Reflectance spectroscopy allows rapid, accurate, and non-destructive estimates of functional traits from pressed leaves

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Running headline: Estimating traits from pressed-leaf spectra

Abstract

1. More than ever, ecologists seek to employ herbarium collections to estimate plant functional traits from the past and across biomes. However, many trait measurements are destructive, which may preclude their use on valuable specimens. Researchers increasingly use reflectance spectroscopy to estimate traits from fresh or ground leaves, and to delimit or identify taxa. Here, we extend this body of work to non-destructive measurements on pressed, intact leaves, like those in herbarium collections.
2. Using a dataset with 618 samples from 68 species, we used partial least-squares regression to build models linking pressed-leaf reflectance spectra to a broad suite of traits, including leaf mass per area (LMA), leaf dry matter content (LDMC), equivalent water thickness, carbon fractions, pigments, and twelve elements. We compared these models to those trained on fresh- or ground-leaf spectra of the same samples.
3. Our pressed-leaf models were best at estimating LMA (R^2 = 0.917; %RMSE = 7.30), C (R^2 = 0.774; %RMSE = 11.8), and N (R^2 = 0.772; %RMSE = 11.5), followed by water-related traits, carbon fractions, and pigments (all R^2 = 0.554-0.763; %RMSE = 12.3-18.6). Other than Ca, Na, and P, remaining elements were predicted poorly (R^2 < 0.5, %RMSE > 20). For most chemical traits, pressed-leaf models performed better than fresh-leaf models, but worse than ground-leaf models. Pressed-leaf models were worse than fresh-leaf models for estimating LMA and LDMC,

but better than ground-leaf models for LMA. Finally, in a subset of samples, we used partial least-squares discriminant analysis to classify specimens among 10 species with near-perfect accuracy from pressed- (>97%), and ground-leaf (>96%) spectra, and slightly lower accuracy from fresh-leaf spectra (>89%).

4. These results show that applying spectroscopy to pressed leaves is a promising way to estimate leaf functional traits and identify species without destructive analysis. Pressed-leaf spectra might combine advantages of fresh and ground leaves: like fresh leaves, they retain some of the spectral expression of leaf structure; but like ground leaves, they avoid the masking effect of water absorption. Our study has far-reaching implications for capturing the wide range of functional and taxonomic information in the world's preserved plant collections.

Keywords: functional traits; herbarium collections; leaf chemistry; partial least-squares regression (PLSR); reflectance spectroscopy; species identification

Introduction

The world's herbaria together contain more than 390 million specimens (Thiers 2020), which are a rich source of information about global plant diversity. Herbarium specimens are collected for many reasons—for example, to document where a species is present, or to serve as vouchers for taxonomic studies. But these specimens are often repurposed for new ends, unforeseen by the collectors (Heberling & Isaac 2017; Meineke et al. 2018). More than ever, ecologists and evolutionary biologists seek to use herbarium specimens to measure functional traits (Heberling 2021): for example, to evaluate long-term changes, often in response to human activity (Willis et al. 2017; Lang et al. 2018); to fill in gaps in sparse trait databases (Perez et al. 2020); or to conduct comparative studies of clades (e.g. Jardine et al. 2020). Measuring functional traits on herbarium specimens carries the promise of letting us reach the inaccessible, including the past or distant parts of the world. Using herbarium specimens also allows researchers to benefit from the taxonomic skill of collectors and curators and refer back to the same specimens for further use—for example, as sources of genetic data, or as references for species identification from new collections (Heberling 2021). By repurposing specimens towards new ends, researchers can address ecological and evolutionary questions that require merging functional, genetic, and distributional data at global scales.

Many functional trait measurements require destructive sampling—for example, by grinding up tissue for chemical analyses. Such measurements include most protocols to determine the elemental or molecular composition of a sample (Pérez-Harguindeguy et al. 2013). Because herbarium specimens are irreplaceable—especially those from historical collections—curators may hesitate to let them be destroyed for ecological research, even in part. Non-destructive techniques to estimate leaf traits from herbarium specimens would make it more feasible to use specimens in functional ecology.

Reflectance spectroscopy is a technique often used to estimate foliar functional traits non-destructively (Curran 1989; Jacquemoud & Ustin 2020). A reflectance spectrum is a measurement of the reflectance of

light from a surface across a range of wavelengths. For leaf tissue, researchers tend to measure reflectance between about 350 and 2500 nm, a range that includes over 98% of solar radiation reaching Earth's surface (American Society for Testing and Materials, 2006). Because the leaf's chemical and structural makeup determines how it absorbs, reflects, and transmits solar radiation, reflectance within this range carries information about many plant traits. These traits are shaped by evolutionary history, such that leaf reflectance spectra can also be used to understand trait evolution or distinguish lineages (Meireles et al. 2020a).

Two main approaches exist to estimate traits from entire reflectance spectra. First, physics-based radiative transfer models like PROSPECT can be inverted on fresh-leaf spectra to estimate a handful of traits whose optical properties are well understood (Jacquemoud & Baret 1990; Féret et al. 2017). Second, statistical models, often created using machine learning techniques like partial least-squares regression (PLSR), can estimate an even wider range of traits, albeit in a less mechanistic (and perhaps less general) way (Serbin & Townsend 2020). Many ecologically important traits lack a unique set of strong absorption features, complicating the development of radiative transfer models. For example, nitrogen is found alike in various proteins (e.g. RuBisCO) and pigments (Wang et al. 2018) in ratios that vary from plant to plant. Moreover, absorption features of compounds often overlap, especially after being broadened by multiple scattering (Curran 1989). A multivariate empirical approach based on techniques like PLSR or stepwise regression gives researchers the flexibility to predict complex traits (Curran 1989). Likewise, multivariate classification techniques like partial least-squares discriminant analysis (PLS-DA) are often used to take advantage of the full spectrum in discriminating species, lineages, or other kinds of biological classes (Clark et al. 2005; Meireles et al. 2020b).

Empirical approaches like PLSR have been widely used to estimate plant traits from spectroscopic data measured on fresh or ground leaves. These traits include leaf N and leaf mass per area (LMA; Asner et al. 2011; Serbin et al. 2014; Serbin et al. 2019; Streher et al. 2020), pigments (Asner et al. 2011; Yang et al.

2016), defense compounds (Couture et al. 2016; Nakaji et al. 2019), non-structural carbohydrates (Ely et al. 2019), and even physiological rates like the maximum rate of RuBP carboxylation in photosynthesis (V_{cmax}; Dechant et al. 2017; Meacham-Hensold et al. 2019; Yan et al. 2021). Leaf-level PLSR models have been used to address such varied ecological topics as defense responses to herbivory (Kula et al. 2020), the role of biodiversity in ecosystem function (Schweiger et al. 2018), and functional trait responses to El Niño (Nunes et al. 2019). Although this multivariate statistical approach is flexible, it is sensitive to the kind of leaf tissue used to train the model. Existing PLSR models have mostly been trained on spectra of fresh leaves (e.g. Serbin et al. 2019) or dried, ground leaves (e.g. Serbin et al. 2014). Such models are not expected to transfer to dried, intact leaves like herbarium specimens because both drying and grinding cause major changes in the reflectance spectrum.

We built PLSR models to estimate traits from the spectra of pressed leaves, like herbarium specimens, and compared their accuracy to models built from fresh or ground leaves. Costa et al. (2018) showed that models based on a related technique, Fourier Transform-Near Infrared Spectroscopy (FT-NIR), could predict five leaf structural traits and a proxy for chlorophyll content from pressed leaves of tropical trees. Here, we explicitly compare the accuracy of trait estimation from the spectra of fresh, pressed, and ground leaves for a wide variety of leaf chemical and structural traits from multiple biomes and functional groups.

For most chemical traits, such as elemental composition or carbon fractions, we conjectured that ground-leaf spectral models would be the most accurate, as prior studies have found (Serbin et al. 2014; Couture et al. 2016; Wang et al. 2020). Both drying and grinding might be important in achieving this high accuracy. Drying may reveal minor absorption features of compounds in dry matter within the short-wave infrared (SWIR) range that, in fresh leaves, are obscured by the dominant effect of water absorption (Peterson et al. 1988; Couture et al. 2016; Wang et al. 2018). Grinding homogenizes the variation in structure and composition throughout the leaf lamina, which may allow us to capture a more even and

representative sample of tissue (Richardson et al. 2021). Since pressed leaves are dried but not ground, we predicted they would yield intermediate accuracy for chemical traits.

For structural traits like LMA, we instead expected fresh and pressed leaves to outperform ground leaves because grinding disrupts the leaf structure. For water-related traits like leaf dry matter content (LDMC; dried mass divided by fresh mass), we expected fresh leaves to outperform both pressed and ground leaves because only they retain the water absorption features that allow direct, quantitative prediction of water content (Carter 1991; Rapaport et al. 2015). Likewise, because pigments tend to degrade after sample collection, we expected to estimate their concentration best from fresh leaves. We also assessed sample discoloration in preparation for mounting or in storage and considered whether it reduces the accuracy of trait estimates, which may indicate whether spectroscopy is useful on old or degraded specimens.

Finally, we asked whether pressed-leaf spectra can be used to identify samples to species. Reflectance spectra often show phylogenetic signal, at least in particular wavelength ranges, because of phylogenetic conservatism in the evolution of their underlying traits (McManus et al. 2016; Diniz et al. 2020; Meireles et al. 2020a). This phylogenetic signal is what often makes it possible to classify species or higher-level taxa from fresh-leaf spectra (Cavender-Bares et al. 2016; Meireles et al. 2020b), but it may or may not be retained in pressed-leaf spectra. Studies with tropical forest species have indeed demonstrated that FT-NIR absorbance spectra of pressed herbarium specimens can be used to classify species or higher-level taxa (Durgante et al. 2013; Lang et al. 2015; Lang et al. 2017; Prata et al. 2018). Based on these studies, Draper et al. (2020) proposed using spectra of herbarium specimens as part of an integrative process of species delimitation and identification. Here, we compare the accuracy of supervised classification from fresh-, pressed-, and ground-leaf spectra among the common species in our dataset. Because they preserve some of the spectral expression of leaf structure (unlike ground leaves) and reveal the distinctive SWIR

absorption features of macromolecules and other compounds (unlike fresh leaves), pressed leaves may represent the best of both worlds for distinguishing species using spectroscopy.

Methods

Sampling

We trained PLSR models on leaf reflectance spectra and traits measured as part of four projects conducted by the Canadian Airborne Biodiversity Observatory (CABO). The projects are:

1. Beauchamp-Rioux: Deciduous forest trees sampled throughout the growing season in 2018 at various sites in southern Québec and Ontario (n = 405; 9 species).
2. Dessain: Forbs, grasses, shrubs, and broadleaf trees from forests and open areas sampled throughout the growing season in 2017 at four sites in southern Québec (n = 72; 59 species).
3. Boucherville: Forbs, shrubs, and grasses sampled throughout the growing season in 2018 from the Parc national des Îles-de-Boucherville in southern Québec (n = 73; 12 species).
4. Warren: A single broadleaf evergreen tree species (*Agonis flexuosa* (Willd.) Sweet) collected across a soil chronosequence (Turner et al. 2018) in southwestern Australia (n = 68).

The majority of samples were from trees (n = 504; 25 species), but herbs (n = 60; 19 species), shrubs (n = 48; 23 species), and vines (n = 6; 1 species) were also represented. The full sampling procedure is described in the Supplementary Materials. We validated the models both internally and on an independent dataset of pressed tree and herb samples collected at Cedar Creek Ecosystem Science Reserve (East Bethel, MN, USA).

Spectral and leaf trait measurements

For each CABO sample, we measured full-range reflectance spectra (350-2500 nm) of the leaves at three stages: (1) freshly sampled, (2) pressed and dried, and (3) ground into a fine powder. We measured fresh-leaf directional-hemispherical reflectance spectra on the adaxial surface of multiple leaves or (for small- or narrow-leaved plants) leaf arrays from each sample using spectroradiometers equipped with integrating

spheres. We pressed a portion of the sample, and measured reflectance spectra on the adaxial surface of pressed leaves between six months and three years later using a spectroradiometer with a leaf clip. Lastly, we measured ground-leaf spectra using a spectroradiometer with a benchtop reflectance probe that pressed loose leaf powder into a smooth, even pellet that we assume has very low transmittance. We trimmed all spectra to 400-2400 nm. Detailed notes on measurement and post-measurement processing of reflectance spectra are found in the Supplementary Materials.

While measuring pressed-leaf spectra, we inspected each pressed specimen by eye to note signs of discoloration in preparation or storage. Typical forms of discoloration include blackening, browning, or the development of a silvery or whitish finish on the leaf surface. We scored each leaf on a discrete scale from 0 to 4. A score of 0 indicates no noticeable discoloration. Scores 1 through 4 indicate increasing discoloration, from 1 (either <5% blackening/browning or development of a silvery finish to the leaf) to 4 (>80% blackening/browning).

We measured the following leaf structural and chemical traits on all CABO samples: Leaf mass per area (LMA; kg m^{-2}), leaf dry matter content (LDMC; mg g^{-1}), equivalent water thickness (EWT; cm), carbon fractions (soluble cell contents, hemicellulose, cellulose, and lignin; %), pigments (chlorophyll *a*, chlorophyll *b*, and total carotenoids; mg g^{-1}), and concentrations of a variety of elements (Al, C, Ca, Cu, Fe, K, Mg, Mn, N, Na, P, Zn; % or mg g^{-1}). Protocol summaries are in the Supplementary Materials. We kept chemical traits on a mass basis for consistency with the usual basis on which they are measured and reported (pigments excepted). However, some traits are most often distributed proportionally to area (Osnas et al. 2013), and retrievals on an area basis may be better for some purposes (Kattenborn et al. 2019).

PLSR modeling for trait estimation

We used a PLSR modeling framework to link the fresh-, pressed-, and ground-leaf spectra to the traits we measured. PLSR is well-suited to handle leaf spectral datasets, which have large numbers of collinear predictors, because it projects the spectral matrix onto a smaller number of orthogonal latent components in a way that maximizes the ability to predict the response variable. We implemented PLSR modeling using *R* package *pls v. 2.7.1* (Mevik et al. 2019). We conducted analyses separately for fresh-, pressed-, and ground-leaf spectra, using the full spectrum (400-2400 nm) to predict each trait. We simply used reflectance values as predictors, since calculating various transformations of reflectance (first- or second-order differences, or pseudoabsorbance defined as –log[reflectance]) made little difference for predictive accuracy in preliminary tests. We also did not perform any transformations to reduce the skew of trait distributions, again finding in preliminary tests that they did not improve predictive accuracy enough to warrant the added complexity. Some studies restrict the wavelengths used in prediction—for example, to ranges known or assumed to contain features relevant to a given trait (e.g. Serbin et al. 2014). Given the potential for photosynthetic pigments to degrade and produce brown pigments in pressed leaves (Fourty et al. 1996), we also built pressed-leaf models restricted to the range 1300-2400 nm, which might be expected to generalize better to datasets that include more degraded leaves. We present these results mainly in the Supplementary Materials.

Our methods for model calibration and validation largely follow Serbin et al. (2014) and Burnett et al. (2021). First, we randomly divided the data into calibration (75%) and validation (25%) datasets, stratified by project. We began by fitting a model for each trait on the calibration dataset. We selected the smallest number of components for which the root mean squared error of prediction (RMSEP) from 10-fold cross-validation was within one standard deviation of the global minimum. We used this number of components—a different number for each trait—in further analyses to predict traits on the internal validation dataset. We calculated the variable importance in projection (VIP) metric for calibration models to see which parts of the spectrum were most important for predicting each trait (Wold et al. 1994).

To test how well we could predict traits on the internal validation subset, we did a jackknife analysis by repeatedly (100×) dividing the 75% calibration data further into random 70% training and 30% testing subsets. For each trait, we trained models on the 70% using the previously determined optimal number of components and predicted the remaining 30%, which gave us a distribution of model performance statistics across iterates (R^2, %RMSE). This distribution reveals how sensitive model performance is to randomly varying sets of training and testing data.

Next, we applied the 100 jackknife models for each trait to the 25% internal validation dataset: for each sample in the validation dataset, we generated a distribution of predictions from the 100 models. We quantified model performance using R^2 and root mean squared error (RMSE) between measurements and mean predictions. We also report the RMSE as a percentage of the 2.5% trimmed range (%RMSE), which we used rather than the full range (as in e.g. Burnett et al. 2021) for robustness to outliers. For each trait, we also tested whether the magnitude of residuals (observed minus predicted) in the validation dataset varied among leaves with different discoloration scores. We performed all statistical analyses in *R v. 3.6.3* (R Core Team 2020).

Finally, to test how well our model would transfer to a fully independent dataset, we applied the ensemble of pressed-leaf models for five traits (LMA, LDMC, EWT, N, and C) to pressed-leaf spectra from Cedar Creek and compared the model-derived trait estimates to measured values. Like most of the CABO dataset, the Cedar Creek dataset comprises trees and herbs from northeastern temperate North America, but it includes an entire functional group (needleleaf conifers) absent among the CABO projects in this study. We collected the spectra with the same instrument and foreoptic as the pressed-leaf spectra in the CABO dataset, but used different sampling, preparation, and trait measurement protocols. Full details on this dataset are provided in Supplementary Materials.

PLS-DA modeling for species classification

We tested the potential to classify species with fresh-, pressed-, and ground-leaf spectra using partial least-squares discriminant analysis (PLS-DA; Barker & Rayens 2003). We took spectra from the ten most common species in our dataset—all of which are deciduous trees except *A. flexuosa*, which is evergreen. Each species was represented by at least 20 specimens (~480 total). For each tissue type, we divided the full dataset into 60% calibration and 40% validation subsets. In the *R* library *caret v. 6.0.84* (Kuhn 2020), we trained models on the calibration subset using 10-fold cross-validation. We chose the number of PLS components during cross-validation by maximizing Cohen's kappa (κ), which describes the agreement between the true and predicted species identities while accounting for the probability of agreement by chance. Imbalanced training data can bias classification algorithms (Sun et al. 2009; Lindström et al. 2011), so we used a two-step procedure to balance classes while maintaining enough training data and avoiding overfitting. First, we downsampled within better-represented species classes at random so that all classes had equal size, then chose the number of components (*n*) that maximized κ. Second, we upsampled from less-represented classes at random with replacement so that classes had equal size—again maximizing κ, but restricting the range of components evaluated to no more than the *n* chosen during the downsampling step. We applied the cross-validated PLS-DA model from the upsampling step to the validation subset and summarized its performance using raw classification accuracy and κ.

Results

Patterns in traits and reflectance spectra

We saw large variation among samples in each of our target traits, ranging from 1.4-fold variation in C to more than 20-fold variation in traits like lignin, P, K, and Zn (Table 1). The ranges of most traits in our dataset covered a large portion of the global distributions in the TRY dataset, but tended to be narrower at both extremes (Kattge et al. 2020). Trees tended to have higher LDMC, C, and lignin than other growth forms. Among the herbs, grasses had particularly high hemicellulose and cellulose and low lignin content, while forbs generally had high N. Some of the trait variation was driven by projects that occupied

particular portions of trait space; for example, *A. flexuosa* in the Warren project tended to have particularly high LMA, Na, and C and low N.

Both pressed and ground leaves had higher median reflectance across nearly the entire spectrum (Fig. 1), as expected based on changes in water content and structure (Carter 1991). Indeed, water absorption features (the largest of which are centered around 1450 and 1930 nm) largely disappeared in pressed and ground leaves. The red edge between the visible and near-infrared (NIR) regions was also blunted by both pressing and grinding, causing the global maximum of median reflectance to shift from 872 nm (fresh) to 954 nm (pressed) to 1313 nm (ground). The coefficient of variation (CV) of reflectance was generally highest where average reflectance was lowest.

PLSR modeling for trait estimation

Tested on the internal validation dataset, pressed-leaf models performed best at predicting LMA (R^2 = 0.917; %RMSE = 7.30), C (R^2 = 0.774; %RMSE = 11.8), and N (R^2 = 0.772; %RMSE = 11.5). These traits were followed by a mixture of water-related traits, carbon fractions, and pigments, as well as Ca (all R^2 = 0.554-0.763; %RMSE = 12.3-18.6). Although some other elements (Na, P) could be estimated with $R^2 > 0.5$ and %RMSE < 20, most models for other elements showed lower accuracy (Table 1; Figs. 2-3 and S1-7). In most cases, the models restricted to 1300-2500 nm had slightly lower predictive accuracy than the full range models (Table S1).

We compared pressed-leaf models to both fresh-leaf and ground-leaf models. The optimal number of components selected to predict each trait was between 3 and 23. For any given trait, ground-leaf models always had the most components, usually followed by pressed-leaf models (Table 1). Fresh-leaf models were best for predicting the structural and water-related traits—LMA, LDMC, and EWT (Figs. 2-3 and S1-7). On the other hand, ground-leaf models were best for predicting chemical traits, like carbon fractions and most elements. Fresh- and ground-leaf models had similar performance for pigments.

Statistics from jackknife analyses showed that model performance was more variable for traits that were predicted less accurately (Figs. S11-S13). There was no correlation between the magnitude of residuals and our discoloration index for any trait ($p > 0.05$).

For all traits except LMA and Fe, the VIP metric for fresh-leaf spectra showed a global maximum between 710 and 720 nm (Fig. 4 and S8-10)—wavelengths slightly longer than the typical inflection point of the red edge (Gitelson et al. 1996). Many traits also show high VIP across the wavelengths ~530-570 nm, corresponding to the green hump. Bands in the NIR range were less important for predicting most traits than much of the visible range. The SWIR range was generally important for predicting LMA, EWT, Na, and pigments, and many other traits showed several local peaks of importance, most prominently at about 1880 nm, but also near 1480 and 1720 nm.

For predicting traits from pressed-leaf spectra, the general trend held that visible reflectance and certain ranges in the SWIR were important for predicting most traits, while the NIR and much of the shorter SWIR (800-1750 nm) were less important (Fig. 4 and S8-10). The red edge peak of importance for most traits was near 705 nm. Other prominent local maxima for many traits lay close to 1440, 1720, 1920, 2130, and 2300 nm. We saw broadly similar patterns in ground-leaf spectra, except that VIP for most traits was lower at longer SWIR wavelengths (2000-2400 nm).

External validation

For most traits, pressed-leaf model performance on the external validation dataset from Cedar Creek was comparable to, but not quite as strong as, the internal validation (Table 2; Fig. 5). The exception was C, for which the models performed very poorly ($R^2 < 0.05$). Among the remaining traits, R^2 ranged from 0.557 (N) to 0.874 (LMA). For N in particular, %RMSE was high (37.8%) due to bias—N concentrations were underestimated for conifers but slightly overestimated for remaining samples.

Since conifers were absent from the CABO training dataset, we considered whether the models we built would extend to this new functional group. For N, models performed better when excluding conifers (R^2 = 0.594, %RMSE = 37.8) than when retaining them (R^2 = 0.557; %RMSE = 29.5). In contrast, for LMA, models performed worse when excluding conifers (R^2 = 0.762; %RMSE = 15.7) than when retaining them (R^2 = 0.874; %RMSE = 10.3), since their LMA values could be predicted well and extended the trait range. Models restricted to 1300-2500 nm did not consistently outperform those trained on the full range—they yielded somewhat better external validation R^2 for N, but worse for LDMC, both including and excluding conifers (Table S2; Fig. S14).

PLS-DA modeling for species classification

PLS-DA models using pressed- and ground-leaf spectra showed near-perfect performance at classifying species (Fig. 6). Models using fresh-leaf spectra were slightly worse but still showed strong performance. The optimal fresh-leaf model, which had 29 PLS components, correctly predicted the taxonomic identity of 170 of the 189 samples in the training dataset ($\kappa = 0.884$; $p < 0.0001$). The best pressed-leaf model, which had 29 PLS components, correctly predicted 184 of the 188 samples ($\kappa = 0.975$; $p < 0.0001$). The best ground-leaf model, which had 30 PLS components, correctly predicted 183 out of 189 samples ($\kappa = 0.963$; $p < 0.0001$). In all cases, the majority (75-100%) of misclassifications were between congenerics.

Discussion

We show that we can estimate a wide range of leaf functional traits for 68 woody and herbaceous species from reflectance spectra of pressed leaves (Table 1; Figs. 2-3 and S1-7). Model performance was highest for LMA, C, and N, followed by a mixture of water-related traits, carbon fractions, pigments, and a few important elements (Ca, Na, and P). Finally, other elements could only be estimated with fairly low accuracy. These results show that pressed-leaf spectra provide an integrative measure of leaf phenotypes, much like fresh-leaf spectra (Cavender-Bares et al. 2017), but with even stronger potential to characterize variation in chemical traits. Perhaps as a result, we could use pressed-leaf spectra to classify species as

accurately as ground-leaf spectra, and better than fresh-leaf spectra. Our results point to the potential that using reflectance spectroscopy on herbarium specimens could yield rapid and non-destructive estimates of many functional traits, enabling more expansive studies of trait variation across space and time.

We compared pressed-leaf models to fresh-leaf and ground-leaf models from the same samples. Our findings about which kind of tissue was best for predicting each trait mostly supported our hypotheses. Ground-leaf spectra showed the strongest performance for chemical traits, likely because grinding homogenizes the lamina and removes the potentially confounding influence of leaf structure (Table 1). Pressed-leaf spectra showed intermediate performance for most chemical traits, perhaps because, like ground leaves, they lack the major water absorption features that mask the smaller features of other compounds in the SWIR range (Peterson et al. 1988). Contrary to our predictions, ground-leaf spectra performed about as well as fresh-leaf spectra, and better than pressed-leaf spectra, for estimating pigment concentrations. The same factors that provided an advantage to ground-leaf spectra in estimating chemical traits—removal of water absorption and leaf structure—also perhaps explain why pressed- and (especially for LMA) ground-leaf spectra performed worse for estimating water-related and structural traits (LMA, LDMC, and EWT). Pressed-leaf models may represent a good compromise in allowing many traits to be estimated with mostly intermediate but nonetheless quite high accuracy.

It may seem perplexing that we could succeed at all in predicting LMA from ground-leaf spectra, or LDMC and EWT from pressed- and ground-leaf spectra. Many nutrients also have weak or no absorption features. The ability to estimate these traits must not result from the optical expression of the traits themselves. We suggest that we instead sense these traits via their correlations with other traits that have a stronger optical expression. This kind of effect—a “constellation effect” (*sensu* Chadwick & Asner 2016; Nunes et al. 2017)—has been invoked to explain the ability to estimate traits like leaf nutrients (Nunes et al. 2017) and $\delta^{15}N$ (Serbin et al. 2014) that are not known to have strong absorption features in the measured range of wavelengths. For example, some nutrients are sensed mainly through symptoms of

deficiency (Jacquemoud & Ustin 2020). However, models that rely on such constellation effects could break down when patterns of trait covariance vary, which could make models for certain traits fail beyond the domain of the training data.

The performance of pressed-leaf models in this study was comparable to the successes obtained here and elsewhere using fresh- and ground-leaf spectra. For example, the ground-leaf models in Serbin et al. (2014) had a validation RMSE of 1.4 and 2.4 for cellulose and lignin percentages. In comparison, our pressed-leaf models achieved an RMSE of 1.52 and 2.30 (Table 1). Our models for LMA also had an RMSE (0.0115 kg m^{-2}) lower than fresh-leaf models from the literature, including Serbin et al. (2019; 0.015 kg m^{-2}), Nakaji et al. (2019; 0.015 kg m^{-2}), and Streher et al. (2020; 0.051 kg m^{-2}). On the other hand, our pressed-leaf model for N performed worse than Serbin et al. (2014; validation RMSE = 0.13 vs 0.273). Although these studies sampled different kinds of vegetation with different trait ranges, such comparisons establish that pressed-leaf models are often comparable to ground- or fresh-leaf models. Our models' error could be within acceptable bounds for addressing a wide range of questions about large-scale ecological or evolutionary patterns that encompass a wide range of trait variation. Moreover, our external validation analyses indicate that our models for some important traits—like LMA, LDMC, EWT, and N, but not C—can transfer reliably to other datasets.

The VIP metric helps us understand what features allow these models to work, but interpreting it can be challenging because reflectance at a given band usually cannot be understood in terms of a single trait. Fresh-leaf VIP for most traits had peaks in the visible and SWIR ranges, with a global maximum at 705 nm (Fig. 4 and S8-10). The pattern of high VIP along the green hump and at the red edge is common in PLSR models from fresh leaves (Ely et al. 2019; Streher et al. 2020; Yan et al. 2021). The red edge (particularly 700-725 nm) may be so important because of its sensitivity to both chlorophyll content and leaf structure (Gitelson et al. 1996; Richardson et al. 2002). Much of the visible range was proportionally even more important for pressed- and ground-leaf models, and most of the NIR was less important

(except for LMA; Fig. 4 and S8-10). There were multiple small peaks in the SWIR, most notably one at 1920 nm that appears similar to a VIP feature in Serbin et al. (2014). Reflectance at 1920 nm is often associated with water absorption in fresh tissue, but any causal link to the leaf's fresh water content is unlikely for pressed and ground leaves. Bands near 1920 nm also include broad absorption features for many components of dry matter, including protein, cellulose, lignin, and starch, any or all of which may contribute to the importance of this region (Curran et al. 1989; Fourty et al. 1996).

With some exceptions, the VIP metric showed that the same bands are often important for predicting different traits. This pattern might be taken as an artifact of trait covariance: For example, the three pigment pools covaried strongly (R^2 = 0.827-0.969) and had nearly identical VIP across the spectrum (Figs. S8-S10). In some cases, similarities in VIP might be taken further to imply that there are a small number of traits whose tight coordination with others underlies the performance of all models through constellation effects. Nevertheless, across the whole dataset, many traits covaried only weakly but still shared important VIP features. For example, EWT, cellulose, N, and K were not tightly coordinated (R^2 = 0.003-0.152) but shared similar patterns of pressed-leaf VIP across the spectrum (Fig. 4), including major peaks at 705 and 1920 nm.

PLS-DA models showed that fresh-, pressed-, and ground-leaf spectra alike could be used to classify species with near-perfect accuracy (>96%) for pressed or ground leaves, and excellent (>89%) accuracy for fresh leaves (Fig. 6), much as in earlier work on tropical flora (e.g. Durgante et al. 2013). In contrast to prior work that deliberately selected many congenerics, our most common species were often distantly related. Among the misidentified samples, most were mistaken for congenerics, which implies that related species are more spectrally similar (Schweiger et al. 2018; Meireles et al 2020a). Hence, if we had included more closely related species, we might have seen a higher error rate. Nevertheless, our analysis reinforces that spectral models can often distinguish species or higher-level phylogenetic groups (Meireles et al. 2020b).

As we predicted, pressed-leaf models showed the highest classification accuracy (>97%). This result is noteworthy, since pressed specimens are the standard way that field botanists preserve plant samples for later identification, but the outcome may be surprising because pressed leaves were never best for predicting any single trait. We conjecture that pressed- and ground-leaf models have an advantage because drying reveals the absorption features of potentially distinctive compounds in the SWIR range. While water content can only vary in one dimension, there are many macromolecules and metabolites whose absorption features in this range might vary independently and allow finer discrimination of species.

The future of spectroscopic trait estimation

Although trait predictions from spectral models are not perfect, they have a few advantages over conventional trait measurements: they (1) can be non-destructive; (2) are fast and require relatively little training; and (3) have very low marginal cost, despite the high capital cost of buying a spectrometer (Costa et al. 2018). These advantages could make it easier to address questions that require large datasets of functional traits. But researchers may be deterred if they must each build their own models tailored to particular uses—and for herbarium specimens, it might not be possible to do the conventional trait measurements needed to train the models. Ideally, spectral models would be general enough that researchers could confidently use them without further validation, but this aim is not easy to achieve: for several reasons, a model trained on any particular spectral dataset may make poor trait predictions on new data. As with any other technique, the goal for spectroscopic trait estimation is to improve model accuracy and generality as much as they can be jointly improved without making the technique harder to use. Below, we discuss some challenges one by one, particularly as they concern herbarium specimens.

One concern is that the new data could be outside the range of traits or optical properties in the training dataset (Schweiger 2020). A fully general model, if such a thing is possible, would need to represent the

full range of leaf functional traits and optical properties. Another kind of concern about model generality concerns sample preparation before spectral measurements. For example, particle size influences ground-leaf spectra (Foley et al. 1998). For pressed leaves, it may be particularly important to prepare samples in consistent ways that preserve the leaves' morphological integrity. In our external validation analyses, we found that pressed-leaf models yielded reasonably accurate predictions of most traits, even though the validation dataset differed in sample preparation protocols and included conifers, which were absent from the training dataset. Nevertheless, even setting aside conifers, external validation for one trait (C) was very poor, and for another (N) showed noticeable bias, such that researchers might need to develop their own correction factors to use the model in practice.

Another class of challenges concerns spectrometers and their foreoptics. Spectra of fresh leaves can be measured with different foreoptics, including integrating spheres, contact probes, or leaf clips. In this study, we used a leaf clip with pressed specimens because mounting delicate pressed leaves in an integrating sphere could damage them. However, leaf clips and contact probes are less likely to produce consistent measurements among instruments or replicate samples due to variation in viewing geometry and anisotropic surface reflectance (Petibon et al. 2021). The logistical constraint of having to use a leaf clip or contact probe could thus make it harder to compare across datasets. Moreover, measuring reflectance with a leaf clip or probe usually requires placing a black absorbing background under the sample to keep transmitted light from being reflected back into the sensor, but many herbarium specimens are glued to a paper backing. When loose leaf material is not available, using spectra from these specimens may require new methods to correct for reflectance from the mounting paper.

Spectrometers and their software may also differ subtly in their sensors and techniques for processing spectra (Castro-Esau et al. 2006), and in some cases, researchers must take steps to reconcile spectra measured from different instruments (Meireles et al. 2020b). Assuming that these kinds of technical challenges can be overcome, our results and others encourage confidence about building general models

to estimate traits from a wide variety of plants (Serbin et al. 2019). The creation of open libraries for spectral data (like EcoSIS, https://ecosis.org/; or the CABO data portal, https://data.caboscience.org/leaf) and spectral models (like EcoSML; https://ecosml.org/) will contribute to this goal.

Even though species classification models are more likely to remain domain-specific, some of the same concerns apply. For example, a training dataset for a species classification model must include strong representation for all the species that one might encounter. Researchers may also want to use existing data, as from open libraries, to train models, which would require resolving concerns about sampling—including phenotypic plasticity—as well as the potential influence of instrumentation and measurement protocols (Meireles et al. 2020b; Draper et al. 2020).

What should herbarium managers do?

Perhaps the most concerning challenge for interpreting spectra from herbarium specimens is that specimens' optical and chemical properties may degrade during preparation or storage, especially light-sensitive pigments. Such degradation could make it hard to distinguish changes in the traits of living plants over time from changes in storage. Even in this study, where no specimens were collected before 2017, many underwent visible changes in color, including browning or blackening; ~12% were scored at 2 or higher. Certain preparation and storage conditions could accelerate changes in optical properties. For example, when collecting herbarium specimens in the wet tropics, researchers often treat leaves with an alcohol solution as a preservative (Blanco et al. 2006). Such treatments could discolor leaves—but so could the spoilage that would occur in their absence. Exposure to high light, temperature, or humidity could also hasten discoloration, which underscores the importance of using best practices for preparation and storage. Some species may also be more susceptible than others; in our dataset, ~42% of *Populus grandidentata* specimens were scored at 2 or higher, but no *Betula papyrifera* specimens were.

Whether such degradation could affect trait estimation is an open empirical question. We find no evidence that it does, even for badly discolored specimens, but our specimens were only collected as early as 2017, while ecologists may want to use specimens collected decades ago. Long-term studies of the same specimens—especially with sets of specimens subjected to varying preparation and storage techniques—would help establish how their optical properties tend to change over time. In any case, it seems likely that estimating traits in old or degraded specimens will require including equally old or degraded specimens in the dataset used to build the model.

Some of the challenges we describe pertain to projects that would seek to measure spectra on samples already collected, but spectroscopy, among countless other novel uses for herbarium specimens (Heberling & Isaac 2017), could also prompt changes in collection practice. The best way to incorporate spectroscopy into the operation of a working herbarium may be to measure incoming specimens as they are pressed, but before they are mounted, which could also mitigate the effects of degradation. Collecting and storing extra leaf material would also circumvent the potential challenge of measuring mounted leaves and aid other novel uses of herbarium specimens (Heberling 2021).

Collections-based research has been accelerated by advances in biodiversity informatics that make it easier to gather and synthesize data (Heberling et al. 2021). If it becomes common practice to measure spectra on herbarium specimens—either at the time of collection (fresh) or after (pressed)—new tools may be needed to link spectral data to specimen metadata and other phenotypic or genetic information (Draper et al. 2020). The hyperdimensionality of the reflectance spectrum could make it hard to accommodate within existing standards for biodiversity informatics such as the Darwin Core (Wieczorek et al. 2012), at least without extensions. One could link records to external databases like EcoSIS or SPECCHIO (Hueni et al. 2020), which are also equipped to store spectral metadata about instrumentation and processing. Coordination between herbarium managers and researchers who use reflectance

spectroscopy could advance protocol development and build agreement about best practices for measurement and curation.

We show that non-destructively measured pressed-leaf spectra retain much of the information about many leaf functional traits found in fresh-leaf spectra. While validating this technique on older specimens will require extensive further research, our findings suggest that leveraging reflectance spectroscopy could allow herbaria to take on a much greater role in plant functional ecology and evolution. Our study has far-reaching implications for capturing the wide range of functional and phenotypic information in the world's preserved plant collections.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

Author contributions

SK, JCB, and EL conceived the ideas and designed the methodology. RBR and SK collected the data. SK analyzed the data and wrote the first draft of the manuscript. All authors made substantial contributions to further drafts and gave final approval for publication.

Tables

Table 1: Summary statistics of PLSR model internal validations from fresh-, pressed-, and ground-leaf spectra. %RMSE is calculated as RMSE divided by the 2.5% trimmed range of data within either the training data set (calibration) or the testing data set (validation). Global ranges are derived from the TRY database (Kattge et al. 2020; accessed 13 April 2021), omitting data with error risk greater than 3. Carbon fractions (solubles, hemicellulose, cellulose, and lignin) and carotenoids are each represented by <1250 datapoints in TRY, which may limit the estimates of the global range. EWT is not available on TRY.

			Fresh				Pressed				Ground			
Trait	Dataset range	Global range	No. comps	R^2	RMSE	%RMSE	No. comps	R^2	RMSE	%RMSE	No. comps	R^2	RMSE	%RMSE
LMA (kg m^{-2})	0.0233-0.217	0.00866-0.354	15	0.960	0.00795	5.05	9	0.917	0.0115	7.30	20	0.824	0.0165	10.5
LDMC (mg g^{-1})	155-573	68.2-857	16	0.853	26.0	8.82	17	0.713	36.4	12.3	20	0.764	33.1	11.2
EWT (cm)	0.00488-0.0282	NA	7	0.871	0.00167	9.99	14	0.763	0.00213	12.8	22	0.691	0.00242	14.5
Solubles (%)	38.5-88.9	20.0-74.6	15	0.438	6.12	19.5	12	0.701	4.31	13.6	23	0.814	3.36	10.6
Hemicellulose (%)	2.77-34.1	8.71-45.0	11	0.483	3.47	22.0	12	0.731	2.48	15.6	20	0.755	2.35	14.6
Cellulose (%)	5.19-25.8	7.65-42.6	20	0.482	2.29	27.4	15	0.735	1.52	18.1	21	0.872	1.04	12.4
Lignin (%)	0.918-21.5	2.27-32.4	10	0.497	2.42	19.6	12	0.554	2.30	18.6	20	0.777	1.65	13.3
C (%)	39.5-53.6	25.5-60.3	17	0.716	1.22	13.4	14	0.774	1.08	11.8	20	0.908	0.689	7.53
N (%)	0.883-5.62	0.14-7.27	10	0.729	0.301	12.6	15	0.772	0.273	11.5	22	0.952	0.127	5.31
Chl *a* (mg g^{-1})	1.24-15.5	1.38-22.1 (total Chl)	5	0.719	1.12	14.1	7	0.626	1.30	16.4	9	0.719	1.14	14.4
Chl *b* (mg g^{-1})	0.486-5.29		5	0.696	0.397	14.7	6	0.555	0.484	17.9	8	0.688	0.410	15.2
Carotenoids (mg g^{-1})	0.193-3.05	0.00102-5.1	5	0.638	0.264	16.9	7	0.615	0.274	17.5	16	0.712	0.240	15.3
Al (mg g^{-1})	0-0.379	0.004-19.7	5	0.220	0.0415	24.5	6	0.243	0.0409	24.1	10	0.593	0.0304	17.9
Ca (mg g^{-1})	1.64-36.9	0.68-69	11	0.425	5.34	19.0	17	0.744	3.57	12.7	23	0.868	2.58	9.16
Cu (mg g^{-1})	0-0.052	0.00154-0.064	8	0.472	0.00577	20.6	10	0.301	0.00653	23.2	7	0.340	0.00641	23.1
Fe (mg g^{-1})	0.0150-0.266	0.0128-1.1	4	0.267	0.0380	20.4	7	0.215	0.0392	21.0	9	0.358	0.0356	19.1
K (mg g^{-1})	1.06-38.2	0.3-78.1	7	0.598	2.92	17.6	14	0.488	3.18	20.1	17	0.694	2.55	15.3
Mg (mg g^{-1})	0.482-7.86	0.277-15.6	7	0.253	0.802	22.1	13	0.373	0.759	20.9	23	0.529	0.656	18.0
Mn (mg g^{-1})	0-1.02	0.00560-5.37	3	0.198	0.158	25.3	12	0.349	0.144	22.9	17	0.655	0.107	18.1
Na (mg g^{-1})	0-4.87	0.00499-48.7	4	0.360	0.612	21.9	9	0.514	0.537	19.2	14	0.426	0.579	20.7

P (mg g^{-1})	0.222-7.18	0.1-9.9	7	0.389	0.866	21.3	19	0.547	0.738	18.1	13	0.598	0.699	17.2
Zn (mg g^{-1})	0.00142-0.647	0.00221-0.242	9	0.250	0.0744	26.2	8	0.310	0.0704	24.8	15	0.437	0.0634	22.3

Table 2: Summary statistics for external validation of pressed-leaf PLSR models. The models were trained on CABO data (see Table 1) and applied to a dataset collected at Cedar Creek.

	Including conifers				Excluding conifers			
Trait	Range	R^2	RMSE	%RMSE	Range	R^2	RMSE	%RMSE
LMA (kg m^{-2})	0.0179-0.229	0.874	0.0186	10.3	0.0179-0.136	0.762	0.0126	15.7
LDMC (mg g^{-1})	133-525	0.559	47.8	16.9	133-504	0.571	49.7	17.2
EWT (cm)	0.00602-0.0372	0.609	0.00449	18.1	0.00602-0.0363	0.567	0.00354	19.6
C (%)	35.7-59.8	0.033	4.42	25.2	35.7-54.1	0.036	4.21	27.1
N (%)	0.75-4.68	0.557	0.725	37.8	0.75-4.68	0.594	0.754	29.5

Figure captions

Fig. 1: Distributions of spectral reflectance and its coefficient of variation (CV) among fresh (A), pressed (B), and ground (C) leaf samples. A solid black line connects the median reflectance at each wavelength, around which dark blue and light blue ribbons denote the middle 50% and 95%. The solid red line shows the coefficient of variation among samples at each wavelength.

Fig. 2: Internal validation results for predictions of LMA, EWT, cellulose, and chlorophyll *a* from fresh- (left), pressed- (middle), and ground-leaf (right) spectra. In each panel, a separate ordinary least-squares regression line is shown for each functional group, overlaid on top of the thick dashed 1:1 line. The error bars for each data point are 95% confidence intervals calculated from the distribution of predictions based on the model coefficients from the 100 jackknife iterations. Figs. 2 and 3 display a subset of traits that represent various functions and span the range of model performance. Plots for remaining traits are shown in Supplementary Materials.

Fig. 3: Internal validation results for predictions of three elements (N, K, and Mn) from fresh- (left), pressed- (middle), and ground-leaf (right) spectra, displayed as in Fig. 2.

Fig. 4: The variable importance of projection (VIP) metric calculated based on fresh- (left), pressed- (middle), and ground-leaf (right) models. As in Figs. 2 and 3, we selected seven traits that represent a range of different patterns in VIP across the tissue types (cell = cellulose). The dashed horizontal line at 0.8 represents the heuristic threshold for importance recommended by Burnett et al. (2021). Plots for traits not shown here are found in Supplementary Materials.

Fig. 5: External validation results for predictions of various traits in Cedar Creek data based on models trained on CABO data. Panels are displayed as in Figs. 2 and 3.

Fig. 6: Confusion matrices for partial least-squares discriminant analysis from fresh- (A), pressed- (B), and ground-leaf (C) spectra. The row specifies the true species identity, while the column specifies the model's prediction. Numbers on the diagonal represent correctly classified species, while off-diagonal numbers represent misclassified species. Full binomials: *Agonis flexuosa*, *Acer rubrum* L., *A. saccharinum* L., *A. saccharum* Marshall, *Betula papyrifera* Marshall, *B. populifolia* Marshall, *Fagus grandifolia* Ehrh., *Populus grandidentata* Michx., *Populus tremuloides* Michx., and *Quercus rubra* L.

Figures

Fig. 1

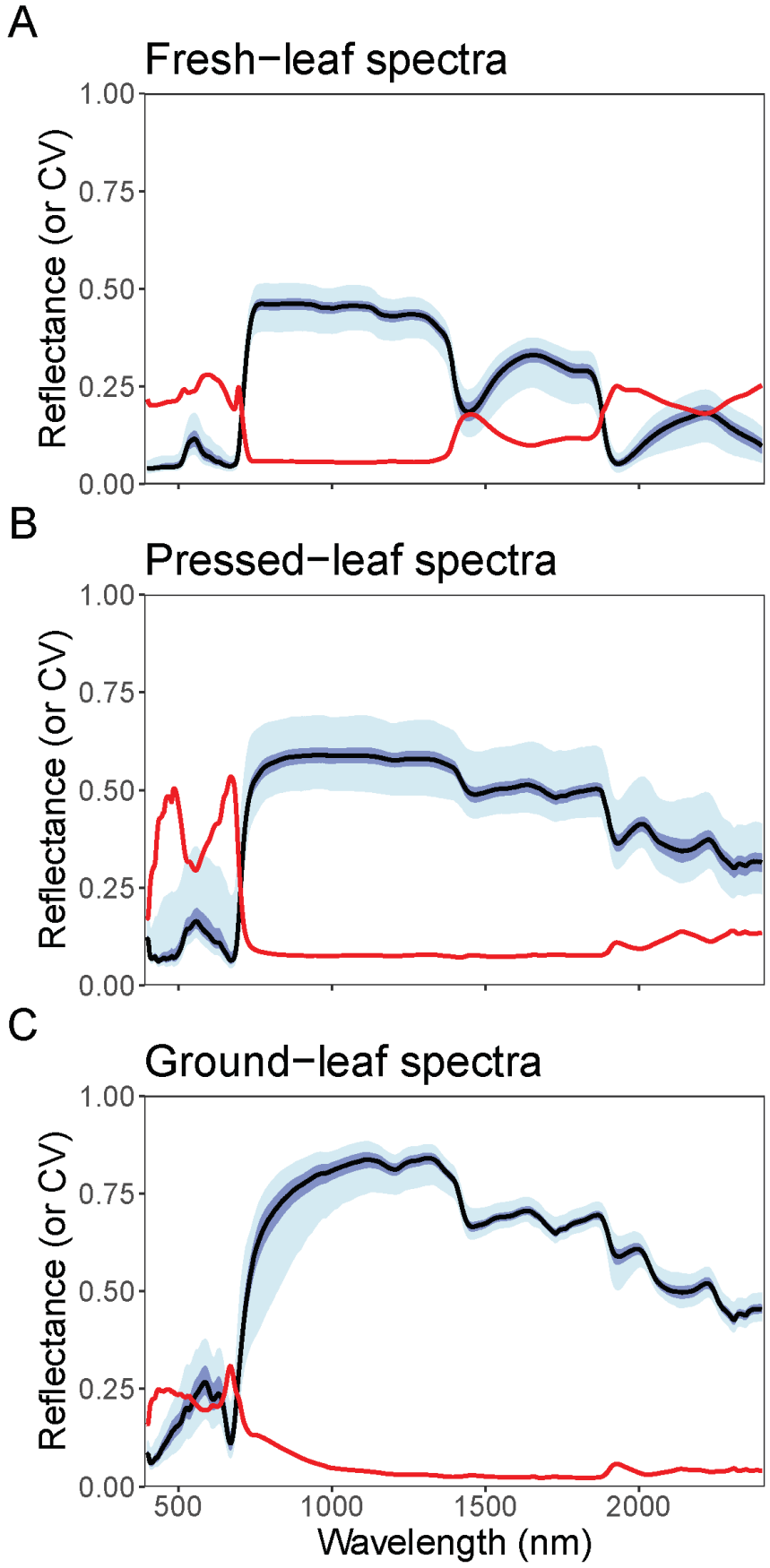


Fig. 2

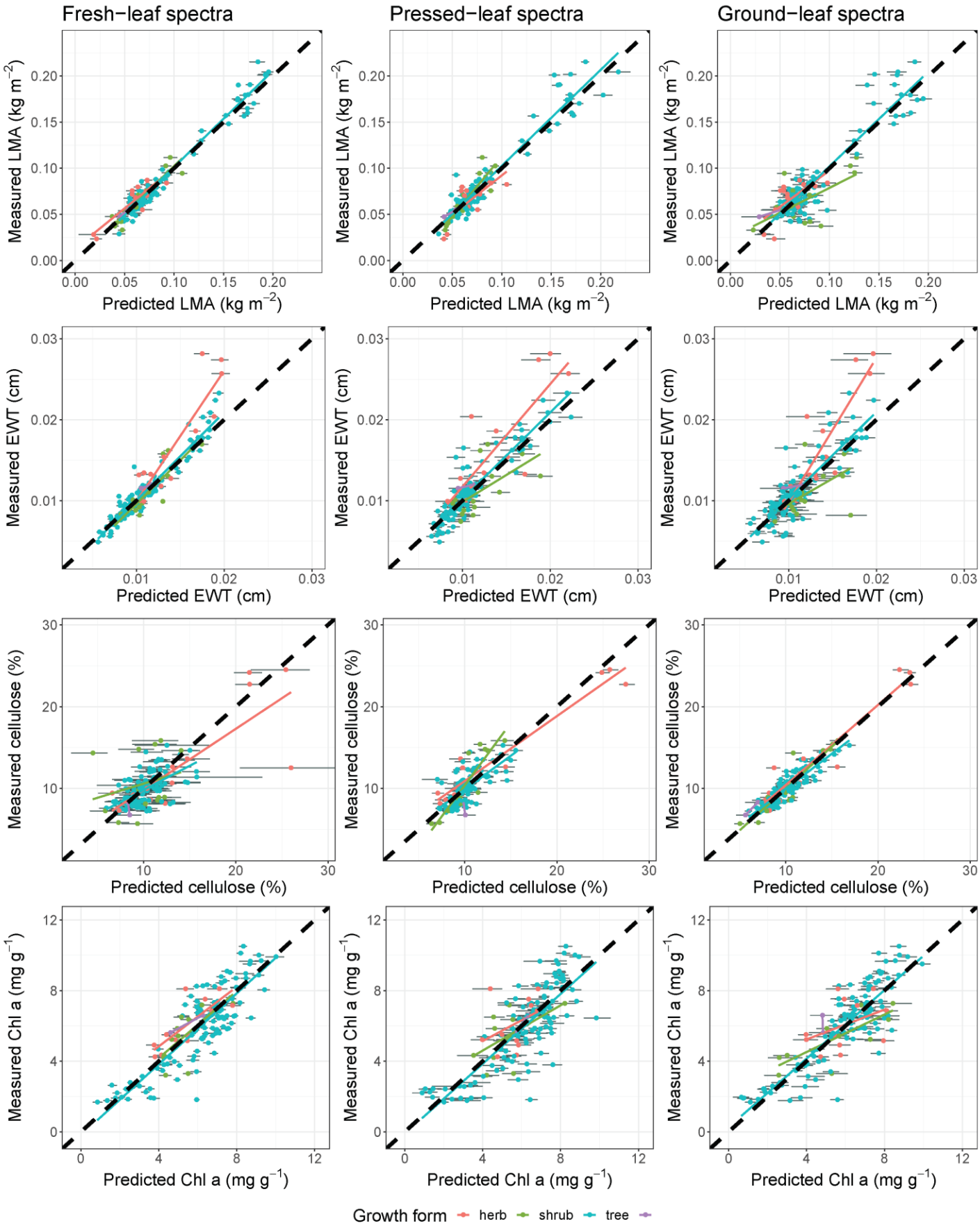

Fresh−leaf spectra
Pressed−leaf spectra
Ground−leaf spectra
Measured LMA (kg m−2)
Predicted LMA (kg m−2)
Measured EWT (cm)
Predicted EWT (cm)
Measured cellulose (%)
Predicted cellulose (%)
Measured Chl a (mg g−1)
Predicted Chl a (mg g−1)
Growth form
herb
shrub
tree

Fig. 3

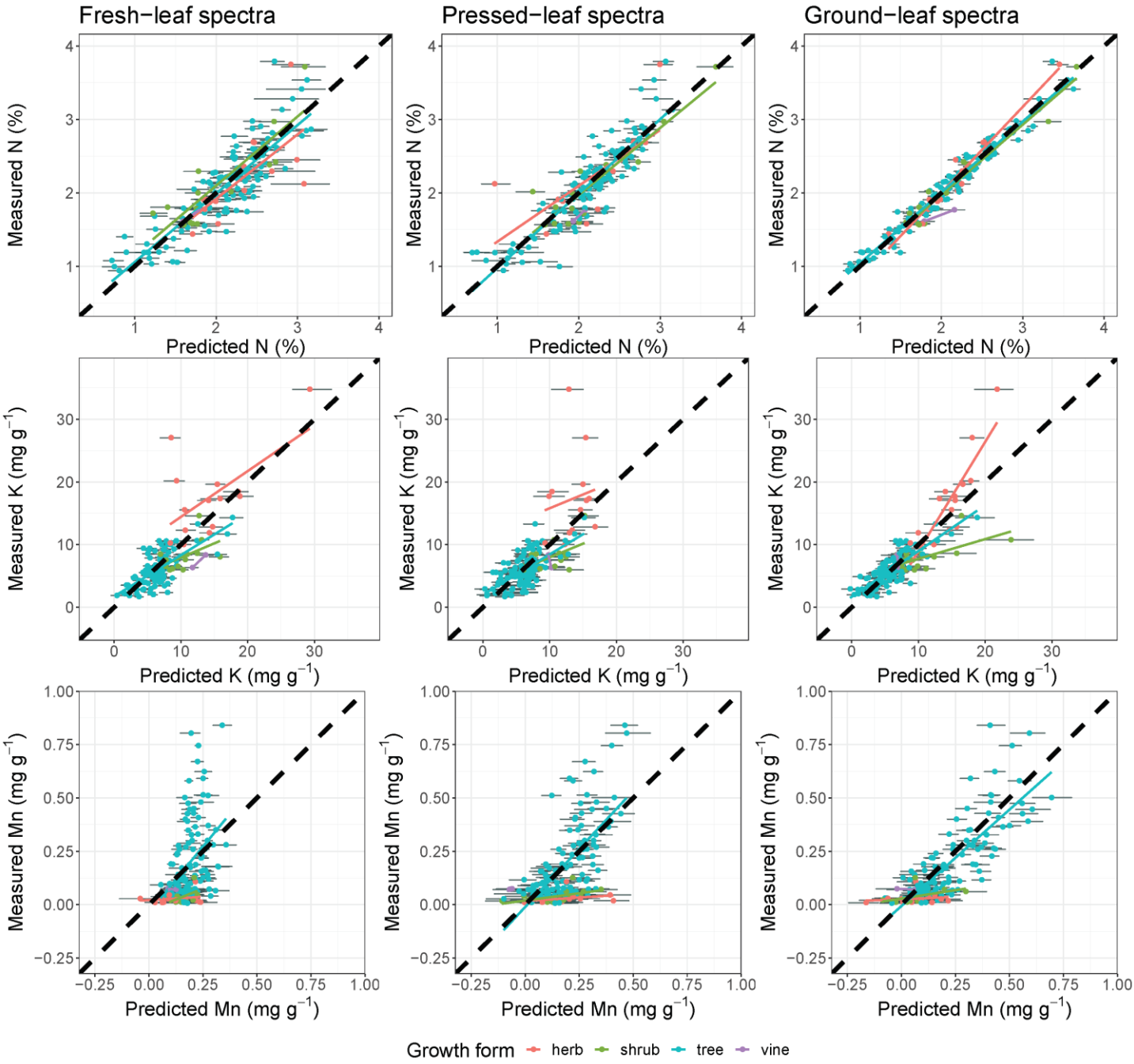

Fresh−leaf spectra
Pressed−leaf spectra
Ground−leaf spectra
Measured N (%)
Predicted N (%)
Measured K (mg g^{-1})
Predicted K (mg g^{-1})
Measured Mn (mg g^{-1})
Predicted Mn (mg g^{-1})
Growth form
herb
shrub
tree
vine

Fig. 4

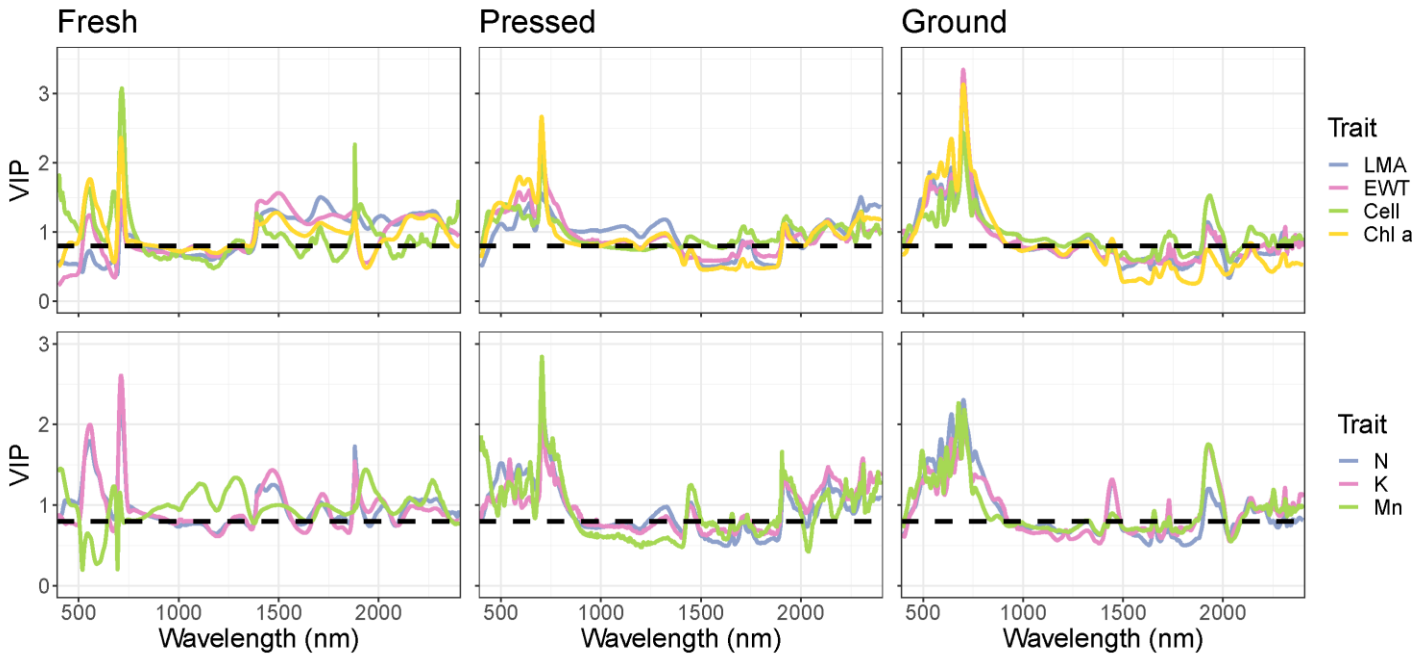

Fresh
Pressed
Ground
VIP
Wavelength (nm)
Trait
LMA
EWT
Cell
Chl a
N
K
Mn

Fig. 5

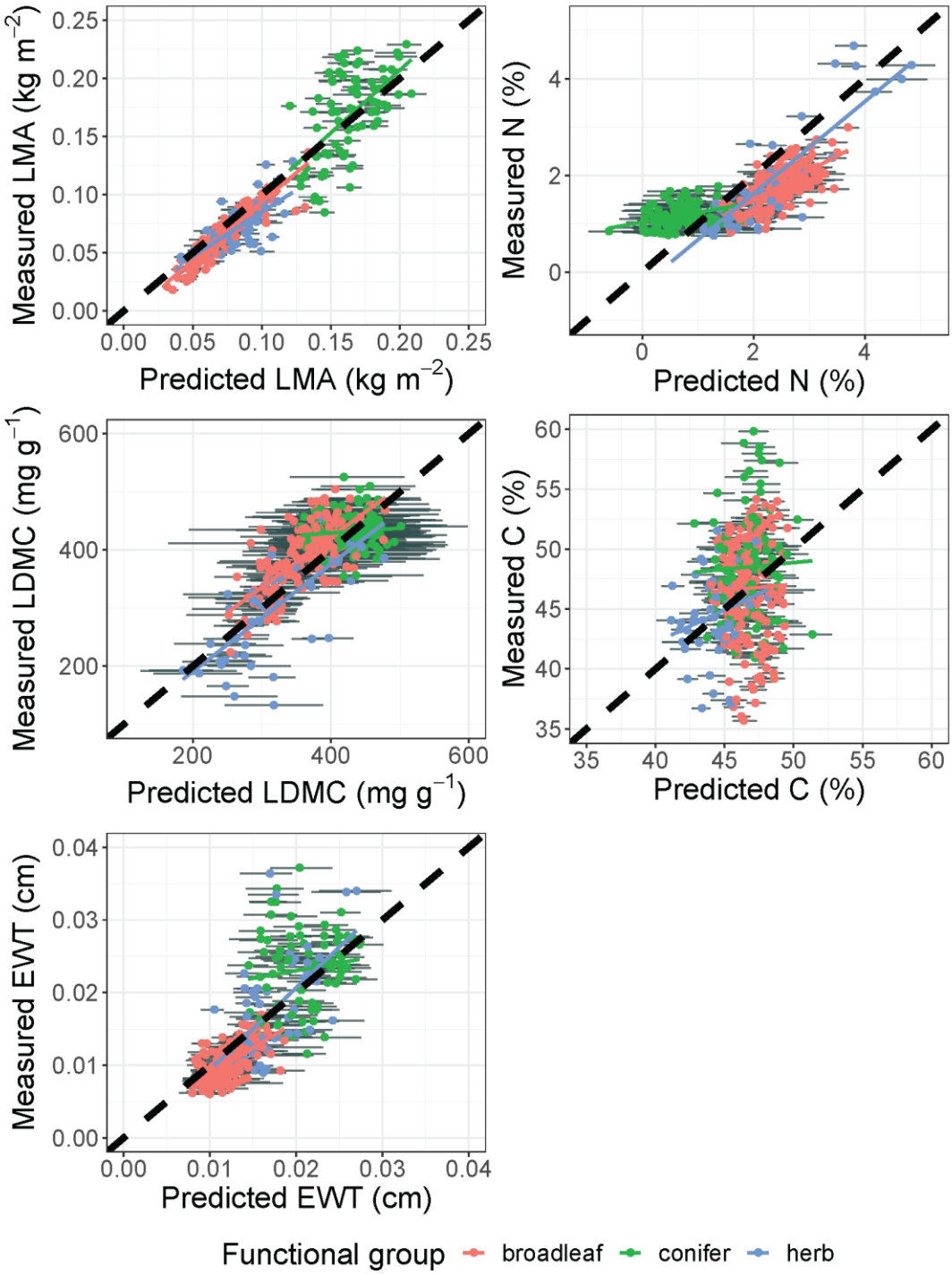
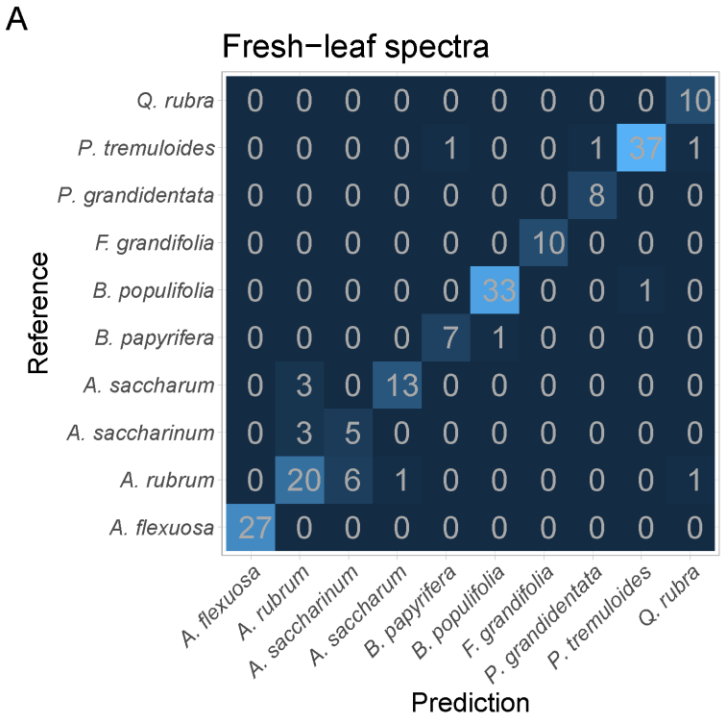
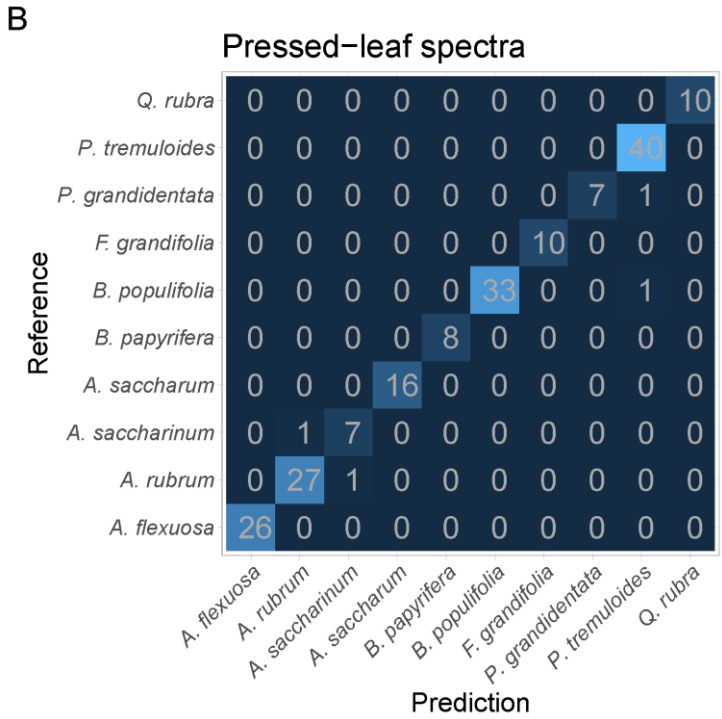
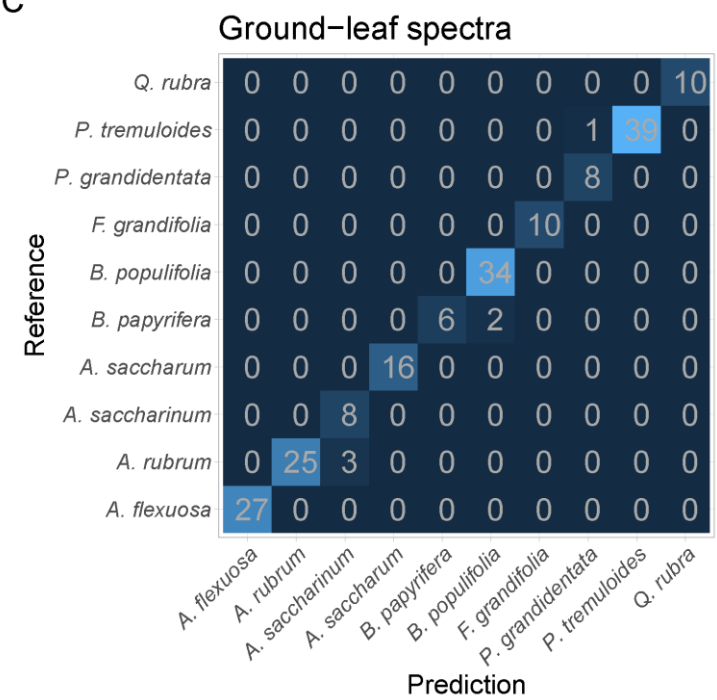

Measured LMA (kg m^{-2})
Predicted LMA (kg m^{-2})
Measured N (%)
Predicted N (%)
Measured LDMC (mg g^{-1})
Predicted LDMC (mg g^{-1})
Measured C (%)
Predicted C (%)
Measured EWT (cm)
Predicted EWT (cm)
Functional group
broadleaf
conifer
herb

Fig. 6


A
Fresh−leaf spectra
Reference
Prediction
Q. rubra 0 0 0 0 0 0 0 0 0 10
P. tremuloides 0 0 0 0 1 0 0 1 37 1
P. grandidentata 0 0 0 0 0 0 0 8 0 0
F. grandifolia 0 0 0 0 0 0 10 0 0 0
B. populifolia 0 0 0 0 0 33 0 0 1 0
B. papyrifera 0 0 0 0 7 1 0 0 0 0
A. saccharum 0 3 0 13 0 0 0 0 0 0
A. saccharinum 0 3 5 0 0 0 0 0 0 0
A. rubrum 0 20 6 1 0 0 0 0 0 1
A. flexuosa 27 0 0 0 0 0 0 0 0 0
A. flexuosa
A. rubrum
A. saccharinum
A. saccharum
B. papyrifera
B. populifolia
F. grandifolia
P. grandidentata
P. tremuloides
Q. rubra


B
Pressed−leaf spectra
Reference
Prediction
Q. rubra 0 0 0 0 0 0 0 0 0 10
P. tremuloides 0 0 0 0 0 0 0 0 40 0
P. grandidentata 0 0 0 0 0 0 0 7 1 0
F. grandifolia 0 0 0 0 0 0 10 0 0 0
B. populifolia 0 0 0 0 0 33 0 0 1 0
B. papyrifera 0 0 0 0 8 0 0 0 0 0
A. saccharum 0 0 0 16 0 0 0 0 0 0
A. saccharinum 0 1 7 0 0 0 0 0 0 0
A. rubrum 0 27 1 0 0 0 0 0 0 0
A. flexuosa 26 0 0 0 0 0 0 0 0 0
A. flexuosa
A. rubrum
A. saccharinum
A. saccharum
B. papyrifera
B. populifolia
F. grandifolia
P. grandidentata
P. tremuloides
Q. rubra

C


Ground−leaf spectra
Reference
Prediction
Q. rubra
P. tremuloides
P. grandidentata
F. grandifolia
B. populifolia
B. papyrifera
A. saccharum
A. saccharinum
A. rubrum
A. flexuosa
0 0 0 0 0 0 0 0 0 10
0 0 0 0 0 0 0 1 39 0
0 0 0 0 0 0 0 8 0 0
0 0 0 0 0 0 10 0 0 0
0 0 0 0 0 34 0 0 0 0
0 0 0 0 6 2 0 0 0 0
0 0 0 16 0 0 0 0 0 0
0 0 8 0 0 0 0 0 0 0
0 25 3 0 0 0 0 0 0 0
27 0 0 0 0 0 0 0 0 0

666

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Supplementary Materials

Sampling

For each sample, we collected a large group of sunlit leaves (>3 h estimated sun exposure per day) at about the same vertical position—for trees, the same branch or neighboring branches from the same individual. We avoided leaves with noticeable damage from herbivores or pathogens. We measured fresh-leaf spectra and fresh-leaf mass in the field, froze leaf disks for pigment analyses, and transported samples in a cooler to the lab. We then measured other whole-leaf traits and divided the remainder of the sample into two groups: (1) a portion to be pressed, and (2) a portion whose leaves were dried at 65 °C for three days, ground using a cyclone mill with 2 mm mesh, redried at 65 °C, and stored for chemical analyses. We measured pressed-leaf spectra on the former portion prior to mounting, and ground-leaf spectra and chemical traits on the latter. Although these are distinct subsets of leaves, we treat them as identical in their trait values since they are divided at random from the larger, relatively homogeneous bulk sample. A minimum of one representative pressed specimen of each species at each site and date is prepared as a specimen at the Marie-Victorin Herbarium in Montréal, Québec, Canada.

We classified species' growth forms following the Database of Vascular Plants of Canada (VASCAN; Desmet & Brouillet 2013). We classified *Agonis flexuosa* as a tree and manually disambiguated some species listed as adopting either shrub or three growth forms.

Spectral measurements

For the Beauchamp-Rioux, Boucherville, and Warren projects, we measured fresh-leaf directional-hemispherical reflectance spectra on each sample in 2018 using an HR-1024i spectroradiometer outfitted with a DC-R/T integrating sphere from Spectra Vista Corporation (SVC; Poughkeepsie, NY, USA). We measured spectra on the adaxial surface of six leaves or, for small or narrow leaves, single-layer leaf arrays following the protocol in Noda et al. (2013) as adapted for our instrument (Laliberté & Soffer

2018a; Laliberté and Soffer 2018b). For the Dessain project, we measured fresh-leaf reflectance spectra in 2017 using a FieldSpec 4 spectroradiometer equipped with an RTS-3ZC integrating sphere from Analytical Spectral Devices, Inc. (ASD; Boulder, CO, USA) following the leaf spectroscopy protocol from the Global Airborne Observatory (https://gao.asu.edu/spectranomics-protocols). We collected spectra from a variable number of leaves or arrays per sample—six in most cases, but as few as three or as many as eighteen from certain samples. For all projects, each reflectance measurement was calibrated against a white Spectralon 99% reflectance panel and corrected for stray light.

For all projects, we measured reflectance spectra on the adaxial surface of pressed, intact leaf samples using a PSR+ 3500 spectroradiometer with a leaf clip foreoptic (Spectral Evolution, Haverhill, MA) between six months and three years after collection. We calibrated reflectance against a white Spectralon reflectance panel and measured 3-7 spectra per sample. Although all samples were pressed in a plant press, some nevertheless dried in a way that created an uneven leaf surface. We noted any visible discoloration that samples underwent during drying or storage, including browning or blackening. In general, we aimed to avoid uneven or discolored areas when measuring spectra, although it was sometimes not entirely possible when discoloration was pervasive.

For all projects, we also measured ground-leaf spectra using the PSR+ 3500 spectroradiometer with a benchtop reflectance probe foreoptic that pressed loose powder into a smooth, even pellet. For each sample, we added at least 0.6 g of leaf powder into a sample tray; in preliminary tests, adding additional material did not change the spectra, suggesting that transmittance was close to zero. We calibrated reflectance against a white Spectralon reflectance panel placed in an identical sample tray. We measured three spectra per sample on the same pellet, turning the sample tray 120° between the first two spectra, and loosening and mixing the powder before reshaping the pellet between the second and third spectra.

A small number of specimens did not have usable spectra at all three stages, usually either due to lack of enough pressed or ground material or to poor spectral quality at any stage. To ensure fair comparisons among the stages, we removed any sample from the dataset that was not available for at least two stages. Among the remaining samples, any sample that was not available for all three stages (<4%) was placed in the training subset for PLSR analyses.

Our spectral processing pipeline varied by the type of data. Although the actual spectral resolution was more variable and coarser, the ASD Field Spec 4 and Spectral Evolution PSR+ 3500 spectrometers' software automatically resampled the spectra to 1 nm resolution and interpolated over the overlap region between sensors (Schweiger & Laliberté 2020). For data from the SVC HR-1024i spectrometer, we performed these steps using linear interpolation. For pressed-leaf spectra only, we detected jumps in reflectance at the sensor overlap region and removed them using the function *match_sensors()* in *R* package *spectrolab v. 0.0.10* (Meireles et al. 2017). Next, we averaged all spectra for a given sample and tissue type (fresh, pressed, or ground). Spectra measured with integrating spheres tend to have greater noise, especially at the ends. To reduce noise in fresh-leaf spectra, we applied a Savitzky-Golay filter using *R* package *signal 0.7.6* (Signal Developers, 2013), with varying order and length: order 3 and length 21 from 350-715 nm, order 3 and length 35 from 715-1390 nm, order 3 and length 75 from 1390-1880 nm, and order 5 and length 175 from 1880-2500 nm. Finally, we trimmed all spectra to 400-2400 nm, removing the extremes where sensors show greater noise. We did all spectral processing in *R v. 3.6.3* (R Core Team 2020) using *spectrolab v. 0.0.10* (Meireles et al. 2017).

Leaf trait measurements

We performed all leaf trait measurements excluding petioles, but including the rachis for compound leaves, since the rachis is functionally analogous to the midrib of a simple leaf. We measured the following leaf structural and chemical traits: Leaf mass per area (LMA; kg m^{-2}), leaf dry matter content (LDMC; mg g^{-1}), equivalent water thickness (EWT; cm), carbon fractions (soluble cell contents,

hemicellulose, cellulose, and lignin) on a mass basis, pigments (chlorophyll *a*, chlorophyll *b*, and total carotenoids) on a mass basis, and concentrations of a variety of elements (Al, C, Ca, Cu, Fe, K, Mg, Mn, N, Na, P, Zn).

From a subset of the leaves for each sample whose fresh-leaf spectra we measured, we used a 7 mm cork borer to punch leaf disks (or cut fragments of narrow leaves) in the field and stored them on ice in the darkness before transferring them to a -80 °C freezer in the lab. We extracted pigments under darkness in an $MgCO_3$-MeOH solution and estimated pigment concentrations using a spectrophotometric protocol on a SPECTROstar Nano microplate reader (BMG LABTECH, Ortenburg, Germany; Girard et al. 2020).

From a separate subset of leaves from each sample, we weighed fresh mass in the field shortly after collection. We then rehydrated the leaves in sealed plastic bags with damp paper towels for at least 12 h and weighed them for rehydrated mass. We scanned leaves and estimated their area using WinFOLIA (Regent Instruments, Québec, QC, CA). Lastly, we dried them for at least 72 h in a drying oven at 65°C before reweighing for dry mass. We calculated LDMC as total dry mass divided by total fresh mass and LMA as total dry mass divided by total rehydrated area (Laliberté 2018). We further calculated EWT from LDMC and LMA.

We analyzed ground bulk tissue samples for N and C using a Vario MICRO Cube combustion analyzer (Elementar, Langenselbold, Germany; Ayotte et al. 2019). We analyzed the same samples for other elements (Al, Ca, Cu, Fe, K, Mg, Mn, Na, P, Zn) using inductively coupled plasma-mass spectrometry (ICP-MS). We removed five data points from the ICP-MS output as extreme outliers that were not representative of the range of concentrations of their respective elements across our samples. We also set values below the detection limit, which sometimes returned as negative concentrations, to zero.

Finally, we analyzed the samples for carbon fractions using an ANKOM 2000 Fiber Analyzer (Ankom Technology, Macedon, New York, USA) to perform a sequence of digestions (Ayotte & Laliberté 2019). The first digestion in a heated neutral detergent washes off soluble cell contents. The second digestion in a heated acidic detergent removes hemicellulose and bound proteins—which, for simplicity, we just refer to as hemicellulose. The third digestion in 70% sulfuric acid removes cellulose, leaving behind lignin and recalcitrant compounds. We used the change in each bag's mass between stages to determine the mass of each fraction. We ashed all samples and used ash content as a correction factor to calculate the ash-free lignin fraction.

External validation dataset

From August to November 2018, we collected 333 samples for an external validation dataset at Cedar Creek Ecosystem Science Reserve (East Bethel, MN, USA). These samples included broadleaf trees (n = 177; 10 species), coniferous needleleaf trees (n = 101; 4 species), and herbs (n = 55; 14 species). Most samples were from the Forests and Biodiversity (FAB) experiment (Grossman et al. 2017), the Biodiversity in Willows and Poplars (BiWaP) experiment (Grossman & Cavender-Bares 2019), and the Big Biodiversity (BioDIV or e120) experiment (Tilman et al. 1997), although some were from other parts of the site.

Each sample included multiple leaves from the same individual, taken when possible from a nearby position on the plant. Unlike in the CABO dataset, we collected samples from any part of an individual, not just sunlit leaves. From each sample, we designated one leaf for fresh mass to be measured immediately following collection; this leaf ('fresh-mass subsample') was stored separately and its spectra were labeled to keep track of its identity. (For conifers or other plants with small or narrow leaves, we often included multiple leaves in this fresh mass sample.) We took and flash-froze 3-5 hole punches from another leaf (or group of leaves) for pigment analyses ('pigment subsample'; pigment data not shown);

this leaf and its spectra were also labeled separately. We collected more leaves to have enough tissue for a bulk chemical sample, but did not keep track of them individually.

In the field, we measured multiple fresh-leaf spectra per sample (not shown here) using a PSR+ 3500 spectroradiometer with a leaf clip foreoptic (Spectral Evolution, Haverhill, MA) and placed the leaves in sealed plastic bags with a damp paper towel. These bags were kept in coolers until the end of the sampling day. We weighed the fresh-mass subsample immediately upon return to the lab and kept all leaves in a refrigerator until we scanned the fresh-mass and pigment subsamples within the next few days. After scanning, we placed all leaves in flat paper bags and pressed them for 2-3 hours in a plant press before removing them and placing them in a drying shed at 40 °C for at least one week. This deviates from most standard herbarium protocols, including the one used in the CABO project, where leaves are kept in the press while drying and subjected to a higher temperature for a shorter time. After we dried the leaves, we weighed the fresh-mass and pigment subsamples. Certain species, especially *Populus tremuloides*, seemed especially prone to discoloration during sample preparation.

In May 2020, we measured pressed-leaf spectra using a PSR+ 3500 spectroradiometer with a leaf clip foreoptic. We did not use a specific protocol for small or narrow leaves, but aimed to arrange leaves in a way that minimized overlaps or gaps. We removed spectra with especially low (<40%) or high (>80%) peak reflectance, and any with major visible gaps in the sensor overlap regions. We measured three spectra per subsample except in cases where getting high-quality spectra proved especially difficult. We then removed the petioles (when applicable) and put leaves in scintillation vials with ball bearings to grind the tissue in a paint shaker, combining all subsamples from a given sample. We then dried samples again and measured C and N with an elemental analyzer (Costech ECS 4010 Analyzer, Valencia, California, USA).

For estimating LMA, LDMC, and EWT, we only used the spectra from the fresh-mass subsample. For C and N, we used all spectra from a given sample, ignoring the subsample labels. As among the CABO pressed-leaf spectra, we first matched sensors, then averaged spectra within the relevant sample or subsample, and lastly trimmed them to 400-2400 nm.

We applied the ensemble of models resulting from our 100× jackknife analysis on the pressed-leaf CABO data and applied them to the Cedar Creek data. We also applied the model ensemble from the pressed-leaf CABO data restricted to 1300-2400 nm to the Cedar Creek data restricted to the same range. We calculated summary statistics and plotted results as in the internal validation.

Supplementary references

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Supplementary tables

Table S1: Summary statistics of PLSR model internal validation from pressed-leaf spectra restricted to 1300-2500 nm. %RMSE is calculated as RMSE divided by the 2.5% trimmed range of data within either the training data set (calibration) or the testing data set (validation).

Trait	No. comps	R^2	RMSE	%RMSE
LMA (kg m^{-2})	7	0.902	0.0125	7.94
LDMC (mg g^{-1})	10	0.694	37.6	12.8
EWT (cm)	10	0.767	0.00222	13.3
Solubles (%)	10	0.712	4.25	13.5
Hemicellulose (%)	10	0.733	2.48	15.6
Cellulose (%)	11	0.745	1.50	17.9
Lignin (%)	11	0.638	2.07	16.7
C (%)	11	0.811	0.987	10.8
N (%)	9	0.772	0.273	11.5
Chl *a* (mg g^{-1})	7	0.582	1.37	17.3
Chl *b* (mg g^{-1})	7	0.514	0.506	18.7
Carotenoids (mg g^{-1})	7	0.599	0.280	17.9
Al (mg g^{-1})	6	0.210	0.0420	24.7
Ca (mg g^{-1})	11	0.723	3.72	13.2
Cu (mg g^{-1})	8	0.243	0.00676	24.0
Fe (mg g^{-1})	4	0.223	0.0391	20.9
K (mg g^{-1})	11	0.566	2.93	18.6
Mg (mg g^{-1})	11	0.413	0.733	20.1
Mn (mg g^{-1})	10	0.368	0.140	22.4
Na (mg g^{-1})	12	0.434	0.577	20.6
P (mg g^{-1})	11	0.507	0.772	19.0
Zn (mg g^{-1})	9	0.295	0.0712	25.1

Table S2: Summary statistics for external validation of pressed-leaf PLSR models, restricting the range of spectra to 1300-2500 nm. The models were trained on CABO data (see Table S1) and applied to a dataset collected at Cedar Creek.

	Including conifers			Excluding conifers		
Trait	R^2	RMSE	%RMSE	R^2	RMSE	%RMSE
LMA (kg m^{-2})	0.867	0.0195	10.8	0.746	0.0111	13.7
LDMC (mg g^{-1})	0.468	51.2	18.1	0.461	53.4	18.5
EWT (cm)	0.608	0.00512	20.7	0.633	0.00318	17.6
C (%)	0.007	4.54	26.0	0.023	4.38	28.2
N (%)	0.622	0.666	34.7	0.614	0.772	30.3

Supplementary figure captions

Fig. S1: Internal validation results for predictions of LMA, LDMC, and EWT from fresh- (left), pressed- (middle), and ground-leaf (right) spectra. In each panel, a separate ordinary least-squares regression line is shown for each functional group, overlaid on top of the thick dashed 1:1 line. The error bars for each data point are 95% confidence intervals calculated from the distribution of predictions based on the model coefficients from the 100 jackknife iterations.

Fig. S2: Internal validation results for predictions of C, N, and P from fresh- (left), pressed- (middle), and ground-leaf (right) spectra, displayed as in Fig. S1.

Fig. S3: Internal validation results for predictions of soluble cell contents, hemicellulose, cellulose, and lignin from fresh- (left), pressed- (middle), and ground-leaf (right) spectra, displayed as in Fig. S1.

Fig. S4: Internal validation results for predictions of chlorophyll *a*, chlorophyll *b*, and total carotenoids from fresh- (left), pressed- (middle), and ground-leaf (right) spectra, displayed as in Fig. S1.

Fig. S5: Internal validation results for predictions of Al, Ca, and Cu from fresh- (left), pressed- (middle), and ground-leaf (right) spectra, displayed as in Fig. S1.

Fig. S6: Internal validation results for predictions of Fe, K, and Mg from fresh- (left), pressed- (middle), and ground-leaf (right) spectra, displayed as in Fig. S1

Fig. S7: Internal validation results for predictions of Mn, Na, and Zn from fresh- (left), pressed- (middle), and ground-leaf (right) spectra, displayed as in Fig. S1

Fig. S8: The variable importance of projection (VIP) metric calculated for all traits from fresh-leaf spectra. Abbreviations: sol = solubles, hemi = hemicellulose, cell = cellulose, lign = lignin, car = total carotenoids. The dashed horizontal line at 0.8 represents the heuristic threshold for importance recommended by Burnett et al. (2021).

Fig. S9: The variable importance of projection (VIP) metric calculated for all traits from pressed-leaf spectra. Abbreviations: sol = solubles, hemi = hemicellulose, cell = cellulose, lign = lignin, car = total carotenoids. The dashed horizontal line at 0.8 represents the heuristic threshold for importance recommended by Burnett et al. (2021).

Fig. S10: The variable importance of projection (VIP) metric calculated for all traits from ground-leaf spectra. Abbreviations: sol = solubles, hemi = hemicellulose, cell = cellulose, lign = lignin, car = total carotenoids. The dashed horizontal line at 0.8 represents the heuristic threshold for importance recommended by Burnett et al. (2021).

Fig. S11: The distributions of fresh-leaf spectral model performance statistics for each trait based on 100 jackknife resamples from the calibration data set. The red dots show the R^2 and %RMSE from applying the ensemble of models to the internal validation subset. Abbreviations: sol = solubles, hemi = hemicellulose, cell = cellulose, lign = lignin, car = total carotenoids.

Fig. S12: The distributions of pressed-leaf spectral model performance statistics for each trait based on 100 jackknife resamples from the calibration data set. The red dots show the R^2 and %RMSE from applying the ensemble of models to the internal validation subset. Abbreviations: sol = solubles, hemi = hemicellulose, cell = cellulose, lign = lignin, car = total carotenoids.

Fig. S13: The distributions of ground-leaf spectral model performance statistics for each trait based on 100 jackknife resamples from the calibration data set. The red dots show the R^2 and %RMSE from applying the ensemble of models to the internal validation subset. Abbreviations: sol = solubles, hemi = hemicellulose, cell = cellulose, lign = lignin, car = total carotenoids.

Fig. S14: External validation results for predictions of various traits in Cedar Creek data based on models trained on CABO data, restricted to 1300-2500 nm. Each panel is displayed as in Figs. S1-7.

Supplementary figures

Fig. S1

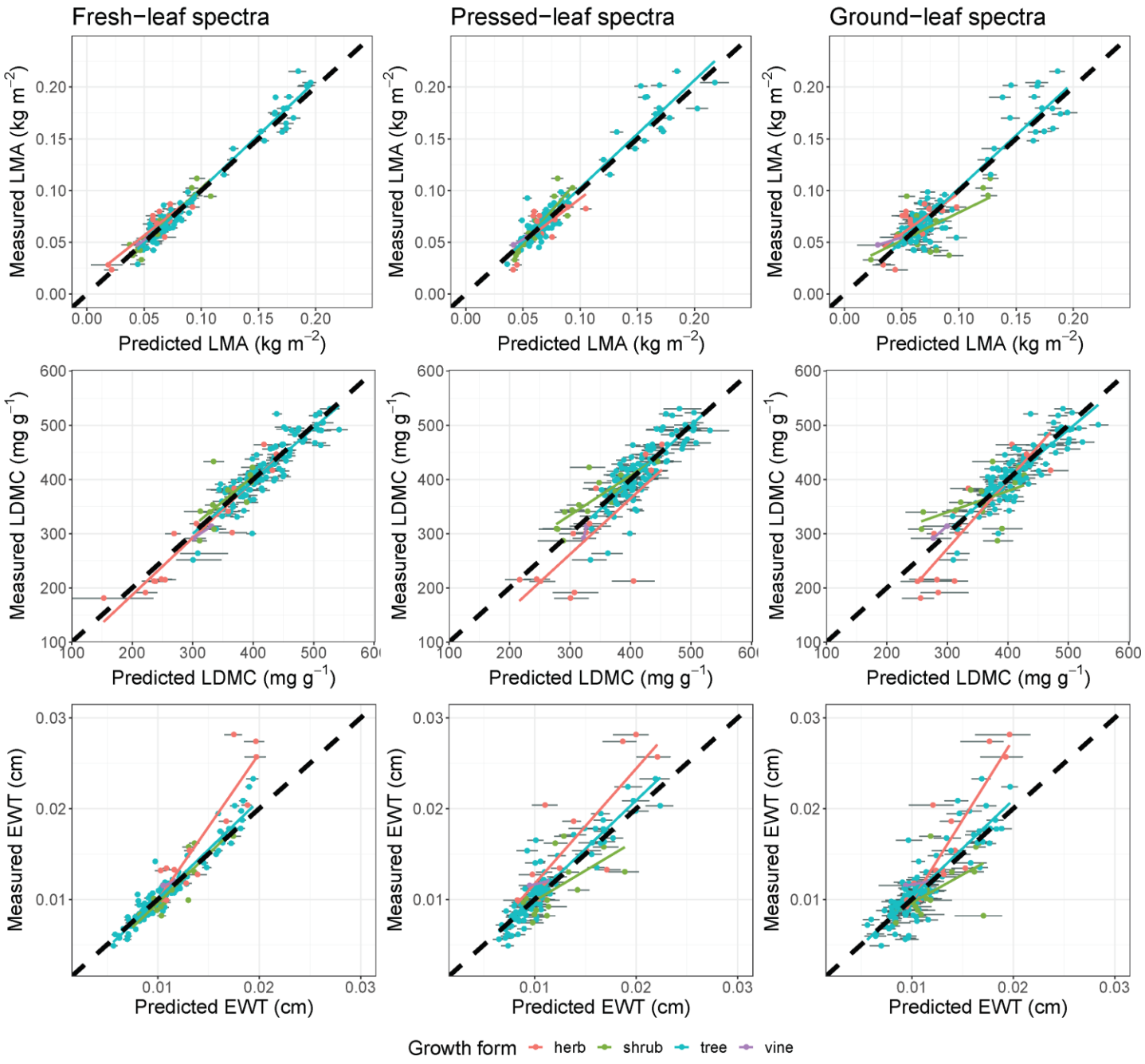


Fig. S2

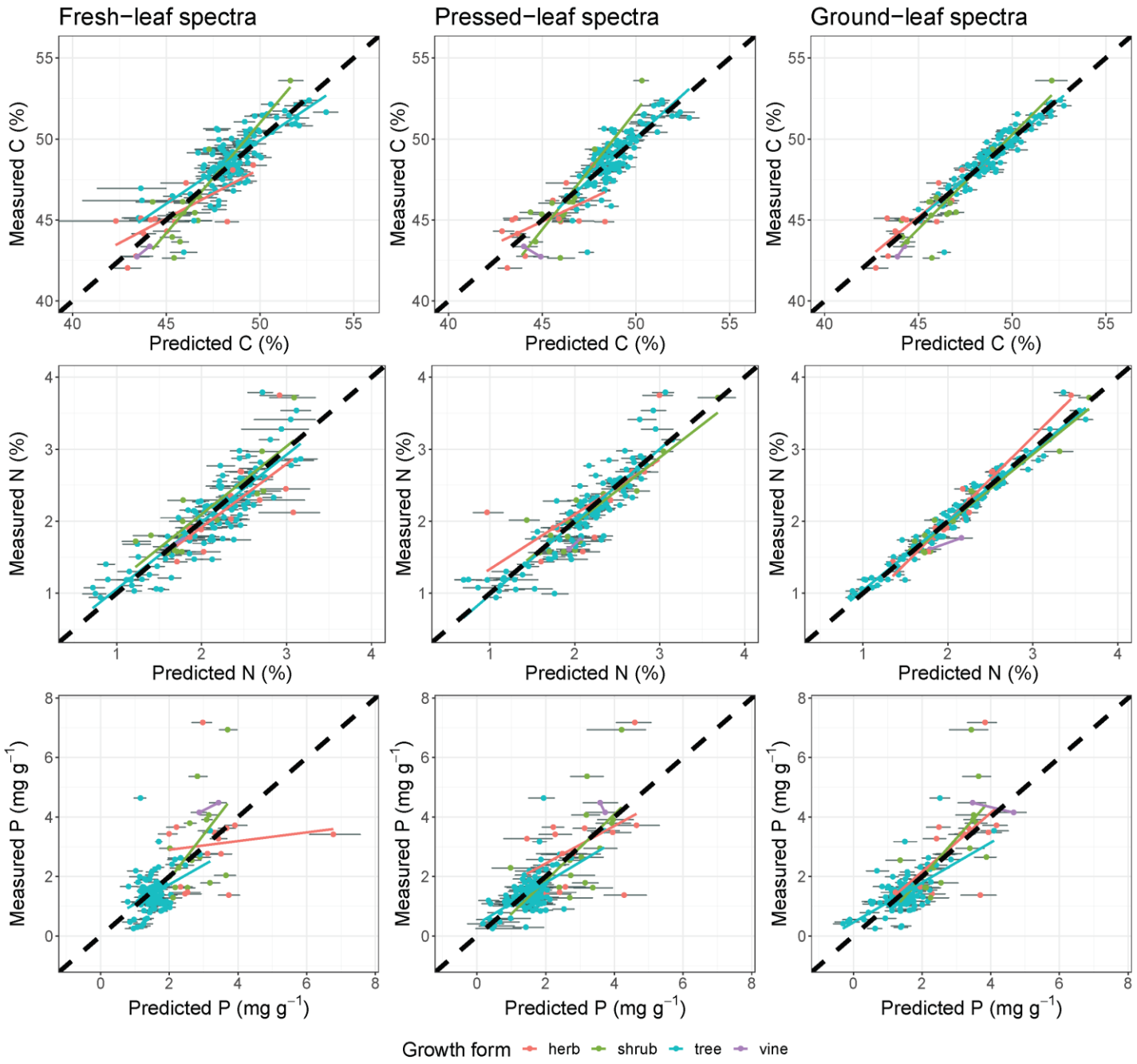

Fresh−leaf spectra
Pressed−leaf spectra
Ground−leaf spectra
Measured C (%)
Predicted C (%)
Measured N (%)
Predicted N (%)
Measured P (mg g^{-1})
Predicted P (mg g^{-1})
Growth form
herb
shrub
tree
vine

Fig. S3

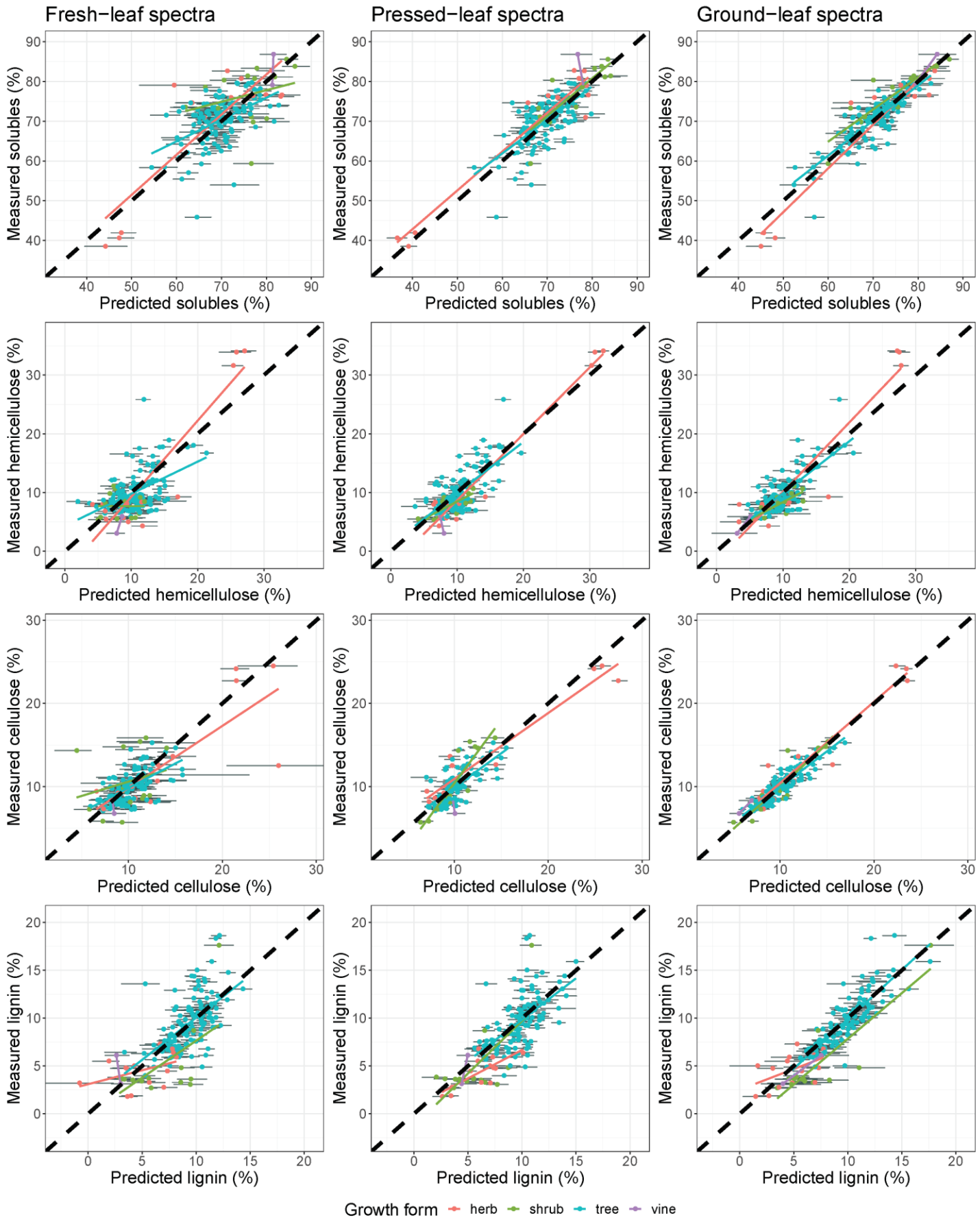

Fresh−leaf spectra
Pressed−leaf spectra
Ground−leaf spectra
Measured solubles (%)
Predicted solubles (%)
Measured hemicellulose (%)
Predicted hemicellulose (%)
Measured cellulose (%)
Predicted cellulose (%)
Measured lignin (%)
Predicted lignin (%)
Growth form
herb
shrub
tree
vine

Fig. S4

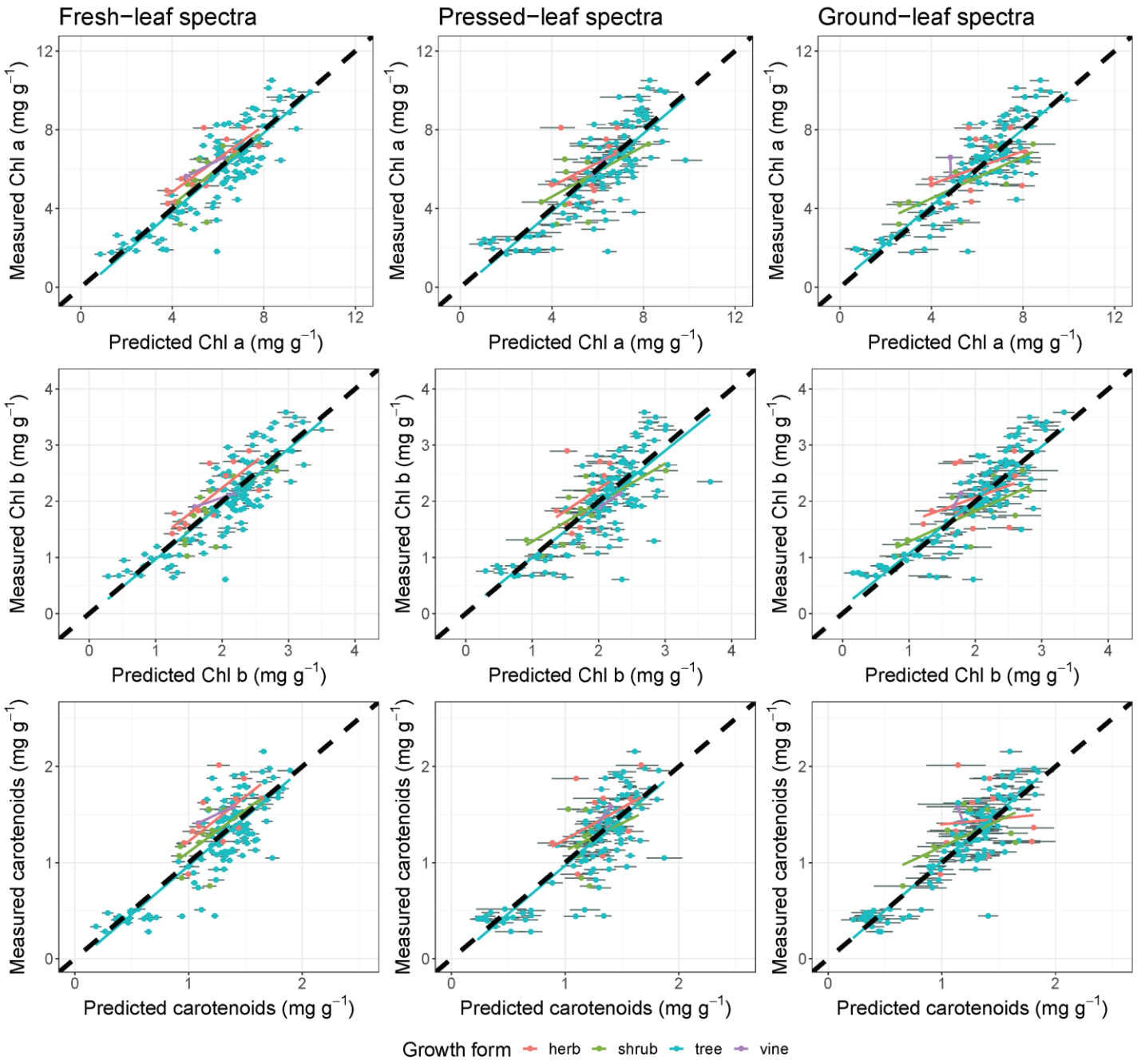

Fresh−leaf spectra
Pressed−leaf spectra
Ground−leaf spectra
Measured Chl a (mg g^{-1})
Predicted Chl a (mg g^{-1})
Measured Chl b (mg g^{-1})
Predicted Chl b (mg g^{-1})
Measured carotenoids (mg g^{-1})
Predicted carotenoids (mg g^{-1})
Growth form
herb
shrub
tree
vine

Fig. S5

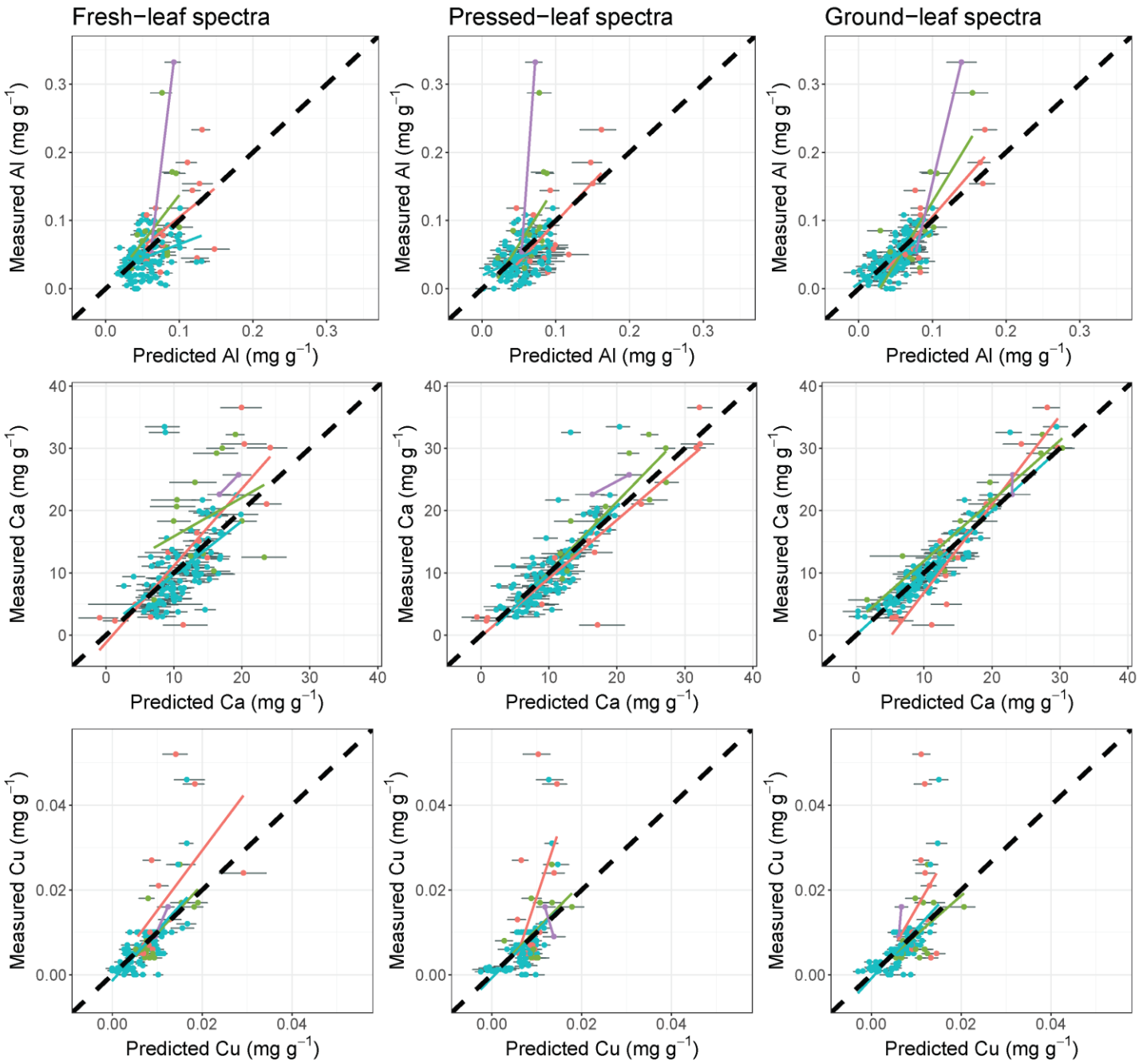

Fresh−leaf spectra
Pressed−leaf spectra
Ground−leaf spectra
Measured Al (mg g^{-1})
Predicted Al (mg g^{-1})
Measured Ca (mg g^{-1})
Predicted Ca (mg g^{-1})
Measured Cu (mg g^{-1})
Predicted Cu (mg g^{-1})

Fig. S6

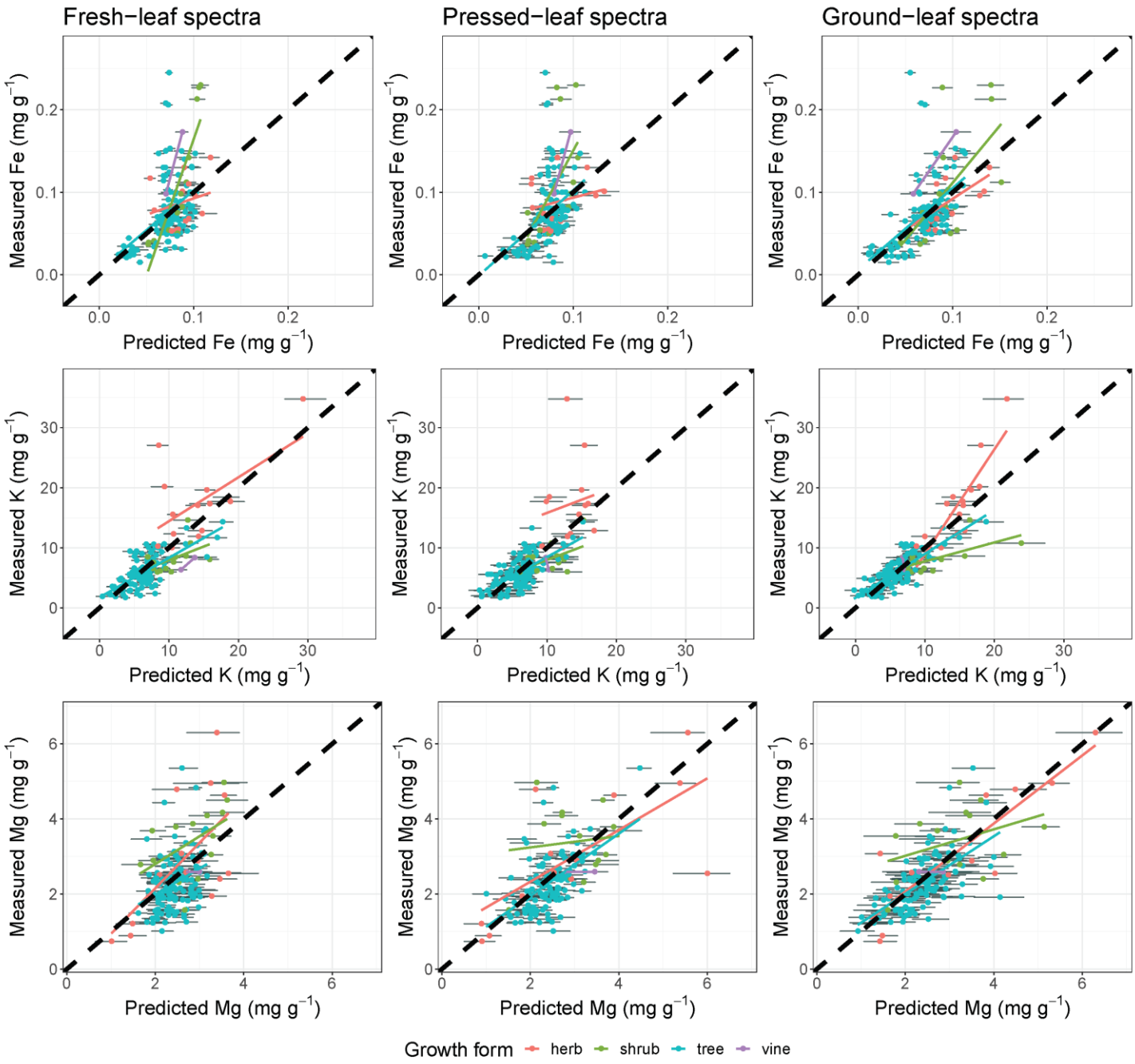

Fresh−leaf spectra
Pressed−leaf spectra
Ground−leaf spectra
Measured Fe (mg g^{-1})
Predicted Fe (mg g^{-1})
Measured K (mg g^{-1})
Predicted K (mg g^{-1})
Measured Mg (mg g^{-1})
Predicted Mg (mg g^{-1})
Growth form
herb
shrub
tree
vine

Fig. S7

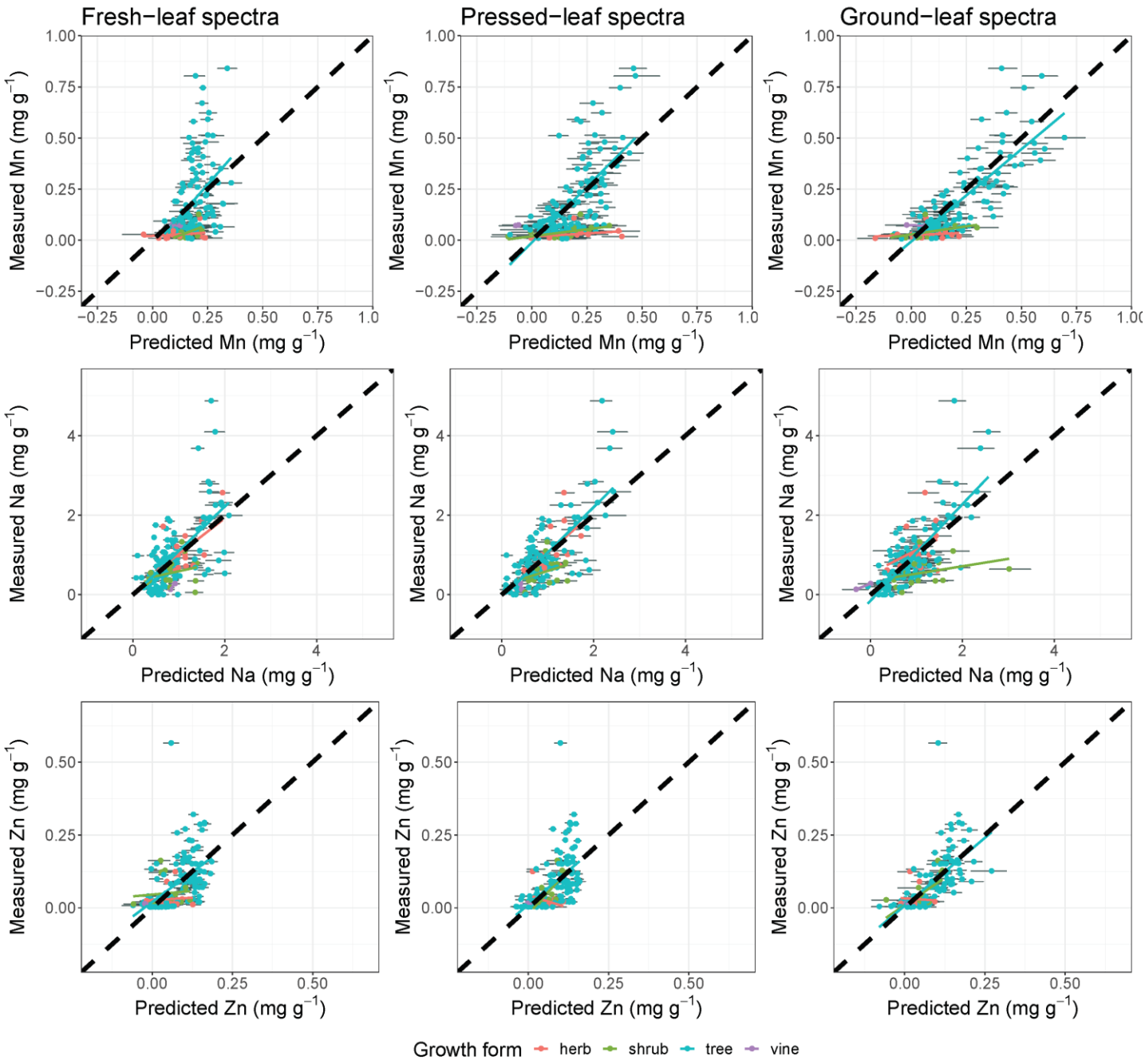

Fresh−leaf spectra
Pressed−leaf spectra
Ground−leaf spectra
Measured Mn (mg g^{-1})
Predicted Mn (mg g^{-1})
Measured Na (mg g^{-1})
Predicted Na (mg g^{-1})
Measured Zn (mg g^{-1})
Predicted Zn (mg g^{-1})
Growth form
herb
shrub
tree
vine

Fig. S8

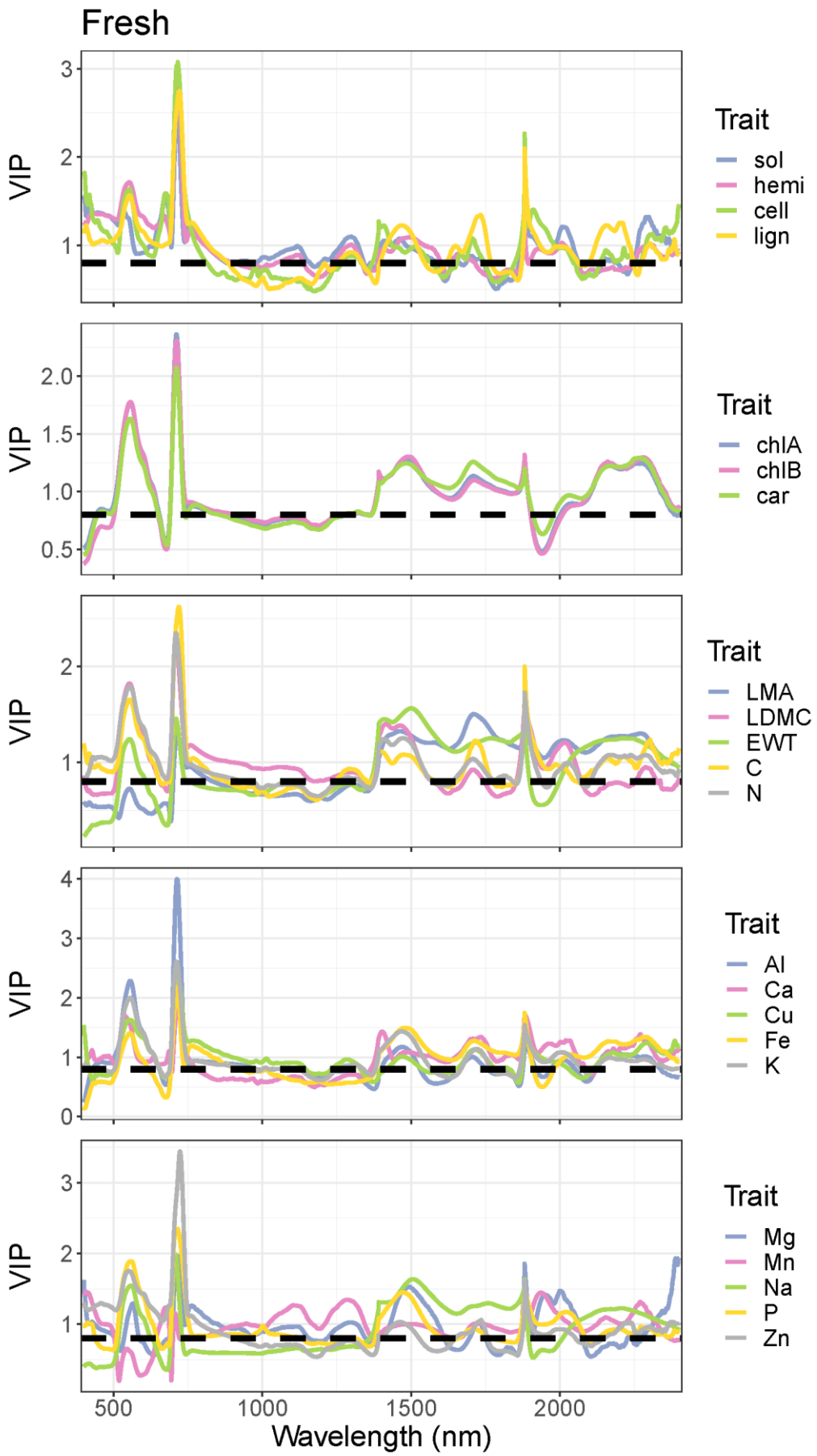
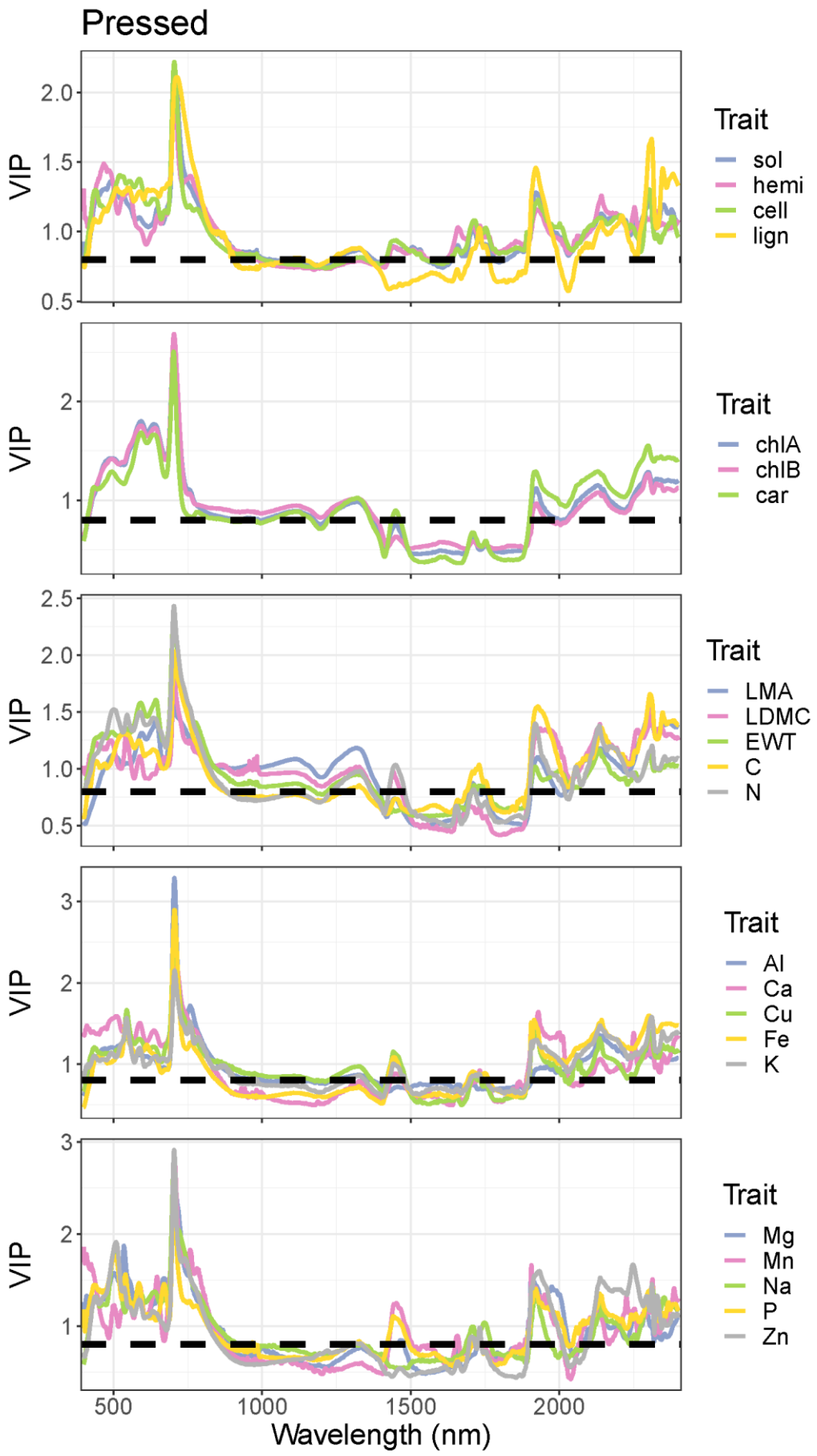

Fresh
VIP
Trait
sol
hemi
cell
lign
chlA
chlB
car
LMA
LDMC
EWT
C
N
Al
Ca
Cu
Fe
K
Mg
Mn
Na
P
Zn
500
1000
1500
2000
Wavelength (nm)

Fig. S9


Pressed
VIP
Trait
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car
LMA
LDMC
EWT
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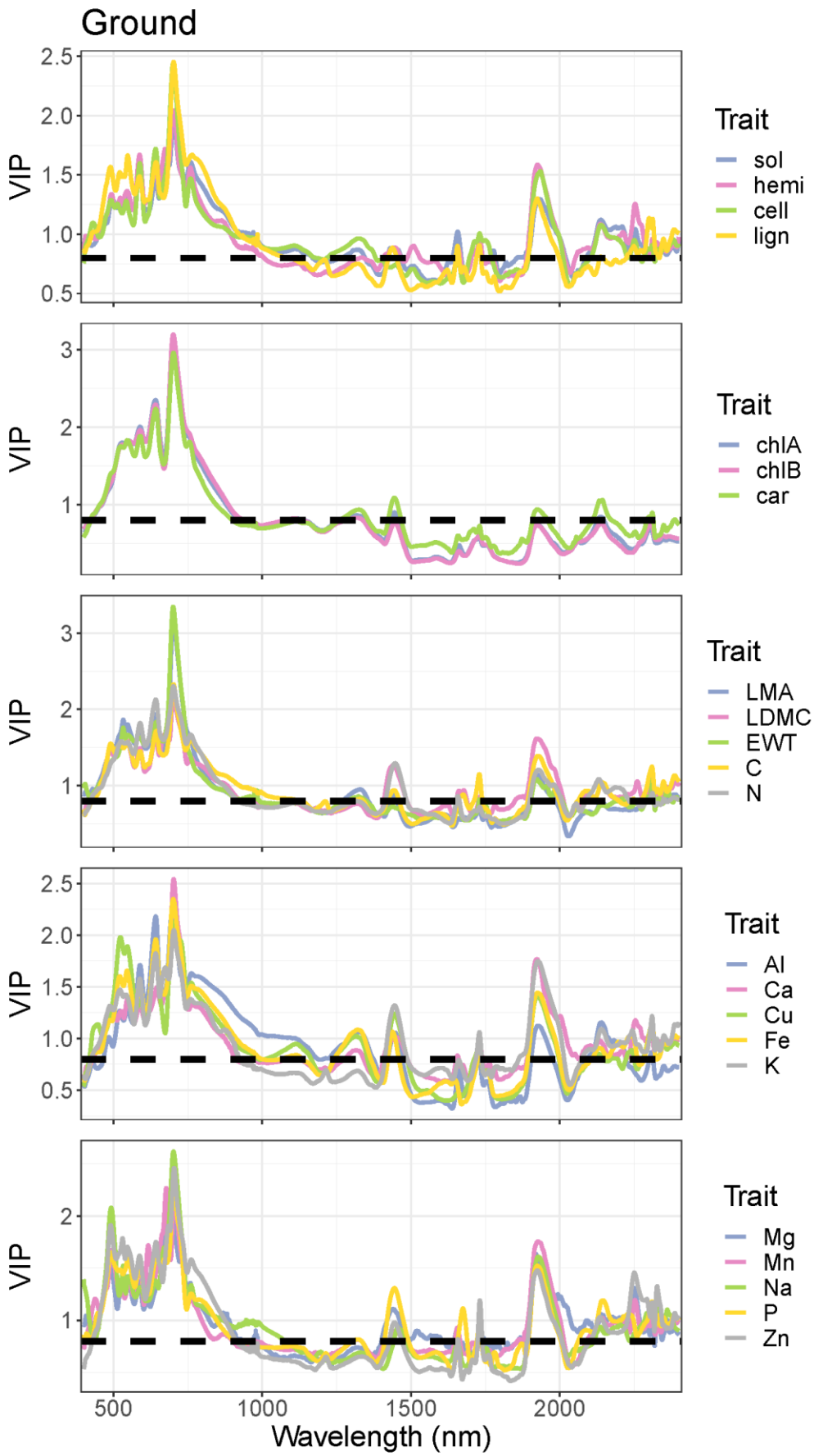
Cu
Fe
K
Mg
Mn
Na
P
Zn
500
1000
1500
2000
Wavelength (nm)

Fig. S10


Ground
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chlB
car
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LDMC
EWT
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Cu
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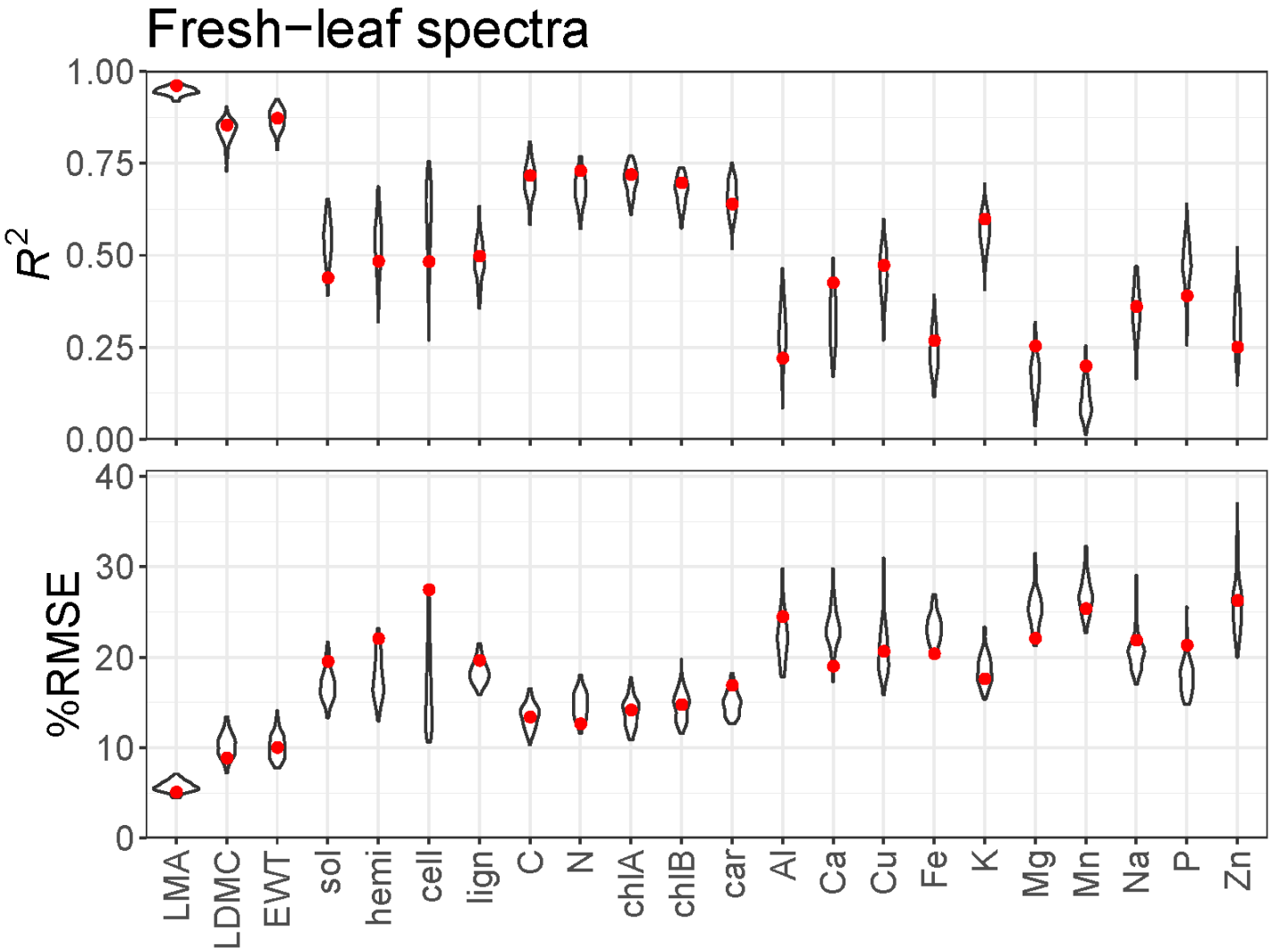
K
Mg
Mn
Na
P
Zn
500
1000
1500
2000
Wavelength (nm)

Fig. S11


Fresh−leaf spectra
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1.00
0.75
0.50
0.25
0.00
%RMSE
40
30
20
10
0
LMA
LDMC
EWT
sol
hemi
cell
lign
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N
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car
Al
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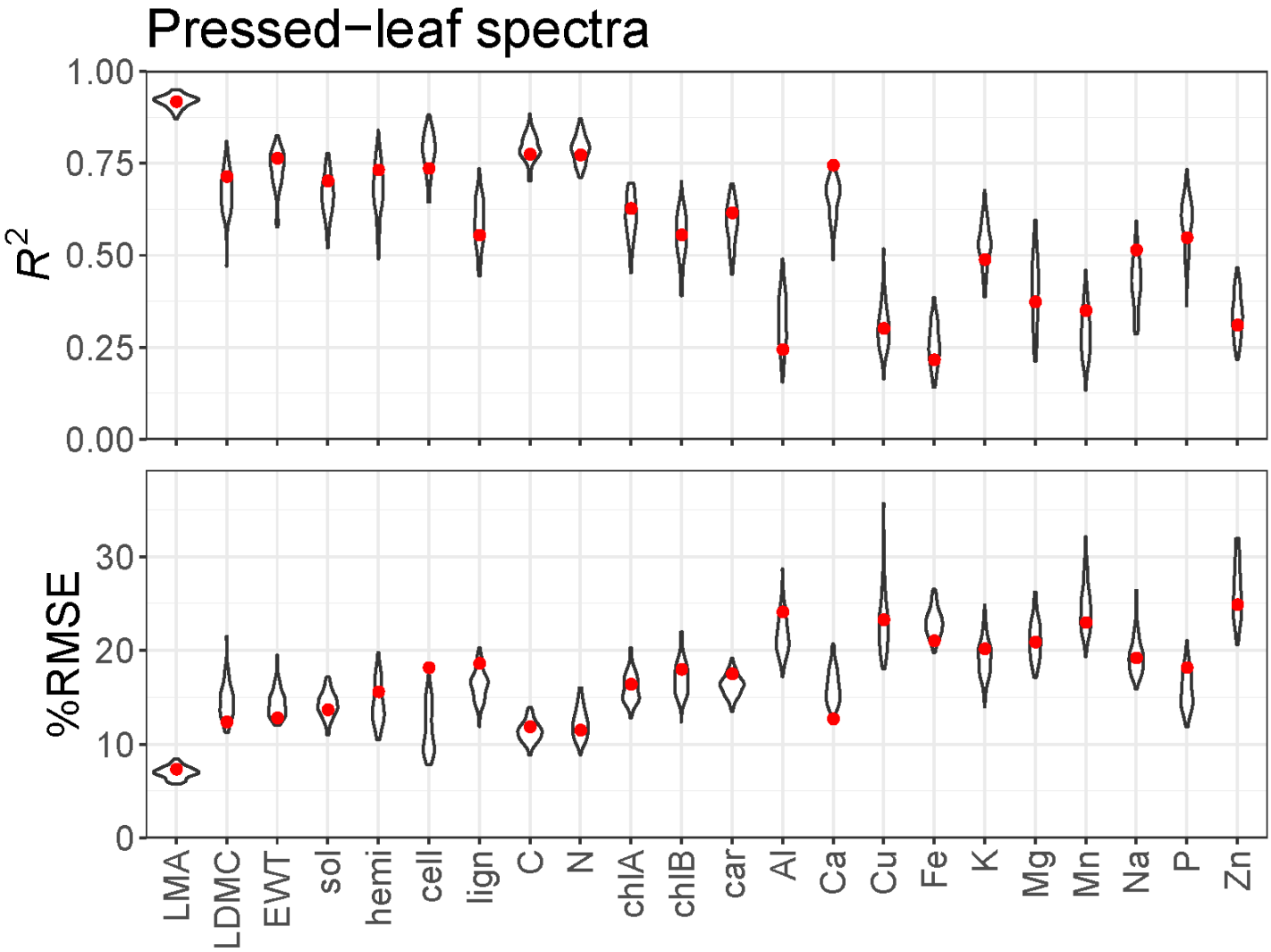
Fe
K
Mg
Mn
Na
P
Zn

Fig. S12


Pressed−leaf spectra
R^2
1.00
0.75
0.50
0.25
0.00
%RMSE
30
20
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sol
hemi
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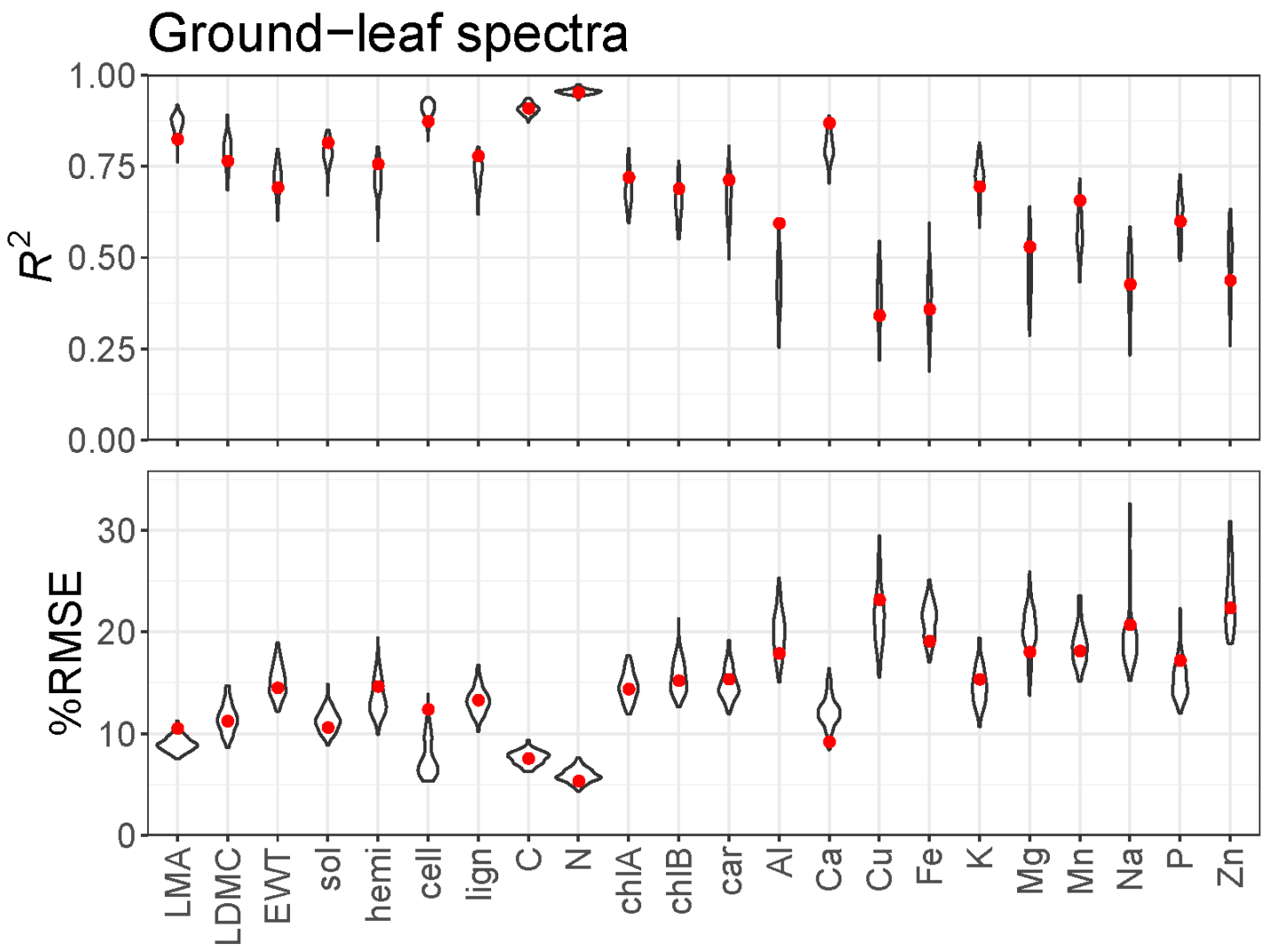
Na
P
Zn

Fig. S13


Ground−leaf spectra
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0.25
0.00
%RMSE
30
20
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LMA
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sol
hemi
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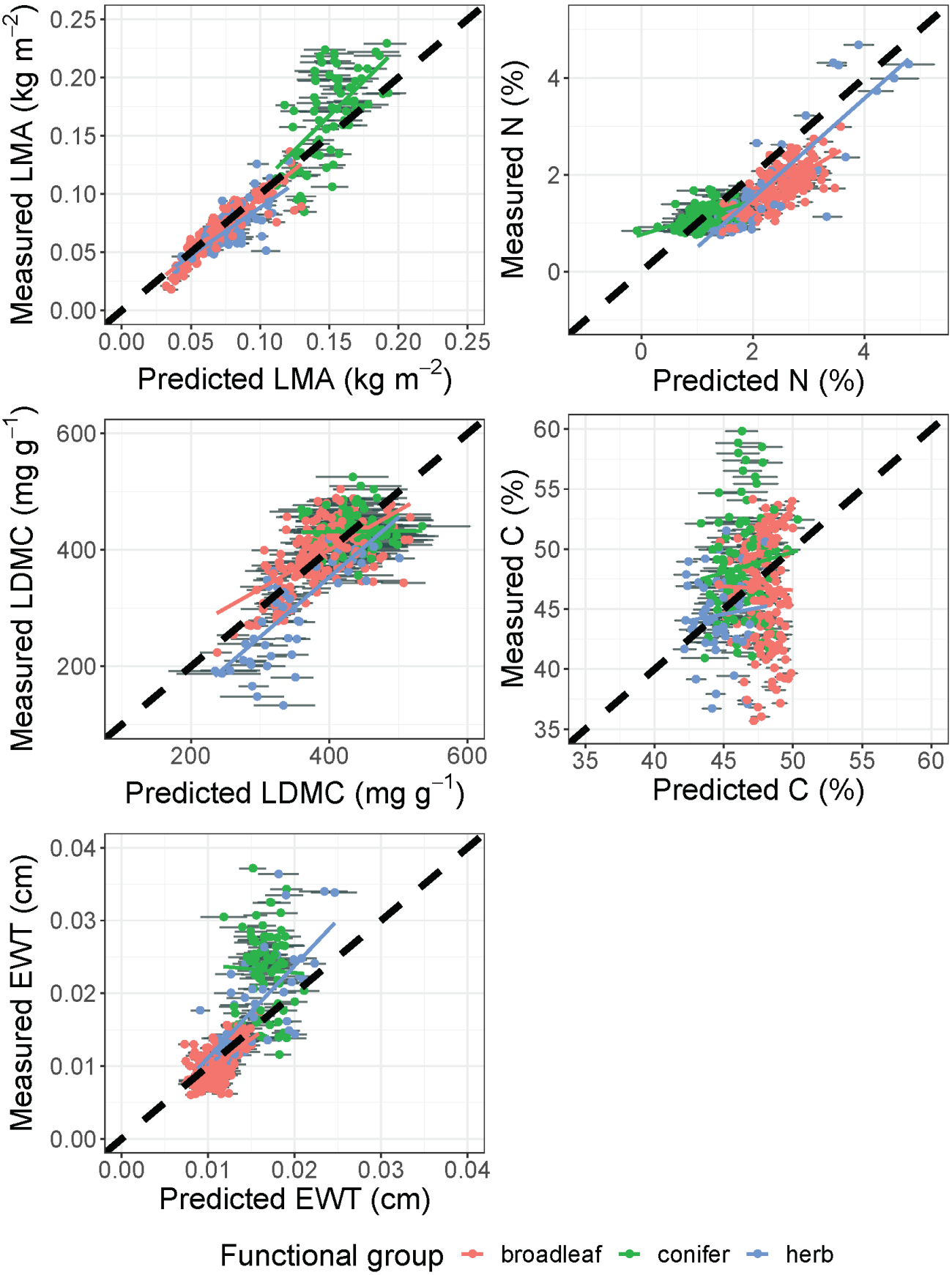
Na
P
Zn

Fig. S14


Measured LMA (kg m^{-2})
Predicted LMA (kg m^{-2})
0.00
0.05
0.10
0.15
0.20
0.25
Measured N (%)
Predicted N (%)
0
2
4
Measured LDMC (mg g^{-1})
Predicted LDMC (mg g^{-1})
200
400
600
Measured C (%)
Predicted C (%)
35
40
45
50
55
60
Measured EWT (cm)
Predicted EWT (cm)
0.00
0.01
0.02
0.03
0.04
Functional group
broadleaf
conifer
herb