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Physiological Responses of Suspension Cultures of *Catharanthus roseus* to Aluminum: Changes in Polyamines and Inorganic Ions[1]

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Summary

The effects of aluminum (Al) treatment on polyamines were studied using suspension cultures of Madagascar periwinkle [*Catharanthus roseus* (L.) G. Don]. The addition of Al (0.2, 0.5, 1.0 mM) to the suspension cultures caused a significant increase in putrescine within 24 h only in freshly transferred cells. By contrast, Al treatment reduced putrescine content at all other times (days 2–7). Further analysis showed that the largest increase in putrescine occurred at 6 h when Al was added at the time of transfer of cells to fresh medium. If Al treatment was delayed to as little as 3 h after transfer to fresh medium, the cellular level of putrescine decreased. While spermidine remained unchanged, spermine was significantly higher in the presence of Al in all cases. The inhibitory effect of Al on DNA synthesis also depended upon the time of its addition. Cellular Ca, Mg, and Mn decreased in response to Al given at the time of transfer to fresh media while Fe increased, and Na, K and P remained unchanged. The results indicate a critical physiological change in the response of cells to Al within a short time (about 3 h) after transfer to fresh medium.

Key words: Arginine decarboxylase, Catharanthus roseus, DNA synthesis, Inorganic ions, Polyamines, Putrescine, Spermidine, Spermine.

Abbreviations: Al = Aluminum; ADC = arginine decarboxylase; 2,4-D = 2,4-dichlorophenoxyacetic acid; FW = fresh weight; ODC = ornithine decarboxylase; PCA = perchloric acid.

Introduction

Polyamines (putrescine, spermidine, and spermine) play a variety of physiological roles in plant growth and development (Bachrach and Heimer, 1989 a, b; Slocum and Flores, 1991). It has been reported that increased polyamine biosynthesis is necessary for completion of the cell cycle in plant cell cultures (Phillips et al., 1987; Pfosser et al., 1990; Maki et al., 1991), and its inhibition halts the process of cell division (Minocha et al., 1991). Among the many suggested roles, participation of polyamines in stress responses has become an active area of research (Flores, 1991, and references therein). An increase in one or more polyamines is generally considered to be an indicator of plant stress (Flores and Galston, 1984; Galiba et al., 1989; Dohmen et al., 1990; Erdei et al., 1990). At cellular pH, polyamines are cationic and, could, therefore, interact with a variety of negatively charged macromolecules.

Aluminum, a major constituent of soil, has recently been suggested as an important factor in forest decline due to its release from soil under conditions of increased acidity (Godbold et al., 1988; Hauhs et al., 1989; Schulze, 1989). Several

hypotheses have been proposed to explain the toxic effects of Al at cellular level in plants. Some of these are: (1) the inhibition of cell division (Ulrich and Clarkson, 1992); (2) blocking of Ca channels in plasmalemma due to the replacement of Ca from exchange sites in root apoplast (Asp et al., 1988; Godbold et al., 1988; Stienen and Bauch, 1988; Rengel and Robinson, 1989); (3) the antagonistic effects of Al on other inorganic cations including Ca and Mg that could interfere with ion uptake and balance (Siegel and Haug, 1983). Additionally, the chemical interaction between Al and P may result in dual effects of Al in plants, i.e., (a) Al may induce P deficiency by causing its precipitation on the root surface or inside the root (Clarkson, 1966; McCormick and Boreden, 1978); and (b) the formation of insoluble Al-phosphate in the soil or on the root surface could lessen Al toxicity (Robson and Pitman, 1983).

Most studies on Al toxicity have dealt with its effects on nutrient uptake in seedlings using sand or hydroponic cultures (Asp et al., 1988; Ohno et al., 1988; Schroder et al., 1988; Schier et al., 1990; Jentschke et al., 1991). Few reports are available on the effects of Al using *in vitro* grown cell cultures (Minocha et al., 1992). The present report deals with the short-term effects of Al on changes in cellular content of both inorganic cations and the polyamines (organic cations). Our results show that the age of cells in culture and the length of the treatment period are critical factors determining the physiological response of cells to Al.

Materials and Methods

Maintenance of suspension cultures

The cell line of *Catharanthus roseus* (L.) G. Don used in this study was obtained from the laboratory of Atsushi Komamine, Tohoku University, Sendai, Japan. Suspension cultures have been maintained in our laboratory for the past four years in modified MS medium on a weekly subculture routine (Kodama et al., 1989; Minocha et al., 1991, 1992). The medium was supplemented with 2.2 μM 2,4-D and 3 % sucrose. Before autoclaving, the pH of medium was adjusted to 6.2. On Friday of each week, 7 mL of suspension were transferred to 43 mL of fresh medium. The flasks were maintained on a gyratory shaker at 120 rpm in the dark at 25 ± 2 °C.

Experimental treatments

Seven-day-old cell suspensions from an appropriate number (4–5) of 250 mL flasks (containing 50 mL cell suspensions) were pooled in a 1 L round-bottomed flask containing fresh medium to obtain the same cell density as for routine subcultures (i.e., 7 mL of 7-d-old cells per 43 mL fresh medium). To achieve homogeneity of suspensions, the cells were constantly stirred at a low speed with a magnetic stirrer during the period of transfer to experimental flasks. Ten milliliters of cell suspension were transferred to individual 50 mL experimental flasks. Concentrations of $AlCl_3$ (0, 0.2, 0.5, 1.0 mM) were chosen for treatments based on previous work in our laboratory (Minocha et al., 1992). Calculated amounts of $AlCl_3$ (100 mM) were added to nine 50-mL flasks (3 replicates per treatment) each day at the same time for 7 d of the culture period. The cells from treated flasks and a set of control flasks were collected for analysis at designated times. One-half milliliter of cell suspension was taken from 3 replicate flasks per treatment to count cell numbers each day during the 7-d culture period. Cell numbers and cell viability were determined according to the procedures described (Minocha et al., 1991, 1992). All treatments were in triplicate, and each experiment was conducted at least three times unless otherwise mentioned.

Polyamine analysis

Cells were collected on Miracloth (Calbiochem Corp., La Jolla, CA) by vacuum filtration and washed twice with deionized distilled water. The cells were placed in ice-cold 5 % PCA in 1.5-mL microfuge tubes (200 mg FW in 0.8 mL PCA). Polyamines were extracted by repeated (3×) freezing (−20 °C) and thawing (room temperature). Detailed studies from our laboratory indicate that extraction of PCA-soluble polyamines by freeze-thawing is equal to or better than that by homogenization with Polytron (Minocha et al., 1994). The homogenate was centrifuged at 16,000 × g for 10 min at 4 °C and the supernatant fraction was used for polyamine analysis. Prior to dansylation, heptanediamine was added to the extracts at a concentration of 0.04 M as an internal standard. Polyamines were dansylated and quantified by HPLC according to the method of Minocha et al. (1990).

Arginine decarboxylase (ADC) assay

Cells (500 mg FW) collected on Miracloth by vacuum filtration were transferred into 15 mL Corex centrifuge tubes containing 1.0 mL of cold extraction buffer [0.05 M Tris-HCl pH 8.4 adjusted at 4 °C, 0.1 mM Na_2-ethylenediamine-tetraacetate, 5.0 mM dithiothreitol, and 0.05 mM pyridoxal-5-phosphate]. The cells were homogenized at 18,000 rpm for 90 s using a Brinkmann Polytron homogenizer (Brinkmann Instrument, Inc., New York). The homogenates were centrifuged at 18,000 × g for 15 min at 4 °C, and the supernatant fractions used for ADC assay according to Robie and Minocha (1989). The reaction mixture contained 200 μL of cell extract and 50 μL of 2 mM cold arginine containing 0.05 μCi DL-1-^{14}C-arginine (Amersham Corp., Arlington Heights, IL; sp. act. 20 mCi/mmol).

Incorporation of ^{3}H-thymidine

At the time of analysis, 0.5 mL of suspensions were removed from each treatment flask and placed in 10 mL test tubes. One μCi of ^{3}H-thymidine (ICN Biochemicals, Irvine, CA, specific activity, 61 Ci/mmol) was added to each tube. The tubes were incubated on a shaker at 50 rpm at 27 °C for 30 min. Procedures for washing free ^{3}H-thymidine and measurement of ^{3}H-incorporation have been described (Minocha et al., 1992).

Determination of cellular inorganic ions

Cells (50 mg FW) collected on Miracloth by vacuum filtration were transferred to 10 mL plastic tubes containing 5 mL of 0.01 N HCl and subjected to repeated (3×) freezing (−20 °C) and thawing (room temperature). The samples were either filtered through a 0.45 μm syringe filter or centrifuged at 18,000 × g for 20 min at 4 °C to remove cell debris. The supernatant fraction was analyzed for inorganic ions with a Beckman Spectrospan V ARL DCP (Direct Current Plasma Emission Spectrometer, Beckman Instruments Inc., New York) according to Minocha et al. (1992).

Replication and statistical analysis

Since the cultures used in this study were asynchronous and the cell numbers changed with time through the duration of the experiments, the effects of Al treatment were statistically analyzed and

compared only within a given time period. Data for each time period and each set of treatments were analyzed as a series of one way analysis of variance (ANOVA) to determine whether statistically significant differences occurred among treatments and control at a given time. If F values for one-way ANOVA were significant, multiple comparisons were performed using the Tukey Test to determine where the significance was. All analyses were performed with Systat™ for Windows, Version 5.0 (Systat Inc. Evanston, IL).

Results

Time course of cell division and polyamines

The first cell division in some cells occurred within 1 to 2 d of culture as indicated by a small increase in cell number (Fig. 1). A larger increase in cell number was observed between days 3 and 6. The rate of cell proliferation slowed after day 6 (Fig. 1).

^{3}H-thymidine incorporation was quite low immediately after subculture (0–6 h) (Fig. 1). DNA synthesis increased 2–3-fold between 6 and 24 h. From the results of two separate sets of experiments (Fig. 1), it was observed that the rate of DNA synthesis peaked at 24 h. During the next 4 d, the rate of DNA synthesis declined gradually to its lowest level on day 7.

Among the three polyamines, changes in putrescine were the greatest during the 7-d growth period (Fig. 2). During the period of 3–9 h, putrescine showed a 5-fold increase. The level of putrescine decreased between 12 and 24 h. A gradual increase in putrescine was seen again during the next 4 d, with a second peak between days 4 and 5. During the next 2 d, putrescine decreased by almost 70 %. Measurement

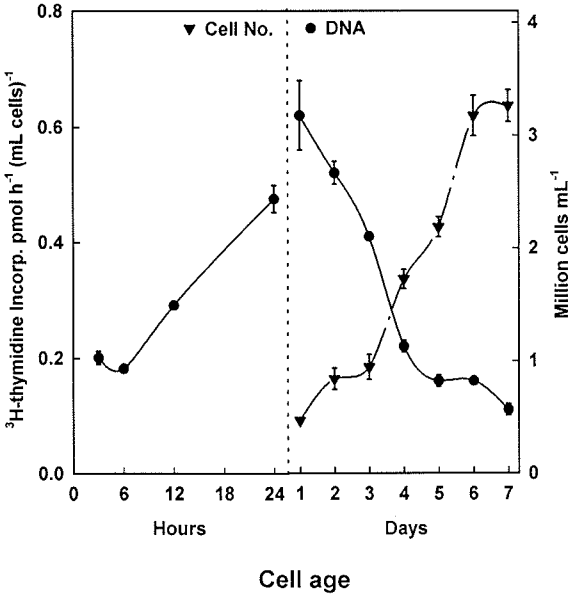

Fig. 1: Time course of changes in DNA synthesis and cell numbers during the 7-d growth period. Data are from two separate experiments: 0–24 h (left), and 1–7 d (right) after transfer to fresh media. Values are Mean ± SE of 3 replicates.

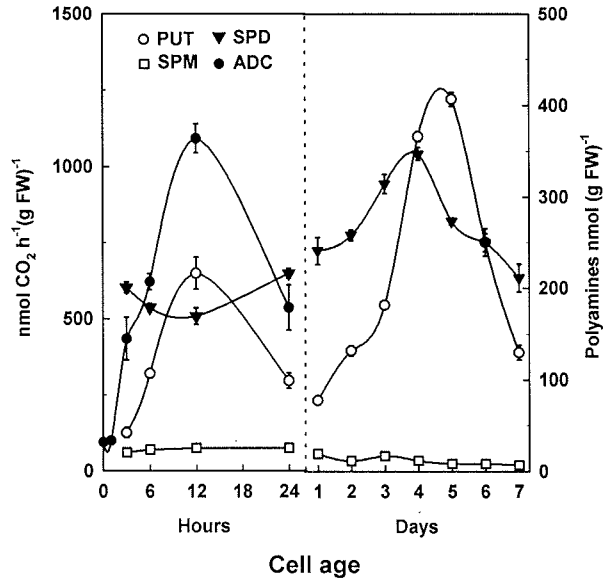

Fig. 2: Time courses of changes in cellular polyamines and ADC activity during the 7-d growth period. Data are from two separate experiments: 0–24 h (left), and 1–7 d (right) after transfer to fresh media. Values are Mean ± SE of 3 replicates. ADC = arginine decarboxylase, Put = putrescine, Spd = spermidine, Spm = spermine.

of ADC activity during the first 24 h following transfer of cells to fresh media showed that this enzyme also increased rapidly and peaked at 12 h, coincident with the first peak of putrescine (Fig. 2). Enzyme activity fell concomitant with the decrease in putrescine.

Spermidine showed a slight decrease during the first 12 h, followed by a consistent increase up to 4 d, when it peaked at a level almost twice as much as that at the time of transfer (Fig. 2). Spermidine declined after 4 d to the same level as that in the beginning of the experiment. Thus, the peak of spermidine occurred slightly ahead of the second peak of putrescine. Spermine content in the cells was always very low and remained unchanged during the 7 d culture period (Fig. 2).

Effects of cell age (culture period) on response to Al

In order to determine the period of maximum sensitivity, Al was added to cells every day of the 7 d culture period at the same time, and the cells analyzed after 24 h of incubation. Data presented in Fig. 3 A show that when Al was added at the time of subculture to fresh medium (zero time), 0.2 and 0.5 mM Al consistently caused a small (30–60 %) but statistically significant increase in cellular putrescine. A slight increase was noted also when cells received 1.0 mM Al treatment. In contrast, the response was different when Al was added at other times. Cells that were more than one day old when treated with Al for 24 h, showed significant decreases in putrescine. Maximum reduction was observed in 3 to 6-d-old cells when putrescine levels were high in the control cells. The decrease was concentration-dependent. The

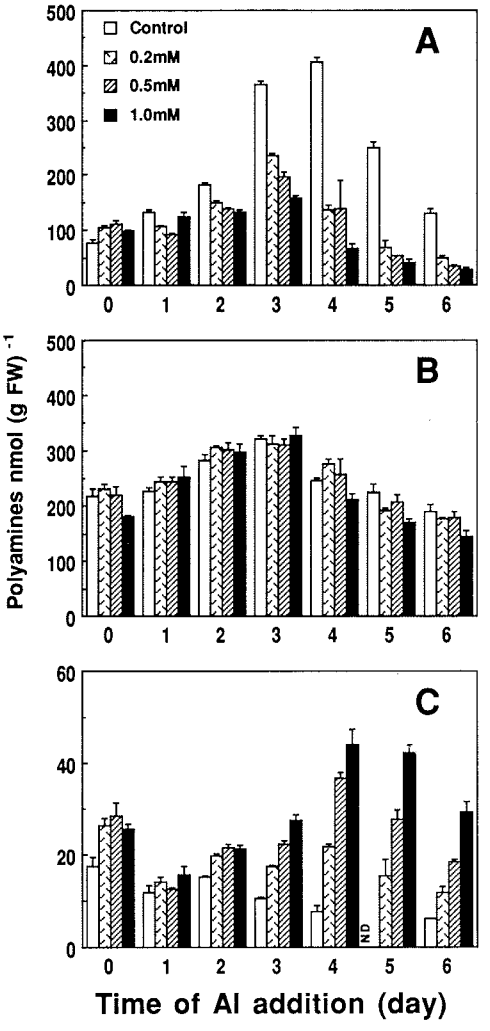

Fig. 3: Effects of Al on cellular levels of putrescine (A), spermidine (B), and spermine (C) when Al was added to 0 to 6-d-old cells and the cells analyzed 24 h thereafter. Values are Mean ± SE of 3 replicates.

inhibitory effect was still noted on day 6 but was much less than that on day 5.

No major difference in spermidine was observed between controls and treated cells, except a 25 % decrease on days 5 and 6 in cells treated with 1 mM Al (Fig. 3 B). In contrast to putrescine, spermine showed an opposite response to Al (Fig. 3 C). That is, spermine increased regardless of when Al was added. The extent of spermine increase depended upon the concentration of Al and the time of its addition. Whereas spermine in the control cells was barely detectable, in 1.0 mM Al-treated cells it increased to as much as 6 % of the total polyamine pool.

The effect of Al on DNA synthesis was also analyzed at the same time that the polyamines were determined in the above experiments. Addition of 0.5 or 1.0 mM Al at the time of transfer caused almost a 50 % reduction of thymidine incorporation after 24 h (Fig. 4). No difference in DNA synthesis between control and 0.2 mM Al treatments was noted at this time. For one day-old cells, only 0.5 mM Al caused a significant reduction of DNA synthesis. From days 2 to 6, thymidine incorporation in 0.5 and 1.0 mM Al-treated cells decreased by 30–50 %. The relative rate of DNA synthesis decreased with the age of cells in culture and with increasing levels of Al.

The data from the above experiments showed that obvious changes in the physiological responses of cells to Al could be observed after the first day of culture. In order to analyze the precise time of this change, the response of cells to Al addition at the time of subculture was studied at shorter time intervals. Two sets of experiments were performed.

In the first of these experiments, freshly-subcultured cells were treated with Al and polyamines determined at various times during the next 36 h. An increase in putrescine was observed as early as 3 h after addition of Al (Fig. 5). At 6 h, pu-

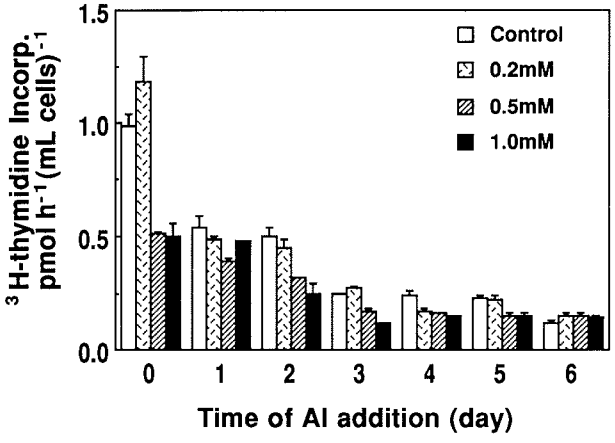

Fig. 4: Effects of Al on DNA synthesis when Al was added to 0 to 6-d-old cells and the cells analyzed 24 h thereafter. Values are Mean ± SE of 3 replicates.

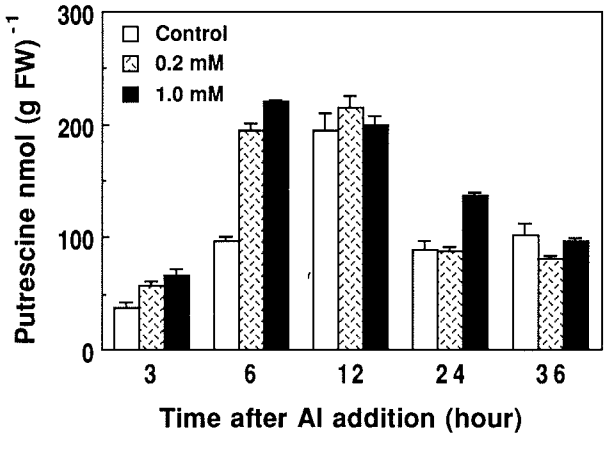

Fig. 5: Effects of Al on cellular levels of putrescine when Al was added at the time of transfer to fresh media, and the cells analyzed at 3, 6, 12, 24, and 36 h thereafter. Values are Mean ± SE of 3 replicates.

trescine levels in the cells treated with Al were almost twice those of control cells. This increase in putrescine was short-lived and was not observed beyond 6 h, except for 1.0 mM Al at 24 h. No increase in putrescine was seen at 36 h. There was no statistically significant change in spermidine within the first 12 h of treatment; however, 1.0 mM Al treatment caused a 20% decrease in spermidine (data not shown). While incubation with Al for 3, 6 and 12 h did not cause a significant change in the level of spermine, a 70% increase in spermine as compared to control cells was evident in 1.0 mM Al-treated cells at 24 and 36 h (data not shown). In parallel experiments, ADC activity was examined at 3 and 6 h after subculture in control and Al-treated cells. No statistically significant change in ADC activity was seen at 3 h, and a slight decrease in ADC occurred in 1.0 mM Al-treated cells at 6 h (Fig. 6).

In the second set of experiments, Al was added at different times during the first 24 h period of culture in the fresh medium, and polyamines were analyzed at 6 h after Al addition. Data presented in Fig. 7 show that an increase in putrescine in response to Al treatment occurred only when Al was added at time zero. Al added at 3, 6, 9, or 15 h after transfer either had no significant effect, or caused a reduction in the cellular putrescine content, except at 15 h. Spermidine and spermine were not affected by this treatment (data not shown).

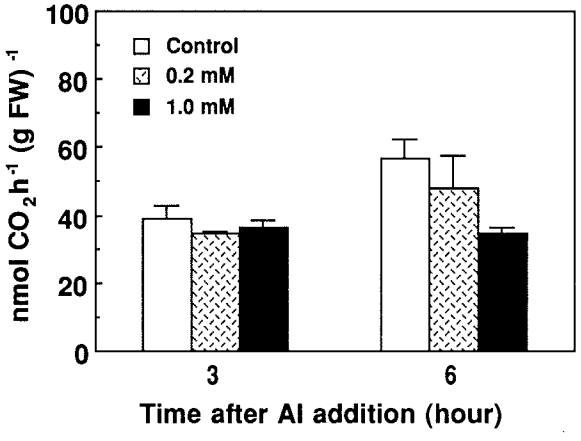

Fig. 6: Effects of Al on ADC activity when Al was added at the time of transfer to fresh media, and the cells analyzed at 3 and 6 h thereafter. Values are Mean ± SE of 3 replicates.

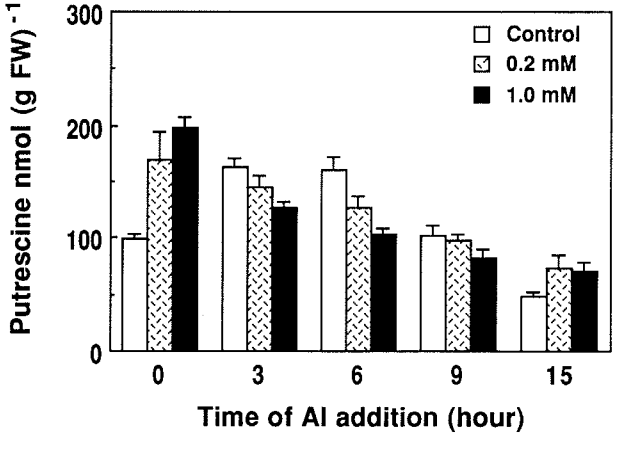

Fig. 7: Effects of Al on cellular levels of putrescine when Al was added at 0, 3, 6, 9, and 15 h after transfer to fresh media, and the cells analyzed at 6 h thereafter. Values are Mean ± SE of 3 replicates.

Effects of Al on inorganic ions

Cells were analyzed for Ca, Mg, Mn, Fe, Na, K, P, and Al at 3, 6, 12, and 24 h after addition of Al to freshly transferred cultures. Al caused a significant decrease in the concentration of Ca, Mn, and Mg (Fig. 8). The decreases were higher with increasing concentration of Al. The decreases in both Ca and Mn were greater at 3 and 6 h than at 12 and 24 h. Over the 24-h period, Ca and Mn showed slight decreases in control cells (on FW basis), whereas cellular Mg increased.

A small increase in Fe concentration was observed as early as 3 h after the cells were treated with 1.0 mM Al. During the remainder of the 24-h period, a several-fold increase in Fe occurred. No difference in Fe was seen between 0.2 mM Al-

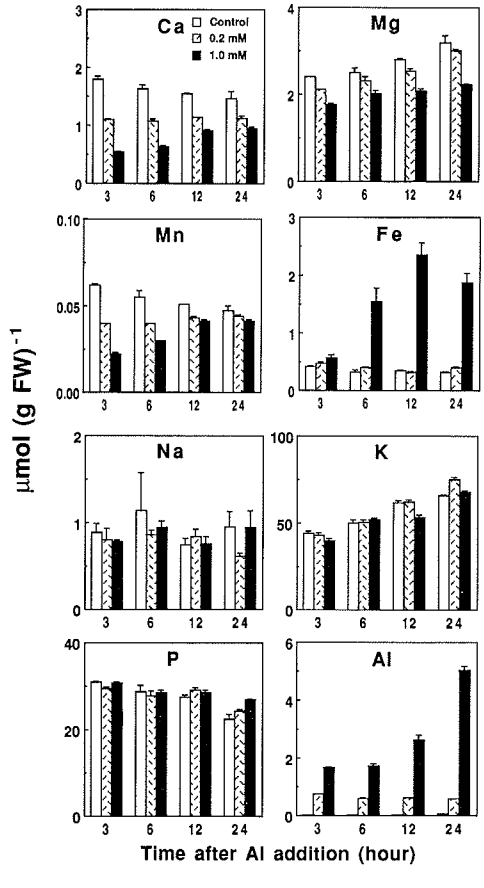

Fig. 8: Effects of Al on cellular levels of different inorganic ions when Al was added at the time of transfer to fresh media, and the cells analyzed at 3, 6, 12, and 24 h thereafter. Values are Mean ± SE of 3 replicates.

treated and control cells. Also, on the basis of FW, the cellular Fe did not change much during 24 h of culture in the control cells.

No significant change was observed in either Na or P concentrations in response to Al treatment. While Na remained unchanged in control cells during the 24 h culture period, P levels showed small decreases with the time of culture. Slight increases in K were observed with time in both the control as well as the Al-treated cells, but Al treatment did not affect K concentration.

The amount of Al was negligible in the control cells as would be expected, since the basal medium contained no added Al. Although treatment with 0.2 mM Al caused a significant accumulation of cellular Al at 3 h, no further change in its concentration was seen beyond this time. Treatment with 1 mM Al resulted in a steady increase of Al from 1.66 μmol $(g\,FW)^{-1}$ at 3 h to 5.02 μmol $(g\,FW)^{-1}$ at 24 h. This translates into approximately a 5-fold accumulation at 24 h as compared to the outside concentration of Al (assuming 1 g FW ≈ 1 mL H_2O).

Discussion

An increase in cellular polyamines before and during nuclear DNA synthesis is a general feature of plant cells including *C. roseus* (Serafini-Francassini, 1980; Maki et al., 1991; Minocha et al., 1991). Kodama et al. (1989) found that the total duration of cell cycle in *C. roseus* was about 32 h with the S phase occurring at 13 to 25 h. In our study, the cells of *C. roseus* were not synchronized. The peak of DNA synthesis observed at 24 h may indicate a high level of synchrony during the first cell cycle following subculture to fresh media. The situation is similar to other cell culture systems where synchrony is observed during the first one or two cell division cycles (Minocha, 1979).

External stress can either result in an increase or a decrease in cellular polyamines, depending upon the type of stress, the plant species, and the time of stress application (Flores, 1991; Minocha et al., 1992; Reggiani et al., 1990, 1993). Among the common polyamines, putrescine appears to be the most sensitive to external stress. Accumulation of putrescine has been observed in response to low pH, high salt concentration, Mg deficiency, NH_4 treatment, exposure to SO_2 and ozone, water stress, chilling, and lack of oxygen (Flores, 1991; Reggiani et al., 1993 and references therein). However, a decrease in putrescine has also been observed under certain stress conditions such as pathogen infection, nutrient deficiency, and Al toxicity. The increase in putrescine under stress conditions is generally preceded or accompanied by an increase in ADC activity.

Two contrasting responses to short-term treatments with Al were observed for putrescine in the present study. When Al was added to 1 to 6-d-old cultures, putrescine levels decreased at 24 h. However, the addition of Al at the time of transfer to fresh media caused an increase in putrescine. It can be hypothesized that the freshly-subcultured cells (0–6 h) may be more sensitive to external stimuli than those acclimated to the medium. Therefore, these cells respond by an increase in putrescine to overcome the stress both from subculture and from Al treatment. The fact that an increase in putrescine at 4 h was observed in a previous study when Al was added to 3-day old cells (Minocha et al., 1992) might also indicate the presence of a short-lived Al stress response even in older cells, which is much weaker than the one noted in the present study with Al treatment given at zero time. Since the observed increases in putrescine at 3 and 6 h were not accompanied by an increase in ADC activity during this short period of treatment, it is possible that the increases were either due to a decrease in conversion of putrescine to spermidine, or the release of putrescine from some conjugated form. Earlier results from Minocha et al. (1992) showed that the decrease in putrescine observed at 24 h after Al addition to 3-d-old cells was probably due to a decrease in ADC activity. Flores and Galston (1982, 1984) found that the accumulation of putrescine in osmotically stressed cereal leaves occurred rapidly, required *de novo* protein synthesis, and was accompanied by a 2–3-fold increase in ADC activity.

The relationship of Al with Ca and Mg has been studied extensively. One of the suggested mechanisms of Al toxicity is the displacement of Ca and Mg in the apoplast by competition at ion exchange sites (Ulrich and Clarkson, 1992). Following Al treatment, Ca and Mg levels were reduced in cortical cell walls of Norway spruce (*Picea abies*) roots (Asp et al., 1988; Stienen and Bauch, 1988; Jentschke et al., 1991) and pitch pine (*Pinus rigida*) needles (Schier et al., 1990). In the present study, addition of Al at zero time caused a decrease in Ca and Mg. By contrast, an increase in putrescine was also noted at the same time. The decline in Mn at 3 and 6 h was similar to earlier reports on the effects of Al on inorganic cations in red spruce (*Picea rubens*) and pitch pine seedlings (Ohno et al., 1988; Schier et al., 1990).

Asp et al. (1988) and Bengtsson et al. (1988) suggested that the stimulatory effect of low concentrations of $AlCl_3$ (0.1–0.3 mM) on P uptake may be due to the blocking of negative surface charges by Al. The fact that similar concentrations of $AlCl_3$ used in our experiments also caused increases in cellular P at 24 h might be related to such an effect. No explanation is available for the dramatic increase in Fe in response to 1.0 mM Al, although similar results have been reported in peach (*Prunus persica*) (Edwards and Horton, 1977) and pitch pine seedlings (Schier et al., 1990), and *C. roseus* cell suspensions (Minocha et al., 1992).

Taylor (1989) reviewed evidence for exclusion of Al from the protoplasts and its immobilization in the cell walls. It is generally accepted that the plasmalemma acts as a barrier for Al to penetrate into the cytoplasm (Hodson and Wilkins, 1991). Adsorption of Al on cation exchange sites in the apoplast can account for a very high percentage of total uptake of Al. It is interesting to note that no change in cellular Al occurred between 3 to 24 h of treatment with 0.2 mM Al. This might indicate rapid equilibrium between the apoplast and the medium at low concentrations of Al. However, the situation was different for 1.0 mM Al; in this case cellular Al increased with time of incubation.

Most of the previous studies on the effects of Al on other inorganic ions have been performed with intact seedlings or whole plants grown in sand or solution cultures. Also, plants were subjected to Al treatments for much longer periods of

time than in the present study. The results with cell suspension cultures of *C. roseus* are in general agreement with the research on seedlings, indicating the suitability of *in vitro* grown cell cultures for such studies.

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