

Effects of Light and Nutrient Availability on Leaf Mechanical Properties of *Plantago major*: A Conceptual Approach

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- **Background and Aims** Leaf mechanical properties, which are important to protect leaves against physical stresses, are thought to change with light and nutrient availabilities. This study aims to understand phenotypic changes of leaf mechanical properties with respect to dry mass allocation and anatomy.
- **Methods** Leaf lamina strength (maximum force per unit area to fracture), toughness (work to fracture) and stiffness (resistance against deformation) were measured by punch-and-die tests, and anatomical and physiological traits were determined in *Plantago major* plants grown at different light and nutrient availabilities. A conceptual approach was developed by which punch strength and related carbon costs can be quantitatively related to the underlying anatomical and morphological traits: leaf thickness, dry-mass allocation to cell walls and cell-wall-specific strength.
- **Key Results** Leaf lamina strength, toughness and stiffness (all expressed on a punch area basis) increased with light availability. By contrast, nutrient availability did not change strength or toughness, but stiffness was higher in low-nutrient plants. Punch strength (maximum force per unit punch area, F_{\max}/area) was analysed as the product of leaf mass per area (LMA) and $F_{\max}/\text{leaf mass}$ (= punch strength/LMA, indicating mass-use efficiency for strength). The greater strength of sun leaves was mainly explained by their higher LMA. Shade leaves, by contrast, had a higher $F_{\max}/\text{leaf mass}$. This greater efficiency in shade leaves was caused by a greater fraction of leaf mass in cell walls and by a greater specific strength of cell walls. These differences are probably because epidermis cells constitute a relatively large fraction of the leaf cross-section in shaded leaves. Although a larger percentage of intercellular spaces were found in shade leaves, this in itself did not reduce 'material' strength (punch strength/thickness); it might, however, be important for increasing distance between upper and lower epidermis per unit mass and thus maintaining flexural stiffness at minimal costs.
- **Conclusions** The consequences of a reduced LMA for punch strength in shaded leaves was partially compensated for by a mechanically more efficient design, which, it is suggested, contributes importantly to resisting mechanical stress under carbon-limited conditions.

Key words: Cell walls, cost–benefit, defence, leaf biomechanics, leaf mass per area, nitrogen, *Plantago major*, punch-and-die test, stiffness, strength, toughness.

INTRODUCTION

Leaves of most plant species are flat and often deployed horizontally, a design that probably serves to maximize light capture per unit resources invested (Givnish, 1979). Under natural conditions, leaves are exposed to various types of mechanical stress, including the gravitational force exerted by their own weight and external forces produced by herbivores, rubbing, rainfall and wind (Niklas, 1992). Thus, leaves must be designed such that they can at least to some extent resist these forces. An increase in leaf strength reduces the damage by these forces and may thus help to limit leaf losses, and contribute to longer leaf life spans (Feeny, 1970; Coley, 1983; Reich *et al.*, 1991; Choong *et al.*, 1992; Turner *et al.*, 1993, 2000; Turner, 1994; Choong, 1996; Wright and Cannon, 2001; Wright and Westoby, 2002; Read *et al.*, 2003; Read and Stokes, 2006).

Light and nutrient availabilities significantly alter leaf morphology and chemistry, which may in turn affect leaf

mechanical properties. Plants typically respond to shading by producing leaves with less mass per unit area (LMA; e.g. Dijkstra, 1989; Hikosaka *et al.*, 1998; Poorter and Evans, 1998; Niinemets and Sack, 2006), which enables greater light capture per unit mass (Hirose and Werger, 1995). Yet reductions in thickness and LMA may also lead to a reduction in leaf mechanical resistance, and therefore leaves may become more vulnerable to mechanical damage. Under light-limited conditions loss of leaf area can be particularly detrimental (Anten *et al.*, 2003), but interestingly shade leaves are often reported to have a longer leaf life span than sun leaves even though they have a lower thickness and LMA (see Westoby *et al.*, 2002). Plants grown at high nutrient availability generally produce larger but shorter-lived leaves with higher nitrogen concentrations per unit area than those at low nutrient availability (e.g. Aerts, 1989; Oikawa *et al.*, 2006), and with greater allocation to photosynthetic proteins (Evans, 1989). These changes may affect the resource allocation to cell walls (Onoda *et al.*, 2004) and consequently leaf strength.

Although there are many studies on phenotypic change in leaf morphology and chemistry in response to different light

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or nutrient conditions, relatively few have measured leaf biomechanics. Most of these used a punch-and-die test (penetrometer) and measured maximum force (or an equivalent measure) per unit punch area to penetrate a leaf, which is appropriately defined as ‘punch strength’ instead of ‘leaf toughness’ in material science (Aranwela *et al.*, 1999; Sanson *et al.*, 2001). Leaves grown at high light intensity typically show either a higher (Dudt and Shure, 1994; Louda and Rodman, 1996; Avalos *et al.*, 2007) or similar punch strength (Rowe and Potter, 2000) compared with shade leaves, while the effect of nutrient availability is less clear: increases (Kerpel *et al.*, 2006), no change (Dudt and Shure, 1994; Floater, 1997; Cornelissen and Stiling, 2006) and reductions (Folgarait and Davidson, 1995) in punch strength with decreasing nutrient availability have all been reported. These changes in punch strength have not been studied in detail, and the mechanisms underlying mechanical adjustments to these environmental changes are therefore poorly understood. Punch strength is a complex trait, which depends on both leaf thickness and material strength (Read and Sanson, 2003). These traits, in turn, are associated with leaf anatomy and chemical composition such as cell-wall fraction. Therefore, a detailed analysis of changes in mechanical strength is needed to understand the mechanisms through which light and nutrient availability can affect leaf strength.

Mechanical strength is mainly provided by cell walls, which account for up to 60% of total leaf carbon (e.g. Blair *et al.*, 1983; Merino *et al.*, 1984; Onoda *et al.*, 2004), indicating that mechanical stability involves considerable carbon costs. The question arises: how efficient is the mechanical design of leaf lamina with respect to the carbon invested? To address this question, a simple factorial model is developed, in which punch strength ($= F_{\max}/\text{punch area}$) is analysed as the product of $F_{\max}/\text{leaf mass}$ (mass use efficiency for strength, equal to punch strength/LMA) and LMA. $F_{\max}/\text{leaf mass}$ in turn can be further analysed as the product of $F_{\max}/\text{cell-wall mass}$ (specific cell-wall strength) and cell-wall mass/leaf mass (dry-mass allocation to cell walls) (see Methods for detail).

The mechanical properties of leaves that were grown at different light and nutrient availabilities were measured to clarify how leaves in different growth conditions utilized biomass with respect to mechanical strength. To this end, by using a punch-and-die test, punch strength, punch toughness (total work to penetrate a leaf) and the initial slope of the force–displacement curve (an index of stiffness) were determined. Punch strength was further analysed with respect to LMA, dry-mass allocation to cell walls and cell-wall-specific strength. Analysis of leaf anatomy and C and N contents in cell walls were also employed for further understanding of the mechanisms underlying changes in leaf mechanical properties. Specifically, the following questions are addressed: (1) how do mechanical properties change with different light and nutrient availability and (2) what mechanisms underlie these changes in mechanical properties? The results are discussed with respect to the ecological significance of changes in leaf mechanical properties.

MATERIALS AND METHODS

Plant material and growth conditions

Plantago major L., a common herbaceous species widely distributed all over Europe, was used for this experiment. This species can grow in a wide range of habitats, from fertile to infertile soils and from moderately shaded to open environment. The physiology and ecology of this species have been extensively studied (e.g. Kuiper and Bos, 1992). Seeds were germinated in pots filled with sand in the greenhouse of the Botanical Garden of Utrecht University on 31 May, 2005. One hundred seedlings were transferred to pots (15 cm diameter \times 15 cm height, one seedling per pot) filled with sieved sand on 6 June. Until 28 June all plants were grown under the same light and nutrient conditions. Each pot was supplied with 50 mL of 250 \times dilution of liquid fertilizer (N:P:K = 7:7:7, Easy GRO, Kemira Agro Rozenburg BV, Rozenburg, the Netherlands) once a week. On 28 June, the pots were randomly assigned to three light treatments and two nutrient treatments (3 \times 2 factorial design) outside of the greenhouse in the botanical garden. The light level was controlled by neutral density shade cloth. High-light (HL), medium-light (ML) and low-light (LL) treatments were created using no, one or two layers of the shade cloth, resulting in light levels that were, respectively, 100, 40 and 16% of natural daylight measured with a light quantum sensor (LI-190SA, Li-Cor, Lincoln, NE, USA). The light environment in each shade house was reasonably homogeneous (as tested by the light sensor), but positions of pots were randomized weekly to minimize spatial effects. The high- (HN) and low-nutrient (LN) treatments consisted of weekly applications of 35 and 7 mg N per pot using the same fertilizer as described above. These different light and nutrient availabilities strongly influenced plant growth. The total plant dry mass (g) at the measurement time was 7.93 (HL-HN), 2.50 (HL-LN), 5.08 (ML-HN), 2.21 (ML-LN), 2.33 (LL-HN) and 1.63 (LL-LN).

Both mechanical and physiological measurements were conducted on one fully expanded young leaf (20 days after emergence) per plant (ten plants per treatment), harvested on the morning of 18 August, 2005 (0800–1000 h). Each harvested leaf was wrapped in a wet paper towel and sealed in a plastic bag to avoid any loss of turgor pressure. Biomechanical measurements were completed within 12 h of the harvest.

Biomechanical measurements

Ecological use of the punch-and-die test (penetrometer) has been criticised by some authors: (1) the incorrect use of terms (see Introduction), (2) friction was not accounted for in most studies and (3) fracture in this test was not controlled in term of shear, compression or tension (Vincent, 1992). However, Aranwela *et al.* (1999) undertook a careful methodological study and developed several ways to improve the technique. These suggestions are followed here. A flat-ended, sharp-edged cylindrical steel punch (diameter = 1.345 mm) and a steel die with a sharp-edged

hole with a small clearance (0.2 mm) were used, which allowed us to control leaf fracture primary by shear force. The punch and die were installed into a general testing machine (5542, Instron, Canton, MA, USA), and the punch was placed to go through the middle of the hole of the die without any friction. The punch speed was kept constant (24.7 mm min^{-1}) and the machine simultaneously recorded load (N) applied to the sample and displacement (mm) of the punch (every 50 ms) so that it is possible to calculate strength, toughness and stiffness (Atkins and Mai, 1985; Aranwela *et al.*, 1999). This punch-and-die test was applied to randomly selected intercostal lamina (between secondary veins) for each leaf (three measurements per each leaf). An example of the measurement is shown in Fig. 1. As most leaves were slightly curved, a small force was required to flatten the leaf (base force, F_b) on the die in the initial stage. When the punch started to compress the leaf, a sharp increase in force was observed. The maximum force (F'_{\max}) was recorded just before the leaf fractured, and after which the force dropped to almost zero.

From force–displacement curves, punch strength and punch toughness were calculated as follows (Aranwela *et al.*, 1999):

$$\text{punch strength (MN m}^{-2}\text{ or MPa)} = F_{\max}/A \quad (1)$$

$$\text{punch toughness (kJ m}^{-2}\text{)} = \int (F_x - F_b) dx/A \quad (2)$$

where F_{\max} is net maximum force ($F'_{\max} = F_{\max} - F_b$) and A is the area of the punch (1.42 mm^2). F_x is the force (F) at any point, x , between the initiation of leaf compression and the end of fracture. A preliminary test in which varying numbers of lamina taken from the same leaves were piled on top of each other showed that punch strength was strictly linear and proportional to the total thickness in the range 0.2–0.8 mm (data not shown). This indicates that if different leaves have the same material properties, punch strength and thickness (and hence LMA) should be linearly related.

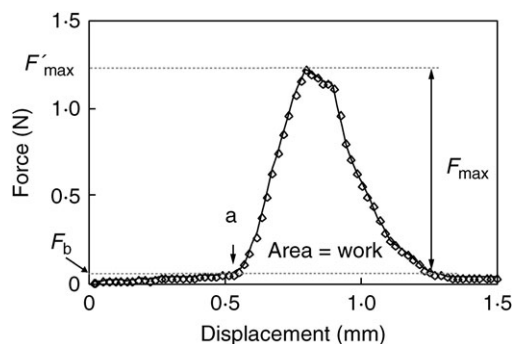


FIG. 1. A force–displacement curve from a punch-and-die test of *Plantago major* leaves. A little force (F_b) is required to flatten the leaf against the die (from 0 displacement to ‘a’). The leaf is assumed to fracture at the maximum force (F_{\max}). After penetration of the punch, force drops to almost zero, but there is still some force because of friction between the punch and leaf.

The initial slope of the force–displacement curve reflects leaf resistance to punch deformation. In this study, this slope (dF_x/dx) was used as an index of stiffness and referred to as punch stiffness:

$$\text{punch stiffness (GN m}^{-3}\text{)} = (dF_x/dx)/A \quad (3)$$

As the initial slope was more or less linear, it was difficult to distinguish between elastic and plastic deformations (Sanson *et al.*, 2001). Punch stiffness was calculated from different parts of the initial slope, and the trend between leaves grown under different treatments was found to be the same irrespective of the part of the slope that was used for the calculation. Therefore, in the present study, we report punch stiffness calculated from the middle part of the initial slope. From a purely mechanical perspective, it is difficult to discern the contributions of shear, compression and bending because leaf deformation occurs by a combination of these forces (Vincent, 1992; Aranwela *et al.*, 1999). If leaves are thicker, deformation by compressive forces may be more dominant than those by shear and bending forces, which may affect the initial slope of the force–displacement curve. Therefore, it was necessary to consider this point in the analysis of the data. Even so, punch stiffness is a useful parameter to understand stiffness of leaves, especially for leaves of similar thickness, as was the case for the leaves from different nutrient treatments (see below).

After the biomechanics measurement, nine leaf discs (diameter = 1.0 cm) were punched out per leaf. Six of them were stored at -80°C until chemical analysis. Three of them were used to measure fresh weight and thereafter oven dried for more than 2 d at 70°C , and used for dry mass measurement. Three small segments (approx. $1 \times 2 \text{ mm}$) of leaf were excised for anatomical analysis and fixed with 2.5 % glutaraldehyde in 100 mM phosphate buffer (pH = 7.0) for more than 1 d.

Chemical analysis

Cell-wall extraction was done following the methods of Lamport (1965), Harris (1983) and Fry (1988). Three frozen leaf discs per leaf were powdered in a pestle and mortar with liquid N_2 , and suspended in 1 mL of 20 mM HEPES buffer (pH 7.0) containing 10 mM dithiothreitol and 1 % Triton X-100. The homogenate was centrifuged at 2500 g at 4°C for 5 min, and the supernatant was removed. The pellet was washed with the HEPES buffer containing 1 % sodium dodecyl sulphate by centrifugation (2500 g at 4°C for 5 min). This procedure was repeated three times. The pellet contained cell-wall material with low-level contamination of starch and cytoplasmic proteins (Lamport, 1965). In order to remove starch, thermostable α -amylase (3 U mL^{-1} ; Magazyme, Wicklow, Ireland) was added and incubated at 80°C for 5 min and left at room temperature for 30 min. To remove cytoplasmic proteins, the pellet was washed with 1 M NaCl by centrifugation (2500 g for 5 min). During these procedures proteins weakly bound to cell walls were removed, and only

tightly bound proteins (structural proteins) remained. Cell-wall material was further washed ($2 \times$ water and $1 \times$ ethanol) by centrifugation (2500 g for 5 min), and dried in an oven (75°C) for 2 d. After drying, the cell-wall materials were weighed and their total C and N concentrations were determined using an element analyser (Carlo Erba 1106, Milan, Italy).

Anatomical analysis

Chemically fixed leaf segments were dehydrated through a graded ethanol series (30, 50, 70, 80, 90, 96 and 100%), and infiltrated and embedded into LR White resin (London Resin Company, Berkshire, UK). The embedded leaf was sliced with a microtome (OMU-3, Leica, Cologne, Germany) at a thickness of $1\ \mu\text{m}$ and stained with 0.5% toluidine blue. Photographs of the sections were taken with a light microscope (AX-LH 100, Olympus, Tokyo, Japan) equipped with a camera (DP70, Olympus).

To calculate average thicknesses of the upper epidermis, palisade, spongy and lower epidermis, the cross-sectional area of each layer was determined and divided by the width of the cross-section. The average thickness of epidermis cell walls (outer side) was calculated from three measurements of walls of randomly selected epidermis cells (for both upper and lower epidermis). Cuticle thickness was not measured because the dehydration process removed some parts of the cuticle (Frost-Christensen *et al.*, 2003). Intercellular air space and area of tertiary veins were evaluated from each leaf cross-section. All these analyses were done using ImageJ 1.34s software (National Institutes of Health, Bethesda, MD, USA). Note that lamina thickness measured with the microscope was slightly lower (10–20%) than that measured by the micrometer probably due to the uneven surface of the leaf and the dehydration process.

Data analysis and statistics

The following conceptual approach was developed to analyse the factors that contribute to leaf strength. Punch strength (F_{\max}/A) was analysed as follows:

$$\text{punch strength} = \frac{F_{\max}}{M_L} \times \frac{M_L}{A} \quad (4)$$

where M_L is leaf mass. F_{\max}/M_L (= punch strength/LMA) can be understood as an index of mass-use efficiency for strength and M_L/A is the same as LMA. F_{\max}/M_L was further analysed as follows:

$$\frac{F_{\max}}{M_L} = \frac{F_{\max}}{M_{\text{CW}}} \times \frac{M_{\text{CW}}}{M_L} \quad (5)$$

where M_{CW} is cell-wall mass. F_{\max}/M_{CW} and M_{CW}/M_L indicate the specific cell-wall strength and dry-mass allocation to cell walls, respectively.

Punch strength and punch toughness were also analysed with respect to thickness. Specific punch strength and

specific punch toughness are calculated as punch strength and punch toughness divided by thickness, respectively (see Read and Sanson, 2003). In the present study, fracture strength and fracture toughness are defined as maximum punch force and total work per unit cross-section area (perimeter \times thickness), respectively. The fracture strength and fracture toughness have a strictly linear relationship with specific punch strength and specific punch toughness, respectively, as long as the same punch rod is used. Fracture strength and fracture toughness may be useful if the data are to be compared with other studies that used different sizes of punch.

Effects of light and nutrient availability were tested by two-way ANOVA. Data were log-transformed when equality of variance was not satisfied between treatments (Levene's test). These analyses were done in STATVIEW 5.0 or SPSS (SAS Institute, Cary, NC, USA). Type II regression [standardized major axis (SMA) tests; Falster *et al.*, 2006] was used to analyse the relationship between LMA and punch strength because both x and y variables were subject to error.

RESULTS

Table 1 shows leaf morphological traits of *P. major* that were grown at three light levels and two nutrient availabilities. Light availability had a strong positive effect on both fresh LMA and dry LMA (referred to simply as LMA hereafter). The higher LMA was the result of both increased leaf thickness and dry mass density (LMA divided by thickness). Nutrient availability did not affect LMA, thickness or dry mass density. The water fraction in fresh leaf was higher in LL than in HL plants irrespective of nutrient conditions.

Punch strength and punch toughness were significantly increased with growth light availability without an effect of nutrient availability (Fig. 2A, B). Both were linearly and strongly correlated with each other across treatments ($y = 0.289x + 0.048$, $R = 0.95$, data not shown). Punch stiffness increased with light availability, but decreased with nutrient availability (Fig. 2C). When punch strength and punch toughness are divided by thickness, there was no difference between the treatments (Table 2).

There was a strong correlation between punch strength and LMA (Fig. 3). SMA slopes for log-transformed data were not significantly different across treatment ($P = 0.95$, common slope = 1.13). However, there were significant shifts along the common slope ($P < 0.001$) and shifts in elevation ($P < 0.001$) between light treatments but not between nutrient treatments, indicating that the relationship was unique for each light treatment (Fig. 3). Both LMA and punch strength increased with light availability, but the relative increase from LL to HL in LMA (131%) was larger than the relative increase in punch strength (64%).

F_{\max}/M_L (= punch strength/LMA, see eqn 4) can be understood as mass-use efficiency for mechanical resistance against external forces. This parameter was 41% higher in LL plants than in HL plants (Fig. 4A) irrespective of nutrient availability. Dry mass allocation to cell walls (M_{CW}/M_L)

TABLE 1. Leaf morphological traits of *Plantago major* grown at three light conditions (100, 40 and 16 %) and two nutrient availabilities (35 and 7 mg N week⁻¹, HN and LN)

Light	Nutrient	Fresh LMA [†] (g m ⁻²)	Dry LMA [†] (g m ⁻²)	Thickness [‡] (mm)	Dry mass density (kg m ⁻³)	Water fraction [‡] (g g ⁻¹)
100 %	HN	340 ± 17	60.9 ± 5.7	0.430 ± 0.018	142 ± 12	0.821 ± 0.010
	LN	305 ± 23	57.0 ± 6.9	0.409 ± 0.040	140 ± 17	0.813 ± 0.014
40 %	HN	225 ± 14	33.6 ± 2.5	0.317 ± 0.022	106 ± 11	0.851 ± 0.012
	LN	220 ± 15	33.8 ± 3.1	0.308 ± 0.028	111 ± 12	0.847 ± 0.008
16 %	HN	194 ± 12	25.8 ± 1.6	0.271 ± 0.013	95 ± 6	0.867 ± 0.007
	LN	193 ± 14	25.1 ± 1.9	0.277 ± 0.024	91 ± 9	0.870 ± 0.011
ANOVA						
Light (L)		****	****	****	****	****
Nutrient (N)		n.s.	n.s.	n.s.	n.s.	n.s.
L × N		n.s.	n.s.	n.s.	n.s.	n.s.

Values are mean ± s.d. ($n = 9-10$). Significance levels (ANOVA): n.s., $P > 0.05$, **** $P < 0.0001$. LMA, leaf mass per unit area.

[†]ANOVA after the data were log-transformed.

[‡]Water fraction is calculated as (fresh mass – dry mass)/fresh mass.

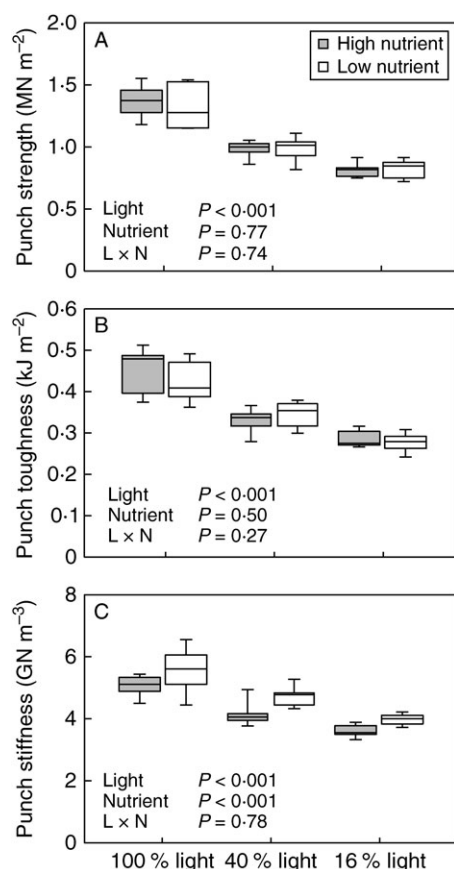


FIG. 2. Box plots of mechanical properties of *Plantago major* leaves grown at three light conditions (100, 40 and 16 %) and two nutrient availabilities (35 and 7 mg N week⁻¹). (A) Punch strength, (B) punch toughness and (C) punch stiffness. The central box in each box plot shows the inter-quartile range and median; whiskers indicate the 10th and 90th percentiles. Effects of light and nutrient availabilities were tested with ANOVA after the data were log-transformed.

increased slightly with decreasing light availability, and also increased slightly with low nutrient availability (Fig. 4B). Specific cell-wall strength (F_{\max}/M_{CW}) increased with low light availability (Fig. 4C).

Figure 5A–C shows representative leaf cross-sections of HL, ML and LL plants grown at high nutrient availability. Lamina thickness decreased considerably with decreasing light availability, with this trend clearly reflecting a trend of reduced thickness of palisade while thicknesses of other layers were more or less constant (Fig. 5D). These results indicate that plants grown under limited light had a relatively higher fraction of epidermis layers (Fig. 6A).

The percentage intercellular air space in the lamina cross-section was not different between nutrient treatments, but increased slightly with low light availability (Fig. 6B). This difference was more pronounced if the intercellular air space was expressed per unit mesophyll cross-section area (17.1, 20.3 and 21.9 % for HL, ML and LL, respectively). The percentage of tertiary veins in the cross-section tended to increase with light availability without an effect of nutrient availability (Fig. 6C). Average thickness of cell walls of upper and lower epidermis was thicker in HL than in LL plants with a marginal effect of nutrient availability (Fig. 6D). Carbon concentration of cell walls was not significantly affected by nutrient availability, but increased with light availability. Nitrogen concentration of cell walls was significantly higher in high nutrient and under low light availability (Fig. 7).

DISCUSSION

Effect of light availability

In addition to the well-known morphological changes in leaf traits (e.g. LMA, thickness) in response to growth light conditions (e.g. Hikosaka *et al.*, 1998; Poorter and Evans, 1998; Niinemets and Sack, 2006), in the present study growth light conditions significantly affected the mechanical properties of leaves. Sun leaves had significantly higher punch strength than shade leaves, consistent with results from previous studies (Dudt and Shure, 1994; Louda and Rodman, 1996; Avalos *et al.*, 2007). Punch toughness and punch stiffness were also higher in sun leaves (Fig. 2). The increase in punch strength with light availability could be attributed to a concomitant increase in LMA (Fig. 3). However, shaded leaves were significantly

TABLE 2. Mechanical properties of *Plantago major* grown at three light conditions (100, 40 and 16%) and two nutrient availabilities (35 and 7 mg N week⁻¹, HN and LN)

Light	100%		40%		16%		ANOVA		
	HN	LN	HN	LN	HN	LN	Light	Nutrient	L × N
Specific punch strength (MN m ⁻² mm ⁻¹)*	3.19 ± 0.28	3.26 ± 0.26	3.10 ± 0.28	3.22 ± 0.40	3.03 ± 0.18	3.00 ± 0.26	n.s.	n.s.	n.s.
Specific punch toughness (kJ m ⁻² mm ⁻¹)*	1.05 ± 0.12	1.04 ± 0.08	1.04 ± 0.09	1.13 ± 0.14	1.06 ± 0.09	1.01 ± 0.12	n.s.	n.s.	n.s.
Fracture strength (MN m ⁻²) [†]	1.07 ± 0.09	1.09 ± 0.09	1.04 ± 0.09	1.08 ± 0.13	1.02 ± 0.06	1.01 ± 0.09	n.s.	n.s.	n.s.
Fracture toughness (kJ m ⁻²) [†]	0.35 ± 0.04	0.35 ± 0.03	0.35 ± 0.03	0.38 ± 0.05	0.36 ± 0.03	0.34 ± 0.04	n.s.	n.s.	n.s.

Values are mean ± s.d. ($n = 9-10$). Significance levels (ANOVA): n.s., $P > 0.05$.

*Specific punch strength and specific punch toughness are calculated as punch strength and punch toughness divided by thickness, respectively.

[†]Fracture strength and fracture toughness are calculated as maximum punch force and total punch work divided by cross-section area (perimeter × thickness), respectively.

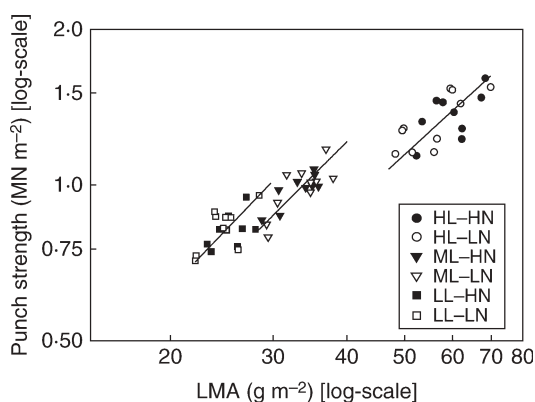


FIG. 3. Relationship between punch strength and leaf mass per area (LMA) of *Plantago major* leaves grown at three light conditions (100, 40 and 16%, HL, ML, LL) and two nutrient availabilities (35 and 7 mg N week⁻¹, HN, LN). A regression line is drawn for each light treatment: $y = 0.0201x^{1.03}$ (100% light), $y = 0.0171x^{1.15}$ (40% light) and $y = 0.0164x^{1.21}$ (16% light). No difference was found in either slope or intercept of regression line with different nutrient treatments (Tukey–Kramer test).

stronger relative to their mass (higher F_{\max}/M_L), which partly compensated for their lower LMA. This greater F_{\max}/M_L of shaded leaves was the result of a greater allocation of dry mass to cell walls (Fig. 4B; Blair *et al.*, 1983; Poorter *et al.*, 2006) and a greater specific cell-wall strength (Fig. 4C). To our knowledge, this is the first study to analyse how components of strength of leaf lamina as a whole change in relation to light environment.

Lucas *et al.* (1991) and Choong (1996) found that fracture toughness of a whole leaf measured by the scissor test was largely determined by vein toughness. As intercostal lamina tissue (between secondary veins) was used in the present study, the contribution of veins was considered to be much smaller than in their study. Furthermore, the fraction of tertiary veins in lamina cross-sections tended to be higher under high light conditions, suggesting that the presence of veins cannot explain the higher allocation of dry mass to cell walls or specific cell-wall strength in shaded leaves. Higher values of these parameters in shade leaves were probably the result of a relatively high fraction of

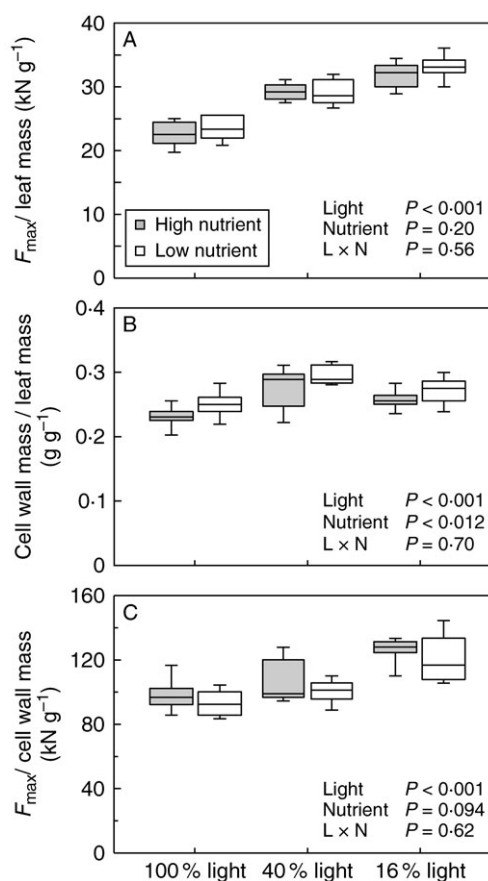


FIG. 4. Box plots of (A) mechanical mass-use efficiency ($F_{\max}/\text{leaf mass}$), (B) dry-mass allocation to cell walls and (C) specific cell-wall strength ($F_{\max}/\text{cell wall mass}$) of *Plantago major* leaves grown at three light conditions (100, 40 and 16%) and two nutrient availabilities (35 and 7 mg N week⁻¹). The central box in each box plot shows the interquartile range and median; whiskers indicate the 10th and 90th percentiles. Effects of light and nutrient availabilities were tested with ANOVA.

epidermis cells in the cross-section (Figs 5 and 6A). The wall thickness of mesophyll cells was considerably thinner (0.1–0.4 μm) than that of epidermis cells (2–4 μm ; Figs 5 and 6D), and the increased fraction of epidermis cells in the cross-section could therefore be a primary

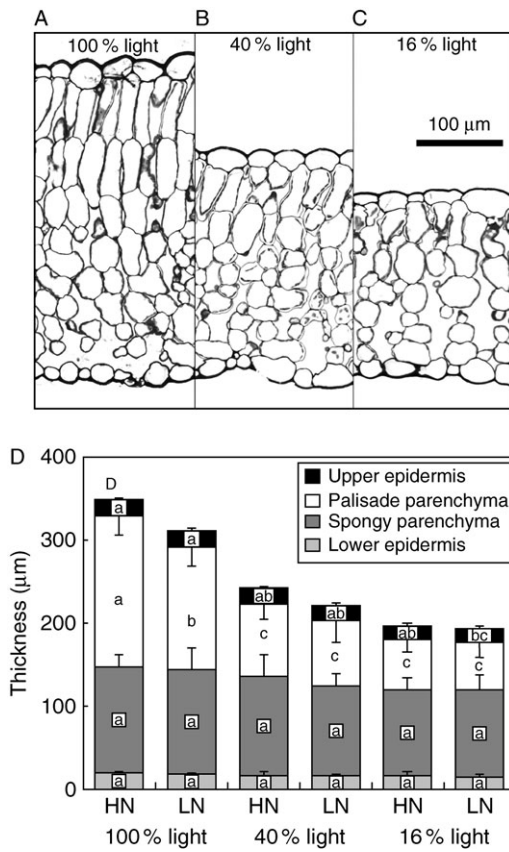


FIG. 5. Cross-sections of leaves of *Plantago major* grown at (A) 100%, (B) 40% and (C) 16% light at a high nutrient availability (35 mg N week⁻¹). Sections were stained with 0.5% toluidine blue. Depth of cross-section is 1 µm. (D) Thickness of upper epidermis, palisade, spongy parenchyma and lower epidermis. High and low nutrient availabilities are indicated by LN and HN, respectively. Values shown are mean ± s.d. ($n = 9-10$). Different letters indicate statistical difference at 5% level (Tukey–Kramer test).

reason for the higher dry-mass allocation to cell walls in the shaded leaves. Furthermore, epidermis cells connect tightly to each other, while mesophyll cells are often separated by intercellular air space. This suggests that the epidermis layer probably resisted a greater punch force per unit cell-wall mass than the mesophyll layer. The importance of the epidermis (and cuticle) to plant mechanical properties has also been suggested in a number of other studies (e.g. Taylor, 1971; Grubb, 1986; Niklas and Paolillo, 1997; Bargel *et al.*, 2006).

Under shaded conditions the relationship between carbon investment and strength in leaves is especially critical as carbon gain is suppressed. Plants respond to this condition by producing leaves with reduced LMA, and an increase in F_{max}/M_L might then be important for maintenance of leaf strength at minimal costs. A high F_{max}/M_L might also be important to discourage herbivores, especially small insects (Choong, 1996; Peeters *et al.*, 2007; S. Gómez, Y. Onoda and J. F. Stuefer, unpubl. res.), because a herbivore would need more force (and energy) to eat a certain amount of biomass. Shaded leaves are found to have longer life spans than sun leaves both within and between

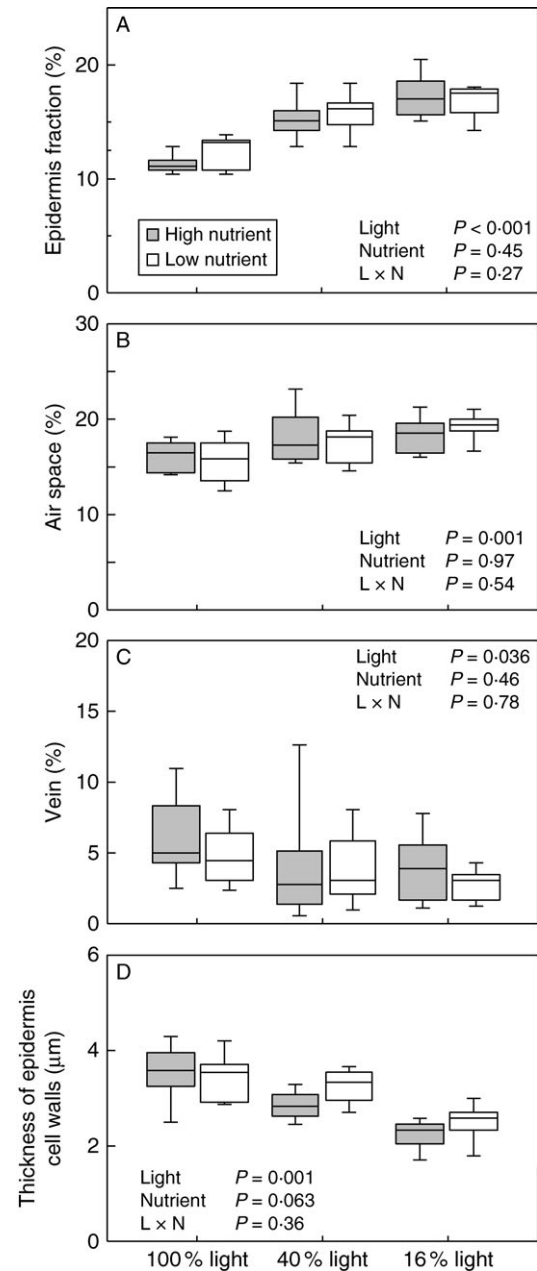


FIG. 6. Box plots of fractions of (A) epidermis layer, (B) intercellular air space and (C) tertiary vein per unit cross-section of leaf lamina. (D) Thickness of epidermis cell walls (upper and lower epidermis averaged). *Plantago major* leaves were grown at three light conditions (100, 40 and 16%) and two nutrient availabilities (35 and 7 mg N week⁻¹). The central box in each box plot shows the interquartile range and median; whiskers indicate the 10th and 90th percentiles. Effects of light and nutrient availabilities were tested with ANOVA after the data were log-transformed if necessary (Levene's test).

species even though they have a lower LMA (e.g. Osada *et al.*, 2001; Westoby *et al.*, 2002), and a larger strength per unit mass might be one factor that contributes to this greater longevity.

Shaded leaves had a greater fraction of intercellular air space in the leaf (Fig. 6B; Lee *et al.*, 2000). Although an increase in air space volume should be expected to reduce

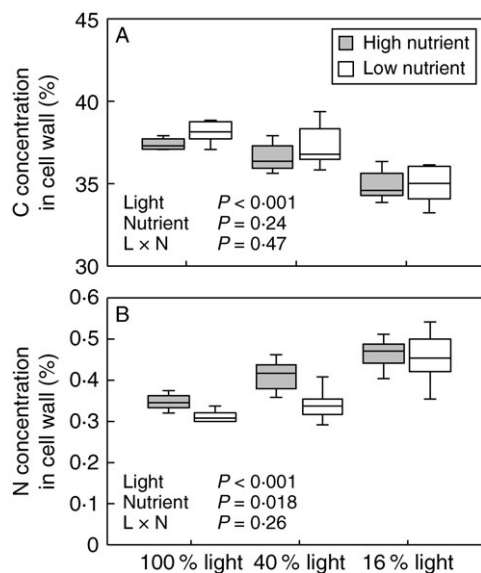


FIG. 7. Box plots of (A) carbon concentration of cell walls and (B) nitrogen concentration of cell walls of *Plantago major* leaves grown at three light conditions (100, 40 and 16%) and two nutrient availabilities (35 and 7 mg N week⁻¹). The central box in each box plot shows the interquartile range and median; whiskers indicate the 10th and 90th percentiles. Effects of light and nutrient availabilities were tested with ANOVA.

specific punch strength (i.e. the strength per unit lamina thickness) as it results in a lower mass density, it did not have an effect in the present study. This might be because air spaces are mainly located in the mesophyll layer while, as suggested above, intercostal lamina strength is mainly provided by the epidermis.

This greater volume of air space might be important to increase bending stiffness at minimal costs. As noted, leaves in the shade become thinner but still need to maintain a horizontal position in order to be able to capture light. Because bending stiffness is proportional to the third power of thickness (Gere and Timoshenko, 1999), a 30% reduction in thickness can lead to a 66% reduction in bending stiffness provided that the material properties of the leaves do not change. Maintaining air spaces in the mesophyll might therefore be a means of increasing the distance between the epidermis layers, the stiffest part of the leaf lamina (Gibson *et al.*, 1988), thus increasing the second moment of area and flexural stiffness of the lamina (Gere and Timoshenko, 1999) at minimal carbon costs. It should be noted that the existence of air spaces in leaves has also been associated with improved CO₂ diffusion conductance, but in most leaves this is not a limiting factor, especially in thin ones (Parkhurst, 1977; Terashima *et al.*, 2001). We therefore propose a role of air spaces in leaves in the maintenance of mechanical stability.

Effect of nutrient availability

A five-fold difference in nutrient availability had a much smaller effect on mechanical properties than a similar difference in light availability. No difference was observed on punch strength, punch toughness or F_{\max}/M_L between

the HN and LN plants. However, HN plants allocated slightly less dry mass to cell walls and had a slightly higher cell-wall-specific strength (Fig. 4). The lower allocation to cell walls could be explained by higher allocation of resources to photosynthetic proteins at high nutrient availability (Evans, 1989).

Although punch strength, toughness and thickness were not different, punch stiffness was increased by low nutrient availability (Fig. 2). As far as we are aware, this is the first observation that nutrient availability affects stiffness of a leaf without changing its strength. No other indices of stiffness (e.g. Young's modulus) were measured in this study, but a separate experiment with the same species observed that higher punch stiffness of leaves grown at low nutrient availability was accompanied by a greater Young's modulus (data not shown). As there was no difference in water content in leaves from the two nutrient treatments, hydrostatic pressure was not likely to be responsible for the difference. Furthermore, most other leaf anatomical parameters, including the vein fraction and the amount of intercellular air space, were also unaffected by nutrient availability (Table 1, Fig. 6), indicating that anatomical traits are not likely to explain the difference in punch stiffness. Changes in chemical composition of cell walls (Fig. 7B), however, might have contributed to differences in stiffness. We speculate that higher protein concentration in cell walls may lead to more elastic leaves, while lower cell-wall protein concentrations (i.e. predominantly carbohydrate skeleton) might result in stiff but relatively brittle leaves. N in cell walls in the present study probably represents structural proteins such as hydroxyproline-rich glycoproteins (Carpita and McCann, 2000) because strong detergents and solvents were used to extract cell walls, and therefore weakly bound proteins (e.g. expansin) were washed out (Lampert, 1965; Showalter, 1993). The exact mechanical function of structural proteins remains to be clarified (Showalter, 2001).

The ecological significance of low stiffness at high nutrient availability might be associated with reducing aerodynamic drag under wind loading (Vogel, 1989; Ennos, 1997). There is generally a positive relationship between nutrient availability and both leaf area growth and plant height, which makes plants grown at high nutrient levels more vulnerable to wind damage. More flexible leaves can reconfigure more easily in the wind, which reduces the exposed area and the drag coefficient through streamlining (Vogel, 1989).

CONCLUDING REMARKS

We have developed a conceptual approach to analyse leaf strength with respect to dry mass and dry-mass allocation to cell walls. Sun leaves were stronger (per unit area) than shade leaves and this difference could mainly be attributed to them having more mass per unit area (higher LMA). Shaded leaves, by contrast, had a greater strength per unit mass (higher F_{\max}/M_L), which was caused by a greater allocation of mass to cell walls and by a greater cell-wall-specific strength. Whether these changes are adaptive or simply a by-product of epidermis thickness being

constant remains to be seen, but the resultant higher F_{\max}/M_L probably contributes to maintenance of leaf lamina strength at minimal cost. Nutrient availability had a much smaller effect on leaf mechanical properties, only lamina tissue stiffness being higher at low nutrients. Although our approach was used to analyse phenotypic responses of single species, it can equally be used in interspecific comparisons, for example to analyse the extent to which leaf biomechanics play a role in the well-known positive correlation between LMA and leaf longevity.

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