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Rhizosphere carbon deposition, oxidative stress and nutritional changes in two poplar species exposed to aluminum

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Summary Species and hybrids in the genus *Populus* have become the focus of investigation for use in biofuels production and their capacity to sequester carbon (C) in the environment. The identification of species resistant to marginal edaphic sites may be important in both of these endeavors. Plant growth, total dissolved organic carbon (TOC) and low molecular weight organic acid (OA) production, antioxidative enzyme activities and mineral content were assessed in *Populus tremuloides* L. and *Populus trichocarpa* Torr. & Gray seedlings under exposure to aluminum (Al). Both species were sensitive to Al, with significant reductions in shoot and root biomass at and above 50 μM Al. Exposure to Al induced 40-fold increases in TOC deposition in *P. tremuloides* and 100-fold increases in *P. trichocarpa*. In *P. tremuloides*, Al treatment induced root exudation of malic and citric acids, while Al increased exudation of citrate and oxalate in *P. trichocarpa*. Organic acids accounted for 20–64% of total C released upon Al exposure, with the proportion of OAs increasing in *P. tremuloides* and decreasing in *P. trichocarpa*. Dose-dependent responses of catalase and ascorbate peroxidase were observed in both root and leaf tissues, indicating that Al exposure induced oxidative stress in poplar. Treatment at and above 100 μM Al reduced the concentrations of calcium (Ca) and magnesium (Mg) in roots and leaves, whereas Al at or above 50 μM reduced root and leaf phosphorous (P) concentrations. The majority of Al taken up was retained in the root system. Even with the induction of OA exudation and accumulation, *P. tremuloides* and *P. trichocarpa* remained sensitive to Al, as evidenced by elevated antioxidative enzyme activities, which may reflect inhibition of Ca or P uptake and destabilization of cell homeostasis in these poplar species. Although plants exhibited reductions in growth and evidence of oxidative and nutritional stress, total C rhizodeposition rates for both species increased with increasing Al exposure concentration. Estimated C deposition rates of 16 mg C $plant^{-1}$ day^{-1} were four-times larger than previously reported values for forest tree species, indicating that edaphic stress plays an important role in C flux to the rhizosphere.

Keywords: exudates, mineral nutrition, organic acids, Populus tremuloides, Populus trichocarpa, reactive oxygen species, total organic carbon.

Introduction

The potential for aluminum (Al) toxicity is widespread in soils of temperate regions and may limit the capture and storage of carbon (C) in forested ecosystems. Soil acidification resulting from natural weathering processes (Halvin et al. 1999) and anthropogenic activities, such as nitrogen deposition (Aber et al. 1998) and surface mining (Karathanasis et al. 1998), increases available and phytotoxic ions in soils and stimulates leaching losses of nutrient cations from these systems. Low soil pH and high Al levels in soils may alter C flow to soils and may be a major factor limiting biological C sequestration strategies that rely on the managed production of tree species (Tuskan and Walsh 2001), such as those in the genus *Populus*. Resistance to Al stress in selected species or hybrids will be important under acidic soil conditions in determining the productivity and C sequestration potential of such systems.

The effects of Al in the rhizosphere on root and shoot growth of forest tree species may be mediated through several direct and indirect pathways. Root growth – cell division and elongation – is directly inhibited by Al (Schaedle et al. 1989). Elevated Al in the root zone also reduces levels of calcium (Ca), magnesium (Mg) and often phosphorous (P) in tree roots and foliage below those required for normal growth and metabolism. Decreased Ca and Mg concentrations in roots and foliage of spruce (Asp et al. 1988), Ca, Mg and P in foliage of tulip poplar (Lux and Cumming 1999, 2001) and Ca in foliage of sugar maple (Schaberg et al. 2006), for example, have been associated with reductions in growth and altered tree physiological function. These effects of Al on trees may be attributed to the influence of Al on ionic interactions in the apoplast (Cronan 1991) or on nutrient transport systems (Cumming et al. 1986), as has been reported for numerous non-woody

species (Kochian et al. 2005). Thus, Al disrupts forest tree nutrient relations, ultimately reducing shoot and root growth and C storage in forested ecosystems (Driscoll et al. 2001, Schaberg et al. 2006).

Differential resistance of forest trees to Al will influence ecosystem composition and C sequestration potential (Aber et al. 1998). One of the well-established physiological mechanisms of Al stress resistance in plants involves the extracellular detoxification and exclusion of Al via root organic acid (OA) exudation and the intracellular chelation and detoxification of Al by OA accumulation. While most studies of OA anion production have been performed on crop plants (Kochian et al. 2005), recent studies on forest tree species, such as *Melaleuca*, *Melastoma* and *Eucalyptus*, similarly indicate that Al-induced root OA exudation and accumulation may be involved in tree response to Al (Watanabe and Osaki 2002, Nguyen et al. 2003, Silva et al. 2004). The exudation (Nguyen et al. 2003) with and without accumulation (Silva et al. 2004) of OAs will lead to the extracellular and intracellular chelation and detoxification of the phytotoxic Al^{3+} ion, reducing its impacts on cell division, cell wall ionic relations, membrane transport activity and root growth, thus facilitating tree growth and C storage on acidic soils.

There is a strong relation between Al exposure and oxidative response in plants (Kochian et al. 2005), which may reflect a fundamental lesion or acclimation response to Al exposure. Increases in the activities of the antioxidant enzymes, such as superoxide dismutase and peroxidase, may indicate that the primary effect of Al on plants may be the generation of free reactive oxygen species (ROS), such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals that alter cell structure and function. The key role of antioxidant enzymes is to reduce or scavenge these ROS. While increased antioxidative enzyme activities as a stress response to Al exposure are well documented in crop species (Basu et al. 2001, Sharma and Dubey 2007, Liu et al. 2008), few studies have reported the role of ROS and Al stress in tree species (Nagy et al. 2004, Takami et al. 2005).

Variation in Al resistance has been reported among and within tree species based on growth and morphological responses (McCormick and Steiner 1978, Schaedle et al. 1989), as well as differences in ionic relations (Nowak and Friend 2005) and exudation (Silva et al. 2004). These studies, although limited in number, suggest that OA production and limited Al accumulation are related to Al resistance in woody tree species. A concerted effort investigating differences in Al resistance within tree species and genotypes would be helpful in understanding the available genetic resources to use in reforestation efforts and C sequestration programs where Al toxicity and acidic soils limit tree growth.

Poplar species and hybrids are intensively cultivated as renewable sources of biomass and for reforestation in the northern United States, Canada and Europe. Because of rapid growth, short rotation cycle, long-term adaptation and ease of propagation, they are used in acidic and polluted soils for reclamation and phytoremediation activities. However, the physiological mechanisms underlying soil stress resistance within these poplar species and hybrids are not yet understood. With the recent sequencing of the poplar genome (see Brunner et al. 2007), the use of these species in physiological stress resistance studies may help elucidate the underlying basis of stress resistance in trees.

Quaking aspen (*Populus tremuloides* L.) is widely distributed across northern North America and is a pioneer species on many disturbed sites. Quaking aspen is most productive on calcium-rich soils (Bates et al. 1992), although its presence on abandoned acidic coalmines in the Appalachian Mountains suggests acid soil resistance in this species (Cumming, personal observation). Black cottonwood (*Populus trichocarpa* Torr. & Gray) is a western North American species and is a dominant species in riparian ecosystems and, in these areas, production is best on soils of neutral pH. Ecotypic variation in cottonwoods based on growth responses is high (Rood et al. 2007), although little has been reported on soil stress resistance. Mineral stresses, especially Al toxicity, are highly dependent on soil conditions and such stresses are likely to have important, complex and poorly understood interactions with carbon sequestration.

The aim of this study was to assess the physiological responses of quaking aspen and black cottonwood to Al in the rhizosphere. We hypothesized that quaking aspen would be more Al resistant based on observations of differences in habitat preferences between the two species. In this study, we compared Al tolerance of seedlings of two different poplar species, *P. tremuloides* and *P. trichocarpa*, exposed to Al between 0 and 500 μM Al in sand culture for 40 days. Measurements of growth, root zone total dissolved organic carbon (TOC) and OA concentrations, root tip accumulation of OAs, tissue antioxidant enzyme activity and root and leaf mineral nutrient content were implemented to gain insight into the mechanisms of Al toxicity and tolerance in these species and patterns of C flux to the rhizosphere that may serve to protect plants from the toxic effects of Al. Investigation of these complex relations between mineral stress and rhizospheric C flux is important because poplar species are being considered as candidate tree species for biomass and C sequestration activities.

Materials and methods

Plant culture

Seeds of *P. tremuloides* and *P. trichocarpa* (purchased from the Sheffield Seed Co., NY) were germinated for 4 days in 4-cm diameter × 21-cm deep pots ('Cone-tainers'™, Stuewe and Sons, Corvallis, OR) containing acid-washed sand (coarse and fine sand mixture ratio 2:1). Seeds were kept moist by watering four-times daily with a 0.1 mM $CaNO_3$ solution at pH 5.6. After 7 days, seedlings were thinned to leave plants of similar size for the experiment. Following

selection, the seedlings of both species were watered four-times daily to field capacity (30 ml per pot) with a nutrient solution containing 1.0 mM NO_3, 0.4 mM NH_4, 0.5 mM K, 0.2 mM Ca, 0.05 mM H_2PO_4, 0.1 mM Mg and SO_4, 50.5 μM Cl, 20 μM Fe, 20 μM B, 2 μM Mn, 2 μM Zn and 0.5 μM Cu, Na, Co and Mo. Solution pH was adjusted to 4.0. After 14 days, Al in the form of $AlCl_3$ was added to deliver treatment solutions containing 0, 50, 100, 200 or 500 μM Al. Aluminum from a 1 M $AlCl_3$ stock, freshly prepared on the day of use, was added with vigorous stirring. All solutions were adjusted to pH 4.0 with 1 N NaOH or 1 N HCl. Both were added slowly with continuous stirring of the solutions to avoid Al precipitation. Seedlings were maintained in a climate-controlled greenhouse with supplemental lighting providing a 14-h photoperiod (mixed metal halide source) and day/night temperatures of 24/19 ± 3 °C. Relative humidity fluctuated with temperature and day. Seedlings were exposed to Al for 40 days.

Growth measurements

Initial shoot height and number of leaves were recorded before seedlings were exposed to Al treatments; these served as growth covariates. Shoot height, number of leaves, leaf area and length of longest root were measured following the 40 days of Al exposure. Shoots were separated from roots and processed for enzyme activity and mineral nutrient concentrations as noted below. Root systems were removed from pots and processed for OA production and content, enzyme activity and mineral nutrient concentrations as noted below. All components were weighed (fresh and dry mass and ratios) to establish total plant biomass.

Determination of TOC and OA exudates in the rhizosphere

After shoots were removed from the root systems, the contents of each pot (roots of one plant and sand mixture) were placed in a beaker. Fifteen milliliters of deionized water were added to the root–sand sample and allowed to stand for 5 min. This water was extracted using a pipette and passed through the root zone sample twice again, removed and filtered (0.45 μM) immediately. The TOC was analyzed using a TOC analyzer (Shimadzu Model TOC-V-CPH). To prepare root zone extracts for OA analysis, 0.8 ml of 10 mM Na_2–EDTA and a drop of 1 M NaOH were added to 15 ml samples of each aqueous root zone extract. This procedure promoted chelation of Al in the filtrate and prevented suppression of OA detection by Al (Cumming et al. 2001). Samples were roto-evaporated and stored at −20 °C until analyzed. Residual salt pellets were dissolved in 1 ml of sterile deionized water and the concentrations of OAs (malate, citrate, formate, succinate and lactate were the predominant acids in these samples) were measured by ion chromatography using a conductivity detector (Dionex ICS-1500, Sunnyvale, CA). For separation of OAs, a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm ID) was employed with minor modifications of Qiu and Jin (2002) and Cumming et al. (2001). The eluent was 2.3 mM heptafluorobutyric acid at a flow rate of 0.6 ml min^{-1}, the suppressant solution was 5 mM tetrabutylammonium hydroxide at a flow rate of 0.6 ml min^{-1} and the analysis time was 20 min. Calibration equations based on peak area for each OA were obtained based on standard OA solutions with varying concentrations and their corresponding peaks. Oxalate concentrations were determined spectrophotometrically using an oxalate diagnostic kit (Trinity Biotech, St. Louis, MO). Oxalate standards were prepared from oxalic acid dihydrate as recommended by the manufacturer. Sample OA concentrations were adjusted by the dilution factor from the extraction to normalize values to those measured at field capacity of the pots. Concentrations of OAs in pots without plants, which were low, were subtracted from values measured for each pot with a plant within the same Al treatment.

Determination of OAs from root apices

Organic acid extraction from root apices was carried out according to Yang et al. (1994) with minor modifications. Root tips (1–1.5 cm long) from each poplar species were excised, rinsed with distilled water, dried with a paper towel, weighed, immediately frozen in liquid nitrogen and stored at −80 °C for OA determination. The frozen roots were ground in a cold mortar and pestle with cold 80% v/v ethanol to form slurry. The mixtures were centrifuged at 4000*g* for 10 min at 4 °C and the pellets were extracted twice with ice-cold water. The supernatant from each of these extractions was roto-evaporated and stored at −20 °C until analyzed. Residual salt pellets were dissolved in 1 ml of sterile deionized water and filtered (0.2 μM) and the concentrations of OAs (malate, citrate, isocitrate, tartrate and succinate were the dominant acids in these samples) were separated and measured using the same method as described previously. Oxalate content was also measured as described previously. The OA concentrations were quantified as μmol g^{-1} FW.

Assay of antioxidant enzymes

Preparation of enzyme extracts Leaves (leaf plastochron index 5) and root samples from the middle portion of the root system were harvested for enzyme assays. Tissue samples were flash-frozen in liquid N_2 immediately after harvesting and stored at −80 °C until extracted. During enzyme extraction, 0.3–0.4 g fresh mass of leaf or root tissue was ground in liquid N_2 in a pre-cooled mortar and pestle to make a fine powder and subsequently homogenized in 2 ml of cold 0.05 M Tris buffer at pH 7.5 containing 4% PVP, 1% BSA and 1% β-mercaptoethanol (Ranade and Feierabend 1991). The homogenate was centrifuged for 10 min at 4 °C at 8000*g*. The supernatant was used for enzyme assays.

Catalase Catalase (CAT) (EC 1.11.1.6) activity was determined by following the consumption of H_2O_2

spectrophotometrically at 240 nm for 3 min using a reaction mixture containing 2.975 ml of 100 mM phosphate buffer at pH 7.0, 24 µl of 30% H_2O_2 and 5 µl of supernatant as an enzyme source (Volk and Feierabend 1989).

Ascorbate peroxidase Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was determined as described by Ievinsh et al. (1995) by measuring the decrease in absorbance due to the conversion of ascorbate to dehydroascorbate spectrophotometrically at 290 nm for 3 min using a reaction mixture containing 150 µl of 100 mM phosphate buffer at pH 7.0, 350 µl of 0.5 mM ascorbic acid, 2.065 ml of sterile water, 300 µl of 1 mM EDTA, 55 µl of 30% H_2O_2 and 80 µl enzyme extract.

Guaiacol-dependent peroxidase Guaiacol-dependent peroxidase (GDP) (EC 1.11.1.7) activity was determined by measuring the increase in absorbance due to the formation of tetra-guaiacol from guaiacol in the presence of H_2O_2 spectrophotometrically at 470 nm for 3 min using a reaction mixture containing 2.48 ml of 100 mM phosphate buffer at pH 7.0, 500 µl of 30 mM guaiacol, 4.8 µl of 30% H_2O_2 and 80 µl of enzyme extract (Volk and Feierabend 1989).

Enzyme activities (CAT, APX and GDP) were expressed as mkat g^{-1} fresh mass.

Plant mineral analysis

After washing the roots and shoots with deionized water, the leaves and roots of each seedling were dried at 60 °C and ground to pass a 20-mesh sieve. Samples of ~100 mg of leaves and roots were digested in a mixture containing 4 ml of 30% H_2O_2 and 5 ml of 70% HNO_3 in 75 ml glass digestion tubes placed in a block digester for 6 h at 135 °C (Jones and Case 1990). After digestion, the digests were brought to a final volume of 75 ml with deionized water. These digested solutions were filtered though No. 44 Whatman filter paper. Concentrations of K, Ca, Mg and Al were measured using a Varian 220FS atomic absorption spectrophotometer (Varian, Inc., Mulgrave, Vic., Australia). Pi concentrations of leaf and root digests were measured spectrophotometrically (Taussky and Shorr 1953).

Statistical analyses

The experiment was a completely randomized blocked, two-way factorial design (6 blocks, 5 Al concentrations × 2 different poplar species) with 10 replicates ($n = 10$, $N = 100$). Blocks accounted for potential environmental gradients within the greenhouse. Initial plant height and leaf number were used as covariates for biomass analyses. Data were log-transformed wherever necessary to achieve homogeneity of variance. The effect of Al concentrations and species on the plant height, leaf number, leaf area and shoot and root biomass were analyzed using a blocked two-way analysis of covariance, with initial growth variables serving as covariates. Effect of Al treatment on rhizosphere TOC and OA concentrations, root tip OA concentrations and plant mineral content were analyzed using one-way analysis of variance (ANOVA) separately by species, followed by Tukey–Kramer's HSD test (significance level of $P < 0.05$) to identify significant differences among treatment means. Statistical analyses were carried out using SAS JMP 7.0 (SAS Institute, Cary, NC).

Results

Growth measurements

The Al sensitivities of quaking aspen and cottonwood were evident in their responses to Al in solution. After 40 days of exposure to $AlCl_3$, the younger leaves and mature leaves had intense anthocyanin pigmentation and mature leaves showed necrosis and chlorosis on leaf margins (data not presented). Roots in the two highest Al treatments (200 and 500 µM) also exhibited classic Al toxicity symptoms (Hirano et al. 2007) and were brown and stunted with numerous thick and short lateral roots (data not presented).

Few significant interactions between species and Al treatment were observed for different growth parameters

Table 1. Results from two-way ANOVA on growth measurements of *P. tremuloides* and *P. trichocarpa* plants grown at 0, 50, 100, 200 and 500 µM Al.

Measurement	Species		Al		Species × Al interaction	
	F	*P*	*F*	*P*	*F*	*P*
Plant height (cm)	6.11	*	16.8	***	0.58	ns
Leaf area (cm^2)	83.26	***	18.2	***	4.75	*
Number of leaves	5.61	*	240.8	***	1.20	ns
Root length (cm)	6.03	*	241.3	***	1.20	ns
Shoot biomass (g)	0.02	ns	32.0	***	1.90	ns
Root biomass (g)	0.54	ns	10.3	***	1.40	ns

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant.
Degrees of freedom are: species 1, Al treatment 4, species × Al treatment 4.

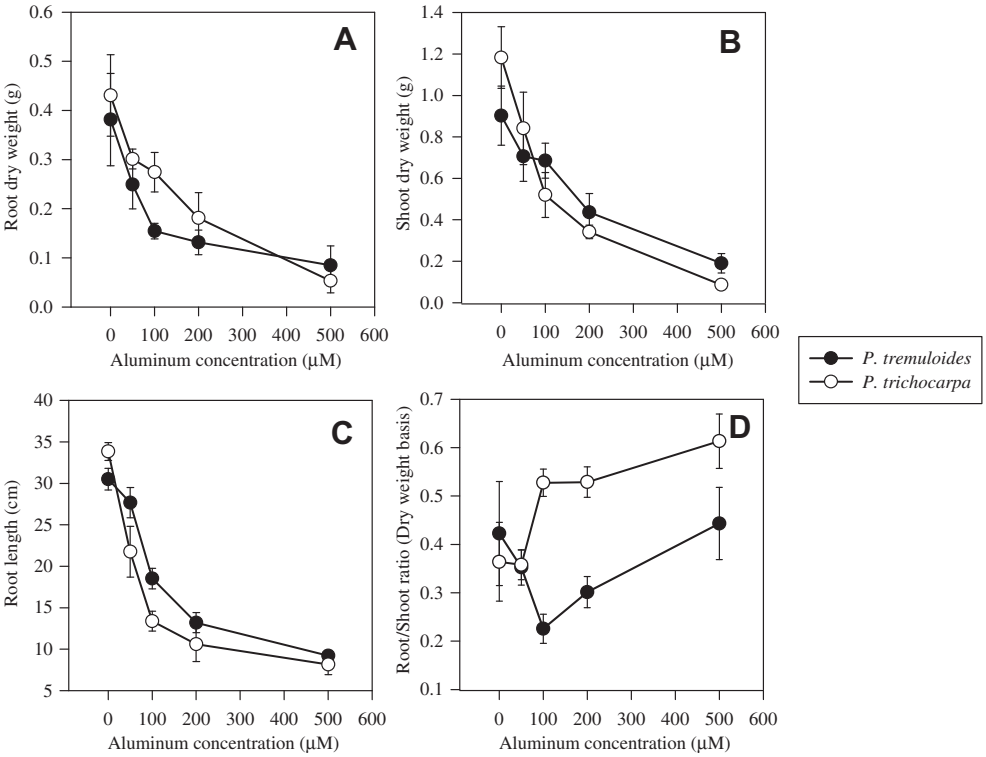

Figure 1. Growth responses (shoot dry weight, root dry weight, root length and root/shoot ratio) of *P. tremuloides* and *P. trichocarpa* plants exposed to Al. Values ($n = 8$) are means ± SD.

assessed (Table 1). Both the species showed substantial reductions in plant height, number of leaves, leaf area, length of the longest root and shoot and root biomass at the lowest Al concentration used. Reductions in growth of roots (Figure 1A and C) and shoots (Figure 1B) resulting from Al treatments were evident at concentrations as low as 50 μM Al in both poplar species. The impacts of Al were more pronounced on root than shoot growth at the lower concentrations of Al for *P. tremuloides*, for which the root/shoot ratio declined precipitously under exposure to Al (Figure 1D). In contrast, the root/shoot ratio of *P. trichocarpa* increased with exposure to Al (Figure 1D).

Exudation of C into the root zone

Exposure to Al induced substantial increases in TOC exudation rates in both species (Figure 2A). Based on measured C washout during the watering cycle, the net dissolved organic carbon produced ranged from 0.4 to 16 mg C plant^{-1} day^{-1}, with greatest production by *P. tremuloides*. The concentrations of TOC increased upon Al stress in both the poplar species. There was a significant difference between rates of C production between species with increasing Al concentration, with production by *P. tremuloides* being greater than that of *P. trichocarpa* at 500 μM Al (Figure 2A). Exposure to Al induced up to a 40-fold increase in TOC deposition in *P. tremuloides* and a 100-fold increase in *P. trichocarpa*.

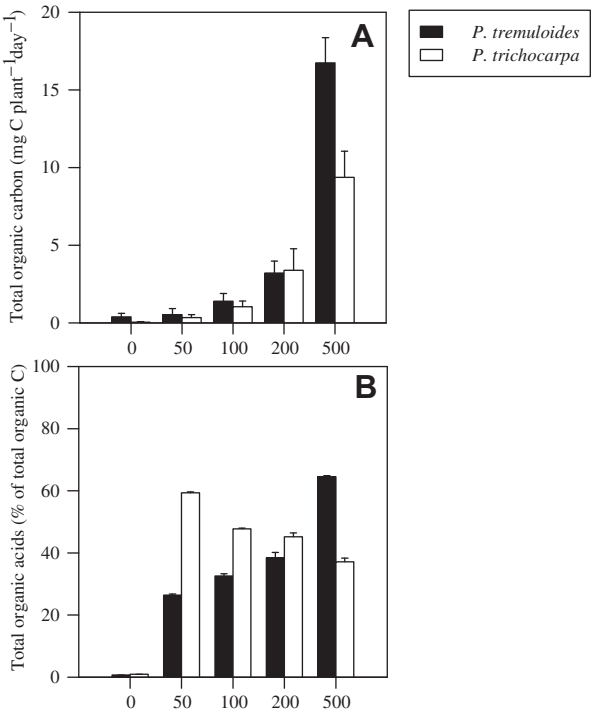

Figure 2. (A) Production of TOC by *P. tremuloides* and *P. trichocarpa* plants exposed to Al. (B) Proportion of OAs in TOC released from the roots. Values ($n = 5$) are means ± SD.

The concentrations of OAs in the rhizospheres of the two poplar species were extremely low in plants not exposed to Al (Table 2). Only lactate was detected for *P. tremuloides* and oxalate for *P. trichocarpa*. However, malate, citrate, formate, oxalate, succinate and lactate were the major organic anions released from roots in the presence of Al

Table 2. Organic acid exudate profiles in the rhizospheres of *P. tremuloides* and *P. trichocarpa* plants exposed to Al. Values ($n = 8$) are means ± SD. Means among treatments within OAs followed by different letters are significantly different at $P \leq 0.05$ (Tukey–Kramer's HSD test). (–) indicates OA concentrations were lower than 0.1 and not significant at $P \leq 0.05$.

Species	Al treatment (μM)	Rhizospheric OA concentration (μM g^{-1} total root FW)					
		Malate	Citrate	Formate	Oxalate	Succinate	Lactate
P. tremuloides	0	–	–	–	–	–	0.1 ± 0.1
	50	0.4 ± 0.1^{b}	0.6 ± 0.1^{b}	1.0 ± 0.1^{b}	0.3 ± 0.1^{c}	0.3 ± 0.1^{b}	0.3 ± 0.1^{b}
	100	1.2 ± 0.1^{b}	2.2 ± 0.1^{b}	0.4 ± 0.1^{c}	0.7 ± 0.1^{c}	0.8 ± 0.1^{b}	0.1 ± 0.1^{b}
	200	4.5 ± 0.3^{b}	7.8 ± 0.6^{b}	3.6 ± 0.2^{b}	2.6 ± 0.2^{bc}	3.8 ± 0.3^{b}	0.8 ± 0.1^{b}
	500	63.0 ± 3.2^{a}	60.1 ± 3.2^{a}	21.7 ± 1.2^{a}	15.7 ± 0.9^{a}	29.3 ± 1.4^{a}	4.7 ± 0.2^{a}
P. trichocarpa	0	–	–	–	0.6 ± 0.01^{b}	–	–
	50	0.2 ± 0.1^{c}	0.2 ± 0.1^{c}	1.0 ± 0.1^{a}	0.2 ± 0.1^{c}	0.1 ± 0.1^{c}	–
	100	0.6 ± 0.1^{c}	0.8 ± 0.1^{c}	0.9 ± 0.1^{b}	0.6 ± 0.1^{c}	0.2 ± 0.1^{c}	0.2 ± 0.1^{b}
	200	3.5 ± 0.3^{b}	4.7 ± 0.1^{b}	0.2 ± 0.1^{b}	5.5 ± 0.4^{b}	2.5 ± 0.2^{b}	17.7 ± 0.9^{a}
	500	18.2 ± 0.9^{a}	51.8 ± 2.6^{a}	1.1 ± 0.1^{a}	53.9 ± 2.7^{a}	28.3 ± 1.4^{c}	2.4 ± 0.2^{b}

Table 3. Production of OA exudates in the rhizospheres of *P. tremuloides* and *P. trichocarpa* plants exposed to Al. Values ($n = 8$) are means ± SD. Means among treatments within OAs followed by different letters are significantly different at $P \leq 0.05$ (Tukey–Kramer's HSD test). (–) indicates OA concentrations were lower than 0.1 and not significant at $P \leq 0.05$.

Species	Al treatment (μM)	Rhizosphere OA production (μmol $seedling^{-1}$ pot field $capacity^{-1}$)					
		Malate	Citrate	Formate	Oxalate	Succinate	Lactate
P. tremuloides	0		–	–	–	–	2 ± 1^{b}
	50	47 ± 21^{c}	60 ± 12^{c}	42 ± 5^{b}	2 ± 1^{b}	11 ± 4^{c}	19 ± 3^{b}
	100	95 ± 43^{c}	121 ± 24^{b}	215 ± 5^{a}	5 ± 2^{b}	22 ± 9^{c}	10 ± 1^{b}
	200	332 ± 1^{b}	287 ± 44^{b}	85 ± 11^{b}	11 ± 1^{a}	146 ± 3^{b}	200 ± 3^{a}
	500	615 ± 1^{a}	594 ± 5^{a}	216 ± 3^{a}	14 ± 3^{a}	322 ± 9^{a}	147 ± 5^{a}
P. trichocarpa	0	–	–	–	2 ± 1^{b}	–	–
	50	83 ± 4^{c}	91 ± 9^{c}	55 ± 1^{c}	136 ± 5^{c}	22 ± 5^{b}	–
	100	143 ± 9^{b}	166 ± 5^{b}	238 ± 2^{b}	305 ± 3^{b}	37 ± 3^{b}	54 ± 4^{c}
	200	235 ± 3^{a}	318 ± 4^{b}	115 ± 2^{b}	517 ± 18^{a}	51 ± 1^{ab}	235 ± 9^{a}
	500	281 ± 9^{a}	738 ± 2^{a}	349 ± 2^{a}	698 ± 19^{a}	54 ± 10^{a}	143 ± 19^{b}

(Table 2). Malate and citrate concentrations (μM g^{-1} FW) exhibited the greatest Al-induced response in *P. tremuloides*, whereas citrate and oxalate were the major OAs produced by *P. trichocarpa* when exposed to Al (Table 2). The exudation of succinate increased to a lesser extent for both poplar species. There were no clear trends for the exudation of lactate and formate in the presence of Al in either species.

Organic acid production was also calculated on a per plant basis (μmol $plant^{-1}$) in terms of field capacity of pots at the end of the experiment. This approach avoided the influence of changes in root mass under exposure to Al on OA production. For both poplar species, Al clearly induced the production of a variety of OAs (Table 3), even though root mass declined with Al exposure. In *P. tremuloides*, malate and citrate were again found to be dominant OAs in the root zone and these increased substantially under exposure to Al (Table 3). Aluminum exposure also stimulated the production of formate, oxalate, succinate and lactate. In *P. trichocarpa*, formate, citrate and oxalate were dominant in the rhizosphere and these, along with malate and succinate, were stimulated by exposure to Al (Table 3).

The proportion of TOC comprised by total OAs indicated divergent responses between the two poplar species in response to Al (Figure 2B). All OAs identified and quantified accounted for 20–64% of total C released upon Al exposure (Figure 2B). In *P. tremuloides*, the proportion increased with increasing concentration of Al, whereas in *P. trichocarpa*, the proportion of TOC exudation as OAs was high under low Al exposures and declined with increasing Al concentrations (Figure 2B). In the case of *P. trichocarpa*, the exudation of other organic C compounds under Al exposure must contribute to the root exudation C budget.

OA concentrations in root tips

Exposure of two different poplar species to Al resulted in the increased production and accumulation of certain OAs in root tips of seedlings (Figure 3). Among the OAs measured, citrate, succinate, malate and oxalate exhibited significant increases in concentration in root tips over the 50–500 μM Al treatment range (Figure 3A and B). The OA with the greatest response in root tips of *P. tremuloides* was malate, which increased 17-fold in response to Al to 390 μmol g^{-1}

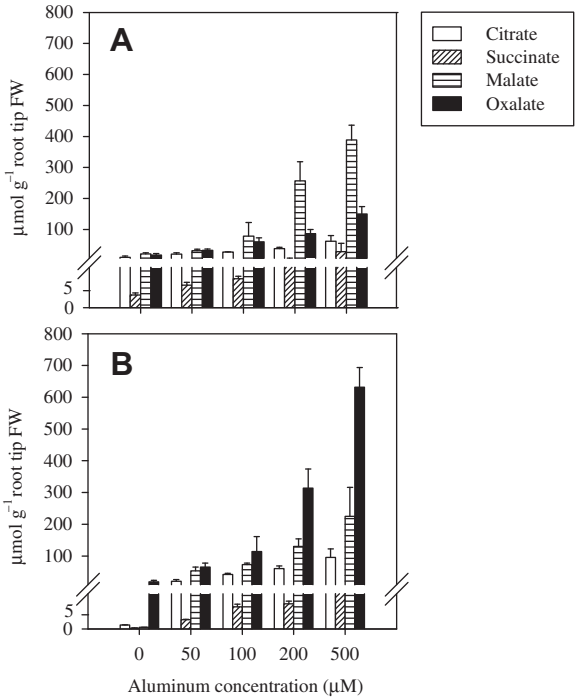

Figure 3. Concentrations of dominant OAs (μmol g^{-1} FW) in root tips (1–1.5 cm) of (A) *P. tremuloides* and (B) *P. trichocarpa* plants exposed to Al. Values (n = 8) are means ± SD.

FW. In contrast, oxalate exhibited an eightfold increase to 650 μmol g^{-1} FW in *P. trichocarpa* under exposure to Al (Figure 3B). The concentration of citrate in roots of *P. tremuloides* increased slightly over the range of Al exposures, whereas Al induced the accumulation of citrate in *P. trichocarpa* (Figure 3A and B). Although stimulated by Al, succinate was found to be the least abundant OA in both the species. We observed various other OAs (aconitate, mevalonate, shikimate, fumarate and malonate) in the absence and presence of Al, but these were found at extremely low concentrations and lacked clear trends in response to treatments (data not presented).

Antioxidant enzyme activity

Exposure to Al generally enhanced the activities of CAT, APX and, to a lesser extent, GDP in both roots and leaves and the activity in root tissues generally increased to greater extent as compared to leaf tissues (Figure 4). Catalase activity was elevated in response to Al in both root and leaf tissues in both the poplar species, with changes occurring under exposure to 50 μM Al in *P. tremuloides* and 100 μM Al in *P. trichocarpa* (Figure 4A and D). Ascorbate peroxidase activity was enhanced in roots at and above 200 μM Al in both species, with little associated change in the activity in leaves (Figure 4B and E). The observed fivefold higher activity in root tissues indicates that APX may be a tissue-dependent oxidative stress response in poplar. Induction of GDP activity was observed at and above 200 μM Al in both roots and leaves of *P. tremuloides*, whereas no consistent pattern of response was evident in *P. trichocarpa*, indicating that the GDP-induced oxidative stress response was species specific (Figure 4C and F).

Plant mineral composition

The Al, Ca, Mg, K and P concentrations (mg g^{-1} DW) in the roots and leaves of *P. tremuloides* and *P. trichocarpa* are presented in Figures 5 and 6, respectively. The concentration of Al significantly increased under exposure to Al in roots and leaves of these poplar species ($P < 0.001$ for both tissues for the Al effect) and the Al concentrations of roots were substantially higher than leaf concentrations (Figures 5A and 6A). Concentrations of Al in roots of *P. tremuloides* increased steadily with Al exposure concentration (Figure 5A), whereas root Al remained low under 0 and 50 μM Al treatments and drastically increased to a plateau at and above 100 μM Al in *P. trichocarpa* (Figure 6A). As with root Al concentrations, the two species exhibited different patterns of translocation of Al from roots to leaves, with *P. tremuloides* increasing leaf Al with exposure and *P. trichocarpa* restricting Al translocation under Al exposures up through 100 μM Al (Figures 5A and 6A). After 40 days of Al treatment, the ratio of Al in the leaves to Al in the roots ranged from 1:10 at 50 μM Al to 1:7 at 500 μM Al in *P. tremuloides*, whereas the ratio in *P. trichocarpa* ranged from 1:13 at 50 μM Al to 1:9 at 500 μM Al.

In *P. tremuloides* exposed to lower Al concentrations (50 and 100 μM Al), there were no significant changes in Ca and Mg content in leaves and roots; Al treatment above 100 μM reduced root and leaf Ca and Mg concentrations (Figure 5B and C). In contrast, Ca and Mg concentrations in the roots and leaves of *P. trichocarpa* declined with increasing supply of Al above 50 μM (Figure 6B and C). In *P. tremuloides*, leaf/root Ca concentration ratio was 1.89 at 50 μM Al and 1.20 at 500 μM Al, indicating that Ca translocation became increasingly inhibited as Al concentration increased in the root zone. In contrast, leaf Ca concentration ratio was 1.42 at 50 μM Al and 2.50 at 500 μM Al in *P. trichocarpa*, indicating that root uptake was affected to a greater extent than translocation in this species. Taken with the tissue concentration data, these patterns indicate that Al differently affected Ca uptake and translocation in *P. tremuloides* and *P. trichocarpa*. Similar patterns were evident for Mg (Figures 5C and 6C).

Significant increases in root and leaf K concentrations were observed with increasing Al treatment over the range of 50–200 μM Al in both poplar species ($P < 0.001$), although concentrations in plants exposed to 500 μM Al tended to decline from concentrations in other Al treatments (Figures 5D and 6D).

As compared to other nutrient responses, reductions in root and leaf P concentrations were more pronounced and occurred at the lowest Al treatment level, 50 μM, in both poplar species (Figures 5E and 6E). The concentrations of P in roots and leaves of both species declined across the full

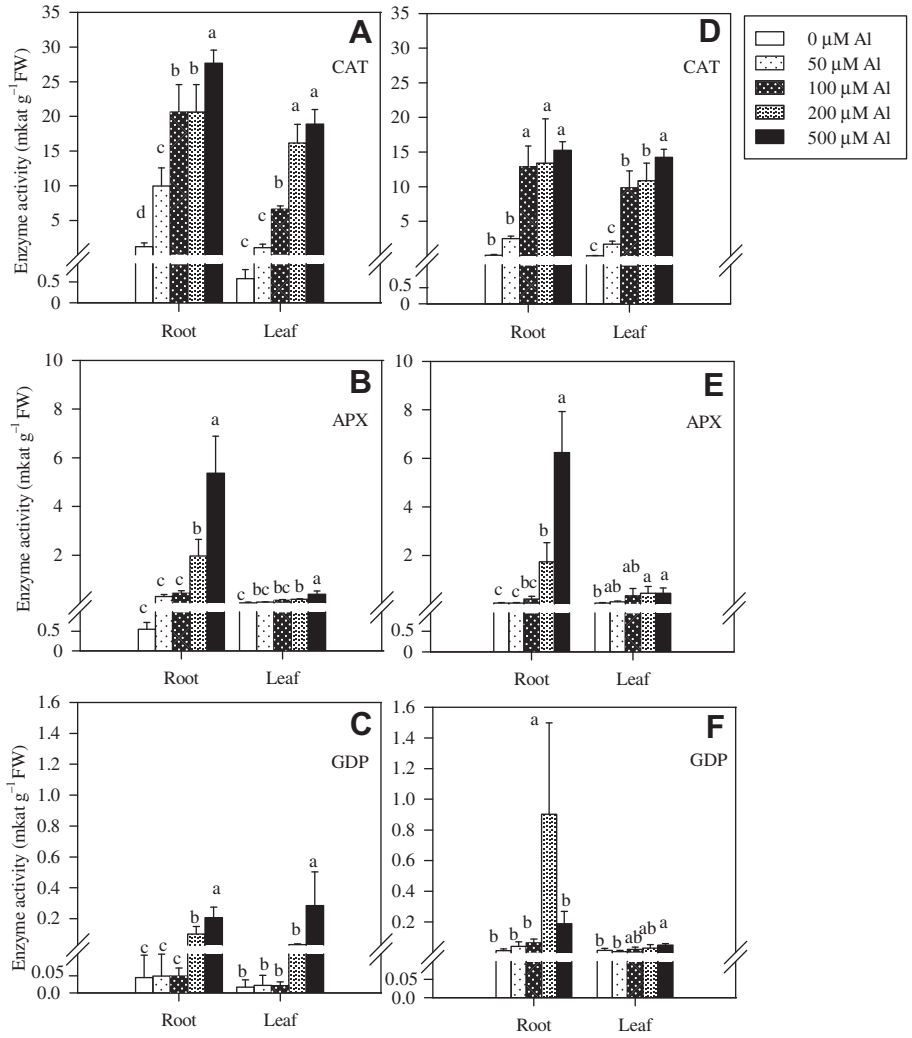

Figure 4. Changes in total enzyme activity (mkat g^{-1} FW) of CAT, APX and GDP in root and leaf tissues of *P. tremuloides* (A, B and C) and *P. trichocarpa* (D, E and F) exposed to Al. Values ($n = 5$) are means ± SD. Different letters within a treatment are significantly different at $P \leq 0.05$ (Tukey–Kramer's HSD test).

range of Al exposures, with reductions in root and foliar P of up to 62 and 73% in *P. tremuloides* and 60 and 70% in *P. trichocarpa*, respectively (Figures 5E and 6E). Across Al treatments, the leaf/root Pi ratio was 2.13 in *P. tremuloides* whereas the ratio was 1.13 in *P. trichocarpa*, indicating higher P translocation efficiency in *P. tremuloides*.

Plant growth responses were influenced by the interactions between Al and cation accumulation in roots. For both poplar species, root length, as measured by the length of the longest root, was highly correlated with the root Al/Ca and the Al/base cation (BC = Ca + Mg + K) ratio (Figure 7). As Al accumulated in roots at the expense of Ca, Mg with or without K, root elongation declined precipitously in poplar seedlings of both species. These associations were stronger for *P. tremuloides* (Figure 7A and B) than for *P. trichocarpa* (Figure 7C and D). The correlations between root or shoot mass and the Ca/Al ratio in *P. tremuloides* ($R^2 = 0.313$ and 0.486 for root and shoot mass, respectively) and *P. trichocarpa* ($R^2 = 0.293$ and 0.258) were weaker with these ionic ratios than those with root length. Taken with patterns of base cation accumulation (Figures 5 and 6), this suggests that root architecture is more sensitive than root or shoot growth to Al.

Discussion

Elevated Al in the environment has numerous effects on the growth and physiology of forest trees, all of which have implications for ecosystem C storage. Aluminum causes a reduction in the number and length of lateral roots and reductions in C allocated to root biomass in various temperate and tropical tree species (Cumming and Weinstein 1990, Godbold and Jentschke 1998, Lux and Cumming 1999, Nguyen et al. 2003, Silva et al. 2004, Hirano et al. 2007). Aluminum-induced changes in tree physiology include alterations in nutrient acquisition and assimilation, increased oxidative stress and changes in photosynthetic gas exchange (Cumming 1996, Silva et al. 2004, Tahara et al. 2005) that will limit C capture and resulting C allocation to ecosystem soil components (roots, exudates, fungal hyphae and bacteria).

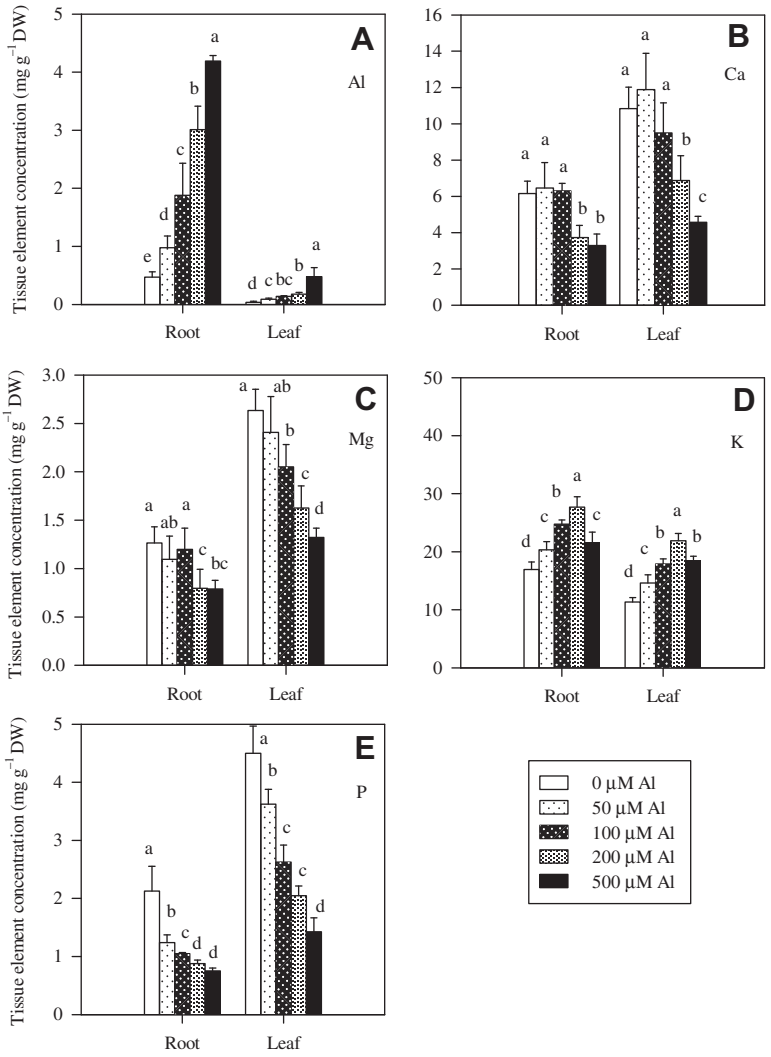

Figure 5. Concentrations of Al, Ca, Mg, K and P in roots and leaves of *P. tremuloides* exposed to Al. Values ($n = 8$) are means ± SD. Different letters within a treatment are significantly different at $P \leq 0.05$ (Tukey–Kramer's HSD test).

In the present study, growth reductions in two poplar species were found at concentrations as low as 50 µM Al (Figure 1), indicating these two species are sensitive to Al in the environment. This finding is supported by the earlier work of McCormick and Steiner (1978) and Lu and Sucoff (2003) that also indicated that poplar is an Al-sensitive tree species according to Schaedle's classification system (Schaedle et al. 1989). Although root morphology and biomass have been considered the most sensitive parameters for Al stress response in tree species (Hirano and Hijii 1998), we also observed significant differences in other growth parameters, such as plant height and leaf area, in the two poplar species at these low Al concentrations (Table 1). The sensitivity of these growth parameters would be important in limiting C capture by poplar used in biological C sequestration management activities and points to the need to identify species or genotypes for reclamation of disturbed sites, such as abandoned coalmines, where soluble Al may limit plant growth. Surprisingly, *P. tremuloides*, a species often found colonizing disturbed and stressful sites in eastern North America (Cumming, personal observation), was sensitive to Al at concentrations as low as 50 µM. This finding suggests that a range of Al sensitivity may exist in this species/genus (Steiner et al. 1984, Casselman et al. 2006) that could be investigated for reclamation and C sequestration purposes.

One of the most important mechanisms of Al resistance in plants functions through the exclusion of Al from roots that occurs with the exudation of carboxylic acids from root tips (see Kochian et al. 2005). Similar systems may function in tree species (Watanabe and Osaki 2002, Nguyen et al. 2003, Silva et al. 2004, Qin et al. 2007). Aliphatic di- and tri-carboxylic acids, such as malate, citrate and oxalate, form complexes with Al^{3+} ions that are less toxic than free Al^{3+} ions. When poplar seedlings were treated with Al in the present study, there was a distinct induction of exudation of a suite of OAs (Table 2). In *P. tremuloides*, the concentrations of OAs produced (on a root mass basis) differs in the order of malate > citrate > succinate > formate > oxalate > lactate, whereas in *P. trichocarpa*, the concentrations of OA followed the order of oxalate > citrate > succinate > malate > lactate > formate (Table 2). On a per

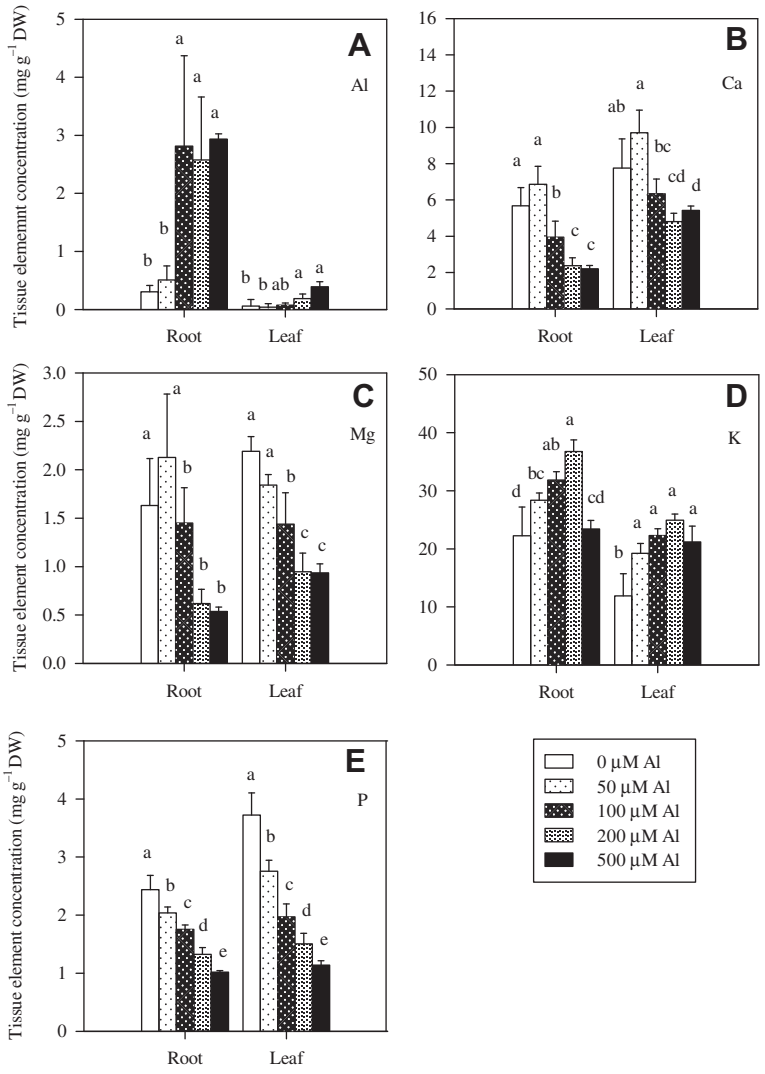

Figure 6. Concentrations of Al, Ca, Mg, K and P in roots and leaves of *P. trichocarpa* exposed to Al. Values ($n = 8$) are means ± SD. Different letters within a treatment are significantly different at $P \leq 0.05$ (Tukey–Kramer's HSD test).

plant or pot basis, citrate is also noted as being significant in the rhizosphere of both poplar species (Table 3). In spite of these increases in OA exudation, roots of both poplar species were sensitive to Al and accumulated Al to high concentrations in their root tissues (Figures 5 and 6).

A comparison of profiles of OA exudates in the rhizosphere of seedlings with the profiles present in root tissues revealed similarities and differences (Table 2; Figure 3). Consistent with patterns of exudation, we found a dose-dependent accumulation of malate in roots of *P. tremuloides* and oxalate in *P. trichocarpa*, respectively. Although citrate and succinate accumulation in root tips also exhibited dose-dependent responses, their concentrations in roots were significantly lower as compared to malate and oxalate. The accumulation of OAs in root tips may reflect an Al resistance mechanism and the formation of Al–OA complexes in roots may limit the translocation of Al to leaves. Based on the observed sequestration of Al in roots (Figures 5 and 6) and accumulation of malate or oxalate in roots (Figure 3), we hypothesize that both poplar species chelate Al internally by producing up to 30-fold greater quantities of total OAs, dominated by malate in *P. tremuloides* and oxalate in *P. trichocarpa*.

The rhizospheric C flux of plants, especially of forest trees, is complex and governed by many edaphic factors (Nguyen 2003, Jones et al. 2004). Best estimates indicate that plant root exudation constitutes about 1–5% of C fixed in photosynthesis and includes a vast array of compounds, such as OAs, carbohydrates, phenolic compounds and amino acids/proteins (Nguyen 2003, Jones et al. 2004). However, under a range of edaphic stresses (e.g., nutrient deficiency, metal stress, drought), the rate of exudation increases significantly, not only as a result of a failure of cell membrane integrity with or without a breakdown in normal cell metabolism (Jones et al. 2004), but also as an acclimation response where the rate of exudation increases to alleviate the stress. In the present study, we observed an extremely pronounced induction of organic C flow into rhizosphere. Our calculated C flux of poplar species (based on TOC analysis and water flux) ranged from 0.4 to 16 mg C

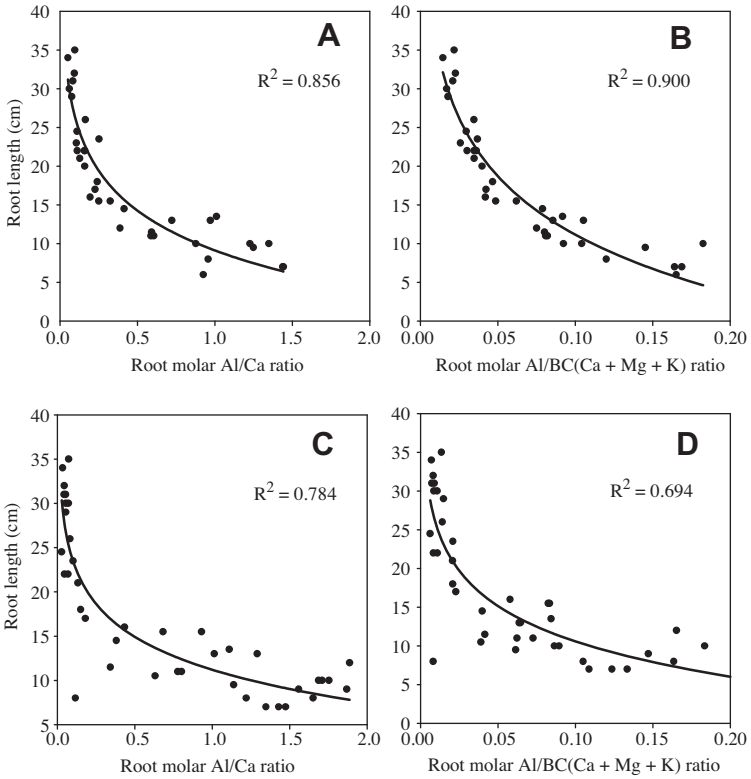

Figure 7. Relations between root length and root molar Ca/Al ratio (A and C) and root molar Al/BC (Ca + Mg + K) ratio (B and D) in roots of *P. tremuloides* (A and B) and *P. trichocarpa* (C and D) plants exposed to Al.

plant^{-1} day^{-1}, with the highest rates of flux occurring in seedlings exposed to Al (Figure 2A). These rates of rhizodeposition are 4- to 12-fold greater than the rates reported for other tree species by Klugh and Cumming (2007) and Uselman et al. (2000).

Studies on exudation response to Al typically measure low molecular weight OAs (e.g., Nguyen et al. 2003, Klugh and Cumming 2007). While such measurements may be useful in understanding the chelation potential of exudates produced by tree roots exposed to Al, they do not contextualize OA exudation in relation to total C flux. In the present study, both OA–C and TOC were induced by exposure to Al. The allocation of C to OAs exhibited species-specific responses to Al, with the proportion increasing in *P. tremuloides* and decreasing following a large allocation under low Al concentrations (Figure 2B). The OAs identified and quantified accounted for between 20% and 64% of total C released upon Al exposure (Figure 2B). The remainder of the unidentified C compounds may consist of carbohydrates or other high molecular weight secondary metabolites, which need further identification and characterization. The high exudation of C upon Al exposure in both poplar species may contribute to a negative influence on growth rate, as C is shunted from growth to stress avoidance metabolism without compensatory shifts in C fixation (Cumming et al. 1992).

The production of ROS in plants is a natural phenomenon. However, oxidative stress in plants represents a breakdown of homeostasis leading to the over-production of ROS. Aluminum-induced oxidative stress has been investigated at the enzyme and gene expression levels and positive correlations between ROS generation and Al stress have been reported in several crop species (Kochian et al. 2005). Limited evidence exists for Al-induced ROS generation in forest trees. In the present study, concentration-dependent relations were observed for antioxidant enzyme activities in roots and leaves of both poplar species (Figure 4). Exposure of poplar seedlings to Al led to the stimulation of CAT and APX at Al concentrations as low as 50 and 200 μM Al, respectively. GDP exhibited lesser activity and did not respond to the extent of the other two systems. While the stimulation in roots may be explained by the local impacts of Al on cell structure and function, the stimulation of antioxidant enzymes in leaf tissue suggests that the observed changes in plant nutrition alter leaf cell integrity or there may be some mechanism that transmits a stress signal from roots to leaves under Al exposure in the root or both. The increase in CAT activity in leaves of poplar due to rhizosphere Al stress is consistent with responses observed in Hinoki cypress (Ogawa et al. 2000*a*, 2000*b*) and in Norway spruce (Nagy et al. 2004).

Disturbances in nutrient uptake caused by Al are commonly interpreted as resulting from the antagonistic effects of Al on cation uptake and acquisition coupled with reductions in root growth that limit soil exploitation (Cronan 1991, Godbold and Jentschke 1998, Lu and Sucoff 2001, Heim et al. 2003). In the present study, the roots of *P. tremuloides* and *P. trichocarpa* exhibited reduced concentrations of Ca and Mg and increased concentrations of Al in root and leaf tissues that became more pronounced as Al exposure increased (Figures 5 and 6). These results are in

agreement with the patterns observed in Al experiments of North American species, European species and Japanese tree species (Hirano et al. 2007). Exposure to Al inhibits the uptake and homeostasis of Ca and Mg, which may be primary lesions involved in Al toxicity to plants (see Kochian et al. 2005). Aluminum may inhibit Ca uptake by blocking Ca^{2+} channels in the plasma membrane and inhibit Mg uptake by blocking binding sites of transport proteins (Kochian et al. 2005). In the present study, Al did not perturb Ca or Mg tissue concentrations until Al treatment concentrations above those inducing reductions in growth (Figures 1, 5 and 6) and, while root Ca and Mg concentrations were sensitive and good predictors of root elongation (Figure 7), foliar Ca and Mg concentrations were not strong predictors of root or shoot growth. This suggests that base cation limitation may not be a limiting factor for growth of poplar under Al exposure. Other than these two major ions influenced by Al stress, foliar and root K concentration increased with increasing Al concentrations (Figures 5 and 6), which we attribute to growth concentration effects – reductions in growth led to increased tissue concentrations when K uptake was not altered. A similar trend was noticed in *Pinus densifolia* Sieb. et Zucc. (Ofei-Manu et al. 2001), but our results are also in contrast with other reports in which K concentrations decreased upon Al exposure (Hirano et al. 2003).

Both root and foliar P concentrations declined in *P. tremuloides* and *P. trichocarpa* upon Al treatment, even at the lowest treatment level of 50 μM (Figures 5 and 6). The possible mechanism could be the inhibition of Pi uptake at the root cell level, with concomitant limitations to translocation to the foliage. This has been observed in many crop plants (see Kochian et al. 2005), but, in forest trees, inconsistent results have been reported. Lux and Cumming (2001) and Klugh and Cumming (2007) noted that Al limited the uptake and translocation of P in *Liriodendron tulipifera*. Other patterns have been observed in which root P increases due to co-precipitation of Pi and Al in the root apoplast (Cumming et al. 1986, Nguyen et al. 2003) or where Al has no effect on plant P status (Hirano et al. 2000, 2003). Clearly, the impacts of Al on tree seedling P nutrition appear to be species specific (see also Schaedle et al. 1989).

Changes in tissue nutrient concentrations and elemental ratios may be good indicators for assessing nutritional consequences of Al exposure. Plant Ca and P and the Al/Ca ratio may be important in discerning Al-induced variation between different species of poplar for unraveling different poplar species/hybrids as Al tolerant and sensitive in future studies and reclamation efforts. While the two species assessed were both sensitive to Al in growth (Figure 1), *P. tremuloides* exhibited a greater partitioning of Ca and P to shoots versus roots in comparison to *P. trichocarpa* and, at the same time, limited the translocation of Al to leaf tissue, at least at intermediate Al exposures (Figures 5 and 6). The maintenance of nutrient transfer to foliage and the limitation of metal translocation may be important characteristics in selecting genotypes for C sequestration strategies on naturally acidic soils or sites damaged by anthropogenic activity.

The toxicity of Al in the soil environment has implications for the capture and storage of C by native and managed forested ecosystems. Species in the genus *Populus* are being investigated in relation to biological C sequestration and biomass production for energy generation. The present work indicates that *P. tremuloides* and *P. trichocarpa* are both sensitive to Al in the soil environment, although different patterns of physiological response suggest the genetic resources to acclimate to acid soil stress may vary substantially within the genus. While baseline levels of root exudation were extremely low, exposure to Al induced the exudation of high concentrations of TOC, which included a suite of OAs dominated by malate in *P. tremuloides* and oxalate in *P. trichocarpa*. As the concentration of Al in the environment increased, root exudation increased and the accumulation of malate and oxalate in the root tips of *P. tremuloides* and *P. trichocarpa*, respectively, became more pronounced. The reductions in growth observed for both poplar species exposed to Al may reflect the diversion of C from growth to rhizospheric and internal pools as possible mechanisms of stress resistance. If plants have the capacity to increase photosynthesis in response, then such compensatory responses would offset reductions in growth (Cumming et al. 1992). However, in the present study, the diversion of C to OA exudation and accumulation in roots did not confer resistance to Al that could be detected. Such changes in C reallocation may underlie Al resistance strategies in woody species, but, in *Populus*, the elucidation of such a phenomena may be species-specific or genotype-specific (Steiner et al. 1984), which needs further investigation. In spite of the exudation and accumulation of OAs in both *P. tremuloides* and *P. trichocarpa*, exposure to Al led to perturbations in Ca and P nutrition and elevated metabolic dysfunction as evidenced by the activation of antioxidant systems, notably CAT and APX, which became more pronounced as plants were placed under increasing Al exposure. The levels of C exudation to the rhizosphere measured in *P. tremuloides* and *P. trichocarpa* exposed to Al far exceed previous reports for other forest species, suggesting that published estimates of C flux to soils extrapolated from controlled studies should be re-examined when considering exudation and C sequestration by forest ecosystems.

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