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Silicon amelioration of aluminium toxicity and cell death in suspension cultures of Norway spruce (*Picea abies* (L.) Karst.)

Subramaniam Prabagar, Martin J. Hodson, David E. Evans*

School of Life Sciences, Oxford Brookes University, Headington Campus, Oxford OX3 0BP, UK

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ABSTRACT

A role for silicon (Si) in the amelioration of aluminium (Al) toxicity in gymnosperms is suggested by their codeposition *in planta*, including within needles. This study was designed to investigate Al/Si interactions at the cellular level using suspension cultures of Norway spruce. Toxic effects of Al were dependent on duration of Al exposure, concentration of Al, and pH. Toxicity was reduced when Si was present, and the effect was enhanced at pH 5.0 compared to pH 4.2. Study of the ultrastructure of Al-treated cells indicated that changes in cell wall thickening, degree of vacuolation, and the degeneration of mitochondria, Golgi bodies, ER and nucleus preceded cell death, and significant amelioration was noted when Si was also present. When the fluorescent dye Morin was employed to localise free Al, cells treated with Al and Si in combination showed less fluorescence than the cells treated with Al alone. Intensity of fluorescence depended on the concentration of Al, duration of treatment and pH. Notably, presence of Si reduced the concentration of free Al in the cell wall in parallel with amelioration of Al toxicity. We therefore propose that formation of aluminosilicate complexes in the wall and apoplasm provide a significant barrier to Al penetration and cell damage in Norway spruce.

1. Introduction

Aluminium (Al) is an important component of many soil minerals and only if soluble, mainly due to low pH, does it become toxic. Its availability depends on its chemical form (largely dependent on pH) and on the formation of complexes, which are of limited solubility and therefore unavailable. Its toxic effects have been reported in monocotyledonous and dicotyledonous angiosperms (Foy, 1988) and in gymnosperms (Schaedle et al., 1989). Globally, Al toxicity is responsible for limiting crop production in tropical and sub-tropical soils (Foy, 1992), and is a potential cause of forest dieback in temperate and boreal forests (Godbold et al., 1988 and Taylor, 1989). In many situations, mechanisms for the amelioration of toxicity are critical for plant growth.

It is now well recognised that silicon (Si) can have ameliorative effects on the phytotoxicity of a variety of metals (Doncheva et al., 2009; Vaculik et al., 2009). Previously, the effect of Si on Al availability in soils and in solution has been studied, together with its effects on plant growth (Hodson and Evans, 1995). Studies have included plant growth in soil (Morikawa and Saigusa, 2004) and hydroponic systems (Cocker et al., 1998a). Most studies have involved monocotyledons (Galvez et al., 1987; Hodson and Sangster, 1993; Hammond et al., 1995; Ma et al., 1997; Hara et al., 1999; Kidd et al., 2001; Kidd and Proctor, 2001; Wang et al., 2004) and a few herbaceous dicotyledons (Li et al., 1989; Baylis et al., 1994). In general, the presence of Si in the culture solution resulted in amelioration of Al toxicity, and only in a few cases was this not observed (Li et al., 1989).

There is considerable evidence to suggest that Al/Si interactions are important in gymnosperms. Conifers transport some Al to their shoot tissues (Hodson and Sangster, 1999). In needles, Al is imported and deposited *via* the transpiration stream for several years before they senesce and abscind. Silicon is also transported through gymnosperms to reach the extremities of the needles. Most studies have involved X-ray microanalysis, especially of Al/Si co-deposits in needles (Hodson and Sangster, 1999, 2002). In white spruce, Al colocalised with Si in the needle epidermis (Hodson and Sangster, 1998) and the tissues with the highest Si (in the tip, the mesophyll and transfusion cells) were also the tissues with the highest Al content (Hodson and Sangster, 1999). A similar pattern was observed in the needles of Eastern hemlock (Sangster et al., 2009). In Norway spruce, codeposition of Al and Si was shown to occur in the tissues interior to the endodermis of damaged needles (Godde et al., 1988); however codeposition is rare in needles of Douglas fir (Sangster et al., 2007). Codeposition of Al and Si has also been observed in the root cell walls of Al-tolerant Norway spruce seedlings (Hodson and Wilkins, 1991). In a study of Norway spruce seedlings in hydroponic culture, Ryder et al. (2003) demonstrated

* Corresponding author.
E-mail address: deevans@brookes.ac.uk (D.E. Evans).

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that Si ameliorated the toxic effect of Al. Speciation analysis indicated that Al^{3+} and hydroxyaluminium species were equally toxic and in both cases, Al toxicity was ameliorated by Si. The effect was pH-dependent with amelioration detected at pH 4.75 and 5.0, but not at pH 4.5 or below.

The present study was designed to explore Al/Si interactions in gymnosperms at the cell level. Cell suspension cultures have been used previously to analyse basic mechanisms involved in Al toxicity at a cellular level (e.g. Ojima et al., 1989; Yamamoto et al., 1994; Staß and Horst, 1995; Minocha et al., 2001; Conner and Meredith, 1985a,b; Ono et al., 1995; Jones et al., 1998; Ramírez-Benítez et al., 2009; Pejchar et al., 2010). These studies reveal that suspension cultures are more susceptible to Al damage in the logarithmic growth phase than in the stationary phase (Yamamoto et al., 2000), and that Al affects cell growth, cell division and viability (Pan et al., 2002) with loss of plasma membrane integrity occurring prior to cell death (Ikegawa et al., 1998).

Suspension culture systems provide an opportunity to explore Al uptake at a cellular level. In carrot suspension cells, Al content increased with increasing external Al concentration (Honda et al., 1997), and was accompanied by inhibition of respiration and decreased ATP content. In tobacco cells, uptake was accompanied by a loss of viability (Yamamoto et al., 1997), while Chang et al. (1999) observed that only about 10% of the Al entered the cytoplasm, the remainder being associated with the cell wall. A similar observation was also made by Schmohl and Horst (2000) in maize.

The subcellular effects of Al have also been studied in suspension cultures, with effects on the cytoskeleton (Sivaguru et al., 1999; Grabski and Schindler, 1995) and membranes (Deleers et al., 1986; Oteiza, 1994; Ono et al., 1995; Ikegawa et al., 2000; Sivaguru et al., 2005). Studies on the effects of Al on gymnosperm suspension cultures have revealed both growth inhibition and effects at a subcellular level. Minocha et al. (1992, 1996, 2001) observed that the addition of $AlCl_3$ to 3-day-old red spruce suspension cultures caused a significant increase in cellular putrescine concentration, increased vacuolar and total cell volume, and increased surface area of Golgi membranes and endoplasmic reticulum. Succinate and oxalate were shown to be secreted into the culture medium (Minocha and Long, 2004). Organic acid secretion has been suggested to be involved in Al tolerance in a number of whole plant systems (Cocker et al., 1998a).

Conducting Al/Si interaction experiments in suspension culture allows the investigation of amelioration phenomena in the absence of the organised tissues of the whole plant. One possible hypothesis is that such organisation is a prerequisite for amelioration to occur. Al/Si interactions have previously been studied in suspension cultures of rice and coffee (Rahman et al., 1999; Quintal-Tun et al., 2007). The presence of Si only marginally reduced the toxic effects of Al in a sensitive variety of rice and had no significant effect in a tolerant variety (Rahman et al., 1999). Quintal-Tun et al. (2007) did not consider cell growth in their coffee suspension cultures, but showed that the toxic effects of Al on the phospholipid signal transduction pathway were ameliorated by silicic acid. It is thus unclear whether amelioration occurs in suspension cultures. The work described in this paper was therefore carried out on Norway spruce suspension cultures to investigate whether Al/Si effects at a cellular level contribute to Al tolerance in this species.

2. Materials and methods

2.1. Cell cultures

Cell suspension cultures of Norway spruce (*Picea abies* (L.) Karst.) were initiated by agitating a fragment of *in vitro* grown embryogenic callus in a volume of liquid medium which was prepared using modified half-strength Litvay's medium (Litvay et al., 1985) containing 1% sucrose, 2 mg L^{-1} 2,4-D, 1 mg L^{-1} benzyladenine (BAP), 500 mg L^{-1} casein hydrolysate, and 250 mg L^{-1} glutamine, at pH 5.7 prior to autoclaving. Rapidly growing embryogenic callus was then transferred to a 250 ml conical flask containing 100 ml of maintenance medium. Cells were subcultured at 9-day intervals (10 ml into 50 ml medium, 250 ml flask) and incubated in the dark at 25 ± 2 °C on a New Brunswick orbital incubator shaker at 120 rpm.

2.2. Aluminium treatments

Aliquots (10 ml) of 9-day-old cell suspensions from 5 culture flasks were transferred with shaking to 250 ml flasks containing 50 ml of fresh medium (pH 4.2) to which $AlCl_3$ had been added to final concentrations of 0, 0.2, 0.5 and 1.0 mM Al (effective concentrations of monomeric Al = 0.09, 0.23 and 0.48 mM, Minocha et al., 1996) in triplicate. They were then shaken in the dark (120 rpm, 25 °C). At 0–48 h after Al (0.2–1.0 mM) addition, 10 ml samples of cells were centrifuged and washed twice with sterile culture medium and then cultured in fresh medium without Al for 19 days before measurement of dry weight (DWT) and packed cell volume (PCV). Data was presented as relative growth (DWT or PCV of Al-treated cells/DWT or PCV of untreated control cells).

Cell viability, determined as loss of plasma membrane integrity, was evaluated by spectrophotometric measurement of retention of Evans blue dye (Baker and Mock, 1994). Cell suspension cultures (10 ml) were treated with 0–1.0 mM Al solution in 250 ml flasks containing 50 ml fresh medium (pH 4.2 or 5.0). After 0 to 48 h, 10 ml samples of the cultures were collected and washed twice with fresh medium. Washed cells were suspended in 2 ml of 0.05% aqueous Evans blue solution and stained for 15 min at room temperature. Cells were sedimented by centrifugation and washed five times with distilled water, after which the dye no longer eluted from the cells. Trapped dye was released by suspending cells in 2 ml of 1% (w/v) aqueous sodium dodecyl sulphate (SDS) and disrupting at room temperature in a sonicator equipped with a microprobe (for 2 min at 44 W; model W-380; Heat Systems-Ultrasonics, Inc., NY, USA). The solution was transferred to an Eppendorf tube and centrifuged at 13,500 × g for 10 min. The optical density of the supernatant was determined at 600 nm.

Percentage of cell death was estimated by dye exclusion. Cells were treated with 0–1.0 mM Al as described above. One drop of Evans blue dye (1% (w/v) aqueous SDS) was mixed with 0.5 ml of cell suspension and incubated for 15 min at room temperature, before viewing by light microscopy. Areas chosen for counting contained a minimum of 25 cells and the percentage of dead cells was calculated.

2.3. Silicon and Al/Si treatments

Silicon and Al/Si treatments were conducted as described above. However, solutions were made by adding various concentrations of $AlCl_3$ solutions (0.2, 0.5 and 1.0 mmol) to 1.0 mM Si at both pH 4.2 and 5.0 and silicic acid was used as Si source. The solutions were then left to sediment for 6 h at room temperature before use.

2.4. Determination of Al and Si content

The Al, Si and Al/Si treated cells were centrifuged and washed twice with sterile culture medium. The cells were suspended in 0.5 ml of acid mixture (H_2SO_4:HNO_3, 1:1, v/v) and were digested using microwave digestion method. Concentrations of Al and Si were determined using atomic absorption spectrophotometry.