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Ecophysiological Study of the Growth of Conifers in Korea in Acidified Soil with Elevated CO₂: the Role of Ectomycorrhizal Infection

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Abstract
Several Pinaceae species (Pinus and Larix sp.) in northeastern Asia, especially Korea, show symptoms of decline due to soil acidification and air pollution. In the industrial area of northwest Korean, growth and physiological activities of Pinus koraiensis and P. rigida are adversely influenced by increased deposition of fluorine (F), chlorine (Cl) and Manganese (Mn), involving both wet and dry deposition; these also have harmful effects on the ectomycorrhizal status. The ectomycorrhizal infection level at the damaged site was 30% less than at a control site. To determine the role of ectomycorrhizal symbiotic association with seedlings of P. densiflora, P. koraiensis, P. rigida and Larix kaempferi during progressive soil acidification, the relation between relative total dry mass (TDM) and the molar ratio of base cation to aluminum (BC/Al) was examined. The TDM of P. koraiensis and L. kaempferi seedlings was approximately 40% at a BC/Al ratio of 1.0, but that of P. rigida and P. densiflora was approximately 50% at the same BC/Al ratio. With increasing amount of H⁺ added to the soil, the water-soluble concentrations of Ca, Mg, K, Al and Mn increased, especially below a soil pH of 3.8. Development of ectomycorrhizae, i.e. Pisolithus tinctorius (Pt), Ectodrench (EC) or Cenococcum geophilum (Cg) in all four test species was significantly greater in the 10 and 30 mmolH⁺·kg⁻¹ treatments than in control treatments. Ectomycorrhizal development in the 60 and 90 mmolH⁺·kg⁻¹ treatments was significantly lower than the other treatments. Atmospheric CO₂ increases yearly that may affect mycorrhizal growth and diversity in forest ecosystems through host plant physiology. The ectomycorrhizal development of each species was significantly higher under elevated CO₂ concentration (720 μmol·mol⁻¹) than at ambient levels (360 μmol·mol⁻¹) in the present study. The P concentration in needles and roots following inoculation with Pt, EC or Cg was significantly higher in all species than without inoculation, at both CO₂ concentrations. Furthermore, no photosynthetic down-regulation was found in any species when inoculated with Pt, EC or Cg, and photosynthesis was significantly higher than in non-inoculated seedlings at both CO₂ concentrations. Stomatal limitation in each species at each CO₂ concentration was less than in non-inoculated seedlings.

Key words: Elevated CO₂, soil acidification, fluoride, chloride, ectomycorrhiza, photosynthetic rate, pine species

Chapter 1
GENERAL INTRODUCTION

1.1 Forest ecosystem in a changing environment
Environmental change, in particular global warming following increasing atmospheric CO₂ and atmospheric acid deposition, is now an important environmental issue. In the last 100 years huge amounts of air pollutants have been released from Europe, North America and Japan, including sulphur dioxide (SO₂), nitrogen oxides (NOₓ), carbon dioxide (CO₂), fluorides (HF), from industrial activities and fossil fuel burning. Moreover, gaseous air pollution has increased rapidly in recent decades because of economic growth, industrial development and urbanization, with consequent increases in energy demand in Asia, Africa and Latin America. Therefore, problem of ‘climate change’ is now worldwide.

Air pollution can impair vegetation by wet and dry deposition, i.e. by damaging canopy foliage and reducing the rate of photosynthesis. Acid deposition reduces the pH of soil by both direct and indirect mechanisms (Ulrich 1990, Okita 1996). Wet and dry depositions of acid substances may gradually lead to acidification of forest soil through increased leaching of base cations (Ca²⁺, Mg²⁺, K⁺, Na⁺) from the rhizosphere soil (Ulrich 1983, Grodziński et al. 1990). Furthermore, dissolution of phytotoxic metals such as aluminum (Al) and manganese (Mn) into the soil accelerates soil acidification (Ulrich et al. 1980, Hallbacken and Tamm 1986). Moderate atmospheric deposition of nitrogen is reportedly beneficial for plants, but excess atmospheric deposition loading onto the forest floor can do long-term harm to forest ecosystems (Nilsson 1987, Moore 1995). Adverse effects include soil acidification (Lamerdorf and Mayer 1993), nutrient imbalance (Havranek et al. 1990, Okochi et al. 1995, Dueck et al. 1998, Grulke et al. 1998), nonoptimal carbohydrate
2

The atmospheric carbon dioxide concentration (CO₂) has continued to rise in recent decades (Houghton et al. 1990, Wyman 1991, Houghton 1995). This trend has indirect influences through global climatic greenhouse effects, and strong direct influences on ecosystems through increased availability of CO₂ for photosynthesis. At the physiological level, elevated CO₂ generally enhances photosynthesis, growth and water-use efficiency (WUE), and reduces the transpiration rate (Bazzaz 1990, Eamus 1991, Poorter 1993, Rogers et al. 1994, Field et al. 1995) and the partitioning of below-ground carbon (Stulen and den Ertog 1993, Rogers et al. 1996). Photosynthesis down-regulation or accumulation occurs with nutrient restriction, however, especially of P or N (Conroy et al. 1986, Coleman et al. 1993, Jach and Ceulemans 2000, Koike et al. 2000, Choi et al. 2005b), and also reduced sink strength (Stitt 1991, Rogers et al. 1998, Griffin et al. 2000).

1.2 Pine species

Pine is a diverse and important tree species for forest rehabilitation and planting of man-made forests in relatively poor soil conditions. There are approximately 100 species of pine, and most are significant for high-quality timber and high biomass productivity (Burns and Honkala 1990). Despite their taxonomic variety and wide distribution in the Northern Hemisphere, all pines have much in common ecologically in the world's forest ecosystems (Barnes et al. 1998).

The pine has a deep main root, and the growing root tips require large soil interstices to permit penetration. Because of the deep main root and poor penetrative ability of the roots, pines do not grow well in poorly drained soils. Consequently, they often have a strong symbiotic relationship with ectomycorrhizae. They are pioneer species in succession, and invade areas where mineral soil has been exposed by major disturbances such as forest fire or degradation by pollutants. Some pine species have high tolerance against drought, poor nutrition and pollutants including acid agents (Choi et al. 2005a, 2006). In Korea, Pinus koraiensis and P. densiflora are the main afforestation species. These pine species are planted in and close to large cities and industrial areas as shade trees. To rehabilitate degraded forested areas, P. rigida and Larix kaempferi were introduced at the start of the 20th century from North America and Japan (Kim 1999).

1.3 Role of ectomycorrhizae in a changing climate

Most tree species are symbiotic with ectomycorrhizae (Brundrett 1991, Smith and Read 1997). Ectomycorrhizae improve a plant’s uptake of water and nutrients, particularly nitrogen and phosphorus from the soil solution. This is because a greater soil volume can be exploited via the widely distributed hyphae (Smith and Read 1997, Lambers et al. 1998). Ectomycorrhizal association can also reduce the toxicity of metals such as Al, Mn, Zn, Cu and Ni; the effect depends on the mycorrhizal fungi and plant host species involved (Willkens 1991, Jentschke and Godbold 2000). Aluminium toxicity has been associated with decreased uptake of base cations (BC) (Ca, Mg and K) (Schaedle et al. 1989, Jentschke and Godbold 2000). These base cations can be taken up by the mycorrhizal mycelium (Finlay 1992), even in plants exposed to high Al concentrations (Finlay 1995). Ectomycorrhizal plants therefore increase the uptake of water and nutrients and protect roots from toxic metals by decreasing the uptake of toxic ions (Kayama et al. 2005). These characteristics of ectomycorrhizae are expected to enhance physiological activity and growth of forest plants under climate change that includes soil acidification due to atmospheric deposition and elevated atmospheric CO₂.

1.4 Objectives and outline of this study

This study consists broadly of two parts; i.e. environmental pollution and its effects on the decline of pines in an industrial region are treated in Chapter 2, and the growth and physiological responses of pines in a changing environment are considered in Chapters 3 and 4. The main concerns of this study are:

What is the most significant factor in the decline of pines in an industrial region?

What is the response of growth and physiological activities in pine trees facing soil acidification and elevated CO₂, especially in regard to their symbiosis with ectomycorrhizae?

Growth and physiological responses of pine forests in both damaged and undamaged areas were examined, and the main factor responsible for the decline of pine forests at the damaged site was identified (Chapter 2). The effect of soil acidification on the growth and physiological responses of pine, and the role of ectomycorrhizae, were investigated (Chapter 3). Elevated CO₂ influences the growth and photosynthetic characteristics of pines; the function of ectomycorrhizae is discussed in Chapter 4. Finally, the results are summarized and the possibility of regeneration under disturbance and changing environment is discussed (Chapter 5).

The following research approach will be used to meet the designed objectives.
Chapter 2
Growth and physiological response of pine species planted in a polluted industrial region

2.1 Introduction

Pine trees are the most popular species for shade tree in cities, roadsides and rehabilitating degraded regions in Korea (Kim 1999). Therefore, Korean pine (Pinus koraiensis Sieb. et Zucc.) and Pitch pine (Pinus rigida Miller) are the most important afforestation tree species in Korean forests, since they can grow in infertile and barren soil, including shallow soil and sandy or gravelly soil. To rehabilitate degraded forested areas, P. rigida was introduced from North America in 1906 (Kim 1999). This species has high resistance to O₃ and soil acidification (Burns and Honkala 1990). In recent years, however, pine forests have been in decline near industrial areas and large cities (Lee et al. 1996, Choi et al. 2003, 2006, Kim et al. 2003).

In most Korean forests the soil is gravely and not particularly fertile, and is easily acidified because it derives from granite (Lee et al. 1998). Even though large amounts of chemicals such as lime or nitrogen fertilizers have been administered to reduce or reverse soil degradation, the decline is continuing (Yoo et al. 1998, Choi 2003).

What are the reasons for the decline in pine forests near industrial areas in Korea? Environmental pollutants (such as SO₂, O₃, NOₓ) usually reduce plant growth (Sucoff 1975, Matyssek et al. 1995a,
According to preliminary surveys, photosynthetic rates at light saturation with ambient CO$_2$ of pine saplings (*Pinus koraiensis* and *P. rigida*) in polluted industrial areas are significantly lower than that in clean University Forests (Choi et al. 2003, Kayama et al. 2004). In polluted areas the soil pH is lower and plants usually contain Mn (Kitao et al. 1997a). Recently, Choi et al. (2005a) found that *P. koraiensis* is more tolerant to soil acidification, based on the BC/Al ratio, than *P. densiflora*. I therefore hypothesize that reduced growth and photosynthetic capacity of pines at the damaged site is due to excess Mn accumulation in pine needles and to the direct effect of F (and other air pollutants). Which pine species are more tolerant to soil acidification and air pollutants?

To answer these questions, I measured the growth performance and the photosynthetic activity *in situ* of pine saplings planted in polluted and clean areas, considering climatic factors.

2.2 Materials and Methods

2.2.1 Study sites

To compare the effect of pollutants on growth and physiological changes, two plantation sites of Korean pine (*Pinus koraiensis* Sieb. et Zucc.) and Pitch pine (*Pinus rigida* Miller) were selected. The control site is located in southeast of Seoul city in Korea, and the damaged site is located in the southwest part of Seoul city. The control sites of *P. koraiensis* and *P. rigida* trees were in the Toi-chon Experimental Forest of Kyunghee University (37.2N, 127.1E), where the air is relatively clean (Kwangju, Kyunggido, Korea). The damaged sites were located in the Ansan industrial area (37.2N, 126.4E) (Ansan, Kyunggido, Korea). This industrial area was established in 1977, and comprises mainly machinery, ceramics, glass, chemicals and dye works industries (Fig. 2.1).

There were three study plots (10 m\times10 m) for the control site, and six for the damaged site. Three of the plots at the damaged site were on two slopes (a

![Fig. 2.1. The two field study sites located in Korea. The opened cycle and closed triangle represent the control site at the Toi-chon Experimental Forest of Kyunghee University and damaged site at the Ansan industrial complex in Korea, respectively.](image-url)
2.2.2 Plant materials
Needles of *Pinus koraiensis* and *P. rigida* were collected from plantations at the control and damaged sites for chemical analysis. Sample needles were taken randomly from sunny branches of five trees per species in early September 2002, and were divided into current-year needles and older needles. The distance between sample trees was approximately 10m. Both sites were reforested 10 years ago, and the trees of each species were 13 years old at the time of sampling (they were planted at three years old). The tree height for each species varied between 1.6 m and 2.0 m.

2.2.3 Analysis of soil chemistry
The soil pH, and the concentrations of nitrogen (N), available phosphorus (P), base cations (Ca, Mg, K), Al and heavy metal (Mn) in soil were measured. The soil pH was determined by a pH meter (CH-8603, Mettler-Toledo, Greifensee, Switzerland) from a soil suspension. The suspension was made by mixing 20 g fresh soil and 50 ml distilled water in a 100 mL beaker, and was stirred by a glass stick every 20 minutes for one hour. The same soil samples were dried at 105°C for 24 h and the N concentration was determined by a CHNS/O analyzer (PE 2400 Series II, Perkin-Elmer, Norwalk, CT). P was extracted with 0.5 M sodium bicarbonate solution (pH = 8.5) (Olsen and Sommers 1982), and exchangeable Ca, Mg and K were extracted from 2.5 g dry soil with 50 ml of 1 M ammonium acetate solution (pH = 7.0) after shaking for 1 h. Al and soluble Mn were extracted from 5.0 g of dry soil with 0.1 N HCl after shaking for 1 h at 30°C. P, Ca, Mg, K, Al and Mn extracted from soil were all measured from these prepared solutions by an ICP (IRIS, Jarrel Ash, Franklin, MA).

2.2.4 Assessments of shoot growth, needle survival ratio and root infection rate
To evaluate shoot growth, the length of leader shoots for 20 samples (4 branches from each of 5 trees) of *P. koraiensis* and *P. rigida* were measured at the control and damaged sites in September 2002, once the leader shoot was observed to be fully grown. And, needle longevity was measured on four branches of five individual trees of each pine species at each study site. The branches were selected from a sunny crown of trees on the forest edge at a height of about 1.2-1.5 m. The main branch was divided up according to shoot age. Twelve shoots of each age from the five trees per species were dried for 4 days at 80°C. After drying, the actual number of needles and the number of needle scars were determined for each shoot. The survival measure of the needles (SN) was calculated from the following formula: SN (%) = RN/TN×100, where RN and TN respectively denote the number of retained and total needles (estimated from multiplying the number of needles borne in bundle by the number of scars) (Kayama et al. 2002).

It dug out 0.07-0.1 m of the tip portion of the roots of both pines at about 0.1 m depth at the control and damaged sites to look for infection by ectomycorrhiza. The root tips were observed and counted the infected and non-infected roots (Quoreshi 2003). The infection rate of ectomycorrhiza (IRE) was determined as IRE (%) = ER/ER + NR)×100, where ER and NR respectively denote the number of ectomycorrhizal and non-ectomycorrhizal roots.

2.2.5 Measurement of photosynthesis
The photosynthetic capacities of five trees of *P. koraiensis* and of *P. rigida* were measured by two methods in September 2002 at the control and damaged sites. In the first method, the photosynthetic light response curve of current year needles was examined in sunny crown trees at the edge of the forest at a height of around 1.3 m. For these measurements, a portable gas analyzer (H4A, ADC BioScientific, Hoddesdon, U.K.) was used; the ambient temperature was 23-25°C and the ambient CO₂ concentration was 355-360 μmol mol⁻¹. Between 15 and 20 needles of two pines were covered with a conifer chamber (137 cm², ADC BioScientific, Hoddesdon, U.K.), and the photosynthetic rate was measured. Supplementary light was provided by a halogen lamp (WALZ, Effeltrich, Germany). The photosynthetic photon flux density (PPFD) was changed from high to low to dark (2000, 1000, 500, 100 and 0 μmol m⁻²s⁻¹) using cloth shades (Krary, Osaka, Japan). After determining the photosynthetic light curve, the width and length of needles were measured using vernier calipers, and then calculated the net photosynthetic rate per unit area. From the resulting data, photosynthetic light response curves were drawn as described by Thornley (1976) using the formula:

\[
P = \frac{\phi I + P_{\text{max}} - \sqrt{((\phi I + P_{\text{max}})^2 - 4\phi I P_{\text{max}})}}{2\phi} - r_d
\]

where \(P\) is the net photosynthetic rate, \(\phi\) is the initial slope of the curve, \(I\) is the incident PPFD, \(P_{\text{max}}\) is the light-saturated rate of gross photosynthesis, \(0\) is the convexity factor of the curve, and \(r_d\) is the dark respiration rate. The light saturation point was taken to be the PPFD value when \(P_n\) reaches 95% of \(A_{\text{max}}\), the non-liner function.

The second method of measuring photosynthetic
capacity looked at the $A/C_i$ ($A=$photosynthetic rate, $C_i$=intercellular $CO_2$ concentration) curves for the same current-year needles, using an open gas exchange system (LI-6400, Li-Cor, Lincoln, NE, USA). The change in the net photosynthetic rate was measured at light saturation, under a photosynthetic photon flux density (PPFD) of 1,000-1,200 μmol m$^{-2}$ s$^{-1}$ provided by a cool halogen lamp (WALZ, Effeltrich, Germany). The leaf temperature was 25℃ and the relative humidity was 50-70%. Leaves were allowed to acclimatize to their surroundings for 10 minutes before measurement began, after which I began the determination with 150-1500 μmol mol$^{-1}$ $CO_2$ concentrations.

Distinct processes regulate the rate of $CO_2$ assimilation at low and high $C_i$ concentrations (Farquhar and Sharkey 1982, Sharkey 1985, Matyssek et al. 1993b). The initial slope of the $A/C_i$ curve is proportional to the carboxylation activity of Rubisco (i.e., the carboxylation efficiency). The net photosynthetic rate at high $CO_2$ concentrations is limited by the regeneration rate of ribulose-1, 5-bisphosphate (RuBP).

2.2.6 Analysis of chlorophyll and chemical elements in needles

Chlorophylls were extracted with dimethyl sulfoxide (DMSO) and were measured spectrophotometrically (Type 100-50, Hitachi, Tokyo, Japan) to determine the concentration of chlorophyll (a and b) from three branches of five trees of each pine species at each study site (Barnes et al. 1992, Shinano et al. 1996). The amount of chlorophyll b increases when the needles grow in shady conditions (Larcher 2003).

The remaining needle samples were dried at 60 ℃ for one week. The dried samples were then ground to a fine powder in a vibrating sample mill (Wonder Blender, Osaka Chemical Co., Osaka, Japan). To determine the concentration of mineral nutrients and heavy metals (Ca, K, Mg, Na, P and Mn), the samples were digested by a microwave digestion system (O·I analytical, College Station, TX) and then underwent ICP analysis. The total amount of precipitation was almost the same at the control and damaged areas, so that it is valid to compare the dose of pollutants from the observed values.

2.2.9 Statistical analysis

Mean values of shoot growth, survival of needles, photosynthetic rate, chlorophyll and concentrations of elements in needles, air, precipitation, soil water and soil were examined between species and sites by the $t$-test (Li 1964) using the Stat View 5.0 software (SAS Institute, Cary, NC, USA). Relations between $P_{sat}$ and each element were estimated using (S)MATR (Falster et al. 2003).

2.3 Results

2.3.1 Concentration of chemical elements in atmosphere, precipitation, soil and soil water

Table 2.1 shows the concentrations of Cl and F in the atmosphere at the control site and the damaged site. No difference was found in the Cl and F concentrations between the two slopes at the damaged site. The concentration of F in the air at the damaged site was about three times higher than at the control site (Table 2.1); this difference is statistically significant ($p<0.05$). The concentration of Cl in the atmosphere behaved similarly ($p<0.01$).

The concentration of Cl and F in the precipitation was significantly higher at the damaged site than at the control site, at least in the growing season ($p<0.05$) (Fig. 2.2). The amount of trapped Cl and F scarcely differed between the two slopes in the damaged area. However, between July and September, there was no significant difference in Cl and F concentrations in the precipitation between the control and damaged sites.

Table 2.2 shows the soil condition at the control and damaged sites. Soil was more acidified at the damaged site than at the control site, however, there was no statistical difference in Al concentration between control and the damaged site. The concentration of Mn in the soil at the damaged site was significantly higher than at the control site ($P<0.01$). No statistical difference in soil conditions was observed between the two slopes at the damaged site. The two slopes as representing the damaged site were averaged. Moreover, exchangeable base cations (Mg, K) and P at the damaged site were significantly lower than at the control site ($P<0.05$). Concentrations of N and Ca were higher at the damaged site than at the control site, however.

Concentrations of Cl and F in soil water showed similar trends to concentrations in precipitation, and
Table 2.1. The concentration of fluorine in the air from the control site and damaged site (Unit= \( \text{F dm}^{-2} \text{LTP/month, mean±S.D.} \)), and the concentration of chlorine. (Unit=ppm, mean±S.D., * P<0.05, ** P<0.01 (\( \text{\$} \) No sample was collected))

<table>
<thead>
<tr>
<th></th>
<th>June</th>
<th>September</th>
<th>October</th>
<th>November</th>
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<tbody>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>29.5±2.41</td>
<td>36.8±6.99</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Damaged</td>
<td>97.0±28.43 **</td>
<td>104±41.52 *</td>
<td>125±22.06</td>
<td>69.2±19.56</td>
</tr>
<tr>
<td>Cl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.28±0.02</td>
<td>0.09±0.03</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Damaged</td>
<td>0.45±0.05 **</td>
<td>0.87±0.30 **</td>
<td>1.13±0.68</td>
<td>-</td>
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</table>

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Table 2.2. The soil pH and concentration of soil chemical elements of A and B horizons at the control and damaged site. (* P<0.05, ** P<0.01, *** P<0.001) (Unit of C and N = mg·100mg\(^{-1}\); Ca, Mg, K, Al and Mn = mg·100g\(^{-1}\))

<table>
<thead>
<tr>
<th></th>
<th>Control A</th>
<th>Control B</th>
<th>Damaged A</th>
<th>Damaged B</th>
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</thead>
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<tr>
<td>pH</td>
<td>5.03±2.20</td>
<td>5.25±2.36</td>
<td>4.69±0.17</td>
<td>4.64±0.07</td>
</tr>
<tr>
<td>N</td>
<td>0.14±0.04</td>
<td>0.06±0.02</td>
<td>0.24±0.12</td>
<td>0.12±0.03 *</td>
</tr>
<tr>
<td>Ca</td>
<td>5.39±0.64</td>
<td>2.78±0.48</td>
<td>6.10±1.84</td>
<td>2.89±1.18</td>
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<tr>
<td>Mg</td>
<td>6.20±0.51</td>
<td>3.84±0.49</td>
<td>3.73±0.15 ***</td>
<td>2.71±0.38 *</td>
</tr>
<tr>
<td>K</td>
<td>40.20±8.25</td>
<td>17.47±3.47</td>
<td>17.40±4.73 **</td>
<td>24.55±7.27</td>
</tr>
<tr>
<td>P</td>
<td>2.42±0.25</td>
<td>2.35±0.21</td>
<td>1.29±0.88 *</td>
<td>0.20±0.11 ***</td>
</tr>
<tr>
<td>Al</td>
<td>20.45±0.76</td>
<td>19.74±2.81</td>
<td>18.69±1.25</td>
<td>20.65±1.26</td>
</tr>
<tr>
<td>Mn</td>
<td>3.16±0.66</td>
<td>1.59±0.44</td>
<td>10.56±3.86 **</td>
<td>13.29±1.55 ***</td>
</tr>
</tbody>
</table>

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Fig 2.2. The concentration of F and Cl in the precipitation from April to November 2003 at the control and damaged site.

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were significantly higher at the damaged site than at the control site \((p<0.05)\) (Fig. 2.3). The concentration of Cl and F in soil water fell with increasing soil depth; this tendency was clearer at the damaged site than at the control site. The F concentration in soil water at depths of 0.1 and 0.3 m from the surface at the damaged site was significantly higher than at the control site, but at 0.6 m depth there was no significant difference between the sites. For Cl, the concentration in soil water at 0.1, 0.3 and 0.6 m down at the damaged site was significantly higher than at the control site \((p<0.001)\).

### 2.3.2 Shoot length, survival of needles and root infection rate

Shoots were shorter at the damaged site than at the control site, being 0.14-0.22 m (damaged site) and 0.26-0.34 m (control site) for *P. koraiensis* and 0.14-0.19 m and 0.23-0.28 m for *P. rigida* (Fig. 2.4). These figures correspond to reductions of 24-47% for *P. koraiensis* and 26-48% for *P. rigida*, and attained statistical significance \((p<0.01)\).

Survival of needles of *P. koraiensis* and *P. rigida* is shown in Figure 2.5. *P. koraiensis* and *P. rigida* both shed their needles earlier at the damaged site than at the control site. *P. koraiensis* retained more than 60% needles of up to 5 years of age at the control site, but at the damaged site it had shed more than 80% of its needles of 3 years of age. Survival of needles of *P. koraiensis* at the damaged site was significantly worse than at the control site \((p<0.05)\). Also, most aged needles of *P. rigida*, more than 3 years old, were shed at both sites.

No infection with ectomycorrhiza was observed in newly formed roots of either pine at the damaged site (data not shown).

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![Fig. 2.3. Example of concentration of F and Cl in the soil water at the control and damaged site.](image)

![Fig. 2.4. Length of shoots at different age classes for *P. koraiensis* and *P. rigida* at the control and damaged sites.](image)

![Fig. 2.5. Survivorship of needles at different age classes for *P. koraiensis* and *P. rigida* at the control and damaged sites.](image)
2.3.3 Concentrations of elements in needles

Table 2.3 shows the concentrations of elements in needles of *P. koraiensis* and *P. rigida* at the control and damaged sites. The concentrations of magnesium (Mg), potassium (K) and phosphorus (P) in 1 to 2-year-old needles of *P. rigida* at the damaged site and in 2 or 3-year-old needles of *P. koraiensis* were significantly lower than the corresponding values at the control site \((p<0.05)\). The K and P concentration in needles at both sites were significantly higher at the damaged site than at the control site \((p<0.05)\). The Cl and F content of needles increased with needle age in both species, especially at the damaged site.

2.3.4 Chlorophyll concentration in needles

Chlorophyll \((a+b)\) and chlorophyll \(b\) concentration in 1 to 3-year-old needles of *P. koraiensis* at the control site showed a similar level of ca. 1.5 \(\mu\text{mol} \cdot \text{g}^{-1}\) \((a+b)\) and ca. 0.25 \(\mu\text{mol} \cdot \text{g}^{-1}\) \((b)\), but concentrations at the damaged site fell rapidly from 1.60 to 0.11 \(\mu\text{mol} \cdot \text{g}^{-1}\) \((a+b)\) and 0.23 to 0.04 \(\mu\text{mol} \cdot \text{g}^{-1}\) \((b)\) with needle age (see Fig. 2.6). Chlorophyll \((a+b)\) and \(b\) concentrations were significantly lower at the damaged site for *P. koraiensis* than at the control site \((p < 0.01)\), except in 1-year-old needles.

Chlorophyll \((a+b)\) and \(b\) concentrations in 1 to 3-year-old needles of *P. rigida* at the control site showed a similar level of ca. 1.5 \(\mu\text{mol} \cdot \text{g}^{-1}\) \((a+b)\) and ca. 0.25 \(\mu\text{mol} \cdot \text{g}^{-1}\) \((b)\), but concentrations at the damaged site fell rapidly from 1.60 to 0.11 \(\mu\text{mol} \cdot \text{g}^{-1}\) \((a+b)\) and 0.23 to 0.04 \(\mu\text{mol} \cdot \text{g}^{-1}\) \((b)\) with needle age (see Fig. 2.6). Chlorophyll \((a+b)\) and \(b\) concentrations were significantly lower at the damaged site for *P. koraiensis* than at the control site \((p < 0.01)\), except in 1-year-old needles.

---

**Table 2.3. Concentration of elements in every age of needles of *P. koraiensis* and *P. rigida*. (Unit = mg·g⁻¹ and \(\mu\text{g} \cdot \text{g}^{-1}\))**

<table>
<thead>
<tr>
<th>Needle age (mg·g⁻¹)</th>
<th><em>P. koraiensis</em></th>
<th><em>P. rigida</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ca C</td>
<td>2.93± 0.83</td>
<td>3.55± 0.89</td>
</tr>
<tr>
<td>Mg D</td>
<td>3.21± 0.27</td>
<td>5.20± 0.33</td>
</tr>
<tr>
<td>K D</td>
<td>5.49± 0.52</td>
<td>4.92± 0.39*</td>
</tr>
<tr>
<td>P C</td>
<td>3.22± 0.46</td>
<td>2.61± 0.38</td>
</tr>
<tr>
<td>Cl D</td>
<td>5.30± 0.35</td>
<td>0.56± 0.20</td>
</tr>
<tr>
<td>F D</td>
<td>13.6± 5.19</td>
<td>20.2± 15.86</td>
</tr>
</tbody>
</table>
| **Table 2.4. Parameter estimates as functions of the A/Ci curves and light curves.**

\(P_{sat}\) is light-saturated net photosynthesis \((\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1})\), \(\Phi\) is apparent quantum yield \((\mu\text{mol} \cdot \text{CO}_2 \cdot \text{m}^{-2} \cdot \text{Pa}^{-1} \cdot \text{CO}_2 \cdot \text{s}^{-1})\), CE is carboxylation efficiency \((\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{Pa}^{-1} \cdot \text{CO}_2 \cdot \text{s}^{-1})\), and RuBP regeneration is the rate of RuBP regeneration \((\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{Pa}^{-1} \cdot \text{CO}_2 \cdot \text{s}^{-1})\). \((*P<0.05, **P<0.01, ***P<0.001)\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th><em>P. koraiensis</em></th>
<th><em>P. rigida</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>(P_{sat}) Control</td>
<td>3.97± 0.16</td>
<td>3.29± 0.36</td>
</tr>
<tr>
<td>(P_{sat}) Damaged</td>
<td>2.55± 0.05</td>
<td>1.38± 0.51</td>
</tr>
<tr>
<td>CE</td>
<td>0.02</td>
<td>0.011</td>
</tr>
<tr>
<td>RuBP regeneration</td>
<td>0.20</td>
<td>0.14</td>
</tr>
</tbody>
</table>

---

(Cited from Choi *et al.* 2006. Environmental Pollution Elsevier with permission)
increased slightly with needle age, but concentrations at the damaged site fell rapidly with needle age, falling to zero in 3-year-old needles. As a result, the chlorophyll (a+b) and b concentrations in *P. rigida* were significantly lower at the damaged site than at the control site (*p* < 0.05).

### 2.3.5 Photosynthetic responses

The net photosynthetic rate of 1-year-old needles saturated at approximately 1300 μmol m⁻² s⁻¹ PPFD for both *P. koraiensis* and *P. rigida* at the control site; at the damaged site saturation was reached at approximately 800 μmol m⁻² s⁻¹ PPFD for *P. koraiensis* and 600 μmol m⁻² s⁻¹ PPFD for *P. rigida* (Fig. 2.7). The

![Photosynthetic light response curves](image1)

*Fig. 2.6. Concentration of chlorophyll (a+b) and b in needles of different age classes at the control and damaged sites for *P. koraiensis* and *P. rigida* (mean±S.D., n=15, FM = fresh mass). *P<0.05, **P<0.01, ***P<0.001.*

*(Cited from Choi et al. 2006. Environmental Pollution Elsevier with permission)*

![Photosynthetic light response curves](image2)

*Fig. 2.7. Photosynthetic light response curves of 1-year-old needles at the control and damaged site for *P. koraiensis* and *P. rigida.*

*(Adopted from Choi et al. 2006. Environmental Pollution Elsevier with permission)*

![CO₂ assimilation (A) response curve](image3)

*Fig. 2.8. The CO₂ assimilation (A) response curve to intercellular CO₂ concentration (Ci) of current year needles at the control and damaged sites for *P. koraiensis* and *P. rigida.*

*(Adopted from Choi et al. 2006. Environmental Pollution Elsevier with permission)*
photosynthetic rate at light saturation \((P_{\text{sat}})\) was significantly lower for both species at the damaged site than at the control site \((P. koraiensis, 3.97 \text{ to } 2.55 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}; P. rigida 3.29 \text{ to } 1.38 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}; p<0.01)\). Moreover, the apparent quantum yield \((\Phi)\) was significantly lower at the damaged site than at the control site (i.e. 0.02 to 0.012 \mu\text{molCO}_2 \cdot \text{m}^{-2} \cdot \text{Pa}^{-1} \cdot \text{CO}_2 \cdot \text{s}^{-1} \text{ for } P. koraiensis, \text{ and } 0.011 \text{ to } 0.007 \mu\text{molCO}_2 \cdot \text{m}^{-2} \cdot \text{Pa}^{-1} \cdot \text{CO}_2 \cdot \text{s}^{-1} \text{ for } P. rigida; \text{ see Table 2.4})

Figure 2.8 shows the photosynthetic dependence of intercellular CO\(_2\) \((C_i)\) at light saturation, \((A/C_i)\), for \(P. koraiensis\) and \(P. rigida\) growing at the control and damaged sites. The net photosynthetic rates \((A)\) of \(P. koraiensis\) and \(P. rigida\) were clearly lower at the damaged site than at the control site, being reduced from 6 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \text{ to } 3 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}.\) Also, the carboxylation efficiency \((CE)\) of the \(A/C_i\) curve for \(P. rigida\) at the damaged site is significantly smaller than at the control site \((0.06 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}/\text{Pa} \text{ and } 0.14 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}/\text{Pa}; p<0.001; \text{ see Table 2.4})\). However, any clear decrease in the CE of the \(A/C_i\) curve of \(P. koraiensis\) at the damaged site was not found relative to the control site. The CE was suppressed more in \(P. rigida\) than in \(P. koraiensis\). Also, the RuBP regeneration rates of \(P. koraiensis\) and \(P. rigida\) at the damaged site were less than at the control site (Table 2.4). From the \(A/C_i\) curve, the reduction in each parameter was smaller in \(P. koraiensis\) than in \(P. rigida\).

### 2.3.6 Relation between photosynthesis and Mn, Cl and F concentrations

\(P_{\text{sat}}\) decreased with increasing Mn concentration in needles in both species (Fig. 2.9). The gradient of the plot of \(P_{\text{sat}}\) versus F and \(P_{\text{sat}}\) versus Cl also showed a similar tendency. In all cases the regression lines fell more sharply for \(P. rigida\). In particular, the gradient of the plot of \(P_{\text{sat}}\) versus Mn differed significantly between \(P. koraiensis\) and \(P. rigida\) \((P<0.01)\).

---

**Fig. 2.9.** The relationship between concentration of Mn, F, Cl, Ca, P, K or Mg in needle and photosynthetic rate at light saturation \((P_{\text{sat}})\) of \(P. koraiensis\) and \(P. rigida\) at the control and damaged sites.
2.4 Discussion

There were no significant difference in soil pH and Al concentration in soils between the control and the damaged sites (Table 2.2). Therefore, this study focused on the factors affecting photosynthetic function (Table 2.4). $P_{\text{sat}}$ decreased with Mn, Cl or F accumulation in the needles of both species. The gradient of the regression lines for $P_{\text{rigida}}$ were steeper than for $P_{\text{koraensis}}$ (Fig. 2.9), although $P_{\text{koraensis}}$ accumulated more of these elements in needles than $P_{\text{rigida}}$. Fluoride is absorbed via the stomata, is transported by transpirational flow in the apoplast, and can accumulate up to toxic levels in the tips and margins of the leaves since there is no removal mechanism. Moreover, fluoride dissolved in water on the leaf surface can be absorbed by diffusion through the cuticle, leading to chlorosis and necrosis of leaf tips and margins (Treshow and Anderson 1989). Chlorine also causes damage to plants from tip and margin burn, necroses, and suppression of physiological activity (Kayama et al. 2003, Larcher 2003). It also reduces the rate of mycorrhizal colonization (Duke et al., 1986) as it was observed in the pine species at the damaged site.

The concentrations of Cl and F in the atmosphere, in precipitation and in soil water were significantly higher at the damaged site than at the control site ($p<0.05$), but did not differ between the sites in precipitation from June to September (Fig. 2.2). This is the rainy season in Korea (Korea Meteorological Administration 2002b, 2003), and concentrations of Cl and F in the precipitation were therefore similar at the control and damaged sites even though the damaged site is quite near to the sea. It is interesting to note that the concentration of Cl and F in precipitation between the control and damaged sites were similar, in spite of that in the atmosphere was significantly higher at the damaged site compared to the control site in September. The concentration of Cl an F in the atmosphere was analyzed after hang up during one month under shelter but with good ventilation, moreover, the concentration of Cl and F in the precipitation was analyzed immediately after rain. Therefore, it is thought that the concentration of Cl and F in the precipitation was diluted since a great quantity of precipitation from June to September. The Cl concentration at the damaged site in the air, precipitation and soil water did not differ between the two slopes, implying that the damaged site is scarcely influenced by Cl from the sea. An increase in air pollutants (i.e. Cl and F) invariably causes the concentration of harmful elements in precipitation and soil water. Enhanced Cl accumulation in needles reduces tree growth; for example, spruce ($P. abies$ and $P. glehnii$) growing along the roadside is damaged by Cl in deicing chemicals (Kayama et al. 2003). The photosynthetic function is also sensitive to chlorine air pollutant in leaves of both native and introduced pine species (Gratani et al. 2000, Zhang et al. 2001). It is well known that F restrains enzyme activity (it is often used as an enzyme repressor), accelerates genetic damage and disrupts the immune system. Accumulation of these harmful elements in plants directly reduces photosynthesis.

It has also been reported that Mn is a co-factor of photosynthesis (Marschner 1995); however, excess Mn in foliage organs usually reduces photosynthetic function at PSII (Nable et al. 1988, Kitao et al. 1997a, 1997b). Enhanced accumulation of Mn, Cl and/or F in needles therefore reduces the carboxylation efficiency in Rubisco, the RuBP regeneration rate, and also $P_{\text{sat}}$, especially in $P_{\text{rigida}}$.

Concentrations of base cations (Mg and K), P ($p<0.01$) and chlorophyll (a+b) and b ($p<0.05$) in needles of both pine species were significantly less at the damaged site than at the control site, except for Ca in $P. koraensis$ (Table 2.3, Fig. 2.6).

Symbiosis with mycorrhizae increases the uptake of nutrients (particularly P) and water, and the resulting vigorous physiological response and increase in growth of host plants enlarges the absorptive surface of the root (Smith and Read 1997, Lambers et al. 1998). At the damaged site, the development of mycorrhizae is liable to be restricted by environmental pollution (Allen 1996), inhibiting the uptake of nutrients (especially P) and water. The reduction of chlorophyll concentration in the needles influences the capture, absorption and conversion of solar energy in photosynthesis; usually, P deficiency in chloroplasts affects photosynthesis through RuBP regeneration (Brooks 1986, Fredeen et al. 1990, Kirschbaum and Tompkins 1990, Jacob and Lawlor 1991, Lewis et al. 1994), and reduces the peak carboxylation velocity or peak capacity of electron transport (Conroy et al. 1986, Lauer et al. 1989, Harley and Sharkey 1991). Nutrient deficiency in plants is accompanied by a reduction in the rate of CO2 assimilation, which in turn reduces shoot growth and accelerates needle loss (Fig. 2.4, 5) (e.g. Field and Mooney 1983). This reduction in shoot length and needle lifespan in both pines at the damaged site is the result of photosynthetic suppression through environmental stressors such as Mn, Cl and F. Needle loss and premature senescence due to air pollutants such as SO2, O3, NOx, or acid deposition, have been reported in pine, birch and poplar (Reich 1983, Matyssek et al. 1993a, 1993b, Reich et al. 1995, Sandermann et al. 1997).

The toxicity of these pollutants is mostly due to their interference with respiration and photosynthetic function (Heber et al. 1995, Matyssek et al. 1995a, 1995b, Sandermann et al. 1997, Lambers et al. 1998, Pukacki 2000). In consequence, the energy status of damaged trees may be reduced (Ernst 1976, Ernst and Joosse-van Damme 1983) by reduction in the uptake of mineral nutrients (Adams 1981, Larcher 2003).

It is concluded that reduction in growth of both pine species at the damaged site is due to various physiological stresses induced by air pollutants. In particular, Cl and F discharged from nearby industrial regions suppressed growth of $P. koraensis$ and $P. rigida$. Moreover, $P. rigida$ is less tolerant to pollutants and excess Mn in needles than $P. koraensis$, and needle loss in $P. rigida$ at the damaged site was accelerated more than for $P. koraensis$ at the damaged site.
Chapter 3
Effect of soil acidification on growth and physiological activities of four pine species seedlings inoculated with ectomycorrhiza

3.1 Introduction
In recent years, growth of Pitch pine (*Pinus rigida*), Korean pine (*Pinus koraiensis*), Red pine (*Pinus densiflora*) and Japanese larch (*Larix kaempferi*), which are the representative pine species for making plantation in Korea, has been suppressed near industrial areas and large cities with inhibition of ectomycorrhiza development (Lee *et al.* 1996, Choi *et al.* 2003, 2006). These declining symptom are partly related to the edaphic condition of Korean forests where soils are originated from granite. Pine species are dominant in the granite derived soils. Many studies show that excess acid precipitation accelerate soil acidification, as a result forest ecosystem has declined (Van Breemen *et al.* 1982, Rollwagen and Zasoski 1988, Larcher 2003). Moreover, rehabilitation practices with pine species are employed near industrial regions, although the initial mortality of planted seedlings is high; this appears to be due to physiological imbalances of plantation seedlings or harsh environmental conditions, especially the soil environment. Excess Mn can prevent photosynthetic activity by reducing Rubisco activity and/or PS II (Kitao *et al.* 1997a, 1997b). However, little is known about the effect of Al and Mn on the development, the nutrient status and the physiological functions of pine species (Hecht-Buchholz *et al.* 1987, Miyake *et al.* 1991, Izuta *et al.* 1995, 1996a, 1996b, Kohno *et al.* 1995, Choi *et al.* 2005a, 2006).

Soil acidification due to dry or wet deposition is generally accompanied by increased leaching of base cations (BC) such as Ca$^{2+}$, Mg$^{2+}$ and K$^{+}$ ions from the soil to the ground water, and an increase in the solubility of phytotoxic metals such as Al and Mn in the rhizosphere (Ulrich *et al.* 1980, Yoshida and Kawahata 1988). Aluminum dissolved in the soil can inhibit the uptake of mineral elements essential for plant growth and absorption of water from the soil, as a result of poor differentiation of root meristems (Foy *et al.* 1978, Rengel 1992). This would cause a disturbance of the nutrient status of forest trees. Tree vitality is strongly influenced by nutrient deficiency, an imbalance of cations, excess accumulation of Al and Mn under high N loading, etc. (Ulrich *et al.* 1980, Van Breemen *et al.* 1982, Ulrich 1989). Declining tree vitality and health are believed to lead poor development of the root system, fungal growth and symbiotic micro-organisms (Dighton and Skelfington 1987, Nakaji 2002).

Infection with ectomycorrhizal fungi can increase the ability of plants to tolerant environmental stresses, especially infertile condition, desiccation, etc. (Brown and Wilkins 1985, Godbld *et al.* 1998, Kayama *et al.* 2005). Ectomycorrhizal association can reduce toxicity of metals such as Al, Mn, Zn, Cu and Ni, which has analysis of the interaction between the mycorrhizal fungi and host plant species (Wilkins 1991, Jentschke and Godbld 2000, Ahonen-Jonnarth *et al.* 2003). Al and/or Mn, increased soil acidity caused by acid precipitation, have been affecting mycorrhizal fungi and uptake of nutrient elements (Thompson and Medve 1984, Jentschke and Godbld 2000). However, symbiosis with ectomycorrhizae that enhances tolerance against heavy metals, such as Al and Mn, should result in an increase in physiological activities, growth and survival of ectomycorrhizal plants on acidified soil (Cumming and Wenstein 1990, Ahonen-Jonnarth *et al.* 2003).

The objective of this chapter is to evaluate the possible role of ectomycorrhiza in modifying the effects of acidified forest soil in nutrient uptake, physiological activity and growth of pine species. For accessing this purpose, three ectomycorrhiza species on four pine species were examined, for estimating sensitivity to soil acidification of four pine species and the tolerance of ectomycorrhizal seedlings. The findings in this study should be useful in improvement in the regeneration and conservation of acid-damaged forest ecosystems.

3.2 Materials and Methods

3.2.1 Plant materials
Seeds of Pitch pine (*Pinus rigida* Miller), Korean pine (*Pinus koraiensis* Sieb. et Zucc.), Red pine (*Pinus densiflora* Sieb. et Zucc.) and Japanese larch (*Larix kaempferi* Sarg.) were collected at the Yongmun Experimental Field of the Korea Forest Service (37.3°N, 127.2°E). After seeds were put in 4°C for 10-day cold treatment, they were surface sterilized with 30% H$_2$O$_2$ for 20 min and rinsed 4-5 times with sterile deionized water, and were then germinated on sterile media, black sand : vermiculate : peat moss = 3:2:1 (volume), in a glasshouse at day/night temperatures 25/20°C with a 16-h photoperiod.

3.2.2 Ectomycorrhiza
Ectomycorrhiza fungi, *Pisolithus tinctorius* (Pers.) Coker et Couch (*Pt*), and the Diehard Ecto drench (EC) - (*Pt* + *Rhizopogon* spp. + *Laccaria* spp. + *Scleroderma* spp.), were obtained from Horticultural Alliance, Inc., USA. *Cenococcum geophilum* Fr. (*Cg*) was isolated from root of *Picea glehnii* (Fr. Schm.) collected from a mixed larch and spruce stand located at the experimental forests of Forestry and Forest Products Research Institute (FFPRI), Sapporo, Japan. The isolated fungal culture and the culture obtained from other laboratory were maintained on modified MMN (Modified Melin-Norkrans) agar medium, and mycorrhizal synthesis was followed further inoculation study (Qu *et al.* 2003). The *Pt* and EC spores were suspended in distilled water. The *Pt*, EC or *Cg* were inoculated directly to roots of each species seedlings and mixed with sterilized brown forest soil of each acidic treatment. The seedlings inoculated with each ectomycorrhiza were naturalized in plastic pots (upper diameter 10.5 cm, height 8.5 cm, lower diameter 7.5 cm) filled with sterilized brown forest soil of each acidic soil treatment.

3.2.3 Soil treatments
Brown forest soil originating from granite was
collected from the organic layer 0-10 cm deep removed from under the stands of pine trees in the town of Shintoku (Hokkaido, Japan). The soil was sieved through a 5-mm mesh immediately after collection to homogenize it. Because of common soil condition in Korea, I used this type of granite soil for this experiment.

An acid solution having anions in the mol ratio as $\text{SO}_4^{2-} : \text{NO}_3^- : \text{Cl}^- = 5 : 3 : 2$, prepared from $\text{H}_2\text{SO}_4$, $\text{HNO}_3$ and $\text{HCl}$, was applied to the soil. This is the forest soil. The concentration of $\text{H}^+$ ions in the soil was 0, 10, 30, 60 and 90 mmolH$^+$kg$^{-1}$. Soil used as a control was not supplemented with $\text{H}^+$. One month after acidification of the soil, seedlings of each pine species were planted individually in plastic pots to simulate rehabilitation practice. In a well-ventilated greenhouse, dry deposition must have occurred. The seedlings were irrigated with distilled water during the growth period for 180 days.

### 3.2.4 Analysis of elements in soil and soil pH

The soil pH was determined using a pH meter (CH-8603, Mettler-Toledo AG) and a soil suspension left undisturbed for one hour. The suspension was made by mixing 20 g of fresh soil and 50 ml of distilled water in a 100-ml beaker with stirring using a glass stick every 20 minutes for 1 hour.

The rest of the soil sample was sieved through a 1-mm mesh after drying in room conditions, and was used for element analysis. The concentration of elements (Ca, Mg, K, Al and Mn) was determined by ICP analysis (IRIS, Jarrel Ash, Franklin, MA, USA) after mixing 20 g of soil with 100ml of distilled water in a 300-ml Erlenmeyer flask, which was then shaken for 1 hour at 25ºC in the shacking incubator.

### 3.2.5 Infection rate with ectomycorrhiza

It was observed that the root tips and counted both infected and non-infected roots (Quoreshi 2003). The inoculation rate of ectomycorrhiza (IRE) was determined according to the following formula : IRE (%) = ER/(ER+NR)×100, where ER and NR respectively denote the number of ectomycorrhizal and non-ectomycorrhizal roots.

### 3.2.6 Measurement of element concentrations in plants

The dried plant tissues were ground to a fine powder using a vibrating sample mill (Wonder Blender, Osaka Chemical Co., Osaka, Japan). To determine the concentrations of elements (P, Al and Mn), dried samples were digested by the microwave digestion system (O-I Analytical, College Station, TX, USA) prior to ICP analysis (IRIS, Jarrel Ash, Franklin, MA, USA). The nitrogen concentration in the shoots and roots was determined with a N/C analyzer (Yanagimoto MT 500W, Tokyo, Japan).

### 3.2.7 Gas exchange characteristics of needles

Net photosynthetic rate of each species was examined using an open gas exchange system (LI-6400, Li-Cor, Lincoln, NE, USA) between 09:00 and 15:00 local time. From 4 to 5 cm of above ground part was covered with a conifer chamber. The change in net photosynthetic rate was measured at PAR saturation, corresponding to a photosynthetic photon flux density (PPFD) of 1000-1200 μmol·m$^{-2}$·s$^{-1}$ which was provided by a cool halogen lamp (Walz, Effeltrich, Germany). The leaf temperature was 25ºC and the relative humidity was 50-70%.

### 3.2.8 Plant growth analysis

To determine the growth rate of the seedlings, five seedlings were randomly sampled from each species and each treatment to determine the total dry mass (TDM) of each organ of the seedlings (shoot, root). The sampled seedlings were washed with distilled water and separated into shoots and roots. All plant organs were dried at 60ºC for 1 week and weighed using an electronic balance (HR-202, A&D, Japan).

The relative total dry mass (TDM) of the seedlings was calculated as follows : relative TDM(%) = (average TDM of each seedling grown at each soil acidified and each ectomycorrhiza treatment) / (average TDM of the seedlings grown in the control soil)×100.

### 3.2.9 Statistical analysis

The plants were arrayed in a randomized complete block design involving all five treatments (control, 10, 30, 60 and 90 mmolH$^+$kg$^{-1}$), with 6 single plant replications of each ectomycorrhiza treatment and each species. Analyses were performed using the Mixed and Genmod procedures of the SAS system (SAS Institute, Inc., 1998). Tukey’s Studentized Range (HSD) test was used to separate means at $p<0.05$.

### 3.3 Results

#### 3.3.1 Soil condition

Table 3.1 shows the pH and concentration of water-soluble elements in the soil solution. Unsurprisingly, the pH of the soil solution decreased with increasing H$^+$ load. The concentration of Al and Mn in water-soluble form increased with decreasing soil pH, with an abrupt increase in Al at soil pH (H$_2$O) = 3.77, 60 mmolH$^+$kg$^{-1}$ treatment. Also, the concentrations of base cations (Ca, Mg, K) in water-soluble increased with decreasing soil pH. The concentration of Ca increased abruptly at soil pH (H$_2$O) 3.77; the increase for K and Mg were less than for Ca.

#### 3.3.2 Mycorrhizal development

Fig. 3.1 shows the inoculation rate of each ectomycorrhiza ($P_t$, EC or $C_g$) for $P$. rifida, $P$. koraiensis, $P$. densiflora and $L$. kaempferi after 180 days grown at each soil treatment. Each ectomycorrhizal development of each pine species was significantly increased at 10 and 30 mmolH$^+$kg$^{-1}$ treatments compared with control (0 mmolH$^+$kg$^{-1}$) treatment ($P<0.05$). However, at the 60 and 90 mmolH$^+$kg$^{-1}$ treatments significantly decreased than
that of control treatment. Moreover, I did not find that any other ectomycorrhiza development in the non-inoculated each seedling grown at each soil treatment.

3.3.3 Condition of chemical elements in plant organs

Fig. 3.2 shows the concentration of nitrogen in needles for each species and each soil treatment. The concentration of N in needle tended to increase with progressive addition of mixed acid solution (H₂SO₄, HNO₃ and HCl). In particular, at the 60 and 90 mmolH⁺·kg⁻¹ treatments at each species and each ectomycorrhiza treatment was significantly greater than in controls (P<0.05).

<table>
<thead>
<tr>
<th>Soil treatment pH (H₂O)</th>
<th>Element concentration (μg·g⁻¹)</th>
<th>(Ca+Mg+K)/Al (mol·mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca</td>
<td>Mg</td>
</tr>
<tr>
<td>control</td>
<td>4.9±0.3</td>
<td>66.2±3.1</td>
</tr>
<tr>
<td>10 mmolH⁺·kg⁻¹ 4.45±0.03</td>
<td>92.9±4.67</td>
<td>6.66±0.02</td>
</tr>
<tr>
<td>30 mmolH⁺·kg⁻¹ 3.85±0.03</td>
<td>157.0±7.90</td>
<td>14.58±0.02</td>
</tr>
<tr>
<td>60 mmolH⁺·kg⁻¹ 3.77±0.02</td>
<td>231.2±11.56</td>
<td>17.91±0.01</td>
</tr>
<tr>
<td>90 mmolH⁺·kg⁻¹ 3.18±0.03</td>
<td>263.6±13.18</td>
<td>21.24±0.01</td>
</tr>
</tbody>
</table>

For ectomycorrhizal seedlings grown with Pt, EC or Cg in acidified soils, the concentration of Al in roots and Mn in needles significantly lower than non-ectomycorrhizal (NE) seedlings, at each species and each soil treatment (P<0.05) (Fig. 3.3, 3.4). And, the concentration of Al in roots and Mn in needles increased with increasing add H⁺, especially 60 and 90 mmolH⁺·kg⁻¹ treatments relative to controls. Addition of solution containing H⁺ to the soil increased the concentration of N and Mn in the needles and Al in the roots. And, I did not find that the difference of Mn concentration in needle for L. kaempferi seedlings grown at 90 mmolH⁺·kg⁻¹ treatment.

Table 3.1. Initial pH and concentration of elements in soil immediately before transplantation planted with each seedling for a growth period of 180 days. Mean of 5 pots ± SE. Data within a series followed by the same small letter do not differ significantly (P<0.05) in each item.

Fig. 3.1. The ectomycorrhiza infection rate of each species grown at control (0), 10, 30, 60 and 90 mmolH⁺·kg⁻¹ treatments after 180 days. Mean of 5 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species. Pt, EC and Cg represented inoculated seedlings by Pisolithus tintorius, Ectodrench or Cenococcum geophilum, respectively.
Fig. 3.2. The concentration of N in needle of each seedling at soil treatment of control (0), 10, 30, 60 and 90 mmolH⁺·Kg⁻¹ grown with each ectomycorrhiza at 180 days. Mean of 5 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species. NE represented non-ectomycorrhizal seedlings and Pt, EC and Cg represented inoculated seedlings by *Pisolithus tintorius*, Ectodrench or *Cenococcum geophilum*, respectively.

Fig. 3.3. The concentration of Al in roots of each seedling at soil treatment of control (0), 10, 30, 60 and 90 mmolH⁺·Kg⁻¹ grown with each ectomycorrhiza at 180 days. Mean of 5 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species. NE represented non-ectomycorrhizal seedlings and Pt, EC and Cg represented inoculated seedlings by *Pisolithus tintorius*, Ectodrench or *Cenococcum geophilum*, respectively.
3.3.4 Photosynthetic response

Fig. 3.5 shows the maximum net photosynthetic rate at saturated CO₂ concentration (P_max) of each seedling at soil treatment of control (0), 10, 30, 60 and 90 mmolH⁺·kg⁻¹ grown with each ectomycorrhiza at 180 days. Mean of 5 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species. NE represented non-ectomycorrhizal seedlings and Pt, EC and Cg represented inoculated seedlings by *Pisolithus* _tintorius_, *Ectodrench* or *Cenococcum* _geophilum_, respectively.

3.3.5 Plant responses to deliberate soil acidification

The dry mass of the whole plant of NE, Pt, EC or Cg at each species increased to 10 or 30 mmolH⁺·kg⁻¹, but I did not find significant difference with control treatment, but *L. kaempferi* seedlings grown with Pt or EC show significant difference (Fig. 3.6). However, the TDM of each species and each ectomycorrhiza treatment decreased significantly for the 60 and 90 mmolH⁺·kg⁻¹ treatments, relative to the control and the 10 and 30 mmolH⁺·kg⁻¹ treatments, even if that of ectomycorrhizal seedlings of each species increased, which was less for NE seedlings.

3.4 Discussion

Most possible factors limiting the growth and nutrient status of plants grown in acid soil are the high soil acidity itself, phyto-toxic metals such as Al or Mn dissolved in the soil solution, and reduced availability of certain elements essential for plant growth (Adams 1981, Ulrich 1989, Kochian 1995). A high concentrations of Al or Mn in nutrient solutions or in acidic soils reduces the uptake of elements essential for growth and the uptake of water through the roots, and reduces the net photosynthetic rate, transpiration rate and leaf chlorophyll concentration of several plants (Suresh et al. 1987, Nable et al. 1988, Macfie and Taylor 1992, Kitao et al. 1997a, 1997b).

The activity of the meristems of the root tip is inhibited by Al (Kelly et al. 1990) or by deficiencies of Ca and Mg induced by antagonism at the root surface (Ericsson et al. 1995), which reduces root development. Moreover, even though Mn is a co-factor of photosynthesis (Marschner 1995), excess Mn in foliage organs usually reduces photosynthetic function at PS II (Kitao et al. 1997a, 1997b). These combined effects of Al in roots and Mn in needles may reduce the photosynthetic rate and growth of *P. rigida*, *P. koraiensis*, *P. densiflora* and *L. kaempferi*. Similar tendencies have been observed in the red pine (Lee et al. 1999) and Japanese cedar (*Cryptomeria japonica*) seedlings treated with H₂SO₄ (Miwa et al. 1994) and AlCl₃ (Izuta et al. 1996b).

The concentrations of elements essential for growth, such as Ca, Mg, K, and P are reduced in conifers grown in a nutrient solution having a pH below 4.0 or in acidic soil (Izuta and Totsuka 1996, Yang et al. 1996). However, in the present study, the nutrient status of each species and each mycorrhizal treatment were not...
Fig. 3.5. Maximum net photosynthetic rate at saturated CO₂ concentration (P<sub>max</sub>) of each seedling at soil treatment of control (0), 10, 30, 60 and 90 mmolH<sup>+</sup>·Kg<sup>-1</sup> grown with each ectomycorrhiza at 180 days. Mean of 5 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species. NE represented non-ectomycorrhizal seedlings and Pt, EC and Cg represented inoculated seedlings by <i>Pisolithus tintorius</i>, Ectodrench or <i>Cenococcum geophilum</i>, respectively.

Fig. 3.6. Effect of soil acidification on dry mass of <i>Pinus rigida</i>, <i>P. koraiensis</i>, <i>P. densiflora</i> and <i>Larix kaempferi</i> seedlings. NE represented non-ectomycorrhizal seedlings and Pt, EC and Cg represented inoculated seedlings by <i>Pisolithus tintorius</i>, Ectodrench or <i>Cenococcum geophilum</i>, respectively. Mean of 5 seedlings ± SE. Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species.
adversely affected by extremely acidified forest soil (data not shown). Similar tendencies have been observed in plant organs grown in low pH solution (Hutchinson 1980, Kim 1986, Izuta et al. 1996b, Choi et al. 2005a). This shows that the ability to take up essential nutrients via the roots of each species was not affected by extremely acidified forest soil. Moreover, the concentration of N in needle at each species was significantly greater than in controls, especially in the 60 and 90 mmolH⁺·kg⁻¹ treatments. Why did the N content in each organ increase significantly with added acid solution compared to the control treatment? The reason is as follows: nitrogen can be absorbed by plants in three distinct forms: nitrate, ammonium, and amino acids (Lamber et al. 1998).

Therefore, it was believed that the seedlings grown in the 60 and 90 mmolH⁺·kg⁻¹ treatments can absorb more nitrogen than controls, because I administered an acid solution comprising H₂SO₄, HNO₃ and HCl. Nitrogen and phosphorus are found to limit tree growth by reducing photosynthetic activity more frequently than any other nutrient (Fisher and Binkley 2000). The present results suggest that the main reason for the growth reduction and photosynthesis depression of each species grown in extremely acidified forest soil (e.g. 60 and 90 mmolH⁺·kg⁻¹ treatments) is accumulation of Al and Mn in root or needle not due to nutrient deficiency.

The molar ratios of cations such as Ca, Mg, and K to Al in soil or nutrient solutions are important factors limiting the growth and nutrient status of woody plants (Hecht-Buchholz et al. 1987, Kruger and Sucoff 1989, Sverdrup et al. 1994, Cronan and Grigal 1995). This study found a strong positive correlation between the relative TDM of each seedling and the molar water-soluble (Ca+Mg+K)/Al (i.e. BC/Al) molar ratio in the soil (Fig. 3.7).

Sverdrup et al. (1990) proposed critical limits for growth: forest soil solution pH lower than 4.4 for the B horizon; labile aluminum higher than 2.0 mg L⁻¹; and BC/Al molar ratio lower than 1.0. Similar relations between plant growth and BC/Al in spruce or pine native to Europe and America have been reported (Rengel 1992, Sverdrup et al. 1994, Cronan and Grigal 1995), and this reduction in growth of pine seedlings grown on acidified soil is due to the effect of nutrient imbalance between Al and (Ca + Mg + K) in the soil.

This study found the reduction of the relative TDM of *P. koraiensis* and *L. kaempferi* seedlings of approximately 40% at a BC/Al ratio of 1.0, but that of *P. rigida* and *P. densiflora* of approximately 50% at a BC/Al ratio of 1.0 (Fig. 3.7). And, also relative TDM of ectomycorrhizal seedlings of each species decreased at a BC/Al ratio of 1.0 compared with their control treatment (i.e. control treatment of each ectomycorrhizal seedling), approximately the same ratio compared with each non-ectomycorrhizal species. However, ectomycorrhizal seedlings of each species increased relative TDM by 20-100% at a BC/Al ratio of 1.0 than NE seedlings of each species, even if those also reduced. My results indicate that *P. koraiensis* and *L. kaempferi* tended more tolerant to BC/Al than *P. rigida* and *P. densiflora*. Moreover, ectomycorrhizal infection significantly increased nutrients uptake and significantly protected plant growth against Al and Mn (Wilkins 1991, Jentschke and Godbold 2000, Ahonen-Jonnarth et al. 2003) compared with...
non-ectomycorrhizal seedlings of each species. As a result, the growth decline at the acidified forest soil was moderated as compared with non-ectomycorrhizal seedlings.

Further studies will be needed to clarify the physiological function and status of pine species with ectomycorrhizae under severe soil acidification, i.e. BC/Al ratio of 0.01-1.0.

Chapter 4
Effect of ectomycorrhizal infection on growth and photosynthetic characteristics of three pine species seedlings grown under elevated CO2 concentrations

4.1 Introduction
Pine species (Pinus rigida, P. koraiensis, P. densiflora and Larix kaempferi) is the dominant conifer in infertile granite regions in the Korean peninsula. These species can survive and grow on the nutrient-poor ridges of mountains after disturbances. These species usually has a symbiotic interaction with ectomycorrhizae allowing for vigorous growth. When there have been several disturbances, i.e. forest decline, fire, typhoon etc., we have been planted pine species as pioneer for forest rehabilitation practices in nutrient-poor soils (e.g. Moroto et al. 1987), because of their fast growth drought tolerant traits. In particular after forest fires, charcoal in soil increases germination rate and surviving living seedlings for increases the aperture of soil, holds the soil moisture and raises soil temperature (Debano et al. 1998).

The pattern and amount of precipitation at middle and high latitudes have recently been predicted to undergo great changes with the increase in atmospheric CO2 concentration [CO2] (IPCC 1996). Precipitation could decrease in middle latitude. Moreover, photosynthetic adjustment in down-wards is frequently observed in plants grown under high [CO2] because of dilution effects of nutrient reduction of enzyme activities, enhanced accumulation of photosynthates in photosynthetic organs and sink strength (Wardlaw 1990, Farrar and Williams 1991, Berntson and Bazzaz 1996).

Symbiotic micro-organisms such as ectomycorrhiza usually act as an efficient root system for absorbing water and essential nutrients (nitrogen, phosphate) (Bolan 1991, Smith and Read 1997, Quoreshi et al. 2003). The activity of the host plants should be enhanced at high [CO2] because symbiotic ectomycorrhiza play an essential role in absorbing water and important elements such as nitrogen and phosphate, and also act as a large carbon sink (Ceulemans and Mousseau 1994, Smith and Read 1997). Therefore, no down-regulation of photosynthesis is predicted following pine species inoculation with ectomycorrhiza at high [CO2] as observed in Pinus densiflora (Choi et al. 2005b).

Several ectomycorrhizae form symbiotic interactions between pine species (Smith and Read 1997). Of these, Pisolithus tinctorius (Pers.) Coker et Couch (Pt) and Cenococcum geophilum Fr. (Cg) are the most common species in pine forests world-wide (Marx et al. 1984, Trappe 1988, Allen 1996). I study here the effect of ectomycorrhizae, e.g. Pt, Cg and mixed mycorrhiza (EC) on the physiological responses of three pine tree seedlings under high [CO2]. My aim is to estimate the effect of ectomycorrhiza association and development on down-regulation of photosynthesis responses and the growth of pine species at increased [CO2] through stomatal limitation and P concentration in needles.

4.2 Materials and methods
4.2.1. Experiment site and design
This experiment with Pinus koraiensis, P. densiflora and Larix kaempferi was conducted at the Hokkaido Research Center, Forestry and Forest Products Research Institute (FFPRI), Sapporo (Japan) in 2002. The seedlings were grown in a FFPRI phytotron with a natural sunlight, day/night temperature range of 26/16 °C and humidity range of 55-75% during the study period lasting 18 weeks. Six cabinets were used for cultivation, with two differing [CO2]. The rhizo-boxes were allocated at random such that half of the seedlings experienced ambient [CO2] (360 μmol mol⁻¹) and the other half experienced elevated [CO2] (720 μmol mol⁻¹) (Koike 1995). At each CO2 concentration (three cabinets of phytotron), the each seedling was inoculated with ectomycorrhiza fungus (Pt, EC or Cg) or non-ectomycorrhizal seedlings (NE). The seedlings number of each treatment in a cabinet was sixteen. Each treatment was repeated in three cabinets, and the subgroups are denoted as follows: (1) 360 μmol mol⁻¹, NE, (2) 360 μmol mol⁻¹, inoculated (Pt, EC or Cg), (3) 720 μmol mol⁻¹, NE, (4) 720 μmol mol⁻¹, inoculated (Pt, EC or Cg).

4.2.2 Plant materials
Seeds of Korean pine (Pinus koraiensis Sieb. et Zucc.), Red pine (Pinus densiflora Sieb. et Zucc.) and Japanese larch (Larix kaempferi Sarg.) were collected at the Yongmun Experimental Field of the Korea Forest Service (37.3ºN, 127.2ºE). After seeds were put in 4°C for 10-day cold treatment, they were surface sterilized with 30% H2O2 for 20 min and rinsed 4-5 times with sterile deionized water, and were then germinated on sterilized media, black sand : vermicululate : peat moss = 3:2:1 (volume), in a glasshouse at day/night temperatures 25/20°C with a 16-h photoperiod.

4.2.3 Ectomycorrhizae
Ectomycorrhiza fungi, Pisolithus tinctorius (Pers.) Coker et Couch (Pt) and Diehard Ecto drench (EC) - (Pt + Rhizopogon spp. + Laccaria spp. + Scleroderma spp.), were obtained from Horticultural Alliance, Inc., USA. Cenococcum geophilum Fr. (Cg) was isolated from root of Picea glehnii (Fr. Schm.) collected from a mixed larch and spruce stand located at the experimental forests of Forestry and Forest Products Research Institute (FFPRI), Sapporo, Japan. The isolated fungal culture and the culture obtained from the laboratory of Forest Resource Biology, Hokkaido University (offered by Dr. Y. Tamai) were maintained on modified MMN agar medium and mycorrhizal synthesis was followed further inoculation study (Qu et al. 2003). The Pt and EC spores were suspended in
distilled water. The Pt, EC or Cg were inoculated directly to roots of each species seedlings and mixed with prepared soil media (see below). The seedlings inoculated with each ectomycorrhiza were naturalized in a rhizo-box (100×230×18 mm) filled with sterilized medium consisting of vermiculate : black sand : peat moss = 2:2:1 (in volume).

4.2.4 Measurement of photosynthesis

The A/Ci (A=net photosynthetic rate, Ci=intercellular CO₂ concentration) curves were examined using an open gas exchange system (LI-6400, Li-Cor, Lincoln, NE, USA) between 09:00 and 15:00 local time. 5 - 7 cm of above ground part was covered with a conifer needle. The change in A was measured at PAR (photosynthetic active radiation) saturation, corresponding to a photosynthetic photon flux density (PPFD) of 1000-1200 μmol m⁻²s⁻¹ which was provided by a cool halogen lamp (Walz, Effeltrich, Germany). The leaf temperature was 25°C and the relative humidity was 50-70%. Foliages were allowed to acclimatize to their surroundings for 10 min prior to measurement, and then determined with 150-1500 μmol mol⁻¹ CO₂ concentrations.

The initial slope of the A/Ci curve is proportional to the carboxylation activity of Rubisco [i.e., the carboxylation efficiency (CE, μmol Pa⁻¹)]. CE was calculated from the increment in intercellular CO₂ partial pressure (Pi) and the net photosynthetic rate of the initial slope of the A/Ci curve (Terashima 1992).

The maximum net photosynthetic rate at CO₂ saturation concentration (Pₚₙₙ, μmol m⁻²s⁻¹) was determined of 1200 μmol mol⁻¹ CO₂. The relative stomatal limitation of photosynthesis (ls, %) was calculated as follows, based on the A to variation in Ci (A/Ci curves) : ls = (1 – Aᵢ/Aᵢ) × 100, where Aᵢ is the actual net photosynthetic rate and Aᵢ is the net photosynthetic rate when stomatal resistance to CO₂ diffusion is zero (Sharkey 1985a). After the gas exchange measurement, the needle projection area was measured with an image scanner (FB636U, Canon, Japan), and then calculated the net photosynthetic rate per unit needle area.

4.2.5 Infection rate of ectomycorrhizae

The root tips was observed and counted both infected and non-infected roots (Quoreshi 2003). The inoculation rate of ectomycorrhizae (IRE) was determined according to the following formula:

IRE (%) = ER/(ER+NR)×100,

where ER and NR respectively denote the number of ectomycorrhizal and non-ectomycorrhizal roots.

4.2.6 Growth rate

Shoot and root dry mass of each seedling was measured after dried at 60°C for 1 week using an electronic balance (HR-202, A&D, Japan).

4.2.7 Phosphorus analysis

The dried samples were then ground to a fine powder in a vibrating sample mill (Wonder Blender, Osaka Chemical Co., Osaka, Japan). To determine the concentration of P, the samples were digested by a microwave digestion system (O/I analytical, College Station, TX) and underwent ICP analysis.

4.2.8 Germination with charcoal

The germination rate of P. densiflora P. rigida and L. kaempferi seeds was examined on sterilized brown forest soil (Chapter 3.2.3) or brown forest soil mixed charcoal. The cold treatment and surface sterilization are follows like up (Chapter 4.2.2). The prepared seeds were germinated in a FPFR phytotron under 360 μmol mol⁻¹ and 720 μmol mol⁻¹ (Chapter 4.2.1). The germinated seedlings tested inoculation rate under the same condition with EC of several ectomycorrhizae (Chapter 4.2.3).

4.2.9 Statistical analysis

Mean values of the inoculation rate, the P concentration in needles, Pₚₙₙ, ls, CE, RuBP regeneration rate and the shoot and root growth were examined by the t-test (Li 1964) using the Stat View 5.0 software (SAS Institute, Cary, NC, USA)

4.3 Results

4.3.1 Shoot and root growth

Different ectomycorrhizal inoculation induced large differences in dry mass between non-ectomycorrhizal seedlings at each species (Fig. 4.1). In P. koraiensis, Pt or EC inoculation significantly increased the shoot and root dry mass relatively to non-ectomycorrhizal seedlings grown at 720 μmol mol⁻¹ [CO₂] (P<0.05), but I did not find that significantly difference between those grown at 360 μmol mol⁻¹ [CO₂]. In P. densiflora, the shoot and root dry mass of ectomycorrhizal seedlings (Pt, EC or Cg) significantly increased relatively to non-ectomycorrhizal seedlings grown at 360 μmol mol⁻¹ [CO₂] and 720 μmol mol⁻¹ [CO₂], with the exception of Pt at 720 μmol mol⁻¹ [CO₂]. In particular, the seedling inoculated with EC was greater than that inoculated with other ectomycorrhizae (e.g. Pt and Cg). Also, dry mass of L. kaempferi seedlings grown with ectomycorrhiza significantly increased compared with non-ectomycorrhizal seedlings grown at 360 μmol mol⁻¹ [CO₂] and 720 μmol mol⁻¹ [CO₂] (P<0.05). Ectomycorrhiza inoculation increased dry mass of P. koraiensis, P. densiflora and L. kaempferi seedlings at both CO₂ concentration and especially EC inoculation more increased dry mass of each species relatively to Pt or Cg inoculation.

4.3.2 Mycorrhizal development and P concentration in needles

Fig. 4.2 shows the infection rate of ectomycorrhiza (Pt, EC and Cg) after 18 weeks growth at both ambient and elevated CO₂ concentrations. Ectomycorrhizal development of EC and Cg for P. koraiensis, P. densiflora and L. kaempferi was significantly affected by the CO₂ concentration (P<0.05). Moreover, Pt development with P. koraiensis was significantly increased grown at 720 μmol mol⁻¹ [CO₂] than that at 360 μmol mol⁻¹ [CO₂], however, that of P. densiflora and L. kaempferi was not significantly increased than...
Fig. 4.1. Effect of ectomycorrhizal inoculation by Pt, EC or Cg on the shoot and root dry mass of P. koraiensis, P. densiflora and L. kaempferi seedlings and non-inoculated (NE) seedlings grown at 360 μmol·mol⁻¹ or 720 μmol·mol⁻¹. Vertical bars represent the standard error of its mean (±SE). Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species. Pt, EC and Cg represented inoculated seedlings by Pisolithus tintorius, Ectodrench or Cenococcum geophilum, respectively.

Fig. 4.2. Ectomycorrhizal infection rate of P. koraiensis, P. densiflora and L. kaempferi seedlings inoculated Pt, EC or Cg seedlings grown at 360 μmol·mol⁻¹ or 720 μmol·mol⁻¹. Vertical bars represent the standard error of its mean (±SE). Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species. Pt, EC and Cg represented inoculated seedlings by Pisolithus tintorius, Ectodrench or Cenococcum geophilum, respectively.
360 μmol·mol⁻¹ [CO₂], even if increased.

The P concentration in needles and roots inoculated with Pt, EC or Cg of each species was significantly higher than without inoculation at both CO₂ concentrations. (*P*<0.05) (Fig. 4.3). Especially, EC or Cg inoculation in *P. densiflora* was significantly increased P concentration in needles and roots than non-ectomycorrhizal seedlings and Pt inoculation seedlings.

### 4.3.3 Photosynthetic response

Fig. 4.4 shows the photosynthetic response to intercellular CO₂ (Ci) at PAR saturation i.e. (A/Ci) for *P. koraiensis*, *P. densiflora* and *L. kaempferi* seedlings grown at 360 μmol·mol⁻¹ [CO₂] and 720 μmol·mol⁻¹ [CO₂]. Values of A of *P. koraiensis*, *P. densiflora* and *L. kaempferi* inoculated with Pt, EC or Cg were significantly higher than those of control seedlings in both [CO₂] treatments. The maximum net photosynthetic rate at saturated CO₂ concentration (*P*ₘₐₓ) of each seedling inoculated with Pt, EC or Cg was higher than for controls (*P*<0.05) (Table 4.1). Moreover, the carboxylation efficiency (CE) and RuBP regeneration rate of the A/Ci curve for each species inoculated with Pt, EC or Cg were significantly higher than for non-inoculated seedlings at both CO₂ concentrations, and especially at 720 μmol·mol⁻¹ [CO₂] except for *P. koraiensis* at 360 μmol·mol⁻¹ [CO₂] (Table 4.1 and Fig. 4.5, *P*<0.05). And, these of non-ectomycorrhizal seedlings of each species show photosynthetic down-regulation, i.e. carboxylation efficiency and RuBP regeneration rate decreased at 720 μmol·mol⁻¹ [CO₂] than 360 μmol·mol⁻¹ [CO₂], but I did not find the tendency at the inoculated seedlings.

#### 4.3.4 Stomatal limitation

Stomatal limitation (ls) of *P. koraiensis* seedlings at each CO₂ concentration and *L. kaempferi* at 720 μmol·mol⁻¹ inoculated with Pt or EC was lower than that of non-inoculated seedlings, but I did not find significant difference (Fig. 4.6). On the other hand, ls of *P. densiflora* at each CO₂ concentration and *L. kaempferi* at 360 μmol·mol⁻¹ inoculated with EC or Cg was significantly lower than that of non-inoculated each seedling (*P*<0.05)

#### 4.3.5 Germination rate and ectomycorrhizal infection rate in soil with mixing charcoal

Germination rate of *P. rigida*, *P. densiflora* and *L. kaempferi* sow seeds in a forest soil mixed charcoal after 21 days was significantly higher than that sow seeds in a forest soil grown at 360 μmol·mol⁻¹ and 720 μmol·mol⁻¹ [CO₂] (*P*<0.05) (Fig. 4.7). However, the ectomycorrhizal infection rate of *P. rigida*, *P. densiflora* and *L. kaempferi* seedlings grown at forest soil mixed charcoal decreased significantly as compared with at forest soil each CO₂ treatment (*P*<0.01) (Fig. 4.8).

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**Table 4.1. Parameter estimates as functions of the A/Ci curve. Maximum net photosynthetic rate at saturated CO₂ concentration (*P*ₘₐₓ) and RuBP regeneration rate of photosynthesis in the needles of *P. koraiensis*, *P. densiflora* and *L. kaempferi* seedlings. Data within a series followed by the same small letter do not differ significantly (*P*<0.05) in each species.**

<table>
<thead>
<tr>
<th>CO₂ treatment (μmol·mol⁻¹)</th>
<th>Inoculation</th>
<th>RuBP Regeneration (μmol·m⁻²·s⁻¹)</th>
<th><em>P</em>ₘₐₓ (μmol·m⁻²·s⁻¹)</th>
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<td><strong>P. koraiensis</strong></td>
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<td>360</td>
<td>NE</td>
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<td>15.36 ± 1.03 a</td>
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<td>Cg</td>
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<td>7.10 ± 0.22 c</td>
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<td></td>
<td>Pt</td>
<td>56.56 ± 0.03 ab</td>
<td>11.13 ± 0.24 a</td>
</tr>
<tr>
<td></td>
<td>EC</td>
<td>103.64 ± 31.35 a</td>
<td>11.24 ± 0.63 a</td>
</tr>
</tbody>
</table>

*: NE represented non-inoculated seedlings. Pt, EC and Cg represented inoculated seedlings by *Pisolithus tintorius*, Ectodrench or *Cenococcum geophilum*, respectively.
Fig. 4.3. Phosphorus concentration of *P. koraiensis*, *P. densiflora* and *L. kaempferi* in shoot and root inoculated Pt, EC or Cg and non-inoculated (NE) seedlings grown at 360 μmol·mol⁻¹ or 720 μmol·mol⁻¹. Vertical bars represent the standard error of its mean (±SE). Data within a series followed by the same small letter do not differ significantly (P<0.05) in each species. Pt, EC and Cg represented inoculated seedlings by *Pisolithus tintorius*, Ectodrench or *Cenococcum geophilum*, respectively.

Fig. 4.4. Relationship between net photosynthetic rate (A) and intercellular CO₂ concentration (Ci) in *P. koraiensis*, *P. densiflora* and *L. kaempferi* seedlings inoculated Pt, EC or Cg and non-inoculated (NE) seedlings grown at 360 μmol·mol⁻¹ or 720 μmol·mol⁻¹. Pt, EC and Cg represented inoculated seedlings by *Pisolithus tintorius*, Ectodrench or *Cenococcum geophilum*, respectively.
Fig. 4.5. The carboxylation efficiency (CE) in *P. koraiensis*, *P. densiflora* and *L. kaempferi* seedlings inoculated *Pt*, *EC* or *Cg* and non-inoculated (NE) seedlings grown at 360 μmol·mol⁻¹ or 720 μmol·mol⁻¹. *Pt*, *EC* and *Cg* represented inoculated seedlings by *Pisolithus tintorius*, Ectodrench or *Cenococcum geophilum*, respectively. Data within a series followed by the same small letter do not differ significantly (*P*<0.05) in each species.

Fig. 4.6. Stomatal limitation (*ls*) in *P. koraiensis*, *P. densiflora* and *L. kaempferi* seedlings inoculated *Pt*, *EC* or *Cg* and non-inoculated (NE) seedlings grown at 360 μmol·mol⁻¹ or 720 μmol·mol⁻¹. *Pt*, *EC* and *Cg* represented inoculated seedlings by *Pisolithus tintorius*, Ectodrench or *Cenococcum geophilum*, respectively. Vertical bars represent the standard error of its mean (±SE). Data within a series followed by the same small letter do not differ significantly (*P*<0.05) in each species.
Fig. 4.7 Germination rate (%) of *P. rigidula, P. densiflora* and *L. kaempferi* sow seeds in forest soil or forest soil mixed charcoal after 21 days grown at 360 μmol·mol⁻¹ or 720 μmol·mol⁻¹. Vertical bars represent the standard error of its mean (±SE). (* P<0.05, ** P<0.01)

Fig. 4.8 Infection rate (%) of *P. rigidula, P. densiflora* and *L. kaempferi* seedlings in forest soil or forest soil mixed charcoal after 18 weeks grown at 360 μmol·mol⁻¹ or 720 μmol·mol⁻¹. Vertical bars represent the standard error of its mean (±SE). (* P<0.05, ** P<0.01, *** P<0.001)
4.4 Discussion

Inoculation with ectomycorrhizae significantly increased the dry mass of seedlings grown at both CO₂ concentrations after 18 weeks (Figs. 4.1). Previous research on the effect of ectomycorrhiza and elevated CO₂ concentration on the biomass growth of forest tree seedlings reveals a varied response (Tissue et al. 1996, Gavito et al. 2000). Growth improvement in ectomycorrhizal plants has been found in several species (Allen 1992, Mousseau et al. 1996, Norby et al. 1996, Tissue et al. 1996, Smith and Read 1997, Rouhier and Read 1998, 1999).

The infection rate of EC or Cg in P. koraiensis, P. densiflora and L. kaempferi seedlings grown at 720 μmol mol⁻¹ [CO₂] was significantly higher than at 360 μmol mol⁻¹ [CO₂]. The infection rate of Pt in P. densiflora and L. kaempferi seedlings grown at 720 μmol mol⁻¹ [CO₂] increased more than that at 360 μmol mol⁻¹ [CO₂], but these were not significant. The significantly high P concentration in needles of Pt, EC or Cg inoculated seedlings is therefore due to the development of ectomycorrhiza (Fig. 4.3). Previous studies have also found that ectomycorrhizal development in seedlings of several tree species at elevated [CO₂] was greater than at ambient [CO₂] (Seegmüller and Rennenberg 1994, Ineichen et al. 1995, Rey and Jarvis 1997 Runion et al. 1997, Rouhier and Read 1998). Ectomycorrhizal development enlarges the absorptive surface of the root, with widely ramified hyphae allowing the release of phosphatase, which enhance the availability of organic phosphate and exude organic acids. These interactions between host plant and ectomycorrhiza increase the use efficiency of limited soluble phosphate and organic N in soil (Smith and Read 1997, Lambers et al. 1998). The A, CE, RuBP regeneration rate and P max of Pt, EC or Cg inoculated P. koraiensis seedlings grown at 720 μmol mol⁻¹ [CO₂] and P. densiflora and L. kaempferi seedlings grown at both CO₂ concentrations were significantly higher than those of non-inoculated seedlings.

The P concentration in needles was also significantly greater in inoculated seedlings (see Table 4.1, Figs. 4.3, 4.5). These photosynthetic changes are regulated by the capacity of Rubisco to fix CO₂ and the capacity to regenerate ribulose-1,5-bisphosphate (RuBP) (Farquhar et al. 1980, Farquhar and Sharkey 1982). Under high and CO₂ saturation, phosphate (Pi) may limit photosynthesis if Pi utilization during CO₂ assimilation and RuBP regeneration exceed the capacity for Pi release during starch and sucrose synthesis (Sharkey 1985b, Lambers et al. 1998).

Ectomycorrhizal plants enhance the rate of photosynthesis over non-ectomycorrhizal plants as a result of improved plant nutritional status, including N and P (Reid et al. 1983, Bolan 1991, Finlay 1992, Smith and Read 1997, Quoreshi 2003, Quoreshi et al. 2003, Choi et al. 2005c), even if the P concentration is the same in needles (Rousseau and Reid 1990). Moreover, ectomycorrhizal improve ls (Guehl et al. 1990, Duñabeitia et al. 2004) by enlarging the soil volume exploited, via widely distributed hyphae (Smith and Read 1997, Lambers et al. 1998). In general, the P concentration in needles affects photosynthesis through RuBP regeneration (Sharkey 1985a, Fredeen et al. 1990, Kirschbaum and Tompkins 1990, Lewis et al. 1994,). through thylakoid (Conroy et al. 1986, Lambers et al. 1998) and through the peak carboxylation velocity or peak capacity of electron transport (Harley and Sharkey 1991). It was concluded that ectomycorrhiza development in inoculated seedlings and the increased P concentration in needles enhance the photosynthetic responses and reduce stomatal limitation relative to non-inoculated seedlings (Smith and Read 1997, Hensen et al. 1998) (Table 4.1, Figs. 4.3, 4.5, 6).

Net photosynthetic rate of non-inoculated seedlings grown at 720 μmol mol⁻¹ CO₂ for 18 weeks tends to be down-regulated, i.e. the seedlings have low CE, RuBP regeneration rate, P max and A/Cl curve; in contrast, ectomycorrhizae inoculated seedlings show no down-regulation at elevated [CO₂]. This phenomenon has been reported in other species raised adequate soil N fertility (Idso and Kimball 1991), or with symbiotic N₂ fixing species even when grown in poor soils (Vogen and Curtis 1995), or with mycorrhizal plants (Staddon et al. 1999). Phosphorus or nitrogen limitation (Conroy et al. 1986, Jach and Ceulemans 2000, Koike et al. 2000, Eguchi et al. 2004) and reduced sink strength (e.g. Rogers et al. 1998, Stitt 1991, Griffin et al. 2000) have both been proposed as mechanisms inducing down-regulation, via reduction of RuBP regeneration capacity and reduced Rubisco activity (Sharkey et al. 1994, Adam et al. 2004).

Therefore, I suggest that Pt, EC or Cg inoculated seedlings have increased the uptake of nutrient and water, which leading to improvement of plant nutritional status and giving rise to more vigorous physiological response, in particular photosynthetic activity; and that these responses delay down-regulation at elevated [CO₂], especially seedlings inoculated with EC.

The present results indicate that ectomycorrhiza inoculation increases uptake of nutrients and water, which resulted in vigorous physiological response significantly increases the dry mass of each seedlings grown at ambient and elevated concentrations of CO₂. Moreover, ectomycorrhizal seedlings moderated down-regulation at elevated CO₂ concentrations. Ectomycorrhiza therefore appear to have strong positive effects on P. koraiensis, P. densiflora and L. kaempferi seedlings at both CO₂ concentrations. Especially, EC, four species ectomycorrhiza mixed, more strongly affected on three pine species for their physiological activity and growth. Moreover, charcoal in soil increases the aperture of soil, as a result holds the soil moisture and raises soil temperature (DeBano et al. 1998). Therefore significantly increase germination rate (Fig. 4.7) and can increase surviving living seedlings. However, these practices of charcoal, lifting the soil pH and hold the soil moisture, and inhibit the ectomycorrhizal development (P<0.01) (Fig. 4.8). It is still question, how to increase ectomycorrhizal development in forest soil mixed charcoal for increase the survival and establishment rate of seedlings.
Chapter 5
GENERAL DISCUSSIONS

5.1 Effects of air pollutants and soil acidification on pine growth

Pine species are one of the important conifers for forest rehabilitation in Korea, since pine species can grow in low-nutrient and barren soil. These species are characterized by their fast growth rate and tolerance to environmental pollution, including air pollution and soil acidification (Burns and Honkala, 1990). For rehabilitating degraded forest areas, P. rigida and L. kaempferi were introduced at the start of the 20th century, from the USA and Japan respectively (Kim 1999). These pine species have declined near large cities and industrial complexes (Lee et al. 1996, Choi et al. 2003, 2005, Kim et al. 2003, Kayama et al. 2004). A continuing increase in soil acidification and atmospheric CO₂ concentration is expected in view of the increasing emission of air pollutants. How do pine trees respond to these environmental conditions?

Decline symptoms of pine forests in industrial complexes were observed. The length of shoots and the survival of needles of P. koraiensis and P. rigida were significantly less at the damaged site than at the control site. Moreover, the physiological responses, specifically the net photosynthetic rate (light and CO₂ response), P₅₀, F, CE and RuBP regeneration rate of P. koraiensis and P. rigida at the damaged site were significantly less than at the control site. The concentrations of chlorophyll (a+b) and chlorophyll b in needles at the damaged site were significantly less than at the control site. There is a reduction in physiological activities of P. koraiensis and P. rigida at the damaged site due to acid deposition. The rise in air pollutants directly damaged the plant physiological responses as a result of increased concentrations of F, Cl in the atmosphere and Mn in the soil and soil water, which finally accumulates in needles.

It is well known that pine species are symbiotic with ectomycorrhizae. Increased harmful elements in soil inhibit the development of ectomycorrhizae at the damaged site. It therefore appears that reduced physiological responses and reduced ectomycorrhizal development due to harmful elements hinder the growth of P. koraiensis and P. rigida. Physiological damage and reduced growth at the damaged site were more evident for P. rigida than for P. koraiensis.

Atmospheric air pollutants gradually cause an increase in soil acidity. Acidified soil increases the leaching of base cations, such as Ca²⁺, Mg²⁺, and K⁺ ions, from soil to the ground water, and increases the solubility of phytotoxic metals such as Al and Mn in the rhizosphere (Ulrich et al., 1980). Acidified soil may therefore harm tree growth not only because of low soil pH, but also through nutrient deficiency and Al and/or Mn in the soil solution. Adequate nutrient and water is vital for tree growth, and to prevent phytotoxic metals from entering acidified soil. It is possible that trees would be helped by symbiosis with ectomycorrhizae in acidified soil.

The concentration of Al and Mn in water increased with decreasing soil pH in the range 5.0 - 4.0, i.e. with progressive soil acidification. Increased Mn and Al in water-soluble form accumulated in tree roots and needles of P. rigida, P. koraiensis, P. densiflora and L. kaempferi seedlings. This phenomenon is clearly related to progressive soil acidification. Most pine species are symbiotic with ectomycorrhizae. Moreover, soil acidification affects ectomycorrhizal development in the rhizosphere. Forest soil that had been acidified artificially (10 and 30 mmolH⁺·kg⁻¹) significantly increased ectomycorrhizal development relative to controls, whereas more strongly acidified forest soil (60 and 90 mmolH⁺·kg⁻¹) significantly reduced ectomycorrhizal development.

Ectomycorrhizal development significantly reduced the concentration of Al in roots and Mn in needles in each species, in a comparison with non-ectomycorrhizal seedlings. Mycorrhizal infection in host plants usually prevents the absorption of heavy metals, such as Ni and Mg (Kayama et al. 2003). The concentration of N in needles increased slightly with progressive soil acidification around the ectomycorrhizal and non-ectomycorrhizal seedlings, since the acid added included HNO₃. It is believed that the reduced photosynthetic activity of P. rigida, P. koraiensis, P. densiflora and L. kaempferi seedlings was a consequence of the accumulation of Mn in needles and Al in roots, rather than nutrient or nitrogen deficiency. Suppression of the rate of photosynthesis affects the growth rate of P. rigida, P. koraiensis, P. densiflora and L. kaempferi seedlings. Moreover, the relative total dry mass (TDM) of P. koraiensis and L. kaempferi seedlings is approximately 40% at a BC/Al ratio (water-soluble) of 1.0, but the value for P. rigida and P. densiflora is approximately 50% at the same BC/Al ratio. Ectomycorrhizal seedlings of each species nevertheless had higher relative TDM value at a BC/Al ratio (water-soluble) of 1.0 than NE seedlings of each species, by 20-100%.

Atmospheric pollutants and soil acidification inhibit physiological responses and plant growth, but ectomycorrhizal infection increases nutrient and water uptake and protects against phytotoxic metals such as Al and Mn. As a result, the decline in growth of pine seedlings in the presence of atmospheric pollutants and soil acidification was less than for non-ectomycorrhizal seedlings. P. koraiensis was less tolerant to atmospheric pollutants and/or soil acidification than P. rigida or P. densiflora. The effect of ectomycorrhizal infection on the seedlings of P. koraiensis was small, however. P. koraiensis has few root hairs, so that it suffers less from soil acidification and gains less benefit from ectomycorrhizal infection. L. kaempferi has greater resistance against soil acidification upon infection of ectomycorrhizal than P. rigida or P. densiflora. Mixed ectomycorrhizae gave better results.
Fig. 5.1. The schematic presentation of climate changes and ecophysiological responses.

Environmental change and ectomycorrhiza
5.2 Effects of elevated CO₂ concentration

Elevated CO₂ concentration significantly increased the infection rate of ectomycorrhiza in seedlings of *P. koraiensis*, *P. densiflora* and *L. kaempferi*. Ectomycorrhizal development at each CO₂ concentration increased the P concentration in needles of each species. Moreover, ectomycorrhizae help to overcome stomatal limitation (ls) by enlarging the surface area of the root system available to soil, via broadly distributed hyphae. Ectomycorrhizal inoculation of seedlings also improves physiological activity (Smith and Read 1997, Lambers et al. 1998).

Photosynthetic activities were down-regulated, specifically the CE, RuBP regeneration rate, P_max and net photosynthetic rate of non-ectomycorrhizal seedlings grown at elevated CO₂ concentrations. Ectomycorrhiza-inoculated seedlings of each species showed no down-regulation at elevated CO₂ concentrations, however. It is believed that ectomycorrhizal infection of seedlings increases the uptake of nutrients and water, generating improved plant nutritional status and a more vigorous physiological response, including photosynthetic activity. These responses will reduce the down-regulation of photosynthesis at elevated CO₂ concentrations, especially with mixed ectomycorrhizal EC.

5.3 Forest rehabilitation after disturbance

The most important factor governing natural regeneration in forests is germination and rooting after natural and/or artificial disturbance. After germination, seedlings ‘plug into’ the network of compatible ectomycorrhizal fungi supported by surviving living trees, gaining the opportunity for early ectomycorrhizal formation and establishment (Amaranthus 1994).

Germination is strongly related to temperature, water and oxygen (Lambers et al. 1998). Most Korean forest soil is granite-derived brown forest soil, generally of low fertility and rapid permeability and easy to compact. Precipitation in the winter-to-spring season is also very low (Korea Meteorological Administration 2002b, 2003). These conditions are not suitable for the germination and rooting of seedlings and ectomycorrhizal development.

Charcoal added to the soil improves the moisture, temperature and physical properties (DeBano et al. 1998), significantly increasing the germination rate (Fig. 4.7) and increasing the survival of living seedlings. Moreover, charcoal can reduce tree damage by soil acidification, by increasing the soil pH of acidified forest soil. Unfortunately, charcoal addition usually inhibits ectomycorrhizal development (Fig. 4.8). The survival and establishment rates of seedlings depend on the rate at which they become ectomycorrhizal (Janos 1980, 1996). If trees become connected to a mycelial network larger than that which they could maintain on their own, this is often highly beneficial (Newman 1988, Perry et al. 1992). It has been suggested that rapid and early integration of seedlings into adult ectomycorrhizal root systems has benefits in carbon transfer from donor to receptor trees (Simard et al. 1997). On the other hand, charcoal inhibits the development of ectomycorrhizae. There remain many open questions about forest rehabilitation practices.

Chapter 6

Synopsis

1. Climate change, specifically global warming because of increasing atmospheric CO₂ and atmospheric acid deposition, is a serious environmental concern not only in Europe, North America and Japan but also in Asia, Africa and Latin America. Since a rapid industrial development and urbanization is taking place in these areas. Air pollution from wet and dry deposition can directly harm plant physiological activity and growth. Moreover, wet and dry deposition of acid substances causes acidification of forest soil by increased leaching of base cations (Ca²⁺, Mg²⁺, K⁺, Na⁺) from the rhizosphere soil, and dissolution of phytotoxic metals such as Al and Mn. Atmospheric deposition can therefore have harmful effects on forest ecosystems, including soil acidification, nutrient imbalances, carbohydrate content and growth. Moreover, the atmospheric carbon dioxide concentration (CO₂) continues to rise, affecting the ecosystem through increased availability of CO₂ for photosynthesis. However, photosynthesis down-regulation occurs by constraints on nutrients, especially P or N and reduced sink strength. Atmospheric deposition, soil acidification and rising atmospheric CO₂ concentration can therefore reduce tree physiological activity and growth. Ectomycorrhizae have a symbiotic relationship with trees. They improve the uptake by plants of water and nutrients, particularly nitrogen and phosphorus, from the soil solution by enlarging the absorptive surface of the root, with widely ramified hyphae. Moreover, ectomycorrhizal associations can reduce the toxicity of metals such as Al, Mn, Zu, Cu and Ni, the effects being dependent on the mycorrhizal fungi and the host species of plant. In view of these environmental changes and ectomycorrhizal characteristics, this study focused on the effects of changing climate on the physiological activity and growth of pine species, and investigated the role of ectomycorrhizae.

2. The concentrations of fluorine (F) and chlorine (Cl) in the atmosphere, precipitation and soil water at the damaged site were all significantly higher than at a control site. Moreover, the concentrations of F, Cl and Mn in needles of the two pine species were significantly higher at the damaged site than at the control site, and concentrations of essential elements (Mg, K, P) and chlorophyll in needles were significantly lower at the damaged site than at the control site. Ectomycorrhizal infection of the two pine species at the control site was 30% higher than at the damaged site. Almost no ectomycorrhiza infection was found in either species at the damaged site. The shoot length and survival statistics of needles of the two pines were significantly lower at the damaged site than at the
control site. In particular, growth of shoots and needle longevity of *P. koraiensis* are larger than for *P. rigida* at the damaged site. The photosynthetic responses (light-saturated net photosynthesis (*P* _{max}), apparent quantum yield (Φ), carboxylation efficiency (CE) and RuBP regeneration rate) of both pines were significantly lower at the damaged site than at the control site. The present comparison of photosynthetic responses and the concentrations of F, Cl and Mn in needles of the two pine species clearly shows that *P. koraiensis* is more tolerant to excess Mn in its needles than *P. rigida*.

3. With increasing amounts of H^+ added to the soil, the water-soluble concentrations of Ca, Mg, K, Al and Mn increased, especially below a soil pH of 3.8. Development of ectomycorrhizae, i.e. *Pisolithus tinctorius* (*Pt*), Ectodrench (EC) or *Cenococcum geophilum* (Cg), with *Pinus densiflora*, *P. koraiensis*, *P. rigida* and *Larix kaempferi* seedlings was significantly greater in the 10 and 30 mmolH^+·kg^{-1} treatments than control treatments. The mycorrhizal development in 60 and 90 mmolH^+·kg^{-1} treatments was significantly lower than the control, 10 and 30 mmolH^+·kg^{-1} treatments. The concentrations of Al in roots and Mn in needles of each species increased with increasing amount of H^+ added to the soil. Also, the concentration of N in needles of each seedling was higher in all treatments than controls. The maximum net photosynthetic rate at saturated CO₂ concentration (*P* _{max}) of each seedling was greater with 10 mmolH^+·kg^{-1} treatment than controls, but it fell above 30 mmolH^+·kg^{-1}, especially in the 60 and 90 mmolH^+·kg^{-1} treatments. However, the concentration of Al in roots and Mn in needles of ectomycorrhizal seedlings of each species was significantly less than corresponding non-ectomycorrhizal (NE) seedlings. The *P* _{max} of ectomycorrhizal seedlings of each species was significantly greater than corresponding non-ectomycorrhizal seedlings. The dry mass of the whole plant of NE, *Pt*, EC or Cg of each species increased in the 10 and 30 mmolH^+·kg^{-1} treatments, but was significantly less in the 60 and 90 mmolH^+·kg^{-1} treatments than other treatments. Moreover, the relative total dry mass (TDM) of *P. koraiensis* and *L. kaempferi* seedlings was approximately 40% at a BC/Al ratio of 1.0, but that of *P. rigida* and *P. densiflora* was approximately 50% at the same BC/Al ratio.

4. The effects of elevated CO₂ concentration on *P. koraiensis*, *P. densiflora* and *L. kaempferi* seedlings were estimated, specifically their physiological responses and growth with ectomycorrhizae, i.e. *Pt*, EC or Cg. Seedlings were grown under ambient (360 μmol·mol⁻¹) or elevated (720 μmol·mol⁻¹) CO₂ concentrations in a phytotron with or without ectomycorrhizae for 18 weeks. The ectomycorrhizal development of each species was significantly higher under elevated CO₂ concentration than at ambient levels. The P concentration in needles and roots following inoculation with *Pt*, EC or Cg was significantly higher in each species than without inoculation at both CO₂ concentrations. Moreover, *P* _{max}, CE and the RuBP regeneration rate for each species inoculated with *Pt*, EC or Cg were significantly higher than for non-inoculated seedlings at both CO₂ concentrations. Stomatal limitation in each species at each CO₂ concentration decreased relative to non-inoculated seedlings, especially in *P. densiflora*. Non-ectomycorrhizal seedlings of each species showed photosynthetic down-regulation, but ectomycorrhizal seedlings did not. Charcoal mixed with forest soil significantly increased the germination rate of *P. rigida*, *P. densiflora* and *L. kaempferi* seeds, but significantly reduced the ectomycorrhizal infection rate of these seedlings.

5. Air pollutants lead to soil acidification and imbalance of soil nutrients. Air pollutants and soil acidification reduce physiological activity and tree growth. Moreover, higher atmospheric CO₂ concentrations lead to photosynthetic down-regulation. In contrast, ectomycorrhizal inoculation increased the uptake of nutrients and water, and protected the plant against phytotoxic metals. Seedlings of each species inoculated with ectomycorrhizae consequently showed increased physiological activities and dry mass growth under soil acidification, and did not show photosynthetic down-regulation under elevated CO₂ concentrations. Mixed ectomycorrhiza, EC, were particularly effective. Also, charcoal mixed with the soil increased the germination rate by helping to maintain the soil moisture and temperature and keeping the soil loose increase aperture of soil, but it obstructs development of ectomycorrhizae.

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