay. The percentage of nitrogen in leaves, root and foliage length, total fresh mass and nitrogenase activity were higher in inoculated plants than in the control ones.

Additional key words: glutamate dehydrogenase, glutamine synthetase, maize, nitrogen content.

Bacteria of genus *Azospirillum* live in association with the roots of several grasses. Many reports have shown positive interaction between *Azospirillum* and grass or grain crop roots (Michiels *et al.* 1989). These bacteria affect plant development through several mechanisms including biological nitrogen fixation (Bulow and Döbereiner 1975) and has been reported to produce phytohormones (Tien *et al.* 1979). The physiology and biochemistry of maize roots and leaves colonized by *Azospirillum* has not been extensively studied. *Azospirillum* provides the plant NH_4^+, glutamine, or other N-compounds when it is growing under nitrogen-fixing conditions. There is evidence indicating an assimilating role for glutamate dehydrogenase (GDH; EC 1.4.1.3.) on the basis of organic nitrogen transported to the shoot, it was suggested that glutamine synthetase (GS; E.C. 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.7.1) may not be sufficient to assimilate NH_4^+ in the roots, and GDH activity in maize roots has been reported to take place with ammonium assimilation (Oaks *et al.* 1980). This study was undertaken to evaluate the effect of *Azospirillum* inoculation on the activities of ammonium assimilating enzymes (GS and GDH), and the general metabolism of nitrogen measured by fresh mass, total nitrogen, root and shoot length and to assign the possible changes to biological nitrogen fixation, in a maize hybrid (DK 664) in interactions with two *Azospirillum* strains.

The experiment was set up in 5 dm^3 pots with a mixture of vermiculite and sand in a 2:1 ratio previously sterilized for 2 h at 120 °C. The seeds of maize (*Zea mays* L.) hybrid DK 664 were sterilized with 1 % *Chloramine T* during 15 min, and then washed twice with sterilized water and once with 34 mM phosphate buffer, pH 6.5. The pots with two maize seedlings were inoculated with 5 cm^3 of a liquid culture containing 1×10^8 CFU $seed^{-1}$. There were two inoculation treatments: *Azospirillum brasilense* strain 42 M (Ab 42M) and *Azospirillum lipoferum* Sp 242 (Al Sp242). These strains were grown for 36 h at 30 °C in NFb liquid medium. All plants were grown for 4 weeks in greenhouse and watered with a

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Abbreviations: Ab 42M - *Azospirillum brasilense* strain 42 M; Al Sp242 - *Azospirillum lipoferum* strain 242; ARA - acetylene reduction assay; CFU - colony forming units; DK 664 - argentine maize genotype Dekalb 664; GS - glutamine syntethase; GDH - glutamate dehydrogenase, PAR - photosyntetic active radiation.

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* Corresponding author; fax: (+54) 1 5248087, e-mail: acura@mail.agro.uba.ar

modified half-strength Hoagland and Arnon (1938) solution, pH 6.0, without nitrogen. Only the fertilized variant received 10 mM NH_4NO_3 as the nitrogen source. The number of replicates was 24 per treatment, 8 replicates were harvested at 10, 20, and 30 d and all measurements were made. Total nitrogen in leaf samples was measured by the micro-Kjeldahl method in triplicate (Lang 1958). For the nitrogenase activity determination, twenty plants per treatment were grown in 100 cm^3 glass tube containing sterilized vermiculite and sand in 2:1 ratio closed with a sterilized cotton stopper. Nitrogenase activity was determined with gas chromatography, by the acetylene reduction assay (ARA) (Hardy *et al.* 1968).

The seeds were inoculated after sowing with 1 cm^3 *Azospirillum* spp. culture, grown overnight (absorbance at 600 nm reached 0.55). The control plants received the same volume of sterilized NFb medium. All plants were kept for 10 d in a growth chamber under 200 µmol $m^{-2}s^{-1}$ PAR (16-h photoperiod) and day/night temperature of 30/25 °C. The leaves and roots (1 g) were homogenized and assayed according to Loyola Vargas and Sánchez de Jimenez (1984) for GDH. GS extraction and activity were carried out according to Yuan *et al.* 1980. The experiment was carried out in duplicate. Proteins were determined according to Bradford (1976).

At end of the experiment (30 d), the roots of 4 plants for each treatment, were taken at random and the number of bacteria per g of roots was determined in triplicate, by the most probable number method (Döbereiner *et al.* 1995). The design was a fully randomized statistical model. Variance analysis of the experimental data was performed using an *ANOVA* software package, and LSD was calculated at $P = 5$ %.

We observed in previous experiments that the combination between Ab 42M and the commercial hybrid DK 664, resulted in an increased root and leaf nitrate reductase activity of inoculated plants, when compared with the non-inoculated plants (Ribaudo *et al.* 1998). In order to understand if *Azospirillum* spp. is able to induce a response in the enzymes involved in the ammonium assimilation of inoculated plants, we decided to investigate the changes in the GDH and GS activity and agronomical parameters throughout assay. Our results show that a potential benefit for maize inoculation does exist. Maize root cell free extracts of inoculated plants show enzyme activity increase over that of the non-inoculated controls (Fig. 1).

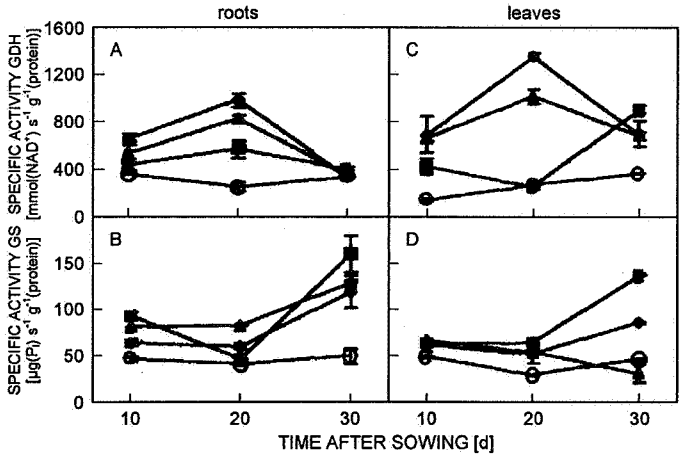

Fig. 1. Specific activity dynamics of glutamate dehydrogenase (GDH) (*A*, *C*) and glutamine syntethase (GS) (*B*, *D*) in roots (*A*, *B*) and leaves (*C*, *D*) of maize plants: *open circles* - control, *squares* - fertilized, *triangles* - inoculated with *Azospirillum brasilense* 42 M, *rhombs* - inoculated with *Azospirillum lipoferum* Sp 242. Means of three replicates. Vertical bars indicate SE and they are drawn only when larger than symbols.

During the second and third weeks (10 and 20 d after sowing) maximum differences were observed in NADH-GDH activity. The GDH activity from plants inoculated with Ab 42M increased between 82.5 and 285 %, and plants inoculated with Al Sp242 increased between 59.4 and 222.5 %, both of them compared with the respective controls. The maximum values obtained from inoculated plants, in comparison with the non-inoculated ones, were 392 % for those inoculated with Ab 42M and 269 % for those plants inoculated with Al Sp242 (Fig. 1*A*,*C*).

The GS leaf activity from inoculated plants showed a greater increase toward the end of the assay (30 d after sowing), tough it was significantly different from the non-inoculated plants in almost all the assay. The values obtained were 83, 86 and 196 % for GS activity from plants treated with Ab 42M, Al Sp242, and fertilized with NH_4NO_3, respectively. The changes in root GS activity were similar to those in leaf GS activity (139, 161 and 222 % for plants inoculated with Ab 42M, Al Sp242 and fertilized with NH_4NO_3, respectively) (Fig. 1*B*,*D*).

The seedlings inoculated with *Azospirillum spp* displayed the maximum peak as regards NADH-GDH activity (roots and leaves) 20 d after sowing whereas GS activity had a small decrease at this point. All groups of

seedlings that received nitrogen as a fertilizer or derived from bacterial nitrogen fixation had the same pattern of curves for NADH-GDH and GS activity along the duration of the experiment. In controls, the activities of these enzymes were lower than in the treatments named before. Magalhães *et al.* (1995) found an increase in GS and NADH-GDH activities in response to NH_4^+. In maize inoculated with *Azospirillum,* Fallik *et al.* (1988) observed changes in GS activity in roots during the first week after sowing. We found that the increase was greater in the fourth week after sowing, but significant differences were seen from 10 until 30 d after sowing. The increase in the activities of both, GDH and GS in inoculated plants, agreed with the nitrogen content present in green tissues. Ten d after sowing, interaction between *Azospirillum* spp. and maize nitrogenase activity was confirmed by ARA. The values obtained were: 3.98 ± 0.59 nmol(C_2H_4) kg^{-1} (d.m) s^{-1} for inoculated plants with Ab 42M and 9.9 ± 1.78 nmol(C_2H_4) kg^{-1}(d.m) s^{-1} for inoculated plants with Al Sp242. The N content in leaves measured at 30 d after sowing, was 0.69 ± 0.036 % for non-inoculated plants, and 2.43 ± 0.078 % for fertilized plants, 1.14 ± 0.077 % for plants inoculated with Ab 42M and 2.63 ± 0.17 % for plants inoculated with Al Sp242 (Table 1).

These values support the idea that bacterial nitrogen fixation had taken place and was the responsible for the better growth of seedlings. The correlation between N percentage in leaves and fresh mass was high in this assay (r = 0.89). The only way for the higher nitrogen content to be in leaves of inoculated plants is that it comes from the nitrogen in the air and that it is incorporated to the green tissues through biological fixation. The reason for this is that the plants in our experiment were grown in an inert medium (vermiculite:sand) and watered with nitrogen-free Hoagland solution. It is possible that, close to the final stage of development, nitrogen requirement were higher and - under limiting conditions of this element - bacterial nitrogen fixation is increased; therefore, N percentage in leaf of inoculated plants is almost the same as that of fertilized plants. In plants inoculated with Ab 42M, we observed the lowest value and this agree with a disminished GS activity in leaves.

Table 1. Effects of inoculation with *Azospirillum* on shoot and root length, fresh mass and nitrogen content in maize plants measured 10, 20 and 30 d after sowing. Means followed by the same letter are not significantly different at $P = 0.05$, $n = 8$.

	Root length [cm]			Shoot length [cm]			Fresh mass [g]			N [% d.m.]
Time after sowing [d]	10	20	30	10	20	30	10	20	30	30
Control	25cd	26cd	28.4cd	16.4e	24.3d	24d	1.5e	2.3e	1.7e	0.69 ± 0.04
Fertilized	23.8d	21.6d	38.6b	16.6e	31.8c	45.5a	1.5e	3.5ed	16.1a	0.43 ± 0.08
A. brasilense 42 M	26cd	33bc	46.5a	27.3d	34.6bc	47.3a	4.5d	5.2d	8.4c	1.14 ± 0.13
A. lipoferum Sp 242	30.6c	34.5bc	42.7ab	25.4d	33.1c	38.4b	3.3ed	6.3d	11.6b	2.63 ± 0.17

The number of microorganisms present in the roots was 2.75 ± 0.45 × 10^8 MPN(cell) g^{-1}(root) for Ab 42M, and 2.50 ± 0.37 × 10^7 MPN(cell) g^{-1}(root)MPN for Al Sp242. The presence of bacteria nitrogen-fixing was confirmed in the control plants, but in a smaller amount (9 ± 0.15 × 10^2 MPN(cell) g^{-1}(root). However, these did not grow a in medium containing 100 μg cm^{-3} of streptomycin sulphate. Neither bacteria could be isolated from fertilized plants. The effect of inoculation on root and shoot length, and shoot fresh mass was monitored during all the experiment. With regard to root length, maximum differences were observed at 30 d after sowing in both inoculation treatments as compared with the non-inoculated plants (Table 1). Plants inoculated with Ab 42M and Al Sp242 displayed differences in fresh mass higher than those of controls as from 10 d after sowing. Kapulnik *et al.* (1985) found an increase in fresh and dry mass of leaves that was conserved during all the assay in maize for forage. The improvement in GDH activity from inoculated plants during the first 3 weeks agrees with significant increase in the fresh mass, length of roots and foliage of inoculated maize. The other enzyme, GS is also capable of responding in a positive manner to the inoculation. Mechanisms other than bacterial nitrogen fixation cannot be excluded, namely hormonal effects, which can increase enzyme activity of GS and GDH in plant growth (Garg and Srivastava 1992).

We would like to point out that all results and observations herein described were obtained from plantelets grown under greenhouse conditions and in poor soil (vermiculite:sand). Results may be completely different under other types of soil and field conditions.

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