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RESEARCH ARTICLE

Functional Traits 2.0: The Power of Metabolomics for Plant Ecology

Root functional traits explain root exudation rate and composition across a range of grassland species

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Abstract

1. Plant root exudation is a crucial means through which plants communicate with soil microbes and influence rhizosphere processes. Exudation can also underlie ecosystem response to changing environmental conditions. Different plant species vary in their root exudate quantity and quality, but our understanding of the plant characteristics that drive these differences is fragmentary. We hypothesised that root exudates would be under phylogenetic control and fit within an exploitative root nutrient uptake strategy, specifically that high rates of root exudation would link to root traits indicative of exploitative growth.
2. We collected root exudates from plants grown in field soil, as well as leachates of the entire plant–soil system, to assess both the quantity and quality of root exudates, and their interaction with the soil metabolome, across 18 common grassland species.
3. We found that exudation varied with plant functional group and that differences were trait dependent. Particularly, root diameter, root tissue density and root nitrogen content explained much of the variation in exudate metabolome, along with plant phylogeny. Specific root exudation rate was highest in forbs and was negatively correlated with root tissue density, a trait indicative of conservative resource-use strategy, and positively correlated with root diameter, which is associated with microbial collaboration and resource uptake 'outsourcing'.
4. *Synthesis*. We provide novel insight into species-specific differences in root exudates and identify root functional traits that might underlie these differences. Our results show that root exudation fits, although not entirely, within current models of the root economic space, with strong positive relationships to outsourcing traits like high root diameter. Determining the role of root exudates as a key facet of the resource-outsourcing strategy necessitates further research into the fundamental controls on root exudation quantity *and* quality, particularly during environmental change.

KEYWORDS

metabolomics, plant–microbe communication, rhizosphere, root exudates, root traits

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1 | INTRODUCTION

Root exudates are the chemical interface through which plants communicate with soil micro-organisms via many classes of primary and secondary metabolites (Badri & Vivanco, 2009). For example, flavonoids exuded through plant roots are known to recruit specific arbuscular mycorrhizal fungi (Steinkellner et al., 2007); colonisation of maize roots, by growth promoting and systemic resistance inducing *Pseudomonas putida*, is driven by exudation of the benzoxazinoid DIMBOA (Neal et al., 2012); and glycerol-3-phosphate in drought-ted maize exudates result in the recruitment of drought-resistance inducing monoderm bacteria (Xu et al., 2018). Moreover, root exudates are a major pathway through which carbon (C) enters the soil (Sokol et al., 2019), and alterations in root exudation have been shown to underlie the response of ecosystems to changing environmental conditions (Hamilton & Frank, 2001; Phillips et al., 2011; de Vries et al., 2019). Among rhizosphere research it has long been established that both the quantity and composition of exudates change during biotic or abiotic stress (Williams & de Vries, 2020; Xu & Coleman-Derr, 2019) and over the life cycle of a plant (Aulakh et al., 2001; Zhalnina et al., 2018), with base exudate composition being dependent on plant genotype (Mönchgesang et al., 2016). Yet, only recently has root exudation been considered a functional trait in its own right, where it has been theoretically central to an exploitative nutrient acquisition strategy within the root economic space, along with competitive traits like high root nitrogen content (Sun et al., 2020). However, most of this research has focussed on root exudation quantity, and it is not clear whether, and how, root exudation rate and quality are part of a root nutrient uptake strategy.

Root exudation processes are gaining momentum as a measurable and informative functional root trait (van Dam & Bouwmeester, 2016). Root traits dictate the functional biology of plants; for instance, roots of legumes that recruit nitrogen fixers in the rhizosphere have high root nitrogen content (Roumet et al., 2006). Root functional traits are dynamic and operate on an economic spectrum of resource use, partially informed by the environment in which the plant is growing (Meier et al., 2020; Sun et al., 2020). This root trait plasticity in the face of changing environmental pressures, over the lifetime of the plant, may be central to plant survival and fitness in different environments; for instance by reducing intraspecific resource competition (Valverde-Barrantes et al., 2017). Root exudation chemistry is no exception, and the exudation of costly metabolites is likely to occur on an economic spectrum, determined by the resource-use strategy of the plant (Miao et al., 2020; Sun et al., 2020). The quantity of metabolites allocated towards root exudates are likely to relate to metabolic activity, and be negatively associated with other, more conservative, plant traits (Bergmann et al., 2020). For example, across six grasses of contrasting nutrient use strategies, high levels of root exudation were associated with high specific root length (SRL) and low root dry matter content (RDMC), traits associated with exploitative growth (Guyonnet, Guillemet, et al., 2018). Interestingly, in woody species high rates of root exudation are found in more active root tissue, so are likely necessary for rapid development; this comes at the cost of structural investment traits, such as root tissue density (Sun et al., 2020). However, root exudation operates relatively independently of other root traits, and composition, rather than exudation rate, may have broader and more ambiguous roles within the rhizosphere, across plants with different strategies of resource acquisition and use.

The chemical composition of exuded metabolites, independent of the exudation rate, has yet to be measured in the context of root functional traits. Exuded metabolites may exist to 'feed' soil micro-organisms, such as those with higher sugar content, which are more often tied to high growth rate and greater microbial 'collaboration' (Bergmann et al., 2020). Indeed, grasses with exploitative growth strategies (with high exudation rates and potentially more diverse exudation chemistry), were associated with a more active microbiota and an increase in rhizosphere denitrification and respiration (Guyonnet, Guillemet, et al., 2018). Alternatively, some exuded compounds are directly involved in acquisition of scarce nutrients, such as metals (Chen et al., 2018) and phosphorus (Randall et al., 2001), which do not require microbial collaboration. This outsourcing gradient is under phylogenetic control (Bergmann et al., 2020), so it stands to reason that the composition of root exudates is also dependent on plant phylogeny. Profiles of root exudates are distinct in different species (for instance *Arabidopsis* and barley; Miao et al., 2020; Pétriacq et al., 2017) and are implicated in shaping the rhizosphere to best promote survival for those species, such as in soils with low nutrient availability (Meier et al., 2020). As root traits and root exudation are intricately linked (Guyonnet, Cantarel, et al., 2018; Li et al., 2018), and root traits also show a strong phylogenetic signal (Roumet et al., 2006) across a range of resource acquisition and use, it is likely that exact chemical components of root exudates will be informative in determining their functional roles within species (Dietz et al., 2020; Herz et al., 2018). For instance, the qualitative composition of root exudates may be more important than other root traits in maximising recovery to damaging abiotic stress, through specific microbial recruitment and subsequently shape future ecosystem diversity (Williams & de Vries, 2020). Despite the importance of multidimensional-phylogenetic frameworks of root traits in building functional ecological models (Bergmann et al., 2020; Valverde-Barrantes & Blackwood, 2016) root exudation processes require further scrutiny to firmly establish their phylogenetic role within these models.

Here, we aimed to test whether phylogeny and plant functional group determines root exudation quantity and quality. Our overall hypothesis was that high root exudation rate and specific composition are compatible with an exploitative growth strategy where outsourcing nutrient acquisition to rhizosphere microbes is paramount. Specifically, we hypothesised: (a) that root traits and root exudation are determined by plant phylogenetic background and functional group; (b) to confirm that root exudation rate is part of a plant 'root exploitative' strategy and is thus higher with high root nitrogen content, and has a negative relationship with root traits indicative of more conservative strategies; and (c) the root metabolome of species with exploitative root traits will be enriched in

compounds associated with high microbial recruitment. We tested these hypotheses by obtaining exudates in 17 common grassland species that represent three functional groups in a controlled environment. A novel hybrid exudation collection approach permitted us to grow plants in natural soil before collecting exudates in a controlled hydroponic system. Moreover, we also sampled the metabolome of the entire root–soil system, to assess the quantity and the quality of root exudates as well as their interaction with soil organic C compounds and microbes.

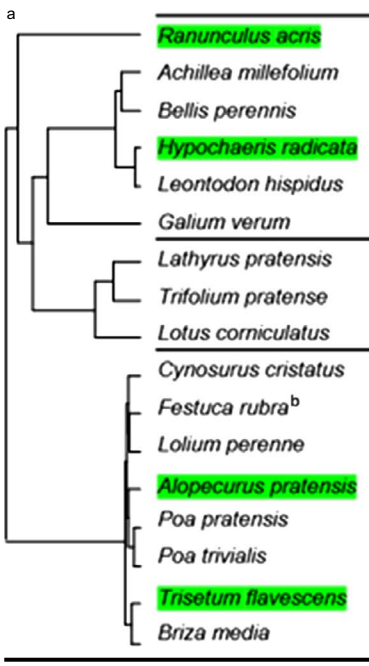
2 | MATERIALS AND METHODS

2.1 | Experimental design

Topsoil (60% clayey brown earth over limestone from the MALHAM 1 association of Eutric Endoleptic Cambisols (Cranfield University 2020); means ± *SD* of %N 0.57 ± 0.02, %C 5.70 ± 0.14%, pH 5.35 ± 0.3) was obtained from Colt Park, a mesotrophic grassland with historically light grazing and minimal fertiliser input situated in northern England (54°11′37.1″ N 2°20′54.9″ W, 348 m a.s.l.) in March 2016. Soil was subsequently sieved (4 mm mesh size), homogenised and stored at 4°C prior to use.

We chose 17 common perennial European grassland species that co-occur in the area where the soil was collected (Sweeney et al. 2020). These represent the functional groups of grasses, forbs and legumes (Table 1), and were grown in two separate experiments: one containing 13 (total number of individuals $n = 140$) and one containing four species ($n = 20$). The first lineage was chosen to cover a large phylogeny and three functional groups including a range of root traits and nutrient acquisition strategies. The second set was chosen to investigate genetic relatedness of the exudate metabolome in more detail and included two more distantly related forbs and two more closely related grasses. Seeds were sourced from Emorsgate Seeds (Norfolk, UK) who produce seed in natural grassland conditions in the UK. Plug trays were seeded after stratification at 4°C and placed in a greenhouse (University of Manchester Firs botanical grounds, Manchester, UK). After 2 weeks, individual seedlings were transplanted into 500 ml experimental pots with one seedling per pot (105 mm diameter, 75 mm depth) with 400 g of processed field-moist soil (~160 g dry soil), and kept in a greenhouse for 3 months in a randomised block design. Plants were watered ad libitum during this time. Plants were grown staggered in four sets; one containing four species, one with five species and a final set with four species. One further set was grown for experiment 2, comprising an additional four species. This set design was chosen due to the labour-intensive nature of root washing and collecting root exudates using the hybrid method, and because of space limitations. These sets were grown from June to September, 2016, March to June, 2017 and June to September, 2017 respectively.

TABLE 1 Information about the phylogeny and attributes of the species used in this study



Species	Abbrev.	Common name	Functional group	Growth rate (week^{-1})[c]
Ranunculus acris	R. acr	Meadow buttercup	Forb	No data
Achillea millefolium	A. mil	Yarrow	Forb	1.5–1.9
Bellis perennis	B. per	Daisy	Forb	1.0–1.4
Hypochaeris radicata	H. rad	Flatweed	Forb	No data
Leontodon hispidus	L. his	Rough hawkbit	Forb	0.5–0.9
Galium verum	G. ver	Lady's bedstraw	Forb	1.0–1.4
Lathyrus pratensis	L. pra	Meadow vetchling	Legume	0.5–1.0
Trifolium pratense	T. pra	Red clover	Legume	<0.5
Lotus corniculatus	L. cor	Bird's-foot trefoil	Legume	1.0–1.4
Cynosurus cristatus	C. cri	Crested dogstail	Grass	1.5–1.9
Festuca rubra[b]	F. rub	Red fescue	Grass	1.0–1.4
Lolium perenne	L. per	Perenial ryegrass	Grass	1.0–1.4
Alopecurus pratensis	A. pra	Meadow foxtail	Grass	1.0–1.4
Poa pratensis	P. pra	Smooth-stalked meadow grass	Grass	No data
Poa trivialis	P. tri	Rough-stalked meadow grass	Grass	1.0–1.4
Trisetum flavescens	T. fla	Yellow oat-grass	Grass	No data
Briza media	B. med	Quaking grass	Grass	1.0–1.4

[a] Phylogeny constructed from Daphne dataset (Durka & Michalski 2012). Species highlighted in green are represented by the second experiment, the rest are in experiment 1.

[b] Only present in the leachate dataset.

[c] Growth rate was obtained from Grime et al. (2007).

After 3 months of growth, all plants were transferred to Percival AR-66L2 climate chambers (CLF PlantClimatics, Wertingen, Germany) set at long day (16:8 hr, light:dark at 16°C night and 18°C day; air relative humidity 65%) to mimic peak growth season and to standardise growth conditions for each species before exudate sampling. Plants were watered by weight to maintain a constant soil moisture of approximately 60% of water-holding capacity to moderate growth conditions for 2 weeks before half being subjected to exudate ($n = 70$) or half for leachate collection ($n = 70$; see Figure S1 for a schematic of the sampling design of this experiment). Exudate collection was performed using a hydroponics-hybrid method, where roots were carefully washed of adhering soil and transferred into 150 ml aerated hydroponics solution. This solution was made from a slurry of 200 g of stored field soil in 1 L of MilliQ water (left to settle and filtered 0.2 mm mesh) that was used to mimic the nutrient supply of the soil plants were grown in. These jars were wrapped in foil to exclude light from reaching the roots, and plants were left for 7 days before root exudate collection. For exudate collection plants were placed in individual sterile glass jars with their roots submerged in 100 ml of collection solution (pure milliQ water) on ice (to minimise turnover of collected exudates), and agitated at 60 rpm on a Stuart Orbital shaker (Cole-Parmer, St. Neots, UK) for 2 hr (to maximise amount of exudate collected over the risk of metabolite turnover, as performed in Dietz et al., 2019) in natural ambient light at 18°C; thereafter the exudate solution was syringe filtered at 0.22 μm (Merck Millipore). Leachate was collected from the intact root–soil system by pouring MilliQ water onto the soil surface and collecting 100 ml on ice. One species, *Festuca rubra*, is only represented in the leachate datasets due to sample damage.

2.2 | Root exudate and leachate collection

Both exudate and leachates samples were syringe filtered at 0.22 μm (Merck Millipore). Samples were concentrated by freeze-drying (Scavac CoolSafe 55-9 Pro; LaboGene, Lynge, Denmark) and re-suspending in liquid chromatography grade water (Sigma-Aldrich, Gillingham, UK), after which one third was used for determination of total carbon using a total organic carbon (TOC) L-series analyser (Shimadzu, Kyoto, Japan) and two thirds were used for metabolite profiling with gas chromatography–mass spectrometry (GC-MS). TOC data were then divided by the root dry biomass and time to discern specific exudation rate. Derivatisation, GC-MS technical specifics and data pre-processing are described in detail in Supp. Materials and Methods S1.

2.3 | Root trait analyses

After exudate collection intact and complete root systems were quickly rinsed with water before separation from the above-ground tissue. Leachate plants were also thoroughly cleaned of adhering soil and separated from above-ground material. Roots from all of these samples, still joined at the root crown, were carefully teased apart and evenly spread out in deionised water on a transparent perspex tray (300 × 200 mm). Root systems were imaged with an Epson Expression 11000XL flatbed scanner system at a resolution of 600 dpi. Using the 2013 WinRHIZO® pro software (Régent Instruments Inc., QC, Canada) structural root traits (total root length, average root diameter and root volume) were analysed with the batch analysis feature for each species enabled. To account for roots crossing an analytical correction was applied along with exclusion of debris smaller than length/width ratio of 4.

After analysis, root fresh weight was determined. Roots were then dried at 60°C for 48 hr before dry weight was also determined. Using the above measurements we calculated specific root length (SRL mm/g) and root tissue density (RTD g/cm^3). Root carbon and nitrogen content (%) were determined on ball-mill ground (MM400 ball mill; Retsch, Hope Valley, UK) dry root material, with a Vario EL Cube (Elementar, Germany).

2.4 | Statistical analyses

To visualise differences in root traits across species and functional groups, we performed principal component analysis (PCA) with projections applied to show which root traits drove the most discrimination between functional groups. Root traits were $\log_{10}$-transformed to meet normality requirements—although root carbon content did not—and data were centred and scaled for PCA. Horn's parallel analysis was used to determine that three components should be retained for each dataset. To measure the strength of the phylogenetic signal within these root traits we calculated Blomberg's K using PHYLOSIGNAL package (Keck et al., 2016) and plotted centred and scaled averages among species to demonstrate which traits displayed significant phylogenetic signals. To measure how well these root traits correlated with specific exudation rate Spearman's rank correlation coefficients were obtained using cor.test in R.

2.5 | Root exudate metabolite analysis

Root exudate composition was analysed using an untargeted GC-MS method (Supp. Materials and Methods S1). Two GC-MS datasets were generated in this study: one containing data from hybrid collected root exudates of the first and second lineages (395 unique features; Table 1): and one for leachate of the same lineage (320 unique features). From the first dataset, a subset of data was made of four species (also 395 unique features).

Although untargeted GC-MS data are semi-quantitative, relative total peak area per species displayed a positive relationship with total exuded C as measured by TOC (Figure S2) indicating that analysed metabolites are representative of the total amount of exuded C. To this end GC-MS data were standardised by dry root weight, after which we performed a PCA to visualise the structure of the data. PCAs were run on $\log_{10}$-transformed data to correct

for heteroscedasticity of widely variable concentrations of the putative metabolites and to aid in intuitive visualisation (Grace & Hudson, 2016). As with the root traits datasets, Horn's parallel analysis determined that no more than three components should be retained for each dataset, and that the third principal component explained very little variation. GC-MS ordinations were plotted first via PCA with centred and scaled data, first uncorrected for root weight and by batch to check for positional bias (Figure S3) before running the root weight corrected data. This was followed by PERMANOVA (adonis function) with species and functional group as main factors, and strata option enabled to constrain permutations by growth set, to assess the impact of species on root traits and root exudate profiles quantified by GC-MS. We then calculated Procrustes distances to model the similarity in ordination between the root traits and metabolomics datasets (VEGAN package; Oksanen et al., 2011).

To visualise the effect of species and root exudate collection method on standardised exudate metabolite profiles, we produced cluster heatmaps, based on *K*means clustering of mean metabolite intensity for each species across the entire exudate metabolome (PHEATMAP package; Kolde, 2012). A dendrogram was generated to illustrate the relative clustering of the metabolomic signature between species and functional groups and measured against the phylogenetic tree by measuring the co-phenetic correlation coefficient (DENDEXTEND package; Galili, 2015) to provide a measure of the phylogenetic signal of the exudate metabolome.

Supervised sparse partial least-square discriminant analyses (sPLS-DAs) were conducted to obtain the metabolites that provide the greatest source of discrimination between functional groups using the MIXOMICS package (Rohart et al., 2017). These were performed on hydroponics and leachate datasets separately. sPLS-DA models were cross-validated for performance using the perf function to provide optimal number of axes (in each case 3) and an optimal number of metabolites that best explain the variation on each axis (Figure S4). Expression of the top 20 significant metabolites was visualised using one matrix Clustered Image Map heatmaps, through hierarchal clustering, to show their relative intensity among species. The relative loadings of each metabolite were presented in a bar chart.

To quantify the relationship between root traits and metabolite expression in the exudates, random forest (RF) analyses were run against the entire dataset (irrespective of the relatedness of the plant species) with root traits that showed significant phylogenetic signal, as the explanatory variables (RANDOMFOREST package; Liaw & Wiener, 2002). A training set of 500 trees was used with 500 variables randomly sampled at each split to provide the lowest error rates. The top 30 metabolites associated with the explanatory variables were annotated.

Identification of all metabolites was putative—level 2 of the Metabolomics Standards Initiative (Sumner et al., 2007)—obtained using the GOLM database, where only annotations of >80% match factor were retained (Kopka et al., 2005). PubChem (Kim et al., 2019) was used to further annotate metabolites for their pathway of origin to display higher level changes in the exudate metabolome.

All analyses and figure preparation were performed in R (v4.0.2; R Core Team, 2013).

3 | RESULTS

We found that functional root traits differed strongly among species, and that there were clear drivers of separation between functional groups (Figure 1; raw data are presented in Figure S5).

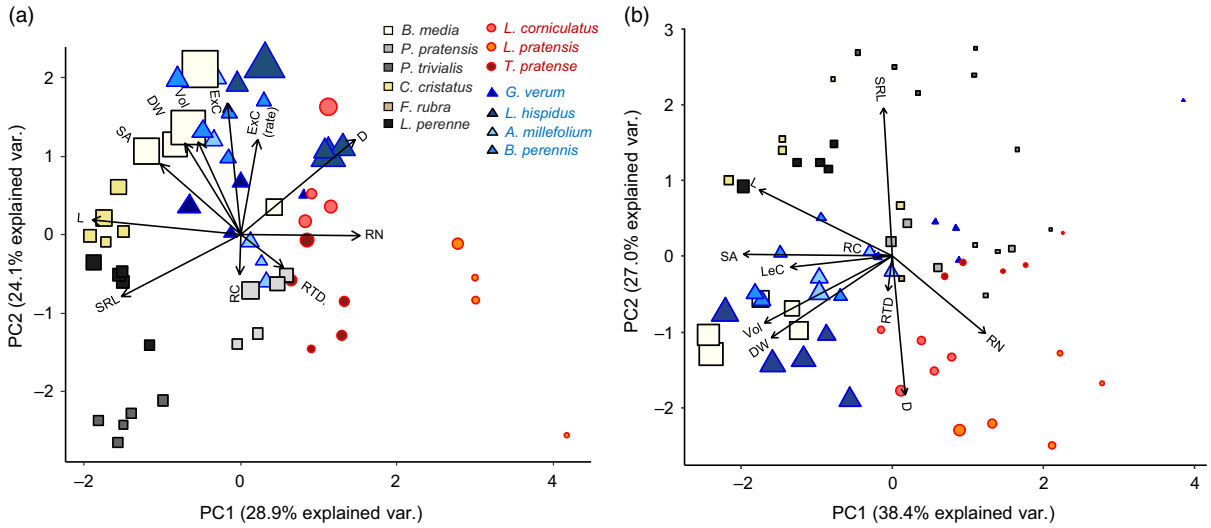


FIGURE 1 Functional root traits of various species of grass, forb and legume. PCA scores plots of root traits from plants grown in soil before transfer to hydroponics (a) and just in soil before leachate collection (b). Arrows on PCA indicate projections for each individual trait. Shapes indicate different functional groups, colours represent species (grey tones are grasses, blues are forbs and reds are legumes). Point size indicates the root dry biomass (g). Root trait annotations are as follows; D, diameter; L, root length; DW, root dry biomass; RTD, root tissue density; SRL, specific root length; RC, root carbon content; RN, root nitrogen content; SA surface area; Vol, root volume; ExC, exuded carbon; ExC rate, hourly exuded carbon per DW; Le C, carbon in the leachate

Legumes were characterised by high root nitrogen content, both in plants used for hybrid and leachate collection, while forbs had the highest specific exudation rate, again both in hybrid (PERMANOVA main effect of functional group $F_{2,45} = 61.1$, $R^2 = 0.40$, $p < 0.001$ and main effect of species $F_{9,45} = 15.4$, $R^2 = 0.45$, $p < 0.001$ – Figure 1a) and leachate-collected root exudates (PERMANOVA main effect of functional group $F_{2,51} = 48.0$, $R^2 = 0.31$, $p < 0.001$ and main effect of species $F_{10,51} = 15.8$, $R^2 = 0.52$, $p < 0.001$ – Figure 1b) respectively. We found the same patterns using Blomberg's K phylosignal analysis, where again legumes displayed higher root nitrogen content ($p = 0.001$ in hybrid and leachate) and forbs had a greater specific exudation rate ($p = 0.004$; Figure 2). We did not find an effect of phylogeny or functional group on any of the other root traits. As specific exudation rate displayed a strong phylogenetic signal, we used Spearman's rank correlation to determine which other root traits it was most correlated with, and found a significant positive correlation with root diameter and a significant negative correlation with root tissue density (Table S1). Root nitrogen content did not show strong relationship with RTD, as expected, among the species tested. Interestingly, by observing just the relationship between root nitrogen content and specific exudation rate, we could quite accurately portray the functional group, which clustered separately along these two axes (Figure S6).

PCA of the root exudate metabolome of hybrid samples distinguished between the three functional groups (Figure 3a; PERMANOVA main effect of functional group $F_{2,51} = 4.01$, $R^2 = 0.08$, $p = 0.032$). Separation between functional groups was much less clear in leachate samples (Figure 3b) and no

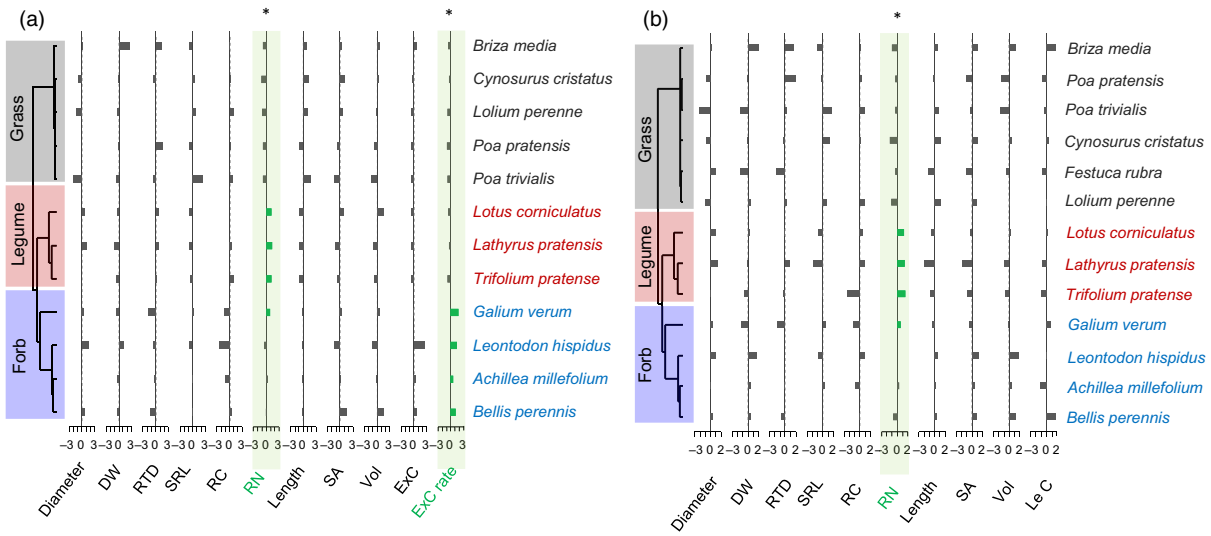


FIGURE 2 Phylogenetic signal of functional root traits of various species of grass, forb and legume. Phylosignal box-plots of root traits from plants grown in soil before transfer to hydroponics (a) and just in soil before leachate collection (b). Boxes indicate the mean strength of the signal for each individual species. Asterisks indicate traits significant for phylosignal (analysis), which are highlighted in green and species that are positive for this trait are also coloured green. Coloured boxes on the dendrogram indicate the functional group (Grass, grey; legume, red; forb, blue). Root trait annotations are as follows; DW, root dry biomass; RTD, root tissue density; SRL, specific root length; RC, root carbon content; RN, root nitrogen content; SA surface area; Vol, root volume; ExC, exuded carbon; ExC rate, hourly exuded carbon per DW; Le C, carbon in the leachate

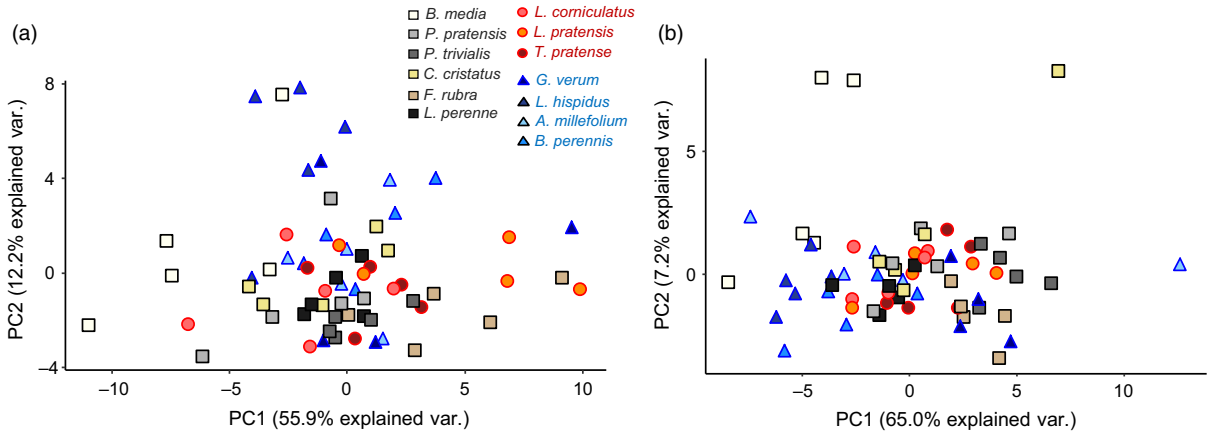


FIGURE 3 PCA scores plots of root exudate profiles across different species. Comparison of the root metabolome profiles between grasses (dark grey squares), legumes (red circles) and forbs (blue triangles) from hydroponic exudates (a) and leachate (b)

longer significant (PERMANOVA main effect of functional group $F_{2,51} = 3.20$, $R^2 = 0.06$, $p = 0.267$). To indicate the strength of the link between metabolome and functional group a cluster heatmap was used on metabolomics data to generate distances between species. From this heatmap it was clear that the metabolomes of grasses tended to cluster closer together, with some forbs clustering separately (*L. hispidus* and *B. perennis*). However, other forbs and legumes were not easily distinguishable (Figure S7). Interestingly, the metabolome of the forb *G. verum* was distinct from the other forbs, which was also apparent in its root traits (Figure 2) and its genetic relatedness to the other forbs (Table 1). To test how well the tree constructed from the metabolome matched that of the phylogenetic tree, a co-phenetic correlation coefficient (CPCC) was used, which showed that there was a fairly strong negative mismatch between the trees ($c = -0.0181$ where values near 0 are not statistically similar and 1 is maximum similarity) driven by a number of species exhibiting a weak phylogenetic signal in their exudate metabolome (Figure S8). CPCC for root traits showed a similar negative mismatch ($c = -0.0112$).

As root trait analysis and exudate metabolome were both significantly affected by functional group, Procrustes analysis was performed to test how close the root traits and metabolome ordinations matched one another (Figure S9) and significance testing with PROTEST found strong and significant correlations of root traits with both hybrid ($m_{12} = 0.75$, $R = 0.50$, $p = 0.01$) and leachate ($m_{12} = 0.69$, $R = 0.56$, $p = 0.01$) metabolomes, despite the variability of the metabolomic data.

Next, we performed a supervised sPLS-DA to assess which metabolites were most responsible for driving variability along each axis. We found that in hybrid-collected exudates, across three components, the majority of the variation between functional groups could be explained (Figure 4). Component 1 (29%) mainly separated

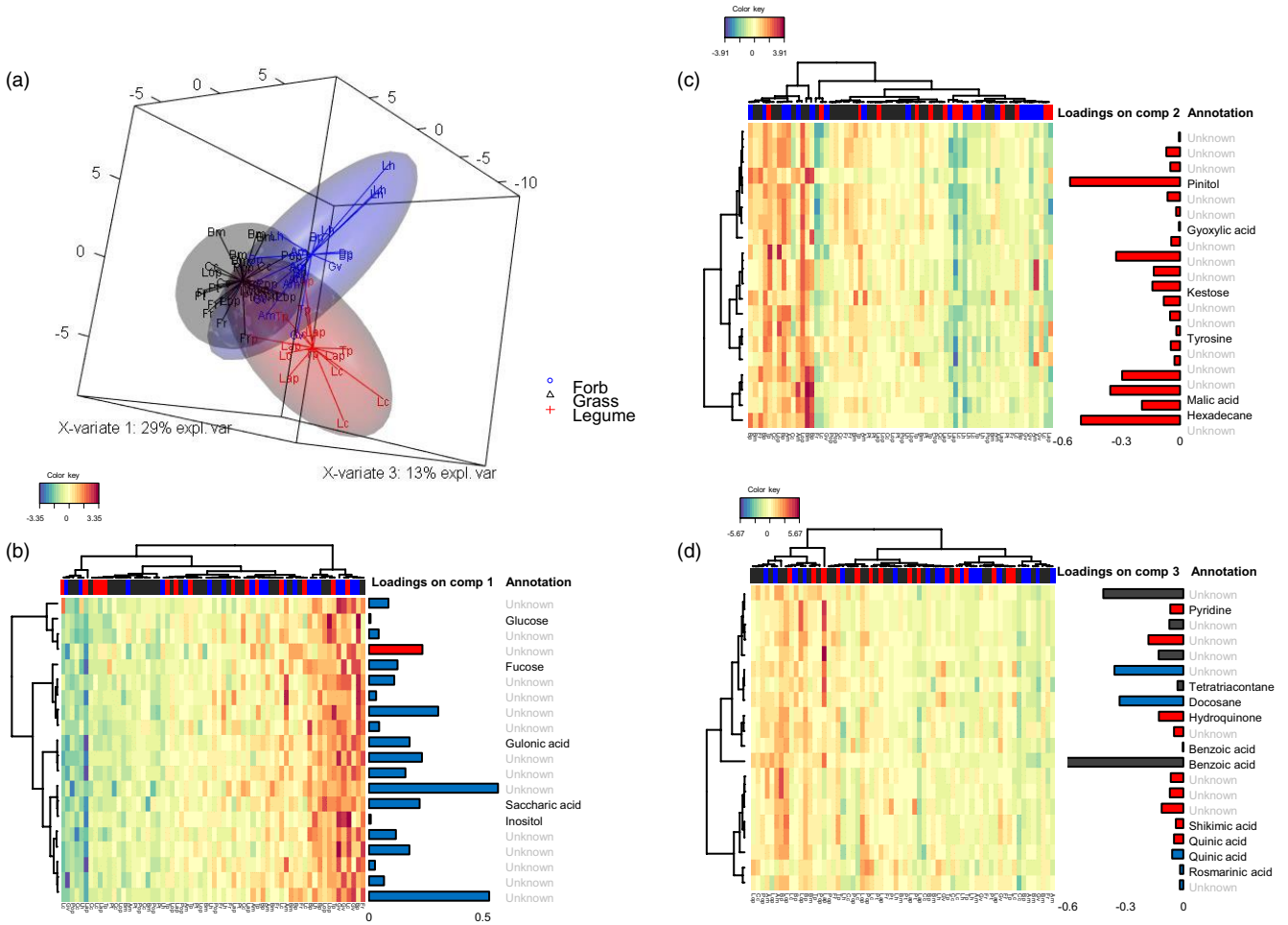


FIGURE 4 sPLS-DA analysis of species-specific root exudate profiles across different functional groups. 3-d ordination plot between grasses, forbs and legumes (a). The top 20 compounds that are most discriminatory between the functional groups are presented for component 1 (b), 2 (c) and 3 (d). The colour within the heatmaps colour indicates the relative intensity for each sample (the colour scale bar is provided within each figure). Functional groups are indicated by coloured boxes below the tree (dark grey, grass; red, legume; blue, forb). Species are labelled underneath the heatmaps. The importance of each metabolite is indicated in graphs to the right of the heatmap—bars are coloured for the functional group each metabolite is enriched in (dark grey, grass; red, legume; blue, forb). Metabolite putative identities (>80% match in GOLM database) are annotated to the right of the heatmap for each axis. Compounds that could not be annotated, or were below the 80% threshold, are annotated 'Unknown' and shaded in grey

legumes from the other functional groups (Figure 4a), as the top 20 metabolites were all enriched in legumes (Figure 4b) and, although level 2 identification was low, these metabolites included sugars (glucose, fucose and inositol) and organic acids (gulonic and saccharic acids). Component 2 (17%) separated forbs, which were enriched in organic acids (gyoxylic and malic acids), sugars (pinitol, kestose), the amino acid tyrosine and hexadecane. Component 3 (13%) mainly separated grasses, which were enriched in benzoic acid and tetratriacontane, but some enrichment of specific metabolites was also seen in legumes (shikimic and quinic acids as well as pyridine, and hydroquinone) and forbs (quinic acid rosmarinic acids and docosane).

The separation between functional groups within leachate samples was less clear than hybrid exudates (Figure 5a) but the sPLS-DA still showed discrimination between the functional groups. Grasses separated most strongly on component 1 (45% of explained variation) with malonic, nonanonic, fumaric and quinic acids as well as ribose and kestose present at enriched concentrations (Figure 5b). Component 2 (18% of explained variation) showed great separation with legumes enriched in hydrocarbons (Figure 5c) and component 3 showed further enrichment in grasses with sugars (sorbose, glycerol and galactose) amines, the amino acid histamine and the riboflavin derivative lumichrome.

As both root nitrogen content (in hybrid and leachate) and exudation rate (in hybrid only) were root traits that exhibited a significant phylogenetic signal, we identified the metabolites most closely associated with these traits using random forest analysis (irrespective of the relatedness of the plant species; Figure 6). In hybrid collected exudates, metabolites that most closely correlated with root nitrogen content included amino acids (methionine, pyridine, norleucine), organic acids (saccharic, malic, succinic and shikimic acids), hydrocarbons (pentadecane, docosane) and sugars (pinitol, sorbose and galactopyranoside). Similar compounds were associated with specific exudation rate, including sugars (galactitol, fucose and glucose) as well as organic acids (gluonic, benzoic, saccharic, lactic and quinic acids), the amino acid serine and hydrocarbons (hexadecane, docosane and tridecane). Although root nitrogen content had a significant phylogenetic signal and was positively associated with legumes

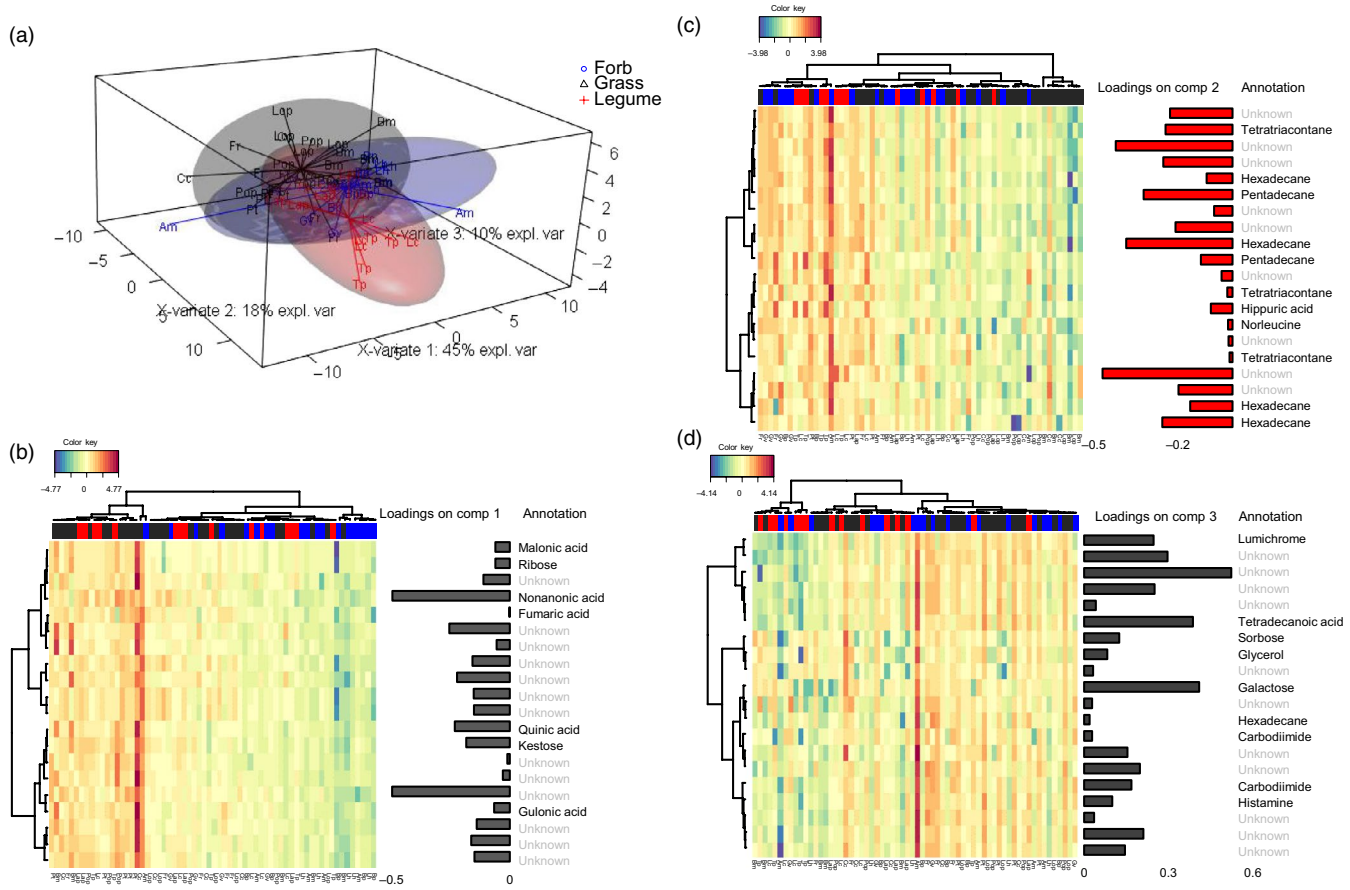


FIGURE 5 sPLS-DA analysis of species-specific leachate profiles across different functional groups. 3-d ordination plot between grasses, forbs and legumes (a). The top 20 compounds that are most discriminatory between the functional groups are presented for component 1 (b), 2 (c) and 3 (d). The colour within the heatmaps colour indicates the relative intensity for each sample (the colour scale bar is provided within each figure). Functional groups are indicated by coloured boxes below the tree (dark grey, grass; red, legume; blue, forb). Species are labelled underneath the heatmaps. The importance of each metabolite is indicated in graphs to the right of the heatmap—bars are coloured for the functional group each metabolite is enriched in (dark grey, grass; red, legume; blue, forb). Metabolite putative identities (>80% match in GOLM database) are annotated to the right of the heatmap for each axis. Compounds that could not be annotated, or were below the 80% threshold, are annotated 'Unknown' and shaded in grey

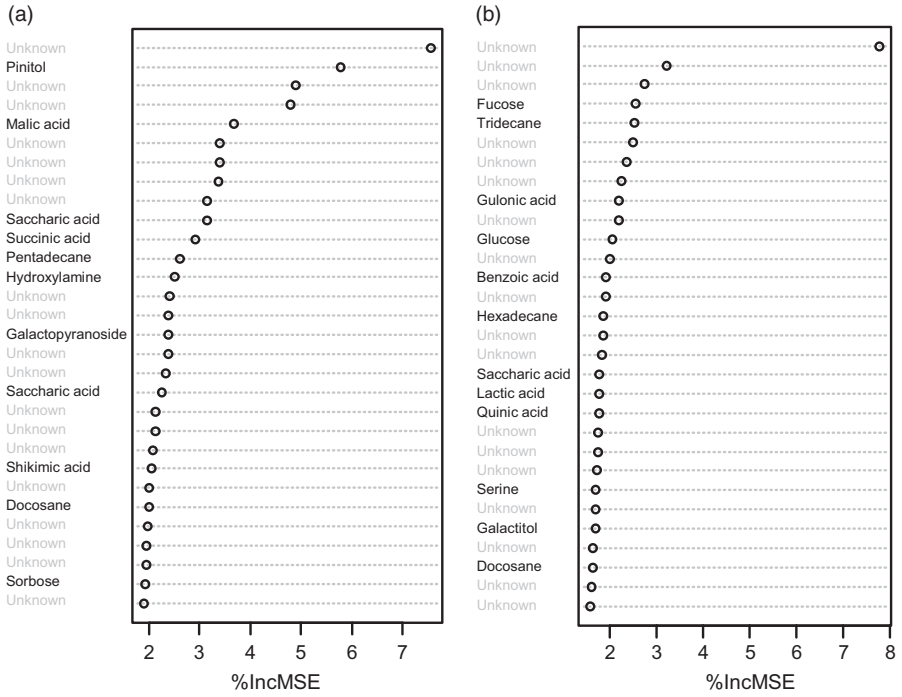


FIGURE 6 Relationship between functional traits and specific metabolites. Metabolites with the highest percentage of influence over the mean standard error from random forest are annotated for root nitrogen and exudation rate in hydroponics (a and b respectively)

in the leachate dataset, the RF model for this dataset was overfitted and thus not performed.

Finally, to test if the phylogenetic signal of the exudate metabolome was conserved in a separate phylogeny, we used a second set of species—two forbs (*H. radicata and R. acris*) and two grasses (*A. pratensis and T. flavescens*) and analysed their exudates only using the hybrid approach (these samples matched well with the original dataset, Figure S10). Within this dataset, there was clear separation between functional group (PERMANOVA main effect of functional group $F_{1,16} = 8.87$, $R^2 = 0.27$, $p < 0.001$; Figure 7a) and species (PERMANOVA main effect of species $F_{2,16} = 4.14$, $R^2 = 0.25$, $p < 0.001$), and the difference of the metabolic profiles of their root exudates between the forbs correlated very well with the difference in their phylogeny, which was larger than between the two grass species (Figure 7b). We verified this pattern using CPCC, which indicated a near identical match between phylogenetic distance and exudate metabolome expression among species, albeit this was likely due to the small dataset and low variability ($c = 0.93$). Within this dataset we could more accurately compare metabolites specific to species using sPLS-DA and found that the separation between species on component 1 was driven by nearly the entire dataset (300 metabolites out of 395 were shown to have large influence). These metabolites fitted into two clusters. The first cluster had higher expression in *H. radicata*, with larger concentrations of sugars, while the other had lower expression in *H. radicata* but higher in the three other species, and consisted of many amino acids, amines and fatty acid alkanes (Figure 7c). Component 2 was driven completely by a small cluster of metabolites that were expressed at higher levels in *R. acris*, including some organic acids (glutaric, gluonic and levulinic acids), sugars (sorbose and kestose) and the rhizosphere signalling molecule calystegine (Figure 7d).

4 | DISCUSSION

We set out to test where root exudation processes fit within current models of plant resource-use strategy and the root economic space. We hypothesised that root exudation is part of an exploitative resource uptake strategy, and thus that root exudation rate would be positively correlated with and that the root exudation metabolome would be linked to, exploitative functional root traits. We found that specific root exudation rate increased with high root diameter and decreased with high root tissue density—the latter fitting with our hypothesis—but we found no relationship with root nitrogen content—a trait generally high in fast-growing annuals (Roumet et al., 2006), and that has been shown to positively correlate with root respiration rate and higher exudation in the fine roots of woody species (Sun et al., 2020). Using whole root systems, we found significant phylogenetic signals for specific exudation rate and root nitrogen content, which had distinct metabolites associated with them. Specifically, higher specific exudation rates occurred in forbs, and were associated with metabolites that play a role in rhizosphere communication. The composition of root exudates did not show a strong phylogenetic signal in a large phylogeny of plants, but the signal was very strong in a smaller phylogeny, suggesting that on wider scales growth strategy may be more important than genetic distance in terms of metabolite expression in root exudates. Finally, we hypothesised that the function of particular metabolites in the exudates, identified through supervised analysis, would be characteristic of, and specific to, certain plant lineages.

We found a negative correlation between specific exudation rate and root tissue density, a trait associated with slower growth through greater investment in structural root development and also reflective of

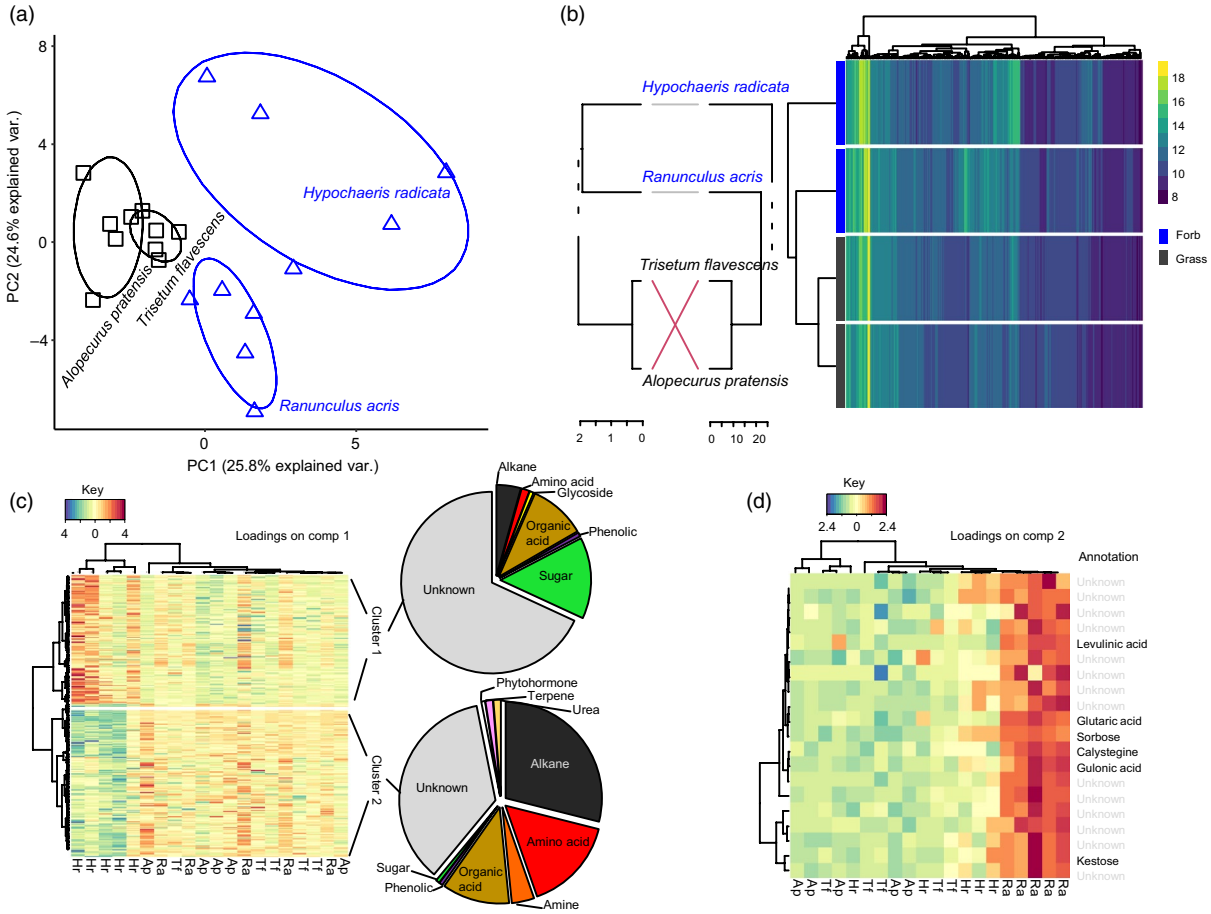


FIGURE 7 Phylogenetic signal within the exudate metabolome between two grasses and two forbs. PCA ordination plot of metabolome, colour indicates functional group, 90% confidence interval presented (a). Relationship between metabolome and phylogeny presented as a tanglegram, heatmap of the metabolome that generated the dendrogram on the right, averaged for each species (b). CIM showing significant loadings on component 1 of sPLS-DA (left), with annotated metabolites from the 2 clusters summarised by their pathway and presented in pie charts (right, (c)). Significant loadings on component 2 illustrated through CIM, with each feature annotated to the left (d). Species are indicated below by abbreviated codes; Hr is *Hypochaeris radicata*, Ra is *Rannunculus acris*, Ap is *Alopecurus pratensis* and Tf is *Trisetum flavescens*

adaptation to low nutrient availability (Kramer-Walter et al., 2016). This finding aligns with the model of exudation rate being an exploitative strategy, as shown previously (Sun et al., 2020). However, no strong relationship between specific exudation rate and root nitrogen content was apparent in our data, nor was there a strong negative relationship with RTD, although the three functional groups clearly differed in their expression of these traits. This may suggest that root exudation rate does not have a de facto role in rapid development and is likely more important along the outsourcing gradient of the root economic space. In line with this we measured a strong positive correlation between high root diameter and high exudation rate. Root diameter displays large degree of influence over other root traits (Ma et al., 2018) and, along with cortex fraction size, is an important driver of 'outsourcing' nutrient acquisition to rhizosphere microbes (Bergmann et al., 2020). Importantly, high root diameter determines the strength of relationship between plants and rhizosphere fungi (Sweeney et al., 2020), including greater association with beneficials and a reduction in pathogens. High specific exudation rate, despite being metabolically costly and hence more associated with exploitative growth, might serve a vital function in providing nutrition to symbionts and potential biocidal exudates to aggressors. This increased rate of exudation is incompatible with conservative growth strategies, illustrated by the negative relationship with RTD, but it is also not truly related to rapid development (high root nitrogen content) or resource mining (high specific root length). Although high root diameter has been previously associated with slow development (Roumet et al., 2006; Sun et al., 2020), given its negative relationship with RTD, its well-established relationship with AMF colonisation (Sweeney et al., 2020) and importance in the collaboration gradient (Bergmann et al., 2020); the positive association we observe between specific exudation rate and diameter demonstrates that exudates are a key component of the collaboration gradient. However, exudation rate may be dynamic over the life cycle of the symbioses, meaning longer term measurements are required to establish the exact role exudation rate plays along a collaborative gradient. Furthermore, the function of particular metabolites in the rhizosphere cannot be determined by measures of exudation rate and requires metabolomic characterisation.

Our data hint that root traits, root exudation rate and the composition of root exudates are under some level of phylogenetic control,

although there was a high amount of variability in the exudate metabolome. In particular, we found that root nitrogen content and specific exudation rate were present at higher levels in legumes and forbs respectively (as previously stated; Ma et al., 2018; Valverde-Barrantes et al., 2017). Root trait syndromes varied with functional group and the ordinations of exudate metabolome and root traits were significantly similar. Thus, specific exudate metabolites were associated with specific root traits irrespective of species relationships, suggesting that the metabolome of the root exudate is achieved through both direct genetic control and indirect genetic control. Root nitrogen content and specific exudation rate may in part determine the exudate metabolome and were associated with greater abundance of metabolites including those known to be chemotactic to rhizosphere microbes (such as malic acid, benzoic acid and possibly succinic acid; Guyonnet et al., 2017; Liu et al., 2015; Rudrappa et al., 2008), and those known to aide in nutrient acquisition (like certain sugars and amino acids; Canarini et al., 2019). However, the composition of the exudate metabolome, among the diverse phylogeny of species we used in this study, could not accurately depict their genetic distance, and some species' metabolic signatures clustered together despite their functional group. This lack of a distinct phylogenetic signal in the root exudate metabolome suggests that a large part of root exudate composition may be universal, irrespective of plant species phylogeny or functional group. This would make sense as many metabolites of root exudates are likely to share essential processes such as for general nutrient acquisition, or microbial recruitment (Badri & Vivanco, 2009), and therefore species-specific differences may be harder to detect or may only exhibit when detrimental growth conditions are apparent, for example when nutrient availability is low (Dakora & Phillips, 2002). However, recent molecular characterisation of root exudates via Fourier-transform infrared spectroscopy has found compelling functional level differences in terms of the molecular components of root exudates, although interspecies variation was not presented (Miao et al., 2020).

Despite a clear phylogenetic signal, more focused analyses revealed distinct chemical signatures in the exudates of the three functional groups which likely have specific roles in the species that exude them. Although forbs and legumes explained most variation across the first two axes, and this variation was driven by various metabolites including sugars and organic acids, identification success of the metabolites was low and our ability to link specific metabolites to functional group was limited. For instance, elevated concentrations of the sugars glucose and fucose in forb exudates may indicate more active mucilage production (Sinha Roy et al., 2002), and enhanced presence of malic acid in legume exudates may be indicative of specific recruitment of microbes to aid in survival, such as *Bacillus subtilis*, a systemic resistance inducing beneficial rhizosphere bacteria and N-fixer (Rudrappa et al., 2008). Even though identification rate was low for specific metabolites, species specificity of the exudate metabolome was very apparent in our second dataset where samples clustered separately on both species and functional group level, and showed a strong CPCC against their genetic relatedness. In this second experiment, the forb *H. radicata* contained much higher levels of sugars in its exudate, perhaps as a food source for rhizosphere recruitment of particular microbes or to aide in phosphorous acquisition (Canarini et al., 2019). However, the forb *R. acris* and the grasses *A. pratensis* and *T. flavescens* contained higher levels of amino acids, which may play an adaptive role in nutrient acquisition and highlight a potential fitness advantage for these species in nutrient poor soils (Carvalhais et al., 2011). While the purpose that these differences serve requires further targeted investigation, it is striking that the phylogenetic distance between *R acris*, *H. radicata* and the grasses were strongly conserved in the exudate metabolome. Previous work suggested that the chemical profile of the root exudate is under fine genetic control and is engineered by the particular plant species or genotype (Mönchgesang et al., 2016) and is sensitive to environmental perturbations (Canarini et al., 2019) where it may exhibit rapid and reactive influence to direct rhizosphere processes that ensure survival (Williams & de Vries, 2020). The chemical signature of functional groups in the leachate was distinct from the exudate which presents an intriguing link between the two. It remains unclear exactly how one influences the other, and further focus is necessary to illuminate the mechanistic link between exuded compounds and their fate in the rhizosphere. Moreover, studies comparing more and less distantly related species will help to develop a more global understanding on the types of metabolites exuded, their conservation among species lineages and potentially their role and reactivity in the rhizosphere.

Understanding how root exudation fits within the root economic space is critical for correctly interpreting their role in ecosystems and their response to global change drivers. Here we show that root exudation rate forms part of both a more exploitative growth strategy and a greater level of outsourcing for nutrient acquisition. As root development itself, and thus root trait expression, may depend on the very presence of root exudates from parent and neighbouring plants (Caffaro et al., 2011), it makes it clear that measuring root exudation processes across a spectrum of root trait expression would be necessary to fully unravel their interconnectivity. Overall, this work highlights the importance of both quality and quantity of root exudates in plant function; quantity of exudate may be vital in establishing a healthy microbiome, and during downstream ecosystem recovery after perturbation, and quality will define exact mechanistic roles that exudates play in the rhizosphere. Further scrutiny of exudation processes, rate, composition and dynamics, will aide in better discerning their functional role, their link to rhizosphere microbial communities and their use in plants with particular growth strategies as well as their potential importance in adaptation and survival during environmental stress.

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AUTHORS' CONTRIBUTIONS

F.T.d.V., R.G. and A.L.S. conceived and designed the experiments; A.L.S., H.M., K.A.H. and H.L. performed the experiments and laboratory analyses; A.W. and F.T.d.V. analysed and interpreted the data;

A.W. wrote the manuscript with contributions from the other authors. No authors have any conflict of interest regarding the production or dissemination of this data.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data are available https://doi.org/10.6084/m9.figshare.13859783 (Williams et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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