Leaf anatomical plasticity of *Brachypodium pinnatum* (L.) Beauv. growing in contrasting microenvironments in a semiarid loess forest-steppe vegetation mosaic

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**Abstract:** After clearcutting xerothermic oakwoods once natural in the forest-steppe loess regions of Hungary, the perennial understory grass *Brachypodium pinnatum* has been persisting for decades by establishing microhabitats from shade to full sun. In this paper, we explore variation in leaf anatomy for plants growing in different microhabitat light regimes (full shade under oak canopy, half shade near shrubs, and in unshaded grassland) in situ, and for plants reciprocally transplanted between these microhabitats. Leaf lamina thickness and mesophyll thickness were about 1.5 times greater in the grassland in situ than in oak subcanopy due to an additional layer of mesophyll cells and to 25-32% taller mesophyll cells. Mesophyll thickness and the proportion of veins plus sclerenchyma were lower for plants transplanted from either full or half shade to full sun than in situ plants in the grassland. Parenchymatous bundle sheath tended to be thicker in the grassland than in the two other microhabitats. Mean intervenial distance remained variable among microsites. These adjustments in leaf anatomy may be a considerable part, but presumably not the dominant component of the medium-term (one year) light acclimation of *B. pinnatum* and the species’ success in microsites with contrasting light climate appearing side-by-side during secondary vegetation succession.

**Abbreviations:** ETR – Electron transport rate, LMA – Leaf mass per area.

**Introduction**

Changes in leaf anatomy are frequent components of plant structural adjustment to environment variability in concert with leaf and plant biochemistry, ultrastructure, biomass allocation, life history, etc. The literature is particularly rich on the subject, although most studies concentrate on interspecific comparisons (e.g., Garnier and Laurent 1994, Van Arendonk and Poorter 1994, Niinemets 1999, Castro-Díez et al. 2000, Choong et al. 1992), and much less attention is paid to within-species variation. Fekete and Szujkó-Lacza (1973) reported anatomical differences for *Quercus pubescens* Willd. leaves in relation to contrasting light and water regime of the habitat. Molnár et al. (2000) found no difference when compared leaf anatomy of the C₄ xerophytic grass *Cleistogenes serotina* (L.) Keng for its rapidly spreading lowland and established colline populations in similar vegetation types. Hilwatika and Bhat (2002) suggest that a great degree of phenotypic plasticity in cuticle thickness and stomatal frequency in forest precursor tree species may contribute to their success of inhabiting both the interior and the margin of scree forest and adjacent fynbos vegetation.

Differences of chloroplast ultrastructure and leaf coarse structure in leaves developed under different light levels have been demonstrated by Boardman (1977), Lambers et al. (1998) and Givnish (1988). In general, the potential for anatomical adjustment to altered environment is higher in newly developing leaves than in mature ones (e.g., Boardman 1977, Sims and Pearcy 1992, Oguchi et al. 2003). There is a considerable variation among species in 1) the capacity for adjustment to changing environment, and 2) the mechanism of such adjustment, i.e., whether it involves modifications in leaf struc-
ture (anatomy and/or ultrastructure), intracellular biochemistry or both. Changes in photosynthetic capacity per unit leaf area in response to high light intensity were mostly attributed to plastic modifications in leaf anatomy for *Alocasia macrorrhiza* (L.) G. Don. (Sims and Pearcy 1992) and *Acer saccharum* Marsh. (Niinemets and Tenhunen 1997). In contrast, when fully-developed leaves of *Chenopodium album* L. were transferred from low to high light, a moderate rise in the photosynthetic rate was not associated with considerable anatomical modifications, but it was primarily due to an increasing area of the chloroplasts facing the intercellular space (Oguchi et al. 2003). Both anatomical and biochemical adjustments were reported to take part in the light acclimation of *Piper sancti-felicis* Trel. (Chazdon and Kaufmann 1993) and *Myrtus communis* L. (Mendes et al. 2001). However, significant or even extensive variations in leaf anatomy (e.g., leaf lamina thickness and mesophyll thickness) were not necessarily associated with photosynthetic light acclimation (Chazdon and Kaufmann 1993, Oguchi et al. 2003, Boardman 1977).

This study aims at assessing the contribution of leaf anatomy variation to the high capacity of *B. pinnatum* to adjust leaf photochemistry to a broad range of microhabitat light levels experienced in the course of secondary succession after forest clearcutting (Mojzes et al. 2003). It is assumed that this species must possess a highly plastic leaf anatomy for such a flexible physiology and morphology. First, we examined leaf anatomical variation for *B. pinnatum* plants growing in situ in three microhabitats with contrasting light regime: in full shade under oak canopy, at the edge of shrubs in grassland (half shade), and in the unshaded grassland. Second, we transplanted plants among these microhabitats to explore the phenotypic plasticity in anatomical structure of leaves emerged in their new microenvironments after one year of transfer.

**Materials and methods**

**Species studied**

The perennial rhizomatous grass *Brachypodium pinnatum* is a characteristic element of seminatural species-rich grasslands in Western and Central Europe. Recently, the increasing dominance of this grass has been serious threat to species diversity (Bobbink and Willems 1987, Bobbink 1991, Hurst and John 1999). Plant characteristics that are considered to play the most important role in this expansion have been extensively studied (see Mojzes et al. 2003, for summary). In contrast, under the semiarid temperate climate of the Carpathian Basin, *B. pinnatum* does not appear to possess such an invasive character (Fekete et al. 1998). It was originally a characteristic understorey species in open xerothermic oakwoods intermingled with steppe grasslands once natural in loess areas of the Hungarian Great Plain and neighbouring foothills as part of the forest-steppe vegetation zone (Zólyomi and Fekete 1994). After clearcutting these oakwoods, this grass has been able to persist for decades and become dominant in the developing xeromesic grassland by successfully colonizing microhabitats from shade to full sun mostly on slopes facing N to E. However, during secondary succession after deforestation, *B. pinnatum* may be replaced by the xeric steppe species, *Festuca rupicola* Heuff. (Fekete et al. 1998, Zólyomi and Fekete 1994). Mojzes et al. (2003) measured a substantial phenotypic adjustment to habitat light environment in leaf and shoot morphology, and in leaf photochemistry for *B. pinnatum*, growing in three contrasting microhabitats (full shade under oak canopy, half shade near shrubs, and full sun in unshaded grassland). In addition to the consolidation strategy (De Kroon and Schieving 1990) and the nonplastic endogenous variation in rhizome length and bud activity of this grass (De Kroon and Knops 1990), this flexibility at (infra)individual level may contribute to the species’ persistence and expansion on microsites with different light regime appearing side by side after deforestation. However, little is known about the role of leaf anatomical adjustment to changing light environment in the plasticity of this species.

**Study area**

The study site is located on a steep, NE-facing slope in a valley near the village Isaszeg 25 km east of Budapest at an altitude of 200-230 m a.s.l. at the margin of the Gödöllö Hills, Hungary. The area falls within the forest-steppe vegetation zone. For a detailed description of the site, see Fekete et al. (1998, 2000) and Mojzes et al. (2003). During the course of secondary succession after clearcutting the former *Quercus pubescens* oakwood in the early 1900’s, several coenological types of *B. pinnatum* grassland appear side by side. These grasslands range from dense and closed swards along the edge of *Crataegus monogyna* Jacq. shrubs – representing an early phase of secondary succession with numerous forest and forest-steppe plant species – through transitional types to the so called steppe-type *B. pinnatum* community, which is not influenced by the shade of shrubs or remnant trees. These types of *B. pinnatum* community are described in detail by Fekete et al. (1998, 2000) and Virágh and Bartha (1998).
Experimental design

Plasticity in leaf anatomy of *B. pinnatum* was tested in the same field experiment as in our previous study for testing the plasticity in shoot morphology and in leaf photochemistry of this grass (Mojzes et al. 2003). The seven treatments were as follows (Fig. 1): plants growing *in situ* 1. under the oak tree canopy (‘Tree’), 2. near *C. monogyna* shrubs (‘Shrub’), 3. in the unshaded grassland (‘Grassland’); and plants transplanted from below the oak canopy 4. to the exposed grassland (‘Tree-Grassland’) or 5. near shrubs (‘Tree-Shrub’), and from the edge of shrubs 6. to the exposed grassland (‘Shrub-Grassland’) and 7. to the oak subcanopy (‘Shrub-Tree’). Plants were transplanted in May 1999. Fifteen monoliths containing the sward and soil were dug out from *B. pinnatum* communities both in the oak subcanopy (shade) and shrub edge (half shade) microhabitats, potted into PVC plastic tubes (25 cm diameter, 20 cm depth), and then divided into three groups of 5 samples. One group from each of these two microhabitats was reciprocally transplanted, another group was transferred into the grassland (full sun) microhabitat, while the third group was used as control and left *in situ*. By using plastic tubes for the control treatment as well we intended to minimize the effect of experimental manipulation on the results, with the exception of the grassland *in situ* treatment, where no plastic tubes were applied. This was because *B. pinnatum* was present in very low abundance in the grassland microhabitat at the date of transplanting. The bottom of plastic tubes was left open and the side wall was perforated to allow lateral movement of water in the soil. On the basis of measurements and statistical analysis for the moisture content within and outside the plastic tubes in 2001, the effect of plastic tubing on soil water status can be neglected (Mojzes et al. 2003). The sods in plastic tubes with 15 to 100 *B. pinnatum* shoots per sod were dug into the soil in a close-to-natural position.

Data collection

In May 2000, when leaf photochemical measurements were performed, the youngest (uppermost) fully-expanded leaf from 5-10 shoots per treatment (1 or 2 shoots per monolith) were removed and transported to laboratory in closed chambers. The middle portion of the leaf blade was severed and fixed in a 1:1:1 mixture (96% alcohol, glycerine and distilled water) until processing. Leaf blade cross sections were obtained by hand cutting without embedding by using elderpith and razor blades. Leaf cross-sections were permanently mounted in the same solution used for sample storage, observed without staining and photographed under light microscope (Nikon Eclipse E400, Nikon Inc., Yokohama, Japan). Measurements and counts were performed by using the Lucia M 3.52a image analysis software (Laboratory Imaging 1997). Lamina thickness in the intervelinal region, distance between vein centers and the area of three component tissues (epidermis, mesophyll, and the sum of vascular tissue and sclerenchyma) were measured on a leaf blade cross section halfway between the central vein and the leaf edge by using a magnification of 100. The outer and inner bundle sheaths were included in the mesophyll and vascular tissues, respectively. Proportional area for each tissue type and average intervelinal distance were calculated. Mesophyll and epidermis thickness, and in the case of mesophyll, the number of cell layers and the height of cells were measured at two points of an intervelinal area. The
thickness of the outer (parenchymatous) bundle sheath was measured on 3-4 randomly selected bundle sheath cells per primary vein. These measurements were performed at a magnification of 400. Seven replicates per treatment were used by averaging the data measured on the same cross section.

**Statistical analysis**

One-way ANOVA with treatments as grouping variable was used with subsequent Tukey-Kramer Multiple Comparisons Test to analyse significant differences among means. Since the variable leaf thickness did not meet the homoscedasticity assumption of ANOVA, a Kruskal-Wallis test with subsequent Dunn’s post hoc test was used instead for multiple comparisons. Linear (Pearson) correlation coefficients were calculated in order to analyze relationships between area proportions of tissue types across all treatments. Each statistics was performed by using GraphPad InStat version 3.05 software (GraphPad Software 2000) and differences were considered significant at p<0.05 level.

**Results**

Leaf lamina thickness was intermediate for ‘Shrub’ and ‘Shrub-Grassland’ plants and did not differ significantly from that of any other treatments (Fig. 2A), although these plants possessed significantly thinner mesophyll than those in the grassland in situ (Fig. 2B). Epidermis thickness did not vary significantly among treatments. Greater leaf mesophyll thickness for ‘Grassland’ plants was due to an additional layer of cells and to a 25-32% taller cells compared with leaves in the full shade microhabitat (Figs. 2C,D, Table 1). ‘Shrub’ and ‘Tree-Grassland’ plants also developed significantly taller mesophyll cells than ‘Tree’ and ‘Shrub-Tree’ plants, nevertheless an opposite trend observed in the number of mesophyll cell layers obliterated the significant differences in mesophyll thickness for these four treatments.

The area proportion of vascular bundles plus sclerenchyma for ‘Grassland’ plants was 18.3%. It was significantly greater than that for other treatments, including ‘Tree-Grassland’ and ‘Shrub-Grassland’ transferred plants as well, which ranged from 11.3% to 15.6% (Fig. 2E). This parameter varied most markedly (61.9%) among in situ treatments (Table 1). By contrast, the proportion of epidermis was significantly lower in the grassland in situ than for plants in the two other microhabitats. The latter ones did not differ from each other in this parameter. The proportional area occupied by vascular and sclerenchymatous tissues together negatively correlated with the proportion of epidermis (r = -0.788; p = 0.0353), but not to that of mesophyll (r = -0.2389; p = 0.6058).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Microhabitat</th>
<th>Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leaf thickness in the intervenial region (μm)</td>
<td>‘Shrub’ ‘Grassland’ ‘Tree’</td>
<td>80.1b 110.7b 73.1a 51.4</td>
</tr>
<tr>
<td>Mesophyll thickness (μm)</td>
<td>68.1a 96.1b</td>
<td>62.6a 53.5</td>
</tr>
<tr>
<td>Average number of mesophyll cells layers</td>
<td>4.4a 6.1b</td>
<td>5.0a 38.6</td>
</tr>
<tr>
<td>Average height of mesophyll cells (μm)</td>
<td>15.9b 15.9b</td>
<td>12.7a 25.2</td>
</tr>
<tr>
<td>Epidermis thickness (μm)</td>
<td>9.7a 9.1a</td>
<td>10.0a 9.9</td>
</tr>
<tr>
<td>Proportional area of veins plus sclerenchyma (%)</td>
<td>11.3a 18.3b</td>
<td>13.5a 61.9</td>
</tr>
<tr>
<td>Proportional area of mesophyll (%)</td>
<td>58.4a 56.0a</td>
<td>55.3a 5.6</td>
</tr>
<tr>
<td>Proportional area of epidermis (%)</td>
<td>30.3b 25.8a</td>
<td>31.2b 20.9</td>
</tr>
<tr>
<td>Mean intervenial distance (μm)</td>
<td>212.9a 181.7a</td>
<td>183.0a 17.2</td>
</tr>
<tr>
<td>Thickness of outer bundle sheath cell layer (μm)</td>
<td>8.2a 8.8a</td>
<td>8.0a 10.0</td>
</tr>
</tbody>
</table>

Table 1. Leaf anatomical parameters of B. pinnatum plants growing in different microhabitats in situ. ‘Tree’, ‘Shrub’ and ‘Grassland’ represent plants growing under the oak canopy, near the shrubs and in the grassland in situ, respectively. Significantly different (p<0.05) microhabitat means in the same row are followed by different letters. The last column presents the difference between the highest and the lowest microhabitat means expressed as percentage of the lowest mean (100*(Max-Min)/Min, %).
Figure 2. Leaf anatomical parameters of transplanted and in situ grown *B. pinnatum* plants in different microhabitat light levels. **A.** Leaf lamina thickness in the intercalary region (μm); **B.** Mesophyll and epidermis thickness (μm); **C.** Average number of mesophyll cell layers; **D.** Average height of mesophyll cells (μm); **E.** Area proportions of epidermis, mesophyll and the sum of vascular tissue and sclerenchyma (%); **F.** Thickness of outer (parenchymatous) bundle sheath cell layer (μm); **G.** Mean intercalary distance (μm). ‘Tree’, ‘Shrub’ and ‘Grassland’ represent plants growing under the oak canopy, near the shrubs and in the grassland *in situ*, respectively. In the case of transplanted plants arrows show the direction of transfers between two microhabitats. Significantly different (p<0.05) means are marked with different letters above or inside the bars.
Both the area and the proportion of veins plus sclerenchyma were significantly greater for ‘Shrub-Tree’ plants than for ‘Shrub’ ones. Treatments varied in the proportion of mesophyll slightly from 53.2% to 58.4%. In ‘Shrub-Tree’ and ‘Tree-Shrub’ leaves, however, it was significantly lower than in leaves on the edge of shrubs in situ, and was similar to that of ‘Tree’ plants (Fig. 2E). The outer (parenchymatous) bundle sheath was 6.8-22.3% thicker for the three treatments in the exposed grassland than those in half or full shade, however, differences remained insignificant except for ‘Tree-Shrub’ plants (Fig. 2F). Mean intervial distance did not differ among treatments apart from ‘Shrub-Grassland’ plants (Fig. 2G).

**Discussion**

**Leaf anatomy of B. pinnatum growing in situ in contrasting microenvironments**

Similarly to shoot morphology and leaf photochemistry, leaf anatomical traits of *B. pinnatum* showed the most pronounced differences between the oak shade and the exposed grassland microhabitats. Microclimate measurements (completed in 2001) indicated an increasing radiation load between these two extremes of microhabitat light and water regimes in late May and early June (Mojzes et al. 2003). As reported in many other intraspecific comparisons, shade leaves were thinner than sun ones due to shorter palisade cells and/or fewer palisade cell layers (e.g., Lambers et al. 1998, Fekete and Szujkó-Lacza 1973, Sims and Pearcy 1992, Oguchi et al. 2003). Variation in the average number of mesophyll cell layers as well as in the mesophyll cell height (Table 1, Figs. 2C,D) indicated that leaves produced by *B. pinnatum* are sun type in the exposed grassland and shade type in the light-limited oak understorey. This may have contributed to the twice greater leaf mass per area (LMA) and the nearly three times higher photosynthetic electron transport rate (ETR) of fully-exposed leaves compared with that in the oak shade (Mojzes et al. 2003). The moderate variation in epidermis thickness among microhabitats contrasts with the results of Mendes et al. (2001) and those of Sims and Pearcy (1992), who observed that the thicker epidermis also contributed to the greater leaf lamina thickness of sun leaves compared with shade ones in *M. communis* and *A. macrorrhiza*.

The similar proportion of mesophyll for the three in situ treatments despite the proportionally greater vascular and sclerenchymatous tissues in the grassland (Table 1, Fig. 2E) as well as the lack of inverse relationship between these two tissue types across the seven treatments imply that a trade-off between productivity and persistence (e.g., that was reported for perennials by Garnier and Laurent 1994) was not reflected in the leaf anatomy of this species. This is consistent with the high ETR of leaves growing in the grassland in situ (Mojzes et al. 2003). *B. pinnatum* exhibited a greater investment in foliar support structures in response to water and temperature stresses and to the presumably higher wind speed of the grassland microsite. This is indicated by the greater proportion of veins plus sclerenchyma at the expense of the proportion of epidermis in the grassland in situ and to the negative correlation between these two tissue types along all the treatments.

Microhabitat differences observed in leaf anatomy of *B. pinnatum*, however, remained much lower than those found for leaf photochemistry and coarse structure (LMA). Furthermore, for plants growing near shrubs in situ, there was a contradiction between both the proportion and thickness of leaf tissue components, which did not differ from those in shade, and leaf photochemical capacity, which was as high as in the full sun microhabitat (Mojzes et al. 2003). These results suggest the importance of cell structural and biochemical variations behind photochemical differences among the three in situ treatments besides the differences observed in leaf structure. Such differences at (ultra)cellular level could concern the number, volume and ultrastructure of chloroplasts, the amount of electron transport components as well as the investment of resources (e.g., nitrogen or chlorophyll) into carbon-assimilating vs. light-harvesting capacity (Lambers et al. 1998, Givnish 1988, Boardman 1977, Oguchi et al. 2003, Niinemets and Tenhunen 1997).

A tendency for thicker outer bundle sheath cell layer for leaves of *B. pinnatum* transplanted into the grassland or growing in this microhabitat in situ, suggests greater temporary water reserves compared with those near shrubs or under the oak canopy (Sage 2001). Similar to our result, water stress did not influence the intervial distance in leaves of *Cynodon dactylon* (L.) Pers. (Utrillas and Alegre 1997).

**Leaf anatomy of transplanted plants**

Compared with the high plasticity of shoot morphology and leaf photochemistry, *B. pinnatum* leaves showed relatively low anatomical modification after one year of transplantation from the source microhabitats of plants to their new microenvironments. Similar to high plasticity in LMA and ETR, the shrub edge to oak canopy transfers appeared to adjust leaf anatomy to a certain extent to the new light regime. The lower proportion of mesophyll in their leaves than in leaves of *in situ* ones near shrubs might reflect a lower allocation to carbon assimilation capacity in the light-limited oak understorey. This may
partly explain the lower electron transport rate of plants transferred to the oak shade compared with those growing near shrubs *in situ* (Mojzes et al. 2003). Expressed as volume proportion, lower and medium leaves of *Elaeagnus angustifolia* L. had a lower amount of mesophyll than upper sun leaves (Klich 2000). Less clear was the interpretation of the greater area and proportion of vascular plus sclerenchymatic tissues in these transplanted plants than in those growing near shrubs *in situ*, which may partly be responsible for the lower proportion of assimilative tissue in the former ones.

The investment in the sum of leaf sclerenchyma and veins was proportionally lower for plants transplanted to the grassland compared with those growing in this microsite *in situ*. However, it does not seem to have negative effects on the photosynthetic performance of these two transfers, at least in May prior to the driest period (Mojzes et al. 2003). Neither the thickness nor the proportion of mesophyll in the leaves of the oak subcanopy to exposed grassland transfer exceeded those obtained under the oak tree *in situ*. Thus, the greater ETR of these transplanted plants compared with those in their source microhabitat *in situ* (Mojzes et al. 2003) seem to be due mainly to biochemical modifications.

In conclusion, leaf anatomical plasticity of *B. pinnatum* appears to be insufficient to explain its high variation in leaf photochemistry. In addition to the substantial plasticity of this grass at the shoot and leaf level, adjustments at cellular level are considered to be important in the species’ successful colonization of microhabitats with different solar radiation appearing side-by-side during the species’ successful colonization of microhabitats with contrasting microhabitat conditions (i.e., half- and full-shade or half-shade and full sun), in a longer period of less contrasting microhabitat conditions (Fekete, G., and J. Szuszó-Laczca. 1973. Leaf anatomical and photosynthetic reactions of *Quercus pubescens* Willd. to environmental factors in various ecosystems. I. Leaf anatomical reactions. Acta Bot. Acad. Sci. Hung. 18(1-2):59-89.


