Aluminium tolerance in the tropical leguminous N_2-fixing shrub *Acaciella angustissima* (Mill.) Britton & Rose inoculated with *Sinorhizobium mexicanum*

Tolerancia al aluminio en la leguminosa arbustiva tropical fijadora de N_2 *Acaciella angustissima* (Mill.) Britton & Rose inoculada con *Sinorhizobium mexicanum*

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ABSTRACT

The tropical legume *Acaciella angustissima* inoculated with or without *Sinorhizobium mexicanum* ITTG R7^T strain was cultivated in an aluminium (Al) spiked soil to study its tolerance towards this metal. Seedlings of *A. angustissima* were grown in soil with 0, 2, 4 or 6 mg Al kg^{-1}. The effect of Al and inoculation on growth, nodulation, and nitrogen and tannins content in the plants was monitored. Height and total and dry root weight of plantlets decreased significantly when soil was spiked with Al, but increased when inoculated. Al decreased the number of nodules. Inoculum increased total N and tannin content of the plantlets. It was found that Al reduced the growth of the tropical leguminous N_2-fixing shrub *A. angustissima*, while inoculation with *S. mexicanum* ITTG R7^T stimulated its growth and increased its tannin and N content.

Keywords: Aluminium tolerance, plant growth, nodulation, nitrogen and tannins content, *Sinorhizobium mexicanum* ITTG R7^T, soil.

RESUMEN

La leguminosa tropical *Acaciella angustissima* inoculada con o sin la cepa *Sinorhizobium mexicanum* ITTG R7^T fue cultivada en un suelo tratado con (Al) para estudiar la tolerancia a este metal. Las plantas de *A. angustissima* fueron crecidas en el suelo con 0, 2, 4 y 6 mg de Al kg^{-1}. Se hizo un seguimiento del efecto de Al e inoculación en el crecimiento, la nodulación, el contenido de nitrogeno y el contenido de taninos en las plantas. La altura, el peso seco total y el peso seco de la raíz de las plantas decrementó significativamente cuando el suelo fue tratado con Al, pero incrementó cuando fue inoculado. El Al disminuyó el número de nódulos. El inóculo incrementó el N total y el contenido de taninos de las plantas. En este experimento fue encontrado que el Al reduce el crecimiento de la leguminosa arbustiva tropical fijadora de N_2 *A. angustissima*, mientras la inoculación con *S. mexicanum* ITTG R7^T estimula el crecimiento e incrementa el contenido de taninos y nitrógeno.

Palabras clave: Tolerancia al aluminio, crecimiento de planta, nodulación, contenido de nitrógeno y taninos, *Sinorhizobium mexicanum* ITTG R7^T, suelo.

INTRODUCTION

Intensive agriculture practices, such as the use of large amounts of N fertilizers, acidifies soil (Izaguirre-Mayoral *et al.* 2002, Poschenrieder *et al.* 2008). Oxidation of NH_4^+ derived from urea or ammonium sulphate acidifies the soil which increases the availability and thus the toxicity of certain metals, especially aluminium (Al) (Kochian *et al.* 2004). Below pH 5, Al becomes soluble and is bound to organic matter or found as Al^{3+}or $Al(OH)_3$ (Kinraide 1991). Aluminium is highly toxic to plants and inhibits their development, decreases biomass production and crop yields (Kinraide & Hagerman 2010). Acid soils can be found in large parts of the world, particularly in the tropics, limiting food production in most developing countries (Poschenrieder *et al.* 2008).

Al toxicity is associated with changes in the physiological and biochemical processes of plants and consequently their productivity (Mora *et al.* 2006, Meriño-Gergichevich *et al.* 2010). The decrease in root growth is one of the initial and most evident symptoms of Al toxicity in plants (Chen *et al.* 2011), thereby reducing the capacity for water and nutrient uptake. Above ground plant parts may also be affected by Al phytotoxicity (Peixoto *et al.* 2002, Garzón *et al.* 2011). At the cellular level, toxic Al triggers an overproduction of oxygen reactive species (ROS) in cells (Ma 2005, Meriño-Gergichevich *et al.* 2010), which alters the functionality of the biomembranes inducing oxidative damage in plants (Boscolo *et al.* 2003, Cristancho *et al.* 2011).

Although exclusion from root tips and restriction of Al transport to upper plant parts seemed to be the most important mechanisms that allow certain crops and wild plants to grow on acid soils with high Al^{3+} availability, other species tolerate relatively high Al concentrations not only in roots (Barcelo & Poschenrieder 2002, Panda & Matsumoto 2007).

Phenolic compounds, such as the tannins, flavonols, flavan-3-ols and anthocyanidins, are characterized by one or more hydroxylated aromatic rings, and they represent a broad range of plant compounds (Tolrà *et al*. 2009). The concentration of phenolic compounds in plants can increase when stressed. For instance, Chen *et al.* (2011) reported that tea plants increased the amount of phenolic compounds, i.e. catechin, when exposed to elevated Al concentrations. Exudation of phenolic compounds by the roots might play a role in the exclusion of Al. Phenolic compounds can form complexes with metals, such as Al, thereby reducing their toxic effects (Kochian *et al.* 2004, Chen *et al.* 2011).

Acaciella angustissima (Mill.) Britton & Rose is a leguminous N_2-fixing shrub that was formerly considered as a *Mimosa* species and later as a member of the *Acacia*, but has now been classified as belonging to the genera *Acaciella* together with other American acacias (Rico-Arce & Rodríguez 1998, Rico-Arce & Bachean 2006). *A. angustissima* can be found in arid and semiarid regions of Mexico (Rzedowski 1978). It is used as firewood, the leaves as forage for goats and sheep and its bark has traditionally been extracted for tannins used to tan hide (Rincón-Rosales & Gutiérrez-Miceli 2008). This legume is characterized by a tuberous main root with numerous lateral ones, abundant foliage and fast growth (Dzowella 1994, Rico-Arce & Bachean 2006). It establishes a symbiosis with N_2-fixing bacteria. Recently, one of the strains that was isolated from *A. angustissima* was *Sinorhizobium mexicanum* ITTG R7^T characterized by a large acidity and salinity tolerance (Lloret *et al.* 2007). *A. angustissima* can grow in nutrient depleted saline or acid soils under semi-arid conditions (Ponce-Mendoza *et al.* 2006). *A. angustissima* could thus easily be used to revegetate eroded soil and as such restore soil fertility. Concordantly, the FAO has developed the Acacia pilot project (http://www.fao.org/news/story/en/item/80060/icode/). Its goal is to promote the use of *Acacia* plants to prevent erosion, restore soil fertility and to show how these trees provide food, fuel, shelter and income during times of hardship.

It remains to be seen, however, if *A. angustissima* tolerates acid soils and large concentrations of Al. Therefore, seedlings of *A. angustissima* were inoculated with or without the strain *S. mexicanum* ITTG R7^T and cultivated in soil spiked with 0, 2, 4 or 6 mg Al kg^{-1}. Growth, nodule formation, total N and tannin content were determined. The objective of this study was to investigate the tolerance of *A. angustissima* inoculated with *S. mexicanum* towards Al.

MATERIALS AND METHODS

Bacterial strain

Acaciella angustissima plants were inoculated with the *S. mexicanum* strain ITTG R7. This strain was isolated recently from nodules of *A. angustissima* (Lloret *et al.* 2007). It is characterized by a high potential to nodule formation and N_2-fixation (Rincón-Rosales *et al.* 2009). The bacteria was grown on PY medium (peptone of casein, 5.0 g; yeast extract, 3.0 g; $CaCl_2$, 0.6 g; distilled water, 1 l) at 28 ºC and preserved at 4 ºC until used (Toledo *et al.* 2003).

Seed treatment and germination

Seeds of *A. angustissima* were scarified with concentrated H_2SO_4 for 10 min, surface sterilized with 1 % (v/v) hypochlorite for 10 min and rinsed eight times with sterile distilled water (Rincón *et al.* 2003). Treated seeds were germinated on 0.8 % agar-water plates at 28 ºC for 48 h (Ruiz-Valdiviezo *et al.* 2009).

Soil characteristics, treatment and experimental design

A clayey soil (type oxisols) was collected at Parral in the municipality of Villacorzo, Chiapas, Mexico (16º 11’ N, 93º 16’ W and 580 m altitude). The soil was cultivated with maize and common bean for > 50 y. The soil with pH H_2O (1:2) 4.6 and EC 0.36 dS m^{-1}, had an organic carbon content of 1.8 g kg^{-1} soil and a cation exchange capacity of 14.36 cmole (p+) kg^{-1} soil. The extractable Al was 3.0 mg kg^{-1} soil.

Two kg air-dried and 2 mm-sieved soil was sterilized to study the specific effects of *S. mexicanum* ITTG R7^T strain and added to polyethylene-lined earth pots to avoid contamination. Fifty ml of an aluminium sulfate solution was added to the soil at four application rates, i.e. 0, 2, 4 or 6 mg Al kg^{-1} soil. The pots were saturated with deionized water and stored for 30 days to equilibrate the applied Al.

Ten seeds scarified as described above were placed in each pot and thinned to five plants when the seedlings lost the cotyledon, i.e. approximately 10 d after sowing. Half of the seedlings were inoculated with 2 ml of a solution with 1×10^6 cells of *S. mexicanum* ITTG R7^T strain ml^{-1} while the other half was left inoculated. A sterile quarter strength Jensen´s medium was added to the plants to provide the basic nutrient requirements (Vincent 1970). Each treatment with six plants was replicated six times in a completely randomized design in a greenhouse. The soil was irrigated to field capacity with deionized water throughout the experiment.

Ten days after Al application, two plants were collected at random from each treatment. Damages to the different plantlets parts were determined using a photonic microscope Carl Zeiss Model K-4 (Germany). Plant height was measured every 10 days. After 120 days, plants were collected and number of nodules counted. The plants were oven-dried at 40°C and the dry matter of leaves, stem and roots was measured. The plants were characterized for total N and tannin content.

Plant characteristics

Total N in the plants was determined using the Kjeldahl method (Bremner 1996). The concentration of Al was determined in a digested 0.5 g plant sub-sample by atomic absorption spectroscopy (Izaguirre-Mayoral 1992). A 30 mg sub-sample of each root and shoot was analyzed for tannin content using the tungsten-molybdenum-phosphorus method (Miranda 2000).

Statistical analysis

Plant characteristics were subjected to one-way analysis of variance using PROC GLM (SAS Institute 1989) to test for significant differences between spiking with aluminium and inoculation with *S. mexicanum* and the least significance difference was then calculated (Table I). The overall effect of the two factors investigated, i.e. aluminium and inoculation, and their interactions were determined (Table II).

Table I. Plant height (cm), total dry plant and root weight (mg), number of nodules and total N and tannin content (mg kg^{-1}) in *Acaciella angustissima* plants cultivated in a soil spiked with different concentrations of Al and inoculated with or without *Sinorhizobium mexicanum*.

Tabla I. Altura de planta (cm), peso seco total de planta (mg), peso seco de raíz (mg), número de nódulos, contenido total de N (mg kg^{-1}) y contenido de taninos (mg g^{-1}) en plantas de *Acaciella angustissima* cultivadas en un suelo adicionado con diferentes concentraciones de Al e inoculado con o sin *Sinorhizobium mexicanum*.

		Aluminium concentration (mg kg^{-1} soil)					
Plant characteristics	Inoculum	0	2	4	6	LSD [a]	P value
Height (cm)	No	13.8[b]	9.5	7.8	6.3	2.1	<0.0001
	Yes	24.5	19.8	18.0	15.5	1.8	<0.0001
	LSD	1.7	2.8	1.8	2.2		
	P value	<0.0001	<0.0001	<0.0001	<0.0001		
Total dry weight (mg)	No	0.80	0.62	0.58	0.48	0.14	0.0016
	Yes	1.32	1.07	0.97	0.91	0.25	0.0218
	LSD	0.16	0.20	0.28	0.26		
	P value	0.0002	0.0017	0.0143	0.0059		
Root dry weight (mg)	No	0.43	0.35	0.34	0.24	0.10	0.0134
	Yes	0.73	0.63	0.50	0.48	0.14	0.0063
	LSD	0.16	0.14	0.15	0.08		
	P value	0.0043	0.0022	0.0446	0.0004		
Number of nodules	No	NA[c]	NA	NA	NA	NA	NA
	Yes	9.5	4.2	5.7	7.5	2.0	0.0005
	LSD	NA	NA	NA	NA		
	P value	NA	NA	NA	NA		
Total N (mg kg^{-1})	No	5.7	5.8	6.7	5.5	1.5	0.3756
	Yes	14.4	10.9	11.2	11.5	1.0	<0.0001
	LSD	0.14	0.09	0.13	0.19		
	P value	<0.0001	<0.0001	0.0002	0.0003		
Tannins (mg kg^{-1})	No	0.37	0.46	0.55	0.56	0.09	0.0031
	Yes	0.82	0.50	0.58	0.85	0.07	<0.0001
	LSD	0.10	0.05	0.09	0.12		
	P value	<0.0001	0.0735	0.4971	0.0010		

[a] LSD: Least significant difference ($P < 0.05$), [b] Mean of six plants, [c] ND: Not applicable. / [a] LSD: Menor diferencia significativa ($P < 0.05$), [b] Promedio de seis plantas, [c] ND: No aplicable.

Table II. Effect of aluminum (ALU) and inoculation with *Sinorhizobium mexicanum* (INO) and their interactions on plant height (cm), total dry plant and root weight (mg), number of nodules and total N and tannin content (mg g^{-1}) in *Acaciella angustissima* plants cultivated in the greenhouse.

Tabla II. Efecto del aluminio (ALU) e inoculación con *Sinorhizobium mexicanum* (INO) y sus interacciones en la altura de planta (cm), el peso seco total de planta (mg), el peso seco de raíz (mg), el número de nódulos, el contenido total de N (mg g^{-1}) y el contenido de taninos (mg g^{-1}) en plantas de *Acaciella angustissima* cultivadas en invernadero.

Factor	Plant height		Total dry plant weight		Root dry weight		Number of nodules		Total N		Tannin content	
ALU	62.53	<0.0001	11.21	<0.0001	11.07	<0.0001	12.62	<0.0001	6.87	0.0017	21.00	<0.0001
INO	517.97	<0.0001	91.46	<0.0001	77.75	<0.0001	NA[a]	NA	425.70	<0.0001	109.26	<0.0001
ALU*INO	0.50	0.6858	0.33	0.8066	1.53	0.2326	NA	NA	9.87	0.0002	26.96	<0.0001

[a] NA: Not applicable/No aplicable.

The relationships between the different plant characteristics were visualized by principal component analysis (PCA). Details of the PCA analysis can be found in Vásquez-Murrieta *et al.* (2007). Briefly, only principal components with Eigenvalues >1 and that explained >10% of the total variance were retained. The matrix of 36 columns (6 treatments soils with six plants) and six lines (variables) was used for PCA. All analyses were performed using the SAS statistical package (SAS 1989).

RESULTS

The *A. angustissima* plants grown in soil spiked with Al, but without *S. mexicanum*, showed visible toxicity symptoms. Microscopic analysis showed that the plants had less lateral roots compared to those cultivated in untreated soil (Fig. 1). The observed roots were thin with slightly damaged tips indicating cellular necrosis. The plants treated with *S. mexicanum* ITTG R7^{T} cultivated in soil spiked with Al showed some damage to the root system, mainly to the lateral roots. The superior part of the roots, however, showed a large number of roothairs without alterations.

The height, the total and root dry weight of *A. angustissima* decreased with increased application of Al independent of inoculation, while the concentration of tannin increased in the uninoculated plantlets (Table I). The number of nodules, total N and the tannin content was also affected by spiking soil with Al, but the effect was not related to the amount applied.

Plant height, total and root dry weight was significantly affected by inoculation and Al added to soil ($P < 0.0001$) (Table II). The number of nodules was significantly affected by the application of Al to soil ($P < 0.0001$). Inoculation, spiking soil with Al and the interaction between them significantly affected the concentration of N and tannin in the plantlets ($P \leq 0.0017$).

The effect of Al on the characteristics of the uninoculated plantlets is clearly shown in scatter plot (Fig. 2). An increased Al application rate reduced plant development, i.e. PC1 becomes more negative when the amount of Al added to soil increased, while the tannin content increased, i.e. PC2 increases. PC1 also decreased for the inoculated plantlets with increased Al content in the soil, but the effect on PC2, i.e. the tannin content was less clear.

The Al concentration was significantly larger in inoculated plants compared to non-inoculated ones and increased with increased Al application rates (Table I). The Al content in the aerial parts of the non-inoculated *A. angustissima* plants cultivated in soil spiked with 2, 4 or 6 mg Al kg^{-1} soil was significantly 1.7, 1.8 and 3.7 times higher than those in cultivated soil with 0 mg Al kg^{-1}, respectively, while in the roots it was 1.6, 1.8 and 4.0 times higher. The Al content was higher in roots of the inoculated and non-inoculated plants compared to that in the aerial parts. The Al content in the inoculated plants roots was 2.5 times higher than in the non-inoculated plant roots.

FIGURE 1. Seedlings of *Acaciella angustissima* grown in aluminum spiked soil and inoculated with *Sinorhizobium mexicanum* ITTG R7^T. Plant: P_1= 0 mg Al kg^{-1}; P_2= 2 mg Al kg^{-1}; P_3= 4 mg Al kg^{-1} and P_4= 6 mg Al kg^{-1}.

FIGURA 1. Plantas de *Acaciella angustissima* crecidas en un suelo adicionado con aluminio e inoculado con *Sinorhizobium mexicanum* ITTG R7^T. Planta: P_1= 0 mg Al kg^{-1}; P_2= 2 mg Al kg^{-1}; P_3= 4 mg Al kg^{-1} y P_4= 6 mg Al kg^{-1}.

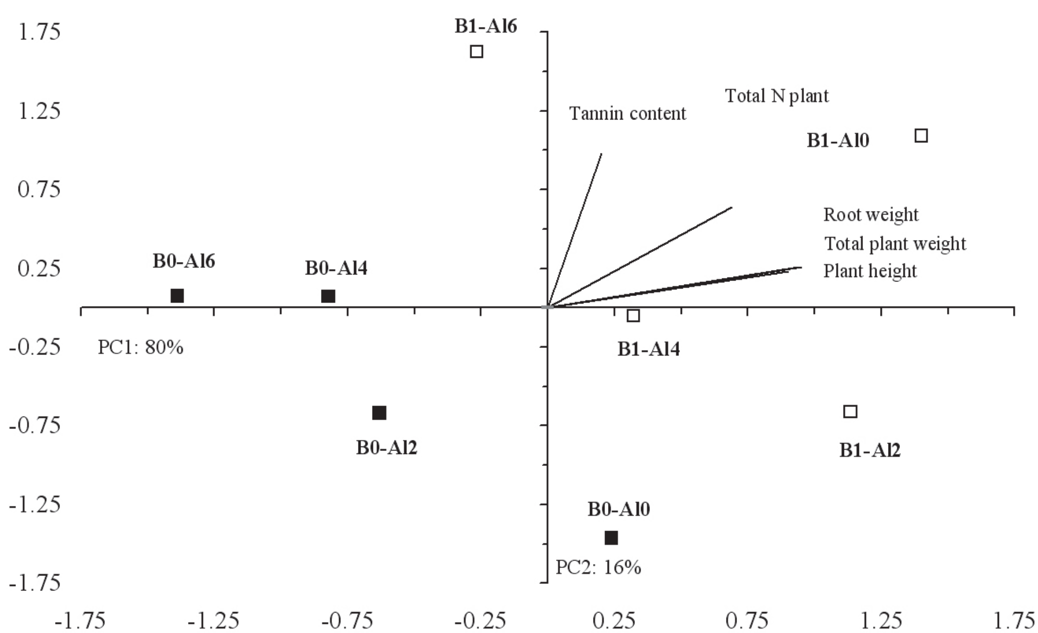

FIGURE 2. Principal component analysis (PCA) performed on characteristics, i.e. tannins content, total N plant, total plant weight, root weight, plant height of *Acaciella angustissima* spiked with aluminum and inoculated with *Sinorhizobium mexicanum* ITTG R7^T.

FIGURA 2. Análisis de components principales (PCA) realizado sobre las características, i.e. contenido de taninos, N total de planta, peso total de planta, peso de raíz y altura de planta de *Acaciella angustissima* (Mill.) Britton & Rose adicionado con aluminio e inoculado con *Sinorhizobium mexicanum* ITTG R7^T.

DISCUSSION

Growth of *A. angustissima* plants was inhibited when cultivated in soil spiked with Al and not inoculated with *S. mexicanum*, leaf fall was faster and stems were intense red-purple and thinner than those of plants cultivated in soil without Al addition. Similar toxicity symptoms have been observed in several species of tropical legumes (Hossain *et al.* 2005, Watanabe *et al.* 2006, Scott *et al.* 2008). Silva *et al.* (2010) reported that the primary symptom of Al toxicity in the plants is a rapid, i.e. within minutes, inhibition of root growth resulting in a reduced and damaged root system, limited water and mineral nutrient uptake and reduced growth.

The amount of root hairs was lower for plants cultivated in Al-contaminated soil. The root hairs are important because they excrete chelating substances that prevent Al^{3+} ions from entering the plant. Root tips of soybean (*Glycine max* L.) release citrate, while wheat (*Triticum aestivum* L.) malate (Kinraide & Hagerman 2010, Xu *et al.* 2010). Flavonoids such as catechin that protect against toxicity of the Al^{3+} (Nguyen *et al.* 2003). The citrate, malate and flavonoid are organic compounds that form a stable complex with Al that are not phytotoxic for the plant (de Andrade *et al*. 2011). Root hairs also facilitate the communication and molecular recognition between the plant and the rhizobia bacteria (Räsänen *et al.* 2001) so that nodules can be formed (Pueppke & Broughton 1999). It has been reported that nodule tissues participate in the exclusion of aluminium and as such protecting the plant against toxic effects of Al (Barcelo & Poschenrieder 2002).

Nodules were formed on the roots of *A. angustissima* inoculated with *Sinorhizobium mexicanum* ITTG R7^T strain cultivated in soil spiked with Al. Shirokikh & Shirokikh (2007) reported that a *Rhizobium leguminosarum* bv. Trifolii 9-4A strain isolated from red clover (*Trifolium pratense* L.) can fix N_2 in spite of the presence of Al. The presence of nodules that fix nitrogen positively affects growth of plants (Räsänen *et al.* 2001). Nodule formation allowed *A. angustissima* to grow taller than plants without nodules as N_2 was fixed and made the plants more resistant to Al toxicity. Al toxicity normally reduces plant growth. Phytotoxic studies on the effect of Al on growth of the legume *Vigna radiate* L. showed that increased Al concentration reduced dry weight of the seedlings significantly (Neogy *et al.* 2002). Du *et al.* (2009) reported that the growth of the seedlings of stylo (*Stylosanthes* spp*.)* was inhibited when the Al^{3+} concentration increased from 50 μM to 100 μM.

All nodules formed on *A. angustissima* showed a red coloration and reduced acetylene so they fixed N_2 (Räsänen *et al.* 2001). Additionally, the ultra-structural analysis of nodules showed the bacteroid with eight rod-shaped bacteroids indicating that the nitrogenase activity was not inhibited. Izaguirre-Mayoral *et al.* (2002) reported that the accumulation of Al in the roots of N_2-fixing legumes species native grown in the Venezuelan savanna, such as *Chamaecrista flexuosa* L.*, Clitoria guianensis* Aubl. Benth.*, Galactia jussiaeana* Kunth*, Zornia curvata* Mohl.*, Centrosema pubescens* Benth.*, Chamaecrista tetraphylla* Benth. and *Phaseolus gracillis* Poepp. ex Benth.*,* did not inhibit their symbiotic capacity. However, Igual *et al.* (1997) reported that the N_2-fixation efficiency of *Casuarina cunninghamiana* L. plants decreased from 0.20 mg N-fixed mg^{-1} nodule dry weight at 0 μM Al to 0.10 mg N-fixed mg^{-1} nodule dry weight at 880 μM Al. Nodules on the roots not only fix N_2 but also help to detoxify the plant (Kochian *et al.* 2005). Izaguirre-Mayoral *et al.* (2002) reported the exclusion of Al from nodule tissues of *Dioclea guianensis* Benth.*,* an Al accumulator legume species native of Venezuelan tropical forests.

Inoculation with *S. mexicanum* ITTG R7^T increased the total N and tannin content in *A. angustissima* compared to plants that were not inoculated. Leguminous species growing in soil with large amounts of Al increase the accumulation of tannins in the bark, root and also in nodules as a protection mechanism (Kochian *et al.* 2005). Small granules of tannins were even found in the nodules suggesting that they might protect the nodules against Al toxicity (Barceló & Poschenrieder 2002, Izaguirre-Mayoral *et al.* 2002). Tannins bind to Al^{3+} ions and exclude it in that way (Barceló & Poschenrieder 2002). This protection mechanism is also observed in *Acacia auriculiformis* A. Cunn. ex Benth. and *Eucalyptus camaldulensis* Dehnh. (Nguyen *et al.* 2003). Rincón-Rosales & Gutiérrez-Miceli (2008) observed that *A. angustissima* shrubs grown in acid soils with Al-concentration >3 mg Al kg^{-1} accumulate up to 25% more tannins that those growing in moderate alkaline soils with Al a concentration >1.2 mg Al kg^{-1}.

The accumulation of Al in the roots of *A. angustissima*, mainly in the secondary ones, classified this species as a root-Al accumulator. The legumes *Chamaecrista flexuosa* L.*, Clitoria guianensis* Aubl.*, Galactia jussiaeane* Kunth and *Zornia curvata* Mohl. are also considered root-Al accumulators (Izaguirre-Mayoral *et al.* 2002). They grow in soils of the Venezuelan savanna with pH 4 and Al content of 8.4 *c*mol kg^{-1} and accumulate Al mainly in their roots.

It was found that the tropical leguminous N_2-fixing shrub *A. angustissima* was tolerant to large amounts of Al in soil so it could be used to restore soil fertility while inoculation with *S. mexicanum* ITTG R7^T will further increase that resistance and improve its growth. Nodules not only stimulated growth and plant-N content, but also stimulated tannin production protecting *A. angustissima* against Al toxicity.

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