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Interactive Effects of Acidity and Aluminium on the Growth, Lipopolysaccharide and Glutathione Contents in Two Nodulating Peanut Rhizobia

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Abstract

Aluminium (Al^{3+}) is an abundant element in the soil, occurring in a wide variety of mineral forms which are largely inert at neutral pH. As the soil pH decreases, aluminium is mobilized into the soil solution and may become toxic to plants and soil microorganisms. In this work we show the effect of the combination of acidity and aluminium on two nodulating peanut rhizobia: *Bradyrhizobium* sp. SEMIA 6144, peanut recommended inoculation, and the native isolate NCHAX obtained from soil Córdoba (Argentina). *Bradyrhizobium* sp. showed a decrease in the growth rate and an increase of glutathione (GSH) content at pH 5.5 and 50 μM aluminium. Although the increase of the GSH content was not enough to prevent the bacterial growth rate decrease, the high level found could be related with a protection against the toxic effects of this metal. The isolate NCHAX growth rate and viability were not affected by the acid pH and the aluminium and the GSH content remained unchanged. However, in both peanut microsymbionts changes were found in the lipopolysaccharide (LPS) contents related to alteration in the cellular hydrophobicity under this stress condition.

Keywords: Rhizobia, peanut, Al^{3+}, LPS, cellular hydrophobicity, GSH

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1. Introduction

Aluminium toxicity is a global problem that limits crop productivity on acidic soils. Large areas of the world contain acidic soils (>30% of the arable land), so aluminium toxicity is a very important worldwide agricultural problem (Von Uexkull and Mutert, 1995). Despite the agronomic importance of this problem, little is known about fundamental mechanisms of aluminium toxicity and resistance.

The combination of acidity and aluminium has damaging effects on the root nodule bacteria growth, whose reduced reproduction further unfavourably influences both soil and host plant root colonization (Keyser and Munns, 1979). Aluminium is an abundant element in the soil, occurring in a wide variety of mineral forms (e.g. aluminosilicates) which are largely inert at neutral pH. As the pH decreases, aluminium is mobilized into the soil solution and may become toxic to plants and soil microorganisms.

Aluminium occurs in the soil solution as a range of ionic species, the balance between them depending on such factors as pH, ionic strength, the type and concentration of organic and inorganic ligands, and the presence of solid phases (Driscoll and Schecher, 1988; Ritchie, 1989). In solutions of aluminium salts at pH <5.5, aluminium is present as the $Al(H_2O)_6^{3+}$ ion which is usually written as Al^{3+} (Martin, 1988).

Although prokaryotes may be affected by fluxes in environmental factors, they are known to harbor adaptive features that enable them to circumvent many of these challenges. The mechanisms underlying the resistance of microbes to a variety of metals have been studied, but few reports on the strategies evoked in response to aluminium stress are available (Hughes and Poole, 1989). While it is tempting to believe that there will be a single major cause of aluminium toxicity common to all bacteria, it should not be forgotten that there may be different mechanisms for different organisms under different conditions (Flis et al., 1992).

There is no reason to believe that the situation is so simple. Firstly, at pH 5.5 and above, little if any aluminium would be in solution and secondly, it is difficult to see how pH would not affect the ratios between the ionic species of monomeric aluminium, even in the presence of the other organic constituents of the medium. Further, there is no a priori reason why only a single form of aluminium should be toxic; it is quite possible that several forms of aluminium may have effects which are different, but which nevertheless result in no growth.

Many possible mechanisms have been proposed to explain the action of aluminium on bacteria (Gracidueñas Piña and Cervantes, 1996). Aluminium binds to membrane components of cell walls and also interferes with iron and

calcium metabolism. Moreover, for its positively charged species may interact with nucleic acids, thus distressing cellular functions (Johnson and Wood, 1990). Appana (1996) demonstrated that the trivalent metal was localized in a phosphatidylethanolamine (PE) containing residue in the strain *Pseudomonas fluorescens* ATCC 13525 during the stationary phase of growth. This study was the first demonstration of the possible involvement of PE in aluminium detoxification. There is also evidence that Al^{3+} toxicity can cause excessive generation of reactive oxygen species (ROS) and an increase in peroxidation and/or breakdown of membrane lipids (Gutteridge et al., 1985).

Biotransformation, modifications in transport systems, and volatilization are some of the other defense strategies evoked by microbes subjected to high levels of metallic elements in their ecological niches (Silver et al., 1989). In *Escherichia coli* has been demonstrated that the molecule glutathione is involved in the binding, transformation and detoxification of a wide variety of compounds (Ferguson et al., 1998).

On the other hand, bacterial resistence to inorganic toxic ions may be determined by genes located in the chromosome or in extrachromosomal elements called plasmids. Functions coded by plasmids are usually dispensable but confer advantageous properties to host bacteria useful for survival under hostile conditions or increasing the cell's metabolic versatility in certain environments.

The study of bacterial resistence to heavy metal (Hg, As, Cd, Cu, Zn) conferred by plasmids has acquired special importance in recent years for its potential use for bioremediation processes as well as for its possible utilization in the recovery of valuable metals (Silver, 1994).

Wood and Cooper (1988) found that 50 μM aluminium caused inhibition of multiplication of the *Rhizobium leguminosarum* bv *trifolii* strains in the pH range 4.6–5.6. Among rhizobia the tolerance to acid and aluminium are independent properties. The study of Kingsely and Bohlool (1992) showed that exopolysaccharides (EPS) production correlated positively with acid tolerance, it was not found to be responsible for aluminium tolerance. Räsänen and Lindström (1997) reported that the acid tolerant *Rhizobium galegae* strain Bg7 having long O-chain LPS and an abundant EPS production turned out to be aluminium sensitive. Thus, in contrast to acid tolerance, aluminium tolerance of *Rhizobium galegae* did not correlate with LPS of the long-chain type and EPS production.

The present study was done in order to evaluate the effects of high concentrations of $AlCl_3$ and low pH (5.5) on the growth and the lipopolysaccharide content in peanut symbionts and also to investigate whether the molecule glutathione is involved in the defense mechanism in this environmental stress.

2. Materials and Methods

Bacterial strain and growth media

Bradyrhizobium sp. SEMIA 6144, able to infect peanut (*Arachis hypogaea* L.), was obtained from MIRCEN (Brazil). The native strain NCHAX was isolated from soil of the province of Córdoba (Taurian et al., 2002). Stock cultures were maintained on YEMA (yeast extract mannitol-agar) supplemented with Congo red (Vincent, 1970). Cultures were grown in the liquid minimal saline medium (MSM) described by Brown and Dilworth (1975) with 10 mM NH_4Cl as the nitrogen source and 10 mM glucose as the carbon source. The medium was buffered with either 20 mM HEPES (N-hydroxyethylpiperazine N'-2-ethanesulphonic acid) at pH 7.0 or 20 mM MES (2-(N-morpholino) ethanesulfonic acid) at pH 5.5. At low pH different $AlCl_3$ concentrations were supplemented (25, 50 and 100 μM) from the beginning of the incubation.

Growth conditions

The starter cultures (pH 7.0 or 5.5) were grown in the medium MSM to early logarithmic phase. Then, they were centrifuged (10,000 x g for 10 min), washed with sterile medium at the same pH and then used to inoculate the experimental cultures. Cultures were incubated at 28°C on a gyratory shaker at 150 rev min^{-1} for the length of the experiment. Growth was measured turbidimetrically at an absorbance of 620 nm and the pH of the medium was monitored. The number of viable cells was determined as cfu (colony-forming units) by the drop-plate method (Somasegaran and Hoben, 1994) on MSM-agar plates (pH 7.0) using bacterial cultures after 48 h of incubation at 28°C.

Protein determination

Total protein content was determined by the procedure of Bradford (1976) with bovine serum albumin as standard.

LPS content determination

LPS were extracted from cellular pellets obtained from bacteria grown in MSM medium for 48 h using the hot-phenol-water method (Westphal and Jann, 1965), then quantified using the anthrone method (Dische, 1962).

Cellular hydrophobicity determination

Cellular hydrophobicity was determined using n-hexadecane as

hydrophobic substance (Rosenberg et al., 1980). Cellular pellet was obtained from bacterial culture at 48 h of incubation by centrifugation at 10,000 g for 30 min. The pellet was washed, resuspended in distilled water (4 ml) and vortexed for 3 min with n-hexadecane (0.8 ml). The aqueous and hydrophobic phases were then allowed to separate at room temperature for 30 min. The absorbance of the aqueous phase was measured at 600 nm against distilled water before and after vortexing, and the results are expressed as the percentage of adhesion to n-hexadecane.

Glutathione content determination

The sum of the reduced and oxidized forms of GSH was determined using a kinetic assay in which catalytic amounts of GSH or GSSG and glutathione reductase bring about the continuous reduction of Ellman reagent (DTNB) by NADPH (Tietze, 1969). Each 1 ml reaction mixture contained 0.1 M phosphate EDTA buffer (pH 7.5), 80 µM DTNB, 0.12 units glutathione reductase, 0.25 mM NADPH and 100 µl cellular extract. The rate of reaction at 25°C was usually expressed as the change in absorbancy per 6 min at 412 nm.

Statistical analysis

Analyses of variance were conducted and least significant differences (LSD) at $P<0.05$ were used in comparisons of results.

3. Results

Effects of acidity and aluminium on bacterial growth rate

Bradyrhizobium sp. SEMIA 6144 showed a decrease in the growth rate and viability at low pH (5.5) and different Al^{3+} concentrations. Bacterial growth rate decreased by 50% at pH 5.5 and 50 µM Al^{3+} respect to value obtained at pH 7.0 (Fig. 1a). The number of viable *Bradyrhizobium* sp. cells determined after 48 h of incubation under different conditions were: 10^{11} cells/ml (at pH 7.0); 10^9 cells/ml (at pH 5.5); 10^8 cells/ml (at pH 5.5 and 25 µM Al^{3+}); 10^7 cells/ml (at pH 5.5 and 50 µM Al^{3+}) and 10^7 cells/ml (at pH 5.5 and 100 µM Al^{3+}). The native isolate of the province of Córdoba NCHAX rate growth was not affected by the acid pH and the aluminium concentration (Fig. 1b) and the number of viable was approximately 10^{10} cells/ml for all the treatments.

Taking into account to the data of *Bradyrhizobium* sp. growth rate, all additional analyses were carried out at the following conditions: a) pH 7.0; b) pH 5.5; c) pH 5.5 plus 50 µM Al^{3+}.

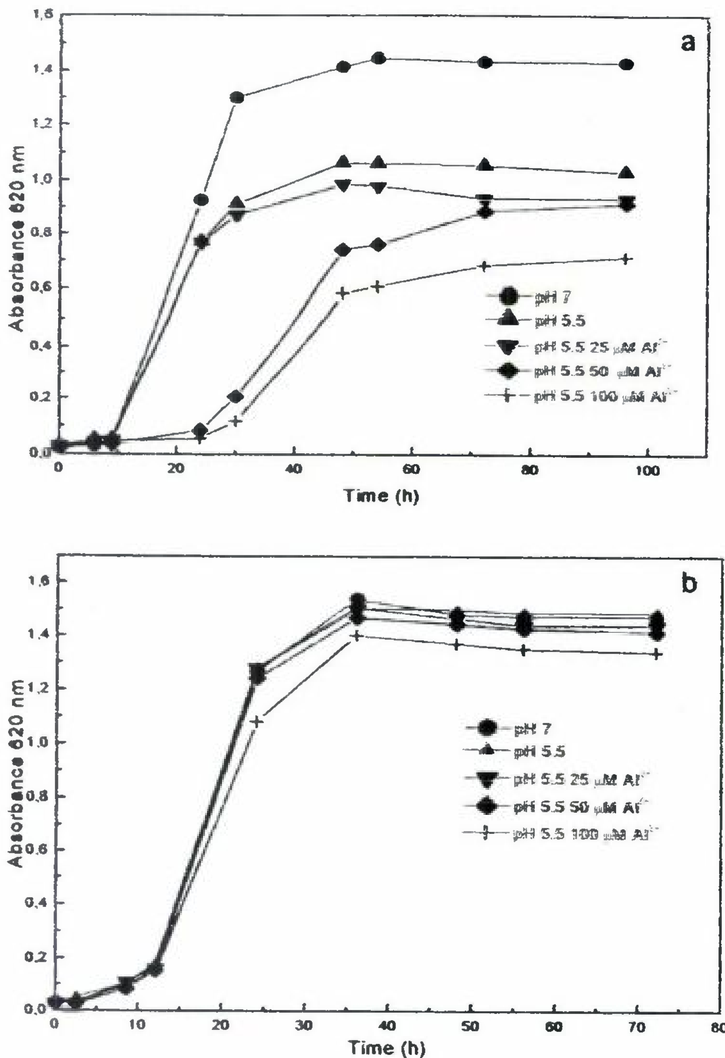

Figure 1. Effect of different pH values and aluminium concentrations on *Bradyrhizobium* sp. SEMIA 6144 (a) and the isolate NCHA-X (b) growth rate. Data are means of three independent experiments.

Effects of acidity and aluminium on LPS content and cellular hydrophobicity

Fig. 2a shows the results of LPS content in *Bradyrhizobium* sp. indicating that there were significant differences between the different treatments, the LPS content at pH 5.5 was lower than those pH 7.0 being the highest LPS content observed at pH 5.5 and 50 µM Al^{3+}. The LPS content in the native

isolate of the province of Córdoba NCHAX showed a significant increment both at pH 5.5 and at pH 5.5 plus 50 μM Al^{3+} compared with pH value 7.0. There were no differences in LPS content in presence or absence of aluminium in the medium of incubation at pH 5.5 (Fig. 2b).

The data of cellular hydrophobicity in *Bradyrhizobium* sp. showed approximately a 15% increase at pH 5.5 plus 50 μM aluminium with regard to pH value 7.0. The lowest adherence to n-hexadecane was observed when *Bradyrhizobium* sp. grew at pH 5.5 (Fig. 3a). The cellular hydrophobicity in the native isolate NCHAX showed variations at different pH values. Thus, a high adherence to n-hexadecane was found at pH 5.5 compared to pH value 7.0. The highest adherence to n-hexadecane was observed when the strain grew at pH 5.5 plus aluminium (Fig. 3b).

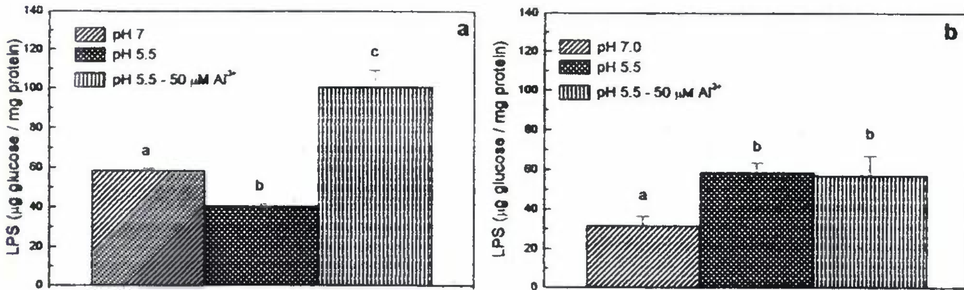

Figure 2. Effect of pH and aluminium on LPS contents in *Bradyrhizobium* sp. SEMIA 6144 (a) and the isolate NCHA-X (b). Data are means ± S.E. of three independent experiments. Different letters in each column indicate significant difference (P<0.05), according to the test LSD.

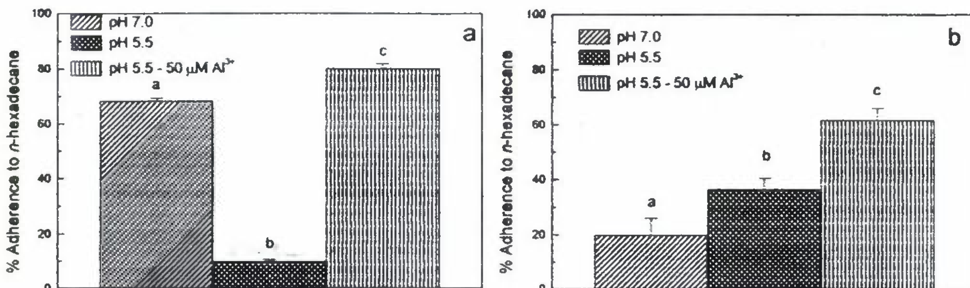

Figure 3. Effect of pH and aluminium on cellular hydrophobicity in *Bradyrhizobium* sp. SEMIA 6144 (a) and the isolate NCHA-X (b). Data are means ± S.E. of three independent experiments. Different letters in each column indicate significant difference (P<0.05), according to the test LSD.

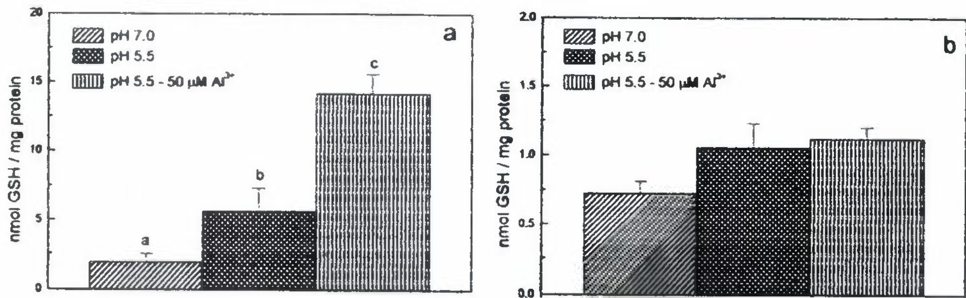

Figure 4. Effect of pH and aluminium on GSH contents in *Bradyrhizobium* sp. SEMIA 6144 (a) and the isolate NCHA-X (b). Data are means ± S.E. of three independent experiments. Different letters in each column indicate significant difference ($P<0.05$), according to the test LSD.

Effects of acidity and aluminium on GSH content

The GSH content in *Bradyrhizobium* sp. showed a significant increment at pH 5.5 in relation to pH value 7.0, whereas a higher GSH content was found when the strain grew in presence of 50 µM aluminium and low pH in the culture medium respect to different pH values. Thus, an increase 10X in the GSH content was observed compared to pH 7.0 and of 4X respect to pH 5.5 (Fig. 4a).

In the isolate NCHAX, there were no significant differences in GSH content at different treatments (Fig. 4b). The GSH content was found to be lower in the native isolate NCHAX than in *Bradyrhizobium* sp. and remained unchanged under stress condition.

4. Discussion

In this research, the estimation of the effect of the acidity on two nodulating peanut rhizobia was performed in connection with the study of the behaviour of these bacteria in aluminium-supplemented media. This combined study is necessary because the tolerance to acidity of a bacterium does not imply spontaneously a tolerance to aluminium (Keyser and Munns, 1979), and also because both conditions affect the multiplication and the survival of these bacteria in the soil (Taylor et al., 1991). Our findings indicate that *Bradyrhizobium* sp. is sensitive to acidity and high level of aluminium in the growing medium whereas the native isolate NCHAX is tolerant at this stress condition.

LPS are major components of the outer membrane of Gram-negative bacteria and are relevant to both outer membrane stability and the interaction of the

bacteria with other organisms and the environment (Kannenberg et al., 1998). Dazzo et al. (1991) demonstrated the importance of LPS in the symbiosis between rhizobia and their host plants. In this study, we demonstrate that the low pH (5.5) produced a diminution in the LPS content in *Bradyrhizobium* sp. strain and an increase in the native isolate NCHXA respect to pH values 7.0. Nevertheless, when *Bradyrhizobium* sp. grew in presence of 50 μM aluminium, the LPS content significantly increased with regard to pH value 5.5. Under the same condition, the LPS content in the native isolate NCHAX remained unchanged. These variations in the LPS content found in *Bradyrhizobium* sp. SEMIA 6144 and the native isolate NCHAX would indicate changes in the membrane permeability depending of stress conditions. In the particular case of aluminium stress, both peanut microsymbionts responded with an increase of LPS content. Similar results were found when *Bradyrhizobium* sp. strain growing in the presence of the fungicide Mancozeb (Angelini et al., 1999).

Purchase et al. (1997) demonstrated that variations in the cellular surface polysaccharides could produce changes in the cellular hydrophobicity of the Gram-negative bacteria. Thus, the increase observed in the adherence of the peanut rhizobia surface to n-hexadecane molecules may be correlated with the high level of LPS content determined at low pH value and high level of aluminium. These findings support the conclusion that the surface characteristics of *Bradyrhizobium* sp. SEMIA 6144 and the native isolate NCHAX are more hydrophobic in this stress condition.

It is well-known that the increase in GSH content in living organisms is associated with the higher defense against toxic compounds (Meister, 1988). Moreover, Ricillo et al. (2000) demonstrated that metabolic production GSH is essential to protect *Rhizobium tropici* CIAT899 strain against environmental stresses that are frequently found in nature, such acidity and osmotic or oxidative shock. Thus, the increase of GSH content in *Bradyrhizobium* sp. cultured in the presence of aluminium and low pH would be a mechanism of defense of the bacteria against the high levels of H^+ and Al^{3+} in the growth medium, although this was not enough to prevent the growth decrease.

Therefore, the role of GSH in bacterial stress protection seems to be shared by a diversity of bacteria, although the molecular basis of its action mechanism remains unknown. For the native isolate NCHAX, the results show that the GSH content did not change with the variations in pH and aluminium, indicating that the tolerance of this isolate would be related with a different mechanism.

In conclusion, the data presented here demonstrate the importance of lipopolysaccharides and glutathione for growth and survival of *Bradyrhizobium* sp. under acidity and aluminium stress. By the contrary, these molecules seems not to be involved in the native strain's tolerance to Al toxicity and acidity. In order to analyse the role of GSH in the response of

Bradyrhizobium sp. to this stress condition, study of glutathione-deficient mutant is being carried out.

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