Effects of nitrogen supply on photosynthetic and anatomical changes in current-year needles of *Pinus koraiensis* seedlings grown under two irradiances

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Abstract

We investigated the responses of photon-saturated photosynthesis rate (P_{sat}) and its simultaneous acclimation of anatomy and nitrogen use patterns of current needles of Korean pine (*Pinus koraiensis*) seedlings grown under factorial combinations of two nitrogen levels and irradiances. Although N supply resulted in a significant increase of N content in needles under both irradiances, the increase of P_{sat} tended to be suppressed only in shade (S). The significant increase of P_{sat} in full sunlight (O) was associated with the increase of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) and chlorophyll (Chl) contents. In contrast, small increase of Chl content and no increase of RuBPCO content were found in S (90 % cut of full irradiance), which would result in a small increase of P_{sat}. This result suggests that extra N is stocked in needles under shade for the growth in next season. With N supply, a significant decrease of specific leaf area (SLA) was detected only in O. This decrease of SLA was due to the increase of density of needle. Furthermore, the increase of needle density was not due to the increased number and size of mesophyll cells, but the increased density of each mesophyll cell. Therefore, although SLA changed in O, the change did not involve anatomical adaptation to use increased N effectively, at least observable by light microscopy. Hence, even though the SLA would change, N deposition will improve the photosynthetic capacity of Korean pine seedlings, not through the development of needle anatomy but through improvement of the allocation of N in both irradiances.

Additional key words: acclimation; anatomical plasticity; evergreen-species; net photosynthetic rate; nitrogen allocation and deposition; ribulose-1,5-bisphosphate carboxylase/oxygenase; specific leaf area.

Introduction

To utilize radiant energy for effective carbon gain, plants change their nitrogen (N) usage along with the irradiation gradients (*e.g.* Šesták *et al.* 1985, Evans 1989, 1996). Not only total foliar N content, the fraction of N investment changes along with irradiance; and in shade (S) there is a lower content and more N selectively invested into chlorophyll (Field and Mooney 1986, Evans 1989, Schulze 1991). In general, plants that grow in low irradiance generally have thin leaves with large specific leaf area (SLA) accompanied by efficiently distributed and well composed chlorophyll (Chl) *a*/*b* ratio (Björkman 1981, Šesták *et al.* 1985, Larcher 2003). Difference in irradiance also results in the change of SLA and anatomical change (Evans and Poorter 2001). Such morphological and anatomical changes are a functional display of foliar N in space and time (*e.g.* Kitaoka and Koike 2004). These anatomical characteristics were also influenced by the change of foliar N content. High N content in a leaf also results in a large SLA, because with increasing thickness the internal leaf porosity increases to reduce diffusion of CO_2 and to use ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) effectively (Šesták *et al.* 1985, Field and Mooney 1986, Koike 1988, Lloyd *et al.* 1992, Terashima *et al.* 2001). These anatomical adaptations are also important for the adaptation to environment and give rise to optimal foliar N partitioning (Hikosaka and Terashima 1995, Niinemets 1999, Evans and Poorter 2001).

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Growth environment for plants is always changing. Nitrogen deposition is increasing along with the recent industrial development in Northeast Asia (Shibata *et al.* 2001, Galloway *et al.* 2004), and the optimal foliar N partitioning is supposed to be affected by N deposition as a fertilizer. In this region, Korean pine with evergreen leaf habit is broadly distributed (Kim 1999). How does such increase of N affect the N use pattern in regenerated tree seedlings through photosynthetic capacity?

Especially in S, such as forest floor, regenerated seedlings have low optimal allocation of N (Reich and Walters 1994, Bazzaz 1996, Kitaoka and Koike 2005). It is considered that high adaptation capacity of photosynthetic function in Korean pine to deep S may be realized both by change in SLA and greater allocation of N to light-harvesting components in needles. In such condition, plant response to increased N deposition may often include symptoms of excess N. Furthermore, such evergreen conifers generally have lower N use efficiency and adaptation mechanisms to keep limited N resources in a long life of leaf (Kikuzawa 1995, Reich *et al.* 1995, Stenberg *et al.* 1995, Walters and Reich 1999, Hikosaka and Hirose 2000), so that the effects of N supply on physiological functions in evergreen conifer would be different from those on herbaceous and deciduous plants which have been well studied (*e.g.* Warren *et al.* 2003).

These mechanisms of adaptation to deep S would be affected by recent N deposition, and as a result, lead to a change of vegetation. To confirm this expectation, we should examine two points: (*1*) How does N supply affect photon-saturated photosynthetic rate (P_{sat}) through the change of N use and anatomy of needles? (*2*) If the changes are different between two irradiances, by what mechanisms does this come about?

We hypothesized as follows: N supply would lead to an increase of N content independently of irradiance. N investment would occur preferentially to RuBPCO in full sunlight (O), but in shade increased N would result in a selective increase of Chl. The SLA would increase, because increased internal porosity would occur to use RuBPCO efficiently in O; in S, the leaf area would increase to use Chl effectively. As a result, these adaptations would lead to an increased P_{sat} in both irradiances.

To reveal these hypotheses, we raised Korean pine seedlings under the conditions of factorial combinations of two irradiances and two N levels (all four combinations) and determined photosynthetic function, N allocation, and anatomical change in needles.

Materials and methods

Experimental design: We set four plantation units (9 m^3) in the Sapporo experimental nursery of Hokkaido University, in northern part of Japan (43 06''N, 141 20'E). In each plantation unit, six Korean pine (*Pinus koraiensis*) seedlings (two-years-old) were planted. The species is characterized by good tolerance in S, slow growth rate, and good tolerance of soil acidification (Choi *et al.* 2006). We set two different irradiances, O (full sunlight) and S in each unit. To create the S condition, we used the neutral shading clothes. In S, the seedlings received a relative 5–10 % photosynthetic photon flux density (PPFD) of full sunlight. We also set two soil N contents (ambient and N-rich condition) by supplying $(NH_4)_2SO_4$; *i.e.* the amount of N supply was 30 kg per ha and year to simulate the total amount of N deposition in northern Japan (Kitaoka 2003). The timing was two weeks after planting. Photosynthetic measurements and samplings for foliar chemical analysis were conducted in early September when the current year needles of Korean pine will get the maximum photosynthetic capacity in all ages of needles (Choi *et al.* 2006).

Photosynthetic measurements: A portable computerized open gas system IRGA (*LI-6400*, *LiCor*, Lincoln, NE, USA) was used for measuring the photon-saturated rate of CO_2 assimilation per unit needle area (P_{sat}/area) of fully expanded mature current-year needles. Five needles were placed in the chamber, with their abaxial surfaces turned upward, and were exposed to PPFD increasing gradually from 0 to 1 600 µmol m^{-2} s^{-1}; the P_{sat}/area value was determined from the irradiance-photosynthesis curve measured at constant CO_2 concentration (360 µmol mol^{-1}), the chamber temperature (25 °C), and saturated PPFD (1 600 µmol m^{-2} s^{-1}). All measurements took place between 500 and 1 000 h on sunny days. The resulting irradiance-photosynthesis curve was fitted by the well known function of Boote and Loomis (1991).

SLA and other anatomical parameters of the needles: We defined the anatomical abaxial surface as a needle surface. Ahead of this study, we checked the total leaf area using cross section. But the ratios between surface area and total surface area were almost the same among all conditions. Therefore, we only used needle abaxial surface area (A) to determine the basic area parameter. The needle projection area was measured with a scanner (*Canon LIDE-80*), using the same needle as for photosynthesis determination. Each needle was put on the scanner, with its abaxial surface turned downward. Thereafter, needle dry mass was determined after drying at 80 ºC to constant mass. SLA was calculated by using the area and mass data. Furthermore, we divided SLA into two parameters, *i.e.* reciprocal value of the product of density (D = dry mass per volume) and volume (V) per surface area (V/S) to investigate the meanings of change in SLA:

$$SLA = S/DM = 1/[(DM/V)\,(V/S)],$$

where DM denotes dry mass. By using the data for SLA and V/S, we calculated the needle density (D). We measured V/S, width, and thickness of a needle to investigate the change of needle shape. We also measured the internal porosity and mesophyll surface area per unit needle area (A_{mes}/A), as main contributing factors to needle and cell density. Also, A_{mes}/A represents the number or size of mesophyll cells. In a Korean pine needle, it is difficult to distinguish between palisade and spongy cells. We therefore identified cells other than bundle sheath cells or epidermal cells as "mesophyll cells". Also, their shapes were too complex to approximate to any model shapes, therefore, we used the simple abaxial surface outline length as A, and the simple total outline length of all mesophyll cells as A_{mes}. For these observations, we studied anatomical cross section of needles. Pieces of needles were infiltrated and fixed in 2.5 % glutaraldehyde in 100 mM phosphate buffer (pH 7.0) for at least overnight at 4 °C. They were then dehydrated in an ethanol series, substituted with *n*-butyl glycidyl ether (QY-1), and were embedded in Spurr's resin. Sections, 2 μm thick, were cut with an ultra-microtome (*Ultracut-N*, *Reichert*, Vienna, Austria) and stained with toluidine blue for light microscopy. Photomicrographs were taken from these sections at a magnification of 20×. Each anatomical parameter was analyzed with *Image J* (National Institutes of Health, Maryland, USA).

Contents of N and other elements in needles: Needle N content was determined with a CHN/O analyzer (*PE 2400* series II, *Perkin-Elmer*, Norwalk, CT, USA), using the same sample as for photosynthesis and SLA determinations. We used needles for Chl measurement that were adjacent to those of photosynthesis measurements. Chl was extracted with dimethyl sulfoxide (DMSO) and measured spectrophotometrically (type *100-50*, *Hitachi*, Tokyo, Japan) to determine the total content of Chl per area. The RuBPCO/area content was determined by the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970, Makino *et al.* 1988). About 100 mg of frozen needles was homogenized in 100 mM sodium phosphate buffer (pH 7.5) containing 1 % (m/v) β-mercaptoethanol and 2 % (m/v) polyvinylpyrrolidone, with a small amount of quartz sand (Kitaoka and Koike 2004). After centrifuging at 9 000×*g* for 1 min, the supernatant fluid was treated with SDS solution [1.0 % (m/v), final concentration] at 100 °C for 5 min. The resulting extract with standard solution made using bovine albumin (*Sigma-Aldrich*, St. Louis, MO, USA), was applied to the SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250. Digital images of gels were obtained using a laser scanner. Absorbance of bands corresponding to the large subunit of RuBPCO was analyzed with *Image J* (National Institutes of Health, Maryland, USA). Each parameter is re-expressed on an area base by using SLA.

Statistical analysis: Differences in the effects of shading treatment, N supply, and their interactions were evaluated by two-way analysis of variance (ANOVA). Differences were considered significant at $p<0.05$. The analysis was performed with the R software, version *2.1.0*.

Results

P_{sat}/area of the seedlings grown in the shade was significantly lower than that for seedlings grown in open condition (Fig. 1). N supply increased P_{sat}/area significantly in both irradiances, but the degree of the increase tended to be suppressed in S (Fig. 1; $p=0.101$).

N, Chl, and RuBPCO per needle area: N/area was significantly lower in S than that in O (Fig. 2*A*). With N supply, N/area increased significantly in both irradiances (Fig. 2*A*). There was no significant difference in the degrees of increases of N/area between two irradiances (Fig. 2*A*). Although the average Chl/area was higher in S than that in O without added N, the effect of shading treatment was not significant (Fig. 2*B*). Whereas N supply led to an increase in Chl/area, the degree of its increase was significantly higher in O than that in S (Fig. 2*B*). RuBPCO/area decreased by shading treatment, and N supply tended to increase the RuBPCO/area only in O (Fig. 2*B*; $p=0.08$).

SLA and other anatomical features: SLA was significantly lower in O than in S (Table 1). Supply of N decreased the SLA in full sunlight, but did not cause significant change in S (Table 1). The width and thickness of needle were significantly less in S, but no effect of N supply was observed in either irradiance (Table 1, Fig. 3). The increase due to shading treatment was similar for width and thickness, so that the needle proportions were unchanged (Fig. 3). Both V/S and A_{mes}/A were significantly lower in S than in O, but no effect of N supply was detectable on either parameter (Table 1, Fig. 3). The internal porosity of needle was almost the same in all four treatments (Table 1, Fig. 3).

Discussion

P_{sat}/area increased in both irradiances with N supply, but the degree of increase tended to be suppressed in the shade. In S, although the optimal N content would be low (Evans and Poorter 2001), there was a significant increase of foliar N content (Fig. 2*A*). From these results, we suggest that the seedlings in S did not use extra N in photosynthesis. Needles passively absorb more N when there is a supply of fixed N, but the N usage pattern in needle changed in different ways in different irradiances.

Physiological adaptation: Whereas the increased N/area would result in both increased Chl/area and RuBPCO/area in O, it would lead to a suppressed increase in Chl/area and no change of RuBPCO/area in S. Adaptation of both the photon absorption and carboxylation capacity occurred in O, accompanied by a significant increase in P_{sat}/area (Figs. 1 and 2*B*). In natural N-limited condition, the relative investment of N shows the trade-off relationship between RuBPCO and Chl contents, *i.e.* light-harvesting protein (Hikosaka and Terashima 1996). However, the RuBPCO and Chl contents would increase to fix CO_2 as much as possible given adequate N and irradiance.

In S, N was allocated selectively into Chl in needles (Fig. 2*B*) to capture photons, which was the most essential factor for photosynthesis in foliar acclimation capacity to environment (*e.g.* Kitaoka and Koike 2004). Of course, allocation of N in needles in shade to RuBPCO/area was very limited. This improvement of the light-harvesting is expected to result in little increase of P_{sat}/area. However, whereas the increase of Chl/area was suppressed, N/area was not suppressed (Fig. 2*A,B*). More N/area, except that allocated to Chl, is expected to be stored as amino acids and soluble protein in readiness for improvement in irradiance due to disturbances, over-wintering, and the growth in the next year (Millard and Proe 1992). This storage mechanism in needles is supposed to be a peculiar adaptation of evergreen species when N uptake from the environment exceeds immediate growth requirements (Warren *et al.* 2003). As well as the change in relative N investment within photosynthetic apparatus, the change including stock mechanism is important. These arguments relate strongly to the changes of P_{sat}/area.

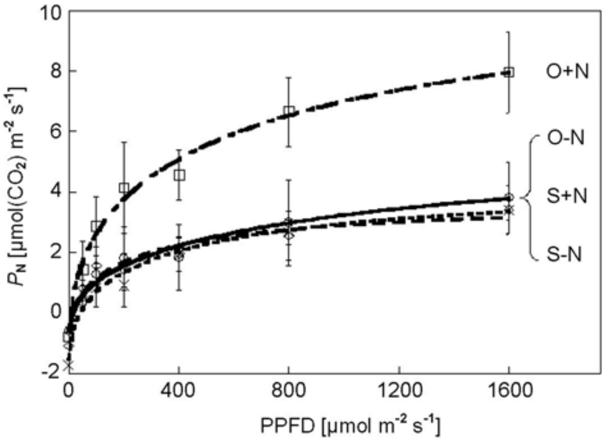

Fig. 1. Examples of irradiance-photosynthesis curves in needles of seedlings grown under full sunlight (O), 5–10 % of full sunlight (S), no nitrogen-supplied (–N), and nitrogen-supplied (+N) conditions expressed as net photosynthetic rate per unit abaxial surface area of needles (P_N). Curves were fitted with asymptotic equation of Boote and Loomis (1993). PPFD = photosynthetic photon flux density during photosynthesis measurements. *Error bars* show SE of mean value of four replications.

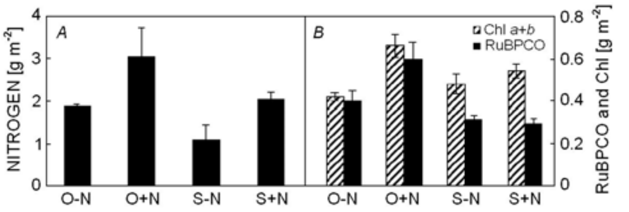

Fig. 2. Contents of nitrogen (*A*), chlorophyll (*B*, *slash bar*), and RuBPCO (*B*, *normal bar*) in needles of seedlings grown under full sunlight (O), 5–10 % of full sunlight (S), no nitrogen-supplied (–N), and nitrogen-supplied (+N) conditions expressed as mass per unit abaxial surface area of needles. *Error bars* show SE of the mean value of four replications.

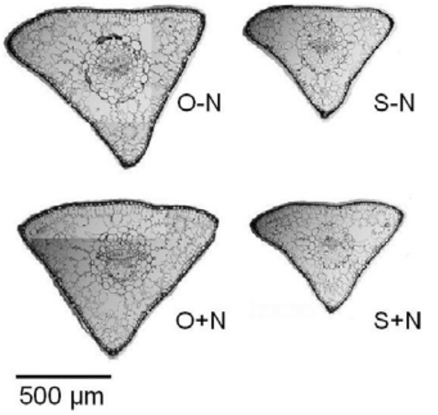

Fig. 3. Needle cross sections of seedlings grown under full sunlight (O), 5–10 % of full sunlight (S), no nitrogen-supplied (–N), and nitrogen-supplied (+N) conditions.

Anatomical adaptations: Whereas SLA was lower in O, no effect on SLA upon N supply was found in S (Table 1, Fig. 3). Since V/S did not change upon supplying N in either irradiance, the lower SLA in O was due to the increase of needle density (D = g/V). This decrease is mainly due to a reduction in the internal porosity of needle, increases in cell density and also in cuticle thickness (Koike 1988), starch accumulation, and increased numbers of grana and thylakoids per granum (Niinemets 1999, Bondada and Syvertsen 2003). In the present study, however, the main contributors (*i.e.* internal porosity and A_{mes}/A) were not changed by N supply. From these results, the change of needle density and SLA with N supply were due to the change of density of each mesophyll cell (intercellular density), as reported by Bondada and Syvertsen (2003). In their study, the development of chloroplast by N supply led to the decrease of SLA. Although we found that the SLA changed by N supply in O, anatomical change did not contribute to

Table 1. Photosynthetic rates and physiological and anatomical parameters in needles of seedlings grown under full sunlight (O), 5–10 % of full sunlight (S), no nitrogen-supply (–N), and nitrogen-supplied conditions (+N) expressed as mass per unit abaxial surface area of needles. Chl = chlorophyll, SLA = specific leaf area, A = needle area. Means± SE of four replications.

Parameter	O–N	O+N	S–N	S+N
P_{sat} [μmol(CO$_2$) m^{-2} s^{-1}]	3.77±1.20	7.27±1.37	3.03±0.53	1.77±0.88
N [g m^{-2}]	1.88±0.03	3.03±0.70	1.06±0.39	2.03±0.16
Chl a+b [mg m^{-2}]	419.77±20.10	663.49±55.02	480.03±47.64	541.62±34.69
RuBPCO [mg m^{-2}]	400.60±43.70	596.00±80.90	309.90±21.70	291.20±24.50
SLA [m^2 kg^{-1}]	1054.40±26.60	871.80±34.60	1617.30±25.00	1653.20±14.7
Needle width [μm]	616.00±19.75	635.24±29.86	471.40±17.13	490.01±13.96
Needle thickness [μm]	480.00±24.26	490.41±25.25	376.00±14.44	376.60±19.03
Internal porosity [%]	16.06±1.96	14.25±1.73	12.89±2.22	14.48±1.64
Volume to surface area ratio	435.12±20.42	398.44±58.74	307.63±19.68	313.69±39.75
A_{mes}/A	15.92±1.00	16.87±1.02	15.29±1.06	16.00±0.40

Table 2. Analyses of variance for all measured parameters in seedlings in response to shading treatment (I) and nitrogen supply (N). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, ns = not significant. The value of p around 0.01 is also shown.

Parameter	I	N	I×N
P_{sat}/area	*	*	0.101
N/area	*	*	ns
Chl a+b/area	ns	**	*
RuBPCO/area	**	0.08	*
SLA	***	**	*
Needle width	***	ns	ns
Needle thickness	***	ns	ns
Internal porosity	ns	ns	ns
Volume to surface area ratio	*	ns	ns
A_{mes}/A	ns	ns	ns

the change of P_{sat}/area (at least according to light microscopy observation). The plant species has specific anatomical plasticity to N conditions (Ishii *et al.* 2001). We concluded that Korean pine seedlings have low anatomical plasticity to the change of N. Contrary to our expectations, this low plasticity led to a decrease in SLA which did not involve the anatomical adaptation.

On the other hand, we could get implication how SLA changes not because change of shape, like thickness and V/S, but because increase in density (D = g/V). In previous studies, increases in foliar N content led to decrease or no change of SLA (Niinemets 1999, Niinemets *et al.* 2001, Gough *et al.* 2004). For instance, Niinemets (1999) reported that the increases of foliar N content and photosynthetic rates were positively correlated with foliage thickness and negatively with SLA. Moreover, he also indicated that these relationships might be critically altered by the variation in foliar density. In our research, the change of needle density contributed to the change in SLA. Moreover, the increase of density was due to the increased intercellular density; although there was no change of shape. The conflicting results would be well explained by taking plasticity of leaf shape into consideration. If the plasticity of leaf shape to the changes in the N supply is low, as found in our study, then the N supply would lead to the increased photosynthetic apparatus. This would in turn give acceleration in photosynthetic rate following development of photosynthetic apparatus in needles, as well as the increased needle density.

Conclusion: The effect of N supply on P_{sat}/area of needles of Korean pine seedlings is positive in both O and S conditions. The changes in P_{sat}/area appear to be mainly due to the different allocation of N between Chl, RuBPCO, and probably form of storage in each irradiance. The decrease in SLA at high N supply is due to an increase in each cell density, but not an increase in the number of mesophyll cells. Our results indicate that N deposition will increase the photosynthetic capacity of Korean pine seedlings, not by development of needle anatomy, but by improvement of the allocation of N, in both S and O conditions.

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