Metabolic Profiles of *Lolium perenne* Are Differentially Affected by Nitrogen Supply, Carbohydrate Content, and Fungal Endophyte Infection[1][W][OA]

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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 analyzed 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 analyzed by analysis of variance. At high N supply nitrogenous compounds, organic acids and lipids were increased; WSCs, chlorogenic acid (CGA), and fibers were decreased. The high-sugar cultivar 'AberDove' had reduced levels of nitrate, most minor amino acids, sulfur, and fibers compared to the control cultivar 'Fennema', whereas WSCs, CGA, and methionine were increased. In plants infected with endophytes, nitrate, several amino acids, and, magnesium were decreased; WSCs, lipids, some organic acids, and CGA were increased. Regrowth of blades was stimulated at high N, and there was a significant endophyte × cultivar interaction on regrowth. 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 carbon/N ratios and in secondary metabolite production as seen in our study is likely to have impacts on herbivore responses.

Symbiotic plant-fungal interactions are of widespread interest to ecological research because 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 aboveground 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% to 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., 2008). The most commonly studied associations are *Neotyphodium lolii-Lolium perenne* in Australasia and *Neotyphodium coenophialum-Lolium arundinaceum* in North America (Christensen et al., 1993) because 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 focused on the biosynthesis, accumulation, and ecological consequences of these fungal alkaloids (for review, 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., 1998b; Malinowski and Belesky, 2000; Hesse et al., 2003, 2005). However, the general mutualistic nature of this particular type of plant-fungus association has been disputed (Saikkonen et al., 2004, 2007; Müller and Krauss, 2005) 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, 2000; Cheplick, 2004, 2007; Hesse et al., 2004). 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

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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' and '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 ergo-Val; AR1 produces only peramine; and AR37 produces only janthitrems) and grew them under high and low nitrogen (N) supply. Concentrations of endophyte, as estimated by quantitative PCR (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 and 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), HPLC, and near-IR reflectance spectroscopy to analyze metabolome-wide impacts of endophyte infection, cultivar differences, and N fertilization. Possible underlying biochemical processes leading to changes in metabolic composition and possible impacts on ecological processes are discussed. The plant material of this 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, 2004) because 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. Because 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 multivaraiate equivalent of the univariate procedure of data transformation. Like transformations for univariate data, factor rotation serves two nonexclusive 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).

We retained the first three rotated factors (RF-1, RF-2, and RF-3) for further analysis using standard ANOVA. The variable loadings for these three RFs are shown in Figure 1. As the RFs increase, those variables that load heavily and positively (loading $\geq$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 sulfur [S]). Variables loading heavily but negatively are carbohydrates and chlorogenic acid (CGA). It should be noted here that low M_r (LMW) WSCs comprise a mixture of Glc, Fru, Suc, and low degree of polymerization (DP) fructans. High M_r (HMW) WSCs are a mixture of high DP fructans (Fru 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), metabolizable energy (ME; indicator of the energy that

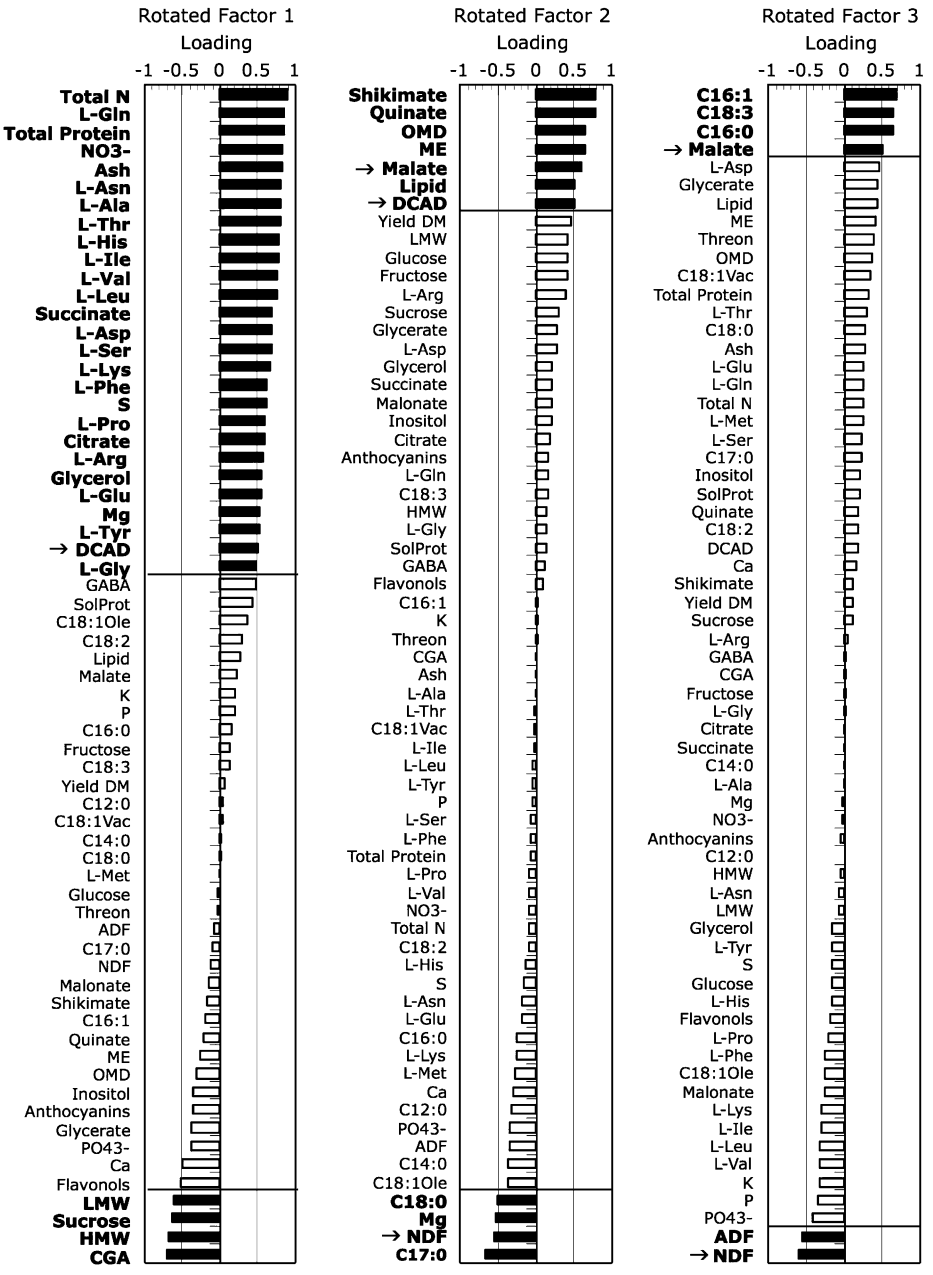

Figure 1. From left to right these graphs show the loadings for each variable onto the first three RFs. The variables loading heavily either positively (loading ≥ 0.5) or negatively (loading ≤ -0.5) are highlighted in black. These multivariate responses can be interpreted as increasing as the positively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

is available to ruminants for maintenance and growth), lipids, and DCAD. Variables loading heavily but negatively include two fatty acids (C17:0 and C18:0), one mineral (magnesium [Mg]), and neutral detergent fiber (NDF; estimates mainly cellulose, hemicellulose, and lignin content).

Variables loading heavily and positively onto RF-3 are three fatty acids (C16:0, C16:1, and C18:3) and malate. Variables loading heavily but negatively onto this axis are acid detergent fiber (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 RF variables was subjected to a three-way ANOVA with N (low, high), cultivar ('Fennema', 'AberDove'), and endophyte (endophyte free [EF], 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 PCA and subsequent factor rotation.

Because 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 supplemental data (Supplemental Table S1). It should be noted that when these variables are analyzed 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 (Supplemental Table S1; Rasmussen et al., 2007). We therefore attached the complete data set as supplemental data (Supplemental Table S2) for those readers who would like to try alternative analyses of these data.

RF 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 SEs of the means are plotted in Figure 2, A, C, and E. 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

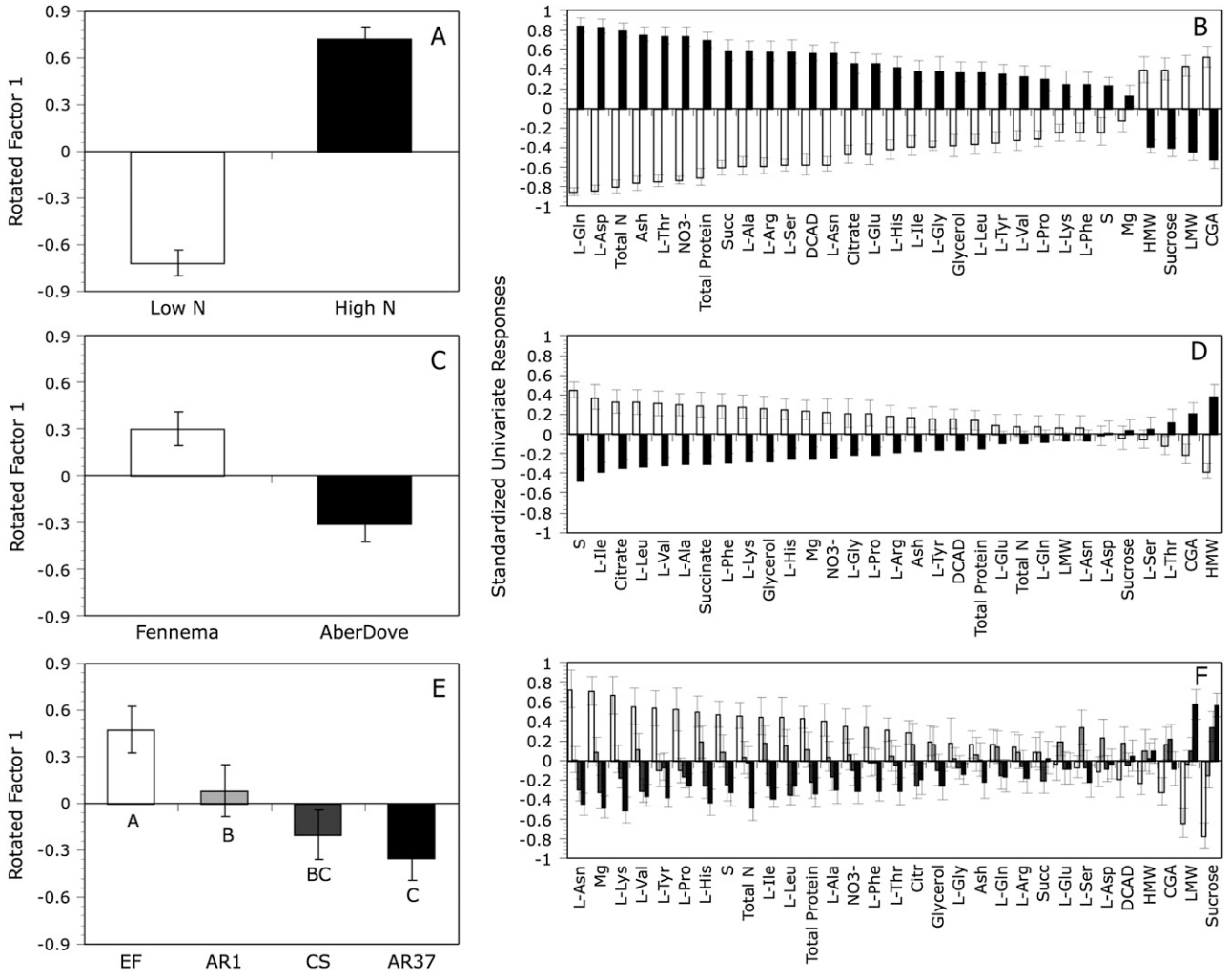

Figure 2. A to F, The mean responses of RF-1 (A, C, and E) and the standardized univariate responses (B, D, and F) to the main effects (all interactions were nonsignificant). A and B, N effect (white bars, low N; black bars, high N). C and D, Cultivar effect (white bars, 'Fennema'; black bars, 'AberDove'). E and F, Endophyte effect (white bars, EF; light gray bars, AR1; dark gray bars, CS; black bars, AR37); treatment levels that share a common letter are not significantly different as determined by a Tukey's HSD mean-separation test.

N; higher in 'AberDove' than in 'Fennema'; and higher in endophyte-infected plants than in EF plants, again in a strain-dependent manner.

The standardized univariate responses of these variables are shown in Figure 2, B, D, and F, 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 ≥ 0.5 and ≤ -0.5). Variable standardization 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 (Supplemental 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 Supplemental Table S2). The SD for nitrate was very high, resulting in relatively smaller differences when standardized. 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 amino acid analyzed here that was not affected by N supply and did not load strongly onto any of the three RFs. 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 amino acids and nitrate (Fig. 2D). Interestingly, S was reduced in this cultivar as well, and we note here that the S-containing amino acid L-Met was significantly increased in 'AberDove' (2.4-fold), when that variable is subjected to a standard univariate analysis (Supplemental 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 standardized univariate responses was strongly strain dependent, with AR1 having the weakest and AR37 the strongest effect on most variables (Fig. 2F). Almost all amino acids were reduced in endophyte-infected plants, but this effect was most apparent for L-Asn and several minor amino acids. Carbohydrates and CGA were increased in infected plants, but the responses were much weaker for HMW WSCs and CGA.

RF 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 RF 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 SEs of the means are plotted in Figure 3, A and C. 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 standardized univariate responses of these variables are shown in Figure 3, B, D, and E, 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 amino acids 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.

RF 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 SEs of the means are plotted in Figure 4, A and C. 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 standardized univariate responses of these variables are shown in Figure 4, B and D; malate and three fatty acids increased at high N compared to low N (Fig. 4B) and in 'AberDove' compared to 'Fennema' (Fig. 4D). The fiber 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 regrown for 2 weeks (Fig. 5). High N supply did result in significantly more regrowth, as expected. There was no difference in regrowth between the two EF cultivars, but the endophytic strain AR37 stimulated regrowth 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

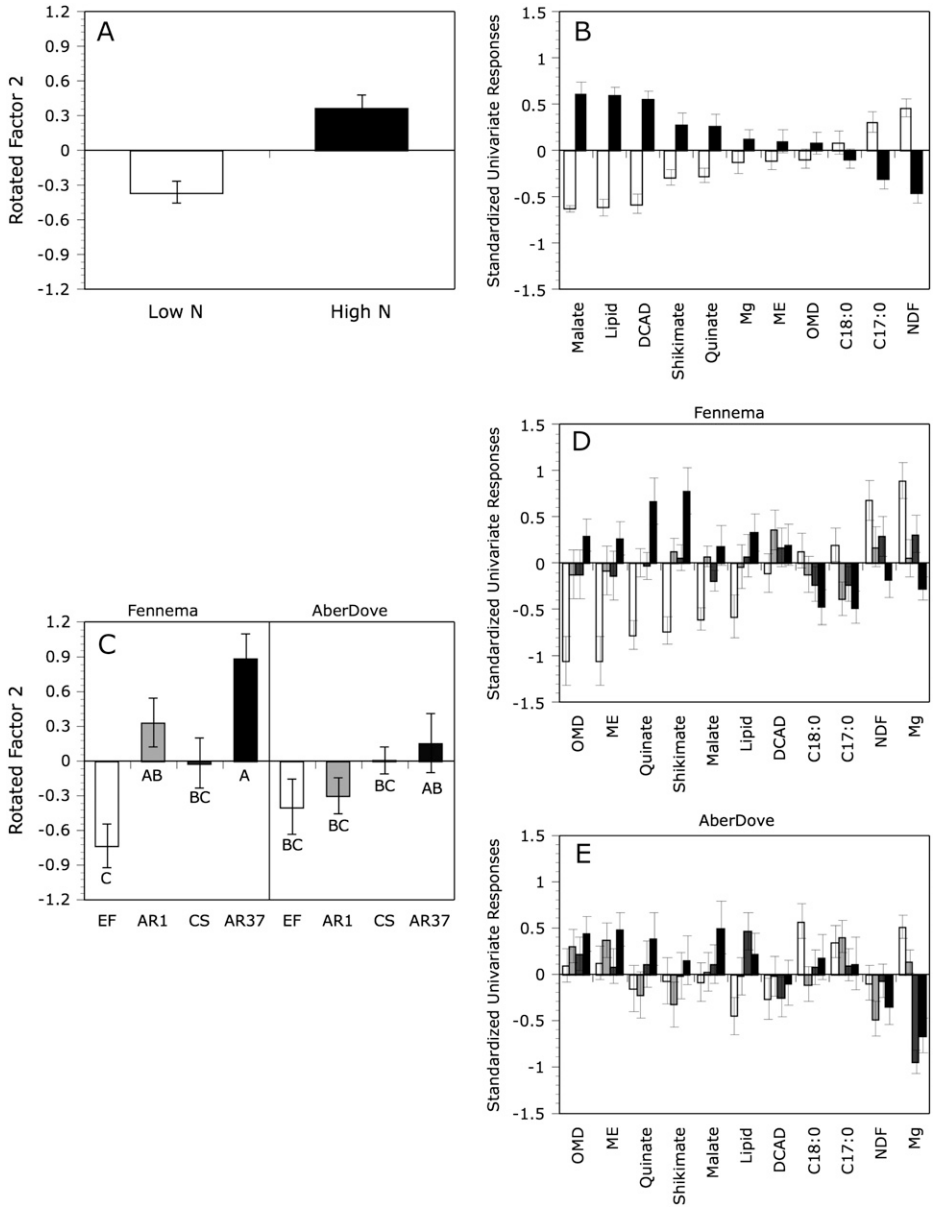

Figure 3. A to E, The mean responses of RF-2 (A and C) and the standardized univariate responses (B, D, and E) to the main N effect and the cultivar × endophyte interactions. A and B, N effect (white bars, low N; black bars, high N). C to E, Cultivar × endophyte interaction ('Fennema' [D]; 'AberDove' [E]; white bars, EF; light gray bars, AR1; dark gray bars, CS; black bars, AR37); treatment levels that share a common letter are not significantly different as determined by a Tukey's HSD mean-separation test.

B, and janthitrems) concentrations against fungal concentrations was highly significant. As reported, fungal concentrations were determined by qPCR of two endophyte-specific genes, chitinase, and a nonribosomal 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 ($F_{1,114} = 190.42$, $P < 0.0001$). The untransformed data are shown in Figure 6.

DISCUSSION

In a previous article (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 analyzed 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

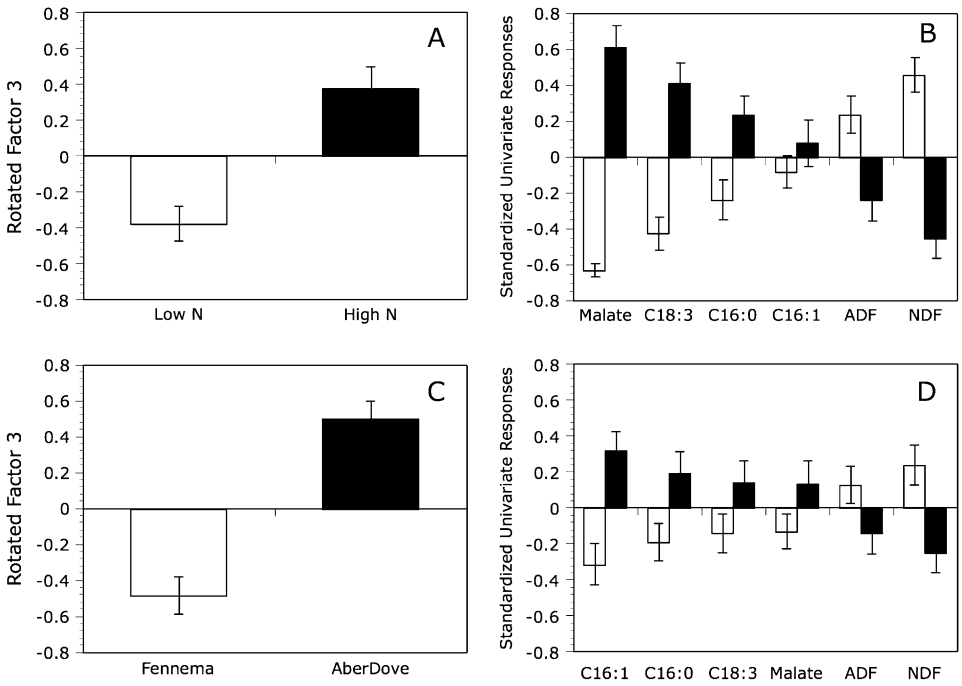

Figure 4. A to D, The mean responses of RF-3 (A and C) and the standardized univariate responses (B and D) to the main effects (all interactions were nonsignificant). A and B, N effect (white bars, low N; black bars, high N). C and D, Cultivar effect (white bars, 'Fennema'; black bars, 'AberDove').

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 (compare with 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 N 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 approximately 9-fold higher in the high N treatment, indicating that nitrate uptake exceeded the plants' capacity for nitrate assimilation. Although 18 out of 19 analyzed amino acids were increased, there was a marked difference in the response of individual amino acids. Major amino acids, which represented approximately 85% of the total free amino acids, were much more affected than minor amino acids. This is in accordance with findings from a variety of crop plants, where mainly major amino acids responded to changes in carbon and N metabolism, whereas minor amino acids were not correlated with these changes and correlated more with each other than with total amino acid pools (Noctor et al., 2002).

Because nitrate assimilation into amino acids 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 this study as well; malate, succinate, and citrate were all increased at high N supply. These organic acids serve several purposes, the major ones being: (1) malate acts as a counteranion to prevent alkalinization (Martinoia and Rentsch, 1994), and (2) organic acids act as carbon precursors for amino acids (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*, like many other cool-season grasses, does not accumulate starch in vegetative organs, but instead water-soluble fructans (Fru polymers) that 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 fiber content and increased lipids in plants, a further indication of a shift from carbohydrates (major component of fibers) to organic acid derived molecules (lipids).

In a discussion of our previous findings that high N supply reduced endophyte concentrations, we hypothesized 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

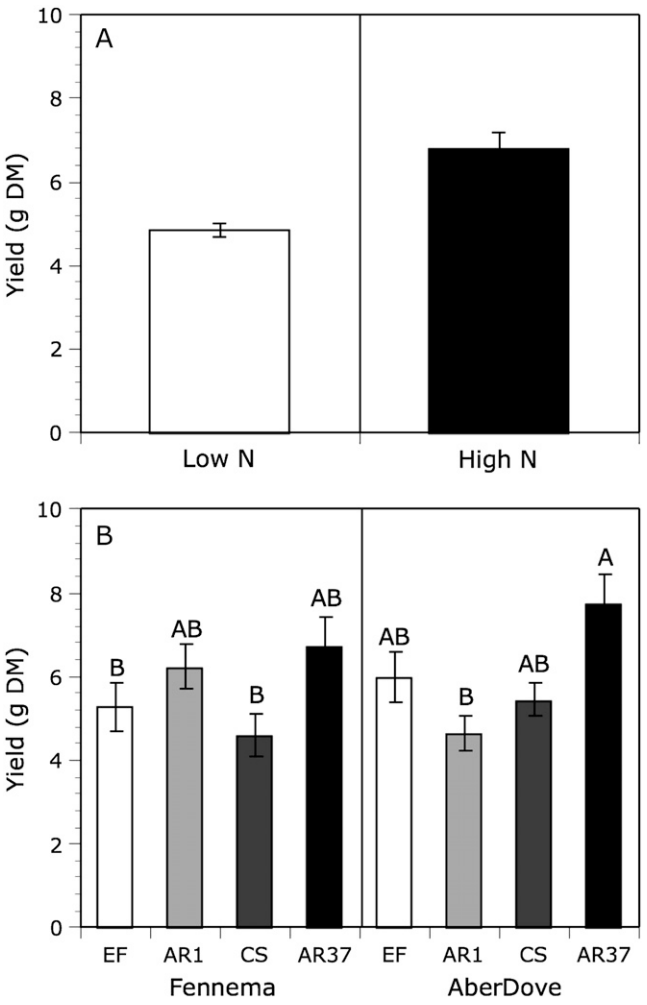

Figure 5. Regrowth of blades in a 2-week period. A, Main effect of N (white bars, low N; black bars, high N). B, Cultivar × endophyte interaction (white bars, EF; light gray bars, AR1; dark gray bars, CS; black bars, AR37); treatment levels that share a common letter are not significantly different as determined by a Tukey's HSD mean-separation test. All other interactions were not significant.

high N did result in significantly increased plant regrowth. 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 Glc, resulting from a cleavage of Suc in the apoplastic regions close to fungal hyphae (Solaiman and Saito, 1997; Wright et al., 1998; Pfeffer et al., 1999). Because high N supply resulted in decreased concentrations of Suc 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 because: (1) 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 (2) 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; it is also unclear if metabolite concentrations in the apoplast are limiting for growth of foliar endophytic fungi at all, especially under conditions favorable 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 ($C_{12}H_{17}N_5O_1$; M_r, 249; Rowan et al., 1986) had a mean concentration of 0.12 μmol g^{-1} dry weight (30 μg/g dry weight) in infected blades (Rasmussen et al., 2007), which represents only 0.03 μmol N g^{-1} dry weight compared to a total N concentration of 2.1 mmol N g^{-1} dry weight (30 mg g^{-1} dry weight) and a nitrate concentration of 11 μmol g^{-1} dry weight (0.74 mg g^{-1} dry weight) in the symbiotic tissue at low N supply. This indicates that endophytes or endophyte alkaloid biosynthesis are rarely N limited and that endophyte and alkaloid concentrations might depend more on carbon availability as discussed above.

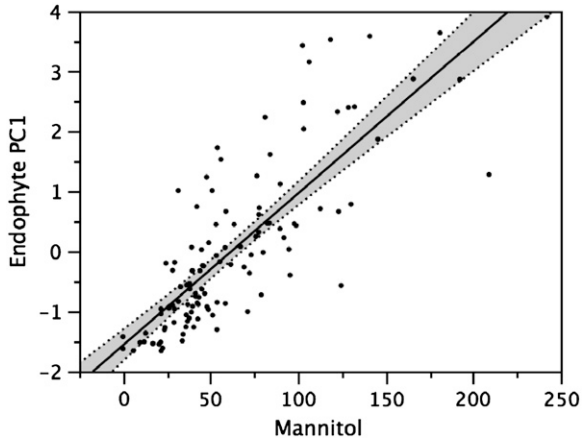

Figure 6. Relationship between mannitol and endophyte concentration. PC-1 is the first axis from a PCA combining two endophyte-specific gene concentrations (as estimated by qPCR). PC-1 explains 95% of the variance in the gene copy numbers. The figure shows that PC-1, our measure of endophyte concentration, is highly correlated ($r = 0.81$) with mannitol concentration (μg mg^{-1} dry weight). Shown is the line of best fit and the 95% confidence intervals around the best fit line. The regression is highly significant ($F_{1,114} = 211$, $P < 0.0001$).

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 Glc, Fru, Suc, 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 fibers. Nitrate levels were almost halved in 'AberDove', but most amino acids were much less affected. None of the amino acids involved in primary N assimilation (L-Glu, L-Gln, L-Asp, and L-Asn) were markedly affected, indicating that N assimilation per se was not impaired in the high-sugar cultivar. However, most minor amino acids 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, because regrowth was not different between the two cultivars. Although it is possible that levels of Glc, Fru, and/or Suc in the apoplastic space (and so available for fungal growth) could have been reduced in 'AberDove', our data do not support this notion because the overall concentration of these sugars differed little between 'AberDove' and the control grass. The relatively small changes in amino acids 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 RFs, was significantly increased in the high-sugar cultivar (2.4-fold). This in itself is an interesting result because 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 pearl millet (*Pennisetum typhoides*) 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 amino acids 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 and milk and meat production, and decreases of N excretion in urine by high-sugar grass-grazing animals (for review, see Edwards et al., 2008 [on high-sugar grasses and their advantages for pasture-fed animals]) 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 harboring 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, 2000; Faeth and Sullivan, 2003; Cheplick, 2004, 2007; Faeth et al., 2004; Hesse et al., 2004; Faeth and Hamilton, 2006). In plants infected with mycorrhizal fungi the increased costs due to carbon flow to the fungus can be offset 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 and Cho, 2003; Hesse et al., 2003; Zabalgogeazcoa et al., 2006; Cheplick, 2007). In our study we saw significant cultivar by endophyte interactions on the regrowth 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. This study is an analysis of combined plant and fungal metabolites and because most metabolites analyzed 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 nonsymbiotic endophyte plants.

A major effect of endophyte infection was an approximately 50% reduction in nitrate levels in the blades, which was accompanied by a reduction of several amino acids, 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. lolii*-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 that colonizes 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 fertilized 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.

Asn was the most reduced amino acid and L-Asn levels are mainly regulated by the carbon (C)/N status of plants. High levels of organic N and low levels of carbon skeletons result in high levels of L-Asn because 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 to 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 amino acids and proteins. We also found reduced levels of fibers 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; furthermore, the tissue we analyzed 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.

Although 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 this 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 localization 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., 1998a), 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 arbuscular mycorrhizal colonized plant roots (Harrison and Dixon, 1993; Volpin et al., 1994; Blee and Anderson, 2002; Hohnjec et al., 2005). Because 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 this study, warrant further investigations of secondary metabolite production and related gene expression profiles in endophyte-infected plants; this might be particularly important to understand the effects of endophyte 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.

CONCLUSION

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 to 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 two grass cultivars, four endophyte treatments (three fungal strains and EF), and two concentrations of N supply.

Plant Material

Details of the experimental setup have been described previously (Rasmussen et al., 2007). In short, we used two *L. perenne* cultivars ('AberDove' and '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 μmol m^{-1} s^{-1}; modified Hoagland nutrient solution containing 9 mM N supplied as nitrate). A total of 160 pots (10 replicates × two cultivars × four endophytes × 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 aboveground 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 2 weeks prior to this were oven dried and weighed for dry matter estimation.

Blades of all plants were cut 6 cm aboveground within 1 h on the same day, 7 h after the start of the daylight period, 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, derivatization procedures, and chromatographic conditions are provided as supplemental data.

Organic acids, sugar alcohols, and fatty acids were extracted, derivatized, and determined by GC-MS as described (Roessner et al., 2000, 2001); for details, see supplemental data. Compounds detected and quantified in the aqueous phase were: citrate, glycerate, malate, malonate, quinate, shikimate, succinate, threonic acid, glycerol, inositol, and mannitol. Fatty acids detected and quantified in the $CHCl_3$ phase were: dodecanoic acid (C12:0), tetradecanoic acid (C14:0), hexadecanoic acid (C16:0), palmitoleic acid (C16:1 Δ^9), heptadecanoic acid (C17:0), octadecanoic acid (18:0), oleic acid (C18:1 Δ^9 Ole), vaccenic acid (C18:1 Δ^{11} Vac), linoleic acid (C18:2), and linolenic acid (C18:3). We also detected traces of palmitvaccenic acid (C16:1 Δ^{11}), which were too low for quantitation. Both palmitvaccenic acid and vaccenic acid have been described as mycorrhizae-specific fatty acids (Schliemann et al., 2008), but we detected palmitvaccenic acid in both endophyte-infected and EF plants.

Free amino acids were derivatized with Waters AccQ-Tag (Millipore), separated by HPLC, and quantified by fluorescence detection (Excitation, 250 nm; Emission, 395 nm) as described (Reverter et al., 1997); for details, see supplemental data. Amino acids detected and quantified were: L-Ala, L-Arg, L-Asn, L-Asp, γ-aminobutyric acid, L-Glu, L-Gln, L-Gly, L-His, L-Ile, L-Leu, L-Lys, L-Met, L-Phe, L-Pro, L-Ser, L-Thr, L-Tyr, and L-Val.

CGA, flavonols, and anthocyanins were extracted with 1 mL of 80% methanol (0.1% acetic acid) using 50 mg of dried plant material, extracts were shaken at room temperature 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 CGA and rutin. Anthocyanins were only analyzed as relative peak areas due to lack of appropriate standards. For details, see supplemental data.

LMW and HMW water soluble carbohydrates were extracted and quantified using anthrone as described (Hunt et al., 2005). Glc, Fru, and Suc were determined by enzymatic methods; for details, see supplemental data.

Nitrate (NO_3^-) and phosphate (PO_4^{3-}) were extracted at room temperature (shaking 1 h) with 50 mL of MilliQ water (Millipore) using 50 mg of plant material. Extracts were filtered through filter paper (2V; Whatman) and analyzed in a FIASTAR 5000 flow injection analyzer (Foss Tecator) following the manufacturer's instructions. Soluble proteins were extracted and determined as described (Bradford, 1976; Hunt et al., 2005).

Plant Quality Parameters

Plant quality parameters were estimated using near-IR reflectance spectroscopy of finely powdered freeze-dried plant material as described (Corson et al., 1999) using a Bruker MPA spectrometer (Bruker Optic GmbH). Parameters estimated were: total protein, ash, S, DCAD, Mg, lipids, potassium, phosphorus, ADF, NDF, dry matter, ME, OMD, and calcium.

Statistical Analysis

All statistical analyses were performed in JMP (version 7.0, SAS Institute, 2007). For the multivariate tests, we performed a PCA 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 RF variables were subjected to a three-way ANOVA with N (low, high), cultivar ('Fennema', 'AberDove'), and endophyte (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 (HSD) mean-separation test to help interpret significant effects (Miller, 1981).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Materials and Methods S1. Analytical methods, including GC-MS analysis of polar and apolar metabolites, HPLC analysis of metabolites, and enzymatic quantification of Glc, Fru, and Suc.

Supplemental 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 SDs of the individual response variables.

Supplemental Table S2. Analytical results for all 66 variables in each sample.

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LITERATURE CITED

Amalric C, Sallanon H, Monnet F, Hitmi A, Coudret A (1999) Gas exchange and chlorophyll fluorescence in symbiotic and non-symbiotic ryegrass under water stress. Photosynthetica **37:** 107–112

Bacetty AA, Snook ME, Glenn AE, Bacon CW, Nagabhyru P, Schardl CL (2007) Nematotoxic effects of endophyte-infected tall fescue toxins and extracts in an in vitro bioassay using the nematode *Pratylenchus scribnery. In* AJ Popay, ER Thorn, eds, Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses. Grassland Research and Practice Series No. 13, NZ Grassland Association, Dunedin, New Zealand, pp 357–361

Bailly J, Debaud JC, Verner MC, Plassard C, Chalot M, Marmeisse R, Fraissinet-Tachet L (2007) How does a symbiotic fungus modulate expression of its host-plant nitrite reductase? New Phytol **175:** 155–165

Belesky DP, Devine OJ, Pallas JE, Stringer WC (1987) Photosynthetic activity of tall fescue as influenced by a fungal endophyte. Photosynthetica **21:** 82–87

Belesky DP, Fedders JM (1996) Does endophyte influence regrowth of tall fescue? Ann Bot (Lond) **78:** 499–505

Belesky DP, Stringer WC, Hill NS (1989) Influence of endophyte and water regime upon tall fescue accessions. I. Growth characteristics. Ann Bot (Lond) **63:** 495–503

Belesky DP, Stuedemann JA, Plattner RD, Wilkinson SR (1988) Ergopeptine alkaloids in grazed tall fescue. Agron J **80:** 209–212

Bianchi G, Gamba A, Limiroli R, Pozzi N, Elster R, Salamini F, Bartels D (1993) The unusual sugar composition in leaves of the resurrection plant *Myrothamnus flabellifolia.* Physiol Plant **87:** 223–226

Blee KA, Anderson AJ (2002) Transcripts for genes encoding soluble acid invertase and sucrose synthase accumulate in root tip and cortical cells containing mycorrhizal arbuscules. Plant Mol Biol **50:** 197–211

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Bush LP, Wilkinson HH, Schardl CL (1997) Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiol **114:** 1–7

Cheplick GP (2004) Recovery from drought stress in *Lolium perenne* (Poaceae): Are fungal endophytes detrimental? Am J Bot **91:** 1960–1968

Cheplick GP (2007) Costs of fungal endophyte infection in *Lolium perenne* genotypes from Eurasia and North Africa under extreme resource limitation. Environ Exp Bot **60:** 202–210

Cheplick GP, Cho R (2003) Interactive effects of fungal endophyte infection and host genotype on growth and storage in *Lolium perenne.* New Phytol **158:** 183–191

Cheplick GP, Clay K, Marks S (1989) Interactions between infection by endophytic fungi and nutrient limitation in the grasses *Lolium perenne* and *Festuca arundinacea.* New Phytol **111:** 89–97

Cheplick GP, Perera A, Koulouris K (2000) Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. Funct Ecol **14:** 657–667

Christensen MJ, Leuchtmann A, Rowan DD, Tapper BA (1993) Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*) and perennial ryegrass (*Lolium perenne*). Mycol Res **97:** 1083–1092

Christensen MJ, Bennett RJ, Ansari HA, Koga H, Johnson RD, Bryan GT, Simpson WR, Koolaard JP, Nickless EM, Voisey CR (2008) Epichloë endophytes grow by intercalary hyphal extension in elongating grass leaves. Fungal Genet Biol **45:** 84–93

Christensen MJ, Bennett RJ, Schmid J (2002) Growth of *Epichloë/Neotyphodium* and p-endophytes in leaves of *Lolium* and *Festuca* grasses. Mycol Res **106:** 93–106

Clay K, Holah J, Rudgers JA (2005) Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. Proc Natl Acad Sci USA **102:** 12465–12470

Clay K, Schardl C (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am Nat **160:** S99–S127

Corson DC, Waghorn GC, Ulyatt MJ, Lee J (1999) NIRS: forage analysis and livestock feeding. Proc NZ Grassland Assoc **51:** 127–132

Dixon RA (2001) Natural products and plant disease resistance. Nature **411:** 843–847

Douds DD, Pfeffer PE, Shachar-Hill Y (2000) Carbon partitioning, cost and metabolism of arbuscular mycorrhizae in arbuscular mycorrhizas: physiology and function. *In* Y Kapulnick, DD Douds Jr, eds, Arbuscular Mycorrhizas: Molecular Biology and Physiology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 107–129

Edwards GR, Parsons AJ, Rasmussen S, Bryant RH (2008) High sugar ryegrasses for livestock systems in New Zealand. Proc NZ Grassland Assoc **69:** 161–172

Faeth SH, Fagan WF (2002) Fungal endophytes: common host plant symbionts but uncommon mutualists. Integr Comp Biol **42:** 360–368

Faeth SH, Hamilton CE (2006) Does an asexual endophyte symbiont alter life stage and long-term survival in a perennial host grass? Microb Ecol **52:** 748–755

Faeth SH, Helander ML, Saikkonen KT (2004) Asexual *Neotyphodium* endophytes in a native grass reduce competitive abilities. Ecol Lett **7:** 304–313

Faeth SH, Sullivan TJ (2003) Mutualistic asexual endophytes in a native grass are usually parasitic. Am Nat **161:** 310–325

Felton GW, Korth KL, Bi JL, Wesley SV, Huhman DV, Mathews MC, Murphy JB, Lamb C, Dixon RA (1999) Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. Curr Biol **9:** 317–320

Finkes LK, Cady AB, Mulroy JC, Clay K, Rudgers JA (2006) Plant-fungus mutualism affects spider composition in successional fields. Ecol Lett **9:** 347–356

Gordon AJ, Ryle GJA, Powell CE, Mitchell DF (1980) Export, mobilization and respiration of assimilates in uniculm barley during light and darkness. J Exp Bot **31:** 461–473

Govindarajulu M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, Bücking H, Lammers PJ, Shachar-Hill Y (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature **435:** 819–823

Graefe H, Gütschow B, Gehring H, Dibbelt L (2003) Sensitive and specific photometric determination of mannitol in human serum. Clin Chem Lab Med **41:** 1049–1055

Graham JH (2000) Assessing costs of arbuscular mycorrhizal symbiosis agroecosystems fungi. *In* GK Podila, DD Douds Jr, eds, Current Advances in Mycorrhizae Research. APS Press, St. Paul, MN, pp 127–140

Hair JF, Anderson RE, Tatham RL, Black WC (1998) Multivariate Data Analysis, Ed 5. Prentice Hall, Upper Saddle River, NJ

Harris PM, Lobley GE (1991) Amino acid metabolism in peripheral tissues. *In* T Tsuda, Y Sasaki, R Kawashima, eds, Physiological Aspects of Digestion and Metabolism in Ruminants. Proceedings of the Seventh International Symposium on Ruminant Physiology. Academic Press, San Diego, pp 201–230

Harrison MJ, Dixon RA (1993) Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular-arbuscular mycorrhizal associations in roots of *Medicago truncatula.* Mol Plant Microbe Interact **6:** 643–654

Harwood VD (1954) Analytical studies on the carbohydrates of grasses and clover. VII. The isolation of D-mannitol from perennial ryegrass (*Lolium perenne* L.). J Sci Food Agric **5:** 453–455

Herrmann KM (1995) The shikimate pathway: early steps in the biosynthesis of aromatic compounds. Plant Cell **7:** 907–919

Herrmann KM, Weaver LM (1999) The shikimate pathway. Annu Rev Plant Physiol Plant Mol Biol **50:** 473–503

Hesse U, Hahn H, Andreeva K, Förster K, Warnstorff K, Schöberlein W, Diepenbrock W (2004) Investigations on the influence of *Neotyphodium* endophytes on plant growth and seed yield of *Lolium perenne* genotypes. Crop Sci **44:** 1689–1695

Hesse U, Schöberlein W, Wittenmayer L, Förster K, Warnstorff K, Diepenbrock W, Merbach W (2003) Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. Grass For Sci **58:** 407–415

Hesse U, Schöberlein W, Wittenmayer L, Förster K, Warnstorff K, Diepenbrock W, Merbach W (2005) Influence of water supply and endophyte infection (*Neotyphodium* spp.) on vegetative and reproductive growth of two *Lolium perenne* L. genotypes. Eur J Agron **22:** 45–54

Hohnjec N, Vieweg MF, Pühler A, Becker A, Küster H (2005) Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different Glomus fungi provide insights into the genetic program activated during arbuscular mycorrhiza. Plant Physiol **137:** 1283–1301

Hunt MG, Rasmussen S, Newton PCD, Parsons AJ, Newman JA (2005) Near-term impacts of elevated CO_2, nitrogen and fungal endophyte-infection on *Lolium perenne* L. growth, chemical composition and alkaloid production. Plant Cell Environ **28:** 1345–1354

Koshino H, Tekada SI, Yoshihara T, Sakamura S, Shimanuki T, Sato T, Tajimi A (1988) Three phenolic acid derivatives from stromata of *Epichloë typhina* on *Phleum pratense*. Phytochemistry **27:** 1333–1338

Krauss J, Harri SA, Bush L, Husi R, Bigler L, Power SA, Müller CB (2007) Effects of fertilizer, fungal endophytes and plant cultivar on the performance of insect herbivores and their natural enemies. Funct Ecol **21:** 107–116

Lam H-M, Coschigano KT, Oliveira IC, Melo-Oliveira R, Coruzzi GM (1996) The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Annu Rev Plant Physiol **47:** 569–593

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol **48:** 251–275

Leuchtmann A (1993) Systematics, distribution, and host specificity of grass endophytes. Nat Toxins **1:** 150–162

Lewis DH, Smith DC (1967) Sugar alcohols (polyols) in fungi and green plants. New Phytol **66:** 143–184

Lyons PC, Evans JJ, Bacon CW (1990) Effects of the fungal endophyte *Acremonium coenophialum* on nitrogen accumulation and metabolism in tall fescue. Plant Physiol **92:** 726–732

Malinowski DP, Alloush GA, Belesky DP (1998a) Evidence for chemical changes on the root surface of tall fescue in response to infection with the fungal endophyte *Neotyphodium coenophialum*. Plant Soil **205:** 1–12

Malinowski DP, Belesky DP (2000) Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci **40:** 923–940

Malinowski DP, Belesky DP (2006) Ecological importance of *Neotyphodium* spp. grass endophytes in agroecosystems. Grassl Sci **52:** 1–14

Malinowski DP, Belesky DP, Hill NS, Baligar VC, Fedders JM (1998b) Influence of phosphorus on the growth and ergot alkaloid content of *Neotyphodium coenophialum*-infected tall fescue (*Festuca arundinacea* Schreb.). Plant Soil **198:** 53–61

Marks S, Clay K (1996) Physiological responses of *Festuca arundinacea* to fungal endophyte infection. New Phytol **133:** 727–733

Martinoia E, Rentsch D (1994) Malate compartmentation—responses to a complex metabolism. Annu Rev Plant Physiol **45:** 447–467

McGarigal K, Cushman S, Stafford S (2000) Multivariate Statistics for Wildlife and Ecology Research. Springer Verlag, New York

Meister B, Krauss J, Haerri SA, Schneider MV, Müller CB (2006) Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. Basic Appl Ecol **7:** 244–252

Miller RG (1981) Simultaneous Statistical Inference, Ed 2. Springer Verlag, New York

Morot-Gaudry JF, Job D, Lea PJ (2001) Amino acid metabolism. *In* PJ Lea, JF Morot-Gaudry, eds, Plant N. Springer Verlag, Berlin, pp 167–211

Morse LJ, Day TA, Faeth SH (2002) Effect of *Neotyphodium* endophyte infection on growth and leaf gas exchange of Arizona fescue under contrasting water availability regimes. Environ Exp Bot **48:** 257–268

Müller CB, Krauss J (2005) Symbiosis between grasses and asexual fungal endophytes. Curr Opin Plant Biol **8:** 450–456

Newman JA, Abner ML, Dado RG, Gibson DJ, Brookings A, Parsons AJ (2003) Effects of elevated CO_2, nitrogen and fungal endophyte-infection on tall fescue: growth, photosynthesis, chemical composition and digestibility. Glob Change Biol **9:** 425–437

Noctor G, Novitskaya L, Lea PJ, Foyer CH (2002) Co-ordination of leaf minor amino acid contents in crop species: significance and interpretation. J Exp Bot **53:** 939–945

Omacini M, Chaneton EJ, Ghersa CM, Müller CB (2001) Symbiotic fungal endophytes control insect host-parasite interaction webs. Nature **409:** 78–81

Ouborg NJ, Vriezen WH (2007) An ecologist's guide to ecogenomics. J Ecol **95:** 8–16

Pavis N, Chatterton NJ, Harrison PA, Baumgartner S, Praznik W, Boucaud J, Prud'homme MP (2001) Structure of fructans in roots and leaf tissues of *Lolium perenne*. New Phytol **150:** 83–95

Pellegrini L, Rohfritsch O, Fritig B, Legrand M (1994) Phenylalanine ammonia-lyase in tobacco. Molecular cloning and gene expression during the hypersensitive reaction to tobacco mosaic virus and the response to fungal elicitor. Plant Physiol **106:** 877–886

Pfeffer PE, Bago B, Shachar-Hill Y (2001) Exploring mycorrhizal function with NMR spectroscopy. New Phytol **150:** 543–553

Pfeffer PE, Douds DD, Bécard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol **120:** 587–598

Pollock CJ, Jones T (1979) Seasonal patterns of fructan metabolism in forage grasses. New Phytol **83:** 9–15

Prud'homme MP, Gonzalez B, Billard JP, Boucaud J (1992) Carbohydrate content, fructan and sucrose enzyme activities in roots, stubble and leaves of ryegrass (*Lolium perenne* L.) as affected by source/ sink modification after cutting. J Plant Physiol **140:** 282–291

Rasmussen S, Parsons AJ, Bassett S, Christensen MJ, Hume DE, Johnson LJ, Johnson RD, Simpson WR, Stacke C, Voisey CR, et al (2007) High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*. New Phytol **173:** 787–797

Reverter M, Lundh T, Lindberg JE (1997) Determination of free amino acids in pig plasma by precolumn derivatization with 6-N-aminoquinolyl-N-hydroxysuccinimidyl carbamate and high performance liquid chromatography. J Chromatogr B Biomed Sci Appl **696:** 1–8

Richardson MD, Chapman GW Jr, Hoveland CS, Bacon CW (1992) Sugar alcohols in endophyte-infected tall fescue under drought. Crop Sci **32:** 1060–1061

Roessner U, Luedemann A, Brust D, Fiehn O, Linke T, Willmitzer L, Fernie AR (2001) Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. Plant Cell **13:** 11–29

Roessner U, Wagner C, Kopka J, Tretheway RN, Willmitzer L (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant J **23:** 131–142

Rowan DD, Hunt MB, Gaynor DL (1986) Peramine, a novel insect feeding deterrent from ryegrass infected with the endophyte *Acremonium loliae*. J Chem Soc Chem Commun **1986:** 935–936

Rudgers JA, Mattingly WB, Koslow JM (2005) Mutualistic fungus promotes plant invasion into diverse communities. Oecologia **144:** 463–471

Ruijter GJG, Bax J, van de Vondervoort PJI, de Vries RP, vanKuyk PA, Visser J (2003) Mannitol is required for stress tolerance in *Aspergillus niger* conidiospores. Eukaryot Cell **2:** 690–698

Saikkonen K, Lehtonen P, Helander M, Koricheva J, Faeth SH (2007) Model systems in ecology: dissecting the endophyte-grass literature. Trends Plant Sci **11:** 428–433

Saikkonen K, Wäli P, Helander M, Faeth SH (2004) Evolution of endophyte-plant symbiosis. Trends Plant Sci **9:** 275–280

Sarosh BR, Sivaramakrishnan S, Shetty HS (2005) Elicitation of defense related enzymes and resistance by L-methionine in pearl millet against downy mildew disease caused by *Sclerospora graminicola*. Plant Physiol Biochem **43:** 808–815

Schardl CL, Leuchtmann A, Spiering MJ (2004) Symbioses of grasses with seedborne fungal endophytes. Annu Rev Plant Biol **55:** 315–340

Schingoethe DJ (1996) Balancing the amino acid needs of the dairy cow. Anim Feed Sci Technol **60:** 153–160

Schliemann W, Ammer C, Strack D (2008) Metabolite profiling of mycorrhizal roots of *Medicago truncatula*. Phytochemistry **69:** 112–146

Singh SB, Bais BS, Singh DR (1972) Effect of different carbon and nitrogen sources on the growth and sporulation of claviceps microcephala (Wallr.) Tul. Mycopathol Mycol Appl **46:** 373–378

Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. Plant Cell Environ **30:** 1126–1149

Smith SE, Dickson S, Smith FA (2001) Nutrient transfer in arbuscular mycorrhizas: How are fungal and plant processes integrated? Aust J Plant Physiol **28:** 683–694

Snoeren TA, de Jong PW, Dicke M (2007) Ecogenomic approach to the role of herbivore-induced plant volatiles in community ecology. J Ecol **95:** 17–26

Solaiman MDZ, Saito M (1997) Use of sugars by intraradical hyphae of arbuscular mycorrhizal fungi revealed by radiorespirometry. New Phytol **136:** 533–538

Solomon PS, Waters ODC, Jörgens CI, Lowe RGT, Rechberger J, Trengove RD, Oliver RP (2006) Mannitol is required for asexual sporulation in the wheat pathogen *Stagonospora nodorum* (glume blotch). Biochem J **399:** 231–239

Solomon PS, Waters ODC, Oliver RP (2007) Decoding the mannitol enigma in filamentous fungi. Trends Microbiol **15:** 257–262

Spiering MJ, Greer DH, Schmid J (2006) Effects of fungal endophyte, *Neotyphodium lolii*, on net photosynthesis and growth rates of perennial ryegrass (*Lolium perenne*) are independent of *in planta* endophyte concentration. Ann Bot (Lond) **98:** 379–387

Stitt M, Müller C, Matt P, Gibon Y, Carillo P, Morcuende R, Scheible WR, Krapp A (2002) Steps towards an integrated view of nitrogen metabolism. J Exp Bot **53:** 959–970

Südekum KH, Wolffram S, Ader P, Robert JC (2004) Bioavailability of three ruminally protected methionine sources in cattle. Anim Feed Sci Technol **113:** 17–25

Takemoto D, Tanaka A, Scott B (2006) A $p67^{Phox}$-like regulator is recruited to control hyphal branching in a fungal-grass mutualistic symbiosis. Plant Cell **18:** 2807–2821

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B (2006) Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell **18:** 1052–1066

Taper ML, Lele SR (2004) The Nature of Scientific Evidence: Statistical, Philosophical and Empirical Considerations. University Chicago Press, Chicago

van der Heijden MGA, Streitwolf-Engel R, Riedl R, Siegrist S, Neudecker A, Ineichen K, Boller T, Wiemken A, Sanders IR (2006) The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytol **172:** 739–752

Vogelsang KM, Reynolds HL, Bever JD (2006) Mycorrhizal fungal identity and richness determine the diversity and productivity of a tallgrass prairie system. New Phytol **172:** 554–562

Volpin H, Elkind Y, Okon Y, Kapulnik Y (1994) A vesicular arbuscular mycorrhizal fungus (*Glomus intraradix*) induces a defense response in alfalfa roots. Plant Physiol **104:** 683–689

Wingler A, Guttenberger M, Hampp R (1993) Determination of mannitol in ectomycorrhizal fungi and ectomycorrhizas by enzymatic microassays. Mycorrhiza **3:** 69–73

Wright DP, Read DJ, Scholes JD (1998) Mycorrhizal sink strength influences whole plant carbon balance of *Trifolium repens* L. Plant Cell Environ **21:** 881–891

Wynn JP, bin Abdul Hamid A, Ratledge C (1999) The role of malic enzyme in the regulation of lipid accumulation in filamentous fungi. Microbiology **145:** 1911–1917

Wynn JP, Ratledge C (1997) Malic enzyme is a major source of NADPH for lipid accumulation by *Aspergillus nidulans*. Microbiology **143:** 253–257

Zabalgogeazcoa Í, Ciudad AG, de Aldana BRV, Criado BG (2006) Effects of the infection by the fungal endophyte Epichloë festucae in the growth and nutrient content of Festuca rubra. Eur J Agron **24:** 374–384

Zhang Y, Adams IP, Ratledge C (2007) Malic enzyme: the controlling activity for lipid production? Overexpression of malic enzyme in *Mucor circinelloides* leads to a 2.5-fold increase in lipid accumulation. Microbiology **153:** 2013–2025