Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 66 (2005) 195–202
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Distribution of the fungal endophyte *Neotyphodium lolii* is not a major determinant of the distribution of fungal alkaloids in *Lolium perenne* plants

Martin J. Spiering [a,*], Geoffrey A. Lane [b], Michael J. Christensen [b], Jan Schmid [a]

[a] *Institute of Molecular BioSciences, College of Sciences, Massey University, Private Bag 11222, Palmerston North, New Zealand*
[b] *AgResearch Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand*

Received 4 September 2004; received in revised form 25 November 2004

Abstract

The relationships of the distributions of the insect and mammalian mycotoxins, lolitrem B and ergovaline, and the insect-feeding deterrent, peramine, with the distribution of fungal mycelium were investigated in three genotypes of perennial ryegrass (*Lolium perenne* L.) infected with the endophyte *Neotyphodium lolii*. In planta levels and distribution of the endophyte and of the three alkaloids were assessed in parallel, and different spatial or temporal concentration gradients were observed for each. Variation in the tissue distribution of the endophyte accounted only for 20%, 6%, and 31% of the variation in ergovaline, lolitrem B, and peramine, respectively. Alkaloid–endophyte ratios, determined in individual grass tissues, showed distinct in planta distribution patterns for each alkaloid and differed in magnitude among genotypes. The ergovaline–endophyte ratio was higher in the very basal plant tissues than in the apical tissues, while the lolitrem B and peramine ratios tended to be higher in apical tissues. The lolitrem B–endophyte ratio increased with leaf age, while no consistent temporal trends were detected for the other alkaloids. The results indicate that endophyte colonisation is a minor determinant of alkaloid levels, and that accumulation of the alkaloids relative to the endophyte mycelium is affected by plant genotype and tissue in a manner specific to each alkaloid. Possible factors in the regulation of alkaloid levels in the grass plant are discussed.

Keywords: *Lolium perenne*; *Neotyphodium lolii*; Poaceae; Alkaloid distribution; Ergot alkaloids; Indole-diterpenoids; Ergovaline; Lolitrem B; Peramine; GUS-reporter gene

1. Introduction

Neotyphodium lolii (Latch, Christensen, & Samuels) Glenn, Bacon & Hanlin is a fungal endophyte symbiotic with perennial ryegrass (*Lolium perenne* L.) (Christensen et al., 1993). In this symbiosis, the biologically active alkaloids, ergovaline, peramine and lolitrem B, are produced (Fig. 1). The ergopeptine, ergovaline, and the indole-diterpenoid, lolitrem B, have been associated with toxic effects on mammals (Bush et al., 1997; Rowan, 1993; Tor-Agbidye et al., 2001) and insects (Ball et al., 1997b; Prestidge and Gallagher, 1988). The pyrrolopyrazine alkaloid, peramine, deters feeding of the Argentine stem weevil (*Listronotus bonariensis*), a major insect pest on perennial ryegrass (Rowan, 1993). Despite the mammalian toxicities associated with ergovaline and lolitrem B, endophyte infection is considered to provide a net benefit in agricultural settings (Bacon, 1993; Bush

* Corresponding author. Present address: Department of Plant Pathology, University of Kentucky, 201F Plant Science Bldg, 1405 Veterans Dr, Lexington, KY 40546, USA. Tel.: +1 859 257 7445x80790; fax: +1 859 323 1961.
E-mail address: mspier2@uky.edu (M.J. Spiering).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.11.021

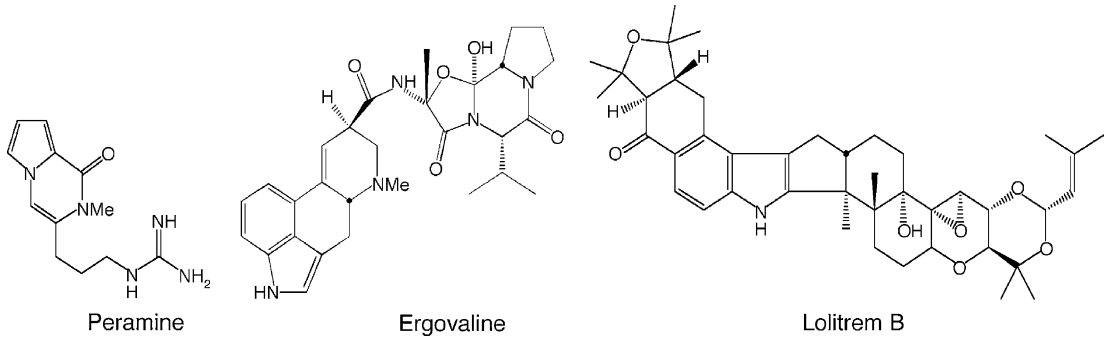

Fig. 1. Structures of the alkaloids produced in *N. lolii*-infected perennial ryegrass.

et al., 1997; Easton, 1999). Therefore, current strategies for forage grass improvement focus on maintaining the benefits of endophyte infection, while minimising negative effects caused by the accumulation of ergovaline and lolitrem B. One approach has been the identification of endophyte strains that do not lead to the accumulation of these toxins in the host plant and inoculation of these strains into endophyte-free grass plants (Bouton et al., 2002; Tapper and Latch, 1999). Perennial ryegrass cultivars infected with endophytes that do not produce the mammalian toxins have been shown to be free from animal toxicity and to maintain significant levels of resistance to insect herbivory (Fletcher, 1999; Popay et al., 1999).

An alternative approach is to manipulate the levels and distribution of alkaloids in the plant, hence, the balance between different alkaloid pathways in the symbiosis, by selection of suitable plant–endophyte associations. Genetic and in vitro studies have indicated that the endophyte is the producer of all three alkaloids (Gurney et al., 1994; Rowan, 1993; Scott, 2001). Both plant genotype and tissue type influence endophyte and alkaloid levels (Easton et al., 2002; Keogh et al., 1996; Roylance et al., 1994), and some correlations between alkaloid levels and endophyte levels were suggested by earlier studies (Ball et al., 1995; Easton et al., 2002; Keogh et al., 1996). It is still unclear, however, whether alkaloid levels in the plant are simply a function of the level of endophyte colonisation, or if the plant genotype/tissue inhabited by the endophyte has an influence on alkaloid levels, for example, by modulating alkaloid biosynthesis in the fungal mycelium or alkaloid degradation and translocation. Until recently, a lack of sensitive high-resolution methods for mapping of endophyte and alkaloid distributions has prevented researchers from addressing this key question. However, such methods have now become available. Tan et al. (2001) have shown that the in planta distribution of endophytes transformed with a constitutively expressed β-glucuronidase (GUS) gene (Jefferson, 1987) can be precisely mapped with quantitative GUS activity assays. We have also recently developed sensitive micro-scale extraction methods for extraction and quantification of ergovaline and peramine (Spiering et al., 2002) and lolitrem B (Tapper and Latch, 1999). Here, we have combined these methods to investigate the relationship between alkaloid and endophyte levels. This study is the first assessing the distributions of all of the major alkaloids produced in *N. lolii*-infected perennial ryegrass together with the distribution of the endophyte. Our findings suggest that levels and distribution of the endophyte is only a minor factor in determining alkaloid levels in a given plant genotype or tissue.

2. Results and discussion

2.1. GUS activity is a quantitative indicator for endophyte levels in different plant genotypes and tissues

Alkaloid levels in endophyte–grass associations are affected by the plant genotype (Adcock et al., 1997; Easton et al., 2002). To represent different spectra of the alkaloids in different endophyte–plant associations, we performed a pre-screening. Tissue concentrations of GUS activity, ergovaline, peramine, and lolitrem B were determined in 15 genotypes infected by the GUS-transformant KS1. Three genotypes, Nui D, Nui UIII, and Nui UIV, showing distinct differences in alkaloid and GUS activity concentrations (data not shown) were selected for detailed dissection. To minimise environmental effects in the experiments, plants of the selected genotypes were introduced into a controlled-environment cabinet.

As shown by Tan et al. (2001), GUS activity closely follows the in planta distribution of KS1 hyphae in the ryegrass genotype Nui D, suggesting that GUS could be used as a quantitative marker for endophyte levels. We confirmed this for all genotypes (Nui D, Nui UIII, and Nui UIV) used in this study. We determined GUS activity and endophyte levels (as hyphal counts in cross-sections) in 29 tissues (sheath and blade from first and third mature leaf) from all three genotypes. Linear regression analysis indicated a first-order relationship

between the two parameters (slope 0.955 ± 0.07; intercept -0.0415 ± 0.158; $r^2 = 0.87$; $P < 0.0001$; data were log-transformed to normalise residuals), and no statistically significant differences in the GUS–hyphal ratio were detected between genotypes or tissues ($P > 0.05$ for all t-tests).

2.2. Alkaloid levels are only weakly correlated with endophyte levels and are influenced by plant genotype, tissue position, or tissue age

Grass tillers were dissected into 16 different grass tissues (Fig. 2), and the concentration of the endophyte (measured as GUS activity concentration) and of the three alkaloids assessed in each tissue. Alkaloid and endophyte levels (Fig. 3) differed significantly ($P < 0.01$; ANOVA) between genotypes and tissues. The distributions of the endophyte and of the alkaloids followed approximate basal–apical gradients regardless of genotype. Similar endophyte and alkaloid gradients have been observed in earlier studies of endophyte-infected ryegrass (Herd et al., 1997; Keogh et al., 1996; Lane et al., 1997; Tan et al., 2001) and tall fescue (Christensen et al., 1998; Siegel et al., 1984).

Linear regression indicated that levels of all alkaloids were significantly ($P \leqslant 0.01$) correlated with levels of the endophyte. However, these correlations were weak: the r^2 values of the regressions indicated that the endophyte accounted only for 20%, 5.7%, and 31% of the in planta variation in ergovaline, lolitrem B, and peramine, respectively.

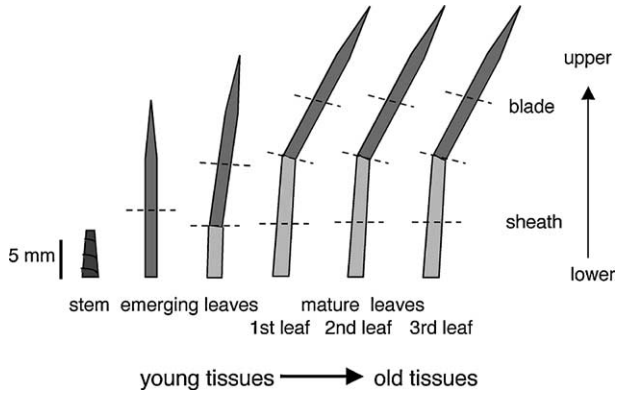

Fig. 2. Schematic of the grass tiller dissection performed in this study. Dashed lines indicate where leaves were cut for sampling. Leaf sheaths are shown in light grey and leaf blades in dark grey. Emerging leaves (at least 20 mm long; younger leaves were discarded) were classified into two types, depending on their developmental stage; one type consisted of blade only, and the other type of both sheath and blade. Mid- and upper leaf blade sections were pooled from the two types of emerging leaves. The mature leaves (1st to 3rd leaf) were separated into sheath and blade, which were each further separated by a median cut into lower and upper tissues. The stem consisted of the small (~5–10 mm long) basal region of the tiller bearing the leaves and roots. Scale bar indicates only the dimension of the true stem.

The steepness of the endophyte gradients differed from that of the alkaloid gradients and the steepness of gradients varied between the alkaloids. For example, ergovaline was more heterogeneously distributed in the tiller than the endophyte, whereas peramine was more homogeneously distributed than both ergovaline and the endophyte. The in planta distribution of ergovaline is of particular interest as there is little detailed information about ergovaline distribution in the literature. Our study shows for the first time that the highest levels of ergovaline are consistently present in the actual stem tissue, a tissue essential for growth and reproduction of the grass plant, and also in the tissues immediately surrounding it.

To quantify the relationship between alkaloid and endophyte distributions, we determined alkaloid to endophyte ratios in each tissue (by dividing the concentration of each alkaloid in a tissue by endophyte concentration in the same tissue). Each alkaloid–endophyte ratio was significantly affected by genotype ($P < 0.02$, <0.005, <0.05 for ergovaline, lolitrem B, and peramine, respectively; ANOVA) and tissue section ($P < 0.001$ for all three alkaloids; ANOVA). This suggested that alkaloid–endophyte ratios were influenced by general genotype effects in all tissues and by general tissue effects in all genotypes. Significant genotype-specific tissue-section effects were also detected (determined as genotype × tissue section interactions; $P < 0.02$, <0.005, <0.001 for ergovaline, lolitrem B, and peramine, respectively), indicating genotype-specific modifiers of the general tissue-section effects. However, we found that these genotype-specific tissue effects (determined as SD; see Section 4) were small: the SD was only 0.21, 0.16, 0.14 for ergovaline, lolitrem B, and peramine, respectively. The general tissue-section effects across the three genotypes were much larger, having an SD of 0.55, 0.59, and 0.28 for ergovaline, lolitrem B, and peramine, respectively.

The analysed grass tissues differed in relative (basal or apical) position in the tiller and age (defined by leaf position in the tiller (Soper and Mitchell, 1956)). This allowed us to determine if tissue effects could be broken down into significant position effects and age effects, as well as their magnitude. By ANOVA, we found significant ($P < 0.01$) effects on all alkaloid–endophyte ratios for tissue position and plant genotype. Leaf age had a significant effect on the lolitrem B–endophyte ratio ($P < 0.001$), but not on the ratios of ergovaline and peramine ($P > 0.05$).

The magnitudes of the effects of plant genotype, tissue position, and leaf age on each alkaloid–endophyte ratio are shown in Fig. 4. The ergovaline–endophyte ratio showed a very heterogeneous vertical distribution. The ratio was much larger in the stem than in the leaf tissues, and also high in the lower leaf sheaths. This might indicate lower mobility of ergovaline and higher rates of

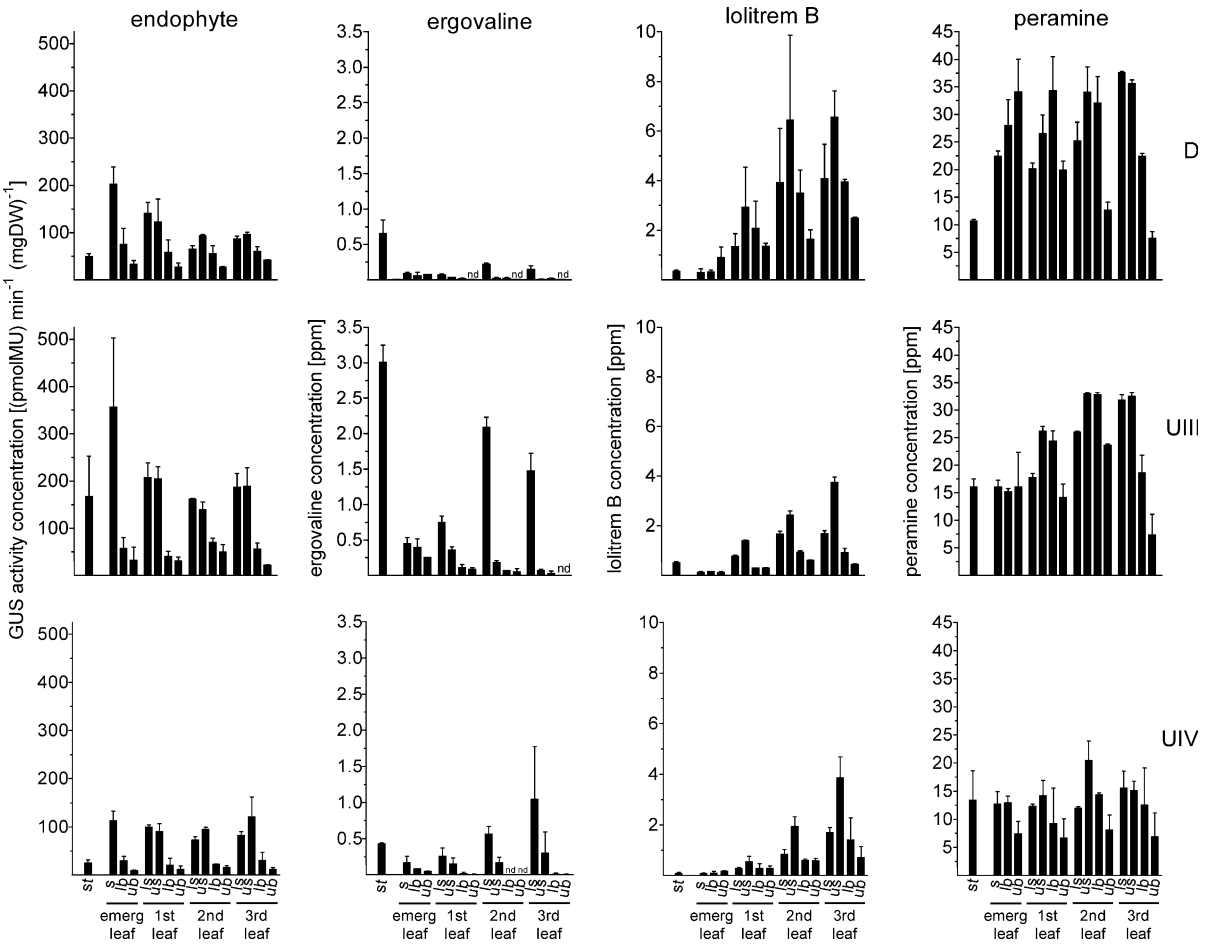

Fig. 3. Distributions of the endophyte (assessed as GUS activity) and of the alkaloids, ergovaline, lolitrem B and peramine, in grass tillers of the three genotypes, Nui D, Nui UIII and Nui UIV, infected by the *N. lolii*-strain KS1. At the top of columns are indicated the parameters measured, and at the far right of each row the plant genotypes assessed. Tissues were dissected as shown in Fig. 2. The stem (st) consisted of the most basal tissues; emerging leaves (emerg leaf) were fractionated into sheath (s), lower blade (lb), and upper blade (ub); the mature leaves (1st to 3rd) were dissected into lower sheath (ls), upper sheath (us), lower blade (lb), and upper blade (ub). GUS activity and alkaloid concentrations were determined in subsamples from tissue samples pooled from 10–12 tillers per replicate plant. Mean concentrations were determined on two replicates; error bars show 1 SD of the mean (nd = not detected).

biosynthesis in the very basal tissues, or more rapid degradation of ergovaline in the apical tissues. In the emerging leaf, ergovaline concentration was low and it showed a more uniform distribution (Fig. 3), perhaps due to translocation in the emerging leaf, despite its hydrophobic nature (Neumann, 1985) or less degradation activity in younger tissues. Leaf age had no general effect on the ergovaline–endophyte ratio (Fig. 4), but there was a significant ($P < 0.05$) 3-fold increase in the ratio in the lower sheath between first and second leaf. Taken together, these observations suggest that ergovaline production in leaves occurs mainly in the sheaths of younger leaves. This was supported by repeated sampling from plants of different age. Under the controlled-environment conditions, ergovaline concentration in tissues tended to be higher in younger plants than in older plants, i.e., ergovaline showed a 3 to 4-fold decline over 40–60 days of plant culture (not shown).

The lolitrem B–endophyte ratio tended to increase gradually along the leaf and varied widely with leaf age, showing a pronounced increase from the emerging to the first leaf and a further increase from first to second leaf (Fig. 4). Increasing lolitrem B accumulation with increasing leaf age is common in *N. lolii*-infected grasses (Ball et al., 1995, 1997a; Keogh et al., 1996). The increase of the lolitrem B–endophyte ratio suggests constitutive production and greater stability of lolitrem B in the plant. This is also supported by results from sampling from plants of different age in the controlled environment, revealing a 1.5- to 2-fold increase of lolitrem B concentration over 40–60 days of plant culture (not shown). The age-dependent increase of lolitrem B might also explain the greater lolitrem B–endophyte ratios in the apical tissues. These tissues are older than the basal ones (Soper and Mitchell, 1956), and observations by one of us have provided evidence that the endophyte

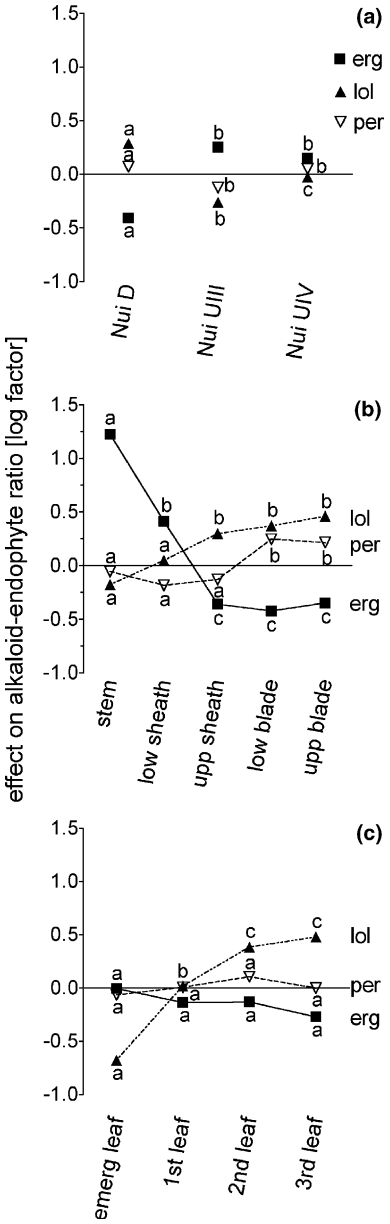

Fig. 4. Effects of (a) plant genotype, (b) tissue, and (c) leaf age on alkaloid–endophyte ratios. Indicated are the effects of the three factors on the alkaloid–endophyte ratios for ergovaline (erg), lolitrem B (lol), and peramine (per). Effects were calculated by subtracting the log-mean of the complete data of an alkaloid–endophyte ratio from the log-mean for individual genotypes, tissue sections, or leaves. Values above zero indicate that a genotype, tissue, or leaf had an alkaloid–endophyte ratio that was higher than the average ratio for a given alkaloid, and vice versa. Note that, except for genotypes, graphs represent data subsets; therefore, plotted effects may not add up to zero. Within each data set, points having a different letter differ significantly from each other at $P = 0.05$ (Fisher's LSD). Abbreviations used: low, lower; upp, upper; emerg, emerging.

hyphae are of the same age as the leaf tissues they inhabit (M.J.C., unpublished data). Translocation of lolitrem B to the apical tissues could also explain its vertical distribution, but this seems less likely given its highly lipophilic properties (Munday-Finch and Gart-hwaite, 1999) and the aqueous environment of the apoplast. Low in planta mobility of lolitrem B is also supported by findings of an earlier study (Ball et al., 1993). The stem, despite being older than most leaf tissues, showed a generally lower lolitrem B–endophyte ratio, suggesting that, similar to ergovaline, accumulation of lolitrem B apparently also depends on the nature of the tissue inhabited by the endophyte.

Mevalonic acid and tryptophan (or tryptophan precursors) are precursors of both ergovaline and lolitrem B (Baxter et al., 1962; Byrne et al., 2002). The ergovaline- and lolitrem B–endophyte ratios could thus be influenced by pathway competition for these two precursors. Indeed genotypes (Fig. 4(a)) and tissues (Fig. 4(b) and (c)) with an elevated ergovaline–endophyte ratio typically had a reduced lolitrem B–endophyte ratio and vice versa. The inverse relationship between ergovaline and lolitrem B ratios in the genotypes might simply be a result of our selection of plants with different alkaloid profiles. However, this selection cannot explain some of the inverse relationships in several plant tissues and with plant age.

The peramine–endophyte ratio was significantly higher in apical than in basal leaf tissues, and exhibited a fairly abrupt change between sheath and blade. This strong sheath-to-blade variation of the peramine–endophyte ratio was mainly due to the relatively uniform distribution of peramine and the heterogeneous distribution of the endophyte in the leaf (Fig. 3). Peramine is relatively hydrophilic (Rowan and Gaynor, 1986), perhaps providing greater mobility of peramine in the plant (Rowan, 1993). Hence, a simple explanation for its uniform distribution is in planta translocation of peramine.

All three alkaloids have been associated with biological activities against herbivores (Gallagher et al., 1981; Prestidge and Gallagher, 1988; Tor-Agbidye et al., 2001), and been implicated in enhanced fitness of endophyte-infected grasses (Bush et al., 1997; Easton, 1999). The different patterns of in planta accumulation of the alkaloids may well reflect adaptation of the symbiosis to feeding pressures by different herbivores (Lane et al., 2000). For optimum protection of the grass plant from herbivory, mechanisms might exist that provide a balance of the levels of all alkaloids in the different parts of the plant. How the in planta alkaloid levels may be controlled in the plant remains to be determined. It is very probable, however, that both alkaloid precursors and regulatory compounds in the immediate in planta environment of the fungus are involved. Using our data as a framework and combining the approach described here with the mapping of expression or disruption of genes recently identified as being involved in alkaloid biosynthesis (Panaccione et al., 2001; Scott, 2001; Wang et al., 2004) should enable further elucidation of these mechanisms.

3. Conclusions

Our data provide a detailed picture of the relationship between alkaloid and endophyte levels, and evidence that alkaloid accumulation in the grass plant is regulated at different levels. First, alkaloid levels in plant tissues depend to some extent on levels of the endophyte mycelium in these tissues. Second, and more importantly, alkaloid levels are affected by the nature of the plant tissue the endophyte inhabits, indicated by varying alkaloid–endophyte ratios in the different tissues, an effect that is further modulated by the plant genotype. This may be due to tissue and genotype differences in rates of alkaloid biosynthesis in the fungal mycelium, in planta translocation of the alkaloids, and varying rates of alkaloid metabolisation/degradation. Third, each of the alkaloids showed a pattern of accumulation different from the other alkaloids, suggesting modes of regulation specific to each alkaloid.

4. Experimental

4.1. Endophyte strains and grass plants used

The endophyte strain KS1 (Tan et al., 2001), a transformant of the *N. lolii* isolate Lp19, naturally occurring in perennial ryegrass (Christensen et al., 1993) was used. KS1 contains the β-glucuronidase (GUS) gene of *E. coli* under control of the constitutive promoter *gpd*A from *Aspergillus nidulans*. The plant genotypes used were of the perennial ryegrass (*L. perenne*) cv. Grasslands Nui.

4.2. Plant selection and growth conditions

KS1 was inoculated into ryegrass seedlings by the method of Latch and Christensen (1985). Inoculated plants were grown for three months in the greenhouse and then analysed for GUS activity and alkaloids (see below) in the pseudostem (comprised of leaf sheath, emerging leaf, and stem tissues). Plants of selected genotypes were grown under controlled-environment conditions (450 ± 30 μmol photons m^{-2} s^{-1} ; 12/24 h light cycle; and 15 ± 2 °C) for >18 months before assessment of GUS and alkaloid distributions. Plants were re-potted every 2–3 months.

4.3. Determination of the relationship between GUS activity and levels of endophyte hyphae in planta

To relate GUS activity in tissues to endophyte levels, GUS activity concentration and hyphal counts in leaf cross-sections were assessed in parallel in grass tissues. Hyphal counts were performed as described by Tan et al. (2001): small sub-sections were cut transversally from either end of a leaf tissue, cleared from leaf pigments, and stained with aniline blue. Stained endophyte hyphae were counted at 400 magnification in 0.5-mm sliced cross-sections cut from each of the sub-sections. Hyphal counts were performed in duplicate on each sub-section. The mean hyphal number in the leaf section was determined from the hyphal numbers in the two sub-sections. The weight of the leaf section was determined, and the section extracted and analysed for GUS activity with a quantitative fluorometric assay (see below). GUS–hyphal ratios were determined by dividing the GUS activity concentration (GUS activity per tissue dry wt) in a tissue section by the average number of hyphae in the same section.

4.4. Grass plant dissection and sample preparations for GUS activity and alkaloid analyses

All plants used in this study were non-reproductive. From each plant (representing an experimental replicate), 10–12 tillers of similar size and having three mature leaves were removed and dissected. An overview of the tiller dissection is given in Fig. 2. Emerging (i.e., growing) leaves were identified by the absence of a fully developed ligule, a small protuberance at the adaxial side of the leaf, separating the leaf sheath from the leaf blade. The stem constituted the very basal tissue, bearing the leaves, roots, and descendant tillers. Roots and young tillers were not included in the analyses. Mature (i.e., non-growing) leaves were dissected as shown in Fig. 2. Dissected tissues of a given tissue fraction were pooled within replicates and ground with a micropestle in liquid nitrogen in 1.5-ml microcentrifuge tubes and freeze-dried for 16–20 h. After freeze-drying, samples were purged with N_2 and stored at −20 °C. Under these storage conditions, no detectable degradation of GUS activity occurred within 10 days (Spiering, 2000). Parallel analyses of GUS and alkaloids were performed on 2–50 mg aliquots taken from each tissue sample. GUS activity concentration was determined within ten days on two to ten milligram samples of grass tissues as described by Tan et al. (2001), and alkaloid analyses (see below) were performed within one to two months (no detectable degradation of alkaloids has been found to occur during this time; GAL and Brian A. Tapper, personal observations).

4.5. Alkaloid analyses

Alkaloids were extracted from 5–50 mg of freeze-dried and ground grass samples. For extraction of lolitrem B, samples were weighed into 2.0-ml plastic vials (Sarstedt; Nürnbrecht, Germany); to the vials was added 1 ml (20–50 mg samples) or 0.5 ml (<20-mg samples) 1,2-dichloroethane:methanol, 9:1 v/v, and the sample–solvent mix agitated for 1 min in a cell disrupter (FP

120 Savant FastPrep, BIO 101 Inc., La Jolla, CA, USA). Samples were then extracted for 1 h at room temperature, in darkness, with gentle agitation on an orbital shaker. Particulate grass material was removed from extracts by a filtration method described by Spiering et al. (2002). Extracts were analysed by HPLC with fluorescent detection as described by Gallagher et al. (1985). Quantitation of lolitrem B was performed with pure lolitrem B, kindly provided by Dr. C.O. Miles, AgResearch Ruakura, Hamilton, New Zealand, as external standard run in the same batch of samples (Gallagher et al., 1985). The lower limit of detection in this assay is 0.1 ppm (Tapper and Latch, 1999). Ergovaline was extracted and analysed by HPLC with fluorescent detection as described by Spiering et al. (2002). Quantitation of ergovaline was performed with the ergopeptine ergotamine added to each sample as an internal standard. The lower limit of detection in this assay is 0.01 ppm (Spiering et al., 2002). Peramine was analysed by HPLC from the same extracts used for the analysis of ergovaline (Spiering et al., 2002), and quantified by UV absorbance using the peramine-analogue homoperamine [3(-4′-guanidinylbutyl)-2-methylpyrrolo[1,2-a]-pyrazin-1(2H)-one] as internal standard. The lower limit of detection in this assay is 2 ppm (Tapper and Latch, 1999).

4.6. Data analyses

Data were log-transformed for statistical analysis, to conform to a normal distribution around the mean; to accommodate values for ergovaline concentration below the level of quantification in some tissues, these were approximated by the lower limit of detection for ergovaline. The dependence of GUS activity on hyphal counts in leaf cross-sections and alkaloid concentrations on endophyte levels (assessed as GUS activity; see Section 2) was tested by linear regression. Alkaloid–endophyte ratios were determined by dividing alkaloid concentration by GUS activity concentration in individual tissue samples. The statistical significance of effects, such as plant genotype and tissue section on GUS activity and alkaloid concentrations, and alkaloid–endophyte ratios, was assessed by analysis of variance (ANOVA). Fisher's LSD was used to test statistical significance of differences between means. In all analyses, a $P < 0.05$ was considered statistically significant for detecting differences between means. The relative magnitude of effects in accounting for the variation in a given alkaloid–endophyte ratio was assessed by determining for each factor the standard deviation about the mean from the ANOVA model (as an example for the calculation: for the ergovaline-endophyte ratio, the effects of the three genotypes (Fig. 4) are −0.41 (D), 0.25 (UIII), 0.15 (UIV) with mean 0 and SD 0.36).

Acknowledgements

We thank Yong Y. Tan and Taha Al-Samarrai for assistance with the plant maintenance. We also thank Elizabeth Davies for valuable assistance with the alkaloid analyses and Zaneta Park-Ng for expert statistical advice. Brian A. Tapper and Susanne Rasmussen provided helpful comments and suggestions in the preparation of this manuscript. This research was supported by the New Zealand Foundation for Research, Science and Technology. M.J.S. was supported by a Massey University Ph.D. scholarship.

References

Adcock, R.A., Hill, N.S., Bouton, J.H., Boerma, H.R., Ware, G.O., 1997. Symbiont regulation and reducing ergot alkaloid concentration by breeding endophyte-infected tall fescue. J. Chem. Ecol. 23, 691–704.

Bacon, C.W., 1993. Abiotic stress tolerances (moisture, nutrients) and photosynthesis in endophyte-infected tall fescue. Agric. Ecosyst. Environ. 44, 123–141.

Ball, O.J.-P., Prestidge, R.A., Sprosen, J.M., 1993. Effect of plant age and endophyte viability on peramine and lolitrem B concentrations in perennial ryegrass seedlings. In: Hume, D.E., Latch, G.C.M., Easton, H.S. (Eds.), Proceedings of the Second International Symposium on *Acremonium*/Grass Interactions. AgResearch Grasslands, Palmerston North, New Zealand, pp. 63–66.

Ball, O.J.-P., Prestidge, R.A., Sprosen, J.M., 1995. Interrelationships between *Acremonium lolii*, peramine and lolitrem B in perennial ryegrass. Appl. Environ. Microbiol. 61, 1527–1533.

Ball, O.J.-P., Barker, G.M., Prestidge, R.A., Sprosen, J.M., 1997a. Distribution and accumulation of the mycotoxin lolitrem B in *Neotyphodium lolii*-infected perennial ryegrass. J. Chem. Ecol. 23, 1435–1449.

Ball, O.J.-P., Miles, C.O., Prestidge, R.A., 1997b. Ergopeptine alkaloids and *Neotyphodium lolii*-mediated resistance in perennial ryegrass against adult *Heteronychus arator* (Coleoptera: Scarabaeidae). J. Econ. Entomol. 90, 1382–1391.

Baxter, R.M., Kandel, S.I., Okany, A., 1962. Biosynthesis of ergot alkaloids – incorporation of mevalonic acid into ergosine. J. Am. Chem. Soc. 84, 2997–2999.

Bouton, J.H., Latch, G.C.M., Hill, N.S., Hoveland, C.S., McCann, M.A., Watson, R.H., Parish, J.A., Hawkins, L.L., Thompson, F.N., 2002. Reinfection of tall fescue cultivars with non-ergot alkaloid-producing endophytes. Agron. J. 94, 567–574.

Bush, L.P., Wilkinson, H.H., Schardl, C.L., 1997. Bioprotective alkaloids of grass–fungal endophyte symbioses. Plant Physiol. 114, 1–7.

Byrne, K.M., Smith, S.K., Ondeyka, J.G., 2002. Biosynthesis of nodulosporic acid A: precursor studies. J. Am. Chem. Soc. 124, 7055–7060.

Christensen, M.J., Leuchtmann, A., Rowan, D.D., Tapper, B.A., 1993. Taxonomy of *Acremonium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*F. pratensis*) and perennial ryegrass (*Lolium perenne*). Mycol. Res. 97, 1083–1092.

Christensen, M.J., Easton, H.S., Simpson, W.R., Tapper, B.A., 1998. Ocurrence of the fungal endophyte *Neotyphodium coenophialum* in leaf blades of tall fescue and implications for stock health. N. Z. J. Agric. Res. 41, 595–602.

Easton, H.S., 1999. Endophyte in New Zealand ryegrass pastures, an overview. In: Woodfield, D.R., Matthew, C. (Eds.), Ryegrass

Endophyte: An Essential New Zealand Symbiosis. New Zealand Grassland Association, Napier, New Zealand, pp. 1–9.

Easton, H.S., Latch, G.C.M., Tapper, B.A., Ball, O.J.-P., 2002. Ryegrass host genetic control of concentrations of endophyte-derived alkaloids. Crop Sci. 42, 51–57.

Fletcher, L., 1999. Non-toxic endophytes in ryegrass and their effects on livestock health and production. In: Woodfield, D.R., Matthew, C. (Eds.), Ryegrass Endophyte: An Essential New Zealand Symbiosis. New Zealand Grassland Association, Napier, New Zealand, pp. 133–139.

Gallagher, R.T., White, E.P., Mortimer, P.H., 1981. Ryegrass staggers: isolation of potent neurotoxins lolitrem A and lolitrem B from staggers-producing pastures. N. Z. Vet. J. 19, 189–190.

Gallagher, R.T., Hawkes, A.D., Stewart, J.M., 1985. Rapid determination of the neurotoxin lolitrem B in perennial ryegrass by high-performance liquid chromatography with fluorescence detection. J. Chromatogr. 321, 217–226.

Gurney, K.A., Mantle, P.G., Penn, J., Garthwaite, I., Towers, N.R., 1994. Loss of toxic metabolites from *Acremonium lolii*, the endophyte of ryegrass, following mutagenesis. Naturwissenschaften 81, 362–365.

Herd, S., Christensen, M.J., Saunders, K., Scott, D.B., Schmid, J., 1997. Quantitative assessment of in planta distribution of metabolic activity and gene expression of an endophytic fungus. Microbiology 143, 267–275.

Jefferson, A.R., 1987. Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. 5, 387–405.

Keogh, R.G., Tapper, B.A., Fletcher, R.H., 1996. Distributions of the fungal endophyte *Acremonium lolii*, and of the alkaloids lolitrem B and peramine within perennial ryegrass. N. Z. J. Agric. Res. 39, 121–127.

Lane, G.A., Ball, O.J.-P., Davies, E., Davidson, C., 1997. Ergovaline distribution in perennial ryegrass naturally infected with endophyte. In: Bacon, C.W., Hill, N.S. (Eds.), *Neotyphodium*/Grass Interactions. Plenum Press, New York, pp. 65–67.

Lane, G.A., Christensen, M.J., Miles, C.O., 2000. Coevolution of fungal endophytes with grasses: the significance of secondary metabolites. In: Bacon, C.W., White, J.F. Jr. (Eds.), Microbial Endophytes. Marcel-Dekker, New York, pp. 341–388.

Latch, G.C.M., Christensen, M.J., 1985. Artificial infection of grasses with endophytes. Ann. Appl. Biol. 107, 17–24.

Munday-Finch, S.C., Garthwaite, I., 1999. Toxicology of ryegrass endophyte in livestock. In: Woodfield, D.R., Matthew, C. (Eds.), Ryegrass Endophyte: An Essential New Zealand Symbiosis. New Zealand Grassland Association, Palmerston North, New Zealand, pp. 63–67.

Neumann, D., 1985. Storage of alkaloids. In: Mothes, K., Schütte, H.R., Luckner, M. (Eds.), Biochemistry of Alkaloids. VCH, Berlin, pp. 49–55.

Panaccione, D.G., Johnson, R.D., Wang, J.H., Young, C.A., Damrongkool, P., Scott, B., Schardl, C.L., 2001. Elimination of ergovaline from a grass–*Neotyphodium* endophyte symbiosis by genetic modification of the endophyte. Proc. Natl. Acad. Sci. USA 98, 12820–12825.

Popay, A.J., Hume, D.E., Baltus, J.G., Latch, G.C.M., Tapper, B.A., Lyons, T.B., Cooper, B.M., Pennell, C.G., Eerens, J.P.J., Marshall, S.L., 1999. Field performance of perennial ryegrass (*Lolium perenne*) infected with toxin-free fungal endophytes (*Neotyphodium* spp.). In: Woodfield, D.R., Matthew, C. (Eds.), Ryegrass Endophyte: An Essential New Zealand Symbiosis. New Zealand Grassland Association, Napier, New Zealand, pp. 113–122.

Prestidge, R.A., Gallagher, R.T., 1988. Endophyte fungus confers resistance to ryegrass: Argentine stem weevil studies. Ecol. Entom. 13, 429–435.

Rowan, D.D., Gaynor, D.L., 1986. Isolation of feeding deterrents against stem weevil from ryegrass infected with the endophyte *Acremonium loliae*. J. Chem. Ecol. 12, 647–658.

Rowan, D.D., 1993. Lolitrems, peramine and paxilline: mycotoxins of the ryegrass/endophyte interaction. Agric. Ecosyst. Environ. 44, 103–122.

Roylance, J.T., Hill, N.S., Agee, C.S., 1994. Ergovaline and peramine production in endophyte-infected tall fescue: independent regulation and effects of plant and endophyte genotype. J. Chem. Ecol. 20, 2171–2183.

Scott, B., 2001. *Epichlo*ë endophytes: fungal symbionts of grasses. Curr. Opin. Microbiol. 4, 393–398.

Siegel, M.R., Johnson, M.C., Varney, D.R., Nesmith, W.C., Buckner, R.C., Bush, L.P., Burrus II, P.B., Jones, A., Boling, J.A., 1984. A fungal endophyte in tall fescue: incidence and dissemination. Phytopathology 74, 932–937.

Soper, K., Mitchell, K.J., 1956. The developmental anatomy of perennial ryegrass (*Lolium perenne* L.). N. Z. J. Sci. Technol. 37, 484–504.

Spiering, M.J., 2000. Distribution of *Neotyphodium lolii*-endophyte metabolic activity in perennial ryegrass (*Lolium perenne* L.) and its implications for alkaloid distribution and photosynthesis. Ph.D. Thesis, Massey University, Palmerston North, New Zealand.

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. J. Agric. Food Chem. 50, 5856–5862.

Tan, Y.Y., Spiering, M.J., Scott, V., Lane, G.A., Christensen, M.J., Schmid, J., 2001. In planta regulation of extension of an endophytic fungus and maintenance of high metabolic rates in its mycelium in the absence of apical extension. Appl. Environ. Microbiol. 67, 5377–5383.

Tapper, B.A., Latch, G.C.M., 1999. Selection against toxin production in endophyte-infected perennial ryegrass. In: Woodfield, D.R., Matthew, C. (Eds.), Ryegrass Endophyte: An Essential New Zealand Symbiosis. New Zealand Grassland Association, Napier, New Zealand, pp. 107–111.

Tor-Agbidye, J., Blythe, L.L., Craig, A.M., 2001. Correlation of endophyte toxins (ergovaline and lolitrem B) with clinical disease: fescue foot and perennial ryegrass staggers. Vet. Hum. Toxicol. 43, 140–146.

Wang, J.H., Machado, C., Panaccione, D.G., Tsai, H.F., Schardl, C.L., 2004. The determinant step in ergot alkaloid biosynthesis by an endophyte of perennial ryegrass. Fung. Genet. Biol. 41, 189–198.