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Research article

The response of high and low polyamine-producing cell lines to aluminum and calcium stress☆

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ABSTRACT

The diamine putrescine (Put) has been shown to accumulate in tree leaves in response to high Al and low Ca in the soil, leading to the suggestion that this response may provide a physiological advantage to leaf cells under conditions of Al stress. The increase in Put is reversed by Ca supplementation in the soil. Using two cell lines of poplar (*Populus nigra* × *maximowiczii*), one with constitutively high Put (resulting from transgenic expression of a mouse *ornithine decarboxylase* – called HP cells) and the other with low Put (control cells), we investigated the effects of reduced Ca (0.2–0.8 mM vs. 4 mM) and treatment with 0.1 mM Al on several biochemical parameters of cells. We found that in the presence of reduced Ca concentration, the HP cells were at a disadvantage as compared to control cells in that they showed greater reduction in mitochondrial activity and a reduction in the yield of cell mass. Upon addition of Al to the medium, the HP cells, however, showed a reversal of low-Ca effects. We conclude that due to increased ROS production in the HP cells, their tolerance to low Ca is compromised. Contrary to the expectation of deleterious effects, the HP cells showed an apparent advantage in the presence of Al in the medium, which could have come from reduced uptake of Al, enhanced extrusion of Al following its accumulation, and perhaps a reduction in Put catabolism as a result of a reduction in its biosynthesis.

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1. Introduction

Aluminum (Al) toxicity in plants has received considerable attention [reviewed in [13,32,39]]; it often occurs in acidic soils, particularly those below a pH of 4.5. The harmful effects of Al include a reduction in DNA replication and restriction of cell division, which result in decreased growth and development, particularly in the roots [12,20,40,43,44]. The toxic effects of Al vary with different tissue types and its uptake depends upon its interaction with other ions in the soil, particularly Ca and phosphate [reviewed in [12,29,30]]. One of the ways in which Al causes cytotoxicity in plants is by blocking Ca^{2+} channels in the plasma membrane thus interfering with cellular Ca homeostasis and impairing the Ca-dependent signal transduction cascades that are essential for cell growth and division [8,12,13,29,30]. Studies of Al tolerance in rice [34] demonstrated that the tolerant varieties were more efficient in the uptake and utilization of Ca and P than the Al sensitive varieties. Thus it appears that many of the effects of Al can be ameliorated by increase in Ca availability to the plants [10,29,30,32,41].

Studies with forest trees have shown that an increase in solubilized Al in the soil (e.g., due to excess nitrate or acidic deposition) negatively impacts the uptake and accumulation of Ca in their foliage, thus causing symptoms of Ca deficiency [12,20,21,23,35,37,45]. At the biochemical level, these symptoms are manifested as changes in foliar polyamines (PAs, particularly the diamine putrescine – Put), amino acids, inorganic ions, soluble proteins, and chlorophyll [11,12,18,23,29,32,41,45]; [R. Minocha, unpublished]. Increased cellular Put and other N-rich amino acids (e.g., Arg and GABA) have been suggested as adaptive responses of plants to Ca deficiency as well as other forms of abiotic stress [1,2,7,14,16], even though this response may come at the expense of other metabolic changes. Several-fold increases in Put accumulation in the foliage of trees in response to high Al and/or low Ca in the soil have lead to the

Abbreviations: Al, Aluminum; γ-EC, γ-glutamyl-cysteine; GSH, Glutathione; HP, high putrescine; ODC, ornithine decarboxylase; PC_2, phytochelatin; PA, polyamine; Put, Putrescine; ROS, Reactive Oxygen Species; Spd, spermidine; Spm, spermine.

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suggestion that this response may actually be a useful biochemical marker of Ca deficiency in forest soils/trees [16,21,23,41]. On the other hand, since only a small fraction of Al is actually transported to the foliage, it can be argued that the observed metabolic changes in the foliage of trees are likely due to decreased cellular Ca or some other signals related to changes in roots that are directly exposed to Al and Ca. Once Al encounters root cells, it precipitates at the cell surface due to the pH difference outside and inside the cell, and inhibits Ca uptake and root growth [11,39]. The exact signal transduction pathway from the roots to the foliage that regulates leaf responses is currently unknown. We suggest that metabolic responses in cell cultures to low Ca and to the presence of Al in the growth medium are analogous to the exposure of roots to these conditions in soil, and may reveal some of the biochemical mechanisms by which roots respond to Al and low Ca in the soil.

The present study was aimed at elucidating the effects of Al, and its interaction with reduced Ca in the medium, on cell cultures of poplar in order to determine how plant cells respond to direct exposure to Al in the presence of low or high Ca with respect to the accumulation of inorganic ions, PAs, and amino acids; and whether the presence of high Put within the cells provides them an adaptive advantage for response to low Ca and/or to the presence of Al.

The two cell lines used in this study, High Put (HP) and control, have been characterized with respect to several metabolic and growth parameters including their protein, PA and amino acid contents, their ability to respond to variations in the concentration and the forms of N, and the activities of several PA biosynthetic enzymes and the expression of their genes [4,15,24,25,27]. They differ significantly in their Put (and to a smaller extent spermidine – Spd) contents and the contents of several amino acids due to the constitutive expression of a mouse *Orn decarboxylase* (m*ODC*) in the HP cells. Thus the HP cell line is akin to an up-regulated *ODC* mutant in comparison with the control cell line used here, which expresses the β-*glucuronidase* (*GUS*) transgene, with no effect on Put.

2. Results

For the duration of the current study, Put contents of the HP cells were four-to-eight fold higher than the control cells while their Spd contents differed by about 40–50% at any time. No significant difference was seen in their spermine (Spm) content. Their growth rates and soluble protein contents during the 7 d culture period followed the same trends as reported earlier [24].

2.1. Effect of Al and Ca on cell growth, mitochondrial activity and membrane integrity

In order to test the interaction of Al with Ca for effects on growth and other biochemical parameters, we first tested if altering the Ca concentration in the medium would by itself affect growth and PA levels in the cells; and also if these responses would be further modified by the addition of Al. Thus the cells were grown in MS medium with (i) normal amounts (4 mM) of Ca – called "control medium", (ii) reduced amounts of Ca (0.8 mM) with no change in other constituents – called 'low-Ca medium', (iii) addition of Al to control medium, and (iv) addition of Al to low-Ca medium. Based upon the effects on growth from initial experiments comparing two concentrations of Al (0.1 and 0.25 mM) added to the control medium, and media with variable concentrations of Ca (0, 0.2, 0.8 and 8 mM Ca – data not shown); 0.1 mM Al and a five-fold reduced amount of Ca (0.8 mM) were selected for detailed experimentation.

Lowering the concentration of Ca in the medium (to 0.8 mM or lower) caused a 50% reduction in fresh weight yield of HP cells after 5 d of growth (Fig. 1A), and doubling the amount of Ca (to 8 mM) in the medium showed positive effects after 4 d of growth (data not shown).

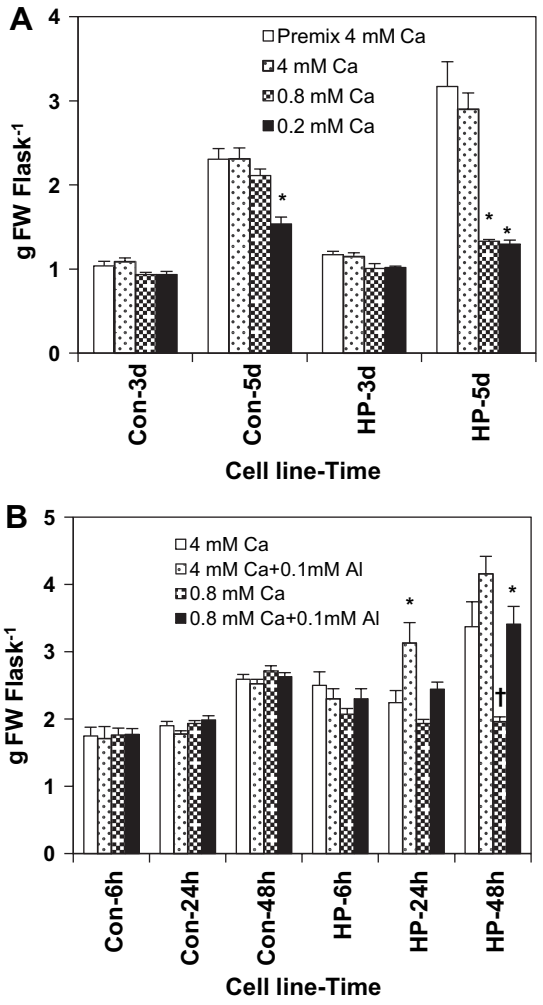

Fig. 1. Yield of fresh mass per flask of control and HP cells grown in different concentrations of Ca (A) and treated with 0.1 mM Al at two concentrations of Ca (B). In (A), cells were grown in different concentrations of Ca starting from time zero and collected on 3 d and 5 d. Each bar represents mean ± SE of 4 replicates from two experiments. Treatment "Premix 4 mM Ca" refers to cells growing in commercial MS medium. An * indicates significant difference ($P \leq 0.05$) of the treatment from the control (4 mM Ca) medium. For (B), cells were treated with 0.1 mM Al after 3 d of growth in normal (4 mM) or low-Ca (0.8 mM) medium. Each bar represents mean ± SE of 6 replicates from two experiments. An * indicates significant difference ($P \leq 0.05$) between plus and minus Al within the same concentration of Ca, and † indicates significant difference ($P \leq 0.05$) between 4 mM and 0.8 mM Ca treatments in the absence of Al. All statistical comparisons were made within the same cell line at the same time of collection.

However, in the control cells, a reduction in Ca concentration down to 0.2 mM was needed to see a 25% decrease in the yield of cell mass in the control cells by 5 d; doubling of Ca had no effect on growth of these cells. Both cell types died within two days of subculture in the absence of Ca. Adding 0.1 mM Al to control medium or to low-Ca medium at 3 d stimulated growth of HP cells within 24 h; i.e., a reversal of the negative effect of low-Ca was apparent (Fig. 1B). Control cells were not affected by Al treatment in either control or low-Ca medium.

The mitochondrial activity of cells was studied by measuring the formation of formazan from DTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [19]. For cells grown in different concentrations of Ca for 3 or 5 d, the HP cells in the control medium (i.e., 4 mM Ca) showed significantly lower mitochondrial activity than the control cells at the same time (Fig. 2A). Whereas in the control cells, mitochondrial activity was adversely affected only after lowering the Ca content twenty-fold (to 0.2 mM) at 5 d of

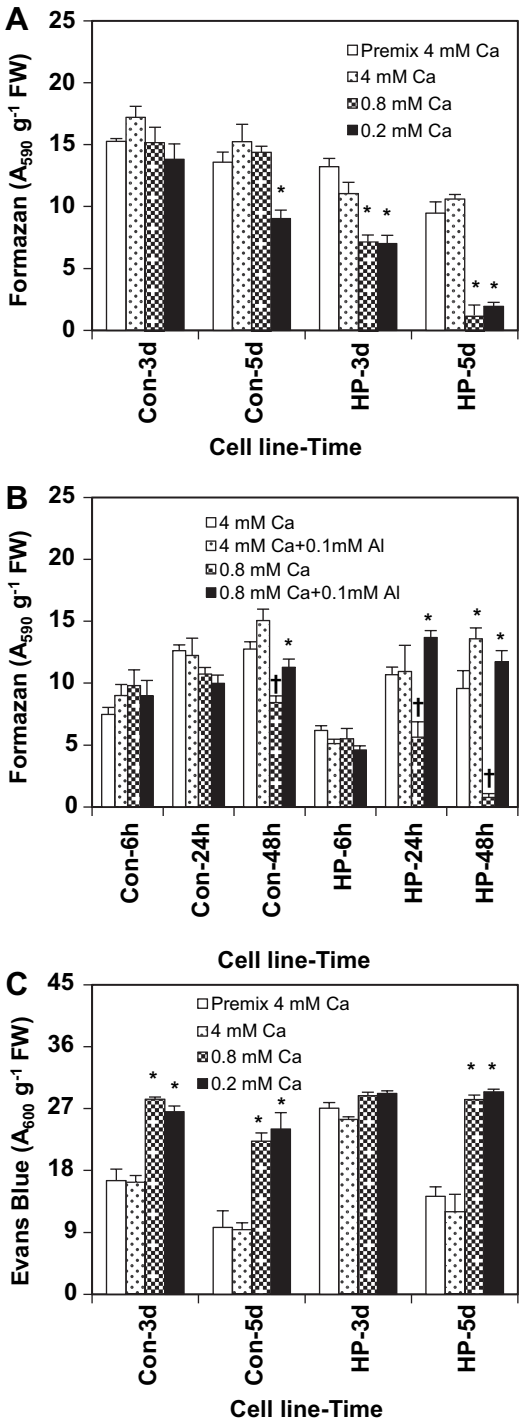

Fig. 2. Absorbance of Formazan produced from MTT (A and B) and the retention of Evans Blue (C) in control and HP cells grown in the presence of different concentrations of Ca from time zero (A and C) and those treated with 0.1 mM Al after growth in normal (4 mM) and low-Ca (0.8 mM) medium for 3 d (B). Each bar represents Mean ± SE of six replicates from two experiments. For further details on A and C, refer to legend of Fig. 1A; and for B, refer to legend of Fig. 1B.

culture, the HP cells showed reduced mitochondrial activity even at a five-fold (0.8 mM) reduction in Ca, and both at 3 and 5 d of analysis; the effect increased with time. Neither the control nor the HP cells (3 d-old at the time of treatment) showed significant change in mitochondrial activity within 24 h of adding 0.1 mM Al to the control medium. However, in the HP cells, a significant increase in mitochondrial activity (vs. no Al) was seen at 48 h after treatment with Al in both normal and low-Ca medium (Fig. 2B), and at 24 h in the low-Ca medium. In the control cells, only a small increase in mitochondrial activity was seen at 48 h in the low-Ca medium supplemented with Al.

In general, the retention of higher amounts of Evans blue stain showed more compromised cell membranes in the HP cells than the control cells grown in the control medium for 3 d (Fig. 2C); cells of both lines were healthier at 5 d as compared to 3 d. A reduction of Ca concentration in the medium had a deleterious effect on membrane integrity of both cell lines; the effect was seen at 3 d as well as at 5 d for control cells and only at 5 d in the HP cells. While the Evans blue retention in control cells was not significantly affected by Al, the HP cells showed an improvement in membrane integrity in response to Al treatment in the control medium (4 mM Ca) as seen by decreased Evans blue retention at 48 h (data not shown).

2.2. Effect of Al and variation in Ca on cellular polyamines

Lowering the amount of Ca in the growth medium by 5- or 20-fold (to 0.8 and 0.2 mM, respectively) caused a significant decrease in cellular Put content in the HP cells after 5 d of growth (Fig. 3A) only the lowest concentration of Ca had an adverse effect on Put in the control cells. No significant change was seen in cellular Spd content as a result of lowering Ca in the medium, except for a small increase in this PA in the HP cells at 5 d with a 20-fold reduction in Ca (Fig. 3B). In 3 d-old cells treated with 0.1 mM Al, cellular Put was lowered to about 50% (in the control as well as low-Ca medium) in both cell lines, the decrease being significant after 24 as well as 48 h (Fig. 3C). Cellular Spd content showed a decrease after Al addition only in the HP cells (Fig. 3D). As expected, cellular Put content was always higher (by as much as 4 to 8 fold) in the HP cells than in the control cells; the differences in Spd were rather small (Fig. 3B, D). Only minor changes in Spm, which was present in relatively small amounts, were seen with these treatments (data not shown).

2.3. Effect of Al and Ca on cellular contents of inorganic ions

When 3 d-old cells were subjected to 0.1 mM Al treatment in the control medium, Al uptake was similar in the two cell lines at 6 h (Fig. 4A). Whereas the control cells between 6 and 48 h of treatment retained, and to some extent accumulated additional Al, the HP cells had more than a 50% loss of Al during this period. As a result, at 48 h, the HP cells had less Al than the control cells, and also less Al than at 6 and 24 h. Lowering Ca concentration in the medium to 0.8 mM did not change the Al accumulation response in either cell line.

The HP cells accumulated somewhat higher amounts of Ca than the control cells at most of the times tested regardless of the presence or absence of Al in the control medium (Fig. 4B). Lower amounts of Ca in the medium resulted in significantly lower Ca accumulation (as compared to that in the control medium) in both cell lines. No further change in Ca accumulation was seen in the two cell lines in response to Al treatment at any time of analysis, regardless of the amount of Ca in the medium.

Cellular contents of Mn, which were similar in the two cell lines in the control medium at any given time of analysis, were enhanced at least two-fold by Al in the HP cells after 24 and 48 h (Fig. 4C). Lower amount of Ca in the medium by itself caused increased Mn accumulation in both cell lines at 6 and 48 h. Adding Al to low-Ca medium caused no further change in Mn content in either cell line except a small reduction in the control cells at 6 h.

Cellular Mg contents in the control and the HP cells were comparable at any given time of analysis when grown in the control medium without Al (Fig. 4D); Mg in the cells increased with time between 6 and 24 h but not thereafter. The presence of Al caused

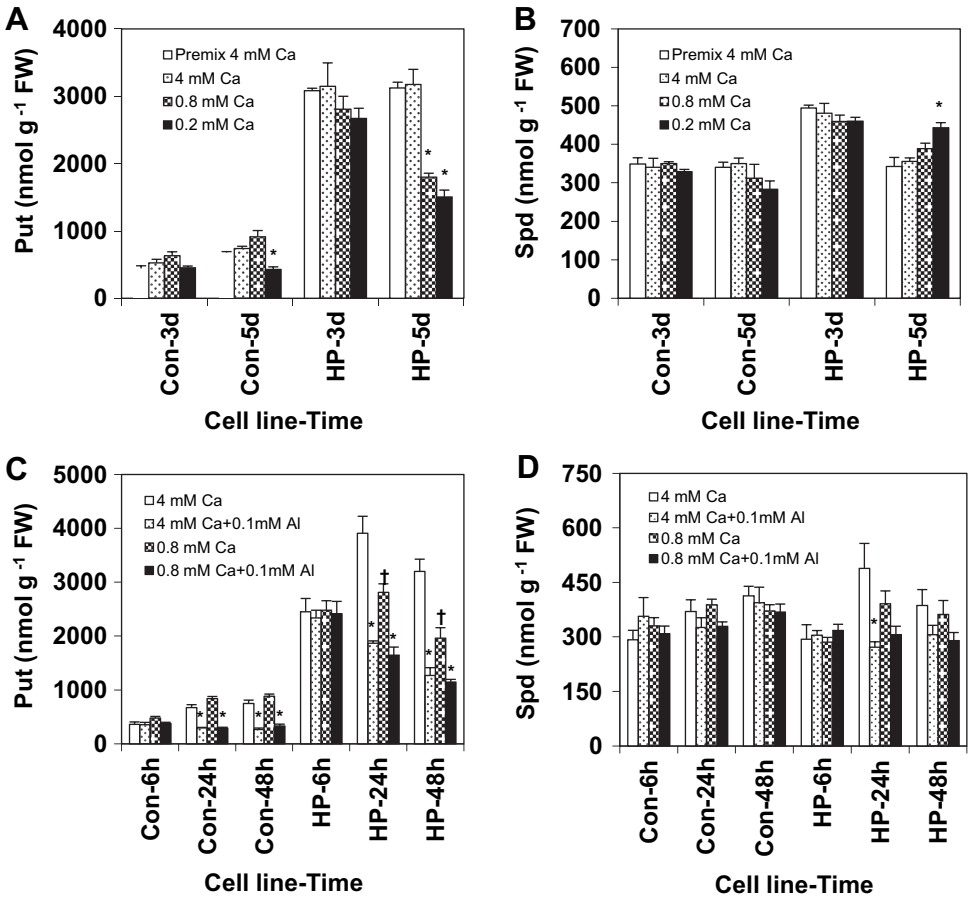

Fig. 3. Cellular contents of putrescine (A, C) and spermidine (B, D) in control and HP cells grown in different concentrations of Ca from time zero (A, B) or treated with 0.1 mM Al after 3 d of growth in normal (4 mM) or low-Ca (0.8 mM) medium (C, D). Each bar represents Mean ± SE of six replicates from two experiments. For further details on A and B, refer to the legend of Fig. 1A; and for C and D, refer to the legend of Fig. 1B.

a small but significant change in Mg accumulation in HP cells at 6 and 48 h. Lower Ca in the medium also caused a transient increase in Mg accumulation at 6 h in both cell lines. Adding Al to low-Ca cultures did not show different effects from those seen for similar treatment at normal Ca.

The accumulation of Fe was not affected by the presence of Al in the control medium except for 48 h after treatment, when an increase in Fe accumulation was seen in the HP cells (Fig. 4E). Lowering the concentration of Ca in the medium had an effect similar to that of adding Al; i.e. higher Fe accumulation was seen in HP cells. Adding Al to the low-Ca medium did not further affect Fe accumulation in either cell line. The accumulation of P, which was similar in the two cell lines, and remained mostly unchanged during the study period, was not affected either by adding Al or reducing Ca in the medium (Fig. 4F).

Whereas Al addition to the medium did not affect K accumulation either at low (0.8 mM) or normal (4 mM) Ca concentration in the control cells, it significantly enhanced K accumulation at both Ca concentrations in the HP cells; the effect was seen at 24 as well as at 48 h (Fig. 4G). The accumulation of Zn was enhanced both by the presence of Al as well as by lower Ca concentration in the medium in both cell lines (Fig. 4H); however, the effects were not consistent at all times.

2.4. Effect of Al on GSH, γ-EC and PC_2

It has been suggested that Al mimics some of the physiological effects of heavy metals in plants; these effects being manifested mostly in relation to Reactive Oxygen Species – ROS [33,43]. Oxidative stress further involves an interaction with Ca [8,31]. We analyzed the effects of Al in the presence of normal (4 mM) Ca concentration in the medium on the cellular contents of several sulfur-rich compounds including PC_2 (a phytochelatin) and its precursors, GSH and γ-EC. As seen from data in Fig. 5, the cellular contents of all three sulfur metabolites in HP cells were lower than those of the control cells on any day of analysis; the differences were about two-fold for GSH (Fig. 5A) and at least 4–5 fold for γ-EC and PC_2 (Fig. 5B, C). The presence of 0.1 mM Al caused a small but significant increase in PC_2 in the control cells at 24 and 48 h (Fig. 5C), but had no effect in other cases.

2.5. Effect of Al and Ca on the cellular free amino acids

Changes in the cellular contents of free amino acids in the control and the HP cells in response to Al treatment and/or reduction in Ca concentration in the medium are summarized in Table 1; detailed data are available in Suppl. Figure S1 (A–N). Although in the HP cells, Orn is the target of increased utilization as a substrate by the mODC and thus the major source of Put biosynthesis, its cellular content was below the detection limit of the HPLC system used here. Previously [25] we have shown that its content is significantly lower in the HP cells as compared to the control cells. Overall differences in the cellular contents of different amino acids between the control and the HP cells were also consistent with the data reported earlier [25] in that the concentrations of most amino acids were lower in the HP cells vs. the

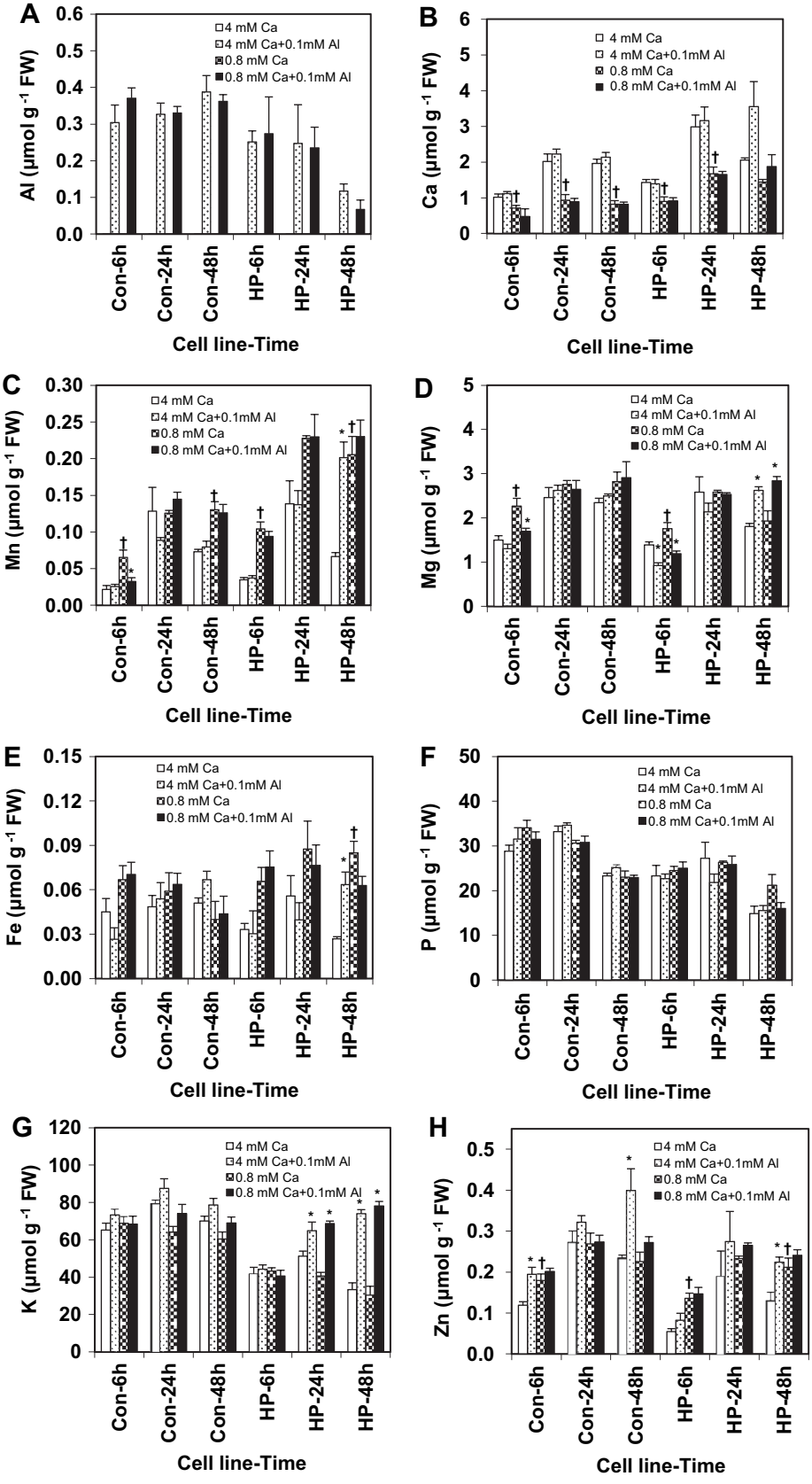

Fig. 4. Cellular contents of inorganic ions in control and HP cells treated with 0.1 mM Al after 3 d of growth in normal Ca (4 mM) or low-Ca (0.8 mM) medium. Each bar represents Mean ± SE of 6 replicates from two experiments; other details and definition of symbols are the same as in Fig. 1B.

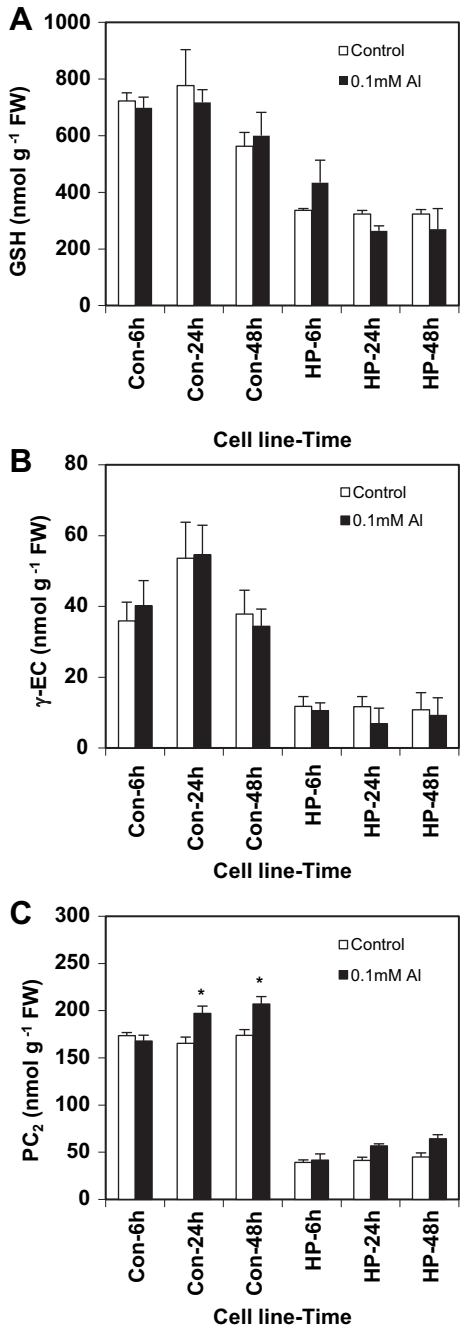

Fig. 5. Cellular contents of (A) GSH, (B) γ-EC, and (C) PC_2 in control and HP cells treated with 0.1 mM Al after 3 d of growth in normal (4 mM Ca) medium. Each bar represents Mean ± SE of six replicates from two experiments. An * indicates significant difference at $P \leq 0.05$.

control cells. In the control cells, Al addition to the medium resulted in significantly lower concentrations of Cys and Phe in normal (i.e., 4 mM Ca) and that of Gln, Arg, Val, Ser, Cys, Phe and Trp in low-Ca medium; the concentration of GABA went up under low-Ca conditions. In the HP cells treated with Al, while Glu and Arg concentrations were higher in the presence of normal Ca, in low-Ca medium, Glu, Gln, Thr, and Ser were increased and GABA was decreased.

Cellular contents of all amino acids except Glu and Arg were enhanced in the control cells by lowering of Ca concentration in the medium (Table 1), but the results were quite different for the HP cells where only the non-protein amino acid (GABA) was higher

Table 1
Summary of results on the changes in amino acids at 48 h after treatment with 0.1 mM Al added after 3 d of growth in normal (4 mM) or low (0.8 mM) Ca medium in control and HP cells of poplar. Direction of arrow shows increase (↑), decrease (↓) or no effect (–) in different comparisons as indicated. 4 mM Ca = control medium, 0.8 mM Ca = Low-Ca medium. Detailed quantitative data are given in Suppl. Figure S1.

Amino acid	4 mM Ca + 0.1 mM Al vs. 4 mM Ca		0.8 mM Ca vs. 4 mM Ca		0.8 mM Ca + 0.1 mM Al vs. 0.8 mM Ca	
	Control	HP	Control	HP	Control	HP
Glu	–	↑	–	↓	–	↑
Gln	–	–	↑	–	↓	↑
Arg	–	↑	–	–	↓	–
Pro	–	–	↑	–	–	–
GABA	–	–	↑	↑	↑	↓
His	–	–	↑	–	–	–
Thr	–	–	↑	–	–	↑
Val	–	–	↑	–	↓	–
Lys	–	–	↑	–	–	–
Ser	–	–	↑	–	↓	↑
Cys	↓	↓	↑	–	↓	–
Gly	–	–	↑	–	–	–
Phe	↓	–	↑	–	↓	–
Trp	–	–	↑	–	↓	–

(Table 1, Suppl. Figure S1). There was no effect of low Ca on any amino acid in HP cells except Glu, which showed a small reduction. In summary, major differences between the two cell lines were seen in response to low Ca; the effects being more dramatic in the control cells and less so in the HP cells.

3. Discussion

The data presented here show that the response of poplar cells to variation in Ca availability in their environment (i.e., in the growth medium) and treatments with Al are different from those seen in the foliage of many tree species subjected to analogous conditions in the soil. A similar conclusion was reached by Jiang et al. [11] with respect to the response of roots vs. the foliage of *Citrus grandis* to phosphorus treatment in the soil. Secondly, while the responses of the two cell lines differ from each other in many ways to lower Ca concentrations in the medium, the presence of several-fold higher amounts of Put in the HP cells provides no adaptive advantage to them for their response to low Ca. In fact, the HP cells are more sensitive to low Ca in terms of mitochondrial activity and growth than the control cells. A possible explanation for this lies in the fact that constitutively high Put production in HP cells is accompanied by an increased Put catabolism [5], which causes oxidative stress via elevated levels of H_2O_2 production [24]. This perhaps is not the situation in the foliage of trees where an increase in the steady-state levels of Put may not be accompanied by increase in its catabolism. Wang and Kao [40], who found increased Put production in response to Al treatment in rice roots also observed inhibition of root growth; perhaps also related to increase in Put degradation and higher H_2O_2 production. The same explanation may be valid for the apparent positive response of HP cells (i.e., increased mitochondrial activity and increased growth) to Al, in that Al caused a reduction in Put in these cells, which may have lead to reduced H_2O_2 production. This argument is supported by the observation that Al treatment caused a major increase in Glu (the primary source of Put) and only a small decrease in GABA (a product of Put catabolism) accompanying a 50% reduction in Put in these cells. Knowing that Put catabolism is closely linked to Put production in these cells [5], it can be surmised that the observed reduction in Put was due to reduced biosynthesis and not increased catabolism.

Putrescine contents in plant cell cultures in response to Al treatment vary with species [3,19–21,45]. A decrease in cellular Put in both poplar cell lines with Al treatment is consistent with an earlier report in *Catharanthus roseus* cell cultures [45]. However, what was not ascertained in either case is if the reduction in Put accumulation was due to its decreased biosynthesis, increased catabolism, and/or extrusion due to disruption of plasma membrane integrity. A likely explanation for the contrasting results between the response of foliage of trees subjected to high Al solubilization in soil [23,41] and the cell cultures in the present study may lie in the fact that in trees, while Ca uptake by roots and its accumulation in the foliage were adversely affected, Al itself did not accumulate in large quantities in the foliage [21,32]. Furthermore, since the symptoms of increased Al availability and Ca deficiency were similar in the leaves [16], it can be argued that increased Put accumulation in the foliage under low soil Ca or high Al may be a response to reduced Ca in the foliage. In the present study with poplar cells, however, no increase in Put was seen either by lowering Ca or by the addition of Al to culture medium. This may be due to the fact that in the culture medium, addition of Al by itself does not affect Ca availability to cells as it happens in the soil for roots. In fact, Al accumulation in the cells caused a decrease in Put. These explanations are consistent with the observations that Ca supplementation of soil concomitantly increases foliar Ca and lowers foliar Put, while at the same time, it decreases the availability of Al in the soil [18,41].

Whereas the increased uptake of ions, especially Ca, may have a protective role in HP cells against Al toxicity, it may also be a response to enhanced ROS activity due to increased H_2O_2 production from high PA turnover as discussed earlier [24]. Increased ROS activate the inwardly rectifying Ca^{2+} channels in plant cells [6,28]. The fact that Al exposure stimulates ROS generation, which causes peroxidative damage to membranes and promotes mitochondrial dysfunction, is well established [13,19,44]. In line with the suggestion of Stark [36], it is also conceivable that increased accumulation of Ca and Mn may in reality be the cause of cell damage in the HP cells.

The early and late responses of HP cells to Al were different from control cells in that there apparently was a slow extrusion of Al from the former between 6 and 48 h. Yamamoto et al. [44] also reported a contrasting effect of Al with time in cultured tobacco cells; a rapid suppression of mitochondrial activity within 6 h of Al addition was followed by an increase in mitochondrial activity for up to 12 h. Enhancement of cell death within 18 h by either lowering of Ca or treatment with Al has since been reported in tobacco cell cultures [3]; the addition of Ca to the Al-containing medium in this study released Al from the cells.

The cellular contents of phytochelatins like PC_2 and its precursor γ-EC typically increase in response to metal toxicity in plant cells [9,33,38]. The HP cells consistently showed lower contents of GSH, PC_2, and γ-EC as compared to control cells (Fig. 5). While Al had no effect on the cellular contents of GSH and γ-EC in either cell line (and only slightly increased PC_2 in the control cells), and PCs do not play a major role in Al binding [39], their reduced contents in the HP cells may be a reflection of lowered Glu, Gly and Cys in them [[25], Suppl. Figure S1]; all of which are precursors of these sulfur-containing metabolites.

Calcium, an essential plant nutrient, is required for numerous physiological and regulatory functions, acts as a counter ion for anions in the cell, and as an intracellular messenger in the cytosol [8,42]. Hence, it is understandable that lowering of Ca in the culture medium would adversely affect the growth of cells. The two cell lines differed more in their response to lower Ca content (0.8 mM) in the culture medium than treatment with Al; particularly striking were the differences in their amino acid contents. For example, while Glu decreased and GABA increased (with no other amino acid changing) in the HP cells grown in low-Ca medium, all amino acids, except Glu and Arg, were higher in the control cells grown in this medium. This response combined with the observed decrease in mitochondrial activity, increase in membrane damage, lowering of Put, increase in Mn uptake, and reduction in overall growth of HP cells in low-Ca medium indicates that they are more sensitive to the lowering of Ca concentration in the medium. It is probably due to a greater reduction in available Ca in the medium of HP cells due to its higher accumulation in these cells under normal conditions. An explanation as to why the HP cells may accumulate more Ca than the control cells is discussed above in relation to increased ROS production, and also in Mohapatra et al. [25].

In conclusion, it appears that due to increased ROS production in the HP cells, their tolerance to low Ca is compromised (particularly in terms of increased damage to mitochondria and cell membranes). However, contrary to the expectation of deleterious effects, the HP cells showed an apparent advantage in the presence of Al in the medium, which could have come from reduced uptake of Al, enhanced extrusion of Al following its accumulation, and perhaps a reduction in Put catabolism as a result of a reduction in its biosynthesis.

4. Materials and methods

4.1. Cell growth and harvest

The high putrescine cell line (called HP) and an isogenic control cell line of *Populus nigra* × *maximowiczii* used here have been previously described [4,5,24,27]. The HP line, referred to earlier as 2E [4,5] expresses a mouse *ODC* transgene, while the control line expresses the β-*glucuronidase* (*GUS*) transgene; both cell lines also express the *NPT*II transgene that allowed selection on kanamycin. An identical 2 × 35S CaMV promoter controlled all three transgenes. Cell cultures were maintained in MS medium [26] as described in Bhatnagar et al. [4,5].

For routine subculture, 7 mL of 7-d old cell suspensions were added to 50 mL fresh medium. For experiments involving treatments with Al, 0.1 mM (final concentration) $AlCl_3$ was added to cell suspensions 3 d after subculture. Subsequently, the cells were collected at different time intervals after addition of Al for various analyses. For experimental variation in Ca concentration, 7-d old cells were subcultured into medium with either normal (i.e., 4 mM; referred to as control medium) or 0.8 mM Ca (called low-Ca) or 0.2 mM or 8.0 mM concentrations of Ca. Medium for these experiments was prepared by mixing individual constituents and not the pre-mixed powder as was the case in experiments involving treatments with Al alone. Cells were also grown in commercially available MS salt mix (Sigma–Aldrich, St. Louis, MO) for comparison of results with medium made from individual constituents.

In experiments involving Ca and Al co-treatment, a similar set-up (as above) was used in terms of subculturing the cells into medium with normal and 0.8 mM Ca concentrations. After 3 d of growth, the cells were either left untreated or $AlCl_3$ was added to a final concentration of 0.1 mM. Collections were done at different time intervals after adding Al.

4.2. Measurement of mitochondrial activity and cell viability

About 100 mg of cells were placed in 1 mL MS medium containing 250 μg MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] for measurement of mitochondrial activity as described in Minocha et al. [19]. Briefly, following gentle mixing at room temperature for 60 min, the cells were harvested by centrifugation (16 000 × g, 10 min), resuspended in 1 mL of 0.04 M HCl in isopropanol, centrifuged for 5 min, and the supernatant used for measurement of A_{590} (Hitachi U-2000, Schaumburg, IL). Cell

viability (loss of membrane integrity) was measured by the Evans blue dye retention method [19].

4.3. Polyamines, amino acids and inorganic ions

Following vacuum filtration, 200 ± 20 mg (FW) of cells were mixed with 4× volume of 5% $HClO_4$ (v/v from stock of 60%; approx. 0.77 N) and frozen (−20 °C) and thawed (room temp) three times before dansylation and quantification of PAs [17] and amino acids [22] by HPLC. The $HClO_4$ fraction was also used for ion analysis by ICP as described in Minocha et al. [17].

4.4. Reduced Glutathione (GSH), phytochelatin (PC_2) and γ-glutamyl-cysteine (γ-EC)

About 100 mg of cells were collected in 500 μL of 6.3 mM diethylenetriamine pentaacetic acid (Fluka) containing 0.1% trifluoroacetic acid, derivatized and analyzed by HPLC [38].

4.5. Statistical analyses

Statistical comparisons (one-way ANOVA) were made for control medium (4 mM Ca) and other treatments in each experiment using SYSTAT 10.1 (Systat, Chicago, IL). All comparisons were made between the respective control and treatment(s) within the same cell line and for the same time of collection. Symbols are described in the Figure legends. All experiments were repeated at least twice and data were pooled for analysis. Significances shown by a symbol or mentioned in the text were calculated at $P \leq 0.05$.

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Appendix. Supplementary material

Cellular content of different amino acids in control and HP cells 48 h after treatment with 0.1 mM Al added after 3 d of growth in normal (4 mM) or low-Ca (0.8 mM) medium. Each bar represents Mean ± SE of six replicates from two experiments; other details and definition of symbols are the same as in Fig. 1B.

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plaphy.2010.04.010.

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