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VICIA FABA – RHIZOBIUM LEGUMINOSARUM SYSTEM SYMBIOTIC RELATIONSHIP UNDER STRESS OF SOIL PH AND ALUMINIUM

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Summary: Aluminium (Al) toxicity is one of the most widespread agronomic problems in world. Selection of pH and Al tolerant strains of *Rhizobium leguminosarum* as well as *Vicia faba* was carried out *in vitro* and *in vivo*. Developing Al-tolerant faba bean cultivar is one approach to overcome this constraint. The growth of the strains was evaluated in term of optical density after 48 h incubation in rotary shaker at 28°C in broth defined basal medium. The investigation showed that *Rhizobium* strain Lóbab Z was the most tolerant strain to pH variations, while HB-3841^{str+} was the most sensitive one. Although the HB-3841^{str+} and E1012 strains were not able to grow at 25 µM $KAl(SO_4)_2$, but they grew at 25 µM $Al(NO_3)_3$. The results showed that the multiplication of *Rhizobium* strains (except E1012) was unaffected by 100 µM $Al_2(SO_4)_3$, while the growth of the strains (except Bükköny 75/4) was affected by 50 µM $AlCl_3$. The inhibitory increasing order of investigated Al compounds was found as following: $Al(NO_3)_3 < Al_2(SO_4)_3 < KAl(SO_4)_2 < AlCl_3$. From the above, it can be concluded that because of the ability of *Rhizobium* strain Lóbab Z to grow *in vitro* conditions containing high concentrations of Al, therefore we suggested using it as reference strain for nodulation potential in soil of high Al content. The effective strains were tested for their symbiotic performance with faba bean cultivar in clay loam brown forest soil with various pH values as well as with different Al doses. The best performance of all the strains was at 6.6 soil pH and between 50 and 100 Al mg kg^{-1} soil at pH was 5.31. The strains Bükköny 75/4 and HB-3841^{str+} were suitable for soils with a pH 6.6. While the strains Lóbab Z and Bükköny 75/4 were suitable for inoculating soil with pH 5.31 and soil containing Al levels between 50 and 100 mg kg^{-1} soil. The final conclusion is that the multiplication of the strains was dependent upon the Al ions and Bükköny 75/4 strain can be recommended for inoculating soil with pH 5.31 and containing Al levels between 50 and 100 mg kg^{-1} soil.

Introduction

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic N_2-fixation.

Aluminium (Al) is an abundant element in Earth's crust; it is believed to be contained in a percentage from 7.5 to 8.1 (Kabata-Pendias and Pendias, 1993). Al is very rare in its free form, it contributes greatly to the soil properties, where it is present mainly as insoluble $Al(OH)_3$. The effects of Al have drawn our attention, mainly due to the acidifying problems. Al may accumulate in plants and cause health problems for animals that consume these plants. Another negative environmental effect of Al is that its ions can react with phosphates, which causes phosphates to be less available to organisms.

Studies of environmental toxicology in recent years have revealed that Al can be a cause of many diseases in humans and animals. It can also exert harmful effects on plant roots (Nowak and Brus 1996). Moreover, soil acidification, resulting from abrupt aggravation of air pollution by acidic N and S oxides, caused the mobilization of toxic Al^{3+} ions, which causes numerous harmful changes in soil environment such as plant poisoning, forest drying or a dramatic decrease in cereal crops cultivated on acidified soils (Gromysz-Kalkowska and Szubratowska 1999).

Many studies indicate that Al can exert a negative effect on many metabolic pathways in the organism, particularly on Ca, PO_4, F and Fe metabolism and show affinity for DNA and RNA, and inhibit enzymes such as hexokinase, acid and alkalic phosphatase, phosphodiesterase and phosphoxidase. The toxic effect can be intensified by acidification of the environment (Graczyk and Dlugaszek 1993).

In acid soils, Al comprises up to 40% of the world's arable land, it becomes soluble as a trivalent cation (Kochian 1995), and is toxic to plants at micromolar concentrations (Kinraide and Parker 1987), which leads to reduced plant growth, and consequently, reduced crop productivity. Al toxicity is a major factor which limits world agricultural production, and becomes more soluble as acidity increases and is often the major toxic element in acidic soils and water (Wakao et al. 2002, Sledge et al. 2005). Therefore, the acidity is considered as a major factor to be detrimental for legume cultivation.

Studies on the effect of low pH upon survival and nodulation by rhizobia are very important. Recently, *in vitro* involving liquid cultures have demonstrated that low pH inhibits nodulation by *R. leguminosarum* (Evans et al. 1980), and *R. phaseoli* (Franco and Munns 1982).

For agriculture the most important forms are active Al. These include mobile and exchangeable Al, assimilable Al and Al contained in water-soluble compounds occurring in solution as Al^{3+} cations. The increased concentrations of these ions and augmented activity of Al fraction is connected with soil acidification ($pH < 5.5$), which is damaging for physico-chemical and biological soil properties and exerts a toxic influence on plants. Al released in acidified soils is the main cause of crop decreases (Adamczyk et al. 1968). Ederson et al. (2009) mentioned that bacterial community structure in Western Amazon soils changed significantly along gradients of base saturation of Al and pH. In agriculture, soil may be acid naturally or may become acidic due to the humans activities. These activities can include farming practices that result in acidification or acid rain as a consequence of industrial processes (Kennedy 1992). While low pH can restrict plant growth in its own right, in most cases it is the dissolution of toxic metals; particularly Al. Göttlein et al. (1999) stated that chemical conditions in the rhizosphere in many respects are different from the bulk soil. In acid forest soils Al chemistry at the soil root interface is of particular interest because of its importance for evaluating the risk of rhizotoxicity. Soil acidity is caused by acid deposition and application of ammonium fertilizers. Based on the Al-pH chemistry, Al species in a medium below pH 3.5 presents predominately as free Al^{3+} ions, which is toxic to plants and microorganisms (MacDonald and Martin 1988). Concerning the mechanisms behind the growth-promoting effects of high Al concentrations as well as tolerance to Al in bacteria, it is presumed that chelating organic compounds e.g., citrate, oxalate and some proteinous substances (Martin 1986) are excreted into the medium to immobilize soluble Al.

Soil acidification is also one of the chemical soil degradation processes, which is responsible for lowering the soil bioproductivity. Al mobilization or accumulation in the soil solution causes this problem. Environmental hazards may be arising because of a high concentration of dissolved Al that is toxic to plant (Wright et al. 1989). In the soil solution, acidic pH increases Al through dissolution of soil mineral surfaces, and solubilization and complexation of Al. It is suggested that soil Al occurs mainly as Al-organic matter complexes rather than as a constituent of clay and clay-like minerals (Boudot et al. 1988). Such interaction reduced the soil C turnover (Parton et al. 1989). Mostly, the microbial activities in soil are optimum in the range of pH 6 to 8. Microbial biomass appears to be not significantly affected by soil acidity at pH range of 4.5-6.5 (Donnelly et al. 1990). However, in acidic pH < 4.5, microbial activity and nutrient turnover are greatly reduced (Santa 2000). The combined impact of H^+ and Al^{3+} on microbial activity and organic matter decomposition could be modelled with ion exchange expression, such as Vanselow expression (Walse et al. 1998).

The primary effect of Al phytotoxicity is the inhibition of root growth; however, the mechanisms involved in this toxicity are far from clear (Matsumoto 2000). One of the most important considerations in legumes-rhizobia symbiotic relationship for optimizing biological N_2-fixation is the response of the microsymbiont and nodule formation to the physical, chemical and biological dynamics of the soil environment. Acid soil is a major factor limiting the performance of the symbiotic biological N_2-fixation in legumes (Graham et al. 1982). Limitations may be due to effects of the macro- and microsymbionts, root infection by especific rhizobial strain, nodule initiation or nodule formation. Kahindi et al. (1997) stated the major factors that determine the population size of rhizobia in absence of their host are environmental stresses such as soil acidity. Helemish and El-Gammal (1987) gave the pH values when the growth of *R. leguminosarum* strain was optimum (5.5), tolerant (8.5) and sensitive (3.5). The optimum pH for the growth of (*Brady*-)*Rhizobium* is around 6.8. Most of rhizobia grow in pH above 4.0 and under 8.5 (Date and Holliday 1979). With respect to the symbioses between (*Brady*-)*Rhizobium* and legumes, Al has been shown to adversely affect the process of nodulation through inhibition of root hair formation and nodule initiation (Flis et al. 1993). The ability of *R. trifolii* to multiply in acidic broth medium with Al concentrations similar to those found in acidic soil solutions has been used to test for tolerance of acid stress (Wood and Shepherd 1987). Whelan and Alexander (1986) mentioned that the root-nodule bacteria grew after a lag period in a culture medium containing 75 µM Al. At 50 µM Al, rhizobial number declined around clover roots and nodules were formed only at pH 4.8 and above. They concluded that Al is a potent inhibitor of rhizobial growth at the expense of excretions. In addition, Chandra and Pareek (1991) established that in acidic soil (pH 5.4), the number and fresh weight of nodules, N-ase activity, shoot dry weight, N-uptake and competitive ability of three strains of chickpea rhizobia were low compared to those of a neutral soil (pH 7.1). Wood et al. (1984a, b) stated that acidity and high Al concentrations are toxic to *Rhizobium*, and inhibit nodule formation. Of all, the fast-growing rhizobia are considered less tolerant to acid pH than bradyrhizobia. *R. tropici* appears to be the most tolerant species of acid soil (Graham et al. 1994). Low pH but greater extent affected the symbiotic development of the plant by higher concentration of Al and Mn (Rai 1992). Recently, *Rhizobium* strains with higher tolerance to pH have been

identified in most *Rhizobium* species (Richardson and Simpson 1989, Wolffe et al. 1991). Evans et al. (1993) and Carter et al. (1994) showed the population of *R. leguminosarum* bv. *viciae* was decreased in very acidic soil. Tripathi and Mishra (1992) isolated acid-tolerant strains of chickpea *Rhizobium* that gave very good nodulation under the low pH conditions. The ability of *Rhizobium* to grow in acidic liquid media with concentrations of Al was similar to those found in acidic stress (Wood and Cooper 1985). Brady et al. (1994) found that streptomycin resistant mutant of *Bradyrhizobium* spp. NC92 in the bulk solution or in the rhizosphere of peanut roots was unaffected by 20 µM Al, Lesueur et al. (1993) identified some *Bradyrhizobium* strains able to grow in the presence of 100 µM $AlCl_3$ and Taylor et al. (1991) identified *B. japonicum* strains tolerant to acidity and Al. Basis on the above, the optimalization of symbiotic biological N_2-fixation is effective only when rhizobial activity is increased. For this reason, the following study determines the effects of different pH values and Al phytotoxicity on *Rhizobium* growth as well as the *Vicia faba – R. leguminosarum* symbiotic relationship.

Materials and method

Microorganisms

Four strains of *Rhizobium leguminosarum* bv. *Viciae;* one originated from Libya (HB-841^{str+} streptomycin resistant mutant) strain, two Hungarian strains (Lóbab Z and Bükköny 75/4), and English strain (E1012) were used in the following investigations. Routinely, strains were maintained at 4°C on yeast extract mannitol agar (Vincent 1970) slants supplemented with 3 g $CaCO_3$ l^{-1}.

Plant

Vicia faba L. seeds of Libyan origin; and characterized as large, smooth, with pale brown coat-containing tannin. Healthy seeds and approximately similar in shape and size were selected. Seeds were surface sterilized with 75 % ethanol for 3 min. followed by 0.2 % acidified mercuric chloride (Vincent 1970) for 5 min., and repeatedly washed with sterile distilled water. The seeds soaked for 36 h at room temperature in sterile distilled water.

Soil samples

Clay loam brown forest soil samples were collected from Gödöllő of original pH 5.31 (H_2O) in which the first 20 mm layer was removed and throughout the next 200 mm depth. Soil was screened to pass a 2 mm stainless steel sieve to remove rocks and soil impurities. Soil pH was measured in a 1:1 soil:water ratio (for 30 min equilibrium) after soil moisture content had been determined in term of the water filled pore space at 30 and 60%. The gravimetric moisture content was determined from the collected samples by drying the soil samples for 24 h at 65°C. The physical and chemical properties of the investigated soil samples are: $pH_{(H2O)}$ 5.31, humus content 1.21% and C:N ratio is 12.4. The soil ingredients are present in mg kg^{-1}: NH_4^+-N (3.2), NO_3^--N (4.5), SO_4^{-2} (4.6), K_2O (123), P_2O_3 (209), Cu (1.86), Mg (206), Mn (195), Cd (0.065), Co (1.57), Pb (8.48), and Zn (7.22).

Aluminium salts

The investigated Al-salts were: aluminium nitrate ($Al(NO_3)_3$), aluminium chloride ($AlCl_3 \times 6H_2O$), aluminium sulphate ($Al_2(SO_4)_3$) and potassium aluminium sulphate ($KAl(SO_4)_2 \times 12H_2O$). The Al-salt solution was added to the culture medium as a filter-sterilized solution according to Makherjee and Asanuma (1998). The used of these salts were according to Wenzl et al. (2001), Wakao et al. (2002), Gromysz-Kalkawska et al. (2004) and Sledge et al. (2005).

In vitro:

Effect of pH on Rhizobium growth

To study the tolerance of the tested strains to pH variations, the pH values of broth medium were adjusted to 4, 4.5, 5.0, 6.6, 7.0, 8.0, 9.0 and 10.0 using the buffer according to Howieson et al. (1988), and the results were compared with pH 7.0 as control. Experiments were carried out with a sterilized, defined basal growth broth medium, which contains the following ingredients litre^{-1} of deionized water according to Johnson and Wood (1987): yeast extract 0.4 g, mannitol 10 g.

The growth dynamics of the four strains throughout 84 h were measured in every 6 h interval using microfermentor technique at 28°C in rotary shaker (150 rpm) and similarly in the presence of 40 μM Al in $Al_2(SO_4)_3$ form. The culture tubes were inoculated with a 125 μl bacterial suspension (number of cells per ml eq. 10^6). Rhizobial growth (in the term of optical density) was measured at λ=550 nm using a UV spectrophotometer.Viable plate count was made to confirm the results, too.

Effect of Al-salts on Rhizobium growth

Similar technique was used to evaluate the survival of the four rhizobial strains to different Al-salts at various concentrations (0, 25, 50, 100, 200 and 400 μM).

In vivo:

The plantation and growth of were carried out in the greenhouse at the Institute of Environmental Science at Szent István University, Gödöllő.

Effect of pH on the plant growth and nodulation potential

The effects of pH on plant growth, nodulation and biological N_2-fixation were carried out as following: The air-dried soil samples was divided into three quarters, first one was remained as control with pH 5.31, and the target soil pH values were adjusted by addition of $CaCO_3$ to the second and third quarters to obtain soil pH 6.6 and 8.3, respectively. Soil samples were mixed well with each of the two different additives. Soil samples were maintained in an optimum soil moisture regime (40%), and average temperature of 27 ± 2°C (day), 19 ± 2°C (night) and 14 h photoperiod for 2 weeks. Soil samples were collected to determine their final soil pH. Sterilized plastic pots of 3 kg capacity (24 cm dam.) were used and filled with sterile (steamed 100°C for 1 h for three consecutive days) soil. Soil of pH 6.6 was kept as control.

Effect of $Al_2(SO_4)_3$ on the plant growth and nodulation potential

At the same time, another set of pot experiment was conducted to investigate the response of symbiotic properties to various concentrations of $Al_2(SO_4)_3$ at different concentrations (0, 50, 100, 200 and 400 mg $Al_2(SO_4)_3$ kg^{-1} soil) in term of plant growth, nodulation potential and N_2-fixation.

Planting and nodulation assessment:

Five seeds were planted/pot and covered with a layer of approximately 20 mm of sterile soil. Pots were loosely covered by cellophane. Nine days after emergence, the pots were thinned to three seedlings. Seedlings were inoculated separately with 10 ml of rhizobial suspension (1.4×10^7 cfu ml^{-1}) and watered with steriled water when required. Plants were grown at 27 ± 2°C (day), 19 ± 2°C (night) and natural illumination (14 h photoperiod) for 8 weeks. After 56 days, plants of all pots were withdrawn and the roots carefully washed several times in tap water followed by distilled water for further investigations. Number of root-nodules was counted, fresh and dry weight of plants and nodules were obtained (drying was carried at 65°C to a constant fix weight and the values expressed as g $plant^{-1}$ and mg root-nodules $plant^{-1}$), total N-content (TNC) was measured as mg $plant^{-1}$ using micro-Kjeldahl method as a criterion of biological N_2-fixation (Burris 1974). The estimated mg N-fixed nodule dry weight was calculated according to Igual et al. (1997). Relative symbiotic effectiveness (RSE) of the strains against Lóbab Z strain was calculated. This is based on the bioproductivity of the plant. For comparison, we used another formula that is based on the TNC $plant^{-1}$.

The experimentation was layout in a complete randomized block design. Means of three replicates per treatment for each strain were analyzed using ANOVA to determine statistical differences among treatment and LSD at $P < 95\%$ was calculated as well as S.D.

Results

Assessment of pH tolerance

Variations in pH tolerance among *R. leguminosarum* bv. *viciae* strains were measured *in vitro*. None of the strains were able to survive at pH 4.0. However, the growth of the strains was maximum at pH 6.6, and higher than the controls (pH 7). There was a linear and significant ($P < 95\%$) decrease in growth potential for all strains below and above the pH values 6.6 – 8.0. The results indicated that none of the strains were able to tolerate pH neither 5.0 nor 10.0 higher than pH ranged from 6.6 to 9.0. *Rhizobium* strains Lóbab Z and E1012 were grew higher than control cultures at pH 8.0 while, strains of HB-3841^{str+} and Bükköny 75/4 preferred pH 6.6. Therefore, it is suggested that *Rhizobium* Lóbab Z strain can be used as faba bean inoculant in various soils having pH values ranging from > 5.0 to < 9.0.

Effect of various Al salts on survival of Rhizobium

Fig. 1 demonstrates the growth dynamic curves of the four *Rhizobium* strains in defined basal broth medium containing 40 µM $Al_2(SO_4)_3$ throughout 84 h.

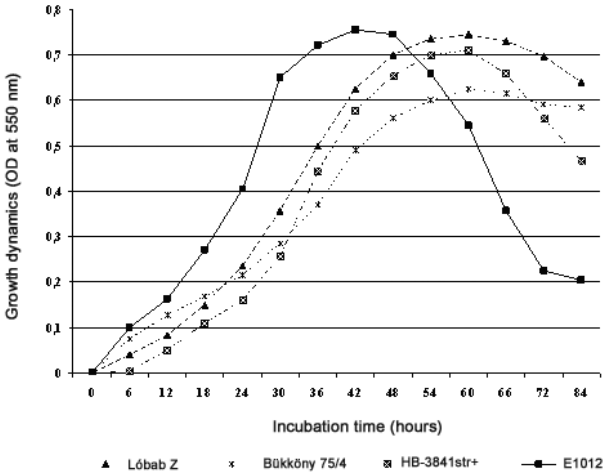

Figure 1. Growth dynamics of different *Rhizobium* strains in liquid medium contaminated by 40 μM of $Al_2(SO_4)_3$ throughout 84 h
1. ábra Különböző *Rhizobium* törzsek szaporodás dinamikája 40 μM $Al_2(SO_4)_3$-t tartalmazó folyadékközegben, 84 órán keresztül

There were no significant differences between the effect of Al on the viable cells number (in term of colony forming unit) and growth dynamics cultures (in broth) of the 4 strains. The investigation illustrated that Al of various forms and concentrations adversely affected the growth of the strains as optical density decreased with increasing Al the concentration. Results showed that at the same concentrations of different Al salts, the growth rates of the strains were varied. Also, it was noted that the strains grew higher than the control cultures at lowest concentrations of all investigated Al salts. The two Hungarian strains were able to tolerate the 100 μM concentration of $Al(NO_3)_3$ and $Al_2(SO_4)_3$. While, the growth rates of the other strains were decreased. Moreover, it was found that *Rhizobium* Lóbab Z and Bükköny 75/4 strains were tolerated 50 μM of $KAl(SO_4)_2$, while, HB-3841^{str+} and E1012 can tolerate 25 μM. Depending on the growth rates of the strains, it was found that $KAl(SO_4)_2$ was more toxic than $AlCl_3$.

Table 1 shows the maximum growth rates of the four *Rhizobium* strains at maximum applied doses of Al salts; also it demonstrates that E1012 is the most sensitive strain to the investigated salts.

Table 1. Maximum concentrations of Al compounds enhancing growth of *R. leguminosarum* strains
1. táblázat A *R. leguminosarum* törzsek szaporodását elősegítő Al vegyületek maximális koncentrációja

Rhizobium strains	*Al concentrations (μM)*			
	$AlCl_3$	$Al(NO_3)_3$	$Al_2(SO_4)_3$	$KAl(SO_4)_2$
Lóbab-Z	50	100	100*	100
Bükköny 75/4	50	50	50*	50*
HB-3841^{str+}	25	50	50*	25
E1012	25	25*	25	25

*Values that are significantly differences with control at least $P < 95\%$.

Table 2 illustrates the significant correlations between the growth rates of the four *Rhizobium* strains at various pH values and two forms $Al(NO_3)_3$ and $KAl(SO_4)_2$ at different concentrations.

Table 2. Correlation between pH and Al compounds affecting the growth of *R. leguminosarum* strains
2. táblázat A *R. Leguminosarum* törzsek szaporodását befolyásoló Al vegyületek és a pH közötti korreláció

Al compounds and concentrations (μM)		*pH values*				
		5.0	*6.6*	*8.0*	*9.0*	*10.0*
$Al(NO_3)_3$	25	0.6293	0.9144	0.6277	0.7102	0.3401
$KAl(SO_4)_2$		0.9690*	0.8288	0.3436	0.5984	0.5322
$Al(NO_3)_3$	50	0.9247	0.4622	0.6304	0.8426	0.9042
$KAl(SO_4)_2$		0.9725*	0.8184	0.5769	0.7577	0.6574
$Al(NO_3)_3$	100	0.9335*	0.6672	0.0399	0.3804	0.4211
$KAl(SO_4)_2$		0.8640	0.9881*	0.2971	0.5122	0.2247
$Al(NO_3)_3$	200	0.0548	-0.1196	-0.8602	-0.6411	-0.3427
$KAl(SO_4)_2$		0.9161	0.9412	0.2707	0.5117	0.3341
$Al(NO_3)_3$	400	0.4562	0.7187	-0.3481	-0.2242	-0.3376
$KAl(SO_4)_2$		0.8604	0.8188	0.0079	0.3277	0.2346

* indicates statistically significant differences ($P < 95\%$) by ANOV.

Data shows that positive significant correlation between pH 5.0 and 25 and 50 μM $KAl(SO_4)_2$ and 100 μM $Al(NO_3)_3$. However, depending on the growth rates of the strains under other environmetal parameters, it was found that no correlation among them except between 6.6 and 100 μM $Al_2(SO_4)_3$. Also, a negative correlation between $Al(NO_3)_3$ at 200 μM and pH values 6.6, 8, 9, and 10 as well as 400 μM $Al(NO_3)_3$ and 8, 9, and 10 pH values.

Table 3 illustrates the correlations among the growth rates of the four *Rhizobium* strains at various pH values and the concentrations of Al salts. The highest correlation coefficient (r = 0.9126) was obtained between Lóbab Z and Bükköny 75/4, while the lowest (r = 0.6951) value was received in the case of strains HB-3841^{str+} and Lóbab Z.

Table 3. ANOVA and correlation among *R. leguminosarum* strains under the pH and Al in different forms and concentrations *in vitro*
3. táblázat R. leguminosarum törzsek között fellépő varianciák (ANOVA) és korrelációk különböző pH viszonyok és az Al eltérő koncentrációi és megjelenési formái mellett, *in vitro* körülmények között

Source	*Prob. level*	*Correlation coefficient (r)*	*Standard error of estimation*	r^2 %
HB-3841^{str+} × Lóbab Z	0.00067	0.7355	30.993	50.40
HB-3841^{str+}×Bükköny 5/4	0.00018	0.7476	28.828	55.89
HB-3841^{str+}× E1012	0.00061	0.6993	30.828	48.90
Lóbab Z × Bükköny 75/4*	0.00000	0.9126	19.865	83.28
Lóbab Z × E1012	0.00001	0.8547	25.295	73.05
Bükköny 75/4 × E1012	0.00000	0.8974	20.067	80.53

*Indicates the process is statistically significant differences ($P < 95\%$) by ANOVA.

Relationship between Soil pH and symbiosis

Table 4a indicates the relationship between the changing of soil pH and symbiotic properties of faba bean plant with it won microsymbionts. In comparison, it was found that the nodule number, nodule dry weight, plant dry weight, TNC and estimated N fixed were lower in soil of pH 5.31 and 8.3 than the control soil of pH 6.6.

Table 4a. Nodulation and symbiotic relationship of *V. faba* - *Rhizobium* system in a sterilized soil at different pH values (Values are means of 3 replicates ± S.D.).
4a. tábla. V. faba – Rhizobium szimbiotikus és gümőképző steril talajon, különböző pH értékek mellett (Értékek 3 ismétlés ± S.D.)

Rhizobium strains	*pH*	*Nodule number*	*Nodule dry weight (mg)*	*Plant dry weight (g)*	*TNC / plant (mg)*	*Estimated N fixed***
Uninoculated (Control)	5.31	-	-	2.80±0.10	164±2.900	-
	6.60	-	-	3.50±0.30	206±2.700	-
	8.30	-	-	1.97±0.4*	112±2.10*	-
Lóbab Z	5.31	98±2.1	221±4.20	3.90±0.12	253±8.300	0.50±0.05
	6.60	138±1.3	299±5.10	4.17±0.10	287±10.50	0.30±0.07
	8.30	93±2.1	184±7.80	3.20±0.06	213±7.100	0.55±0.09
Bükköny 75/4	5.31	118±3.9	236±7.40	3.70±0.10	248±4.600	0.36±0.03
	6.60	159±2.6	320±2.50	4.70±0.30	305±5.300	0.31±0.02
	8.30	116±3.0	214±3.60	3.50±0.21	227±2.400	0.54±0.06
E1012	5.31	45±0.6	81±7.30	2.1±0.13*	184±0.41*	0.30±0.02
	6.60	86±1.6	167±2.60	3.06±0.40	213±0.720	0.05±0.0*
	8.30	71±2.7	138±2.10	2.01±0.2*	139±0.51*	0.38±0.05
HB-3841^{str+}	5.31	75±3.3	141±3.30	3.5±0.110	234±4.500	0.40±0.07
	6.60	141±6.6	272±4.70	3.89±0.21	382±6.300	0.65±0.03
	8.30	102±3.3	201±2.40	3.6±0.17*	225±4.70	0.56±0.04

*Are not significantly different from control at $P < 95\%$.
** Estimated mg N-fixed mg^{-1} nodule dry weight =

$$\frac{\text{TNC of inoculated plant - TNC of uninoculated plant}}{\text{Total nodule dry weight}}$$

The English strain E1012 was unable to be established under this environment. The Bükköny 75/4 and HB-3841^{str+} strains can be recommended as inoculants under this soil condition. It was found that Lóbab Z stain was more effective for nodulation in acid soil than HB-3841^{str+} strain which preferred the alkaline soil for nodulation.

Effect of $Al_2(SO_4)_3$ on plant growth, nodulation potential

Results in Table 4b indicates that 50 mg $Al_2(SO_4)_3$ kg^{-1} soil did not stimulate the symbiotic properties more than the inoculated plant grew in soil with 0 mg $Al_2(SO_4)_3$ kg^{-1} soil. However, nodulation potential and TNC / plant were reduced by increasing the concentrations of $Al_2(SO_4)_3$. Also, it was found that pots inoculated by the strains Lóbab

Z or Bükköny 74/5 at the Al concentrations 50 and 100 mg $Al_2(SO_4)_3$ kg^{-1} soil were more adapted to this environmental stress than those inoculated by HB-3841^{str+} and E1012. The results showed that the symbiotic properties of plants inoculated by the strain HB-3841^{str+} were higher than those obtained in the pots inoculated by strain E1012.

Table 4b. Faba bean - *R. leguminosarum* relationships at different Al concentrations after 56 days plantation.
4b. tábla. Lóbab - *R. leguminosarum* közötti kölcsönhatások különböző Al koncentrációk mellett, 56 napos ültetvényben

Rhizobium strains	*Al (µM / kg soil)*	*Nodule number*	*Nodule dry weight (mg)*	*Plant dry weight (g)*	*TNC / plant (mg)*	*Estimated N fixed***
Uninoculated	0	-	-	2.80±0.100	124±2.9	-
	50	-	-	3.28±0.200	164±2.1*	-
	100	-	-	2.65±0.14*	124±1.3*	-
	200	-	-	2.43±0.20*	116±1.7*	-
	400	-	-	2.07±0.30*	94±1.1*	-
Lóbab Z	0	157±2.4	451±1.80	3.47±2.900	143±1.60	0.44±0.07
	50	140±3.4	442±8.20	5.05±0.210	312±3.40	0.34±0.02
	100	117±2.4	425±5.40	4.78±0.430	294±2.60	0.40±0.07
	200	80±6.40	279±3.10	3.90±0.320	245±1.90	0.46±0.06
	400	57±5.10	243±2.10	3.03±0.240	185±1.80	0.38±0.04
Bükköny 75/4	0	161±2.9	474±1.81	3.62±1.700	162±1.40	0.49±0.03
	50	135±3.4	407±3.70	4.71±0.110	293±4.50	0.32±0.02
	100	110±3.7	396±9.40	4.23±0.260	264±3.70	0.35±0.04
	200	75±1.10	328±2.80	3.89±0.310	231±5.60	0.35±0.03
	400	43±2.40	188±2.40	3.50±0.360	205±6.70	0.59±0.05
E1012	0	92±1.20	174±2.21	3.24±1.720	221±2.40	0.29±0.06
	50	63±2.20	279±9.10	3.66±0.270	233±6.20	0.25±0.01
	100	40±2.40	189±5.50	3.08±0.190	192±2.50	0.36±0.04
	200	25±1.7*	135±3.10	2.82±0.17*	165±3.4*	0.36±0.02
	400	16±1.1*	89±4.1*	2.41±0.22*	139±3.6*	0.51±0.06
HB-3841^{str+}	0	143±2.1	284±1.21	3.75±2.110	373±1.40	0.48±0.02
	50	130±1.3	393±7.40	4.01±0.390	257±5.10	0.24±0.03
	100	82±1.70	307±6.60	3.79±0.270	236±1.20	0.37±0.03
	200	60±1.10	227±4.40	3.23±0.250	195±5.60	0.35±0.02
	400	37±1.2*	114±4.90	2.86±0.14*	169±2.1*	0.66±0.07

*Are not significantly different from control at $P < 95\%$.
**Estimated mg N-fixed mg^{-1} nodule dry weight =

$$\frac{\textit{TNC of inoculated plant - TNC of uninoculated plant}}{\textit{Total nodule drz weight}}$$

Overall, it can be conclude that the Al tolerance decreasing order of the strains was Lóbab Z > Bükköny 75/4 > HB-3841^{str+} > E1012.

The results recorded in Tables 5a. and 5b. showed that the RSE based on the dry weights and TNC at different soil pH and Al concentrations, respectively. When Lóbab Z strain was used as reference, Bükköny 74/5 strain is the superior strain at pH 6.6 in both formulae.

Table 5a. A comparison of the relative symbiotic effectiveness based on the dry weight or TNC / plant inoculated by *Rhizobium* strains in sterilized soils at different pH values after 56 days plantation.
5a. tábla A szárazsúlyon alapuló, illetve a *Rhizobium* törzsekkel beoltott nitrogéntartalmának/növény szimbiózis hatékonyságának összehasonlítása steril talajon, különböző pH értékek mellett, 56 napos ültetvényben

Rhizobium strains	*pH*					
	5.31		*6.6*		*8.3*	
	Dwt[a]	TNC[b]	Dwt[a]	TNC[b]	Dwt[a]	TNC[b]
Lóbab Z**	100	100	100	100	100	100
Bükköny 75/4	94.9	98	112.7	106.3	97.2	106.6
E1012	53.9	72.7	73.4	74.2	55.8	65.3
HB-3841^{str+}	89.7	92.5	93.3	133.1	88.9	105.6

Table 5b. A comparison of the relative symbiotic effectiveness based on the dry weight or TNC / plant inoculated by *Rhizobium* strains in sterilized soils at different Al^{3+} concentrations after 56 days plantation.
5b. tábla. A szárazsúlyon alapuló, illetve a *Rhizobium* törzsekkel beoltott nitrogéntartalmának/növény szimbiózis hatékonyságának összehasonlítása steril talajon, különböző Al^{3+} koncentráció mellett, 56 napos ültetvényben

Rhizobium strains	*Al concentrations in $Al_2(SO_4)_3$ kg^{-1} soil*							
	50		*100*		*200*		*400*	
	Dwt[a]	TNC[b]	Dwt[a]	TNC[b]	Dwt[a]	TNC[b]	Dwt[a]	TNC[b]
Lóbab Z**	100	100	100	100	100	100	100	100
Bükköny 75/4	97.3	97.8	94.4	95.8	99.2	114.9	129.6	124.8
E1012	73.9	76.0	63.7	64.8	67.5	63.1	87.5	83.2
HB-3841^{str+}	90.9	92.8	80.2	81.1	79.5	76.4	105.7	101.9

*Are not significantly different from control at $P < 95\%$
**The strain Lóbab Z as reference

$$^{a}RSE = \frac{\text{Dry weight of inoculated plant X 100}}{\text{Dry weight of inoculated plant with referenced strain}}$$

$$^{b}RSE = \frac{\text{TNC of inoculated plant X 100}}{\text{TNC of inoculated plant with referenced strain}}$$

At pH 8.3, Bükköny 75/4 was superior only in the case of TNC formula and HB-3841^{str+} strain was superior at pH 6.6. The Bükköny 75/4 strain was superior only at 200 mg $Al_2(SO_4)_3$ kg^{-1} soil by applying the TNC formula. Meanwhile, both strains Bükköny 75/4 and HB-3841^{str+} were superiors in the pots of 400 mg $Al_2(SO_4)_3$ kg^{-1} soil concentration in both applied formulae.

Discussion

Numerous studies conducted for many years have shown that Al can be both beneficial and harmful for plants, causing even death. The beneficial effect of Al on plants consists of the stimulation of Fe absorption by root system, increased absorption of P, prevention of toxic effects of Cu and Mn and plant protection against phytopathogenic fungi.

On the other hand, detrimental Al effect on plants manifests itself as crop decrease which is caused by changes in the morphology of root system, inhibition of its elongative growth, root callosity, reduced number of rootlets, and dying away of growth cone. The inhibitory effects of Al on plants and microorganisms are well known but not yet fully understood in their physiological and biochemical aspects. *R. leguminosarum* was reported to excrete Al-induced protein or siderophore under neutral and alkaline conditions (Rogers et al. 2001). Rogers et al. (2001) mentioned that acid rain solubilises Al which can exert toxic effects on *R. leguminosarum* bv. *viciae* synthesizes the hydroxamate siderophore vicibactin in response to Fe limitation. It was found that $Al(NO_3)_3$ completely inhibited bacterial growth at 25 μM concentration. When Al and vicibactin solutions were added separately to growing cultures, growth was partly inhibited at 25 μM concentrations, but fully inhibited was at 50 μM concentrations. According to our investigations, we are in agreement with this result in case of E1012 but not with other strains (Table 1). Rogers et al. (2001) gave explane the possibility that: the Al-vicibactin complex is not taken up by the cell; the complex is taken up but Al is not released from vicibactin; Al is released in the cell but is precipitated immediately. However, vicibactin reduces the toxicity of Al by complexing it outside the cell. These explanation could be accepted by us.

The concentration of Al^{3+} ions in soil solution is high unless the pH < 5.0 (Schmohl and Horts 2002, Zhang et al. 2007). Inostroza-Blancheteau et al. (2008) studied the main physiological mechanisms of Al resistance and the genetic and molecular bases and explain the degree of resistance between different cereals species. Soil acidification due to fertilizers or acid rain caused by industrial pollution is an increasing threat to agricultural and natural ecosystems Al toxicity is very dependent on the pH of the soil. Availability of highly toxic Al^{3+} ion in soil is remarkably affected by pH. The results obtained from this investigation are in accord with Batzil et al. (1992) who found that most *Rhizobium* strains preferred neutral pH to acidic pH. Moreover, the results agreed with Merbach et al. (1990) who indicated that Al toxicity was an essential part of the detriments of low

pH values for *R. trifolii*. Our results are in agreement with this report. Helemish et al. (1993) determined the minimum, optimum and maximum pH values affecting the growth of *R. sesbaniae* which were 2, 8 and 10 respectively, showing that the local strain could tolerate a wide range of pH values (5-10) with maximum growth at pH 8. Also, the pH values from 5-7 supported the growth, while pH of 10 was not lethal for its growth. Furthermore, they showed that pH values less than 5.0 (3-4) did not support the growth. These results were in full agreement with the results of Pandhler and Kahlon (1978) who observed that *Rhizobium* of *P. sativum* fail to grow at pH 3.0 and growth was attained at pH (6.5-8.0). This demonstration supports our investigations, too. It was found (Wood and Cooper 1988) that 50 µM Al decreased the number of acid-tolerant strains at pH 4.5 and acid-sensitive strains of *R. trifolii* at pH 5.5. Also, this concentration was more toxic to acid-sensitive strains in the log phase than in the stationary phase. Wisniewski and Delmotte (1996) stated that the attachment of *Bradyrhizobium* sp. (*Lupinus*) to lupine roots was pH dependent, with an optimal pH at 6.6 for binding. This result documented by our results, too. We have also shown that the best nodulation potential and N_2-fixation were in the soil of pH at 6.6. We have to mention here that our strains (the Hungarians) can tolerate the same dose (100 µM of $Al(NO_3)_3$ and $KAl(SO_4)_2$) which was reported by Wood and Cooper (1988). Whelan and Alexander (1986) established that *R. trifolii* could grow in cultures containing 75 µM Al, and this result is supported by our findings as well. The inhibitory increasing order of investigated Al compounds was found as following: $Al(NO_3)_3 < Al_2(SO_4)_3 < KAl(SO_4)_2 < AlCl_3$. Acid rain solubilises Al which can exert toxic effects on soil bacteria. The root nodule bacterium *R. leguminosarum* bv. *viciae* synthesises the hydroxamate siderophore vicibactin in response to Fe limitation. There are several recent reviews that discuss mechanisms of Al tolerance and toxicity in plants. These include reviews by Kochian (1995), Ma et al. (2001), and Ryan et al. (2001). Paudyal et al. (2007) studied the effect of $AlCl_3$ at 0, 25, 50, 75 and 100 µM on two strains of rhizobia. The effects were assessed for bacterial growth in culture and in symbiotic parameters such as biomass production and nodulation in host plant. Al was found to have detrimental effect in both *in vitro* and *in vivo* conditions in all its concentration.

Johnson and Wood (1987) developed a method to study Al toxicity towards *Rhizobium*. This involved growth in broth followed by washing and measurement of cell viability in deionized distilled water plus Al. The results illustrated the high degree of sensitivity and rapid response of *R. leguminosarum* bv. *trifolii* and *R. loti* to Al under acid conditions but confirm earlier results on the relative tolerance of these two species.

Previous work on A1 toxicity to *Rhizobium* has used defined media (Keyser and Munns 1979, Wood and Cooper 1984, 1985) which contain both phosphate (5–500 µmol l^{-1}) and sulphate (30–500 µmol l^{-1}). It seems likely, therefore, that some of the A1 would be neutralized by these anions. Such methods may therefore overestimate the tolerance of rhizobia to A1 as has been suggested by Alva et al. (1986) for plant nutrient solutions. To overcome this problem a method has been devised in which the response of *Rhizobium* to A1 is measured in deionized distilled (DD) water.

Neogy et al. (2002) studied the effect of Al toxicity on growth of mungbean (*Vigna radiata* L. Wilczek) seedlings. It was found that seed germination declined with increased content of $Al_2(SO_4)_3$, while promotive effect was observed at very low dosage. In our investigations, we found that 50 mg $Al_2(SO_4)_3$ kg_{-1} soil improved the symbiotic properties

(see Table 4b). Roy and Chakrabartty (2000) investigated the growth of *Rhizobium* sp. strain BICC 651 in the presence of 100 μM Al and produced a threefold higher level of siderophore than in the control culture under Fe limitation during the stationary phase. Al in increasing concentrations resulted in decreased growth, and the effect was alleviated by the addition of Fe. Siderophore production decreased gradually in Al-treated culture as well as in the control with the addition of increasing concentrations of Fe, and at 50 μM Fe the level of siderophore was practically undetectable. The siderophore binds Fe and also Al. For some extend, it may be happened in our studied too, that under different conditions of Al treatments, siderophore was produced and this is our further task.

Marziah et al. (1995) stated that Al toxicity is a complex growth-limiting factor. Glasshouse studies using solution culture were carried out to examine the effects of Al on growth and nodulation of groundnut. Results showed that root elongation and dry weight in Al-treated plants were significantly reduced. At 20 and 30 days after planting, there was a decrease in nodule number (30%) and dry weight (45%) with an increase in the sum of activities of monomeric Al from 10 to 30 μM. The results confirmed our results obtained (see Table 4b).

Barabasz et al. (2002) concluded that although the mechanisms underlying Al toxicity have not been fully elucidated yet, we are already aware that environmental pollution and acidification of large amounts of soil and surface waters, and also drinking water, can be compared to the opening of "Pandora's box" and releasing a poison, which slow can be harmful for the whole population of humans, animals and plants.

One way to reduce the toxic effect of Al is to neutralize the acidity with calcareous amendments. However, this practice is demanding and not very effective. An alternative is the search for genetic variability in the genome of cropping grasses and/or their wild relatives to resist Al.

From the above, it can be conclude, that Bükköny 75/4 strain almost was able to multiply in microenvironment conditions containing high concentrations of Al, therefore, our suggestion is to use this strain for nodulation potential in soil of high Al content. From eco-agricultural point of view, the pH of rhizosphere environment is the most important parameters that could affect the attachment and invasion processes as the first steps for nodulation. In conclusion, successful growth of faba bean in acid soils would suggest that the faba bean-*Rhizobium* symbiosis is tolerant of acid infertility factors. A solution to the problem of soil acidity and Al toxicity is that some chemical substances or natural organic residues can be added to soil.

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VICIA FABA - RHIZOBIUM LEGUMINOSARUM SZIMBIOTIKUS KAPCSOLATA A TALAJ KÉMHATÁSÁNAK ÉS ALUMÍNIUM KONCENTRÁCIÓJÁNAK FÜGGVÉNYÉBEN

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Kulcsszavak: *Rhizobium leguminosarum*, talaj kémhatás és Al^{3+} koncentráció, szimbiotikus kapcsolatok, *Vicia faba*

Összegzés: Az alumínium (Al) által okozott toxicitás egyike a legelterjedtebb mezőgazdasági problémáknak. pH és Al toleráns *Rhizobium leguminosarum* és *Vicia faba* törzseket szelektáltunk *in vitro* és *in vivo* körülmények között. Az alumínium toleráns *V. faba* törzsek tenyésztése egyike azon tényezőknek, melyek megoldást nyújthatnak az alumínium által okozott talajszennyezettségre. A törzsek szaporodását táplevesben, 28°C-on, 48 órán keresztül történő inkubációt követően értékeltük, optikai denzitásuk (sűrűségük) alapján. Tanulmányaink azt igazolták, hogy a pH változásokra leginkább toleráns törzs a *Rhizobium* Lóbab Z volt, a legérzékenyebb pedig a HB-3841^{str+}. Habár a HB-3841^{str+} és E1012 törzsek nem szaporodtak 25 µM $KAl(SO_4)_2$-t tartalmazó táptalajon, 25 µM $Al(NO_3)_3$-t tartalmazó közegben már igen. Az eredmények azt igazolták, hogy 100 µM $Al_2(SO_4)_3$ koncentráció nem volt hatással a *Rhizobium* törzsek szaporodására (kivéve az E1012 törzset), ellenben 50 µM $AlCl_3$ koncentráció már gátolta a törzsek szaporodását (kivéve a Bükköny 75/4 törzsét). A vizsgált alumínium vegyületek növekvő sorrendje a gátló hatásuk függvényében: $Al(NO_3)_3 < Al_2(SO_4)_3 < KAl(SO_4)_2 < AlCl_3$. A fentiekből levonható a következtetés, hogy mivel a *Rhizobium* Lóbab Z törzs képes nagy koncentrációjú Al tartalmú közegben, *in vitro* körülmények között szaporodni, e törzs alkalmas lehet magas alumínium tartalmú talajok gümőképző képességének növelésére. A törzsek lóbabbal történő szimbiotikus képességét vizsgáltuk agyagbemosódásos barna erdőtalajon (Gödöllő), különböző pH és Al koncentráció mellett. A legjobb eredményt 6,6 pH mellett, az alumínium 50–100 mg kg^{-1} koncentrációjánál kaptuk, a pH 5,31 volt. A Bükköny 75/4 és HB-3841^{str+} törzsek 6,6 pH-jú talajon kiválóan tenyészthetőek. Lóbab Z és Bükköny 75/4, 5,31 pH-jú, 50-100 mg kg^{-1} Al-t tartalmazó táptalajon tenyészthető. Végső következtetésként levonható, hogy az egyes törzsek szaporodása az Al koncentrációtól függött, és a Bükköny 75/4 törzs kiválóan alkalmazható 5,31 pH-jú, az alumíniumot 50–100 mg kg^{-1} koncentrációban tartalmazó talajok oltására.