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Leaf structure and anatomy as related to leaf mass per area variation in seedlings of a wide range of woody plant species and types

Abstract The structural causes of variation in leaf mass per area, and of variations in leaf structure accounted for by leaf habit and life form, were explored in a set of laboratory-grown seedlings of 52 European woody species. The leaf traits analysed included density, thickness, saturated mass/dry mass, and leaf nitrogen per mass and per area. Other traits described the anatomy of leaves, most of them relating to the lamina (proportions of palisade and spongy parenchymata, epidermis, air space and sclerified tissues, expressed as volume per leaf area, and per-cell transversal areas of epidermis and parenchymata), and another referring to the mid rib (transversal section of sclerified tissues). Across the whole set of species leaf mass per area was correlated with leaf density but not with thickness, and this was confirmed by taxonomic relatedness tests. Denser leaves corresponded with greater proportion of sclerified tissues in the lamina, smaller cells and lower water and N contents, but no relation was found with the proportion of air space in the lamina. Taxonomic relatedness analysis statistically supported the negative association of leaf density with saturated to dry leaf mass ratio. Thicker leaves also exhibited greater volume per leaf area and greater individual cell area in each of the tissues, particularly parenchyma. Mean leaf mass per area and leaf thickness were lower in deciduous than in evergreen species, but no significant differences in leaf density, proportion of sclerified tissues in the lamina or cell area were found between the two groups. Leaf mass per area was higher in trees and subshrubs than in shrubs and climbers-plus-scramblers, this rank being equal for leaf density and proportion of sclerified tissues in the lamina, and reversed for cell area. Given the standardised environment and ontogenetic phase of the seedlings, we conclude that variation in leaf structure and anatomy among species and species groups has a strong genetic basis, and is already expressed early in the development of woody plants. From an ecological viewpoint, we can interpret greater leaf mass per area across this species set as greater allocation to support and defence functions, as shown predominantly by species from resource-poor environments.

Introduction

Leaves are the main organs of assimilation in most higher plants. Leaf photosynthetic capacity is connected with plant potential primary production (Reich et al. 1997), which to a great extent determines the plant's competive ability (Grime 1977). However, environmental constraints have limited the tendency to maximise photosynthetic capacity through plant evolution, as resources must also meet other plant functions. For example, leaves must defend themselves against herbivores and other physical hazards (Coley 1983; Herms and Mattson 1992), or they must store their assimilates to be consumed during future unfavorable periods (Bloom et al. 1985; Meletiou-Christou et al. 1992). Each species' pattern of allocation between protection and production must reflect the balance between different selective forces which have acted on the whole-plant life strategy through evolution. Therefore, the search for leaf traits indicative of leaf performance is crucial to the understanding of the functional ecology of plant species.

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The ratio between the dry mass and the area of plant leaves (leaf mass per area, LMA), and its inverse, have been used as easy-to-assess indexes of leaf and plant function. They are implicated in many functional aspects of plants, such as gas exchange (Field and Mooney 1983; Oren et al. 1986), potential photosynthetic rate (Reich et al. 1997) decomposition rate (Cornelissen and Thompson 1997; Cornelissen et al. 1999), leaf toughness (Lucas and Pereira 1990; Choong et al. 1992) and relative growth rate (Lambers and Poorter 1992; Reich et al. 1992; Cornelissen et al. 1996). Some authors have considered LMA as an indicator of the position of a species along an axis between resource-rich and resource-poor environments (Reich et al. 1992; Cornelissen et al. 1996; Westoby 1998), and others as a contributor to the regulation of growth and production from the leaf to the ecosystem levels (Reich et al. 1997). But does genotypic LMA variation among species always reflect the same structural variation, and therefore the same functional significance?

Previous studies have suggested a negative answer to this question. Dijkstra (1990) and Witkowski and Lamont (1991) pointed out that both leaf thickness and density may account for changes in LMA and both traits may vary independently. Moreover, anatomical structure underlying variation in leaf density and thickness may differ depending on the nature of the species and their environment (Garnier and Laurent 1994; Van Arendonk and Poorter 1994; Westoby et al. 1998). Across laboratory-grown species contrasting in the nutrient or water availability of their natural habitats, high LMA corresponded with high proportions of support tissues, small cells (Garnier and Laurent 1994; Van Arendonk and Poorter 1994), high carbon and low nitrogen contents (Niemann et al. 1992; Poorter and Bergkotte 1992; Van Arendonk and Poorter 1994). However, these studies only included small herbaceous plants, so that we do not know whether their findings have general validity across plant groups.

Woody plants ensure a longer life span and a taller stature through a carbon-expensive structure, i.e. wood. Therefore, the possession of a woody stem affects the whole-plant life strategy and may impose other constraints on the relationships among leaf traits. We found in woody species that seedlings of slow-growers showed a higher LMA (Cornelissen et al. 1996) and lower nitrogen content (Cornelissen et al. 1997) than those of fast growers. In this paper we attempt to unravel the structural anatomy underlying LMA variation among seedlings of 52 diverse woody species common in the floras of Great Britain and north-east Spain (cf. Cornelissen et al. 1996). A second aim is to compare innate leaf structural variation in different life forms and leaf habit groups (deciduous/ evergreen). We test and discuss our findings in relation to the taxonomic relatedness and ecology of the species.

Materials and methods

Plant material

The study species were selected from the commonest native woody plants in temperate and Mediterranean Europe. A few common naturalised or garden-cultured species were added to increase the range of contrast in terms of leaf life spans, life forms, habitat preferences and taxa. However, constraints posed by seed availability, germination and anatomy protocols limited the number of species to 52. Among the life forms, climbers and scramblers were considered together as they shared the trait of using external support. Nomenclature follows Stace (1991) for British and garden species, and Flora Iberica (Castroviejo et al. 1986–1999) for Spanish species.

The plant material for leaf analysis was taken from populations of seedlings grown in a growth chamber. These populations are the same as those used in a previous work to estimate the potential RGR of the species, so the detailed germination and growth protocols can be found in Cornelissen et al. (1996). In summary, seedlings were grown from seeds collected in the field in natural environments, mostly in Great Britain and north-east Spain. After germination, seedlings were transplanted into experimental 300- or 400-ml pots and filled with quarried, prewashed silica sand. The pots were put in a growth chamber with standard environmental conditions at the Unit of Comparative Plant Ecology, Sheffield University (details in Hendry and Grime 1993). The light regime was 135 μmol m^{-2} s^{-1} of photosynthetic photon flux density (red/far ratio 1.4) for 14 h day^{-1}. This adds up to a daily total of 6.8 mol m^{-2} day^{-1}, which is similar to the daily total at 19% of full light outdoors in summer and can be classified as partial shade (see Cornelissen et al. 1998). Day and night temperatures were 20–22 and 15–17°C respectively. To avoid gradients of light and temperature inside the chamber affecting the development of seedlings, all the pots were moved to new randomly chosen positions in the chamber every 2 days.

For each species, the starting point of the growth analysis period was when the modal plant of the population had opened the first true leaf (pair), as plants then started to gain carbon by true leaves. Other standardisation criteria, such as time from germination or plant size, would lead to great ontogenetic differences between species. The seedling population was then grown for 21 days, supplied every 2 days with 0.25 ml solution ml^{-1} sand volume of full-strength Rorison nutrient solution (N, P and K at 56, 31 and 78 mg l^{-1}, respectively, plus Ca, Mg, Fe and trace elements) (Hendry and Grime 1993). At the end of this period the whole population was harvested. Three to four seedlings per species were randomly selected and one fully expanded leaf per seedling pickled for anatomical studies (Hendry and Grime 1993).

Anatomical preparation

The leaf samples were taken in the middle of the lamina, cutting the mid-rib transversely. Due to sampling limitations the maximum sample width was 5 mm, so that whole leaves were cut across when their widths were less than 5 mm, whereas only a part of the lamina, including the mid-rib, was considered for larger leaves. The samples were embedded in 5% agar and progressively dehydrated in 50, 70 and 95% ethanol (2 h per solution). The small blocks of agar were then infiltrated for 15 days with resin JB 4 (Polysciences). After polymerisation of the resin 2-μm-thick cross-sections were obtained with a glass ultra-microtome; then they were stained with 5% toluidine blue and permanently mounted onto slides with DPX (further details in Castro-Díez et al. 1998).

Data collection

The leaf cross-sections were studied with a light microscope equipped with a drawing tube. The lamina thickness (LTh) was

found to be quite regular throughout the lamina width except its borders and the protruding veins areas, therefore the sampling points avoided both regions. A part of the lamina beside the mid rib that included minor veins, was drawn, including the contours of each tissue. Then, the whole area and those occupied by each tissue were measured from the drawing using a Delta-T leaf area meter and corrected for the scale. The tissues considered included adaxial plus abaxial epidermis, palisade and spongy parenchymata and the sclerified tissues of the lamina (STL), the latter consisting of the xylem and the sclerenchyma of minor veins. From these measurements, we estimated the proportion of each tissue in the transversal section area of the lamina. The proportion of air space in the leaf lamina (AS) was calculated as follows. One portion of lamina per leaf section was drawn with the contours of the intercellular area. By placing at random an acetate with a grid of equidistant points over the drawing, we calculated the ratio of points falling in intercellular space to the total number of points. This ratio indicates the proportion of air space in the leaf transversal section. According to Delesse's principle, all these measurements can be expressed either as a proportion of the cross-sectional area ($\mu m^2\ \mu m^{-2}$), or of the whole leaf volume ($\mu m^3\ \mu m^{-3}$) (Garnier and Laurent 1994). In order to express all these proportions on the same basis as LMA, i.e. amount per leaf area, we transformed the volume proportion of each lamina component ($\mu m^3\ \mu m^{-3}$) to volume per leaf area ($\mu m^3\ \mu m^{-2}$) by multiplying the former by the thickness of the lamina (μm).

The mean cell size of each of the epidermal layers and parenchymata was calculated by measuring a small area of each tissue in the cross section and counting its number of cells. In the case of palisade and spongy parenchymata the total area of space was subtracted from the measured surface. By dividing each tissue area by its cell number we obtained an average transversal cell area for each tissue. As cell areas of these four tissues were highly correlated with each other (all $r>0.45$, $P<0.001$) and exhibited very similar trends, only the cell area of the palisade parenchyma tissue is shown in the tables.

Finally, the area occupied by the sclerified tissues in the midrib (STMR), i.e. xylem plus sclerenchyma, was measured as a crude indication of the degree of investment in foliar support tissues, together with STL. However, we could not express this parameter as volume per area, as for most of the species we lacked an entire lamina section on which to measure the leaf width.

The average leaf mass per area and leaf water content of the same seedling populations were taken from Cornelissen et al. (1996). They calculated the former trait as the ratio between the leaf dry mass of the whole plant and its total leaf area, and the latter as the ratio between total saturated and dry leaf masses of each plant (SM/DM). Leaf density (LD) was obtained according to Witkowski and Lamont (1991) as the ratio between LMA and LTh. Leaf N contents of the same seedling populations, both on a mass (N_{mass}) and on a leaf area basis (N_{area}) were taken from Cornelissen et al. (1997).

Although some variability in anatomical traits will exist along the width of leaf laminas of certain species, we assume that the interspecific component must account for most of the trait variation across such a wide range of species.

Statistical analysis

Pearson's correlation coefficients were calculated between LMA, LD and LTh and all the anatomical parameters in the whole set of species. Most traits (except LMA) were natural-log transformed to normalise their distributions. For the most important correlations, a taxonomic relatedness analysis was performed. For each set of two or more subtaxa belonging to the same taxon, it was recorded whether the tested relationship showed a positive (+) or negative (−) trend. The average value of the lower taxa could then be used for a similar comparison of the next level up, up to the class level (Kelly and Beerling 1995). A chi-square test was used to assess whether the total number of pluses or minuses was greater than by chance.

A two-way ANOVA was performed to assess the effects of life form and leaf habit on the variation of the explored leaf traits. This analysis excluded climbers-plus-scramblers and subshrubs, as they were represented by only one or two taxa in one of the leaf habit groups. Taxonomic relatedness tests comparing leaf traits between species groups were not performed due to the low numbers of contrasts.

Results

Leaf trait variation across the whole set of species

The mean values for LMA, LTh, LD and the anatomical traits are shown in Table 1. LMA values ranged from 17.5 in *Salix caprea* to 105.9 g m^{-2} in *Quercus ilex* subsp. *ilex*. The thinnest leaves were those of *Fagus sylvatica* (95 μm) and the thickest those of *Prunus laurocerasus* (308 μm). *Buddleja davidii* was the species with the least dense leaves (101 g dm^{-3}), while *Q. ilex* subsp. *ilex* showed the densest ones (603 g dm^{-3}).

Across all 52 species LMA was correlated with LD but not with leaf thickness (Fig. 1, Table 2). The lamina components whose volumes per leaf area increased with LMA were firstly the sclerified tissues of secondary veins, and secondly the palisade parenchyma. Denser leaves possessed higher volume of sclerified tissues per leaf area unit, but lower of air space, epidermis, palisade and spongy parenchymata. However, when these proportions were expressed as volume percentage, LD was only correlated with STL (data not shown). Denser leaves also possessed thinner laminas and smaller cells in all the tissues (correlation coefficients of LD versus cell area of the four lamina tissues varying from $r=-0.55$ for spongy parenchyma to $r=-0.61$ for both upper and lower epidermis, $P<0.001$ in all the cases). Leaf water and mass-based N contents were strongly and negatively correlated with LD and less strongly with LMA. Variations in the thickness of leaf laminas were accounted for by variation in the volumes per leaf area of all the tissue layers, mainly those of both parenchymata (Table 2). The cell areas of the four lamina tissues, especially parenchyma, were positively correlated with LTh (correlation coefficients varied from $r=0.46$ for adaxial epidermis to $r=0.72$ for spongy parenchyma, $P<0.001$ in all cases). Leaf N was higher in thicker leaves, mainly when expressed on an area basis. The transversal area of the sclerified tissues of the mid-rib was not correlated with either LMA or with its components, LD and LTh (Table 2).

Taxonomic relatedness tests performed for the most striking correlations (Table 3) confirmed the consistency of the positive correlations of LD with LMA, and the negative one between LD and SM/DM. LD versus LTh and versus palisade cell area were positive almost as often as they were negative over the whole set of contrasts. LMA versus LTh, which was non-significant in the Pearson's test, showed a higher frequency of positive trends among the taxonomic contrasts. The other correlations, although not significant, showed an apparently higher frequency of the sign found in the Pearson's correlations.

Table 1 List of the species analysed in this study, provenance of seeds (*S* Spain, *B* Britain) and several traits (life form: *T* trees, *S* shrubs, *SS* subshrubs, *C+Sc* climbers and scramblers; leaf habit: *D* deciduous, *E* evergreen; *LMA* leaf mass per area, *LTh* lamina thickness, *LD* leaf density, *SM/DM* saturated to dry leaf mass ratio; volume per leaf area unit of the following lamina components: *AS* air space, *STL* sclerified tissues, *Ep* abaxial plus adaxial epidermis, *PP* palisade parenchyma, *SP* spongy parenchyma; CA_{pp} cell area of the palisade parenchyma, *STMR* area of xylem plus sclerenchyma in the mid-rib transversal section). The means given are based on three or four replicate plants per species

Species	Seed provenance	Life form	Leaf habit	LMA[a] g m^{-2}	LTh μm	LD g dm^{-3}	SM/DM[a]	AS μm^3 μm^{-2}	STL μm^3 μm^{-2}	Ep μm^3 μm^{-2}	PP μm^3 μm^{-2}	SP μm^3 μm^{-2}	CA_{pp} μm^2	STMR μm^2
Acer platanoides	B	T	D	36.36	124.8	291.3	3.05	55.8	2.02	20.6	33.3	71.0	254	34838
Aesculus hippocastanum	B	T	D	45.24	143.0	316.4	3.30	34.1	5.69	35.9	40.4	66.6	463	293560
Alnus glutinosa	B	T	D	21.13	100.6	210.1	5.32	29.1	0.33	30.3	25.7	44.6	260	7134
Arbutus unedo	S	T	E	49.09	138.0	355.8	2.82	36.4	1.24	30.1	42.0	65.8	658	3157
Berberis vulgaris	B	S	D	35.98	130.6	275.6	3.67	32.2	2.74	22.8	42.9	64.8	650	5684
Betula pendula	B	T	D	35.36	111.7	316.7	3.57	20.4	0.99	28.2	26.5	56.9	182	2738
Buddleja davidii	B	S	D	19.42	191.9	101.2	11.41	51.4	1.54	32.1	70.1	89.6	1017	3137
Buxus sempervirens	S	S	E	51.15	167.8	304.8	3.07	42.6	2.92	17.7	66.8	83.3	384	3684
Calluna vulgaris	B	SS	E	40.65	138.9	292.7	2.86	55.9		28.1	33.9	76.9	374	301
Castanea sativa	B	T	D	42.45	135.9	312.3	2.76	51.9		24.5	35.7	75.7	310	114990
Cornus sanguinea	B	S	D	34.65	162.2	213.6	2.82	46.1	2.79	29.5	50.5	82.2	431	4650
Corylus avellana	B	S	D	31.16	98.7	315.7	3.27	39.8	0.51	23.1	26.5	49.1	220	58223
Cytisus scoparius	B	S	E	27.72	167.8	165.2	6.78	60.0	0.40	42.9	52.9	72.0	527	2666
Dryas octopetala	B	SS	E	40.74	197.0	206.8	4.67	71.2	0.48	46.2	57.3	93.6	502	1123
Empetrum nigrum	B	SS	E	50.30	100.0	503.0	3.45	22.3		30.3	24.2	45.6	256	378
Erica cinerea	B	SS	E	60.86	125.9	483.3	3.30	26.7		40.4	34.7	50.8	281	591
Fagus sylvatica	B	T	D	37.31	94.8	393.5	2.18	32.8	7.18	15.4	28.6	50.9	219	39645
Frangula alnus	B	S	D	30.28	112.8	268.5	3.47	27.7	0.49	27.1	34.4	51.4	320	3283
Hebe ×franciscana	B	S	E	30.86	191.9	160.9	7.54	27.9	0.83	24.3	54.3	113.3	758	2547
Hedera helix	B	C+Sc	E	47.34	204.8	231.1	3.51	68.3	2.23	19.9	54.4	130.5	679	18421
Helianthemum nummularium	B	SS	E	77.22	213.3	362.0	3.59	32.9		32.0	98.8	82.5	542	3940
Hippophae rhamnoides	B	S	D	38.08	252.2	151.0	5.45	117.5	2.40	33.0	111.4	107.9	610	5895
Ilex aquifolium	B	T	E	53.83	188.9	285.0	3.87	54.2	1.36	23.3	54.7	110.9	406	6906
Juglans regia	B	T	D	38.31	120.2	318.8	3.37	31.0	0.90	25.7	33.5	61.0	319	43642
Laburnum anagyroides	B	T	D	41.92	161.9	259.0	4.22	44.8	1.22	40.6	44.8	76.5	661	7302
Ligustrum vulgare	S	S	E	47.75	164.3	290.7	3.52	52.0	1.16	25.1	55.3	84.0	1010	5586
Lonicera implexa	S	C+Sc	E	26.64	170.0	156.7	5.24	50.6	1.33	44.2	52.0	73.8	885	4608
L. periclymenum	B	C+Sc	D	27.28	135.6	201.2	4.95	35.2	0.21	35.9	51.1	48.5	951	3551
Malus sylvestris	B	T	D	39.70	181.5	218.8	3.33	98.3	1.38	22.6	42.7	116.2	372	23994
Prunus laurocerasus	S	S	E	60.01	307.8	195.0	3.74	111.1	1.53	49.0	74.0	184.7	893	13868
P. lusitanica	B	S	E	42.50	203.7	208.7	3.55	80.0	0.71	33.0	34.1	136.6	571	12132
P. spinosa	B	S	D	27.32	151.1	180.8	4.80	47.0	2.03	41.9	37.3	71.9	402	8692
Quercus cerris	B	T	D	51.36	100.0	513.6	2.49	20.3	2.58	14.7	46.0	39.2	361	88171
Q. ilex subsp. *ilex*	S	T	E	105.85	175.6	603.0		38.5	14.07	17.1	80.5	78.0	392	63637
Q. petraea	B	T	D	40.55	113.0	359.0	2.56	38.0	5.51	19.6	35.1	58.3	288	59064
Q. robur	B	T	D	53.41	110.6	483.1	2.49	26.3	8.33	18.8	37.6	54.1	328	106646
Q. suber	B	T	E	69.99	156.5	447.3		35.0	12.67	20.8	52.0	83.7	364	68539
Rhamnus alaternus	S	S	E	46.60	155.4	299.9	3.19	38.1	1.74	35.4	55.6	64.4	299	5654
R. cathartica	B	S	D	37.66	130.9	287.6	3.37	43.1	0.97	19.8	39.1	72.1	281	4471
Rhododendron ponticum	S	S	E	32.44	172.8	187.7	3.82	54.4	0.00	30.6	47.3	94.9	1022	284
Ribes nigrum	B	S	D	19.31	119.1	162.1	5.41	32.6	0.58	33.1	31.1	54.9	392	4537
Rosa arvensis	B	C+Sc	D	24.40	129.6	188.2	4.43	56.0	0.44	25.6	31.2	72.9	296	4328
Rubus fruticosus	B	C+Sc	D	27.56	136.7	201.7	5.24	43.9	0.59	37.2	38.3	61.2	400	13137
Salix caprea	B	T	D	17.51	103.3	169.4	7.54	20.4	0.68	26.2	32.5	44.6	419	5003

Table 1 (continued)

| Species	Seed provenance	Life form	Leaf habit	LMA[a] $g\,m^{-2}$	LTh μm	LD $g\,dm^{-3}$	SM/DM[a]	AS $\mu m^3\,\mu m^{-2}$	STL $\mu m^3\,\mu m^{-2}$	Ep $\mu m^3\,\mu m^{-2}$	PP $\mu m^3\,\mu m^{-2}$	SP $\mu m^3\,\mu m^{-2}$	CA$_{pp}$ μm^2	STMR μm^2
Sambucus nigra	B	S	D	30.66	224.8	136.4	5.98	46.1	0.96	51.5	53.4	119.9	1573	11100
Solanum dulcamara	B	C+Sc	D	19.36	134.4	144.0	8.50	29.3	0.26	25.5	49.9	59.1	882	16659
Thymus polystrichus	B	SS	E	44.49	232.6	191.3	5.12	57.3	4.32	31.5	112.8	88.3	1229	1567
Ulex europaeus	B	S	E	49.55	257.4	192.5	7.91	66.6	2.28	50.4	76.5	130.5	830	5807
Ulmus glabra	B	T	D	33.62	103.0	326.5	3.61	28.4	0.57	28.6	28.9	45.5	255	6578
Vaccinium myrtillus	B	SS	D	44.79	132.8	337.3	3.13	23.6		22.1	38.3	72.3	558	1021
V. vitis-idaea	B	SS	E	37.68	136.9	275.3	3.73	42.8		22.4	35.0	79.4	475	1054
Viburnum opulus	B	S	D	44.57	105.0	424.4	3.19	22.1	2.27	28.1	21.1	55.8	511	3639

[a] Data from Cornelissen et al. (1996)
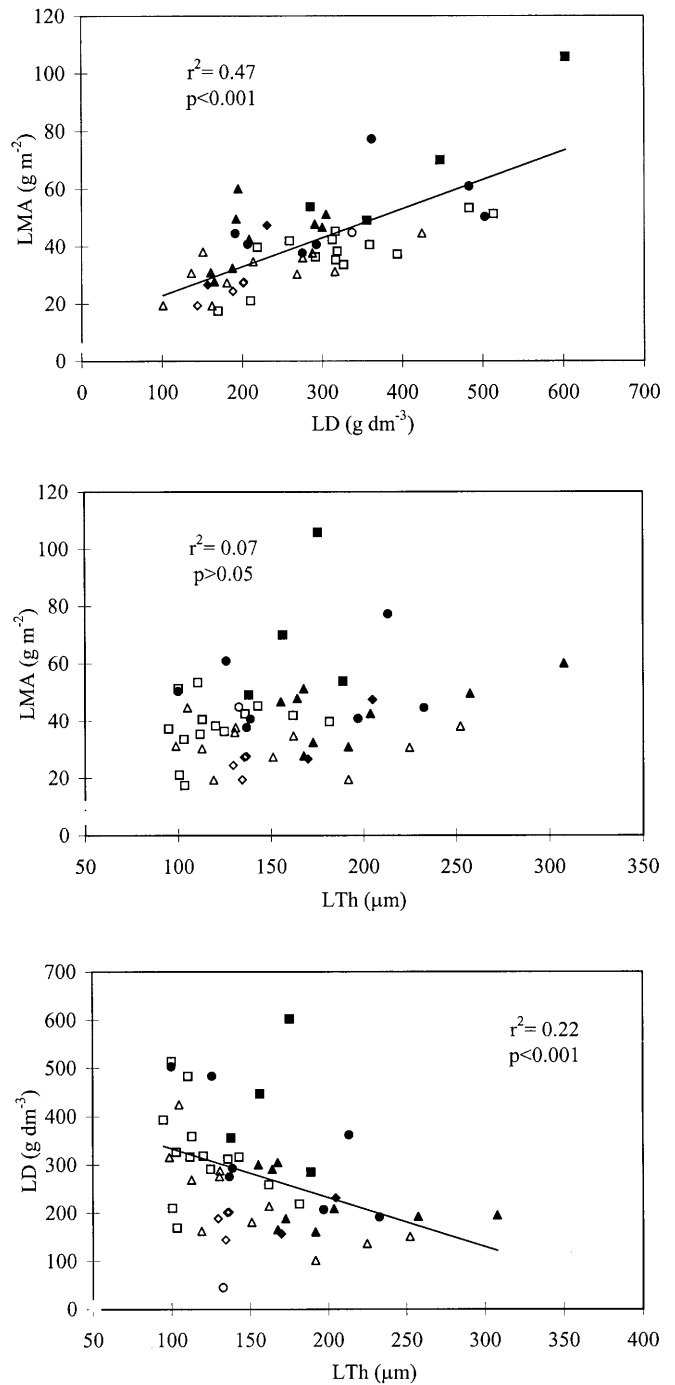
Fig. 1 Regressions between leaf mass per area (*LMA*), lamina thickness (*LTh*) and leaf density (*LD*). *Graphs* represent natural values of the variables, but regression coefficients were calculated using natural-logarithm transformations of LTh and LD (*open symbols* deciduous species, *closed symbols* evergreens, *squares* trees, *triangles* shrubs, *circles* subshrubs, *diamonds* climbers+scramblers)

Variation in leaf traits between life forms and leaf habits

Table 4 shows the effects of life form (trees versus shrubs) and leaf habit (deciduous versus evergreen) on the leaf trait variation, on the basis of a two-way

ANOVA. Both factors affected LMA, their interaction being significant. Figure 2 shows a higher LMA in trees than in shrubs, the difference being much greater among evergreen than among deciduous species. It also shows that evergreens had more leaf mass per area than decidu-ous, but the difference in this case was much greater among trees than among shrubs. LD only varied between life forms, being higher in trees than in shrubs. Shrubs and evergreens exhibited thicker leaves than trees and deciduous species, respectively. The epidermis volume

Table 2 Pearson correlation coefficients of LMA and its compo-nents with the anatomical traits (abbreviations as in Table 1; N_{mass} leaf nitrogen on mass basis, N_{area} leaf nitrogen on area basis). All variables were ln-transformed except LMA

	LMA	LD	LTh
LD	0.68***		
LTh	0.27 ns	–0.47***	
SM/DM	–0.54***	–0.83***	0.38**
AS	–0.02 ns	–0.47***	0.75***
STL	0.72***	0.60***	–0.06 ns
Ep	–0.23 ns	–0.53**	0.46**
PP	0.37**	–0.30*	0.83***
SP	0.23 ns	–0.41**	0.91***
CA_{PP}	–0.06 ns	–0.57***	0.68***
STMR	0.19 ns	0.24 ns	–0.13 ns
N_{mass}	–0.54***	–0.78***	0.31*
N_{area}	0.37*	–0.16	0.69***

*$0.005 \geq P > 0.01$, **$0.01 \geq P > 0.001$, ***$P \leq 0.001$, *ns* not significant

Table 4 Summarised results of the two-way ANOVA testing the effect of leaf habit (*D* deciduous, *E* evergreen) and life form (*S* shrub, *T* tree) on the leaf traits

	Life form (S×T)	Leaf habit (D×E)	Interaction
LMA	***	***	*
LD	***	ns	ns
LTh	*	**	ns
SM/DM	ns	ns	ns
AS	ns	ns	ns
STL	**	ns	ns
Ep	**	ns	ns
PP	ns	**	ns
SP	ns	**	ns
CApp	*	ns	ns
STMR	**	ns	ns

All the variables except LMA were ln-transformed

Table 3 Taxonomic relatedness analysis of the main relationships between LMA, LTh, LD and the anatomical and chemical traits. The null hypothesis states that these relationships show either pos-itive (+) or negative (–) trends no more often than chance levels across the contrasts at all taxonomic levels (one-tailed chi-square tests). The number of taxa used in each contrast is in parentheses. Abbreviations and significance levels as in Tables 1, 2

|	LMA vs. LD	LD vs. LTh	LMA vs. LTh	LMA vs. STL	LD vs. STL	LD vs. CA_{PP}	LD vs. SM/DM	LD vs. N_{mass}	LTh vs. N_{area}
Species within Genera									
Lonicera (2)	+	–	–	–	–	+	–	+	–
Prunus (3)	+	+	+	–	–	+	–	–	+
Quercus (5)	+	+	+	+	+	+	–	–	+
Rhamnus (2)	+	+	+	+	+	+	–	+	+
Vaccinium (2)	+	–	–	+	–				
Genera within Families									
Betulaceae (3)	+	+	+	+	+	–	–	–	–
Caprifoliaceae (3)	+	–	–	+	+	–	–	–	+
Ericaceae (5)	+	–	–	+	+	–	–	+	–
Fabaceae (3)	+	–	+	+	+	+	–	–	+
Fagaceae (3)	+	–	+	+	+	+	–	+	+
Rhamnaceae (4)	+	+	+	+	+	–	–	–	–
Rosaceae (5)	+	+	+	+	+	+	–	–	+
Families within Orders									
Ericales (2)	+	–	–	–	+	–	+		
Fagales (2)	+	+	+	+	+	+	–	–	+
Rosales (3)	+	+	+	+	+	+	–	+	+
Sapindales (2)	+	+	+	+	+	+	+	–	–
Scrophulariales (3)	+	–	–	–	–	+	–	–	–
Orders within Subclasses									
Asteridae (4)	+	+	+	+	+	+	–	–	+
Dilleniidae (3)	+	+	+	–	–	+	–	–	+
Hamamelidae (3)	–	–	+	+	+	–	–	–	+
Rosidae (8)	+	–	+	+	+	–	–	–	+
Subclasses within Class									
Magnoliopsida (5)	+	–	–	–	–	–	–	–	+
χ^2	11.45	0.00	1.50	2.67	2.67	0.60	8.84	3.09	2.02
Significance	**	ns	ns	ns	ns	ns	**	ns	ns
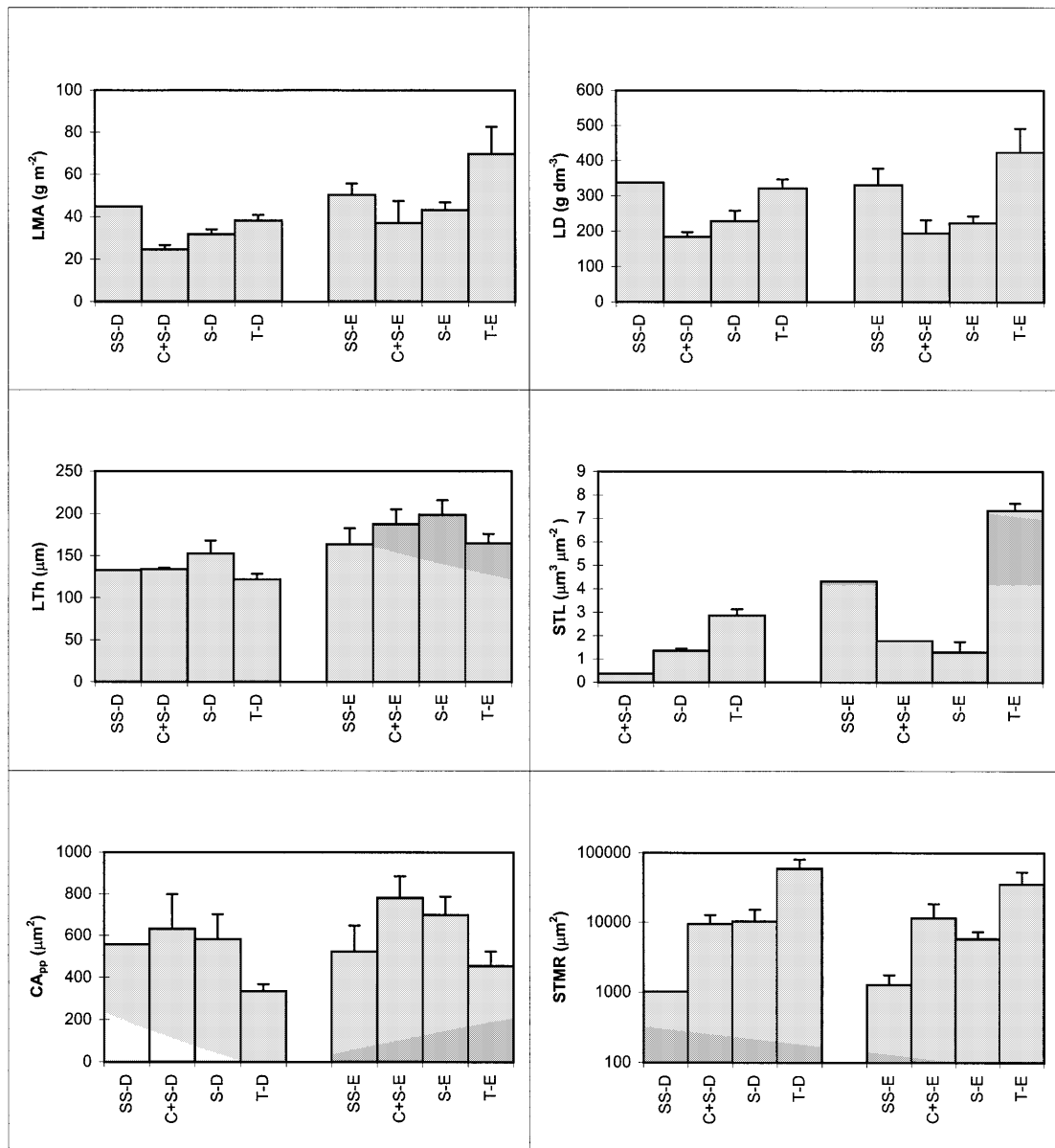
Fig. 2 Average leaf traits (*LMA* leaf mass per area, *LD* leaf density, *LTh* lamina thickness, *STL* sclerified tissues of the lamina, *CA_pp* cell area of the palisade parenchyma, *STMR* area of xylem plus sclerenchyma in the mid-rib transverse section) of species groups differing in life form and leaf habit. *Error bars* represent SE. STMR is represented in a logarithmic scale (life forms: *T* trees, *S* shrubs, *SS* subshrubs, *C+S* climbers and scramblers; leaf habits: *D* deciduous, *E* evergreen)

per leaf area was greater in shrubs than in trees, while those of both palisade and spongy parenchymata were higher in evergreen than in deciduous species (data not shown). Neither the amount of air space in the lamina nor the leaf water content differed between life forms or leaf habits. Shrub cells exhibited a greater transverse area than those of trees for all the tissues studied. Finally, STMR was significantly higher in trees than in shrubs, but did not vary between leaf habits.

Although the low number of species representing subshrubs and climbers-plus-scramblers did not allow them to be included in the statistical analysis, Fig. 2 represents the average values of some leaf traits for all the life forms. Subshrubs exhibited average values of LMA, LD and STL similar to those of trees, while climbers-plus-scramblers showed the lowest averages of the three leaf traits among the four life forms. The highest cell area average appeared in the non-self-supporting life forms.

Discussion

Relationships of LMA and its components
with anatomical traits

The 52 woody species from western Europe exhibited 6-fold variation in LMA. A higher biomass per unit of leaf

area may be achieved by possessing more cells, and/or by individual cells having more biomass. If all tissues have extra cells in similar proportions, a higher mass per leaf area will be accompanied by greater thickness but similar density. However, if cells are more numerous mainly in the densest tissues, both density and thickness of leaves will be greater. Higher cell biomass may result from greater accumulation of secondary compounds in the cell wall, in the cytoplasm or in the vacuoles, the three alternatives giving denser leaves, but no noticeable difference in lamina thickness. Greater average cell wall thickness may be accounted for by a higher proportion of the thickest-walled (support and conductive) cells or by a greater deposition of cell wall material throughout the tissues.

The different kinds of relationship among LMA, LD and LTh reported by different authors (Körner and Diemer 1987; Dijkstra 1990; Witkowski and Lamont 1991; Choong et al. 1992), suggest that all the above alternatives are possible. In the present data set LD was the main component of LMA, and this relationship stood up to the taxonomic relatedness test. The slight negative cross-species correlation between thickness and density of leaves was not confirmed by the taxonomic relatedness test. This finding accords with those of Wilson et al. (1999) and Westoby et al. (1998) on British and Australian species, respectively. Species with denser leaves also exhibited lower volumes per leaf area of epidermis and parenchymata, and consequently lower air space per area, but higher volumes of sclerified tissues. This result is consistent with those of Van Arendonk and Poorter (1994) and Garnier and Laurent (1994), who reported that LMA of grass species was correlated with the amount of sclerenchyma. Smaller cells in all the tissues, and the consequent higher proportion of cell walls per cell volume, also corresponded with denser leaves. Leaf density was associated neither with cell size nor with sclerified tissues per leaf area through the taxonomic tree. However, when this last trait was expressed as percentage of lamina volume, its correlation with LD became greater ($r=0.68$, $P>0.001$) and stood up to the taxonomic relatedness test ($\chi^2=5.04$, $P<0.05$). This finding suggests that STL has been more important than cell size in determining the density of leaves throughout plant evolution.

Leaf density may also vary as a consequence of differences in cell mass, but we have not assessed cytoplasm or vacuole storage, or cell wall thickness. Some authors have reported that the accumulation of starch may account for up to 30–40% of total leaf dry mass (McDonald et al. 1986; Rufty et al. 1988). However, this was a consequence of a reduction in nutrient availability, which was not the case in the growth conditions of our seedlings. Therefore interspecific variation in cell wall thickness is more likely to have contributed to additional LD variation in our study.

The lack of correlation between STMR and LD or LMA, may indicate either that the mid rib is not denser than the lamina, or that a larger investment in the dense mid-rib is offset by a larger, less dense lamina. The thick-walled cells constituting the sclerified tissues of the mid rib and their tight package, appear to support the latter argument (see also Grubb 1986). In addition, by using data for individual leaf areas from Cornelissen et al. (1999) and J.H.C Cornelissen and P. Castro-Díez (unpublished work), which were measured in the same populations as the ones used here, we found that STMR increased allometrically with leaf area ($r=0.86$, $P<0.0001$).

A higher proportion of secondary veins in the lamina, expressed in STL, may contribute to higher leaf resistance to physical and herbivore damage, as well as to a shorter water diffusion pathway from the vein endings to the mesophyll cells. This may be an important trait when the cells are tightly packed, as lateral conduction of water is poor through the palisade parenchyma (Wylie 1946; Esau 1977). Most of the highest STL values were found in the Fagaceae species, which are also among the biggest-seeded of the data set (cf. Cornelissen et al. 1996). By using seed weight data from Cornelissen et al. (1996), we obtained a positive correlation between STL and seed weight ($r=0.45$ $P=0.002$, both traits were natural-logarithm-transformed). These result suggests that both traits might have been selected for in the stressed habitats where these species tend to grow: highly sclerified leaves would allow longer leaf life, and big seeds would facilitate seedling establishment in stressful regeneration environments.

All the above structural differences related to high LD ultimately resulted from a higher proportion of cell wall mass across the leaf. As cell walls are N- and water-poor (Niemann et al. 1992), a higher proportion of cell wall should be reflected in lower leaf water and N contents. Indeed, LD was highly and negatively correlated with both SM/DM and N_{mass}, and so did LMA although less strongly (see also Cornelissen et al. 1997). Comparable results were reported by different authors for both herbaceous and woody species sets (Choong et al. 1992; Garnier and Laurent 1994; Shipley 1995).

Cross-species variation in LTh involved all four tissue layers, but mainly spongy and palisade parenchymata, according to the findings of Choong et al. (1992) for adult trees in Singapore. As cell size increased with leaf thickness, but lamina air space percentage remained constant [Pearson correlation coefficient between Ln (LTh) and Ln (%AS)=0.12, $P>0.05$], cell wall proportions should be lower in thicker than in thinner leaves, which explains the positive correlation between LTh and water and N contents on a mass basis. The high volumes per leaf area of the N-richest tissues, i.e. both parenchymata, exhibited by thicker leaves, explains the tight correlation between leaf thickness and leaf N per area (cf. Cornelissen et al. 1997).

The cross-species pattern of trait correlation may have been distorted by the fact that the light regime was not equally optimal for all the species. According to the published literature on leaf anatomy responses to varying light regimes (Wylie 1951; Chabot and Chabot 1977; Smith and Nobel 1978; Witkowski and Lamont 1991), species grown under a suboptimal light regime would

develop leaves with lower mass per area as a consequence of a thinner lamina. However, in this analysis no correlation appeared between LMA and LTh, so our results appear little affected by this source of variability.

The ecological interpretation of LMA variation must focus on the associated traits. Poor environments limit the assimilation rate of the leaves of the plants which inhabit them. In such conditions there is a premium on resource conservation as opposed to fast growth (Bloom et al. 1985; Aerts 1995). Therefore, traits which allow leaves to extend their photosynthate pay-back period may have been selected (Ryser 1996; Kikuzawa 1995). Such traits are related to leaf resistance and anti-herbivore defence. Leaf toughness seems to depend mainly on the vein density, as leaf veins have been found to be around 20 times tougher than the surrounding matrix in some woody species (Lucas et al. 1991; Choong et al. 1992). Therefore, as both LMA and LD were correlated with STL, they may be expected to be good predictors of leaf toughness. In fact, leaf fracture toughness of adult plants was found to correlate with LD in 42 Singapore trees (Choong et al. 1992) and with LMA in both 16 Argentine and 23 British woody plants (Cornelissen et al. 1999). In addition, the low N_{mass} and water contents of high LMA and LD leaves, must reduce their nutritional quality to herbivores (Coley 1983). Indeed, the less dense leaves of our data set belonged predominantly to species from nutrient-rich environments, while the densest ones belonged mostly to those from oligotrophic soils, Mediterranean lands or late successional shaded communities.

The evolutionary price to pay for constructing dense leaves of high mass per area is a lower photosynthetic capacity. Firstly, a high investment in cell walls or storage leaves less biomass available for photosynthetic cell compartments. Secondly, the internal gas conductance of leaves (g_i) has been found in some species to be inversely proportional to tissue density and directly to mesophyll cell size (Sylversten et al. 1995). Although g_i is just one component of the total CO_2 conductance from the extra-foliar environment to the chloroplasts, Parkhurst (1994) suggested that low g_i may be an important constraint for CO_2 uptake.

Leaf habit and life form as related to leaf anatomy

Variations in LMA among life forms were paralleled by variations in those leaf traits which were correlated with LMA across species. Life forms may be situated along an axis of variation between two opposed leaf structures. Dense, small-celled and highly sclerified leaves are seen particularly in seedlings of trees, while big-celled, less sclerified and less dense leaves are more common among shrubs. Although the other life forms could not be reliably compared, subshrub leaves appeared to be similar to those of trees, while climbers and scramblers exhibited yet lower LMA, less dense, less sclerified and bigger-celled leaves than shrubs. These results might be interpreted in terms of successional stage and resource availability. Subshrubs tend to occur in stressful environments, such as heathlands and semi-arid Mediterranean lands (Specht 1979). Here, selective forces must have favoured a longer use of each resource unit to compensate for their low availability. This may be attained through a long leaf life span (Reich et al. 1992; Aerts 1995), and therefore through a tough leaf structure. In the less stressed environments shrubs tend to be early-successional compared to trees (Specht 1979). The former must grow fast (see Cornelissen et al. 1996) to quickly occupy the available space, particularly as seedlings. Leaf structure associated with fast growth involves allocation to photosynthetic components to the detriment of support and protection inside the leaf, and therefore gives rise to less dense, little sclerified and big-celled tissues. Many trees feature later in succession and regenerate below more closed canopies, where carbon gain is constrained by relatively low resource levels. In this situation, trees may maximise their long-term carbon gain through the efficient use of foliar nutrients, i.e. through longer-lived, well protected leaves (Aerts 1995). Finally, climbers and scramblers are often associated with heterogeneous light environments (Teramura et al. 1991). The possession of short-lived, highly productive leaves allow them to efficiently exploit temporal light gaps. Again, this strategy requires a leaf structure which favours photosynthesis over leaf defence and persistence.

It has been reported that LMA of evergreens is higher than that of deciduous species, both in adults (Coley 1988; Reich et al. 1992; Cornelissen et al. 1999) and in seedlings (Cornelissen et al. 1996). This difference has been related to the greater physical resistance required by evergreen leaves to live longer and through unfavourable periods for survival (Chabot and Hicks 1982; Kikuzawa 1995). However, our analysis has revealed that the higher LMA of evergreen seedlings was neither parallelled by higher density nor sclerification in leaves, but it was by thicker mesophyll, a trait which does not necessarily imply greater leaf toughness (Choong et al. 1992). Therefore, it seems that evergreen leaves acquire their physical resistance through their life, a process which may be part of the ontogenetic changes from seedling to adult phase (see Cornelissen 1999), and/or induced by changes of the environments in which the plants grow (Wylie 1951). The thicker mesophyll of evergreen seedling leaves was not reflected in bigger cells, and it was probably a consequence of a larger number of cell layers. Thick mesophylls provide the leaf with higher photosynthetic capacity per unit of area, but also enhance the competition among cells for CO_2 and light. Givnish (1979) predicted thick leaves to be selected in environments with high availability of both resources and/or where their absorption rates by the mesophyll cells were low. This would happen when irradiation is high, when transpiration is high (as it is linked to CO_2 uptake), and when photosynthesis is low (as CO_2 depletion rate is low). The natural environments of most of our evergreen species fulfil one or more of these condi-

tions. Several of them came from Mediterranean areas, characterised by high irradiation through the year and long dry summers. The British ones came mostly from light-exposed moorlands and heathlands, two typically oligotrophic environments, where low nutrient availability has a negative effect upon photosynthesitic capacity.

In conclusion, this work has shown that the structural attributes underlying LMA variation across species are mainly related to differences in leaf density, which result from variations of the degree of sclerification in the lamina. These relationships are similar to those found by other authors for herbaceous species. Innate variation in LMA between life forms and leaf habit groups already appears in the seedling phase, and may reflect environmental and successional affinities of each functional group. The tougher structure reported for evergreen leaves of adult plants, as compared with those of deciduous plants, was not apparent in the seedling phase. It seems that this develops later in the plant's life as a product of the plant's ontogeny and its growth environment.

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