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Infection by a foliar endophyte elicits novel arabidopside-based plant defence reactions in its host, *Cirsium arvense.*

S.E. Hartley[1], R. Eschen[2], J.M. Horwood[1], A.C. Gange[3], E.M. Hill[4*]

[1] Department of Biology, University of York, Heslington, York YO10 5DD, UK.
[2]CABI Europe-CH, Rue des Grillons 1, CH-2800 Delémont, Switzerland.
[3] School of Biological Sciences, Royal Holloway, University of London, Egham Hill, Egham TW20 0EX, UK.
[4] School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK.

***** Author for correspondence:
Tel: (00) 44 (0)1273 678382
Email: e.m.hill@sussex.ac.uk

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Summary

- Endophytic fungi live asymptomatically within plants. They are usually regarded as non-pathogenic or even mutualistic, but whether plants respond antagonistically to their presence remains unclear, particularly in the little-studied associations between endophytes and non-graminoid herbaceous plants.
- We investigated the effects of the endophyte *Chaetomium cochlioides* on leaf chemistry in *Cirsium arvense*. Plants were sprayed with spores; leaf material from both subsequent new growth and the sprayed leaves was analysed two weeks later. Infection frequency was 91% and 63% for sprayed and new growth respectively, indicating *C. cochlioides* rapidly infects new foliage.
- Metabolomic analyses revealed marked changes in leaf chemistry with infection, especially in new growth. Changes in several novel oxylipin metabolites were detected, including arabidopsides reported here for the first time in a plant species other than *Arabidopsis,* and a jasmonate-containing galactolipid.
- The production of these metabolites in response to endophyte presence, particularly in newly infected foliage, suggests endophytes elicit similar chemical responses in plants to those usually produced following wounding, herbivory and pathogen invasion. Whether endophytes benefit their hosts may depend on a complex series of chemically mediated interactions between the plant, the endophyte, other microbial colonists and natural enemies.

Introduction

Endophytic fungi are present in most, if not all plant species and appear to be ubiquitous in plants in natural ecosystems (Petrini 1986; Stone *et al.* 2000). This widespread distribution, together with their long history of association with land plants (Arnold *et al.* 2003; Krings *et al.* 2007) suggests they have ecological and evolutionary significance for plants (Rodriguez *et al.* 2009). However, the precise nature of this significance and the mechanisms underpinning it remain unclear in many cases, particularly for endophytes within forbs (Hartley & Gange 2009); far more is known about the behaviour of endophytes in symbiotic associations with grasses (Saikkonen *et al.* 2006; Rodriguez *et al.* 2009). Endophytes usually live asymptomatically within healthy plant tissues; they may be closely related to virulent pathogens but typically cause no pathogenic effects themselves. As such they have not generally been considered antagonistic to their host plants, and indeed are often viewed as mutualistic, although any benefits to their hosts are likely to depend on a range of factors, including the species identity of both host and fungus, the phylogeny and life history strategy of the endophyte, particularly its mode of transmission, and the physiological status of the host (Hartley & Gange 2009).

The vertically transmitted Clavicipitceaous endophytes infecting grasses have been proposed as defensive mutualists of their hosts (Cheplick & Clay 1988) because they are associated with the production of toxic alkaloids (Siegel & Bush 1996) which enhance host resistance to herbivory (e.g. Wilkinson *et al.* 2000, Tanaka *et al.* 2005). However, the benefits to the host of endophytes transmitted horizontally by spores are less well-studied and remain unclear. In forbs, the role of horizontally transmitted endophytes as defensive mutualists against herbivores is particularly poorly known, but there is some evidence to suggest that infection can increase resistance to herbivores (McGee 2002; Gange *et al.* 2012). This effect, like the anti-herbivore benefits conferred by grass-infecting endophytes, is thought to be due at least in part to their production of secondary metabolites (Schulz *et al.* 1999), yet to date there is no clear experimental demonstration of such anti-herbivore metabolite production in the plant tissue of forbs which has been shown to result from endophyte infection or growth. Although many endophytes appear to be closely related to pathogens (Carroll 1988) and are known to increase resistance to pathogen antagonists by mechanisms including the production of secondary metabolites, competitive exclusion and

induction of systemic resistance (Arnold *et al.* 2003; Rodriguez *et al.* 2009), it has also been demonstrated that asymptomatic endophytes do not induce anti-pathogen plant defence systems until other pathogens have attacked (Redman *et al.* 1999).

It seems the interactions between endophytes and their host plants are able to lie anywhere on a continuum from latent pathogen to mutualistic symbiont (Carroll 1988; Schulz & Boyle 2005), but in all cases the chemical responses of the host plant to the presence of the endophyte are likely to be critical to any host benefits, as are any chemicals produced by the endophytes themselves, whether these act to promote virulence or provide mutualistic protection against other organisms to their hosts. Endophytes are known to produce an array of biologically active secondary metabolites in culture (Schulz *et al.* 1999; Schulz *et al.* 2002), but whether these antagonistic substances are also synthesised *in vivo*, and if so, the role they play in the interaction between plant and fungi remains unknown (Schulz & Boyle 2005). More detailed knowledge of the chemical responses of the host in response to infection is needed to understand whether the plant responses to initial endophyte infection resemble those of the plant to invasion of pathogenic fungi, or whether the endophyte somehow manages to avoid inducing these responses.

In this study we examined the nature and extent of the chemical changes in the host in response to infection and whether such changes were of plant or fungal origin or both. Our key questions were:
(i) Does the host respond initially as if endophytes are pathogenic and induce defences which are subsequently overcome by the endophyte, or do endophytes never induce such defensive responses?
(ii) If defensive responses are induced, are they local (i.e. restricted to colonised tissue) or systemic (i.e. spread to new leaves produced subsequent to infection)?
(iii) Does endophyte infection lead to changes in the levels of chemical defences which potentially make the host more resistant to subsequent herbivore or pathogen attack?
To address these questions we used a metabolomic approach, i.e. a non-targeted analytical approach which allows the simultaneous profiling of a wide variety of in metabolites in plant tissues (Fiehn 2002; Macel *et al.* 2010).

We investigated the chemical basis of the interactions between a common forb, *Cirsium arvense* (creeping thistle), recognised as one of the world's most problematic weeds (Tiley 2010) and a

foliar endophyte. *Chaetomium cochlioides* was chosen as our model endophyte as it is a predominant species detected in *C. arvense* in natural populations (Gange *et al.* 2007). Plants were infected with the endophyte, and two weeks after infection, the metabolite profiles of extracts from the originally infected tissue and from new leaves formed since infection were compared with similarly aged leaves from uninfected plants. We profiled metabolite extracts of leaf tissues to determine if endophyte infection leads to the *de-novo* production of metabolites in infected tissues and, if so, whether these compounds were of fungal or plant origin. We hypothesised that infected plants would show an altered chemical profile compared with uninfected individuals; if the plant is initially antagonistic to the endophyte, we predicted that this altered profile would include plant defence metabolites known to be active against pathogen invasion, and potentially other invading organisms, explaining our prior observation that endophytes affect insect herbivores feeding on this plant (Gange *et al.* 2012). Our second objective was to determine if the host's chemical response to infection is systemic or local. Given that the response of insects to the presence of endophytes can be detected systemically (Gange *et al.* 2012), even though endophyte growth may not be throughout the plant (Sánchez Márquez *et al*. 2012), we hypothesised that any defensive compounds produced would be transmitted systemically to leaves formed since infection.

Methods

Plant material and endophyte infection

Cirsium arvense seeds were obtained from a commercial supplier (Herbiseed, West End, Twyford, UK). Plants were grown from surface-sterilized seed (30 min in 10 % commercial bleach solution, then rinsed with ultrapure water), sown in sterile potting soil (autoclaved twice for 1 h at 121 °C). At the first leaf stage, the seedlings were planted into 13 cm diameter pots with John Innes No. 3 soil. The plants were kept in an unlit, unheated greenhouse and watered as needed. After eight weeks, half of the plants (15 replicates) were treated with *C. cochlioides*, originally isolated from *C. arvense* and maintained on potato dextrose agar. The fungus was applied as a 1 mL water-oil (1:1) suspension containing ca. 18,000 spores. The untreated plants were sprayed with an equal amount of water-oil mixture without spores. To minimize the number of spores reaching the soil, the soil around each plant was covered with tissues while the treatments were applied. At the time of spraying, the number and total length of the stems, and the number of leaves on each plant were recorded and a stick was placed next to each plant to monitor plant growth. Two weeks thereafter, the plants were harvested and the number and total length of stems and the number of leaves were recorded. Then, with a cork borer, one 8-mm disk was taken of each of three leaves that were present when the treatments were applied and of three leaves that had grown since (old and new leaves, respectively). Simultaneously, additional intact leaf and stem material of the old (sprayed) and newly infected plant parts was snap-frozen in liquid nitrogen and stored at -80 ^{0}C prior to metabolomic analysis.

Analysis of endophyte infection

The leaf disks were surface-sterilized to remove epiphytes with a modified technique based on method 3 in Schulz et al. (1993). The sterilized disks were placed abaxial surface downwards on potato dextrose agar (3.9 % w/v, Oxoid Ltd., Basingstoke, Hampshire, UK) and the emerging fungi were subcultured on potato carrot agar (2.0 % w/v) to stimulate sporulation. 60 mg L^{-1} Penicillin G and 80 mg L^{-1} streptomycin sulphate salt (Sigma-Aldrich Co., St Louis, MO) were added to the agars to prevent bacterial growth. Cultures were grouped based on colony morphology and representative isolates of each group were identified by Dr B.C. Sutton.

Metabolomic analyses

Plant extraction: An aliquot of ground frozen material (0.1g) was extracted in a ball mill using 2ml of a multi solvent composition of ice cold isopropanol: acetonitrile: water (3:3:2) according to Sana *et al.* (2010). Deuterated internal standards (10ng) were added to the tissue extracts prior to solvent extraction for quality control to monitor sample recovery, MS analytical sensitivity and for chromatogram alignment. These comprised progesterone-d9 as a standard for analysis using positive electrospray ionization (+ESI) and 17β-estradiol-d4-sulfate for analysis using negative (-) ESI mode (CDN Isotopes, Quebec, Canada). The tissue extracts were centrifuged, the supernatant removed, and the pellet extracted with a further 2 ml of multi solvent mixture. The supernatants were combined and a 0.5 ml aliquot was dried down under vacuum and resuspended in 300 µL 70% methanol water, and filtered (0.2µm) prior to mass spectrometry analyses.

Extraction of C. cochlioides *cultures:* Aliquots (100-200mg) of *C. cochlioides* cultures, grown on potato dextrose agar, were extracted with the same multi solvent mixture (2 x 0.5mL) used for plant extracts. After addition of internal standards, the extracts were vortexed, filtered (0.2µm), the solvent removed from the supernatant, and the residues dissolved in 100 µL 70% methanol water for mass spectrometry analyses.

Metabolomic profiling: Plant extracts were profiled using ultra high performance liquid chromatography (UPLC) linked quadrupole time-of-flight mass spectrometry (QTOFMS). Samples were analysed in random order and an aliquot (15 µL) of each sample was injected onto an Acquity UPLC BEH C18 column (1.7 µm particle size, 2.1 × 50 mm, Waters, UK) maintained at 20^0C. Mobile phase solvent A consisted of 0.1% formic acid in water (5% acetonitrile) and B 0.1% formic acid in acetonitrile. The flow rate was 0.3 ml min^{-1} and the gradient program was 0-9 min from 0 to 100% B; 9-29 min 100% B.

Analytes were detected with a Micromass (Waters, Manchester, UK) QTOF-MS system. Samples were analysed in both +ESI and –ESI modes to detect as many metabolites as possible. Capillary voltage was 2.60 kV in positive mode and between –2.60 kV and -2.75 kV in negative mode. Argon was used as collision gas at TOF penning pressures of 4.83 x 10^{-7} to 5.73 x10^{-7} mbar, the cone and multiplier voltages were set at 35 and 550 V respectively and the collision energy was 10 eV. A baffled lockspray source enabled on line calibration of mass ions. Sulfadimethoxine (5 pg μL^{-1} in

1:1 v/v methanol/water, plus, in positive mode only, 0.1% formic acid) was used as internal lockmass infused at 40 µL min^{-1} via a lockspray interface (baffling frequency: 0.2 s^{-1}). For both positive and negative modes the source temperature was 100ºC, the desolvation temperature was 250ºC and the desolvation nitrogen flow 300 l h^{-1}. The mass spectrometer was tuned to 9000 mass resolution and spectra were collected in full scan mode from 100 to 1200 m/z.

Multivariate analyses. Spectral peaks were deconvoluted and aligned using the MarkerLynx V 4.1 software package (Waters Corporation, Milford, MA, USA). The parameters used for detecting spectral peaks were optimised so that the internal standard signals were detected with the minimum noise level possible. Isotopic peaks were removed and the data was binned using 0.03 Da mass and 0.1 min retention time windows. The area intensities of all the resulting spectral peaks were normalised so that the sum of peak intensities for each sample was 10,000 and the dataset exported to SIMCA-P software for multivariate analysis (Umetrics UK Ltd., Winkfield, Windsor, Berkshire, UK).

The datasets were pareto-scaled and log transformed prior to principal component analysis (PCA) and any outliers were detected from the 95 % confidence area of the Hotelling's T^2 tolerance area. The datasets were then analysed by the supervised partial least squares-discriminant analysis (PLS-DA) to find class separating differences. PLS-DA maximises the covariance between X, the LC-MS data, and Y, the treatment class. Cross validation (CV) was used to determine the significant components of the models and thus minimise overfitting. During each CV round, one sample from each class was excluded (in random order), and the classification of the excluded samples predicted from modelling the remaining dataset. The performance of the models was then described by the explained variation (R^2X for PCA and R^2Y for PLS-DA) and the predictive ability (Q^2X or Q^2Y) parameters of the models.

In order to identify the loading variables influencing sample classification, models with high predictive ability ($Q^2Y > 0.5$) were analysed using orthogonal PLS-DA (OPLS-DA) to select data that was only due to class separation. The most discriminative variables (retention time (RT) x m/z) between control and infected plants were visualized using an 'S'-plot which is a scatter plot of covariance and correlation loading profiles resulting from the OPLS-DA model (see Wiklund *et al.*

2008). Discriminatory variables (metabolite markers) between control and treated plants were confirmed using the Students t-test and metabolite markers were ranked according to their *p* value. Non-normally distributed data (determined by the Kolmogorov-Smirnov test) were log transformed prior to statistical analysis. However, because the number of false positives (type 1 errors) was likely to be high due to the abundance of variables being tested, a false discovery rate (FDR) of 5% was applied to the *p* values to establish the significance of each metabolite marker (Benjamini *et al.* 2001).

Structural identification of metabolites

Metabolites were analysed in accordance with the standards set by the Metabolomics Standards Initiative (Sumner *et al.* 2007). The observed masses of discriminatory metabolites (i.e. the loading variables identified influencing sample classification in the OPLS-DA models) were used to calculate theoretical masses of candidate molecules with the elemental composition tool (Version 4.00) of the MassLynx software. Formulae were calculated from a mass accuracy of <10ppm, with elemental constraints of $C_{100}H_{300}O_{50}N_{10}P_{10}S_{10}Na_2$, and the metabolite formula determined from a combination of mass accuracy and isotopic fit data. For further structural identification of the plant metabolites, extracts were analysed by UPLC-QTOFMS at collision energies of 20-40eV to obtain fragmentation data obtained from collision induced dissociation (CID) analyses. The identity of discriminatory metabolites was then determined from their accurate mass composition, their isotopic fit and fragmentation. Mass spectra and fragmentation data for identified compounds were compared with those published in journals, metlin or massbank metabolite databases (http://metlin.scripps.edu/, http://www.massbank.jp).

Statistical Analyses

The effect of *C. cochlioides* infection on total stem length and the number of leaves on each plant was analysed using generalised linear models with *C. cochlioides* treatment as a fixed factor. Leaf count data were assumed to follow a Poisson distribution. Endophyte infection frequency was calculated by dividing the total number of isolates (separate colonies) per plant of each fungal species by the total number of isolations (leaf segments) for that plant (Gange *et al.* 2007). The effect of *C. cochlioides* treatment and the difference between old and new growth on the number of isolates, the number and diversity indices (Shannon and Simpson diversity) of endophyte species in

each plant was tested using generalised linear models with *C. cochlioides* treatment and leaf age as fixed factors and an assumed Poisson data distribution. A two way ANOVA was used to analyse the effect of leaf age, endophyte infection and the interaction of age x infection on metabolite abundance. The relative concentration of each metabolite in each sample was determined from the ratio of the peak area of the molecular ion to that of the internal standard. Data was transformed Log_{10} (X + 1) to ensure homogeneity of variance (Levene's test) prior to analysis. Spearman correlations were used to investigate the effect of underlying infection with endophyte species other than *C. cochliodes* on metabolite concentrations.

Results

Endophyte infection

There were no effects of *C. cochlioides* treatment on the number of leaves or the total stem length of the plants (both $p > 0.25$; average 29.9 ± 2.1 leaves and 79.9 ± 5.4 cm stem per plant). For treated plants, the infection frequency of *C. cochlioides* was 91.1 ± 3.6% and 62.7 ± 5.6 for the sprayed leaves and for new leaves respectively (Table 1). The presence of *C. cochlioides* in new leaves indicated movement of the endophyte from infected parts of the plant to sites of new growth of plant tissue. In contrast, in the uninfected control plants no *C. cochlioides* was detected in new leaves and there was only a 2.4 ± 1.7% frequency of infection present in the old leaves. An additional 15 fungal species were also found in either the control or *C. cochlioides* treated plants (Table 1). However, the highest infection frequency of these species was 31% in control plants and 11% in *C. cochlioides* treated plants, so although other endophytic fungi were present in treated plants, the infection frequency was relatively low and the relative occurrence of individual species was not related to the *C. cochlioides* treatment. Excluding *C. cochlioides* itself, 14 different fungi were isolated from the leaves of control plants, compared with only seven from *C. cochlioides* treated plants, a significant difference ($p < 0.001$). The endophyte diversity in *C. cochlioides* treated plants was lower than that in control plants (0.31 ± 0.07 vs. 0.87 ± 0.08 and 0.22 ± 0.05 vs. 0.61 ± 0.04 for Shannon and Simpson diversity, respectively; both $p < 0.001$). There was no difference in the number or diversity of fungal species between old and new growth in either untreated or *C. cochlioides* treated plants (all $p > 0.25$).

Metabolite p*rofiling*

After initial processing (see methods) of the MS datasets, 52,144 and 16,889 signals were detected in +ESI and –ESI mode respectively. Analysis of these datasets revealed that the relative standard deviation of the peak areas of the internal standards used for quality control was between 6 and 10% for either ESI mode, indicating little drift in MS sensitivity during the analyses. PCA of the +ESI MS mode data revealed distinct separation between the new leaves analysed from control and *C. cochlioides* treated plants and the variability accounted by the first two components of the PCA model was 25% (Figure 1A). Further analysis using supervised PLS-DA of the same dataset

revealed a model with good predictivity (Q2Y >0.52) and a high degree of explained variation (a total R2Y of 0.84) modelled for the first 3 latent variables (Figure 1B). The first latent variable explained 26.8% of the Y variation and was related to biochemical differences between the old and new control leaves. The second and third latent variables explained 28.7% and 28.4% of the Y variation respectively, and were related to *C. cochlioides* treatment in either the old or the new leaves. This model revealed large chemical space between the infected and control treatments of new leaves, but less so between the treatments from old leaves. Hence in both models, the greatest difference in leaf biochemistry in response to *C. cochlioides* infection appeared to reside in the younger (unsprayed) leaves harvested 2 weeks after infection, and there was less separation between older infected and control leaves. Analysis of the -ESI MS mode data revealed similar patterns to the +ESI mode (data not shown).

Identification of metabolite markers of endophyte infection

The datasets were further modelled separately using OPLS: 'S' plot analyses were used to detect metabolite signals that had changed significantly (judged using FDR corrected *p* value) in response to *C. cochlioides* infection. Only 10 significant markers of *C. cochlioides* infection were detected in +ESI analysis of extracts of new leaves, and 9 metabolite structures were common to both + and –ESI datasets (Table 2 and fragment data given in supporting information Table S1). In +ESI mode the markers were detected as the sodium adducts, whereas in –ESI they were present as the M-H ion, and for some compounds as the formate adduct too. In extracts from old leaves only one marker of *C. cochlioides* infection was detected after correction for the FDR, and this was also present as a marker in the extracts from new leaves (metabolite III). The relative abundances of these metabolites and significant differences between treatment groups are presented in Table 3.

The main metabolite markers that were responsible for the discrimination between the control and *C. cochlioides* treated plants were a series of free or esterified oxidised lipids (oxylipins). Metabolites I and II were identified as free trihydroxy fatty acids with MS fragments consistent with hydroxylation on the C9, C11 and C12 positions, and the same structures were identified from metabolite databases (metlin) as oxidation products of octadecatrienoic acid (α-linolenic acid) and octadecadienoic acid (linoleic acid) respectively. In addition, Metabolite II coeluted with, and showed the same MS as an authentic standard of 9,12,13-trihydroxyoctadecenoic acid (Larodan

Fine Chemicals, Malmo, Sweden). Metabolite III was identified as an oxylipin esterified to a galactolipid which comprises both galactose and glycerolipid groups (see core structure in Figure 2). The ion at m/z 211.1338 was consistent with an oxidised C12 fatty acid structure. Further loss of CO_2 from m/z 211.1338 gave rise to m/z 167.1440 ($C_{11}H_{19}O$) which is consistent with a fragment of a saturated C11 group containing a cyclopentyl moiety and a common fragment for dihydrojasmonic acid (metlin/massbank databases). The other acyl group (m/z 277.2162) was octadecatrienoic acid. Fragments at m/z 447.2231 and 513.3069 corresponded to loss of the C18 and C12 acyl groups from the galactosylglycerol backbone respectively. Fragment ions corresponding to galactosylglycerol (m/z 253.0925) and to galactose (m/z 179.0569) were also observed. To our knowledge this study is the first identification of a C12 oxylipin galactolipid ester in plant tissues.

The identity of Metabolite IV is currently unknown, and based on its empirical formula the compound was not present in plant metabolite databases and it's concentrations in the plant extracts were too low for further analyses. Metabolites V and VI were identified as oxophytodienoic acid (OPDA) ester derivatives of digalactosyl and monogalactosyl glycerols respectively. Fragments at m/z 291.1960 indicated the presence of an oxidised C18 acyl ester group, m/z 181.1226 revealed further cleavage between C11 and C12, and m/z 165.1281 corresponded to loss of CO_2 from a fragment of $C_{12}H_{17}O_3$ which was consistent with a ketone group on C12 and a common fragment of the OPDA structure (metlin database). The ion at m/z 415.1453 in the mass spectrum of metabolite V indicated loss of OPDA from the digalactosyl glycerol moiety of the molecule, and similarly for metabolite VI m/z 253.0921 corresponded to loss of OPDA from a monogalactosylglycerol group. In addition, the mass spectra of both metabolites V and VI were the same as those previously reported for these compounds (Glauser *et al.* 2008). Metabolites VIII, X and IX had already been detected in leaves from wounded Arabidopsis plants (Buseman *et al* 2006; Ibrahim *et al* 2011). Metabolite VIII was identified as Arabidopside D, an OPDA diester of digalactosyl glycerol with a mass spectrum similar to that previously reported for this compound (Buseman *et al.* 2006). Fragments at m/z 291.1962 and 165.1285 confirmed the OPDA moiety, and m/z 689.3386 and 415.1449 and were consistent with the loss of 1 and 2 OPDA groups respectively from the molecular ion. Metabolite VII eluted from the UPLC 0.03 mins prior to metabolite VIII and was a structure closely related to VIII. The mass spectrum of VII comprised ions at m/z 167.1442 and

293.2123 consistent with the loss of a double bond from the cyclopentyl ring of OPDA and which corresponded to a structure of the oxophytoenoic acid diester of digalactosyl glycerol. The mass spectrum of metabolite IX was consistent with a structure comprising OPDA and octadecatrienoic acid (m/z 277.2162) esters of monogalactosyl glycerol and was the same as that previously reported for this compound (Buseman *et al.* 2006). Metabolite X eluted 0.09 mins later than IX, and the mass spectrum indicated it was the OPDA and octadecadienoic acid ester analogue of IX. The structures corresponding to these esterified oxylipin metabolites are given in Figure 2.

Analysis of the relative abundance of all metabolites, including the identified esterified oxylipins, revealed that they were always detected in control old and new leaves, although their levels altered significantly primarily in new growth in response to *C. cochlioides* infection (Table 3). In new leaves, levels of the free hydroxy fatty acids (metabolites I, II) increased by 4-6 fold in infected plants, and levels of the esterified jasmonate metabolite (III) by 48 fold. In contrast, the levels of the other oxylipins which were esterified with either OPDA or dihydroOPDA (V-X) decreased by 2-33 fold in new growth of *C. cochlioides* treated plants. Examples of the relative concentrations of the different esterified oxylipins in leaves from control and infected plants are given in Figure 3. Analysis of metabolite abundance by ANOVA revealed significant effects of *C. cochlioides* infection (for all metabolites) and of leaf age (all metabolites except V, VI and VII). However, for all metabolites there was also a significant interaction of leaf age x infection on abundance, and (with the exception of metabolite IV) the effect size (η^2) of this interaction was comparable or greater than that of either leaf age or infection alone, confirming that the effects of *C. cochlioides* infection on metabolite levels were primarily associated with leaf age (new growth). In contrast, the effect of leaf age x infection interaction was less important for IV where levels of this metabolite were reduced in both old and new growth as a result of *C. cochlioides* infection.

The effect of the frequency of infection with fungal species other than *C. cochlioides* on the abundance of individual metabolites in either control or in treated plants was examined using Spearman correlation analyses. There was no significant correlation between the frequency of infection of non-*C. cochlioides* fungi, analysed either as individual species or combined as a total, and metabolite abundance ($p \leq 0.05$, two-tailed test).

To investigate the source of the metabolites associated with *C. cochlioides* infection, extracts were prepared from *C. cochlioides* cultured on agar plates. None of the arabidopside or trihydroxyfatty acid metabolites identified in the plant extracts were detected in extracts of ex-situ *C. cochlioides* cultures, suggesting that these metabolites were most likely to be of plant origin produced in response to colonisation, but we cannot rule out the possibility they are produced by the fungi once it is present in-situ, since we cannot separate fungal and plant compounds from colonised leaf material.

Discussion

Effect of endophyte infection on metabolite profiles

The presence of *C. cochlioides* caused large changes in plant metabolism in infected tissue, notably in compounds associated with defensive reactions to wounding and invasion. The most significant changes, both in terms of magnitude of the changes in response to infection and in terms of the potential consequences for the endophyte-host interaction, were in oxylipins esterified to galactolipids. These types of galactolipids have previously only been detected in species of the genus *Arabidopsis,* hence these metabolites have been termed 'arabidopsides', and they usually contain an ester of OPDA, which is an oxidised C18 fatty acid and a precursor of jasmonates (Gobel & Feussner, 2009; Dave & Graham, 2012). This study is the first to identify these arabidopside structures in a plant species other than *Arabidopsis,* a novel finding. The use of a metabolomic non-targeted analytical approach has identified arabidopside-related metabolites in another completely unrelated species which would not have been detected using targeted analyses and has provided new information on the responses of plants to endophyte infection.

In *Cirsium*, oxylipin metabolites whose concentrations changed significantly following infection included esters of dihydrojasmonic acid and 12-oxophytodienoic acid (OPDA), common lipoxygenase metabolites of the jasmonate pathway, which controls plant responses to wounding by herbivory and other organisms including pathogens (Farmer *et al.* 2003; Wu & Baldwin 2010). OPDA is a precursor of jasmonates and is formed from the action of 13-lipoxygenase on octadecatrienoic acid (α-linolenic acid). However, it is still unclear whether the rapid accumulation of free jasmonates in response to wounding in other plant species results from metabolism of OPDA pools derived from free or esterified OPDA (Koo *et al.* 2009). Recent studies have revealed that OPDA can be formed from lipoxygenase action on octadecatrienoic acids esterified to galactolipids in *Arabidopsis* tissues, indicating that formation and release of esterified OPDA may be the first step in synthesis of free jasmonates (Nilsson *et al.* 2012). Reduction of OPDA to dihydroOPDA is the next step in jasmonate synthesis and esterified dihydroOPDA (metabolite VII) was also detected in our analyses, indicating possible further metabolism of esterified OPDA. The production of free jasmonates was not detected, though the identification of an esterified dihydrojasmonate metabolite could indicate that they may have been biosynthesised and subsequently sequestered as a result of endophyte infection. Levels of esterified OPDA were significantly reduced in newly infected leaves of endophyte-treated plants, indicating that these esterified metabolites may be important precursor pool for jasmonate metabolism. In contrast, levels of the jasmonate ester, a wound signalling compound, were increased by endophyte infection in new leaves, but decreased in older leaves after infection, suggesting either that biosynthesis of this metabolite is transient after initial infection, or that it is further metabolised to other (undetected) structures.

Interestingly, we found that the largest biochemical differences between control and endophyte treated plants occurred in the youngest leaves, i.e. ones which had been produced since infection. Thus changes in the abundance of esterified oxylipins were most pronounced in the new leaves of endophyte-treated plants, where endophyte infection had spread from the older leaves originally exposed to *C. cochlioides*. These jasmonate metabolites and OPDA- esterified galactolipids are usually formed rapidly after wounding of tissue as signalling molecules associated with damage and are not detected in intact tissues (Busemann *et al.* 2006; Nilsson *et al.* 2012). To cause such wounding responses suggests that endophytes may disrupt cellular integrity to trigger these responses. This suggestion would repay further investigation, since the evidence to date suggests that endophyte growth within plants is intercellular, with no impact on plant cell structure (Rodriguez *et al.* 2009).

All 10 metabolites identified as markers of endophyte in infection in *Cirsium* were detected in control leaves from plants that had not been sprayed with spores of *C. cochlioides*. It is unlikely that they were present as a result of tissue damage, as leaves and stem material were immediately snap frozen after removal from the plant. However, the presence of oxylipins in leaves from control plants is not completely unexpected as these compounds are central to plant responses to a range of biotic stresses. A number of endophyte species (other than *C. cochlioides*) were detected in control leaves (Table 1), which may have been responsible for eliciting their production, although our analysis indicates that their presence was not significantly correlated with the concentration of any of the metabolites. Given that endophytes in forbs are transmitted horizontally, it would be impossible, and indeed unrealistic, to grow plants that are endophyte-free and some 'background' endophytes will always be present (Van Bael *et al.* 2009). In natural ecosystems, endophytes are present in all plant tissues examined to date (Arnold *et al.* 2003; Rodrigueuz *et al.* 2009) and our results suggest that even if these fungi occur at very low density in plants, they could still affect the chemical composition of the host tissues with consequences for the interactions between hosts and other organisms, potentially making endophytes a major structuring force in ecological communities.

The role of the compounds induced by infection

Our study has identified increases in esterified oxylipins in endophyte infected host plants. Previous work has demonstrated that these compounds are associated with wounding, herbivory (Kourtchenko *et al.* 2007; Glauser *et al.* 2008; Walters *et al.* 2006; Taki *et al.* 2005; Stintzi *et al.* 2001), or attack by pathogenic fungi (Goebel *et al.* 2002; Martin-Arjol *et al.* 2010; Walters *et al.* 2006). In *Arabidopsis*, levels of oxylipins have been shown to increase in the hypersensitivity response of the plant to avirulence proteins isolated from the bacterial pathogen *Pseudomonas syringae*; in vitro they inhibited growth of bacterial pathogens and of other plants (Hisamatsu *et al.* 2005; Andersson *et al.* 2006). The detection of these compounds in *Cirsium* in response to endophyte infection suggests that plants may "experience" endophytes as pathogenic, since at least initially, *Cirsium* produces the same sort of chemical in response to the endophyte as it does in response to wounding, insect herbivory or pathogen invasion. However, the production of the arabidopsides and other oxylipin esters is clearly insufficient to prevent the endophyte from becoming established within the plant tissues. Further work is needed to establish whether this is because the plant defensive response is too transitory or of insufficient magnitude to prevent colonisation by the fungus, or indeed its spread to new tissues.

The chemical responses to endophyte infection we have identified are part of the jasmonate signalling pathway which underpins induced systemic resistance in plants (Landgraf *et. al.* 2002; Wu & Baldwin 2010), suggesting endophyte-infected plants may be able to respond more effectively to subsequent attack by natural enemies. These mechanisms may help to explain the observation that foliar endophytes can provide plants with protection against pathogens; application of the endophytes *Alternaria cucumaria* or *Cladosporium fulvum* to the leaves of cucumber plants induced systemic resistance and reduced infection by the powdery mildew *Sphaerotheca fuliginea* (Reuveni and Reuveni (2000). Similarly, fungal endophytes protected a tropical tree species against a major pathogen, *Phytophthora sp*. (Arnold *et al.* 2003). Hence, although our results suggest that the plants perceived the endophytes as antagonists at the time of the infection, the impact of the infection may in the long term be positive if endophytes are able to play a role in host defence, whether against pathogens or herbivores (Gange *et al.* 2012). Indeed, fungal diversity and the average number of endophyte fungal species in our study was lower in plants that had been treated with *C. cochlioides*. Once *C. cochlioides* became established within plant tissues, it reduced infection of treated plants with other endophytic fungi, pointing towards a potential mechanism for

mutualism. The mechanisms underpinning this effect may relate to physical exclusion, or the induction of systemic defences which "prime" the plant to repel other invaders (Van Hulten, *et al.* 2006).

Systemic growth by the fungus and plant responses in new and old leaves

C. cochlioides was not restricted to treated leaves, but spread to new tissues. This was a surprising result as colonisation of shoots by foliar endophytes has traditionally been considered to be primarily local (Stone *et al.* 1994). Previous infection studies of foliar endophytes have failed to detect much, if any, systemic growth (Jaber & Vidal 2010; Gange *et al.* 2012), whilst Sánchez Márquez *et al.* (2012) suggest that they are incapable of systemic growth in grasses. Not only was the fungus able to grow systemically, but the impact of infection on foliar chemical composition altered as it spread: the response to endophyte infection was far greater in newly colonised leaves compared with the older tissue originally infected. Ten compounds showed a significant change in response to infection in new leaves compared with only one in older tissue. The greater response in new leaves was not due to higher levels of *C. cochlioides* infection in these tissues compared with older ones; newer leaves had somewhat lower levels of infection (see Table 1). It may reflect the fact that the plant mounts a more vigorous defensive response to endophytes in new leaves, either because those leaves are more "valuable" to the plant and hence worth defending more actively (McKey 1974; McCall & Fordyce 2010), or because the younger leaves are more physiologically able to mount such a defensive response (Koricheva & Barton 2012).

Differences in local and distant responses to attack may provide an alternative explanation for the stronger defence reaction in new leaves. In *Nicotiana*, the production of jasmonate in damaged leaves, in response to a local signal such as herbivore attack, and the detection of jasmonate in distal leaves is necessary for the induction of systemic responses (Wu and Baldwin 2010). However, it was shown that in *Arabidopsis*, this process is independent of the production of jasmonate in the damaged leaves and was reliant on transport of another unidentified wound signalling compound (Koo et al. 2009). It is possible that a similar mechanism may take place in *Cirsium arvense*, whereby the production of signalling compounds, independent of jasmonate production, in treated leaves may induce systemic defences and localised production of jasmonate in distal leaves.

Whatever the explanation for the greater defence response of new leaves, it was clearly an unsuccessful defence against *C. cochlioides* as the endophyte was able to become established in these tissues. The finding that foliar endophytes do spread rapidly between tissues, and that they elicit the production of defence reactions in those tissues, clearly has implications for the other organisms attacking endophytic plants, whether those are herbivorous insects, or other fungi, pathogenic or not. This was illustrated in our study by the reduced number of endophyte species in *C. cochlioides* treated plants, which may demonstrate the "priming" effect of *C. cochlioides* infection on plant defences. Such priming has been found in other studies demonstrating that endophyte infection can confer systemic resistance to herbivores and pathogens (Vega *et al.* 2008; Ownley *et al.* 2010).

Conclusion

In summary, this study has revealed for the first time the presence of arabidopside based galactolipids in a plant species other than *Arabidopsis,* suggesting their production maybe more widespread than currently thought. Furthermore, our work revealed the production of novel compounds in infected tissues and that the metabolites induced in response to endophyte presence are those typically associated with plant responses to attack by pathogens or wounding by herbivores. The production of these compounds occurs to the greatest extent in newly infected leaves in association with systemic infection of the endophyte in areas of new growth. These results provide some evidence at the biochemical level to support the idea that plants "fight back" against endophyte colonists. However, the endophyte-induced production of compounds used by plants as systematic signals of wounding, herbivory and pathogen attack, suggest that endophytes also have the potential to benefit their hosts by conferring protection or priming the host plant against attack by herbivore or pathogens. Endophytes can thus be considered central to a complex chemically mediated series of interactions between host plants, their microbial colonists and their natural enemies.

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Table 1. The mean (S.E.) % infection frequency of the foliar endophytic fungal species, and the total number of cultured fungi, in leaves from *C. cochlioides* -treated and untreated plants.

Endophyte species	Control - untreated		*C. cochlioides cochlioides*-treated	
	Old leaves	New leaves	Old leaves	New leaves
Acremonium strictum	9.5 (12.5)	0	0	4.4 (9.1)
Acrodontium griseum	4.8 (9.7)	23.8 (22.1)	0	0
Aspergillus sp.	0	7.1 (11.4)	0	0
Black yeast	0	2.4 (7.1)	2.2 (6.7)	0
Botrytis cinerea	2.4 (7.1)	0	0	0
Chaetomium cochlioides	**2.4 (7.1)**	**0**	**91.1 (15.3)**	**66.7 (23.9)**
Chaetomium fimeti	4.8 (9.7)	2.4 (7.1)	2.2 (6.7)	11.1 (21.1)
Cladosporium cladosporioides	23.8 (19.4)	9.5 (16.3)	4.4 (9.1)	0
Geotrichum candidum	0	4.8 (9.7)	0	0
Mucor hiemalis	0	0	0	2.2 (6.7)
Penicillium sp.	31.0 (22.2)	23.8 (24.4)	2.2 (6.7)	2.2 (6.7)
Periconia minutissima	2.4 (7.1)	0	2.2 (6.7)	0
Phialophora sp.	0	2.4 (7.1)	0	0
Stemphylium botryosum state of *Pleospora herbarum*	4.8 (9.7)	0	0	0
Sordaria fimicola	2.4 (7.1)	0	0	0
Trichothecium roseum	2.4 (7.1)	0	0	0
Unidentified	19.0 (17.3)	23.8 (24.4)	0	4.4 (9.1)

Table 2. Metabolite markers identified in leaves of *Cirsium arvense* infected with the endophyte *C. cochlioides.*

Metabolite number	Retention time	*m/z* observed ion	Ion species	Formula of ion	*m/z* calculated ion	ppm difference	Identity of metabolite
I	3.99	351.2149	M+Na	$C_{18}H_{32}O_5Na$	351.2147	0.6	9,12,13-trihydroxyoctadecadien acid
	3.99	327.2152	M-H	$C_{18}H_{31}O_5$	327.2171	-5.8	
II	4.23	353.2308	M+Na	$C_{18}H_{34}O_5Na$	353.2305	1.1	9,12,13-trihydroxyoctadeceno acid
	4.23	329.2315	M-H	$C_{18}H_{33}O_5$	329.2328	-3.9	
III	8.52	731.4340	M+Na	$C_{39}H_{64}O_{11}Na$	731.4346	-0.8	dihydrojasmonic/octadecatrienc acid ester of monogalactosyldiacylglycero
	8.52	707.4334	M-H	$C_{39}H_{63}O_{11}$	707.4370	-5.1	
IV	4.30	462.2852	M+H	$C_{26}H_{40}NO_6$	462.2856	-0.8	unidentified metabolite
V	4.60	713.3348	M+Na	$C_{33}H_{54}O_{15}Na$	713.3360	-1.6	oxophytodienoic acid ester of digalactosylmonoacylglycero
	4.60	689.3381	M-H	$C_{33}H_{53}O_{15}$	689.3384	-0.4	
VI	5.05	551.2803	M+Na	$C_{27}H_{44}O_{10}Na$	551.2832	-5.2	oxophytodienoic acid ester of monogalactosylmonoacylglyce
	5.05	527.2857	M-H	$C_{27}H_{43}O_{10}$			
VII	8.73	991.5542	M+Na	$C_{51}H_{84}O_{17}Na$	991.5606	-6.4	oxophytoenoic acid ester of digalactosyldiacylglycerol
	8.73	967.5612	M-H	$C_{51}H_{83}O_{17}$	967.5630	-1.8	
VIII	8.76	987.5350	M+Na	$C_{51}H_{82}O_{17}Na$	987.5293	5.7	oxophytodienoic acid ester of digalactosyldiacylglycerol
	8.76	963.5322	M-H	$C_{51}H_{79}O_{17}$	963.5317	0.5	
IX	9.78	811.4983	M+Na	$C_{45}H_{72}O_{11}Na$	811.4972	1.3	oxophytodienoic/octadecatrienc acid esters of monogalactosyldiacylglycero
	9.78	787.5012	M-H	$C_{45}H_{71}O_{11}$	787.4996	0.2	
X	9.87	813.5162	M+Na	$C_{45}H_{74}O_{11}Na$	813.5129	4.0	oxophytodienoic/octadecadienc acid esters of monogalactosyldiacylglycero
	9.87	789.5156	M-H	$C_{45}H_{73}O_{11}$	789.5153	0.3	

Extracts of plant tissues were analysed by UPLC-QTOFMS in +ESI and –ESI modes. Fragment data were obtained using collision-induced dissociation and are given in supporting information Table S1.

Table 3. Changes in the relative abundance of metabolite markers identified in leaves of *Cirsium arvense* after infection with the endophyte

Metabolite number	Identity	Relative abundance				Ratio met abunda control/in plan
		Old leaves		New leaves		
		Control	Treated	Control	Treated	Old leaves
I	9,12,13-TriHODE	1.34±2.05	0.74±1.21	1.39±1.80	8.56±2.67	0.55
II	9,12,13-TriHOME	2.54±1.50	2.98±2.74	2.05±0.77	8.64±3.91	1.17
III	MGDG(dihydroJA/18:3)	1.70±1.64	0.08±0.20	0.15±0.23	7.26±6.09	0.05
IV	-	10.92±26.45	0.53±0.95	64.97±45.53	4.40±4.96	0.05
V	DGMG(OPDA/H)	2.21±3.61	4.23±8.07	12.72±6.95	0.34±1.25	1.91
VI	MGMG(OPDA/H)	4.29±5.64	7.94±13.31	20.36±8.31	1.54±2.61	1.85
VII	DGDG(dihydroOPDA/dihydroOPDA)	22.86±28.21	29.65±34.56	65.96±31.87	9.73±16.96	1.3
VIII	DGDG(OPDA/OPDA)	81.00±36.22	99.45±22.37	103.09±16.20	39.20±12.09	1.2
IX	MGDG(OPDA/18:3)	2.22±2.35	1.44±1.85	6.95±3.98	0.56±0.74	0.65
X	MGDG(OPDA/18:2)	3.98±4.04	2.65±3.40	13.42±8.19	1.06±1.10	0.67

C. cochlioides.

Figure legends.

Figure 1. PCA and PLS-DA scores plot of the effect of infection with the endophyte *C. cochlioides* on the metabolome profile of leaf biochemistry in *Cirsium arvense.* Leaf extracts were analysed by UPLC-TOFMS in +ESI mode. A) PCA plot; the percentages of explained variation (R2X) modelled for the first two latent variables (t1, t2) are displayed on the related axes. B) PLS-DA plot; the percentages of explained variation (R2Y) modelled for the first three latent variables (t1, t2, and t3) are displayed on the related axes.

Figure 2. Structures of galactolipids identified in *Cirsium arvense.*
Metabolite numbers refer to those in Table 2. Gal= galactosyl moiety. The position of acyl groups was undetermined; for structures containing diacyl groups the most abundant acyl ion in the mass spectrum was allocated the *sn*-1 position (Guella *et al.* 2003).

Figure 3. Box and whisker plots of the relative abundance of esterified oxylipins in leaves of *Cirsium arvense* from control and endophyte-treated plants.
Relative abundance was calculated as the ratio of the signals (M-H ion) of the analyte:internal standard signal in 0.6 mg plant tissue.

Figure 1.

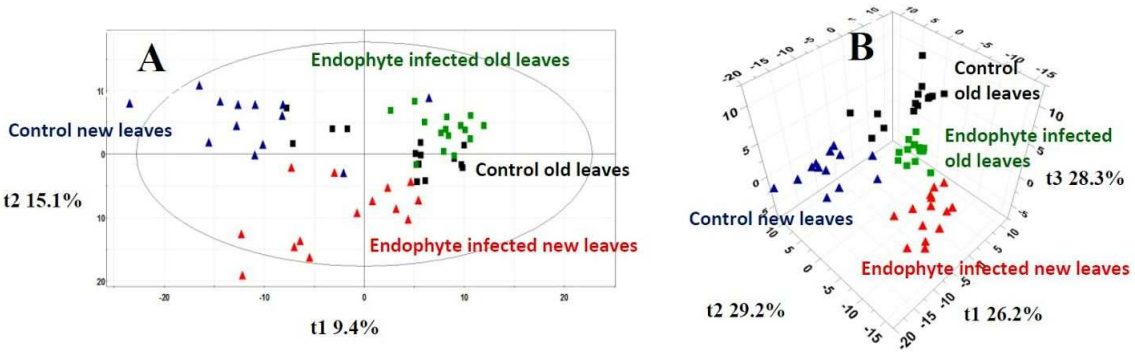
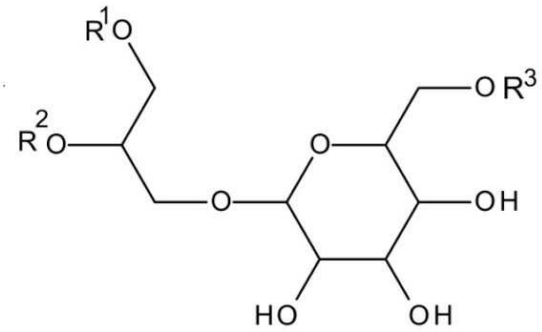
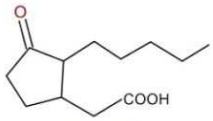

A
Endophyte infected old leaves
Control new leaves
Control old leaves
Endophyte infected new leaves
t2 15.1%
t1 9.4%
B
Control
old leaves
Endophyte infected
old leaves
t3 28.3%
Control new leaves
Endophyte infected new leaves
t2 29.2%
t1 26.2%

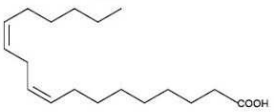
Figure 2.



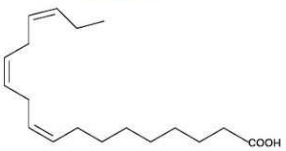
Arabidopside generic structure



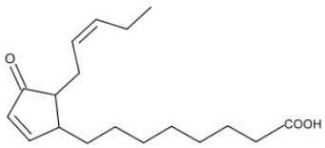
dihydroJA



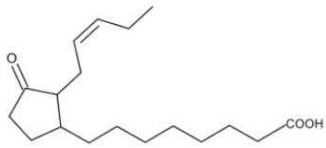
C18:2



C18:3



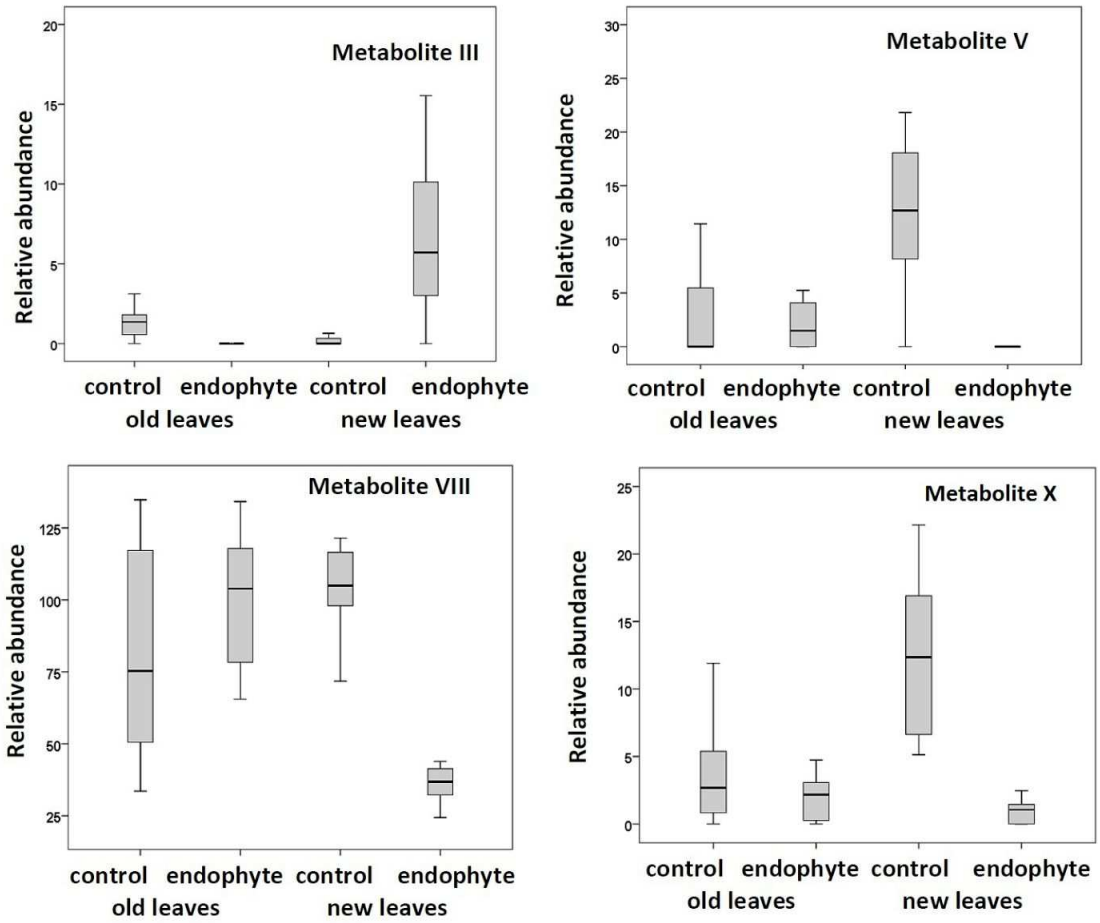
OPDA



dihydroOPDA

Metabolite	**R^1**	**R^2**	**R^3**
III	dihydroJA	C18:3	H
V	OPDA	H	Gal
VI	OPDA	H	H
VII	dihydroOPDA	dihydroOPDA	Gal
VIII	OPDA	OPDA	Gal
IX	OPDA	C18:3	H
X	OPDA	C18:2	H

Figure 3.


Metabolite III
Relative abundance
20
15
10
5
0
control
endophyte
control
endophyte
old leaves
new leaves
Metabolite V
Relative abundance
30
25
20
15
10
5
0
control
endophyte
control
endophyte
old leaves
new leaves
Metabolite VIII
Relative abundance
125
100
75
50
25
control
endophyte
control
endophyte
old leaves
new leaves
Metabolite X
Relative abundance
25
20
15
10
5
0
control
endophyte
control
endophyte
old leaves
new leaves