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Short Communication

Root herbivore performance suppressed when feeding on a jasmonate induced pasture grass

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Running title: Jasmonate suppression of root herbivores

Abstract

1. Plants defend themselves from insect herbivore attack using a range of physical and chemical defences which are in many cases regulated by phytohormones such as jasmonates. While much more is known about how jasmonates regulate defence against aboveground herbivores (e.g. herbivores of leaves), there is increasing interest in how they influence belowground defences.
2. For the Poaceae, most belowground studies focus on highly domesticated cereals. Here we demonstrate how exogenous application of methyl jasmonate (MeJA) to the leaf blades of a non-domesticated pasture grass (*Microlaena stipoides*) caused a more than two-fold decrease in relative growth rates (RGR) of a root-feeding chafer (*Dermolepida albohirtum*). MeJA treatment did not affect root consumption rates, but substantially reduced the efficiency of conversion of ingested food to body mass.
3. Non-targeted metabolomics identified significant changes in the metabolome of MeJA-induced plants, with three compounds (a galactolipid, a trihydroxy fatty acid and a lysophospholipid) found to be correlated with herbivore RGR, although their roles in herbivore defence remain uncertain.

4. This study suggests that an important Australian pasture grass can become better defended against root herbivores via enhanced jasmonate activity.

Key Words – belowground herbivore; grass; jasmonic acid; metabolomics; *Microlaena stipoides*; root herbivory.

Introduction

In the last decade, we have seen significant advances in our understanding of how plant hormones regulate plant defences against insect herbivores (Wu & Baldwin, 2010). Jasmonates are recognised as key regulators of chemical pathways that activate genes associated with herbivore defence aboveground (Jander & Howe, 2008). Compared to herbivores feeding aboveground, we know comparatively less about how jasmonate-regulated defences operate belowground against root herbivores (Erb *et al.*, 2012a; 2012b). While this situation is improving, the majority of studies addressing phytohormone induction and root herbivores are mainly concerned with domesticated plants, particularly cereals (Erb *et al.*, 2012a; Erb *et al.*, 2013; Lu *et al.*, 2015). These studies suggest that jasmonates, while less inducible than in the shoots, regulate root resistance to herbivores. Lower induction of jasmonates indicate that defensive machinery of the roots may be more sensitive of jasmonate activity or synergistic signals are involved. Salicylic acid, for example, which can dampen jasmonate-based defence, does not appear to be induced by root herbivores so its deficiency may indirectly boost jasmonate activity (Johnson *et al.*, 2016). In addition to being crop pests, root herbivores can be important within natural ecosystems because of their sheer abundance. The collective mass of pasture scarab beetles, for example, can outweigh that even of domesticated mammals grazing aboveground when considered per unit area (Frew *et al.*, 2016a).

A first step towards understanding jasmonate-regulated defences against root herbivores in pasture grasses would be to establish whether exposure of plant tissues to jasmonsates affects herbivore performance. Exogenous application of methyl jasmonic acid (MeJA) is a common approach for achieving this (Erb *et al.*, 2013; Lu *et al.*, 2015). We investigated whether MeJA application to an important pasture grass native to Australia, *Microlaena stipoides* (syn. *Ehrharta stipoides*), affects the performance and feeding behaviour of a root-feeding chafer insect. We undertook non-targeted metabolomic screening of MeJA-treated and untreated root tissue to explore whether changes in the root metabolome were related to root herbivore performance.

Methods and Materials

Plants

Forty weeping meadow grass (*Microlaena stipoides*) plants were grown from seed (Native Seeds, VIC, Australia) in all-purpose potting mix (Richgro, WA, Australia) in pots (70 mm diameter, 135 mm deep) for c. 32 weeks. The study was conducted in a naturally-lit glasshouse chamber (3 × 5× 3 m; width × length × height) with UV transparent plexiglass walls and roof. Air temperature was regulated at 30 °C (±4 °C) and fell to 22 °C (±4 °C) at night. Humidity was controlled at 60 % (±6 %). Plants were irrigated with c. 70mL of water three times a week and provided with a single dose (2g) of Osmocote Controlled Release fertilizer. Twenty plants were selected at random and were treated with MeJA (95%, Sigma Aldrich) by applying 1mL of a solution 100µg/mL MeJA (Sigma Aldrich) in Tween 20 ± 0.1% to the leaf blades, just above the soil surface, with a pipette. Control plants received 1mL of Tween 20 ± 0.1% only. This was repeated 24 and 48 hours later. Plants were then separated from the soil and were washed free of soil for feeding assays.

Feeding Assays

We used the greyback chafer (*Dermolepida albohirtum*) as a model grass-feeding root herbivore. This species is native to Australia and feeds in pastures but has also become a significant pest of sugarcane (Frew *et al.*, 2016a). Feeding assays were conducted as described by Frew *et al.* (2016b). In summary, individual third instar larvae were starved for 24 h, weighed and placed in a Petri dish (14 cm diam) with c. 4 g of fresh root material taken from one of 10 plants treated with MeJA or 10 control plants, each selected at random. Larvae fed for 24 h, then starved for 12 h to ensure all frass was expelled, before being reweighed. Evaporative water loss from roots during the assay was accounted for (Frew *et al.*, 2016b).

Relative growth rate (RGR) of larvae was calculated as: mass gained (g)/ initial mass (g)/time (days). Relative consumption (RC) is an estimate of the mass of root material ingested over the 24 h period relative to initial body mass. It was calculated as: food ingested (mg change in dry mass) / mean body mass over experimental period (mg fresh mass). Efficiency of conversion of ingested food (ECI) was calculated as: mass gained (mg change in fresh body mass)/food ingested (mg change in dry mass) × 100.

Metabolomics sample preparation

Root material not used for the feeding assays was snap frozen before being freeze dried and ball-milled to a fine powder. Five glass beads were added to 20 mg of each ground root sample, which was then shaken in a tissue lyser for 4 min in 500 µL methanol:milliq water:formic acid (75:24.5:0.5 v/v) at 30 Hz. This was centrifuged and the supernatant was extracted for analysis.

Untargeted profiling was carried out by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry according to a protocol adapted from Gaillard *et al.* (2018). The separation was performed at a flow rate of 0.5 mL/min using a Waters Acquity UPLC HSS T3 column (2.1 × 100mm, 1.7 µm particle size) maintained at 40°C. The following gradient of mobile phase A (water+0.05% formic acid) and B (acetonitrile+0.05% formic acid) was applied: 0-60% B in 6.0 min, 60-100% B in 2.5 min, holding at 100% for 2.0 min and reequilibration at 0% B for 3.0 min. The injection volume was of 2.5 µL. Mass spectrometric detection was performed in negative electrospray over a mass range of 85-1200 Da using independent data acquisition (MS^E). A pool of all samples was injected as quality control using the following sequence: two injections at the beginning of the batch, then one injection every 20 samples and at the end of the batch. Peak selection was performed as described by Gaillard *et al.* (2018). Compounds that were significantly up/down-regulated by JA were further selected for identification. Putative identification was achieved on the basis of (i) determination of molecular formulae from accurate mass measurements and (ii) mass spectral fragmentation characteristics and comparison with existing databases.

Statistical analysis

RGR was log transformed and analysed with a one-way ANOVA with MeJA-induction as the fixed factor. RC and ECI were analysed with Kruskal-Wallis tests. Compounds of potential interest relating to MeJA-induction were initially identified using partial least squares discriminant analysis (PLS-DA). Compounds with a PLS-DA PC1 score > 0.1 (i.e. the compounds that contribute to the majority of the dataset variance associated with MeJA-induction) were selected, before being analysed with one-way ANOVAs. Potential associations between concentrations of these compounds and RGR were examined with Pearson's correlation tests. All analysis was conducted with the R statistical package.

Results and Discussion

The RGR of larvae feeding on MeJA-induced grass roots was significantly lower than for those feeding on untreated plants (Fig. 1a; $F_{1,18} = 12.24$, $P = 0.003$), with most larvae losing mass when feeding on MeJA-induced plants. There was no significant difference in RC with herbivores eating similar amounts of root tissue from MeJA-induced and non-treated plants (Fig. 1b; $H_1 = 1.463$, $P = 0.226$). As a result of this, ECI was much lower (-27.15 ± 12.12) when feeding on MeJA-induced plants compared to non-treated plants (1.18 ± 15.68) (median values ± inter-quartiles; $H_1 = 10.57$ $P < 0.001$). In short, herbivores were feeding at similar rates on MeJA-induced and non-induced *M. stipoides*, but were unable to obtain adequate nutrition when feeding on MeJA-induced plants. This indicated that herbivores were not deterred from feeding on MeJA-induced plants (at least in the short-term) but their performance was being adversely affected by consuming such root tissue, which is at least compatible with enhanced production of defensive compounds in the roots. Assays that aim to quantify feeding metrics of insect herbivores are often standardised to 24 hr (e.g. Slansky, 1985; Frew *et al.*, 2016b), which was sufficient to detect suppression of herbivore performance in this study. We should, however, be cautious about extrapolating our findings over longer periods since larval *D. albohirtum* can live for over a year (Frew *et al.*, 2016a) and it is unknown how long the effects of MeJA-induction persist for in *M. stipoides*. Nonetheless, exogenous MeJA application to rice resulted in comparable reductions in the growth of root herbivores (cucumber beetle, -50% and rice water weevil, -100%) when feeding for 7 and 20 d, respectively (Lu *et al.*, 2015).

In total, 14 primary and secondary metabolites in the roots of *M. stipoides* either increased or decreased in response to MeJA-induction, three of which were significantly, though not strongly, correlated with root herbivore RGR (Table 1). MeJA-induction caused concentrations of a galactolipid (a digalactosylmonoacylglyceride, 18:2-0:0-DGMG; $C_{33}H_{58}O_{14}$) to decrease, and this compound was positively correlated with RGR (Table 1). Galactolipids, including their aldehydes, can be major sources of essential fatty acids (Ohlsson, 2000) but can sometimes act as defences against insect and echindoderm herbivores (Deal *et al.*, 2003). MeJA-induction also caused significant increases in $C_{19}H_{36}O_5$, (a monounsaturated trihydroxy fatty acid) and $C_{27}H_{52}NO_9P$ (a linoleoyl-lyso phosphocholine), both of which were negatively correlated with insect RGR (Table 1). Unsaturated fatty acids can be toxic to insects in bioassays (Harada *et al.*, 2000), including root herbivores (Bernklau *et al.*, 2016). However, a saturated trihydroxy fatty acid, phloionolic acid ($C_{18}H_{36}O_5$) is a known constituent of suberin in cork (Pereira, 2007), and the monounsaturated $C_{19}H_{36}O_5$ trihydroxy fatty acid observed here may therefore reflect an increase in suberinisation of roots. Mono- and digalactosyldiacylglycerols also play roles in the regulation of systemic acquired resistance in plants (Gao *et al.*, 2014). Perhaps the best candidate to explain the decreased herbivore performance is the linoleoyl lysophosphocholine, as some compounds in this class have been shown to be highly cytotoxic to human cancer cell lines (Niezgoda *et al.*, 2015).

To our knowledge, this is the first study to show that exogenous application of MeJA to a non-domesticated pasture grass has negative impacts on a root herbivore. Using metabolomics, we established that the concentrations of three compounds both changed in response to MeJA-induction and were correlated (either positively or negatively) with root herbivore performance. The exact structures of these compounds could not be resolved, and regardless, are relatively unstudied so it is unproven whether these are linked to the observed declines in herbivore performance and feeding efficiency. Further work and different analytical approaches such as detailed lipid profiling may shed light on this. Nonetheless, we were able to establish that MeJA-induction impaired root herbivore growth and thus resulted in roots that were potentially better defended; this was previously unknown for non-domesticated grasses. In reporting these findings, we aim to stimulate further interest in characterising belowground plant defences in non-domesticated as well as domesticated grass species.

Author contributions. Project design: SNJ, IH, BDM. Data collection and statistical analysis: JMWR. Metabolomics analysis: GG. All authors contributed manuscript preparation.

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