Biol Invasions (2026) 28:70
https://doi.org/10.1007/s10530-026-03768-9

ORIGINAL PAPER

Low foliar nitrogen and elevated defensive metabolites in the invasive weed *Calluna vulgaris* (heather) may impair biocontrol agent performance

D. Paul Barrett · Maria A. Minor · Paul Peterson · Simon V. Fowler · Arvind K. Subbaraj · David J. Lun · Andrea Clavijo McCormick

Received: 18 July 2025 / Accepted: 23 January 2026 / Published online: 21 February 2026
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Abstract Introducing insect biocontrol agents to manage invasive plants is an effective and sustainable management option. However, biocontrol agents sometimes fail to establish or are ineffective in the new range. Heather beetle *Lochmaea suturalis,* (Thomson, 1866) (Coleoptera: Chrysomelidae) sourced from the United Kingdom (UK), was introduced into New Zealand's North Island Central Plateau (CP) to control invasive *Calluna vulgaris* (L.) Hull (heather). The beetle was difficult to establish and the few populations that did, achieved poor population growth rates and expansion in contrast to the rapid rates associated with its conspecifics in the UK. We hypothesize that low soil nutrients such as nitrogen and phosphorous (N and P) on the CP may be linked to changes in *C. vulgaris'* primary nutritional and secondary defensive metabolites, leading to poor beetle performance. Testing this hypothesis involved application of N and P to soil in *C. vulgaris* field plots, measurement of foliar N and Carbon (C), plus using non-targeted metabolomics, exploring differences in the primary and secondary metabolites between treatments. Raising soil nutrients amplified several primary metabolites including N-based amino acids and concomitantly reduced the majority of phenylpropanoids, a secondary metabolite group containing many defensive compounds. This work seeks to better understand, how abiotic conditions in a new environment, influence invasive plant metabolism,

Communicated by Evangelina Schwindt,.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10530-026-03768-9.

D. P. Barrett (✉) · M. A. Minor · D. J. Lun · A. Clavijo McCormick
College of Sciences, Massey University, PB 11 222, Palmerston North 4410, New Zealand
e-mail: d.p.barrett@massey.ac.nz

M. A. Minor
e-mail: M.A.Minor@massey.ac.nz

A. Clavijo McCormick
e-mail: A.C.McCormick@massey.ac.nz

P. Peterson
Bioeconomy Science Institute, Manaaki Whenua - Landcare Research, Massey University, Riddet Rd, Palmerston North 4472, New Zealand
e-mail: petersonp@landcareresearch.co.nz

S. V. Fowler
Bioeconomy Science Institute, Manaaki Whenua - Landcare Research, 74 Gerald St, Lincoln 7608, New Zealand
e-mail: fowlers@landcareresearch.co.nz

A. K. Subbaraj
Bioeconomy Science Institute, AgResearch Ltd., 1365 Springs Road, Lincoln 7608, New Zealand
e-mail: arvind.subbaraj@agresearch.co.nz

potentially altering the efficiency of foliage assimilation which may impair biocontrol agent establishment and effectiveness. Such understanding may be applied to mitigate potential foliar deficiencies at release sites and contribute to improving success rates of biocontrol as a management strategy for invasive plants.

Keywords Invasive plants · *Calluna vulgaris* · Foliar nitrogen · Metabolomics · Phenylpropanoids · Biological control

Introduction

Sustainable long-term management of invasive plants is often achieved using specialist phytophagous insects as classical biological control agents (Hayes et al. 2013; Schwarzländer et al. 2018). Not all biological control agents achieve the expected outcome due to either a failure to establish (McFadyen 2000; Hayes et al. 2013), by being ineffective or achieving only variable effectiveness when they do (McClay and Balciunas 2005; Raghu and Dhileepan 2005). Biocontrol agent establishment and/or effectiveness is thought to be influenced by abiotic (Grevstad 1999; Harms et al. 2020) and/or biotic factors (Syrett 1983; Briese 1986; Schulz et al. 2019). While target plant assimilability or nutritional value has been considered for some aquatic plants e.g. Room and Thomas (1985), the same parameters in terrestrial plants have, until recently, seldom been considered as a potential limitation on introduced insect control agents (but see Hinz and Müller-Schärer 2000; Van Hezewijk et al. 2008; Uyi et al. 2016).

Soil nutrient availability is known to influence the levels of both primary and secondary plant metabolites. For example, soil phosphate deficiency influences plants by reprogramming them from primary to secondary metabolism which can result in increased levels of a range of secondary metabolites such as phenylpropanoids (Gershenzon 1984; Malhotra et al. 2018; Wang et al. 2023).

Phenylpropanoids are the largest class of carbon (C) -rich plant secondary metabolites, which include phenolic acids (e.g. benzoic and cinnamic acids) and flavonoids (Kováčik et al. 2007; Ruan et al. 2010) plus flavones, coumarins (Kováčik and Klejdus 2014), quinic acids (Giorgi et al. 2009), anthocyanins, flavonoid glycosides, isoflavones (Gershenzon 1984), stilbenes, terpenoids and tannins, many of which are involved in plant defence against insect herbivores (Bassman 2004; Zhang and Bjorn 2009; Escobar-Bravo et al. 2017; Kumar et al. 2020). Thus, elevated levels of these compounds can result in impaired insect herbivore performance (Izaguirre et al. 2007; Kuhlmann and Müller 2010; Qi et al. 2018), while reduced levels, can result in improved herbivore performance and/or increased feeding rates (Rousseaux et al. 2001; Ballaré et al. 2012; Dinh et al. 2013; Ballaré 2014).

Soil N deficiency, similar to P, imposes limits on plant metabolism which results in reduced growth and/or lower plant yields (Giorgi et al. 2009). This deficiency induces reprograming of primary and secondary metabolism with a reduction of N-rich amino acids, proteins and enzymes and an increase in C rich phenolic compounds and may also result in a shift of the plants carbon to nitrogen ratio (C:N) (Kováčik and Bačkor 2007; Rubio-Wilhelmi et al. 2012). Nitrogen is an essential component of the insect diet but at low levels is a limiting factor for growth, survival and reproduction (Mattson 1980; Ren et al. 2022). The primary source of nitrogen for insects are plant proteins which comprise of amino acids—compounds with amino (-NH_2), carboxyl (–COOH) groups, and a specific chain–which, when assimilated are broken down into amino acid components and used as structural proteins, enzymes or receptor molecules (Kraus et al. 2022).

The C:N ratio in plant tissue is important for herbivores as reflected in the Efficiency of Conversion of Ingested food (ECI) model which implies that high ECI foods for herbivores have a higher concentration of N-containing amino acid and proteins coupled with adequate carbohydrates as a proportion of the concentration of C-rich defensive metabolites (Mattson 1980; Henn and Schopf 2001). Evidence also suggests that there are foliar N threshold values above which insect growth may increase with increasing concentration but below which they cease to develop because the N required to build structural proteins is insufficient (Mattson 1980). For example, the chrysomelid beetle *Paropsis atomaria* (Olivier, 1807) (Coleoptera: Chrysomelidae) feeding on *Eucalyptus blakelyi*, (Maiden) (Myrtales: Myrtaceae) larval weight gain decreased and development time increased when foliar N fell below 1.7% dry weight and at 0.8% dry weight the

larvae died before reaching 3rd instar (Ohmart et al. 1985; Ohmart 1991).

Heather beetle *Lochmaea suturalis* (Thomson, 1866) (Coleoptera: Chrysomelidae) was released in New Zealand in 1996 as a biocontrol agent for the highly invasive shrub, heather, *Calluna vulgaris* (L.) Hull. On the North Island Central Plateau most releases failed, and for those that did establish, population growth and expansion rates were poor relative to the rates recorded in its native UK range (Pakeman et al. 2002; Peterson et al. 2007, 2024; Rosenburgh and Marrs 2010; Fowler et al. 2015). This is in contrast to three releases in the Rotorua region where all three established on *C. vulgaris* with % foliar N approximately 50% higher than that of the CP region (Peterson et al. 2011). Now, some two and a half decades later, beetle populations in the CP region are well established and causing widespread damage to heather, and recent findings (Barrett unpublished data) suggests population densities are attaining levels closer to those recorded in the UK (Pakeman et al. 2002; Rosenburgh and Marrs 2010). Damage to *C. vulgaris* to most areas in this CP region is usually severe, but in some areas, beetle effectiveness is variable, with many plants remaining viable.

Soil types of the CP region are very low in N and extremely low in P which can impair the uptake of available soil N (Xia et al. 2023). Heather foliar N levels in the region recorded through the years 2008 to 2010 had a mean value of ~ 1.1% N gm^{-1} DM (Peterson et al. 2015) whereas foliar N levels in the UK averaged ~ 1.48% N gm^{-1} DM (Peterson et al. 2011). Previous trials with *L. suturalis* larvae and adults when fed foliage from N and P fertilised CP heather, resulted in improved larval survival and increased adult body size and female fecundity (Peterson et al. 2024). A series of field experiments using un-fertilised and fertilised heather plots at new release sites also demonstrated improved population growth rates and establishment success of *L. suturalis* in fertilized plots compared to unfertilized plots (Peterson et al. 2024). This indicates a direct link between soil nutrients and biocontrol agent performance. However, how N and P fertilization changes the C:N ratio of CP heather foliage and the balance and interaction of N-containing primary and C-rich secondary metabolites on assimilation efficiency for *L. suturalis* remains unclear.

To explore these questions, we applied N and P fertilizer to naturally growing plants in a field-based experiment, and compared foliar N, C and primary and secondary metabolites, between fertilised and non-fertilised plants. For quantification of foliar N and C content we used a standard Dumas combustion method and for primary and secondary metabolites, UHPLC-MS/MS non-targeted metabolomics. The balance of primary N-containing and secondary metabolites was altered in line with the "growth-defence trade-off" principle for plants under nutrient stress and we discuss these results in the context of improved assimilability and improved control agent performance, and establishment rates. We posit that understanding abiotic influences on targeted invasive plants which result in altered assimilability may provide information with which to mitigate these changes before agent releases and improve biocontrol success rates and management outcomes.

Materials and methods

Field experiment, sample collection and storage

On 22nd of Oct 2021 on the North Island CP, New Zealand, a site between Lat. -39.228816, Lon. 175.717081 and Lat -39.230145, Lon. 175.715263. with heather of similar age and architecture and no heather beetle present was established. Eight plots (four times two treatments), each 5 × 5 m^2 were set out, then three soil cores (30 mm dia.) to the depth of 15 cm were collected from random positions within each plot. These were analysed post setup, to assess any variation in soil nutrients between plots. After core collection, plots were randomly allocated to fertiliser (treatment) or no fertilizer (controls) and seven *C. vulgaris* plants tagged within each. Fertiliser was applied as 325.5 g Urea and 1217.5 g Triple Superphosphate per 25 m^2 using the application protocol detailed in Peterson et al. (2024).

On 30th Dec 2021, sprigs of foliage ~ 20 mm long were collected from the tip of ~ 20–30 stems of each tagged plant in both treatments for foliar nitrogen (N), carbon (C) and metabolite analyses. These samples were immediately cryo-frozen in nitrogen vapour then stored at − 80 °C. All plants in both treatments were sampled for herbivorous invertebrates to assess potential amplification of secondary metabolites

induced due to insect herbivores. Further information and results are available in Supplemental Information Doc.1 (1.1).

Analytical protocols

Soil cores were air dried (18–25 °C) to zero mass change, passed through a 1 mm Endicott precision sieve, homogenised and then analysed for pH, Olsen P (Ols P) and total N (tN) at Hills Laboratories Ltd., Hamilton, NZ. Phosphorus for Olsen P was obtained using $NaHCO_3$ (0.5 M, pH8.5) extractant and total N obtained using the Dumas combustion method in a VarioMAX CN Macro Elementar analyser. For pH, soils were slurried (1:2 v/v) soil:H_2O and analysed using a glass bulb pH probe.

Before analysis, foliage samples were freeze dried, stored for 10 days at −20 °C, before grinding to ~150–50 µm particle size. For foliar total N and C analyses, 30 ± 0.5 mg of each ground sample was weighed, combined with an equal weight of tungsten (VI) oxide and pressed into foil envelopes for analysis. Using the Dumas combustion method protocol for foliage, samples were analysed at Massey University using a Vario Macro Cube Elementar analyser with furnace temperature 1150 °C, reduction tube temperature 850 °°C and helium flow 600 µl min.$^{-1}$

Extraction of metabolites for UHPLC-MS/MS analysis are fully detailed in Supplemental Information Doc.1 (1.2). Briefly 50 ± 0.5 mg of ground sample was weighed into 2 mL microcentrifuge tubes, 800 µL of pre-chilled chloroform:methanol ($CHCl_3$:CH_3OH; 1:1 v/v) was added, then homogenised for 5 min and stored for 1 h at − 20 °C. Then, 400 ml of H_2O was added to each, homogenised again, and centrifuged to create a biphasic layer. 200 µl aliquots of the upper layer were pipetted into microcentrifuge tubes, one each for C18 and HILIC chromatography analysis and a final 200 µl aliquot added to larger tube to form a homogenous mix for quality controls (QCs). All samples were dried under a continuous flow of N_2 and immediately stored at − 80 °C until reconstitution prior to analysis. Immediately prior to LC–MS analysis, all samples plus QCs were reconstituted by adding 200 µl of reconstitution solvent, vortexed until dissolved then transferred to an autosampler vial, capped and chilled until loading for analysis.

Chromatography and tandem mass-spectrometry analysis of polar and semi-polar compounds were achieved using a Thermo LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) consisting of a Dionex UltiMate 3000 UHPLC coupled with a high-resolution Q Exactive Focus Quadrupole-Orbitrap mass spectrometer utilising heated electrospray ionisation run in both positive and negative modes. For semi-polar compounds, samples were injected into a 1.9 µm Thermo Hypersil GOLD™ C18 column (UHPLC, 100 mm × 2.1 mm, Thermo Fisher Scientific, USA) at 25 °C and for polar compounds, injected into a 5 µm ZIC®-pHILIC column (100 mm × 2.1 mm, Merck Darmstadt, Germany) also run at 25 °C. Details of all chromatographic and mass spectral conditions are detailed in Supplemental Information Doc.1 (1.3).

Thermo derived.raw files for each stream i.e., C18 pos, C18 neg, HILIC pos and HILIC neg modes, were converted to mzML format using MSConvert-GUI (Adusumilli and Mallick 2017), uploaded into MZmine (Pluskal et al. 2020) to determine the appropriate baseline noise threshold and then into XCMS online https://xcmsonline.scripps.edu/ for feature detection, alignment and exploratory data analysis (Domingo-Almenara and Siuzdak 2020). Feature detection parameters for C18 data were: *m/z* deviation 10 ppm, min and max peak width 5 and 20 respectively, mzdiff 0.001, s/n threshold 20, Prefilter intensity 1e4 and noise filter 2.5e4. For HILIC data the same parameters were 10 ppm, 10 and 60, 0.001, 20, 1e4 and 8e3. After downloading the output, raw mass spectrometry data were organised into data matrices comprising the mean *m/z* and retention times and the corresponding ion intensity measurements for each feature making it suitable for statistical analysis.

Data analysis

We used non-parametric Kruskal–Wallis analysis to test for heterogeneity of soil nutrient parameters (pH, Ols P and tN) between the eight experimental plots prior to fertilization while foliar N levels were analysed using one-way ANOVA.

Analysis of primary and secondary metabolites involves a series of steps beginning with reduction of background variability in the data matrix for each stream using a QC vs Blank t-test thus allowing subtraction of features with p values > 0.05, and

secondly, t- values corresponding to any features high in the blanks. These data matrices were each uploaded into MetaboAnalyst 6.0 (MA ver 6.0) (Pang et al. 2021) and data integrity checked to confirm the number of samples, number of peaks, missing values, and the number of treatment groups. No missing values were detected in any of the data sets. For filtration of variables showing low repeatability, the threshold to remove those with high percent relative standard deviation (RSD) was set at 30% to that of the QCs and the data normalised by auto-scaling (mean-centred and divided by the standard deviation of each variable). Gaussian distribution was confirmed so that feature mass intensities are comparable.

For each stream, using MA ver 6.0, we explored these data matrices, and compared treatment (fertilizer) and control (no fertilizer) data by subjecting them to orthogonal partial least squares discriminant analysis (OPLS-DA); the separation between treatments was statistically confirmed using 20 permutations to provide acceptable R^2, Q^2 and p values. Based on a 95% confidence interval, one outlier (the same plant sample) was removed from all streams except for C18pos which had two removed, before final analysis. Features for annotation were identified by applying a paired t-test to these data matrices and retaining all the features less than the false discovery rate threshold value of FDR < 0.05 (Benjamini and Hochberg 1995).

Annotations were conducted and confidence levels confirmed (Sumner et al. 2007) for each metabolite by interrogating the original.raw files using Xcalibur Freestyle. Formula matches were confirmed, and mass accuracy parameters were set within 10 ppm (i.e. +/– 5.0). For all C18 features, confidence levels were set according to Sumner et al. (2007) with level 2 (parent ion plus at least one fragment) and level 3 (the parent ion only) being confirmed using the MassBank.eu (https://massbank.eu/) spectral database. Where the same annotated and confirmed compound (ion) appeared in both positive and negative modes, the one with the highest signal intensity (arbitrary units; au) was included in the final data table. For further verification of the annotated compounds and the potential to discover additional ones not available in the MassBank spectral library, a QC file was uploaded into XCMS-METLIN and spectral matches > 0.9 deemed level 2 confidence. Fold change values were calculated for all annotated compounds.

Using the intensity data of all the annotated metabolites listed in Tables 1 and 2 pathways enrichment analysis was performed using the platform in MA ver 6.0. Metabolic pathways that are significantly influenced by the fertilizer treatment were assessed using the upland cotton (*Gossypium hirsutum*) pathway library as a reference.

For all streams, a literature search provided information on the physiological or defensive functions of a range of both primary and secondary annotated compounds. We then used linear regression analysis to test for any predictive relationship between total foliar N and the intensity values for each compound identified as impacting the pathway analysis and also those identified from the literature as being involved in insect herbivore defence.

Results

Soil nutrients and foliar N and C

Analysis of the soil nutrient parameters sampled from all eight plots prior to establishment, showed very low total N levels in all plots (0.22 ± 0.04; min 15, max 32). Kruskal–Wallis analysis showed a significant difference, $H(7) = 15.38$, $p = 0.031$, between plots but post-hoc analysis (Dunns test with Bonferroni adjustment for multiple comparisons) could not reveal which plots differed at $\alpha = 0.05$. Olsen P revealed extremely low levels (2.04 ± 0.55; min 1, max 3) with no significant difference, $H(7) = 11.93$, $p = 0.103$, between plots. Similarly, pH levels (5.77 ± 0.12; min 5.60, max 6.00) between plots showed no significant difference, $H(7) = 9.96$, $p = 0.191$.

Mean foliar total N for fertilized plants (1.49% DW) was significantly higher than that for control plants (1.38% DW), t(52) = -3.263, $p = < 0.001$ (Fig. 1a). Despite the variability in soil N between plots, analysis of foliar total N as % dry weight (% DW) using one-way ANOVA demonstrated no significant difference between the four plots within the treatment experiment (data not shown). The mean foliar carbon to nitrogen (C:N) ratio was 36.75 for control and 33.56 for fertilizer treatment, also significantly different, t(52) = 3.799, $p = < 0.001$, see Fig. 1b.

Table 1 Primary and secondary metabolites including seven containing nitrogen ions, annotated from the HILIC and C18 streams.

P/S	Class or sub class	Name	Formula	KEGG	STR	A/R	t- stat	FC	CL	m/z	rt.	Fragments
1°	Pyrrolidine	2-Pyrrolidineacetic acid [a]	$C_6H_{11}NO_2$		HIL +	↓	− 4.9223	2.17	2	130.0859	13.70	MS 1.0
1°	Pyridine	Pyridoxine	$C_8H_{11}NO_3$	C00314	HIL -	↓	− 3.1595	1.35	3	168.0656	15.09	
1°	Alpha Amino Acid	L-Valine [b, e, f]	$C_5H_{11}NO_2$	C00183	HIL -	↑	3.0871	1.28	3	116.0706	14.72	
1°	Alcohol polyol	Pantothenic acid [c, d]	$C_9H_{17}NO_5$	C00864	HIL +	↑	8.4034	2.16	3	220.1172	13.70	
1°	Pyrrolidine	Pyrrolidine	C_4H_9N		HIL +	↑	2.9117	1.20	3	72.0809	14.75	
2°	Indole	1-methoxy-3-formylindole	$C_{10}H_9NO_2$		C18 +	↑	3.3148	1.90	2	176.0703	7.09	148.0778
2°	Phenylalanine deriv	N-Fructosyl phenylalanine	$C_{15}H_{21}NO_7$		C18 +	↑	3.1907	1.50	2	328.1383	9.50	311.1168, 311.1092
1°	Carbohydrate	Galactaric acid	$C_6H_{10}O_8$	C00879	HIL -	↑	6.547	1.60	3	209.0295	16.96	
1°	" " " "	a,a-Trehalose [g]	$C_{12}H_{22}O_{11}$	C01083	HIL +	↑	3.3114	1.15	3	343.1223	15.81	
2°	Monoterpenoid	Camphor [h, i]	$C_{10}H_{16}O$	C00808	C18 +	↓	− 4.3251	2.18	3	153.1271	9.99	
2°	Chalcone	3,4,2',4',6'-Pentamethoxychalcone	$C_{20}H_{22}O_6$		C18 +	↓	− 4.0992	1.58	3	359.1481	10.34	
2°	Terpene Lactone	Costunolide	$C_{15}H_{20}O_2$	C09382	C18 +	↑	4.5116	1.58	2	233.1531	10.14	137.0599 187.1482 214.1431
2°	Med-chain Aldehyde	trans-2-Hexenal	$C_6H_{10}O$	C08497	C18 +	↑	4.0411	1.72	3	99.0805	7.41	

All t- test threshold values are FDR < 0.05. Superscript letters refer to plant physiological or effect on insect references. P/S = Primary or secondary, KEGG = compound database, STR = stream, A/R = Amplified or reduced, t- stat. = t statistic, FC = fold change, CL = confidence level, m/z = Parent ion, rt. = retention time, Fragments with MS are annotated via XCMS-METLIN with a spectral match score > 0.9

Plant physiological references: Singh and Shaner, (1995)[b], Miret and Munné-Bosch (2014)[c]

Effect on insect references: Huang et al. (2011)[a], Levinson et al. (1967)[d], Kim and Mullin (1998)[e], Genc (2006)[f], Tellis et al. (2023)[g], Kleine and Müller (2011)[h], Lazarević et al. (2022)[i]

Table 2 Secondary phenylpropanoid and benzenoid compounds annotated from the HILIC and C18 streams.

Ph/Bz	Class or sub class	Name		KEGG	STR	A/R	t- stat	FC	CL	m/z	rt.	Fragments
Ph	Flavonoid Glycoside	Naringin	$C_{27}H_{32}O_{14}$	C09789	C18 +	↑	3.3452	2.29	2	581.1849	9.11	563.1660 435.1210
Ph	" " " " "	Apigenin 7-O-glucoside	$C_{21}H_{20}O_{10}$	C04608	HIL -	↓	− 2.9338	2.02	3	431.0978	2.51	
Ph	" " " " "	Quercetin 3-(6"-acetylglucoside)	$C_{23}H_{22}O_{13}$		C18-	↓	− 3.0586	1.60	2	505.0989	10.01	301.0402 300.0304
Ph	" " " " "	Apigenin 7-glucuronide	$C_{21}H_{18}O_{11}$		C18-	↓	− 3.0513	1.41	3	445.0776	10.10	
Ph	" " " " "	Kaempferol-3-O-Rhamnoside	$C_{21}H_{20}O_{10}$		C18 +	↓	− 3.1122	1.80	2	433.1121	9.90	287.0511 288.0575 129.0539
Ph	Phenolic Glucoside	Dihydrobenzoic acid pentose	$C_{12}H_{13}O_8$		C18-	↓	− 3.7425	1.96	3	285.0615	2.77	
Ph	Flavonol Glucoside	Kaempferol-3-Glucoside-2''-p-coumaroyl	$C_{30}H_{26}O_{13}$		C18-	↓	− 3.9152	1.41	3	593.1303	6.84	
Ph	Flavonoid Glucoside	Myricetin-3-Xyloside	$C_{20}H_{18}O_{12}$		C18 +	↑	3.9611	1.57	2	451.0862	8.49	320.0488 319.0434
Ph	Flavone	Quercetin [j, k]	$C_{15}H_{10}O_7$	C00389	C18-	↓	− 3.4841	1.51	3	301.0353	9.87	
Ph	" " "	Apigenin	$C_{15}H_{10}O_5$	C01477	C18 +	↓	− 3.1233	1.70	3	271.0592	10.66	
Ph	Flavan	(+)-Gallocatechin	$C_{15}H_{14}O_7$	C12127	C18 +	↓	− 3.3654	1.52	2	307.0807	6.30	MS 0.947
Ph	Isoflavonoid	Formononetin	$C_{16}H_{12}O_4$	C00858	C18-	↑	5.1222	1.81	3	267.0639	4.17	
Ph	Flavanonol	Dihydroquercetin	$C_{15}H_{12}O_7$	C01617	C18-	↑	3.2741	1.41	3	303.0509	7.00	
Ph	Flavone	Tricin	$C_{17}H_{14}O_7$	C10193	C18-	↑	3.265	2.96	3	329.0666	9.59	
Ph	Coumarin	3,4-Dihydrocoumarin	$C_9H_8O_2$	C02274	C18 +	↑	3.113	1.46	2	149.0594	9.50	103.0533
Ph	Hydroxycoumarin	Scopoletin [l]	$C_{10}H_8O_4$	C01752	C18 +	↑	3.6905	2.49	2	193.0492	7.49	MS 0.934
Ph	Cinnamic acid	4-Methoxycinnamic acid	$C_{10}H_{10}0_3$		HIL +	↓	− 2.7308	1.43	3	179.0697	13.61	
Ph	Cinnamic acid	trans-Cinnamic acid [m]	$C_9H_8O_2$	C00423	C18-	↓	− 3.4846	2.76	3	147.0443	8.46	
Bz	Benzenediol	3,4-Dihydroxymandelic acid	$C_8H_8O_5$	C05580	C18-	↓	− 3.365	1.24	2	183.0292	8.91	139.0400 137.0242
Bz	Benzenoid	2,4,5-Trimethoxybenzaldehyde [n]	$C_{10}H_{12}O_4$		HIL +	↓	− 2.7546	1.42	3	197.0802	13.63	
Bz	" " " "	2,5-dihydroxybenzoic acid	$C_7H_6O_4$	C00628	C18-	↓	− 4.0177	1.49	2	153.0185	2.92	109.0294
Bz	" " " "	Diethylphthalic acid [o]	$C_{12}H_{14}O_4$	C14175	C18 +	↓	− 7.6783	2.14	3	223.0961	6.00	

All t- test threshold values are FDR < 0.05. Superscript letters refer to references regarding defensive antiherbivore function. Ph/Bz = Phenylpropanoid or Benzenoid, KEGG = compound database, STR = Stream, A/R = Amplified or reduced, t- stat. = t statistic, FC = fold change, CL = confidence level, m/z = Parent ion, rt. = retention time, Fragments with MS are annotated via XCMS-METLIN with a spectral match score > 0.9

Effect on insect references: Wang et al. (2019)[j], Gao et al. (2022)[k], Westcott et al. (1992)[l], Dixit et al. (2017)[m], Gu et al. (2025)[n], Huang et al. (2021)[o]

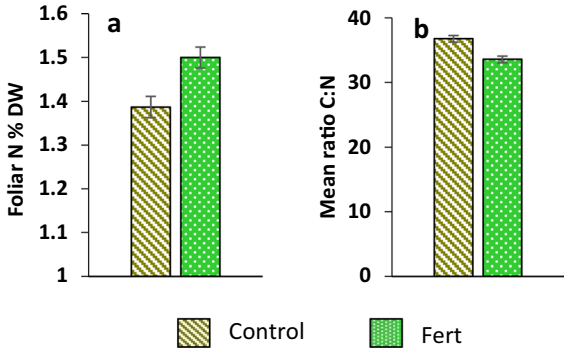


Fig. 1 Total N % dry weight level (**a**) and carbon to nitrogen (C:N) ratio (**b**) in *C. vulgaris* foliage from plants growing in control vs N and P fertilized soil (applied 10 weeks prior to sampling). Both foliar N and the mean C:N ratios differed significantly ($p < 0.001$) between treatments. Error bars = one SE

Metabolite responses to treatments

OPLS-DA demonstrated significant separation between the metabolite profiles of *C. vulgaris* from the control (no fertilizer) and fertilizer groups. Confirmation statistics were for the C18 positive stream: $R^2 = 0.955$, $Q^2 = 0.715$, $p < 0.05$ and for C18 negative stream: $R^2 = 0.967$, $Q^2 = 0.735$, $p < 0.05$. For the HILIC positive stream, $R^2 = 0.969$, $Q^2 = 0.81$, $p < 0.05$ and the HILIC negative stream, $R^2 = 0.939$, $Q^2 = 0.769$, $p < 0.05$ (Fig. 2).

A total of thirty-five compounds were annotated from all four streams (Tables 1 and 2). Over both tables, thirteen compounds are allocated level two confidence and the remainder at level three. Of the level two compounds XCMS-METLIN provided three spectral matches not available via the MassBank library. All

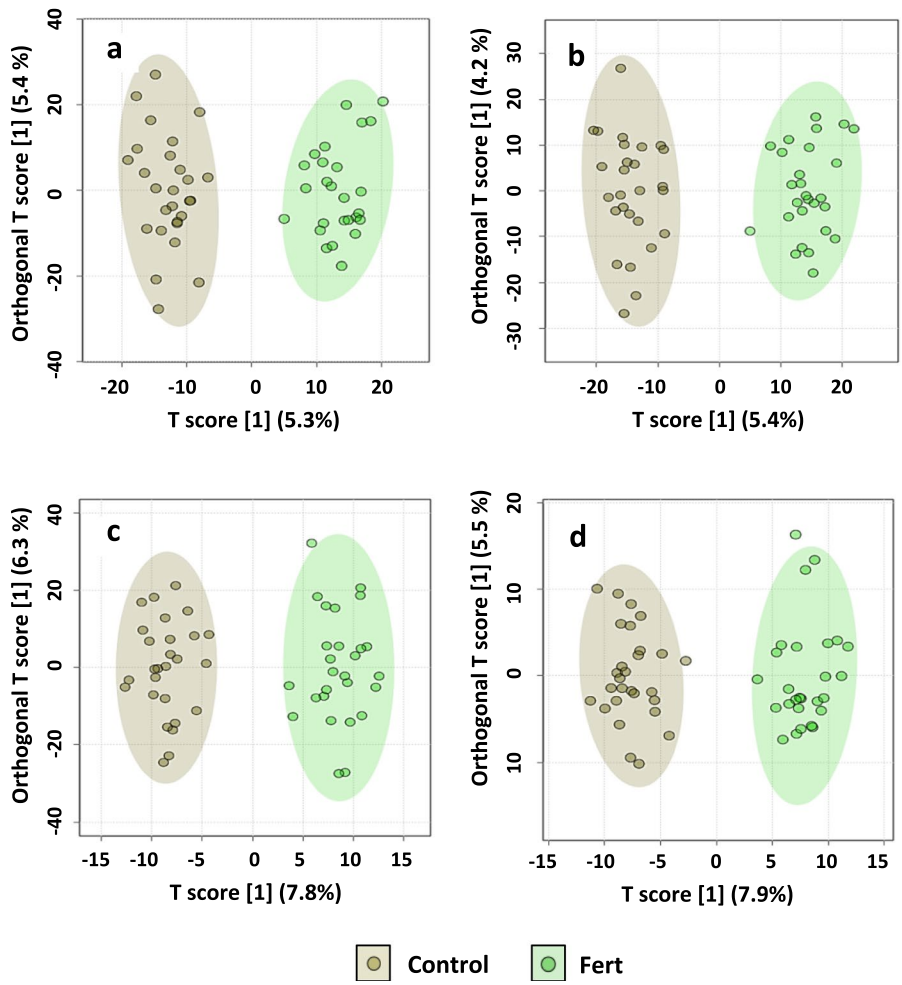


Fig. 2 Differences in metabolite profiles of heather (*Calluna vulgaris*) growing in control soil vs soil after N and P fertilizer (Fert) application 10 weeks prior to sampling. OPLS-DA for significant separation of clusters = $p < 0.05$. for all four HPLC–MS/MS streams. C18 pos (**a**), C18 neg (**b**), HILIC pos (**c**) and HILIC neg (**d**). Dots within each cluster represent the sampled plants in each treatment and ellipses represent 95% confidence. C18 pos control had two outliers removed and all others had one removed

annotated compound confirmation data is provided in Supplemental Information Doc. 2.

Changes in metabolite intensity expressed as fold change (FC) implies that a FC of 1 = no change, FC of 1.50 = 50% amplification/reduction, or a FC of 2.5 = 150% amplification/reduction etc. Seven metabolites in the top section of Table 1 are N-containing compounds, five of which were amplified in the fertilizer treatment. Seven compounds in the top section of Table 1 are also primary metabolites identified from the HILIC streams, two of which are common carbohydrates i.e. galactaric acid and a,a-trehalose which are both similarly amplified. Four secondary compounds in the bottom section of Table 1 comprise two terpenes (including camphor), a chalcone and an aldehyde.

In Table 2, twenty-two phenylpropanoids show a range of phenolic glycosides, glucosides and various flavonoids, coumarins and cinnamic acids plus four benzenoids. The fertilizer treatment has resulted in fifteen (68%) phenylpropanoids having reduced intensities and seven being amplified with the four benzenoids all reduced.

Pathway analyses show impact on flavone and flavonol biosynthesis

Pathway enrichment analysis identified five major pathways that were upregulated in the fertilized plants. The greatest impacts were on the flavone and flavonol biosynthesis pathway (with apigenin and quercetin as the main contributors) and on the biosynthesis of various plant secondary metabolite pathway (with scopoletin as the main contributor) each with an impact factor of 0.25. The pantothenate and CoA biosynthesis pathway (with pantothenic acid and L-valine as main contributors) was the next most impacted at 0.13, followed by the flavonoid biosynthesis pathway (with apigenin, taxifolin, quercetin and (+)-gallocatechin with a factor of 0.072. Vitamin B6 metabolism had the lowest impact factor of 0.024 due to the presence of pyridoxine. For further details on this analysis see Supplemental Information Doc. 1 (Fig. 1).

Relationships between foliar N and primary and secondary metabolites

Simple linear regression analysis revealed significant positive correlations between total foliar N and two primary metabolites – pantothenic acid ($r^2 = 0.18$, $F(1,25) = 5.75$, $p = 0.02$ and $r^2 = 0.33$, $F(1,26) = 13.25$, $p = 0.001$) in fertilizer and control treatments, respectively) and L-valine ($r^2 = 0.32$, $F(1,25) = 11.78$, $p = 0.002$ and $r^2 = 0.38$, $F(1,26) = 16.31$, $p = 0.001$) in fertilizer and control treatments, respectively (Fig. 3a, b). For secondary metabolites, total foliar N was not significantly correlated with diethylphathalic acid in the fertilizer treatment but a significant negative correlation was evident in the control plants ($r^2 = 0.17$, $F(1,25) = 5.36$, $p = 0.02$ (Fig. 3d). A metabolite of real interest, trans-cinnamic acid, which is the key precursor of all phenylpropanoid derived metabolites, shows no correlation with % foliar N but displays considerably less variation with fertilizer treatment (Fig. 3c).

Discussion

Soil fertilizer application affects the Calluna vulgaris *metabolome*

All four streams used in the HPLC–MS/MS analysis provided a comprehensive assessment of changes to both the primary and secondary metabolites associated with the two treatments. Our results show that application of fertilizer to the soil significantly influences the total foliar N content of *C. vulgaris*, with N % DW increases in the fertilizer treated plants (Fig. 1a). We also demonstrate a significant reduction in the C:N ratio (Fig. 1b) and changes to both of these parameters resulting from enhanced soil nutrients are in line with earlier reports from the literature (Carroll et al. 1999; Power and Collins 2010; Peterson et al. 2024). The mean foliage % N in the control treatments (1.38%) is in the higher range reported for this region (1.00–1.42%) (Peterson et al. 2024) but was the only heather beetle-free site accessible for the experiment. Changes in foliar N and the C:N ratio with fertilizer application reported here do appear modest by comparison to earlier data (Peterson et al. 2024) but we contend this change sufficiently demonstrates the significant foliar metabolic effects in heather resulting from fertiliser application. The magnitude of foliar % N difference between fertilized and control treatments is ~ 7.4% which we consider to be biologically meaningful given the critical importance of foliar N for insect growth and development (Mattson 1980). Support for this can be extrapolated from the data of Fox

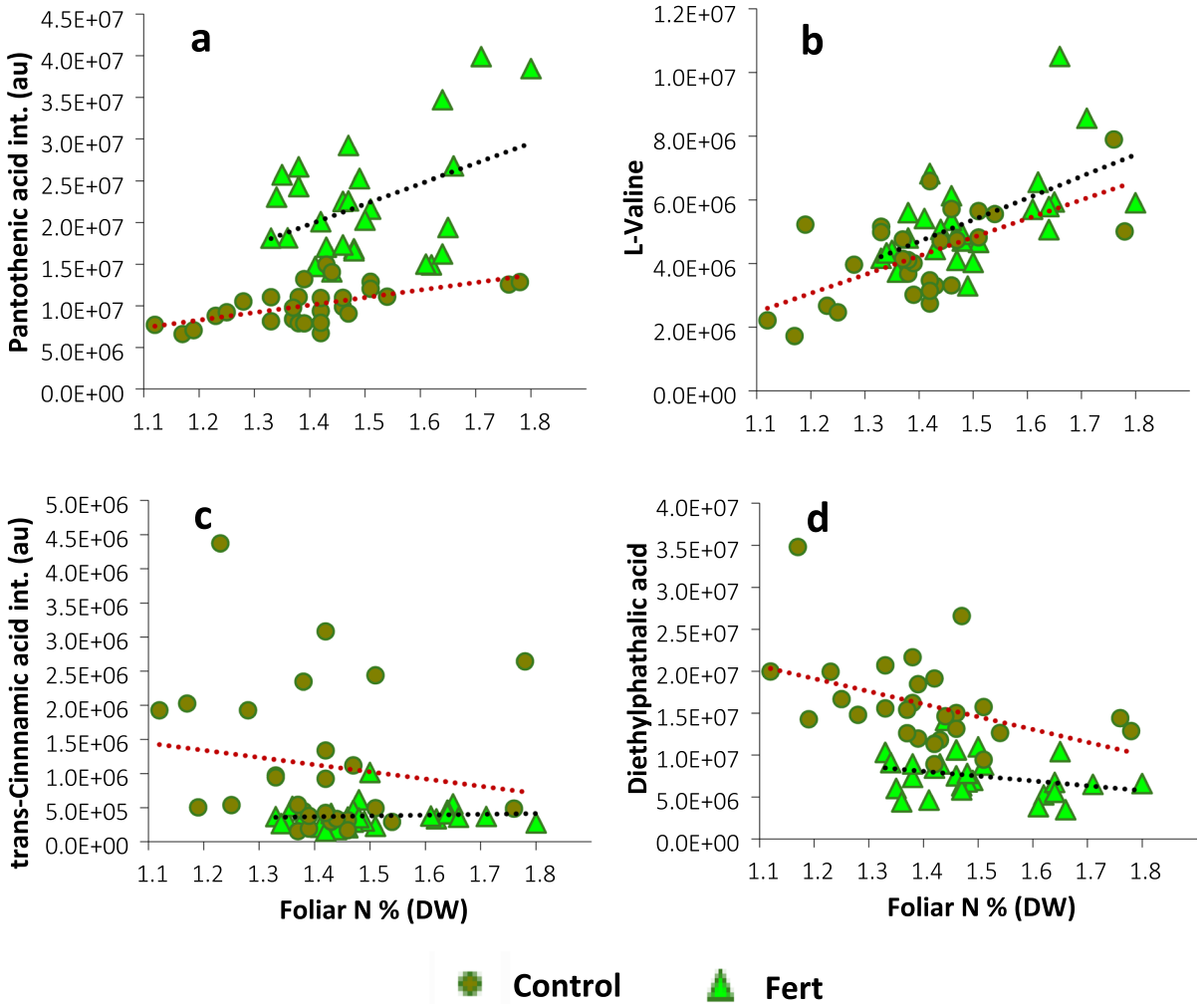


Fig. 3 Total foliar N (% dry weight) vs metabolite signal intensity, arbitrary units (au) in *Calluna vulgaris* growing in control soil vs soil after N and P fertilizer (Fert) application 10 weeks prior to sampling. Primary metabolites pantothenic acid (**a**) and L-valine (**b**) show a significantly positive correlation with foliar N in both treatments. The secondary metabolite trans-cinnamic acid (**c**) has no significant relationship with N but indicates a reduction in mean fold change of 2.76 and a marked reduction in variability in the fertilizer treatment. Diethylphthalic acid (**d**) is reduced in the fertilizer treatment by 2.14-fold change and shows a significant negative correlation with N in the control treatment. All listed R^2 values significant, $p < 0.05$

and Macauley (1977). That study used various *Eucalyptus* spp. with foliar N differences ranging from 3 to 19%, all of which provided measurable differences in the efficiency of conversion of ingested food (ECI) for larvae of the chrysomelid leaf feeder *P. atomaria.*

A total of thirty-five compounds were annotated from these analyses which includes seven N containing metabolites, five of which display FC increases. Also, of the thirty-five, twenty-two (~68%) secondary metabolite phenylpropanoids display a FC decrease in these C-rich compounds. Thus, an increase in N containing and a concomitant reduction in many of the C-rich compounds with fertilizer application reduced the C:N ratio in *C. vulgaris* foliage. This result is also consistent with the work reported by Power and Collins (2010) who demonstrated changes to the C:N ratio of *C. vulgaris* due to differential atmospheric N deposition in the UK.

Improved performance of the heather beetle *L. suturalis* has been linked to increased foliar N (due to

atmospheric deposition) previously, from work done in the UK and Europe (Brunsting and Heil 1985; Bobbink and Heil 1993; Cuesta et al. 2008). Those reports, however, did not attempt to analyse, the metabolite profile of *C. vulgaris* (i.e., plant primary N-containing and secondary metabolites) to assess possible changes in foliage assimilability and gain deeper understanding of the mechanisms involved. It is well documented that plants are highly sensitive to nutrient supply and in either N, P or K nutrient stress, many, including woody shrubs, in line with the "growth-defense-trade-off" principle (Li et al. 2024), modulate phenylpropanoids and terpenoids. This response, results in elevated levels of secondary metabolites, thus increasing their resistance to herbivores (Gershenzon 1984; Zheng et al. 2021).

Variation of phenylpropanoids including quercetin and trans-Cinnamic acid (Barrett et al. 2024) and a range of volatile terpenoids (Effah et al. 2020) under varying soil nutrients, has previously been established for *C. vulgaris* in the NZ CP environment. When phenylpropanoids are elevated, trans-cinnamic acid, a key metabolite in the shikimate- phenylpropanoid pathway, can also be elevated (Kováčk and Bačkor 2007). Interestingly, this is seen in our results where trans-cinnamic acid in the control plants was elevated and highly variable, perhaps indicating the sensitivity to marginal levels of soil nutrients but markedly reduced and less variable with increased soil N and P (Fig. 3c). This trend was also apparent in camphor, a monoterpenoid synthesised via the entirely separate, mevalonic acid pathway, again perhaps indicating high sensitivity to nutrient stress and suggesting that these metabolites in *C. vulgaris,* with further confirmation, might be useful bio-markers as indicators of nutrient stress.

Primary metabolite function and effects

Pathways analysis revealed pantothenic acid and L-valine as the two key metabolites that drive upregulation of the pantothenic CoA (Coenzyme A) biosynthetic pathway, but they are also important for insect nutrition. Pantothenic acid is a key precursor for CoA which has a central role in plant metabolism, especially for carbohydrate and fatty acid synthesis (Miret and Munné-Bosch 2014) but is also an important source of vitamin B5 which, if absent from an insect diet, severely impairs development and survival as demonstrated in the dermestid beetle *Dermestes maculatus* (De Geer, 1774) (Coleoptera: Dermestidae) (Levinson et al. 1967).

L-valine is an essential alpha amino acid which plays a central role in plant metabolism, growth and development and is a building block for proteins (Singh and Shaner 1995). It is also an essential amino acid for insect growth and development without which they fail to develop and, it has also been shown to be phagostimulatory to the western corn rootworm *Diabrotica virgifera* (LeConte, 1886) (Coleoptera: Chrysomelidae) (Kim and Mullin 1998; Genc 2006). Considerable FC reduction in the non-protein amino acid (NPAA), 2-pyrrolidineacetic acid was also evident for the fertilized plants (Table 1.) Many NPAAs are directly toxic to insect herbivores by obstructing primary metabolism and/or interfering with neurological processes. Additionally, NPAAs store nitrogen in a form that is metabolically unavailable to herbivores (Huang et al. 2011) and this FC decrease may potentially reduce the defensive capacity in fertilized *C. vulgaris*. The relatively small FC increase in a,a-trehalose may also be important as it is a carbohydrate which circulates in insect haemolymph providing instant energy and is also associated with insect growth and development (Tellis et al. 2023).

Secondary metabolite effects and detoxification

Secondary metabolites in *C. vulgaris* were dominated by the twenty-two phenylpropanoid derived compounds which include four phenolic acid benzenoids. Of these phenylpropanoids, fifteen (68%) were reduced in the fertilizer treatment. The literature provides evidence that metabolites such as 2,4,5-trimethoxybenzaldehyde, camphor and the phthalic acid ester—diethylphthalic acid (Fig. 3d)—as well as trans-cinnamic acid, scopoletin and quercetin, all function as defensive metabolites against herbivorous insects.

The benzenoid 2,4,5-trimethoxybenzaldehyde has been shown to impact growth and development of *Chilo suppressalis* (Walker, 1863) (Lepidoptera: Crambidae), feeding on rice (*Oryza sativa*) (L) (Gu et al. 2025) and camphor is also considered a growth inhibitor and feeding deterrent (Kleine and Müller 2011). In a controlled trial, camphor significantly reduced the feeding rate of Colorado potato beetle (*Leptinotarsa decemlineata*) (Say, 1824) (Coleoptera:

Chrysomelidae) at all concentrations when compared with controls (Lazarević et al. 2022). Less is known about diethylphthalic acid; however, phthalate esters are found in several plant families, have considerable antibiotic, insecticidal and allelopathic capabilities and therefore cannot be discounted from our assessment of *C. vulgaris'* defensive arsenal (Huang et al. 2021).

Trans-cinnamic acid is toxic to larvae of *Helicoverpa armigera* (Hübner, 1808) and *Spodoptera litura* (Fabricius, 1775) (lepidoptera: noctuidae) feeding on cotton (*Gossypium* sp.) (Malvales: Malvaceae) (Dixit et al. 2017). Scopoletin, a metabolite amplified in the fertilizer treatment is effective in reducing survival and mean weight of *Melanoplus sanguinipes* (Fabricius, 1798) nymphs (Orthoptera: Acrididae) (Westcott et al. 1992). Quercetin, a toxin, impairs growth, development and survival in lepidopteran noctuid larvae, including larval development *of S. exigua*, pupation duration of *H. armigera* and *S. litura* and causes mortality in *S. frugiperda* (Gao et al. 2022). Larval mortality due to quercetin is also recorded in *Lymantria dispar* (Linnaeus, 1758) (Lepidoptera: Lymantriidae) and *Oedaleus asiaticus* (Bei-Bienko, 1941) (Orthoptera: Acrididae) (Wang et al. 2019; Gao et al. 2022).

The toxicity of trans-cinnamic acid, scopoletin and quercetin to coleoptera species however is less certain. It is likely though, that enzymic proteins such as cytochrome P450, which catalyse reactions to detoxify terpinen-4-ol when ingested by *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Chrysomelidae), or glutathione-S-transferases (GST) that conjugate phenolic compounds in the emerald ash borer *Agrilus planipennis* (Fairmaire, 1888) (Coleoptera: Chrysomelidae) would be upregulated. These enzymes are released into the insect mid-gut in response to elevated phytotoxins and are likely a significant metabolic cost which can negatively impact insect performance (Rajarapu and Mittapalli 2013; Wang et al. 2019; Gao et al. 2022). A recent report by Kshatriya and Gershenzon (2024) suggests that cytochrome P450 and GSTs are a widespread strategy used by all major orders of insect herbivores (including specialists) for the detoxification of many plant metabolites including phenolic based compounds (Schuler 2011; Lu et al. 2021). Further, a report by Pokharel (2023), suggests that whether sequestration or detoxification is the resistance strategy used by insects to nullify plant defences there is likely a metabolic trade off and cost to growth and/or reproduction.

Effects on the ECI

The Efficiency of Conversion of Ingested food (ECI) model in relation to herbivorous insects, demonstrates that the highest ECI's for insect herbivores would be for those feeding on higher concentrations of N-containing amino acid and proteins, coupled with adequate carbohydrates and low concentrations of defensive metabolites (Mattson 1980; Henn and Schopf 2001). For the primary metabolites, increased levels of pantothenic acid could be an important contributor to an improved ECI due not only to it providing N-containing amino acids but as a source of vitamin B5 critical to insect growth and development. Similarly, L-valine is likely to elevate the ECI by providing increased levels of amino acids. There is less certainty around the contribution of reduced levels of the NPAA 2-pyrrolidineacetic acid, however. Any N is likely to be inaccessible from this metabolite but with a FC reduction of 2.17 the potential for reduced toxicity may be relevant but requires controlled feeding trials focusing on the direct effects of this metabolite to gain a better understanding.

We are uncertain about the decrease in many other secondary metabolites and what degree of influence they may have on the ECI for *L. suturalis*. This is because the magnitude of fold reduction for most of these metabolites is relatively small. Fold difference changes of comparable magnitude for a similar range of secondary metabolites showed no effect in a bioassay measuring *L. suturalis* larval survival and pre-pupal weights (Barrett et al. unpublished data). It seems reasonable to assume that any reduction in the metabolic cost for detoxification for an insect herbivore should improve the conversion and assimilation efficiency of the food ingested. We posit therefore that amplification of N-containing primary metabolites in conjunction with ~ 68% decrease in carbon-rich phenylpropanoids in fertilized *C. vulgaris* should improve the ECI for *L. suturalis*. Such changes should increase the availability of N while at the same time reducing the metabolic costs of digestion for both larvae and adults and provide the impetus for increased larval growth and development and adult female fecundity as demonstrated in our earlier laboratory and field trials (Peterson et al. 2024).

Conclusions

There are previous reports demonstrating negative impacts of low foliar N in biocontrol agent performance in terrestrial weed biocontrol scenarios (Hinz and Müller-Schärer 2000; Wheeler 2001; Van Hezewijk et al. 2008; Uyi et al. 2016). The poor establishment and population growth of *L. suturalis* reported in Peterson et al. (2024) also confirmed a negative impact of low foliar N on this biocontrol agent in the CP environment. Changes to both primary N-containing and secondary metabolites identified with the application of metabolomics reported here, are the most likely drivers of this scenario. It is apparent that changes to target plant quality resulting in poor ECI's in the invaded environment could be an important factor to consider when predicting or assessing biocontrol agent success.

Early assessment and comparison of foliar total N and C between native and introduced ranges may provide important information to address any release site imbalances due to soil nutrient status and provide an enhanced ECI to improve introduced biocontrol agent establishment success. The population thereafter however has still to contend with poor quality foliage while dispersing. Our earlier metabolomics studies identified metabolites which may be involved in plant defence that are elevated in the CP range possibly due to the influence of high ultraviolet (UV) light compared to the native UK range (Barrett et al. 2024) and the results reported here revealed some of those are not reduced with fertilizer application. Thus, the initial poor performance of *L. suturalis* on the CP may well have been due to a combination of low foliar N and UV elevated defensive metabolites both contributing to the unexpected low population densities and variable effectiveness of this control agent. We contend therefore, the application of metabolomics may enhance our understanding of the mechanisms underlying failed or suboptimal biocontrol outcomes.

Acknowledgements We wish to acknowledge Bubs Smith and the board of Ngāti Tūwharetoa for assisting with access to the Central Plateau field sites. Also, Evans Effah, Ben Pearson, and Fern Kumeroa for assistance with field sampling.

Author contributions DPB, conceived all questions and objectives of the investigation. Conducted field trials, sampling, sample processing, analysis, annotation and interpretation of data. Primary author of the manuscript. MM, advised on aspects of experimental design, analysis and interpretation of data. PP advised on experimental design, provided historical data, and assisted with field trials. SVF, advised on heather nutrient status and prior experimental data. AKS, advised on metabolomic data analysis. DJL ran all samples using UHPLC-MS. ACM, secured funding and principal investigator. Advised on concept, design and interpretation of investigations. All coauthors revised the final manuscript.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions. We wish to thank the following organisations for funding this project. The QE II technicians study award, awarded to Paul Barrett. Manaaki Whenua-Landcare Research, for MBIE—Strategic Scientific Investment Funding allocated to Paul Barrett. The Royal Society, Fast Start Marsden Grant "Plant Communication in times of rapid environmental change" awarded to ACM.

Data availability All relevant.raw spectral data sets are available upon reasonable request.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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