

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

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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 understood, but little is known regarding the relationship between anatomical trade-offs and ecological strategies (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 mid-vein portion it reflects the mechanical constraints inherent to large leaves (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 spectrum was that of economics (S-R selection) characterised by a mechanical/ICAS vs. chlorenchyma/epidermis trade-off in the midvein, but contrary to expectations ICAS and chlorenchyma did not trade-off in the lamina. The secondary axis involved a trade-off between larger leaved competitors with extensive mechanical/fibrovacular tissue and small-leaved species (S and R-selected) with proportionally greater ICAS, chlorenchyma and epidermis. Our results confirm the expectation that ecological strategy variation has a basis in underlying trade-offs between tissues with contrasting physiological and structural roles.

Introduction

The 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 'leaf economics spectrum' or 'plant resource economics spectrum' (Wright et al. 2004; Reich 2014). 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).

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 structural trade-offs have been investigated only in the context of specific leaf economics traits, usually 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 CO₂ diffusion, limiting photosynthetic rates (see Ye et al. (2020) and references therein). It is also clear that the relative extent of tissue types comprising leaves varies with environmental factors, such as elevation (Liu et al. 2021). However internal anatomical variation has not been placed in the context of the wider 'global spectrum' trade-off between economics and size.

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 CO₂ 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?

Currently, the only 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). 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 CO₂ 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 in particular exhibit lamina and mid-vein anatomies optimised for contrasting functions, with the lamina exhibiting a greater relative extent of chlorenchyma and the midvein specialised in terms of mechanical and vascular tissues (the division of functions being less evident for S- and R-selected species).

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, wide phylogenetic coverage (i.e. representing major clades of both dicots and monocots). 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 $\sim 4^{\circ}\text{C}$ (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 \times or 20 \times 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 8 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^2), 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). 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), chlrenchyma (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.

Analysis of data

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.3.130; 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 71.8% of variability in the dataset, and represented a main axis of variability in leaf economics, the strongest Pearson's correlations (r) with PCA1 being with S and R selection ($r = -0.918$ to 0.927 , respectively). This was associated with variation between the extent of mechanical and fibro-vascular tissues (negative correlations, alongside S-selection) and the extent of epidermis and chlorenchyma (positive correlations, together with R-selection). PCA2 was an axis of size, between C-selection at one extreme and S- and R-selection at the other, and while variation in mechanical and fibro-vascular tissues was evident (i.e., a greater extent with increasing C-selection) these relationships were much weaker than for PCA1 (Fig. 4A).

For the mid-vein portion, PCA axes 1 and 2 accounted for 77.6% of the variability in the dataset (Fig. 4B). PCA1 was an axis of economics (S to R selection) characterized by variation in intercellular airspace and to a lesser extent epidermis and chlorenchyma, but PCA2 was an axis of size characterized by C-selected (large-leaved) species with greater mechanical tissues and less intercellular airspace, compared to smaller-leaved (S and R-selected) species with greater airspace, chlorenchyma and epidermis, and fewer mechanical tissues in the midvein portion (Fig. 4B). Thus lamina and midvein portions differed, but particularly for C-selected species, where a trade-off between photosynthetic (chlorenchyma and intercellular airspace) and supportive (mechanical and fibro-vascular) tissues was more pronounced.

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

Differential investment between tissue types is apparent between CSR strategies. As hypothesized, 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 leaf laminae and extensive mesophyll tissues, particularly in the midvein portion. 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, but in the midvein region it is characterised by extensive investment in fibro-vascular and mechanical tissues which trade-off against photosynthetic tissues (chlorenchyma as the site of photosynthetic CO_2 fixation, and intercellular airspace as the pathway between stomata and chlorenchyma cells and thus chloroplasts). 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.

The single correlations between CSR strategy scores and the extent of tissue types are statistically significant but highly variable. This 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 large numbers of 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.

However, the results largely confirm expectations and suggest that Li et al.'s (2017) functional modules are a conceptually important system for considering the internal functions underpinning macroscopic scale ecological strategies, at least at the level of the leaf. Indeed, the results demonstrate 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.

In terms of a mechanism underpinning this differential investment, the established field of carbon partitioning is undoubtedly of great relevance. Whereas functional ecology typically measures plant growth in terms of biomass accumulation (productivity) and the final investments of fixed carbon in the masses of different organs, carbon partitioning relies on a set of methodologies to investigate the temporal dynamics of carbon source-sink relationships and to calculate carbon budgets. This involves gas-exchange measurements, carbohydrate contents, gene upregulation and radio-isotope tracing of carbohydrate movement (e.g., Farrar 1996; Dilkes et al. 2004). This can discern the relatively instantaneous processes of carbon allocation, in terms of allocation to structures but also in terms of carbon forms and modification (polymerization/depolymerisation) for different roles (e.g., sucrose during transport, glucose for respiratory use, starch for storage). Investment in ephemeral and highly metabolically active leaves (R-selection) is likely to be associated with carbon partitioning based on instantaneous sucrose mobilization and conversion to glucose and fructose in sinks, rather than investment in long-chain polymers such as cell wall carbohydrates or storage polysaccharides. The division of photosynthetic vs. supportive functions in portions of C-selected leaves is likely to involve differential fluxes of carbon that could be quantified using a carbon partitioning framework. Integration of physiological data on carbon forms and fluxes could also include the chaperone sugars involved in protecting cellular components against structural damage as part of the cellular stress response (Pierce et al. 2005). Furthermore, it is likely that differential allocation involves other essential elements, such as macronutrients, which also participate in the trade-offs that determine plant strategies (Grime and Pierce 2012), and which can be quantified and investigated using similar techniques to those used for carbon partitioning. Finally, while carbon partitioning may seem an overly physiological approach in the context of broader plant ecology, it has been used to trace the movement of photosynthates between plants and pathogens, between hosts and parasites (particularly parasitic plants), the rhizosphere and mycorrhizae (see Pollock et al. 1992; recent examples include Shinde et al. 2018; Salmeron-Santiago et al. 2022). It is thus essential to the mechanics of many ecological relationships between primary producers and a range of organisms, and shows great promise for the mechanistic explanation of plant functional relationships in general, linking genetic expression, anatomical traits and macroscopic functional traits to wider aspects of plant ecology.

In conclusion, our data confirm the expectation that ecological strategy variation has a basis in underlying trade-offs between tissues with contrasting physiological and structural roles, which represent internal functional constraints to resource (carbon and mineral) deployment between tissue types.

Declarations

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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.

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Figures

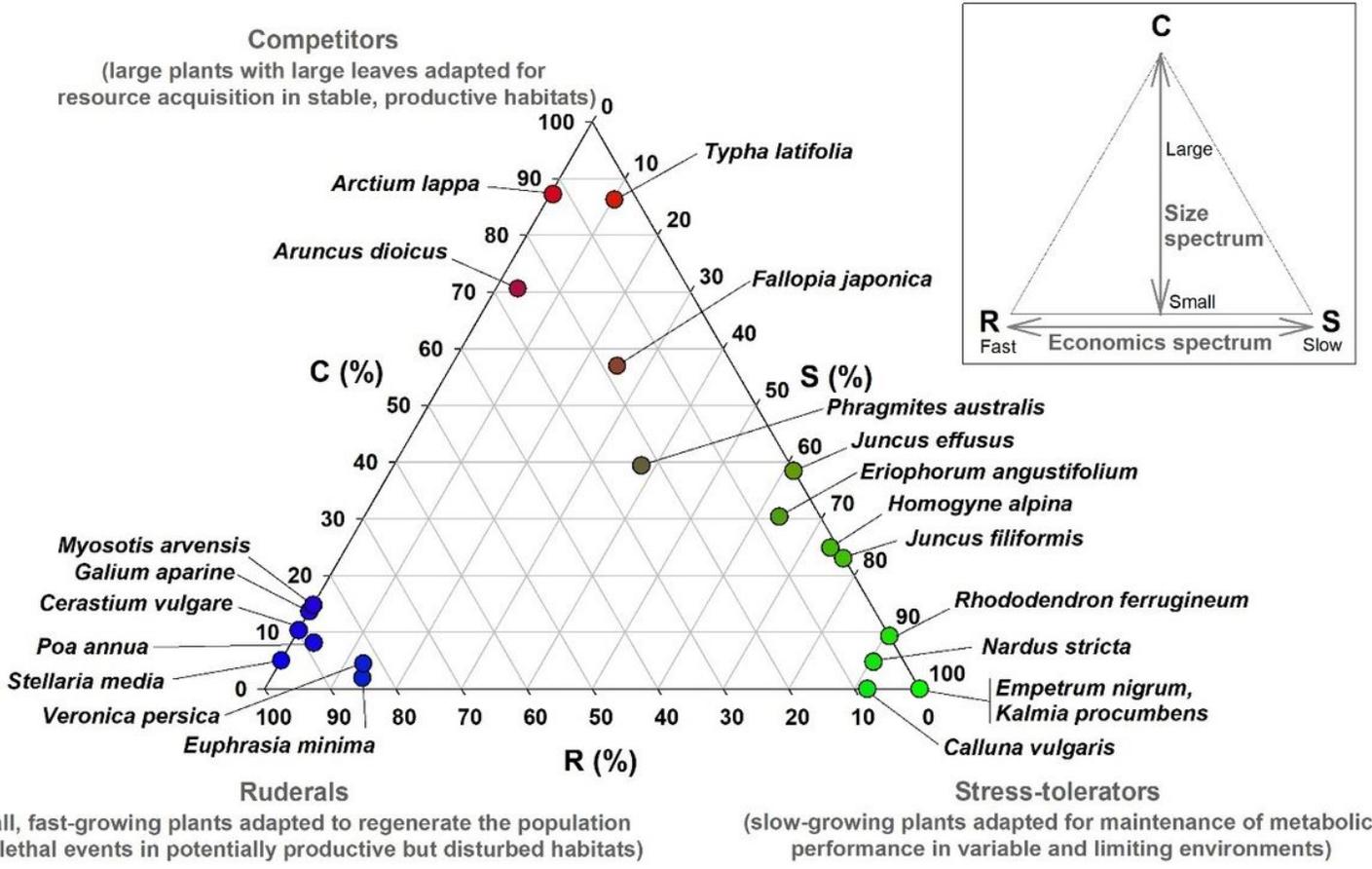


Figure 1

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.

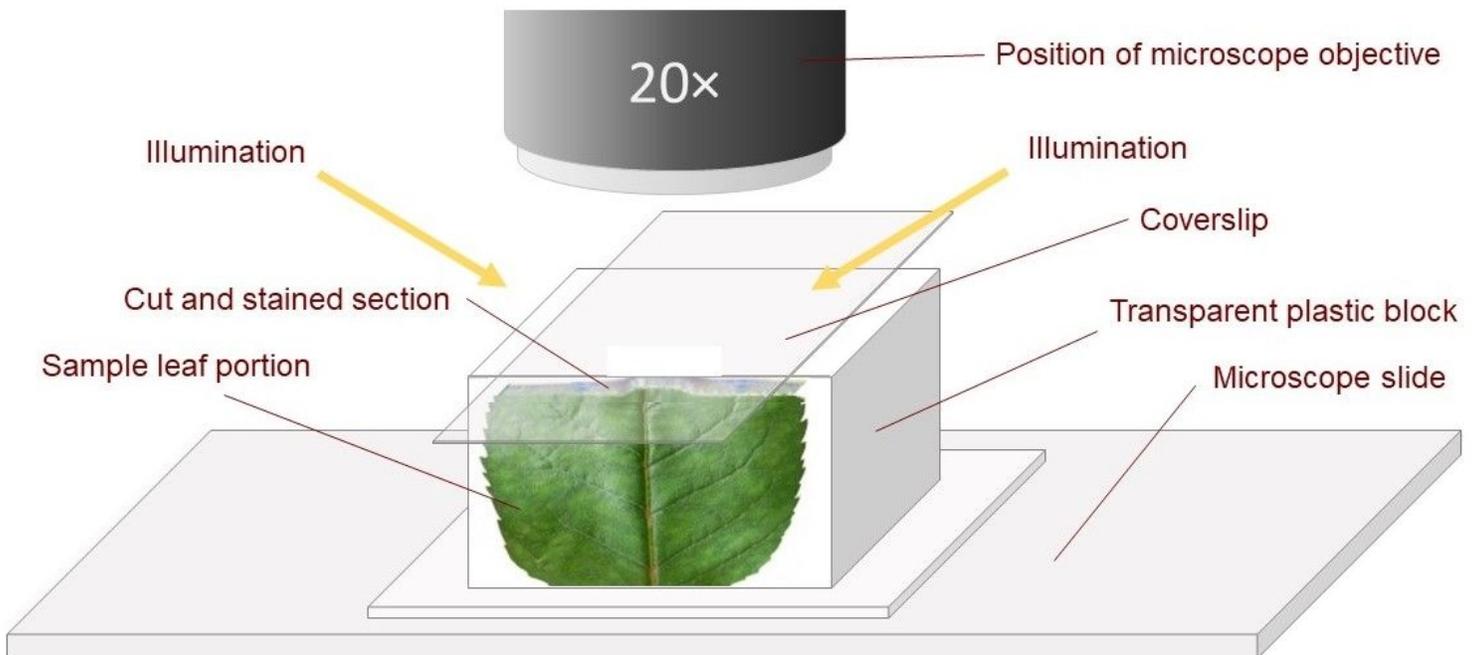


Figure 2

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 (~2×0.5 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.

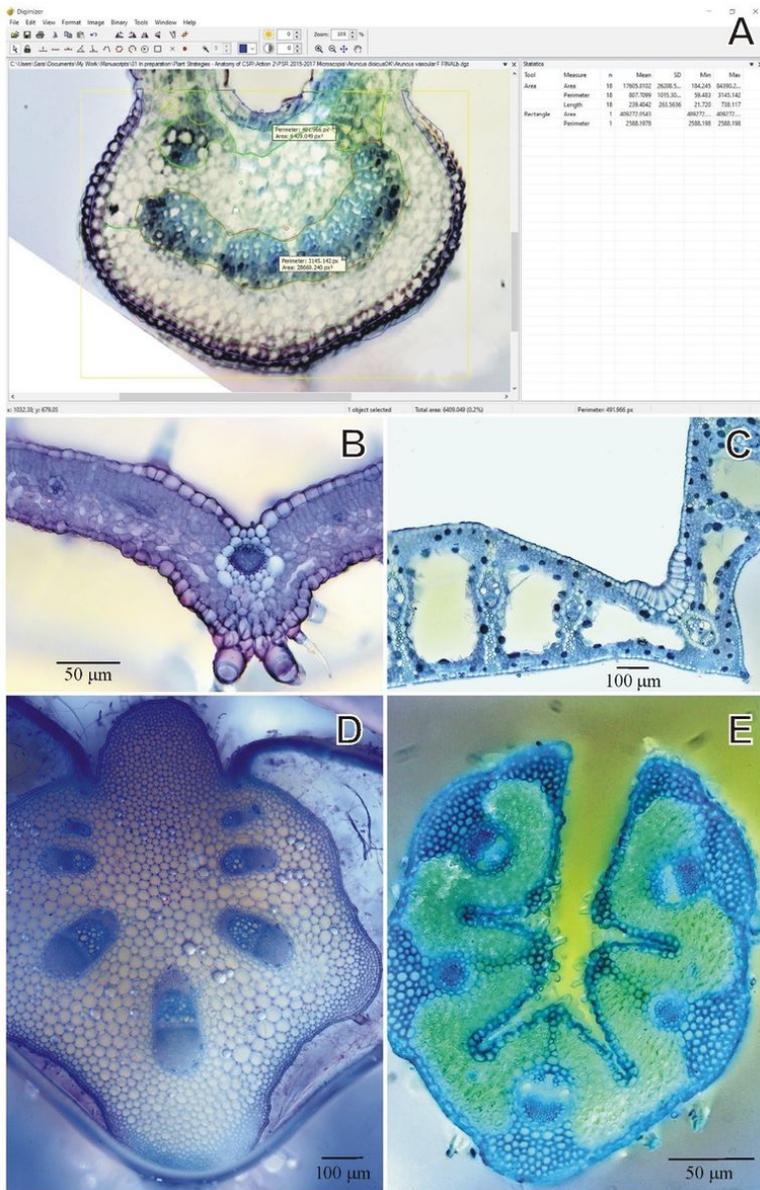


Figure 3

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). Further photomicrographs represent examples of transverse sections used in this study, and include (B). *Cerastium vulgare*, (C). *Eriophorum vaginatum*, (D). *Arctium lappa* (midvein section only), (E). *Nardus stricta*.

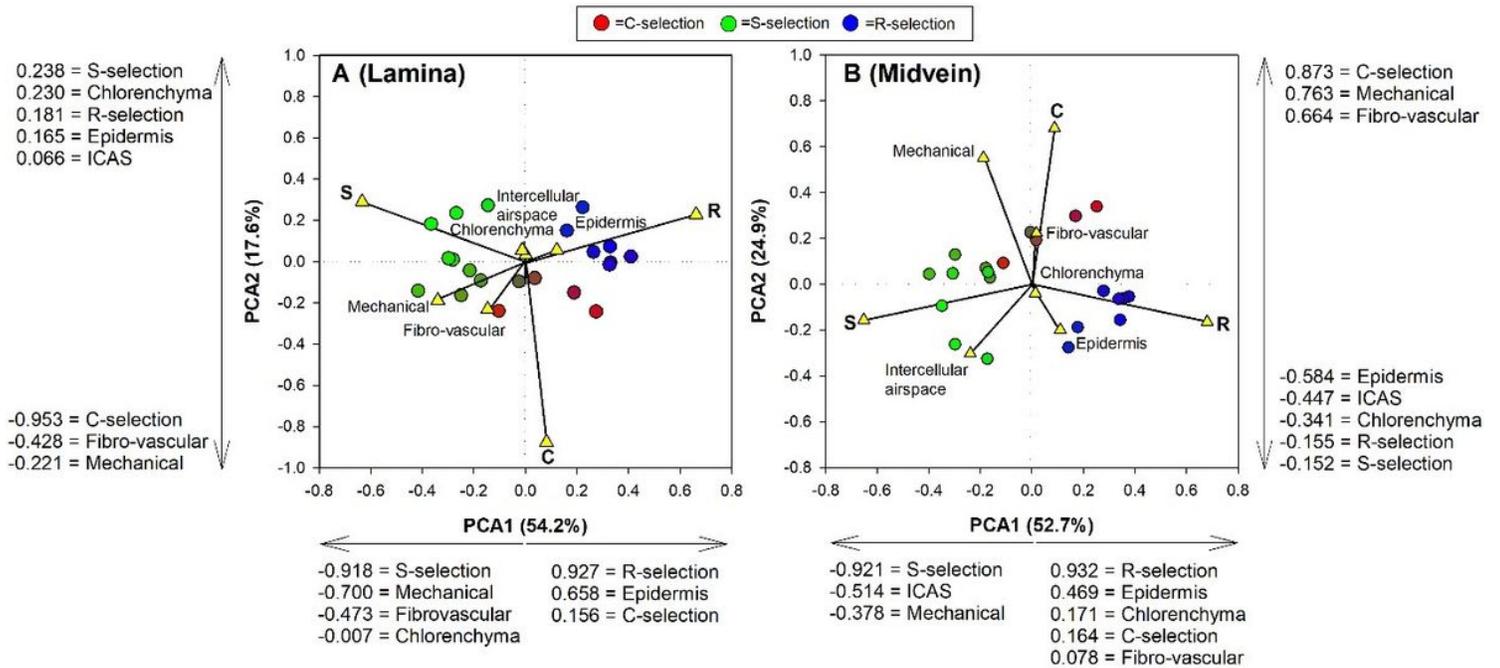


Figure 4

Principal Components Analysis (PCA) demonstrating the associations between leaf tissue types (the relative extent of epidermis, mesophyll chlorenchyma, mechanical, fibro-vascular and intercellular airspace [ICAS]) and Grime's competitor, stress-tolerator, ruderal (CSR) strategies, from transverse sections of the leaf lamina (A) and the midvein section (B) for a range of ecologically contrasting species. Values along axes represent Pearson's correlation coefficients (r) between measured parameters and PCA axes (1.000=perfect positive correlation, 0.000=no correlation, -1.000=perfect negative correlation).

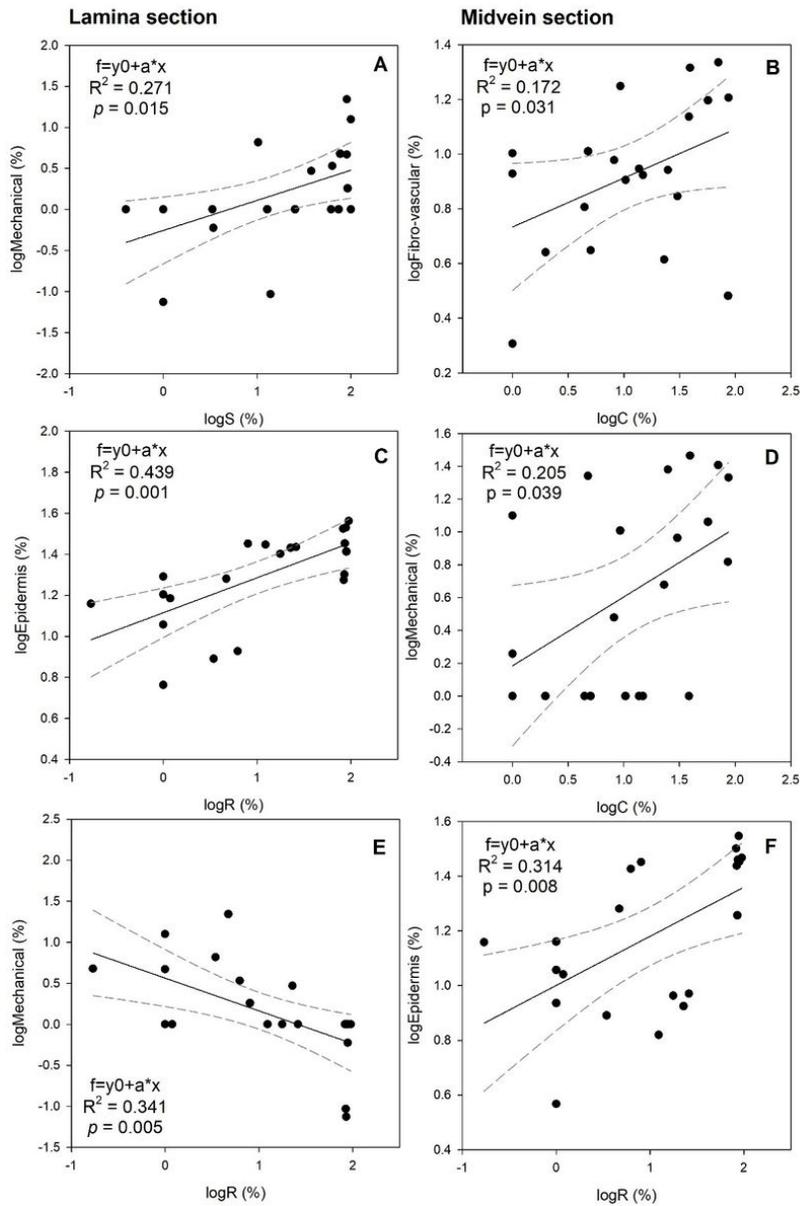


Figure 5

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).

Supplementary Files

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- [Pierceetal2022PlantEcologyTableS1.pdf](#)