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Metabolomics analysis of the *Lolium perenne–Neotyphodium lolii* symbiosis: more than just alkaloids?

Susanne Rasmussen · Anthony J. Parsons · Jonathan A. Newman

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Abstract Above ground plant parts of *Lolium perenne* often harbour endophytic *Neotyphodium lolii* fungi. These occur both naturally and commercially, as variant strains are introduced to modify the grass metabolic profile. They reside in the apoplastic spaces and rarely cause visible symptoms of infection. The vast majority of literature has focussed on the biosynthesis, accumulation, and ecological relevance of a limited number of alkaloids produced by *N. lolii* which have been shown to negatively affect insect pests and vertebrate herbivores. Much less is known about the effects of other metabolites in these interactions or the role of resource supply on metabolic profiles, nor critically on the metabolic consequences of differences in the amount (concentration) of endophyte present. Here, we provide a synthesis of some of our recently published studies on effects of resource supply (nitrogen, carbohydrates) on concentrations of endophytes and endophyte specific metabolites in the *L. perenne–N. lolii* association. We present results of both quantitative PCR and targeted metabolomics studies, using contrasting endophyte strains in two perennial ryegrass cultivars. We also present and discuss a hypothetical schematic representation of possible links between plant and fungal metabolic networks. A multiple regression analysis of numerical insect responses and metabolic profiles indicates that effects of endophyte infection on insect population sizes could be predicted by concentrations of a range of metabolites other than alkaloids and depended on insect species, fungal strain, and nitrogen supply.

Keywords Ryegrass · Carbohydrates · Nitrogen · Herbivory · Metabolomics

S. Rasmussen (✉) · A. J. Parsons
AgResearch Grasslands, Tennent Drive, Palmerston North 4442, New Zealand
e-mail: Susanne.rasmussen@agresearch.co.nz

J. A. Newman
Department of Environmental Biology, University of Guelph, Guelph N1G 2W1, Canada

Introduction

Temperate and tropical grasses are often infected with endophytic fungi belonging to the genus Clavicipitaceae (Leuchtmann 1993). The best studied symbiotic associations of this type are *Neotyphodium lolii–Lolium perenne* (ryegrass) and the *N. coenophialum–Schedonorus arundinaceus* (syn. *L. arundinaceum*, tall fescue), as these are of relevance to agricultural pasture-based animal production systems especially in Australia, New Zealand, and the USA (Christensen et al. 1993). In contrast to some plant mutualists (e.g. the well researched mycorrhizae) these endophytes do not extend outside the plant; they reside almost entirely in the apoplastic spaces

(between plant cells) of above ground plant parts (Fig. 1a), although very small amounts of fungi can also be detected in root tips (Christensen et al. 2008). While some *Epichloë* spp. have sexual stages that can cause damage to plant seed heads (choke disease), their asexual *Neotyphodium* spp. descendants are asexual and rarely cause any visible symptoms of disease. Unlike many biotrophic fungal pathogens and mycorrhizae which invade plant cells and form specific intracellular structures (haustoria and arbuscules, respectively) and membrane interfaces for nutrient transport between host and fungus (Hall and Williams 2000; Hahn and Mendgen 2001 and references therein), the *Neotyphodium*/*Epichloë* endophytes do not invade cells and no specific membrane interfaces are known to be formed in this association. However, it has been shown that fungal hyphae are firmly attached to plant cells (Fig. 1; Christensen et al. 2008), and it is possible that nutrients are transported from plant to fungal cells at the site of attachment, although the prevalent hypothesis is that nutrients are taken up by the fungus from the apoplastic fluid in the intercellular space in which it resides (Hinton and Bacon 1985). Of importance to recognise here is that to the best of current understanding, this means that all resources required by the fungus must be obtained solely from the host plant.

Neotyphodium/*Epichloë* fungi synthesize a range of alkaloids, mainly the pyrrolopyrazine peramine, the indole-diterpene lolitrem B, the peptide alkaloid ergovaline, and, in the case of *N. coenophialum*, several lolines (Bush et al. 1997; Lane et al. 2000; Schardl et al. 2004). The alkaloids can confer resistance against a range of insects to the host plants and so improve plant productivity (Rowan et al. 1986; Fletcher and Easton 1997; Schardl 2001; Clay and Schardl 2002). However, some alkaloids (e.g. ergovaline and lolitrem B) have detrimental effects on grazing mammals, causing e.g. fescue toxicosis (Lyons et al. 1986; Strickland et al. 1996) and ryegrass 'staggers' (Gallagher et al. 1984) in cattle and sheep, resulting in production losses, and welfare concerns, for the meat and dairy industry. Recently, several new fungal strains lacking (or very low in) lolitrem B and ergovaline have been commercialised and are used to infect ryegrass (AR1, AR37) and tall fescue (AR542, known commercially as Max Q, and also as Max P). While AR1 produces peramine as the major alkaloid, AR37 does not produce any of the commonly known and extensively studied alkaloids, but synthesizes epoxy-janthitrems instead (Tapper and Lane 2004). Concentrations of alkaloids found in infected plant tissues have been reported to depend on a variety of factors like e.g. nutrient availability or host genotype. To date, it is not clear to what extent these differences in alkaloid concentrations depend on the metabolic activity of the fungus per se (e.g. as the result of changes in fungal gene expression or enzyme activities); or if low/high alkaloid levels are due in some circumstances to different and changeable concentrations of fungal tissue in the fungus/host association.

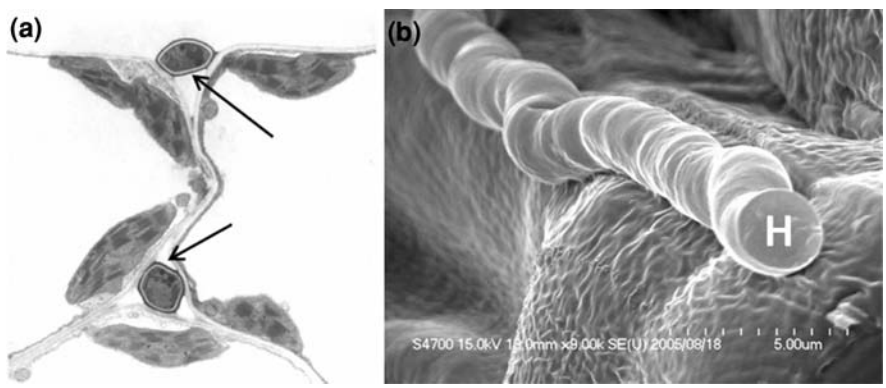

Fig. 1 Micrographs of *Neotyphodium* endophytic hyphae in planta. **a** Aniline-blue stained *N. lolii* hyphae (*arrows*) in ryegrass leaf sheath. **a** TEM micrograph showing *N. lolii* hyphae (*arrows*) in the intercellular spaces of a young ryegrass leaf blade. **b** Freeze-fracture SEM micrograph showing a hyphae (H) of *N. coenophialum* in close contact with tall fescue leaf cells. Micrographs have been reproduced from Christensen et al. (2008) (Fig. 1) with permission from Elsevier

Apart from negative effects of endophyte alkaloids on herbivores, it has also been shown that endophytes can have beneficial effects on other plant fitness parameters e.g. improving drought and mineral stress tolerance (Malinowski et al. 1998a; Amalric et al. 1999; Malinowski and Belesky 2000; Hesse et al. 2003, 2005), although the mechanisms responsible for these benefits remain largely unresolved. The host/fungal association has been viewed generally as mutualistic, though this concept has been challenged recently (Cheplick 2004; Saikkonen et al. 2004, 2007; Müller and Krauss 2005). The heterotrophic endophyte depends on substrates provided by the autotrophic host plant and a metabolic cost to the host has therefore been postulated (Cheplick 2007), though very few studies attempting to quantify these costs have been published so far. The balance of endophyte effects on plant performance has been shown to depend e.g. on resource supply (Cheplick et al. 1989; Malinowski et al. 1998b), and evidence of negative effects of high resource supply in the host, on endophyte abundance, would favour seeing the association as a mutualism–parasitism continuum.

Previous transcriptomics and metabolomics studies of the rhizospheric rhizobium—legume, and arbuscular mycorrhiza—plant, mutualistic associations have revealed that such endosymbionts can induce a wide range of changes in gene transcript and metabolite profiles and concentrations (Hohnjec et al. 2005; Schliemann et al. 2008). Recent metabolomics and transcriptomics studies on effects of parasitic fungal biotrophs in maize (*Zea mays*; Doehlemann et al. 2008) and *Brassica rapa* (Abdel-Farid et al. 2009) have also shown widespread reprogramming of host plants' metabolism. Here, we provide a synthesis of several controlled environment experiments that we used to alter resource availability, to analyse endophyte concentrations and metabolic profiles in *N. lolii* infected ryegrass plants using both quantitative PCR (qPCR) and targeted metabolic profiling methods (Rasmussen et al. 2007, 2008b). The same plants were also exposed to insect herbivores, and a multiple regression analysis was performed to resolve differences between resource treatments in metabolic profiles, and to link the numbers of insects found on infected and non-infected plants to specific metabolites. This revealed that a range of non-alkaloid metabolites, and different balances between metabolites, might be involved in insect herbivore responses to endophyte infected ryegrass plants (Rasmussen et al. 2008a).

Effects of resource supply on endophyte and alkaloid concentrations

Endophyte produced alkaloids are critical in pasture based animal production systems: (1) for the protection of forage grasses from insect damage, but also (2) for their potential to harm grazing livestock. It would therefore be important to test, if, and how, changes in pasture management (e.g. nitrogen fertilisation) or the use of different cultivars (e.g. with intrinsically different carbohydrate contents) affect the synthesis and concentration of these alkaloids, given these external factors are altering the fundamental supply of resources to the fungus. Although a great number of field based studies has been performed to test for effects of season, weather, and management on alkaloid profiles and concentrations (for *L. perenne* see: Keogh 1983; Ball et al. 1995; Salminen and Grewal 2002; Hume and Barker 2005; for *S. arundinaceus* see: Lyons et al. 1986; Belesky et al. 1988; Arechevaleta et al. 1992; Malinowski et al. 1998b), controlled studies on individual environmental factors that would allow to predict effects of management practices like e.g. fertilisation, are unfortunately relatively rare. In tall fescue increased N availability tends to increase alkaloid concentrations (Lyons and Bacon 1984; Belesky et al. 1988; Arechevaleta et al. 1992), whereas in ryegrass, N supply seems to have no consistent effect (Stewart 1986; Lane et al. 1997; Hunt et al. 2005; Krauss et al. 2007). Almost no information is available on the effects of specific grass cultivars with intrinsically contrasting metabolic composition on consequent alkaloid concentrations (in the presence of endophytes). This is so even though it is well established that endophyte content and alkaloid accumulation can be affected by host genotypes (Easton et al. 2002).

In our controlled environment studies we tested the effects of carbohydrate levels in the host, and nitrogen availability on the concentrations of both the endophyte and alkaloids, using a combination of host, endophyte and resource supply conditions. We used two *L. perenne* cultivars differing intrinsically in carbohydrate content: 'AberDove' (high sugar levels) and 'Fennema' (normal sugar levels). These cultivars

mainly differ in the concentrations of fructans, which are medium to high molecular weight water soluble carbohydrates (HMW WSCs), and the major storage sugar in vegetative tissues of ryegrass (Pollock and Jones 1979; Pavis et al 2001; Parsons et al. 2004). Individual seedlings of each cultivar were infected with one of three strains of endophytes: CS—common strain LP19 (produces peramine, lolitrem B, and ergovaline), AR1 (produces peramine only), and AR37 (produces none of these three alkaloids, but epoxy-janthitrems), and compared with each other and endophyte-free plants. For a detailed description of experimental conditions and analytical methods see Rasmussen et al. (2007). Plants were grown at two different N levels resulting in a total blade N content of 3% in the 'low' N, and of 4% in the 'high' N treatment. These N concentrations are representative for characteristically unfertilised ryegrass pastures grown extensively in a mixed sward with the N-fixing legume *Trifolium repens*, and mineral N-fertilised intensive pastures, respectively (Parsons et al. 1991).

As changes in alkaloid concentrations at different resource availabilities could be due to changes in alkaloid production per unit endophyte (specific activity of fungal strain) or to changes in the amount of fungus per unit of tissue (endophyte concentration per se), we quantified fungal concentrations in the infected plants, using qPCR of endophyte specific genes, and fungal specific metabolites.

A major outcome of that study was that both endophyte and alkaloid (here we show peramine only) concentrations were strongly reduced (halved) in the high sugar cultivar 'AberDove' compared to 'Fennema' (Fig. 2a, b). High N levels had a similar effect (Fig. 2d, e), and these effects were additive, resulting in an overall reduction of endophyte, and alkaloid concentrations, of up to 75% in the high sugar cultivar when grown at high N (Rasmussen et al. 2007). Likewise mannitol, a very common polyol in fungi (Lewis and Smith 1967), and described in both endophyte infected tall fescue (Richardson et al. 1992) and ryegrass (Harwood 1954), was also reduced in 'AberDove' relative to 'Fennema' and at high N (Fig. 2c, f; Rasmussen et al. 2008b).

In our studies we demonstrated 'direct' linear relationships between fungal metabolite concentrations and the concentration of fungi in the same tissues (Rasmussen et al. 2007, 2008b). Spiering et al. (2005) reported only a weak relationship between alkaloid and endophyte content, but these authors were looking across a range of tissues and tissue ages. Endophyte content is much higher in some tissues, e.g. sheaths, compared to mature blades, whereas, e.g. peramine concentrations are almost the same in these two tissues (Ball et al. 1997). Linear relationships between fungal metabolite concentrations and fungal amounts might be observable *within* a given tissue type, but would be less likely to be expected *across different tissues*. More general relationships between endophyte and alkaloid concentrations and endophyte/grass responses to environmental factors

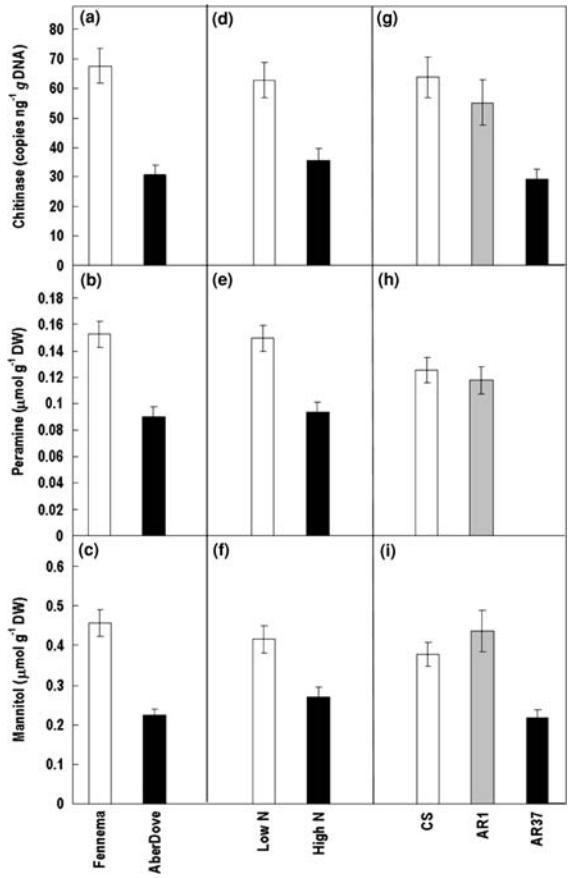

Fig. 2 Main effects of cultivar (**a**, **b**, **c**; means of high and low N plants infected with CS, AR1, or AR37), N supply (**d**, **e**, **f**; means of plants from both cultivars infected with CS, AR1, or AR37) and endophytic strain (**g**, **h**, **i**; note for **h**: AR37 does not produce peramine; means of both cultivars grown at high and low N) on concentrations of endophyte (chitinase copies; **a**, **d**, **g**), peramine (**b**, **e**, **h**) and mannitol (**c**, **f**, **i**) in mature blades of endophyte infected *L. perenne* plants (*bars* SE)

require far more attention, than has been common place, to the actual concentrations of endophytes, and to linking endophyte concentrations to alkaloid concentrations within a given tissue.

As to possible explanations for resource supply effects on endophyte concentrations we hypothesized that high nitrogen supply might increase plant growth more than fungal growth and so result in an overall 'dilution effect' (Rasmussen et al. 2008b). It would be interesting to test if other plant growth affecting nutrients (e.g. phosphorous) or treatments (e.g. temperature, light), have similar effects on endophyte concentrations. In contrast, this kind of 'dilution effect' seems not to be related to reduced endophyte concentrations in the high sugar cultivar 'AberDove' compared to 'Fennema', as we did not detect any differences in plant growth between the two cultivars (Rasmussen et al. 2008b). It could be argued that high sugar cultivars, which, as stated above, mainly differ in the concentrations of high molecular weight fructans, might have lower concentrations of low molecular weight sugars like glucose, fructose, and sucrose compared to 'Fennema'. These sugars are more likely to be transported to the apoplastic space and, analogous to models described for mycorrhizal and pathogenic fungi (Pfeffer et al. 1999; Hall and Williams 2000) might be the main source of carbon skeletons and energy for the heterotrophic endophytic fungus. Lower levels of these sugars could therefore impair fungal nutrition and growth, but we note here that, to date, it is not known (1) if endophytic *Neotyphodium*/*Epichloë* fungi take up nutrients from the apoplastic space and/or from plant cells they are attached to, and (2) which metabolites are taken up by these fungi. Although we did not measure sugar concentrations in the apoplast, we showed that overall concentrations of glucose, fructose, and sucrose in the symbiotic tissue were quite similar in the two cultivars tested (Rasmussen et al. 2008b).

Another possible explanation might be cultivar-specific effects per se, as strong host × endophyte specificities have been shown previously to be determining factors for endophyte abundance/alkaloid accumulation (Easton et al. 2002; Saikkonen et al. 2004), but our experiments were not designed to test this hypothesis. However, our detailed analysis of metabolic profiles revealed that one of the 18 quantified free amino acids, L-methionine, was significantly higher in the high sugar cultivar 'AberDove' (2.4 fold) compared to 'Fennema' (Rasmussen et al. 2008b). L-methionine has been reported to act as an inhibitor of *Claviceps microcephala* growth and sporulation (Singh et al. 1972) and to induce resistance against *Sclerospora graminicola* in pearl millet (Sarosh et al. 2005). Clearly, further studies are needed to decipher mechanisms regulating endophytic growth and metabolite production in their host plants.

Effects of *N. lolii* endophytes on host plant metabolic composition

The endophyte studied by us is a heterotrophic organism which depends completely on macro- and micronutrients and energy produced or taken up by its autotrophic host, and so all metabolites produced by the endophyte are ultimately derived from plant metabolites. It is often argued that, based on total amounts of DNA, the endophyte represents only between 0.5 and 2 % of the association (Young et al. 2005) implying that endophyte effects on host metabolism will be small. However, as pointed out earlier by us (Rasmussen et al. 2007), the haploid *N. lolii* genome is very small (the genome size of its sexual congener *E. festucae* has been estimated to be approximately 30 Mb; Kuldau et al. 1999) compared with the diploid ryegrass genome (6.18×10^3 Mb); any ratios determined based on total DNA are therefore heavily biased towards plant DNA. We have shown previously that the ratios of fungal compartments (single nuclei per fungal compartment) to plant cells determined by qPCR of copy numbers of endophyte specific single copy genes (one copy = one nuclei) can be as high as 1:2 in infected mature blades (Rasmussen et al. 2007). The 40 Mb genome of the filamentous fungus *Neurospora crassa* has been estimated to code for approximately 10,000 genes (Mannhaupt et al. 2003), but likewise the much larger plant genomes have been estimated to code for 10,000–60,000 genes (Messing et al. 2004). It is therefore likely that the presence of the endophyte and its metabolic activity can contribute substantially to the metabolism of the association. To test this hypothesis, we performed a metabolomics analysis in the same material as described above, and in which we quantified 66 major metabolic variables and compared the profiles in blades of each of the three

endophyte infected grasses with each other and with those in the non-infected grasses (Rasmussen et al. 2008b). It is important to note here that all analyses were performed on whole blade tissue; it is therefore not possible to distinguish between fungal and plant metabolites except for those metabolites known to be produced by endophytes only (alkaloids, mannitol) or by plants only (plant secondary metabolites like e.g. chlorogenic acid).

A detailed description of the statistical analysis of our results based on principal components analysis with subsequent factor rotation can be found in Rasmussen et al. (2008b). Concentrations of 41 of the 66 metabolic variables were significantly different in endophyte infected compared to uninfected mature blades. A schematic representation of possible metabolic links between plant and fungal metabolites is shown in Fig. 5 and will be discussed below. Overall, endophyte infection resulted in a reduction of nitrogenous compounds. Seventeen out of 18 free amino acids, as well as nitrate, total N, and total proteins were reduced; reductions of nitrate, L-asparagine and L-proline were very strong and are shown in Fig. 3a–c. Concomitantly, carbon compounds were increased in infected tissues (Rasmussen et al. 2008b), most strongly affected were sugars (shown as total water soluble carbohydrates; Fig. 4a), organic acids (especially quinate and shikimate, the precursors of aromatic acids; Fig. 4b), and phenolics (Fig. 4c; main phenolic in ryegrass blades is chlorogenic acid, the quinate ester of caffeic acid, Rasmussen et al. 2008b). A reduction of nitrate levels has been shown previously for endophyte infected tall fescue (Lyons et al. 1990; Belesky and Fedders 1996) and ryegrass (Hunt et al. 2005), but it is unclear which mechanisms lead to this reduction. Mycorrhizal fungi have been shown to regulate host plant N assimilation (Govindarajulu et al. 2005; Bailly et al. 2007), but since *Neotyphodium* endophytes largely are absent from the roots, other, probably more indirect, mechanisms seem to operate here. Although we have not analysed overall C/N ratios, the much lower levels of L-asparagine in endophyte infected tissues are an indication of a higher C to N ratio compared to uninfected tissues, as this amino acid acts as an inert and stable N reserve (Lam et al. 1996) and is especially synthesized and stored in tissues with low C to N ratios (here: uninfected tissue).

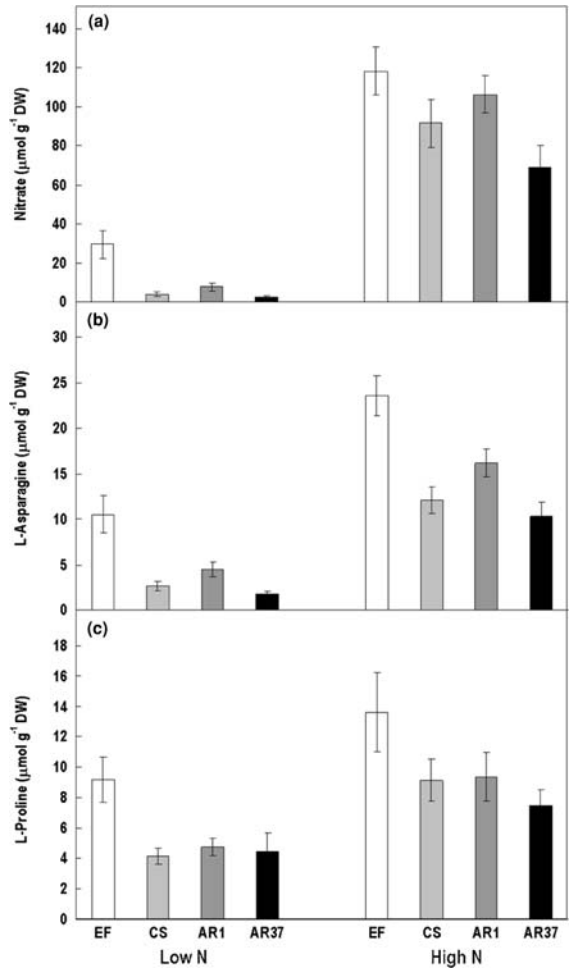

Fig. 3 Effects of endophyte infection at low and high N supply on concentrations of nitrate (**a**), L-asparagine (**b**), and L-proline (**c**) in mature blades of *L. perenne* plants (*bars* SE)

It is possible that the increase in carbon compounds is just due to reduced nitrate availability, and so reduced synthesis of nitrogenous compounds, in the tissue tested. However, at low N availability the effects of endophyte infection on the metabolic composition of the symbiotic tissue were generally stronger (Figs. 3, 4). At low N supply endophyte concentrations were also much higher (Fig. 2d; Rasmussen et al. 2007), it is therefore possible that the changes in metabolic composition are (also) due to increased sink strength of the heterotrophic endophyte on metabolites produced by its host. Increased sink strength has been shown to increase photosynthetic activity in host plants' source tissue and to be one of the drivers for changes in metabolic host plant

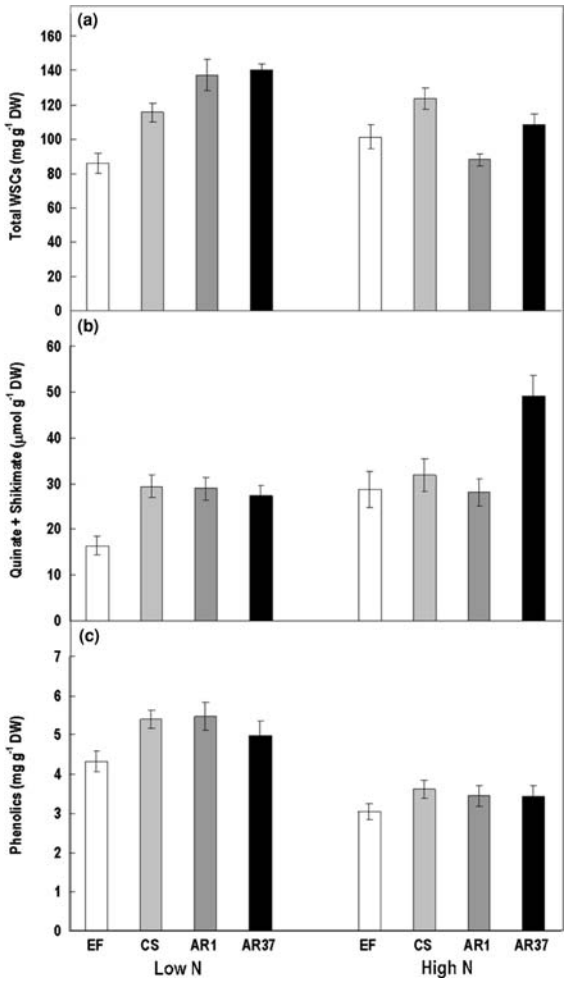

Fig. 4 Effects of endophyte infection at low and high N supply on concentrations of total WSCs (**a**), quinate and shikimate (**b**), and phenolics (**c**) in mature blades of *L. perenne* plants

composition in arbuscular mycorrhizal associations (Wright et al. 1998; Douds et al. 2000; Graham 2000; Pfeffer et al. 2001), but a major difference between *Neotyphodium*/*Epichloë* and arbuscular mycorrhizal associations is the presence of an extensive extraradical hyphal net in the latter, resulting in a much larger total biomass of the heterotrophic mycorrhizae and so a much higher sink strength compared to the fungal associations studied here. In addition, the tissues analysed in our studies represent both source (plants' photosynthetic carbon fixation) and sink (heterotrophic fungus) tissue, making it very difficult to distinguish between source and sink processes. Nevertheless, negative impacts of *Neotyphodium* endophytes on plant performance and growth in natural ecosystems, especially under severe resource limitations (Cheplick et al. 1989, 2000; Faeth and Sullivan 2003; Cheplick 2004, 2007; Hesse et al. 2004; Faeth and Hamilton 2006), could result in higher concentrations of endophytes under these conditions (as seen in our studies), resulting in increased metabolic demands by endophytes on their host plants.

The organic acids quinate and shikimate are precursors for aromatic acids (Herrmann and Weaver 1999), which are required for phenylpropanoid biosynthesis. We have shown that quinate/shikimate as well as phenolics (mainly chlorogenic acid, Rasmussen et al. 2008b) were increased in endophyte infected ryegrass, consistent with reports from infected tall fescue (Malinowski et al. 1998a). It has also been reported that *N. coenophialum* infection of above ground tall fescue tissue affects concentrations and patterns of phenylpropanoids in the roots and that this improves plant resistance to soil nematode (*Pratylenchus scribneri*) infection (Bacetty et al. 2007). At this stage a discussion on possible mechanisms for increased levels of phenolics in endophyte infected tissues is very speculative, but it has been suggested that endophytes might induce a weak resistance response in their host plants (Malinowski and Belesky 2000), comparable to effects of mycorrhizal colonisation (Harrison and Dixon 1993; Volpin et al. 1994; Hohnjec et al. 2005), thereby increasing the biosynthesis of phenylpropanoids. A strong induction of host plant secondary metabolites has also been shown in *Ustilago maydis* infected maize (Doehlemann et al. 2008), and in *Brassica rapa* plants infected with a range of pathogenic fungi (Abdel-Farid et al. 2009). Associated with the induction of secondary metabolites and resistance responses in plants is the production of reactive oxygen species (ROS; Torres and Dangl 2005; Takemoto et al. 2007). Recent studies of the association between *Epichloë festucae* and perennial ryegrass have shown that ROS production plays a critical role in the regulation of *in planta* endophytic hyphal morphogenesis and growth (Tanaka et al. 2006; Tanaka et al. 2008).

Joint plant and fungal metabolic networks

A hypothetical schematic representation of endophyte metabolic effects and possible metabolic network connections in ryegrass blades infected with *N. lolii*

strain Lp19 (CS) is shown in Fig. 5. Please note that this hypothetical scheme only shows those metabolites and plant quality parameters which were actually measured in our studies (except for acetyl CoA, which we have not analysed, but which is central to most processes described below). Many metabolites known to be important intermediates of metabolic pathways, like e.g. phosphorylated sugars and CoA esters, as well as metabolites usually present in very low concentrations, like e.g. phytohormones, were not detected by our analytical methods. The arrows and lines in Fig. 5 do not represent a direct biosynthetic

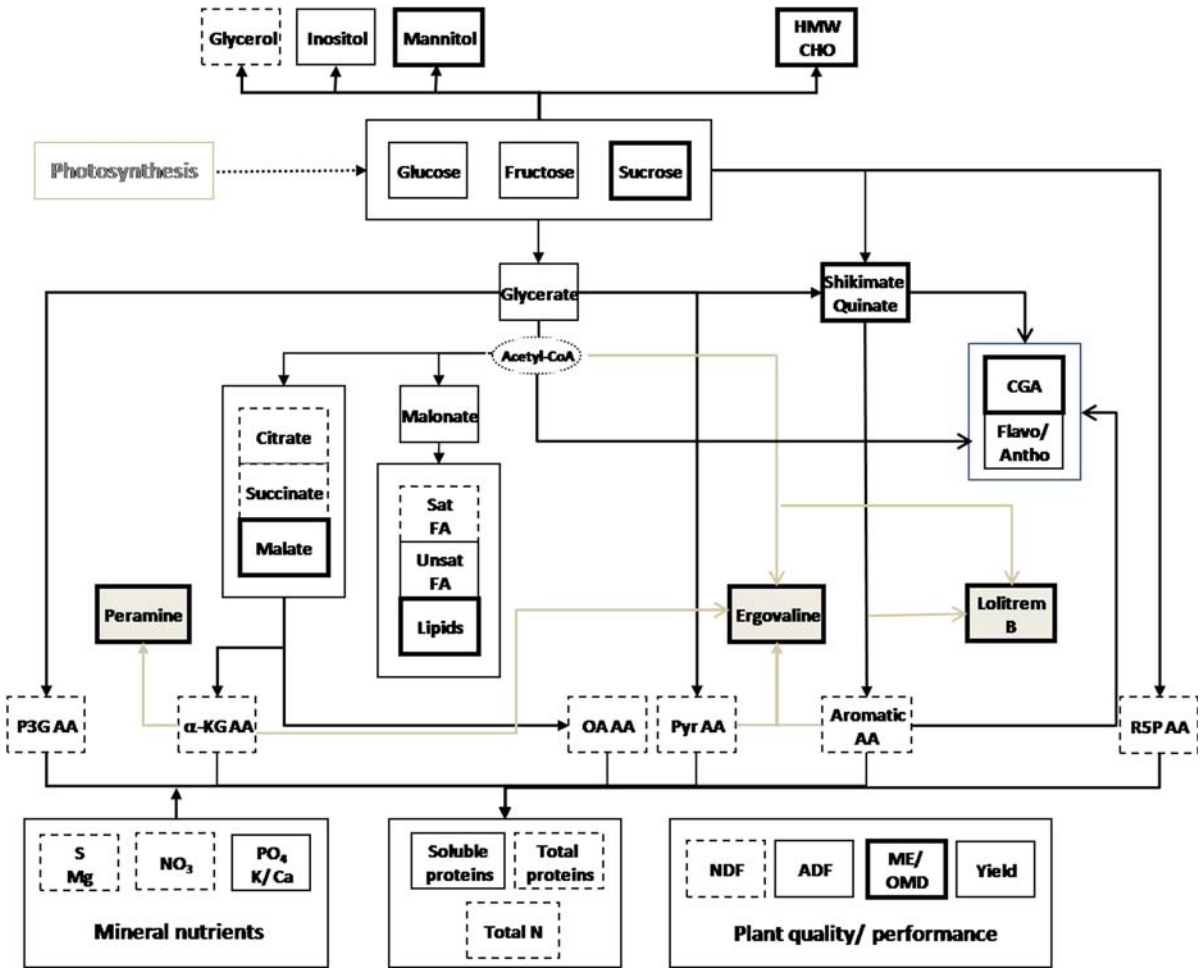

Fig. 5 Hypothetical schematic representation of metabolic endophyte effects and possible metabolic network connections in *L. perenne* mature blades infected with *N. lolii* Lp19 (CS) strain. Up-regulated metabolites are shown in *bold solid boxes*, down-regulated metabolites in dashed boxes, and unchanged metabolites in *fine solid boxes* (for details of analytical methods see Rasmussen et al. 2008b). Shown are only metabolites/quality parameters that were quantified in our analyses, except for acetyl CoA (*dotted circle*). *Arrows* and *lines* do not represent direct biochemical relationships, but rather indicate possible connections between those metabolites. Metabolites produced exclusively by the endophytic fungus and connections to them are *highlighted* in *grey*. Amino acids synthesized from the same precursor were grouped: P3G AA—L-serine, L-cysteine (not quantified), L-glycine derived from 3-phosphoglycerate; α-KG AA—L-glutamate, L-glutamine, L-proline, L-arginine derived from α-ketoglutarate; OA AA—L-aspartate, L-asparagine, L-methionine, L-threonine, L-lysine, L-isoleucine derived from oxaloacetate; Pyr AA—L-alanine, L-valine, L-leucine derived from pyruvate; PEP + E4P AA—L-phenylalanine, L-tyrosine, L-tryptophan derived from erythrose 4-phosphate and phosphoenolpyruvate (Stryer 2000). Fatty acids were grouped into saturated fatty acids (Sat FA; only C17:0 and C18:0 were down-regulated) and unsaturated fatty acids (Unsat FA). Plant quality parameters were analysed by near-infrared spectroscopy: *NDF* neutral detergent fibre; *ADF* acid detergent fibre; *ME* metabolisable energy; *OMD* organic matter digestibility

relationship between the depicted metabolites, but rather a simplified scheme of possible metabolic network connections. To further simplify this scheme, we have grouped some of the analysed metabolites like e.g. saturated and unsaturated fatty acids (Sat FA, Unsat FA), and amino acids (based on their common precursor; Stryer 2000).

As stated above, our analyses does not enable us to define if and to what extent the individual metabolites are produced by the plant or the fungus, and we can also make no statements as to which plant metabolites are taken up by the endophytic fungi and further metabolised. Detailed studies on metabolite uptake have been performed for mycorrhizal associations (Shachar-Hill et al. 1995; Solaiman and Saito 1997; Pfeffer et al. 1999), and for some biotrophic fungal pathogens (Clark and Hall 1998; Hall et al. 1992; Hall and Williams 2000) indicating that glucose is the preferred sugar to be taken up by these fungi, but unfortunately this kind of information is still lacking for *Neotyphodium/Epichloë*—grass associations. The only metabolites analysed here known to be produced exclusively by the endophytic fungal strain *N. lolii* Lp19 are the alkaloids peramine, lolitrem B, and ergovaline (highlighted in grey in Fig. 5). Mannitol is most likely a fungal metabolite and was found in high concentrations in endophyte infected blades, but we also detected traces of this polyol in some uninfected samples. Therefore, we cannot exclude that ryegrass itself can produce this compound, and it is known to accumulate in more than 50 families of the Angiosperms (Lewis and Smith 1967).

The unusual pyrrolopyrazine peramine has so far been shown to be produced only by *Neotyphodium/Epichloë* fungi (Siegel et al. 1990). Although no experimental data are available for the biosynthetic precursors of this alkaloid, its structure suggests that it is derived from the two amino acids arginine and proline (Rowan 1993), both belonging to the α-ketoglutarate amino acid family (α-KG AA). Recently, a non-ribosomal peptide synthetase catalysing peramine formation has been isolated from *N. lolii*, and the knock-out of the encoding gene (*perA*) in *E. festucae* has been shown to result in a loss of peramine (and associated feeding deterrent activity against Argentine stem weevil—ASW) in plants infected with the mutant strain (Tanaka et al. 2005).

Although the alkaloid lolitrem B seems to be specific for *Neotyphodium/Epichloë* fungi, other indole-diterpenoids like paspaline and paxilline are produced by other fungal genera as well (Springer et al. 1975; Springer and Clardy 1980). Indole-diterpenoids have a common cyclic diterpene core, derived from geranylgeranyl diphosphate (GGDP), and an indole group (Saikia et al. 2008). The starting compound for GGDP is isopentenyl diphosphate, which is produced from acetyl CoA (indicated as a grey arrow from acetyl CoA to lolitrem B in Fig. 5). The indole ring is most likely derived from a precursor of tryptophan (an aromatic amino acid; de Jesus et al. 1983). The enzymatic biosynthesis of lolitrem B in *E. festucae* and *N. lolii* is encoded by a gene cluster of at least 10 genes, which also contains a gene for GGDP synthase (Young et al. 2005, 2006), making it likely that GGDP is produced by the fungus itself. The knock-out of one of the genes (*ltmM*) in *E. festucae* resulted in a loss of lolitrem B in plants infected with the mutant strain (Young et al. 2005).

Ergot alkaloids have been shown to be produced via isoprenylation of the aromatic amino acid tryptophan with dimethylallylpyrophosphate (synthesized from acetyl CoA) to form dimethylallyltryptophan (DMAT), which is converted to lysergic acid (Lane et al. 2000; Tudzynski et al. 2001). A gene coding for DMAT synthase has been cloned from *Neotyphodium* sp. isolate Lp1, and the knock-out of this gene abolished ergovaline production in ryegrass plants infected with the mutant strain (Wang et al. 2004). Ergovaline, the main ergotpeptide found in *Neotyphodium/Epichloë* infected plants, is produced by transfer of the amino acids L-alanine, L-valine (both derived from pyruvate) and L-proline (from α-ketoglutarate) to lysergic acid by a multifunctional enzyme complex, which has also been identified from *Neotyphodium*. sp. Lp1 (Panaccione et al. 2003).

Although endophyte alkaloid biosynthesis can be seen as a sink for carbon and nitrogen compounds produced by the host plant, a closer look at actual changes of metabolite concentrations and total alkaloid concentrations indicates that this sink is most probably very small. In the case of peramine ($C_{12}H_{17}N_5O$), the most abundant of all analysed alkaloids, we found that its concentration at low N was 0.15 μmol/g DW (Fig. 2e), but the concentrations of L-arginine and L-proline (likely precursors of peramine) were much more reduced in endophyte (CS) infected compared to uninfected plants by

approximately 5 μmol/g DW each (Rasmussen et al. 2008b; L-arginine from 18 to 13 μmol/g DW; L-proline from 9 to 4 μmol/g DW, Fig. 3c). Compared to peramine, ergovaline ($C_{29}H_{35}N_5O_5$) and lolitrem B ($C_{42}H_{55}NO_7$) are present in infected mature blades in much lower concentrations of approximately 0.5 nmol/g DW and 1.2 nmol/g DW, respectively (Rasmussen et al. 2008b). We suggest therefore that the changes in metabolic profiles seen in our study are not predominantly caused by alkaloid biosynthesis, but rather by more general metabolic processes occurring in the fungus like e.g. biosynthesis of proteins, lipids, and cell walls, and maintenance and growth respiration.

Many open questions remain as to how and to what extent the endophyte affects plant metabolism. Some of the most important questions which need to be addressed in future studies are: (1) Which metabolites are taken up by the endophyte? (2) Are these metabolites taken up from the apoplast or from plant cells the endophyte is attached to? (3) Which transporters are involved? (4) Does the endophyte affect nitrate uptake/transport from host roots? (5) Does endophyte infection induce host genes involved in secondary plant metabolite production (e.g. genes of the phenylpropanoid pathway)?

Effects of *N. lolii* endophytes on insect herbivores

Resistance against herbivory, notably damage by potentially devastating pest attacks e.g. by Argentine Stem Weevil, is an unquestionable benefit of endophyte infection to plant fitness, in both natural and commercial (agricultural) contexts. Usually, this fitness benefit is ascribed to the production of anti-herbivorous alkaloids (Siegel et al. 1990; Rowan and Latch 1994; Bush et al. 1997; Schardl et al. 2007). However, the widespread changes seen in metabolic profiles, and especially the shift to higher carbon/nitrogenous metabolite ratios, and potential stimulation of defense compounds make it arguable that effects on insect herbivores might be causally related to metabolic factors other than the alkaloids alone (Brown et al. 2002; Douglas 2006; Douglas et al. 2006), or at least that other factors can modulate alkaloid impacts.. We exposed the same plant material used in the studies discussed above (Rasmussen et al. 2007, 2008b) to a suite of insects (aphid spp., thrips, mites) and assessed the impacts on the insect populations (Rasmussen et al. 2008a). Different insect species responded very differently to endophyte infected plants. Aphids belonging to the genus *Rhopalosiphum* were much more abundant on AR1 infected plants than on endophyte free, CS or AR37 infected plants (Fig. 6a). In contrast, *Sitobion frageriae* aphids were much more abundant on AR37 infected plants (Fig. 6b). This clearly shows that different insect herbivores (here phloem sucking aphids) may be affected in a different way, even when feeding on the same plant tissue. Numbers of both, *S. frageriae* and mites, were highest on AR37 infected and lowest on endophyte free plants (Fig. 6b, c).

We regressed numerical responses of the individual insect populations to the analysed metabolites to link each of these responses to endophyte infection (Rasmussen et al. 2008a). We found that apterate *Rhopalosiphum* spp. correlated negatively to lolitrem B, lipids, and fibre content, and positively to peramine and malonate. In contrast, apterate *S. frageriae* correlated negatively to peramine and histidine, and positively to sugars. The responses of mites could not be linked to any of the alkaloids, but was positively correlated with shikimate, L-serine, and the fatty acid C16:0. In general, we found more thrips and mites on plants grown with high N supply, while numbers of aphids were slightly reduced on these plants (Rasmussen et al. 2008a). As would be expected (despite common perceptions) aphid populations were not affected by higher sugar levels in 'AberDove' compared to 'Fennema' (Rasmussen et al. 2008a). Although this type of analysis does not allow the establishment of direct and causal relationships between a certain metabolite and insect population responses, the differential pattern of these responses is intriguing and warrants further metabolomics studies on the effects of non-alkaloid metabolites on insect and other herbivores. A recent study also performed in our lab and using untargeted direct infusion ion trap mass spectrometry revealed the presence of formerly unknown compounds produced in endophyte infected plants (Cao et al. 2008). Some of these compounds belong to the class of cyclic oligopeptides, which have been shown to be potentially antimicrobial (Arai et al. 1973, Strobel and Hess 1997; Seto et al. 2007) and it is therefore possible that some of the plant protecting effects seen in

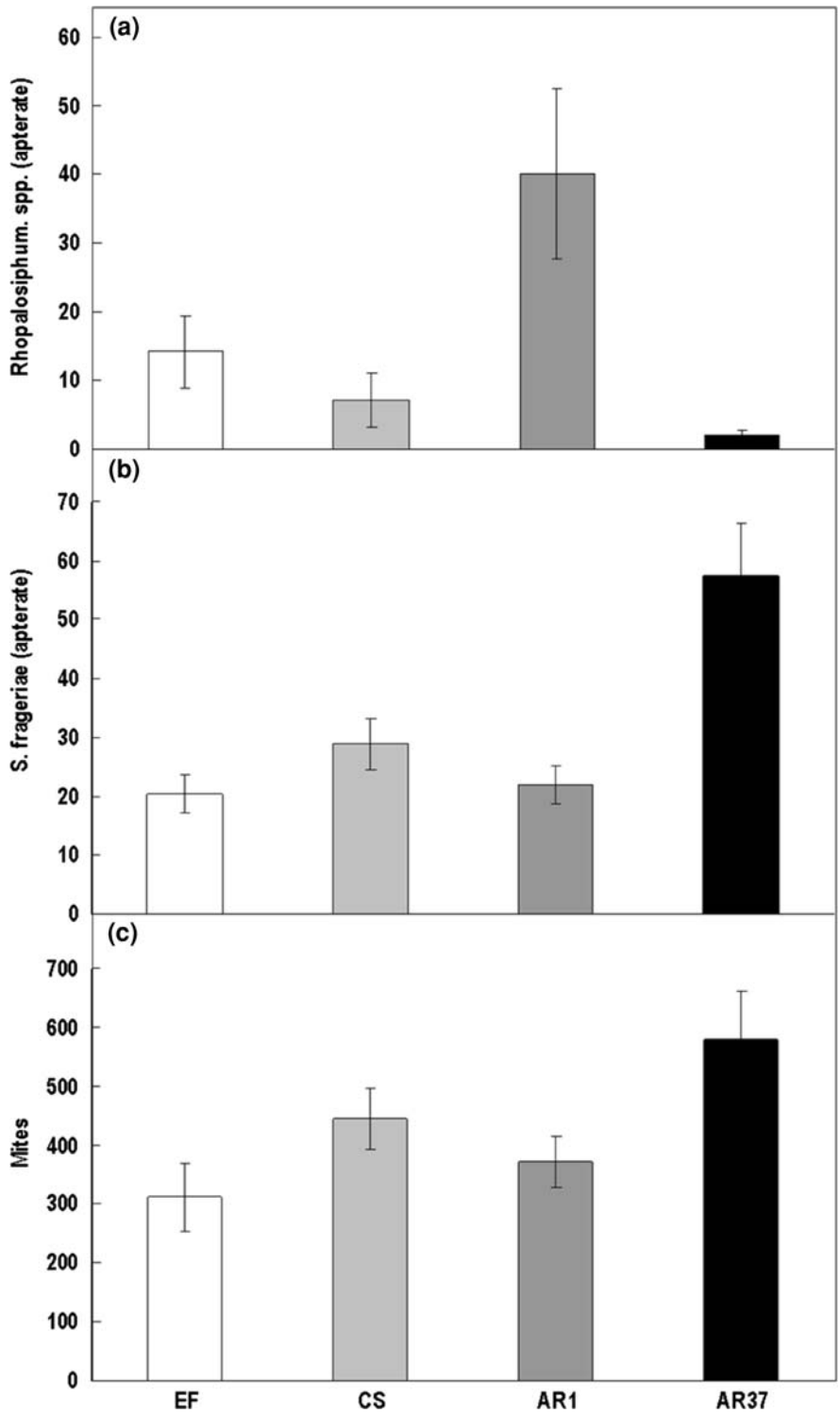

Fig. 6 Effects of endophyte infection on numbers of apterate *Rhopalosiphum* spp. (**a**), apterate *S. frageriae* (**b**), and mites (**c**) on shoots of *L. perenne* plants

endophyte infected plants are due to these or other, still completely unknown, endophyte specific compounds.

Endosymbioses present a particular challenge to using metabolic profiling to characterise metabolic processes in the endosymbiotic organisms: (1)

because more than one organism genome/metabolome is involved, (2) many metabolites are identical in both organisms, (3) it is unclear to what extent one organism is using (in effect) the other's metabolic network, to synthesise a given metabolite, and (4) because there is uncertainty over the abundance of the endosymbiont in the host population. The combined metabolic profile of the host/endosymbiont association as reported by us is arguably only descriptive, and we have proposed, from our studies, how metabolic profiles can be presented as simply another measured variable in a conventional critical experimental design where a series of treatments/factors have been used to perturb the system. This may reveal extra insights into the fitness consequences that a given profile ascribes.

But because differences in fitness between individuals in a population will mean shifts in the prevalence of e.g. endophyte infected individuals, we propose it is important to report wherever possible, the quantity (e.g. as a concentration) of endosymbiont present within any one plant's tissue, as well as across the population.

Biotechnological advances increasingly aim to modify plant metabolic composition through the introduction of e.g. endophytic strains with novel alkaloids. It is therefore critical to ensure that e.g. a toxic compound usually accumulated at low levels, does not become unexpectedly a highly accumulated compound, due purely to an increased population of e.g. the endosymbiont within one organisms' tissues, or an increased prevalence of that symbiosis at the population level brought about e.g. by unintended (and unrelated to the alkaloids of interest) effects on the metabolic profiles of the symbiotic association. Combinations of metabolomics (and other 'omics' approaches) and ecological sciences are valuable to assess the wider impacts of shifts in metabolic profiles.

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