COMPENSATION EFFECT OF BACTERIUM CONTAINING BIOFERTILIZER ON THE GROWTH OF *CUCUMIS SATIVUS L.* UNDER AL-STRESS CONDITIONS

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Abstracts

Biofertilizers are used to improve soil fertility and plant production in sustainable agriculture. However, their applicability depends on several environmental parameters. The aim of our study was to evaluate the effect of free-living bacteria containing fertilizer on the growth of cucumber (*Cucumis sativus* L. *cvs. Delicates*) under aluminium (Al) stress. Different responses to Al stress of cucumber growth parameters were examined in terms of root elongation and physiological traits, such as Spad index (relative chlorophyll value), biomass accumulation of root and shoot, Al uptake and selected element contents (Fe, Mn, Zn, Mg) of leaves and root. The applied bacteria containing biofertilizer contains *Azotobacter chroococcum* and *Bacillus megaterium*.

The dry weights of cucumber shoots and roots decreased in line with the increasing Al concentration. Due to different Al treatments (10^{-3}M, 10^{-4}M) higher Al concentration was observed in the leaves, while the amounts of other elements (Fe, Mn, Zn, Mg) decreased. This high Al content of the leaves decreased below the control value when biofertilizer was applied. In the case of the roots the additional biofertilizer treatments compensated the effect of Al. The relative chlorophyll content was reduced during Al-stress in older plants and the biofertilizer moderated this effect. The root/shoot ratio was decreased in all the Al-treatments in comparison to the control. The living bacteria containing fertilizer also had a modifying effect. The root/shoot ratio increased at the 10^{-4} M $Al_2(SO_4)_2$ + biofertilizer and 10^{-4} M $Al(NO_3)_3$+ biofertilizer treatments compared to the control and Al-treatments.

According to our results the biofertilizer is an alternative nutrient supply for replacing chemical fertilizers because it enhances dry matter production. Biofertilizer usage is also offered under Al polluted environmental conditions. Although, the nutrient solution is a clean system where we can examine the main processes without other effects of natural soils. The soil can modify the results, e.g. the soil born microorganisms affect on nutrient availability, and also can modify the harmful effects of different heavy metals. The understanding of basic processes will help us to know more about the soil behaviour.

Keywords: aluminium, biofertilizer, plant growth, root/shoot ratio, root length

INTRODUCTION

Our environment is more or less polluted with different heavy metals which have an effect on agricultural productivity. The intensity of pollution depends on locality and the use of land. It is well-known that the contamination is higher in the surroundings of mining territories than other areas. Therefore, these are the primary sources of the wider spread of different polluting agents.

Although numerous metals are essential for the normal functioning of all organisms, no biochemical role has been assigned to aluminium thus far. However, this trivalent element, which is the most widespread metal in the crust of the earth, has attracted significant attention due to its toxic influence on most living systems [9].

Some heavy metal-like ions are constituents of the upper soil layer in large amounts, as is the Al. The lowering soil pH makes these compounds more soluble [26]. In crop production, aluminium toxicity is one of the major growth limiting factors in acidic soils.

The toxic effects of aluminium are primarily root-related [34, 35]. The root system becomes stubby as a result of inhibition of elongation of the main axis and lateral roots [23]. The severity of inhibition of root growth is an acceptable indicator of genotypic differences in aluminium toxicity [12, 13]. The aluminium toxicity often expressed simultaneously in two ways, namely induced deficiency of mineral nutrients, and inhibition in root elongation. Inhibition of root growth by aluminium should further increase the risk of phosphorus deficiency; aluminium toxicity may inhibit the shoot growth by limiting supply of nutrients and water by poorer subsoil penetration or lower root hydraulic conductivity [24].

Aluminium interferes with cell division in root tips and lateral roots, increases cell wall rigidity by cross linking pectins, reduces DNA replication by increasing the rigidity of the DNA double helix, fixes phosphorous in less available forms in soils and on root surfaces, decreases root respiration, interferes with enzyme activity governing sugar phosphorylation

and the deposition of cell wall polysaccharides and the uptake, transport and also use of several essential nutrients (Ca, Mg, K, P and Fe) [15]. Excess Al even induces iron deficiency symptoms in rice (*Oryza sativa* L.), sorghum and wheat [4, 16]. Pereira et al. [31] and Corrales et al. [6] examined the effect of Al on the growth of *Cucumis sativus,* such as one of the Al-sensitive plants.

Many studies have examined the effect of different bacteria on the compensation of Al-stress [20, 29, 18]. The use of plant growth promoting rhizobacteria (PGPR) including phosphate and potassium solubilising bacteria as a biofertilizer was suggested as a sustainable solution to improve plant nutrition and production [36]. These bacteria vary in their mechanisms of plant growth promotion but generally influence growth via P solubilisation, nutrient uptake enhancement, or plant growth hormone production [32]. Bacteria are common inhabitants of metal-contaminated sites, where they accumulate and immobilize heavy metals. The cell walls of gram-positive bacteria have strong metal-binding properties [2]. Some bacteria also produce extracellular polysaccharide sheaths that bind metals [27]. Binding of the siderophore to a heavy metal dramatically changes the free metal concentration. The effect on metal uptake and toxicity are dependent on this siderophore-metal complex being recognized by an uptake receptor [5]. Siderophore production is used *in situ* as a protective mechanism against heavy metal toxicity [11].

The aim of this study was to examine the compensation effect of living bacteria containing fertilizer which contains two bacteria *Azotobacter croccoccum* and *Bacillus megaterium* under Al stress conditions. Our hypothesis was that living bacteria containing fertilizer depending on the applied Al concentration could compensate the effect of different Al-treatments. Special interest was given the changes in dry matter accumulation of root and shoot and Al uptake, as well as to the risk associated with the increase in the content of Al.

MATERIALS AND METHODS

The experimental plant was cucumber (*Cucumis sativus* L. *cv. Delicates*). The seeds were soaked in 10 mM $CaSO_4$ for 4 hours after sterilization and then germinated on moistened filter paper at 25 ^oC. The seedlings were transferred to continuously aerated nutrient solution of the following composition: 2.0 mM $Ca(NO_3)_2$, 0.7 mM K_2SO_4, 0.5 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.1 mM KCl, 1µM H_3BO_3, 1µM $MnSO_4$, 10 µM $ZnSO_4$, 0.25 µM $CuSO_4$, 0.01 µM $(NH_4)_6Mo_7O_{24}$. Iron was added to the nutrient solution as Fe(III)-EDTA at a concentration of 10 µM. The pH of nutrient solution was 6.2.

The seedlings were grown under controlled environmental conditions (light/dark regime 10/14 h at 24/20^oC, relative humidity of 65–70% and a photosynthetic photon flux of 300 µmol m^{-2} s^{-1}) in growth chamber. The volume of experiment pots were 1.0 L, with one pot containing 1 plant.

The dry matter content was measured by thermogravimetric method. The samples were dried for two days at 85 ^oC. The dry matter of the shoots and roots of three plants was measured. The root/shoot ratio was calculated from the dry weight. Root length was measured by placing the root on covered millimetre paper. The number of repetition was three. The root length was measured in the 20^{th}, 30^{th}, 40^{th}, 50^{th}, 60^{th} and 70^{th} hours of the experiment.

The element (Al, Fe, Mn, Zn) contents of plants were determined using an OPTIMA 3300DV ICP-OA spectrophotometer. Ten ml HNO_3 (65v/v%) were added to each gram of the samples for overnight incubation. Then, the samples were pre-digested for 30 min at 60°C. Finally, 3 ml H_2O_2 (30m/m%) were added for a 90 min. boiling at 120°C. The solution were filled up to 50 ml, homogenized and filtered through MN 640 W filter paper. The number of laboratory readings for ICP was the mean of three samples.

The relative chlorophyll contents (Spad index) of the 2^{nd} leaves of the cucumber were measured (n=14) using a Chlorophyll Meter, SPAD - 502 (Minolta).

The applied biofertilizer contains *Azotobacter chroococcum* and *Bacillus megaterium*. Both bacteria play an important role in nature. The *Azotobacter chroococcum* bounds atmospheric and extracting nitrogen in the form of ammonium ions in the soil. *Bacillus megaterium* is a phosphate solubilising bacteria having capability of solubilising insoluble phosphate in the soil and make them available to the plants. The dose of biofertilizer was 1ml dm^{-3}. The nutrient solution was completed with $Al_2(SO_4)_3$, $Al(NO_3)_3$, $AlCl_3$ (10^{-4}M and 10^{-3} M) when Al-stress was examined. The Al-compounds and biofertilizer were added to the nutrient solution at the beginning of the experiments and at every nutrient solution change. The experiment was finished on the 23rd day of experiment.

Microsoft Office Excel 2003 and Sigma Plot 8.0 version were used to the statistical analysis.

RESULTS

Usually, plant growth is the most sensitive to heavy metals because of the complexity of the physiological processes involved. First, the toxic effect of Al was observed at the $Al_2(SO_4)_3$ treatments (Table 1). The dry weights of cucumber shoots and roots decreased under Al treatments. The effect of $Al_2(SO_4)_3$ on the dry weight depends on the applied concentration. The lower Al concentration caused moderate dry weight reduction both in the shoot and root. The reduction effect was higher in the case of root (14 %) than in the shoot (6 %). The 10^{-3}M Al treatments resulted in less (40-50 %) dry matter production, than 10^{-4}M Al treatment compared to the control values. The root was more sensitive to the higher Al concentration, as the same affect was experienced in the case of lower Al concentration. The 10^{-3}M Al treatments caused a more than 50 % decline in the dry weight of the root. As we can see in Table 1, the microorganism containing nutrient solution moderated the toxic effect of Al. The additional applied living bacteria containing fertilizer significantly increased the dry matter of cucumber roots compared to the simple Al treatment. The dry matter of shoots and roots

increased with 40-50% at the 10^{-4} $Al_2(SO_4)_3$ treatment due to additional biofertilizer treatments. In the case of 10^{-3}M $Al2(SO_4)_3$ the biofertilizer treatment did not induce any changes.

Examining the favourable impact of biofertilizer on the uptake of Al and other elements, the contents of elements both in the shoot and root were measured during Al-stress (Table 2 and Table 3). The amounts of Fe, Mn and Zn were analyzed, because they have very important role in the redox, detoxification and energy transformation processes. Due to two Al treatments, higher Al concentration was observed both in the shoot and root, while the amounts of other elements (Fe, Mn, Zn) decreased. The Al-content of shoot and root decreased when living bacteria containing fertilizer was added to the Al-treatments and in the case of the shoots, it declined below the control value. The contents of Fe, Mn and Zn increased during the living bacteria containing fertilizer treatment compared to the single Al-treatment. The highest amounts of Al and Fe remained in the roots, as a consequence of the retarded root-to-shoot transport.

The element composition of leaves may influence chlorophyll contents. The toxicity and growth inhibition effect of Al is related to decreased photosynthesis and decreased organic matter production. The relative chlorophyll contents of cucumber leaves can be seen in Table 4. The relative chlorophyll content decreased by 9-14 % under Al-stress on the17^{th} day of treatment. This decrease of relative chlorophyll contents was also experienced compared to the control value when biofertilizer was applied on the 17^{th} day of the experiments. In older plants (23^{rd} day), the relative chlorophyll content was significantly higher when biofertilizer was added to the Al treated plant.

The effect of the three Al compounds ($Al_2(SO_4)_3$, $Al(NO_3)_3$, $AlCl_3$) in 10^{-3}M and 10^{-4}M concentration was examined on the root growth (Figure 1). In all the cases the impact of living bacteria-containing fertilizer was investigated.

The root growth decreased for all Al treatments depending on concentration. The inhibition of root growth was higher at higher Al concentrations. There were small differences between the different Al-form treatments at the 10^{-3} M concentration but at the 10^{-4} M concentration the order is: $Al_2(SO_4)_3$ > $Al(NO_3)_3$ > $AlCl_3$. The root length was longer with 25 % in the 20th hour of the experiment compared to the control roots when living bacteria-containing fertilizer was applied. Moreover, the biofertilizer could compensate the effect of $Al_2(SO_4)_3$, $Al(NO_3)_3$ and $AlCl_3$ when 10^{-4}M concentration were applied, while this effect was not pronounced at higher concentrations. The living bacteria based fertilizer had only a slight compensation effect when 10^{-3}M $Al(NO_3)_3$ and $Al_2(SO_4)_3$ treatments were applied. The biofertilizer could not compensate the effect of 10^{-3}M $AlCl_3$. Moreover, the root growth decreased when living bacteria-containing fertilizer was applied, in comparison to the $AlCl_3$ treatment.

The root/shoot ratio was measured when the first foliage-leaf appeared (4th day) and on the 14th days of the experiment, when the second foliage-leaf was full developed. The root/shoot ratio was decreased due to the Al-treatments in comparison to the control (Figure 2). The living bacteria-containing fertilizer modified the root/shoot ratio when 10^{-4}M $Al_2(SO_4)_3$ and 10^{-4}M $Al(NO_3)_3$ treatments were applied. In these cases, the root/shoot ratio was higher than the control, even when only living bacteria-containing fertilizer was used. The biofertilizer could compensate the effect of 10^{-3} M $Al_2(SO_4)_3$ because the root/shoot ratio was higher than without biofertilizer. On the other hand, the biofertilizer could not compensate the effect of 10^{-3}M $Al(NO_3)_3$ and $AlCl_3$.

DISCUSSION

Several previous studies have been published about Al toxicity, its physiological and biochemical mechanisms. It is known, that complex forming ability of organic acids (released

by roots, or microorganisms) reduces Al-toxicity [3, 19]. Useful soil microorganisms can exudates organic acids, which may have a role in the counteraction of toxic Al.

Our work presents, results about the Al and living bacteria-containing fertilizer single and combined effect on cucumber dry matter production. The dry weights of cucumber shoots and roots decreased in line with the increasing Al concentration. Pereira et al. [31] also observed *in vivo* that aluminium strongly interferes with *Cucumis sativus* growth. The effect on plant growth is a very complex process and the Al-induced inhibition of growth can have multiple causes. The exclusion mechanism is primarily mediated by Al-activated exudation of organic acids, such as malate, citrate or oxalate from the root apex and the site of Al toxicity [25]. In the case of higher Al concentration, the root damage was very pronounced. According to previous studies [34, 35], the toxic effects of aluminium are primarily root-related. Additional biofertilizer treatment can compensate the Al toxicity effect, thus producing higher dry matter results. Organic acids excreted by microorganisms of biofertilizer, chelate Al in the rhizosphere reducing the concentration and toxicity of Al at growing root tip [25]. One of the applied microorganisms is a *Bacillus megaterium*, which has a phosphate solubilising capability. According to Pellet et al. [30], phosphate has also been identified as a form of root exudates which has significant role in cation chelation, and therefore it can also be considered a potential source in Al exclusion from the root tip.

Due to two Al treatments, higher Al concentration was observed both in the shoot and root, while the amounts of other elements (Fe, Mn and Zn) decreased. The content of Al decreased when living bacteria containing fertilizer was applied in all the cases, and it remained below the control value. In Al sensitive plants – such as cucumber – Al was considerably deposited in the root-tips; the root elongation was retarded and the top growth was inhibited. Nalewajko and Paul [28] demonstrated that the Al (250 mg l^{-1}) significantly decreased the microbial phosphate uptake in water samples from two Canadian lakes. DeGraaf et al. [7] stated that the

more Al concentration was given to the nutrient solution, the highest the Al concentration was in the plants.

There is no convincing evidence that Al is an essential mineral element even for accumulator species. However, there are many reports on the beneficial effects of low Al concentrations in the soil or nutrient solution on plant growth [14]. Because of the similarity in size and change between Al^{3+} and Fe^{3+}, aluminium readily forms complexes with siderophores [17], and these aluminium-sideophore complexes can be transported into the cell [8]. *Bacillus megaterium* provides an excellent system to study the effects of such siderophore formation and transport on heavy metal toxicity [20].

The relative chlorophyll content decreased during Al-stress and the biofertilizer application could not compensate this effect over a short time period. According to Fagerai et al. [10], the plant under Al-stress, *Cucumis sativus*, showed a significant decrease in the amount of organic matter, indicating a decrease in photosynthesis, which could be the consequence of the reduction in chlorophyll content. Moreover, Barker [1] showed that aluminium directly affects the photosynthesis rate and indirectly affects the synthesis of enzymes, pigments and essential cofactors for the process. In case of high amounts of organic acids release by microorganisms the nutrients will be more available in the rhizosphere therefore the roots should not release much organic compounds therefore the dry matter loss will be reduced.

The root/shoot ratio was decreased due to the Al-treatments in comparison to the control. Over the inhibition of root growth under aluminium toxication, other obvious symptoms were detected: lateral roots are getting brown and thinner (21, 33 also based on our experiences). The living bacteria-containing fertilizer modified the root/shoot ratio when 10^{-4} M $Al_2(SO_4)_3$ and 10^{-4} M $Al(NO_3)_3$ treatments were applied.

The shoot and root growth ratio varies widely between plant species during their ontogenesis and is strongly modified by external factors. When parts of the shoots are removed, plants

tend to compensate this by lower root growth and returning to a ratio characteristic for the species. However, there is some controversy as to whether this reflects functional equilibrium between roots and shoots [22]. The root/shoot ratio helps to assess the overall health of plants. The normal root/shoot ratio for each is the control. Any changes from this level would be an indication of a change in the overall health of plants.

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Tables

Table 1. Effects of different concentrations on the dry weight of cucumber shoots and roots (n=3± s.e.) (g plant^{-1}) Significant differences compared to the control: *p<0.05, and Al treatment to biofertilizer application: ^{a}p<0.05

Treatments	Dry weight of shoot	Dry weight of root
Control	0.17± 0.07	0.15± 0.01
Biofertilizer	0.31± 0.07*	0.21± 0.07*
10^{-4} M $Al_2(SO_4)_3$	0.16± 0.06	0.13± 0.06
10^{-4} M $Al_2(SO_4)_3$+biofertilizer	0.23± 0.06*^{a}	0.19± 0.04*^{a}
10^{-3} M $Al_2(SO_4)_3$	0.10± 0.09	0.07± 0.01*
10^{-3} M $Al_2(SO_4)_3$+biofertilizer	0.11± 0.04	0.05± 0.01*

Table 2. Concentration of examined elements in the second leaves of cucumber under Al-stress and treated using bio-fertilizer (mg kg^{-1}) n=3± s.e Significant differences compared to the control: *p<0.05;

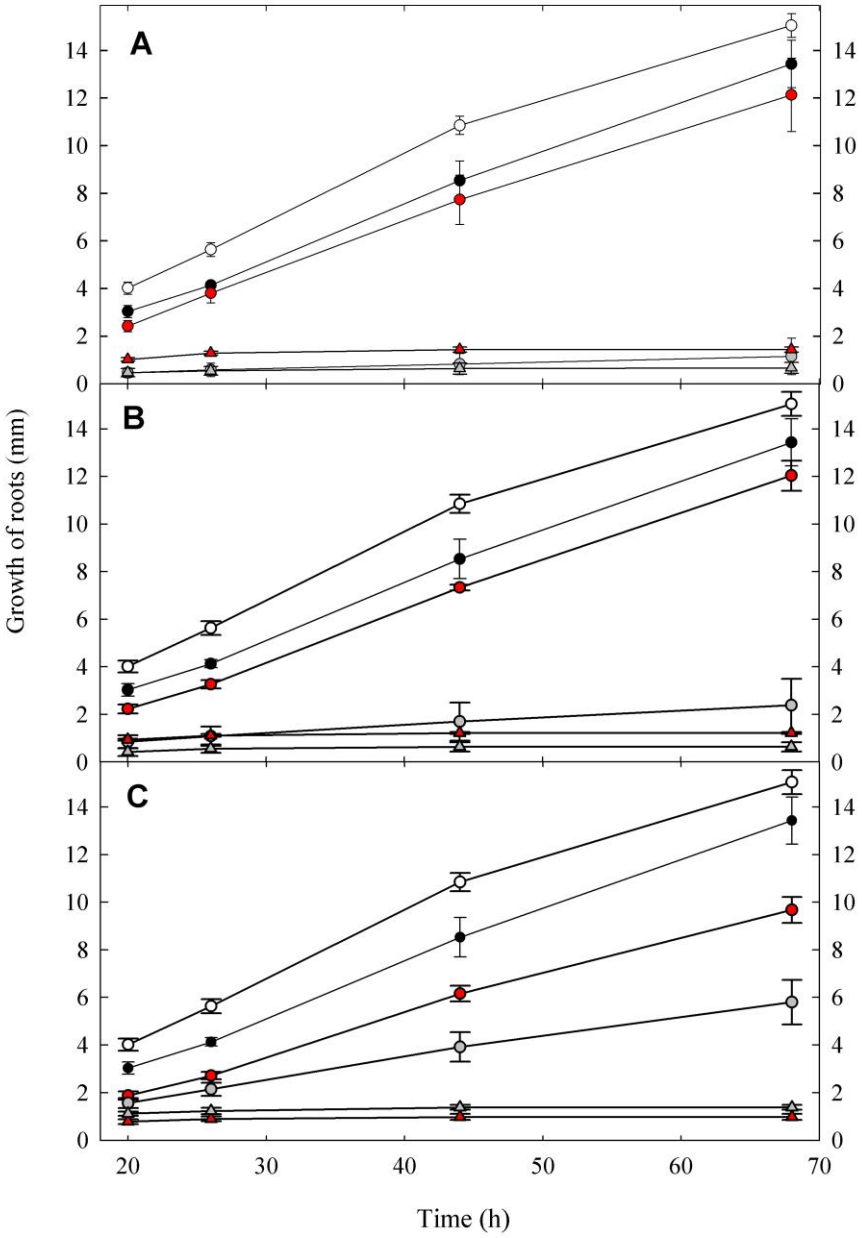
	Treatments		
Elements	Control	10^{-4} $Al_2(SO_4)_3$	Al+Biofertilizer
Al	24.20± 1.7	68.40± 3.8*	23.90± 3.0
Fe	194.29± 8.5	139.10± 9.3*	143.50± 10.1
Mn	61.30± 4.7	17.60± 1.8*	51.90± 6.1
Zn	75.60± 5.8	52.10± 4.7*	67.80± 4.9

Table 3. Concentration of examined elements in the roots of cucumber under Al-stress and treated using biofertilizer (mg kg^{-1}) n=3± s.e Significant differences compared to the control: *p<0.05; **p<0.01;***p<0.001.

	Treatments		
Elements	Control	10^{-4} $Al_2(SO_4)_3$	Al+Biofertilizer
Al	131.7± 7.5	8057.1± 564***	4577.0± 325***
Fe	1046.8± 95.7	974.3± 87.0	1140.8± 65.1
Mn	30.9± 1.1	8.7± 0.01**	23.7± 1.2
Zn	40.1± 1.7	34.7± 1.8*	66.1± 5.1*

Table 4. Effect of different treatments on the relative chlorophyll content (Spad index) in the second leaves of cucumber. n=15± s.e. Significant differences compared to the control: *p<0.05; and Al treatment to biofertilizer application: [a]p<0.05

Treatments	17th day	20th day	23rd day
Control	48.9± 0.70	44.2± 0.92	38.8± 1.10
10^{-4} $Al_2(SO_4)_3$	44.8± 1.55	44.3± 1.60	36.6± 1.05
10^{-4} $Al_2(SO_4)_3$ + Biofertilizer	42.3± 0.81	40.8± 1.19*	40.0± 1.01*[a]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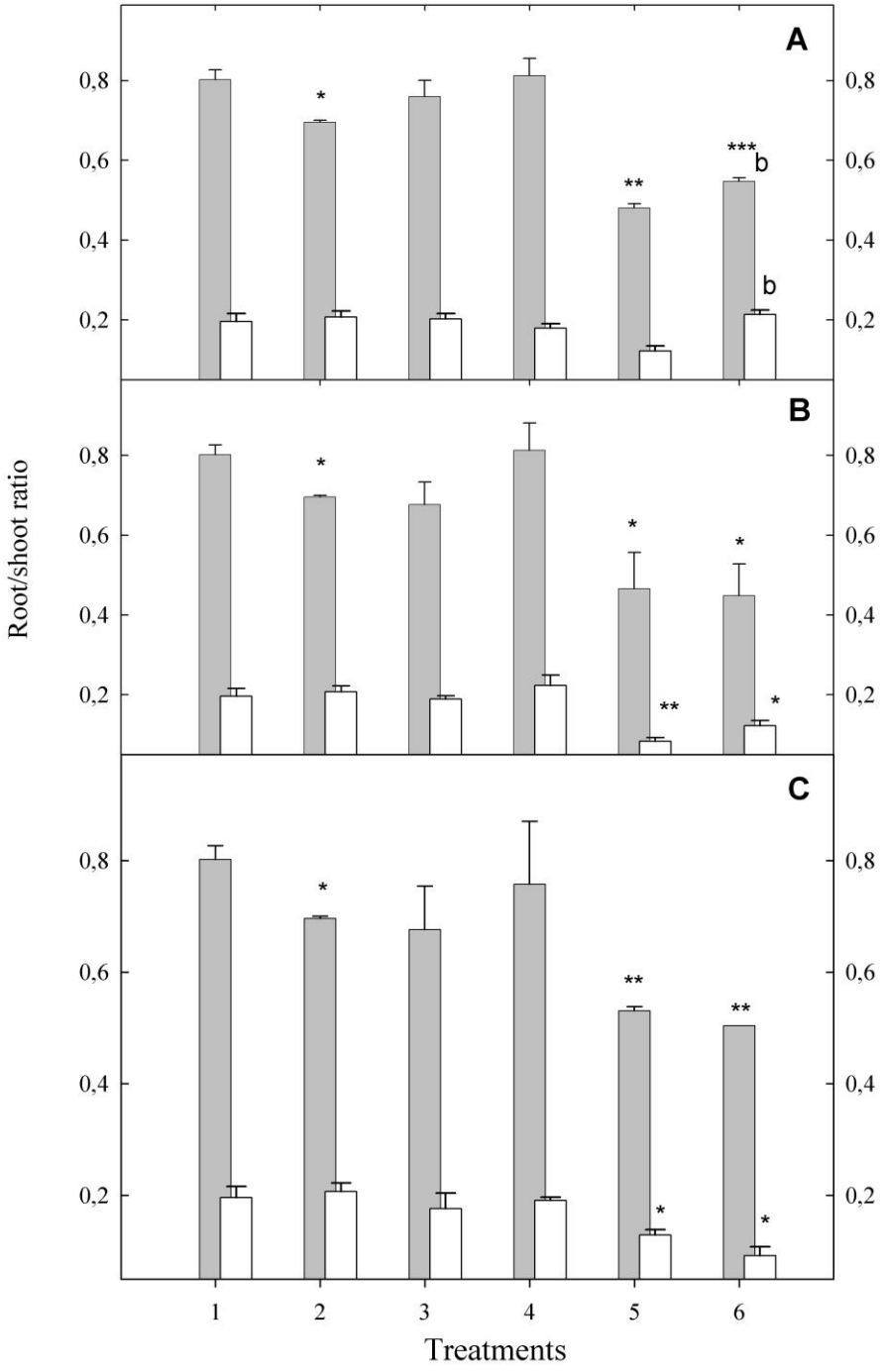
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6
Treatments

Figure 1. Effect of different Al compound ($Al_2(SO_4)_3$, $Al(NO_3)_3$, $AlCl_3$) and biofertilizer on the growth of cucumber roots (mm). All data are significantly different from the control at the $p<0.05$ level. Values represented mean ±s.e.

Marks: A: Control, Biofertilizer, $Al_2(SO_4)^3$ 10^{-4} M+biofertilizer, $Al_2(SO_4)^3$ 10^{-4} M, $Al_2(SO_4)^3$ 10^{-3} M+biofertilizer, $Al_2(SO_4)^3$ 10^{-3} M.

B: A: Control, Bio-fertilizer, $Al(NO_3)_3$ 10^{-4} M+biofertilizer, . $Al(NO_3)_3$ 10^{-4} M, $Al(NO_3)_3$ 10^{-3} M+biofertilizer, $Al(NO_3)_3$ 10^{-3} M.

C: Control, Biofertilizer, $AlCl_3$ 10^{-4} M +biofertilizer $AlCl_3$ 10^{-4} M, $AlCl_3$ 10^{-3} M +biofertilizer, $AlCl_3$ 10^{-3} M.

Figure 2. Effect of different Al compound ($Al_2(SO_4)_3$, $Al(NO_3)_3$, $AlCl_3$) and biofertilizer on the root/shoot ratio of cucumber. Significant differences compared to the control: *p<0.05; **p<0.01;***p<0.001, and Al treatment to biofertilizer application: ^{b}p<0.01. Marks: A : 1. Control, 2. Biofertilizer, 3. $Al_2(SO_4)^3$ 10^{-4} M, 4. $Al_2(SO_4)^3$ 10^{-4} M+biofertilizer, 5. $Al_2(SO_4)^3$ 10^{-3} M, 6. $Al_2(SO_4)^3$ 10^{-3} M+biofertilizer. B: 1. Control, 2. Biofertilizer, 3. $Al(NO_3)_3$ 10^{-4} M, 4. $Al(NO_3)_3$ 10^{-4} M+biofertilizer, 5. $Al(NO_3)_3$ 10^{-3} M, 6. $Al(NO_3)_3$ 10^{-3} M+biofertilizer. C: 1. Control, 2. Biofertilizer, 3. $AlCl_3$ 10^{-4} M, 4. $AlCl_3$ 10^{-4} M+biofertilizer, 5. $AlCl_3$ 10^{-3} M, 6. $AlCl_3$ 10^{-3} M+biofertilizer. 4^{th} day of the treatment , 14^{th} day of the treatment