Ryan, G. D., Rasmussen, S., Xue, H., Parsons, A. J., & Newman, J. A. (2014). Metabolite analysis of the effects of elevated CO2 and nitrogen fertilization on the association between tall fescue (*Schedonorus arundinaceus*) and its fungal symbiont *Neotyphodium coenophialum*. *Plant, cell & environment*, 37(1), 204-212.

Corresponding author: Geraldine D. Ryan (gryan@uoguelph.ca)

ABSTRACT

Atmospheric CO_2 is expected to increase to between 550 ppm and 1000 ppm in the next century. CO_2-induced changes in plant physiology can have ecosystem-wide implications and may alter plant-plant, plant-herbivore and plant-symbiont interactions. We examined the effects of three concentrations of CO_2 (390ppm, 800ppm and 1000ppm) and two concentrations of nitrogen fertilizer (0.004gN/week vs. 0.2gN/week) on the physiological response of *Neotyphodium* fungal endophyte-infected and uninfected tall fescue plants. We used quantitative PCR to estimate the concentration of endophyte under altered CO_2 and N conditions. We found that elevated CO_2 increased the concentration of water-soluble carbohydrates and decreased the concentration of plant total amino acids in plants. Fungal-derived alkaloids decreased in response to elevated CO_2 and increased in response to nitrogen fertilization. Endophyte concentration (expressed as the number of copies of an endophyte-specific gene per total genomic DNA) increased under elevated CO_2 and nitrogen fertilization. The correlation between endophyte concentration and alkaloid production observed at ambient conditions was not observed under elevated CO_2. These results suggest that nutrient exchange dynamics important for maintaining the symbiotic relationship between fungal endophytes and their grass hosts may be altered by changes in environmental variables such as CO_2 and nitrogen fertilization.

INTRODUCTION

Atmospheric CO_2 is expected to rise from a current ambient concentration of 390ppm to between 550 and 1000ppm by the year 2100 (IPCC 2007). The effects of elevated CO_2 on plant growth, physiology, metabolism, morphology and competition have been well studied (Bazazz 1990; Robinson, Ryan & Newman 2012). CO_2 enrichment has been shown to increase plant photosynthetic rates resulting in increased growth and biomass production and physiological changes brought about by altered resource allocation. A commonly observed CO_2-induced change is an increase in carbohydrate metabolites and a decrease in nitrogen metabolites such as amino acids and soluble protein, resulting in an increased plant C:N ratio (Robinson *et al.* 2012). Such changes in resource allocation are also expected to alter plant defensive chemistry. The carbon-nutrient balance (CNB) hypothesis has often been employed as a model of allocation to defensive compounds under elevated CO_2 and predicts that carbon products in excess of those needed for primary metabolic functions will result in increased carbon-based secondary metabolites and subsequent decreased N-based secondary metabolites (Bryant, Chapin & Klein 1983). While there is some evidence that supports this outcome on average (Robinson *et al.* 2012) generalizations concerning these changes have been difficult to formulate and may be subject to complex regulation by phytohormones (Ryan, Rasmussen & Newman 2010; DeLucia *et al.*, 2012). Such alterations in plant primary and secondary metabolism are expected to have bottom-up trophic effects that are likely to affect broader community interactions. While these effects have been relatively well studied in the case of herbivores and their host plants, very little work exists on the broader foodweb impacts of elevated CO_2.

The perennial grass *Schedonorus arundinaceus* (Schreb; tall fescue) is an agricultural pasture grass that is commonly associated with the mutualistic clavicipitaceous fungus *Neotyphodium coenophialum* (Glenn, Bacon, & Hanlin). *Neotyphodium* endophytes are asexual, vertically transmitted fungi whose hyphae grow in the intercellular spaces of host grasses (Christensen *et al.* 2008). Grasses themselves are relatively poor producers of defensive compounds and the presence of mutualistic *Neotyphodium* fungi has been shown to directly increase host plant resistance to both vertebrate and invertebrate herbivores through the production of toxic alkaloids (Clay 1988). The presence of endophytic fungi can alter the outcome of competitive interactions between plants by providing increased herbivore resistance, drought tolerance, disease resistance, nutrient stress tolerance and reproductive vigour in host plants (Kuldau & Bacon 2008; Cheplick & Faeth 2009). As such, endophytes have a critical role in shaping community composition.

Recent studies have suggested that the degree of mutual benefit for endophytic fungi and their hosts may be conditional on environmental factors such as nutrient availability (Saikkonen *et al.* 2006). Heterotrophic microorganisms such as fungal

endophytes and mycorrhizae depend on carbon and energy provided by their autotrophic host plants. It is not entirely clear which organism controls such interactions and to what extent abiotic mechanisms contribute to these dynamics. Though these mechanisms have yet to be elucidated, nutrient exchange between plant and fungal partners seems to play an important role (Kiers & Denison 2008; Ryan *et al.* 2008; Draper, Rasmussen & Zubair 2011). Thus, changes in environmental variables that can alter plant resource allocation may have implications for the way in which symbiotic partners interact. Both elevated CO_2 and nitrogen fertilization can alter plant nutrient allocation and may affect nutrient exchange in ways that impact the production of N-based mycotoxins and carbon energy sources for endophytic growth. Few studies have examined the interaction between fungal endophytes and host plants under elevated CO_2 (but see: Marks & Clay 1990; Groppe *et al.* 1999; Newman *et al.* 2003; Hunt *et al.* 2005). Hunt *et al.* (2005) found that *N. lolii*-derived alkaloids increased in perennial ryegrass grown under elevated CO_2, but only when plants were well fertilized. CO_2-induced increases in defensive compounds are likely to impact herbivores in a high CO_2 atmosphere. Studies have also shown that endophyte infection can buffer CO_2-induced decreases in plant protein production in both tall fescue (Newman *et al.* 2003) and perennial ryegrass (Hunt *et al.* 2005), which may affect herbage quality for herbivores beyond changes in defensive chemistry.

Changes in fungal growth in response to altered environmental variables may have implications for the strength and direction of plant-endophyte interactions, particularly in cases where nutrient dynamics are altered. The growth responses of endophytes to changes in plant physiology have been difficult to study due to the fact that endophyte and plant tissues cannot be physically separated. As such, most studies in the endophyte literature report only fungal presence/absence data in host grasses. However, quantitative PCR (qPCR) provides a promising technique for estimating endophyte concentration by quantifying the number of copies of endophyte-specific genes found in the total (plant + fungus) genomic DNA (Rasmussen *et al.* 2007; Liu *et al.* 2011).

In this study we examine the effects of elevated CO_2, nitrogen fertilization and endophyte status on plant physiological parameters associated with carbon and nitrogen metabolism. In endophyte-infected plants, we examine the effects of CO_2, nitrogen fertilization and their interaction on endophyte-derived alkaloids. We use qPCR to examine N and CO_2 effects on the concentration of *N. coenophialum* in plant tissues. To our knowledge this is the first time this technique has been used to examine *in planta* fungal growth of *N. coenophialum* in response to altered atmospheric CO_2 concentration.

MATERIALS AND METHODS

CO_2-controlled chambers

Nine plexiglass chambers housed in a greenhouse in a 3 x 3 square pattern were used to control CO_2. Chambers were constructed and operated according to Grodzinski *et al.* (1999). An Argus Greenhouse Control System was used to control and monitor the concentration of CO_2, relative humidity and temperature. We used three CO_2 concentrations (ambient (390 ppm), 800 ppm and 1000 ppm) with three replicates per concentration, which were blocked according to a light gradient within the greenhouse (one of each CO_2 concentration per block, arranged as a latin squares design). These elevated CO_2 concentrations are within the range of the projected increase by the year 2100. Chambers were maintained at a relative humidity of ~40% and a temperature of 23°C. The light regime was set to an 18:6 L/D cycle.

Plant material

Experimental *S. arundinaceus* (tall fescue) plants were taken from a group of long-standing nursery plants where each plant was the product of a single seed and thus represented a single genotype. Georgia 5 seed lines were originally obtained from Donald Wood (University of Georgia, USA). Seed stock was originally infected with the common strain (CS) of *N. coenophialum* fungal endophyte. Following one year of seed storage at room temperature, some seeds lost the endophyte infection. Thus, all plant genotypes used were compatible with endophyte infection. Six E+ genotypes and six E- genotypes were randomly selected from plants grown from these seed stocks for the experiment. Prior to the experiment, endophyte infection status was confirmed for each genotype using the immunoblot method (Agrinostics Ltd.). Two tillers were removed from the appropriate parent plants and were cut back to 5-6 cm (above- and below-ground tissue) and repotted in a mixture of 50% Sunshine Mix #2 (Sun Gro Horticulture) and 50% vermiculite (2A; Thermorock). Pots were constructed from 0.635 cm thick PVC piping cut to 9 cm x 38 cm (D x H). Each genotype (6 E- and 6 E+) was replicated twice per chamber (one set of replicate genotypes per level of nitrogen fertilization) for a total of 216 pots. To avoid unwanted insect colonization prior to the beginning of the experiment, plants were sprayed with insecticidal soap after placement in the CO_2-controlled chambers.

Plants were watered as necessary with de-ionized water. Plants were fertilized weekly with either a low N (0.004 gN/week) or high N (0.2 gN/week) treatment, delivered as 40 ml of a 0.3 g/L NH_4NO_3 or 14.7 g/L NH_4NO_3 solution respectively. All plants received 40 ml of a micronutrient solution weekly (nitrogen-free Long Ashton solution; Ponder *et al.* 2000). Plants were cut back to 10 cm above soil level weekly over the whole experimental period and the cuttings were dried and weighed. In order to test the resulting plant nitrogen status, we measured %N in blade tissues of above-

ground material at two time points, pooled across endophyte and CO_2. Low N plants contained 2.69 ± 0.12 %N, while high N plants contained 4.24 ± 0.10% N; these values reasonably approximate N-limited and N-rich pastures respectively (Parsons, Harvey & Woledge 1991). Plants were grown in treatment conditions for 6 months, at which point two 4th or 5th instar Rhopalosiphum padi aphids were released onto each plant. Details of aphid population dynamics and phloem biochemistry will be published in a separate manuscript.

Alkaloid extraction and analysis

All alkaloid analyses were performed on above-ground plant tissues (blade and sheath tissues combined). Ergovaline extraction was performed using a method modified from Spiering *et al.* (2002). A sample of lyophilized grass tissue (50 mg) was extracted for one hour with 1ml of extraction solvent (50% isopropanol, 1% lactic acid with 1.16 ng/ml ergotamine tartrate as internal standard). The sample was then centrifuged (8000 g, 5 min) and a 500 µl aliquot of the supernatant transferred to an HPLC vial for analysis.

Extraction and analysis of lolitrem-B was performed using a method modified from Spiering *et al.* (2005). Samples of lyophilized grass tissue (50 mg) were extracted for one hour with 1 ml of the extraction solvent (9:1 dichloroethane/methanol). Samples were centrifuged for 5 min at 8000 g and 500 µl aliquots of the supernatant were transferred to HPLC vials for analysis.

Loline alkaloids (N-acetyl loline, N-formyl loline and N-acetyl norloline) were measured using gas chromatography (GC) from a method modified from Kennedy & Bush (1983) and Yates, Fenster & Bartelt (1989). A sample of lyophilized grass tissue (100 mg) was extracted for 1 hour with 50 µl of 40% methanol/5% ammonia and 1ml of 1,2-dichloroethane (containing 53.7 ng/ml 4-phenylmorpholine as internal standard). The tubes were then centrifuged for 5 min at 8000 g, and the supernatant transferred to a glass GC vial via a 10 µm filter for analysis. The analysis was conducted on a GC-flame ionization detector (GC-FID) equipped with a ZB-5 capillary column (30m x 0.32mm x 0.25µm film; Phenomenex, Torrance, CA, USA).

Peramine was extracted using a method modified from Spiering *et al.* (2002). A sample of lyophilized grass tissue (50 mg) was extracted for one hour with 1ml of extraction solvent (50% methanol with 2.064 ng/ml homoperamine nitrate as internal standard). The sample was then centrifuged (8000 g, 5 min) and a 500 µl aliquot of the supernatant transferred to an HPLC vial for analysis.

Endophyte concentration

The concentration of *N. coenophialum* endophyte in plant tissue was estimated based on qPCR of genomic DNA (gDNA) isolated from infected plants using the Roche

LightCycler® 480 system (Roche Diagnostics). DNA was extracted from 25 mg of freeze-dried powdered plant material (pseudostem + blade) using DNeasy® Plant Mini kit (Qiagen, Bio-Strategy Ltd., Auckland, New Zealand) following the manufacturer's handbook. Primers (forward: 5'-cacgtactgactgaagcgtagc-3'; reverse: 5'-caatgcagcgagtgaacatc-3') suitable for qPCR were designed for a fragment of a *N. coenophialum*-specific gene, encoding the translation elongation factor 1-alpha (Takach *et al.* 2012; GenBank Acc. # JX028264). The gel-purified PCR products were cloned into pCR®2.1-TOPO® vectors (Invitrogen NZ Ltd., Auckland, New Zealand) and transformed into One Shot® Escherichia coli cells by chemical transformation. Single colonies were grown in liquid culture and plasmids extracted and purified using a QIAprep®Miniprep kit (Qiagen, Bio-strategy Ltd., Auckland, New Zealand). A dilution range of the plasmids from 2 $\times 10^1$ to 2 $\times$ 10^6 copies was used to generate a standard curve. Three nanograms of gDNA isolated from infected and uninfected plant material were mixed with LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics), primer pairs and water, for a total volume of 15 µl per qPCR reaction. The PCR protocol was as follows: pre-incubation: one cycle of 95°C for 5 min; amplification: 45 cycles of 95°C for 10 s, 64°C for 10 s and 72°C for 10 s; the formation of single PCR products in the assays was confirmed by melting analysis: 95°C for 5 sec, 65°C for 1 min and a continuous melting curve fluorescent acquisition at 97°C; cooling: 40°C for 30 sec. The concentration of endophyte present in the grass is expressed as the number of copies of fungal-specific gene per ng total (plant + fungal) genomic DNA.

Water-soluble carbohydrates, protein and amino acids

Low molecular weight (LMW) carbohydrates (mostly glucose, fructose and sucrose) and high molecular weight (HMW) carbohydrates (mainly fructans) were extracted and quantified from above-ground tissues as previously described (Hunt *et al.* 2005; Rasmussen *et al.* 2007). Briefly, 25 mg of dried tissue (blade + sheath) were extracted with 2 × 1 ml of 80% ethanol (LMW sugars) and subsequently with 2 × 1 ml water (HMW sugars) for 30 min at 60° C. Extracts were centrifuged, and supernatants of each fraction were analyzed separately using anthrone as a colorimetric reagent (Jermyn 1956). Standard calibration curves were generated using sucrose and inulin for LMW and HMW sugars respectively. Absorbance was measured at 620 nm.

Total free amino acids were analyzed from the ethanol fraction used for carbohydrate extraction and were determined colorimetrically with ninhydrin, as described previously (Yemm 1955). L-glutamine was used as a standard and absorbance was measured at 570 nm.

Soluble proteins were extracted using 0.1% mercaptoethanol in 100 mM potassium phosphate buffer. Extracts were centrifuged and the resulting supernatant

was analyzed according to Bradford (1976) with absorbance measured at 595 nm using Bovine Serum Albumin (BSA) as a standard.

Statistics

We used a blocked split-plot experimental design with CO_2 as the whole-plot factor and nitrogen and endophyte as sub-plot factors. All analyzes were performed using a mixed effects ANOVA model with CO_2, nitrogen and endophyte as fixed factors and plant genotype as a random factor. We also included a block term but as we had no a priori hypothesis about block x treatment interactions, we excluded these interactions from our model and collapsed these terms into the residuals (Newman, Bergelson & Grafen 1997). Table 1 shows the degrees of freedom for the analysis. For endophyte and alkaloid concentrations, we used a reduced version of the model that excluded "endophyte" as a factor, since these are only found in endophyte-infected plants.

All analyses were conducted using JMP statistical software version 8. A Box-Cox transformation was used to homogenize the residual variance and an examination of the residuals following transformations suggested that the assumptions of the ANOVA were met. The untransformed means, and standard error of the means, are reported as measures of response magnitude and data dispersion.

RESULTS

Water-soluble carbohydrates

LMW-WSC concentration increased under elevated CO_2 ($F_{2,4} = 49.76$, $p < 0.0001$) and decreased in the high nitrogen treatment ($F_{1,142} = 10.08$, $p < 0.01$). However there was a significant CO_2 x N interaction ($F_{2,142} = 10.23$, $p < 0.0001$; Fig. 1a), whereby the increase in LMW concentration under elevated CO_2 was observed only in the high N treatment. The effect of CO_2 on LMW concentration was also dependent on endophyte (CO_2 x endophyte interaction: $F_{2,142} = 8.15$, $p < 0.01$; Fig. 1b) and the magnitude of the CO_2-induced increase in LMWs was higher in endophyte-infected plants.

The concentration of HMW-WSCs also increased under elevated CO_2 ($F_{2,4} = 43.23$, $p < 0.0001$) and decreased under high N ($F_{1,142} = 80.05$, $p < 0.0001$). Again the effects of elevated CO_2 were dependent on a CO_2 x N interaction ($F_{2,142} = 7.63$, $p < 0.001$; Fig. 1c) but unlike LMW response, the CO_2-induced increase in HMWs was observed only in the low N condition. There was no significant effect of endophyte infection on HMW WSCs.

Nitrogen metabolites

Total soluble protein increased under high N fertilization ($F_{1,142} = 59.45$, $p < 0.0001$; Fig. 2a). Total amino acids also increased under high N fertilization ($F_{1,142} = 441.93$, $p < 0.0001$). However, there was a significant interaction between CO_2 and N fertilization

($F_{2,142}$ = 6.52, p < 0.01; Fig. 2b) whereby amino acids decreased under elevated CO_2, but only in the low N condition. There was also a significant N x endophyte interaction ($F_{2,142}$ = 12.78, p < 0.01; Fig. 2c); under high N conditions, E+ plants had a greater concentration of amino acids than E-, but this was not the case under low N conditions.

Endophyte and alkaloid concentrations

Total lolines ($F_{1,70}$ = 78.56, p < 0.001; Fig. 3a), ergovaline ($F_{1,70}$ = 188.60, p < 0.0001; Fig. 3c) and peramine ($F_{1,70}$ = 200.10, p < 0.0001; Fig. 3e) all increased with high N fertilization. Total lolines decreased significantly under elevated CO_2 ($F_{2,4}$ = 14.67, p = 0.001; Fig. 3b), while there was a marginally significant (p < 0.1) decrease in ergovaline under elevated CO_2 ($F_{2,4}$ = 3.41, p < 0.1; Fig. 3d).

The concentration of endophyte (expressed as the number of copies of an endophyte-specific gene per ng of total genomic DNA) increased under elevated relative to ambient CO_2 ($F_{2,4}$ = 5.72, p < 0.05; Fig. 4a), but this difference was seen only in the 800 ppm condition and not in the 1000ppm condition. Endophyte concentration increased in the high N treatment ($F_{1,70}$ = 37.37, p < 0.01; Fig. 4b).

In order to examine differences in the production of alkaloids by endophyte under our treatment conditions, we expressed alkaloid concentration (µg/gDM) per unit endophyte (copies/ng gDNA) and subjected the per unit endophyte production of all alkaloids individually to the ANOVA model above. We found that the production of alkaloids per unit endophyte significantly decreased for all three alkaloids under elevated CO_2 (Lolines: $F_{1,70}$ = 9.22, p < 0.01; Peramine: $F_{1,70}$ = 5.23, p < 0.05; Ergovaline: $F_{1,70}$ = 6.44, p < 0.05). The reduction in loline production per unit endophyte is shown in Figure 5a. This CO_2-induced reduction was similar for both peramine (390 ppm: 0.21 ± 0.01; 800 ppm: 0.09 ± 0.01; 1000 ppm: 0.10 ± 0.01) and ergovaline (390 ppm: 0.027 ± 0.003; 800 ppm: 0.018 ± 0.002; 1000 ppm: 0.023 ± 0.003).

In order to examine whether endophyte concentration was a good predictor of alkaloid concentration in general, we regressed endophyte content against alkaloid concentration. There was a significant positive correlation between endophyte and alkaloid concentration, but only under ambient conditions. This relationship became less significant as CO_2 increased. Figure 5b, c and d shows the correlation for loline alkaloids (390 ppm: R^2 = 0.38, $F_{1,32}$ = 19.58, P < 0.0001; 800ppm: R^2 = 0.11, $F_{1,32}$ = 4.13, P < 0.1; 1000 ppm: R^2 = 0.001, $F_{1,32}$ = 0.032, P > 0.1). The relationship for peramine (390 ppm: R^2 = 0.32, $F_{1,32}$ = 14.99, P < 0.001; 800ppm: R^2 = 0.11, $F_{1,32}$ = 3.94, P < 0.1; 1000 ppm: R^2 = 0.02, $F_{1,32}$ = 0.667, P > 0.1) and ergovaline (390 ppm: R^2 = 0.23, $F_{1,32}$ = 9.55, P < 0.01; 800ppm: R^2 = 0.07, $F_{1,32}$ = 2.47, P > 0.1; 1000 ppm: R^2 = 0.001, $F_{1,32}$ = 0.019, P > 0.1) were similar to that of lolines shown in figure 5.

DISCUSSION

Plant metabolite responses

Plant carbohydrate responses in the present study were similar to those seen in other studies, in that carbohydrates (here LMW and HMW sugars) decreased in high N (Pettersson, McDonald & Stadenberg 1993; Skinner, Morgan & Hanson 1999; Curtis *et al.* 2000; Rasmussen *et al.* 2007, 2008) and increased under elevated CO_2 (Pettersson *et al.* 1993; Skinner *et al.* 1999; Curtis *et al.* 2000; Robinson *et al.* 2012). For nitrogen metabolites, only total amino acids changed under elevated CO_2, with the concentration decreasing under elevated CO_2 in the low nitrogen treatment (nitrogen x CO_2 interaction). We found no CO_2 x endophyte interaction for amino acids and soluble protein concentrations. This is inconsistent with the results of Hunt *et al.* (2005) and Newman *et al.* (2003) who found that a CO_2-induced reduction in protein in uninfected perennial ryegrass and tall fescue plants respectively, disappeared when plants were infected with endophyte. We found no evidence here of this buffering effect of endophyte in tall fescue. It is difficult to say why this discrepancy might occur, but we note that both previous studies where conducted under field conditions, and thus in very different environments than the present study. Additionally, the Newman *et al.* (2003) study used the cultivar "Kentucky-31" and analyzed cuttings from the "grazed horizon" (5cm above soil level) combined over three time points, each of which had a higher regrowth interval (4 weeks) than the plants used here (1 week). The Hunt *et al.* (2005) study used harvested leaf and sheath tissues of perennial ryegrass combined together, but in 50-day post-germination plants. Thus, discrepancies may be accounted for by differences in growth conditions, genetic background, sampling intervals and/or plant age.

Endophyte and alkaloid responses

Our observation that alkaloid concentrations more than doubled in the high N treatment suggests that the production of alkaloids in tall fescue is N-limited. The importance of plant nitrogen status on the production of N-based alkaloids may explain the slight CO_2-induced decrease in alkaloids seen here for both lolines and ergovaline. A general hypothesis has emerged in the CO_2 literature which predicts that increased carbohydrate accumulation under elevated CO_2 results in a dilution of nitrogen which may result in a subsequent reduction in N-based metabolites (Cortrufo, Ineson & Scott 1998; Ryan *et al.* 2010; Robinson *et al.* 2012). This study shows that CO_2-induced nitrogen limitation may also affect symbiont-derived metabolites, which may have implications for nutrient exchange between symbiotic partners. Decreased alkaloid concentrations under elevated CO_2 may result in reduced herbivore resistance, which may decrease the competitive advantage of endophyte infection in a high CO_2 atmosphere.

Endophyte concentration (copies/g gDNA) increased in the current study under elevated CO_2, but only for the 800ppm treatment. This may be due to increased carbohydrate resources being shunted to endophyte growth due to increased photosynthetic output. However, the dynamics of resource exchange in grass-endophyte symbioses are not well understood. Rasmussen *et al.* (2007) found that the concentration of the closely related N. *lolii* endophyte in perennial ryegrass decreased in plants that were selectively bred to produce high levels of water-soluble carbohydrates. More recent work suggests that such changes may be brought about in part by carbohydrates but are also strongly dependent on the genetic background of host plants (Ryan *et al.* unpublished).

The grass-endophyte mutualism and elevated CO_2

We observed that alkaloid production per unit endophyte decreased in elevated CO_2 (Fig. 5a). Interestingly, we also found that a linear correlation between endophyte and alkaloid concentration in ambient conditions disappeared in elevated CO_2, which may indicate that the symbiotic nutrient economy may be disrupted to some degree in a high CO_2 atmosphere (Fig.5 b,c,d). The relationship between alkaloid and endophyte concentration in plants grown under elevated CO_2 appears to be quadratic, with very low levels of alkaloids in plants with either very low or very high endophyte concentrations, and this was particularly apparent in the 1000 ppm treatment. This may suggest that plants undergoing the largest CO_2-induced changes in resource allocation have a higher endophyte concentration due to excess carbohydrates, and the lowest alkaloid concentrations due to simultaneous N dilution. If this interpretation is correct then we would expect that plants where the C:N ratio are highest (i.e. strong CO_2-induced C/N allocation responses) would have the lowest alkaloid per unit endophyte concentrations. While there was some support for this here (Log[lolines per unit endophyte] = 3.17 – 0.38 (Log[C:N]); R^2 = 0.06, $F_{1,102}$ = 7.03, $P < 0.01$), the C:N ratio explained only a small amount (~6%) of the variation in the response. It is unknown whether host plants can exert control over the degree of endophyte growth (Ryan *et al.* 2008).

The current study suggests that nutrient exchange may in part be a passive process: an "overflow" mechanism whereby nutrient exchange between symbiotic partners is subject to availability. Our results point to the intriguing possibility that the degree of benefit conferred by endophyte infection and associated anti-herbivore toxin production for the host plant may decrease in a high CO_2 atmosphere by shunting nutrients toward endophyte growth and away from alkaloid production. The idea that the strength and/or direction of the symbiosis might be altered in high CO_2 could be more appropriately tested by examining the fitness responses of endophyte-infected vs. endophyte-free plants in elevated CO_2, and the degree to which they are

dependent on the presence of herbivores. In practice, lifetime fitness is difficult to measure and vegetative reproduction and biomass are often used as proxies (Cheplick & Faeth 2009). However, it is worth noting that there was no CO_2 x endophyte interaction in the present study for plant growth parameters, suggesting that the relative difference between E+ and E- plants was similar across all CO_2 treatments. Endophyte-infected plants consistently produced more tillers and above- and below-ground biomass across all treatments (data not shown).

However, endophytes may provide a range of benefits to host plants outside of herbivore resistance (Clay 1990) and the changes in resource allocation observed here will be dependent on the response of other factors affecting this mutualism. Increased tillering and biomass production in endophyte-infected grasses could be due to e.g. increased production of growth affecting phytohormones such as gibberellins and auxin (Depuydt & Hardtke 2011). It has been shown previously that clavicipitaceous endophytes can produce auxin (indole-3-acetic acid and related compounds) in culture (Porter *et al.* 1985; De Battista *et al.* 1990; Yue *et al.* 2000; Tanaka *et al.* 2003), but it is unclear if fungal auxin production affects plant growth in vivo (De Battista *et al.* 1990). It is also possible that endophyte infection regulates plant hormone production and a transcriptome study of L. perenne infected with a mutated strain of E. festucae impaired in a stress-activated mitogen-activated protein kinase showed significant effects on a number of plant genes related to phytohormone, including auxin biosynthesis and translocation in vivo (Eaton *et al.* 2010).

How generalizable are endophyte responses to CO_2 and N fertilization?

Research on the nutrient dynamics of grass-endophyte interactions in response to altered environmental variables suggests that results may not be generalizable across *Neotyphodium* species. In *N. coenophialum*-infected tall fescue, increased nitrogen fertilization has been shown to result in increased alkaloid production (Lyons, Plattner & Bacon. 1986; Arechavaleta *et al.* 1992) and the current study suggests that N fertilization also results in increased endophyte concentration. On the other hand, in N. *lolii*-infected perennial ryegrass, increased nitrogen input appears to cause a dilution effect resulting in decreased alkaloid (Hunt *et al.* 2005; Rasmussen *et al.* 2007) and endophyte (Rasmussen *et al.* 2007) concentrations. While we observed a decrease in alkaloid concentration under elevated CO_2 in the present study using tall fescue, endophyte-derived alkaloids have been shown to increase under elevated CO_2 in perennial ryegrass (Hunt *et al.* 2005). Taken together, these results suggest that treatment-induced changes in plant physiology do not induce similar responses in different *Neotyphodium*–grass associations. Continued research on how changes in plant physiology affect growth, physiology and nutrient dynamics in fungal endophytes may illuminate some of the mechanisms underlying this interaction. In particular,

variables which alter the balance of storage and growth in plants, such as manipulation of CO_2 levels, nutrient fertilization and selective plant breeding, may be useful tools for the study of plant-fungal endophyte interactions. Additionally, comparisons in plants sampled at similar ages, growth conditions and regrowth intervals may illuminate the extent to which such differences are truly divergent rather than the product of sampling differences.

ACKNOWLEDGMENTS

We gratefully acknowledge Lui Qianhe (AgResearch) for advice on qPCR, Wade Mace and Heike Schwendel (AgResearch) for assistance with HPLC and GCMS (alkaloid analysis) and Kruti Shukla for assistance with plant maintenance. GR was supported by a fellowship from the Natural Science and Engineering Research Council (NSERC) Canada. Funding to support this research was provided by grants from NSERC and the Ontario Ministry of Agriculture, Food and Rural Affairs, to JN.

REFERENCES

Arechavaleta M., Bacon C.W., Plattner R.D., Hoveland C.S. & Radcliffe D.E. (1992) Accumulation of ergopeptide alkaloids in symbiotic tall fescue grown under deficits of soil water and nitrogen fertilizer. Applied and Environmental Microbiology 58, 857–861.

Bazzaz F.A. (1990) Response of natural ecosystems to the rising global CO_2 level. Annual Review of Ecology, Evolution and Systematics 21, 167–196.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Bryant J.P., Chapin F.S. III & Klein D.R. (1983) Carbon/ nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40, 357–368.

Cheplick G.P. & Faeth S. (2009) Ecology and evolution of the grass-endophyte symbiosis. Oxford University Press, New York.

Christensen M.J., Bennett R.J., Ansari H.A., Koga H., Johnson R.D., Bryan G.T., Simpson W.R., Koolaard J.P., Nickless E.M. & Voisey C.R. (2008) Epichloë endophytes grow by intercalary hyphal extension in elongating grass leaves. Fungal Genetics and Biology 45, 84–93.

Clay K. (1988) Fungal Endophytes of Grasses: A Defensive Mutualism between Plants and Fungi. Ecology 69, 10–16.

Clay K. (1990) Fungal endophytes of grasses. Annual Reviews of Ecology and Systematics 21, 275–297.

Cortrufo M., Ineson P. & Scott A. (1998) Elevated CO2 reduces the nitrogen concentration of plant tissues. Global Change Biology 4, 43–51.

Curtis P.S., Vogel C.S., Wang X., Pregitzer K.S., Zak D.R., Lussenhop J., Kubiske M. & Teeri J.A. (2000) Gas exchange, leaf nitrogen, and growth efficiency of Populus tremuloides in a CO_2-enriched atmosphere. Ecological Applications 10, 3–17.

De Battista J.P., Bacon C.W., Severson R., Plattner R.D. & Bouton J.H. (1990) Indole acetic acid production by the fungal endophyte of tall fescue. Agronomy Journal 82, 878-880.

DeLucia E.H., Nabity P.D., Zavala J.A. & Berenbaum M.R. (2012) Climate change: resetting plant-insect interactions. Plant Physiology 160, 1677–1685.

Depuydt S. & Hardtke C.S. (2011) Hormone signalling crosstalk in plant growth regulation. Current Biology 21, R365-R373.

Draper J., Rasmussen S. & Zubair H. (2011) Metabolite analysis and metabolomics in the study of biotrophic interactions between plants and microbes. In: Biology of plant metabolomics. Annual Plant Reviews, Vol. 43 (ed R.D. Hall), pp. 25–59. Blackwell Publishing, Oxford.

Eaton C.J., Cox M.P., Ambrose B., Becker M., Hesse U., Schardl C.L. & Scott B. (2010) Disruption of signaling in a fungal-grass symbiosis leads to pathogenesis. Plant Physiology 153, 1780-1794.

Grodzinski B., Schmidt J.M., Watts B., Taylor J., Bates S., Dixon M.A.& Staines H. (1999) Regulating plant/insect interactions using CO_2 enrichment in model ecosystems. Advances in Space Research 24, 281–291.

Groppe K., Steinger T., Sanders I., Schmid B., Wiemken A. & Boller T. (1999) Interaction between the endophytic fungus Epichloë bromicola and the grass Bromus erectus: effects of endophyte infection, fungal concentration and environment on grass growth and flowering. Molecular Ecology 8, 1827–1835.

Hunt M.G., Rasmussen S., Newton P.C.D., Parsons A.J. & Newman J.A. (2005) Near-term impacts of elevated CO2, nitrogen and fungal endophyte-infection on Lolium perenne L. growth, chemical composition and alkaloid production. Plant, Cell and Environment 28, 1345–1354.

IPCC (2007) Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change (eds S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor & H.L. Miller). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jermyn M.A. (1956) A new method for determining ketohexoses in the presence of aldohexoses. Nature 177, 38–39.

Kennedy C.W. & Bush L.P. (1983) Effect of environment and management factors on the accumulation of N-acetyl and N-formyl loline alkaloids in tall fescue. Crop Science 23, 547–552.

Kiers E.T. & Denison R.F. (2008) Sanctions, cooperation, and the stability of plant–rhizosphere mutualisms. Annual Review of Ecology Evolution and Systematics 39, 215–236.

Kuldau G. & Bacon C. (2008) Clavicipitaceous endophytes: Their ability to enhance resistance of grasses to multiple stresses. Biological Control 46, 57–71.

Liu Q., Parsons A.J., Xue H., Fraser K., Ryan G.D., Newman J.A. & Rasmussen S. (2011) Competition between foliar *Neotyphodium lolii* endophytes and mycorrhizal Glomus spp. fungi in Lolium perenne depends on resource supply and host carbohydrate content. Functional Ecology 25, 910–920.

Lyons P.C., Plattner R.D. & Bacon C.W. (1986) Occurrence of peptide and clavine ergot alkaloids in tall fescue grass. Science 232, 487–489.

Marks S. & Clay K. (1990) Effects of CO_2 enrichment, nutrient addition, and fungal endophyte-infection on the growth of two grasses. Oecologia 84, 207–214.

Mattson W.J. (1980) Herbivory in relation to plant nitrogen content. Annual Review of Ecology, Evolution and Systematics 11, 119–161.

Newman J.A., Bergelson J. & Grafen A. (1997) Blocking factors and hypothesis tests in ecology: Is your statistics text wrong? Ecology 78, 1312–1320.

Newman J.A., Abner M.L., Dado R.G., Gibson D.J., Brookings A. & Parsons A.J. (2003) Effects of elevated CO_2, nitrogen and fungal endophyte infection on tall fescue: Growth, photosynthesis, chemical composition and digestibility. Global Change Biology 9, 425–437.

Parsons A.J., Harvey A. & Woledge J. (1991) Plant/animal interactions in continuously grazed mixtures. I. Differences in the physiology of leaf expansion and the fate of leaves of grasses and clover. Journal of Applied Ecology 28, 619–634.

Pettersson R., McDonald A.J.S. & Stadenberg I. (1993) Response of small birch plants (Betula pendula Roth.) to elevated CO2 and nitrogen supply. Plant, Cell and Environment 16, 1115–1121.

Ponder K.L., Pritchard J., Harrington R. & Bale J.S. (2000) Difficulties in location and acceptance of phloem sap combined with reduced concentration of phloem amino acids explain lowered performance of the aphid Rhopalosiphum padi on nitrogen deficient barley. Entomologia Experimentalis et Applicata 97, 203–210.

Porter J.K., Bacon C.W., Cutler H.G., Arrendale R.F. & Robbins J.D. (1985) In vitro auxin production by Balansia epichloë. Phytochemistry 24, 1429-1431.

Rasmussen S., Parsons A.J., Bassett S., *et al.* (2007) High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in Lolium perenne. New Phytologist 173, 787–797.

Rasmussen S., Parsons A.J., Fraser K., Xue H. & Newman J.A. (2008) Metabolic profiles of Lolium perenne are differentially affected by nitrogen supply, carbohydrate content, and fungal endophyte infection. Plant Physiology 146, 1440–1453.

Robinson E.A., Ryan G.D. & Newman J.A. (2012) A meta-analytical review of the effects of elevated CO_2 on plant-arthropod interactions highlights the importance of interacting environmental and biological variables. New Phytologist 194, 321–336.

Ryan G.D., Parsons A.J., Rasmussen S. & Newman J.A. (2008) Can optimality models and an "optimality research program" help us understand some plant-fungal relationships? Fungal Ecology 1, 115–123.

Ryan G.D., Rasmussen S. & Newman J.A. (2010) Global Atmospheric Change and Trophic Interactions: Are There Any General Responses? In: Plant Communication from an Ecological Perspective (eds F. Baluška &V. Ninkovic), pp. 179–214. Springer-Verlag, Berlin.

Saikkonen K., Lehtonen P., Helander M., Koricheva J. & Faeth S.H. (2006) Model systems in ecology: dissecting the endophyte-grass literature. Trends in Plant Science 11, 428–433.

Skinner R., Morgan J.A. & Hanson J.D. (1999) Carbon and nitrogen reserve remobilization following defoliation: Nitrogen and elevated CO_2 effects. Crop Science 39, 1749–1756.

Spiering M.J., Davies E., Tapper B.A., Schmid J. & Lane G.A. (2002) Simplified extraction of ergovaline and peramine for analysis of tissue distribution in endophyte-infected grass tillers. Journal of Agriculture and Food Chemistry 50, 5856–5862.

Spiering M.J., Lane G.A., Christensen M.J. & Schmid J. (2005) Distribution of the fungal endophyte *Neotyphodium lolii* is not a major determinant of the distribution of fungal alkaloids in Lolium perenne plants. Phytochemistry 66, 195–202.

Takach J.E., Mittal S., Swoboda G.A., Bright S.K., Trammell M.A., Hopkins A.A. & Young C.A. (2012) Genotypic and chemotypic diversity of *Neotyphodium* endophytes in tall fescue from Greece. Applied and Environmental Microbiology 78, 5501–5510.

Tanaka E., Tanaka C, Ishihara A., Kuwahara Y. & Tsuda M. (2003) Indole-3-acetic acid biosynthesis in Aciculosporium take, a causal agent of witches' broom of bamboo. Journal of General Plant Pathology 69, 2003.

Yates S.G., Fenster J.C. & Bartelt R.J. (1989) Assay of tall fescue seed extracts, fractions and alkaloids using the large milkweed bug. Journal of Agriculture and Food Chemistry 37, 354–357.

Yemm E.W. (1955) The determination of amino acids with ninhydrin. Analyst 80, 209–213.

Yu Q., Miller C.J., White Jr. J.F. & Richardson M.D. (2000) Isolation and characterization of fungal inhibitors from Epichloë festucae. Journal of Agricultural and Food Chemistry 48, 4687-4692.

Table 1: ANOVA model and degrees of freedom used for all analyses in this experiment with the exception of alkaloid and endophyte concentration responses. Since these are found only in endophyte-infected plants, we used a reduced model that excluded "endophyte" as a factor.

Source	df
Block	2
CO_2	2
Whole-Plot Error	4
Endophyte	1
Genotype(Endophyte)	10
CO_2 x Endophyte	2
Genotype(Endophyte) x CO_2	20
Nitrogen	1
Nitrogen x Endophyte	1
Genotype(Endophyte) x Nitrogen	10
Nitrogen x CO_2	2
Nitrogen x CO_2 x Endophyte	2
Genotype(Endophyte) x Nitrogen x CO_2	20
Sub-Plot Error	142

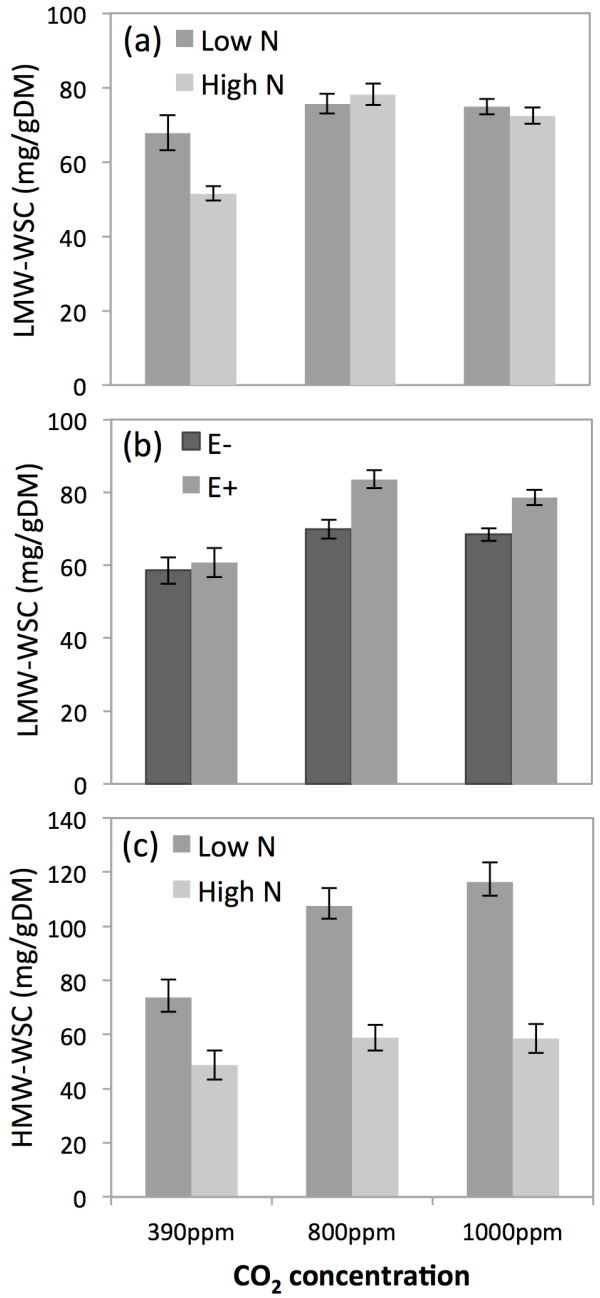

Figure 1: Carbohydrate responses to treatments showing the interactive effects of (a) nitrogen and CO_2 on LMW carbohydrate, (b) endophyte and CO_2 on LMW carbohydrate, and (c) nitrogen and CO_2 on HMW carbohydrate (means ± SE).

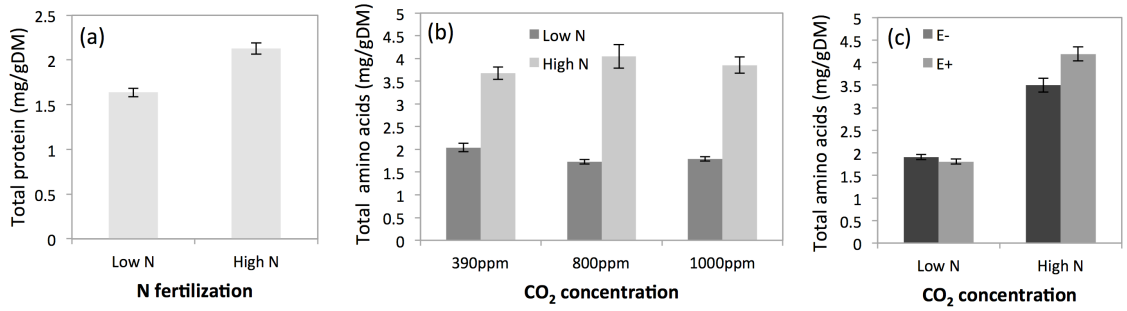

Figure 2: Treatment effects on plant nitrogen metabolites showing (a) the effect of nitrogen fertilization on total protein concentration (b) the interactive effects of nitrogen and CO_2 on total amino acid concentration, and (c) the interactive effects of endophyte status and N fertilization on total amino acids (means ± SE).

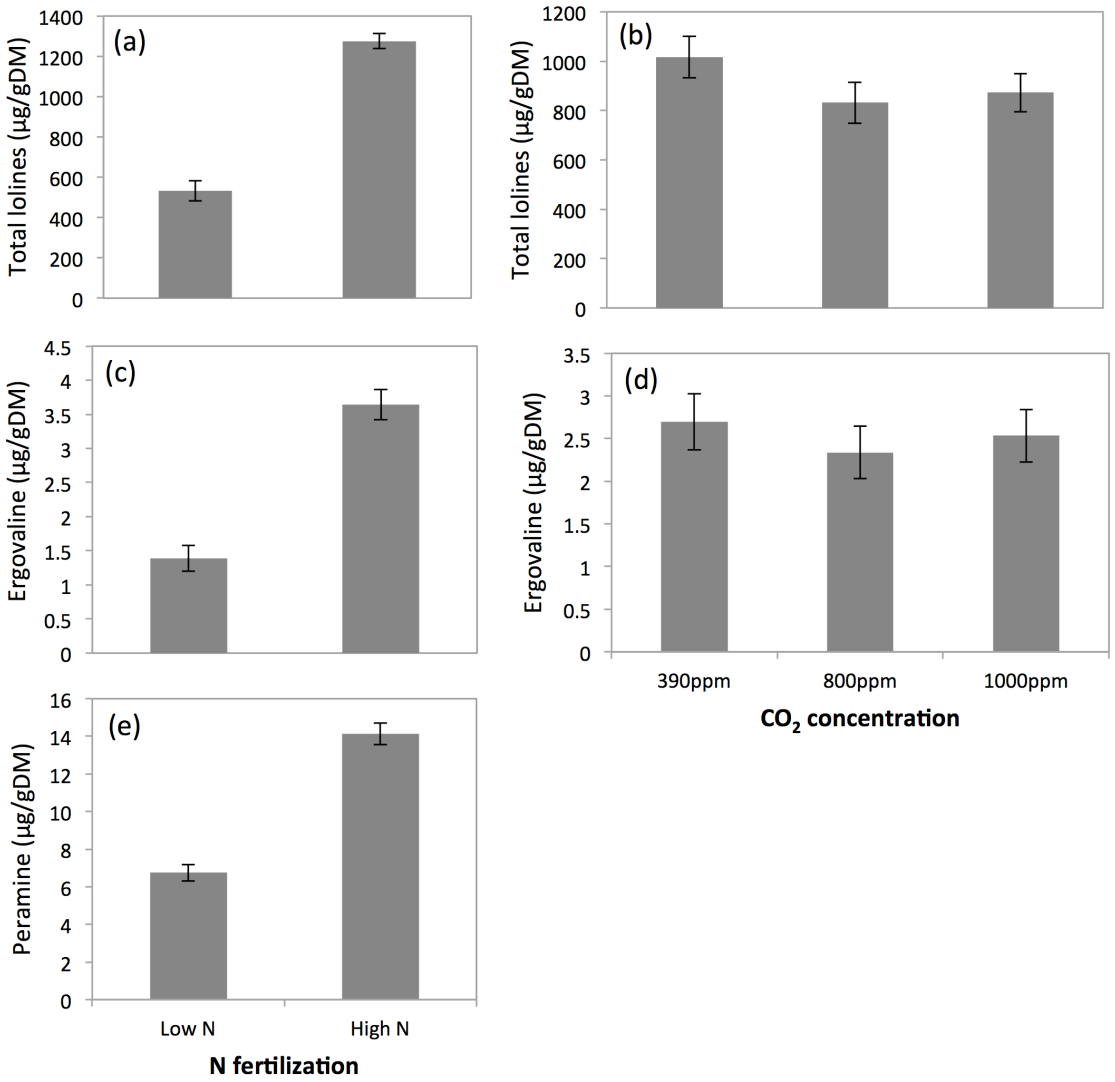

Figure 3: Treatments effects on the concentration of endophyte-derived alkaloids showing the main effect of nitrogen on (a) lolines, (c) ergovaline and, (e) peramine, and the main effect of CO_2 on (b) lolines and (d) ergovaline (means ± SE).

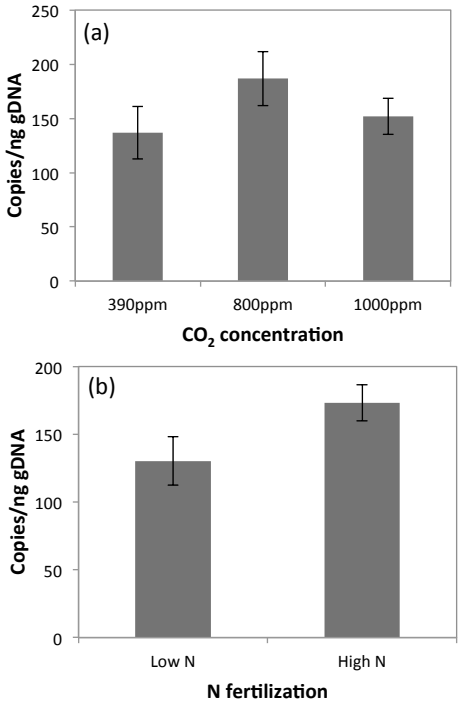

Figure 4: The main effects of (a) elevated CO_2 and (b) nitrogen fertilization on the concentration of endophyte, expressed as the number of copies of an endophyte-specific gene per total genomic (plant + fungal) DNA (means ± SE).

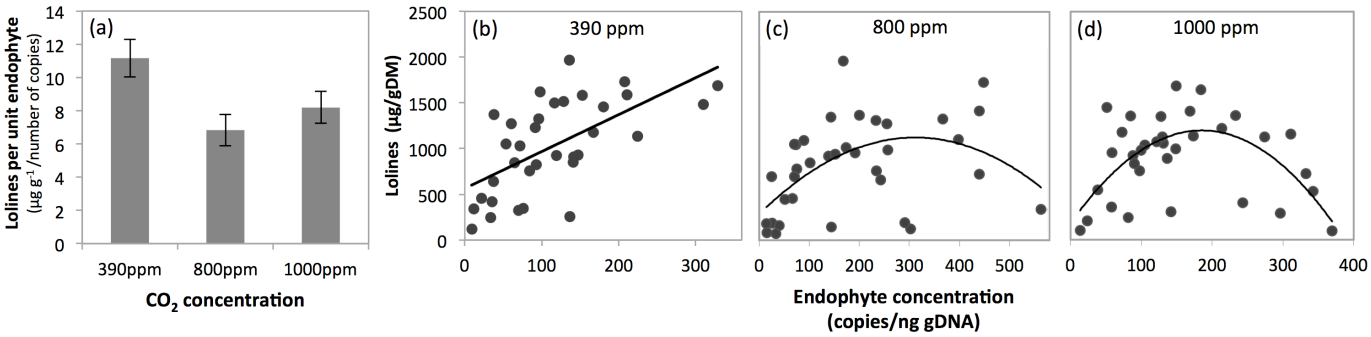

Figure 5: The effects of elevated CO_2 on alkaloid production by endophytes showing (a) loline alkaloid production per unit endophyte (means ± SE), and the correlation between endophyte and alkaloid concentration at (b) 390 ppm (Loline concentration = 555.06925 + 4.0332141*Endophyte concentration; $R^2 = 0.38$, $F_{1,32} = 19.57$, $P < 0.0001$), (c) 800 ppm (Loline concentration = 591.93237 + 2.1535393*Endophyte concentration - 0.0085847*(Endophyte concentration -186.642)^2; $R^2 = 0.26$, $F_{1,32} = 5.66$, $P < 0.01$), and (d) 1000 ppm (Loline concentration = 867.63768 + 1.9432844*Endophyte concentration - 0.0294447*(Endophyte concentration -151.988)^2; $R^2 = 0.37$, $F_{1,32} = 9.27$, $P < 0.001$).