Title:
The leaf anatomy of a broad-leaved evergreen allows an increase in leaf nitrogen content in winter.

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Abstract

In temperate regions, evergreen species are exposed to large seasonal changes in air temperature and irradiance. They change photosynthetic characteristics of leaves responding to such environmental changes. Recent studies have suggested that photosynthetic acclimation is strongly constrained by leaf anatomy such as leaf thickness, mesophyll and chloroplast surface facing the intercellular space, and the chloroplast volume. We studied how these parameters of leaf anatomy are related with photosynthetic seasonal acclimation. We evaluated differential effects of winter and summer irradiance on leaf anatomy and photosynthesis. Using a broad-leaved evergreen *Aucuba japonica*, we performed a transfer experiment in which irradiance regimes were changed at the beginning of autumn and of spring. We found that a vacant space on mesophyll surface in summer enabled chloroplast volume to increase in winter. The leaf nitrogen and Rubisco content were higher in winter than in summer. They were correlated significantly with chloroplast volume and with chloroplast surface area facing the intercellular space. Thus, summer leaves were thicker than needed to accommodate mesophyll surface chloroplasts at this time of year but this allowed for increases in mesophyll surface chloroplasts in the winter. It appears that summer leaf anatomical characteristics help facilitate photosynthetic acclimation to winter conditions. Photosynthetic capacity and photosynthetic nitrogen use efficiency were lower in winter than in summer but it appears that these reductions were partially compensated by higher Rubisco contents and mesophyll surface chloroplast area in winter foliage.
**Introduction**

Evergreens inhabiting the understory in a temperate deciduous forest are exposed to large changes in temperature and light over the year. Air temperature is around the freezing point in winter and about 25°C in summer (Röhrig 1991). The irradiance in the understory changes dramatically with shedding and flushing of canopy leaves. Daily integrated PPFD under an open canopy is three to tenfold higher in winter than in summer despite the shorter day length (Skillman *et al.* 1996; Muller *et al.* 2005).

Leaves acclimate to changes in temperature and irradiance (e.g. Berry and Björkman 1981; Björkman 1981; Huner *et al.* 1998). In evergreens several leaf traits change seasonally: they have more nitrogen and Rubisco, a higher Chl a/b ratio, a higher Rubisco/Chl ratio, and a lower Chl/nitrogen ratio in winter (Skillman *et al.* 1996). These parameters of photosynthesis are correlated to ambient temperature and irradiance (Muller *et al.* 2005), and may represent adaptive responses to the seasonal changes in environmental conditions.

Leaf anatomy has been shown to play an important role in photosynthetic acclimation in herbaceous and deciduous tree species. Leaves are thicker with a higher leaf nitrogen content and a higher $P_{\text{max}}$ when grown at high than at low irradiance (Terashima *et al.* 2001). Thicker leaves allow for a larger chloroplast surface area facing the intercellular space ($S_c$), which is essential for efficient CO$_2$ diffusion to the carboxylation site in the chloroplasts (von Caemmerer and Evans 1991; Evans *et al.* 1994; Terashima *et al.* 2001). However, mature leaves in most species do not have flexibility in leaf anatomy when environmental conditions change (Sims and Pearcy 1992). Recently Oguchi *et al.* (2003, 2005, 2006) showed that shade leaves in an annual herb and several deciduous tree species had vacant space along the mesophyll cell surface, which allowed for an increase in $S_c$ and $P_{\text{max}}$ when irradiance increased. On the other hand, shade leaves of *Fagus crenata* had less vacant space and changed neither leaf nitrogen content nor $P_{\text{max}}$ when irradiance increased (Oguchi *et al.* 2005). These results suggest that, to increase $P_{\text{max}}$, leaves need to be thicker than the minimum to arrange all chloroplasts to fill the mesophyll surface (Oguchi *et al.* 2005). Although thick shade leaves are costly in terms of biomass investment, an increase in carbon gain with photosynthetic acclimation is greater than the invested biomass to allow acclimation if gaps are formed (Oguchi *et al.* 2008).
Although several studies showed that mature leaves of evergreen species change photosynthetic components in response to seasonal environmental changes (Skillman et al. 1996, Miyazawa and Kikuzawa 2005), there seems no study on the role of leaf anatomy, particularly of the vacant space in those changes. Because evergreen species in the understory of a deciduous forest are exposed to a large increase in irradiance and a decrease in temperature from summer to winter, we hypothesize that they have a large vacant space in summer to allow an increase in chloroplast volume towards winter. Alternatively, the leaf anatomy may limit an increase in $S_e$ and an increase in chloroplast volume would not increase the light saturated photosynthetic rate at ambient CO$_2$ ($P_{\text{max}}$). This might have been the case for the studies that showed a decrease in $P_{\text{max}}$ in spite of an increase in leaf nitrogen content in winter (Muller et al. 2005, Miyazawa and Kikuzawa 2005). If the increase in leaf nitrogen during winter was solely for a non-functional storage (Warren and Adams 2004) the leaf anatomy would maximize photosynthesis in summer and not in winter, and there would be no abundant vacant space along the mesophyll cell surface walls.

The aim of the study was to elucidate how leaf anatomy relates to the photosynthetic acclimation to the seasonal environment. More specifically, our question was whether leaf anatomy was to maximize photosynthesis in summer or in winter. To answer this question, we performed a transfer experiment in which irradiance of the plants was changed at the beginning of autumn and of spring. If leaf anatomy was to maximize photosynthesis in winter, plants that were exposed to high irradiance in winter would have greater leaf thickness irrespective of irradiance in summer. If it was to minimize biomass investment, leaf thickness would be determined by irradiance in summer. We raised *Aucuba japonica*, an evergreen shrub in temperate forests in Japan, for two years at different irradiance regimes under the natural seasonal changes in temperature. Seasonal changes in photosynthetic and anatomical traits were studied on new leaves in the second year of the experiment.

**Materials and methods**

*Aucuba japonica* Thunb. is a common understory shrub in Japan. It is evergreen and dioecious with relatively large and long-lived leaves. Under natural conditions in central Japan the mean life span of
leaves was 2.6 years (Yamamura 1992). After bud break in April it flowers, expands new leaves and shed the oldest leaves within about one month.

In August 2001 we collected saplings in the forest dominated by *Quercus serrata* adjacent to the experimental garden (38°15’ N 140°50’ E) of Tohoku University. The saplings were about 20 cm high and 3-4 years old. They were not branched and had 2-4 current-year leaves and 2-4 previous-year leaves. *A. japonica* had relatively thick roots that were easily excavated from the humus layer of the forest soil without visible damage to the roots. The saplings were potted individually in 4-litre pots in a 16:3 mixture of kiryusuna (Japanese volcanic sand) and pelapon (cocos fibre). Each pot received 20 ml of a commercial nutrient solution (1.8 mM [N], Hyponex NPK = 6:10:5) weekly. Additional water was provided when needed. In winter, nutrients were given every two weeks. The plants were grown under one or three layers of neutral shading cloth, high and low irradiance, respectively, in a vinyl house with open sides. Irradiance (PPFD) was measured with a quantum sensor (LI190-SA, Li-Cor Inc, Lincoln, NE, USA) connected to a data logger (LI1000, Li-Cor) at one-hour intervals. Temperature in the vinyl house was measured with Onset temperature loggers (StowAway Tidbit Temperature logger, Onset, MA, USA).

Plants were allotted randomly to four sets with different irradiance regimes to test the effect of irradiance in relation to temperature over the years. One set of plants was grown at high irradiance (*I*<sub>high</sub>) and the second at low irradiance (*I*<sub>low</sub>) throughout for two years. The third set of plants was grown at high irradiance in winter and low irradiance in summer (*I*<sub>dec</sub>) simulating the deciduous understory conditions. The fourth set was inverse to the previous one with low irradiance in winter and high irradiance in summer (*I*<sub>inv</sub>). *I*<sub>dec</sub> and *I*<sub>inv</sub> sets of plants were transferred between high and low irradiance treatments on 20 November 2002, 3 May 2003, 10 November 2003 and 25 April 2004 (Fig. 1), approximately at the time when the surrounding deciduous forests shed old leaves (November) or flushed new leaves (April/May). Photosynthetic measurements were taken in the second year from summer 2003 to spring 2004.

Three healthy plants were selected from each of the 4 irradiance regimes (*I*<sub>high</sub>, *I*<sub>low</sub>, *I*<sub>dec</sub>, *I*<sub>inv</sub>) for photosynthesis measurement and biochemical analyses every season. Measurements were taken in the laboratory within about 2 weeks: summer, 15-30 August 2003; autumn, 26 November–5 December
2003; winter, 30 January –12 February 2004; spring 20 May- 30 May 2004. Thus measurements were taken more than 2 weeks after transfer in \( I_{dec} \) and \( I_{inv} \). One non-shaded leaf per plant was selected for photosynthesis measurements on the attached leaf using a portable gas exchange system (Li-6400-40, LiCor). The light-saturated rate of photosynthesis (\( P_{\text{max}} \)) was determined at a CO\(_2\) concentration of 370 \( \mu \text{mol mol}^{-1} \), PPFD of 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), leaf temperature of 15 °C and 80% relative humidity. After photosynthesis measurements in a controlled temperature room the plants were returned to their respective irradiance regime outside. Within 1-2 days the leaves used for photosynthesis were harvested in the morning and subjected to biochemical analysis and leaf anatomy. For these analyses at least 5 leaf discs of 10 mm in diameter were punched out per leaf in the laboratory.

Three leaf discs were dried at 80°C for more than 3 days. After dry mass determination, nitrogen (N) content was determined with an NC analyzer (NC-80, Shimadzu, Kyoto, Japan). At least 2 leaf discs were stored at -80°C for determination of Rubisco as described in Muller et al. (2005). Of the remaining leaf, sections of about 1 x 2 mm were cut out and directly prepared for light microscopy as described by Oguchi et al. (2003). The fixated leaves were embedded in Spurr’s resin and 0.8 \( \mu \text{m} \) thick cross-sections were cut. One or two cross-sections per leaf were analysed using the image processing software ImageJ v1.36b (NIH, MD, USA). The surface area of mesophyll cells facing the intercellular space (\( S_{\text{mes}} \)), the area of chloroplast surfaces facing the intercellular space (\( S_{c} \)) and the total chloroplast volume per unit leaf area (\( V_{\text{chl}} \)) were calculated. To convert length in cross-section to surface area, a curvature factor (\( F \)) was determined assuming that the shape of the palisade cells was a cylinder with flat ends and that the sponge cell was a spheroid (Thain 1983). \( F \) was 1.26-1.35 in palisade and 1.12 -1.23 in spongy cells.

The value of \( P_{\text{max}} \) was analysed as the product of leaf nitrogen content per leaf area (\( N_{\text{area}} \)) and the photosynthetic nitrogen use efficiency (PNUE):

\[
P_{\text{max}} = N_{\text{area}} \cdot \text{PNUE} \tag{1}
\]
Considering that most leaf N is in chloroplasts (Evans 1989) and that arrangement of chloroplasts along mesophyll surface is affected by various morphological parameters, N\textsubscript{area} was analyzed as follows (Oguchi \textit{et al.} 2005):

\[ N_{\text{area}} = \frac{N_{\text{area}}}{V_{\text{chl}}} \cdot V_{\text{chl}}/S_c \cdot S_c/S_{\text{mes}} \cdot S_{\text{mes}} \quad (2) \]

\( N_{\text{area}}/V_{\text{chl}} \) indicates the N concentration per unit chloroplast volume, if a fixed fraction of leaf N is in chloroplasts (Evans 1989), \( V_{\text{chl}}/S_c \) indicates chloroplast thickness, \( S_c/S_{\text{mes}} \) is the fraction of chloroplast area facing the intercellular space.

PNUE was analysed as the product of the following factors:

\[ \text{PNUE} = \frac{P_{\text{max}}}{\text{Rubisco}} \cdot \text{Rubisco}/N_{\text{area}} \quad (3) \]

where Rubisco is the Rubisco content per leaf area. \( P_{\text{max}}/\text{Rubisco} \) may decrease when the CO\textsubscript{2} concentration in the chloroplast decreases (Hikosaka \textit{et al.} 1998), when the activation state of Rubisco and of other photosynthetic proteins decreases (Mott and Woodrow 2000), or when photoinhibition occurs (Adams \textit{et al.} 2004). \( \text{Rubisco}/N_{\text{area}} \) indicates the nitrogen partitioning between photosynthetic and non-photosynthetic components within leaves (Hikosaka 2004).

The effects of irradiance and temperature on leaf traits over the year were tested by ANOVA. The effects of summer and winter irradiance were tested by grouping the sets of plants with the same irradiance in summer and winter, i.e. \( I_{\text{high}} \) and \( I_{\text{inv}} \) (high irradiance in summer) versus \( I_{\text{low}} \) and \( I_{\text{dec}} \) (low irradiance in summer) to test the ‘summer-irradiance’ effect and \( I_{\text{high}} \) and \( I_{\text{dec}} \) (high irradiance in winter) versus \( I_{\text{low}} \) and \( I_{\text{inv}} \) (low irradiance in winter) to test the ‘winter-irradiance’ effect by ANOVA.

Statistical analyses were done using SYSTAT (Systat Software Inc., San Jose).

**Results**

**Growth conditions**
The daily average PPFD was on average 5.1 mol m^{-2} day^{-1} (ranging from 2.2 to 11.8 over the year) in high irradiance and 1.4 mol m^{-2} day^{-1} (ranging from 0.5 to 3.0 over the year, Fig. 1a) in low irradiance. In both irradiance levels the daily average PPFD decreased gradually from summer to winter and increased sharply towards spring as a result of the increase in the solar elevation and the predominance of a clear sky. The seasonal change in temperature ranged from a daily average temperature of 21.2 °C in August to -0.5 °C in January (Fig. 1b). The monthly maximum and minimum temperatures were on average 10.1°C higher and 6.8°C lower, respectively, than the daily average temperature. The two irradiance regimes had no discernible effect on daily average, minimum, or maximum temperatures during the year (data not shown).

**Seasonal change in physiological and morphological traits**

There was a significant seasonal change in $P_{\text{max}}$, which tended to decrease from summer to winter (Fig. 2a, Table 1). Although growth irradiance did not significantly affect $P_{\text{max}}$, there was a significant interaction between season and growth irradiance in the effect on $P_{\text{max}}$ (Table 1). In summer, $P_{\text{max}}$ tended to be higher in high irradiance (I$_{\text{high}}$ and I$_{\text{inv}}$) than in low irradiance regimes (I$_{\text{low}}$ and I$_{\text{dec}}$), while in winter, an opposite pattern was observed in which $P_{\text{max}}$ was higher in low irradiance regimes (I$_{\text{low}}$ and I$_{\text{inv}}$). $N_{\text{area}}$ also showed a significant seasonal change (Table 1). $N_{\text{area}}$ increased from summer to autumn in I$_{\text{low}}$ and I$_{\text{dec}}$, or from summer to winter in I$_{\text{high}}$ and I$_{\text{inv}}$ (Fig. 2b). In spring $N_{\text{area}}$ decreased to similar values of the previous summer. $N_{\text{area}}$ tended to be higher in leaves exposed to high irradiance in winter (I$_{\text{high}}$ and I$_{\text{dec}}$) than those in low irradiance in winter (I$_{\text{low}}$ and I$_{\text{inv}}$) through the year (Fig. 2b). PNUE decreased from summer to winter in parallel to changes in $P_{\text{max}}$. In summer and autumn PNUE was higher in plants grown at high summer-irradiance regimes (I$_{\text{high}}$ and I$_{\text{inv}}$), whereas in winter PNUE was highest in the plants growing at low irradiance (I$_{\text{low}}$ and I$_{\text{inv}}$).

The Rubisco content changed significantly in a similar pattern to $N_{\text{area}}$ and was highest in autumn (I$_{\text{high}}$ and I$_{\text{dec}}$) or winter (I$_{\text{low}}$ and I$_{\text{inv}}$) (Table 2). $C_i$ showed significant seasonal changes (Table 1). $C_i$ first decreased from summer to autumn, and then increased or remained similar (I$_{\text{inv}}$) in winter.
and thereafter decreased to its lowest values of the year in spring (Table 2). Leaf mass per area (LMA) showed a significant seasonal change and was also significantly different between irradiance regimes (Table 1). LMA increased from summer to winter and was higher at high irradiance regimes from summer to winter (Table 2).

Cross sections of $I_{\text{high}}$ and $I_{\text{low}}$ leaves in summer and winter are illustrated by light microscopic photographs (Fig. 3). Although $I_{\text{high}}$ leaves seem to have one more palisade layer than $I_{\text{low}}$, there was no significant effect of season and growth irradiance on leaf thickness (Fig. 4) and $S_{\text{mes}}$ (mesophyll surface area, Table 3) (Table 1). On the other hand, there were significant effects of season and growth irradiance on chloroplast morphologies. $V_{\text{chl}}$ (chloroplast volume per leaf area) changed seasonally and $S_{\text{c}}$ (chloroplast surface area) was affected by growth irradiance (Table 1). Both $V_{\text{chl}}$ and $S_{\text{c}}$ were higher in autumn/winter and at high irradiance (Fig. 4b, c). Reflecting the changes in $S_{\text{c}}$, $S_{\text{c}}/S_{\text{mes}}$ tended to be higher in autumn/winter (Table 3). $V_{\text{chl}}/S_{\text{c}}$ (chloroplast thickness) also showed a seasonal change and tended to increase in winter (Tables 1, 3). However, $N_{\text{area}}/V_{\text{chl}}$, which indicates the protein concentration in chloroplasts, was not affected by season and growth irradiance (Tables 1, 3).

**Relationships among parameters**

The chloroplast related traits were correlated significantly with leaf nitrogen content except $S_{\text{mes}}$ (Fig. 5). $V_{\text{chl}}$, $V_{\text{chl}}/S_{\text{c}}$ and $S_{\text{c}}$ all increased with $N_{\text{area}}$ with no differences between seasons (ANOVA among slopes P= 0.50, 0.33 and 0.92, among intercepts P= 0.47, 0.06 and 0.23 for $V_{\text{chl}}$, $V_{\text{chl}}/S_{\text{c}}$ and $S_{\text{c}}$, respectively). There was a positive linear relation between the Rubisco content and $N_{\text{area}}$ (Fig. 6b) that differed between seasons (ANOVA: among slopes P=0.48 and among intercepts P=0.03). The Rubisco content at a given $N_{\text{area}}$ increased from summer to spring. $P_{\text{max}}$ was positively correlated with the Rubisco content in autumn and winter but was not in the other seasons (Fig. 6a). The $P_{\text{max}}$/Rubisco ratio was correlated negatively with $V_{\text{chl}}/S_{\text{c}}$ (Fig. 7a) and positively with $C_{i}$ (Fig. 7b) across all seasons.

**Effects of summer and winter irradiance**
Summer irradiance had significant effects on \( P_{\text{max}} \) and PNUE (Table 1): Both \( P_{\text{max}} \) and PNUE in summer were higher in leaves that were in high irradiance in summer (Fig. 2). However, other traits were less affected by summer irradiance. On the other hand, winter irradiance affected more photosynthetic traits (Table 1). \( N_{\text{area}} \), \( C_{\text{i}} \), \( V_{\text{chl}} \) and \( S_{c} \) were higher in leaves that were exposed to high irradiance in winter (Fig. 2, 4, Table 1, 2). LMA and \( S_{\text{mes}} \) were also higher in leaves that were exposed to high irradiance in winter (Tables 2, 3). These results suggest that leaf morphology was more strongly determined by winter than summer irradiance.

**Discussion**

**Morphology and function**

We found that there was a large vacant space for chloroplast arrangement along mesophyll surface (i.e. \( 1 - S_{c}/S_{\text{mes}} \)) in summer (Table 3). This enabled \( S_{c} \) to increase with increasing \( N_{\text{area}} \) in winter (Figure 2, 4, 5) in spite of constant \( S_{\text{mes}} \) (Table 3). This change in \( S_{c} \) from summer to winter was similar to that observed in an annual and several deciduous tree species when their shade leaves were transferred to high irradiance (Oguchi et al. 2003, 2005, 2006). Just as the leaf anatomy of shade-tolerant plants has been shown to allow for increases in \( S_{c} \) following gap formation, results presented here indicate that leaves were thicker than needed in the summer which facilitated acclimation-related increases in \( S_{c} \) in the winter.

Nevertheless, the increase in \( S_{c} \) from summer to winter did not contribute to increasing photosynthetic rates. Rather, \( P_{\text{max}} \) decreased substantially from summer to winter/spring when PNUE also decreased (Fig. 2). As the Rubisco content per unit nitrogen did not decrease (Fig. 6), the reduction in \( P_{\text{max}} \) and PNUE should be ascribed to a decrease in \( P_{\text{max}} \) per unit Rubisco (Fig. 6, see Eqn. 3). The decrease in \( P_{\text{max}} \) per Rubisco from summer to autumn has also been observed in *Tiarella cordifolia* and *Daphniphyllum humile* growing in the understory (Rothstein and Zak 2001; Katahata et al. 2005). The change in \( P_{\text{max}}/\text{Rubisco} \) ratio is attributable to (1) changes in properties of Rubisco (i.e.
decreased Rubisco activation state), (2) inhibition of the photosynthetic apparatus, or (3) limited supply of CO$_2$. The first possibility recalls the argument that evergreen leaves accumulate Rubisco as an inactive storage protein in winter (Warren and Adams 2004). However, it does not explain the increase in Rubisco in spring, when Rubisco if stored would be used for new leaf formation (Table 2).

The second and third possibilities for the decrease in P$_{\text{max}}$ per Rubisco are related to stress in low temperature and high irradiance in winter. Recent studies have shown that the rate of Rubisco-limited photosynthesis decreases with increasing photoinhibition (Hikosaka et al. 2004; Hirotsu et al. 2005, Miyazawa et al. 2007). The plants grown at high irradiance in winter had a high C$_i$ but lower P$_{\text{max}}$ and PNUE values than plants growing at low irradiance (Fig. 2), suggesting an involvement of photoinhibition. Photoinhibition by high irradiance in winter has been well documented (e.g. Adams et al. 2004). The third possibility, CO$_2$ diffusion, might have involved in *A. japonica* leaves. Except for winter, P$_{\text{max}}$ per Rubisco was well correlated with C$_i$, suggesting that stomatal conductance was responsible. Experimental studies have shown that leaves grown at lower temperatures tend to have lower C$_i$ (Hikosaka et al. 1999, 2006; Yamori et al. 2006). Additionally we found that the P$_{\text{max}}$/Rubisco ratio was negatively correlated with chloroplast thickness (V$_{\text{chl}}$/S$_c$, Fig. 7). The length of the diffusion path to Rubisco increases with increasing chloroplast thickness (Terashima et al. 2001, 2006), which might have lowered the CO$_2$ concentration at the carboxylation site and thus the P$_{\text{max}}$/Rubisco ratio. If the decrease in the P$_{\text{max}}$/Rubisco ratio was a result of photoinhibitory damage and/or of an acclimatory response of stomata to prevent water loss, the complementary increase in V$_{\text{chl}}$ and S$_c$ rather suggests an increase in the total photosynthetic machinery to function at low temperature, although actual P$_{\text{max}}$ was low. Warren and Adams (2004) also suggested the possibility that the larger amounts of Rubisco are effective for photosynthesis in suboptimal conditions.

It should be noted, however, that all the mesophyll surface was not filled by chloroplasts in winter (Fig. 3, Table 3). It may be because leaves are constructed to acclimate to further increase in irradiance or to further decrease in temperature. Substantial vacant space may also be necessary for other functions such as chloroplast movement (Kasahara et al. 2002, Tholen et al. 2008).

How do environmental conditions affect leaf traits?
N\text{area} of *A. japonica* leaves increased in autumn to winter (Fig. 2), as has been shown for *A. japonica* grown under natural conditions (Yamamura and Kimura 1992; Muller *et al.* 2005). This has also been shown in perennial evergreen herbs (Skillman *et al.* 1996), broadleaved evergreens (Miyazawa and Kikuzawa 2005) and in evergreen conifers (Warren and Adams 2001; Han *et al.* 2004). Under natural conditions the irradiance in the understory increases strongly with shedding of canopy leaves when air temperature decreases in autumn. Experimental studies have shown that both temperature and irradiance potentially affect N\text{area}: N\text{area} tends to be higher when grown at higher irradiance (e.g. Field 1983, Evans 1989) or at low temperature (Weih and Karlsson 2001; Yamori *et al.* 2005; Hikosaka 2005). In the natural environment, N\text{area} in *A. japonica* correlated with both irradiance and temperature (Muller *et al.* 2005). In the present study, where plants were exposed to natural changes in temperature with the controlled irradiances, N\text{area} was affected by seasonal changes in temperature rather than by growth irradiance (Table 1). This suggests that temperature rather than irradiance primarily determines the changes in N\text{area} in natural conditions for this species.

S\text{mes} significantly differed depending on winter irradiance (Table 1, 3). As new leaves of *A. japonica* that are in bud in winter emerge with the flush of canopy trees, winter irradiance may be detected by the winter bud or by older mature leaves. In *Fagus crenata*, *F. japonica* (Uemura *et al.* 2000) and *Chenopodium album* (Yano and Terashima 2001), the number of palisade cell layers was determined by the irradiance of mature leaves and not of developing new leaves. Similar mechanisms may be involved in the evergreen species *A. japonica*.

**Conclusion**

The present study showed that leaf anatomy is constructed to allow an increase in leaf nitrogen and Rubisco in winter; i.e. mesophyll surface area determined by winter irradiance is large enough to accommodate increased chloroplast volume in winter. If we see leaves only in summer, they look too thick in terms of biomass investment. Although the increase in leaf nitrogen and Rubisco content did
not directly increase photosynthetic capacity in winter, it may compensate the decrease in photosynthetic nitrogen use efficiency due to stressful environment in winter.

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Literature


Miyazawa Y, Kikuzawa K, Otsuki K (2007) Decrease in the capacity for RuBP carboxylation and regeneration with the progression of cold-induced photoinhibition during winter in evergreen broadleaf tree species in a temperate forest. Functional Plant Biology 34:393-401


**Figure legends**

Fig. 1. Changes in PPFD (a) and temperature (b) over the year with a scheme of the irradiance regimes on top. The open and closed bars indicate high and low irradiance regimes, respectively. The dotted lines indicate when photosynthesis measurements were performed and the arrows when plants were transferred. The 10 day average PPFD of the high (open symbols) and low (closed symbols) irradiance regime (a) and the 10 day mean average temperature and absolute minimum and maximum values from August 2003 to May 2004 (b). Values are means ± SE.

Fig. 2. Seasonal changes in photosynthetic capacity measured at 15°C ($P_{\text{max}}$), $N_{\text{area}}$ and photosynthetic nitrogen use efficiency (PNUE). The average is shown for *A. japonica* grown all the year at high ($I_{\text{high}}$, open squares, solid line) and low ($I_{\text{low}}$, closed squares, dotted line) irradiance and plants transferred in autumn from low to high ($I_{\text{dec}}$, open circles, broken line) or from low to high ($I_{\text{inv}}$, closed circles, broken dotted line) irradiance and vice versa in spring. Values are mean ± SD (n=3). Arrows indicate the time when plants were transferred.

Fig. 3. Leaf cross sections of *A. japonica* grown at high irradiance ($I_{\text{high}}$) in summer (a) and in winter (b) or at low irradiance ($I_{\text{low}}$) in summer (c) and in winter (d).

Fig. 4. Seasonal changes in leaf thickness, chloroplast volume ($V_{\text{chl}}$) and chloroplast surface area facing the intercellular space ($S_{\text{c}}$). The average is shown. Values are means ± SD (n=3). For symbols see Fig. 2.
Fig. 5. The relation between N\textsubscript{area} and (a) V\textsubscript{chl}, (b) V\textsubscript{chl}/S\textsubscript{c}, (c) S\textsubscript{c} and (d) S\textsubscript{mes} for individual leaves in summer (open diamonds), autumn (closed diamonds) winter (open triangles) and spring (closed triangles). Linear regressions of all data: (a) y = 0.34x + 2.85 P<0.001 r\textsuperscript{2}=0.31; (b) y=0.008x+2.73, P<0.05, r\textsuperscript{2}=0.14; (c) y=0.05x+6.65, P<0.001, r\textsuperscript{2}=0.27; (d) ns.

Fig. 6. The relationship between P\textsubscript{max} and Rubisco (a) and between Rubisco and N\textsubscript{area} (b) for individual leaves. Significant linear regression for P\textsubscript{max} and Rubisco (a): autumn (chain), y=0.76+2.62, P<0.05, r\textsuperscript{2}=0.45 and winter (dotted line), y=1.7x+3.2, P<0.01, r\textsuperscript{2}=0.65. Linear regression lines for Rubisco and N\textsubscript{area} (b): summer (solid line), y=0.024x-1.7, P<0.001, r\textsuperscript{2}=0.79; autumn (chain), y=0.025x-1.34, P<0.05, r\textsuperscript{2}=0.45; winter (dotted line), y=0.016x+0.28, P<0.05, r\textsuperscript{2}=0.47; spring (broken line), y=0.01x+1.34, ns. For symbols see Fig. 5.

Fig. 7. The relationship between P\textsubscript{max} per unit Rubisco (P\textsubscript{max}/Rubisco,) and chloroplast thickness (V\textsubscript{chl}/S\textsubscript{c}) (a), and intercellular CO\textsubscript{2} concentration (C\textsubscript{i}) (b) for individual leaves. Regression lines:(a) y=-0.72x+4.70, P<0.01, r\textsuperscript{2}=0.20 and (b) y=0.019x-1.64, P<0.001, r\textsuperscript{2}=0.43, excluding high irradiance in winter (open squares). For symbols see Fig. 5.
Table 1. Effects of season and irradiance on leaf anatomy and physiology traits

The effect on leaf physiological and anatomical traits of season and growth irradiance (irradiance plants are exposed to, i.e. $I_{\text{high}}$, $I_{\text{low}}$, $I_{\text{dec}}$, $I_{\text{inv}}$, see text and Fig. 1), summer irradiance (irradiance plants are exposed to in summer, i.e. $I_{\text{high}}$ and $I_{\text{inv}}$ vs $I_{\text{low}}$ and $I_{\text{dec}}$) or winter irradiance (irradiance plants are exposed to in winter, i.e $I_{\text{high}}$ and $I_{\text{dec}}$ vs $I_{\text{low}}$ and $I_{\text{inv}}$), tested by ANOVA. P values are shown.

<table>
<thead>
<tr>
<th>Variable</th>
<th>season</th>
<th>growth-irradiance</th>
<th>interaction</th>
<th>season</th>
<th>summer-irradiance</th>
<th>interaction</th>
<th>season</th>
<th>winter-irradiance</th>
<th>interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{\text{max}}$ (µmol m$^{-2}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.100</td>
<td>0.001</td>
<td>0.000</td>
<td>0.005</td>
<td>0.119</td>
<td>0.000</td>
<td>0.902</td>
<td>0.139</td>
</tr>
<tr>
<td>$N_{\text{area}}$ (mmol m$^{-2}$)</td>
<td>0.006</td>
<td>0.215</td>
<td>0.763</td>
<td>0.004</td>
<td>0.112</td>
<td>0.442</td>
<td>0.003</td>
<td>0.556</td>
<td>0.449</td>
</tr>
<tr>
<td>PNUE (mmol mol$^{-1}$ s$^{-1}$)</td>
<td>0.000</td>
<td>0.095</td>
<td>0.002</td>
<td>0.000</td>
<td>0.008</td>
<td>0.078</td>
<td>0.000</td>
<td>0.556</td>
<td>0.449</td>
</tr>
<tr>
<td>Leaf thickness ($\mu$m)</td>
<td>0.290</td>
<td>0.423</td>
<td>0.931</td>
<td>0.280</td>
<td>0.991</td>
<td>0.276</td>
<td>0.297</td>
<td>0.617</td>
<td>0.971</td>
</tr>
<tr>
<td>$V_{\text{ch}}$ (m$^3$ m$^{-2}$)</td>
<td>0.007</td>
<td>0.057</td>
<td>0.313</td>
<td>0.018</td>
<td>0.761</td>
<td>0.299</td>
<td>0.003</td>
<td>0.001</td>
<td>0.584</td>
</tr>
<tr>
<td>$S_{\text{c}}$ (m$^2$ m$^{-2}$)</td>
<td>0.050</td>
<td><strong>0.021</strong></td>
<td>0.724</td>
<td>0.105</td>
<td>0.293</td>
<td>0.796</td>
<td><strong>0.046</strong></td>
<td><strong>0.007</strong></td>
<td>0.645</td>
</tr>
<tr>
<td>$C_{\text{i}}$ (µmol mol$^{-1}$)</td>
<td>0.000</td>
<td>0.907</td>
<td>0.113</td>
<td>0.000</td>
<td>0.621</td>
<td>0.615</td>
<td>0.000</td>
<td>0.004</td>
<td>0.485</td>
</tr>
<tr>
<td>Rubisco (g m$^{-2}$)</td>
<td>0.000</td>
<td>0.532</td>
<td>0.665</td>
<td>0.000</td>
<td>0.170</td>
<td>0.949</td>
<td>0.000</td>
<td>0.064</td>
<td>0.302</td>
</tr>
<tr>
<td>LMA (g m$^{-2}$)</td>
<td>0.000</td>
<td><strong>0.002</strong></td>
<td>0.224</td>
<td>0.000</td>
<td>0.124</td>
<td>0.892</td>
<td>0.000</td>
<td><strong>0.000</strong></td>
<td>0.162</td>
</tr>
<tr>
<td>$N_{\text{area}}/V_{\text{ch}}$ (mmol m$^{-3}$)</td>
<td>0.585</td>
<td>0.340</td>
<td>0.459</td>
<td>0.660</td>
<td>0.575</td>
<td><strong>0.035</strong></td>
<td>0.593</td>
<td>0.160</td>
<td>0.970</td>
</tr>
<tr>
<td>$V_{\text{ch}}/S_{\text{c}}$ ($\mu$m)</td>
<td><strong>0.010</strong></td>
<td>0.657</td>
<td>0.089</td>
<td><strong>0.011</strong></td>
<td>0.330</td>
<td><strong>0.035</strong></td>
<td><strong>0.006</strong></td>
<td><strong>0.004</strong></td>
<td>0.572</td>
</tr>
<tr>
<td>$S_{\text{c}}/S_{\text{mes}}$</td>
<td>0.001</td>
<td>0.260</td>
<td>0.655</td>
<td><strong>0.001</strong></td>
<td>0.391</td>
<td>0.447</td>
<td><strong>0.001</strong></td>
<td>0.231</td>
<td>0.874</td>
</tr>
<tr>
<td>$S_{\text{mes}}$ (m$^2$ m$^{-3}$)</td>
<td>0.404</td>
<td>0.165</td>
<td>0.204</td>
<td>0.452</td>
<td>0.779</td>
<td>0.940</td>
<td>0.393</td>
<td><strong>0.049</strong></td>
<td>0.286</td>
</tr>
</tbody>
</table>
Table 2. Changes in leaf traits in four irradiance regimes

Seasonal changes per irradiance regime of $C_i$ (intercellular CO$_2$ concentration), Rubisco (Rubisco per leaf area) and LMA (leaf mass per area). Values are the mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>summer</th>
<th>autumn</th>
<th>winter</th>
<th>spring</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_i$ (µmol mol$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{high}$</td>
<td>217.3 ± 10.2</td>
<td>175.9 ± 37.6</td>
<td>250.8 ± 59.0</td>
<td>162.8 ± 14.5</td>
</tr>
<tr>
<td>$I_{low}$</td>
<td>199.0 ± 9.8</td>
<td>178.6 ± 39.0</td>
<td>194.6 ± 25.2</td>
<td>173.1 ± 29.4</td>
</tr>
<tr>
<td>$I_{dec}$</td>
<td>254.0 ± 6.3</td>
<td>174.2 ± 34.8</td>
<td>205.2 ± 53.7</td>
<td>153.7 ± 42.1</td>
</tr>
<tr>
<td>$I_{inv}$</td>
<td>214.4 ± 1.8</td>
<td>182.9 ± 29.8</td>
<td>182.7 ± 27.0</td>
<td>136.0 ± 20.3</td>
</tr>
<tr>
<td>Rubisco (g m$^{-2}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{high}$</td>
<td>2.79 ± 0.49</td>
<td>4.14 ± 0.82</td>
<td>2.81 ± 0.12</td>
<td>3.41 ± 1.13</td>
</tr>
<tr>
<td>$I_{low}$</td>
<td>2.05 ± 0.47</td>
<td>2.34 ± 0.66</td>
<td>2.90 ± 0.63</td>
<td>2.78 ± 0.50</td>
</tr>
<tr>
<td>$I_{dec}$</td>
<td>2.01 ± 0.42</td>
<td>3.51 ± 0.64</td>
<td>3.35 ± 0.31</td>
<td>3.38 ± 0.32</td>
</tr>
<tr>
<td>$I_{inv}$</td>
<td>1.89 ± 0.11</td>
<td>2.40 ± 1.21</td>
<td>3.84 ± 0.76</td>
<td>2.75 ± 0.44</td>
</tr>
<tr>
<td>LMA (g m$^{-2}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{high}$</td>
<td>77.4 ± 11.4</td>
<td>76.1 ± 7.0</td>
<td>98.7 ± 1.2</td>
<td>99.9 ± 17.0</td>
</tr>
<tr>
<td>$I_{low}$</td>
<td>62.8 ± 3.2</td>
<td>65.9 ± 1.9</td>
<td>74.5 ± 1.6</td>
<td>79.2 ± 15.4</td>
</tr>
<tr>
<td>$I_{dec}$</td>
<td>65.3 ± 11.3</td>
<td>80.0 ± 3.2</td>
<td>100.2 ± 5.3</td>
<td>90.7 ± 12.9</td>
</tr>
<tr>
<td>$I_{inv}$</td>
<td>66.5 ± 1.8</td>
<td>72.5 ± 3.2</td>
<td>83.1 ± 2.6</td>
<td>76.9 ± 2.4</td>
</tr>
</tbody>
</table>
Table 3. Factors explaining the seasonal change in N\textsubscript{area}

The factors that explain the seasonal changes in N\textsubscript{area} (N\textsubscript{area}/V\textsubscript{chl}, V\textsubscript{chl}/S\textsubscript{c}, S\textsubscript{c}/S\textsubscript{mes} and S\textsubscript{mes}) in different light treatments (I\textsubscript{high}, I\textsubscript{low}, I\textsubscript{dec}, I\textsubscript{inv}). Values are mean ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>summer</th>
<th>autumn</th>
<th>winter</th>
<th>spring</th>
</tr>
</thead>
<tbody>
<tr>
<td>N\textsubscript{area}/V\textsubscript{chl} (mmol m\textsuperscript{-3})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I\textsubscript{high}</td>
<td>3.28 ± 0.93</td>
<td>2.45 ± 0.53</td>
<td>3.24 ± 1.48</td>
<td>2.73 ± 0.88</td>
</tr>
<tr>
<td>I\textsubscript{low}</td>
<td>3.41 ± 0.41</td>
<td>3.31 ± 0.20</td>
<td>2.45 ± 0.52</td>
<td>4.05 ± 0.66</td>
</tr>
<tr>
<td>I\textsubscript{dec}</td>
<td>2.79 ± 0.48</td>
<td>2.86 ± 0.25</td>
<td>2.32 ± 0.33</td>
<td>2.82 ± 0.89</td>
</tr>
<tr>
<td>I\textsubscript{inv}</td>
<td>3.11 ± 0.17</td>
<td>2.88 ± 0.61</td>
<td>3.40 ± 0.37</td>
<td>2.69 ± 0.09</td>
</tr>
<tr>
<td>V\textsubscript{chl}/S\textsubscript{c} (µm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I\textsubscript{high}</td>
<td>3.85 ± 0.48</td>
<td>4.25 ± 0.28</td>
<td>4.46 ± 1.02</td>
<td>5.00 ± 0.96</td>
</tr>
<tr>
<td>I\textsubscript{low}</td>
<td>3.81 ± 0.17</td>
<td>3.61 ± 0.25</td>
<td>4.80 ± 0.63</td>
<td>3.36 ± 1.05</td>
</tr>
<tr>
<td>I\textsubscript{dec}</td>
<td>4.21 ± 0.21</td>
<td>4.40 ± 0.40</td>
<td>4.81 ± 0.31</td>
<td>4.20 ± 0.50</td>
</tr>
<tr>
<td>I\textsubscript{inv}</td>
<td>3.50 ± 0.02</td>
<td>3.88 ± 0.15</td>
<td>4.01 ± 0.25</td>
<td>4.16 ± 0.19</td>
</tr>
<tr>
<td>S\textsubscript{c}/S\textsubscript{mes}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I\textsubscript{high}</td>
<td>0.71 ± 0.02</td>
<td>0.77 ± 0.02</td>
<td>0.66 ± 0.20</td>
<td>0.69 ± 0.16</td>
</tr>
<tr>
<td>I\textsubscript{low}</td>
<td>0.61 ± 0.07</td>
<td>0.72 ± 0.04</td>
<td>0.72 ± 0.09</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>I\textsubscript{dec}</td>
<td>0.59 ± 0.02</td>
<td>0.72 ± 0.03</td>
<td>0.81 ± 0.02</td>
<td>0.73 ± 0.11</td>
</tr>
<tr>
<td>I\textsubscript{inv}</td>
<td>0.61 ± 0.04</td>
<td>0.66 ± 0.04</td>
<td>0.79 ± 0.04</td>
<td>0.68 ± 0.03</td>
</tr>
<tr>
<td>S\textsubscript{mes} (m\textsuperscript{2} m\textsuperscript{-2})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I\textsubscript{high}</td>
<td>20.35 ± 3.40</td>
<td>23.22 ± 0.97</td>
<td>23.66 ± 0.50</td>
<td>21.33 ± 0.32</td>
</tr>
<tr>
<td>I\textsubscript{low}</td>
<td>20.39 ± 4.51</td>
<td>19.86 ± 1.04</td>
<td>18.71 ± 1.41</td>
<td>18.71 ± 3.57</td>
</tr>
<tr>
<td>I\textsubscript{dec}</td>
<td>23.08 ± 0.85</td>
<td>24.14 ± 2.28</td>
<td>22.22 ± 3.67</td>
<td>20.51 ± 1.32</td>
</tr>
<tr>
<td>I\textsubscript{inv}</td>
<td>22.61 ± 0.80</td>
<td>20.85 ± 2.25</td>
<td>19.20 ± 1.33</td>
<td>19.69 ± 2.10</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
**Figure 4**

(a) Leaf thickness (μm) measured in different seasons.
(b) Chlorophyll concentration ($V_{chl} \text{ (m}^3\text{m}^{-2})$) across seasons.
(c) Leaf area ($S_{l} \text{ (m}^2\text{m}^{-2})$) in various seasons.
Figure 5

- **Figure 5a:** 
  - $V_{vol}$ (m$^3$ m$^{-2}$) vs. $N_{area}$ (mmol m$^{-2}$)

- **Figure 5b:** 
  - $V_{vol}/S_{um}$ vs. $N_{area}$ (mmol m$^{-2}$)

- **Figure 5c:** 
  - $S_{um}$ (m$^2$ m$^{-2}$) vs. $N_{area}$ (mmol m$^{-2}$)

- **Figure 5d:** 
  - $S_{area}$ (m$^2$ m$^{-2}$) vs. $N_{area}$ (mmol m$^{-2}$)

The graphs illustrate the relationship between volume, area, and concentration as a function of the area parameter.
Figure 6
Figure 7