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Effect of low pH on the enzyme activities of the ammonium assimilation pathways in the symbiotic association *Bradyrhizobium* sp. peanut (*Arachis hypogaea* L.)

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Abstract
The activities of glutamine synthetase (GS, E.C. 6.3.1.2) and glutamate synthase (GOGAT, E.C.1.4.1.13) were determined in leaves, stems, roots and nodules of peanut plants inoculated with *Bradyrhizobium* sp. SEMIA 6144 and grown in soils with neutral reaction (pH 7.0) or acid reaction (pH 5.5). Leaves, stems and nodules showed no significant differences in those activities between the different trial groups. A high increase in the activities of these enzymes was observed in inoculated roots as compared to non-inoculated ones. The maximum increase of the specific activity of GS and GOGAT was found in inoculated roots grown in soils with acid reaction. Since inoculation and low pH of soil were necessary for a maximum in the activity of the ammonium assimilation enzymes, we suggest that *Bradyrhizobium* sp. SEMIA 6144 strain is the most sensitive part of the symbiotic association to soil acidity.

Keywords: *Arachis hypogaea*, glutamine synthetase, glutamate synthase, ammonium assimilation

1. Introduction

The ammonium available for plants can derive from the atmospheric nitrogen fixed by the Rhizobiaceae bacteria in the legume nodules or from the nitrate absorbed from the soil. NH_4^+ and organic nitrogen are also taken up from soil. Whatever its origin may be, the ammonium is then assimilated and incorporated into the nitrogenous compounds. The enzymes glutamine synthetase and glutamate synthase participate in this process by catalyzing the reactions:

L-Glutamate + NH_4^+ + ATP –> L-Glutamine + ADP + P_i
L-Glutamine + 2-oxoglutarate + NADPH (orFdx.red) –>
2L-Glutamate + NADP + (or Fdx.ox)

The two major classes of GOGAT enzymes in higher plants are a ferredoxin-dependent GOGAT (Fdx-GOGAT) and a NADPH-dependent GOGAT (NADPH-GOGAT), both plastid localized. Fdx-GOGAT is the predominant GOGAT isoenzyme in leaves and plays a major role in the reassimilation of photorespiratory ammonia. Whereas the NADPH-GOGAT isoenzyme is present in low amounts in leaves, but constitutes the main isoenzyme in nonphotosynthetic tissues, NADPH-GOGAT may function predominantly in primary nitrogen assimilation.

Large areas of arable land in the central region of Argentina are known to have progressively acidified over the last 10 to 20 years. Continuous cultivation without crop rotation has been identified as one of the main factors favoring soil acidification (Michelena et al., 1989). Thus, the loss of soil fertility and the instability in the yields of crops have been a growing concern. Hampp et al. (1997) showed that these soils have low contents of organic matter and a rather low pH on the superficial horizons. Under these conditions, the nodulation of the legume roots was poor even in those cases where inoculated seeds were used. Although the effects of the acid pH of soil on nodulation of legumes have been studied in depth, it is ignored how this soil property affects the assimilation of NH_4^+.

Peanut (*Arachis hypogaea* L.) is an important crop all over the world, since it provides direct subsistence food and several other food products. Argentina, one of the major producers in the world, concentrates about 96% of its production in the central and southern region of the province of Córdoba. Previously, we reported that *Bradyrhizobium* strains able to infect peanut roots showed a growth and viability decrease in culture medium at low pH (Taurian et al., 1998; Macció et al., 2000; Ponsone et al., 2004).

In this work, the effects of inoculation with

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Bradyrhizobium sp. on the enzymes involved in the ammonium assimilation pathways were studied in different organs of peanut plants grown in neutral (pH 7.0) or moderately acid (pH 5.5) soils.

2. Material and Methods

Bacterial strain and growth conditions

Bradyrhizobium sp. SEMIA 6144, able to infect peanut (*Arachis hypogaea* L.), was obtained from MIRCEN (Brazil). Stock cultures were maintained on YEMA (yeast extract mannitol-agar) supplemented with Congo red (Vincent, 1970). Cultures were grown in YEM medium at 28°C on a gyratory shaker at 150 rpm. The number of viable cells was determined as cfu (colony-forming units) by the drop-plate method on YEMA plates, using bacterial cultures after 48 h of incubation (Somasegaran and Hoben, 1994).

Plant growth and inoculation

Peanut seeds (*Arachis hypogaea* L. cv Tegua runner type) were kindly supplied by the nursery El Carmen S.A, General Cabrera, Córdoba, Argentina. Seeds were surface-sterilized following the method described by Vincent (1970). Sterilized seeds were germinated at 28°C in Petri dishes on one layer of Whatman N° 1 filter paper and moist cotton until the radicle reached 2–3 cm. Individual seedlings growing from sterile seeds were aseptically transferred to pots containing sterile soils of the central-southern region of the province of Córdoba (pH 7.0 or 5.5, respectively). The soil was sterilized by autoclaving at 121°C for 1 h during three consecutive days and the pH was determined using a soil/water (1/1 w v^{-1}) suspension. Two experimental groups of plants were used: a) control without inoculation, b) plants inoculated with 4 ml of *Bradyrhizobium* sp. SEMIA 6144 (1×10^8 cfu ml^{-1}). The pots were placed in a greenhouse under controlled-environment conditions (light intensity of 200 μE m^{-2} sec^{-1}, 16-h day/8-h night cycle, at a constant temperature of 28°C and a relative humidity of 50%) and watered regularly with sterile tap water according to soil field capacity (14%). Experimental samples were harvested at growth stage R1 (flowering: approximately 40 days after planting (DAP)) and used for the determinations of shoot dry weight, shoot nitrogen content, number of nodules and ammonium assimilation enzymes. For the dry weight determination, the shoots were dried at 65°C during 72 h. The nitrogen content in shoots was determined according to the procedure proposed by Nelson and Sommers (1973).

Preparation of plant protein extracts

Root, leaf and stem samples were homogenized as described in Tanimoto and Harada (1989) and the homogenates were centrifuged at 3,000 g for 20 min. Nodules (100 mg fresh weight) were detached from roots of several plants and crushed, using a pestle and mortar on ice in 2 ml of the following extraction medium: Mg-phosphate buffer (2.5 mM $MgCl_2$, 50 mM potassium phosphate, pH 6.8) and 10% (w v^{-1}) polyvinylpolypyrrolidone. The homogenate was centrifuged at 600 g for 5 min and the nodule debris were removed. All supernatants obtained were used to measure the GS activity. For GOGAT activity, the supernatants were filtrated in columns of Sephadex G-25 and the fractions with the greatest protein concentrations were used. Protein was estimated by the Coomassie blue staining method with bovine serum albumin as standard (Bradford, 1976).

Enzyme assays

Glutamine synthetase activity was measured by the synthetase assay from the amount of γ-glutamylhydroxamate (γ-GH) produced in the reaction (Bielawski, 1994). Specific activity for GS was expressed as μmol γ-glutamylhydroxamate (γ-GH) produced min^{-1} mg^{-1} protein. Glutamate synthase was determined by the method of Ratti et al. (1985). Specific activities were expressed as nmol NADPH oxidized min^{-1} mg^{-1} protein.

Statistical analysis

Data were analyzed statistically by one-way ANOVA and means were compared with Duncan's multiple mean comparison test. A level of $p<0.05$ was accepted as significant.

3. Results and Discussion

Effect of the inoculation on the peanut growth

A favorable rhizosphere environment is highly important to the interaction between root hairs and *Rhizobium*, as it not only encourages the growth and multiplication of rhizobia but also ensures the healthy development of root hairs. Any environmental stress affecting these processes is also likely to influence infection and nodulation (Alexander, 1984). Most studies on environmental stress have been focused on the *Sinorhizobium meliloti*-alfalfa and *Rhizobium phaseoli*-bean associations. Segundo et al. (1999) reported that *S. meliloti* isolates, obtained from acid soils from Argentina and Uruguay, were able to nodule alfalfa at pH 5.6 and showed a delayed nodulation and decreased nodule number. Surprisingly, neither the dry matter nor the relative concentration of nitrogen was significantly reduced at this condition. Khradi et al. (2001) found that the reduction of the nitrogen fixation and plant growth of common bean (*Phaseolus vulgaris*) due to salt

stress was accompanied by changes in enzymatic activities and in reduced nitrogen demand of the host plant (ureides and amino acid in nodules). In these symbiotic associations rhizobia infect legumes by a mechanism involving infection threads formation. However, there is little information available on the effect of environmental stress on peanut-rhizobia interaction. This association is especially interesting because there are no cell-to-cell infection threads. Instead, infection through the epidermis involves intercellular penetration (crack entry), i.e. entry at the point of emergency of lateral roots (Boogerd and van Rossum, 1997).

In this work, the inoculation of peanut roots with *Bradyrhizobium* sp. SEMIA 6144 showed a beneficial effect on the plant growth, because neither the shoot dry weight nor the nitrogen content were affected by the acid soil. However, the nodule number decreased at acid pH in respect to neutral pH (Table 1). In spite of the lower nodule number found in plant inoculated with SEMIA 6144 at pH 5.5, the symbiotic effectiveness (shoot nitrogen content) was not affected. By comparing shoot nitrogen content with number of nodules, similar patterns were found (r=0.95) at pH 7.0 and (r=1) at pH 5.5. Thus, fewer nodules have to provide the host plant nitrogen it needs, a demand that is perhaps met by its higher effectiveness. Similar results were obtained by our group (Angelini et al., 2005) in the study of the nodulation kinetics of peanut plants inoculated with *Bradyrhizobium* sp. SEMIA 6144 in the medium BMM (Dudley and Jacob, 1987) buffered with 20 mM MES at pH 5.5; which showed a decrease of the nodules number and nodule dry weight from 15 to 60 DAP without alteration of symbiotic effectiveness at 60 DAP.

The results obtained suggest that the acidity does not act directly, but does so to a large extent by increasing the reaction to inoculation. Our findings show that the rhizobial strain is more acid-sensitive than the plant as previously suggested by Tang and Thomson (1996). Besides, the rhizobial infection is the acid sensitive event in the *Lens culinaris* nodulation, while the growth and survival of the rhizobia are not affected (Lie, 1981).

Ammonium assimilation in peanut plants

Assays of the enzymes involved in glutamate synthesis in *Bradyrhizobium* sp. SEMIA 6144 growing at pH 5.5 showed that the activities of GS/NADPH-GOGAT increased in correlation to the glutamate content. This would support the contention that the GS/NADPH-GOGAT pathway contributes to the increase of glutamate synthesis being one possible bacterial response to acid stress (Natera et al., 2002). To go deep in the knowledge of the effect of low pH, we analyzed the ammonium assimilation enzymes in the symbiosis *Bradyrhizobium* sp. peanut.

The specific activity of GS determined in the roots of plants inoculated with *Bradyrhizobium* sp. showed a significant increase with respect to non-inoculated roots

Table 1. Estimation of growth, nodulation and total nitrogen content of peanut plants under different treatments.

Treatments	Shoot dry weight mg plant^{-1}	Shoot nitrogen content mg (shoot dry weight)$^{-1}$	Number of nodules per plant
pH 7.0			
Control	360.00±20.84a	16.60±0.86a	–
Inoculated	556.67±23.36b	30.09±0.75b	18.00±0.57a
pH 5.5			
Control	356.67±29.66a	16.42±1.24a	–
Inoculated	540.66±21.32b	29.09±0.95b	13.66±0.88b

Data are means ± S.E of three independent experiments with ten replicates each per treatment. Different letters in each column indicate significant differences ($P<0.05$), according to the Duncan's test.

(Fig. 1). Although it is known that the GS activity increases by inoculation (Smith and Gallon, 1993), it reached its maximum in inoculated roots cultivated in acid soil (pH 5.5). These results suggest that the soil acidity can stimulate an increase of the root GS activity only in inoculated plants. In the nodule extract, the GS activity did not show differences at both pHs (7.0 and 5.5). On the other hand, the comparison of the GS activity and symbiotic effectiveness in nodulated roots and nodules revealed a high correlation between them (r=1) at both pHs. It is supposed that GS plays a leading role in both the primary assimilation of ammonium produced during symbiotic fixation of molecular nitrogen in nodules, and in its secondary assimilation in roots, in order to keep constant the shoot nitrogen content in peanut plants growing under acid condition.

The specific activity of GOGAT followed a similar pattern. The highest activities of this enzyme, as well as those of GS, were observed in inoculated roots grown in acid soil (Fig. 2). This coincidence between the behavior of GS and GOGAT is in accord with the joint participation of both enzymes in the assimilation of ammonia.

No significant differences in relation to the GS and GOGAT activities were observed in stems, nodules or leaves under acid stress conditions. Although the GS activity in leaves is considerable, it is known that it largely depends on the chloroplastic enzyme involved in the secondary assimilation of NH_4^+, which mainly derives from the photorespiration and is not related to the primary assimilation. The specific activities of GS and GOGAT were considerably higher in roots compared to other plant organs.

The results obtained on the ammonium assimilation enzymes in peanut at 40 DAP differ from other legumes such as lentil where GS and GOGAT activities were much higher in nodules than in roots from 40 to 90 DAP (Chropa et al., 2002). It is possible to suggest that the more

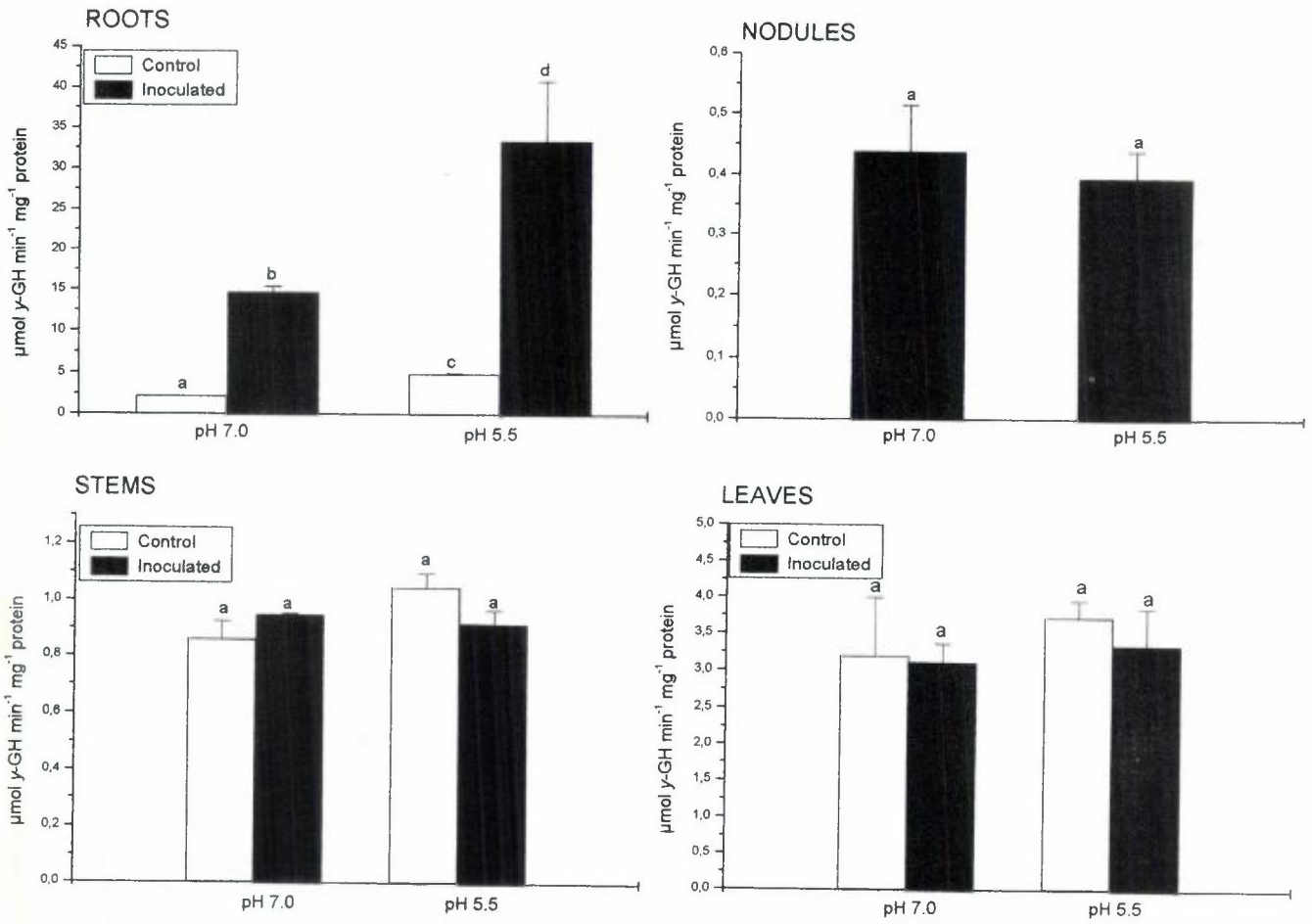

Figure 1. Glutamine synthetase specific activity of roots, stems, leaves and nodules of peanut plants. Data are means ± S.E of four independent determinations. Different letters in each column indicate significant differences ($P<0.05$), according to the Duncan's test.

availability of fixed N may modulate the enzyme gene expression in the nodule, considering that nodule GS and GOGAT are plant gene products whose expression can be influenced by the stage of the nodule development and effectiveness (Vance et al., 1988; Suganuma et al., 1999). In contrast to the result found in peanut nodules by acid stress, in faba bean nodules the GS and GOGAT activities were inhibited by salinity (Cordovilla et al., 1999), indicating that the response of GS and GOGAT activities in nodules are also subjected to the influence of different environmental stresses.

It has been found in soybean that the increased expression of at least one of the genes codifying GS1 (GS cytosolic isoenzyme), is controlled by the level of nitrogen externally provided or obtained as result of the N_2 fixing in the nodules, while in *Phaseolus vulgaris*, the ammonium by itself does not seem to act as a signal molecule (Cren and Hirel, 1999). According to our results, the stimulus (or signal) causing the increase in specific activities of GS and GOGAT in peanut roots apparently would not be directly related to the concentration of the ammonium available for the assimilation.

Because the ammonium assimilation is involved in the synthesis of amino acids, the increase observed in the GS and GOGAT activities is probably a reaction to the stress caused by the acidity of soil. It is well demonstrated that amino acids are accumulated, together with other compatible osmolytes, in response to the osmotic stress. Although a protective role of these compounds, distinct from their participation in osmotic adjustment (for example, preventing the effects of ions or scavenging hydroxyl radicals), had been suggested (Bray et al., 2000). Further studies are being carried out to elucidate the mechanism of regulation of these enzymes in response to stress condition.

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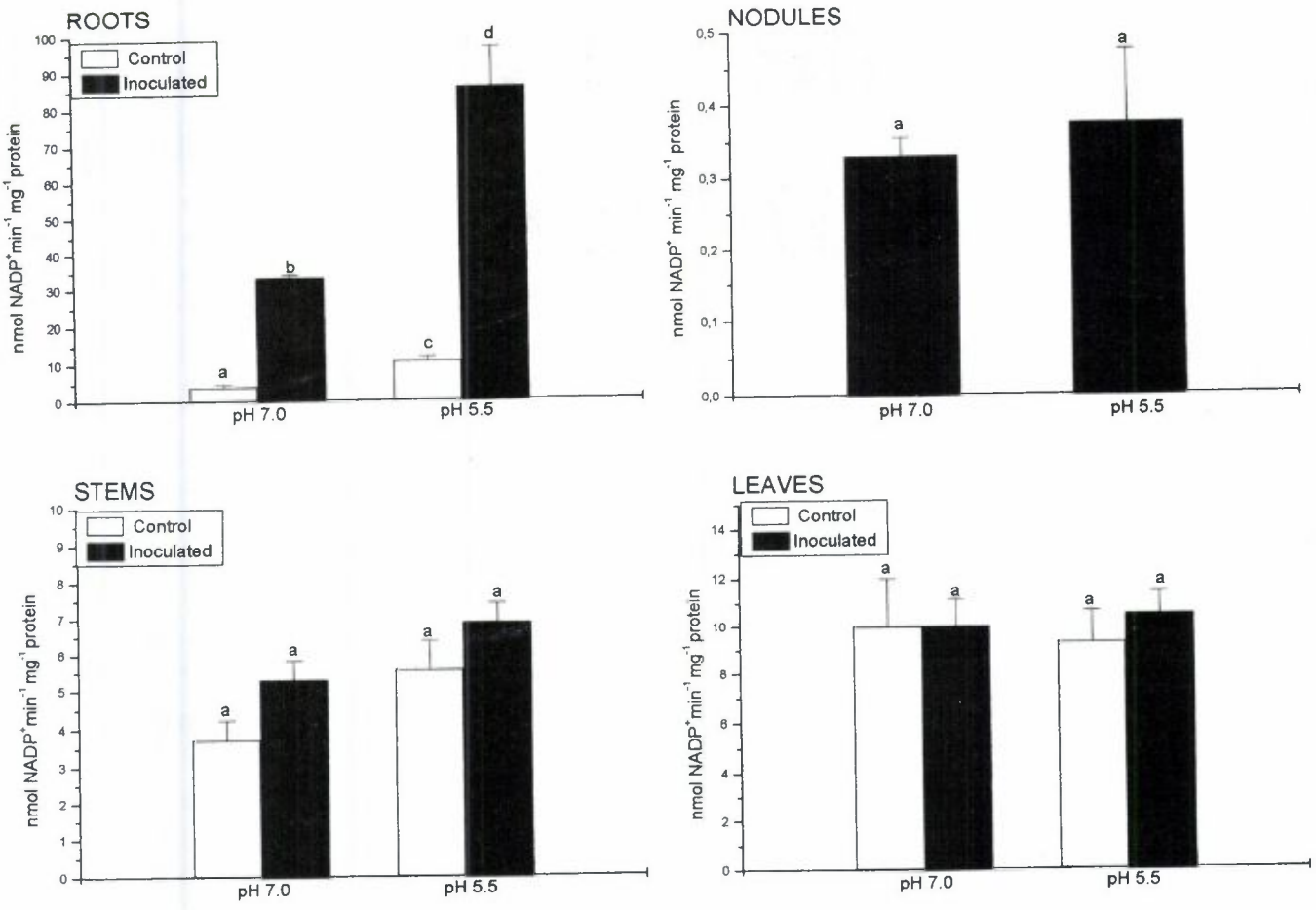

Figure 2. Glutamate synthase specific activity of roots, stems, leaves and nodules of peanut plants. Data are means ± S.E of four independent determinations. Different letters in each column indicate significant differences ($P<0.05$), according to the Duncan's test.

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