

# **The leaf anatomical trade-offs associated with plant ecological strategy variation**

**Simon Pierce<sup>1\*</sup>, Dario Maffi<sup>1</sup>, Franco Faoro<sup>1</sup>, Bruno E.L. Cerabolini<sup>2</sup>, Alberto Spada<sup>1</sup>**

<sup>1</sup>Department of Agricultural and Environmental Sciences – University of Milan, via Celoria 2, 20133 Milano, Italy.

<sup>2</sup>Department of Biotechnologies and Life Sciences (DBSV), University of Insubria, via J.H. Dunant 3, I-21100 Varese, Italy.

\* **corresponding author:** [simon.pierce@unimi.it](mailto:simon.pierce@unimi.it)

## **16-digit ORCID of the authors**

Simon Pierce: 0000-0003-1182-987X

Dario Maffi: n.a.

Franco Faoro: 0000-0003-2620-9171

Bruno E.L. Cerabolini: 0000-0002-3793-0733

Alberto Spada: 0000-0001-7665-0446

## Abstract

Plant functioning depends on variation in resource economics traits (acquisition vs. conservation) and the size of plants and their parts (the ‘global spectrum of plant form and function’). The anatomical basis of single traits (e.g., leaf mass per area; LMA) is generally understood, but little is known regarding the relationship between anatomical trade-offs and ecological strategies (representing integrated suites of traits). We hypothesised correlations between the relative extent of leaf tissue types and Grime’s Competitor Stress-tolerator Ruderal (CSR) strategies, principally a trade-off between structural (mechanical/fibro-vascular) vs. photosynthetic (chlorenchyma and intercellular airspace; ICAS) tissues, for ecologically-contrasting herbaceous angiosperms in northern Italy. Specifically, that in the lamina portion the trade-off represents the economics spectrum (S-R selection), but in the midvein portion it reflects the mechanical constraints inherent to supporting large leaves (i.e., varies with C-selection). We used microscopy and image analysis to determine the relative cross-sectional area of tissues from transverse leaf sections (lamina and midvein portions) of angiosperms of contrasting CSR strategies. Principal components analysis (PCA) determined that the main trade-off (PCA1) was between mechanical/fibrovascular tissues vs. ICAS/epidermis/chlorenchyma, but that this was associated with the economics spectrum (R- to S-selection) in the lamina, and with size (C-selection) for the midvein. A secondary trade-off in both lamina and midvein portions involved ICAS (i.e., the internal gas diffusion pathway) vs. chlorenchyma/epidermis (light capture), associated with S- to R-selection, respectively. Our results confirm the expectation that ecological strategy variation has a basis in underlying trade-offs between tissues with contrasting metabolic/architectural (i.e., economics/size-related) roles.

**Keywords** CSR theory, global spectrum, Grime, leaf economics, plant defence/defense, plant functional type, universal adaptive strategy theory

## Declarations

**Funding:** This project was funded with the aid of grant number 19628 (RV\_RIC\_AT16SPIER; *Piano di Sostegno alla Ricerca* 2015-2017) awarded to S.P. from the Department of Agricultural and Environmental Sciences (DiSAA), University of Milan, Italy.

**Conflicts of interest/Competing interests:** SP is an Editorial Board Member.

**Data availability:** The dataset is available in Microsoft Excel format as Table S1 (Online Resource 1).

**Code availability:** N/A

**Authors' contribution:** SP, BELC and AS conceived the study, SP collected samples from the field, DM and FF conducted leaf sectioning and performed microscopy with help from SP and AS, AS performed image analysis with help from SP, SP conducted statistical analyses and wrote the manuscript, and all authors were involved in manuscript editing.

### **Acknowledgments**

The authors thank the Native Flora Center of the Lombardy Region (*Centro Flora Autoctona*; CFA) and Parco Monte Barro for permission to collect leaf material in the Lombardy region.

## Introduction

Vascular plant adaptive radiation involves the evolution of various aspects of phenotype operating across a range of scales, from molecular, cellular, organ and whole plant life history characters. Adaptive or functional traits (characters that affect survival) and plant ecological strategies are typically measured and analysed at the level of organs (leaves, roots, stems, flowers and dispersules) and the whole plant (e.g. integrated suites of traits including canopy height, biomass production and relative growth rate, and the timing of reproductive events). At this scale, differential expression of traits reflects fundamental trade-offs in resource and biomass investment (Grime 1965), the importance of which has been confirmed world-wide (Díaz et al. 2016) and is known to limit plant adaptation to tenable trait combinations. The principal trade-off involves “*attributes conferring an ability for high rates of resource acquisition in productive habitats and those responsible for retention of resource capital in unproductive conditions*” (Grime et al. 1997), resulting in “*a spectrum of plant functional types which in terms of resource processing range from ‘the acquisitive’ to ‘the retentive’ and correspond respectively to highly productive and chronically unproductive vegetation*” Grime and Mackey (2002). This is now widely recognised as the ‘plant resource economics spectrum’ (Freschet et al. 2010) or ‘plant structural economics spectrum’ (Verbeeck et al. 2019), which is represented in different organs as the ‘leaf economics spectrum’ (Wright et al. 2004; Reich 2014), the ‘root economics spectrum’ (e.g. de la Riva et al. 2021) and the ‘wood economics spectrum’ (Chave et al. 2009). The second main axis of functional trait variability involves plant and organ size traits, which together with the economics spectrum form the ‘*global spectrum of plant form and function*’ (Díaz et al. 2016). Beyond this ‘primary’ trade-off, ‘proximal’ traits are specific characters in response to selection pressures acting at particular moments of the life cycle: for instance, reproductive traits such as the pollination syndrome or seed dormancy breaking requirements (Grime and Pierce 2012). Integration of functional traits into ecological strategies is also a key concept because plant fitness may ultimately depend on the optimization of trait integration along the trade-off axes more than it does on the performance of any single trait (Guo et al. 2018). Indeed, while investigation of single traits can reveal the variability of each trait, only when traits are considered together as suites or ‘strategies’ can the trade-offs underpinning the functional response and adaptation of species be discerned (discussed by Grime & Pierce 2012).

Crucially, the extent to which plant and organ-level trade-offs are evident at smaller scales, such as tissues or cells, is little understood, although the partitioning of essential hydrocarbons

and minerals between tissues with contrasting roles is likely to be fundamental to plant functioning (Grime and Pierce 2012). To date these fine-scale physiological and anatomical/structural trade-offs have been investigated only in the context of specific traits, including sized-related traits such as leaf area (Wright et al. 2017), leaf venation and the hydraulic capacity of differently sized leaves (Sack & Frole 2006; Sack & Scoffoni 2013) and, separately, leaf economics traits such as leaf mass per area (LMA) or its inverse, specific leaf area (SLA) (Hassiotou et al. 2010; Villar et al. 2013; del la Riva et al. 2016; Xiong et al. 2016; Guo et al. 2017; John et al. 2017; Onoda et al. 2017; Belluau and Shipley 2018). For instance, greater LMA of deciduous trees is related to the ratio of mesophyll (chlorenchyma tissue) to intercellular airspace and epidermis (Villar et al. 2013). Reduced intercellular airspace is known to reduce mesophyll conductance to internal CO<sub>2</sub> diffusion, limiting photosynthetic rates (see Ye et al. (2020) and references therein). Indeed, LMA is a product of the density and thickness of leaves (Niinemets 2001), specifically due to the extent of investment in cell wall material and the trade-off between a cell size and number, expected to “be affected by the proportion of different types of tissues in the leaf” (Shipley et al. 2006). LMA responds to environmental parameters such as solar irradiance, temperature, precipitation and, by extension, site aridity (Niinemets 2001; see also Grubb et al. 2015). It is also clear that the relative extent of tissue types comprising leaves varies with environmental factors, such as elevation (Liu et al. 2021), temperature, aridity and soil nutrient availability (Tian et al. 2016). Some size-related traits, particularly involving leaf venation (vein length per unit leaf area) are involved in determining hydraulic conductance and thus influence physiological traits involved in the leaf economics spectrum, such as mass-based photosynthetic rates and stomatal conductance (Sack et al. 2013). However, variation in the extent of different tissue types and thus functions has not been explicitly investigated in the context of the wider ‘global spectrum’ trade-off between economics and size (i.e. how anatomical features and roles trade off against one another as part of the general adaptive strategy of each species).

Notably, while resource economics variation is a property of the entire leaf, large leaves exhibit extensive lamina area but must be physically supported by prominent mechanical/vascular venation, in particular the main mid-vein, suggesting a division of ‘photosynthetic’ and ‘supportive’ roles between the lamina *sensu stricto* and venous portions. Thus, is it reasonable to predict a prominent division of tissue types (mesophyll chlorenchyma vs. mechanical/vascular) between lamina and venous leaf portions for species with larger leaves. Crucially, this generally agrees with Li et al.’s (2017) concept of modular function, whereby leaf tissue types reflect three main ‘modules’: the light capture module (essentially

chlorenchyma), water-nutrient flow (vascular tissues), and gas-exchange (intercellular airspace and the CO<sub>2</sub> diffusion pathway to stomata). Mechanical tissues could be said to represent a fourth ‘support’ module, but are often integrated with vascular bundles as xylary reinforcement.

How can we relate anatomical trade-offs to macroscopic functional trait trade-offs and plant adaptive/ecological strategies?

A leading theory of plant strategies that can provide a general explanation for the evolution of the joint economics and size trade-off (i.e. that considers the context of the natural selection pressures that delimit plant functioning) is Grime’s (1974) CSR (Competitor, Stress-tolerator, Ruderal) theory (discussed by Pierce and Cerabolini 2018; also Grime and Pierce 2012, Pierce and Fridley 2021). Competitor species dominate stable, resource rich habitats by pre-empting resources using traits permitting rapid growth to large size (i.e. large ‘size of whole plants and their parts’, *sensu* Díaz et al. 2016). Stress-tolerators dominate in habitats where limiting and variable abiotic factors constrain metabolic performance, and are robust and slow-growing (conservative resource economics), some eventually becoming large. Ruderals dominate in habitats where biomass is periodically destroyed (disturbance) and are characterised by rapid growth using extremely ephemeral leaves (acquisitive economics), with populations persisting as propagules rather than mature individuals. Aside from this theoretical context, the CSR scheme also provides a practical quantitative framework for the comparison of individuals and species. In practice, quantification of CSR strategies is performed using leaf functional traits that represent resource economics and size trade-offs (Cerabolini et al. 2010; Pierce et al. 2012, 2013). Specifically, the resource economics spectrum (S to R-selection) is determined from leaf fresh and dry mass and area measurements that allow calculation of structural/photosynthetic tissue density traits. A leaf size axis arising perpendicular to this terminates in the extreme of C-selection (Pierce et al. 2013). The CSR score represents not economics or size *per se*, but the trade-off between these multiple functions.

Calculation of CSR strategies is relevant to ecology across a range of scales encompassing the centimetre-scale of species coexistence within communities (Pierce et al. 2014) to biomes at the global scale (Pierce et al. 2017). Indeed, CSR analysis has been used to predict vegetation responses such as local scale shifts in plant community composition along succession gradients (Zanzottera et al. 2020), regional scale functional shifts in response to climate and soil (Dalle Fratte et al. 2019a; Zhang and Wang 2021) and can help explain species global and native range sizes (Liao et al. 2021). Specifically, the CSR analysis method of Pierce et al. (2017), calibrated using the global-scale economics/size trade-off, is now a well-established method applied worldwide to explain intra- and interspecific functional variability and environmental responses

for wild plants in natural circumstances (e.g. Dayrell et al. 2018, Vasseur et al. 2018; Dalle Fratte et al. 2019b; Dudova et al. 2019; Baltieri et al. 2020; Behroozian et al. 2020; Giupponi 2020; Ferré et al. 2020; Escobedo et al. 2021; Fernandes et al. 2021; Han et al. 2021; Hooftman et al. 2021; Lazzaro et al. 2021; Mugnai et al. 2021; Tameirão et al. 2021; Watkins et al. 2021; Bricca et al. 2022; Fletcher et al. 2022; Han et al. 2022; Seele-Dilbat et al. 2022; Yu et al. 2022). It provides both a dependable method and theoretical context for comparing plant functional variability.

To what extent are different tissue types with different functions associated with CSR strategy variation? To a limited extent this has been investigated within the Poaceae family (Pierce et al. 2007), for which greater intracellular airspace (decreased resistance to CO<sub>2</sub> diffusion for photosynthesis) was positively associated with R-selection (and negatively with S-selection). However, beyond this single family, we hypothesise that anatomical/ecological strategy associations may be generally evident across a broader taxonomic range of Angiosperms. Here we quantify the relative extent of leaf tissue types for species with known, measured CSR strategies from a range of flowering plant families, to investigate the specific hypotheses that: 1). R- to S-variation is associated with an increasing extent of mechanical and fibro-vascular tissues and decreasing relative extent of mesophyll chlorenchyma, and 2). C-selected species exhibit a particularly strong contrast between lamina (optimised for metabolic function) and midvein (optimised for mechanical support) leaf portions.

## **Materials and methods**

### **Plant material**

Herbaceous angiosperm species were chosen based on several criteria: availability of quantitative CSR strategy scores (the extent of C-, S-, and R-selection expressed in %) in the global dataset of Pierce et al. (2017), prevalence (and/or dominance) of the species in contrasting habitats (productive, unproductive or disturbed), tendency towards an extreme of the CSR triangle, the local availability of wild plant material, phylogenetic coverage (i.e. representing major clades of both dicots and monocots; see One Thousand Plant Transcriptomes Initiative (2019) for a recent and comprehensive plant phylogeny). These criteria underpinned the choice of 21 species that broadly represent CSR strategy variation (Fig. 1), for which the more labour-intensive work of anatomical investigation was conducted. Full

species names, including authorities, are available in Table S1, alongside information on the collection location for each set of leaf samples for each species.

CSR scores from Pierce et al. (2017) were previously calculated based on the trade-off between values of specific leaf area (SLA), leaf dry matter content (LDMC) and leaf area (LA) (calculated from leaf fresh weight, dry weight and area measurements), which represent positive and negative extremes, respectively, of leaf economics, and size variation. The precise method is detailed in two methodological papers (Pierce et al. 2013; 2017), see Table S1 for details of the C, S and R scores of each species used in the present study. These measurements were not repeated here: data from Pierce et al. (2017), from the same geographic area and populations as the present study, were used directly.

For each species, eight undamaged, young and fully expanded leaves were collected, each leaf representing a separate individual plant. Leaves were collected towards the end of spring, from individuals still in the vegetative, rather than reproductive, phase of the life cycle. Leaves were excised at the base and placed in a fixative solution of formalin-acetic-80% alcohol (1:1:8; FAA) at ~4 °C (in the field, in a cool-bag over icepacks, in the laboratory, in a refrigerator). After 24 h samples were transferred to 70% ethanol which was replaced with fresh 70% ethanol after a further 24 h, followed by longer-term storage at 4 °C before sample sectioning.

## Histology

To rapidly and economically process a large number of samples a simple and original method for the visualization of leaf tissues was set up. Fixed leaves were washed thoroughly with distilled water and a leaf portion of about 2 × 0.5 cm was excised from approximately half-way along the length of the leaf lamina with a sharp razor blade. For small leaves not exceeding 2 cm in width, a transverse section of the whole lamina was cut, perfectly perpendicular to the lamina. Leaf portions were then affixed with double-sided tape on the side of a transparent plastic block mounted on a microscope slide, aligning the cut edge of the leaf with the edge of the block. The cut edge was then stained by exposure to 0.2% toluidine blue O (Merck) micro-pipetted onto the sectioned surface for 5-15 minutes, depending on the plant species, followed by washing with distilled water and placing of a coverslip (Fig. 2). Finally, the block was mounted on a microscope slide and observed by an Olympus BX50 microscope (Tokyo, Japan), illuminating the cut surface of the leaf with fiber optics in proximity to the objective. Observations were carried out with a 10× or 20× objective and digital images recorded. Due to the large scale of many of the leaf samples and depth of field limitations (and thus difficulty in



focussing across the sample), a focus stacking technique was employed: a *z*-axis stack of photomicrograph frames was produced for each section, to ensure that focussed image portions were available for the entire field of view. CombineZP image stacking software (Alan Hadley; alan@micropics.org.uk) was used to automatically stitch together focussed portions of frames for each leaf section, using the ‘pyramid weighted average’ method. As multiple images were obtained per section (10 frames per stack), two leaf portions were analysed (lamina and mid-vein) with eight replicates for 21 species, a total of 3360 images were used in the present study.

### Image analysis

Digimizer software (v.4.6.1; MedCalc Software Ltd., Ostend, Belgium) was used to quantify the relative cross-sectional area of each tissue type in each transverse section (i.e., for each digital image, the perimeter of each tissue type was traced by hand and then measured in units of square pixels, or px<sup>2</sup>), for: 1). an area of leaf lamina, and 2). an area encompassing (and limited to) the main leaf vein portion (see Fig. 3 for details and examples). The mid-vein portion was identified based on the presence of the largest vascular bundle within the leaf, and delimited by placing a rectangular mask over the section (*sensu* Fig. 3C), within which the relative extent of different tissue types was determined. Tissue type classes were: epidermis (adaxial plus abaxial epidermis, including cuticle), mechanical (extraxylary schlerenchyma plus collenchyma), fibro-vascular (vascular tissues plus associated xylary schlerenchyma and collenchyma), chlorenchyma (chlorophyllous mesophyll parenchyma) and intercellular airspace (ICAS). The mean ( $\pm$  S.E.) proportion of leaf sections comprised of each tissue type was calculated for lamina and for mid-vein portions.

### Data analysis

All data (CSR scores and tissue proportions) were log transformed as a standard means of normalising data of disparate types prior to multivariate analysis (principal components analysis; PCA) and regression analysis. PCA was performed using Multi-Variate Statistical Package (MVSP v.3.13o; Kovach Computing Services, Anglesey, Wales), Pearson’s correlations matrices were produced using SYSTAT 12 (Systat Software, Chicago, IL, USA), and linear regressions and associated analysis of variance (ANOVA) were performed with SigmaPlot 10 (Systat Software).

## Results

Examples of leaf sections used in the study are presented in Fig. 3. The main multivariate analysis, a PCA of interspecific variation in CSR strategy and the extent of tissue types, is presented in Fig. 4. With regard to the lamina portion of the leaf (Fig. 4A), the first two axes of variability (PCA1 and 2) accounted for 86.5% of variability in the dataset, and represented a main axis of variability in leaf economics, positively correlated with S-selection (Pearson's correlation coefficient ( $r$ ) = 0.491) and negatively with R-selection ( $r$  = -0.492). This was associated with variation between the extent of mechanical and fibro-vascular tissues (positively along PCA1) and the extent of intercellular airspace, epidermis and chlorenchyma (negatively). PCA2 was associated again with S- and R-selection (positively and negatively, respectively) but with variation mainly in intercellular airspace (with S-selection) and the extent of epidermal tissues (with R-selection). A secondary trade-off (PCA2; Fig. 4A) involved intercellular airspace (positively) vs. chlorenchyma/epidermis (negatively), associated with S- to R-selection, respectively.

For the midvein portion, PCA axes 1 and 2 accounted for 91.6% of the variability in the dataset (Fig. 4B). PCA1 was an axis of size characterized by variation in greater mechanical tissues and less intercellular airspace (associated with C-selection;  $r$  = 0.518), compared to smaller-leaved (R-selected) species with greater airspace and epidermis, and fewer mechanical tissues in the midvein portion (Fig. 4B). As with the lamina portion, a secondary trade-off involving intercellular airspace (positively) vs. chlorenchyma/epidermis (negatively), associated with S- to R-selection (respectively), was also evident for the midvein portion (PCA2; Fig. 4B). Thus, lamina and midvein portions differed, the main trade-off in the lamina being one of economics (and R- to S-selection) and the main trade-off in the midvein portion reflecting the degree of mechanical support vs. physiological function, and C-selection. Notably, the secondary trade-off (PCA2) was between internal space for gas diffusion vs. physiologically active cells.

The trade-offs evident in the multivariate analysis were also evident as single correlations between pairs of characters (Fig. 5). These single correlations were highly variable but statistically significant. For instance, the positive correlation between the extent of mechanical tissues in the leaf lamina and the extent of S-selection was highly variable ( $R^2=0.271$ ) but with a high degree of confidence that an increase in S-selection is associated with more extensive

mechanical tissues ( $p=0.015$ ; Fig. 5A). Thus, while it is possible to state that these relationships exist, a great deal of caution should be exercised if attempting to use values of one parameter to directly predict or calculate values of another.

## Discussion

The results support the expectation that differential investment between tissue types is apparent between CSR strategies. As hypothesized, and supported by Figs. 4 & 5, S-selection is associated with relatively tough lamina portions (i.e., with extensive extra-xylary mechanical tissue and fibro-vascular tissue). This is in agreement with Grime's (1974) suggestion that the leaves of stress-tolerators are inherently tough and well defended against herbivory ('constitutive defence'; see Pierce et al. 2005). R-selection is associated with soft leaves (i.e. fewer mechanical tissues) and extensive mesophyll, both in the lamina and midvein portions. This represents an adaptation to extremely rapid growth without much capacity to support the leaf, either in terms of constitutive defence or physical support to allow overtopping of competing plants. C-selection is not associated with any particular investment trade-off in the lamina portion (it exhibits extremely weak correlations with axes of variability; Fig. 4), but in the midvein region it is characterised by extensive investment in fibro-vascular and mechanical tissues which trade-off against photosynthetic tissues (intercellular airspace as the pathway between stomata and chlorenchyma cells and thus chloroplasts, chlorenchyma as the site of photosynthetic CO<sub>2</sub> fixation, and epidermis). This reflects the adaptation of C-selected species to produce large leaves that must be mechanically well supported, but also include regions of thin, parenchymatous lamina to facilitate gas exchange and light acquisition, with different portions of the leaf optimised for these photosynthetic and supportive functions.

Thus, the tissues of the lamina portion exhibit a trade-off involving 'soft' to 'hard' leaves and R- to S-selection, but not C-selection. This suggests that the lamina portion is governed by resource economics (i.e. acquisitive to conservative physiology, respectively). In contrast, the midvein exhibited a strong gradient of mechanical and fibro-vascular tissues associated with C-selection, suggesting that the leaf size spectrum is specifically associated with variation in supporting veins and is governed by the development mainly of the midvein region. Despite attempts to understand how leaf size and particularly venation traits could be linked to the leaf economics spectrum (e.g. Sack et al. 2013), these results suggest a degree of separation between

‘metabolic’ and ‘support’ functions, and that Li et al.’s (2017) functional modules (i.e. the light capture module, the water-nutrient flow module and the gas exchange module) are a realistic way of conceptualising leaves. Li et al. (2017) proposed this system because different species can share a similar position along the leaf economics spectrum but differ in particular traits, and functional modules provide an alternative point of view for considering the evolution of leaf functioning that can account for internal anatomical differences, rather than the ‘whole leaf’ measurements typically used to investigate the leaf economics spectrum. In other words, different internal functions and tissues are subject to different natural selection pressures. Our results suggest that both leaf economics and leaf functional module approaches are useful: leaves are comprised of functional modules which are more (or less) involved in managing resource economics. We should add that in addition to Li et al.’s (2017) three modules our suggestion (see Introduction) that it may be useful to add a specific ‘structural’ module delimited by mechanical tissues found some support from the fact that the main trade-off seen in the lamina was between mechanical tissues and intercellular airspace (much less so for chlorenchyma; Fig. 4). Indeed, Li et al. (2017) restricted their considerations to the lamina portion of a typical dicotyledonous dorsoventral leaf (i.e. with distinct palisade and spongy chlorenchymas), rather than the leaf as a whole (i.e. including midvein and petiole regions). Further complications could arise from the fact that most monocots exhibit isobilateral leaves, the internal differences being more between chlorenchyma externally *vs.* hydrenchyma or aerenchyma internally. Rather than Li et al.’s (2017) ‘water-nutrient flow’ module, we found that mechanical tissues co-varied with fibro-vascular tissues (Fig. 4), suggesting that it would be more precise to state that mechanical and vascular tissues together form a ‘physiological and physical support’ module. Indeed, because xylem elements are necessarily rigid (to maintain form and function despite internal pressure changes), xylem has always had a structural support role and a strong influence on the evolution of vascular plant architectural traits. Mechanical tissues often add their support directly next to vascular tissues (but not in the case of ‘extra-xylary’ mechanical tissues such as most collenchyma and some fibre bundles), and it is evident that ‘water-nutrient flow’ is intimately linked to physical support. Certainly, for C-selected species, the ‘support module’ represents one of the principle axes of adaptation, underpinning the size spectrum of Díaz et al. (2016) and the leaf venation/size relationships that are evident in the global flora (Sack & Scoffoni 2013). Ultimately, these results confirm that ecological strategy spectra do have a basis in differential investment at the level of tissues – something that has largely been assumed (e.g., Pierce et al. 2005; Grime and Pierce 2012) rather than directly investigated.

As a potential complication, note that the single correlations between CSR strategy scores and the extent of tissue types (Fig. 5) are statistically significant but highly variable. This variability can be interpreted in two ways: that the integration of multiple traits is more crucial to plant functioning than any single trait (Guo et al. 2018), or that this variability simply represents limitations to the dataset. Indeed, the analysis is based on ‘hard’ traits (Hodgson et al. 1999), i.e., traits representing a fundamental property of plant functioning but that are labour intensive to acquire. In this case, sample preparation, microscopy and image analysis represent three sequential time-consuming processes prior to data analysis, leading to a compromise regarding the number of species that can be included considering the human resources available. Additionally, C-selected species, by definition, exclude other species, leading to low species richness in competitor-dominated plant communities (Cerabolini et al. 2016). This means that while a plant community dominated by stress-tolerators or ruderals may provide various examples of S- or R-selected species, it is relatively difficult to encounter strongly C-selected species, limiting the choice of species that can be collected and used. Indeed, in the present study species do not represent the extremes of C- and S-selection as two simple groups, rather they represent a gradient from strongly S-selected to strongly C-selected species, with variation between these extremes (Fig. 1). This is sufficient, as results of the multivariate analysis are statistically significant and support the hypotheses, but a well-funded study with sufficient resources could undoubtedly investigate a much broader range of species, reducing variability in the dataset and improving precision.

In conclusion, our data confirm the expectation that ecological strategy variation has a basis in underlying trade-offs between tissues with contrasting metabolic and structural roles.

## References

- Baltieri M, Fantinato E, Del Vecchio S, Buffa G (2020) Intraspecific variability of leaf traits and functional strategy of *Himantoglossum adriaticum* H. Baumann. *Plant Sociol* 57(2):105-112.
- Behroozian M, Ejtehadi H, Memariani F, Pierce S, Mesdaghi M (2020) Are endemic species necessarily ecological specialists? Functional variability and niche differentiation of two threatened *Dianthus* species in the montane steppes of northeastern Iran. *Sci Rep* 10:11774. <https://doi.org/10.1038/s41598-020-68618-7>.

- Belluau M, Shipley B (2018) Linking hard and soft traits: Physiology, morphology and anatomy interact to determine habitat affinities to soil water availability in herbaceous dicots. *PLoS ONE* 13(3): e0193130. <https://doi.org/10.1371/journal.pone.0193130>.
- Bricca A, Di Musciano M, Ferrara A, Theurillat J-P, Cutini M (2022) Community assembly along climatic gradient: contrasting pattern between- and within- species. *Perspectives in Plant Ecology, Evolution and Systematics* 56:125675.
- Cerabolini BEL, Brusa G, Ceriani RM, De Andreis R, Luzzaro A, Pierce S (2010) Can CSR classification be generally applied outside Britain? *Plant Ecol* 210:253-261. <https://doi.org/10.1007/s11258-010-9753-6>.
- Cerabolini BEL, Pierce S, Verginella A, Brusa G, Ceriani RM, Armiraglio S (2016) Why are many anthropogenic agroecosystems particularly species-rich? *Plant Biosyst* 150(3): 550-557. doi: 10.1080/11263504.2014.987848
- Chave J, Coomes D, Jansen S, Lewis SL, Swenson NG, Zanne AE (2009) Towards a worldwide wood economics spectrum. *Ecol Lett* 12:351-366.
- Dalle Fratte M, Brusa G, Pierce S, Zanzottera M, Cerabolini BEL (2019a) Plant trait variation along environmental indicators to infer global change impacts. *Flora* 254:113-121. <https://doi.org/10.1016/j.flora.2018.12.004>.
- Dalle Fratte M, Bolpagni R, Brusa G, Caccianiga M, Pierce S, Zanzottera M, Cerabolini BEL (2019b) Alien plant species invade by occupying similar functional species to native species. *Flora* 257:151419.
- Dayrell RLC, Arruda AJ, Pierce S, Negreiros D, Meyer PB, Lambers H, Silveira FAO (2018) Ontogenetic shifts in plant ecological strategies. *Funct Ecol* 32:2730-2741. <https://doi.org/10.1111/1365-2435.13221>.
- de la Riva EG, Olmo M, Poorter H, Ubera JL, Villar R (2016) Leaf Mass per Area (LMA) and its relationship with leaf structure and anatomy in 34 Mediterranean woody species along a water availability gradient. *PLoS ONE* 11(2): e0148788. doi:10.1371/journal.pone.0148788.
- de la Riva EG, Querejeta JI, Villar R, Pérez-Ramos IM, Marañón T, Galán Díaz J, de Tomás Marín S, Prieto I (2021) The economics spectrum drives root trait strategies in mediterranean vegetation. *Front Plant Sci* 12:773118. doi: 10.3389/fpls.2021.773118
- Díaz S, Kattge J, Cornelissen JHC, Wright IJ, Lavorel S, Dray S, Reu B, Kleyer M, Wirth C, Prentice IC, Garnier E, Bönisch G, Westoby M, Poorter H, Reich PB, Moles AT, Dickie J, Gillison AN, Zanne AE, Chave J, Wright SJ, Sheremet'ev SN, Jactel H, Baraloto C, Cerabolini B, Pierce S, Shipley B, Kirkup D, Casanoves F, Joswig JS, Günther A,

- Falczuk V, Rüger N, Mahecha MD, Gorné LD (2016) The global spectrum of plant form and function. *Nature* 529:167–171. <http://doi.org/10.1038/nature16489>.
- Dudova KV, Dzhatdоеva TM, Dudov SV, Akhmetzhanova AA, Tekeev DK, Onipchenko VG (2019) Competitive strategy of subalpine tall-grass species of the northwestern Caucasus. *Moscow University Biological Sciences Bulletin* 74(3):140-146.
- Escobedo VM, Rios RS, Gianoli E (2021) Interactive effects of shading and disturbance on plant invasion in an arid shrubland: assembly processes and CSR-strategies. *J Ecol* 109:2405-2420.
- Fernandes GW, Tameirão LBS, Costa AR, Ribeiro DG, Neves MN, Souza-e-Brito BG, Negreiros D (2021) Functional traits of three major invasive grasses in a threatened tropical mountain grassland. *Anals da Academia Brasileira de Ciências* 93(2):e20200119.
- Ferré C, Caccianiga M, Zanzottera M, Comolli R (2020) Soil-plant interactions in a pasture of the Italian Alps. *J Plant Interact* 15(1):39-49.
- Fletcher J, Willby N, Oliver DM, Quilliam RS (2022) Resource recovery and freshwater ecosystem restoration - prospecting for phytoremediation potential in wild macrophyte stands. *Resources, Environment and Sustainability* 7:100050.
- Freschet GT, Cornelissen JHC, van Logtestijn RSP, Aerts R (2010) Evidence of the ‘plant economics spectrum’ in a subarctic flora. *J Ecol* 98:362-373.
- Giupponi L (2020) Intraspecific variation in functional strategy and leaf shape of *Campanula elatinoidea* reveals adaptation to climate. *Flora* 268:151605.
- Guo C, Ma L, Yuan S, Wang R (2017) Morphological, physiological and anatomical traits of plant functional types in temperate grasslands along a large-scale aridity gradient in northeastern China. *Sci Rep* 7:40900. <https://doi.org/10.1038/srep40900>
- Guo WY, van Kleunen M, Winter M, Weigelt P, Stein A, Pierce S, Pergl J, Moser D, Maurel N, Lenzner B, Kreft H, Essl F, Dawson W, Pyšek P (2018) The role of adaptive strategies in plant naturalization. *Ecol Lett* 21:1380–1389. <https://doi.org/10.1111/ele.13104>.
- Grime JP (1974) Vegetation classification by reference to strategies. *Nature* 250(5461):26-31. <https://doi.org/10.1038/250026a0>.
- Grime JP, Thompson K, Hunt R, Hodgson JG, Cornelissen JHC, Rorison IH, Hendry GAF, Ashenden TW, Askew AP, Band SR, Booth RE, Bossard CC, Campbell BD, Cooper JEL, Davison AW, Gupta PL, Hall W, Hand DW, Hannah MA, Hillier SH, Hodgkinson DJ, Jalili A, Liu Z, Mackey JML, Matthews N, Mowforth MA, Neal AM, Reader RJ, Reiling K, Ross-Fraser W, Spencer RE, Sutton F, Tasker DE, Thorpe PC, Whitehouse J (1997)

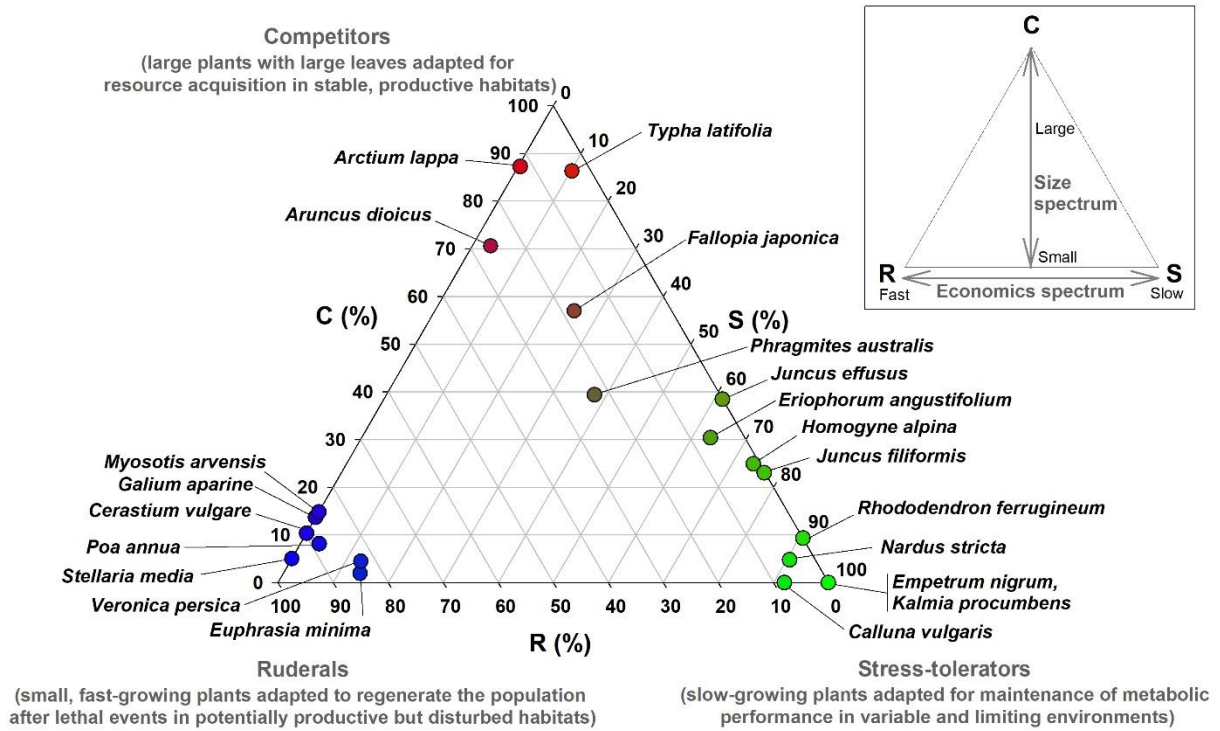
- Integrated screening validates primary axes of specialisation in plants. *Oikos* 79(2):259-281. <https://doi.org/10.2307/3546011>.
- Grime JP, Mackey JML (2002) The role of plasticity in resource capture by plants. *Evol Ecol* 16(3):299-307. <https://doi.org/10.1023/A:1019640813676>.
- Grime JP, Pierce S (2012) *The Evolutionary Strategies that Shape Ecosystems*. Wiley-Blackwell, Chichester, UK. pp. 264. ISBN: 978-0470674819
- Grubb PJ, Marañón T, Pugnaire FI, Sack L (2015) Relationships between specific leaf area and leaf composition in succulent and non-succulent species of contrasting semi-desert communities in south-eastern Spain. *J Arid Env* 118:69-83.
- Han X, Huang J, Yao J, Xu Y, Ding Y, Zang R (2021) Effects of logging on the ecological strategy spectrum of a tropical montane rain forest. *Ecol Indic* 128: 107812.
- Han X, Huang J, Zang R (2022) Soil nutrients and climate seasonality drive differentiation of ecological strategies of species in forests across four climatic zones. *Plant Soil* 473:517–531.
- Hassiotou F, Renton M, Ludwig M, Evans JR, Veneklaas EJ (2010) Photosynthesis at an extreme end of the leaf trait spectrum: how does it relate to high leaf dry mass per area and associated structural parameters? *J Exp Bot* 61(11):3015-28. doi:10.1093/jxb/erq128.
- Hodgson JG, Wilson PJ, Hunt R, Grime JP, Thompson K (1999) Allocating C-S-R plant functional types: a soft approach to a hard problem. *Oikos* 85(2):282–294. <https://doi.org/10.2307/3546494>.
- Hooftman D, Kimberley A, Cousins, SAO, Escibano-Avila G, Honnay O, Krickl P, Plue J, Poschlod P, Traveset A, Bullock JM (2021) Dispersal limitation, eutrophication and propagule pressure constrain the conservation value of grassland green infrastructure. *Biol Conserv* 258:109152.
- John GP, Scoffoni C, Buckley TN, Villar R, Poorter H, Sack L (2017) The anatomical and compositional basis of leaf mass per area. *Ecol Lett* 20: 412–425. doi: 10.1111/ele.12739
- Lazzaro L, Colzi I, Ciampi D, Gonnelli C, Lastrucci L, Bazihizina N, Viciani D, Coppi A (2021) Intraspecific trait variability and genetic diversity in the adaptive strategies of serpentine and non-serpentine populations of *Silene paradoxa* L. *Plant Soil* 460:105-121.
- Li L, Ma Z, Niinemets Ü, Guo D (2017) Three key sub-leaf modules and the diversity of leaf designs. *Front Plant Sci* 8:1542. doi: 10.3389/fpls.2017.01542.
- Liao H, Li D, Zhou T, Huang B, Zhang H, Chen B, Peng S (2021) The role of functional strategies in global plant distribution. *Ecography* 44:493-503.



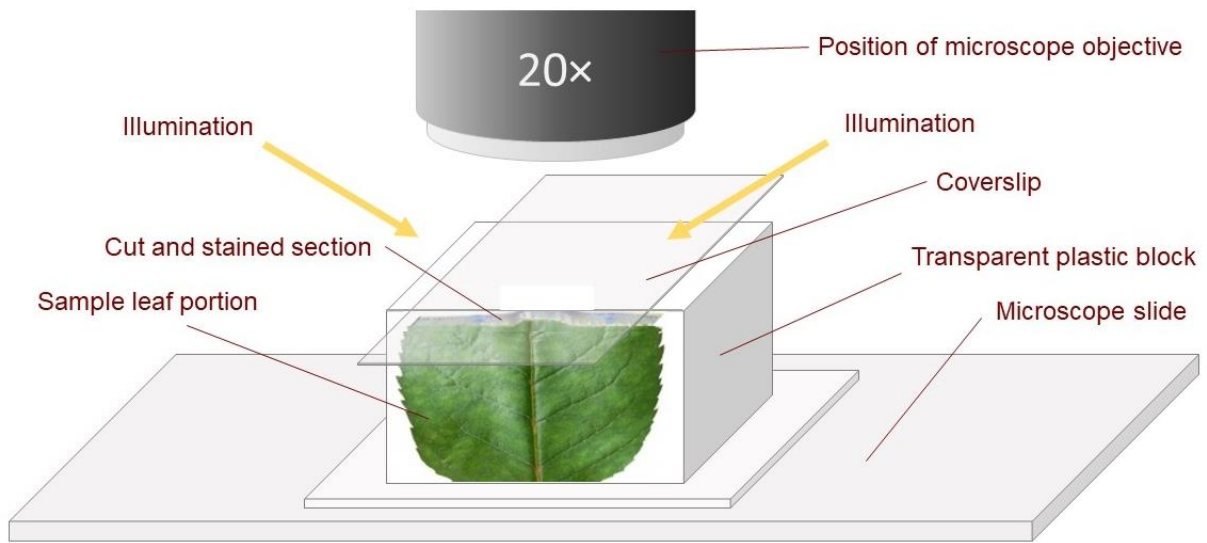
- Liu X, Chen H, Sun T, Li D, Wang X, Mo W, Wang R, Zhang S (2021) Variation in woody leaf anatomical traits along the altitudinal gradient in Taibai Mountain, China. *Global Ecol Conserv* 26: e01523. <https://doi.org/10.1016/j.gecco.2021.e01523>.
- Lux A, Morita S, Abe J, Ito K (2005) An improved method for clearing and staining free-hand sections and whole-mount samples. *Ann Bot* 96: 989-996.
- Mugnai M, Frasconi Wendt C, Balzani P, Ferretti G, Dal Cin M, Masoni A, Frizzi F, Santini G, Viciani D, Foggi B, Lazzaro L (2021) Small-scale drivers on plant and ant diversity in a grassland habitat through a multifaceted approach. *PeerJ* 9: e12517.
- Niinemets Ü (2001) Global-scale climatic controls of leaf dry mass per area, density, and thickness in trees and shrubs. *Ecol* 82(2):453-469.
- One Thousand Plant Transcriptomes Initiative (2019) One thousand plant transcriptomes and the phylogenomics of green plants. *Nature* 574:679–685.
- Onoda Y, Wright IJ, Evans JR, Hikosaka K, Kitajima K, Niinemets Ü, Poorter H, Tosens T, Westoby M (2017) Physiological and structural tradeoffs underlying the leaf economics spectrum. *New Phytol* 214:1447-1463.
- Pierce S, Cerabolini BEL (2018) Plant economics and size spectra are both explained by one theory. Milan, The Plant Press.  
<http://www.researchgate.net/publication/326069336> Plant economics and size trait spectra are both explained by one theory. Accessed 25/01/2022
- Pierce S, Fridley JD (2021) John Philip Grime 30 April 1935 — 19 April 2021. *Biogr Mem Fell R Soc* 71:249-270.
- Pierce S, Ceriani RM, De Andreis R, Luzzaro A, Cerabolini B (2007) The leaf economics spectrum of Poaceae reflects variation in survival strategies. *Plant Biosyst* 141(3): 337-343.
- Pierce S, Brusa G, Sartori M, Cerabolini BEL (2012) Combined use of leaf size and economics traits allows direct comparison of hydrophyte and terrestrial herbaceous adaptive strategies. *Ann Bot* 109(5):1047-1053. <https://doi.org/10.1093/aob/mcs021>.
- Pierce S, Brusa G, Vagge I, Cerabolini BEL (2013) Allocating CSR plant functional types: the use of leaf economics and size traits to classify woody and herbaceous vascular plants. *Funct Ecol* 27(4):1002-1010. <https://doi.org/10.1111/1365-2435.12095>.
- Pierce S, Negreiros D, Cerabolini BEL, Kattge J, Díaz S, Kleyer M, Shipley B, Wright SJ, Soudzilovskaia NA, Onipchenko VG, van Bodegom PM, Frenette-Dussault C, Weiher E, Pinho BX, Cornelissen JHC, Grime JP, Thompson K, Hunt R, Wilson PJ, Buffa G, Nyakunga OC, Reich PB, Caccianiga M, Mangili F, Ceriani RM, Luzzaro A, Brusa G,

- Siefert A, Barbosa NPU, Chapin III FS, Cornwell WK, Fang J, Fernandes GW, Garnier E, Le Stradic S, Peñuelas J, Melo FPL, Slaviero A, Tabarelli M, Tampucci D (2017) A global method for calculating plant CSR ecological strategies applied across biomes worldwide. *Funct Ecol* 31(2):444-457. <https://doi.org/10.1111/1365-2435.12722>.
- Pierce S, Vagge I, Brusa G, Cerabolini BEL (2014) The intimacy between sexual traits and Grime's CSR strategies for orchids coexisting in semi-natural calcareous grassland at the Olive Lawn. *Plant Ecol* 215:495-505. <https://doi.org/10.1007/s11258-014-0318-y>.
- Pierce S, Vianelli A, Cerabolini B (2005) From ancient genes to modern communities: the cellular stress response and the evolution of plant strategies. *Funct Ecol* 19(5): 763-776.
- Pinho BX, Tabarelli M, Arroyo-Rodríguez V, Engelbrecht BMJ, Pierce S, Hietz P, Müller S, Lohbeck M, Bongers F, Niinemets Ü, Santos BA, Wright IJ, Slot M, Benchimol M, Swenson N, de Lima RAF, Pillar VD, Slik F, Jansen S, Peres CA, Barlow J, Mendes G, Hernández-Ruedas MA, Martínez-Ramos M, Faria D, Kraft N, Chacon E, Júnior V, Mariano-Neto E, Kattge J, Melo FPL (2020) Functional biogeography of Neotropical rainforests: trait-climate relationships and assembly patterns of tree communities. *Glob Ecol Biogeogr* 30(7):1430-1446. <https://doi.org/10.1111/geb.13309>
- Reich PB (2014) The world-wide 'fast-slow' plant economics spectrum: a traits manifesto. *J Ecol* 102:275–301. <https://doi.org/10.1111/1365-2745.12211>.
- Ruzin SE (1999) *Plant Microtechnique and Microscopy*. New York: Oxford University Press.
- Sack L, Frole K (2006) Leaf structural diversity is related to hydraulic capacity in tropical rainforest trees. *Ecol* 87(2):483-491.
- Sack L, Scoffoni C (2013) Leaf venation: structure, function, development, evolution, ecology and applications in the past, present and future. *New Phytol* 198(4):983-1000.
- Sack L, Scoffoni C, John GP, Poorter H, Mason CM, Mendez-Alonzo R, Donovan LA (2013) How do leaf veins influence the worldwide leaf economics spectrum? Review and synthesis. *J Exp Bot* 64(13):4053-4080.
- Seele-Dilbat C, Kretz L, Wirth C (2022) Vegetation of natural and stabilized riverbanks and early effects of removal of bank fixation. *Int Rev Hydrobiol* 107:88–99.
- Shipley B, Lechowicz MJ, Wright I, Reich PB (2006) Fundamental trade-offs generating the worldwide leaf economics spectrum. *Ecol* 87(3):535-541.
- Tameirão LBS, Paiva DC, Negreiros DC, Veloso MDM, Berbara RLL, Dias LE, Pierce S, Fernandes GW (2021) Role of environmental filtering and functional traits for species coexistence in a harsh tropical montane ecosystem. *Biol J Linn Soc* 133:546–560.

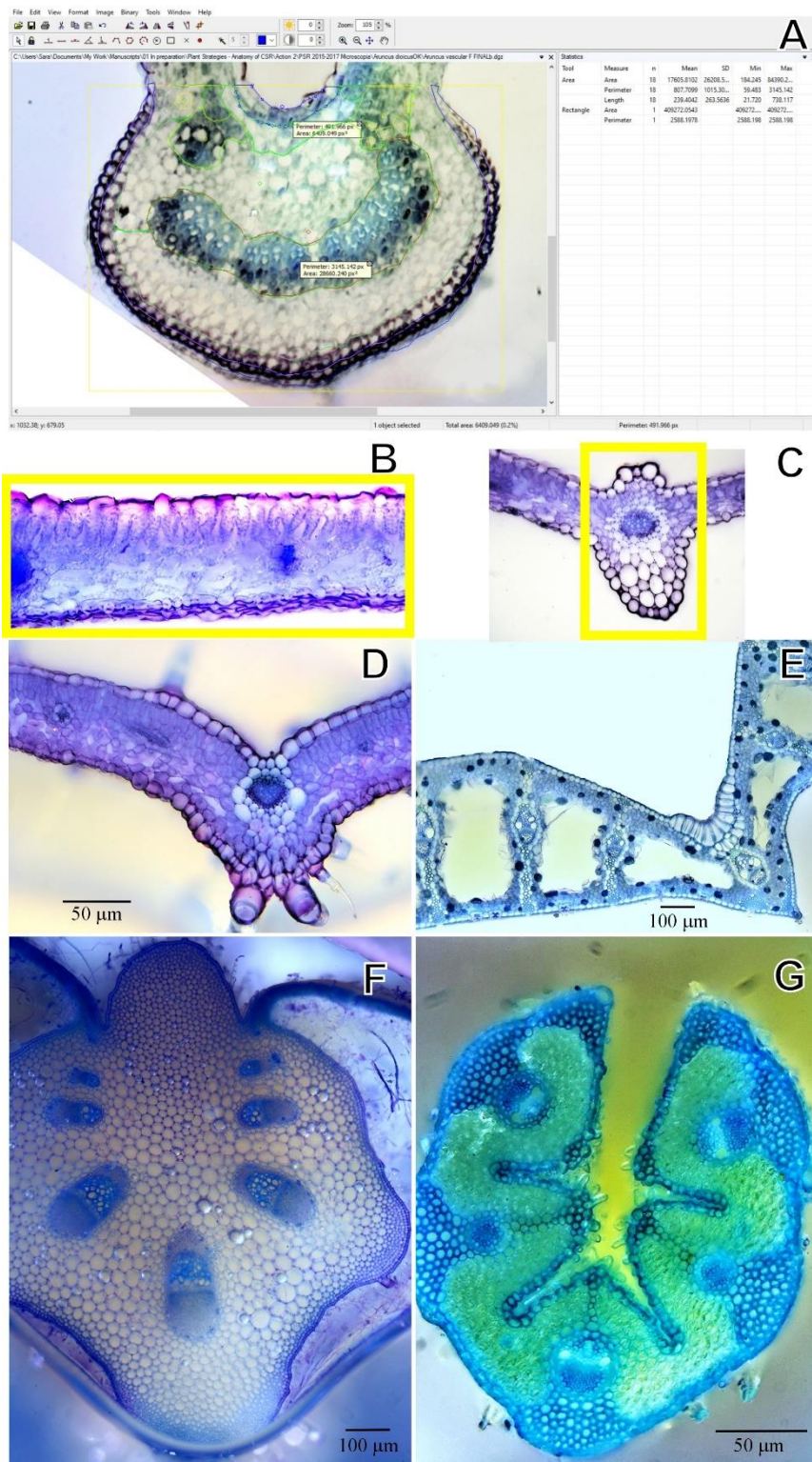
- Tian M, Yu G, He N, Hou J (2016) Leaf morphological and anatomical traits from tropical to temperate coniferous forests: mechanisms and influencing factors. *Sci Rep* 6:19703.
- Vasseur F, Sartori K, Baron E, Fort F, Kazakou E, Segrestin J, Garnier E, Vile D, Violle C (2018) Climate as a driver of adaptive variations in ecological strategies in *Arabidopsis thaliana*. *Ann Bot* 122:935-945.
- Verbeeck H, Bauters M, Jackson T, Shenkin A, Disney M, Calders K (2019) Time for a Plant Structural Economics Spectrum. *Front For Glob Change* 2:43. doi: 10.3389/ffgc.2019.00043
- Villar R, Ruiz-Robledo J, Uberta JL, Poorter H (2013) Exploring variation in leaf mass per area (LMA) from leaf to cell: an anatomical analysis of 26 woody species. *Am J Bot* 100:1969–1980. doi: 10.3732/ajb.1200562
- Watkins H, Hirons A, Sjöman H, Cameron R, Hitchmough JD (2021) Can trait-based schemes be used to select species in urban forestry? *Frontiers in Sustainable Cities* 3:Art.654618.
- Wright IJ, Reich PB, Westoby M, Ackerley DD, Baruch Z, Bongers F, Cavender-Bares J, Chapin T, Cornelissen JHC, Diemer M, Flexas J, Garnier E, Groom PK, Gulias J, Hikosaka K, Lamont BB, Lee T, Lee W, Lusk C, Midgley JJ, Navas M-L, Niinemets Ü, Oleksyn J, Osada N, Poorter H, Poot P, Prior L, Pyankov VI, Roumet C, Thomas SC, Tjoelker MG, Veneklaas EJ, Villar R (2004) The worldwide leaf economics spectrum. *Nature* 428:821-827. <https://doi.org/10.1038/nature02403>.
- Wright IJ, Dong N, Maire V, Prentice IC, Westoby M, Díaz S, Gallagher RV, Jacobs BF, Kooyman R, Law EA, Leishman MR, Niinemets Ü, Reich PB, Sack L, Villar R, Wang H, Wilf P (2017) Global climatic drivers of leaf size. *Science* 357(6354):917-921.
- Ye M, Zhang Z, Huang G, Xiong Z, Peng S, Li Y (2020) High leaf mass per area *Oryza* genotypes invest more leaf mass to cell wall and show a low mesophyll conductance. *AoB PLANTS* 12(4): plaa028. <https://doi.org/10.1093/aobpla/plaa028>.
- Yu J, Hou G, Zhou T, Shi P, Zong N, Sun J (2022) Variation of plant CSR strategies across a precipitation gradient in the alpine grasslands on the northern Tibet Plateau. *Science of the Total Environment* 838:156512.
- Zanzottera M, Dalle Fratte M, Caccianiga M, Pierce S, Cerabolini BEL (2020) Community-level variation in plant functional traits and ecological strategies shapes habitat structure along successional gradients in an alpine environment. *Community Ecol* 21:55-65. <https://doi.org/10.1007/s42974-020-00012-9>.
- Zhang X, Wang S (2021) Joint control of plant ecological strategy by climate, regeneration mode, and ontogeny in northeastern Chinese forests. *Ecol Evol* 11:6703-6715.



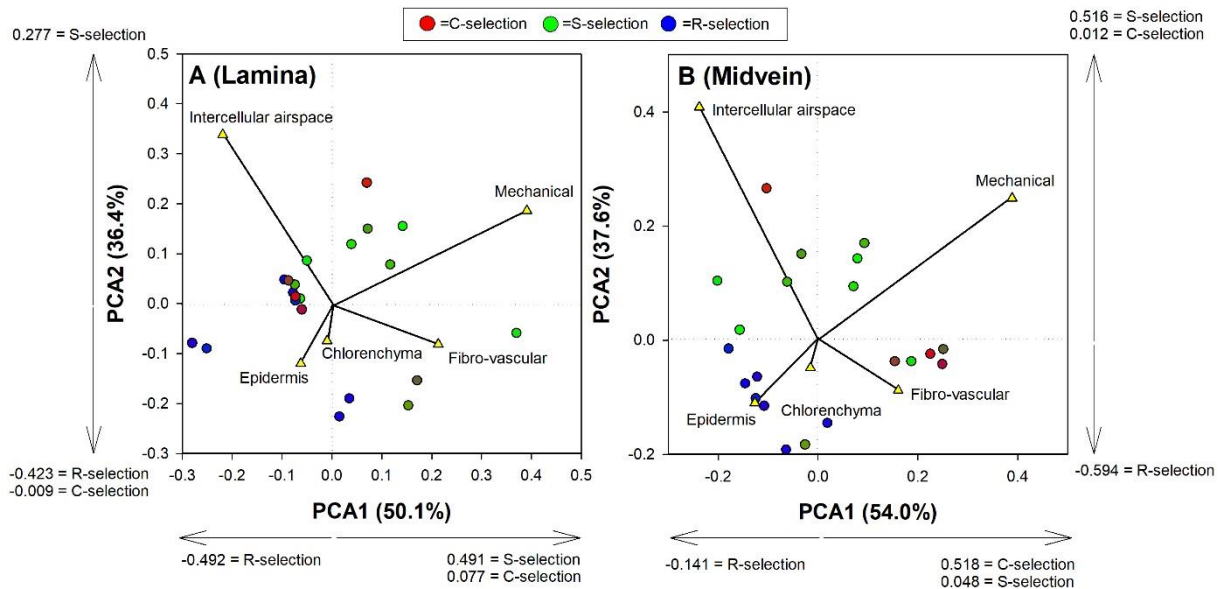
**Fig. 1** The CSR ecological strategies of the study species encompass the three extremes of Grime’s CSR triangle, as determined by the StrateFy CSR classification tool of Pierce et al. (2017). Inset shows the CSR triangle in the context of the global spectrum of plant form and function (Díaz et al. 2016), comprising the resource economics spectrum and the plant/organ size spectrum.



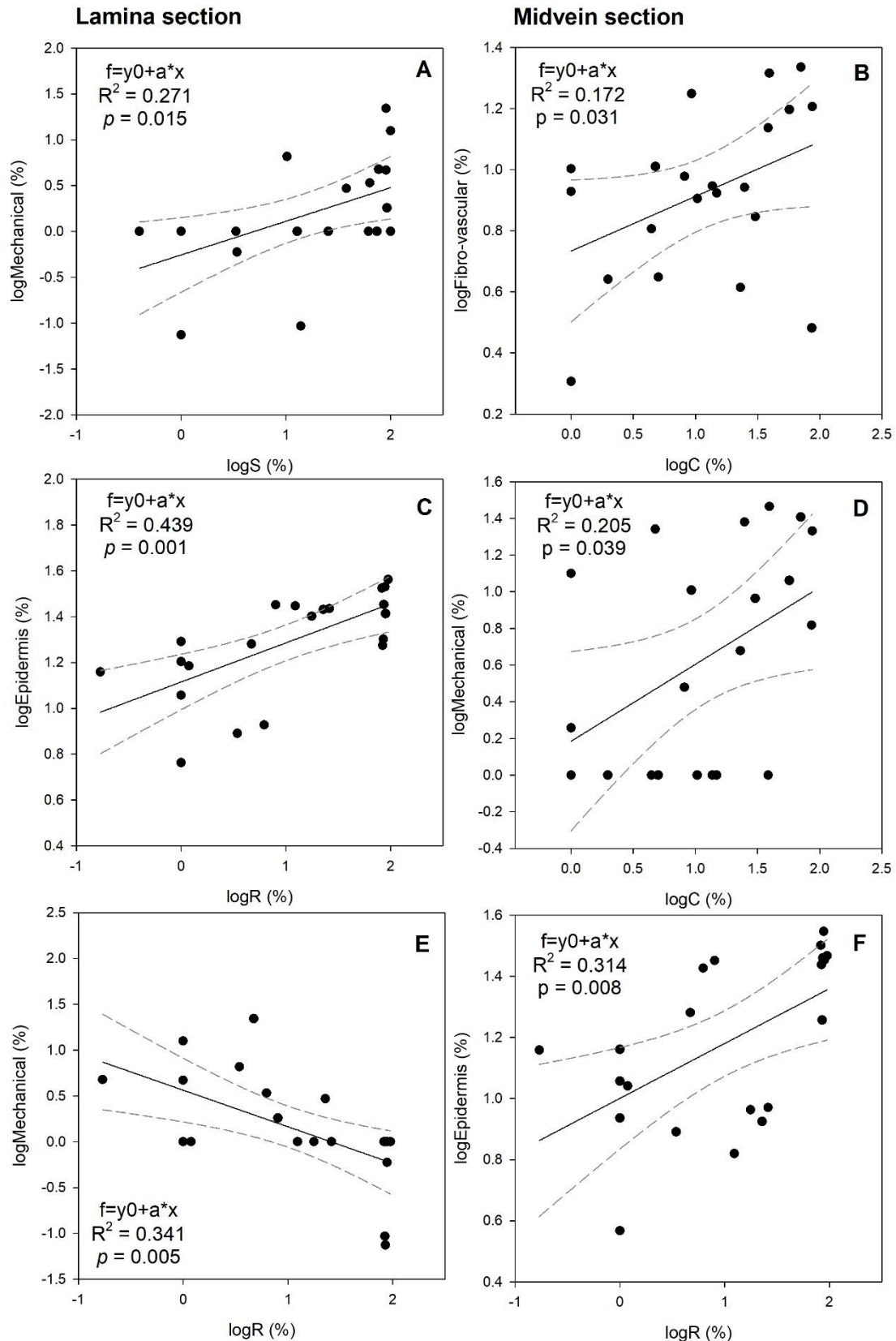
**Fig. 2** Explanatory illustration of the novel set up for the rapid yet precise processing of hand cut transverse leaf sections. For each species, replicate leaf portions ( $\sim 2 \times 0.5$  cm) excised from approx. half-way along the length of the leaf lamina were affixed with double-sided tape to the side of a transparent plastic block mounted on a microscope slide. The cut edge was stained with 0.2% toluidine blue O micro-pipetted onto the sectioned surface for 5-15 min followed by washing with distilled water and placing of a coverslip.



**Fig. 3** The use of Digimazer software to determine the relative cross-sectional area occupied by different tissues, in this case a transverse section of the midvein portion of a leaf of *Aruncus dioicus* (A). (B) and (C) show how lamina and midvein portions, respectively, were defined (i.e. the relative extent of tissue types was determined within the area delimited by the yellow box), for the example of *Galium aparine*. Further photomicrographs represent examples of transverse sections used in this study, and include (D). *Cerastium vulgare*, (E). *Eriophorum vaginatum*, (F). *Arctium lappa* (midvein section only), (G). *Nardus stricta*.



**Fig. 4** Principal Components Analysis (PCA) demonstrating the associations between leaf tissue types (the relative extent of epidermis, mesophyll chlorenchyma, mechanical, fibro-vascular and intercellular airspace) from transverse sections of the leaf lamina (A) and the midvein section (B) for a range of ecologically-contrasting species from northern Italy. Values along axes represent Pearson's correlation coefficients ( $r$ ) between C-, S- and R-selection and PCA axes (1.000 = perfect positive correlation, 0.000 = no correlation, -1.000 = perfect negative correlation).



**Fig. 5** Examples of statistically significant single correlations between C, S and R scores and the extent of different leaf tissues in lamina (A, C, E) and midvein (B, D, F) portions of leaf for species of contrasting ecological strategy. Solid line represents linear regression and dashed lines represent 95% confidence intervals (ANOVA).