Running title: Metabolic profiles of endophyte-infected ryegrass

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Research Area: Plant-Microbe Interactions
Metabolic profiles of *Lolium perenne* are differentially affected by nitrogen supply, carbohydrate content and fungal endophyte infection

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ABSTRACT

*Lolium perenne* cultivars differing in their capacity to accumulate water soluble carbohydrates (WSCs) were infected with three strains of fungal *Neotyphodium lolii* endophytes or left uninfected. The endophyte strains differed in their alkaloid profiles. Plants were grown at two different levels of nitrogen (N) supply in a controlled environment. Metabolic profiles of blades were analysed using a variety of analytical methods. A total of 66 response variables were subjected to a principle components analysis and factor rotation. The first three rotated factors (46 % of the total variance) were subsequently analysed by ANOVA.

At high N supply nitrogenous compounds, organic acids and lipids were increased; WSCs, chlorogenic acid (CGA) and fibres were decreased. The high sugar cultivar AberDove had reduced levels of nitrate, most minor amino acids (AAs), sulphur and fibres compared to the control cultivar Fennema, whereas WSCs, CGA, and methionine were increased. In plants infected with endophytes, nitrate, several AAs, and Mg were decreased; WSCs, lipids, some organic acids and CGA were increased. Re-growth of blades was stimulated at high N; and there was a significant endophyte x cultivar interaction on re-growth. Mannitol, a fungal specific sugar alcohol, was significantly correlated with fungal biomass.

Our findings suggest that effects of endophytes on metabolic profiles of *L. perenne* can be considerable, depending on host plant characteristics and nutrient supply, and we propose that a shift in C/N ratios and in secondary metabolite production as seen in our study is likely to have impacts on herbivore responses.
INTRODUCTION

Symbiotic plant-fungal interactions are of widespread interest to ecological research as they can influence important ecosystem processes, including plant productivity, plant diversity, and plant-herbivore interactions (Omacini et al., 2001; van der Heijden et al., 2006; Vogelsang et al., 2006). Recently, associations of grass plants with fungal endophytes belonging to the Clavicipitaceae family and residing in above ground plant parts, have become the subject of detailed ecological studies (Clay et al., 2005; Rudgers et al., 2005; Finkes et al., 2006; Meister et al., 2006). These fungi occur in 20-30 % of all grass species (Leuchtmann 1993) with their hyphae growing in the apoplastic space without penetrating into plant cells and generally causing no visible symptoms of infection (Christensen et al., 2007). The most commonly studied associations are *Neotyphodium lolii* - *Lolium perenne* in Australasia and *N. coenophialum* - *L. arundinaceum* in North America (Christensen et al., 1993), as these are of particular importance to agricultural pastoral systems.

*Neotyphodium* spp. endophytes can confer a range of benefits to their grass hosts, mainly by producing specific alkaloids that deter feeding by herbivores (Bush et al., 1997). Considerable research efforts have been focussed on the biosynthesis, accumulation and ecological consequences of these fungal alkaloids (for reviews see: Clay and Schardl, 2002; Schardl et al., 2004). Much less is known about the impacts of fungal endophytes on other plant performance parameters and metabolism, although endophyte infection has been implicated in increasing drought and mineral stress tolerance (Malinowski et al., 1998; Malinowski and Belesky, 2000; Hesse et al., 2003; Hesse et al., 2005). However, the general mutualistic nature of this particular type of plant-fungus association has been disputed (Saikkonen et al., 2004; Müller and Krauss, 2005; Saikkonen et al., 2007) and it was shown that positive effects of the endophyte on plant performance depend on genetic variation in the host and endophyte, and on nutrient availability (Cheplick et al., 1989; Cheplick et al., 2000; Cheplick 2004; Hesse et al. 2004; Cheplick 2007). This link between resource availability and beneficial or neutral versus detrimental effects on plant performance suggests a metabolic cost of the endophyte to the host plant and recent publications indicate that metabolic effects of endophyte infection beyond the accumulation of alkaloids are of importance to the
understanding of ecosystem wide impacts of this symbiosis (Hunt et al., 2005; Krauss et al., 2007; Rasmussen et al., 2007).

In a recent study (Rasmussen et al., 2007), we infected two L. perenne cultivars (AberDove, Fennema) that differed in their water soluble carbohydrate (WSC) content with three strains of N. lolii (common strain CS, AR1, and AR37) that differed in their alkaloid profiles (CS produces: peramine, lolitrem B, and ergovaline; AR1 only peramine; AR37 only janthitrems) and grew them under high and low nitrogen (N) supply. Concentrations of endophyte, as estimated by quantitative polymerase chain reaction (qPCR), in infected plant tissue were shown to be reduced by 40% under high N supply, and by 50% in the higher sugar cultivar; these effects were additive and alkaloid concentrations were linearly related to endophyte concentrations. We also reported a significant interaction between endophyte infection and L. perenne cultivar on sugar and soluble protein accumulation, indicating that introducing new cultivars, novel endophyte strains or increasing N inputs might affect the role of endophytes in grassland ecosystems.

It has been suggested that the integration of ‘-omics’ technologies such as transcriptomics or metabolomics with traditional ecological research in the emerging research field of ‘ecogenomics’ might help to understand the mechanistic basis for community processes and especially plant-pathogen and plant-herbivore interactions (Ouborg and Vriezen, 2007; Snoeren et al., 2007). Here, we report a detailed study on metabolic profiles of the same plant material as described in Rasmussen et al. (2007) using several analytical techniques including gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), and near infrared reflectance spectroscopy (NIRS) to analyse metabolome-wide impacts of endophyte infection, cultivar differences and N fertilisation. Possible underlying biochemical processes leading to changes in metabolic composition and possible impacts on ecological processes are discussed. The plant material of the present study was subsequently used to test if endophyte-induced changes in metabolic composition affected insect herbivore performance and results will be published in a separate report.

RESULTS

Principal components analysis of metabolite responses
The analysis of 66 response variables (as measured in this study) presents a problem for the conventional ‘frequentist’ approach to statistical analysis (Taper and Lele, 2005), as the experiment-wide likelihood of committing a ‘Type 1 error’ (detecting ‘false’ treatment effects) increases with the number of variables. Metabolic data are often complex and the correlated and constrained nature of some metabolites, coupled with the problem of α-inflation due to ‘Type 1 errors’, strongly argues for a multivariate statistical approach revealing how sets of metabolites, rather than individual metabolites, respond to treatments. Here, we used principal components analysis (PCA) to reduce the number of metabolic response variables to a set of new ‘composite’ variables (McGarigal et al., 2000), which are now uncorrelated with each other. As these components themselves are often difficult to biologically interpret we subsequently subjected them to ‘factor rotation’ to cause individual variables to load more heavily onto a single axis (Hair et al., 1998).

Factor rotation is a method for rotating the principal component axes around the centroid. The centroid is the equivalent of the sample mean when the response has more than one dimension. Factor rotation can be understood as the multivariate equivalent of the univariate procedure of data transformation. Like transformations for univariate data, factor rotation serves two non-exclusive purposes. First, like univariate transformations, factor rotation is necessary when the multivariate normality assumption of the data is not met. Second, like some forms of univariate transformations (e.g. logarithms) factor rotation can aid in the interpretation of the principal component space. The effect of factor rotation is to redistribute some of the variance from earlier components to later components. We used the most common form of factor rotation, varimax rotation, which increases the magnitude of variables that load heavily (positively or negatively) onto an axis, and simultaneously, decreases the values of variables that do not load heavily onto an axis. In doing so, the varimax rotation yields a multivariate structure that is easier to understand because it increases the distinction between the large and small loading variables and so makes the biological interpretation of the axes simpler. For further discussion and references on factor rotation, the interested reader should see McGarigal et al. (2000) pages 58-60.

We retained the first three rotated factors (RF-1, RF-2, RF-3) for further analysis using standard ANOVA. The variable loadings for these three RFs are shown in Figure 1. As the rotated factors increase, those variables that load heavily and positively (loading ≥ 0.5) also increase; and those variables that load heavily but...
negatively (loading ≤ -0.5) decrease. As can be seen in Figure 1, variables loading heavily and positively onto RF-1 include mainly nitrogenous compounds, two organic acids, two minerals, glycerol, ash (inorganic or mineral component of plant material), and dietary cation anion difference (DCAD; indicator of difference between sodium and potassium versus chloride and S). Variables loading heavily but negatively are carbohydrates and chlorogenic acid (CGA). It should be noted here that LMW (low molecular weight) WSCs comprise a mixture of glucose, fructose, sucrose and low degree of polymerisation (DP) fructans. HMW (high molecular weight) WSCs are a mixture of high DP fructans (fructose polymers), the major reserve carbohydrates in *L. perenne* (Pavis et al., 2001).

Variables loading heavily and positively onto RF-2 include organic acids, *in vitro* organic matter digestibility (OMD; estimates total nutrients digestible by ruminants), metabolisable energy (ME; indicator of the energy that is available to ruminants for maintenance and growth), lipids and DCAD. Variables loading heavily but negatively include two fatty acids (C17:0, C18:0), one mineral (Mg), and neutral detergent fibre (NDF; estimates mainly cellulose, hemicellulose, and lignin content).

Variables loading heavily and positively onto RF-3 are three fatty acids (C16:0, C16:1, C18:3) and malate. Variables loading heavily but negatively onto this axis are acid detergent fibre (ADF; estimates mainly cellulose and lignin content) and NDF.

Note that malate, DCAD, and NDF (denoted with arrows in Fig. 1) load heavily onto two axes, complicating the interpretation of these responses. This is because each of the univariate responses follows part of the response pattern for each of the multivariate response variables. Consequently interpreting them within the context of the multivariate variables is difficult.

Each of the three rotated factor variables were subjected to a three-way ANOVA with N (low, high), cultivar (Fennema, AberDove), and endophyte (endophyte free - EF, common strain - CS, AR1, AR37) as the main effects and all two- and three-way interactions. Examination of the residuals suggests that the assumptions of homogeneity and normality were met without the need to further transform these variables. With 160 samples this design should have an error degrees of freedom (df) of 144. In all of the F-tests shown below the error df is 138 due to missing samples in one or more of the 66 metabolites used in the principal components analysis and subsequent factor rotation.

As some readers might feel uneasy with this type of multivariate approach we also present the results of an ANOVA of all (Box-Cox transformed) individual response
variables as supplementary material (Table S1). It should be noted, that when these variables are analysed as univariate response variables rather than as part of a principal component axis, some of them show significant interactions that are not readily apparent in the multivariate approach, e.g. LMW and HMW WSCs (Table S1; Rasmussen et al., 2007). We therefore attached the complete data set as supplementary material (Table S2) for those readers who would like to try alternative analyses of these data.

Rotated factor 1

N (F $1,138=257.01$, P<0.0001), cultivar (F $1,138=46.01$, P<0.0001), and endophyte infection (F $3,138=16.02$, P<0.0001) induced significant variation in the variables comprising RF-1. These three effects accounted for 71% of the total variance of RF-1. None of the interactions were significant. The mean responses of RF-1 and standard errors of the means are plotted in Figure 2a, 2c, and 2e. They can be interpreted as follows: Concentrations of variables with positive loadings were higher in high N than in low N; lower in AberDove than in Fennema; and lower in endophyte-infected plants than in EF plants. The endophyte effect was strain dependent with AR1 showing the smallest and AR37 the largest effect. Concentrations of variables loading negatively onto RF-1 were lower in high N than in low N; higher in AberDove than in Fennema; and higher in endophyte infected plants than in EF plants, again in a strain dependent manner.

The standardised univariate responses of these variables are shown in Figure 2b, 2d, and 2f as support for the interpretation of the multivariate responses and to allow a closer inspection of those variables loading heavily onto RF-1 (i.e. loadings $\geq 0.5$ and $\leq -0.5$). Variable standardisation allows direct comparison of the response magnitudes for variables with either very different concentrations, or variables with different units of measurement. As can be seen (Fig. 2b), the effect of high N supply was most prominent on major amino acids (L-Gln, L-Asp, L-Thr, L-Ala, L-Asn, L-Arg, L-Ser, L-Asn, and L-Glu), which represent 85% of the total free amino acid pool across all treatments (Table S1). Nitrate, total N and total protein were also considerably increased at high N supply and we note here that nitrate was increased almost 9-fold, whereas all other variables were increased less than 3.2 fold (based on untransformed data, see also Table S2). The standard deviation for nitrate was very high, resulting in relatively smaller differences when standardised. Minor amino acids (L-His, L-Gly, L-Ile, L-Leu, L-Tyr,
L-Val, L-Pro, L-Lys, and L-Phe) were much less affected by increased N supply. L-Met was the only AA analysed here that was not affected by N supply and did not load strongly onto any of the three rotated factors. All variables loading negatively onto RF-1 (loadings ≤ -0.5; carbohydrates and CGA) were decreased at high N supply (Fig. 2b).

Most variables (loadings on RF-1 ≥ 0.5) were in fact decreased in the high sugar cultivar AberDove compared to Fennema, but this effect was most prominent on minor AAs and nitrate (Fig. 2d). Interestingly, S was reduced in this cultivar as well, and we note here that the S containing AA L-Met was significantly increased in AberDove (2.4-fold), when that variable is subjected to a standard univariate analysis (Table S1). From the negatively loading variables (loadings ≤ -0.5) only HMW WSCs and CGA were considerably increased in AberDove (Fig. 2d).

Effects of endophyte infection on the magnitude of the standardised univariate responses was strongly strain dependent, with AR1 having the weakest and AR37 the strongest effect on most variables (Fig. 2f). Almost all AAs were reduced in endophyte infected plants, but this effect was most apparent for L-Asn and several minor AAs. Carbohydrates and CGA were increased in infected plants, but the responses were much weaker for HMW WSCs and CGA.

Rotated factor 2

N (F 1,138=31.24, P<0.0001), cultivar (F 1,138=12.26, P<0.0001), endophyte infection (F 3,138=3.85, P<0.05), and the cultivar by endophyte interaction (F 3,138=4.13, P=0.008) all induced significant variation in the variables comprising rotated factor 2. These four effects accounted for 39% of the total variance of RF-2. None of the other interactions were significant. The mean responses and standard errors of the means are plotted in Figures 3a and 3c. Concentrations of variables with positive loadings were higher in high N than in low N; and higher in endophyte infected Fennema plants than in EF Fennema, but in AberDove plants there was little variation in these variables. Concentrations of variables with negative loadings on RF-2 show the reverse pattern.

The standardised univariate responses of these variables are shown in Figures 3b, 3d and 3e as support for the interpretation of the multivariate responses. The effect of high N supply was most prominent on malate, lipid and DCAD (concentrations increased; Fig. 3b), and C17:0 and NDF (both decreased, Fig. 3b).

The precursors of aromatic AAs and phenylpropanoids (shikimate and quinate), OMD and ME were strongly increased in Fennema infected with endophyte (especially
with AR37; Fig. 3d); this effect was much weaker in AberDove (Fig. 3e). Lipids and malate were increased by endophyte infection in both cultivars to the same degree. The two fatty acids (C17:0 and C18:0), NDF and Mg were reduced in endophyte infected plants, but the magnitude of that effect was dependent on the cultivar.

**Rotated factor 3**

N (F 1,138=36.82, P<0.0001) and cultivar (F 1,138=61.13, P<0.0001), but not endophyte infection induced significant variation in the variables loading onto RF-3. Together these two effects accounted for 42% of the total variation in RF-3. None of the interactions were significant. The mean responses and standard errors of the means are plotted in Figures 4a and 4c. Concentrations of variables with positive loadings were higher in high N than in low N (Fig. 4a), and higher in AberDove than in Fennema (Fig. 4c).

The standardised univariate responses of these variables are shown in Figures 4b and 4d; malate and three fatty acids increased at high N compared to low N (Fig. 4b) and in AberDove compared to Fennema (Fig. 4d). The fibre components ADF and NDF decreased at high N (Fig. 4b) and were lower in AberDove (Fig. 4d).

**Plant growth**

N (F 1,144=19.62, P<0.0001), endophyte (F 3,138=5.99, P<0.001) and the endophyte by cultivar interaction (F 3,138=3.34, P<0.05), induced significant variation in the dry weight of blades re-grown for two weeks (Fig. 5). High N supply did result in significantly more re-growth, as expected. There was no difference in re-growth between the two EF cultivars, but the endophytic strain AR37 stimulated re-growth more in AberDove compared to AR1 infected plants, and also compared to EF and CS infected Fennema.

**Mannitol**

Previously (Rasmussen et al., 2007), we reported that a regression of endophyte alkaloid (peramine, lolitrem B, janthitrems) concentrations against fungal concentrations was highly significant. As reported, fungal concentrations were determined by qPCR of two endophyte specific genes, chitinase and a non-ribosomal peptide synthetase, which were highly correlated and condensed to a single principal component PC1. Here, we regressed another fungal metabolite, the sugar alcohol
mannitol, to the same PC1 and also found the regression to be highly significant (F₁,₁₁₄=190.42, P<0.0001). The untransformed data are shown in Figure 6.

**DISCUSSION**

In a previous paper (Rasmussen et al., 2007) we described some major effects of N and a sugar accumulating ryegrass cultivar on the concentration and alkaloid production of fungal *N. lolii* strains. To recap briefly, both high N supply and the high sugar cultivar (AberDove) substantially reduced endophyte presence (expressed as a concentration of endophyte specific DNA in total fungal and plant genomic DNA). Both treatments each reduced endophyte concentration by between 40 and 50 %, and the effects were additive (leading to an overall 75 % reduction in high sugar plants at high N supply). Endophytic alkaloid concentrations were affected in the same way and were, in fact, highly correlated with endophyte concentrations. The same plant material used in that previous study was analysed here in detail for metabolic responses to the different treatments (high N supply, high sugar cultivar, and endophyte infection). We discuss possible mechanisms of how these treatments might have caused the described reduction in endophyte concentrations based on the different metabolite profiles. We also discuss the extent to which the observed changes support the notion that endophytes might be a drain (net cost) on plant metabolism or might up-regulate metabolism (cf. sink stimulation). A change in metabolic profiles per se may provide insights into the nature of the grass-endophyte association and may also be critical to understand further multitrophic interactions, e.g. the response of herbivores (insects and grazers) to the ryegrass-endophyte association.

**Effects of high nitrogen supply**

The effects of high N supply on metabolic profiles in ryegrass blades were most prominent on nitrogenous compounds, as expected. Nitrate levels in blades were approx. 9-fold higher in the high N treatment, indicating that nitrate uptake exceeded the plants’ capacity for nitrate assimilation. Although 18 out of 19 analysed amino acids were increased, there was a marked difference in the response of individual amino acids. Major AAs, which represented approx. 85 % of the total free AAs, were much more affected than minor AAs. This is in accordance with findings from a variety of crop plants, where mainly major AAs responded to changes in carbon and nitrogen
metabolism, whereas minor AAs were not correlated with these changes and correlated more with each other than with total AA pools (Noctor et al., 2002).

As nitrate assimilation into AAs requires reductants (10 electrons per molecule nitrate), energy (ATP) and carbon skeletons this process is tightly linked with photosynthesis and carbon metabolism (Stitt et al., 2002; Smith and Stitt, 2007). Nitrate supply results in decreased carbohydrate synthesis and accumulation, and a large proportion of carbon is converted via glycolysis and citric acid cycle into organic acids, as was seen in the present study as well - malate, succinate and citrate were all increased at high N supply. These organic acids serve several purposes, the major ones being: (i) malate acts as a counter-anion to prevent alkalinisation (Martinioa and Rentsch, 1994) and (ii) organic acids act as carbon precursors for AAs (Morot-Gaudry et al., 2001). Most of these studies have been performed in starch accumulating plants like Arabidopsis, and it should be noted here that L. perenne, as many other cool-season grasses, does not accumulate starch in vegetative organs, but instead water-soluble fructans (fructose polymers) which are stored in the vacuole and serve as the major reserve carbohydrate (Pollock and Jones, 1979; Gordon et al., 1980; Prud’homme et al., 1992; Pavis et al., 2001). The fact that we also see a decrease in these carbohydrates at high nitrate supply suggests that comparable processes as described for starch accumulators are operating in fructan accumulating plants as well. High N supply also reduced the fibre content and increased lipids in plants, a further indication of a shift from carbohydrates (major component of fibres) to organic acid derived molecules (lipids).

In a discussion of our previous findings that high N supply reduced endophyte concentrations, we hypothesised that this might be due to a ‘dilution’ effect, i.e. plant growth is increased more than fungal growth under these conditions. This hypothesis is supported by the data set on yield presented here (Fig. 5a), as high N did result in significantly increased plant re-growth. However, other factors might have contributed to the reduction in endophytic concentrations. The best studied endophytic fungi residing in plant parts are mycorrhizal fungi, for which it has been shown that up to 20% of carbon fixed by the host plant can be allocated to the fungus (Douds et al., 2000; Graham 2000; Pfeffer et al., 2001). The major form of carbon transported to these fungi is glucose, resulting from a cleavage of sucrose in the apoplastic regions close to fungal hyphae (Solaiman and Saito, 1997; Wright et al., 1998; Pfeffer et al., 1999). As high N supply resulted in decreased concentrations of sucrose and LMW WSCs in our
study, it is possible that this treatment also caused a reduced availability of sugars for the endophytic fungi, resulting in reduced fungal growth. However, caution must be applied here, as (i) the measured sugar concentrations are averaged across the whole blade tissue and do not allow us to make statements about sugar concentrations in the apoplastic space (where the endophytic fungi reside), and (ii) the endophytic biomass present in *Neotyphodium*-infected grass blades is much lower compared to that of mycorrhizal fungi, which have an extensive net of extraradical hyphae drawing on plant carbon resources. Related to (ii), it is also unclear if metabolite concentrations in the apoplast are limiting for growth of foliar endophytic fungi at all, especially under conditions favourable for photosynthesis as in our study.

As previously reported, endophyte alkaloids were reduced in infected plants grown at high N (Rasmussen et al., 2007), this result was counterintuitive, as it has been suggested that alkaloid concentrations should increase with increased levels of N availability because these compounds require N for their biosynthesis (Belesky et al., 1988; Faeth and Fagan, 2002). However, it should be noted that even the most abundant and N-rich alkaloid peramine (C_{12}H_{17}N_{5}O_{1}, MW 249; Rowan et al., 1986) had a mean concentration of 0.12 μmol g^{-1} DW (30 μg/ g DW) in infected blades (Rasmussen et al., 2007), which represents only 0.03 μmol N g^{-1} DW compared to a total nitrogen concentration of 2.1 mmol N g^{-1} DW (30 mg g^{-1} DW) and a nitrate concentration of 11 μmol g^{-1} DW (0.74 mg g^{-1} DW) in the symbiotic tissue at low N supply. This indicates, that endophytes or endophyte alkaloid biosynthesis are rarely nitrogen limited and that endophyte and alkaloid concentrations might depend more on carbon availability as discussed above.

**Effects of high sugar cultivar**
The high sugar cultivar AberDove had, as reported earlier (Rasmussen et al., 2007) higher levels of carbohydrates in the blades compared to the control cultivar Fennema. However, only the HMW WSC fraction (consisting of high DP fructans) was affected, whereas glucose, fructose, sucrose and LMW WSCs were not different between the cultivars. In general, the higher sugar levels were accompanied by reduced levels of nitrogenous compounds, succinate, citrate and fibres. Nitrate levels were almost halved in AberDove, but most AAs were much less affected. None of the AAs involved in primary N assimilation (L-Glu, L-Gln, L-Asp, L-Asn) were markedly affected,
indicating that N assimilation per se was not impaired in the high sugar cultivar. However, most minor AAs were reduced, which might indicate a lack of carbon skeletons provided by glycolysis and the citric acid cycle.

As stated above, endophyte concentrations were halved in the high sugar cultivar and as discussed previously (Rasmussen et al., 2007) this was unlikely to be the result of ‘dilution’, as re-growth was not different between the two cultivars. Although it is possible that levels of glucose, fructose and/or sucrose in the apoplastic space (and so available for fungal growth) could have been reduced in AberDove, our data do not support this notion, as the overall concentration of these sugars differed little between AberDove and the control grass. The relatively small changes in AAs as seen in this study also do not seem to support the notion of N limitation for fungal growth. However, L-Met, although not loading heavily onto any of the rotated factors, was significantly increased in the high sugar cultivar (2.4-fold). This in itself is an interesting result, as increased L-Met could be related to reduced endophyte concentrations in the high sugar cultivar. L-Met has been reported to act as a fungal growth and sporulation inhibitor in cultures of *Claviceps microcephala*, a fungus infecting ear heads of *Pennisetum typhoides* (pearl millet) and causing ‘ergot disease’ (Singh et al., 1972). L-Met also induced resistance against *Sclerospora graminicola* (causing downy mildew disease) in pearl millet (Sarosh et al., 2005). This amino acid may also have wider implications for herbivores. L-Met is considered to be one of the most limiting AAs for protein synthesis in growing ruminants, lactating dairy cows and wool producing sheep (Harris and Lobley, 1991; Schingoethe, 1996) and is often supplemented to decrease protein and amino acid degradation in the rumen (Südekum et al., 2004). We do not know yet if high sugar cultivars in general accumulate more L-Met, but if this were the case, reported increases in protein availability, milk and meat production, and decreases of N excretion in urine by high sugar grass grazing animals (for a review on high sugar grasses and their advantages for pasture fed animals, see Edwards et al., 2007) might not only be caused by higher sugar levels, but rather by a combination of increased sugar and L-Met levels.

**Effects of endophyte infection**

Metabolic costs for host plants harbouring foliar endophytes have been implied to be the cause of negative impacts on plant performance and growth seen especially in natural ecosystems or under severe resource limitations (Cheplick et al., 1989; Cheplick
et al., 2000; Faeth and Sullivan, 2003; Cheplick 2004; Faeth et al., 2004; Hesse et al., 2004; Faeth and Hamilton, 2006; Cheplick 2007). In plants infected with mycorrhizal fungi the increased costs due to carbon flow to the fungus can be off-set by increases in photosynthesis (Wright et al., 1998) and improved plant nutrition (Smith et al., 2001). Studies of photosynthetic processes in grasses infected with foliar endophytes are not conclusive and rates of net photosynthesis can be increased (Belesky et al., 1987; Amalric et al., 1999), unchanged or decreased (Spiering et al., 2006), depending on the growth phase of the host plants, nutrient status and environmental conditions. Marks and Clay (1996) demonstrated an endophyte by temperature interaction, and Newman et al. (2003) found an endophyte by N interaction on photosynthetic rates. Effects of endophyte infection on growth also strongly depend on host genotype, resource availability and environmental stress (Belesky et al., 1989; Malinowski and Belesky, 2006; Morse et al., 2002; Cheplick & Cho, 2003; Hesse et al., 2003; Zabalgogeazcoa et al., 2006; Cheplick 2007). In our study we saw significant cultivar by endophyte interactions on the re-growth of blades, which was only stimulated in AberDove plants infected with the endophyte strain AR37, clearly demonstrating the importance of specific host plant – endophyte strain interactions and environmental conditions on overall physiological outcomes of the association. The present study is an analysis of combined plant and fungal metabolites and as most metabolites analysed here are likely to be present in both organisms it is impossible to make statements about impacts on plant metabolism only. We can therefore only discuss overall effects of endophyte infection on the symbiotic metabolism as compared to the non-symbiotic endophyte plants.

A major effect of endophyte infection was an approx. 50% reduction in nitrate levels in the blades, which was accompanied by a reduction of several AAs, total N and total protein; such a reduction of nitrogenous compounds has been described earlier for *N. coenophialum* infected tall fescue (Belesky and Fedders, 1996) and for *N. loli* infected ryegrass (Hunt et al., 2005). The impact of endophyte in reducing nitrate levels in grass leaves has also been shown by Lyons et al. (1990), who used an *N. coenophialum* mutant strain which colonises exclusively sheath tissues and not blades, of tall fescue plants. Lyons et al. (1990) saw infection by endophyte caused a reduction in nitrate levels in both sheaths and blades, in plants fertilised either with nitrate or ammonium. It is known that both ectomycorrhizal and arbuscular mycorrhizal fungi can regulate plant N assimilation (Govindarajulu et al., 2005; Bailly et al., 2007), but as the foliar
endophytes studied here are absent from the roots, their impacts on plant N uptake and transport are probably more indirect. A study of nitrate transporters, nitrate reductase and root metabolites in endophyte infected plants is needed to understand the mechanisms by which foliar endophytes reduce nitrogenous compounds.

Asparagine was the most reduced amino acid and L-Asn levels are mainly regulated by the C/N status of plants. High levels of organic N and low levels of carbon skeletons result in high levels of L-Asn as this amino acid has a high N to C ratio and acts as an inert and stable N-reserve (Lam et al., 1996). In our study, endophyte infection resulted in an increased C/N ratio - more soluble sugars and less organic N – and this might have negatively affected L-Asn biosynthesis.

As pointed out, sugar levels were increased in endophyte infected plants, it is possible that this increase is caused simply by reduced use of carbon skeletons for AAs and proteins; we also found reduced levels of fibres in endophyte infected plants, which could mean that more of the fixed carbon remains soluble and is not incorporated into cell walls. But higher sugar levels might also, at least partially, be a result of increased ‘sink strength’ as seen in plants infected with mycorrhizal fungi (Wright et al., 1998; Douds et al., 2000; Graham 2000; Pfeffer et al., 2001). However, as stated above, mycorrhizae have a much higher biomass compared to the foliar endophytes studied here; and furthermore, the tissue we analysed is both, source (photosynthetically active plant tissue) and sink (heterotrophic fungal tissue), at the same time and it is therefore difficult to distinguish specific sink effects.

While the organic acids citrate and succinate were decreased in endophyte infected plants, malate was increased. It has been shown that malate plays a critical role in lipid biosynthesis in filamentous fungi, where it is irreversibly decarboxylated to pyruvate by malic enzyme with the formation of NADPH. Malic enzyme is suggested to be the major NADPH-generating enzyme required for providing reducing power for fatty acid synthase in Aspergillus nidulans and other lipid storing fungi (Wynn and Ratledge, 1997; Wynn et al., 1999; Zhang et al., 2007). Light microscopy studies have shown that N. lolii hyphae accumulate lipid bodies in its hyphae in planta (Christensen et al., 2002), and in the present study lipids were increased in endophyte infected plants. A gene coding for malic enzyme has not been identified in Neotyphodium spp., but it is likely that the identification of this gene and subsequent expression and localisation studies will give further insights into fungal metabolic processes that are linked to the C and N economy of the host plant.
Shikimate and quinate, precursors for the aromatic amino acids L-Phe, L-Tyr and L-Trp (Herrmann 1995; Herrmann and Weaver, 1999), were increased in endophyte infected plants, particularly in Fennema. L-Phe and L-Tyr are the major aromatic acid precursors for phenylpropanoids, which are involved in plant defense responses in many plants (Dixon 2001), and the pathways leading to the production of these secondary metabolites are often induced by pathogen and herbivore attack (Pellegrini et al., 1994; Felton et al., 1999). We found the major phenylpropanoid accumulating in ryegrass blades, CGA, to be increased in endophyte infected plants. Only very few studies refer to phenylpropanoids in endophyte infected grass plants (Koshino et al., 1988), but it has been reported that *N. coenophialum* infected tall fescue plants accumulated more phenolics in shoots and roots (Malinowski et al., 1998) and it was suggested that this might be relevant for nematicidal effects of root extracts on the root nematode *Pratylenchus scribnery* (Bacetty et al., 2007). Nothing is known about possible mechanisms by which endophyte infection leads to an increase of these compounds, but it is tempting to speculate that endophytes, although usually not causing any disease effects, induce a weak resistance response in infected plants, as was previously suggested (Malinowski and Belesky, 2000), and was shown for AM colonised plant roots (Harrison and Dixon, 1993; Volpin et al., 1994; Blee and Anderson, 1996; Hohnjet et al., 2005). As reactive oxygen species (ROS) like superoxide and hydrogen peroxide are also involved in inducing defense reactions and downstream secondary metabolite pathways (Lamb and Dixon, 1997), it should be noted that ROS production by a fungal NADPH oxidase has been shown to be critical for the mutualistic interaction of *Epichloë festucae* (*Epichloë* spp. are the sexual variants of *Neotyphodium* spp.) and *L. perenne* (Takemoto et al., 2006; Tanaka et al., 2006). These findings, taken together with the results from the present study, warrant further investigations of secondary metabolite production and related gene expression profiles in EP infected plants; this might be particularly important to understand effects of EP infection on pathogens and herbivores independent of fungal alkaloids.

**Mannitol and fungal biomass**

Mannitol accumulated only in endophyte infected *N. lolii* plants and was linearly correlated with fungal biomass. We have previously shown that the endophytic alkaloids peramine, lolitrem B and janthitrems were highly correlated with fungal biomass as well (Rasmussen et al., 2007). Mannitol is a very common polyol in fungi
(Lewis and Smith, 1967) and has been described earlier to accumulate in endophyte infected tall fescue (Richardson et al., 1992) and ryegrass plants (Harwood 1954). Although mannitol has been implicated as an osmoprotectant in the resurrection plant *Myrothamnus flabellifolia* (Bianchi et al., 1993), a study in *N. coenophialum* infected tall fescue indicates that mannitol levels are not increased by drought (Richardson et al., 1992). A recent review (Solomon et al., 2007) also questions this role for mannitol and concludes that the role and requirement of mannitol seem to differ depending on the fungus. In *Aspergillus niger*, mannitol is involved in conidial oxidative and high temperature stress protection (Ruijter et al., 2003), and in the wheat pathogen *Stagonospora nodorum* it is required for asexual sporulation (Solomon et al., 2006). Clearly, more studies are needed to dissect the function of mannitol in endophyte-infected grasses, but the fact that mannitol was correlated with fungal biomass might offer an attractive alternative for the estimation of endophytic abundance in infected tissues. We are currently developing a high-throughput spectrophotometric assay for the quantification of mannitol in plant tissues (Wingler et al., 1993; Graefe et al., 2003) to test its applicability to a wide range of experimental conditions and to ensure that mannitol is in fact related to fungal biomass in general and can be used as an estimate of it.

**CONCLUSIONS**

We have shown that fungal endophyte infection significantly affects both primary and secondary metabolism of its host plant, clearly demonstrating the need for wider metabolic studies beyond alkaloid accumulation to understand ecosystem functions of this association. We have identified a range of biochemical responses and future molecular studies should focus on the dissection of the underlying mechanisms. Both, a shift in C/N ratios and in secondary metabolite production due to endophyte infection, are likely to have impacts on herbivore and plant pathogen responses to grasses infected with *Neotyphodium* spp. These impacts can be of an indirect nature linked to the nutritional value of plants and/or of a more direct nature linked to toxicity of secondary metabolites beyond fungal alkaloids. Our study also shows that metabolic traits of specific grass cultivars/populations and nutrient availability can be critical factors in determining metabolic and physiological outcomes of the grass-endophyte association and must therefore be taken into consideration for future experiments.
MATERIALS AND METHODS

The fundamental design of the experiment was a three-way ANOVA comprising of two grass cultivars, four endophyte treatments (three fungal strains and EF), and two concentrations of N supply.

Plant material

Details of the experimental set-up have been described previously (Rasmussen et al., 2007). In short, we used two *L. perenne* L. cultivars (AberDove, Fennema) differing in their WSC content. Seedlings were inoculated with three *N. lolii* strains differing in their alkaloid profiles: AR1, common strain Lp19 (CS), and AR37, and one set of plants was left uninfected (EF). Plants were grown for several months in soil outdoors and subsequently transferred to pots containing a 1:1 mix of vermiculite and perlite medium and grown in controlled climate chambers (14 h light, 20°C; 10 h dark, 10°C; light intensity 620 μmols m⁻¹ s⁻¹; modified Hoagland nutrient solution containing 9 mM N supplied as nitrate). A total of 160 pots (10 replicates x two CVs x four EPs x two N supplementations) representing 320 genotypes (two per pot) were kept under these conditions in two chambers in a random setup for 18 weeks. Plants were cut back to 6 cm above ground fortnightly throughout the experiment. After 18 weeks half of the pots received a nutrient solution containing 2.25 mM N, the other half received a nutrient solution containing 9 mM N, and 8 weeks later plant material was harvested for metabolite analysis. Cuttings two weeks prior to this were oven dried and weighed for dry matter estimation.

Blades of all plants were cut 6 cm above ground within 1 h on the same day, 7 h after the start of the daylight period and immediately frozen in liquid N and subsequently freeze-dried. The material was stored at -20°C until further analysis.

Metabolite analysis

Detailed information on instrumentation, derivatisation procedures and chromatographic conditions are provided as Supplementary Material (SM1).
Organic acids, sugar alcohols, and fatty acids were extracted, derivatised and determined by GC-MS as described (Roessner et al., 2000, 2001); for details see SM 1a. Compounds detected and quantified in the aqueous phase were: citrate, glycerate, malate, malonate, quinate, shikimate, succinate, threonic acid (Threon), glycerol, inositol, and mannitol. Fatty acids detected and quantified in the CHCl₃ phase were: Dodecanoic acid (C12:0), tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), palmitoleic acid (C16:1 Δ⁹), heptadecanoic acid (C17:0), octadecanoic acid (18:0), oleic acid (C18:1 Δ⁹ Ole), vaccenic acid (C18:1Δ¹¹ Vac), linoleic acid (C18:2), and linolenic acid (C18:3). We also detected traces of C16:1 Δ¹¹ (palmitvaccenic acid), which were too low for quantitation. Both, palmitvaccenic and vaccenic acid have been described as mycorrhizae specific fatty acids (Schliemann et al., 2007), but we detected palmitvaccenic acid in both endophyte infected and EF plants.

Free amino acids were derivatised with Waters AccQ-Tag (Millipore Corporation, MA), separated by HPLC, and quantified by fluorescence detection (Ex: 250 nm, Em: 395 nm) as described (Reverter et al., 1997); for details see SM 1b. Amino acids detected and quantified were: L-alanine (L-Ala), L-arginine (L-Arg), L-asparagine (L-Asn), L-aspartate (L-Asp), γ-amino butyric acid (GABA), L-glutamate (L-Glu), L-glutamine (L-Gln), L-glycine (L-Gly), L-histidine (L-His), L-isoleucine (L-Ile), L-leucine (L-Leu), L-lysine (L-Lys), L-methionine (L-Met), L-phenylalanine (L-Phe), L-proline (L-Pro), L-serine (L-Ser), L-threonine (L-Thr), L-tyrosine (L-Tyr), and L-valine (L-Val).

Chlorogenic acid (CGA), flavonols (Flavo), and anthocyanins (Antho) were extracted with 1 mL 80% methanol (0.1 % acetic acid) using 50 mg dried plant material, extracts were shaken at RT for 30 min, centrifuged (30 min, 13,000 g) and the supernatant transferred into HPLC vials. Extracts were separated by HPLC and quantified using photodiode array detection based on calibration curves obtained from pure chlorogenic acid and rutin. Anthocyanins were only analysed as relative peak areas due to lack of appropriate standards. For details see SM 1b.

Low molecular weight (LMW) and high molecular weight (HMW) water soluble carbohydrates were extracted and quantified using anthrone as described (Hunt et al., 2005). Glucose, fructose, and sucrose were determined by enzymatic methods; for details see SM 1c.
Nitrate (NO$_3^-$) and phosphate (PO$_4^{3-}$) were extracted at room temperature (shaking, 1 hr) with 50 mL MilliQ water using 50 mg plant material. Extracts were filtered through filter paper (2V, Whatman) and analysed in a FIASTAR 5000 flow injection analyser (Foss Tecator, Sweden) following the manufacturer’s instructions. Soluble proteins were extracted and determined as described (Bradford et al., 1976; Hunt et al., 2005).

**Plant quality parameters**

Plant quality parameters were estimated using NIRS of finely powdered freeze-dried plant material as described (Corson et al., 1999) using a Bruker MPA spectrometer (Bruker Optic GmbH, Ettlingen, Germany). Parameters estimated were: Total protein (TotProt), ash, sulphur (S), dietary cation anion difference (DCAD), magnesium (Mg), lipids (Lipid), potassium (K), phosphorus (P), acid detergent fibre (ADF), neutral detergent fibre (NDF), dry matter (DM), metabolisable energy (ME), organic matter digestibility (OMD), and calcium (Ca).

**Statistical analysis**

All statistical analyses were performed in JMP (version 7.0, SAS Institute, 2007). For the multivariate tests, we performed a Principal Components Analysis on the correlations among the 66 response variables. We then performed factor rotation on the first three axes using the Varimax method (Hair et al., 1998). The rotated factor variables were subjected to a three-way ANOVA with N (low, high), CV (Fennema, AberDove) and EP (EF, AR1, CS, AR37) as the factors. We included and retained all two- and three-way interactions throughout. Analysis of the residuals suggested that all of the assumptions of the ANOVA were met without the need for further transformation. We used Tukey’s Honestly Significant Difference test to help interpret significant effects (Miller 1981).
Supplementary Material

1. ANALYTICAL METHODS

1a. GC-MS analysis of polar and apolar metabolites

1b. HPLC analysis of metabolites

1c. Enzymatic quantification of glucose, fructose, and sucrose

2. TABLES

2a. Table S1: Results of an ANOVA of all individual univariate response variables after Box-Cox-transformation. *** - P<0.001; ** - P<0.01; * - P<0.05. Last three columns show all untransformed means, units and standard deviations of the individual response variables.

2b. Table S2: Analytical results for all 66 variables in each sample.
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FIGURE LEGENDS

**Figure 1:** From left to right these graphs show the loadings for each variable onto the first three rotated factors. The variables loading heavily either positively (loading ≥ 0.5) or negatively (loading ≤ -0.5) are highlighted in black. These multivariate responses can be interpreted as increasing as the positively loading variables increase and decreasing as the negatively loading variables increase.

**Figure 2:** The mean responses of RF-1 (a, c, e) and the standardised univariate responses (b, d, f) to the main effects (all interactions were non-significant). (a, b) - N effect (white bars - low N; black bars - high N); (c, d) - cultivar effect (white bars - Fennema; black bars - AberDove); (e, f) - endophyte effect (white bars - EF; light grey bars - AR1; dark grey bars - CS; black bars - AR37); treatment levels that share a common letter are not significantly different as determined by a Tukey’s HSD test.

**Figure 3:** The mean responses of RF-2 (a, c) and the standardised univariate responses (b, d, e) to the main N effect and the cultivar x endophyte interactions. (a, b) - N effect (white bars - low N; black bars - high N); (c, d, e) - cultivar x endophyte interaction (d - Fennema; e - AberDove; white bars - EF; light grey bars - AR1; dark grey bars - CS; black bars - AR37); treatment levels that share a common letter are not significantly different as determined by a Tukey’s HSD test.

**Figure 4:** The mean responses of RF-3 (a, c) and the standardised univariate responses (b, d) to the main effects (all interactions were non-significant). (a, b) - N effect (white bars - low N; black bars - high N); (c, d) - cultivar effect (white bars - Fennema; black bars - AberDove)

**Figure 5:** Re-growth of blades in a two-week period. (a) – Main effect of N (white bars - low N; black bars - high N); (b) – cultivar x endophyte interaction (white bars - EF; light grey bars - AR1; dark grey bars - CS; black bars - AR37); treatment levels that share a common letter are not significantly different as determined by a Tukey’s HSD test. All other interactions were not significant.
Figure 6: Relationship between mannitol and endophyte concentration. PC-1 is the first axis from a principal components analysis combining two endophyte specific gene concentrations (as estimated by qPCR). PC-1 explains 95% of the variance in the gene copy numbers. The figure shows that PC-1, our measure of endophyte concentration is highly correlated ($r = 0.81$) with mannitol concentration ($\mu g\ mg^{-1}\ DW$). Shown is the line of best fit and the 95% confidence intervals around the best fit line. The regression is highly significant ($F_{1,114} = 211, P < 0.0001$).
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