Effects of calcium on seed germination, seedling growth and photosynthesis of six forest tree species under simulated acid rain

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We selected six tree species, Pinus massoniana Lamb., Cryptomeria fortunei Hooibr. ex Otto et Dietr., Cunninghamia lanceolata (Lamb.) Hook., Liquidambar formosana Hance, Pinus armandii Franch. and Castanopsis chinensis Hance, which are widely distributed as dominant species in the forest of southern China where acid deposition is becoming more and more serious in recent years. We investigated the effects and potential interactions between simulated acid rain (SiAR) and three calcium (Ca) levels on seed germination, radicle length, seedling growth, chlorophyll content, photosynthesis and Ca content in leaves of these six species. We found that the six species showed different responses to SiAR and different Ca levels. Pinus armandii and C. chinensis were very tolerant to SiAR, whereas the others were more sensitive. The results of significant SiAR × Ca interactions on different physiological parameters of the six species demonstrate that additional Ca had a dramatic rescue effect on the seed germination and seedling growth for the sensitive species under SiAR. Altogether, we conclude that the negative effects of SiAR on seed germination, seedling growth and photosynthesis of the four sensitive species could be ameliorated by Ca addition. In contrast, the physiological processes of the two tolerant species were much less affected by both SiAR and Ca treatments. This conclusion implies that the degree of forest decline caused by long-term acid deposition may be attributed not only to the sensitivity of tree species to acid deposition, but also to the Ca level in the soil.

Keywords: acid rain sensitive, acid rain tolerant, chlorophyll content, leaf calcium content, photosynthesis rate, tree species.

Introduction

Acid rain, or acid deposition, has been a serious environmental problem worldwide for several decades. Along with Europe and North America, China has become one of the regions of the world most severely polluted by acid rain (Menz and Seip 2004, SEPA 2010). According to the study of Feng (2000), Pinus massoniana Lamb. forest and Cunninghamia lanceolata (Lamb.) Hook. forest were largely destroyed by acid rain in Sichuan, China. Other reports also found severe defoliation in several forests in other areas of southern China (Larssen and Carmichael 2000).

Acid deposition can deplete soil base cations from pools, especially calcium (Ca), and limit the uptake and incorporation of Ca in trees (Watmough and Dillon 2003a, 2003b, Drouet et al. 2005). Acid deposition can also leach cations from foliage (DeHayes et al. 1999), with Ca having the greatest rate of efflux (Likens et al. 1998). When the availability of soil Ca is low, acid-induced foliar leaching may lead to the aggravation of Ca deficiencies (Juice et al. 2006). Furthermore, other Ca-mediated processes that support foliar stress responses, such as carbohydrate storage, photosynthesis, chlorophyll content and antioxidant enzyme activity, may also be influenced by soil Ca depletion (Kobe et al. 2002, Gabara et al. 2003). Meanwhile, it has been shown that local vegetation was restored with higher foliar Ca concentration and healthier growth after Ca additions were made to Ca-poor soil.

Soil Ca depletion is a more serious issue in southern China as compared with North America and Europe because the main soil type in the acid rain area in southern China is lateritic soil, which is acidic, base-poor soil and can be even further acidified when exposed to a high H+ input (Zeng et al. 2005). Liu et al. (2007) reported that nearly half of available soil Ca in the monsoon evergreen broad-leaved forest of the Dinghushan Nature Reserve in Guangdong in southern China was depleted by acid rain. In addition, the growth of P. massoniana in the field was improved by the addition of lime (Huang et al. 2006).

Ca is an essential and major plant nutrient, and a stable Ca level is required to maintain cell wall structure and membrane function. Species may also vary widely in their inherent growth requirement for Ca (Thompson et al. 1997). White and Broady (2003) have classified plant species into calcifuges, which grow preferably in acid soil with low Ca, and calcicoles, which are tolerant to calcareous soils. They point out that the Ca contents of calcifuge and calcicole plants growing in their natural habitats differ dramatically. Calcifuges generally grow well at low Ca level in the rhizosphere and hardly respond to increased soil Ca content, which may even inhibit growth. Conversely, calcicole plants with relatively low foliar Ca show restricted growth at low Ca level. Some studies demonstrate that different tree species often show various capacities of tolerance to acid rain due to their different Ca requirements, indicating that Ca availability is very important in predicting the effect of acid rain on trees (Kobe et al. 2002, Moore and Ouimet 2006, Moore et al. 2008).

However, to the best of our knowledge, neither the Ca requirements nor the acid rain tolerance among forest tree species growing in the acid rain area in southern China under different Ca levels have been clarified. This paper focuses on whether the tolerance of tree species to acid rain changes at different Ca levels, and whether the changes, if any, may lead to changes in seedling regeneration and species composition change at the community level. Six dominant tree species found in areas affected by acid rain in southern China were selected for study: P. massoniana, Cryptomeria fortunei Hoolbr. ex Otto et Dietr., C. lanceolata, Liquidambar formosana Hance, Pinus armandii Franch. and C. chinensis Hance. All of the species are widely distributed and commonly used in reforestation in southern China (Yeh et al. 1994, Feng 2000, Akiba and Nakamura 2005, Dai et al. 2009, Wu and Zhao 2010), and some of them have been seriously damaged by acid rain (Feng 2000). The objective of this study is to highlight the effects of Ca on seed germination, seedling growth and photosynthesis for these six forest tree species under the influence of acid rain. We also hypothesized that the effect of Ca may differ among the six species due to their variable tolerance to acid rain.

**Materials and methods**

**Seed germination experiment**

Seeds of woody species P. massoniana, C. fortunei, C. lanceolata, L. formosana, P. armandii and C. chinensis were purchased from Tree Seed Centre in Shuyang in Jiangsu province of China. Seeds were surface-sterilized with a 0.1% mercurial chloride solution for 10 min and washed with distilled water. Then seeds were placed in culture dishes with three layers of filter sheets and kept at a constant temperature of 25 °C in a culture chamber.

The simulated acid rain (SiAR) solution was prepared as described by Fan and Wang (2000) using a solution of 1 mmol l−1 H2SO4 and 1 mmol l−1 HNO3 in a ratio of 5:1 by chemical equivalents, similar to the general anion composition of rainfall in southern China. The pH of SiAR was adjusted to pH 3.0. We also prepared three Ca concentration solutions: 0.1 mmol l−1 for low Ca level (L), 2.0 mmol l−1 for medium Ca level (M) and 20.0 mmol l−1 for high Ca level (H).

The filter sheets in the dishes were maintained in a wet condition by spraying with SiAR or distilled water as control (CK), as described by Fan and Wang (2000) and other published documents (Porter and Sheridan 1981, Xie et al. 2009, Jin et al. 2010). Ca at three levels was also applied to dishes in both SiAR and CK treatments. Four replicates of each of the six treatments resulted in 24 dishes for each species. For the six species, a total of 144 dishes were used in our experiment. Germination was specified when the radicle emerged as described by Bradford et al. (2008). Seedlings were removed from the culture dishes in order to avoid interference with further germination. Newly germinated seeds were counted daily. The experiment was terminated after 30 days, when no more germination was observed over a period of 2 consecutive days. The length of seed radicle was measured. Germination percentage was calculated as total number of germinated seeds divided by total number of seeds.

**Seedling growth experiment**

**Soil pretreatment**

Three soil columns (diameter = 25 cm, length = 100 cm) were filled with lateritic soil, the typical soil type in the acid rain area in southern China. In our experiment, the treatment procedure was as follows. First, the soil was leached by SiAR for 6 h. The pH of SiAR was adjusted to pH 3.0. We also prepared three Ca concentration solutions: 0.1 mmol l−1 for low Ca level (L), 2.0 mmol l−1 for medium Ca level (M) and 20.0 mmol l−1 for high Ca level (H).

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**Soil pretreatment**

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A solution containing 1 mmol l$^{-1}$ NH$_4$OAc was used to extract soil exchangeable cations. Then the cation concentrations were measured by ICP-MS (PerkinElmer Inc., Elan DRC-e, Waltham, MA, USA). The results are displayed in Table 1. After the leaching and recovery treatment, the final actual exchangeable Ca content in the soil treated with different Ca levels are shown. Soil pH is measured by an Orion pH meter using redistilled water. Ammonium acetate is used to determine soil exchangeable cations. Values are means ± SE (standard error).

<table>
<thead>
<tr>
<th>Ca level</th>
<th>Soil pH</th>
<th>Exchangeable cation content in soil (mmol kg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>5.01 ± 0.15</td>
<td>Ca$^{2+}$ 1.852 ± 0.253, Mg$^{2+}$ 2.300 ± 0.672, Mn$^{2+}$ 0.442 ± 0.018, K$^+$ 0.817 ± 0.216, Na$^+$ 1.768 ± 0.524, Al$^{3+}$ 1.876 ± 0.621</td>
</tr>
<tr>
<td>M</td>
<td>4.82 ± 0.08</td>
<td>Ca$^{2+}$ 19.655 ± 4.189, Mg$^{2+}$ 2.291 ± 0.154, Mn$^{2+}$ 0.450 ± 0.067, K$^+$ 0.829 ± 0.179, Na$^+$ 1.791 ± 0.415, Al$^{3+}$ 1.892 ± 0.384</td>
</tr>
<tr>
<td>H</td>
<td>4.68 ± 0.11</td>
<td>Ca$^{2+}$ 107.080 ± 8.847, Mg$^{2+}$ 3.254 ± 1.131, Mn$^{2+}$ 0.518 ± 0.039, K$^+$ 1.052 ± 0.339, Na$^+$ 2.083 ± 0.327, Al$^{3+}$ 2.036 ± 0.293</td>
</tr>
</tbody>
</table>

The value of pH and content of exchangeable cations in the soil treated with different Ca levels are shown. Soil pH is measured by an Orion pH meter using redistilled water. Ammonium acetate is used to determine soil exchangeable cations. Values are means ± SE (standard error).

### Plant culture, sampling and analysis

For each species, 6-month-old healthy and uniformly sized seedlings were selected and transplanted into plastic pots with a diameter of 30 cm and a height of 20 cm for 2 weeks of recovery. Each pot was filled with ~6 kg of the soil containing high (H), medium (M) or low (L) Ca, which was pretreated as described above. For each parameter, we set up four replicates, and a total of 144 plastic pots were used.

The seedlings were sprayed once each day either with a SiAR solution (pH 3.0) for the SiAR treatment or with distilled water for the CK treatment, following Fan and Wang (2000) and other published studies (Porter and Sheridan 1981, Xie et al. 2009, Jin et al. 2010). After 2 months of treatment, the seedlings were prepared for further experiments. Seedling performance was assessed in terms of height and biomass separated into aerial and root portions. The height of the aerial part was measured as the distance from the soil surface to the shoot apex, and the root was measured from the soil surface to the root apex. Plant materials were oven-dried (70 °C) to constant weight (Du and Huang 2008).

Leaves were photographed with a digital camera, and necrotic spot and total leaf areas were quantified using Adobe Photoshop 7.0 (Adobe Systems, Inc., San Jose, CA, USA). The visible necrosis percentage was expressed as the ratio of necrotic area to total leaf area as described previously (Xiao et al. 2005).

Chlorophyll was extracted with ice-cold 80% acetone. Absorption of the extract was measured at 663 and 645 nm with a spectrometer (Varian Cary 50 UV-VIS) and the concentration of chlorophyll was calculated with formulae proposed by Amon (1949).

Leaf net photosynthesis ($P_n$) was measured with a portable photosynthesis system (PP System CIRAS-2) under natural conditions. According to references, the saturating point of *P. massoniana* seedling is 800 µmol m$^{-2}$ s$^{-1}$ (Yang et al. 2006); *C. lanceolata*, 800 µmol m$^{-2}$ s$^{-1}$ (Xie et al. 2002); *P. armandii*, 680 µmol m$^{-2}$ s$^{-1}$ (Liang et al. 2009); *L. formosana*, 800 µmol m$^{-2}$ s$^{-1}$ (Xie et al. 2004); and *C. chinensis*, 750 µmol m$^{-2}$ s$^{-1}$ (Wang and Guo 2007). For uniformity, we used a common PAR of 800 µmol m$^{-2}$ s$^{-1}$ for all species with an internal LED light source. Three seedlings exposed to sunlight for at least 5 min prior to measurement were used in the $P_n$ measurements. For each seedling, at least three leaves were sampled. Finally, the mean value was obtained from the nine replicates.

After completion of the photosynthetic measurements, leaves were harvested for elemental analysis. Leaf tissue was dried for 72 h at 80 °C. The dried leaves were pulverized by an electric mill. Samples were ashed at 550 °C and dissolved in a 1 mmol l$^{-1}$ HNO$_3$ solution. Finally, the concentration of Ca in the leaf was measured by ICP-MS and expressed as grams per kilogram of dry weight.

### Statistical analyses

One-way analysis of variance (ANOVA) with Tukey’s honestly significant difference (HSD) was used to determine significant differences in the germination rate and radicle length of seeds due to Ca and SiAR treatments using the SPSS 11.0 statistical package (SPSS, Inc., Chicago, IL, USA). Similarly, significant differences in response of seedling leaves to SiAR and soil Ca levels were also determined using this statistical method. Two-factor ANOVA with Tukey’s HSD using the General Linear Model procedure was performed to test the significance of the interactive effects of SiAR and Ca treatments on each physiological parameter. The visible necrosis percentages were log$_{10}$ transformed before analysis because their distributions were right-skewed. Linear correlation analysis was used to investigate the relationships among all variables, including visible necrosis percentage, root–shoot ratio, chlorophyll content, photosynthetic rate and Ca content in leaves and soil, by the automatic linear fit function of Origin 7.5 (OriginLab Corporation, Hampton, MA, USA).
Results

Seed germination rate and radicle length

Our data (Table 2) show that SiAR significantly reduced the seed germination of *P. massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana* by ∼30% (*P < 0.05*) at the low Ca level. However, at the high Ca level, SiAR had less effect on germination. For example, the germination of *L. formosana* seeds in the high Ca level treatment was reduced by only 7% under SiAR. Furthermore, the inhibition of SiAR on seed germination was reversed at the high Ca level for *C. fortunei* and *C. lanceolata*. Significant SiAR × Ca treatment interactions (*P < 0.05*) were observed in three sensitive species: *P. massoniana*, *C. lanceolata* and *L. formosana* (Table 2 and Figure S1 available as Supplementary Data at *Tree Physiology* Online). However, for the species *C. fortunei*, *P. armandii* and *C. chinensis*, no interaction of SiAR × Ca on germination was found.

Similar to the results from the germination study, radicle length showed no difference between SiAR and CK under high Ca level for each species (Figure 1), indicating the moderating role of Ca under SiAR. Furthermore, the SiAR treatment had no effect on *C. chinensis* at any Ca level, suggesting its high tolerance to SiAR. Significant differences (*P < 0.05*) in radicle length shown as follows: SiAR, SiAR effect; Ca, Ca effect; and S & C, SiAR and Ca interactive effect.

Table 2. Effects of different Ca concentrations (L, M and H) on the germination rate of seeds for six forest tree species under SiAR treatment.

<table>
<thead>
<tr>
<th>SiAR treatment</th>
<th>Ca level</th>
<th>Species</th>
<th>P. massoniana</th>
<th>C. fortunei</th>
<th>C. lanceolata</th>
<th>L. formosana</th>
<th>P. armandii</th>
<th>C. chinensis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>L</td>
<td>0.765 ± 0.042Aa</td>
<td>0.622 ± 0.034Aa</td>
<td>0.506 ± 0.031Aa</td>
<td>0.934 ± 0.043Aa</td>
<td>0.536 ± 0.019Aa</td>
<td>0.650 ± 0.018Aa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.827 ± 0.061Ba</td>
<td>0.652 ± 0.016Aa</td>
<td>0.594 ± 0.014Ba</td>
<td>0.919 ± 0.021Aa</td>
<td>0.575 ± 0.031Aa</td>
<td>0.700 ± 0.020Aa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>0.910 ± 0.027Ca</td>
<td>0.683 ± 0.015Ba</td>
<td>0.611 ± 0.011Ca</td>
<td>0.859 ± 0.042Aa</td>
<td>0.556 ± 0.027Aa</td>
<td>0.650 ± 0.013Aa</td>
<td></td>
</tr>
<tr>
<td>SiAR</td>
<td>L</td>
<td>0.506 ± 0.033Ab</td>
<td>0.572 ± 0.017Ab</td>
<td>0.366 ± 0.026Ab</td>
<td>0.723 ± 0.039Ab</td>
<td>0.514 ± 0.025Ba</td>
<td>0.600 ± 0.017Aa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.749 ± 0.044Bb</td>
<td>0.619 ± 0.015Ba</td>
<td>0.523 ± 0.045Ba</td>
<td>0.776 ± 0.017Aa</td>
<td>0.511 ± 0.034Ba</td>
<td>0.625 ± 0.028Aa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>0.875 ± 0.054Ca</td>
<td>0.660 ± 0.004Ca</td>
<td>0.644 ± 0.039Ca</td>
<td>0.881 ± 0.032Ba</td>
<td>0.510 ± 0.027Aa</td>
<td>0.575 ± 0.039Aa</td>
<td></td>
</tr>
<tr>
<td>SiAR × Ca</td>
<td>P &lt; 0.05</td>
<td>ns</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>ns</td>
<td>ns</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Calcium concentrations were 0.1 mmol l⁻¹ for low Ca level (L), 2.0 mmol l⁻¹ for medium Ca level (M) and 20.0 mmol l⁻¹ for high Ca level (H). Germinated seeds were counted after 7 days.

1Different capital letters denote significant differences between means for the variable in the same SiAR treatment (CK or SiAR) at different Ca levels, and different lower case letters denote significant differences between means for the variable under CK and SiAR treatments at the same Ca level as evaluated by one-way ANOVA. The species × treatment interaction was conducted by two-way ANOVA (Tukey, *P < 0.05*). ns, not significant.

Figure 1. Effects of SiAR and Ca levels (L, M and H) on the radicle length of six forest tree species. Data were obtained by measuring the radicle length of 14-day-old seedlings. The actual Ca concentrations were the same as in Table 2. Different capital letters denote significant differences between means for the variable in the same SiAR treatment (CK or SiAR) as measured under different Ca levels, and different lower case letters denote significant differences between means for the variable under CK and SiAR treatments at the same Ca level as evaluated by one-way ANOVA (Tukey, *P < 0.05*). The species × treatment interaction was conducted by two-way ANOVA (Tukey, *P < 0.05*). The significance of the factorial analysis (ANOVA) is shown as follows: SiAR, SiAR effect; Ca, Ca effect; and S & C, SiAR and Ca interactive effect.

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**Ca effects on germination, growth and photosynthesis under simulated acid rain**

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length between SiAR- and CK-treated seedlings of *C. lanceolata* and *L. formosana* under low and medium Ca levels were found, implying the susceptibility of these two species to SiAR. In contrast to the results from the germination study, SiAR × Ca interactions were only found in *C. lanceolata* (Figure 1 and Figure S1 available as Supplementary Data at *Tree Physiology* Online), indicating that it is extremely sensitive to Ca supply under SiAR (P < 0.05).

**Plant growth**

After 2 months of SiAR treatment, we divided the whole seedlings into aerial portions and roots, and then measured the length and weight of each section. As shown in Table 3, the seedling length and weight of *P. massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana* tended to be sensitive (but not statistically significantly different) to SiAR. For these species, SiAR affected root length and weight more significantly than the aerial portion. Interestingly, high Ca level also significantly accelerated root growth more than the aerial portion; e.g., for *C. fortunei* and *L. formosana*, the root length was at least twice as long at high Ca level as at low Ca level. The aerial portion showed no significant difference for each treatment (P > 0.05). Dry weights of both the aerial portions and the roots were reduced significantly with SiAR at the low Ca level for *P. massoniana* and *C. fortunei* (P < 0.05). Consistently, SiAR led to the reduction of the shoot/root ratio at low and medium Ca levels for sensitive species. We found that SiAR did not affect the growth of shoot and root of seedlings grown at high Ca level (Table 3). For *P. armandii* and *C. chinensis*, there were no significant differences between any treatments. Further, no significant interaction of SiAR and soil Ca was observed for seedling height, root length or dry weight, except for the ratio of root to shoot for *P. massoniana* and *L. formosana* (Table 3 and Figure S1 available as Supplementary Data at *Tree Physiology* Online).

**Leaf necrosis**

Leaf necrotic area was negatively related to soil Ca content under SiAR. At low Ca level, the visible leaf necrosis percentages were 56, 22, 18 and 27% for *P. massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana*, respectively. In contrast, the average visible leaf necrosis percentage for each species at high Ca level was <10% (Figure 2). It is clear that leaf development and growth were effectively protected against SiAR treatment by the high level of Ca.

**Chlorophyll content and net photosynthetic rate**

After 2 months of SiAR and Ca treatments, the benefit of Ca in preventing the decline of chlorophyll content caused by SiAR was observed in most of the selected tree species (Table 4). Notably, chlorophyll content was higher in SiAR than in CK under high Ca level for both *C. fortunei* and *C. lanceolata*. We found significant SiAR and soil Ca interactive effects in the four sensitive species (*P. massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana*), but none for the tolerant species (*P. armandii* and *C. chinensis*). We also found that the ratio of chlorophyll a to b decreased significantly with SiAR treatment at the low Ca level for three species (*P. massoniana*, *C. lanceolata* and *L. formosana*), but this decline was ameliorated under higher Ca levels. The significant interaction of SiAR and Ca on the ratio of chlorophyll a to b was also observed in *P. massoniana* and *L. formosana* (P < 0.05) (Table 4 and Figure S1 available as Supplementary Data at *Tree Physiology* Online). However, no differences were found between SiAR and CK under any Ca level for chlorophyll a + b or a/b in *P. armandii* and *C. chinensis* (Table 4 and Figure S1 available as Supplementary Data at *Tree Physiology* Online), suggesting their high tolerance to SiAR.

A similar trend was also observed in net photosynthetic rate (Pn). The decline in Pn caused by SiAR treatment was recovered with the increase in Ca. As shown in Figure 3, the reduction of Pn by SiAR is around 35% for *P. massoniana* and 22% for *L. formosana* at the low Ca level, but <10% when the seedlings were grown at the high Ca level. We also found that Pn of *P. armandii* was reduced by ~33% with the SiAR treatment under low Ca conditions, although its seeds had a relatively high tolerance to SiAR. For *C. chinensis*, there was no significant difference (P > 0.05) in Pn among the different Ca and SiAR treatments. By analyzing the interactive effect of SiAR and soil Ca on Pn (Figure 3 and Figure S1 available as Supplementary Data at *Tree Physiology* Online), we also found significant effects in all species except *C. chinensis* (P < 0.05), implying that Ca can reduce the stress caused by SiAR.

**Leaf Ca content**

Variation in foliar Ca content among different tree species was significant (Figure 4). We found that Ca content was twofold higher for *P. massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana* when grown in soil with high Ca level, as compared with low or medium Ca level. The foliar Ca content of *P. massoniana*, *L. formosana* and *P. armandii* was decreased significantly by SiAR at low Ca level (P < 0.05). Even for *P. armandii*, the combined effect of SiAR and low soil Ca reduced the foliar Ca content by 18%. The foliar Ca content in *C. chinensis* was still stable after SiAR and Ca treatments, implying that this species has a very high tolerance to acid rain. We also noted the significant interaction of SiAR and soil Ca (Figure 4 and Figure S1 available as Supplementary Data at *Tree Physiology* Online) in the four sensitive species (P < 0.05). For the tolerant species of *C. chinensis*, however, we did not find any interactive effect.

**Discussion**

**Responses of six forest tree species to SiAR**

Acid rain can cause leaf damage, including chlorotic mottling (Bussotti et al. 1997), marginal necrosis (Fan and Wang 2000),
Table 3. Effects of different soil Ca levels on the seedling growth of six forest tree species under 2-month SiAR treatment.

<table>
<thead>
<tr>
<th>Species</th>
<th>Seedling height (cm)</th>
<th>Root/shoot</th>
<th>Root length (cm)</th>
<th>Dry weight of the aerial part (g seeding⁻¹)</th>
<th>Dry weight of the root (g seeding⁻¹)</th>
<th>Root/shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CK</td>
<td>SIAR</td>
<td>CK</td>
<td>SIAR</td>
<td>CK</td>
<td>SIAR</td>
</tr>
<tr>
<td>P. massoniana</td>
<td>L 6.2 ± 1.5Aa¹</td>
<td>5.2 ± 1.8Aa</td>
<td>7.5 ± 2.4Aa</td>
<td>6.7 ± 1.7Aa</td>
<td>0.19 ± 0.04Aa</td>
<td>0.30 ± 0.00Ab</td>
</tr>
<tr>
<td></td>
<td>M 6.8 ± 0.9Aa</td>
<td>5.4 ± 1.2Aa</td>
<td>7.4 ± 2.8Aa</td>
<td>6.5 ± 2.1Aa</td>
<td>0.20 ± 0.03Aa</td>
<td>0.05 ± 0.01Aa</td>
</tr>
<tr>
<td></td>
<td>H 6.5 ± 1.5Aa</td>
<td>6.6 ± 1.4Aa</td>
<td>7.8 ± 2.1Aa</td>
<td>8.2 ± 1.7Aa</td>
<td>0.22 ± 0.01Aa</td>
<td>0.06 ± 0.00Aa</td>
</tr>
<tr>
<td>C. fortunei</td>
<td>L 8.5 ± 3.4Aa</td>
<td>6.1 ± 2.6Aa</td>
<td>8.5 ± 2.2Aa</td>
<td>7.2 ± 2.1Aa</td>
<td>1.71 ± 0.46Aa</td>
<td>0.23 ± 0.01Aa</td>
</tr>
<tr>
<td></td>
<td>M 9.4 ± 3.8Aa</td>
<td>7.8 ± 2.4Aa</td>
<td>14.6 ± 2.4Ba</td>
<td>12.2 ± 2.5Ba</td>
<td>2.25 ± 0.35Ba</td>
<td>0.30 ± 0.08Ba</td>
</tr>
<tr>
<td></td>
<td>H 8.8 ± 2.4Aa</td>
<td>9.1 ± 3.8Aa</td>
<td>15.6 ± 3.1Ba</td>
<td>18.3 ± 4.4Aa</td>
<td>2.11 ± 0.21Ba</td>
<td>0.32 ± 0.03Ba</td>
</tr>
<tr>
<td>C. lanceolata</td>
<td>L 5.3 ± 1.7Aa</td>
<td>5.5 ± 1.6Aa</td>
<td>5.3 ± 1.3Aa</td>
<td>3.1 ± 0.4Ab</td>
<td>0.16 ± 0.04Aa</td>
<td>0.09 ± 0.02Aa</td>
</tr>
<tr>
<td></td>
<td>M 5.5 ± 2.3Aa</td>
<td>5.8 ± 0.9Aa</td>
<td>6.8 ± 1.9Aa</td>
<td>4.2 ± 0.6Ba</td>
<td>0.18 ± 0.02Aa</td>
<td>0.13 ± 0.01Ba</td>
</tr>
<tr>
<td></td>
<td>H 5.8 ± 0.4Aa</td>
<td>5.4 ± 1.5Aa</td>
<td>7.4 ± 2.8Aa</td>
<td>6.8 ± 2.7Ca</td>
<td>0.20 ± 0.02Aa</td>
<td>0.15 ± 0.03Ba</td>
</tr>
<tr>
<td>L. formosana</td>
<td>L 5.8 ± 1.1Aa</td>
<td>6.3 ± 2.3Aa</td>
<td>5.8 ± 1.7Aa</td>
<td>2.8 ± 0.9Aa</td>
<td>0.15 ± 0.06Aa</td>
<td>0.07 ± 0.02Aa</td>
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<tr>
<td></td>
<td>M 6.1 ± 2.5Aa</td>
<td>7.7 ± 1.4Aa</td>
<td>12.6 ± 3.7Ba</td>
<td>9.6 ± 3.1Ba</td>
<td>0.16 ± 0.06Aa</td>
<td>0.11 ± 0.04Aa</td>
</tr>
<tr>
<td></td>
<td>H 6.4 ± 2.2Aa</td>
<td>6.8 ± 1.4Aa</td>
<td>13.8 ± 4.3Ba</td>
<td>12.9 ± 4.1Ba</td>
<td>0.14 ± 0.06Aa</td>
<td>0.14 ± 0.01Ba</td>
</tr>
<tr>
<td>P. amandii</td>
<td>L 7.9 ± 2.7Aa</td>
<td>7.5 ± 1.8Aa</td>
<td>16.3 ± 3.9Aa</td>
<td>14.8 ± 6.4Aa</td>
<td>0.98 ± 0.26Aa</td>
<td>0.11 ± 0.04Aa</td>
</tr>
<tr>
<td></td>
<td>M 7.4 ± 1.6Aa</td>
<td>7.1 ± 2.5Aa</td>
<td>19.6 ± 6.3Aa</td>
<td>18.4 ± 5.8Aa</td>
<td>1.15 ± 0.70Aa</td>
<td>0.15 ± 0.03Aa</td>
</tr>
<tr>
<td></td>
<td>H 7.6 ± 2.8Aa</td>
<td>7.8 ± 1.3Aa</td>
<td>18.2 ± 4.4Aa</td>
<td>17.6 ± 6.1Aa</td>
<td>1.20 ± 0.22Aa</td>
<td>0.14 ± 0.05Aa</td>
</tr>
<tr>
<td>C. chinensis</td>
<td>L 10.9 ± 3.1Aa</td>
<td>12.9 ± 5.6Aa</td>
<td>15.6 ± 6.2Aa</td>
<td>18.7 ± 6.9Aa</td>
<td>1.72 ± 0.25Aa</td>
<td>0.56 ± 0.08Aa</td>
</tr>
<tr>
<td></td>
<td>M 15.3 ± 4.3Aa</td>
<td>16.8 ± 5.4Aa</td>
<td>17.9 ± 5.5Aa</td>
<td>19.2 ± 6.3Aa</td>
<td>1.83 ± 0.55Aa</td>
<td>0.63 ± 0.07Aa</td>
</tr>
<tr>
<td></td>
<td>H 13.8 ± 2.7Aa</td>
<td>15.4 ± 3.9Aa</td>
<td>19.2 ± 6.4Aa</td>
<td>21.6 ± 8.2Aa</td>
<td>1.75 ± 0.66Aa</td>
<td>0.65 ± 0.10Aa</td>
</tr>
</tbody>
</table>

The actual Ca levels in the soil were the same as in Table 1.

¹Different capital letters denote significant differences between means for the variable in the same SiAR treatment (CK or SiAR) as measured at different Ca levels, and different lower case letters denote significant differences between means for the variable under CK and SiAR treatments at the same Ca level as evaluated by one-way ANOVA (Tukey, P < 0.05). The species x treatment interaction was conducted by two-way ANOVA (Tukey, P < 0.05). ns, not significant.
cuticular damage (Shan et al. 1997), reduction in photosynthesis (Flagler et al. 1994, Velikova et al. 1997) and defoliation (Larssen and Carmichael 2000), which further lead to decreases in vertical growth, stem incremental growth and total tree biomass (Fan and Wang 2000, Feng 2000). Our results also showed that the reduction in chlorophyll content (Table 4) could lead to a decrease in $P_n$ to some extent under SiAR (Figure 3). However, the reductions varied between species. For example, $P_n$ was reduced by around 35% under SiAR in *P. massoniana*, but by <10% in *C. chinensis* (Figure 3).

Figure 2. Visible necrosis percentage of leaves for six forest tree species grown under SiAR for 2 months at different soil Ca levels. The actual Ca levels in the soil were the same as in Table 1. The visible necrosis percentage was expressed as the ratio of necrotic area to total leaf area. Different lower case letters denote significant differences under SiAR treatment as evaluated by one-way ANOVA (Tukey, $P < 0.01$).

Table 4. Effects of different soil Ca levels on chlorophyll content and chlorophyll $a/b$ ratio of seedling leaves for six forest tree species under SiAR treatment.

<table>
<thead>
<tr>
<th>SiAR treatment</th>
<th>Ca level</th>
<th>Species</th>
<th>Chlorophyll $a + b$ (mg g$^{-1}$)</th>
<th>$P_{n}$ $a/b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. massoniana</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CK</td>
<td>L</td>
<td>2.121 ± 0.098Aa</td>
<td>2.742 ± 0.115Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>2.435 ± 0.256Aa</td>
<td>2.254 ± 0.183Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>2.886 ± 0.138Ba</td>
<td>2.686 ± 0.158Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>SiAR</td>
<td>L</td>
<td>1.741 ± 0.133Ab</td>
<td>1.786 ± 0.116Ab</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1.990 ± 0.113Ab</td>
<td>1.797 ± 0.258Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>2.501 ± 0.081Ba</td>
<td>2.928 ± 0.248Ba</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. fortunei</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.090 ± 0.070Aa</td>
<td>1.038 ± 0.094Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.208 ± 0.124Aa</td>
<td>1.155 ± 0.100Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.183 ± 0.127Ba</td>
<td>1.218 ± 0.130Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. lanceolata</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.332 ± 0.102B a</td>
<td>1.378 ± 0.131Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.432 ± 0.111Aa</td>
<td>1.482 ± 0.134Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.445 ± 0.112Ba</td>
<td>1.495 ± 0.135Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>L. formosana</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.300 ± 0.080B a</td>
<td>1.350 ± 0.100Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.400 ± 0.110Aa</td>
<td>1.450 ± 0.120Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.410 ± 0.111Ba</td>
<td>1.460 ± 0.121Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P. armandii</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.100 ± 0.060Aa</td>
<td>1.150 ± 0.090Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.200 ± 0.100Aa</td>
<td>1.250 ± 0.120Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.200 ± 0.101Ba</td>
<td>1.250 ± 0.121Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. chinensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.000 ± 0.040Aa</td>
<td>1.050 ± 0.060Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.100 ± 0.080Aa</td>
<td>1.150 ± 0.090Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.100 ± 0.085Ba</td>
<td>1.150 ± 0.095Aa</td>
<td>$P &lt; 0.05$</td>
</tr>
</tbody>
</table>

The selected six species in our experiments showed different responses in seed germination and seedling growth to SiAR. For *P. armandii* and *C. chinensis*, seed germination and seedling growth exhibited less sensitivity to SiAR. However, SiAR negatively influenced both seed germination and seedling growth for the four sensitive species *P. massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana* (Table 2, Figure 1). Our results were consistent with the study in three legumes subjected to SiAR (Ashenden and Bell 1989). A similar case was also observed in a study on beech and sugar maple in North America, which found that sugar maple is much more sensitive than beech when impacted by acid rain (Park and Yanai 2009).

Effect of Ca on the responses of six forest tree species to SiAR

Responses of seed germination and seedling growth

Calcium plays an important role in enhancing the stability and structural integrity of biological tissues at scales ranging from intercellular membranes to the cell walls of woody stems (Sanders et al. 1999). By placing tree seeds in dishes at three Ca levels (low, medium and high), we found that germination increased along with the increase in Ca content ($P < 0.05$) for three of the six species (Table 2). This result is consistent with that found in the experiment using lime addition in Drayton Valley, Canada (van den Driessche et al. 2005). Researchers found that the application of lime increased aspen (*Populus tremuloides*) seed germination by 29% in Ca-poor soil where

Chlorophyll was extracted with 80% acetone and then measured by a spectrophotometer. The actual Ca levels in the soil were the same as in Table 1. Different capital letters denote significant differences between means for each variable under the same SiAR treatment (CK or SiAR) as measured at different Ca levels, and different lower case letters denote significant differences between means for each variable from CK and SiAR at the same Ca level as evaluated by one-way ANOVA (Tukey, $P < 0.05$). The species × treatment interaction was conducted by two-way ANOVA (Tukey, $P < 0.05$). ns, not significant.
the Ca concentration is <67 mmol kg\(^{-1}\) in surface soil. However, there were no positive effects of lime application for the other two sites with Ca concentrations of >150 mmol kg\(^{-1}\), implying that the dramatic effects of Ca addition only occur in Ca-poor soil. Besides, faster growth of the radicles was found at high Ca level after germination in all species except for *C. chinensis* (Figure 1), suggesting that Ca can promote root establishment.

In the seedling growth experiment, we found that the six tree species had variable responses to SiAR under different Ca levels. Calcium has a different impact on underground versus aboveground portions of the plant (Table 3). A significant variation in root growth, but not in shoot growth, was observed. Similarly, Shevtsova and Neuvonen (1997) reported no remarkable changes in shoot growth under acid rain for *Quercus rubra* in northern Finland. Another study of *P. massoniana* also...
showed that root growth is more sensitive to acid rain stimulus than shoot growth (Kong et al. 2000). Moreover, our experiment showed that the ratio of root to shoot weight increased at the high Ca level under CK or SiAR treatment in acid rain-sensitive species, i.e., *P. massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana* (Table 3). However, for the acid rain-tolerant species, i.e., *P. armandii* and *C. chinensis*, the ratio of root to shoot weight showed no significant difference among various Ca levels under CK or SiAR treatment. These data indicated that the seedling growth of acid rain-sensitive species can be restored significantly by additional Ca supply under SiAR treatment, whereas this benefit was not obvious for tolerant tree species under either CK or SiAR.

**Characteristics of seedling leaf response**

Foliar Ca deficiencies have become an important factor of integrated physiological studies aimed at understanding the causes, mechanisms and symptoms of declining growth in both Norway spruce in Europe (Schulze 1989) and red spruce in the USA (Schaberg et al. 2000). Comparing the Ca content in leaves, we found that there were significant differences between the species (Figure 4), perhaps due to species-specific characteristics, such as differences in their Ca metabolism (Broadley et al. 2003, 2004). Many studies have shown that Ca was more consistently associated with soil pH compared with other nutrients, and the total foliar Ca content generally increased with increasing soil Ca (Kobe et al. 2002, Juice et al. 2006, Moore and Ouimet 2006, Halman et al. 2008, Moore et al. 2008). Here, we found that leaf Ca of plants in high Ca soil was up to twice as high as those in low Ca soil, which is consistent with the results in the recent literature (Juice et al. 2006). Moreover, foliar Ca content was positively
correlated with growth (Kobe et al. 2002). Compared with plants grown at high Ca soil, more Ca was leached more seriously from plants grown at low Ca level after SiAR treatment (Figure 4). Furthermore, the leaf chlorophyll a to b ratio in seedlings grown at low Ca soil also decreased for three of the six species under SiAR treatment (Table 3), which was different from the results of Fan and Wang (2000). This disagreement may be caused by the combination of acid rain and low Ca content, which led to a more serious effect on leaf pigment.

Some reports suggested that Ca may protect plant leaves by influencing antioxidant enzyme activity (Velikova et al. 1997, Cakmak 2005, Wang 2010) or the formation of a wound-repair agent at the higher Ca level (McLaughlin and Wimmer 1999). This would explain the reduction in visible necrosis at high Ca levels (Figure 2). The addition of lime had a positive effect on $P_a$ and the sugar content in the leaves of red spruce (McLaughlin and Wimmer 1999), thereby increasing plant tolerance (Halman et al. 2008). All these results suggest that high Ca levels indeed enhance the tolerance of plants to SiAR.

**Relationship between soil Ca content and physiological and growth indexes**

Reduced nutrient supply from the soil as indicated by lower xylem sap concentrations of Ca (Osonubi et al. 1988) can reduce overall canopy production efficiency, which may lead to the depletion of reserve carbohydrates and has been identified as a symptom of acid rain stress in declining trees (Kobe et al. 2002). We used linear regression to describe the relationships among soil Ca content, leaf Ca content and physiological indexes under various treatments. In order to eliminate the inter-specific difference, Bullen and Bailey (2005) expressed the data in terms of a ‘delta’ factor that gave the absolute change value compared with the control value. Following this method, we used the relative rate of change between the SiAR and CK groups under three Ca levels (Figure 5). The six woody species showed two types of regression features. One group contains *P. massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana*. Their leaf Ca content declined with soil Ca reduction, suggesting that these species were highly sensitive to SiAR. However, the other group, *P. armandii* and *C. chinensis*, was significantly different because the leaf Ca content was stable, indicating strong resistance to SiAR. Consistently, the significant interactions of SiAR and Ca on seed germination and seedling growth were also only observed in the sensitive species ($P < 0.05$), suggesting the dramatic rescue effect of Ca under SiAR treatment in these species, whereas no interactive effect of SiAR and Ca treatment in the two acid rain-tolerant species ($P > 0.05$) was observed. The result further proved the varied response to SiAR under different Ca levels among our selected species. Bigelow and Canham (2007) also found variation in growth response to Ca among several tree species in a northern hardwood forest. Likewise, we found that the relative change rate of other physiological indexes varied among species as shown in Figure 6, indicating two distinct regression trends. However, the mechanisms underlying differences in the regulation of Ca uptake and translocation among tree species are still not well understood (McLaughlin and Wimmer 1999, Reich et al. 2005).

**Ecological implications**

The biogeochemical fate of Ca is interwoven with the structure and function of forest ecosystems at the community and ecosystem levels. Calcium availability is also associated with community composition (Van Breemen et al. 1997, Kobe et al. 2002). Based on our laboratory study, we found that seeds and seedlings of the six tree species responded differently to SiAR and Ca levels. Our preliminary data from a field study revealed that the seedling density of the sensitive species *P. massoniana* was much lower than that of the tolerant species *C. chinensis* in an acid rain-affected area of southern China, while there was no difference between the two species in a relatively clean area (data not shown). So we presumed that the different responses of various species to Ca depletion may result in changes in species composition over long-term exposure to acid rain in southern China. This hypothesis is supported by long-term research in Quebec, Canada, in which two dominant species, sugar maple and America beech, underwent changes in structure and composition due to their different responses to acid deposition (Duchesne et al. 2005). Moreover, a recent study in North America also found that sugar maple is susceptible to acid rain and may ultimately be replaced by American beech in regions of low pH and base cation depletion by continued anthropogenic acid deposition (Park and Yanai 2009). Altogether, this suggests that seedling regeneration can be altered by acid rain, tolerant species may replace the sensitive ones after long-term acid rain exposure and severe acid rain may accelerate forest succession in Ca-poor soils.

**Conclusions**

Our study indicates that high levels of Ca in the soil can efficiently protect plants against injury under SiAR by reducing SiAR's negative effect on seed germination, seedling growth, leaf chlorophyll content, photosynthesis and leaf Ca content. Conversely, low Ca content in soils aggravates the damage caused by SiAR. In addition, we found that the six selected tree species have different responses to the SiAR and Ca treatments. *Pinus massoniana*, *C. fortunei*, *C. lanceolata* and *L. formosana* were sensitive to SiAR and Ca treatment, while *C. chinensis* was the most tolerant. Also, we analyzed the interactive effects of SiAR and Ca treatment for the six species by two-way ANOVA. A significant interactive effect was found only in the four sensitive species. Thus, we hypothesized that forest community
composition and structure of a forest can be changed by long-term acid rain impact due to variable tolerance of different species to acid rain. Results from our preliminary field study and long-term monitoring in North America and Canada, and our present research confirm our hypotheses, whereby the seedling regeneration of sensitive species was much lower in the acid rain area as compared with the area unaffected by acid rain, and the negative effects of acid rain on sensitive tree species can be ameliorated by the addition of Ca.

Supplementary data

Supplementary data for this article are available at Tree Physiology Online.

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References


