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Effects of High Nitrogen Load and Ozone on Forest Tree Species

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Abstract

Increases in nitrogen (N) deposition from the atmosphere and tropospheric ozone (O₃) concentration are considered to be environmental stresses affecting forest ecosystems. In this paper, we summarized the background of the studies on the effects of N load and O₃ on forest tree species, and introduced two experimental studies on the effects of excessive N load and O₃, singly and in combination, on growth, physiological functions such as photosynthesis and nutrient status of Japanese red pine (*Pinus densiflora*). The present situation of atmospheric N deposition and the results of experimental studies suggest that further increase of N deposition to Japanese forest ecosystems at more than the current levels does not always increase tree growth, but depletes the health of sensitive tree species such as Japanese red pine. Furthermore, there is the possibility that increases in the atmospheric N deposition and tropospheric O₃ concentration will adversely affect the health of sensitive tree species such as Japanese red pine in the near future.

Key words: nitrogen, ozone, Japanese red pine, growth, photosynthesis, nutrient status

Introduction

Forest decline is one of the serious environmental problems worldwide. Although the causes and/or mechanisms of these phenomena are different among the sites, acid deposition with excessive nitrogen (N) and tropospheric ozone (O₃) are considered to be environmental stresses relating to these phenomena not only in Europe and North America, but also in Asia (Krause *et al.*, 1986; Sandermann *et al.*, 1997; Izuta, 1998).

In this paper, we summarize the background of the studies on the effects of nitrogen load and O₃ on forest tree species, and introduce our experimental studies on the effects of high N load and O₃, singly and in combination, on growth, physiological functions and nutrient status of Japanese red pine (*Pinus densiflora*).

Nitrogen

1. Nitrogen deposition

Atmospheric deposition of nitrogen (N) to terrestrial ecosystems has been increasing with elevated anthropogenic emissions of nitrogen oxide (NO_x) and reduced N (NH_y) since the industrial revolution (IPCC, 2001; Galloway, 2001; Amann, 2001). In general, optimal amounts of N input to forest ecosystems increase primary production of trees (Ingestad *et al.*, 1986; Read, 1991; Tamm, 1991). However, excessive N deposition from the atmosphere plays as phytotoxicant which causes soil acidification and eutrophication in forest ecosystems (Schütt and Cowling, 1985; Nihlgård, 1985; Schulze, 1989). Furthermore, an increase in the emission of NO_x

induces increases in acid deposition and tropospheric ozone (O₃) levels (Penkett, 1988).

Nitrogen oxide mainly consists of nitrogen dioxide (NO₂) and nitric oxide (NO), and NH_y is composed of ammonia (NH₃) and ammonium (NH₄⁺) (Mészáros, 1992; Erisman and Draaijers, 1995). These N compounds are emitted mainly from anthropogenic sources such as industry, traffic and live stock breeding (Erisman and Draaijers, 1995). Recently, global emissions of NO_x and NH_y were estimated to be approximately 52 and 109–131 Tg N year⁻¹, respectively (Erisman and Draaijers, 1995; IPCC, 2001). Because of increase in consumption of fossil fuels, the major sources of NO_x, continuous increase in NO_x emission has been predicted on global scale (IPCC, 2001; Prather *et al.*, 2001). In Japan, because the emission of SO_x has been reducing from the 1980s as a result of technical and political regulations, atmospheric SO₂ concentration maintains at relatively low level at the present time. However, NO_x emission is predicted to be increasing in the 21st century (Science and Technology Agency, 1991). This indicates that a major acidic air pollutant in Japan is NO_x rather than SO_x.

Main sources of anthropogenic NH_y emission are livestock management (80–90%) and N fertilizer (Hov *et al.*, 1987; Mészáros, 1992). Because of continuous increases in livestock breeding and fertilization use, NH_y deposition and its impacts on forest ecosystems are considered to increase worldwide (Zhao and Wang, 1994; Whelpdale *et al.*, 1997; Klimont, *et al.* 2001). Although limited information is available on the emission of NH_y in Japan (Ishii and Komeiji, 1993;

Murano *et al.*, 1995), NH_3 emission and emission ratio of NH_3 to NO_x in 1994 were estimated to be 0.43 Tg N year^{-1} and 0.84 mol mol⁻¹, respectively (Kannari *et al.*, 2001). The average molar ratio of NH_4^+ to nitrate (NO_3^-) in the current precipitation ranged from 1.2 to 1.4 (Katoh *et al.*, 1990; Hara, 1992) and that in the sum of throughfall and stemflow were between 0.62 and 1.1 in suburban forests (Okita *et al.*, 1993; Kobayashi *et al.*, 1995). This indicates that Japanese forest ecosystems are influenced not only by NO_x , but also by relatively high NH_y deposition from the atmosphere.

2. Effects of nitrogen deposition on forest ecosystems

Nitrogen is a limiting nutrient for tree growth in some temperate coniferous forests (Ingestad and Kähr 1985; Crane and Banks 1992). In forest ecosystems where the N limits primary production of trees, because most of N supplied by atmospheric deposition is absorbed and assimilated by microbe and plants, N output as nitrate (NO_3^-) by stream water is lower than N input by atmospheric deposition (Stoddard, 1994). However, several researchers observed relatively high N output by stream water as compared with atmospheric N input, and suggested that N status in some forests of Central Europe and North America are changing from 'N-limited' to 'N-saturated' (Skeffington and Wilson, 1988; Stoddard, 1994; Fenn *et al.*, 1996).

Over-nutrition of N in N-saturated forests reduces tree vitality by many N-related processes such as accelerated soil acidification, deteriorated nutrient status such as excessive accumulation of manganese (Mn) or deficiencies of phosphate (P) and magnesium (Mg) in leaves, and increased sensitivity to other environmental stresses such as drought and frost (Nihlgård, 1985; Cowling *et al.*, 1988; Schulze, 1989). Because a clear correlation was observed between regional variation of atmospheric NH_4^+ deposition and forest decline (Den Boer and Van den Tweel 1985), excessive N input is considered to be one of the major environmental stresses possibly leading to forest decline in Central Europe (e.g. Schütt and Cowling, 1985). Moreover, increase in N deposition is predicted to affect successional dynamics and biodiversity of forest ecosystems (Skeffington and Wilson, 1988; Van Breemen and Van Dijk, 1988). Vegetation structure may be changed according to the sensitivity of plant species to N, and as a consequence, habitat of plant species tolerant to excessive N or favor relatively high N supply would expand at the expense of N-sensitive plant species.

In Europe, it is recently suggested that the thresholds of N load for appearance of N-saturation and forest damage are approximately 10 kg ha⁻¹ year⁻¹ and 25 kg ha⁻¹ year⁻¹, respectively (Wright *et al.*, 1995). In Japan, relatively high atmospheric N deposition, which is similar to that in Central Europe and North America, has been observed. Annual average N deposition by precipitation over Japan was from 7 to 10 kg ha⁻¹ yr⁻¹ during the past few decades (Katoh *et al.* 1990; Hara, 1992). In Japanese coniferous forests at suburban areas, N input by wet deposition (bulk precipitation) and that

by throughfall and stemflow ranged from 10 to 20 kg ha⁻¹ year⁻¹ (Iwatsubo *et al.* 1997, Ohruai and Mitchell 1997) and 12 to 40 kg ha⁻¹ year⁻¹ (Okita *et al.* 1993, Kobayashi *et al.* 1995; Baba and Okazaki, 1998), respectively. These N deposition rates exceed the threshold value of 10 kg ha⁻¹ year⁻¹ which could induce N-saturation in European forest ecosystems (Wright *et al.*, 1995). Current field studies showed that inorganic N output by stream water is higher than N input as bulk precipitation in some forested areas, indicating that N-saturation has already been occurring in Japanese forest ecosystems (Ohruai and Mitchell, 1997; Mitchell *et al.* 1997; Baba and Okazaki, 1998; Izuta, 2001).

3. Toxicity of excessive nitrogen to forest tree species

It is well known that dry deposition of NO_x reduces plant growth possibly by cellular acidification, enhanced levels of NO_2^- and NH_4^+ in plant tissue, and direct interference of free radical (N=O) to photosynthetic enzymes (Wellburn, 1990). However, adverse effects on growth and physiological functions of plants are generally induced by exposure to relatively high concentrations of atmospheric NO_x at several ppm ($\mu\text{mol mol}^{-1}$), direct toxicity of the current atmospheric levels of NO_2 or NO below 30 ppb (nmol mol^{-1}) may be negligible for tree health in Japan. On the other hand, high accumulation of NH_y has detrimental effects on plants by uncoupling of electron transport, membrane dysfunction and appearance of visible foliar injury (Fangmeier *et al.*, 1994). It has been reported that 10-month-fumigation with NH_3 above 138 ppb reduced bud burst, apical shoot growth and frost hardiness of Scots pine (*Pinus sylvestris*) seedlings (Dueck *et al.*, 1990). Short-term exposures for 1 to 7 weeks to relatively high levels of NH_3 above 330 ppb induced visible needle injury and loss of needles in Scots pine seedlings (Van der Eerden, 1982; Hauk *et al.*, 1990; Fangmeier *et al.*, 1994). In European forests, critical (no-adverse effect) levels for NH_3 related to acute effects by short-term exposures (one day or less) and to chronic effects by long-term exposures (one year) were defined as 355 ppb and 10.5 ppb, respectively (Ashmore and Wilson, 1994; De Vries *et al.*, 2000). In Japan, although there is no information concerning the long-term monitoring of atmospheric NH_3 concentration in forested areas, the annual average concentration of NH_3 in the urban atmosphere ranged between 6.6 and 7.6 ppb, suggesting that acute injury of NH_3 to forest tree species does not occur at the present time.

Wet deposition of HNO_3 and NH_4^+ plays as acidic substance for forest ecosystems. High load of these N compounds causes soil acidification by H^+ originated from HNO_3 and nitrification process of NH_4^+ (Van Breemen *et al.*, 1982; Ulrich, 1991). Because soil acidification leads to leaching of base cations such as K, Ca and Mg from soil to watershed and increase in the solubility of Mn and Al in the rhizosphere, tree vitality may be declined by nutrient deficiency, imbalance of cations and excessive accumulations of Mn and Al under high N load (Van Breemen *et al.*, 1982; Ulrich, 1989; Ulrich and Sumner 1991). These negative impacts

induced by high N deposition have already been observed in some polluted forests of Central Europe (Den Boer and Van den Tweel 1985; Van Dijk and Roelofs, 1988; Flückiger and Braun, 1998). The harmful influences of increasing N deposition to Japanese forest ecosystems seem to be occurred by its kinetics between soil-plant systems rather than by direct toxicity of gaseous NO₂ or NH₃. However, although there are many studies on the relationships between Japanese forest production and nutrient status or cultivation techniques since the 1960s (e.g. Tsutsumi 1962, Kawada 1968, Akama 1993), limited information is available on the responses of Japanese forest trees to high N load (Nakaji and Izuta, 2001; Nakaji *et al.*, 2001; Nakaji *et al.*, 2002).

Ozone

1. Tropospheric ozone

Ozone (O₃) in the troposphere has been emerged as major gaseous air pollutant which adversely affects terrestrial ecosystems (Sandermann *et al.*, 1997). Although O₃ in the upper stratosphere plays an important role for biosphere by reducing the penetration of biologically damaging UV radiation, the same gas in the troposphere plays as a main component of photochemical oxidants, and causes severe damages on physiological functions of organisms by its high oxidative reactivity. The major sources of tropospheric O₃ are intrusion from the stratosphere and photochemical production in the troposphere (Hov, 1984; Thompson, 1992; Wakamatsu *et al.*, 1989). Human activities mainly influence photochemical production (Ashmore and Bell, 1991). With increasing energy consumption and motor vehicle use, increasing amounts of O₃ precursor trace gases such as NO_x, carbon monoxide and hydrocarbons are released into the atmosphere. These trace gases alter the equilibrium of atmospheric reaction (NO₂ + O₂ → NO + O₃) in favor of O₃ production.

Tropospheric O₃ concentrations in the Northern Hemisphere have been increasing (Oltmans *et al.*, 1998; Logan *et al.*, 1999). In East Asia, relatively high background O₃ concentration above 50 ppb, which is similar to those at polluted areas in North America and Europe, have been observed (Akimoto, 1997; Oltmans *et al.*, 1998; Logan *et al.*, 1999). Moreover, further increases of ground-level O₃ are expected in many parts of the world as a result of continuing rise in the emissions of precursor gases such as NO_x (Chameides *et al.*, 1994; Fowler *et al.*, 1999; Emberson *et al.*, 2001; Prather *et al.*, 2001).

2. Effects of ozone on forest ecosystems

Ozone is known to decline growth and vitality of trees by reductions in net photosynthesis and foliar longevity (i.e. acceleration of senescence), imbalance of carbon allocation to plant organs (i.e. reduced root biomass and low root/shoot ratio) and enhanced sensitivity to other environmental stresses such as drought, parasite attack, fungal disease and other air pollutants (Reich, 1987; Manning and Tiedemann, 1995; Izuta *et al.*, 2001).

Since the 1960s, severe forest decline has been observed in Central Europe and North America (e.g. Krahl-Urban *et al.*, 1988). In the USA, several field studies and experimental studies using fumigation chambers (i.e. open-top chambers) revealed that exposure to ambient levels of O₃ could induce visible foliar injuries in many native tree species such as red maple (*Acer rubrum*), black cherry (*Prunus serotina*), yellow-poplar (*Liriodendron tulipifera*) and table mountain pine (*Pinus pungens*), and these O₃-specific symptoms are similar to those observed at mountainous areas with forest decline (Kress and Skelly, 1982; Duchelle *et al.*, 1982; Skelly *et al.*, 1983; Neufeld *et al.*, 1992). From the results of several experimental studies conducted by National Acid Precipitation Assessment Program (NAPAP), main cause of some forest decline in North America, for example, pine species in Sierra Nevada Mountains and red spruce (*Picea rubens*) in Appalachian Mountains, is considered to be high O₃ pollution (Irving, 1991; Chevone and Linzon, 1988; McLaughlin and Percy, 1999). Furthermore, several model studies suggested that forestry production in North America has already been reduced in the range of 0-10% per year by the current levels of O₃ (Pye, 1988; Hogsett *et al.*, 1997; Chappelka and Samuelson, 1998). Also in Central Europe, elevated O₃ is one of the major environmental stresses which reduce forest vitality and possibly accelerates forest decline together with increasing acid deposition (e.g. Krause *et al.*, 1986; Schulze *et al.*, 1989). In European countries, therefore, several research projects are widely proceeding to assess the potential risk of O₃ to crops, trees and semi-natural vegetation and to define the guideline for protecting terrestrial ecosystems from adverse effects of O₃ (Fuhrer *et al.*, 1997; Fuhrer and Achermann, 1999).

In Central Europe and North America, mean maximum O₃ concentrations during the growing season of crops in 1990 were above 60 ppb (Emberson *et al.*, 2001). In Japan, since 1988, mean hourly oxidant (O_x) concentration above 60 ppb and that above 120 ppb have continuously been recorded at more than 98% and 30% of General Environment Air Monitoring Stations, which are established at more than 1000 sites of urban and suburban areas (Environment Agency, 1989-2000). Ozone is one of the main ingredients (app. 90%) of O_x. Also in rural mountainous areas, O_x pollution is widely distributed mainly by transport of polluted air from urban areas (Kurita *et al.*, 1985; Wakamatsu, 1997, 2001). For example, mean O₃ concentrations between May and August at Mt. Dodaira in Saitama Prefecture, which is located 63 km far northwest from Tokyo, ranged from 50 to 85 ppb during the past decade (Ogawa *et al.*, 2000; Muto and Umezawa, 2001). Chang *et al.* (1990) explained that this severe O₃ pollution in inland areas is mainly caused by transported air pollutants with sea breeze from Tokyo metropolis. At several rural forest areas such as Oku-Nikko and Ohyama, where severe forest decline or dieback of deciduous and coniferous tree species can be seen, high hourly mean O₃ concentrations above 100 ppb have been observed, suggesting that negative impacts of O₃ on Japanese forest ecosystems can not be

negligible (Hatakeyama and Murano, 1996; Totsuka *et al.*, 1997; Aso *et al.*, 2001). However, limited information is available for evaluation and risk assessment of the current or future effects of ground-level O₃ on Japanese forest tree species (Izuta *et al.*, 1996; Matsumura *et al.*, 1996; Matsumura, 2001; Yonekura *et al.*, 2001a; Yonekura *et al.*, 2001b).

3. Toxicity of ozone to forest tree species

Several experimental studies conducted in the USA and Europe revealed that chronic fumigation with ambient levels of O₃ induces growth reduction and disturbance of many physiological processes such as photosynthesis with or without visible foliar injuries (Chappelka and Chevone, 1992; Skärby *et al.*, 1998). The O₃-induced reduction in photosynthesis is considered to be mainly due to reduced carboxylation capacity in the chloroplasts, linking to depleted biosynthesis and proteolysis of photosynthetic enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) rather than photochemical capacity of PS II or stomatal limitation (Sasek and Richardson, 1989; Wallin *et al.*, 1992; Pell *et al.*, 1994; Reichenauer *et al.*, 1997).

It is known that growth and photosynthetic responses of plants to O₃ can be changed by several biotic (species, genotypes, plant age) and abiotic factors (light, temperature, soil nutrient status and other pollutants) (Izuta *et al.*, 1988; Izuta *et al.*, 1991; Izuta *et al.*, 2001). Especially, soil N status strongly influences the sensitivity of trees to O₃ (Pell *et al.*, 1995; Matyssek *et al.*, 1995). Pääkkönen and Holopainen (1995) found that increasing N availability can confer European birch (*Betula pendula*) seedlings with greater resistance to O₃, whereas opposite responses have been reported in the seedlings of loblolly pine (*Pinus taeda*), Scots pine (*Pinus sylvestris*) and aspen (*Populus tremuloides*) by Tjoelker and Luxmoore (1991), Utriainen and Holopainen (2001) and Bielenberg *et al.* (2001). These previous studies suggest that combined effects of high N load and O₃ are quite different among the tree species.

Experimental studies on the effects of high N load and O₃ on Japanese red pine seedlings

1. Objective

Japanese red pine is one of the most representative Japanese coniferous tree species. Since the early 1960s, the decline of Japanese red pine was observed mainly in the western part of Japan (Photo. 1). Several field studies and pathological experiments indicated that mortality of pine trees (*Pinus densiflora* and *Pinus thunbergii*) was mainly due to pine wilt disease by epidemic attacks of beetle-vectored nematode, *Bursaphelenchus* sp. (Kiyohara and Tokushige, 1971; Morimoto and Iwasaki, 1972; Fukuda, 1997). While, several studies indicated that air pollutants such as SO_x, NO_x and O_x are considered to accelerate pine forest decline (Yokobori, 1986; Naemura *et al.*, 1996; Hirano and Morimoto, 1999). Yokobori (1986) reported that low tree-ring growth of Japanese red pine trees during the 1970s was well in agreement with the sum of NO_x and O_x concentrations. Tsuchiya and Naemura (1998)

revealed a positive correlation between air pollution by NO_x and decline of Japanese red pine. Recently, high atmospheric N deposition is considered to be a major stress factor which accelerates decline of Japanese red pine (Kume *et al.* 2000). Therefore, the objective of our experimental studies is to clarify the effects of high N load and/or O₃ on growth, physiological functions and nutrient status of Japanese red pine and its mechanisms (Nakaji and Izuta, 2001; Nakaji *et al.*, 2001; Nakaji *et al.*, 2002).

2. Effects of high N load on Japanese red pine seedlings

We investigated the effects of high N load on growth, photosynthesis and nutrient status of Japanese red pine seedlings. One-year-old seedlings were transplanted into 2.5-L plastic pots containing 2.0-L of brown forest soil and were grown under field conditions at the experimental field of Tokyo University of Agriculture and Technology (Fuchu, Tokyo, Japan), as shown in Photo. 2. From 25 May to 10 June 1999, N was added as 25 mM NH₄NO₃ solution to the potted soil surface at 0 (N0), 25 (N25), 50 (N50), 100 (N100) and 300 (N300) mg l⁻¹ fresh soil volume. The amounts of N added to the soil were equivalent to 0, 28, 57, 113 and 340 kg ha⁻¹ on the basis of potted soil surface area, respectively. Because the current ratio of NH₄⁺ to NO₃⁻ in throughfall and stemflow has been reported to be 0.98 (Okita *et al.*, 1993), NH₄NO₃ was used in this study. The seedlings were grown for 2 growing seasons from May 1999 to October 2000. During the two growing seasons, the seedlings were irrigated with deionized water as necessary. To avoid the natural N input from wet deposition, all the seedlings were protected from natural precipitation by a transparent polyvinyl chloride roof on rainy days.

Change in the concentration of NO₃⁻ in the soil solution after the addition of NH₄NO₃ solution to potted brown forest soil during the 17-month growth period of Japanese red pine seedlings is shown in Fig. 1. The concentration of NO₃⁻ in the soil solutions increased with increasing the amount of N added as NH₄NO₃ solution to the soil. The concentration of NH₄⁺ in the soil solutions was rapidly decreased probably due to plant uptake and consumption in nitrification (data not shown).

Dry mass of Japanese red pine seedlings grown in the five N treatments after the 5-month (25 October 1999) and 17-month growth periods (28 October 2000) is shown in Fig. 2. In the N300 treatment in the first growing season and the N100 and N300 treatments in the second growing season, high N load caused significant reductions in the needle, fine root and whole-plant dry masses as compared with those in the N0 treatment. Although there was no significant treatment effect on the root/shoot ratio, the ratio of fine root dry mass to total root dry mass was significantly reduced by the highest N treatment (N300).

Net photosynthetic rate at 350 μmol mol⁻¹ CO₂ in the '99 needles of Japanese red pine seedlings grown in the five N treatments is shown in Fig. 3. The N treatment significantly reduced net photosynthetic rate

and stomatal diffusive conductance to CO₂, and increased the ratio of intercellular CO₂ concentration to atmospheric CO₂ concentration in the '99 needles. During the first growing season, the treatments of N300, N100 and N50 significantly reduced net photosynthetic rate from July, September and December 1999 to February 2000 as compared with the N0 treatment, respectively. The negative N effects on net photosynthetic rate remained during the second growing season.

The concentration of Rubisco in the '99 needles of Japanese red pine seedlings grown in the five N treatments is shown in Fig. 4. The N treatment significantly reduced Rubisco concentrations from August in the first growing season to September in the second growing season.

The concentrations of elements in the '99 needles of Japanese red pine seedlings grown in the five N treatments on 25 October 1999 (5-month-old needles) and 28 October 2000 (17-month-old needles) are shown in Fig. 5. Needle concentrations of N and Mn were significantly increased by the N treatments in October 1999 and 2000. Needle concentrations of P and Mg were significantly reduced by the N treatments in October 1999. In October 2000, needle concentrations of P and Mg tended to be reduced by the N treatments. The N/P and N/Mg ratios in the needles were significantly increased by the N treatments in October of the both growing seasons.

The relationships between ectomycorrhizal infection (Photo. 3) and needle P or Mg concentration in Japanese red pine seedlings in October 2000 are shown in Fig. 6. Needle P and Mg concentrations were positively correlated with the ratio of mycorrhizal-infected root tips to total root tips (mycorrhizal infection rate).

The relationship between Mn/Mg ratio and specific Rubisco activity in the '99 needles of Japanese red pine seedlings in September 1999 is shown in Fig. 7. The highest Mn/Mg ratio of 0.88 g g⁻¹ was found in the seedlings grown in the highest N treatment (N300), and the specific Rubisco activity in this treatment was approximately 15% lower than that in the N0 treatment. This result suggests that relatively high Mn accumulation with lowered Mg concentration inhibits Rubisco activation in the needles of Japanese red pine seedlings.

Frost hardiness as evaluated by electrolyte leakage rate of Japanese red pine seedlings grown in the five N treatments is shown in Fig. 8. The electrolyte leakage rate (*k*) in the needles increased with reducing the freezing temperature. The highest N treatment caused a significant increase in the *k* at -5 °C and below. The highest *k* of 0.0027 was detected at -30 °C temperature regime in the seedlings grown in the treatment of N300. This value is lower than the threshold value of 0.004 for the appearance of irreversible needle damage in red spruce (*Picea rubens* and *Picea rubra*) seedlings (Murray *et al.* 1989).

In conclusion, Japanese red pine is relatively sensitive to high N load as compared to other Japanese conifers such as Japanese cedar (*Cryptomeria japonica*)

(Nakaji *et al.*, 2001). Relatively high N load induces visible needle injuries and reductions in the whole-plant dry mass, net photosynthetic rate and frost hardiness. Main cause of high N load-induced reduction in net photosynthetic rate is inhibition of CO₂ fixation in the chloroplasts due to lowered activity and concentration of Rubisco in the needles. High N load-induced reductions in the needle concentration and activity of Rubisco are accompanied with lowered concentrations of P and Mg in the needles, increased concentration of Mn in the needles and poor ectomycorrhizal infection in the roots.

3. Effects of high N load and O₃ on Japanese red pine seedlings

We investigated the effects of high N load and O₃, singly and in combinations, on growth, photosynthesis and nutrient status of Japanese red pine seedlings. On 28 April, one-year-old seedlings were transplanted into 1.5-L plastic pots filled with brown forest soil, and N was added as NH₄NO₃ to the potted soil at 0 (N0), 100 (N100), 300 (N300) and 500 mg l⁻¹ fresh soil volume (N500). The amounts of N added to the potted soil were equivalent to 0, 135, 405 and 675 kg ha⁻¹ on the basis of soil surface area, respectively. Then, the seedlings were grown for 173 days from 22 May to 11 November in naturally lit phytotrons (Photo. 4) at Tokyo University of Agriculture and Technology (Fuchu, Tokyo, Japan). In phytotrons, half of the seedlings were exposed to charcoal-filtered air. The remaining half of the seedlings were exposed to 60 nl l⁻¹ (ppb) O₃ for 8 hours a day (9:00~17:00). All the seedlings were irrigated with deionized water as necessary. During the experimental period, we measured pH and element concentrations of soil solution, plant dry mass, needle gas exchange rates and needle concentrations of N, chlorophyll, total soluble protein and Rubisco.

The pH and concentrations of NH₄⁺ and NO₃⁻ in the soil solution during the growth period of Japanese red pine seedlings are shown in Fig. 9. The soil solution pH decreased with increasing the amount of N added to potted brown forest soil, but in no case to a value less than 4.0. The N load increased the concentrations of NH₄⁺ and NO₃⁻ in the soil solution. During the growth period of Japanese red pine seedlings, NH₄⁺ concentration was rapidly decreased due to plant uptake and consumption in nitrification, whereas NO₃⁻ concentration remained at relatively high levels, reflecting the N supply to the soil.

The effects of N load and O₃ on dry mass of Japanese red pine seedlings are shown in Fig. 10. The N load at 100 to 500 mg l⁻¹ or exposure to 60 ppb O₃ caused a significant reduction in the height of the seedlings. No significant interactive effects of N load and O₃ were detected on the height of the seedlings. The whole-plant dry mass of the seedling was reduced by the N load or exposure to O₃. Although the whole-plant dry mass of the O₃-exposed seedlings grown in the soil treatment of N500 showed the least value, there were no significant interactive effects of O₃ and soil N on the whole-plant dry mass of the seedlings.

The effects of N load and O₃ on net photosynthetic rate at 350 μmol mol⁻¹ CO₂ (*A*₃₅₀), gaseous phase diffusive conductance to CO₂ (*G*_s), carboxylation efficiency (*CE*) and maximum net photosynthetic rate at saturated CO₂ and PPF (A_{max}) of Japanese red pine seedlings are shown in Fig. 11. The N load at 100 to 500 mg l⁻¹ or exposure to 60 ppb O₃ caused significant reductions in the *A*₃₅₀ and *CE* of the seedlings. The N load induced significant reductions in the *G*_s and A_{max} of the seedlings. No significant interactive effects of N load and O₃ were detected on these parameters of the seedlings.

The concentrations of N, chlorophyll, total soluble protein and Rubisco in the current-year needles of Japanese red pine seedlings are shown in Fig. 12. The concentrations of N, chlorophyll, total soluble protein (TSP) and Rubisco were significantly reduced by the N load. The exposure to O₃ significantly reduced Rubisco concentration. No significant interactive effects of N load and O₃ were detected on the concentrations of these needle components.

The relationships between relative growth rate (RGR) of Japanese red pine seedlings and NO₃⁻ concentration in the soil solution or N load in the two experimental studies are shown in Fig. 13. Based on the results obtained from the two experimental studies, RGR of the seedlings reduces when soil NO₃⁻ concentration and N load are above 5 mM and 57 kg ha⁻¹, respectively. Ozone does not affect the sensitivity of the seedlings to increasing N load.

In conclusion, although there are no statistical significant interactions between N load and O₃ on any growth and photosynthetic parameters, the combination of excessive soil N and O₃ greatly reduces dry mass and net photosynthesis of Japanese red pine seedlings. High N load at 100 mg l⁻¹ fresh soil volume (135 kg N ha⁻¹) does not ameliorate O₃ damage of Japanese red pine seedlings unlike other researches for European tree species, indicating that the combined effects of N load and O₃ are different among the tree species. The

reduction in dry weight growth of Japanese red pine seedlings by high N load or exposure to O₃ is caused by the reduction in net photosynthetic rate mainly due to decreased Rubisco quantity in the chloroplasts.

Conclusions

Based on the results obtained from our experimental studies, it is suggested that further increase of N deposition to Japanese red pine forest at more than the current levels does not always increase tree growth, but depletes the health of this tree species. Furthermore, the increase in tropospheric O₃ concentration with high N deposition will adversely affect the health of Japanese red pine forest in the future.

To evaluate and predict the effects of acid deposition with excessive N and gaseous air pollutants such as O₃ on forest ecosystem, it is necessary not only to conduct field surveys, but also to experimentally investigate growth and physiological responses of forest tree species to these environmental stresses. We must clarify the species-specific difference in the response to increasing N deposition for evaluating the future situation of forest ecosystems. Furthermore, we must investigate the effects of O₃ on growth, physiological functions such as photosynthesis and nutrient status of many forest tree species.

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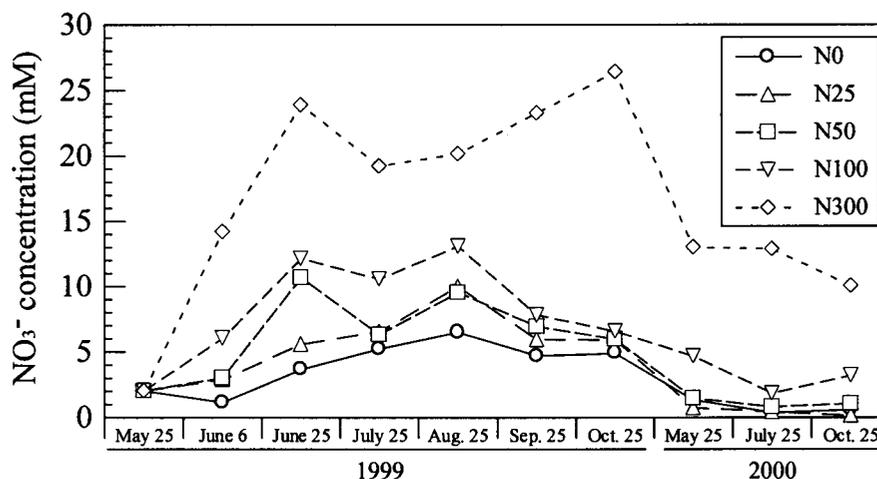


Fig. 1. Change in NO₃⁻ concentration of soil solution after the addition of NH₄NO₃ solution to potted brown forest soil during the 17-month growth period of *Pinus densiflora* seedlings. Each value is the mean of 4 determinations.

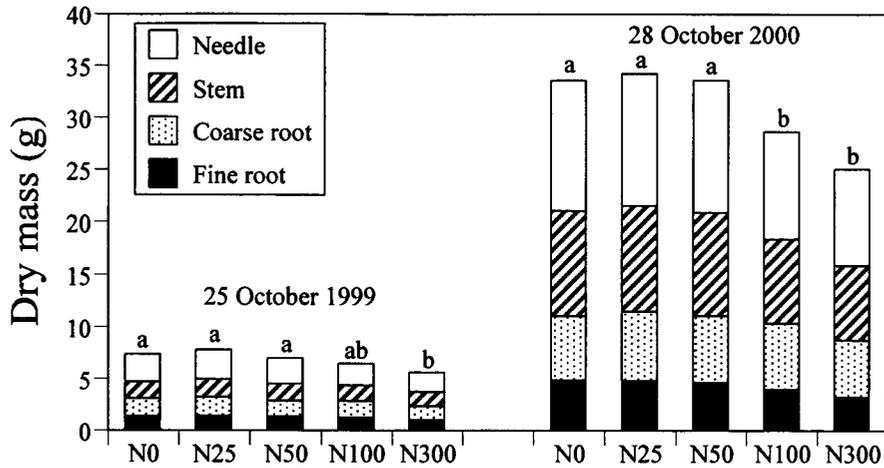


Fig. 2. Dry mass of *Pinus densiflora* seedlings grown in five N treatments after the 5-month (25 October 1999) and 17-month growth periods (28 October 2000). Each value is the mean of 12 determinations. Different letters above a bar indicate significant differences among the N treatments (Duncan's multiple range test; $p < 0.05$).

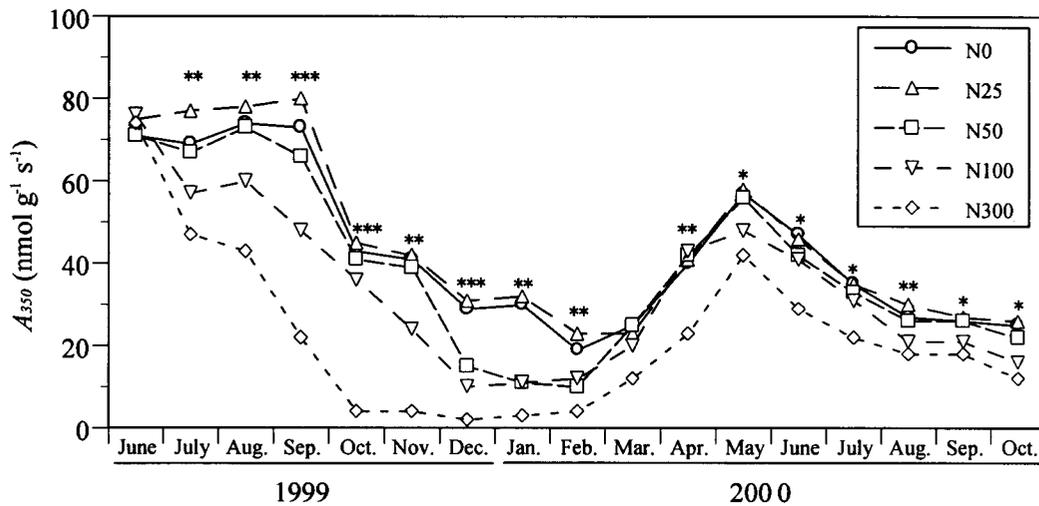


Fig. 3. Net photosynthetic rate at $350 \mu\text{mol mol}^{-1} \text{CO}_2$ (A_{350}) in the '99 needles of *Pinus densiflora* seedlings grown in five N treatments. Each value is the mean of 4 determinations. Significant effects of N load on A_{350} at each observation date were indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA).

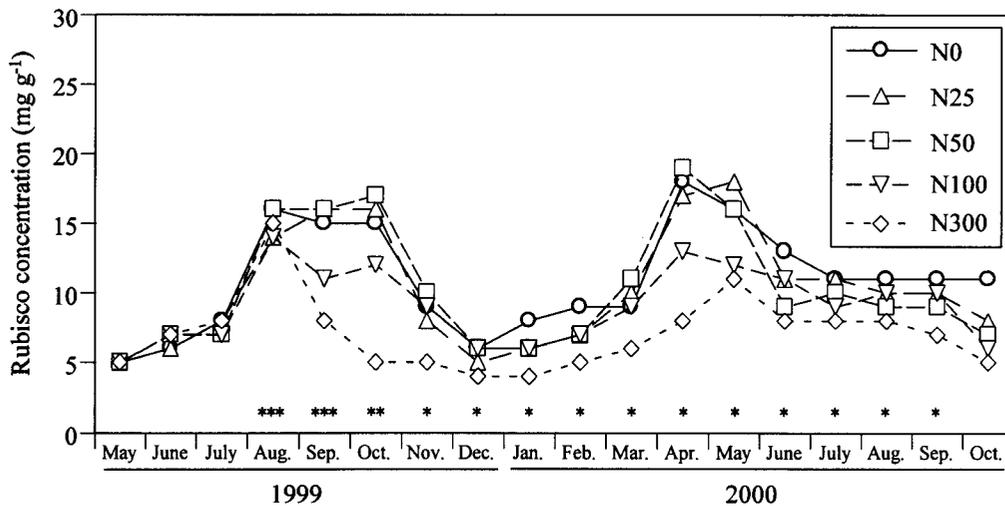


Fig. 4. The concentration of Rubisco in the '99 needles of *Pinus densiflora* seedlings grown in five N treatments. Each value is the mean of 4 determinations. Significant effects of N load on Rubisco concentration at each observation date were indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA).

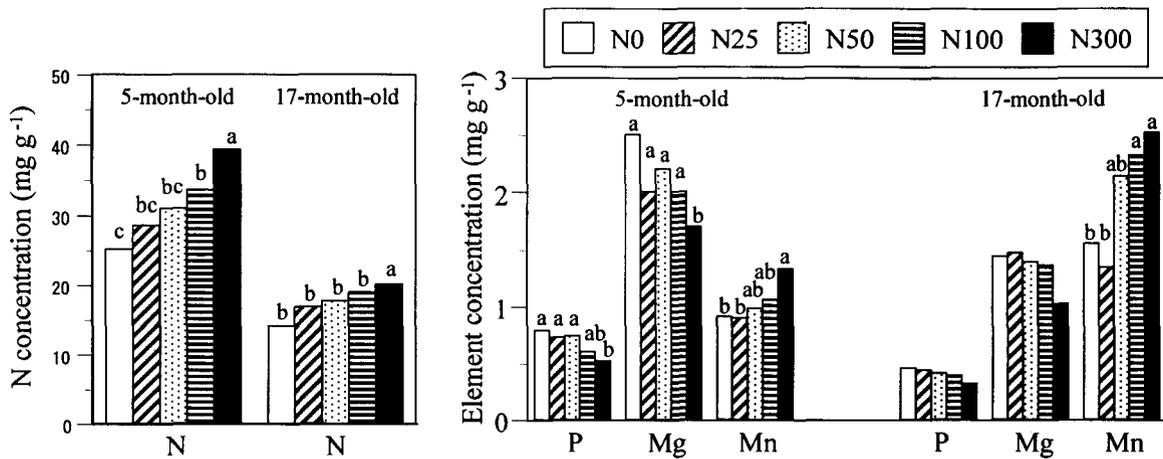


Fig. 5. The concentrations of elements in the '99 needles of *Pinus densiflora* seedlings grown in five N treatments on 25 October 1999 (5-month-old needles) and 28 October 2000 (17-month-old needles). Each value is the mean of 6 determinations. Different letters above the bars indicate significant differences among the N treatments (Duncan's multiple range test; $p < 0.05$).

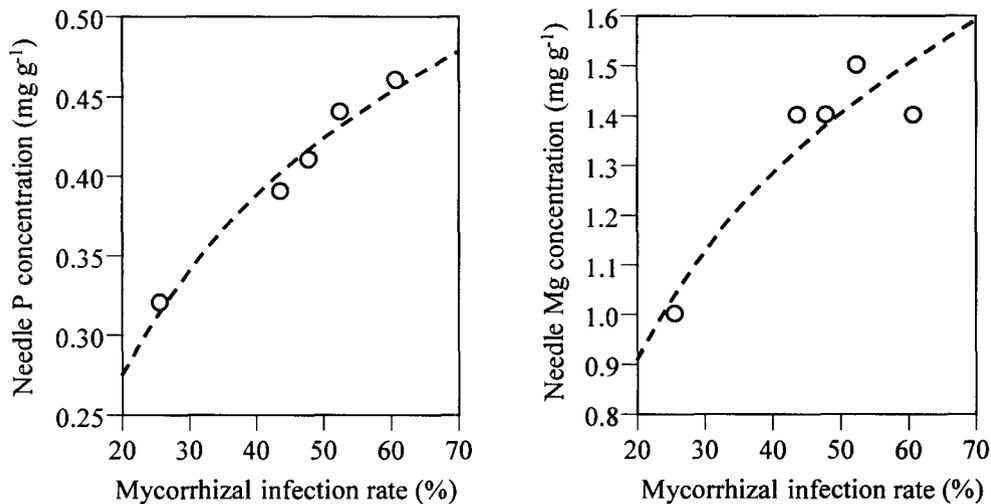


Fig. 6. The relationships between ectomycorrhizal infection and needle P or Mg concentration in *Pinus densiflora* seedlings in October, 2000. Each symbol shows the mean value in each N treatment (n=6).

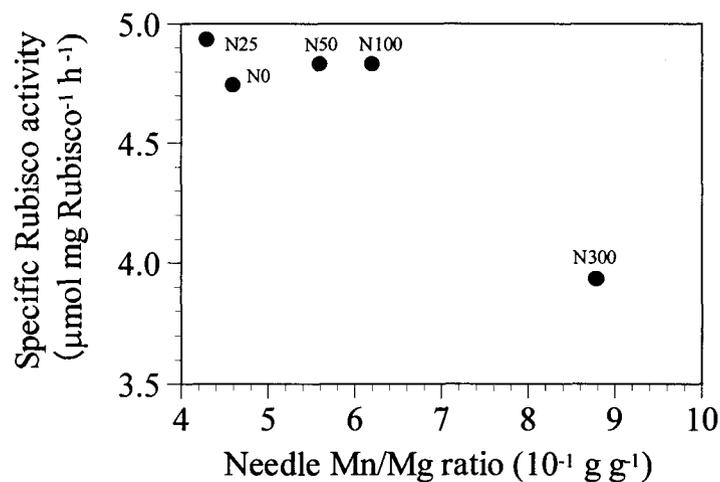


Fig. 7. The relationship between Mn/Mg ratio and specific Rubisco activity in the '99 needles of *Pinus densiflora* seedlings in September, 1999. Each symbol shows the mean value in each N treatment (n=5).

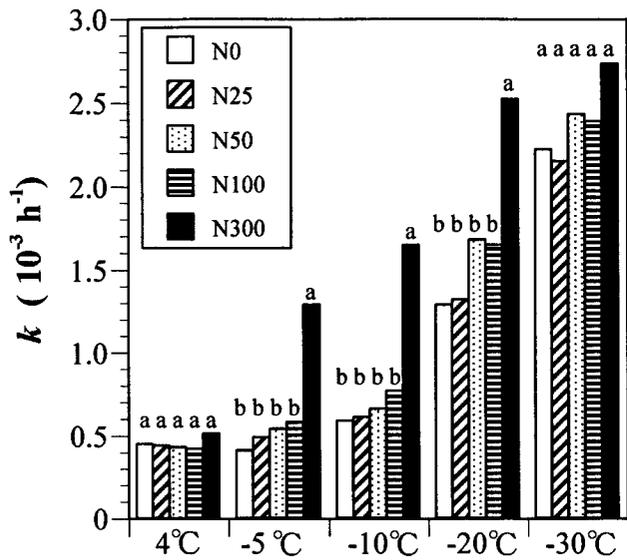


Fig. 8. Frost hardness as evaluated by electrolyte leakage rate (k) in the needles of *Pinus densiflora* seedlings grown in five N treatments. Each value is the mean of 4 determinations. Different letters above the bars indicate significant differences among the N treatments at each temperature (Duncan's multiple range test; $p < 0.05$).

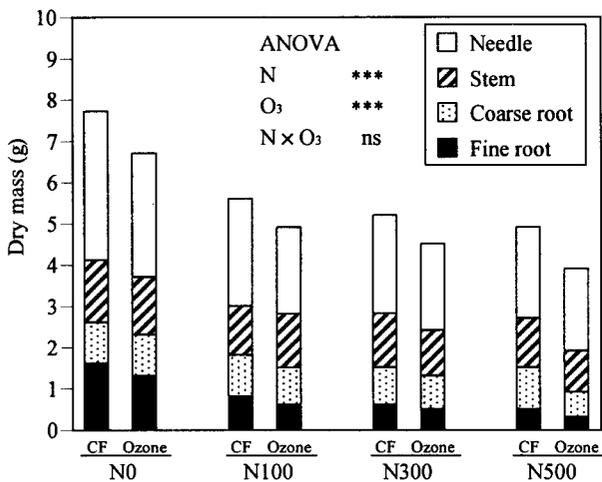


Fig. 10. The effects of N load and/or O₃ on dry mass of *Pinus densiflora* seedlings. Each value is the mean of 8 determinations. Two-way ANOVA: *** $p < 0.001$, ns=not significant.

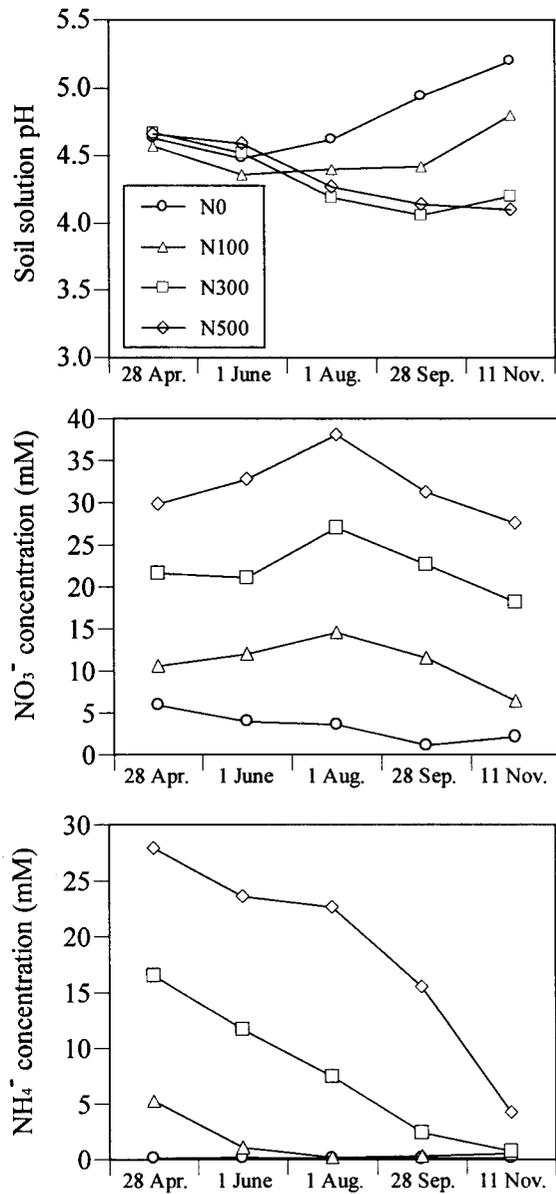


Fig. 9. The pH and concentrations of NH₄⁺ and NO₃⁻ in the soil solution during the growth period of *Pinus densiflora* seedlings. The data were pooled across the gas treatments, and each value is the mean of 8 determinations.

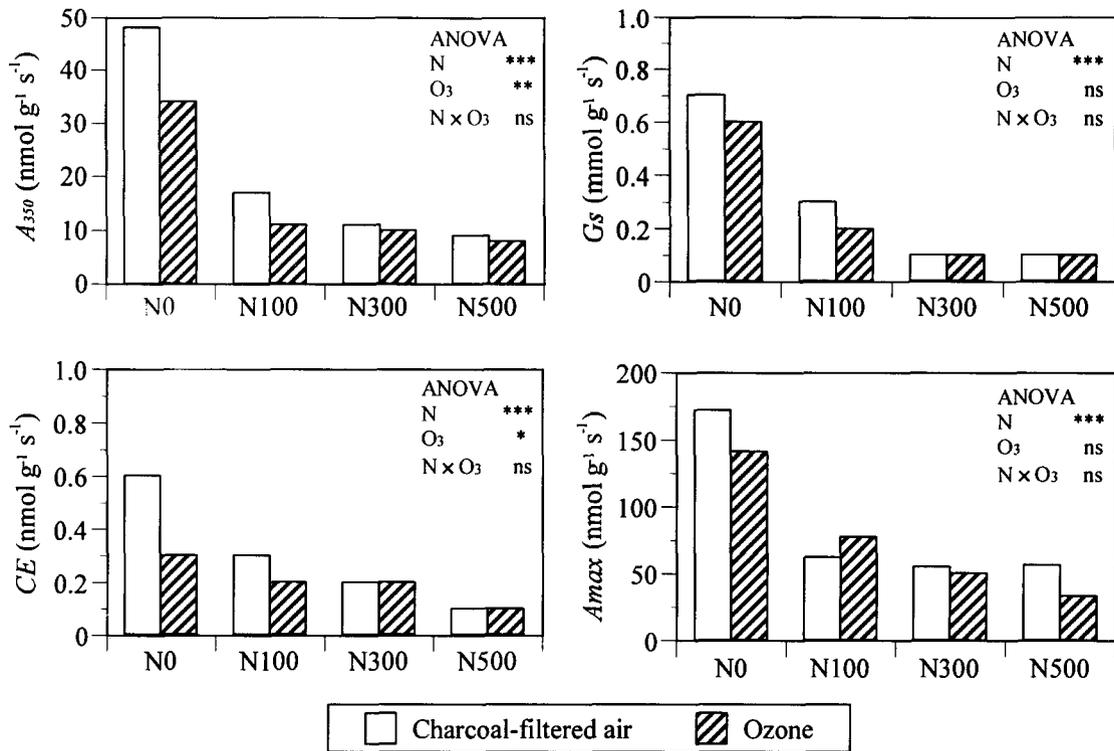


Fig. 11. The effects of N load and O₃ on net photosynthetic rate at 350 μmol mol⁻¹ (A_{350}), stomatal diffusive conductance to CO₂ (G_s), carboxylation efficiency (CE) and maximum net photosynthetic rate at saturated CO₂ and PPFD (A_{max}) of *Pinus densiflora* seedlings. Each value is the mean of 4 determinations. Two-way ANOVA: * p <0.05, ** p <0.01, *** p <0.001, ns=not significant.

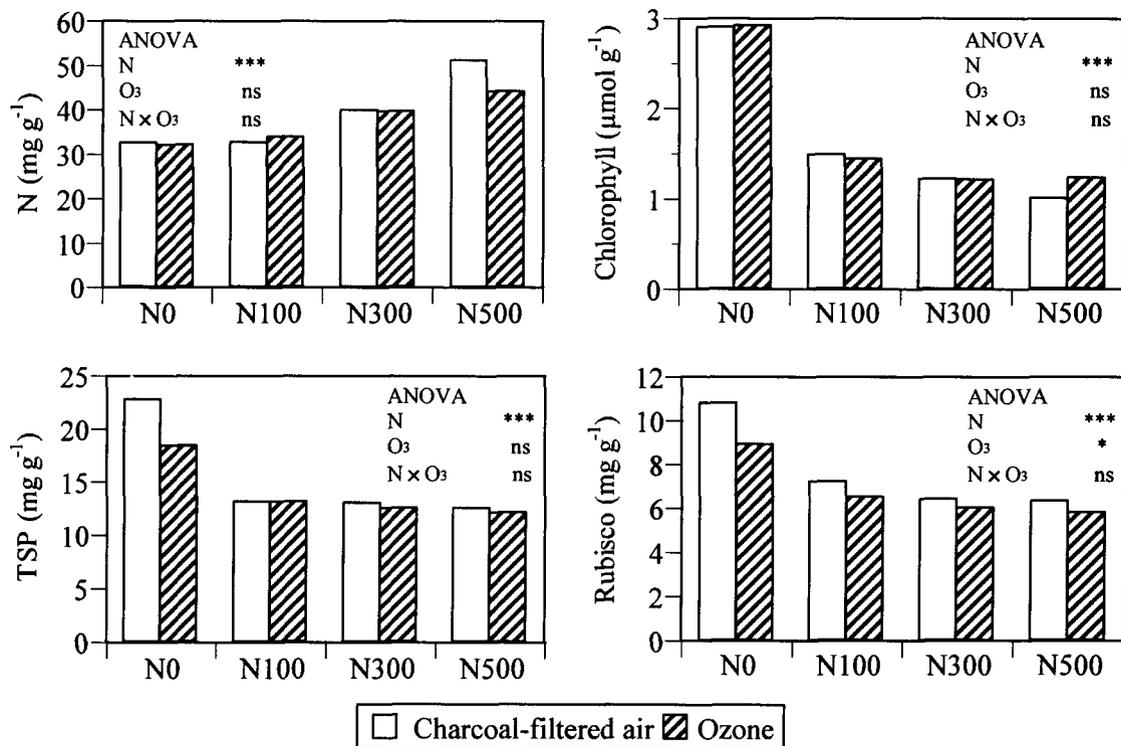


Fig. 12. The concentrations of N, chlorophyll, total soluble protein (TSP) and Rubisco in the current-year needles of *Pinus densiflora* seedlings. Each value is the mean of 4 determinations. Two-way ANOVA: * p <0.05, *** p <0.001, ns=not significant.

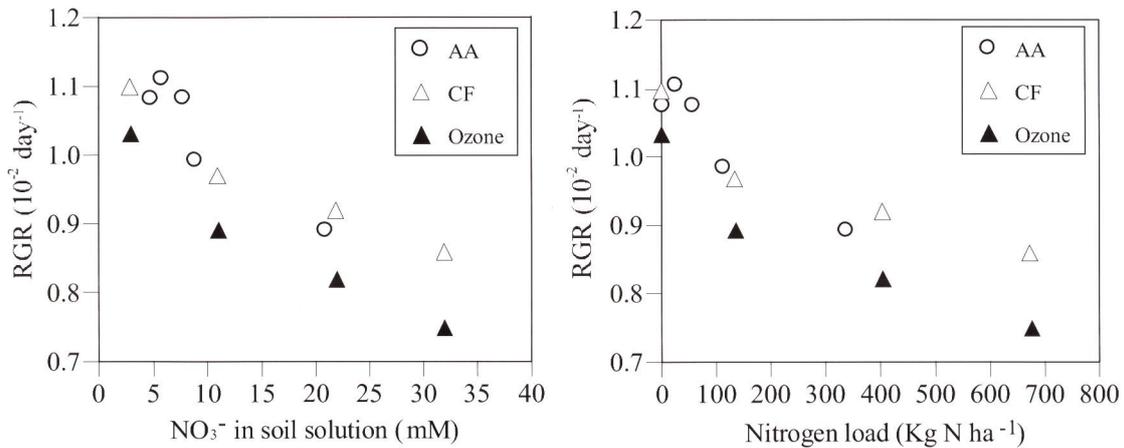


Fig. 13. The relationships between relative growth rate (RGR) of *Pinus densiflora* seedlings and NO_3^- concentration in the soil solution or N load in the two experimental studies. Nitrate concentrations in the soil solution are mean values of periodical determinations during the growing season of the seedlings. Nitrogen loads are expressed on the basis of surface area of potted brown forest soil. The RGRs of the seedlings exposed to ambient air in the first experiment (AA), charcoal-filtered air (CF) and 60 ppb O_3 in the second experiment were calculated by the following equation: $\text{RGR} (\text{day}^{-1}) = (\ln M_2 - \ln M_1) / dt$, where M_1 =initial whole-plant dry mass (g), M_2 =final whole-plant dry mass (g), dt =experimental period of one growing season (153 days in the first experiment and 173 days in the second experiment).



Photo. 1. Decline of *Pinus densiflora* in the western part of Japan (Hiroshima Prefecture).



Photo. 2. *Pinus densiflora* seedlings grown under field conditions at the experimental field of Tokyo University of Agriculture and Technology (Fuchu, Tokyo, Japan).

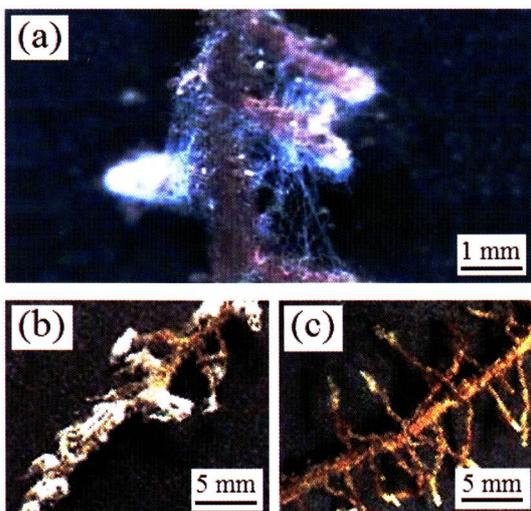


Photo. 3. Mycorrhizal root tips of *Pinus densiflora* seedlings grown in the N_0 treatment (a), and fine root of the seedlings grown in the N_0 (b) or N_{300} (c) treatment on 28 October, 2000.

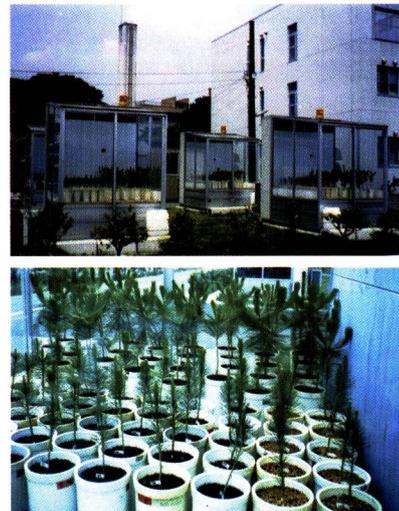


Photo. 4. Phytotrons at Tokyo University of Agriculture and Technology (Fuchu, Tokyo, Japan) and *Pinus densiflora* seedlings in the phytotron.

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