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Aluminium toxicity effects on *Cucumis melo* and response of diphosphonucleoside kinases

Lazaros Symeonidis[1], Mohamad M. Abou Auda[1] & Traianos Yupsanis[2]*

[1] *School of Biology, Dept. of Botany, Aristotle University, Thessaloniky, Greece*
[2] *School of Chemistry, Dept. of Biochemistry, Aristotle University, GR-54124 Thessaloniki, Greece; tel.: +302310997744, fax: +302310997689, e-mail : yupsanis@chem.auth.gr*

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Low pH level (4.0) decreased leaf area and dry mass of shoot and root in comparison to 6.5 pH. Aluminium (Al) concentrations of 74–296 μmol L^{-1}, added to five days old seedlings which were left to grow for two weeks, at pH level 4.0, increased leaf area, dry mass of shoot and root and chlorophyll content of leaves of a Greek melon variety (*Cucumis melo* L.). Increased Al concentrations (74 to 296 μmol L^{-1}) in nutrient solution promoted calcium (Ca) and magnesium (Mg) concentration in the shoot in contrast to the root, while potassium (K) concentration was the same in shoot and root and almost unaffected at low Al concentrations. Iron (Fe) concentration, in contrast to Ca, Mg or K, was higher (about four times) in root than in shoot. Increased values of measured parameters, in low Al concentrations, in relation to the control, probably denoted the beneficial effect of Al^{3+} on H^+ ions. Values of measured parameters started to decrease at 592 μmol L^{-1} Al concentration, in nutrient solution, characterizing the studied material as an Al-tolerant variety. After SDS-PAGE the autoradiograms of shoot and root revealed two phosphorylated bands of low molecular mass of about 14 and 17 kDa. Thin layer chromatography revealed that both extracted protein bands possessed diphosphonucleoside kinase activities using GDP as substrate. Endogenous protein phosphorylation slightly increased (by about 15%, $P \leq 0.05$) at low concentrations of Al in both root and shoot and then decreased.

Key words: *Cucumis melo* L., aluminium toxicity, diphosphonucleoside kinases, protein phosphorylation.

Abbreviations: GDP – guanisinodiphosphonucleoside; NAD – nicotinamide adenine dinucleotide; NDP kinase – nucleoside diphosphate kinase; TLC – thin layer chromatography

Introduction

Aluminium toxicity is considered the most important metal toxicity problem and the major factor limiting crop productivity in acid soils (Foy, 1988; Kochian, 1995; Matsumoto, 2000 and references therein). The first and most rapid symptom of Al toxicity is reduction of root growth,

* Corresponding author

that has been associated with a decrease in mitotic activity (MATSUMOTO & MORIMURA, 1980; ROY et al., 1988). The exact mechanisms of this Al induced inhibition of root growth are not fully understood and a variety of physiological processes and functions may be involved (FOY, 1988; KOCHIAN, 1995; MATSUMOTO, 2000). Aluminium is known to modify the elemental composition of plant tissue by interfering with ion uptake and translocation (FOY et al., 1978; TAYLOR, 1988).

Increase, decrease or no influence on chlorophyll content (as a non-specific biomarker) in response to Al have been reported (GREGER et al., 1992; SIMON et al., 1994; ZAVAS et al., 1996).

The rhizotoxic Al^{3+} ions, prevailing in acid solutions, often enhanced growth at low concentration under acidic conditions that reduced root elongation (ANDERSSON & BRUNET, 1993; KINRAIDE, 1993, 1997; LLUGANY et al., 1995).

H^+ ion toxicity is additionally referred as a limiting factor for plant growth (ANDERSSON & BRUNET, 1993; BRUNET, 1994; KINRAIDE, 1993, 1997).

Regarding proteins and enzymes induced in Al-treated plants, different changes in cytoplasmic and membrane proteins were induced by Al-treatment of maize and barley seedlings (HUTTOVÁ et al., 1998; MISTRÍK et al., 2000; TAMÁS et al., 2000). Phenylalanine ammonia lyase, proteinase inhibitor and metallothionine-like protein have also been proposed to be products of seven wheat genes induced by Al (SNOWDEN & GARDNER, 1993; RICHARDS et al., 1998). It is suggested that these genes represent a suite of stress genes induced by different stressors such as toxic metals, plant pathogens, wounding and so on (CRUZ-ORTEGA & OWNBY, 1993; DIDIER-JEAN et al., 1996). Yet, NAD-kinases (SLASKI, 1989; 1990) and protein kinases (MOUSTAKAS et al., 1992) are involved in the response of Al toxicity to wheat. To our knowledge there are no data available concerning the response of nucleoside diphosphate kinases (NDP-kinase, E.C. 2.7.4.6) to Al toxicity. NDP-kinases catalyse the transfer of the terminal phosphate of 5′ triphosphate nucleotides (NTPs) to 5′ diphosphate nucleotides (NDPs). This transfer is associated with the regulation of intracellular di-and tri-phosphonucleotide levels and it may also participate in the regulation of growth development and signal-transduction processes (RANDAZZO et al., 1991; SOMMER & SONG, 1994; ZHANG et al., 1995). Furthermore, recently NDP-kinases have been proposed as biochemical markers to identify biochemical characteristics among parental species and their hybrids (YUPSANIS & SYMENOIDIS, 2001).

In the present work the effects of aluminium on biomass, nutrients, chlorophyll content and NDP-kinase in *Cucumis melo* were studied.

Material and methods

Plant material and growth conditions

Five days old seedlings of *Cucumis melo* L. were grown for two weeks on half-strength (only for macronutrients) Hoagland nutrient solution, pH 4.0 ±0.2, at different Al concentrations. The appropriate amounts of Al were added into the nutrient solution as $Al_2(SO_4)_3 \cdot 18\ H_2O$ to achieve the 0, 74, 148, 296, 592, and 888 μmol L^{-1} Al concentrations.

All the experiments were carried out in growth chamber at 23 ± 1 ℃ and 60 ± 3% relative air humidity during the light period (16 h ∼200 μmol m^{-2} s^{-1}), 19 ± 1 ℃, and 75 ± 3% relative air humidity during the dark (8 h). The nutrient solutions were changed every second day.

Metal content determination

Fifteen days after the addition of Al the roots and shoots of the plants were separated, washed, dried in a forced- air oven at 80 ℃, weighted and digested in a nitric-perchloric 4:1 solution. Root and shoot samples were analyzed for Ca, Mg, K and Fe by Perkin Elmer Atomic Absorption Spectrophotometer.

Chlorophyll content determination

Plant leaves of each treatment (second leaf from the bottom) were homogenized with 80% acetone. Chlorophyll content was determined by measuring the absorption of extract in a spectrophotometer and calculated according to LICHTENTHALER (1987).

Leaf area measurement

Leaf area (second leaf from the bottom) was measured using a Mk2 area meter connected to a Tc 7000 Series Camera.

Protein determination

Protein concentration was determined according to BEARDEN (1978) using bovine serum albumin as a standard.

Extraction of enzymes

The 15^{th} day roots and shoots were ground in a pestle and mortar with 3 volumes of 100 mmol L^{-1}tris-acetate buffer pH 7. The homogenate was clarified by centrifugation (15 min, 13 000 *g*). Samples dialysed against homogenization buffer were used as enzyme sources.

Protein kinase assay

Protein kinase activity was assayed by incubating 25 μg protein of the enzyme extraction in a total volume 50 μL at 37 ℃ for 2 min in the presence of 0.1 pmol ($\gamma-^{32}P$) ATP (6000 Ci/mmol), 33 mmol L^{-1} tris-HCl pH 7.6, 1 mmol L^{-1} Mg^{2+} and 10 mmol L^{-1} K-Pi pH 7.5 (MOUSTAKAS et al., 1992). Phosphorylation was quantified by liquid scintillation counting according to YUPSANIS et al. (1989).

Endogenous protein phosphorylation
The phosphorylated endogenous proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) according to the LAEMMLI (1970) system. The gels were dried under vacuum and autoradiographed using Kodak-O-Mat-X-ray film (YUPSANIS et al., 1989).

Detection of NDP-kinase activity
After SDS-PAGE and autoradiography, the main labelled bands were excised. Every excised band was washed to remove SDS and the ability of NDP-kinase to transfer (^{32}P)- phosphate from (γ–^{32}P) ATP to nucleoside diphosphate, using GDP as substrate, was assayed and detected by thin layer chromatography (TLC) according to YUPSANIS & SYMENOIDIS (2001).

Statistical analysis
All experiments were repeated 3 times. Means and standard error (SE) are shown in the Figs 1, 2, 3, 4 and 5. Statistical analysis was performed on the data using an ANOVA model (completely randomized block) and Duncan's criterion ($a = 0.05$).

Results and discussion

At low pH (4.0) there was a significant ($P \leq 0.05$) reduction of leaf area (52%), dry shoot (44.5%) and root (36.4 %) mass, in comparison to the leaf area and shoot and root dry mass, respectively, at 6.5 pH. Low pH also decreased shoot but not root Ca, Mg, K, and Fe concentrations (data not shown).

The plant growth reduction observed at low pH shows the toxicity of H^+ which is in accordance with the findings of ANDERSSON & BRUNET (1993), KINRAIDE (1993, 1997), BRUNET (1994), ZAVAS et al. (1996).

Cucumis melo showed good growth or even growth stimulation at low Al concentrations, and pH 4.0, in the nutrient solution, which is in agreement with the reported data in different plants (ANDERSSON & BRUNET, 1993; KINRAIDE, 1993; CLUNE & COPELAND, 1999). Aluminium concentrations of 74 to 296 μmol L^{-1} in the nutrient solution promoted leaf area (Fig. 1), while shoot and root dry mass increased or remain unaffected (Fig. 2). Increased leaf area and shoot and root dry weights in comparison to the control, may be the results of alleviating of proton toxicity by Al^{3+} (ANDERSSON & BRUNET, 1993; KINRAIDE, 1993, 1997; OSAKI et al., 1997 and references therein). A different explanation for the stimulation of plant growth by low Al concentrations may be based on speciation effects related to the complex solution chemistry of Al that may have influenced the bioavailability of other ions in a way that

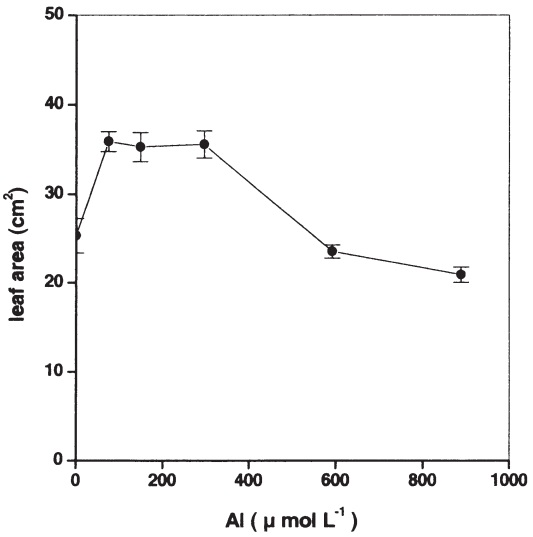

Fig. 1. Effect of different Al concentrations (0, 74, 148, 296, 592, 888 μmol L^{-1}), at pH 4.0, on leaf area of *Cucumis melo* grown in nutrient solution.

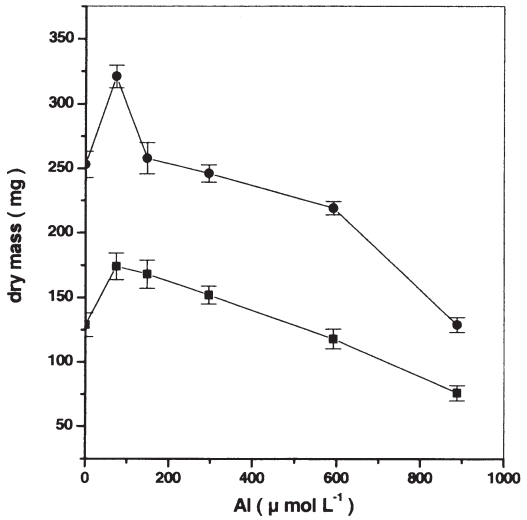

Fig. 2. Effect of different Al concentrations (0, 74, 148, 296, 592, 888 μmol L^{-1}), at pH 4.0, on shoot (●) and root (■) dry weight of *Cucumis melo* grown in nutrient solution.

stimulated plant growth (CLUNE & COPELAND, 1999). Fig. 3 shows that low (74–148 μmol L^{-1}) Al concentrations in the nutrient solution increased chlorophyll a, b and a+b contents. The increase in chlorophyll content in the presence of Al may

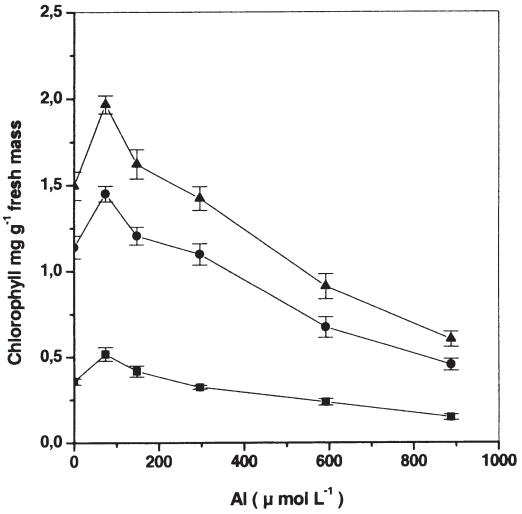

Fig. 3. Effect of different Al concentrations (0, 74, 148, 296, 592, 888 μmol L^{-1}), at pH 4.0, on leaf chlorophyll content (chlorophyll a ●, b ■, and a+b ▲) of *Cucumis melo* grown in nutrient solution.

be due to the Al induced increased level of Mg (Fig. 4b) in the shoot (Greger et al., 1992; Zavas et al., 1996) since Mg is necessary for chlorophyll formation.

Calcium and magnesium concentrations in shoot and root, as affected by Al, are shown in Figs 4a and b. At 74 to 592 μmol L^{-1} Al concentrations, Ca and Mg concentrations of shoot were increased, while root Ca and Mg concentrations were decreased in comparison to the control. In all used Al concentrations, Ca and Mg concentrations of root were lower of about twice or more in comparison to the shoot.

The increased accumulation of Ca and Mg in the shoot suggested that translocation of these elements to the shoot was unaltered by Al (Jan, 1991).

Decreased amount of Ca and Mg in the roots (Figs 4a, b) in contrast to the shoots, at different Al concentrations suggested a continuous transfer

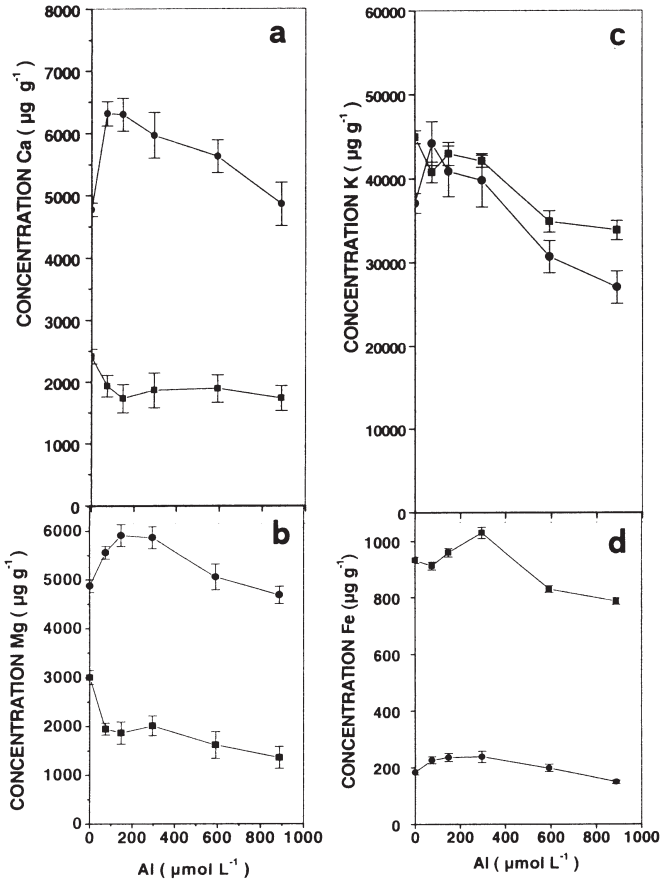

Fig. 4. Concentrations of Ca (a), Mg (b), K (c) and Fe (d) in shoot (●) and root (■) of *Cucumis melo* grown in nutrient solution with different Al concentrations (0, 74, 148, 296, 592, 888 μmol L^{-1}) at pH 4.0.

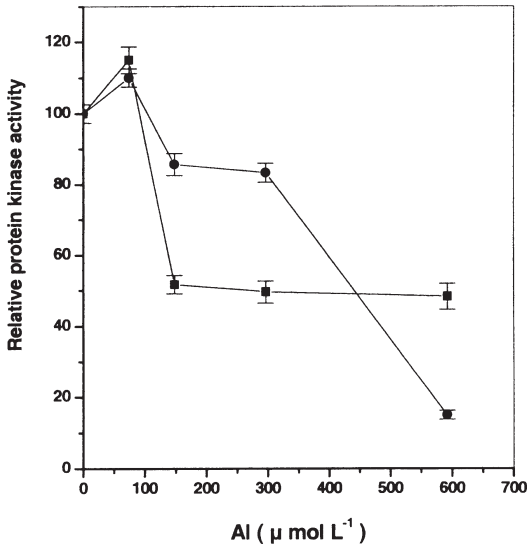

Fig. 5. Effect of different Al concentrations (0, 74, 148, 296, 592 μmol L^{-1}) in the nutrient solution, on relative protein kinase activity (in percent of control) of 19 days old *Cucumis melo* shoot (●) and root (■) at pH 4.0.

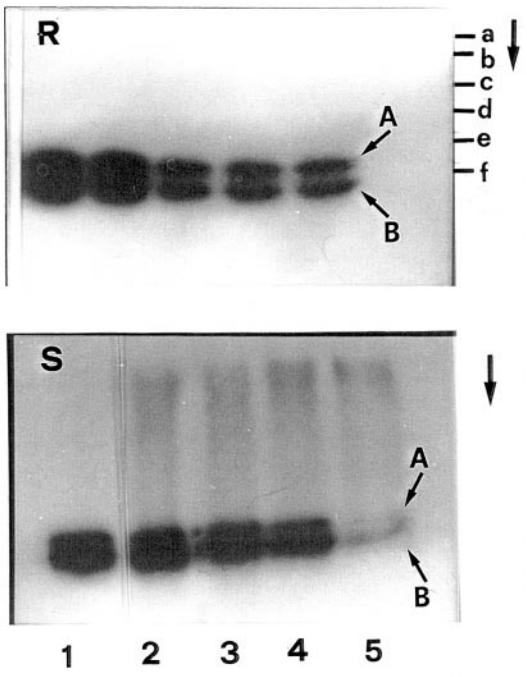

Fig. 6. Analysis by SDS-PAGE and autoradiography of the endogenous proteins of the extractions prepared from *Cucumis melo* root (R) and shoot (S), grown in different Al concentrations (0, 74, 148, 296, 592 μmol L^{-1}), at pH 4.0 in the nutrient solution. Exposure time 24 h. Pre-stained molecular markers: a phosphorylase b (Mr ~ 130000); b bovine serum albumin (Mr ~ 75000); c ovalbumin (Mr ~50000); d carbonic anhydrase (Mr ~ 39000); e soybean trypsin inhibitor (Mr ~ 27000); f lysozyme (Mr ~ 17000). The arrows A and B indicate the position of the catalytic subunits of the NDP kinases.

of Ca and Mg from roots to the shoots (JAN, 1991).

Potassium (K) concentration (Fig. 4c) was almost equal in shoots and roots and at low Al concentrations it was even unaffected.

In contrast to macronutrients, the lower concentration of iron (Fe) in shoots than in roots (Fig. 4d) indicated a retardation of translocation of Fe from roots to the shoots (JAN, 1991).

It is now well known that Al inhibits plant growth by interfering with many physiological processes (KOCHIAN, 1995; MATSUMOTO, 2000). The response of the studied material to low pH (4.0) and different Al concentrations may be in accordance with KINRAIDE's (1993, 1997) hypothesis visualized Al^{3+} and H^+ competing for common apoplastic binding sites. Thus Al^{3+} (prevailing in acidic medium) would be capable of alleviating proton toxicity and protons would be capable of alleviating Al^{3+} toxicity.

Decreased values for almost all measured parameters were observed at 592–888 μmol L^{-1} Al concentrations (Figs 1–4), suggesting that the used melon variety may be Al-tolerant.

The effect of Al on protein kinases activity and endogenous protein phosphorylation was studied at 74, 148, 296 and 592 μmol L^{-1} Al concentrations. Both root and shoot kinase activities were slightly increased by about 15% ($P \leq 0.05$) at 74 μmol L^{-1}Al in comparison to the control and then decreased, by about 50% in the roots over 148 μmol L^{-1} Al and by about 15% in the shoots at 148–296 μmol L^{-1} Al and then decreased rapidly to about 85% at 592 μmol L^{-1} Al concentration ($P \leq 0.05$) (Fig. 5).

Aluminium (Al^{3+}) is known to interact with enzymes that utilise ATP as substrate in plant cells such as protein kinases, NAD-kinases and phosphatases (MARTIN, 1988; SLASKI, 1989, 1990; MOUSTAKAS et al., 1992). For example in a less sensitive durum wheat variety protein kinase activity decreased at all Al concentrations while it increased in the most sensitive (MOUSTAKAS et al., 1992). SLASKI (1990) also reported an increase of NAD-kinase activity in root apical meristems of various crops under Al stress.

SDS electrophoresis followed by autoradiography revealed that two low-molecular-weight (LMW) bands (about 14 kDa and 17 kDa) were mainly phosphorylated in both shoot and root (Fig. 6). These bands were present in the control (-Al) as well as in the Al-treated plants but their intensities decreased with increasing Al concentration (Figs 5, 6).

Thin layer chromatography (TLC) (not shown) revealed that the gel extracted LMW phosphorylated bands of *Cucumis melo* were similar to those in *Pisum sativum* (WHITE et al., 1993), spinach (ZHANG et al., 1995), barley (GEORGATSOS & FISENTZIDES, 1996) and *Thinopyrum ponticum*, *Triticum aestivum* and their hybrids (YUPSANIS & SYMEONIDIS, 2001), indicating that they were catalytic subunits of NDP-kinases.

It is suggested that one form of NDP-kinase is a cytosolic enzyme and may be responsible for processes like DNA and RNA synthesis which require deoxy-and ribo-triphosphonucleosides, respectively. The second NDP-kinase form, bound with membranes, may be responsible for the cycling of GDP for signal transduction (RANDAZZO et al., 1991; WHITE et al., 1993) and is possibly to involved in the interconversion of UDP to UTP during the synthesis of UDP-glucose for cellulose synthesis. However, the exact physiological role of these enzymes under Al-stress remains to be studied.

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