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Effects of aluminum on DNA synthesis, cellular polyamines, polyamine biosynthetic enzymes and inorganic ions in cell suspension cultures of a woody plant, *Catharanthus roseus*

Rakesh Minocha, Subhash C. Minocha, Stephanie L. Long and Walter C. Shortle

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Increased aluminum (Al) solubility in soil waters due to acid precipitation has aroused considerable interest in the problem of Al toxicity in plants. In the present study, an in vitro suspension culture system of *Catharanthus roseus* (L.) G. Don was used to analyze the effects of aluminum on several biochemical processes in these cells. The aliphatic polyamines, spermine and spermidine, and their precursor, putrescine, have been implicated in a number of stress responses of plants. Addition of 0.2, 0.5 or 1.0 m*M* $AlCl_3$ to cells cultured for 3 days caused a small but significant increase in cellular levels of putrescine at 4 h followed by a sharp decline by 16 h. There was no further decline in levels of putrescine during the next 32 h. Spermidine levels did not change appreciably compared to those in the control cultures. However, spermine levels increased by 2–3–fold at 24 and 48 h. Cellular activities of arginine decarboxylase (ADC; EC 4.1.1.19) and S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) were both inhibited by 20–25% at 4 and 7 h. Ornithine decarboxylase (ODC; EC 4.1.1.17) was less than 10% of ADC activity at all times. Whereas all concentrations of Al caused a slight decrease in total cell number, cell viability was affected only by 1.0 m*M* Al. There was a decrease in the cellular levels of Ca, Mg, Na, K, Mn, P and Fe in the cells treated with Al at 4 h, but a significant increase by 16 and 24 h. The results presented here suggest that both the absolute amounts of Al and the length of exposure to it are important for cell toxicity.

Key words – Aluminum, arginine decarboxylase, calcium, *Catharanthus roseus*, ions, magnesium, polyamines, S-adenosylmethionine decarboxylase.

R. Minocha (corresponding author) and W. C. Shortle, USDA Forest Service, NEFES, P. O. Box 640, Durham, NH 03824, USA; S. C. Minocha and S. L. Long, Dept of Plant Biology, Univ. of New Hampshire, Durham, NH 03824, USA.

Introduction

Aluminum (Al), bound as oxides and complex aluminosilicates, is the most abundant metal in the earth's crust. Until recently, surface water concentrations of Al ion (Al^{3+}) have remained minimal because of the insolubility of aluminum hydroxide complexes at neutral pH (Macdonald and Martin 1988). In many of our forests, the amount of introduced acidity far exceeds the rates of acid buffering by weathering of minerals. This causes a lowering of the soil pH due to increased soil acidity. This results in the loss of exchangeable cations (Ca, Mg and K) and release of normally sparingly soluble Al and heavy metals into the soil solution (Godbold et al. 1988). High concentrations of aluminum have been known for some time to impair growth of crop plants (Foy et al. 1978, Asp et al. 1988). Initially, Ulrich et al. (1979) proposed that Al toxicity may also be the main cause for forest decline. The evidence to support this hypothesis comes from several laboratories (Godbold et al. 1988, Shortle and Smith 1988, Ulrich 1989). The degree to which Al toxicity symptoms are expressed by

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plants depends on a number of factors including: amount and species of Al, amount of other ions present, amount and form of organic material and the plant genotype (Karr et al. 1984). At pH below 5.0, Al^{3+} is the principal mononuclear species (assuming OH^- is the only ligand available) and $Al(OH)^{2+}$ and $Al(OH)_2^+$ are present in very low amounts. The occurrence of several Al species in the artificial culture media (due to the presence of other ligands) makes it difficult to investigate, in isolation, the toxic effects of individual species (Kinraide 1991). The biochemical processes and the molecular mechanisms through which Al may exert its toxic effects are not well understood.

The aliphatic polyamines spermine and spermidine, and their precursor putrescine, which are found in all animal and plant cells, appear to play an important role in cell proliferation and differentiation (Smith 1985, Bachrach and Heimer 1989a,b). The cellular polyamine content is highly regulated and a variety of stimuli such as Ca and Mg deprivation (Smith 1973), high salinity (Strogonov et al. 1972), SO_2 fumigation (Priebe et al. 1978), pathogenesis (Cohen at al. 1981, Greenland and Lewis 1984), osmotic stress (Flores and Galston 1982, 1984, Matsuda 1984), ozone and acid stress (Dohmen et al. 1990) all lead to an accumulation of one or more of the polyamines. This increase, with some exceptions, is accompanied by increased activity of their key biosynthetic enzymes. While both the intact plants and cell/tissue cultures have been used, most of the studies published to date have involved herbaceous plants. The data from these studies suggest that polyamine biosynthesis could play an important role in the survival of plants under stress.

The present study is an attempt to understand the effects of Al stress on DNA synthesis, cellular polyamine levels and their biosynthetic enzymes, and uptake of inorganic ions in suspension cultures of a woody plant, *Catharanthus roseus*. Cell cultures of *C. roseus* are highly suited for such studies because of the availability of a vast amount of biochemical and molecular data on these cells.

Abbreviations – ADC, arginine decarboxylase; ODC, ornithine decarboxylase; PCA, perchloric acid; SAMDC, S-adenosylmethionine decarboxylase.

Materials and methods

Culture conditions

Suspension cultures of *Catharanthus roseus* (L.) G. Don were obtained from the laboratory of Professor Atsushi Komamine, Tohoku University, Sendai, Japan, and maintained in MS medium (Murashige and Skoog 1962) containing 3% sucrose and 2.2 μM 2,4-dichlorophenoxy acetic acid (2,4-D) as described by Kodama et al. (1991a). The medium was adjusted to pH 6.2 before it was autoclaved. Whereas 2,4-D was added before autoclaving, $AlCl_3$ was filter sterilized. Cells were subcultured at 7-day intervals by transferring 7 ml of cell suspension into 43 ml of fresh medium in 250-ml Erlenmeyer flasks. The flasks were kept in the dark at 27°C on a gyratory shaker at 150 rpm. Various concentrations of Al were selected on the basis of preliminary studies on their effects on cell viability in this tissue (R. Minocha, unpublished data).

For experimental treatments, 10-ml samples of cells were transferred from 3-day-old batch cultures to 50-ml flasks. Three different concentrations of $AlCl_3$ were added at this stage and the flasks were kept on a shaker until the time of analysis. The pH of the medium was not adjusted during the period of each experiment. By 3 days, the pH dropped to 4.2 ± 0.3 and stayed within this range for the next 4–5 days. Each treatment was run in triplicate and each experiment was run at least 3 times. For determination of cell numbers, protoplasts were prepared from samples of cell suspensions and counted in a hemacytometer as described by Minocha et al. (1991a). Cell viability was determined by staining with erythrosin B stain (0.4% in Ca-Mg-free saline containing 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g glucose per liter of water).

Determination of cellular inorganic ion content

Cells were collected on Miracloth (Calbiochem-Behring Corp., La Jolla, CA, USA) and thoroughly washed with deionized distilled water. After fresh weights were taken, the cells were homogenized for 90 s in 0.01 *M* HCl at 20 000 rpm with a Brinkmann Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY, USA). Extracts were centrifuged immediately at 18 000 *g* for 20 min at 4°C and the supernatant solutions were sent to USDA Forest Service, NEFES, Berea, KY, where the ion contents were analyzed with a Beckman Spectrospan V ARL DCP (Direct Coupling Plasma Emmision Spectrometer, Beckman Instruments Inc., Fullerton, GA, USA) using the Environmental Protection Agency's (EPA) method number 66–AE0029 (1986).

Incorporation of ^{3}H-thymidine

At appropriate intervals, 37 kBq of [methyl-^{3}H] thymidine (specific activity 1.59 TBq mol^{-1}, New England Nuclear, Boston, MA, USA) were added to a 0.5-ml cell suspension in a 12-ml centrifuge tube. Following 30 min of incubation at 27°C with constant shaking, 3 ml of ice-cold 4% perchloric acid (PCA) were added to each tube and the tubes were vortexed for 10 s. After 15 min of incubation on ice, the cells were filtered through a GFC glass fiber filter (24 mm; Whatman International Ltd, Maidstone, UK) premoistened with 4% PCA using a multifilter manifold under vacuum (Millipore Corp., Bedford, MA, USA). The filters were washed successively with 3 ml of 4% PCA, 15 ml of cold 80% ethanol and 15 ml of cold 100% ethanol. The filters were dried at 45°C for 2 h and counted for radioactivity in 10 ml of

Tab. 1. Effects of Al on cell division and cell viability in suspension cultures of *Catharanthus roseus*. Different concentrations of $AlCl_3$ were added to 3-day-old cultures. Numbers in parentheses represent percent of control. nd, Not determined.

Treatment	Cells ml^{-1} (x 10^6)	Viability (%)	Cell mass (mg ml^{-1})
24 h			
Control	1.9 (100)	89.3 (100)	71.8 (100)
0.2 m*M*	1.5 (80)	88.9 (99.6)	57.2 (80)
0.5 m*M*	1.4 (73)	77.8 (87.1)	50.0 (70)
1.0 m*M*	1.6 (84)	58.3 (65.3)	38.1 (53)
48 h			
Control	2.8 (100)	90.4 (100)	96.7 (100)
0.2 m*M*	2.1 (75)	87.1 (96.4)	83.3 (86)
0.5 m*M*	nd	79.5 (87.9)	72.2 (75)
1.0 m*M*	nd	55.5 (61.4)	62.8 (65)

ACS (Amersham Corp., Arlington Heights, IL, USA) scintillation fluid.

Polyamine analysis

Cells were collected on Miracloth and thoroughly washed with deionized distilled water. After fresh weights were taken, the cells were homogenized for 90 s at 20000 rpm in 5% cold PCA with a Brinkmann Polytron homogenizer. Extracts were kept on ice for 1 h and then centrifuged at 18000 *g* for 30 min at 4°C. The supernatant fraction was used for dansylation and quantification of polyamines by HPLC (Minocha et al. 1990). Each treatment was run in triplicate and each experiment was repeated at least twice.

Enzyme assays

For preparation of extracts, cells were collected on Miracloth by vacuum filtration, washed with deionized distilled water, and then homogenized in the extraction buffer [2 ml (g fresh weight)$^{-1}$] for 90 s at 20000 rpm using a Brinkmann Polytron homogenizer. The extraction buffer for ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) contained 50 m*M* Tris-HCl, pH 8.4, adjusted at 4°C, 50 μ*M* pyridoxal-5-phosphate, 0.1 m*M* Na_2-EDTA and 5 m*M* dithiothreitol (DTT). For S-adenosylmethionine decarboxylase (SAMDC), the extraction buffer contained 100 m*M* potassium phosphate, pH 7.5, 3 m*M* putrescine and 1 m*M* DTT. The homogenates were centrifuged at 18000 *g* for 20 min at 4°C and the supernatant fractions were used for enzyme assays. ODC and ADC were assayed according to the procedure of Robie and Minocha (1989) and SAMDC according to Minocha et al. (1991b).

Statistical analysis

Since the batch cultures were asynchronous and the cell numbers changed within the duration of the experiments, the effects of Al treatments were statistically analyzed only for given time periods. Data for each time period and for each set of treatments were analyzed as a series of one way analysis of variance designs for randomized complete block, blocked by experiment. Whenever F values for ANOVA were significant, the means were compared by Tukey's Test. All analyses were done by using Systat version 5.0.

Results

Effects of Al on cell division and cell viability

All concentrations of Al inhibited cell division by 20–25% within 24 h of treatment (Tab. 1). Whereas the cell mass [mg cells (ml culture)$^{-1}$] was proportionate to the cell numbers for 0.2 and 0.5 m*M* Al, it was significantly lower for 1.0 m*M* Al, indicating more severe effects on cell growth at this concentration. Addition of 0.2 m*M* Al had no effects on cell viability by 24 h whereas 0.5 and 1.0 m*M* Al caused 13 and 35% cell mortality, respectively. By 48 h, the cells tended to clump together in the 0.5 and 1.0 m*M* Al treatments. These loose clumps contained live as well as dead cells. Aluminum-treated cells generally appeared to be more rounded and plump than the controls.

Effects of Al on cellular inorganic ion content

There was an overall decline in the cellular content of Ca 4 h after the addition of Al, but it was significant for only the 0.5 and 1.0 m*M* Al treatments (Fig. 1). Levels of Mg, Mn and Fe were also significantly lower in all Al-treated cells compared to cells from the control treatment at 4 h. This decrease was in proportion to the amount of Al added in the cases of Fe and Mn, but in the case of Mg the decrease was similar irrespective of the dose. Cellular levels of K, Na and P showed no significant change at 4 h. By 16 h, the cellular levels of most of these ions were either equal to or higher than those in the control cells. At 24 h, all the inorganic ions were present in significantly higher amounts in both the 0.5 m*M* and 1.0 m*M* Al-treated cells than in the control cells. In most cases this increase was proportionate to the amount of Al in the medium. The extracellular supply of Al caused an increase in the cellular concentrations of Al by 6-, 15-, and 20-fold with 0.2, 0.5 and 1.0 m*M* Al, respectively, at 4 h. Whereas these levels did not change substantially for 0.2 m*M* Al at 16 and 24 h, there was a significant increase in Al uptake over time for the other two concentrations.

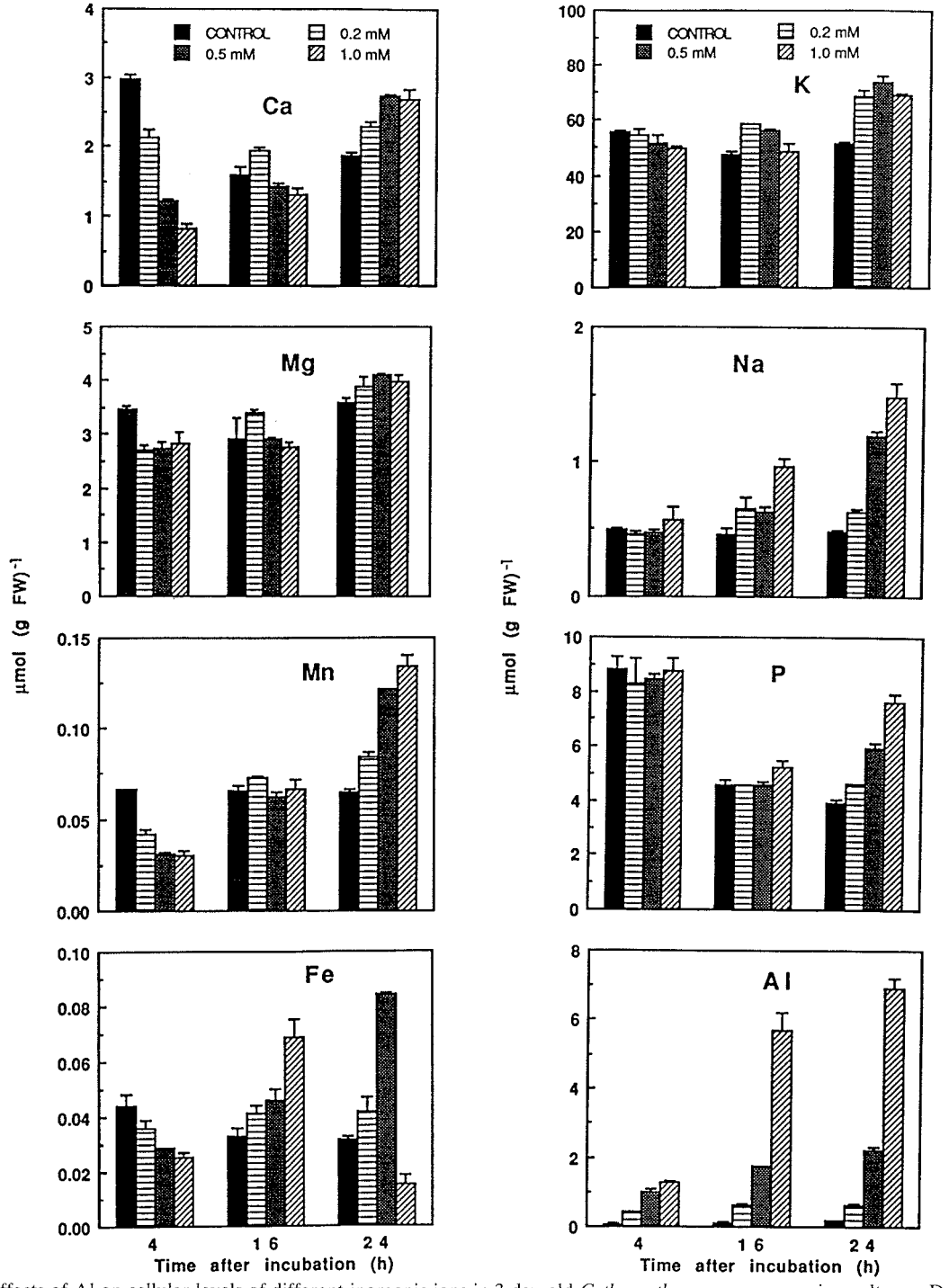

Fig. 1. Effects of Al on cellular levels of different inorganic ions in 3-day-old *Catharanthus roseus* suspension cultures. Data are mean ± SE of 3 replicates.

Effects of Al on DNA synthesis

DNA synthesis was studied by using cells grown in batch cultures for 3 days. As shown in Fig. 2, the rate of incorporation of ^{3}H-thymidine into the PCA-insoluble fraction varied considerably in the control cultures at different times because of the asynchronous nature of the cultures. There was a 20–35% increase in the rate of incorporation of thymidine within 4 h of addition of Al. Whereas the increase caused by 0.5 m*M* Al at 4 h was

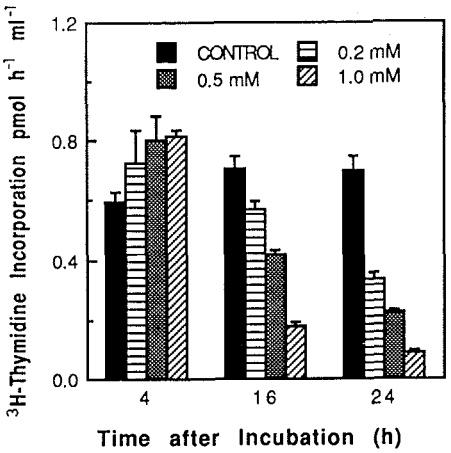

Fig. 2. Effects of Al on ^{3}H-thymidine incorpotation into PCA-insoluble material in 3-day-old *Catharanthus roseus* suspension cultures. Data are mean ± SE of 3 replicates.

significantly higher than the increase caused by 0.2 m*M* Al, 1.0 m*M* Al had the same effect as 0.5 m*M*. By 16 h, there was a strong inhibition of DNA synthetic activity, the level of inhibition being proportionate to the concentration of Al added. At 24 h, there was more than 90% inhibition in the incorparation of ^{3}H-thymidine in cells treated with 1.0 m*M* Al as compared to the control cells.

Effects of Al on cellular polyamines

Figure 3 illustrates the effects of Al on cellular levels of the 3 major polyamines tested. Cellular putrescine levels increased significantly (20–25%) within 4 h in the presence of 0.2 and 0.5 m*M* Al, while there was no such increase in the presence of 1.0 m*M* Al. By 24 h, all concentrations of Al caused a substantial (60–70%) reduction in cellular putrescine. At 48 h, this decline was proportionate to the dose of Al added. Cellular spermidine was not affected significantly by 24 h, but at 48 h the spermidine level in 1.0 m*M* Al-treated cells was significantly lower compared to the control cells; here again, as in the case of putrescine, the decline was proportionate to the concentration of Al in the medium. Cellular spermine increased at least 2-fold at 24 and 48 h in the presence of Al.

Effects of Al on ADC, ODC and SAMDC activities

Both ADC and SAMDC activities were inhibited by about 20–25% in the presence of Al at all times (Figs 4 and 5). The effect of Al was proportionate to its concentration in the medium in most cases. The activity of ODC was extremely low in these cells, and never exceeded 10% of the ADC activity.

Discussion

Most of the previous studies on the effects of Al in plants have used either seedlings grown in soil/nutrient solutions or roots grown in vitro. Althought comparisons between cell suspension cultures and whole plants (and/or organs) must be interpreted with caution, data available so far indicate that some valid conclusions can be drawn by using either one of the systems. Godbold et al. (1988) and Schier et al. (1990) observed that exposure to Al caused a reduction in growth of spruce seed-

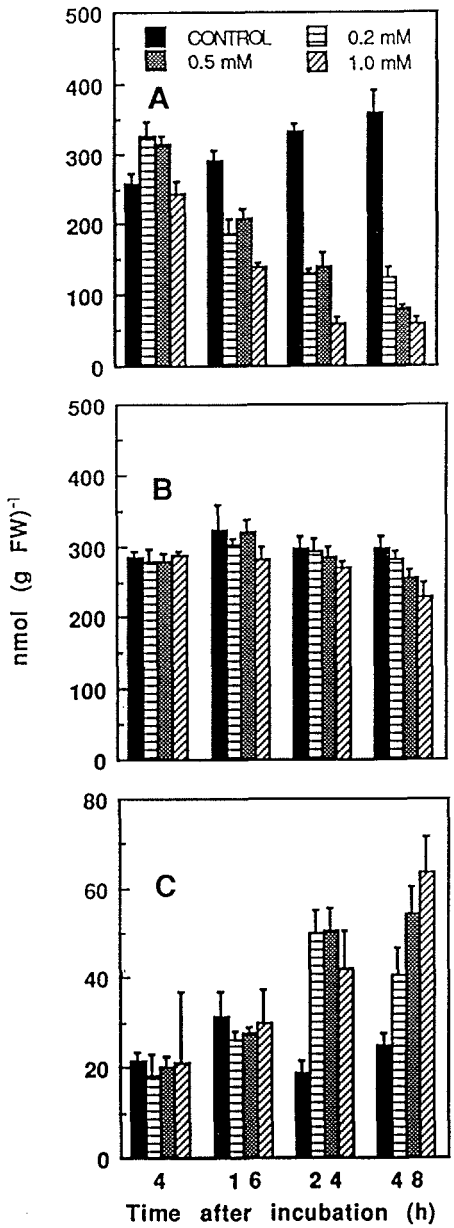

Fig. 3 A-C. Effects of Al on cellular levels of putrescine (A), spermidine (B), and spermine (C) in 3-day-old cultures of *Catharanthus roseus*. Data are mean ± SE of 6 replicates.

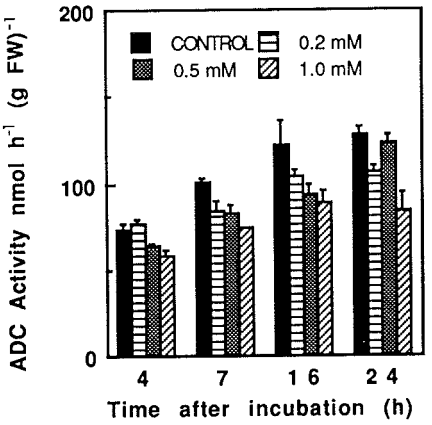

Fig. 4. Effects of Al on the cellular levels of ADC activity in 3-day-old cell cultures of *Catharanthus roseus*. Enzyme activity = nmol CO_2 released h^{-1} (g FW)$^{-1}$. Data are mean ± SE of 3 replicates.

lings as measured by several parameters. McQuattie and Schier (1990) reported that Al caused premature vacuolation and the accumulation of electron-dense material between the cell wall and the plasmalemma. Clarkson (1965) concluded, based on studies with onion root cultures, that some mechanism associated with cell division was highly sensitive to Al and was permanently damaged by short exposures. Matsumoto et al. (1977) suggested the binding of Al to DNA to be a potential cause for inhibition of cell division. Data presented here are consistent with observations by others on the inhibitory effects of Al on DNA synthesis, cell division and cell mass. While causing a slight but consistent increase in DNA synthesis at 4 h, Al treatment resulted in a severe inhibition of DNA synthesis at 16 and 24 h.

Putrescine has been known to increase in relation to the rate of DNA synthesis and cell division in *Catharanthus* as well as in other plant tissues (Serafini-Fracassini et al. 1980, Smith 1985, Minocha et al. 1991a). Cellular putrescine has also been shown to accumulate in response to a variety of chemical agents causing stress in plants (Flores and Galston 1982, Dohmen et al. 1990). This increase was generally severalfold and was often accompanied by increased activity of ADC. Most of these studies were, however, limited to monocots. In the present case, concomitant with the increase in DNA synthesis at 4 h, there was a small but significant increase in the level of cellular putrescine. This accumulation of putrescine at 4 h after Al treatment was, however, accompanied by a decrease in cellular ADC activity. The increase in putrescine levels, therefore, could be due to its reduced utilization for the biosynthesis of spermidine, since Al caused a significant reduction in SAMDC activity. In *Catharanthus*, the alternate pathway for putrescine biosynthesis (i.e. ODC) is present at low levels, never exceeding 10% of the ADC activity (see also Minocha et al. 1991a). At 24 h and thereafter there was a decline both in putrescine and spermidine coincident with the reduction of ADC and SAMDC, as well as an inhibition of DNA synthesis. The increase in ADC and SAMDC activities seen in control cultures during the 24 h period may be due to the fact that the cells are in log phase of growth between day 3 and 4 after subculture (R. Minocha, unpublished data).

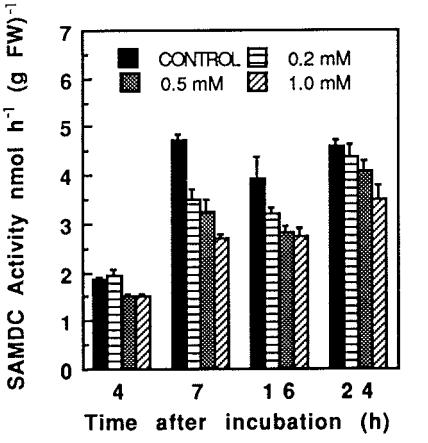

Fig. 5. Effects of Al on the cellular levels of SAMDC activity in 3-day-old cell cultures of *Catharanthus roseus*. Enzyme activity = nmol CO_2 released h^{-1} (g FW)$^{-1}$. Data are mean ± SE of 3 replicates.

It is interesting to note that the cellular inorganic ion concentrations decreased in the presence of Al as compared to the controls at 4 h, but by 24 h the cellular ion content was higher in the Al-treated cells. This accumulation was in proportion to the dose of Al. The finding of a reduction in the cellular levels of inorganic ions at 4 h is similar to the findings of Stienen and Bauch (1988) and Schier et al. (1990) on the uptake of Ca and Mg in the presence of Al in conifer seedlings. It should, however, be pointed out that most of the past studies on Al effects in woody plants involved relatively long-term treatments to intact seedlings where the transport of ions to the shoots was dependent on the uptake of ions by the roots. In the present study, the cells were in direct contact with the medium and therefore the effects of Al could be seen within 2 h (data not presented).

Evidence for the exclusion of Al from protoplasts and its immobilization in the cell wall has recently been reviewed (Godbold et al. 1988, Tayler 1989). There is reasonable evidence to support the hypothesis that the plasma membrane acts as a barrier to the entry of Al into the cytoplasm. Alternatively, Al may react with the silicates present in the cell wall to form aluminosilicates (Hodson and Wilkins 1991). This could explain the initial blockage of cation exchange sites on the membrane which could cause a decrease in the uptake of other inorganic ions into the cytoplasm at 4 h. Increased levels of putrescine at this time may compensate for the loss of inorganic cations. At later stages (24 and 48 h), however, when the cellular levels of putrescine decline, the inorganic cations accumulate in the cells. Asp et al. (1988) have demonstrated that not only is Al bound in

the free spaces in roots, but it also crosses the plasma membrane. In a 9-week-long study with spruce seedlings they found that Al concentrations increased in the shoots in proportion to the dose of Al applied. Results presented here also show that if cells are grown in the presence of Al for longer periods, the cellular concentrations of Al increase with time, the increase being proportionate to the concentrations of Al in the medium.

There is ample evidence in the literature indicating that Al alters membrane properties. One of the few initial studies on the effects of Al on membrane functions showed leakage of K from the plasma membrane (Woolhouse 1969). Viestra and Haug (1978) showed a dramatic decrease of membrane lipid fluidity by Al in *Thermoplasma acidophilum*. Later Suhayda and Haug (1986) demonstrated decreased Mg-ATPase activity by Al in plasma membrane-enriched microsomal fractions of maize roots in the presence of Al.

The question as to whether the toxic effects of Al are due to alterations in the plasma membrane properties or are related to the accumulation of Al in the cytoplasm still remains unanswered. The data presented here suggest that: 1) the effects of short-term treatments (4 h) with Al, which probably represent interaction with the plasma membrane, are quite different from its effects over a longer period (24 h) which may be due to cellular accumulation, and 2) even the lowest concentration of Al which promotes DNA synthesis and putrescine accumulation over short periods became inhibitory over longer periods (Fig. 1). This indicates that both the absolute amounts of Al and the length of exposure to it are important for cell toxicity. It should also be noted that while 1.0 m*M* Al in the medium caused a substantial loss of viability within 24 h, 0.2 m*M* Al had little effect on cell viability even at 48 h, despite the fact that it had caused a severe inhibition of DNA synthesis.

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